USING NEAR INFRARED REFLECTANCE SPECTROSCOPY AS AN ALTERNATIVE TO ANIMAL BIOASSAYS FOR DETERMINING DIGESTIBLE AMINO ACID CONTENT OF DIETARY INGREDIENTS USED IN POULTRY DIETS

by

MITCHELL LAKE DAVIS

(Under the Direction of Adam Davis)

ABSTRACT

Because feed constitutes approximately 70% of the cost of broiler live production, developing diets that meet the needs of maintenance, growth, and production while minimizing nutrient excess, is one way to maximize profit and animal health. Protein, as a supply of digestible, essential amino acids, supports the rapid muscle growth of broilers and the prolific egg production of laying hens. Determining the digestible amino acid content of feedstuffs requires an animal bioassay and laboratory analyses, which are time consuming and expensive. In commercial poultry production, the results from these analyses are not available before the tested ingredient is fed. Near infrared reflectance spectroscopy is a rapid analysis technique involving calibration of a multi-purpose analyzer to detect the reflectance of near infrared light from a feed ingredient to predict the nutrient content of a sample. The current research strives to create a calibration curve for the prediction of digestible amino acid content for each of the 10 essential amino acids for poultry in feed ingredients used in poultry diets. Initially, a calibration curve for detection of each of the 10 essential amino acids was created and validated for use in any conventional or alternative feed ingredient. While the R^2 values of the validation curves for the 10 calibrations ranged from 0.86-0.96, 23-53% of samples in these validations had predicted values that deviated 10% or more from their bioassay determined values. Subsequently, utilizing near infrared reflectance spectroscopy to create calibrations that were ingredient specific improved prediction accuracy for the 10 essential amino acids. For example, ingredient specific calibration curves for dried distillers' grains improved R^2 values to above 0.93 for all 10 essential amino acid validation curves and resulted in 16% or less of samples having predicted values deviating plus or minus 10% or greater from their bioassay determined values for all essential amino acids except lysine and tryptophan. These results indicate that near infrared reflectance spectroscopy has the potential to accurately predict the digestible amino acid content of feed ingredients, especially if ingredient specific calibration models are created for each amino acid.

INDEX WORDS: Essential amino acids, Broiler, Nutrition, Poultry production, Alternative feed ingredients

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DEDICATION

This dissertation is dedicated to my grandfather, Mr. John Marvin “Bud” Moon, or as he was known to me, Pop. Pop had little formal education past middle school and went straight to work as a teenager. He was the definition of hard work and grit, growing up and building a family during some of the hardest times in rural America. His dedication to his family was shown through his marriage of 65 years to his wife, Evelyene, his work ethic, his willingness to help anyone he could, and his thriftiness. Pop was never a rich man, but he provided for his family, spent very little on himself, and built up a savings for retirement. Pop was independent, to a fault. He wanted to help others but did not like needing help for himself. While attending the “school of hard knocks,” he saw how education helped others around him, and instilled in his three daughters and his seven grandchildren the value of education. I remember him telling me of friends and acquaintances who had doctorates and advanced degrees, working in the medical field and in education. He always wanted me to continue my path, earning degrees, and would always tell me to “keep going son,” because he knew that education had helped so many he knew. My grandfather had the ability to look beyond his own world view, outside of his daily life, and even without the internet, help direct us down paths that would utilize our strengths to be most successful. Of his seven grandchildren, four have college degrees, three have post-secondary training and education, and one served our country in the Marine corps. Four of us also own or have owned businesses that contribute partially or fully to our income. A short dedication is the least I can do to honor his name, but the length of this work would not be enough to adequately describe how this man influenced my life. Thank you for your time, your wisdom, your training, and your love, for without you, this work and this life would not be possible.

To my Pop, Mr. John Marvin “Bud” Moon

January 3rd, 1935- January 1st, 2022

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CHAPTER 1

INTRODUCTION

Poultry diet ingredients

Poultry products are some of the most affordable protein sources in the United States and have become a preferred source of protein for many citizens. As the human population in the U.S. increases, demand for affordable protein increases as well. Zuidhof et al. (2014) stated:

> "Between 1960 and 2004, the US consumer price index for poultry products increased at half the rate of all other products (USDA, Economic Research Service, 2004), due to improvements in growth and efficiency. This has likely been a major factor contributing to higher per capita consumption of chicken meat between 1950 (9.4 kg) and 2005 (39.2 kg; USDA, Economic Research Service, 2014)."(p.2970)

The United States has also increased exportation of poultry products. From 1994 to 2013, the U.S. increased poultry exports from 1.5 million metric tons to 4.1 million metric tons, or at an increase of 5.4% annually (Zhuang and Moore, 2015). The domestic increase in demand coupled with increased demand internationally for poultry products led to an annual production of 17.0 million metric tons of poultry products in the U.S. in 2013, a growth of 2.4% annually since 1994 (Zhuang and Moore, 2015). The U.S. must continue to increase the efficiency of poultry production to minimize the costs and resource utilization while meeting the increase in poultry product demand.

Broilers can gain approximately one pound of meat per one and a half pounds of feed consumed (Cobb-Vantress, 2018; Aviagen, 2022). Laying hens can produce approximately one egg per day during egg production (Lohmann, 2020). These high yields are attributed to improved genetics and quality nutrition (Havenstein et al., 2003). The feed for a broiler operation is the largest expense for broiler producers with a variety of factors such as commodity price fluctuations, governmental regulations, and use of commodities for alternative fuel production influencing that value (Kocak et al., 2022). Minimizing the cost of feed may be accomplished by minimizing nutrient excesses in the feed by efficiently using feed ingredients to match the nutritional requirements for growth, maintenance, and production of the bird.

In general, poultry diets are composed of a cereal grain to provide energy, a concentrated source of protein such as soybean meal (SBM) or animal byproduct meal, and a fat/oil to provide additional energy, increase palatability, and reduce the dustiness of the feed. These main ingredients are supplemented with synthetic amino acids and vitamin and mineral premixes to make a diet that meets the birds' nutrient requirements. Traditionally, diets for U.S. poultry production are based on corn and SBM. In other parts of the world, cereal grains such as wheat, barley, and sorghum may be used rather than corn when they are more available (Leeson and Summers, 2019). Additionally, SBM can be partially replaced with other concentrated protein sources including sunflower meal, canola meal, dried distillers' grains with solubles, and/ or animal by- product meals such as meat and bone meal, fish meal, and feather meal (Caires et al., 2010).

Alternative ingredients. While SBM is the most common protein source used in animal feeds worldwide, the availability of amino acids can be negatively impacted by the processing of SBM, which may include insufficient heating or excessive heating in an attempt to destroy naturally occurring trypsin inhibitor (Evans and McGinnis, 1946; Clandinin et al., 1947; McGinnis and Evans, 1947). The use of alternative ingredients as replacements for SBM in animal feed has grown, driven by attempts to lower feed costs. Animal byproduct meals can be used as a protein source to partially replace SBM in commercial diets. Animal byproducts are processed feed ingredients derived from animal protein sources or byproducts of animal meat processing (Leeson and Summers, 2019). These rendered animal protein products may include meat meal, meat meal tankage, meat and bone meal, poultry meal, animal by-product meal, dried animal blood, blood meal, feather meal, and egg-shell meal (Sapkota et al., 2007). Meat and bone meals are the most common dietary ingredient for poultry made from animal byproducts (Leeson and Summers, 2019). Meat and bone meals can have a variable nutrient profile related to the species of animal used, the ratio of soft tissue to bone, and the amount of fat in the meal, and this variability is a concern for nutritionists (Parsons et al., 1997; Mahmoudnia et al., 2011).

Another alternative feed ingredient has resulted from ethanol fuel production. In the U.S., over 14 billion gallons of ethanol fuel is produced annually using corn as the main source for fermentation (Hoekman and Broch, 2018). The use of corn for alternative fuel production directs much of the U.S. corn to fuel production, thereby lowering the supply of corn for animal feed markets (Kocak et al., 2022). Ethanol production does provide feed grade byproducts known as dried distillers' grains and dried distillers' grains with solubles (DDG and DDGS). The nutrients that remain after fermentation and extraction of the corn alcohol are more concentrated than in the original starch, making DDGS an improved source of protein at 25-30% crude protein and increasing the concentration of fat to 9-13% (Pahm et al., 2009). Recently, higher crude protein DDGS products have become available, and when coupled with improved oil extraction processes, these ingredients can offer greater than 35% crude protein and less than 4% fat (Fries-Craft and Bobeck, 2019). However, the nutrient profile and availability of nutrients in DDGS can be affected by the processing techniques, especially the drying procedure, used after the alcohol has been extracted from the DDGS solid fraction (Parsons et al., 2006).

There are by-products of the baking industry that have been successfully utilized as alternative ingredients in poultry diets (Damron et al., 1965). Bakery products include breads, cookies, crackers, cakes, doughs, and other baked snack foods discarded as waste from baked food companies (Damron et al., 1965; Day and Dilworth, 1968). Companies collect this by-product, filter out trash and extra material, dry, and blend the bakery products to create a by-product meal that is suitable for animal feed (Day and Dilworth, 1968). The resulting product has a crude protein level and amino acid composition similar to or greater than corn, with a higher fat content and corresponding higher energy content than that of corn (Miller et al., 1987). Bakery products can have higher levels of salt (Miller et al., 1987). More recent studies have shown variability in the nutrient composition of bakery meal products, including crude protein ranges of 9.3-15.3% (Waldroup et al., 1982), well above the 6-8% for corn. Nutrient variability of the blended bakery products has been a concern for commercial implementation as the supply of bakery by-products has increased (Waldroup et al., 1982; Dale, 1986; Dale and Fuller, 1987). Price, supply availability, and nutrient consistency are all factors to consider when incorporating these bakery products into least cost formulation (Saleh et al., 1996). Analysis of these baked products would help nutritionists identify these differences and incorporate the products more accurately in formulation.

