ANALYSIS OF FEED INGREDIENTS IN POULTRY AND SWINE

by

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(Under the Direction of Gene M. Pesti and Michael Azain)

ABSRACT

The effect of feed ingredients and its impact on poultry and swine will be investigated in this dissertation. Four studies were conducted to understand feed better and feed components poultry and swine. The first study examined the relationship of standardized ileal digestibility (SID) and amino acid concentration in feed ingredients for swine. The second study determined a method for quantifying total choline in feed ingredients. The third study investigated the bioavailability of choline in organic feed ingredients for broiler chickens. The fourth study looked at quantifying betaine and betaine bioavailability of organic feed ingredients in broiler chickens. The fifth study determined the amino acid digestibility of organic feed ingredients in broiler chicks.

It was determined that there was an increase in SID as amino acid concentration increased in swine. This confirmed the results in a mirror study done in chickens. Choline can be extracted from feed ingredients in two-part chemical digestion. This method was developed specifically for feed ingredients and can be applied to a wide variety of feed ingredients. Total choline varied depending feed ingredient analysis. Choline bioavailability varied depending on feed ingredient. Betaine can be quantified in feed ingredients. Bioavailability of betaine also varied depending on feed ingredient. Betaine content was highly variable depending on feed ingredient. Amino acid

digestibility of organic feed ingredients is highly variable between feed ingredients. Amino acid digestibility of organic feed ingredients is similar to conventionally grown feed ingredients.

INDEX WORDS: Swine, Amino acid, HPLC, Broilers, Choline, Betaine, Organic

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CHAPTER 1

INTRODUCTION

Feed ingredients are integral to animal nutrition. Without knowing how to feed ingredients to animals properly, the animal nutrition industry will not progress. This is important when feeding for niche markets like the organic market. This means that more novel feed ingredients that are not typically fed to animals may be used in organic livestock production. It is important to know how to feed these novel feed ingredients to get the most out of the feed ingredient used as well as the best performance of the animal under the production system used.

In organic production, crystalline methionine is fed in limited quantities to poultry and is entirely banned in other livestock. Methionine is a limited amino acid in most animal diets. Feeding ingredients that are high in methionine are vital for organic poultry production. Even when feeding high methionine feed ingredients, there still may not be enough methionine to meet an animal's methionine requirement. There are specific nutrients, choline and betaine, have been shown to spare methionine. This means that less methionine is needed in the diet to meet the animal's requirement. For choline and betaine to be used in organic production, to spare methionine, there must be a reliable analysis of total and bioavailable choline and betaine in feed ingredients.

Traditional assumptions about how to standardize ileal digestibility (SID) may not be as correct as we assume. A paper by Pesti and Tahir in 2012 showed that SID in chickens is not constant with amino acid concentration and this is an assumption that is needed for SID. A similar study was performed with swine to determine if the conclusions from Tahir and Pesti 2012 are applicable in swine and well.

Feed ingredient analysis is essential to determine nutrient composition. Traditional choline analysis was developed for baby formulas and choline supplements. These methods would not be the best for feed ingredients. Feed ingredients have proteins, fats, and fibers that could impede the extraction of compounds like choline.

Betaine is a compound that is naturally occurring in plants. Betaine determination in feed ingredients is limited. Betaine in feed ingredients can vary due to various growing conditions, which affect water uptake in plants. Determination of betaine digestibility in livestock is limited as well.

CHAPTER 2

LITERATURE REVIEW

Organic poultry production

Organic food production has increased in popularity recently in the United States. The National Organic Program (NOP) was created in 2002. Since the NOP has been established the amount of organic production has dramatically increased in the United States since 2006 (USDA, 2015, OTA, 2016, Figure 2-1). According to the USDA, the most recent definition of organic production is "a production system that… responds to site-specific conditions by integrating cultural, biological, and mechanical practices that foster cycling of resources, promote ecological balance, and conserve biodiversity" (USDA, 2017). Organic production has little to do with the types of commodities being grown and more to do with a whole farm management approach of producing agricultural products (USDA, 2015).

Raising animals in this way could cause some contradictions. Mackenzie et al. (2016) formulated swine diets under different environmental constrictions that were least cost (that was used as a control), least non-renewable resource use (NRRU), least eutrophication (EP), least global warming potential (GWP), and least environmental impact (EI). Results showed that the NRRU diet formulation used no crystalline amino acids, yet were higher in protein than all other diets. The NRRU diet may be a good choice for organic producers to formulate their diets on since crystalline amino acids cannot be used (Coffey and Baier, 2012). The NRRU diets used ingredients like meat meal and high protein soybean meal; however, these ingredients would not be practical for organic producers to source. The EP diet has the lowest protein, used crystalline amino acids,

and used no meat meal. This diet would have the lowest impact on water runoff. The GWP diet only used the crystalline amino acid methionine and fed the second highest protein level. This shows that there is no best way to formulate a diet, every diet would have environmental tradeoffs, including organic type diets.

Organic poultry production is different from conventional poultry production in that organic poultry must have access to the outside (Fanatico et al., 2009). Also, feed ingredients that are used in conventional production systems cannot be used in organic production, and this includes genetically modified crops and crystalline amino acids. The only exception is for methionine which can be added to the poultry diet at 2, 2, and 3 pounds per ton for layers, broilers, and turkeys respectively (Coffey and Baier, 2012). Crystalline amino acids cannot be used in organic diets for any other type of livestock. Organic production of all sectors is increasing (Figure 2-1). Meat, fish, and poultry are a smaller portion of organic sales compared to produce (fruits and vegetables). However, broilers and layers are the most numerous when looking at the number of animals raised with approximately 21.7 million broilers and 17.5 million layers in 2016 (Figure 2-2). Organic meat production is the fastest growing section of the organic sector, with organic poultry being the most substantial of that segment (Fanatico et al., 2009, Figure 2).

Organic poultry production is an area where research needs to be pursued to ensure that the animals are being raised under the best conditions for that production system. For organic poultry production to succeed it must be profitable. It has been shown that organic broiler production yielded higher profits and was more economical than conventionally raised broilers (Cobanoglu et al., 2014, Bokkers and deBoer, 2009). Increased in profits, however, is not the direct goal of organic production according to Fanatico et al. (2009). Both variable and fixed cost for organic production are higher than conventional production (Cobanoglu et al., 2014). Fanatico et

al. (2009) state that organic production focuses on sound environmental practices, animal health, and overall product quality. Organic production should be less of a focus on maximizing production which may not reduce costs or be the most economical. This may not be how most organic poultry in the United States are raised. Most organic production in the United States is currently raised in a large-scale, intensive type management systems where industries of scale help maximize profits (Fanatico et al., 2009).

Organically produced poultry must be allowed to exercise, have access to the outdoors, and exhibit natural behavior. These natural behaviors could include scratching and dust bathing (Fanatico et al., 2009). Organic regulations do not require how much time or space birds need to be outside, and this is left up to the organic certifying organizations. Animals can be confined for a limited period of time depending on the stage of production or weather conditions. There is also no requirement on what types of birds to raise (Fanatico et al., 2009, USDA 2015). National Organic Program (NOP) does suggest using animals that are best suited for the climate, and animals that are more disease resistant (USDA, 2015). However, specific breed recommendations are not mentioned

A perceived benefit of organic production is increased animal health. Preventive health management is vital for organic animal management. Extra attention must be on ventilation, good nutrition, vaccines, good biosecurity, minimizing stress exposure to prevent animals from getting sick, since organic regulations do not permit the use of antibiotics (USDA, 2015, Fanatico et al., 2009). These practices should be done with conventionally raised poultry as well. Bokkers and deBoer (2009) showed that organic broiler production spends more on bird health than conventional broiler production. Organic broilers produce more excreta and ammonia than

conventionally raised broilers (Bokkers and deBoer, 2009). This could lead to lower air quality for the broilers and more pollution for the environment.

Antibiotics of any kind are not permitted for any animals to be marketed as USDA organic. There are alternative growth promoters that can be used in organic animal production. Probiotics can be used in place of antibiotic growth promoters. Probiotics are good bacteria that increase gut and overall health. Probiotics can colonize the gut, and this would keep harmful bacteria in small numbers, so they would not be able to colonize the gut and cause disease (Radfar and Farhoomand, 2008). Prebiotics are also permitted in organic animal production. Prebiotics are food for good bacteria to consume. Prebiotics are substances that allow good bacteria to proliferate while limiting bad bacteria to grow (Javier et al., 2017). Even though animals that are given antibiotics are not permitted, antibiotics must be given if the animal is clinically sick, and antibiotics are the only effective alternative. Doing this will cause the animal to lose organic status, and the animal must be marketed in other non-organic markets (Coffey and Baier, 2012). Since antibiotics are not permitted, mortality and necrotic enteritis may be higher in organic broilers (Fanatico et al., 2009). It has been shown that broilers that are not given antibiotic growth promoters have increased mortality (Sun et al., 2005).

Another way to reduce disease would be proper biosecurity methods. This would be limiting unnecessary foot traffic. Farm staff and visitors should use appropriate biosecurity equipment. This would include boot bags, boot washes, truck, and equipment washes. Pest management is also crucial for biosecurity. Removing pests removes a vector for diseases to enter the herd and flock. Pest should be managed in a way that is within the guidelines of organic management. This includes non-synthetic lures, traps, and repellents (Coffey and Bair, 2012). Diseases from wildlife can be transmitted to organic animals and make them sick. Methods to

remove pests that are within the organic guidelines are removal habitat and food sources for the pest in question. If these methods do not work, synthetic and non-synthetic substances that are permitted for use by the National List of Allowed and Prohibited Substances (Coffey and Bair, 2012).

Feeding animals organic diets could change the composition of meat for the consumer. Srednicka- Tober et al. (2016) did a meta-analysis of organic and conventionally raised meat and fat quality composition studies of various kinds of meat including beef, pork, and poultry. Overall, there was an increase in the organically raised meat of both polyunsaturated fatty acids, omega three fatty acids, and omega six fatty acids. Organically raised meat has less monounsaturated fatty acids. However, when looking at specific animal groups of meat, (poultry, beef, pork, and lamb and goat) poultry was the only meat that saw these changes. It was concluded that differences in fat quality were small when comparing conventional and organically raised poultry.

Methionine

Methionine is typically the first limiting amino acid in poultry fed corn and soybean meal (SBM) diets (Blair et al., 1986). Methionine is an important amino acid for many reasons. Methionine is a building block for proteins, a sulfur source, and is important for labile methyl transfer (Pesti et al., 2005, Figure 3-4). Methionine is typically a limiting amino acid in common the United States livestock diets. There are many companies that sell supplemental methionine, as a crystalline amino acid or a keto analog. Methionine is limited in organic diets, and this poses challenges for organic poultry producers. An alternative for an organic system would be to have a lifetime limit on methionine instead of an absolute limit. This would allow birds to have increase methionine when the birds are younger and have higher methionine requirements. As the bird ages, the methionine requirement would decrease and added methionine would decrease as well (Burley

et al., 2016). This would mirror the requirements of birds, so birds are less deficient when birds are younger and have a higher demand for methionine and protein in general. This has the potential to help the bird's overall health for later stages since methionine is needed for immune functions (Bunchasak, 2009).

Methionine alternatives

Genetic changes of livestock

Raising slower growing livestock would reduce the nutrient density that the animal would need in a day. A slower growing animal will not need the same nutrient density to reach maximum growth performance and genetic potential. Diets that are lower in protein could still be adequate for an animal that has a slower maximum growth performance, and would not have the environmental impact of high protein diets (Burley et al., 2016). Poultry with slower growing genetics may also have slower gut passage rate, which would make feeding forages a more viable option. If a poultry digestive tract is slowed, then there may be more time for microbial digestion in the ceca and make the overall diet fed more efficient (Lumpkins et al., 2010).

Fast-growing strains of birds have been defined as birds that achieve market weight, of four pounds, in 7 weeks, with genetic selection on high breast yield. Slower growing strains are defined as birds that reach market weight in 12 weeks with less genetic pressure on breast yield (Fanatico et al., 2009). When directly comparing a fast-growing versus a slow-growing strain, fast-growing birds were heavier, even though feed consumption was the same between strains. Feed conversion was worst for slow-growing strains. Slow-growing birds ate more forage when given access to forage, compared to the fast-growing strain of birds. The slower growing strain had lower mortality (Fanatico et al., 2005). However, within genotype (slow vs. fast growth) outdoor access did not affect body weight. Access to the outside did decrease feed conversion ratio (FCR) of both

genotypes. Genotype appears to influence growth more than outdoor access. Broilers with access to the outside have a lower FCR then similar birds raised inside. When comparing daily methionine requirement slower growing birds do not appear to have a lower requirement than fast-growing birds (Fanatico et al., 2009).

High protein diets

One way to meet the methionine requirement of a broiler would be to increase the proportion of protein in the diet until the first limiting amino acid requirement is met. This would mean increasing the protein in poultry feed until the methionine requirement is meant. This would increase diet prices because protein typically is the most expensive feed component (NRC, 2012). Having high protein diets may not be a good animal welfare choice for animals themselves. Increasing protein in the diet would increase ammonia and decrease air quality while causing footpad lesions in poultry (Mackenzie et al., 2016, Ward et al., 1975). Increasing protein in the diet would not be good for the environment either. Increasing protein would increase nitrogen in the feces and the excreta. If this highly nitrogenous waste reached a watershed, there could be an increased chance of algae blooms and dead zones in the ocean (Mackenzie et al., 2016). Even though high protein diets are a method permitted by USDA organic guidelines, feeding high protein diets that have the potential to harm waterways is not in the spirit of the organic guidelines. A repeated concept that is stated in the *Guide for Organic Producers* is that the soil and water quality should be maintained or improved with organic practices; however, if high protein diets are fed to animals, this would not be the case (Coffey, and Baier, 2012). Water and soil quality could decrease with organic practices. This is shown by Mackenzie at al. (2016) who assigned different types of diets different sustainability scores. Diets that had the lowest eutrophication, an

indicator for water pollution, had lower digestible crude protein; however, these diets also used more crystalline amino acids, which is not permitted in organic production.

A higher crude protein diet may also increase the methionine requirement (Garcia-Neto et al., 2000), which would be counterproductive when trying to meet the methionine requirement by increasing the total protein. Increasing protein to meet methionine needs would not be a good management system for organic livestock since methionine cannot be economically fed in excess. This shows that methionine alternatives like choline, betaine, folate, vitamin B_{12}, and other labile methyl groups should be used to meet labile methyl needs, and to not used methionine alone.

Outdoor grazing

Poultry are not typically thought of as grazing animals. Organic guidelines mandate outdoor access for all animals (USDA, 2015). However, the guidelines do not mandate the quality of the pasture the livestock have access to. Animals could make up for nutrient deficiencies in the feed if they have access to pasture. However, due to the large moisture content of forages, overcoming severe deficiencies is unlikely (Burley et al., 2015). Forages are high in fiber which is not very digestible for poultry, and forages could make other parts of the diet less digestible. It is unlikely that forages would increase the protein quality of the diet, so deficiencies like methionine would still be a problem for nutritionists to overcome if forages are used (Walker and Gordon, 2002). Birds may not want to eat the forage provided (Wood, 1956, Walker and Gordon, 2002). If the bird will not eat the forage, the nutrient content of that forage is irrelevant. It has been shown that adding a nonstarch polysaccharide enzyme while pasture raising roosters will increase energy digestibility (Buchanan et al., 2007). This could be a way to increase pasture utilization. However, if the enzyme used is genetically modified then the chickens used would not be able to be marketed as organic (Coffey and Bair, 2012).

Broilers may have an increase in growth performance and carcass yield when giving access to fresh clover (Ponte et al., 2008a). This means that when birds have access to pasture, they would consume either forage or scratch for insects, and consume extra energy for growth. There appears to be a trial/ season effect on growth performance with the autumn trial having greater body weight gains than the spring trial. The control birds had no pasture access, also had an increase in body weight in the autumn trial, as well (Ponte, 2008a). In this experiment, meat quality and sensory measurements were taken as well. In the spring trial lightness score of the meat increased in pastured reared birds, compared to the control. In the autumn the lightness score decreased in pastured reared birds, compared to the control. In both the fall and the spring trials, redness score decreased in birds given pasture access. Tenderness, juiciness, and flavor of the meat where unaffected. There would be no improvement in consumer experience when consuming pasture-raised broilers. There was an increase consumer appreciation of the pasture reared poultry showing that there may be an increase in consumer satisfaction of broilers raised on pasture (Pointe et al., 2008a).

Pasture-raised broilers could have different fatty acid profiles due to the forage consumed. Control birds had higher levels of total lipids in the breast muscle compared to pasture-raised birds. Control birds had more linoleic acid, compared to pasture-raised birds. Pasture-raised broilers had higher levels of linolenic acid. There were seasonal effects of the fatty acid profile, with autumn raised pasture birds having a lower total lipid value in the breast muscle compared to birds raised in the spring (Ponte et al., 2008b).

Having broilers on pasture does not guarantee that the birds will get eat the pasture that they are given access too. Restricting grain intake, with pasture access, may increase consumption of forage. Ponte et al. (2008c) did a study where grain was restricted by 75 and 50%, with and

without pasture access. Restricting grain access reduced weight gain, and access to pasture did not restore weight gain to ad libitum weights. This shows that pasture access would not be a substitute for a complete feed (Ponte et al., 2008c).

There is an assumption that poultry on pasture will be scavengers and forage for insects. However, this may not be the case. A study by Clark and Gage (1997), showed that arachnids decrease in areas that poultry had pasture access to; however, beetle and earthworm populations remained unchanged. Assuming that poultry will eat insects while on pasture is not a guarantee, they may not ingest enough insects to reduce a wide variety of insect populations. Poultry given access to pasture may not actually eat insects.

A study by Karsten et al. (2010) had layers that had access to outdoor pasture and control hens in cages. At the end of the study egg production of the pasture hens were 13% lower than the caged hens. Pasture hens also weighed less than caged hens. Pasture hens had higher omega-3 fatty acids, vitamin A, and vitamin E in the eggs than caged birds' eggs. Pasture-raised hens could have more nutrient-rich eggs, and it may come at the cost of egg production.

High methionine feed ingredients

A way to reduce protein of feed ingredients while supplying adequate amounts of methionine is to add ingredients that contain high amounts of methionine. Most common grains and protein sources do not have enough methionine to meet the requirement for most poultry without overfeeding protein (Burley et al., 2015). Most feed ingredients that contain high methionine levels are less common alternative feed ingredients. Brazil nut meal has one of the highest levels of methionine with a methionine content of 3.35% on an as-is basis. This is almost five times as much methionine as solvent extracted soybean meal (SBM). Dehulled expeller extracted sunflower meal has 1.04% methionine. The methionine of dehulled expeller sunflower

meal is almost double of SBM. Sunflower is grown in certain areas of the United States, which could make sunflower meal a good feed ingredient choice regionally.

Specific animal products can have high levels of methionine in them. For example, spray-dried egg white and casein have a methionine content of3.06 and 2.65%, making them good sources of methionine if allowed to be used (Burley et al., 2015). Fishmeal is a good source of methionine and does not need to be considered organic because under the regulations fishmeal is considered a supplement and not a feed ingredient (Coffey and Bair, 2012). However, the fishmeal used must be free of ethoxyquin or similar preservatives to be added to organic diets. Sourcing fishmeal without prohibited preservatives can be difficult (Fanatico et al., 2009). One disadvantage of organic meat marketing is it may also be tied to other niche markets like 'all vegetarian fed' diet labeling as well, and this would make these sources of methionine not permitted to be used. However, there seems to be less of a consumer objection to poultry eating insects, so insect and insect meals may be used. Classes of insects *Lepidoptera* (butterflies and moths) and *Hemiptera* (true bugs) have very high methionine contents of 4.7 and 4.3% respectively. Crickets also have a high methionine content of 1.93% this would make these ingredients good methionine sources. A high content of insects may put off flavors in the eggs and meat, and this would not be favorable for the consumer (Burley et al., 2015). However, studies about feeding poultry controlled amount of insect meals are limited in poultry. Insect meal for poultry is considered an animal by-product and would not be permitted in for certified organic markets (Fanatico et al., 2016). If poultry eat insects because of scavenging behavior than the animal is still considered organic.

Commercially, organically grown seaweed could be a way to supplement amino acids, including methionine; however, the cultivar of seaweed used is very important. Not all seaweeds would be a good source of methionine. Seaweed may also be a source of fiber which could decrease

digestion. Cultivating conditions of the seaweed are very important because seaweed could accumulate heavy metals that would not be good for the animals consuming the seaweed, or the people consuming the animals (Angell et al., 2016).

Organic supplements

A study by Filho et al. (2015a) studied the growth performance of a natural form of methionine purified from soybean meal. The supplement is proprietary, so composition was not discussed in the paper. A naturally derived supplement would be a way to increase methionine in the diet without overfeeding protein. If the supplement is not synthetically made, it could be approved for organic production. The results from this study showed that a vegetable source of methionine had lower average daily gain, final body weight, and feed intake. Another study by Filho et al. (2015b) showed that this supplement does not increase flock uniformity to DL-methionine levels. A uniform flock is needed for large-scale poultry production when harvesting and marketing the birds.

Kinds of Choline

Choline is in many forms in food and feedstuffs (Patterson et al., 2008, Zeisel et al., 2003). Choline has important functions for both plant and animals (Zeisel et al., 2003). Choline can be incorporated into water and fat-soluble forms in both plants and animals (Crawford et al., 1968, Pond et al., 2005). Choline is used for maintaining cell membranes, neurotransmitters, and in methyl group metabolism (Koc et al., 2002). Since choline has so many functions in various organisms, there are many different forms of choline that are used by both plants and animals (Zeisel et al., 2003). Choline can be endogenously synthesized from the amino acid glycine, or choline can also be consumed in feedstuffs that contain choline as well (Ratriyanto et al., 2009).

The common forms of choline are acetylcholine, glycerophosphocholine, phosphocholine, CDP-choline, phosphatidylcholine, and sphingomyelin.

Choline

The molecule choline, or free choline, is a water-soluble molecule that has been supplemented in animal feeds to increase performance. Choline is claimed to be an essential nutrient in adult animals (Barroeta et al., 2013, Figure 3-3). Choline is required for young animals including poultry (Nesheim et al., 1971) Choline has been supplemented to poultry for many years, and the NRC has a nutrient requirement for choline for broilers and layers (NRC, 1994). It also appears that choline in soybean meal is heat stable (Aburto et al., 1998).

A study by Jose et al. (1970) showed that choline deficiency could decrease brain and liver weights in chicks fed diets low in choline. In the brain, the deficiency is most likely caused by sphingomyelin production being limited since sphingomyelin contains choline. The decrease liver weight is most likely due to the decreased liver function, like labile methyl metabolism being inhibited due to lack of choline.

However, there is still not a consensus if choline is actually needed to be supplemented in a practical diet, or if an adult animal makes enough choline endogenously. Also, there is enough choline in practical feed ingredients to meet the needs of animals (Barroeta et al., 2013). A study was done by Nesheim et al. (1971) fed purified diets to layer hens that had no added choline. These diets were designed to make the layers deficient in choline. Layers fed a diet with no added choline did lose weight during the trial compared to birds that had choline or methionine added. Layers that did not have any supplemented choline had increase liver fat compared to layers with supplemented choline. Nesheim et al. (1971) did determine that choline was essential for layer

hens. However, this did not determine if choline in feed ingredients was enough to meet the requirement of layers.

A study done by Parsons and Leeper (1984) showed that a corn and soybean diet with 1.0 g/kg of supplemented choline chloride increased both egg weight and egg yield in Cornell randombred hens. This study would indicate that choline supplied in practical diets is inadequate, and this could be due to not enough choline provided in the diet or the choline in the feed ingredients is not available to the bird. However, this study is not conclusive. First, the study used randombred layers, not commercial layers. Randombred birds may have different choline needs compared to commercial layers. Second, this study is older, both bird genetics and crop genetics have changed since the 30+ years this study was published. This could affect whether choline needs to be supplemented in today's practical diets using modern genetics. A study by Keshavarz and Austic (1985) used commercial layers and investigated the inclusion of choline on layer performance. They found that commercial layers that were in production for over nine months did not benefit from supplemental choline. However, younger birds that have only been in production for two months had increased egg size when choline was supplemented to the diet. This shows that choline required by layers may be dependent on age or stage of production.

A study in catfish done by Zhang and Wilson (1999) showed that choline is needed for commercial catfish. Feeding choline up to 400 mg/kg showed an increase in weight gain in semi-purified diets compared to diets with no choline added.

One possible reason why choline supplementation research is inconsistent is that choline may improve immune function. If the animals are not stressed, there may be no benefit to adding choline; however, if the animals have their immune systems taxed there would be a benefit to adding choline. The animals would be able to maintain growth performance with supplemental

choline, under stressed conditions. A study by Swain and Johri (2000) showed that the addition of choline did not improve growth performance. However, leukocyte migration inhibition test increased with increasing choline. This would indicate that immune function increases with increasing choline. Hemagglutination and ELISA titers were both increased with an additional 2000 mg/kg of added choline to the diet. This effect was still significant even 21 days post immunization. This data would suggest that there is an increase in immune response parameters when choline is added to the diet. This response may be dose-dependent. The supplementation of choline did not increase growth performance. The birds may not have been challenged enough to cause a decrease in growth performance that choline supplementation would help prevent through improvements in immune function. That there was no interaction of choline and methionine supplementation on immune function (Swain and Johri, 2000).

A more recent study by Gul et al. (2005) investigated if choline supplementation could offset the antinutritional effects of feeding vetch seed. Choline chloride was added to the diet at 0.47%. Choline supplementation did not restore performance to positive control which did not contain vetch. Choline did not reduce performance compared to the negative control which contained 22% vetch.

There are different forms of choline supplementation that affect the bioavailability and therefore the performance of the animal if choline is a marginally deficient nutrient in the diet. A study by Gangane et al. (2010) compared an herbal form of choline to a chemical derived form of choline (choline chloride) in growing broilers. The study determined no difference in the type of choline (herbal or synthetic) on serum cholesterol or serum triacylglycerides. A study done by Rama-Rao et al. (2001) found no difference in broiler breeder fertility, egg weight, and total numbers of eggs when choline was fed from three different sources. However, the final body

weight of the corn choline source group was 200 grams less compared to the pearl millet and broken rice choline groups. Choline may be less available in corn than other cereal grains. If a bird is marginally deficient in choline egg parameters including fertility may be maintained at the expense of body weight or egg size.

Betaine

Betaine is structurally similar to choline (Figure 2-3). Betaine has a carboxylic acid group while choline is an alcohol group. Both compounds have a positively charged nitrogen with three methyl groups; this is the functional part of choline and betaine. Betaine is also called trimethylglycine and can be made from glycine or choline in animals (Kettunen et al., 2001, Kempson et al., 2013, Ratriyanto et al., 2009). Betaine has multiple uses in both plants and animals. Betaine is important to the osmotic regulation of plants (Carnicelli et al., 2017). In animals, betaine can also be absorbed through the intestines (Zwart et al., 2003). Betaine also has a function in the intestines, which could increase digestibility of nutrients (Eklund et al.,2006a, Xu and Yu, 2000, Amerah and Ravindran, 2015). This increase in digestibility with increasing NDF and ADF digestibility by increasing microbial activity (Eklund et al., 2006b).

Betaine content in plants can increase up to three times the normal level during drought conditions (Storey and Jones, 1975, deZwart et al., 2003). Betaine is in high concentration in sugar beets, and sugar beet by-products, wheat and wheat by-products, and alfalfa meal (Ratriyanto et al., 2009, Eklund et al., 2005). Betaine is also in common food products including chicken, beans, and spinach (de Zwart et al., 2003). Choline can be irreversibly converted to betaine (Day and Kempson, 2016, Zeisel et al., 2003, Figure 2-4). Not all plants have the pathway to produce betaine, and this could make these plants more susceptible to drought stress (Chen and Murata, 2001, Rontein et al., 2002).

In plants, choline is converted to betaine in two steps, and a similar process occurs in mammals as well. First choline is converted to betaine aldehyde by the enzyme choline monooxygenase (CMO). The last step is to convert betaine aldehyde to betaine by the enzyme betaine aldehyde dehydrogenase. Choline monooxygenase is thought to be the limiting in step in betaine production in plants (Werertilnyk and Hanson, 1989). However, more recent studies show that CMO may not be the limiting step in genetically modified drought-tolerant plants (Yamada et al., 2015).

In animals, betaine can be transported into the kidney cells by specific protein, betaine/ γ-aminobutyric acid GABA transport system (BGT1). BGT1 increases in the kidney during dehydration and other osmotic stresses. There is also a liver-specific form of BGT1, and this allows betaine to enter in the liver (Kempson et al., 2013). This is where betaine is used for labile methyl metabolism (Pesti et al., 2005). Betaine is needed for the enzyme betaine-homocysteine S-methyltransferase (BHMT) (Figure 2-4). This enzyme converts homocysteine to methionine. Methionine is then converted to S-Adenosylmethionine (SAM). SAM can be used as a methyl donor for various compounds (Kempson et al., 2013).

Betaine is also present in the kidney of mammals as an osmoprotection this can protect an animal from dehydration by helping water reabsorption (Ratriyanto et al., 2009, Kempson et al., 2013). It is thought that betaine's osmoprotective properties are due to its ability to accumulate in cells and during ionic stress, like dehydration, betaine can replace inorganic ions and protect enzymes and cell membranes (Ratriyanto et al., 2009, Petronini et al., 1992). This similar property could be seen in chickens as well. Even though chickens do not make urine, they make uric acid which is solid. When broilers were given increased salt, supplemental betaine increased growth performance (Honarbakhsh et al., 2007). Adding supplemental betaine to layer hens that are heat

stressed has been shown to increase egg production compared a diet without betaine (Ryu et al., 2002).

In humans, supplemental betaine is given to reduce levels of homocysteine in the blood (Day and Kempson, 2016). Lowering homocysteine has the potential to reduce cardiovascular risks (Hankey G. J. and Eikelboom, 1999, Rajaie and Esmaillzadeh, 2011). The amount of betaine to be effective could be as high as 6 g/day, which is much higher than the 0.1-0.3 g/day which the average American consumes. In humans, betaine has also been used to help alleviate the symptoms of fatty liver disease (Day and Kempson 2016). This is mostly due to a study in mice. When BHMT has been knocked out in mice, there is increase fat accumulation in the liver (Teng et al., 2011).

In practical livestock diets, there could be betaine that would be available for the animal to absorb and utilize in typically fed feedstuffs. Betaine may also be able to increase digestibility of other nutrients (Eklund et al., 2006a). Betaine can also be made from choline, and therefore supplementing betaine has the potential to spare choline (Day and Kempson, 2016, Figure 2-4). Supplemental betaine may or may not be needed (Sales, 2011). The reaction to convert choline to betaine, two enzymes collectively called choline oxidase, may be rate-limiting (Eklund et al., 2005). To convert choline to betaine is a two-step process with a betaine aldehyde intermediate. The first enzyme is choline monooxygenase, which converts choline to betaine aldehyde. The second enzyme is an aldehyde dehydrogenase that converts betaine aldehyde to betaine. Even if there are adequate amounts of choline available to an animal, supplemental betaine may still be more effective than choline alone. The betaine would be in the active form as opposed to choline which would have to be converted to betaine before it would be available for betaine functions in the body.

A study done by Harms and Russel (2002) showed that there was no increase in egg weight, egg content, total egg numbers of commercial layers when betaine was supplemented to commercial type diets that had adequate amounts of choline. This study used supplemental betaine but did not use feed ingredients that were devoid of betaine. So, there may have been adequate levels of betaine already in the diet.

There are some reports of betaine being used as a supplement for poultry. Alirezaei et al. (2012) investigated betaine as a possible antioxidant. This study showed that there was increased in antioxidant enzymes glutathione peroxidase and catalase, and lower thiobarbituric acid reactive substances (TBARS) in breast muscle of birds fed betaine supplemented diets. TBARS are an indicator of lipid peroxidation and free radical formation (Czerska et al., 2015). This would indicate that betaine may help improve the antioxidant status of broiler chickens. However, Fu et al. (2016) found lower levels of glutathione peroxidase in the breast muscle of birds fed a diet where betaine completely replaced methionine. Indicating that oxidative stress may increase when replacing methionine with betaine.

There are mixed reports on the efficacy of supplemented betaine to coccidiosis challenged broilers. Supplemented betaine may reduce the negative effects of a coccidiosis challenge. Kettunen et al. (2001) reported that broilers were challenged with coccidiosis and supplemented with betaine had a higher plasma, jejunal, and ileal betaine than broilers not supplemented with betaine. Non-coccidiosis challenged birds did not see an increase in betaine in intestinal tissues when betaine was supplemented. Waldenstedt et al. (1999) showed that betaine increase broiler live weight when birds were challenged with coccidiosis, and this effect was not additive with the coccidiostat Narasin. A study by Amerah and Ravindran (2015) reported that betaine supplementation decreased the impact of coccidiosis challenge and increased nutrient digestibility.

In contrast, research done by Matthews and Southern (2000) showed that dietary betaine supplementation had no effect on *Eimeria acervulina* infection in broilers.