Regionally available products may also be available and utilized in poultry diets. Peanut meal, cottonseed meal, and sunflower meal are examples of protein sources in poultry diets that can be utilized when available and priced favorably. Peanut meal is the solid fraction of crushed peanuts formed as a by-product during the oil extraction process of peanuts. Similar to soybean meal, peanut meal can be a product of the expelling and/or of the solvent extraction process of oil, as raw peanuts are approximately 35-40% oil (Batal et al., 2005). The resulting peanut meal contains 40-50% protein. Grau (1946) reported peanut meal to be low in the amino acids methionine and lysine, which are the two most widely used synthetic amino acids today, allowing peanut meal to be better utilized in broiler diets when supplemented with synthetic amino acids. The presence of aflatoxin, a product of fungi, in peanut meal is a concern for animal nutrition, and the United States Department of Agriculture sets the maximum aflatoxin limit at 20 parts per billion (ppb) for feed ingredients. Heat processing during the extraction process manages aflatoxin contamination (Mann et al., 1967). Previous methods included treatment with aqueous ammonia, hydrogen peroxide, sodium hydroxide, acetone, and ozonation (Dollear et al., 1968; Dwarakanath et al., 1968; Price et al., 1982). The heat treatment process can have an impact on the availability of amino acids in the peanut meal, specifically lysine (Zhang and Parsons, 1996).

Cottonseed meal is the byproduct of oil extraction of cottonseed. Cottonseed meal contains 30-50% crude protein, approximately 25% crude fiber, and is low in the amino acids lysine and methionine (Phelps, 1966). Lysine is generally low due to effects of heat treatment and due to the binding of lysine to free gossypol in the meal (Altschul, 1954; Fernandez and Parsons, 1996). Gossypol is an antinutritional factor in cottonseed meal, produced by the cotton plant. Gossypol binds free lysine and iron, making it inaccessible to the animal (Braham et al., 1967; Fernandez et al., 1995). Cottonseed meal protein has also been shown to be less digestible than corn and SBM in poultry (Fernandez et al., 1995). Sunflower meal is another protein meal that is regionally available for poultry diets and is a by-product of the oil extraction process of sunflower seeds. The high oil producing sunflower seeds contain 40-50% oil. After extraction, the resulting meal contains 24-44% protein, and has no antinutritional factors that can impair animal growth and production, but sunflower meal has a high and variable fiber content of 12-25% depending on processing procedures (Senkoylu and Dale, 1999). Lysine is the limiting amino acid in sunflower meal (McGinnis et al., 1948) and lysine availability is sensitive to the heat processing of the meal (Alexander and Hill, 1952).

Consumer trends. A shift in consumer preferences in recent years, especially in developed nations, has also influenced poultry feed ingredient utilization and diet formulation. Consumer demand for all natural, organically grown, and antibiotic free animal products has risen. In 2009, the organic food category was growing seven times faster than any other food category and was increasing its growth by 15% per year (Crandall et al., 2009). The United States Department of Agriculture or USDA defines organic food as:

> "Organic food is produced by farmers who emphasize the use of renewable resources and the conservation of soil and water to enhance environmental quality for future generations. Organic meat, poultry, eggs, and dairy products come from animals that are given no antibiotics or growth hormones. Organic food is produced without using most conventional pesticides; fertilizers made with synthetic ingredients or sewage sludge; bioengineering; or ionizing radiation. Before a product can be labeled 'organic,' a government-approved certifier inspects the farm where the food is grown to make sure the farmer is following all the rules necessary to meet USDA organic standards. Companies that handle or process organic food before it gets to your local supermarket or restaurant must be certified, too (USDA, 2008)."

All-natural food is another product category that has seen similar growth trends as organic food and is often lumped into the same category when discussing these markets. The USDA defines this product group as:

> “A product labeled "natural" is a product containing no artificial ingredient or added color and is only minimally processed. Minimal processing means that the product was processed in a manner that does not fundamentally alter the product (USDA, 2008).”

To produce organic or all natural meat products, the animals must be fed organic or all naturally grown feed ingredients. When considering plant based ingredients like corn and SBM, varieties that perform best under organic conditions, without pesticides and other crop protections in the field, are generally different than the varieties that are grown traditionally using pesticides, herbicides, miticides, and selected fertilizers (Buchanan et al., 2007). Organically grown feedstuffs have developed innate mechanisms to protect themselves from pests, which may include higher concentrations of antinutritional factors such as non-starch polysaccharides (Buchanan et al., 2007). These varieties will have lower nutrient digestibility than the traditionally grown varieties due to this increase in antinutritional factors (Buchanan et al., 2007; Van Krimpen et al., 2016).

Amino acid definitions

As analysis for amino acids in feedstuffs became more available, nutritionists began focusing on the amino acid levels of diets and meeting the requirements for individual amino acids in poultry, rather than adhering to a level of crude protein in formulating diets (Baker and Han, 1994). In 1943, it was recognized that approximately 21% crude protein diets produced the best growth in chicks, but it was also noticed that the addition of the individual amino acid methionine to diets would improve broiler growth (Bird, 1947). The following was published by the USDA, written by Bird (1947):

> “Considerable progress has been made at the California Agricultural Experiment Station in expressing the protein requirements of growing in terms of amino acids, which constitute proteins. More information on this vital subject and on the distribution of amino acids in feeds will help us to predict the feeding value of feedstuffs and feed mixtures from the results of chemical analyses. It is too much to expect, however, that the value of the usual protein supplements will be predictable entirely on the basis of their amino acid content, because they also contain vitamins that poultry need.”

During this time, nutritionists were working with mostly vegetable based feedstuffs and were not using supplementary amino acids or vitamins in animal diets, leading to difficulty isolating the effects of vitamin deficiency and amino acid deficiency. Other supplements including milk byproducts, cattle manure, and fish meal also showed efficacy to improve growth of poultry on vegetable diets. The use of these alternative products was related to the lack of meat byproducts due to the war effort of World War II (Hammond and Titus, 1944; Bird, 1947).

In the 20 years following World War II, nutrient research for poultry would blossom, with research regarding vitamins, minerals, energy, and amino acids, including the interactions among many of these elements, increasing significantly (Anderson and Dobson, 1959; Leong et al., 1959; Lewis et al., 1962). Amino acids were of particular concern, as the use of exogenous amino acids helped determine many of the limiting amino acids in poultry diets, even if these exogenous amino acids were not available in sufficient quantities for commercial feed production (Rosenberg, 1957). Pesti (2009) demonstrated mathematically that to meet the methionine need of a modern broiler without supplementation of exogenous methionine, a diet would need to contain 70.86% soybean meal and have a crude protein level of 35.60%, but excessively high levels of protein can limit bird performance (Harper, 1956; Harper et al., 1970; Smith and Austic, 1978; Harper et al., 1984).

In the 1960s, researchers began working to balance the amino acids in poultry diets to maximize growth and development (Dean and Scott, 1965; Huston and Scott, 1968; Sugahara et al., 1969). This early research would lay the foundation of the ideal protein concept, or the idea that the balancing of all amino acids to a reference amino acid would allow for standardization of diet amino acid content. Lysine was chosen as the reference amino acid because it is generally the first or second limiting amino acid in practical poultry diets, it is used primarily in protein accretion and maintenance in the bird, laboratory analysis for lysine is rather straight forward and accurate, and lysine is one of the most researched amino acids meaning that a variety of requirements under different circumstances had been established (Emmert and Baker, 1997). Using wet chemistry analysis for amino acids, developing ratios of amino acids in diets relative to lysine, and using commercially available, purified amino acids allowed nutritionist to formulate poultry diets with more accuracy, using the ideal protein concept, thereby increasing poultry growth and performance (Emmert and Baker, 1997; Baker et al., 2002; Corzo et al., 2002; Dari et al., 2005; Corzo, 2012; Belloir et al., 2017; Chrystal et al., 2020).

Essential amino acids in chickens

By 1944, the essentiality of amino acids in poultry diets had been pretty well outlined (Block and Bolling, 1944). A dietary essential amino acid is an amino acid that cannot be synthesized in the body in sufficient quantities by the bird to meet their requirement. As a result, these amino acids must be obtained in the diet. The ten essential amino acids in poultry are methionine, lysine, threonine, valine, arginine, isoleucine, tryptophan, leucine, histidine, and phenylalanine.

Methionine is a sulphur containing amino acid (Osborne, 1902; Mueller, 1923; Rose et al., 1936). Supplementation of poultry diets with methionine can increase white meat yield and decrease carcass fat (Wallis, 1999). Methionine content of many plant protein sources is limited, requiring supplementation with synthetic methionine to meet the requirement for poultry (Rose et al., 1936; Rose, 1937). Biochemically, methionine can serve as a methyl group donor by its metabolism into the coenzyme S- adenosyl methionine (Cantoni, 1953). Once the methyl group has been donated, the resulting product can be converted into homocysteine, which can be remethylated back into methionine or transsulfurated into cysteine (Finkelstein, 1990). Due to its ability to form disulfide bonds, cysteine supplied through dietary intake and from the metabolism of methionine, is important in collagen and keratin synthesis in the production of scales and feathers in poultry (Ackerson et al., 1928; Elsworth and Phillips, 1938). Methionine is also important in protein synthesis as one of the 20 protein forming amino acids and in muscle accretion in poultry (Wallis, 1999; Liu et al., 2007). Egg laying birds require sufficient amounts of sulphur amino acids in the diet for the formation of albumin in the egg, which is high in methionine (Schutte and van Weerden, 1978). There is evidence that methionine acts as an antioxidant inside the cell and can increase antioxidant response to heat stress (Luo and Levine, 2009; Elnesr et al., 2019).

Almquist and Mecchi (1942) were the first to study lysine requirements in poultry. When evaluating lysine requirements of broilers, lysine seems to be important in the development of white meat products, and requirements may vary for growth parameters such as bodyweight gain, breast meat yield, and feed conversion ratio (Acar et al., 1991). Since all lysine catabolism takes place in the liver, and few other metabolic pathways exist for lysine to be metabolized into another product, most intact lysine is deposited in the muscle, and conserved in the body after digestion (Flodin, 1997). If lysine must be catabolized, it will become glutamate and acetyl CoA for cellular processes (Grove and Roghair, 1971).