In a study done by Fu et al. (2016) methionine was replaced with betaine to determine differences in growth performance and carcass composition in broilers. The diets used had choline chloride added to the vitamin premix. The study found no differences in growth performance or carcass yield in any of the diets. This would indicate that betaine could replace methionine. However, pH and water holding capacity decreased in the betaine only diet. This may affect meat quality and juiciness of the breast muscle. A study was done by Zhan et al., (2007) fed a methionine deficient diet, a methionine supplemented diet, and a betaine supplemented diet. Both the methionine supplemented, and the betaine supplemented diet had similar weight and feed conversion compared the diet without supplementation. Both the methionine and betaine supplemented diet had similar carcass yields and higher carcass yields than a diet with no supplemented methionine. This study shows that betaine has the potential to spare methionine.

A study was done in Cobb 500 males fed supplemental betaine to a no methionine, low methionine, and adequate methionine diets. Even though added betaine had no significant differences on methionine, there still may be a benefit to added betaine to a diet (McDevitt et al., 2010). However, betaine treatments had decreased the standard deviation of the broilers. Another study by Zhan et al. (2007) showed that betaine supplemented betaine had decrease standard deviations compared to a diet that supplemented just methionine. This would indicate that adding betaine increases flock uniformity. Even if diets are adequate for methionine, there still may be a benefit to supplement betaine if it means an increase in flock uniformity.

A study done in pigs by Albuquerque et al. (2016) investigated betaine supplementation and the effect of fat deposition in obese type pigs. The study found supplemental betaine had no

effect on growth parameters or carcass characteristics. However, there was an increase in intermuscular fat for the diet that had supplemental betaine. Both muscles, *longissimus lumborum* and *biceps femoris*, had increased gene transcription lipogenic genes acetyl-CoA carboxylase, fatty acid synthase, stearoyl-CoA desaturase. The lipolysis gene lipoprotein lipase was up-regulated in the betaine supplemented pigs. However, a gene that indicates lipolysis, adiponectin, was down-regulated. Fatty acid transport genes, heart-type fatty acid binding protein, and 3-hydroxy-3-methyl -glutaryl- CoA reductase where up-regulated in the betaine supplemented diet. Betaine supplementation could be used in fatty type pigs where increases in marbling are desired. However, a meta-analysis by Sales (2011) showed that overall there was a decrease in carcass fat with supplementation of betaine. This could indicate that betaine affects different genetic types different ways.

Glycerophosphocholine

Glycerophosphocholine (GPC) is a glycerol with choline attached (Figure 2-3). This compound could be important for nonheme iron absorption in lean meat. Even nonheme iron is better absorbed when meat is present, and there has not been a compound identified in meat that would explain this phenomenon (Armah et al., 2008). A study by Armah et al. (2008) may have found why nonheme iron is better absorbed when meat is present. The study took lean beef and digested it using simulated stomach and small intestine juices, and enzymes. The digested material was analyzed for compounds based on molecular weight. Different fractions were added to cell cultures for iron uptake measurements. The compound that increased iron uptake was analyzed using mass spectrometry and compared to L-α-glycerophosphocholine. The spectra were a matched to L -α glycerophosphocholine. L-α-glycerophosphocholine was then fed to people with

a vegetarian lasagna. Iron absorption was higher in people that consumed the food with L-α-glycerophosphocholine. This suggests that GPC could be important for iron absorption.

A study done in people by Kawamura et al. (2012) showed that GPC could improve fat metabolism when ingested. GPC increase free choline, growth hormone, and fatty acids in the blood. Ketone bodies were increased 2 hours after ingestion of GPC. This indicates that orally administered GPC may have the potential to increase fat oxidation in people. This study only used healthy participants with normal fat metabolism. This may not be applicable to people that have an abnormal fat metabolism.

Phosphatidylcholine

Phosphatidylcholine (PTC) is an amphiphilic molecule that is both water and fat soluble and is a component of cell membranes. Phosphatidylcholine is also referred to as lecithin, which is a class of compounds that also contains phosphatidylinositol and phosphatidylethanolamine (Jansen et al., 2015). Phosphatidylcholine contains choline and fatty acids attached by a glycerol backbone (Patterson et al., 2008, Figure 2-3). Phosphatidylcholine can be broken down by a pancreatic enzyme called phospholipase D. This is an enzymatic process may not be complete depending on intestinal conditions (Frohman et al., 1999). Free choline may not have the same availability as PTC since the PTC must first be broken down to free choline. Feeding phospholipids like PTC can have health benefits, in humans, like lowering serum cholesterol (Kim et al., 2008).

A study by Filho et al. (2015) found that feeding a PTC choline supplement had the same performance as choline chloride indicating that PTC is just as available as free choline. Soybean meal as a high content of PTC. A broiler study done by Menten et al. (1997) found that the availability of choline in SBM can range from 97-105%, so the authors concluded that choline in

SBM is highly available to growing broiler chick. This means that PTC can be a very effective source of choline for livestock.

Phosphatidylcholine can have a single fatty acid removed enzymatically and became lysolecithin. This molecule still contains choline that could be available to the animal. A broiler study by Jansen et al. (2015) showed that when PTC was enzymatically changed to lysolecithin, there was still more PTC that lysolecithin. Feed intake was lower when birds were supplemented with rapeseed lysolecithin, but not soybean lysolecithin or a diet with no lysolecithin. This agrees with March and MacMillen (1980) that fed both soybean and rapeseed meal and found that broilers fed rapeseed meal had worst performance than broilers fed a similar quantity of soybean meal.

A study by An et al. (1997) fed safflower phospholipids to layer hens. The phospholipid has a PTC component. Safflower phospholipid was in two different treatments: phospholipid and triglyceride mix (crude mix), and purified phospholipids, both treatments contain PTC. There was no difference in bird weight or feed efficiency at the end of the study. However, birds on the crude mix of phospholipids have increased egg weights compared to all other groups. Liver phospholipid content was not affected by treatment; both phospholipid groups have lower liver cholesterol than layers fed tallow. Phosphatidylcholine was lower in the yolk of both groups fed phospholipids compared to the birds fed tallow. Yolk PTC content could be lower when layers are supplemented phospholipids, and this seems odd since the phospholipid contained PTC.

A study by Jin et al. (1998) fed tallow, and tallow with the lecithin to nursery pigs. Feeding tallow with lecithin increased average daily gain across all phases compared to tallow alone. Overall, there was an increase in feed efficiency as well. Feeding tallow with lecithin increased nutrient digestibility of dry matter, nitrogen, and crude fat, compared to tallow alone. When comparing tallow with lecithin, there was no difference between corn or soybean oil. Adding

lecithin to tallow increases gain, and nutrient absorption compared to tallow alone. Unfortunately, this study did not use lecithin with corn oil or soy oil.

A study by Kim et al. (2008) fed increasing levels of lecithin to finisher pigs and found an increase in average daily gain and feed conversion when lecithin was added to the diet. However, there was no change in digestibility values. Feeding lecithin decreased total blood cholesterol, and blood LDL cholesterol. Adding lecithin to the diet increased blood triglycerides. Feeding lecithin had no effect on carcass characteristics or TBARS in the muscle.

(Cytidine diphosphocholine) CDP-choline

CDP-choline is an intermediate of PTC. Phosphatidylcholine is formed in the Kennedy or CDP-choline pathway. Phosphatidylcholine can be formed different ways in bacteria. The Kennedy pathway is not essential to form PTC, and this pathway does seem more prolific if cells are turning over at a high rate (Fagone and Jackowski, 2012). This pathway is more predominate if choline is supplemented (Dowd et al., 2000). Phosphatidylcholine in the Kennedy pathway is not the endpoint, but PTC is an intermediate in the CDP-choline cycle. This pathway can turn PTC into, phosphatidylserine, or lysophosphatidylcholine. Converting CDP-choline to PTC is the rate-limiting step of the cycle. The enzyme that converts CDP choline to PTC is called phosphocholine cytidylyltransferase. The Kennedy pathway is a way to transfer lipid movement in the cells (Fagone and Jackowski (2012).

Sphingomyelin

Sphingomyelin is important for the function of nerves and membranes (Garrett and Grisham, 2013). A study was done in mice to see if there was a dose-dependence effect of sphingomyelin on the epidermis of mice. Feeding mice sphingomyelin did not affect feed intake or body weight. Feeding sphingomyelin did increase skin hydration and decrease the skins ability

to dry out. There was not a dose effect of sphingomyelin on skin hydration (Haruta-ono et al., 2012).

Phosophocholine

Phosophocholine is formed when choline gets phosphorylated, and this process uses 1 ATP. Choline gets trapped in the cell when it is phosphorylated. This allows cells to convert and use choline (Fagone and Jackowski, 2012). Phosophocholine can also occur when sphingomyelin gets degraded (Jansen et al., 2001).

Acetylcholine

Acetylcholine is a neurotransmitter in animals (Pond et al., 2005). Acetylcholine is not typically supplemented in diets.

High-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) is a way to quantify chemicals. HPLC is commonly used for quality control in the pharmaceutical industry. HPLC can be used to find specific compounds in food as well (deZwart e al., 2003). HPLC methods may be chosen over other analytical ways to analyze compounds because HPLC is relatively inexpensive compared to mass spectroscopy or NMR spectroscopy (deZwart et al., 2003, Hefni et al., 2015).

Choline Analysis

The Association of Official Analytical Chemists (AOAC) approved methods for choline analysis may not be the best analysis for feed ingredients. The AOAC method for choline was designed for infant formula and later expanded to choline and lecithin supplements (Fu et al., 2012, Rader et al., 2004). These compounds are semi-purified and may not have many compounds that would interfere with extraction of choline. Compounds that would impede extraction of choline would be cell walls, that would not allow choline to be removed and therefore not quantified. This

can make extraction of choline from whole foods more difficult (Graham et al., 2009). March and MacMillian, (1980) reported total choline and free choline, showing that choline is in multiple forms in one feedstuff. Free choline does not have natural ultraviolet (UV) or fluorescence so a derivatizing agent must be used to quantify choline using a UV or fluorescence detector method (McEntyre et al., 2009).

Hefnie et al. (2015) stated that most choline in food products is esterified in phospholipids. However, Patterson et al. (2008) and Zeisel et al. (2003) shows this is not the case. Most milk products have more free choline than PTC. Egg and egg products have high levels of PTC and levels of free choline as well. Grains and cereals can also have considerable levels of free choline that could be equal to PTC. Being able to have a choline method that can measure total choline is important.

One way to extract total choline is to heat a sample at 70°C in 1M HCl for 3 hours. The sample is then cooled, and the pH is adjusted to 3.5-4. The sample is diluted to 50mL with water and then filtered. Once the sample is filtered, the sample is ready for analysis which includes phospholipase D to cleave choline from glycerol (Woollard and Indyk, 2000). A method by Fu et al. (2012) had a similar method Woollard and Indyk (2000). However Fu et al. (2012) used an internal standard and pH adjusted to 3.0-3.3 before using phospholipase D. Acid hydrolysis seems to be better suited for samples containing free choline, and while samples containing lecithin samples seem to do better with an alkaline hydrolysis (Rader et al., 2004). This poses a challenge when trying to quantify choline from a complex source, like feed ingredients, which has both free choline and lecithin. The AOAC method is only recommended for infant formula and choline supplements (AOAC, 2016). The AOAC method 2012.20 for determining total choline uses suppressed conductivity. The AOAC method 2012.18 uses ultra-high-pressure liquid

chromatography with mass spectroscopy. These methods for quantifying choline may not be suitable for feed ingredients since they are only approved for infant formula and dietary supplements.

A method by Hefni et al. (2015) extracted choline by homogenizing samples and using the Bligh and Dyer (1959) extraction three times, which included 1:2:0.8 ratio of chloroform: methanol: water. The liquid collected was recorded prior to acid hydrolysis. The acid hydrolysis was 1M hydrochloric acid in 90% acetonitrile (AcN) 10% water (vol./vol.) at 115°C for 30 minutes. Samples were then cooled and neutralized using 10M sodium hydroxide (NaOH). Samples were brought to a final volume of 5mL using water. Samples were then treated with phospholipase D to ensure complete digestion. It was determined that there was no difference when phospholipase D was used, so it was determined that there was complete digestion of the lecithin. Samples were then derivatized according to McEntyre et al. (2009). To summarize, 20 μL samples were then derivatized in 1mL of AcN 60mL of 1M NaOH, about 80mg of magnesium oxide, and 20μL of 1-napthyl isocyanate, the derivatizing agent. Samples were shaken for 15 minutes at 25°C. Once samples were derivatized, 60μL of water was added, and samples were centrifuged and ready for HPLC. The mobile phase was 10mmol/L of tetramethylammonium hydroxide and 20mmol/L of glycolic acid in 15% water and 85% AcN. The flow rate was 1mL/ minute. Fluorescence detection was used to quantify choline at 220nm excitation /350nm emittance. This method was verified using liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance. This method had an accuracy of recovery between 88-110% depending on sample measure, and coefficient of variance of about 8%. This could be a method that could be used to quantify choline without an enzymatic treatment added. This method may miss the free choline in foods. Choline can be in the form of PTC which is esterified; however,

depending on the individual ingredient, there may be more free choline than PTC (Patterson et al., 2008).

Choline quantification and extraction methods have been investigated previously. Menten and Pesti, (1998) determined choline content of feed ingredients using choline kinase. This method used a Goldfisch apparatus. The samples are refluxed for at least 2 hours. Samples were pH adjusted to 6.0-6.5 and brought to 100mL. Samples were enzymatically digested with choline kinase. This enzyme breaks down choline to NADH which is determined by spectrophotometry. The extraction was KOH in methanol. This would extract the free choline and the phosphatidylcholine (PTC) in solution. The heat and basic conditions would be adequate to break down the PTC and liberate choline. Unfortunately, the choline kinase in used in this method is not commercially available anymore.

Betaine analysis

Betaine analysis is limited since betaine is not considered an essential nutrient, so methods that quantify betaine in feedstuffs are limited. Betaine is in hair, and an extraction quantification of betaine in hair has been done for the cosmetic industry (Pulliainen et al., 2009). In recent years, there has been increased interested in betaine for human health, so methods to quantify betaine in foods have been increasing (deZwart et al., 2003, Zesial et al., 2014). Betaine is water soluble which is useful for analysis. Betaine, in animals, is not in multiple forms, unlike choline. Extraction procedures are about making sure betaine is in an aqueous solution, and samples are adequately ground or treated so that the betaine can be released from the cells (deZwaert et al., 2003).

A method by deZwart et al. (2003) uses a food processor and water to homogenize samples. The next step is to shake the samples for five minutes. Samples were then centrifuged, and the aqueous layer was removed, and dichloromethane was added to remove fat. Once betaine was

extracted from the samples, samples were then derivatized and quantified for betaine using HPLC. (Hefni et al., 2016).

A method by Mar et al. (1995) used an HPLC method that was more sensitive than previous methods that converted betaine to betaine periodide. These methods had sensitivities of about 50 nmol of betaine. This is not ideal for betaine quantification in tissues, because betaine is in small quantities in tissues. Samples were extracted in a 2:1 solution of methanol and chloroform. Samples were extracted according to Bligh and Dyer (1959). Samples were then vortexed and left at -20°C for one hour. The supernatant was transferred to a new tube, and the extraction procedure was repeated, and the supernatants were combined. 1:1 solution of water chloroform was added to make two phases. The aqueous layer was then dried, reconstituted to a set volume of 10μL of water and 125μL of methanol. The betaine fraction was collected, dried, and derivatized. Derivatization procedure was dissolving dried betaine fraction in 20μL of methanol, 180 μL of acetonitrile (AcN), and 20μL of a 10% suspension of magnesium oxide in water. After mixing derivation solution (100mM 4'-bromo-phenacyl in AcN) was added. Samples were vortexed for 5 minutes, then centrifugated for 5 minutes. The supernatant was injected into the HPLC. Mobile phase was 80% can, 6.8% ethanol, 2% acetic acid, 3% Ammonium acetate,12.7% water, 1.0% 0.1 M sodium phosphate, vol./vol. This method is effective and had low sensitivity; however, explosive nature of diazomethane, which is needed to make the 4'- bromo-2-diazoacetophenone makes this procedure not practical for feed ingredients laboratory.

A paper by Hefni et al. (2016) used that same extraction as deZwart et al. (2003), that was explained previously. However, the derivatization method for the betaine was different. Samples were devitalized using 2-bromo-2' acetonaphthone. The mobile phase was 94% AcN with 6% water with 15mmol/L triethylamine and 30 mmol/L of succinic acid with a flow rate of 1.2 mL/

minute. Hefni et al. (2016) also had a derivation procedure using 2'-naphthacyl triflate with a mobile phase of 95% AcN and 5% water with 15mmol/L triethylamine and 30mmol/L succinic acid with a flow rate of 1 mL/minutes. Both of these methods were validated using LC-MS and were comparable LC-MS average recoveries were 98.5% while the 2-bromo-2' acetonaphthone derivation average recovery was 101%. The 2'-naphthacyl triflate average recovery was 95%.

(Meta) Analysis of Choline in Foodstuffs

Choline is in a variety of feed and food ingredients (Patterson et al., 2008). The meta-analysis of choline and choline types from Patterson et al. (2008) was done to show if choline and choline types were distributed in certain foodstuffs. Patterson et al. (2008) classified free choline (Fcho), glycerophosphocholine (GPC), phosphocholine (Pcho), phosphatidylcholine (PTC), sphingomyelin (SM), total choline (Tcho), and betaine (bet) of 632 foodstuffs. A large number of foodstuffs used by Patterson et al. (2008) makes it a good dataset to look at the relationship of total choline to choline components of foodstuffs. Feed ingredients were also categorized in various food groups. The categories include dairy and eggs, spices and herbs, baby food, fats and oils, chicken and turkey, soups, sauces and gravies, sausage and luncheon meats, breakfast cereals, fruits and fruit products, pork products, vegetables and vegetable products, nuts and seeds products, beef and beef products, beverages, finfish and shellfish products, legume and legume products, lamb products, baked products, sugars and sweets, cereal grains and pastas, fast foods, mixed dishes, snacks, and ethnic foods. All analysis was done using JMP Pro 13. Free choline, GPC, Pcho, PTC, SM, Tcho, and BET was not normally distributed using a Q-Q plot. Transformations were performed and verified using a Q-Q plot. Ln((X/0.1) +1) was used for Fcho, GPC, Pcho, SM, and BET. Log(x+1) was used for PTC and Tcho. Using normalized data there is no correlation of Tcho to any choline component, including betaine, and correlation coefficients

are very low (Table 2-1). There was a significant relationship of Tcho to choline components using GLM procedure. The model that was used was Normalized Tcho = FCho+ GPC + Pcho + PTC + SM+ residual. However, this could be a confounding effect since Tcho was calculated using the unrounded values of the choline components (Patterson et al., 2008). All the choline components should equal Tcho because that is how total choline was determined in Patterson et al., (2008).

When Fcho was used as the response, free choline is a precursor to other compounds that contain choline, so this was done to reduce confounding effects since the sum of choline components would not equal free choline. Yet there is a possibility that Fcho could be related to other choline components since choline would be a precursor for GPC, Pcho, SM, and PTC. This analysis was done to test the hypothesis that Fcho influenced choline components in human foodstuffs. There was a significant main effect of Fcho to bet when food classification was used as a block ($P<0.001$). Blocking has a significant impact. There was a negative response to Fcho and bet in beef and lamb products ($P<0.0001$). There was a negative effect of sausage and lunch meat on the relationship of Fcho and bet ($P<0.01$). There was a trend for increase betaine with increasing Fcho in pork ($P=0.07$). There was no effect of poultry products on bet and Fcho ($P=0.47$). Breakfast cereals had a positive response to betaine ($P<0.0001$) (Table 2-2).

Overall R^2 was not high among food groups. Lamb products had the highest R^2, of 0.97, of all the food groups. This high R^2 is most likely due to chance and the small sample size of that food group (n=4). Overall, the R^2 was low meaning that the model did not fit the data well, little of the variation was accounted for in the model. This would indicate that betaine is not a good predictor of free choline in foodstuffs. Even though there appears to be a relationship of Fcho to bet, and this relationship would not be well suited for predicting Fcho.

Free choline is significantly associated to many choline components except SM ($P<0.001$, Table 2- 3). However, the relationship of free choline to choline components may be significant, and this relationship does not account for the majority of the variation of the different components, and this is indicated in the R^2 of the relationships (Table 2-3). All of the R^2s' are below 0.12 meaning that only about 12% of the variation can be due to the factor in question. In conclusion, there may be a relationship of free choline and different choline components in foodstuffs, and this relationship is small and may not be the best predictor of choline components.

Digestibility and availability of nutrients

Nutritionists commonly refer to digestibility and availability as similar concepts. Digestibility is both the breakdown and disappearance of nutrients from the gastrointestinal tract (GIT). When nutrients disappear from the GIT, it is thought that nutrients are absorbed and available for the animal to use. For purposes of this review digestibility and availability with both be used as an indicator of absorption. The nomenclature used will be determined by the author of the original paper cited.

Choline

Soybean meal is a good source of choline due to its lecithin content (Aburto et al., 1998). Choline is in SBM as the form of phosphatidylcholine (PTC) (Patterson et al., 2008, Figure 2-3). For choline to be available from PTC, the choline must be cleaved from the glycerol backbone. This can be done by a pancreatic enzyme called phospholipase D. This breakdown is an enzymatic process may not be complete depending on intestinal conditions (Frohman et al., 2009). A study done by Menten et al. (1997) found that the availability of choline in SBM can range from 97-105%, so the authors concluded that choline in SBM is highly available to growing broiler chicks. However, a study by Molitoris and Baker (1976) found that choline availability in chickens for

SBM ranged from 56.6-76.2% in chickens. Zhang and Wilson (1999) found that choline from SBM was 73% available in catfish. Availability of choline in different feed ingredients is highly variable.

Emmert et al. (1996) developed a method to determine the bioavailability of choline from various types of soybean lecithin. Choline deficient diet in chickens was made using soy protein isolate that contains no soybean lecithin. This diet was designed to only give a response to only choline and not methionine or betaine. This diet was compared to a crystalline amino acid diet that contained no soy products. The two choline deficient diets gave similar results for choline deficient and choline bioavailability. It was found that different lecithin products gave similar bioavailabilities and were about 100% bioavailable.

A study by Emmert et al. (1996) was used to determine choline bioavailability of overheated SBM, canola meal, and peanut meal. Overheating protein sources is known to decrease the availability of amino acids. The impact of overheating on other nutrients is not well studied. Traditional SBM had choline that was 90% available while overheated SBM was 95% bioavailable. Canola meal had low choline bioavailability of 24%, while overheated canola meal was 32% bioavailable. Peanut meal had a choline bioavailability of 78% while overheated choline was 82% bioavailable. In all cases, choline was more bioavailable in the overheated protein products. Even though overheating protein meals may affect the bioavailability of amino acids, choline bioavailability appears to be unaffected.

Choline bioavailability is low in canola meal which is supported by March and MacMillian (1980). Choline is very bioavailable in SBM, and this is supported by Emmert et al. (1997) and Menten et al. (1997). Canola and rapeseed meal (RSM) also contain choline in the form of lecithin (Jansen et al., 2015). Rapeseed meal contains antinutritional factors that may affect digestion

(March and MacMillian, 1980). Canola meal and RSM contain high levels of choline (March and MacMillian, 1980, NRC 2012). Due to the antinutritional factors availability of choline in RSM may be lower than other feed ingredients. Low bioavailability of choline in canola meal could be due to a certain choline containing compound called sinapine, which is also a known antinutritional compound in canola meal (Khajali and Slominski, 2012, Mailer et al., 2008). Certain varieties of canola meal can be up to 2% sinapine (Emrani et al., 2015). This would partially explain canola meal's high choline content and low bioavailability. Sinapine appears in the feces, meaning the choline in sinapine was not utilized by the animals (Qiao et al., 2008).

A study was done by March and MacMillen (1980) determined the availability of RSM in broiler chicks. In this study, both free and total choline was analyzed. Choline availability in RSM was compared to SBM. RSM had higher levels of choline than SBM; however, perosis was higher in birds fed RSM. Even when choline was supplemented to the RSM diets perosis was still higher than soybean meal diets. This would indicate that even though RSM has higher levels of choline than SBM, there is something antinutritional that binds the choline and makes it unavailable to the bird. When looking at the intestinal contents SBM had about 85% of the choline in the free form; however, in RSM, only 50% of the choline was in the free form. The reduced availability could be due to the antinutritional factors in RSM, like glycosylates. Another feed ingredient known for its antinutritional factors is cottonseed meal (Lindsey et al., 1980). These antinutritional factors may make choline less available. The availability of choline in cottonseed meal was 55% in catfish (Zhang and Wilson, 1999). There have not been many studies in other types of animals to determine the choline availability in cottonseed or cottonseed meal.

A study was done by Rama-Rao et al. (2001) fed diets with different grain sources, corn, pearl millet, and broken rice with no supplemental choline. All diets had approximately the same

level of total choline. There was no difference in egg properties during the study; however, birds on the corn diet did weigh 200 grams less compared to the other diets. This could indicate that corn may have lower availability of choline compared to other grains. Zhang and Wilson (1990) found that availability of choline in catfish of cornmeal and cooked cornmeal was 69 and 78% respectively. Availability of choline in wheat middlings was low in catfish with a choline availability of only 54%. Different grains have different choline availability, and this could affect animal performance.

Choline availability is not widely reported in animal sourced products. A study done in catfish appears that animal products have high choline availabilities of 90-92% for meat and bone meal and fish meal. These choline availabilities are higher than the plant choline availabilities reported that ranged from 54-78% (Zhang and Wilson, 1999). There may be differences in choline availability depending on if the choline comes from a plant or animal source.

Protein (Source of essential amino acids)

Protein is a very important macronutrient in animal nutrition and has been studied for many decades. How much and what kinds of proteins to feed animals has been a research topic of interest since for at least the past hundred years (Grindley, 1917). Protein is always a topic for animal nutritionists to research, because protein is typically the most expensive component of the diet (NRC, 2012). Being able to accurately determine amino acid digestibility is essential for determining accurate requirements for animals (Adeola et al., 2016). How to formulate diets for animals has changed as knowledge about protein digestibility has increased. Proteins are made up of amino acids (Pesti et al., 2005). Diets are formulated on an amino acid basis. Diets were formulated on a total amino acid basis (Erickson et al., 1980). Over time research demonstrated that amino acids were not completely digested (NRC, 2012). An assay was developed to determine

how much of the amino acid disappeared from the digestive tract. Microbial fermentation could alter the amino acid content of feces, and amino acids are only absorbed in the small intestine, ileal fermentation is the preferred method to determine amino acid digestibility in both swine and chickens (Sauer and Ozimek, 1986).

There are still different ways to determine endogenous losses from the small intestine. Endogenous losses occur in the intestine where protein, or other compounds, are secreted into the GIT and not reabsorbed. These compounds are digestive enzymes and other proteins like mucin (Adeola et al., 2016). There are specific and nonspecific, also called basal endogenous, losses. Basal endogenous losses occur with normal intestinal turnover and are not dependent on the composition of the diet (McDonald et al., 2011, Stein et al., 2007). Specific endogenous losses are dependent on the composition of the diet (Cowieson and Ravindran, 2007). Fiber can increase specific endogenous losses by affecting gut viscosity, and passage time (Parsons e al., 1983). Increasing protein can increase specific endogenous losses due to additional enzymes being secreted into the GIT (Hodgkinson et al., 2000). Phytate can increase specific endogenous losses, including phytase can reduce endogenous losses (Cowieson and Ravindran, 2007).

Endogenous losses can be calculated by collecting the digesta from a nitrogen-free diet, a diet with amino acids that are 100% digested, or by regression analysis. Using a nitrogen-free diet or a highly digestible amino acid diet will correct for AID using endogenous losses that are collected experimentally from an animal (Stein et al., 2007). Calculated endogenous losses by regression use computer models. Using regression typically yields higher standardized digestibility values, than diet specifically fed for endogenous losses. Spindler et al. (2014) recommend using regression for determining endogenous losses. However, Adeola et al. (2016) suggest using a nitrogen-free diet, because of experimental effects like animals and diets.

Apparent ileal digestibility (AID) is defined as "the net disappearance of ingested dietary amino acids from the digestive tract proximal to the distal ileum" (Stein et al., 2007). AID does not account for the endogenous losses of the gut that include enzymes, intestinal cells, mucus, and other GIT secretions (Nyachoti et al., 1997). When accounting for endogenous losses standardized ileal digestibility (SID) can be calculated by removing the basal endogenous losses from the ileal outflow. SID does not account for specific endogenous losses from feeding specific feed ingredients (Stein et al., 2007). These factors that affect specific endogenous losses are fiber content, retention time, and antinutritional factors in the feed ingredient (Le Gall et al., 2005, Cowieson et al., 2009). True ileal digestibility (TID) is the undigested portion of amino acids in the distal ileum. TID can be difficult to determine because specific endogenous losses are not easily measured (Stein et al., 2007).

If TID easily could be determined then, and this may be the best way to determine amino acid digestibility since TID accounts for specific and endogenous losses. A way to determine TID would be to convert lysine to homoarginine. However, in certain feed ingredients, and this can overestimate threonine digestibility give threonine TID values of 104 to 113% in pigs (Eklund et al., 2012). None of the reported SID values was over 100% (Eklund et al., 2012). However, overestimation between amino acids can occur with SID as well (NRC, 2012). This is not ideal since threonine can be limiting an amino acid in most swine diets. Overestimation of SID is common as well in the amino acid proline (NRC, 2012). Overestimation of amino acids is not ideal when formulating diets and can increase pollution in the environment.

AID will increase as amino acids content in the diet increases. This makes AID amino acid concentration dependent. So, AID is not the best measure of digestibility of individual feed ingredients, since amino acid concentration will affect the digestibility coefficient. SID has

become the accepted measure of digestibility since it is assumed not to be dependent on amino acid concentration (Stein et al., 2007, Urbaityte et al.,2009).

Studies by Fan et al. (1995) and Eklund et al. (2005) showed that SID is independent of CP and AA contents. This would mean that SID values are additive Stein et al. (2005) showed just this, SID values of multiple feedstuffs are additive. Some insights into the practical implications may be evident from the re-examination of data from a paper investigating the additivity of values for SID values. Stein et al. (2005) fed diets based on corn, soybean meal, or canola meal, and their blends to pigs and measured SIDs for amino acids. Standard ileal digestibility values of the blends were predicted from the determined values of the ingredients. Based on a series of weak t-tests, Stein et al. (2005) concluded: "with few exceptions, no differences between predicted and measured values for SID were observed". When the measurements were, all taken with the CP near 19%, the additivity was excellent. However, the additivity was not as clear as when corn, whose SID values were determined at approximately 9% CP was blended with the higher protein ingredients. SID may be relatively easy to measure in pigs. When feed ingredients are being fed that have large differences in protein levels, digestibility values may not be as additive as previously thought.

A study by Kim et al. (2009) compared the digestibility of a high protein distillers grain with solubles (HPDGS) to traditional SBM which both had similar crude protein values. The HPDGS had consistently lower SID values for amino acids than the SBM. The ingredients were not corrected for fiber. The HPDGS had five times more neutral detergent fiber (NDF) fraction than the SBM. This could be a reason HPDGS had lower SID since fiber can decrease amino acid digestibility (Stein et al., 2007). When SBM was gradually replaced with HPDGS with solubles, there was no difference in growth parameters.

Tahir and Pesti (2012) showed that there is a persistent correlation between amino acid concentration and SID. This was seen in both a broiler chick and a cockerel assays, which are two different ways to determine SID in chickens. A similar correlation was found in pigs as well (Bloxham et al., 2016). This was a study by Zhai and Adeola (2011) where protein content of the diet affected the SID of the diet. This study showed that SID decreased with protein content. This study agrees with Lagos and Stein (2017) that had the lowest crude protein SID values for SBM that had the highest crude protein content. AID increased with increasing protein content this agrees with Stein et al. (2007). SID is usually calculated from AID. This could affect the accuracy of SID since it's directly measured from AID. This has the potential to confound any problems of AID with SID.

A study by Baker and Stein (2009) investigated the SID of different kinds of soybean meal in pigs. They had high protein, low-oligosaccharide, and conventional soybean meal (SBM). The different kinds of SBM were both hexane extracted and extruded. Overall, the extruded soybean meals had higher SID values than extracted SBM. Low oligosaccharide SBM had higher SID than high protein or conventional SBM, and this could indicate that fiber content has more of an effect on SID than protein level.

A SID study done by Bandean et al. (2011) showed that the there was less variation of digestibility coefficients in multiple samples of field peas, which had a higher digestibility, than flaxseed, which had a lower digestibility. There was less variation of the amino acid analysis of the field peas than in the flaxseed. This could indicate that there is variation within feed ingredients leads to more variation in SID.

A study by Presto et al. (2011) performed SID assays in pigs using organically grown rapeseed, linseed, and hemp seed. SID for crude protein ranged from 81.7 to 85.9% between the

different oil seeds and faba beans. Rapeseed has the lowest digestibility and faba beans having the highest. Lysine SID ranged from 82.0-91.9% with Linseed having the lowest lysine SID and Faba beans being the highest. The 2012 swine NRC had no hemp seed or linseed to compare to. Faba beans in this study were more digestible than the NRC's values with SID crude protein (CP) value 79% compared to 85.9% in Presto et al. (2011). Faba beans had SID lysine 85% and 91.9% for NRC and Presto et al. (2011) respectively. The SID CP in rapeseed meal in Presto et al. (2011) was higher at 81.7% than the different kinds of canola meal in the NRC which ranged from 64-75%, and lysine values ranged from 71-74%, respectively (NRC, 2012). In Presto et al. (2011) the SID lysine value was 87.3%. Amino Dat 4.0 reported the SID lysine value of rapeseed meal to be 74%, which is lower than reported in Presto et al. (2011). The values in the 2012 NRC and Amino Dat 4.0 are non-organic values, and there is no physiological reason that organic feedstuffs would be more digestible than conventionally grown feedstuffs.

There are different ways to perform the standardized digestibility assay in chicken. One way is a broiler chick assay. This is where young broiler chicks are fed a diet for a series of days ad libitum and at the end of the trial typically between 7-21 days the chickens are euthanized, and ileal contents are collected and analyzed (Garcia et al., 2007). Another method is to use cecectomized roosters. This procedure involves using adult birds that have had their ceca surgically removed. This means that microbial fermentation is limited. These birds are then fasted for a set period of time. Fed typically 100 grams of feed ingredient or diet of interest is given to the bird. Excreta is then collected and analyzed. It has been shown that the cecectomized rooster assay has higher digestibility than the chick assay (Tahir and Pesti, 2012, Garcia et al., 2007). There could be many reasons why this occurs. The cecectomized roosters are in a fasted state, and this had the possibility of increasing digestibility. The broiler assay uses younger birds while the

cecectomize rooster assay uses older birds. Digestibility could be a function of age. This makes formulating diets difficult since differences in digestibility coefficient will cause differences in final formulation. This will affect overall performance. In pigs, there is an effect of feed on SID (Goerke et al., 2011). This means that how much food that is consumed has an effect on digestibility. This could partially explain the difference in rooster and chick assays in poultry.

Labile methyl groups

Labile methyl groups have a variety of functions in the body. Labile methyl transfer one carbon groups from one compound to another. This is important for making purines for deoxyribonucleic acid (DNA) (Pesti et al., 2005). One important function of labile transfer is to methylate DNA for epigenetic modification. S-adenosine methionine (SAM) is important for this transfer (Yang et al., 2011). SAM gets its labile methyl group from betaine (Figure 2-4). Labile methyl is considered a futile cycle in the body because regardless of need the cycle still runs and expels energy this may be to help excrete heavy metals from the body (Pesti et al., 2005).

Labile methyl groups have been called "protein stores" (Swick and Benevenga, 1976). This is a misleading term because the body does not store protein. The body can repurpose amino acids during times of protein deficiency. Even though the animal is not ingesting nitrogen, nitrogen will still be excreted. Even Swick and Benevenga (1976) acknowledge this. "Protein storage" is more about protein plasticity and less about a physiological system able to store amino acids for a time of nitrogen depletion.

An issue when studying labile methyl requirement and is that the source of the nutrient does not need to be filled by the same compound (or two). Nutrients that have been linked to labile methyl metabolism are methionine, betaine, choline, serine, threonine, 5-methlytetrahydrofolate, and cobalamin (Figure 2-4). This makes finding a direct effect of one nutrient in particular difficult.

To have a labile methyl deficiency you have to be low, or marginally low, in some or all of those nutrients. Overfeed one and the labile methyl requirement could be met. Labile methyl groups do not have any specific deficiency symptoms of their own, so a deficiency could go unnoticed or attributed to something else other than labile methyl groups (Pesti et al., 2005).

In recent years labile methyl groups or one carbon metabolism has been recognized to be important for epigenetics. Epigenetics are heritable characteristics that are not directly due to genetic information. These modifications are commonly due to methylation of histones, which are designed to organize DNA in the chromosome. Labile methyl cycle is very important for epigenetic modifications because the methylation of homocysteine to S-adenosyl-methionine (SAM) is methyl donor for DNA methylation (Friso et al., 2017). Folate is commonly considered the most important DNA methyl donor of embryonic development (Choi and Friso, 2005). However, in mice, it has been shown that a decrease in choline of the mother, affected methylation and fetal brain development of the mouse pups (Niculescu et al., 2006). Folic acid is associated with neural tube defects. Over the years many nutrients related to labile methyl groups have been related to neural tube defects including choline, betaine, and vitamin B_{12} (Li et al., 2016).

Interrelationships of nutrients

Methionine, choline, and betaine work in the labile methyl group cycle (Pesti et al., 2005, Figure 2-4). If one nutrient is deficient, for example, choline and this could affect the whole pathway. This has the potential to cause deficiencies of other nutrients as well. An excess of one nutrient could keep the pathway going even if the other nutrients are in marginal quantities. This effect will be referred to as ‘sparing’.

Choline may have the ability to spare labile methyl donors. However, literature is mixed. Blair et al. (1986) found no evidence that choline could spare methionine in turkey poults fed corn

SBM diets. Other studies have shown that other nutrients may spare choline. Augspurger et al. (2005) showed that S- methlymethionine had the ability to spare choline in 8 day old chicks. Klose and Almquist (1940) showed that a combination of choline and homocysteine could replace the methionine requirement in leghorn chicks, and this was before the discovery of vitamin B_{12}.

A study performed by Pillai et al. (2006) fed increasing levels of DL-methionine and a single level of hydroxy analog of methionine with either supplemented betaine or choline to starter broilers. When choline was added to the diet, there was an increase in body weight gain with no added methionine. Betaine supplementation did not increase in body weight gain, but there was an increase in feed efficiency with no methionine added. When the diet contained 0.03% DL-methionine, both choline and betaine had increased body weight gains. There was not an effect of body weight gain when 0.11% of DL-methionine was added. When the experiment was repeated, there was an effect of choline and betaine on body weight gain, feed intake, and feed efficiency when no methionine was added to the diet. However, there was no effect with increasing levels of DL-methionine. When 0.10% of a hydroxy analog was added, there was a decrease in body weight gain when choline and betaine were added. There are differences when choline and betaine are added to the diet; however, these results are not consistent even within experimental groups. This is a problem of labile methyl research, with many different factors intertwined finding consistent, repeatable differences are difficult to obtain.

In a study of birds by Baker et al. (1982) studied whether there was an interdependence of choline and methionine diets. This was done in two parts a purified diet and a corn SBM diet. The purified diets were with both increasing methionine and increasing choline. With the purified diets the birds with 0.04% methionine and 912 mg/kg of choline had the best gain of 127 grams by the end of the study. The birds with 0.35% methionine and 912 mg/ kg of choline had the best feed

efficiency with a gain: feed ratio of 0.721. However, this study does not seem to indicate that choline can spare methionine and increase growth. The corn SBM diet supported the same conclusion. It would seem that the substitution should be studied by response surface techniques. Picking points to compare is not always helpful to find an intertwined biological phenomenon.

Methionine not only supplies methionine groups, and methionine also supplies sulfur for metabolic processes in the body (Pond et al., 2005). Not only could there be an interrelationship of methionine and methyl group donors, but there could be a possibility to supply non-methionine sulfur to spare methionine as well, and this would allow methionine to be used for methyl group transfer. A study by Blair et al. (1980) invested whether choline or potassium sulfate could spare methionine and reduce the addition of crystalline methionine. The study concluded that no interactions of methionine, choline, or potassium sulfate in corn SBM diets. However, a study by Harms and Miles (1984) showed in turkey poults choline increased body weight when no methionine was added to the diet. This study also saw no effect of potassium sulfate. It appears that sulfur is not a 'sparing' compound for methionine.

Glycine and serine are amino acids that are a part of the labile methyl cycle (Pesti et al., 2005, Figure 2-4). Glycine and serine could influence the requirement of choline and vice versa. A study by Siegert et al. (2015) showed that gain: feed ratio improved with increasing choline, or glycine. Choline intake had an effect of required glycine intake showing that these nutrient requirements are interrelated. It makes sense that choline and glycine requirement would be interrelated since glycine can be converted to choline (Kettunen et al., 2001, Kempson et al., 2013, Ratriyanto et al., 2009).

A study by Pesti et al. (1980) showed that choline and methionine requirement are interrelated. This would due to labile methyl groups as the nutrient that would be limiting, not the

methionine or choline themselves. Finding how to use these compounds in a consistent practical way is one of the challenges of animal nutrition.

There is also conflicting evidence on supplementing betaine has a methionine sparing effect. Schutte et al. (1997) showed that betaine could not be used to fully replace DL-methionine in commercial diets. A study was done by Garcia-Neto et al. (2000) fed low and high protein diets with and without supplemented betaine and methionine. In diets that were marginally deficient in methyl groups, there was an effect of supplemental betaine on body gain and feed conversion ratio. The effects of supplemental betaine were still less than when methionine was added to the diet. Betaine can spar methionine. In this study, it does appear that betaine can spare some methionine. A study by Rostagno and Pack (1996) found that betaine could not be used to spare methionine; however, the diets used had adequate choline for adequate methyl group supply extra betaine may not have been needed.

5-methyltetrahydrofolate (5-MTHF) a form of folate is another methyl donor in the body (Friso et al., 2017). Feeding different levels of folate with betaine and methionine could affect performance. A study by El-Husseiny et al. (2007) performed a study in broilers that looked at feeding different levels of betaine, folate, and methionine. When methionine was fed at 0.33% of the diet adding 0.75 g/kg of betaine increased body weight and lowered feed conversion. The same effect was seen if 1.00 g/kg of folic acid was added to a diet with 0.33% methionine. When methionine was increased to 0.45% of the diet, there was no effect of adding betaine. Adding 1.00g/kg of folic acid did increase weight gain; however, feed conversion was unaffected. Adding betaine to the diet that had 0.33% methionine increased digestibility of organic matter, crude protein, and crude fat. This increase in digestibility was not seen when methionine in the diet was 0.45%. This shows that betaine and folate could have a sparing effect of methionine when

methionine is marginal in the diet. The effect does not affect growth performance, but it affects digestibility as well. Studies by Ryu et al. (1995 a, b) showed that folic acid requirement in broilers is affected by dietary methionine and choline as well. Requirements for micronutrients like methionine, folate, and choline are interdependent and difficult to determine individually.

There are different forms of folate that are biologically relevant. 5-methlytetrahydrofolate is a methyl donor for the methionine synthase enzyme, which is an alternative way to convert homocysteine to methionine. Methionine synthase is a vitamin B_{12} dependent enzyme. When 5-MTHF donates its methyl group, it becomes tetrahydrofolate or (THF). If there is a vitamin B12 deficiency in methionine synthase will not function. A buildup of 5-MTHF will occur with a decrease in THF. This processed is called the "methyl folate trap" (Barak et al., 1993). A vitamin B_{12} deficiency can cause deficiency like symptoms even when adequate folate is present. This can put extra pressure on BHMT to convert homocysteine to methionine. This could deplete betaine and decrease methionine in the liver.

Arginine is an essential amino acid in birds (NRC, 1994). Arginine is necessary for creatine production. Labile methyl groups are needed to make creatine as well. This means that there is a potential for arginine and labile methyl groups to be interrelated. The presence or absence of arginine or creatine could affect performance. A study by Chamruspollert et al. (2002) did an experiment to show a relationship between labile methyl donors and arginine. Two experiments were done that added increasing levels of arginine with either methionine, betaine, or both. In the first experiment, there was an arginine by methionine interaction for feed conversion. In the second experiment, there was an interaction of methyl source and arginine for feed conversion this shows that methyl groups can affect the arginine requirement. However, this was only seen for feed

conversion and no other growth parameter. Methionine or betaine did not affect muscle creatine. Overall methyl groups seemed to have little influence on arginine requirements in young broilers.

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Table 2-1. Correlation Coefficients of Total choline on choline components

	Tcho	Betaine	FCho	GPC	Pcho	PTC	SM
Tcho	1.000	-0.115	-0.556	-0.269	-0.078	-0.133	0.0719
Betaine	-0.115	1.000	-0.254	-0.168	0.1902	-0.160	-0.166
FCho	-0.556	-0.254	1.000	-0.184	-0.364	-0.081	0.3228
GPC	-0.269	-0.168	-0.184	1.000	-0.064	-0.084	-0.274
Pcho	-0.078	0.1902	-0.364	-0.064	1.000	-0.188	-0.202
PTC	-0.133	-0.160	-0.081	-0.084	0.188	1.000	-0.560
SM	0.0719	-0.166	0.3228	-0.274	-0.202	-0.560	1.000

All correlation values are nonsignificant

Table 2-2. Estimates of slopes of betaine as function of free choline from Patterson et al. (2008)

Food group	Slope	P>F	R^2
Dairy and eggs	1.15	**0.001**	0.35
Spices and herbs	0.23	0.07	0.20
Baby food	0.05	0.82	0.005
Fats and oil	0.59	0.12	0.19
Chicken and Turkey	-0.18	0.47	0.02
Soups, sauces, and gravy	-0.17	0.58	0.08
Sausage and luncheon meats	-1.50	0.009	0.36
Breakfast cereals	0.37	**<0.0001**	0.59
Fruit and fruit products	0.71	**0.02**	0.13
Pork products	0.28	0.07	0.08
Vegetables and vegetable products	-0.09	0.16	0.03
Nuts and seeds	0.20	**0.04**	0.23
Beef and beef products	-2.28	**<0.0001**	0.47
Beverages	0.81	**0.05**	0.26
Finfish and shellfish products	-0.04	0.91	0.0009
Legume and legume products	0.12	0.35	0.04
Lamb products	-2.75	**0.01**	0.97
Baked products	0.17	**0.01**	0.136
Sugar and sweets	0.78	**0.03**	0.23
Cereal grains and pasta	0.16	0.08	0.13
Fast foods	0.03	0.63	0.005
Mixed dishes	0.03	0.82	0.005
Snack	0.14	0.09	0.17
Ethnic Food	0.36	**0.002**	0.23
Main effects	0.16	**<0.0001**	0.07

Bolded P values indicate a significant relationship of free choline to betaine.

Table 2-3. Relationship of Free choline on different choline components

	Slope	P value	R^2
Betaine	0.16	0.0001	0.07
Glycerophosphocholine	0.22	0.0001	0.06
Phosphocholine	0.40	0.0001	0.12
Phosphatidylcholine	0.31	0.0001	0.02
Sphingomyelin	-0.01	0.6698	0.0003

Table 2-4. Pounds grown in 2016 of selected organic and conventional crops

Crops	Pounds grown (organic crops)	Pounds grown (conventional)
Flaxseed	5,589,060	519,360,000
Oats	97,640,512	2,072,640,000
Peas	9,023,200	277,620,000
Millet	9,112,950	627,900,000
Sorghum[1]	35,547,736	8,342,000,000
Soybean	276,142,560	257,765,160,000
Sunflower	8,273,968	2,651,635,000
Corn	1,431,517,024	848,290,128,000
Barley[1]	130,453,008	199,941,000
Canola[2]	305,000	3,086,340,000
Wheat[1,3]	633,098,580	138,523,380,000

[1] Includes both grain and seed

[2] Edible

[3] Includes Durham, Winter, and Spring varieties

Table 2-5. Value of sales (in US Dollars) of organic livestock in 2016

Livestock	Values of sales ($)
Milk cows	57,801,386
Beef Cattle	10,531,380
Layers	2,462,123
Broiler	749,929,661
Turkeys	83,129,395
Pigs	6,891,039

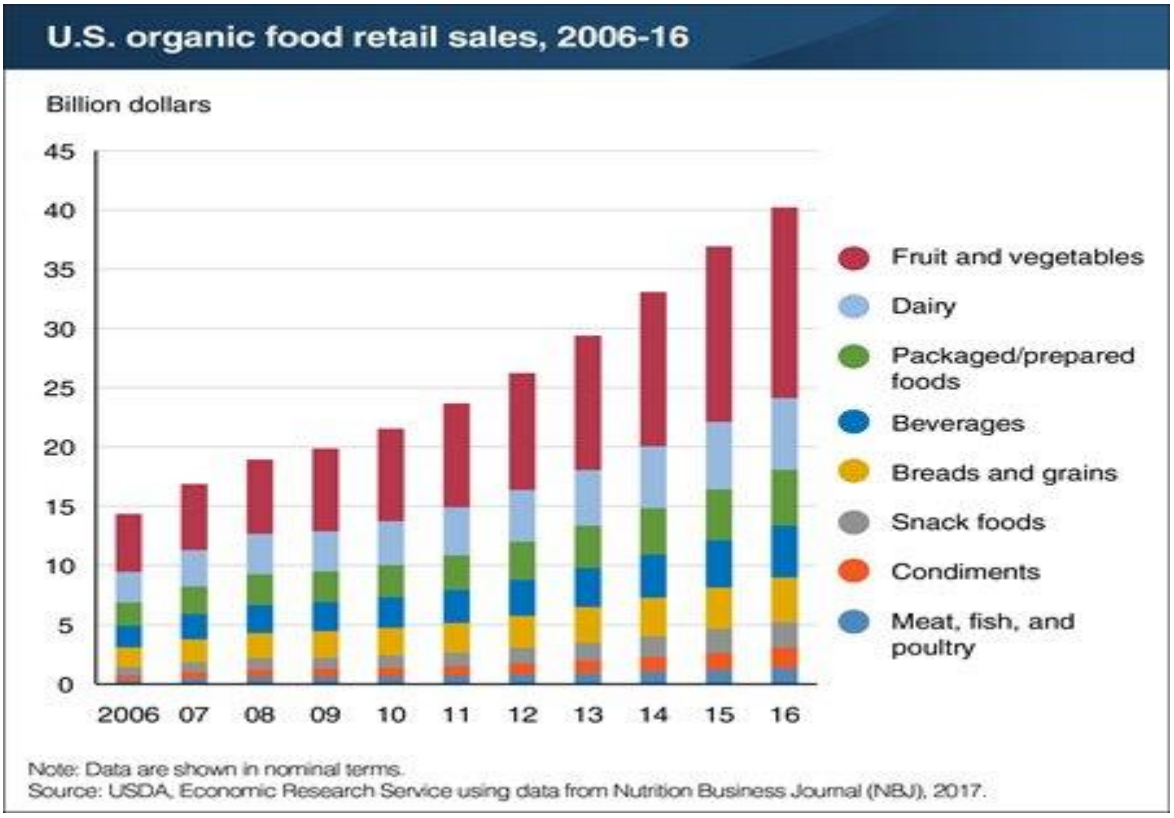


Figure 2-1. Organic food production in the United States

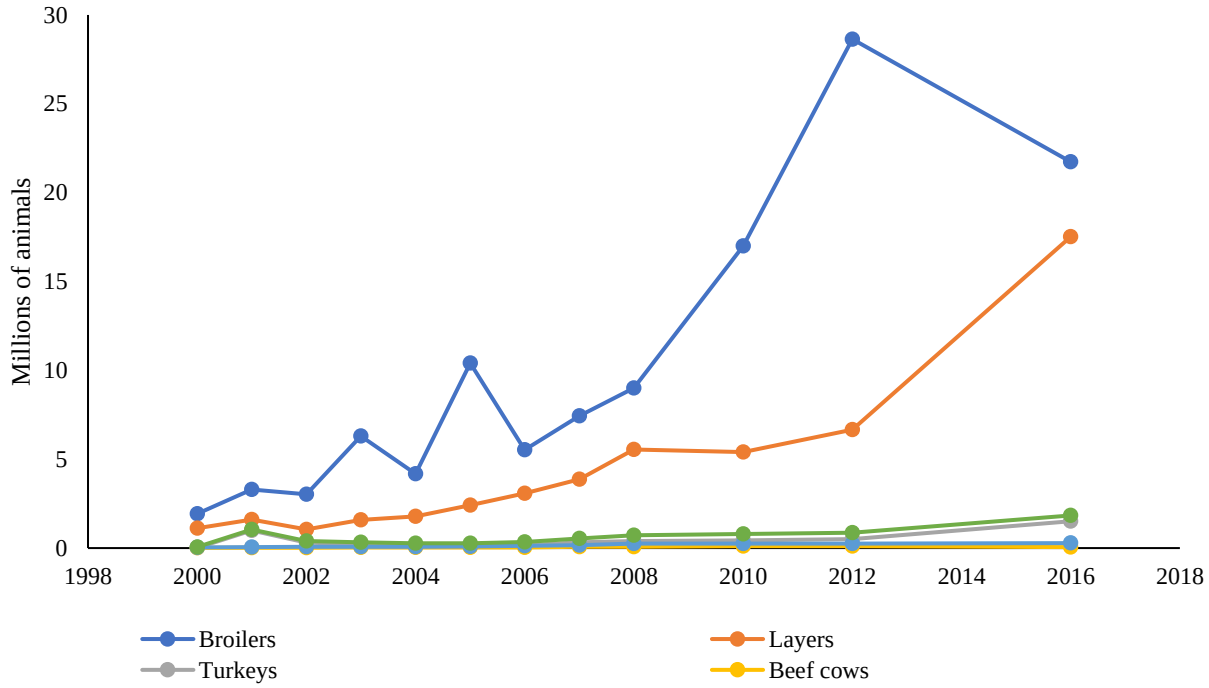


Figure 2-2. Organic livestock in the United States from years 2000-2016

https://www.ers.usda.gov/Data-products/organic-production.aspx

http://usda.mannlib.cornell.edu/usda/current/OrganicProduction/OrganicProduction-09-20-2017_correction.pdf

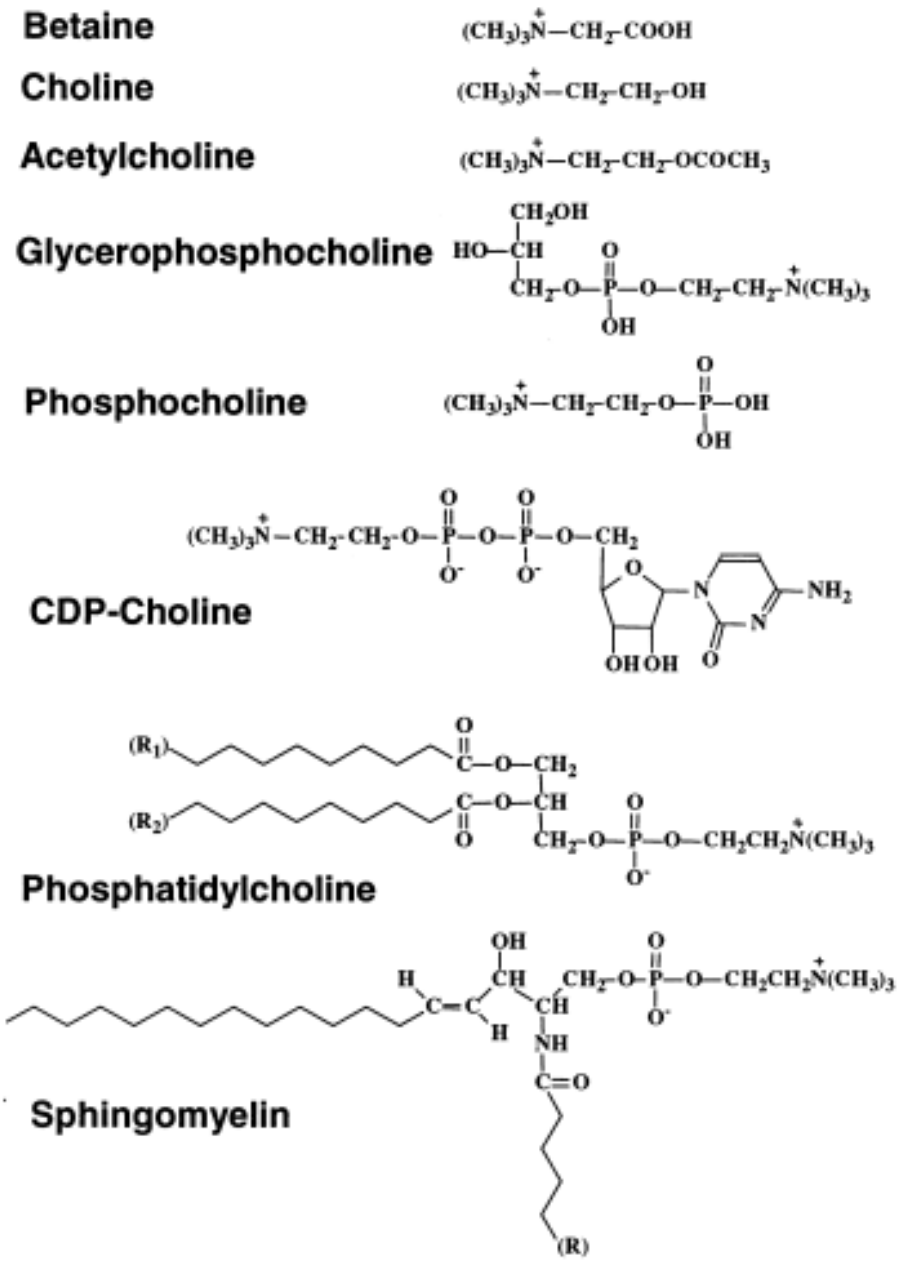
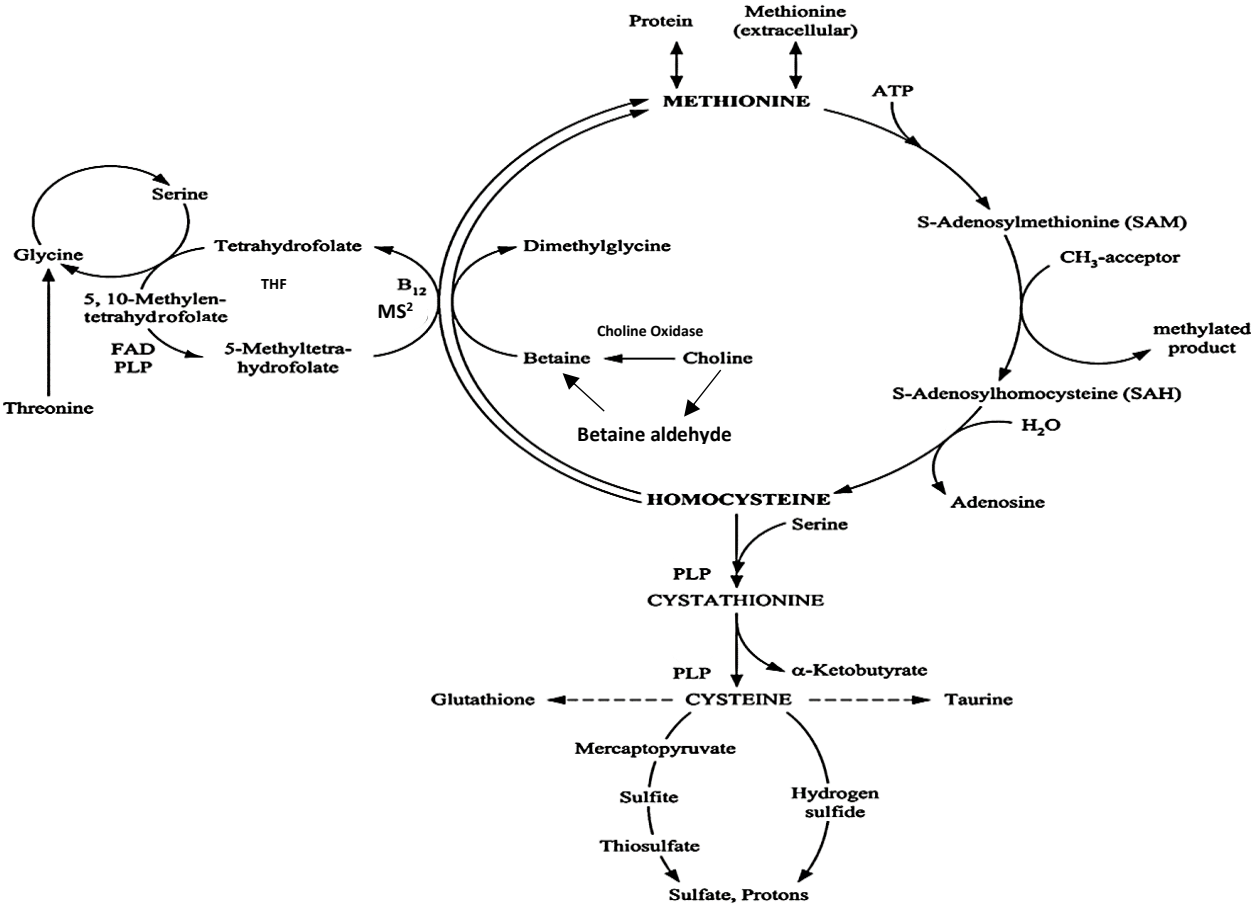


Figure 2-3. Choline and different compounds containing choline Koc et al. (2002).



[1] BHMT Betaine homocysteine methyltransferase

[2] Methionine Synthase

Figure 2-4. Labile methyl transfer pathway

CHAPTER 3

COMPARISON OF DIGESTIBLE AMINO ACIDS DATABASES: RELATIONSHIP BETWEEN STANDARDIZED ILEAL AMINO ACID DIGESTIBILITY AND CONCENTRATION FROM ASSAYS WITH SWINE FROM NATIONAL RESEARCH COUNCIL AND EVONIK INDUSTRIES DATABASES

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Abstract: Databases containing total amino acid (AA) profiles and standardized ileal digestibility (SID) values of 20 feedstuffs commonly fed to pigs from two sources, EI (Evonik Industries, 2010) and the Nutrient Requirements of Swine, 12th edition (NRC, 2012). These databases were used to compare AA concentration effects on standardized ileal digestibility coefficients. Databases were compared with all available AA, and individual AA: lysine and methionine. Data were separated into plant, or animal derived categories. The total AA values were similar for the two databases with an R^2 of 0.997, $P < 0.001$. The linear relationship between digestibility coefficients from the two databases was still significant, an R^2 of 0.658, $P < 0.001$. Both databases had increasing SID values with increasing protein content of the feed ingredient when all AA were included (correlation coefficients (CC) = 0.362 and 0.349 EI and NRC, respectively $P < 0.001$). Total lysine was positively correlated with increasing total lysine and increasing SID lysine (CC = 0.556 and 0.618, EI and NRC, respectively $P < 0.001$). Total methionine also had increasing SID (CC = 0.577 and 0.666 $P < 0.01$) for EI and NRC databases respectively. Differences in AA profiles and standard ileal digestibility profiles would affect diet formulation, cost, and pig performance.

1. Introduction

Standardized ileal digestibility (SID) has become the accepted standard to measure amino acid (AA) digestibility (Stein et al., 2007 and Levesque et al., 2010). In nutrition, apparent ileal AA digestibility increases with dietary AA concentration while true ileal digestibility (TID) and standardized ileal digestibility (SID) are assumed to be independent of AA concentration (Stein et al., 2007a). The digestibility of SID AA in feed ingredients is important to the swine industry, and many peer-reviewed studies were conducted to find the SID of various feed ingredients (Jacela et al., 2010 and Li et al., 2015). The results from these studies are compiled in to databases for nutritionists to use when formulating diets on a digestible basis (Stein et al., 2007a, NRC, 2012).

Swine diets that are formulated on a digestible basis increase nitrogen (N) absorption this decreases pollution and dietary costs (Lee et al., 2017). SID should be an indicator of what the animal can use for maintenance and growth, so having reliable digestibility values for feed ingredients is necessary to formulate cost-effective diets, and maximize growth performance (Stein et al., 2007b). Variations in different SID values from different sources can lead to different formulations, which could change to animal performance and affect diet costs.

There are various factors that can affect digestibility of AA. Heat treatment of certain feed ingredients like soybean meal, field peas, or peanut meal may decrease antinutritional factors (ANF) while improving the digestibility of the feed ingredients overall. However, the digestibility of certain AA like lysine can decrease when feed ingredients are heat treated (Herkelman et al., 1992, Barneveld et al., 1993).

Other ways that have been determined to measure AA digestibility are apparent ileal digestibility (AID) the total disappearance of amino acids from the digestive tract to the ileum. The AID assay does not account for any endogenous AA contributions from the digestive tract. AID is simple to measure since no corrections for endogenous losses are needed. Since AID does not correct for endogenous contributions from the digestive tract, AID underestimates AA digestion (Stein et al., 2007a, NRC, 2012). AID is correlated to lean muscle gain, while total tract digestibility is not correlated to lean muscle growth (Just et al., 1985). AID advantages and disadvantages are beyond the scope of this paper will not be discussed since this topic has been addressed in other articles (Hodgkinson and Moughan, 2000 and Sauer et al., 2000).

True ileal digestibility (TID) reflects total AA disappearance from the digestive tract to the distal ileum. All AA collected during TID are undigested AA from the diet fed since all endogenous losses are corrected for. TID has also been referred to real ileal digestibility. However,

to clarify terms only TID, not real ileal digestibility, will be referred to in this paper. TID corrects for endogenous losses due to specific feed ingredient differences, as well as basal losses from the digestive tract. Specific endogenous losses can change due to feed ingredients being fed. Specific endogenous losses can increase due to fiber or other antinutritional factors (ANF). Specific endogenous losses can increase which increasing neutral detergent fiber (NDF) and acid detergent fiber (ADF). This can increase AA digestibility of proline and glycine disproportional high compared to other AA (Nyachoti et al., 1997). TID not typically used since specific endogenous losses can be difficult to measure and time consuming to measure. Since SID and TID are assumed to be parallel, improving TID collection and analyzation of TID may not give an added benefit (Stein et al., 2007a). The newest edition of the Nutrient Requirements of Swine does not report any TID values (NRC, 2012). However, if SID and TID are not parallel then, then ways to improve TID should be investigated further. The purpose of this paper is not to investigate TID, but to investigate if SID is independent of AA concentration.