In the 1940s, threonine was determined to be an essential amino acid for protein synthesis, growth, and development in poultry (Almquist and Grau, 1944; Hegsted, 1944; Grau, 1949). Commercial threonine was introduced in the 1980s and this availability stimulated an increase in research in the 1980s and 1990s (Kidd, 2000). Threonine metabolism includes the formation of glycine, allowing threonine to be glycine sparing and allowing for marginal glycine deficiencies in low crude protein diets (Baker et al., 1972). Threonine has roles in feather formation (as feather protein is 20% threonine and serine, and serine is a metabolic end-product of threonine catabolism) (Baker et al., 1972; Stilborn et al., 1997), uric acid formation (Krasna et al., 1952), and formation of poultry gamma globulins (Tenenhouse and Deutsch, 1966). The largest quantity of dietary threonine is used for the growth and maintenance of the gastrointestinal tract, as it is estimated that 61% of first pass dietary threonine is used in the gastrointestinal tract, according to research done in piglets (Stoll et al., 1998). Mucin is secreted by goblet cells and coats the intestinal tract, to provide lubrication and protection of the epithelial cells against abrasion, pathogenic bacteria, and antinutritional factors (Horn et al., 2009; Duangnumsawang et al., 2021). Mucin protein is 28-40% threonine (Carlstedt et al., 1993) and though secreted constantly, this threonine remains unavailable for absorption due to mucin’s resistance to digestion (Fuller, 1994). Research has also suggested that threonine may have a role in intestinal cell development, modulating villi height and crypt depth in the avian gastrointestinal tract (Zhang et al., 2016; Chen et al., 2017).

The branched chain amino acids valine, isoleucine, and leucine are all essential amino acids in poultry and contribute heavily to muscle accretion, as approximately one-third of muscle weight is attributed to the branched chain amino acids (Kim et al., 2022). Leucine is generally not limiting in corn and SBM diets (NRC, 1994; Waldroup et al., 2002), but the dietary balance of all three branched chain amino acids and the interactions that occur from their imbalances is of importance (Harper et al., 1954; Harper, 1956; Harper et al., 1970). For example, the relatively high concentration of leucine in corn and SBM diets can initiate enzymatic degradation of the excess leucine, but because all branched chain amino acids are catabolized by the same metabolic pathway, this increase in activity can lead to the unintentional degradation of valine and isoleucine, affecting the availability of those amino acids to the bird (Allen and Baker, 1972). With feed-grade commercial valine being introduced to the market in 2008, followed subsequently by isoleucine (Kidd et al., 2013), research on branched chain amino acid antagonisms and the mitigation of these antagonisms has increased (Lee et al., 2020). Beyond muscle growth in poultry, branched chain amino acids also have roles in promoting immune response through development of the thymus and bursa of Fabricius (Konashi et al., 2000), and preventing proteolysis of muscle (Busquets et al., 2000).

Arginine is a nutritionally essential amino acid in young mammals but not adult mammals (Rose, 1937; Borman et al., 1946). However, in avian species, it is essential throughout their lifespan because, being uricotelic, birds cannot perform de novo synthesis of arginine (Tamir and Ratner, 1963). Arginine is used in the synthesis of proteins, but also as a precursor to other metabolically active molecules including ornithine (Nesheim, 1968; Austic and Nesheim, 1970), which can subsequently form products including polyamines and the amino acid proline (Austic, 1973; Morris Jr., 2007). Arginine supplementation in poultry has been associated with increased lymphoid development, especially the thymus and spleen (Kwak et al., 1999), and increased heterophil counts (Lee et al., 2002). Dietert et al. (1994) reported that even moderate deficiencies in arginine could lead to depressed antibody titers, reduced populations of lymphocytes, and increased susceptibility to infection in poultry. Arginine is used for nitric oxide (NO) production, and nitric oxide is linked to collagen synthesis, wound healing, tumor suppression, hormone release, vasodilation, and immune response, as reviewed by Wu et al. (1999). Supplementation of poultry diets with arginine and the production of NO has also been linked to attenuation of pulmonary hypertension syndrome in broilers (Wideman et al., 1995). Requirements for arginine in poultry were last established in 1994 (NRC, 1994), but subsequent research indicates that the published dietary requirement may only be sufficient for maximal growth, and that the requirement necessary for optimal immune system development and function may be greater than the NRC requirement (Dietert et al., 1994; Wideman et al., 1995; Wideman et al., 1996; Ruiz-Feria et al., 2001).

Tryptophan is a dietary essential amino acid in poultry (Almquist and Mecchi, 1941; Grau and Almquist, 1944; Almquist, 1947). Tryptophan is involved with protein synthesis, and given that tryptophan is retained in low concentrations in the body and is provided in low concentrations from feed sources, can become limiting for protein anabolism (Sainio et al., 1996). Corticosterone and heat shock protein 70 were both decreased significantly in tryptophan supplemented birds (Bello, Idrus, Meng, Awad, et al., 2018), demonstrating a role of tryptophan in stress management in poultry. It was reported that tryptophan supplementation in laying hens reduced negative behaviors including aggression, feather picking, and cannibalism (Fouad et al., 2021). Tryptophan supplementation in diets has been shown to attenuate aggressive behaviors of both male and female broiler breeders on skip-a-day feeding programs, and was shown to lower corticosterone levels and increase serotonin levels in birds under these feeding conditions (Shea et al., 1990; Mench, 1991; Bello, Idrus, Meng, Narayan, et al., 2018). Tryptophan serves as a precursor for the hormones serotonin and melatonin that regulate animal circadian rhythm (Morin, 1999)and these hormones also modulate blood pressure, body temperature, feed intake, and tissue growth and repair (Hamm and Menaker, 1980; Lacy et al., 1986; Corzo et al., 2005). Serotonin has been linked to voluntary feed intake in production animals, and supplementation of dietary tryptophan was shown to increase feed intake in the pig (Henry et al., 1992; Bello, Idrus, Meng, Narayan, et al., 2018). Supplementation with tryptophan has also been shown to increase muscle insulin like growth factor 1 (IGF-1), offering a possible explanation to improved growth in tryptophan supplemented birds (Pan et al., 2013; Dukes et al., 2015).

Phenylalanine and histidine are two essential amino acids that have historically received little attention in diet formulation, as they were thought to be of little concern in practical poultry diet formulation (Kidd et al., 2021). Phenylalanine is tied closely to tyrosine when considering poultry diets and may be reported as a tyrosine + phenylalanine requirement, or the total aromatic amino acids (Grau, 1947; Armstrong, 1955). Phenylalanine has roles in the synthesis of catecholamines, including dopamine, epinephrine, and norepinephrine (Fernstrom and Fernstrom, 2007). Studies have also indicated that phenylalanine may help in mitigating the negative effects of mycotoxins found in feed ingredients (Gibson et al., 1990). Histidine can serve in antioxidant roles through conversion to carnosine, anserine, and urocanic acid (Mehler and Tabor, 1953; Taylor et al., 1991). Histidine also has roles in metabolism and conversion to histamines for a variety of roles from regulation of inflammation to wound healing (Branco et al., 2018; Moro et al., 2020). Histidine depletion in poultry diets has been shown to decrease growth and meat yield (Kai et al., 2015).

Determining amino acid digestibility

The total amino acid content of feed ingredients indicates how much of an amino acid is present in that feed ingredient, but it does not guarantee that all of it is available for use by the bird. Digestible amino acid content describes the percentage of that total amino acid content that is absorbed across the entire intestinal mucosa and can be utilized by the bird. Formulating broiler diets using the digestible amino acid content of feed ingredients rather than the total amino acid content can result in better growth and feed efficiency of broilers, especially in diets using feedstuffs with low amino acid digestibility coefficients (Fernandez et al., 1995; Dari et al., 2005).

Determination of the digestible amino acid content of feedstuffs is animal specific and must be done using animal bioassays. In poultry, the two most common animal bioassays for determination of amino acid digestibility are the broiler chick standardized ileal digestibility assay (Payne et al., 1968; Ravindran, Hew, et al., 1999) and the precision fed cecectomized rooster assay (Parsons, 1985). Both methods evaluate digestible amino acid content of feed ingredients by comparing the total amino acid content of the feed to the amino acid content of the resulting excreta or digesta, and employ correction factors for endogenous losses (Sibbald, 1979).

Broiler chick ileal assay. Broiler chick ileal sampling in poultry was introduced as a procedure for digestible nutrient evaluation by Payne et al. (1968), but a variety of modifications to the original procedure have been made in the years since, leading to the more widely accepted procedure presented by Ravindran, Hew, et al. (1999). The assay does not require surgery to be performed on the birds before use for the assay, so it is an accessible assay for groups with the facilities to house birds and the resources to perform collection. In brief, for the standardized ileal digestibility assay, four to six chicks per replication, with 3-4 replications per sample are typically raised until 14 or 21 days of age, using a starter diet or a standardized basal diet (Soaees and Kifer, 1971). Using older birds at 35 days of age, Ravindran, Hew, et al. (1999) used 4 birds per replicate to obtain and adequate amount of digesta. The assay begins when the chicks are switched from being fed the starter diet to being fed the test diet (ad libitum) for 5-7 days (Ravindran, Cabahug, et al., 1999). The test diet is considered nutritionally complete as it contains a purified carbohydrate source such as dextrose, an oil as a fat source, added vitamins and minerals, and a single protein ingredient as a source of amino acids (Soaees and Kifer, 1971; Ravindran, Hew, et al., 1999).

Another set of birds will also serve as an endogenous control group to determine the endogenous loss correction factor that will be applied to yield standardized ileal digestibility (SID) from apparent ileal digestibility (AID) (Payne et al., 1968; Soaees and Kifer, 1971; Ravindran, Hew, et al., 1999). Two methods are widely used for determination of endogenous losses in the chick ileal assay. First, a nitrogen free diet can be fed for the feeding period and thus all amino acids present in the ileum will be of endogenous origin (Soaees and Kifer, 1971). Second, a diet formulated similarly to the test diet, but with a highly digestible protein source such as casein (essentially 100% digestible protein), replacing the test ingredient in this diet, is fed. This method assumes that no undigested amino acids from casein will be present in the digesta in the ileum and all amino acids present in the ileum will be of endogenous origin (Gargallo and Zimmerman, 1981).

For the broiler chick ileal assay, the test diets contain a known percent of a non-digestible, inert marker to determine the amount of feed consumed (Soaees and Kifer, 1971). After consuming the diet, the bird will digest and absorb the components of the diet that are digestible, leaving behind the non-digestible marker. Since this marker is mixed at a specific ratio in the diet, the amount of feed consumed can be mathematically calculated from the amount of recovered marker (Summers and Robblee, 1985). Chromic oxide and titanium dioxide are the two most common markers used in poultry nutrition (Fenton and Fenton, 1979), but acid insoluble ash is also used (McCarthy et al., 1974; Ravindran, Cabahug, et al., 1999). It has been demonstrated that the movement of inert markers may not match that of the digesta, leading to inaccuracies in determining nutrient digestibility using inert markers (Oberleas et al., 1990).

At the completion of the 5-7 day feeding period, birds are then euthanized, and the ileum is resected from each bird. Specifically, the distal portion of the ileum from Meckel's diverticulum to approximately 4cm before the ileo- cecal junction will be removed and the digesta will be rinsed from the intestine using distilled water (Ravindran, Hew, et al., 1999). With this method, the digesta is removed before amino acids could be digested by the bacteria present in the large intestine (Payne et al., 1968) while also avoiding amino acids of urinary origin that are added with the intestinal waste at the cloaca (O'dell et al., 1960). The digesta from the endogenous control birds and from birds fed the test diet is dried using freeze drying or oven drying before being sent to a laboratory, along with a sample of the test diet, for analysis of non-digestible marker content and amino acid content. From the laboratory reported values, the digestibility of each individual amino acid can be calculated.

Criticisms of the chick standardized ileal assay include feeding on a free access basis, a diet containing the test ingredient as the sole source of amino acids for 5-7 days, because the test ingredient will not provide a balanced profile of amino acids and can depress feed intake (Tobin and Boorman, 1979). As different broilers are used for each ingredient determination, different experimental conditions will exist such as potential intestinal challenges, potential disease challenges, broiler genetics, and environmental management conditions, that have a larger impact on young birds, still developing their digestive capabilities and this variability makes comparing digestibility results across multiple experiments more variable (Kim and Corzo, 2012). In addition, from an animal welfare perspective, concerns have been raised about the number of chicks that are killed for each feed ingredient digestibility determination (Kim et al., 2011). To complete 3-4 replications per feed ingredient, 12-24 will be sacrificed for digestible amino acid determination. In addition, the use of broiler chicks serving as the model for digestibility data applied to layers and broiler breeders has been questioned (Kim and Corzo, 2012). Finally, the chick ileal digestibility assays are also more time consuming and more expensive when compared to the cecectomized rooster assay method that will be discussed next (Parsons, 2020).

Cecectomized rooster assay. In the cecectomized rooster assay, adult white leghorn roosters are cecectomized so that the bacteria in the ceca are not able to digest and utilize undigested amino acids from the small intestine of the bird, and thus artificially inflate amino acid digestibility values (Soaees and Kifer, 1971; Parsons, 1985). It was reported that the microbial population in the chicken hindgut has a large impact on amino acid digestibility, and up to approximately 25% of the amino acid content of excreta from intact birds comes from microbial origin, which suggests that the microbes heavily metabolize amino acids that are not absorbed by the bird (Salter and Coates, 1971; Parsons et al., 1982). Once cecectomized, these roosters can be fed repeatedly throughout their life. In this bioassay, 10 cecectomized roosters per feed ingredient (Parsons, 1985) are fasted for 24 hours before being tube fed 30g of the test feed ingredient and subsequently being placed in a cage with a collection tray for 48 hours (Sibbald, 1979; Parsons, 1985). Excreta with uric acid is collected and dried via freeze drying or using an oven, before being sent for amino acid analysis (Dale et al., 1985; Parsons, 1985). One set of fasted roosters are not fed the test ingredient, but are also placed in the collection cage for the 48-hour window (Sibbald, 1979). Since they were not fed, their feces will reflect endogenous losses of amino acids from intestinal secretions and sloughed off intestinal cells and will be used as correction factor to apparent amino acid values to yield true digestibility values of the feed ingredient. Endogenous losses can be determined through the fasted bird procedure or through feeding a nitrogen free control diet, however, research indicates that the nitrogen free diet method increases endogenous losses of amino acids (Parsons, 2020). It is therefore preferred to determine endogenous losses with the fasted bird procedure.

Criticism of the precision fed cecectomized rooster assay includes animal welfare concerns related to the cecectomy surgery of the roosters, the force- feeding procedure, and the fasting of the roosters for an extended time (Lemme et al., 2004). Other criticisms include the feeding of a single protein ingredient at a time to the birds, which is not a nutritionally complete diet, and the use of adult birds in determining nutrient digestibility of ingredients for younger birds (Lemme et al., 2004). Studies including Lei and Lixian (2012) have demonstrated a change in enzyme activity after 24 hours of fasting, indicating that fasting can affect the amounts and types of enzymes released during digestion. The fasting method associated with endogenous control determination receives criticism for ignoring the impact of dry matter intake on basal endogenous losses as feed moves through the intestine (Lemme et al., 2004). Butts et al. (1993) reported increased endogenous amino acid excretion correlated to increased dry matter intake in the pig. However, in comparative studies between the cecectomized rooster assay and the broiler ileal bioassay, digestibility calculations were shown to be similar for all samples, with few samples differing significantly when tested with both methods (Garcia et al., 2007; Adedokun et al., 2009; Parsons, 2020). Samples that were significantly different tended to be related to age differences, specifically when evaluating young chicks less than 14 days of age (Garcia et al., 2007). Therefore, it was determined that both methods could be considered valid measures for the determination of amino acid digestibility in birds over 14 days of age (Garcia et al., 2007). Overall, the precision fed cecectomized rooster assay can be preferred over the broiler chick SID assay as it is considered to produce results that are comparable to the broiler chick SID assay while remaining less expensive and less labor intensive (Garcia et al., 2007).

Both the broiler chick bioassay and the cecectomized rooster bioassay are expensive and time consuming. The cost per ingredient sample for each bioassay will be 800 – 1500 dollars and results will not be available for 2 to 4 weeks from the initiation of procedures. Thus, poultry nutritionists must depend on previously determined digestible amino acid values for ingredients and if those are not available, they use total amino acid content values of the ingredient. Early research in poultry nutrition identified the importance of meeting the dietary amino acid requirements for optimum growth and reproduction (Almquist, 1952). Because amino acid digestibility values vary in a given ingredient based on things such as cultivar genetics (Kluth et al., 2005; del Alamo et al., 2008; Perera et al., 2019), growing conditions (Wilcox and Shibles, 2001; Karr-Lilienthal et al., 2004) and processing procedures (Parsons et al., 1992), when formulating diets, poultry nutritionists will use conservative average amino acid digestibility values in order to minimize the chance of the diet having an amino acid deficiency. For ingredients in which only total amino acid concentrations are known, the nutritionists must make an informed guess based on similar ingredients as to how much of the total amino acids are digestible, and again, in order to ensure bird performance, the estimates will be conservative and often lower than the actual digestibility values, leading to nutrient excesses in the feed above the requirement of the bird (Van Kempen and Simmins, 1997).

Excess amino acids. Formulating diets with excess protein, and therefore excess amino acids, increases the cost of production, as protein (amino acid) sources are among the costliest of the dietary ingredients around the world (Kidd et al., 2021; Sumbule et al., 2021; Banday et al., 2023). Once the amino acid needs for growth, maintenance, and production are met in the bird, excess amino acids are catabolized, yielding carbon skeletons that can be utilized in energy production (Szepesi et al., 1970) and the nitrogenous waste component, ammonium. The ammonium by- product of amino acid catabolism must be excreted, as it is toxic to the bird (Visek, 1968). Ammonium is generally transported via glutamine in the bloodstream (Souba, 1991) to the liver, the major site of uric acid synthesis (Badenoch-jones and Buttery, 1975). In poultry, a majority of this ammonium is excreted as uric acid (Davis, 1927; Creek and Vasaitis, 1961), while smaller fractions of excreted nitrogen come from urea, creatine, and ammonium (Davis, 1927).

Ammonia. Once excreted, the nitrogenous waste products uric acid and urea can both be converted to gaseous ammonia (NH_3) in the poultry house, and microbial breakdown of uric acid in the litter is the main source of NH_3 production in the poultry house (Schefferle, 1965). The actions of microbial uricase and urease in the presence of uric acid, water, and oxygen convert the excreted uric acid from the bird into urea, and subsequently, the urea excreted from the bird and the urea formed from uric acid degradation, into carbon dioxide (CO_2) and NH_3 (Bachrach, 1957; Schefferle, 1965). The NH_3 generated in poultry houses has bird health and performance implications, as reviewed by Kristensen and Wathes (2000). Miles et al. (2004) reported a decrease in bodyweight of 6% and 9% in broilers exposed to 50ppm and 75ppm ammonia level treatments, respectively, when compared to the control (0ppm ammonia) group, but no significant bodyweight difference in 25ppm ammonia level treatment group, which agreed with the suggestion by Kristensen and Wathes (2000) that ammonia in poultry production facilities should not exceed 25ppm. Increased mortality was seen in birds receiving the 75ppm treatment (Miles et al., 2004).

Near infrared reflectance spectroscopy

A rapid analysis procedure used on site to accurately analyze feed ingredients for digestible amino acid content before using the ingredients in diets would allow nutritionists to formulate diets with minimized nutrient excess using the values obtained from each load of ingredient they are receiving at the mill. Near infrared reflectance spectroscopy (NIRS) could provide this rapid analysis procedure. Near infrared reflectance spectroscopy is a rapid chemical analysis technique utilizing electromagnetic radiation in the near infrared spectrum of 730nm to 2500nm (wave numbers 4000-12500cm^{-1}). This technique has advantages over the previously mentioned animal bioassays as it takes very little time to complete, requires only a small amount of sample, requires no chemical reagents, and produces no waste. This technique was first described by Norris and Hart (1965) as a potential technique for chemical analysis of feedstuffs but was not applied to feedstuff and forage quality analysis until 1976 (Norris et al., 1976). In NIRS, light in the near infrared spectrum is projected onto functional groups that have vibration frequencies within the same range, and the interactions among these groups and the light are plotted by measuring the amount of light that is reflected back to the detector (Smith et al., 2001). Light of similar wavelengths is absorbed by bonds in the functional groups matching the vibration frequency of that wavelength. Photosensitive materials such as lead sulfide or silicon on the detector converts the reflected light into electrical signals that can be recorded and analyzed by computer software programs (Givens et al., 1997). In the near infrared spectrum there are few functional groups that will have exclusive absorbance, so statical analysis is applied to identify secondary relationships within the spectral data that align with the determined reference values (Foley et al., 1998). Initially, NIRS technology was limited by computer processing power as it was quickly realized that reflectance spectroscopy and multiple regression techniques were both key to identifying interactions and interference between functional groups (Norris, 1992). As computer processing power has improved, the utilization of NIRS has increased and its accuracy in prediction has improved, increasing its popularity and creditability (Moughan et al., 2000).