The SID technique should account for basal endogenous losses that are not influenced by the type of test material or feed ingredient. The SID assay is not designed to measure such losses (Saucer and Ozimek, 1986). Even though the SID assay is not ideal, this digestibility procedure has become widely accepted for swine research due to time, labor, pig to pig variation, and monetary considerations (Urbaityte et al., 2009 and Stein et al., 2007a). TID and SID are thought to be parallel even though SID is consistently lower than TID (Stein et al., 2007a). The most common ileal fistula procedure for swine has become the T- cannula (Friedman, 1989). Standardized ileal digestibility has been accepted as an accurate measure of digestibility in pigs, assuming that AA digestibility is independent of the concentration of AA in the finished feed (Stein et al., 2005). SID is widely accepted as the easiest, most economical, and a highly repeatable way

to measure AA digestibility this paper will address how SID is affected by the AA concentration in various feed ingredients from two AA databases.

The relationship between AA concentration and SID in databases compiled from poultry databases found excellent agreement for total AA in common feed ingredients (Tahir and Pesti, 2012). The total AA values were similar for the same feed ingredients in different databases. Pesti and Tahir, (2012) found a positive correlation between increasing SID with increasing AA concentration. Similar correlations were found in both a broiler chick assay and a ceacomized rooster assay databases (Tahir and Pesti, 2012). This finding was unexpected since SID is thought to correct for basal endogenous losses, and a feedstuff should have a constant digestibility regardless of AA concentration being fed (Pesti and Tahir 2012, Stein et al., 2007a, and Stein et al., 2007b). This suggests that digestibility is a property of the assay and not the property of the feed ingredient (Sibbald, 1987, Pesti and Tahir, 2012). The objective of this study was to determine if this same relationship as observed in Pesti and Tahir (2012) in seen swine databases as well. The Nutrient Requirements of Swine, 12th edition (NRC, 2012) and Evonik Industries (EI, 2010) databases were used for this comparison because nutritionists use both databases to formulate diets for swine. The NRC database has values that are a compilation of values from various peer-reviewed studies, studies included into the NRC are chosen by an expert committee, and the EI database is based on their internal research and from what is available the from peer-reviewed literature. The nutrient values used in both databases are a compilation of feed ingredients that are from various sources and would be a better compilation of feed ingredients than individual studies since individual research studies can have outliers feed ingredients that would not show a representative example of the feed ingredient. Using database AA values are a better representation of the AA in feed ingredient, than a single study. The study reported here tested the null hypothesis

that there would be no effect of AA concentration on SID. The null hypothesis that there is no difference in total AA or SID values between databases will also be tested.

2. Materials and methods

For the purposes of this study, SID is defined as ileal digesta that corrects for basal endogenous losses (Stein et al., 2007a). Data were compiled from the AMINODat® 4.0 (Evonik Industries, 2010) database and Nutrient Requirements for Swine, 12th edition (NRC, 2012). Twenty common feed ingredients were chosen for this analysis based on corresponding ingredients for both the NRC and EI. Feed ingredients were also chosen on having the most available data in both the NRC and EI (Table 3-1). Amino acid concentration data were analyzed using PROC GLM procedure of and correlations were determined using PROC CORR SAS (9.4) (SAS Institute, Inc., Cary, NC).

Total AA were analyzed as one group. Further comparisons were made as well with common limiting AA, Lys and Met. Feed ingredients were also separated based on animal or plant origins. When sorghum, fish meal, and cottonseed meal appeared to be outliers, separate analyses without sorghum, fish meal, and cottonseed meal tested the influence of these ingredients to measure if these ingredients had a large influence in the results. Main effects remain unaffected by the removal sorghum, fish meal, and cottonseed meal, so it was determined that these ingredients were not outliers and left in the analysis. Because of this other outlier and leverage tests were not determined to be necessary.

Analysis for this study was similar to Pesti and Tahir, 2012. Slopes for AA digestibility = *f* (AA concentration) were found for each AA in each ingredient (Table 3-1). The models were then fitted, with AA digestibility = *f* [AA concentration, database (NRC vs. EI), AA, source (plant

vs. animal), and 2-way interactions]. 3- way interactions were tested for; no 3- way interactions were found and, 3- way interactions were removed from the analysis.

To confirm that the observed phenomena were observed in individual research papers, data were compiled from Almeida et al., 2011 and Stein et al., 2005. These ingredients were analyzed in a similar way with the model AA digestibility = *f* (AA concentration, ingredient, source, interactions).

3. Results

There were positive correlations of AA concentration and SID with correlation coefficients (CC) of 0.362 for EI and 0.349 NRC respectively ($P < 0.001$). SID lysine was positively correlated with total lysine and increasing SID lysine (CC = 0.556 EI and 0.618 NRC ($P < 0.01$)). Total and digestible methionine were positively correlated with CC of 0.577 and 0.666 ($P < 0.01$) for EI and NRC databases respectively.

The total AA values of both databases were only 0.5% different (Figure 3-1, $R^2 = 0.997$). The intercept of the regression line was not statistically different from zero ($P > 0.05$). The slope was also not statistically different from 1. There is no difference in the total AA content of the 20 feed ingredients between the NRC and EI databases. Figure 3-1 shows the relationship between total AA_{NRC} and total AA_{EI}. If both databases had equivalent values, then there should be a slope of 1, and all AA values should be on the line. The is slope is statistically the same as 1 with a slope of 0.997.

The total AA profile of corn was the most similar between NRC and EI databases with values of 0.45% and 0.44% for AA_{NRC} and AA_{EI}, respectively (Table 3-1). Corn gluten meal had

the largest difference in total AA profile between the databases, with an average difference of 0.32. Casein had the highest average AA value for NRC and EI (5.07% and 5.24%, respectively).

Digestibility coefficients (DC) had a slope of 0.78 with all AA included in the line of best fit ($R^2 = 0.658$). This slope is shifted, in part, by a difference in the DC of sorghum between DC_{NRC} and DC_{EI} (Figure 3-2). When sorghum was removed from the analysis the slope was increased to 0.85 and R^2 was increased to 0.82 (Figure 3-2). However, the removal of sorghum did not affect the main effects. The DC_{NRC} values of sorghum are 16% less than and DC_{EI} sorghum values (Table 3-1). Cottonseed meal and fish meal also had larger than average differences between DC_{EI} and DC_{NRC} with a difference in DC of -7.4 and -5.6, respectively (Table 3-1). Again, the removal of these ingredients did not affect the main effects.

The slopes of the SID concentration as a function of AA of all feed ingredients of both databases are all positive. Ten of the 40 slopes are significantly different from zero ($P < 0.05$) and seven of the 40 slopes are trends ($P < 0.10$, Table 3-2). Removing ingredients (sorghum, fish meal, and cottonseed meal) that had high differences in DC between NRC and EI did not affect the significance of main effects of the AA concentration of the ingredient or coefficient interactions (Table 3-3).

As AA content increases, SID increases ($P < 0.05$, Figure 3-3). The NRC has a lower slope (1.33) than EI (1.94), but similar intercepts (79.4 and 81.0 for NRC, respectively). There is a significant difference between plant and animal sources ($P < 0.05$, Figure 3-4). There was an overall effect of crude protein (CP) (data not shown), this was not data was not reported since CP is highly correlated to overall AA concentration. AA concentration is a better indicator of true protein, which is what the animals use for growth (Marriotti et al., 2008).

The Lys and Met were analyzed separately as well, due to the economic importance of these AA. Lys concentration increases, there was an increase in Lys SID. There was also a significant difference between plant and animal products SID ($P < 0.05$) and AA concentration ($P < 0.05$) (Figure 3-5). There was no difference between NRC and EI lysine AA and SID ($P > 0.05$, data not shown). As total methionine increased, SID methionine increased as well. There was a significant difference between plant and animal sources of Met ($P < 0.05$). The relationship between Met AA concentration and SID between NRC and EI databases was similar ($P > 0.05$).

4. Discussion

These data demonstrate the null hypothesis: that there is not a relationship of AA concentration on SID with increasing AA concentration should be rejected ($P < 0.05$). There is a relationship of AA concentration on with increasing SID. The null hypothesis that there is no difference between databases of total AA was not be rejected. However, the null hypothesis that there was no difference between SID database values was rejected.

SID and AA concentration are highly correlated. Correlation is not causation, yet correlation is the first step to identify potential issues that need to be investigated and studied further. The correlation is persistent and cannot yet be corrected for when considering protein source, feed ingredient, or database. A meta-analysis by Zeng et al. (2017) showed that there was a relationship between AA content and SID, this study was limited to various kinds of distillers dried grains (DDGS), and no other kinds of feed ingredients. Zeng et al. (2017) showed that there was a negative relationship between NDF and AA digestibility. A similar meta-analysis by Messad et al. (2017) showed a similar relationship between NDF and AA SID in oilseed meals. This current study showed a consistent relationship between AA concentration and SID in 20 feed ingredients. Only two of the feed ingredients in the current study were DDGS varieties. Four of the feed

ingredients in this study were soy-based products. However, all feed ingredients in this study followed the relationship of increasing SID with increasing AA concentration.

This study shows that the AA SID assay results with swine are similar to those results with poultry (Pesti and Tahir, 2012). Increasing SID with increasing AA concentration is seen in poultry and swine assays. This shows that the phenomena of increasing AA concentration with increasing SID are a consequence of the assay procedure used and not the specie that the assay is performed with. Both poultry and swine assays calculate SID in similar manners (Bandegan et al., 2010, Stein et al., 2007a). Increasing SID with increasing AA concentration may also be a property of the feed ingredient as well (Sauer and Ozimek, 1986).

Figure 3-1 has a slope of 0.997, meaning that the NRC database values are on average were 0.3 percent higher than the EI database values, for the feed ingredients analyzed. However, this difference was small and nonsignificant. The slope in figure 3-1 is statistically equal to 1, which is expected if total AA in NRC and EI databases are equal. This also agrees with Pesti and Tahir, 2012, that total AA is similar among the same feed ingredients in different databases. The relationship of the total AA of both AA_{NRC} and AA_{EI} is extremely high. This would indicate that the method of determining total AA levels is extremely accurate and reproducible (AOAC, 2000). In Figure 3-3, there is more variation in digestibility of AA when total AA content is small than when total AA is large. This is most likely due to small variation in AA quantification having a higher impact on digestibility when total AA in the feed and digesta are small. There is very limited variation in the feed ingredients chosen for the total AA assay. Total AA profiles were not statistically different between 20 feed ingredients chosen the NRC and EI databases. Differences between the NRC and EI databases do not come from the total AA values, but from differences in digestibility coefficients.

Removing sorghum from the statistical analysis decreased the amount of variation in DC. However, main effects remained unchanged; therefore sorghum is not considered an outlier. Large differences in feed ingredient DC are not unexpected. In Pesti and Tahir (2012) feather meal also had a DC difference of 16 percentage points when comparing the two databases of that study. In the current study, there was a 16% difference in the DC of sorghum between DC_{NRC} DC_{EI} databases. In both studies, 16% was the largest difference observed. Apparently, there will be certain feedstuffs that will have large differences in DC when comparing databases, that may just be from variation in the number of samples tested. Certain kinds of feed ingredients could be highly variable, due to a variety of reasons, including processing, regional differences, and cultivar differences. Database source when using digestibility coefficients of different feed ingredients may be considerably different this would affect the formation of the final diet. For example, regionally, sorghum may be fed instead of corn. The 16% difference in digestibility coefficients would cause a difference in diet formulation when using data from the two databases. This would cause a difference in diet cost and performance of pigs. The source of the digestibility data used to formulate the diet would affect the final diet composition and animal performance. For example, in sorghum and soybean meal based diet, the final diet formulation would be different depending on the digestibility values used. Sorghum would be included at a lower rate in the NRC diet formulation with a higher soybean meal inclusion, compared to the EI diet formulation. Sorghum digestibility is lower in NRC database compared to the EI database. The total AA profiles are the same for both databases.

All the slopes of feed ingredients in Table 3-2 are positive. If there was no relationship between AA concentration and SID half of the slopes would be expected to be negative. However, this is not the case, all the slopes are positive, some ingredients have a significant relationship

between AA concentration and SID ($P < 0.05$). The overall relationship is significant ($P < 0.04$, Table 3-3). Other feed ingredients do not have a significant relationship, but all the slopes are numerically positive. The overwhelming incidences of positive coefficients indicate the lack of significance is due to a weak statistical test. This was also seen when an individual journal article was analyzed in a similar manner, and similar results were found (Almeida et al., 2011). Both in Table 3-1 and 3-2 and Figures 3-1 to 3-5 and in Almeida et al. (2011) (Figure 3-8) all show positive slopes of all feed ingredients when comparing AA concentration versus SID contents. This is different than the results of Stein et al. 2007a who reports that AID increases to an asymptote to SID, while SID remains consent regardless of AA concentration (Figure 3-6). The observations of Almeida et al., 2011 are not a result of combing research from various sources, but the result of the assay from which these values are obtained.

It is clear that measured AA digestibilities are dependent on the concentration of the AA in the test ingredients (Table 3-2 and Figure 3-3). Some insights into the practical implications may be evident from the re-examination of data from a paper investigating the additivity of values for SID values. Stein et al. (2005) fed diets based on corn, soybean meal, or canola meal, and their blends to pigs and measured SIDs for amino acids. Standard ileal digestibility values of the blends were predicted from the determined values of the ingredients.

Based on a series of relatively weak t-tests, Stein et al. (2005) concluded "with few exceptions, no differences between predicted and measured values for SID were observed". Regression analysis of the same data (Figure 3-9) shows excellent agreement between predicted and measured SID values when two high protein feeds are blended (soybean and canola meals). The estimate of the slope of $SID_{predicted} = f(SID_{measured})$ is very close to one, and the intercept is clearly very close to zero. When the measurements were all taken feed ingredients with a crude

protein of near 19%, the additivity was excellent. If feed ingredients used have similar AA concentration, digestibility of the final diet is similar to the digestibility values predicted in the feed ingredients. However, the additivity was not as clear as when corn, whose SID values were determined at approximately 9% crude protein was blended with the higher protein ingredients, like soybean meal (Figure 3-9). This could cause differences in the AA digestibility of the final diet.

Almeida et al., 2011, measured SID levels and AA digestibility in several diets. There was no diet by measured SID level interaction, and a common slope was fitted (Figure 3-8). A positive slope of 1.13 confirms the conclusion above that AA level is indeed affecting SID estimates. The $SID_{predicted}$ values are greater than $SID_{measured}$ ones by 13%. However, the negative intercepts offset much of the difference due to the slope so that average values of deviations were less than 2% for the four diets (0.63 to 1.67). When the blends were measured, the variation in SID predicted minus SID measured increased as indicated by lower R^2's. The range of SID values was increased, and the SID of proline exceeded 100% in two of the diets.

Animal product ingredient sources are thought to be more digestible, which is why they are typically used in nursery pig diets (Zier et al., 2004). However, data from this current database comparison does not confirm this. This could be due to the small sample size of animal protein sources since we compared 16 plant feed ingredients but only four animal source ingredients. Processing of animal sources has an effect on digestibility (Williams, 1995). Meat meal, and meat and bone meal were heated and processed in a way that may influence digestibility. Most of the animal source ingredients analyzed could be considered "heavily processed". Casein was an animal source that was highly digestible, and there was no any plant protein that had a comparable protein level or a comparable digestibility.

The reason for the large values for proline is not clear, but similar values are listed for some ingredients in the NRC (2012). The NRC, 48% CP SBM had Pro SID of 113%, Soy protein concentrate had a Pro SID of 102%, and hard wheat had a Pro SID of 105% (NRC, 2012). If one ingredient stimulates specific endogenous losses with more proline for instance, then all the ingredients in a mixed feed could be affected. EI does not report SID coefficients for proline. A possible reason for increased proline digestibility above 100% could be due to pectin or other gel forming compounds present in feed ingredients may increases in the gut when pectin is fed, which bind the bile and not allow for reabsorption (deLange et al., 1989). Bile is high in Pro and Gly (Low, 1982). This could explain why AA like Gly and Pro have SID reported to be over 100% in the NRC (NRC, 2012). Feed ingredients that increase mucin production can increase the amount of proline and glycine in the gut (Nyachoti et al., 1997). Increasing mucin production in certain feed ingredients would increase the specific endogenous contributions, which is not taken into account when calculating SID (Stein et al., 2007b). SID only accounts for basal endogenous losses not due feed ingredients; AA like proline and glycine show basal endogenous losses can overestimate AA digestibility. Proline and glycine could increase due to specific endogenous losses in the diet from changes do to feed ingredients. This is not accounted for in the SID assay.

The persistent correlation of increasing SID with increasing total AA appears more like what is expected from AID than SID (Stein et al., 2007a, Figure 3-7). This is not how an ideal AA SID assay should be, especially since SID should correct for basal endogenous losses. It is accepted that sometimes AA digestibility is greater than 100 % because of specific or differential endogenous losses between ingredients. Neither the effects of specific excretions in mixed diets nor if other ingredients induce similar changes in endogenous losses have been established. The obvious examples, Pro and Gly, have been researched because they have SID reported greater than

100%, but other AA that do not reach that threshold could still have similar problems that go undetected (NRC, 2012 and Nyachoti et al., 1997).

There are different ways to directly measure endogenous losses. Protein-free diets are fed for a period of time, usually between 3 to days, and any AA that are collected are thought to be from the gut and not the diet since there was no protein added to the diet (Gowalock et al., 2016, and Kong and Adeola, 2014). Nitrogen (N)- free diets are not ideal for measuring endogenous losses. This would be a protein deficient state compared to an animal is eating a diet that is an adequate protein (Low et al., 1980). If endogenous losses are found using an N-free diet, then animals are in a negative protein balance and may not secrete the same endogenous losses as a diet that contains protein (Urbaityte et al., 2009, Maughan and Rutherfurd, 2012). Another way to calculate endogenous losses is to feed an enzyme hydrolyzed diet using a highly digestible feed ingredient like casein. When comparing these two assays enzyme hydrolyzed diets have consistently had higher DC than a protein-free diet (Yin et al., 2008). The N-free diet method is the most widely accepted method for determining SID, for swine, despite the well-known shortcomings (Kong and Adeola, 2014). Also, the N-free method overestimates Pro and Gly and underestimates all other AA (Urbaityte et al., 2009). Feeding a diet with highly digestible nitrogen sources, like casein, wheat gluten, and crystalline AA may alter the endogenous losses to better mimic the effect of a typical diet (Jansman et al., 2002, Pedersen and Boisen, 2002). The ingredients in this type of endogenous loss diets may not be as highly digestible as assumed. Therefore, this would overestimate endogenous losses (Nyachoti et al., 1997). Endogenous losses determined by an N-free diet or regression method can underestimate endogenous losses (Sauer and Ozimek, 1986). How endogenous losses are calculated is also not stated by the NRC or EI, and no recommendation is made on which method for determining endogenous losses is preferred.

Further studies are needed to determine the causation for increase SID with increasing AA concentration. Methods need to be developed that more accurately determine endogenous losses, so digestibility can be measured as accurately as possible. This could make the relationship between SID and AA concentration less pronounced.

The ileal sampling or surgical techniques are not stated in the NRC or AMNIODat® 4.0 (NRC, 2012, Evonik Industries, 2010). Standardized ileal digestibility was assumed to be cannulated in a similar way to Cunningham et al. (1962) or Friedmean (1989). The NRC compiles data on based multiple peer-reviewed studies (NRC, 2012). Neither EI or the NRC discloses whether SID is calculated on direct collection method or regression. EI does gather data on feed ingredients themselves and uses peer-reviewed papers as well. Laboratory variation due to slightly different lab conditions and techniques can cause differences in digestibility values as previously mentioned by Williams (1995). It was suggested to not average digestibility values from different laboratories, which is how the NRC values are reported (NRC, 2012).

Future comparisons could incorporate more animal-based sources. Finding more comparable feed ingredients is mostly due to the limited to the small study numbers used in the NRC. For example, yellow dent corn had approximately 127 samples for the total amino acids profile in the NRC, while EI had 918 for the for the same ingredient (NRC, 2012, Evonik Industries, 2010). The sample numbers in the NRC database decreased dramatically when comparing less common feed ingredients. For example, corn gluten meal (CGM), the NRC only lists 6 sample sources for CGM for the total AA profile, while EI lists 582 for the total AA profile. Evonik Industries always had more samples for each feedstuff than the NRC.

Ideally, digestibility assays should measure absorption and utilization. This is commonly referred to as digestibility. Disappearance from the digestive tract is assumed to be absorption from

the digestive tract. Therefore, the nutrients are available for the animal to use (Sibbald 1987). From this study, digestibility appears to a function of the diet and not the feed ingredient fed. Assays should be developed that determine digestibility similar to how the feed ingredient is fed in the diet.

True ileal digestibility is not commonly used to determine digestibility, due to the cost of determining TID. True ileal digestibility does account for specific endogenous losses, even specific losses caused by feeding various feed ingredients (Stein et al., 2007a, Stein et al., 2007b). One way to improve future variation within feed ingredients would be to tailor TID and SID methods to certain types of feed ingredients. However, these changes would be time consuming and costly (Mosenthin et al., 2000).

What this study does not consider are nonnutritive components that might affect AA digestibility. These anti-nutritional components include fiber, mycotoxins, and phytate are known to decrease digestibility of AA (Sibbald, 1987). Phytase supplementation has been shown to increase AA digestibility (Biehl and Baker, 1996). Quantifying how these components affect protein digestibility is difficult, and may have more of an impact on individual feedstuff variation and are less likely to impact feed ingredient comparisons (Sauer and Ozimek, 1986). Fiber is known to decrease protein digestibility by various mechanisms (Friedman, 1989). Zeng et al. (2017) and Messad et al. (2016) have shown that NDF and ADF are negatively correlated to AA digestibility. Feed ingredients with high NDF, ADF, and other antinutritional factors (ANF) may lower AA digestibility due to physically interacting with AA and impeding AA absorption, keeping the AA in the digestive system, unabsorbed. This study did not discuss ANF of feed ingredients since AMINODAT 4.0 only reported AA values and no other nutrient or ANF. Adding in ANF like NDF and ADF to the model for the EI feed ingredients would come from the non EI

sources and this would not reflect the ANF in EI feed ingredients. If the NRC 2012 AA digestibility values are different, then it is reasonable to assume that different components of the feed ingredients would be different as well. When comparing both the NRC and EI database values, it was important to use values both databases compared to not impose criteria on to the data that might lead to confounding, like using NRC NDF values for the EI database.

Metabolic bioavailability or indicator amino acid oxidation (IAAO) could be an alternative to SID; however, the use of radioactive isotopes could be a hindrance to making this method more widely used (Levesque et al., 2010). However, IAAO has been used more to determine AA requirements and less for AA bioavailability (Moehn et al., 2008, Ewing et al., 2001, Myrie et al., 2014). Research studies that determine AA digestibility of feed ingredients using IAAO are limited (Moehn et al., 2005). Further studies are needed using IAAO to determine feed ingredient AA digestibility.

The relationship between SID and AA concentration may be biological; further studies are needed to determine this. If this relationship is biological, then digestibility needs to be determined on a concentration basis not a feed ingredient basis. Digestibility should be determined under normal feeding conditions. When determining SID of specific feed ingredients, the feed ingredients are not fed under practical feeding conditions. Digestibility of nutrients should be assayed under the same conditions in which the feed ingredients are fed. This would ensure that research performed is applicable to the situation it is meant for.

5. Conclusions

Feed ingredients with higher AA content have higher SID AA digestibilities. Amino acid digestibility may change with different inclusion levels of feed ingredients because AA

digestibility is co-dependent with protein level in the diet. Digestibility of AA and amount of AA in a feedstuff are not interchangeable between databases. There is a very strong relationship between predicted and measured SID values when measured ingredient have similar AA concentration.

Taken together, the results presented here and those of Messad et al. (2016) and Zeng et al. (2017) raise serious questions about the additivity of digestible AA values. Messad et al. (2016) demonstrated that the fiber in oilseed meals affects the digestibility of AA in the oilseed meals. Zeng et al. (2017) showed that fiber affects AA digestibility in DDGS as well. Should the fiber in soybean meal, for instance, not then also affect the AA digestibility of AA in the maize in a mixed soy-maize diet? Should not then the fiber and AA concentration in the mixed diet be the critical variables that need to be studied? Further studies, could develop the expected AA digestibilities from constituents of the ingredients and concentration fed in mixed diets.

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Table 3-1

A comparison of the average amino acid (AA) and digestibility coefficients in feedstuffs for swine[1]

	Average AA %			Average digestibility coefficient %		
Ingredient	NRC	Evonik	Difference	NRC	Evonik	Difference
Barley	0.58	0.56	0.02	80.2	81.1	-0.9
Canola meal	1.88	1.79	0.09	76.8	76.2	0.6
Casein	5.07	5.24	-0.17	96.0	98.0	-2.0
Corn	0.45	0.44	0.01	82.0	84.2	-2.2
Corn distillers grain	1.56	1.39	0.17	76.0	76.9	-0.9
Corn gluten meal	3.13	3.45	-0.32	89.0	89.8	-0.8
Cottonseed meal	1.98	1.82	0.16	73.4	80.8	-7.4
Field peas	1.21	1.15	0.06	79.9	78.3	1.6
Fish meal	3.25	3.10	0.15	83.1	88.7	-5.6
Full fat soybeans	1.92	2.05	-0.12	79.9	82.3	-2.4
Hard wheat	0.74	0.82	-0.07	87.7	89.1	-1.4
Lupins	1.79	1.93	-0.15	84.4	87.3	-2.9
Meat and bone meal	2.68	2.59	0.08	74.6	77.2	-2.6
Meat meal	2.88	2.86	0.02	77.9	80.7	-2.8
Sorghum	0.51	0.52	-0.01	77.7	93.8	-16.1
Soy protein concentrate	3.67	3.40	0.27	90.5	88.6	1.9
Soybean meal (44%)	2.44	2.46	-0.02	87.3	88.6	-1.3
Soybean meal (48%)	2.64	2.65	-0.01	89.1	88.6	0.5
Sunflower meal	1.64	1.47	0.17	83.9	83.1	0.8
Wheat distillers grain	1.87	1.66	0.21	74.4	77.1	-2.7
Average	2.17	2.06	0.11	82.19	84.52	-2.33

[1]The original data were compiled from AMINODat®.4.0 (Evonik Industries (EI), 2010) and The Nutrient Requirements of Swine 2012 edition (NRC).

Table 3-2

Estimates of the slopes of digestible amino acids as functions of the concertation of amino acid in feed ingredients common to the of two similar data sets[1]

	EI		NRC	
Ingredient	Slope	P>F	Slope	P>F
Barley	5.73	0.22	3.80	0.03
Canola meal	1.78	0.49	1.53	0.15
Casein	0.36	0.23	0.39	0.07
Corn	11.47	0.07	5.25	0.08
Corn distillers grain	3.37	0.16	1.84	0.16
Corn gluten meal	0.32	0.78	0.87	0.03
Cottonseed meal	2.68	0.05	2.30	0.01
Field peas	7.88	0.01	3.70	0.04
Fish meal	2.63	0.03	0.58	0.48
Full fat soybeans	0.10	0.96	0.62	0.55
Hard wheat	2.74	0.39	2.50	0.09
Lupins	2.42	0.01	1.22	0.04
Meat and bone meal	4.87	0.06	1.67	0.07
Meat meal	1.45	0.20	0.81	0.25
Sorghum	1.68	0.63	5.25	0.04
Soy protein concentrate	0.69	0.24	0.31	0.46
Soybean meal (44%)	0.97	0.25	0.20	0.70
Soybean meal (48%)	1.38	0.10	0.14	0.87
Sunflower meal	3.32	0.17	0.90	0.31
Wheat distillers grain	3.85	0.48	1.87	0.09
Average	2.98		1.79	

[1]The original data were compiled from AMINODat®.4.0 (Evonik Industries (EI), 2010) and The Nutrient Requirements of Swine 2012 edition (NRC).

Table 3-3

Analysis of variance results comparing the digestibility of amino acid (AA) in 20 common ingredients between the National Requirements of Swine and Evonik Industries databases[1]

	All AA			-Sorghum		-Fish Meal		-Cottonseed Meal	
Source	df	Type III Sum of Squares	P>F	df	P>F	df		df	
Database[2]	1	215.5	<0.0001	1	<0.0001	1	<0.0001	1	<0.0001
Concentration[3]	1	0.4	0.72	1	0.45	1	0.46	1	0.5536
Ingredient	19	59.9	0.39	18	0.91	17	0.90	16	0.91
Type of AA	9	17.5	0.74	9	0.71	9	0.68	9	0.73
Type of AA*Ingredient	171	2032.8	<0.0001	162	<0.0001	153	<0.0001	144	<0.0001
Database*Ingredient	19	1061.8	<0.0001	18	<0.0001	17	<0.0001	16	0.004
Concentration*Database	1	12.7	0.04	1	0.04	1	0.05	1	0.04
Error	399			379		359		339	

[1]The original data were compiled from AMINODat®.4.0 (Evonik Industries (EI), 2010) and The Nutrient Requirements of Swine 2012 edition (NRC).

[2] Databases used are NRC or EI

[3] Concentration is the individual amino acid (AA) level

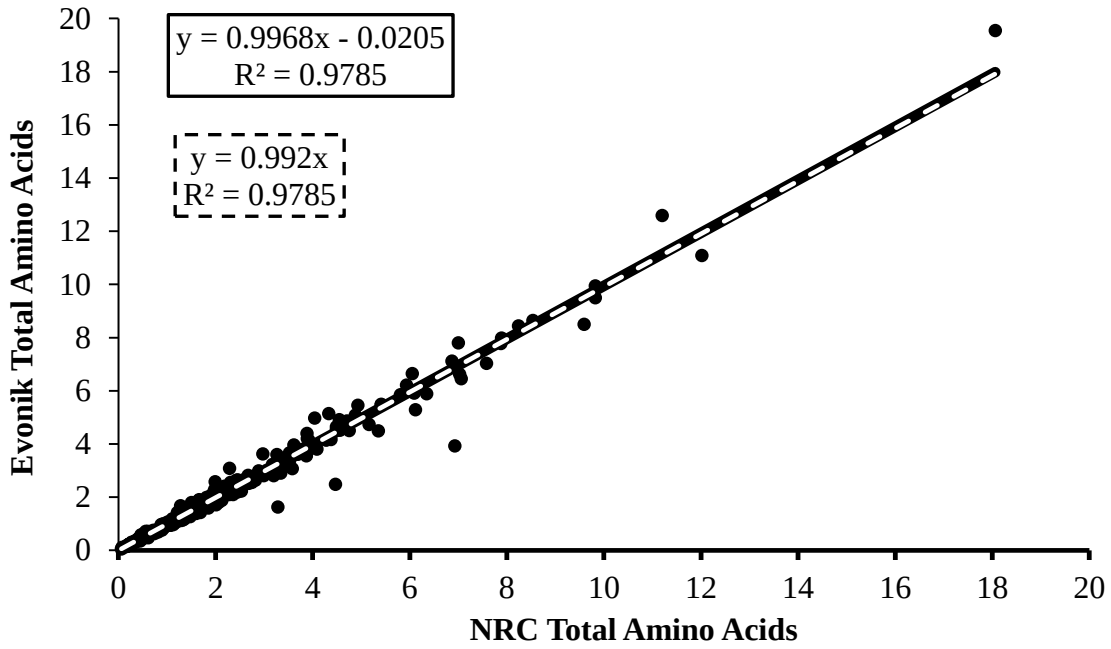

Figure 3-1. Fit ratio between total (essential and nonessential) amino acids (% of ingredient) in (NRC, 2012, Evonik Industries, 2010).

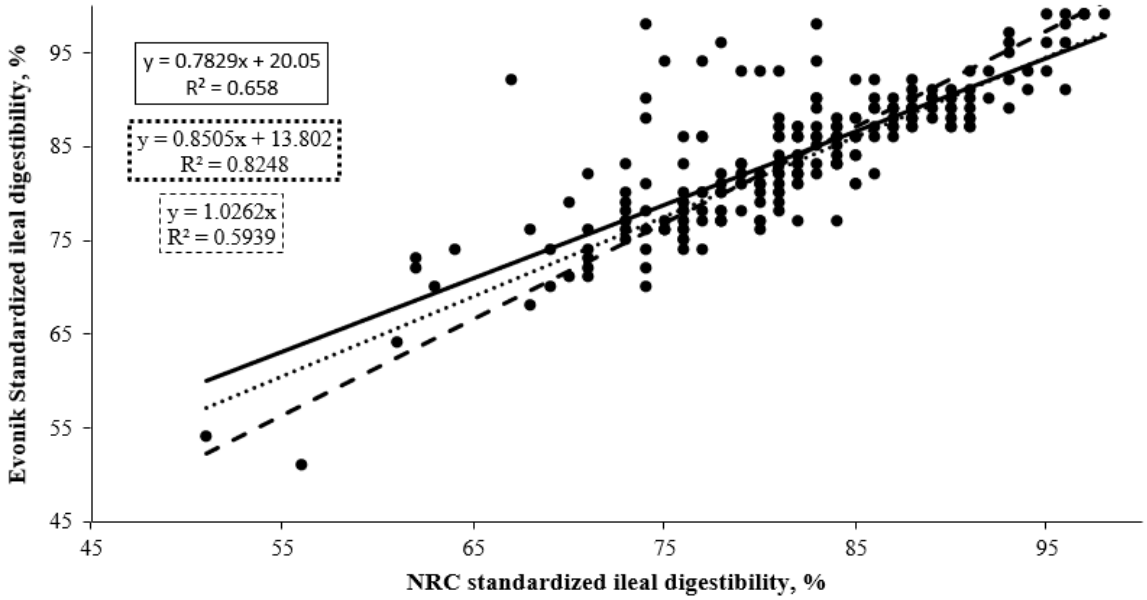

Figure 3-2. The relationship between total (digestibility coefficients) amino acids as % of ingredient of 2 databases with 20 feed ingredients in swine Both assay used ileal cannulas. (NRC, 2012, Evonik Industries, 2010). Solid line is all data. Dotted line is all data except sorghum. Dashed lined is all data with an intercept of zero.