Feedstuffs used in animal nutrition consist primarily of organic matter with chemical bonds mainly between carbon, oxygen, nitrogen, sulfur, and phosphorus. Bonds containing these atoms will vibrate at specific frequencies depending on bond length, bond strength, and the surrounding constituents, causing them to absorb light at specific frequencies in the near infrared spectrum (Van Kempen and Bodin, 1998). In NIRS feed analysis, a light source projects the light onto a finely ground feed sample and the reflected light is then measured by the detector. As feed is opaque, reflectance spectroscopy is the preferred method for analysis rather than transmittance (Givens et al., 1997). The reflected light is then compared to the wavelengths and intensity of light initially emitted and the difference between the intensity at each wavelength is plotted in an absorption spectrum. Chemical bonds within the sample including carbon to hydrogen (C-H), nitrogen to hydrogen (N-H), and oxygen to hydrogen (O-H) bonds will absorb light at specific wavelengths, returning the unabsorbed light at that wavelength to the detector. As different molecules all have unique arrangements of atoms and corresponding chemical bonds, each molecule within a sample will have a unique NIRS “fingerprint” that can be identified and subsequently quantified (Givens et al., 1997). The quantification of these bonds and interactions among their functional groups, allows for the quantification of larger molecules like sugars, proteins, lipids, and other nutritionally relevant molecules (Givens et al., 1997). Many molecules in feedstuffs will not be individual and free, but rather will be associated or even chemically bond to other molecules present in the heterogenous mixture. Thus, the integrated molecules will need to be distinguished by their 'overtones' and 'combination bands' which are variations in the bond lengths of chemical groups causing them to be in a different energy state when excited by the NIR light source than the same group would have been in the original molecule (Givens et al., 1997).

Light scattering is another issue associated with NIRS. When the NIR radiation is reflected, refracted, or diffracted against random particles or a medium contained within the sample, the change in light can change the detection of the light from that molecule. Light scattering is most affected by the particle grind size, temperature of the sample, and moisture content of the sample (Givens et al., 1997). Understandably, there is no mathematical law describing light scattering in a heterogenous mixture of absorbing constituents as seen in feed ingredients. Therefore, an NIRS machine must be calibrated with a selection of samples that account for the reasonable physical property differentiation that would occur in the population one seeks to measure. These samples must be as similar as reasonably possible for the population and be processed and analyzed in a uniform manner (Givens et al., 1997).

The ultimate goal of NIRS analysis is to establish and validate a calibration model that correlates data in the spectral scans of a specific group of samples to the known concentrations of the nutrient of interest. The known nutrient content of that sample may be determined through chemical analysis, biological analysis, or a combination of the two like the cecectomized rooster bioassay to determine the digestible amino acid content of feed ingredients. Once a calibration curve using these samples has been established that has a high correlation between the spectral data and the wet chemistry data, the calibration curve can be validated and then used to predict the nutrient content of interest in other samples not used in the creation of the calibration and validation curves.

In creating NIRS models for nutrient prediction, the spectral analysis may need to be transformed mathematically to best standardize the spectral plots for accurate correlation to wet chemistry values and accurate determination of the nutrient of interest. Mathematical treatments of this plot include taking the log, 1st derivative, 2nd derivative, or utilizing x and y shifts to standardize all spectral analyses, however, there is no, one, ideal mathematical transformation that works for all molecules within a heterogenous mixture (Rinnan et al., 2009; Huang et al., 2010). Curve smoothing may also be employed to minimize noise, minimize the effects of light scattering, and reduce the effect of overlapping bands (Givens et al., 1997). Additionally, as the number of sample scans available to create a calibration model decreases, sample preparation uniformity becomes more critical in constructing a useful model (Givens et al., 1997).

Sample preparation for NIRS

Sample preparation is key to uniform spectral data collection and utilization in feed ingredient analysis (Starr et al., 1985). Before analysis using the NIRS machine, the instrument should be allowed to warm up for a minimum of 15 minutes, as the spectral analysis of samples is very sensitive to temperature changes (Westerhaus, 1989a). The warming up of the electronics inside the instrument including the lamp and the detector creates a temperature gradient that can affect the temperature of the sample compartment, affecting the spectral readings (Workman, 2008).

Grinding. Variation in sample particle size due to sample grinding will have the largest effect on reflection and absorbance as grinding increases the surface area of the product for exposure to near infrared (NIR) light. Feed ingredients should be reduced to a particle size that ensures that all ingredient nutrients are uniformly exposed to the NIRS light source. It has been determined experimentally that the accuracy of an NIRS calibration model is increased by uniform grinding (Fontaine et al., 2001). Ideally, NIRS analysis of the ground sample would take place immediately after sample preparation, but also after a rest period to dissipate any added heat from grinding. After samples are analyzed on the NIR instrument, these ground samples should be stored immediately to limit degradation and to be available for future use. It is recommended that the samples be stored in a freezer in glass bottles (Valdes and Leeson, 1992), polyethylene bottles (Fontaine et al., 2002), or plastic bags (Smith et al., 2001; Tahir et al., 2012), as to minimize oxidation of the product and other chemical changes caused by improper long term storage (Abrams, 1989).

There are two general types of laboratory feed grinders: shearing- type and impacting-type. Shearing- type mills like the Wiley mill, have knives that first reduce particle size then force the sample through a sieve into a collection vessel. Impact grinders force the feed through a wall impregnated with silicon carbide before passing through a sieve. Significant differences have been reported in sample analysis of whole soybeans ground with a Cyclotec (impact) grinder versus a Wiley (shearing) grinder (Bakalli et al., 2000). It was also found that the Cyclotec grinder caused a 3% sample moisture loss due to increased air exposure, leading to less experimental error when preparing samples using this grinder (Bakalli et al., 2000). Grinding can create heat that warms the sample and has an impact on NIRS analysis (Hunt et al., 1978).

Different sized sieves or screens have been reported throughout the literature, but 1 mm Screens (Valdes, Young, Leeson, et al., 1985; Van Kempen and Bodin, 1998; Bakalli et al., 2000) and 0.5 mm screens (Bakalli et al., 2000; Smith et al., 2001; Fontaine et al., 2002) are roughly, equally represented in current literature. Regardless if a 0.5 mm or 1 mm screen is used, it is imperative that the same screen is used for all samples of a calibration model and all samples ground for analysis using this calibration, as the near infrared spectrometer is sensitive to the sample surface area exposed for analysis, and differences in average particle size has even been shown to account for up to 90% of spectral variance among samples (Givens et al., 1997; Foley et al., 1998).

Temperature. A change in sample temperature can also cause bond deformation, leading to distortions in the spectral analysis that are not generally confined to a unique region of the spectrum (Hansen et al., 2000). Temperature variation among prepared samples should be minimized by allowing samples to rest after grinding, dissipating any heat imparted during the grinding process. Temperature variation among samples will affect bonding of organic elements within the sample, resulting in variation among absorbance bands and overtones detected within the sample (Hansen et al., 2000).

Moisture. Excess water in samples used for NIRS analysis creates issues including O-H bond stretch and O-H bond deformation in the 950-1075nm, 1340-1450, and 1810-2000nm regions of the NIR spectrum. These deformations can cause detector saturation in these regions, distorting the analysis (Baker et al., 1994; Givens et al., 1997). As these regions correspond with regions common in feed analysis for protein and C-H bonds, the presence of moisture and the associated detector saturation in a spectra can obscure regions of the NIRS spectra needed for the prediction of nutrients (Givens et al., 1997).

Pre- processing. Pre-processing techniques applied to the raw spectra file can be classified as scatter correction methods or spectral derivatives (Conzen, 2014). Standard Normal Variate (SNV) is the most common technique applied for scatter correction. Scatter correction helps remove undesirable scatter effects in the spectra before the data is modeled (Conzen, 2014). Correction coefficients are calculated and applied to the multivariate equation to minimize noise. The correction coefficient is determined by taking the original spectra value, subtracting the overall mean for the spectral image, and dividing that value by the standard deviation of spectrum (Rinnan et al., 2009). The noise in the spectral curve can detract from the main absorption spectrum and cause variation in the spectral line including peaks of the spectral image.

Multiplicative Scatter Correction (MSC) is another scatter correction technique that is common in NIRS analysis (Conzen, 2014). This technique uses a similar calculation to SNV, but it compares the spectrum in question to an “ideal” spectrum with no scattering effects, though finding a single “ideal” spectrum is rather unreasonable when dealing with feed ingredients. The program can mathematically find ideal sections of multiple spectra and use them to “stitch” together an ideal spectrum for comparison (Windig et al., 2008). This then modifies the scale and creates an offset to apply to all spectra in the model which corrects for noise using baseline shifts and changes to peaks in the spectra, unlike SNV which has less impact on spectral peaks (Windig et al., 2008). Normalization uses a scaling procedure similar to MSC but instead of searching for an “ideal” spectrum, this technique searches for anchoring sections of all spectra that are constant among all spectra in the model. These anchoring points are then used to align all spectra and a scaling is performed to closely align these points. Like MSC, this includes baseline shifts and adjustments to spectral peaks (Rinnan et al., 2009).

Spectral derivative pre-processing may also be applied to spectral images. Taking the derivative of a spectral image helps standardize spectra by evaluating the derivative of the point rather than its original x and y position in the plot. This prevents error caused by baseline shifts and peak changes that can occur in scatter correction techniques. This method is now evaluating the slopes of points along the line rather than the location of those points. First and second derivatives are the most common spectral derivatives applied in NIRS analysis (Huang et al., 2010). Derivative pre-treatment is used to resolve peak overlaps and baseline shift issues in spectra. It is also important to note that both scatter correction and spectral derivative preprocessing techniques can be combined for the evaluation of a set of spectra (Conzen, 2014). Examples include first derivative plus vector normalization and first derivative plus MSC.

NIRS calibration curve creation and validation

After samples have been prepared and scanned using the NIRS instrument, samples can then be selected for incorporation into a calibration curve that correlates the spectral image to the determined concentration value of the molecule of interest in the sample. The spectral image for each sample will be entered into the NIRS program and assigned the known chemical value determined through wet lab analysis or biological assay analysis of the sample. The program will create a calibration curve that uses specific wavenumbers within the range of 4000-12500 cm^{-1} to best identify peaks or unique spectral characteristics that may correspond with the concentration of the chemical being measured. The selection of calibration samples is critical as the spectra of the samples used should cover the entire range of expected chemical values for a given product and should mirror the expected variability for that product (Fontaine et al., 2002).

The number of samples used in a calibration curve must also be considered. Windham et al. (1989) recommended that a minimum of 50 samples should be present in the calibration curve. Other sources recommend 10-50% of the total population of samples should be in the calibration model (Westerhaus, 1989b; Conzen, 2014). The sample spectra selected for the calibration curve will undergo pre-processing, allowing for standardization of spectral images and selection of NIR spectral regions that best correlate to the known chemical values (Conzen, 2014). The calibration curve will then be presented to the user as a regression model of true vs predicted values. Based on this regression model, the user can begin adding samples, removing samples, and evaluating possible outlying samples in the dataset to improve the calibration curve. If a possible outlier is identified through the NIRS program, the user should evaluate the sample to ensure that it was not a preparation or scanning error. If this is not the issue, the outlying sample could be an atypical sample or a sample with poor characteristics such as mold contamination or degradation (Van Kempen and Bodin, 1998; Fontaine et al., 2001). The user must then determine whether outlier samples should be kept in the calibration curve, used in the validation curve, or excluded from the NIRS model completely.

Once the calibration curve is created, it must be validated with an independent set of samples. The chemical concentration of the validation samples and the physical characteristics of these samples should be representative of the samples used in the calibration curve (Westerhaus, 1989b). The validation samples are prepared and scanned in the same manner as the calibration samples. The content of the molecule of interest is predicted in the validation samples using the calibration curve. The predicted values of the validations samples are then compared to the known true bioassay chemical reference values by the software, allowing the predictive abilities of the calibration curve to be determined (validated). The validation results can indicate areas of weakness in the calibration curve including where samples may need to be added or removed for better predictability.

Statistical evaluation. There are a variety of statistical methods described for the evaluation of the accuracy of calibration and validation curves. The coefficient of determination or R^2 is the simplest technique used on both the calibration curve and the validation curve. This statistic compares the residuals of the NIRS prediction back to the total sum of squares of the range of true values for the samples. It indicates how closely the predicted values compare to the true values. Similar to R^2 is the residual predictive deviation (RPD). The RPD is calculated similarly to the R^2, but the denominator is the error of prediction of the model, allowing the range of values to increase well above 1.0. A ratio greater than 3.0 is considered a significant measure of accuracy when using the RPD (Fontaine et al., 2002; Losada et al., 2009). The root mean square error of cross validation (RMSECV) and the root mean square error of prediction (RMSEP) are the statistical measures of error in the calibration curve and validation curve, respectively. These values should be minimized in the calibration model, and to compare the calibration and validation curves, the values should be similar (Valdes and Leeson, 1992; Fontaine et al., 2002). Practical considerations when evaluating the statistical accuracy of the validation curve would include calculating the percentage deviation of the predicted value from the true value for each validation sample. This measure would better identify accuracy of the models, specifically at the lowest concentrations, as the calculation references the true value of each individual sample rather than an average error or average mean of the group (Cope, 2021).

NIRS application in animal nutrition

Unlike wet chemistry analysis techniques and animal bioassays, NIRS requires no special reagents and produces no waste, other than the dust created from grinding (Bakalli et al., 2000). Regarding efficiency, once individual calibration models have been developed for each nutrient, a single spectral scan can be used to determine the nutrient concentration of multiple nutrients simultaneously, using any calibration model available to the user (Fontaine et al., 2001).

The NIRS technology has been successfully calibrated to determine dry matter, crude protein, fat, total amino acids, and other feed parameters in feed ingredients and diets (Valdes and Leeson, 1992, 1994; Garnsworthy et al., 2000; Wu et al., 2002; Kovalenko et al., 2006; Reid, 2017). In comparing NIRS to nitrogen-based regression analysis for predicting crude protein concentrations, NIRS was shown to explain 21-58% more variation in each feedstuff category than nitrogen-based regression analysis (Van Kempen and Bodin, 1998). Other research has reported correlation coefficients ranging from 0.91-0.99 for calibrations predicting crude protein (Valdes, Young, McMillan, et al., 1985; Aureli et al., 2017). While NIRS has been proven effective in predicting total nutrient content of multiple nutrients in feed ingredients (Rahman et al., 2015), its use in the prediction of digestible nutrient content including digestible amino acid content and metabolizable energy content is not well developed, as reviewed by Cope (2021).

Though there are many perceived benefits of NIRS for analysis of feed ingredients, there are some disadvantages to implementing an NIRS program. The initial cost of an NIRS machine with software for feed analysis can be prohibitive, with initial costs exceeding $100,000 (Brown et al., 2020). The cost of operation of this instrument is low, in terms of electricity and hourly operation costs, but feed production facilities may need to invest in a dedicated employee to operate their NIRS analysis program, depending on the frequency of sampling and scanning that must be performed. The operator should be familiar with laboratory protocols from prior training or with an associate degree in a laboratory field and should be overseen by a nutritionist or maintain a close relationship with the technical team at their specific NIRS manufacturer. In the long term, the instrument does require maintenance every 3-5 years for the light source and any associated software updates (Bruker Optics, Billerica, MA). The use of the machine is predicated upon the availability of the needed calibration models for analysis of the nutrient of interest for each ingredient. After the initial cost of the instrument, if an operator has the calibration models needed, the cost of each individual sample analysis is low (Bastianelli, 2013). If calibration models need to be developed, the nutrient content of ingredients will need to be determined, whether through wet lab chemistry analysis or the use of animal bioassays, and the owner must absorb the expense of generating the needed samples and values.

Optic differences, especially between different NIRS machine brands, but even between machines of the same brand, as well as differences in sample grinding and laboratory conditions all create variation in spectral analyses that can be present in the NIRS calibration, making transfer between machines difficult (Givens et al., 1997). As samples are scanned on one machine, the characteristics of that machine may contribute to the accuracy of the model. This is one reason why scatter correction and spectral derivative treatments, as reviewed earlier, are necessary in NIRS analysis, as they help buffer against individual machine biases. To mitigate machine biases, calibration samples can be stored and scanned on the new machine. Once scanned on the new instrument, the wet chemistry values can be associated with the spectra and the calibration model can be recreated using the same samples but using the unique scans created by that machine.

Summary

Over time, meeting the amino acid requirements of poultry has evolved from formulating diets on a crude protein basis, to a total amino acid basis, and more recently, to a digestible amino acid basis. These transitions have allowed diet formulations to better match the amino acid requirements of the bird being fed to what is available in the diet, which has improved feed efficiency and decreased production costs. However, formulating on a digestible amino acid basis is currently hampered by the lack of accurate digestible amino acid concentrations for traditional feed ingredients that have variations due to cultivar genetics, growing conditions, and processing procedures. Newer, alternative feed ingredients have simply not had digestible amino acid values determined. The lack of digestible amino acid values for feed ingredients is because the bioassays used to determine the values are expensive and time consuming. However, NIRS technology could solve this problem if digestible amino acid calibration curves could be successfully developed and validated.

CHAPTER 2

STATEMENT OF PURPOSE

Digestible amino acids are an area of increased focus in the poultry industry and are important nutrients to consider when formulating poultry diets. In poultry, there are 10 essential amino acids that cannot be synthesized by the bird in sufficient quantities for growth, maintenance, and production, and therefore must be obtained from the diet. Evaluating protein sources used in poultry diets for digestible, essential amino acids is preferred over the crude protein method and the total amino acid method as the digestible content of an amino acid source represents what is available to the bird for maintenance, growth, and production. To properly formulate these rations with high accuracy and efficiency, a nutritionist must know the digestible amino acid concentration of feed ingredients. Presently, the best method for determining the digestible amino acid content of feed ingredients is through the cecectomized rooster bioassay and broiler chick ileal sampling bioassay and accompanying laboratory wet chemistry analyses. Though accurate, these processes are time consuming and expensive. In a commercial feed mill setting, the animal bioassay with lab analyses cannot be completed before the sampled ingredient is milled and fed to the animals, limiting its usefulness in diet formulation.

Near infrared reflectance spectroscopy is a rapid analysis method that can be used to predict nutrient composition of feedstuffs by using a multipurpose analyzer that is calibrated to analyze the reflectance of near infrared light from specific feed ingredients. Initially, samples must be collected, prepared, and subsequently scanned using the NIRS system, then associated within the software with known values determined using the animal bioassay and laboratory analyses. From this data, a calibration curve can be developed that can predict specific nutritional components in unknown samples without further wet chemistry analysis. The accuracy of this calibration curve is then validated with a separate set of samples in the validation curve, using the equation determined by development of the calibration curve. This method of evaluation is quick, requires no chemicals, creates no additional waste other than the feed used to scan, and is relatively easy to perform. The NIRS analysis method has been used in the feed industry for estimation of crude protein, vitamins, minerals, total amino acids, moisture, ash, and gross energy in feed ingredients, but there is a lack of published research using NIRS for the direct determination of digestible nutrients including digestible amino acids. As the digestible amino acids are most useful to nutritionists for accurate feed formulation, the direct, accurate determination of digestible amino acids with NIRS would provide the most benefit in commercial poultry production. Therefore, the goal of the current research is to create and validate NIRS calibrations for the determination of digestible amino acid content for each of the 10 essential amino acids in feed ingredients used in poultry diets and to have these calibrations predict 95% of ingredient samples for each of the 10 essential amino acids in feed ingredients to within plus or minus 5% of the actual bioassay determined value.