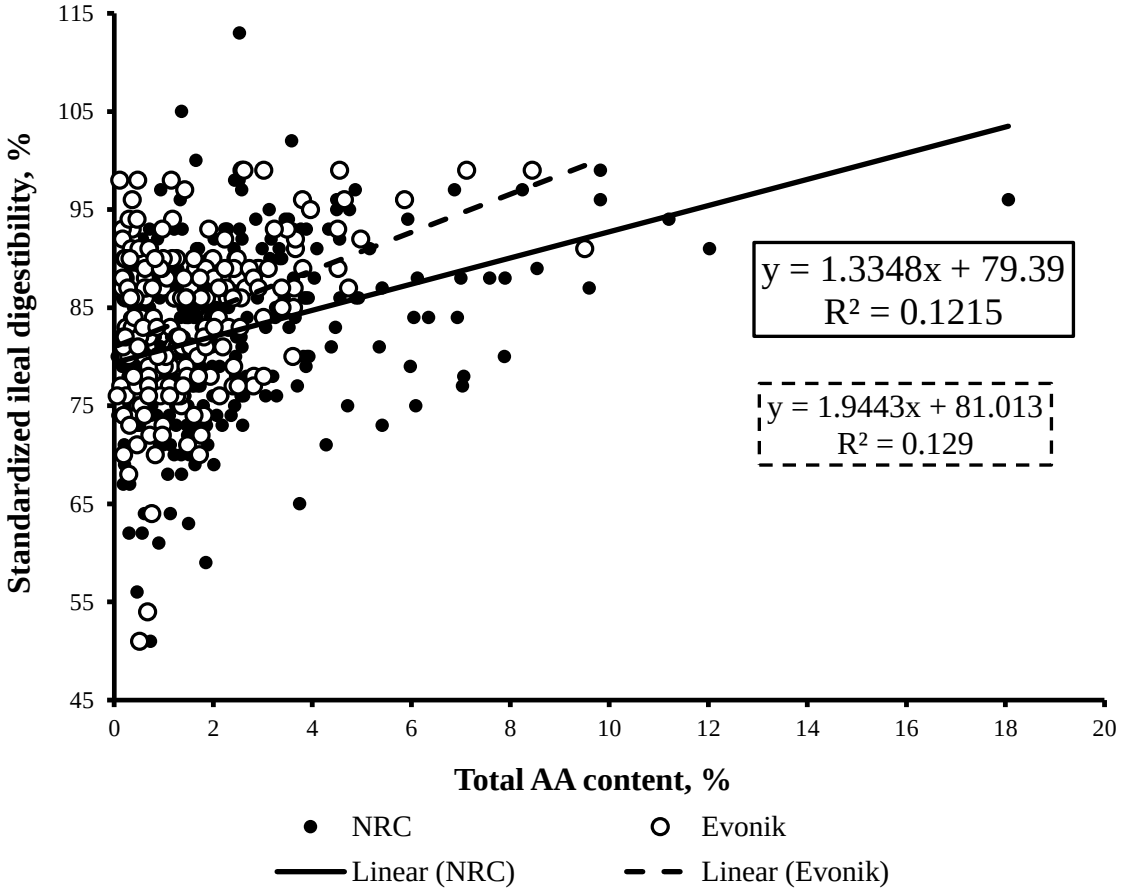

Figure 3-3. Comparison amino acid content versus standardized ileal digestibility (SID) for the NRC and Evonik. The relationship between total amino acids (% of ingredient) and SID in two databases with common feed ingredient (NRC, 2012, Evonik Industries, 2010).

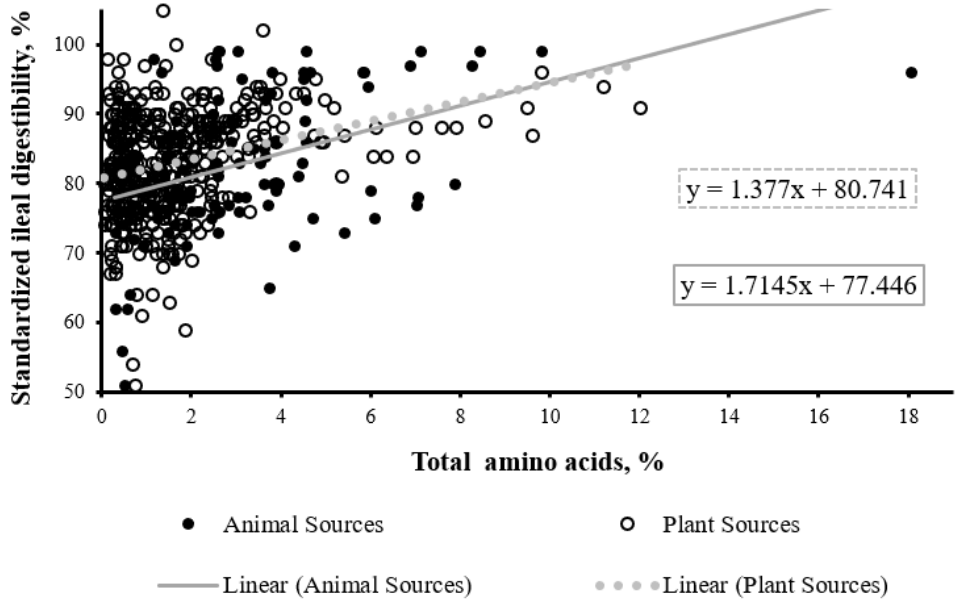

Figure 3-4. Comparison of plant and animal sources. The relationship between total amino acids (% of ingredient) and standardized ileal digestibility (SID) in two databases with common feed ingredient (NRC, 2012, Evonik Industries, 2010).

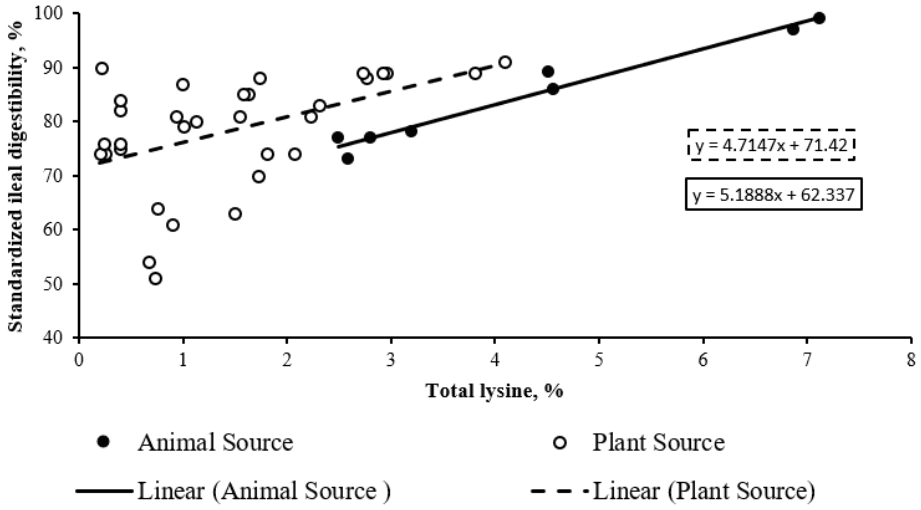

Figure 3-5. Comparison of lysine between plant and animal sources. The relationship between total amino acids (% of ingredient) and standardized ileal digestibility (SID) in two databases with common feed ingredient (NRC, 2012, Evonik Industries, 2010) ($P<0.01$ for both slopes).

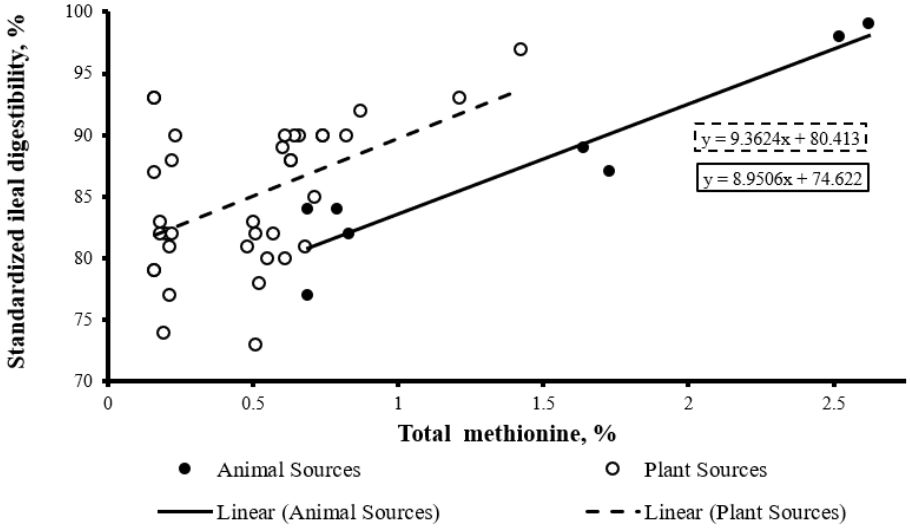

Figure 3-6. The relationship between total methionine (% of ingredient) and standardized ileal digestibility (SID) of methionine in two databases with common feed ingredient. Comparing the differences in plant and animal feedstuff sources (NRC, 2012, Evonik Industries, 2010) ($P < 0.05$ for both slopes).

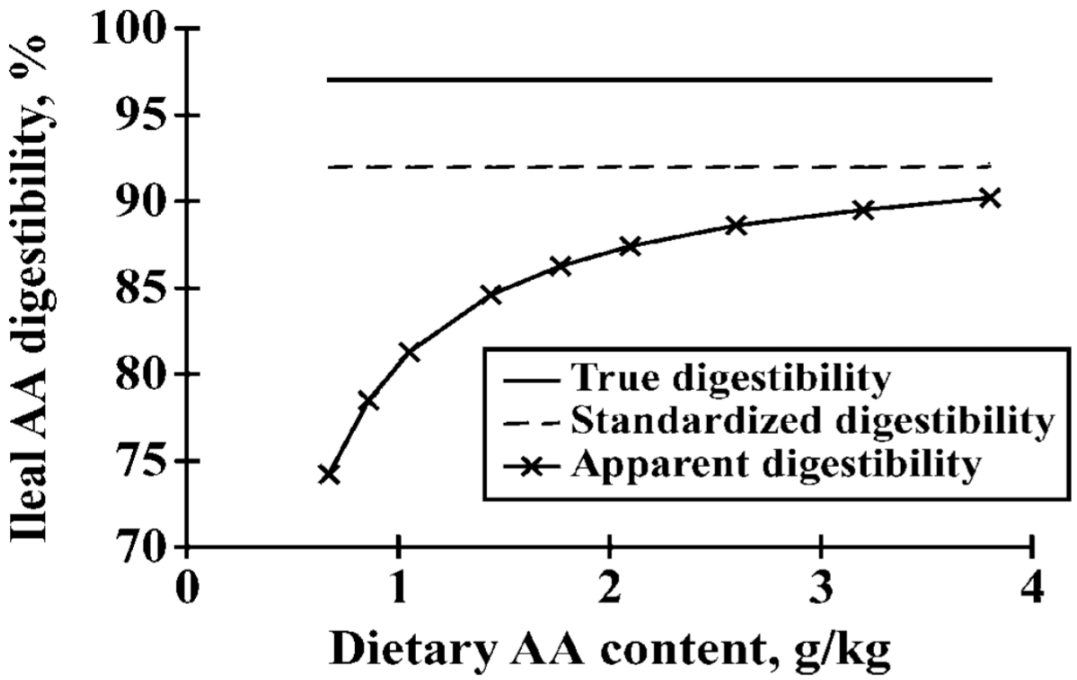

Figure 3-7. Chart from Stein et al. 2005 to show relationship of true, standardized, apparent digestibility

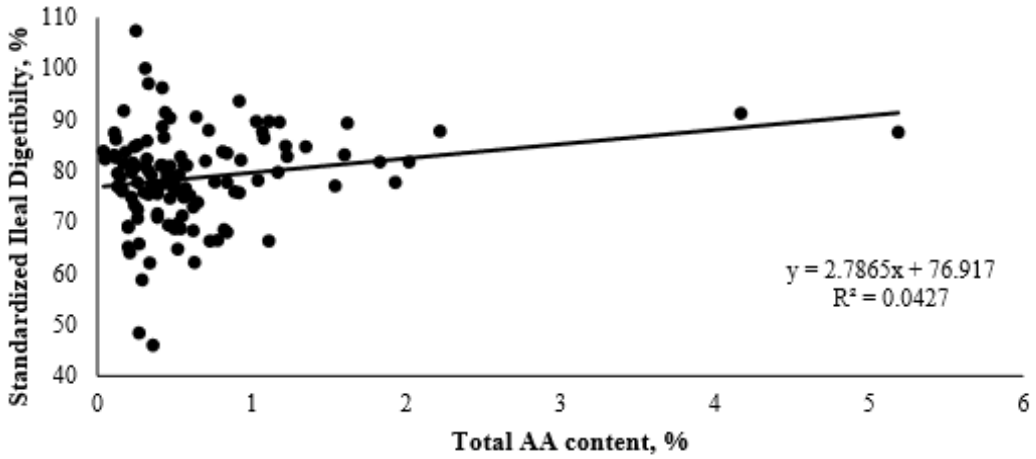

Figure 3-8. Comparison of amino acid content versus standardized ileal digestibility (SID) for Almedia et al., 2011. The relationship between total amino acids (% of ingredient) and SID in journal paper with seven feed ingredients.

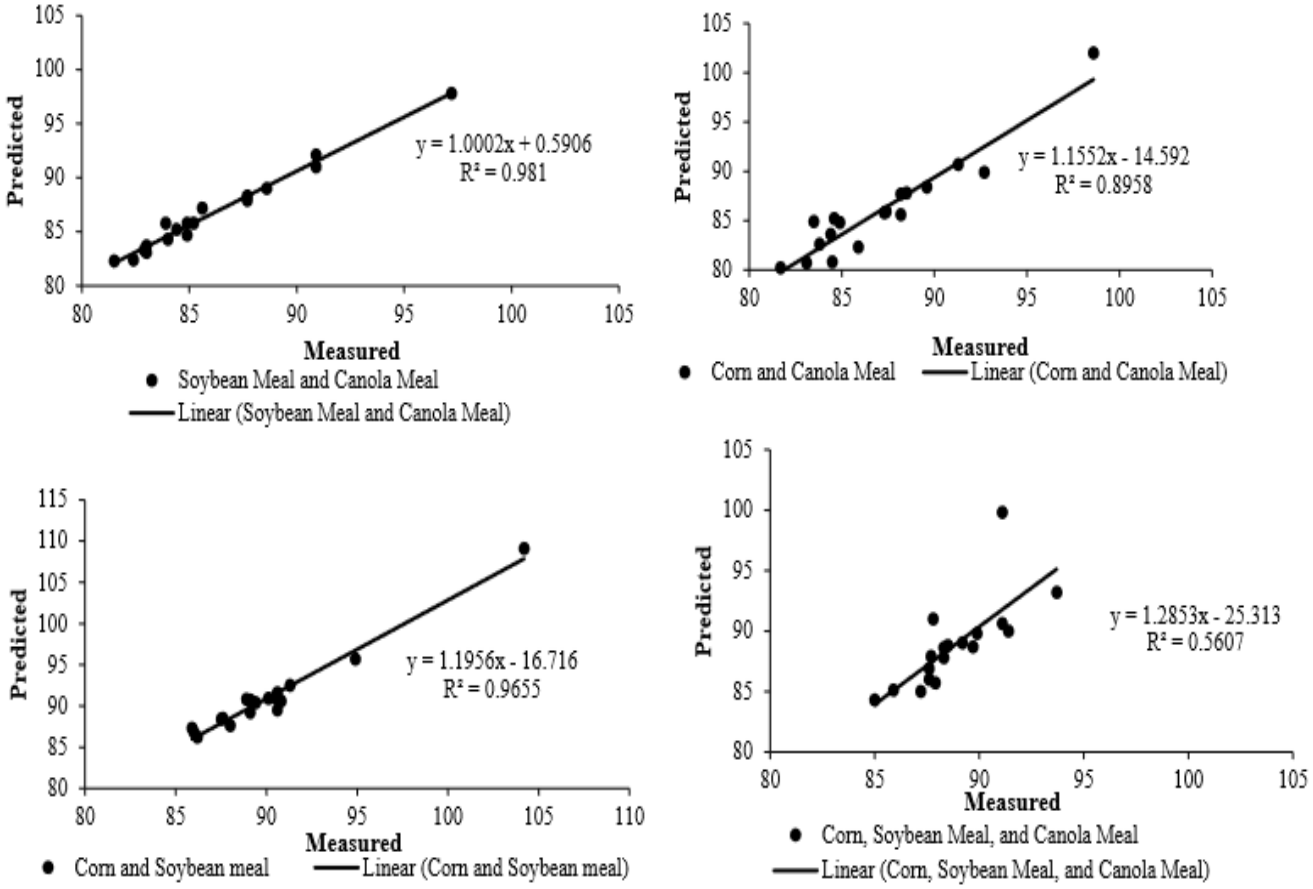

Figure 3-9. Comparison of amino acid content versus standardized ileal digestibility (SID) for Stein et al., 2005. The relationship between total amino acids (% of ingredient) and SID in journal paper with mixed feed ingredients

CHAPTER 4

EXTRACTION AND QUANTIFICATION OF CHOLINE IN LIVESTOCK FEEDSTUFFS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

BACKGROUND: Choline assays that have be developed may not be suited for feed ingredients. Choline is a quasi like vitamin that is increasing in interest in organic livestock production. Choline may have the ability to spare methionine, where methionine supplementation is limited.

RESULTS: This method can be used to be applied different kinds of feed ingredients. To determine total choline in feed ingredients.

CONCLUSION: This choline extraction method can be applied to many different kinds of feedstuffs.

INTRODUCTION

Choline has many functions in the body of animals. Choline is vital for cell membrane integrity, nerve function, and methyl transfer (Garrett and Grisham, 2013, Pillai et al., 2006) Choline is a nutrient that has been of increasing interest in organic livestock production since choline has the potential to spare methionine[2]. Methionine is a limiting nutrient in organic livestock production (Atwal et al., 1980)

Choline can be in many forms in plants including free choline and bound choline (Cabezas et al., 2016). Bound choline is chemically bonded to another compound. A bound form of choline that is common in plants is phosphatidylcholine (PTC) which is choline attached to a glycerol backbone, with two fatty acids bonded to the glycerol. Free choline is water soluble (Patterson et al., 2008. While PTC is amphiphilic, common feedstuffs in livestock, like soybean meal and canola meal, contain PTC, which contain two non-polar fatty acid tails (Cabezas et al., 2016, Patterson et al., 2008). Phosphatidylcholine is also referred to as lecithin (Cabezas et al., 2016). Other forms of choline commonly found in plants include free choline and phosphocholine (Toyosawa et al., 1966).

Most methods for extracting choline were not designed for feed ingredients (AOAC, 2016). Most choline assays are designed for infant formula and dietary supplements (AOAC, 2016, Fu et al., 2012, Rader et al., 2004). Extraction of choline, in plants, can be difficult since choline is in multiple forms of choline in various feed ingredients. For total choline to be quantified, fat-soluble forms of choline must be liberated to be free choline. Phosphatidylcholine can be hydrolyzed to free choline in both acidic and basic solutions (Rader et al., 2004). There are also plant matrixes like cell walls and other compounds that can impede the extraction of choline that would not be a problem in infant formula or dietary supplements (Patterson et al., 2008).

There is a colorimetric method designed for quantifying total choline for feed ingredients (AACC, 2004). However, this method was determined that it underestimated choline content. This was modified with a nitric acid digestion (AACC, 2004). Limit of detection for this method according to the American Association of Cereal Chemists (AACC) is low with a detection limit of 2mg kg^{-1} (Atwal et al., 1980, AACC, 2004). This method appears to be adequate for choline quantification, in feed ingredients. For complete digestion of choline to occur there needs to be a strongly acidic or basic environment (AOAC, 2016 Fu et al., 2012).

A method for total choline quantification for feed ingredients requires PTC to be hydrolyzed, to liberate free choline, while not degrading the free choline present in feed ingredients. Previous studies do indicate that there is free choline present in some feed ingredients (March and MacMillian 1980). There are many kinds of choline in food ingredients, for people, so it is reasonable to assume that there is free choline in livestock feed ingredients as well (Atwal et al., 1980, March and MacMilliam 1980). There are few studies that publish values of total choline in livestock feed ingredients. Values that are published for livestock do not say the method used to quantify choline (NRC 1994, NRC 2012). Values of total plant components including

choline may be dated. Cultivars and varieties of plants used may change over time (March and Mac Millian, 1980, NRC 1994, NRC 2012, Bratestam et al., 2007). This would make reported values of choline different than current values of choline in feed ingredients. To the authors' knowledge, there is no publish choline extraction method that uses a two-part, strong acid followed by a strong base, extraction method.

Choline does not have natural fluorescence absorption, and the ultraviolet (UV) absorption of choline is near the UV cut-off of many common high-performance liquid chromatography (HPLC) solvents, which means that quantifying choline without a derivatizing compound would not be ideal. An effective HPLC method would be more cost-effective than a mass spectroscopy/ liquid chromatography procedure. High performance liquid chromatography can is a more economical option for quantifying compounds compared to mass spectroscopy/ liquid chromatography. HPLC methods would quantify total choline, which is standard for livestock reference books (NRC 1994, NRC 2012).

MATERIALS AND METHODS

Reagents and equipment

The following reagents were used and purchased from Sigma-Aldrich (St. Louis, MO, USA): High Performance Liquid Chromatography (HPLC) grade acetonitrile >99.9%, L-α-Phosphatidylcholine, which was purified from egg yolk, and phospholipase D.

The following reagents were bought from Acros Organics (Geel, BE): choline bitartrate 97%), 1-naphthyl isocyanate 99%, magnesium oxide 98%, and glycolic acid 99%.

The following reagents were used and purchased from Alfa Aesar (Reston, VA, USA) tetramethylammonium hydroxide 98%.

The following reagents were supplied by Fisher Scientific (Phillipsburg, NJ, USA): HPLC grade water >99.9, Sodium hydroxide pellets 99.5-100.5%, hydrochloric acid 36.5-38.0%. HPLC was an Agilent 1260 Infinity model with a fluorescent detector (Agilent Technologies) and autosampler (Agilent Technologies). The pump was a single pump The column was an Agilent bio 5 μm strong cation exchange column 250 mm × 4.6mm (Agilent Technology, Santa Clara, CA, USA) in a column compartment that was set to a constant temperature of 40°C. Software used ChemStation for LC Systems (G2170BA).

The mobile phase contained 10 mM tetramethylammonium hydroxide and 20mM of glycolic acid in 15% water and 85% acetonitrile. The flow rate was 0.5 mL min^{-1}. Excitation of 220nm and 350 nm emission was used.[5] Choline peak was seen at 5.25 min (Figure 4-1).

Feedstuffs

Feed ingredients included canola meal, soybean meal, corn, alfalfa meal, flaxseed, and oats. These feedstuffs were chosen because these feedstuffs represent a wide variety of plant matrixes. This includes different levels of fat, protein, and fiber. These feedstuffs were sourced from three organic distributors. The flaxseed, oats, canola meal, and an alfalfa meal came from Organics Unlimited Inc (Atglen, PA, USA). A soybean meal and an alfalfa meal came from Herbrucks Poultry Farm (Saranac, MI, USA). A second soybean meal was sourced from Cargill Feed and Nutrition (Minneapolis, MN, USA).

Extraction of choline

Choline extraction entailed a two-part digestion. All samples were ground through a 1mm sieve (Wiley-mill, Thomas Scientific, Swedesboro, NJ, USA). All samples were run as triplicates. The analyte was extracted from 0.1g test portion with 3mL of 1M sodium hydroxide at 115°C±5 in a capped test tube for 30 minutes (min) using a hot plant and pierced aluminum heating block.

Samples were cooled slightly and 6mL of 1M hydrochloric acid at 115°C±5 in a capped test tube 14mL for an additional 30 min with a hot plate and pierced aluminum heating block.

Samples were cooled and neutralized to a pH of 7±0.5 with NaOH and HCl solutions, as required. Samples of the same ingredient were all brought to the same volume using HPLC grade water. This step is not necessary; however, this will make calculating the final concentration of choline simpler. This was typically between 10-10.5 mL. Samples were filtered through a 0.22 μm nylon syringe filter (Celltreat Pepperell, MA, USA). Filtered samples were frozen at -20°C until analysis, which was within two weeks.

Phospholipase D treatment

Phospholipase D was according to Fu *et al.*[9] Briefly as follows samples were enzymatically digested by adding 400μL of a test sample to a 1.5mL microcentrifuge tube. Then 100μL of the enzyme solution was added (approximately 600units/mL in a pH 8 Tris/HCl buffered solution) and heated at 37°C for 30 min in a rotating water bath at 100 rpm. Samples are either derivatized immediately or stored in the-20°C freezer until derivatization on the next day. After completing the enzymatic digestion (Table 4-1).

Derivatization

Derivatization processed was started by thawing samples to room temperature. All samples were kept away from fluorescent lighting during the procedure. This method was developed by McEntyre *et al.* [16] In summary, in a 1.5 mL microcentrifuge tube 20μL sample was added to 1mL of acetonitrile, 60 μL of 1M sodium hydroxide, and approximately 80mg of magnesium oxide. Magnesium oxide was added first followed by the acetonitrile 1M NaOH and sample. Magnesium oxide was added to remove any water present. Finally, 20 μL of 1-naphthyl-isocyanate was added. Samples were vortexed between each step. Samples were shaken 100 rpm at 25°C in a water bath

(Lab Line Instruments, Model R3545, USA) for 15min. 60μL of HPLC grade water was added to sample after samples were removed from the water bath. Samples were vortexed and sat covered for 2 h covered in a cupboard from fluorescent light. Letting the sample sit for 2 hours allowed any excess 1-naphthyl-isocyanate to react such that crystal formation would take place in the vials for the HPLC. Samples were filtered through a 0.22 μm nylon syringe filter before loading into 2mL HPLC vials. All samples were analyzed by HPLC within 48 hours. The limit of detection was 2.5 μM and Limit of quantification was 2.9 μM.

Mobile Phase

HPLC was an Agilent 1260 Infinity model with a fluorescent detector (Agilent Technologies) and autosampler (Agilent Technologies). The pump was a single pump The column was an Agilent bio 5 μm strong cation exchange column 250 mm × 4.6mm (Agilent Technology, Santa Clara, CA, USA) in a column compartment that was set to a constant temperature of 40°C. Software used ChemStation for LC Systems (G2170BA). Flow rate was 0.5 mL min^{-1} .

Calibration curve

Choline bitartrate was used to construct a standard curve over a concentration of 0-1000μM. These working standards were derivatized as described above. A calibration curve was performed during each run. Choline standards were prepared and stored for two weeks or thawed three times before discarding.

Statistical analysis

Statistical tests were done to compare means. Results were expressed as the mean± standard deviation. Differences were determined using a t-test in JMP 13.

RESULTS

Samples were run in triplicate, and standard curves with an R^2 less than of 0.98 were rejected (Figure 4-2).

Table 4-1 shows that the method can hydrolyze bound choline to free choline in whole feed ingredients and in purified PTC, which was used for spiking. Since choline can be in both water-soluble and fat-soluble forms, it was determined that spiking was needed to show that both kinds of choline could be recovered. Choline bitartrate (chol-bit) and PTC was used. Chol-bit was used as free choline, PTC simulated bound choline. Doubling was used as a recovery method as well to ensure that a complete digestion was being performed, this indicated there were enough acid and base units for a total hydrolysis of choline to occur at the desired sample weight of 0.1 g (Table 4-3).

To ensure that there was a complete hydrolysis of choline the extraction/ hydrolysis was done over different time points to show that 15 minutes of base followed by acids was too short while 60 minutes was too long and could possibly begin to degrade some of the choline (Table 4-2).

Different feed ingredients were used to ensure the method could be used across various feed ingredients that had different plant matrixes. These different plant matrixes could change how the choline extraction works in different types of feed ingredients. It was necessary to show that this method could be applied to various feed ingredients that have different compositions. Since livestock feeds can have a wide range of compositions, canola meal and soybean meal are known to contain choline as lecithin (McEntyre et al., 2009, Aburto et al., 1998). This method shows consistent recoveries in soybean and canola meal. Whole flaxseed includes the highest percentage of fat of the ingredients tested, with a crude fat value of 15%. This is a fat content for livestock

feed ingredients. This method can be used to quantify choline in a high fat feed ingredient, like flaxseed. Oats is a high fiber feed ingredient with a neutral detergent fiber content of 36%. The fibrous cell walls could make extraction of choline into solution harder. Using a 1mm grind appears to be adequate to break up the outer hull for the choline to be extracted. Alfalfa meal was chosen since this feed ingredient has a moderate amount of both protein and fiber. Since there were multiple samples of alfalfa meal this also made alfalfa meal an ideal feed ingredient to investigate the variation of choline within feed ingredients, soybean meal was used for this purpose as well.

The calibration curve was highly repeatable and was used to determine the choline content of the feed ingredients (Figure 4-1). The response method was a linear range over 2.5 μM to 1000 μM of choline bitartrate. The limit of detection was 2.5 μM. This would be a choline content of 43.0 mg kg^{-1} with a 0.1g sample with a final volume of 10mL. Limit of quantification was 2.9 μM of choline which would be 45.3 mg kg^{-1} for a 0.1g sample with a final volume of 10 mL (Shrivastave et al., 2011).

Choline was done on a wide variety of feed ingredients with feed ingredients that are used in organic production (Table 4-4). Choline content can vary between feed ingredients. Feed ingredients like canola meal and soybean meal contain lecithin (Aburto et al., 1998, Jensen et al., 2015). Lecithin contains bound choline, so feed ingredients like canola meal, soybean meal, and sunflower are high in choline this could be due to lecithin content in these oilseed meals (Cabezas et al., 2016). Canola meal, sunflower meal, and soybean meal have the highest choline contents (3208, 2531, and 2862 mg kg^{-1}, respectively), this makes sense since these three ingredients contain lecithin. Corn and sorghum had lower values of choline with a total choline value of 556 and 531 mg kg^{-1}, respectively. All other values of choline had intermediate amounts of choline with a range from 1074-1565 mg kg^{-1}.

DISCUSSION

The extraction method and quantification shown in this paper can be used to quantify total choline in ingredients that are fed to livestock using HPLC. A previous method by Awtal et al. (1980) for quantifying total choline in feed ingredients focused on heating the sample in methanol, with an updated modification of using a heated acid hydrolysis. For total choline to be extracted from feed ingredients, it appears that there needs to be a chemical breakdown, either a strong acid or base, and heat, for total hydrolysis of choline (Atwal et al., 1980, AOAC 2016, Hefni et al., 2015). Bound choline can be digested to free choline using different aqueous chemical conditions. Methods for quantifying choline differ on the type of sample the method is designed for. Infant formula and choline supplements have different properties than livestock feed ingredients. Different extraction methods should be developed. Laboratories have different equipment needs and therefore may not have certain kinds of equipment like a mass spectrophotometer but may have equipment like a HPLC.

The AACC method for quantifying total choline does not specify how samples should be ground or processed to ensure that solvents and acid have the adequate surface area to interact with the feed ingredient. However, the procedure does acknowledge that adding pumice to the sample to decrease clumping. Atwal et al. (1980) indicate that some feed samples were processed through a roller mill before extraction. This method does not state what ingredients should be prepared with a roller mill. Average particle size or screen size used for grinding was not reported. Particle size can be an indicator to ensure that there is adequate surface area to interact with the chemical solution (Drakos et al., 2017). For the choline extraction reported in this paper, the authors recommend a 1 mm grind to ensure small enough particles to adequately interact with the feed ingredient being digested in an aqueous solution.

Atwal et al. (1980) procedure modifies the AACC procedure and only takes into account feed ingredients interactions that are protein based. This does not account for other macromolecules, like fiber and fat, that could interfere with the extraction of choline. Fiber and cell wall structure effects can be minimized by grinding samples before extraction procedure. Grinding samples reduces particle size, increases surface area, and mechanically breaks down the cell wall so cell contents can be exposed to digestion solution. The digestion process breaks down the glycerol backbone and allows for the digestion of PTC which liberates the choline.

Since a National Institute of Standards and Technology (NIST) standard could not be obtained that had been previously analyzed for choline that had a similar composition to the feed ingredients used. A NIST standard was not used. It was important for the authors to thoroughly use various recovery methods (Table 4-2). Utilizing both chol-bit and PTC ensured that both the water and fat-soluble portions of choline could be quantified. This was to ensure that common forms of choline in livestock feed ingredients could be quantified. Known amounts of chol-bit and PTC were derivatized and quantified. A 0.7 M solution of PTC was extracted and was quantified to contain the correct amount of choline. A similar procedure was done using chol-bit to ensure that water-soluble choline could be accurately measured as well.