CHAPTER 3

MATERIALS AND METHODS

Cecectomized rooster bioassay

All animal procedures were approved by the University of Georgia Animal Care and Use Committee. The cecectomized rooster bioassay was performed similarly to the method established by Parsons (1985). Annually, 400 day of hatch Hy-Line W36 Single Comb White Leghorn male chicks were obtained from the Hy-Line North America, LLC. The chicks are placed in a room measuring 7.31 by 6.14 meters containing six plastic pan feeders (0.14 meters^2 each) and two water lines each equipped with 20 nipple drinkers. A Chore-Time ChoreTronics Model 18 controller (Chore-Time, a division of Chore-Time Brock International, Inc., Milford, IN) regulated a single natural gas-fired furnace, one exhaust fan (45.7 centimeter), and one ventilation fan (25.4 centimeter) for air exchange. Inlet air was brought in from a common hallway equipped with a separate ChoreTronics Model 18 controller regulating two gas fired furnaces and an evaporative cooling system. Ambient room temperature was set at 34°C on Day 1 and decreased by 0.28°C per day until 24°C was reached and maintained through 20 weeks of age. The lighting intensity was set at 20 lux from 0-14 days of age then reduced to 2 lux from Day 14 to 20 weeks of age. The cockerels received 23.5 hours of light on the day of placement and light time was reduced by 30 minutes daily until 10 hours of light per day was reached and maintained until 20 weeks of age. The cockerels were provided with feed and water ad libitum throughout this period with all diets formulated to meet or exceed guidelines established by the NRC (1994).

At 20 weeks of age, roosters were relocated to a single room containing six, 64 individual cage battery units with cages measuring 35.5 centimeters wide, 45.7 centimeters deep, and 61.0 centimeters tall. Each cage was equipped with a single nipple drinker and had access to a feed trough. The room measured 12.4 meters wide by 14.5 meters long and was ventilated by a positive pressure ventilation system. The system provided central heating and cooling via a natural gas fired furnace and an integrated evaporative cooling system. The temperature and ventilation were controlled by a standalone Invensys brand controller (Invensys Building Systems, Loves Park, Illinois, 61111, USA). There were also 3 circulation fans controlled by an independent timer, set to circulate air for one minute out of each five-minute cycle. Lighting intensity was maintained at 20 lux and light was provided for 16 hours per day.

For determination of digestible amino acid content, cecectomized roosters were utilized to avoid cecal bacterial digestion and utilization of undigested amino acids by the bird confounding the results. Cecectomy surgery occurred after roosters acclimated to their individual cages when the roosters were between 21 and 26 weeks of age. After a 21-day recovery period from surgery, the roosters were utilized for feeding trials. A total of 160 roosters were cecectomized for the amino acid digestibility bioassay. Cecectomized roosters were utilized for ingredient feeding trials until they reach 80 weeks of age, at which point they were replaced with the next group of 20-week-old roosters.

When a sample was ready to feed, 8-10 roosters were fed per sample. Roosters were removed from feed 24 hours prior to feeding an ingredient sample, to allow the digestive tract to empty (Sibbald, 1979). Most commonly, each fasted rooster was fed 35 grams of the feed ingredient. However, for ingredient samples that were less dense and bulky like wheat middlings and soybean hulls, only 25 grams was fed to reduce the chance of crop impaction. The measured sample was precision fed through direct placement of a tube into the crop. After feeding, each rooster was housed in an individual collection cage measuring 30.5 centimeters wide, 45.7 centimeters deep, and 50.8 centimeters tall, for the next 42 hours with ad libitum access to water. A stainless-steel collection pan was placed under the bird's cage during this time to collect excreta. In addition to birds fed the ingredient samples, another group of 10 fasted roosters were not fed, but placed in collection cages for the next 42 hours to serve as endogenous control birds. After 42 hours, the birds were returned to their original cage and given full access to feed and water again. The excreta from each rooster was scraped from the stainless-steel pans and dried at 60°C for 18-30 hours (Dale et al., 1985). After the samples were dry, they were individually weighed. Weighed samples were then combined for three to five roosters to create two to three replicates of excreta samples for each feed ingredient that was fed. The individual replicate samples were ground using a KitchenAid® (KitchenAid®, Benton Harbor, MI) coffee grinder, sifted through a 1-millimeter sieve, and placed into labeled sample bags for subsequent total amino acid analysis. Also, 100 gram and 50-gram aliquots of each feed ingredient fed was saved for subsequent NIRS analysis, and total amino acid determination, respectively.

Chemical reference methods

Nitrogen content of samples was assessed with a LECO combustion analyzer (LECO Corporation, St. Joseph, MI) using the combustion technique (AOAC, 2006b). Nitrogen correction and conversion to crude protein percentage was done using the 6.25 correction factor. Complete amino acid analysis was completed by high performance liquid chromatography using the multi-step procedure outlined in AOAC Official Method 982.30 (AOAC, 2006a).

Digestible amino acid calculation

Once amino acid analysis had been performed on the feed and excreta samples, these values were utilized to calculate digestible amino acid values for each amino acid for each sample using the following equation:

> [[Total amino acid content in grams of the amino acid of interest in the feed ingredient– (total amino acid content in grams of the amino acid of interest in the excreta sample from cecectomized roosters fed the sample ingredient– total amino acid content in grams of the amino acid of interest in the excreta sample from endogenous control cecectomized roosters)] divided by the total amino acid content in grams of the amino acid of interest in the feed ingredient sample] multiplied by 100 to yield the percent digestibility of that amino acid in that feed sample. Digestibility values for the two to three replicate excreta samples for each ingredient sample were then averaged.

NIRS sample preparation

Ingredient samples stored at room temperature were ground at 16,000 rotations per minute through a 1-millimeter screen with a Retsch type ZM200 centrifugal laboratory grinder (Retsch GmbH, Haan, Germany). The sample was then bagged in a labeled sterile, plastic sample bag (Thermo Fisher Scientific, Waltham, MA) and allowed to re-acclimate to room temperature for 30 minutes to ensure no excess heat was present before scanning. The grinder components were thoroughly cleaned between samples using a professional grade wet/ dry vacuum (Rigid, model WD 14500, St. Elyria, OH) and absorbent towelettes (Kimwipes, Kimberly-Clark, Roswell, GA). Samples that already had a consistent particle size below the 1-millimeter screen threshold were not ground. After scanning with the NIRS analyzer, samples were returned to their individual labeled sample bag and stored at -20°C.

Bruker Multipurpose Analyzer (MPA)

A Bruker multipurpose analyzer, Fourier transform, near infrared spectrometer (Bruker MPA: FT-NIR, Bruker Optics, Billerica, MA) was used for near infrared reflectance analysis of feed samples. This machine was outfitted with a sample rotator to ensure uniform exposure of the feed sample to the NIR light source. The machine was kept in a climate-controlled laboratory at room temperature. The machine was turned on 30 minutes prior to use and had its dried desiccant beads replaced at this time. These actions ensured the machine's electronics and infrared lamp had time to warm up and stabilize, and that the sample chamber within the machine had excess moisture removed before operation. A background light scan was performed before analysis of each sample to ensure calibration of the machine before samples were scanned. Samples were placed in a quartz bottomed analysis cup and lightly packed by shaking the cup, not by pressing the sample. The cup was then placed on the rotator, a background scan was performed, and then the final NIRS scan was performed to generate a spectral image of the sample. The MPA system included OPUS® version 8.5 software (Bruker Optics, Billerica, MA) for operation of the MPA NIR machine, analysis of the resulting spectral images, and building of the calibration models using the spectral images.

Samples for the calibration and validation curves were selected from the same pool of samples, but the samples used in the calibration curve were independent from the samples used in the validation curve. In all, 1,034 samples were used for the digestible amino acid dataset. These samples were from 58 unique ingredient categories (Table 3.1). Crude protein content of samples ranged from 2.1% up to 95.7% (Table 3.2). Samples were independently assigned to the calibration and validation curves with a goal of 20-30% or fewer of samples in the calibration set with the remaining samples in the validation curve. To create a calibration curve, 50% of available samples were assigned to the calibration curve using random assignment by percentage nutrient content or via principal component analysis (PCA). Samples were then revised by removing samples from the calibration curve and placing them in the validation curve until the 30% or less margin was reached. Samples by ingredient category were proportionately represented in the calibration curve and the validation curve. The goal was to develop a calibration curve for each essential amino acid using the fewest number of samples while maintaining the maximum predictive capacity. Having the fewest number of calibration samples facilitates recreating the calibration curves on NIRS machines produced by different manufacturers when it is necessary to take physical samples for scanning on a new machine.

After full spectral analysis for all ingredient samples was completed on the Bruker MPA, the OPUS® software package (Billerica, MA) was used to determine the appropriate wavelength frequency ranges and spectral pre-processing to apply to each calibration model for optimum prediction accuracy. Frequency regions for the best detection of each amino acid were selected within the overall range from wave number 9000 to wave number 4200. These wave numbers were used in lieu of the standard frequency range values because the wave number system allows for more divisions across the frequency range for improved accuracy. The wave numbers ramge 9000-4200 is equivalent to the frequency range of approximately 1050-2400 nanometers.

Table 3.1. Feed ingredient samples used in near infrared reflectance spectroscopy evaluation of digestible, essential amino acid content.

Ingredient type	Sample count	Ingredient type	Sample count
	n		n
AA manufacture waste[1]	11	Hominy	3
Alfalfa meal	1	Isolated DDGS products[7]	3
Algae	3	Isolated soy protein	11
Almond shells	4	Jatropha meal	1
Unidentified animal meal[2]	19	Meat and bone meal	355
Arrow root leaves	1	Millet	2
Bakery meal	20	Moringa powder	1
Barley	1	Offal & waste	2
Blood meal	20	Poultry by-product meal	98
Brazil nut powder	1	Pea meal	2
BSFL[3]	5	Peanut meal	10
Calliandra leaves	1	Pongamia meal	1
Canola meal	19	Rice bran	2
Carinata meal	2	Rice hulls	4
Cassava leaves	1	Rice protein	4
Corn	47	Rye	1
Corn germ meal	5	Soybean meal	130
Corn gluten feed	2	Sorghum	8
Cottonseed meal	1	Soybean hulls	1
DAF skimmings[4]	2	Sprouted seeds[8]	5
DDGS[5]	108	Sugar beet root powder	1
Dried porcine mucosa	1	Sunflower meal	3
Feather meal	29	Supermarket waste	6
Fermented products[6]	18	Sweet Potato leaves	1
Fish meal	6	Poultry tankage	26
Flaxseed meal	2	Wheat	7
Hatchery waste	2	Wheat DDGS	1
Hemp leaves by-product	2	Wheat middlings	7
Hemp seed	2	Yeast	2
		Total	1034

[1] Amino acid manufacturing by-products.
[2] Animal by- product meals whose composition was not defined.
[3] Black soldier fly larvae.
[4] Dissolved air floatation skimmings.
[5] Dried distillers' grains with solubles.
[6] Fermented soybean meals, fermented DDGS and unidentified fermented products.
[7] Isolated products from DDGS production.
[8] Seed meals of sprouted corn, soybean, barley, and wheat.