It was determined that there was no need to include a phospholipase D treatment in the final method. The samples treated with phospholipase D were numerically lower than that samples that were only digested. The decrease in choline is most likely caused when the enzyme step was included. This diluted the samples and decreased the sensitivity of the analysis. This was another reason that the phospholipase D treatment was not included in the final procedure. This agrees with Hefni et al. (2015), which also determined that phospholipase D treatment was unnecessary.

The calibration curve is highly repeatable. There was a strong linear relationship between luminosity units and μmoles of choline in the sample. A solution of PTC with a known molarity could be calculated using this method indicates that the reaction of 1-napthyl isocyanate was effective at binding to choline and allowing choline to be quantified using a fluorescence detector. The limit of detection seems high with a limit of detection of 43 mg kg^{-1} and limit of quantification was 45.3 mg kg^{-1}; however, no samples ran had a choline content lower than 500 mg kg^{-1} so getting a lower limit of detection would not be necessary. The limit of detection was 2.5 μM for the calibration curve, with an upper limit of 1000μM of choline. The large linear range of the line can quantify choline in samples up to 10200 mg kg^{-1} without having to dilute the samples. Even though the limit of detection was higher than Awal et al. (1980) choline in feed ingredients is not in small quantities, so a low limit of detection is not necessary for this procedure. Conversely running a small sample weight below 0.1 g is also not needed. In a commercial setting feed samples are typically collected in large quantities, like kilograms, so running a small sample size to limit excessive use of the collected sample is not necessary.

The analyzed values of corn, soybean, alfalfa meal were similar to previously reported values (NRC 1994, NRC 2012). The value of oats (1218 mg kg^{-1}) was greater than reported value of oats (946 mg kg^{-1}) (NRC 2012). The analyzed value of canola meal was lower than the reported value canola meal (3509 vs. 6700 mg kg^{-1}) (NRC 1994). However, looking through the literature of analyzed choline values in canola meal do not report total choline values in canola meal as high as the published book values (March and MaMillian 1980, Emmert and Baker 1997). Choline content of canola meal could be highly variable due to sinapine (Mailer et al., 2008). Sinapine is an antinutritional choline containing phenol (Mailer et al., 2008, Khajiali and Slominski, 2012). [26] Since sinapine is an antinutritional factor for livestock feed, there has been recent pressure to

reduce sinapine in canola meal (Emrani et al., Harloff et al., 2012, Jeske et al., 2013). A reduction of sinapine in canola plants could reduce total choline content of canola meal, making historical book values of total choline in canola and canola byproducts inaccurate.

The objective of this work to make a total choline extraction method, specifically for livestock, was achieved. This method is different from other methods for choline extraction methods because this method uses a two-part base than acid extraction. This is unique compared to other methods of choline extraction that only use acid only or base only.[9] Having a base digestion followed by an acid digestion could help increase the hydrolysis of the choline.

CONCLUSION

This method can be applied to extract total choline in a wide variety of feed ingredients for livestock. This is important since feed ingredients have different types of plant matrix material that would impede the extraction of choline. Patterson et al. (2008) showed that choline is in many different forms in human food. It is reasonable to assume that choline is in various forms in livestock feed ingredients as well. When quantifying choline, it is essential to consider the different kinds of choline present.

With increasing production of organically raised meat in the United States, there is pressure to find alternatives to supplemental methionine (Bokkers et al., 2009, Pesti et al., 1980). Choline could be one of those alternatives, so there is a need to be able to accurately quantify choline in livestock feeds (Coffey and Baier 2016). This procedure can be used to quantify choline in livestock feed ingredients.

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Table 4-1. Choline determination of selected in mg/kg with and without using phospholipase D[1]

Ingredient	Choline (mg kg^{-1})	P value
Soybean meal	2639±175.3	>0.08[2]
Soybean meal with enzyme	2393±143.2	
Phosphatidylcholine	104.8 ±15.1	>0.01[3]
Phosphatidylcholine with enzyme	75.4 ±13.3	

[1] All sample were run as triplicates

[2] T- test between soybean meal and soybean meal with enzyme

[3] T- test between phosphatidylcholine and phosphatidylcholine enzyme

Table 4-2. Effect of time on choline content in representative feed ingredients[1,2]

	Amount of choline in sample (mg kg^{-1})	P value
15 min	1891± 97.6	>0.05
30 min	2981± 140.4	-
45 min	2863± 173.1	<0.05
60 min	2580± 195.8	>0.10

[1] This is a representative sample of soybean meal. This pattern was since in all feed ingredients tested

[2] All samples were run as triplicates

Table 4-3. Average recoveries for choline using doubling, choline bitartrate and PTC[1,2]

Ingredient	Standard used	Choline added	Choline in sample (mg kg^{-1})	Choline recovered (mg kg^{-1})	Recovery
Corn	Double sample size	0.2g of sample	558 ±39.6	560±39.3	99.6 ± 4.3
	Choline bitartrate	20 µL of 0.1M Chol-bit	558 ±39.6	2611±157.41	115.1 ± 2.7
	PTC	30 µL of 0.07M PTC	558 ±39.6	2648±212.0	109.2 ±9.2
Canola meal	Double sample size	0.2g of sample	3509 ±363.8	3809±203.1	91.7 ±2.2
	Choline bitartrate	20 µL of 0.1M Chol-bit	3509 ±363.8	5228±466.5	117.5 ±14.2
	PTC	30 µL of 0.07M PTC	3509 ±363.8	5768±266.5	111.5 ±5.8
Flaxseed	Double sample size	0.2g of sample	1166.5±108.1	1074±34.4	85.4±3.3
	Choline bitartrate	20 µL of 0.1M Chol-bit	1166.5±108.1	2933.5±345.2	124.7±11.9
	PTC	30 µL of 0.07M PTC	1166.5±108.1	2803.0±95.8	129.6±6.7
Oats	Double sample size	0.2g of sample	1218.6±165.6	1324.8±109.9	84.0±4.6
	Choline bitartrate	20 µL of 0.1M Chol-bit	1218.6±165.6	3625.9±303.8	113.8±10.8
	PTC	30 µL of 0.07M PTC	1218.6±165.6	2969.1±192.9	112.1±4.35
Soybean meal (1)	Double sample size	0.2g of sample	2698.7±100.1	2645.8±105.1	104.0±1.1
	Choline bitartrate	20 µL of 0.1M Chol-bit	2698.7±100.1	4979.3±36.4	130.9±3.4

	PTC	30 μL of 0.07M PTC	2698.7±100.1	4346.4±320.7	98.2±26.6
Soybean meal (2)	Double sample size	0.2g of sample	2997.6±278.3	3022.8±396.6	98.3±5.3
	Choline bitartrate	20 μL of 0.1M Chol-bit	2997.6±278.3	4755.9±554.4	104.2±19.1
	PTC	30 μL of 0.07M PTC	2997.6±278.3	4514.6±240.3	89.6±7.6
Alfalfa meal (1)	Double sample size	0.2g of sample	1305.1±348.5	1371.6±369.8	90.3±4.0
	Choline bitartrate	20 μL of 0.1M Chol-bit	1305.1±348.5	3717.2±116.3	116.5±12.4
	PTC	30 μL of 0.07M PTC	1305.1±348.5	2875.2±221.2	87.4±5.2
Alfalfa meal (2)	Double sample size	0.2g of sample	1095.5±254.7	989.9±27.8	110.3±10.3
	Choline bitartrate	20 μL of 0.1M Chol-bit	1095.5±245.7	3353.0±36.7	91.7±5.9
	PTC	30 μL of 0.07M PTC	1095±245.7	2510.1±188.0	82.3±12.4

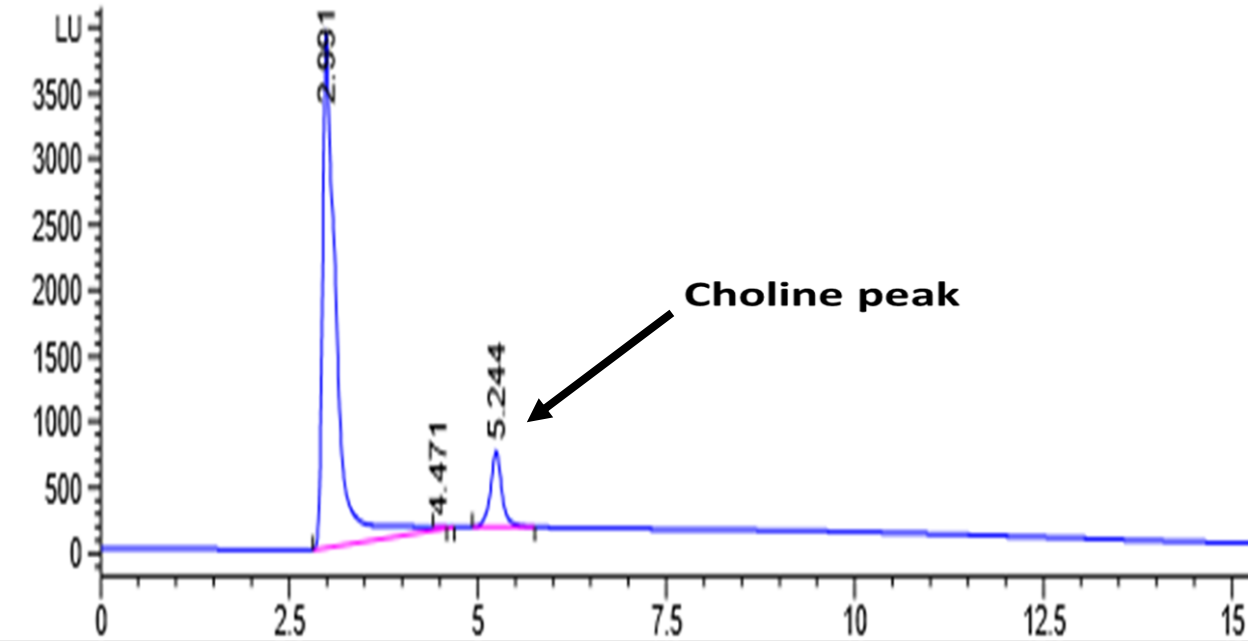
[1] All samples were run as triplicates

[2] PTC= Phosphatidylcholine

Table 4-4. Total choline content for various organically grown feed ingredients [1]

Ingredient	Choline (mg/kg)	Standard deviation
Alfalfa meal	1220	195
Soybean meal	2862	189
Sunflower meal	2531	74
Canola meal	3208	156
Corn	556	41
Hominy	1408	78
Wheat midds	1549	89
Millet	1248	77
Oats	1112	122
Flaxseed	1074	43
Sorghum	531	26
Barley	1565	88
Field peas	1165	123

[1] All samples ran as triplicates



[1] HPLC used was Agilent 1260 Infinity with fluorescent detector (Agilent Technologies). Mobile phase was 10 mmolL-1 tetramethylammonium hydroxide and 20mmolL-1 of glycolic acid in 15% water and 85% acetonitrile. The flow rate was 0.5 mLmin-1. Excitation of 220nm and 350 nm emission

Figure 4-1. Choline chromatograph of 200 μM of choline bitartrate eludes at 5.24 minutes[1]

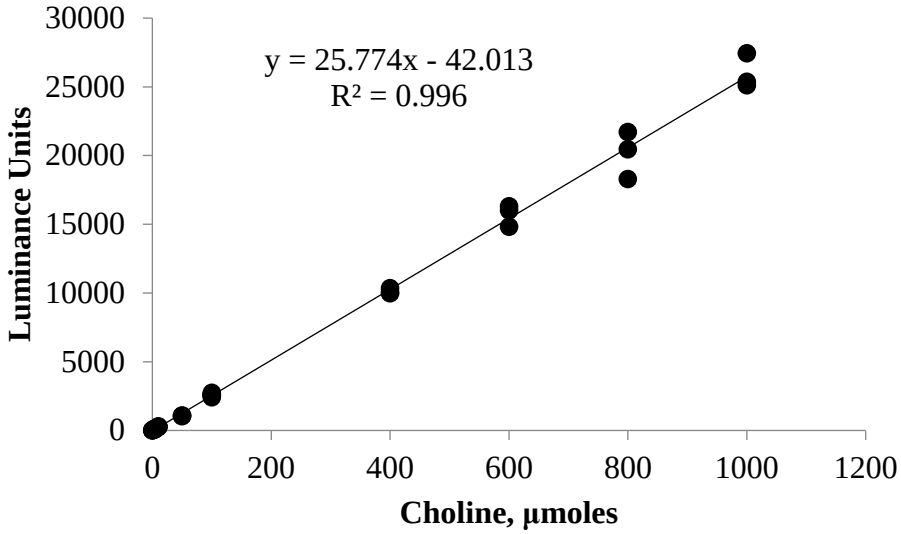


Figure 4-2. Choline calibration curve, using choline bitartrate

CHAPTER 5

CHOLINE DIGESTIBILITY OF ORGANIC FEED INGREDIENTS IN BROILER CHICKS

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SUMMARY

Organic poultry has been an emerging market in the United States in recent years. The organic industry has different regulations and management practices compared to conventionally raised poultry. This means different feed ingredients could be utilized in organically raised poultry compared to conventionally raised poultry. Organic poultry production may incorporate feedstuffs that have not been extensively fed to poultry, and therefore there is limited research on how to feed these novel feed ingredients to poultry. Research is needed to determine how available the nutrient components are in poultry. This study was done to evaluate the digestibility of choline of organic feed ingredients in broiler chickens. Six hundred and forty Cobb 500 broilers were fed nine organic feed ingredients in two trials. The ingredients were oats, flaxseed, field peas, millet, alfalfa meal, canola meal, sunflower meal, sorghum, and barley. There was also a basal diet to determine endogenous losses for choline. Ileal samples were collected at fourteen days of age. Canola meal and flaxseed have the lowest choline digestibility. Sunflower meal had intermediate digestibility. Most feed ingredients, oats, field peas, millet, alfalfa meal, sorghum, and barley, have high choline digestibility. It appears that oilseed meals have lower choline digestibility than grains.

DESCRIPTION OF PROBLEM

Organic poultry production is increasing in the United States (OTC 2016, USDA 2015). Feed ingredients used in organic production may not have been commonly fed to conventionally raised poultry, so research is needed to learn how to feed these novel feed ingredients in poultry. This will optimize the performance of organically grown poultry.

In organic production, methionine is fed in limited quantities of two pounds per ton for broilers (Coffey and Bair 2012). Additional methionine is typically supplemented in conventionally raised poultry. Supplementing crystalline methionine or a DL-2- hydroxy-(4-

methylthio) butanoic acid (HMTBA) in poultry diets can decrease overall protein levels, improve air quality around the chickens, and decrease the cost of the diet (Bokker and DeBoer, 2009). The amount of supplemental methionine allowed by the National Organic Program (NOP) of the United States does not supply enough methionine to meet the bird's requirement at certain ages (Burley et al., 2016). If adequate methionine cannot be supplemented, then other methods, of methionine sparing must be used. A methionine sparing technique that can be used is to have excess choline maximize homocysteine re-methylation to form methionine de-novo thus to reducing the amount of dietary methionine needed (Pesti et al., 1980). Depending on the choline content of the feed ingredients used in the diets, choline in feed ingredients could be used to spare methionine.

There needs to be a way to quantify choline in feed ingredients and digesta to decide if a choline supplement is necessary. A feed ingredient high in choline may not have available choline for the bird to utilize. There could be antinutritional factors that could affect the digestibility of nutrients, including choline (Khajali and Slominski 2012). Choline availability in feed ingredients has been studied in selected ingredients (March and MacMillian, 1980, Emmert et al., 1996, Rama Rao et al., 2001). Expanding choline availability data for various feed ingredients is important when feeding novel feed ingredients to poultry.

Previous studies suggest that choline availability is high in grains and soybean meal and low for canola meal (March and MacMillian, 1980, Emmert et al., 1996, Rama Rao et al., 2001). There is not much information in the literature about how digestibility choline is in a wide variety of feed ingredients for poultry. The objective of this study is to determine the digestibility of choline, in broilers, in organic feed ingredients.

MATERIALS AND METHODS

Experimental protocols were approved by the University of Georgia (UGA) Institutional Animal Care and Use Committee (UGA Animal Care and Use A2015 09-011-Y1-A0) (Athens, Ga).

Six-hundred and forty day old Cobb 500 males chicks were allotted, eight birds per pen, to Petersime battery brooder cages (Petersime Incubator Co., Gettysburg, OH, USA). Chicks were evenly split between two trials. All chickens were fed a common starter diet without antibiotics for 1-10 d (Table 5-1). On d 10 diets were changed to the test diets. Test diets were fed from 10-14 d. Water and feed were available *ad libitum* during the entire experiment. The room was kept at a constant temperature of 27.5°C throughout the entire experiment. Battery brooder heaters were set to a temperature of 30°C for one week. After the end of the first week, brooder heaters were turned off.

Diets

Feed ingredients were obtained from Organics Unlimited (Atglen, PA, USA). Unground feed ingredients, alfalfa meal, canola meal, and sunflower meal, were ground before diet mixing, Flaxseed, field peas, millet, and sorghum were ground through a 2mm screen (Wiley Mill Thomas Scientific, Swedesboro, NJ, USA). Oats and barley were ground through a 5mm screen to crack the hull on the oats and barley. Alfalfa meal, canola meal, and sunflower meal were pre-ground and were not additionally ground. Diet composition is shown in Table 5-2.

Test and reference (dextrose) diets were formulated according to protein content (Table 5-3). High protein feed ingredients canola meal, flaxseed, sunflower meal, field peas, and alfalfa meal were formulated to contain 15% crude protein. Low protein feed ingredients oats, barley,

millet, and sorghum contained 77% of the feed ingredient in the final diet. TiO_2 was added at 0.3%, as an indigestible marker diet to determine the digestibility of choline (Myers et al., 2004).

Collection of ileal samples

On d 14 birds were euthanized by CO_2 asphyxiation. Immediately after killing entire ileal contents, from the Meckel's diverticulum to the beginning of the cecum were collected and pool per pen (Garcia et al., 2007). Ileal samples were frozen and freeze-dried (Labconco, Labconco Corporation, Kansas City, MO, USA). Endogenous losses were determined using a diet with only supplemented choline chloride, (reference diet, 880 mg/kg choline). Endogenous losses were calculated with the dextrose-based reference diet, were the only source of choline came from choline chloride which was assumed from previous research to have 100% bioavailability (Emmert et al., 1996).

Choline analysis

All feed ingredients were ground through a 1 mm screen before analysis (Wiley Mill Thomas Scientific, Swedesboro, NJ, USA). Ileal samples were ground and homogenized using a mortar and pestle. Feed ingredients, diet, and ileal samples were analyzed for total choline using the method of Bloxham et al. (2017). Choline analysis was performed using high-performance liquid chromatography (HPLC). Mobile phase contained 10mM tetramethylammonium hydroxide and 20mM of glycolic acid in 15% water and 85% acetonitrile. The flow rate was 0.5 $mLmin^{-1}$. Excitation of 220nm and 350 nm emission was used using fluorescent detector (Agilent Technologies model 1200 Waldronn, Germany). The column was an Agilent bio 5 μm strong cation exchange column 250 mm × 4.6mm (Agilent Technologies model 1200 Waldronn, Germany).

Summary of the methods is as follows; 0.2 g samples were digested at 115°C in 3mL of a 1M solution of NaOH for 30 min. Samples were slightly cooled then 6mL of 1M HCl was added, and samples were heated at 115°C for an additional 30 min. Samples were then cooled and neutralized to a pH to 7. Samples were frozen until choline analysis, which was with 2 weeks.

Choline derivatization is as follows; in a 1.5 mL microcentrifuge tube 20µL sample was added to 1mL of acetonitrile, 60 µL of sodium hydroxide, and approximately 80mg of magnesium oxide. 20 µL of 1-naphthyl-isocyanate was added last. Samples were shaken 100 rpm at 25°C for 15min. 60µL of HPLC grade water was added to sample after samples were removed from the water bath. Samples were vortexed and sat for 2 hours covered away from fluorescent light. Samples were filtered through a 0.22 µm filter before loading into an HPLC. All samples were analyzed by HPLC within 48 hours (Hefni et al., 2015).

There was one modification when analyzing the ileal contents: digestion solutions had twice the molarity (2M versus 1M) and half the volume to give the same amount of acid and base units yet allowed the samples to be concentrated enough to quantify choline using HPLC.

Choline was determined using the equation (Stein et al., 2007):

Standardized choline digestibility = apparent ileal digestibility + [(basal choline endogenous / Choline diet) x 100]

Statistical analysis

Data were analyzed using SAS Proc GLM, with diet as a class variable (SAS Institute 9.4, Cary, North Carolina). There was a test for a diet by trial interaction. There was not a diet by trial interaction, so the interaction was removed from the analysis. Differences between ingredients were determined by Tukey's test. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Canola meal had the lowest digestibility with a choline availability of 36%. This is similar to previous research by March and MacMillian 1980 that shows that choline digestibility of canola meal is low. There were similar results to Emmert and Baker 1997 that determined choline availability in canola meal ranged from 24-32% . The low choline availability could be due to general antinutritional factors in canola meal (Burley et al., 2016). These known anti-nutritional factors in canola meal would include glucosinolates, tannins, phytic acid, and fiber (Khajali and Slominski, 2012, Mailer et al., 2008). These antinutritional factors possibly have a general effect by binding to choline and decreasing absorption.

Low digestibility of choline in canola meal could be due to a choline containing compound called sinapine, which is also a known antinutritional compound in canola meal (Khajali and Slominski, 2012 , Emrani et al., 2015). Certain varieties of canola meal can be up to 2% sinapine (Emrani et al., 2015). This would partially explain canola meal's high choline content yet low digestibility. Sinapine appears in the feces, meaning the choline in sinapine was not utilized by the animal (Menten et al., 1997). Canola meal also has phosphatidylcholine, and this compound also contains choline. It appears that only plants from the *Brassicaceae* family have a high content of sinapine in the plant and seed portions (Claub et al., 2008). Canola meal is the only plant of the Brassicaceae family that is commonly fed to livestock in the United States (Boucherean et al., 1991). Sinapine is thought to be a way for the seed to store choline for phosphatidylcholine synthesis (Bouchereau and Strack, 2010). However, soybean contains high levels of choline and phosphatidylcholine without containing sinapine. However, phosphatidylcholine does not appear to be antinutritional, since choline bioavailability is high in soybean meal (Menten et al., 1997).

Sinapine would not only cause canola meal to have a lower feeding value for other nutrients but make the choline in canola meal have a poor digestibility as well.

In recent years there has been a genetic selection of the canola plant to reduce sinapine levels (Milkowski et al., 2010, Harloff et al., 2012, Jesske et al., 2013). Use of these cultivars could reduce the choline content of canola meal; however, this has not been investigated. This selection has the potential to increase the overall digestibility content of other nutrients of canola meal, not just the choline. These plants were developed through genetic selection, not genetic modification, so these cultivars of canola could be included in an organic diet for chickens or other organically raised livestock. There is also genetically engineered canola that has been engineered specifically for reduced sinapine (Milkowski et al., 2010). These cultivars would be considered genetically modified and would not be suitable for organic production (Coffey and Baier et al., 2012).

Flaxseed also has low choline digestibility, with a digestibility of 51 ±16%. There have not been studies of choline digestibility in flaxseed. However, there have been studies that show that ground unprocessed flaxseed does have low protein digestibility, and flaxseed inclusion will lower growth performance in poultry (Harloff et al., 2012, Jesske et al., 2013, Jia et al., 2010, Shen et al., 2005, Bandegan et al., 2010). Flaxseed does have general antinutritional compounds like phenols that would lower the feeding values of whole flaxseed (Wojengo et al., 2016).

Grains like oats, barley, sorghum, and millet all have high choline bioavailability (Table 5-4). There was a study by Rama Rao et al. (2001) that indicated that bioavailability of choline in millet and other grains was high, similar to, the current study where millet had a digestibility of 98.4%. Other grains, in this study, like oats, barley, and sorghum had a high digestibility of choline as well (88.84±7.3, 84.45±3.9, 94.6±2.5%, respectively).

Sunflower meal may be used in alternative management methods and where sunflower meal is regionally more available that soybean meal or other protein sources (Oliveira et al., 2016). It is a particularly good source of methionine; sunflower meal may be added to a broiler diet without losing growth performance (Knudsen 2014). Sunflower meal does have some general antinutritional factors that may affect poultry including fiber content and phenol compounds (Woyengo et al., 2016, Knudsen 2014). These compounds may affect digestibility of nutrients including choline. However, there have been no other studies of choline digestibility in sunflower meal in poultry.

Other potential protein sources like alfalfa meal and field peas have high choline digestibility (Table 5-4). The results from this study indicate that the choline in alfalfa meal is high with a digestibility of 94.75%. Field peas have not been extensively studied as a feed ingredient in poultry. Studies that are available show that field peas could be a fed to poultry without affecting growth rate (Dotas et al., 2014). This indicates that field peas may be able to be incorporated in organic diets, without losses in growth rate (Leinonen et al., 2013).

Oilseed and oilseed by-products are generally known for having anti-nutritional factors that would lower their feeding value of the feed: certain kinds of oilseeds can have low digestibilities of amino acids (Woyengo et al., 2016, Lomascolo et al., 2012). This study indicates that other nutrients, besides amino acids, seem to have lower feeding values as well. Choline seems to follow this trend for lower digestibility in certain kinds of oilseeds.

CONCLUSIONS AND APPLICATIONS

1. Choline digestibility is variable across feed ingredients. Canola meal had a low choline digestibility value of 36.7%. This could be due to choline containing antinutritional compounds. Sunflower meal and flaxseed had low choline

digestibility as well. This could be due phenol containing antinutritional factors that are in oilseeds. Choline digestibility appears to be in high in grains, in agreement with previous research

2. Further research should be to expand the feed ingredients tested, so there is a larger database on how digestible choline is in a wide variety of feed ingredients.

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Table 5-1. Starter diet fed for broilers fed to chicks from 1-14 day

Ingredients	Percent
Corn	56.125
Soybean meal	37.50
Soybean oil	3.07
Limestone	0.73
Salt	0.30
Defluorinated phosphate	1.75
Trace mineral premix[1]	0.075
Vitamin premix[2]	0.25
DL methionine	0.20

[1] Supplied per kilogram of diet: Mn, 107.2 mg; Zn, 85.6 mg; Mg, 21.44 mg; Fe, 21.04; Cu, 3.2 mg; I, 0.8 mg; Se, 0.32 mg.

[2] Supplied per kilogram of diet: vitamin A, 110229 IU; vitamin D_3, 22000 IU; Vitamin E, 220 IU; .25 mg vitamin B_{12}, 5 ppm; Biotin, 0.11 mg; Menadione, 1.1 mg; Thiamine, 2.21 mg; Riboflavin, 4.41 mg; d-Pantothenic, Acid, 11.02 mg; Vitamin B_6, 2.21 mg; Niacin, 44.09 mg; Folic Acid, 0.011 mg; Choline 38 mg

Table 5-2. Choline content of individual feed ingredients reported in mg/kg[1]

Ingredient	Choline, mg/kg	Standard deviation
Oats	1112	122
Flaxseed	1074	43
Field peas	1165	59
Millet	1248	77
Alfalfa meal	1201	63
Canola meal	3208	156
Sunflower meal	2531	74
Sorghum	531	26
Barley	1565	88
Dextrose	Not detected	N/A

[1] All samples were performed in triplicate

Table 5-3. Composition of the diets used in the choline digestibility assays

Ingredient, %	Dextrose	Oats	Flaxseed	Field Peas	Millet	Alfalfa Meal	Canola Meal	Sunflower meal	Sorghum	Barley
Dextrose	92.85	17.27	25.15	17.57	16.93	15.38	34.45	34.63	17.03	17.24
Soybean oil	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.000
Salt	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix[1]	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Mineral premix[2]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Dicalcium phosphorus	2.40	1.54	1.82	2.27	1.80	1.27	1.30	1.50	1.75	1.49
Limestone	0.90	1.06	1.06	0.89	1.14	0.00	0.37	0.74	1.09	1.14
TiO_2	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Oats	0	77.00	0	0	0	0	0	0	0	0
Flaxseed	0	0	68.81	0	0	0	0	0	0	0
Field Peas	0	0	0	76.14	0	0	0	0	0	0
Millet	0	0	0	0	77.00	0	0	0	0	0
Alfalfa Meal	0	0	0	0	0	80.21	0	0	0	0

Canola Meal	0	0	0	0	0	0	60.75	0	0	0
Sunflower Meal	0	0	0	0	0	0	0	60.00	0	0
Sorghum	0	0	0	0	0	0	0	0	77.00	0
Barley	0	0	0	0	0	0	0	0	0	77.00
Analyzed value, mg/kg										
Choline	880	1637	909	1292	1098	1367	3254	2427	920	1620
Calculated value, %										
Crude protein, %	0.00	8.47	15.00	15.00	12.76	15.00	15.00	15.00	7.87	9.72

[1] Supplied per kilogram of diet: vitamin A, 110229 IU; vitamin D_3, 22000 IU; Vitamin E, 220 IU; .25 mg vitamin B_{12}, 5 ppm; Biotin, 0.11 mg; Menadione, 1.1 mg; Thiamine, 2.21 mg; Riboflavin, 4.41 mg; d-Pantothenic, Acid, 11.02 mg; Vitamin B_6, 2.21 mg; Niacin, 44.09 mg; Folic Acid, 0.011 mg; Choline 38 mg.

[2] Supplied per kilogram of diet: Mn, 107.2 mg; Zn, 85.6 mg; Mg, 21.44 mg; Fe, 21.04; Cu, 3.2 mg; I, 0.8 mg; Se, 0.32 mg

Table 5-4. Digestibility of choline in organic feed ingredients[1]

Diet[2]	SID	Standard error
Oats	88.84[a]	7.27
Flaxseed	50.79[bc]	16.07
Field Peas	87.73[a]	2.45
Millet	98.42[a]	0.94
Alfalfa Meal	94.04[a]	1.86
Canola Meal	36.70[c]	4.26
Sunflower Meal	61.75[b]	13.42
Sorghum	94.75[a]	2.47
Barley	84.45[a]	3.91

[1] Means in a column not sharing a common superscript are different ($P < 0.05$)

[2] Diets represent 8 replicates per feed ingredient

CHAPTER 6

DIGESTIBILITY AND ANALYSIS OF BETAINE FOR ORGANIC FEEDSTUFFS IN BROILER CHICKS

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SUMMARY

Betaine is an important compound for animal metabolism. Animals can benefit from betaine under heat stress or low methionine diets. This is important in organic poultry diets, where synthetic methionine is limited by law, and the typical corn and soybean meal based diet would not supply adequate methionine. The objective of this study is to quantify total betaine in feedstuffs and the determine the bioavailability of betaine in organic feed ingredients in broilers. Fifteen ingredients were used to determine total betaine content in each feedstuff: alfalfa meal (n=2), soybean meal (n=2), wheat middlings, hominy, corn, barley, canola meal, field peas, sorghum, sunflower meal, oats, flaxseed, and millet. Ingredients with no detectable betaine were: alfalfa meal (1), soybean meal (1), hominy, corn, field peas, and flaxseed. Ingredients with low levels of betaine which was approximately 1 mg/kg were soybean meal (2), canola meal, and oats. The ingredients with quantifiable levels of betaine were alfalfa meal (2), barley, sorghum, wheat middlings, millet and sunflower meal. Feed ingredients that were obtained in high enough quantities and contained quantifiable betaine were fed to broiler chicks to determine the digestibility of betaine were: barley, millet, and sunflower meal. Barley had the lowest betaine digestibility, followed by sunflower meal. Millet had the greatest digestibility. All digestibility values were statistically different from each other. In conclusion, betaine content in feed ingredients and digestibilities are highly variable. Betaine content within feed ingredients is variable as well.

DESCRIPTION OF PROBLEM

Betaine (BET), also called trimethylglycine or glycine betaine, has been used as a feed additive for poultry for many years, either as a methyl donor (Eklund et al., 2005, Zhan et al., 2006), to reduce the effects of coccidiosis challenge (Amerah and Ravindran, 2015),

or reduce the effects of heat stress (Honarbakhsh et al., 2007). In mammals, BET has been linked to various benefits, including reducing the effects of heat stress (Ryu et al., 2002). Heat stress can cause BET to accumulate in the kidneys (Kempson et al., 2013). Betaine has also been shown to increase nutrient digestibility in poultry (Amerah and Ravindran, 2015). Betaine has been most studied, in poultry, for its potential as a labile methyl donor (Eklund et al., 2005, Zhan et al., 2006, Garcia et al., 2000). Betaine has been studied as a methionine replacement in livestock diets. Studies in poultry showing the efficacy of BET have been mixed due to variation in the BET as a methionine replacement (Garcia et al., 2000, Rostagno and Pack, 1996).

Betaine in plants is a naturally occurring compound to reduce the effects of drought stress (Honarbakhsh et al., 2007). Not all have plants have the pathway to produce BET (Yamada et al., 2015, Peel et al., 2010). Plants that do not have the ability make BET may not be as drought tolerant as plants that can produce BET (Peel et al., 2010). Increase salt in the soil can increase BET in plants if the BET pathway is present (Yamada et al., 2015). Plants that have the ability to produce BET may not have measurable BET due to growing conditions, such as excessive rainfall during the growing season (Sakamoto and Murata, 2002). This can cause BET content to be highly variable within cultivars of plants (Sakamoto and Murata, 2002). This can make finding a representative sample of BET within plant species difficult. Betaine is also variable between plant species, as well (Peel et al., 2010). Wheat, wheat byproducts, beet, and beet byproducts are known to have high amounts of BET (Yamada et al., 2015, Peel et al., 2010, Sakamoto and Murata, 2002).

There are cultivars such as corn and sorghum that can either contain or be totally void of BET (Peel et al., 2010). Betaine will accumulate in certain plant tissues depending on the

growing conditions of the plant (Yamada et al., 2015). Betaine may accumulate differently in different plant tissues (Sakamoto and Murata, 2002, Likes et al., 2007). For example, BET may accumulate in the leaves of corn, yet, BET may not be in the corn grain, and therefore, corn may not be a good source of BET for non-ruminants Sakamoto and Murata, 2002, Chendrimada et al., 2002).

In recent years, organic poultry production has been increasing in the United States (USA) (Mench et al., 2011). The addition of crystalline methionine is limited by law to 2, 2, 3 pounds per US ton in broilers, layers, and turkeys, respectively, according to the National Organic Program (Coffey and Baier, 2012). With these restrictions, methionine would be a limiting amino acid in a typical corn and soybean meal diets for poultry (Burley et al., 2016). Betaine may serve to spare methionine. Methionine alternatives are needed for organically raised poultry to be fed efficiently (Burley et al., 2015). BET may be a supplemental methionine alternative in organic diets since BET can re-methylate homocysteine and reduce methionine catabolism *in vivo* (Zhan et al., 2007). Feeding ingredients that are naturally high in BET could be a strategy used to supplement BET instead of feeding chemically synthesized BET. Organic poultry diets would be ideal conditions for methionine sparing to have a measurable effect on poultry performance. There have been no studies published that have investigated the digestibility of BET from feed ingredients in poultry. The objective of this study was to determine the BET content of various feed ingredients used in organic poultry production and to determine BET digestibility in selected feed ingredients.

MATERIALS AND METHODS

Experimental protocols were approved by the University of Georgia (UGA) Institutional Animal Care and Use Committee (UGA Animal Care and Use A2015 09-011-

Y1-A0) (Athens, Ga). Two hundred fifty-six day old Cobb 500 males chicks were allotted to 8 birds per pen, to Petersime battery brooder cages (Petersime Incubator Co., Gettysburg, OH, USA). Eight pens per treatment. Chicks were evenly split between two trials. All chicks were fed a common starter diet without antibiotics for 1-10 d (Table 6-1). On d 10 diets were changed to the test diets (Table 6-2). Water and feed were available *ad libitum* during the entire experiment. On d 10 birds were switched to BET digestibility diets. BET digestibility diets were fed from 10-14 d (Garcia et al., 2000). The room was kept at a constant temperature of 27.5°C throughout the entire experiment. Battery brooder heaters were set to a temperature of 30°C for one week. After the end of the first week, brooder heaters were turned off.

Feed ingredients and Diets

The flaxseed, oats, canola meal, sorghum, field peas (FP), millet, barley, sunflower meal (SFM), and an alfalfa meal (1) came from Organics Unlimited Inc (Organics Unlimited Atglen, PA, USA). Soybean meal (1) (SBM), wheat middlings, hominy, and a second alfalfa meal (2) came from Herbrucks Poultry Farm (Saranac, MI, USA). A second SBM (2) and corn were sourced from Cargill Feed and Nutrition (Mineapolis, MN, USA).

Ingredients that had a measurable amount of BET and were in large enough quantities to run a chick digestibility trial were barley, millet, and SFM. Test diets were formulated according to protein content (Table 6-2). Reference diet (dextrose) contained no protein and no BET and was used to determine endogenous losses of BET. Sunflower meal was formulated to contain 15% crude protein. Low protein ingredients, barley, and millet contained 77% of the feed ingredient in the final diet. TiO_2 was added at 0.3%, of the diet to determine the digestibility of BET. TiO_2 was analyzed using the method Myers et al. (2004).

Millet was ground through a 2mm screen (Wiley Mill Thomas Scientific, Swedesboro, NJ, USA). Barley was ground through a 5mm screen to crack the hull. Sunflower meal came pre-ground and was not additionally ground. Diet composition is shown in Table 6-1. Diets were formulated to meet or exceed the starter requirement of Cobb 500 broilers (Cobb 500 Management guide).

Collection of ileal samples

On d 14, birds were euthanized by CO_2 asphyxiation. Immediately after euthanizing, entire ileal contents, from the Meckel's diverticulum to the beginning of the cecum were collected and pooled per pen (Garcia et al., 2007). Ileal samples were frozen and freeze-dried (Labconco, Labconco Corporation, Kansas City, MO, USA). Endogenous losses were determined using a diet with no betaine, (reference diet, 0 mg/kg BET).

Betaine analysis

Feed ingredients, diets, and ileal samples were analyzed for BET using the method of Hefni et al. (2016). All feed ingredients were ground through a 1 mm screen before analysis (Wiley Mill Thomas Scientific, Swedesboro, NJ, USA). Ileal samples were ground and homogenized using a mortar and pestle.

The extraction procedure for BET analysis was modified from Hefni et al. (2016): Instead of the sample in a final volume of 5 mL of water, a final volume of sample in 3 mL of water was used. Also, if BET content was found to be more than 500 mg/kg, then samples were re-extracted with a total of 4 extractions, instead of the two extractions, with an increase in the final volume of 6 mL with the sample was used (Table 6-4). This was done for alfalfa meal (1), millet, millet diet, and wheat middlings.

The analysis was performed using high performance liquid chromatography (HPLC) (Phenomenex Incorporated Luna Torrance CA, USA). The mobile phase was 94% acetonitrile with 14.3 mM triethylamine and 30mM of succinic acid. The column dimensions were 5µM strong cation exchange 100Å 150 *x* 4.6 mm (Hefni et al., 2016). The flow rate was 1.0 ml min^{-1} column temperature was set to 40°C. Betaine was measured using UV vis with a wavelength of 249 nm. The linear range for betaine was 0-3000µM, with a lower limit of detection 50µM.

BET digestibility was determined using the equation: (Stein et al., 2007)

Standardized ilea BET digestibility = apparent ileal digestibility + [(basal BET $_{endogenous}$ / BET $_{diet}$) x 100]

Statistical analysis

Data were analyzed using SAS Proc GLM, with diet as a class variable (SAS Institute 9.4). There was a test for a diet by trial interaction. There was no diet by trial interactions; therefore, the interaction was removed from the analysis. Differences between ingredients were determined by Tukey's multiple range test. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Flaxseed, field peas, SBM (1), hominy, corn, and alfalfa meal (2) had no detectable BET (Table 6-3). Previous studies have also reported no detectable BET in corn (Eklund et al., 2005, Chendrimade et al., 2002). A previous study found BET in trace amounts of 2 mg/kg in corn grain (Storey and Jones, 1975). No studies have reported BET in hominy, other corn grain byproducts such as corn gluten meal have been determined to have no BET, as well (Eklund et al., 2005, Chendrimada et al., 2002). Hominy is corn grain byproduct, so it

is reasonable to assume that hominy would contain no BET, such as corn gluten meal. When corn was genetically manipulated to accumulate BET, BET would only accumulate under high salt conditions over a long period of time in the leaves. Other plant tissues such as the corn kernel were not analyzed for BET (Peel et al., 2010). Betaine can accumulate in different tissues of the same plant at different rates (Storey and Jones, 1975).

Oats, SBM (2), and canola meal had trace levels of BET detected of 1 mg/kg (Table 6-3). Trace amounts of BET could be due to bin contamination, the cultivar variety grown, or growing conditions. There have been no previous studies that found BET in SBM. Eklund et al. [1] reported 2 SBM samples with no detectable BET. Eklund et al. (2005) also found no detectable levels of BET in rapeseed meal, as well. Canola meal is similar to rapeseed meal; however, plant and BET metabolism in canola could be different than in rapeseed. Eklund et al. (2005) reported 590 mg/kg of BET in oats. Oats in this study only had trace levels of BET (Table 6-3). Servillo et al. (2018) found that oats contained 53 mg/kg of BET. Oats in this study could be low in BET due to growing conditions that did not allow for BET accumulation. Hefni et al. (2018) found that BET in oats can range from 28-44 mg/100g. Low levels of BET in oats is not unexpected. This study agrees with previous studies that suggest BET can vary in oats by over a factor of ten. This is supported by Sima et al. (2013) where there was an observed increase of BET in forage millet by over a factor of ten when the same forages were grown in high salt conditions compared to no salt conditions.

Wheat middlings contained 4874 mg/kg of BET (Table 6-3). This is within the range of reported by Chendrimada et al. (2002) which ranged from 4440-5750 mg/kg. However, BET values from this study and Chendrimada et al. (2002) are higher than wheat middlings from Likes et al. (2007) who report BET in wheat middlings of 3607 mg/kg. The wheat

middlings in this was within the range of Eklund et al. [1], their samples ranged from 2675-4980 mg/kg. Wheat and wheat byproducts can be highly variable in BET content, and this could be due to growing conditions or cultivars used. All wheat and wheat by-product do appear to contain BET.

There is considerable variation in the BET content of alfalfa and alfalfa meal [13, 35]. Plants that are not grown under drought or salt stress conditions, BET may not be produced Sakamoto and Murata, 2002). The two kinds of alfalfa meal in this study that were highly variable. Alfalfa meal (1) from Herbrucks Poultry Farm in Michigan, USA had a BET content of 759±67 mg/kg, and alfalfa meal (2) from Organics Unlimited Atglen, Pennsylvania, USA had no detectable BET. In another study, BET levels in alfalfa meal ranging from 1770-3850 mg/kg (Eklund et al., 2005). Clearly BET in alfalfa meal is highly variable and average values of BET in feed formulation would not be useful.

Alfalfa plants are known to utilize different compounds during drought stress including proline, proline BET, and trigonelline; all have a similar purpose as BET in plants (Sim et al., 2013, Wood et al., 1991). Depending on the cultivar used, BET may not be the primary compound utilized by the plant during salt stress. Growing conditions were not known for each alfalfa meal used in this study. Betaine levels in alfalfa meal are variable and should be measured when considering alfalfa meal as a BET source. Wood et al. (1991) measured fresh alfalfa leaves of different cultivars grown in the same greenhouse, BET ranged from 249-2201 nmol of BET. These samples are on a wet matter basis, not a dry matter basis, so that BET would be less concentrated per gram of wet sample per gram than dry samples. There is a lot of variation in BET in alfalfa depending on the cultivar used, even when growing conditions are the same.

Grains sorghum, barley, and millet had BET contents of 32, 399, 1069 mg/kg respectively (Table 6-2). Sorghum is a drought tolerant plant, having measurable BET in sorghum would be expected. However, studies have shown that depending on cultivar there may or may not be measurable BET in sorghum [10]. Kidd et al. [39] reported a barley BET content of 730 mg/kg which is higher than the organic sample in this study (Table 6-2). However, Servillo et al. (2018) found 255 mg/kg BET in a barley sample, less than the barley sample in Table 6-2. Henfi et al. (2018) reported Swedish barley BET content ranging from 40-100mg/100g.The BET concentration in barley can vary greatly, and betaine can increase in barley under drought stress (Hattori et al., 2009). No studies were found that reported values of BET for millet grain, however, a study by Sima et al. (2013) found that forage millet leaves can contain up to 300 mmol/ gram of BET grown in high salt conditions.

No previous studies to determine BET digestibility in feed ingredients. The present study found BET digestibility was highest in millet with a bet digestibility of 95.7 ± 0.4%. Sunflower meal had a BET digestibility of 67.4 ± 8.4%. Barley had the lowest barley digestibility of 43.7 ± 12.1% (Table 6-5).

The endogenous contribution of BET needs to be accounted for if BET digestibility is to be determined in broiler diets. The diet to determine endogenous losses did not have any measurable BET; however, there was measurable BET in the ileal contents of 6.4 mg/kg. This suggests that there is betaine in gastrointestinal secretions or sloughed cells or the gastrointestinal bacteria have the ability to change nutrients in the digestive tract (Holmstom et al., 1994). There were no betaine or amino acids in the endogenous loss diet; however, there was choline chloride, and this choline could have been oxidized to BET. However, this specific type of reaction has not been documented in poultry, these gastrointestinal conditions

exist and are studied in ruminants (McAllister et al., 1996). Bacteria can change choline into BET. It may be valuable to for bacteria to change compounds such as choline into BET since BET has properties like being more oxidized than choline, reducing the effects of osmotic stress, in addition to being a labile methyl donor.

Betaine metabolism in plants is complex. BET in plants not only depends on growing conditions but also depends on enzymes that convert choline or glycine into BET. Experimentally there are crops, such as corn, being developed that have a BET pathway (Peel et al., 2010). Betaine is not typically in corn grain, however, depending on economics and growing conditions, these cultivars of corn could be used. Increased BET in corn and sorghum may reduce total choline in these plants indicating that BET may be made from choline (Peel et al., 2010). In order to be valuable in feed formulation, either cultivars with consistent levels of betaine need to be developed, or methods to measure BET in ingredients at the feed mill need to be developed.

CONCLUSIONS AND APPLICATIONS

1. BET is highly variable in feed ingredients used in organic poultry (and other species) production.
2. BET digestibility is highly variable in feed ingredients.
3. Endogenous losses in broilers contain betaine and must be accounted when determining digestibly.

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Table 6-1. Common starter diet to chicks fed from 1-10 day

Ingredients	Percent
Corn	56.125
Soybean meal	37.50
Soybean oil	3.07
Limestone	0.73
Salt	0.30
Defluorinated phosphate	1.75
Trace mineral premix[1]	0.075
Vitamin premix[2]	0.25
DL methionine	0.20

[1] Supplied per kilogram of diet: Mn, 107.2 mg; Zn, 85.6 mg; Mg, 21.44 mg; Fe, 21.04; Cu, 3.2 mg; I, 0.8 mg; Se, 0.32 mg

[2] Supplied per kilogram of diet: vitamin A, 110229 IU; vitamin D_3, 22000 IU; Vitamin E, 220 IU; .25 mg vitamin B_{12}, 5 ppm; Biotin, 0.11 mg; Menadione, 1.1 mg; Thiamine, 2.21 mg; Riboflavin, 4.41 mg; d-Pantothenic, Acid, 11.02 mg; Vitamin B_6, 2.21 mg; Niacin, 44.09 mg; Folic Acid, 0.011 mg; Choline 38 mg.

Table 6-2. Composition of diet used in the betaine digestibility of select feed ingredients fed from 10-14 days[3]

Ingredient, %	Dextrose	Millet	Sunflower meal	Barley
Dextrose	92.85	16.93	34.63	17.24
Soybean oil	2.00	2.00	2.00	2.000
Salt	0.50	0.50	0.50	0.50
Vitamin premix[1]	0.80	0.80	0.80	0.80
Mineral premix[2]	0.25	0.25	0.25	0.25
Dicalcium phosphorus	2.40	1.80	1.50	1.49
Limestone	0.90	1.14	0.74	1.14
TiO_2	0.30	0.30	0.30	0.30
Oats	0	0	0	0
Flaxseed	0	0	0	0
Field Peas	0	0	0	0
Millet	0	77.00	0	0
Alfalfa meal	0	0	0	0
Canola Meal	0	0	0	0
Sunflower Meal	0	0	60.00	0
Sorghum	0	0	0	0
Barley	0	0	0	77.00
Analyzed value, mg/kg				
Betaine	0	570	117	134
Calculated value, %				

Crude protein, %	0.00	12.76	15.00	9.72

[1] Supplied per kilogram of diet: vitamin A, 110229 IU; vitamin D_3, 22000 IU; Vitamin E, 220 IU; .25 mg vitamin B_{12}, 5 ppm; Biotin, 0.11 mg; Menadione, 1.1 mg; Thiamine, 2.21 mg; Riboflavin, 4.41 mg; d-Pantothenic, Acid, 11.02 mg; Vitamin B_6, 2.21 mg; Niacin, 44.09 mg; Folic Acid, 0.011 mg; Choline 38 mg.

[2] Supplied per kilogram of diet: Mn, 107.2 mg; Zn, 85.6 mg; Mg, 21.44 mg; Fe, 21.04; Cu, 3.2 mg; I, 0.8 mg; Se, 0.32 mg

[3] 8 replicates per diet fed

Table 6-3. Total betaine content of organic feed ingredients fed from 10-14 d[1]

Ingredient	Betaine, mg/kg	Standard deviation
Oats	1	N/A
Flaxseed	Not detected	N/A
Field peas	Not detected	N/A
Millet	1069	120
Soybean meal (1)	Not detected	N/A
Soybean meal (2)	1	N/A
Wheat middlings	4874	123
Hominy	Not detected	N/A
Corn	Not detected	N/A
Alfalfa meal (1)	759	67
Alfalfa meal (2)	Not detected	N/A
Canola meal	1	N/A
Sunflower meal	337	82
Sorghum	32	12
Barley	399	86
Dextrose	Not detected	N/A

1. All samples were run as triplicates

Table 6-4. Extraction of betaine in feed ingredients[1]

Ingredient	Sample amount (g)	Volume of water (mL)	Number of extractions	Betaine per extraction	Total Betaine (mg/kg)
Wheat	0.2±0.1	2	5	3553±52	4804±52
Middlings		1		821±7	
		1		419±5	
		1		11±2	
		1		~1	
Alfalfa	0.2±0.1	2	5	514±29	756±29
meal		1		173±14	
		1		67±12	
		1		2±1	
		1		~1	
Soybean	0.2±0.1	2	3	~1	~1
Meal		1		Not detected	
		1		Not detected	

Oats	0.2±0.1	2	3	~1	~1
		1		Not detected	
		1		Not detected	
Sorghum	0.2±0.1	2	3	28±7	31±7
		1		3±1	
		1		Not detected	

[1] Values are means of triplicate analysis

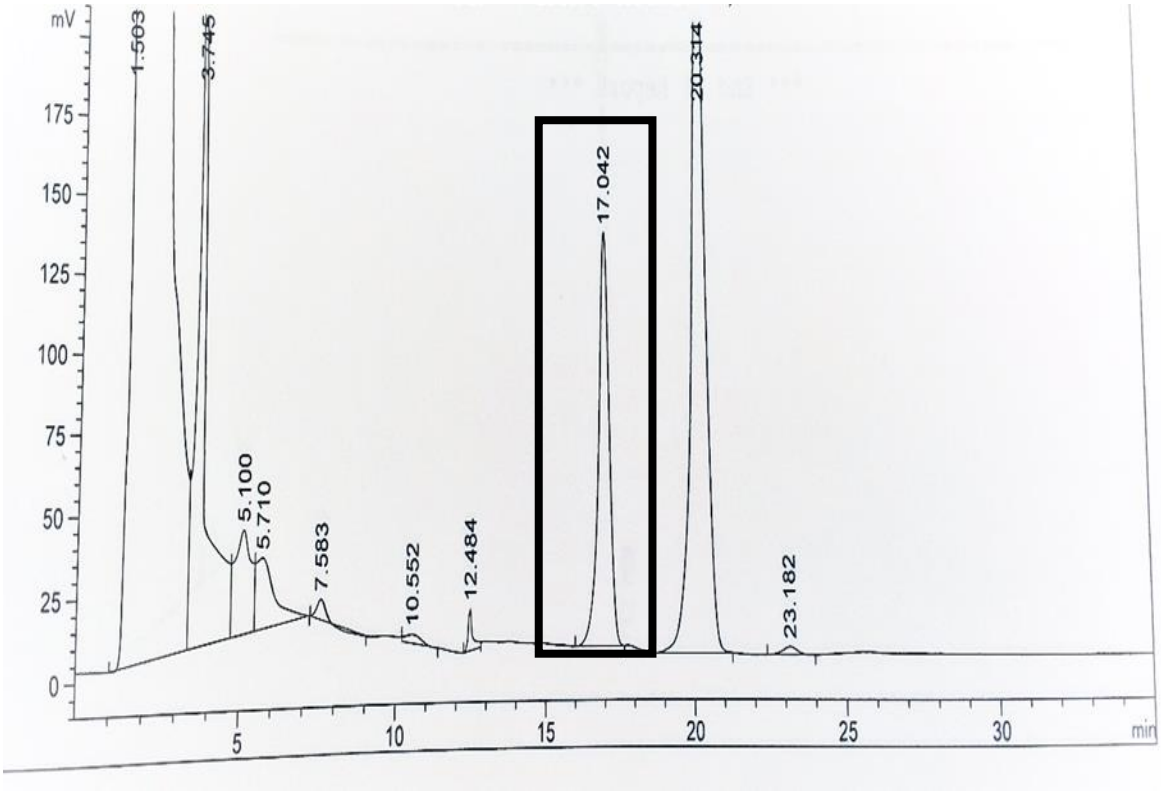
Table 6-5. Standardized ileal digestibility of betaine in selected feed ingredients

Diet[3]	SID[1,2]	Standard Error
Millet	95.7[a]	0.4
Sunflower meal	67.4[b]	8.4
Barley	43.7[c]	12.1

[1] Means in a column not sharing a common superscript are different ($P < 0.05$)

[2] SID = standardized ileal digestibility

[3] Each value represents 8 replicates



[1] Betaine peak at 17.0 minutes is shown in the black box

Figure 6-1. Betaine chromatograph of 2000 μM [1]

CHAPTER 7

DETERMINATION OF STANDARDIZE ILEAL DIGESTIBILITY OF ORGANIC FEED INGREDIENTS IN BROILER CHICKS IN

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SUMMARY

The organic poultry industry is a growing segment in the United States. There is limited research on how to grow poultry that is to be marketed as organic. Determining digestibility is important to feed poultry efficiency. Protein is an important macronutrient for poultry. Determining the amino acid digestibility of organic feed ingredients is needed to feed poultry. Standardized ileal digestibility was highest for field peas and millet, and lowest for flaxseed. Correlations have been observed between levels amino acids and digestibility, in ingredients databases. This same correlation was seen in this study. Total amino acid profiles for the organic feed ingredients were generally lower than previously established book values for organic feed ingredients. However, there was a better agreement between organic feed ingredients in this study and previously established organic feed ingredients. In conclusion, the standardized ileal digestibility of organic feed ingredients is highly variable between feed ingredients.

DESCRIPTION OF PROBLEM

Organic food production is increasing in the United States (USDA, 2017). Broiler chickens are the largest segment of the meat sector with $749 million in revenue in 2016 (USDA, 2016, Fanatico et al., 2009). Organic livestock needs to be grown under different conditions than conventionally raised livestock, and this can cause different ingredients to be used in diets. There are multiple restrictions on what kinds of ingredients can be incorporated into organic diets for poultry. Feed ingredients for organic production cannot be classified genetically modified (Coffey and Bair, 2012). These diets are typically limited in the amino acid (AA) methionine, and synthetic sources of methionine are limited due to organic regulations (Burley et al., 2015, Burley et al., 2015). Organic feed ingredients can be combined in such a way to meet the methionine

requirement of poultry. Alternative grain and protein sources can be used to supply more methionine than a typical corn and soybean meal basal blend diet.

There are many reasons to choose different feed ingredients when sourcing ingredients for a diet. Canola meal has been fed to conventional-raised poultry, despite the known antinutritional factors (Khajali and Slominski, 2012). The methionine content of canola meal could make canola meal a source of methionine for organic production. Flaxseed can be used in both conventional and organic diets to increase omega-3 fatty acids in meat and eggs to make these foods more nutritious for the consumer (Jacob, 2007, Lee et al., 1995). Alfalfa meal can be used in layer diets as a natural pigment enhancer for the yolk (Sullivan and Holleman, 1962). Sorghum and millet can be used as an alternative to corn when feeding livestock and regionally may be more economical than corn (USDA, 2016). Sunflower meal can be fed to livestock in place of soybean meal since sunflower meal has higher methionine than soybean meal, this is needed for organic diets (NRC, 2012).

Digestibility data of organic feed ingredients is limited (Presto et al., 2015). Organic feed ingredients may not be the same as conventionally grown feed ingredients. This could be due to differences in cultivar used, fertilizer, or herbicide use. Research needs to be performed to determine if organic feed ingredient digestibility is similar to previously established values of corresponding feed ingredients. The objective of this study is to determine the amino acid standardized ileal digestibility of various organic feed ingredients.

MATERIALS AND METHODS

Experimental protocols were approved by the University of Georgia (UGA) Institutional Animal Care and Use Committee (UGA Animal Care and Use A2015 09-011-Y1-A0) (Athens, Ga).

Six-hundred and forty day-old Cobb 500 males chicks were allotted, eight birds per pen, to Petersime battery brooder cages (Petersime Incubator Co., Gettysburg, OH, USA). Chicks were evenly split between two trials. All chickens were fed a common starter diet without antibiotics for 1-10 days (Table 7-1). On day 10 diets were changed to the test diets. Test diets were fed from 10-14 days. Water and feed were available *ad libitum* during the entire experiment. (Table 7-3) The room was kept at a constant temperature of 27.5°C during the entire experiment. Battery brooder heaters were set to a temperature of 30°C for one week. After the end of the first week, brooder heaters were turned off.

Diets

Feed ingredients were obtained from Organics Unlimited (Atglen, PA, USA). Unground feed ingredients, alfalfa meal, canola meal, and sunflower meal, were ground before diet mixing, Flaxseed, field peas, millet, and sorghum were ground through a 2mm screen Wiley Mill Thomas Scientific, Swedesboro, NJ, USA. Oats and barley were ground through a 5mm screen to crack the hull on the oats and barley. Alfalfa meal, canola meal, and sunflower meal were pre-ground and were not additionally ground. Diet composition is shown in Table 7-3.

Test and reference (dextrose) diets were formulated according to protein content (Table 7-3). High protein feed ingredients canola meal, flaxseed, sunflower meal, field peas, and alfalfa meal were formulated to contain 15% crude protein. Low protein feed ingredients oats, barley, millet, and sorghum contained 77% of the feed ingredient in the final diet. TiO_2 was added at 0.3%, in the diet as an indigestible marker to determine the digestibility of the amino acid (Myers et al., 2004).

Collection of ileal samples

On day 14 birds were euthanized by CO_2 asphyxiation. Immediately after killing entire ileal contents, from the Meckel's diverticulum to the beginning of the cecum were collected and pooled by pen (Garcia et al., 2007). Ileal samples were frozen, and freeze-dried (Labconco, Labconco Corporation, Kansas City, MO, USA). Endogenous losses were determined using a diet an amino acid free diet. Endogenous losses were calculated with the dextrose-based reference diet.

Amino acid analysis

All feed ingredients were ground through a 1 mm screen before analysis [16]. Ileal samples were ground and homogenized using a mortar and pestle. Feed ingredients, diets, and ileal samples were analyzed by University of Missouri – Columbia, Office of the Missouri State Chemist, Analytical Services (Table 7-2 and 7-4).

Standardized ileal amino acid digestibility was determined using the equation Stein et al., 2007:

Standardized AA digestibility = apparent ileal digestibility + [(basal $AA_{endogenous}$ / AA_{diet}) x 100]

Statistical analysis

Data were analyzed using SAS Proc GLM, with diet as a class variable (SAS Institute, Cary, NC, USA). There was a test for a diet by trial interaction. There was not a diet by trial interaction, so the interaction was removed from the analysis. Differences between ingredients were determined by Tukey's studentized test. Differences were considered significant at $p < 0.05$.

A previous meta-analysis by Tahir and Pesti (2012) indicated a relationship between amino acids and SID. The data in this study were analyzed to determine if the relationship between amino

acids and SID is similar to previous studies by Tahir and Pesti (2012). The models for as-fed and total amino acids concentrations and digestibilities: Slopes for AA digestibility = f (AA concentration) were found for each AA in each ingredient. The models were then fitted, with AA digestibility = *f* [AA concentration, kind of AA, ingredient, and 2- and 3-way interactions].

RESULTS AND DISCUSSION

There are only two studies about the composition and digestibility of organic feed ingredients (Jacob 2007, Presto et al., 2011). A Swedish study of organic rapeseed had lower crude protein and overall lower amino acid values compared to the organic canola meal of this study (Presto et al., 2011). The digestibility of canola meal in this study is similar to a sample to a sample in Lee et al. (1995); measured with ceacomized roosters, while the current study used a broiler-chick assay. A study by Presto et al. (2011), found that SID digestibility of organic rapeseed in pigs was the lowest when compared to other protein sources of organic hemp seed, linseed, faba beans, and rapeseed. The SID digestibility of organic, (Swedish) rapeseed was 81.7% in pigs, which is higher than the sample in Table 7-5 with a SID digestibility of 79.5% in organic canola meal (Presto et al., 2011).

The crude protein of barley in this study was 10.1% which was lower than the range reported in Bandegan et al. (2011). Bandegan et al. (2011) reported the range for crude protein of barley was between 12.1-18.0%. All the amino acids in this study were lower than the individual amino acids reported by Bandegan et al. (2011). The barley in that study was conventionally grown barley. Jacob (2007) found organic barley crude protein ranged from 9.9%-15.7%, the barley in Table 7-2 is within the same range.

Barley digestibility in this study is similar to a previous study by Bandegan et al. 2011. In that study crude protein SID ranged from 74-83%, while the current study found CP SID of barley

was 75.3% (Table 7-5). All the individual essential amino acids SID agreed with Bandegan et al. (2011) except for isoleucine which had a lower SID of 75.6% compared to the 77-87% from Bandegan et al. (2011). The non-essential amino acids, alanine, aspartate, cysteine, glycine, and serine had lower SID values in the samples studied here than Bandegan et al. (2011) (Table 7-5). Glutamate (Table 7-5) had higher SID than reported in Bandegan et al. (2011) with SID glutamate of 93.1% in the current study compared to a range of 83- 91% in Bandergan et al. (2011).

Millet had the highest amino acid SID when compared to all other feed ingredients (Table 5). Sorghum digestibility in this study was significantly lower ($P < 0.05$) than millet; however, sorghum digestibility was still high with an average SID value of 79.7%. Millet was more digestible than sorghum (Table 7-5), and this is similar to a previous study by Batonon-Alvao et al. (2016), which showed similar results that crude protein digestibility of millet was consistently higher than crude protein digestibility of sorghum. Sorghum digestibility can be highly variable by year, as shown by Perez-Maldonado and Rodrigues [26] apparent digestibility from one year to the next ranged from 65% to 77%. This indicates that sorghum digestibility is highly variable depending on growing conditions.

The crude protein of flaxseed in this study was higher than crude protein of flaxseed as reported by Bandergan et al. (2011) (21.8% vs. 20.1). The flaxseed studied by Bandergan et al. (2011) was conventionally raised. The flaxseed in Jacob (2007) was similar to other organically grown flaxseeds with a crude protein range of 23.13-18.34%.

The flaxseed in Table 7-5 was full-fat ground flaxseed, and all amino acids in flaxseed had low SID. Low digestibility of flaxseed has been reported in young birds (Gonzalez-Esquerra and Leeson, 2000), this is similar to the observation that is seen in a study where amino acid SID was lowest in flaxseed. Gonzalex-Esquerria and Leeson (2000) also reported lower overall digestibility

when feeding full-fat flaxseed as a mash, compared to pellet or crumbled diets. A growth performance study supplementing increasing levels of flaxseed from 0-10% of flaxseed inclusion showed that increasing flaxseed decreased growth in broilers (Mridula et al., 2015). The amino acid digestibility of a flaxseed sample was higher in Lee et al. (1995) than in the current study. A study by Jia and Slominski (2010) found that adding pelleting and exogenous enzymes increased digestibility of flaxseed in broilers. However; this would not be permitted in organic production. The same study found no effect of grinding the flaxseed on digestibility (Gonzalex-Esquerria and Leeson, 2000). Flaxseed in the current study (Table 7-5) was ground, and no other processing was done. The SID flaxseed in this study was lower than the SID of flaxseed reported by Bandegan et al. (2011), with SIDs ranging from 50-59%, while the current study had an average SID of 36.5%.

The average sunflower meal digestibility was 79.9%, showing that sunflower meal is a relatively digestible feedstuff. Sunflower meal is a good protein source and can be used to reduce the inclusion of soybean meal (Rama Roa et al., 2006). Previous reports show that sunflower meal has higher methionine than soybean meal, so the inclusion of sunflower meal could be helpful in organic production where methionine supplementation is needed (Burley et al., 2015).

A study by Han and Parsons (1990) found that alfalfa meal average standardized amino acid digestibility in cecectomized roosters was 67.6%. Very similar to a current study with an average SID amino acids value of 67.9%. Higher amino acid digestibility in Liang et al. (2015) was for a sample in growing pigs.

The SID of field peas in Table 7-5 was the highest compared to all the protein sources (Table 7-5). Farrell et al. (1999) fed field peas and different legumes to broilers. The field peas had the highest feed conversion ratio, body weight, and feed intake compared to faba beans and lupins. The inclusion of field peas did not affect eggs per hen. Field peas do not affect poultry

performance, and if determined to be an economical feed ingredient could be incorporated into organic diets as an alternative protein source (Perez-Maldonado, 1999). The SID of field peas in pigs was 81%, which is lower than in Table 7-5 with a SID of 87% (Cavigelli et al., 2009) (Table 7-5). The SID of field peas in broilers is similar to Bandegan et al. (2011). The samples studied by Bandegan et al. (2011) the SID of field peas ranged from 86-89%, and the current study (Table 7-5) had a SID of 87%. The crude protein of field peas in this study was 19.7%, which is lower than reported values of Bandegan et al. (2011) which reports samples of field pea crude protein that ranged of 20.6-26.3%. As expected, individual amino acids in field peas in Table 7-5 were lower than reported amino acid values of field peas in Bandegan et al. (2011). The field pea crude protein value was within the range reported by Jacob (2007), which were reported to be 19.1-26.4%.

Feed ingredients in this study typically had lower amino acid content compared to conventionally raised feed ingredients (Jacob, 2007, NRC, 1994, Preso et al., 2011, Bandegan et al., 2011). This could be due to growing conditions that are different in organic and conventional systems. Organic production is restricted to the kinds of fertilizer and herbicide used; this could cause plants not to reach their genetic potential (Cavigelli et al., 2009). Figure 2 shows the relationship of the amino acid profile of feed ingredients used in this study compared to the 1994 NRC. A slope of 1.35 indicates that NRC feed ingredients have greater amino content (Figure 7-2). The NRC has conventionally grown feed ingredients. When comparing the feed ingredients in this study to organic feed ingredients analyzed by Jacob (2007) they are similar. This indicates that organic feed ingredients are more similar to each other than to conventional feedstuffs.

A meta-analysis by Tahir and Pesti (2012) that demonstrated the relationship between amino acid in the diet and digestibility of that amino acid in two commonly used amino acid databases. A significant ($P<0.001$) correlation between amino acid level and SID of 0.152. Even

though this relationship is weak it is significant. Figure 7-1 shows a relationship of amino acid in the diet on the x-axis versus the digestibility of that amino acid on the y-axis. This shows that there is a positive relationship between amino content versus digestibility in an individual study (Figure 7-1), which affirms that the conclusions of Tahir and Pesti (2012) are not limited to database values. The phenomena that increasing amino acid content increases digestibility can be applied to individual studies as well. This was even seen with the ingredient flaxseed, which had the lowest digestibility compared to all of the feed ingredients (Table 7-5). Even with the low digestibility of flaxseed compared to the other feed ingredients, amino acids with higher amino acid level have higher digestibilities. This phenomenon is the result of so called "digestibility" assays really measuring the functions of digestion and absorption. The contribution of difference in absorption on SID values has yet to be determined. This would be expected if different proteins with different digestibilities had different amino acid levels.

Kind of amino acid could have an impact on digestibility as well as the type of feed ingredient. Table 7-6 where glutamic acid is the amino acid present in the highest quantity, and the highest digestibility. The branched-chain amino acid, leucine, is typically present in higher quantities than other branched-chain amino acids and has the highest digestibility (Table 7-6). Branched-chain amino acids are known to antagonize each other (Chalova et al., 2016). This demonstrates that amino acids are indeed absorbed according to Michaelis-Menten kinetics (1913). This is also seen with arginine and lysine antagonism; arginine is present in greater amounts than lysine and arginine digestibility is generally greater than lysine digestibility.

Organic feed ingredients can be chosen to meet the requirements for the animals while complying with organic production regulations. Organic crops can be grown for their low environmental impact. However, growing alternative plant proteins for poultry production does

not appear to reduce the global warming potential, so growing alternative plant proteins to minimize environmental impact may not be necessary (Leinonen et al., 2013).

Diets should be formulated to best meet the requirement of the bird; however, due to organic regulations, this can be difficult. Restrictions can cause increased nitrogen in the diet, that is then the excreted by the bird. Excess nitrogen can leach into the water and cause pollution. Reducing the total amount of nitrogen in organic poultry diets, while maintaining enough methionine to meet the requirement of the bird is difficult, to accomplish due to organic regulations (NRC, 2012).

CONCLUSIONS AND APPLICATIONS

1. The amino acid content of organic feed ingredients are more similar to each other than to amino acids from established works like the NRC. Millet and field peas had the greatest digestibility, while flaxseed had the lowest digestibility.
2. Digestibility of organic feed ingredients is highly variable. Further research needs to be done to establish more digestibly values for organic feed ingredients.
3. This study demonstrates previous works that amino acid content and digestibility are correlated.

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Table 7-1. Common starter diet feed to chicks from day 1-10

Ingredients	Percent
Corn	56.125
Soybean meal	37.50
Soybean oil	3.07
Limestone	0.73
Salt	0.30
Defluorinated phosphate	1.75
Trace mineral premix[1]	0.008
Vitamin premix[2]	0.25
DL methionine	0.20

[1] Supplied per kilogram of diet: Mn, 107.2 mg; Zn, 85.6 mg; Mg, 21.44 mg; Fe, 21.04; Cu, 3.2 mg; I, 0.8 mg; Se, 0.32 mg

[2] Supplied per kilogram of diet: vitamin A, 110229 IU; vitamin D_3, 22000 IU; Vitamin E, 220 IU; .25 mg vitamin B_{12}, 5 ppm; Biotin, 0.11 mg; Menadione, 1.1 mg; Thiamine, 2.21 mg; Riboflavin, 4.41 mg; d-Pantothenic, Acid, 11.02 mg; Vitamin B_6, 2.21 mg; Niacin, 44.09 mg; Folic Acid, 0.011 mg; Choline 38 mg.

Table 7-2. Amino acid composition of organic feed ingredients[1]

	Barley	Canola meal[2]	Field Peas	Alfalfa meal	Sorghum	Sunflower meal[2]	Oats	Flaxseed[3]	Millet
Indispensable									
Arginine	0.52	1.48	1.53	0.77	0.33	1.60	0.54	1.97	0.61
Histidine	0.25	0.74	0.49	0.41	0.21	0.59	0.19	0.50	0.32
Isoleucine	0.33	0.87	0.70	0.61	0.31	0.79	0.27	0.75	0.45
Leucine	0.70	1.80	1.38	1.20	1.12	1.47	0.62	1.21	1.40
Lysine	0.43	1.45	1.41	0.95	0.24	1.02	0.41	0.88	0.42
Methionine	0.19	0.53	0.21	0.27	0.17	0.50	0.15	0.38	0.28
Phenylalanine	0.50	0.95	0.86	0.75	0.42	0.91	0.41	0.92	0.63
Threonine	0.34	1.09	0.70	0.69	0.29	0.87	0.28	0.75	0.40
Tryptophan	0.11	0.38	0.18	0.23	0.08	0.32	0.09	0.36	0.16
Valine	0.47	1.16	0.82	0.81	0.40	1.00	0.39	0.96	0.58
Dispensable									
Alanine	0.44	1.18	0.86	0.88	0.79	1.05	0.42	0.98	1.13
Aspartic Acid	0.65	1.93	2.14	2.41	0.60	2.02	0.69	1.96	0.95
Cysteine	0.21	0.57	0.27	0.20	0.15	0.40	0.23	0.34	0.20
Glutamic Acid	2.28	4.19	3.12	1.77	1.69	4.07	1.61	3.85	2.60
Glycine	0.43	1.28	0.88	0.78	0.30	1.36	0.43	1.24	0.44
Proline	1.06	1.54	0.78	1.23	0.68	1.04	0.42	0.75	0.86
Serine	0.41	1.06	0.85	0.71	0.38	0.98	0.39	0.95	0.74
Tyrosine	0.31	0.76	0.63	0.58	0.33	0.58	0.27	0.54	0.43
Crude Protein	10.1	24.7	19.7	18.7	8.2	25.1	8.75	21.8	13.3

[1]Samples were analyzed by University of Missouri – Columbia, Office of the Missouri State Chemist, Analytical Services

[2] Expeller pressed

[3] Full-fat

Table 7-3. Diet composition of reference and organic feed ingredients diets fed to broilers between 10-14 days

Ingredient, %	Dextrose	Oats	Flaxseed	Field Peas	Millet	Alfalfa Meal	Canola Meal	Sunflower meal	Sorghum	Barley
Dextrose	92.85	17.27	25.15	17.57	16.93	15.38	34.45	34.63	17.03	17.24
Soybean oil	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.000
Salt	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix[1]	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Mineral premix[2]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Dicalcium phosphorus	2.40	1.54	1.82	2.27	1.80	1.27	1.30	1.50	1.75	1.49
Limestone	0.90	1.06	1.06	0.89	1.14	0.00	0.37	0.74	1.09	1.14
TiO_2	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Oats	0	77.00	0	0	0	0	0	0	0	0
Flaxseed	0	0	68.81	0	0	0	0	0	0	0
Field Peas	0	0	0	76.14	0	0	0	0	0	0
Millet	0	0	0	0	77.00	0	0	0	0	0
Alfalfa Meal	0	0	0	0	0	80.21	0	0	0	0
Canola Meal	0	0	0	0	0	0	60.75	0	0	0
Sunflower Meal	0	0	0	0	0	0	0	60.00	0	0
Sorghum	0	0	0	0	0	0	0	0	77.00	0

Barley	0	0	0	0	0	0	0	0	0	77.00
Analyzed value										
Crude Protein	0.45	7.08	15.77	15.22	10.52	15.24	15.93	14.55	6.79	8.34
Calculated value, %										
Crude protein, %	0.00	8.47	15.00	15.00	12.76	15.00	15.00	15.00	7.87	9.72

[1] Supplied per kilogram of diet: vitamin A, 110229 IU; vitamin D_3, 22000 IU; Vitamin E, 220 IU; .25 mg vitamin B_{12}, 05 ppm; Biotin, 0.11 mg; Menadione, 1.1 mg; Thiamine, 2.21 mg; Riboflavin, 4.41 mg; d-Pantothenic, Acid, 11.02 mg; Vitamin B_6, 2.21 mg; Niacin, 44.09 mg; Folic Acid, 0.55 mg; Choline, 38 mg.

[2] Supplied per kilogram of diet: Mn, 107.2 mg; Zn, 85.6 mg; Mg, 21.44 mg; Fe, 21.04; Cu, 3.2 mg; I, 0.8 mg; Se, 0.32 mg

[4] Crude Protein was analyzed by University of Missouri – Columbia, Office of the Missouri State Chemist, Analytical Services

Table 7-4. Amino acid[1] composition of organic diets fed to broiler chicks from 10-14 days

	Dextrose	Oats	Flaxseed	Field Peas	Millet	Alfalfa Meal	Canola Meal	Sunflower meal	Sorghum	Barley
Indispensable										
Arginine	0.01	0.44	1.41	1.23	0.44	0.60	0.94	1.00	0.24	0.39
Histidine	0.01	0.16	0.33	0.39	0.24	0.31	0.44	0.38	0.15	0.18
Isoleucine	0.01	0.28	0.68	0.70	0.43	0.61	0.68	0.63	0.29	0.30
Leucine	0.04	0.53	0.90	1.14	1.14	1.00	1.17	0.99	0.86	0.54
Lysine	0.02	0.31	0.58	1.16	0.27	0.69	0.88	0.61	0.16	0.31
Methionine	0.00	0.10	0.27	0.15	0.21	0.21	0.32	0.30	0.10	0.12
Phenylalanine	0.02	0.37	0.73	0.76	0.55	0.67	0.66	0.66	0.35	0.41
Threonine	0.02	0.24	0.56	0.56	0.31	0.54	0.68	0.55	0.22	0.26
Tryptophan	<0.02	0.07	0.18	0.13	0.09	0.17	0.20	0.17	0.06	0.08
Valine	0.01	0.38	0.79	0.77	0.53	0.75	0.86	0.77	0.35	0.40
Dispensable										
Alanine	0.02	0.34	0.69	0.68	0.92	0.68	0.74	0.66	0.59	0.33
Aspartic Acid	0.03	0.57	1.42	1.73	0.71	1.90	1.21	1.25	0.45	0.49
Cysteine	0.01	0.21	0.28	0.23	0.16	0.19	0.42	0.27	0.11	0.18
Glutamic Acid	0.03	1.35	2.82	2.58	2.11	1.39	2.72	2.61	1.31	1.79
Glycine	0.03	0.37	0.90	0.71	0.32	0.62	0.82	0.87	0.23	0.33
Proline	0.02	0.39	0.55	0.70	0.74	1.01	1.05	0.75	0.56	0.87
Serine	0.02	0.30	0.64	0.62	0.51	0.50	0.60	0.54	0.26	0.29
Tyrosine	0.01	0.17	0.34	0.43	0.28	0.38	0.40	0.32	0.17	0.18

[1]Samples were analyzed by University of Missouri – Columbia, Office of the Missouri State Chemist, Analytical Services

Table 7-5. SID of broilers fed organic feed ingredients from 10-14 d[3]

Amino acid	Oats	Flaxseed	Field Peas	Millet	Alfalfa meal	Canola meal	Sunflower meal	Sorghum	Barley
Indispensable									
Arginine	80.4±3.9	52.5±3.0	95.1±1.9	89.2±1.4	77.4±2.7	87.9±2.2	89.3±5.1	81.6±1.2	75.7±4.5
Histidine	72.2±5.1	32.8±3.2	87.6±1.8	84.5±2.0	60.8±1.9	81.4±2.8	82.9±12.6	75.8±1.1	74.0±3.9
Isoleucine	71.4±3.7	33.4±3.1	88.8±3.1	87.2±2.8	69.7±1.6	76.8±2.3	81.4±7.4	83.6±1.3	75.6±3.8
Leucine	76.3±5.3	35.3±2.3	89.5±5.4	92.0±2.8	73.6±2.0	83.0±3.2	79.7±11.5	91.5±0.64	79.6±3.9
Lysine	60.2±9.1	30.3±6.3	92.6±3.1	80.5±2.8	66.2±3.2	76.3±3.3	77.3±17.2	68.4±1.4	69.6±7.0
Methionine	65.2±9.1	32.4±3.6	80.8±2.7	87.1±3.1	68.8±3.3	83.9±3.5	85.3±12.4	80.9±0.74	73.6±5.8
Phenylalanine	78.7±3.0	37.1±2.2	88.1±2.3	88.6±2.8	70.8±1.4	80.9±3.3	84.3±10.0	85.9±0.64	79.8±3.1
Threonine	55.4±4.3	19.9±3.0	82.2±2.7	77.2±3.7	61.4±2.9	70.6±4.5	74.4±16.5	67.4±.79	62.6±4.4
Tryptophan	64.9±7.6	67.1±3.9	81.2±2.4	82.2±3.3	68.6±2.0	85.9±3.3	87.1±10.3	81.6±1.1	81.1±4.6
Valine	71.6±3.2	30.2±2.9	86.1±2.8	85.9±3.0	68.7±2.4	77.0±3.3	80.1±9.7	80.5±0.88	74.3±3.9
Dispensable									
Alanine	68.8±4.5	32.9±3.3	86.9±3.0	89.9±3.5	70.2±1.8	79.7±3.0	81.0±11.2	88.6±0.63	68.9±4.7
Aspartic Acid	73.1±3.5	40.7±2.3	91.5±2.6	87.0±2.4	81.6±1.4	79.9±3.5	74.6±8.4	80.3±0.94	69.9±4.7
Cysteine	66.6±2.0	35.8±1.2	75.2±2.9	77.4±2.4	34.6±2.4	76.1±2.7	73.8±18.7	63.7±2.6	75.5±2.8
Glutamic Acid	90.1±0.81	53.9±1.9	98.6±3.9	97.0±2.9	77.8±2.7	93.2±1.6	84.2±14.8	95.4±2.7	93.1±2.5
Glycine	67.7±2.3	39.8±2.5	85.1±2.7	78.4±2.0	59.0±1.5	77.3±3.0	68.2±7.7	69.0±0.8	68.0±4.5
Proline	73.6±2.1	23.1±2.2	85.2±2.5	88.7±2.6	77.6±1.0	75.3±2.4	79.8±8.7	84.2±1.0	88.4±1.6
Serine	63.3±.4.1	31.8±3.7	85.4±1.8	85.8±4.2	58.1±5.1	72.3±5.9	73.4±16.5	77.5±0.62	70.9±4.3
Tyrosine	63.9±3.5	28.0±2.9	86.7±1.6	85.3±3.4	77.9±1.4	74.3±4.0	81.3±16.1	78.9±0.78	75.6±2.4
Average AA[1]	70.2±4.6[d]	36.5±2.7[e]	87.0±3.3[a]	85.8±3.5[a]	67.9±1.5[d]	79.5±4.0[b]	79.9±10.4[b]	79.7±0.84[b]	75.3±3.0[c]

[1] Means in a column not sharing a common superscript are different (P < 0.05)

[2] SID= standardized ileal digestibly

[3] 8 replicates per pen

Table 7-6. Average amino acid content and SID[1] for individual amino acids in organic feed ingredients fed to broiler chicks from 10-14 days[2]

Amino Acid	Amino acid, %	Standard Error	CV	SID	Standard Error	CV
Indispensable						
Arginine	1.04	0.33	55.36	81.0^{ab}	2.03	15.06
Histidine	0.41	0.03	43.35	72.4^{bcd}	2.79	23.15
Isoleucine	0.56	0.04	38.75	74.2^{abcd}	2.76	22.30
Leucine	1.21	0.06	29.23	77.8^{ab}	2.80	21.63
Lysine	0.80	0.07	53.69	69.1^{bcd}	2.97	25.77
Methionine	0.30	0.02	45.43	73.1^{bcd}	2.85	23.40
Phenylalanine	0.71	0.04	29.9	77.15^{abc}	2.62	20.35
Threonine	0.60	0.05	45.68	63.46^{d}	3.07	29.03
Tryptophan	0.21	0.02	52.48	77.7^{ab}	1.54	11.89
Valine	0.73	0.05	36.93	72.7^{bcd}	2.78	22.95
Dispensable						
Alanine	0.85	0.042	28.65	74.1^{abcd}	2.89	23.40
Aspartic Acid	1.48	0.118	47.77	75.4^{abcd}	2.40	19.13
Cysteine	0.29	0.021	44.14	64.3^{cd}	2.90	27.03
Glutamic Acid	2.80	0.167	35.78	87.0^{a}	2.38	35.78
Glycine	0.79	0.67	50.37	68.0^{bcd}	2.15	18.92
Proline	0.93	0.05	34.01	75.1^{abcd}	3.26	26.12
Serine	0.72	0.04	35.63	68.7^{bcd}	2.81	24.57
Tyrosine	0.49	0.03	32.30	72.4^{bcd}	2.99	24.80

[1] SID= Standardized Ileal Digestibility

[2] Ingredients include oats, flaxseed, field peas, millet, alfalfa meal, canola meal, sunflower meal, sorghum and barley

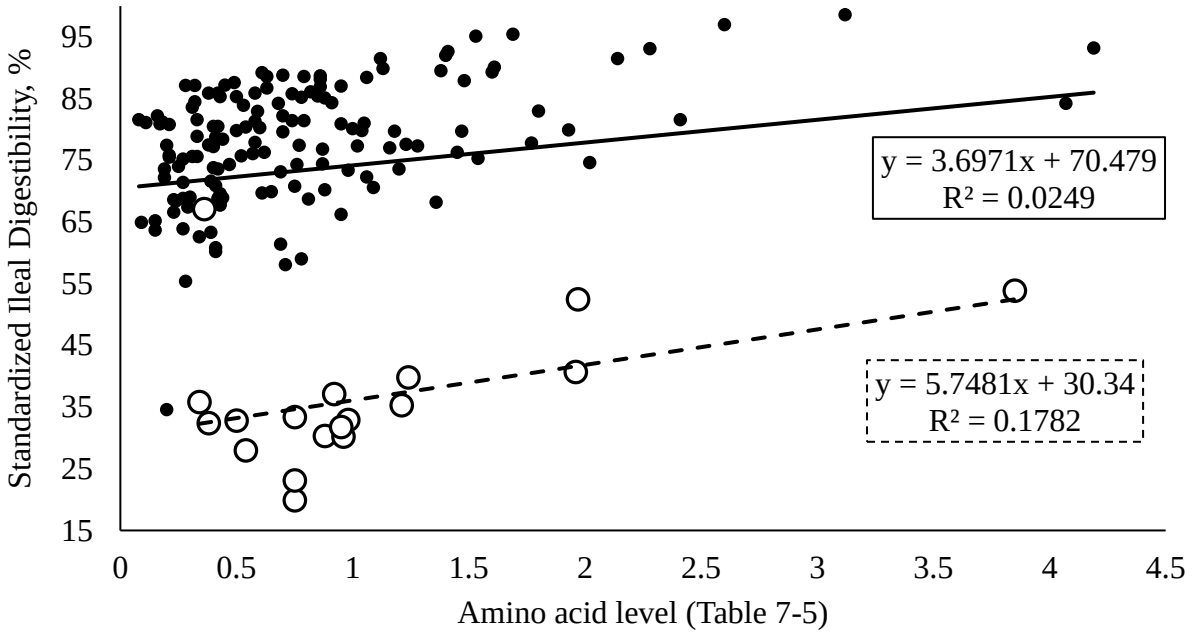

Figure 7-1. Individual amino acid level as a function of level from Table 7-5[1].

[1]Filled points and sold line is fitted for all data points including flaxseed. Open points and dotted line are fitted for flaxseed only.

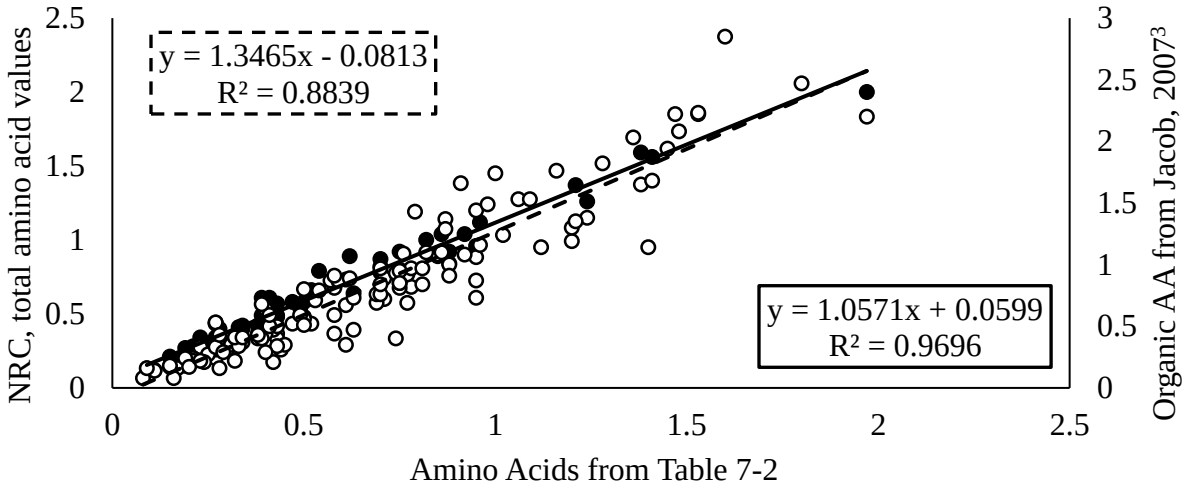

Figure 7-2. Organic amino acids as function of NRC total amino acids, and total amino acid from selected organic feed ingredients from Jacob, 2007

[1]All NRC amino acid values are from 1994 NRC, except for flaxseed, which came from 2012 NRC

[2] Open points dotted line denote organic amino acids as a function of NRC [11, 39]

[3] Solid points and solid line denote organic amino acids as a function of Jacob, 2007 [6]

CHAPTER 8

CONCLUSIONS

The results from these works show that there that is research that stills need to be done when deciding what feed ingredients to feed to livestock. Organic production will continue to grow in the United States regardless of scientific consensus on the lowest environmental impact for environmental production. Betaine supplementation in plants is an unreliable way supplement betaine for animals. The amount of betaine in plants is determined more on growing conditions, than on weather conditions. Choline is needed for livestock of all kinds and will continue to be an area of research. Establishing an assay for choline in feed ingredients is important when studying the effects of choline. It is important to expand the number of feed ingredients that have had choline digestibility determined on them. This is needed for choline availability and to determine if any antinutritional factors could affect choline digestibly.

Amino acids are an important macronutrient for livestock nutrition. There is always improvement needed to better determine digestibly of feed ingredients to better meet the requirement of the animal. It has been shown that amino acid level positively affects digestibility, this is seen in both swine and poultry. Data from this dissertation data seems to imply that amino acids are absorbed according to Michaelis-Menten kinetics. This adds a new layer of complexity to determining what to feed livestock.

APPENDICES

PUFFF 1.0 – PIG USER FRIENDLY FEED FORMULATION

Workbook v. 1.0

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PUFFF.xls workbook is a feed formulation tool that can help researchers formulate diets for all ages and types of pigs. And it should work just fine for smaller producers with just a few diets as well.

PUFFF.xls needs the solver add-in and macros enabled for the workbook to function properly.

Why is it important to have a feed formulation program specifically for swine?

Digestibility of amino acids and energy values are species dependent. Having a feed formulation that is swine specific is convenient, so swine specific values are all in one place.

The National Research Council has published the 11th Edition of the *Nutrients requirements of Swine* in 2012 and there was no feed formulation program that used their values to formulate diets. ***PUFFF! Here it is!***

What does the PUFFF workbook do?

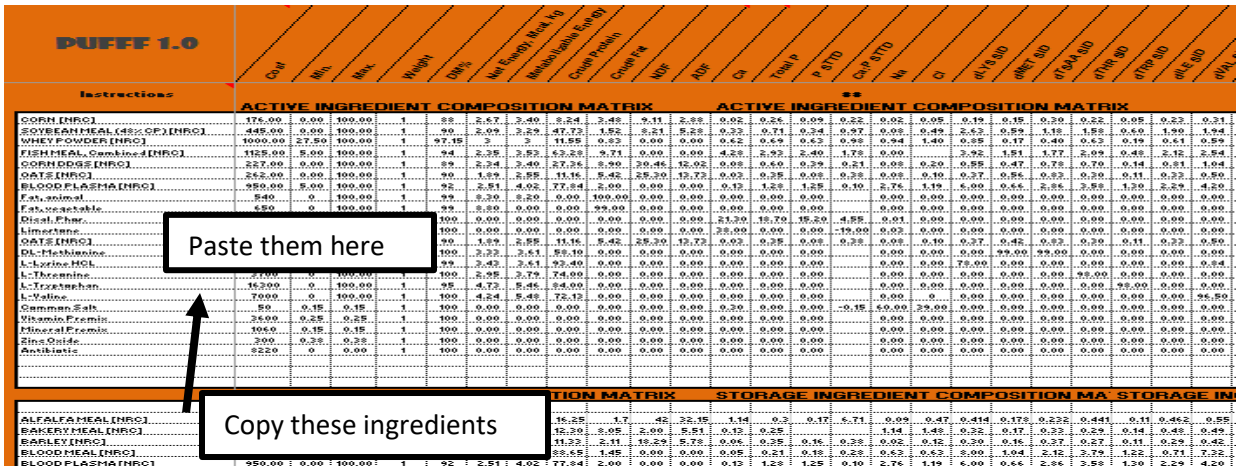
The PUFFF workbook allows a researcher or a nutritionist to formulate diets specifically for pigs in a least cost feed formulation way. The PUFFF 1.0 workbook comes with the ability to adjust minimums and maximums for each ingredient and nutrient. The 'Requirements' spreadsheet has requirements for 45 types of pigs to make diets for. PUFFF 1.0 uses least-cost formulations from the specifications entered on the 'Ingredients' and 'Requirements' spreadsheets, simply click 'Formulate Now' button on the 'Formulate' spreadsheet. Prices, level of feed inclusion, and level of nutrients can all be adjusted to meet the needs of the nutritionist formulating the diet.

What does the PUFFF 1.0 workbook consist of?

The PUFFF 1.0 workbook has 11 spreadsheets. There is a 'Title' spreadsheet. The PUFFF 1.0 workbook consists of an 'Ingredients' spreadsheet, this is where the feedstuffs are stored. There is an active ingredient composition matrix, these are the feed ingredients that are used to formulate the diet. There can be up to 25 ingredients in the active feed ingredients matrix. In addition, there is also a storage ingredient composition matrix that can store as many feed ingredients as you want. If you want to modify the amino acid content of a feed ingredient, this must be done in 'Coefficients' spreadsheet. In the 'Coefficients' spreadsheet both the total amino acid level and the digestibility value can be changed. Values from the 'Coefficients' spreadsheet will automatically update in the 'Ingredients' spreadsheet. There is a 'Requirements' spreadsheet

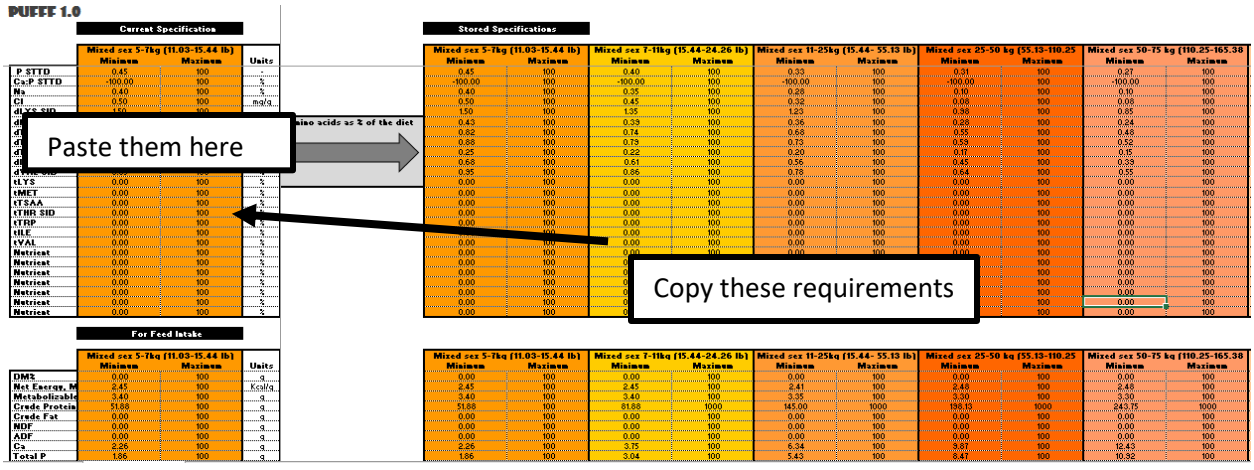
that has different nutrient requirements for different levels of production in pigs. The ‘Formulate’ spreadsheet is where the diet gets formulated, this is also where a sensitivity report can be calculated and shadow price can be determined. The ‘Sensitivity Report’ spreadsheet will appear when the button ‘Get Sensitivity Report’ is clicked. There are both ‘Feed Spec’ (feed specification) and ‘Mixing sheet’ spreadsheets. The mixing sheet spreadsheet can be given to the feed mill make the diet. There is a ‘Graphs’ spreadsheet to compare the requirement to the nutrient levels in the diet. There is also a ‘Feed intake’ spreadsheet to estimate feed consumption. Finally, there is a conclusion tab.

Step 1: Choose the ingredients



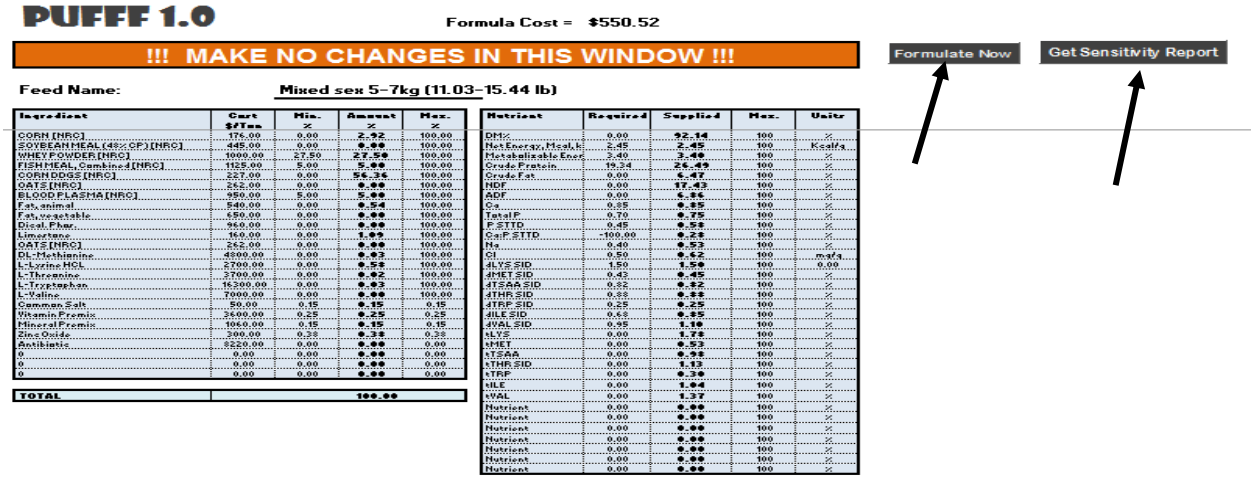
Move the ingredients that you want to use for diet from the storage ingredient composition to the active ingredient composition. Minimums and maximums for ingredients can be changed in the active ingredient composition matrix space as well.

Step 2: Choose feed requirements



Copy and paste the feed specification you want to formulate a diet for to the ‘Current Specification’ space.

Step 3: Formulate!



When both the ingredients and requirements are entered, click the "Formulate now" button to formulate the diet. When the diet is formulated then you can click the "Get Sensitivity Report" to get a sensitivity report and from this one can calculate shadow prices from this report.

How is the PUFFF 1.0 workbook used?

The PUFFF 1.0 workbook can be used to formulate diets for various types of pigs. This workbook can formulate diets for young pigs, gilts, barrows, gestating, and lactating sows. The PUFFF workbook uses requirements and the ingredients from the *Nutrient Requirements of Swine*, 11th edition. Feed ingredients can be easily added and changed to fit the needs of the person that is formulating the diet. The feed ingredients in PUFFF 1.0 are for reference only, make not reflect individual analysis of certain feed ingredients

References

National Research Council, 2012. Nutrient Requirements of Swine, 10th Edition. National Academy Press, Washington DC.