Table 3.2. Crude protein values for feed ingredient samples used in near infrared reflectance spectroscopy evaluation of digestible amino acid content.

Ingredient type	Sample count	Crude protein values		
		Mean	Minimum	Maximum
	n		%	
AA man. waste[1]	11	78 ± 2.8	73	84
Animal by-products[2]	530	58 ± 10.2	24	96
Bakery meal	7	13 ± 2.3	10	16
Canola	19	36 ± 2.7	28	41
Corn	43	8 ± 0.8	6	10
DDGS[3]	86	34 ± 9.6	22	57
Fermented products[4]	18	64 ± 13.4	29	77
Isolated soy protein	11	59 ± 5.3	53	67
Other ingredients[5]	72	27 ± 16.7	2	63
Peanut meal	8	47 ± 5.1	42	55
Soybean meal	122	48 ± 5.6	33	69
Sorghum	8	12 ± 2.9	9	16
Wheat	6	13 ± 1.4	11	15
Wheat middlings	5	16 ± 0.8	15	17
Total	946[6]			

[1] Amino acid manufacturing by-products.

[2] Animal by-products- includes unidentified animal by-product meal, blood meal, dissolved air flotation skimmings, dried porcine mucosa, feather meal, fish meal, hatchery waste, meat and bone meal, offal and waste, poultry by-product meal, and poultry tankage.

[3] Dried distillers' grains with solubles.

[4] Fermented soybean meals, fermented DDGS, and unidentified fermented products

[5] Other ingredients/ all products with 6 or less total samples- includes alfalfa meal, algae, almond shells, arrow root leaves, barley, Brazil nut powder, black soldier fly larvae, calliandra leaves, carinata meal, cassava leaves, corn germ meal, corn gluten feed, cottonseed meal, flaxseed meal, hemp by-products, hemp seed, hominy, isolated DDGS products, jatropha, millet, moringa powder, pea meal, pongamia meal, rice bran, rice hulls, rice protein, rye, soy hulls, sprouted seeds, sugar beet root powder, sunflower meal, supermarket waste, sweet potato leaves, wheat DDGS, and yeast.

[6] Only 946 samples of the total 1034 samples used in NIRS evaluation of digestible amino acid content had values for crude protein content.

All feed ingredient samples were scanned in their “as is” state. Although drying the samples would limit the moisture content of samples and minimize the influence of moisture on the spectral image, as reviewed in Chapter 1, the developed NIRS digestible amino acid calibrations are intended to be used in commercial feed mill settings where the ingredients are used on an “as is” basis and not on a dry matter basis. In addition, poultry diets are formulated with ingredients that are subsequently fed to birds on an “as is” basis.

Experiment 1- Universal feed ingredient model

All 1,034 ingredient samples (Table 3.1) were used to develop calibration and validation curves for predicting the digestible amino acid content of the 10 essential amino acids. Individual calibration curves were created for each essential amino acid using approximately 150 samples per curve, leaving all the remaining samples to be used in the validation curve. The calibration curves for the 10 amino acids arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine were identified as Experiments 1a- 1j, respectively.

Experiment 2- Common animal by- products model

Experiment two used a subset of samples from experiment 1. The 533 samples used were animal by- products that are commonly used in poultry diets, which included feather meals, fish meals, meat and bone meals (MBM), poultry by-product meals (PBM), poultry tankage, and unidentified animal by-product meal products. Each of the 10 calibration curves for predicting the essential digestible amino acid content of common animal by- product meals contained approximately 145 samples, leaving the remaining samples for use in the validation curve. The calibration and validations curves for the 10 essential amino acids were identified as Experiments 2a- 2j.

Experiment 3- DDGS products model

In the final experiment, only the DDGS samples from Experiment 1 were used to create and validate calibration curves for digestible, essential amino acid content determination. There were 108 DDGS samples, so each specific amino acid calibration curve was created using approximately 33 samples, leaving the remaining samples in the validation curve. The calibration curves and their validations for the 10 essential amino acids arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine were identified as Experiments 3a- 3j, respectively.

Statistical Analysis

Statistical analysis of the calibration and validation curves was completed using the Bruker OPUS® software package, version 8.5. Calibration and validation curves had initial robustness evaluated with the coefficient of determination (R^2) and the residual predictive deviation (RPD). The R^2 value represents how closely the data fits the model. It is calculated as one minus the quotient of the sum of residuals squared and the total sum of squares. It shows how well the model explains the variance of the data set by comparing the true values to the predicted values and how well the resulting points align with the regression model, often called the goodness of fit. The RPD value is calculated by dividing the standard deviation of the observed values by the bias-corrected mean error of prediction. The RPD is more qualitative than the R^2 as the denominator in the RPD is reflective of the error in the model and as that error is lowered, the value lowers which allows the RPD to increase, reflective of the model improvement. The resulting value is a representation of the models' predictive capacity with values over 3.0 considered meaningful, and higher values serving as a better indicator of predictive accuracy (Conzen, 2014). Values greater than five are considered good for quality control while values greater than eight are considered excellent for analytical tasks (Conzen, 2014).

The OPUS® software also calculated the standard error of the estimate (SEE) for calibration curves. The SEE represents the average distance the observed values fall from the regression line established by the calibration model (Conzen, 2014). In the validation curve, the root mean square error of the prediction (RMSEP) and the bias of the resulting regression line were two validation specific metrics. The RMSEP is the quantitative measure for the preciseness of estimation of the test set samples (Conzen, 2014). Smaller values of RMSEP are preferred as they indicate a smaller range of error in the model. This value will be reflective of the range of the model so there are no “good” or “bad” values or ranges. The goal is to minimize the RMSEP when developing a model. In practice, approximately 75-85% of samples will fall within the RMSEP when evaluating the absolute value of their residuals, but this value does not specify what ranges the model is most accurate or least accurate in predicting values. The bias of a validation model is the deviation of the line established by the model from the ideal line of x=y (Conzen, 2014). The model should attempt to minimize bias when establishing a regression line of true to predicted values.

In addition to OPUS® statistical evaluations, evaluation of calibration curve accuracy when broken down by individual ingredient was done using JMP® Pro 26.0 (JMP Statistical Discovery, LLC, Cary, NC). Practical evaluation of calibration models was also done by calculating the absolute deviation of the predicted value from the true value. Samples in the validation curve whose predicted value deviated from their lab determined true values by less than or equal to 2.5%, 5%, 10%, 15%, and 20% were quantified as done by Cope (2021).

CHAPTER 4

RESULTS

Calibration curves were created and validated for the prediction of digestible amino acid content for the 10 essential amino acids in poultry feed ingredients. Pre-processing techniques were determined using the OPUS® software package and applied to ingredient sample spectra. Although the pre- processing methods utilized varied across amino acid calibration curves, vector normalization was most commonly applied (Table 4.1).

Experiment 1- Universal feed ingredients calibration

Experiment 1a. The universal calibration curve for digestible arginine was calibrated for a range of 0.02% to 6.53% digestible arginine content (Table 4.2). While the validation curve had an R^2 of 0.93 and an RPD of 3.85, only 67% of samples were predicted with an error rate of 10% or less of their true value (Table 4.3).

Experiment 1b. The calibration curve for digestible histidine used 148 ingredient samples with a content range of 0.00% to 6.62% (Table 4.4). The R^2 and RPD of the histidine validation curve were 0.96 and 5.18, respectively. However, only 62% of samples were predicted with an error rate of 10% or less of their true value (Table 4.3).

Experiment 1c. The calibration curve for digestible isoleucine utilized 149 ingredient samples that had a digestible isoleucine content range of 0.04% to 3.97% (Table 4.5). While the isoleucine validation curve had an R^2 of 0.93, only 34% of samples had predicted values that had an absolute percent deviation of 5% or less from their actual value (Table 4.3).

Table 4.1. Near infrared reflectance spectroscopy pre-processing techniques used in each calibration curve created for the determination of digestible amino acid content of the essential amino acids in poultry feed ingredients (Experiments 1-3).

Amino acid	Universal	Common animal products	DDGS products
	Pre- processing technique		
Arginine	1st derivative + VN[1]	Vector normalization	Vector normalization
Histidine	Vector normalization	Vector normalization	1st derivative + MSC[2]
Isoleucine	Vector normalization	Vector normalization	Vector normalization
Leucine	Vector normalization	Vector normalization	1st derivative
Lysine	Vector normalization	Vector normalization	1st derivative + MSC[2]
Methionine	Vector normalization	1st derivative + VN[1]	1st derivative
Phenylalanine	Vector normalization	Vector normalization	1st derivative + VN[1]
Threonine	1st derivative + VN[1]	Vector normalization	Vector normalization
Tryptophan	Vector normalization	1st derivative + MSC[2]	1st derivative + VN[1]
Valine	Vector normalization	Vector normalization	1st derivative + SLS[3]

[1] First derivative + vector normalization

[2] First derivative + multiplicative scatter correction

[3] First derivative + straight line subtraction

Table 4.2. Digestible arginine content of ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 1a).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n	%			n	%		
AA man.by-product[1]	2	3.64 ± 0.862	3.03	4.24	9	3.93 ± 0.595	2.69	4.51
Animal by-product[2]	58	3.27 ± 0.981	1.04	5.49	502	3.17 ± 0.529	1.84	5.24
Bakery meal	2	0.43 ± 0.056	0.39	0.47	18	0.55 ± 0.105	0.36	0.74
Canola	4	1.25 ± 0.742	0.59	1.91	15	1.71 ± 0.420	0.85	2.31
Corn	5	0.33 ± 0.029	0.30	0.38	42	0.32 ± 0.048	0.25	0.46
DDGS[3]	11	1.33 ± 0.570	0.53	2.18	97	1.25 ± 0. 534	0.53	2.38
Fermented product	8	3.89 ± 0.516	3.13	4.47	10	3.63 ± 1.021	1.08	4.40
Isolated soy	0	-	-	-	11	3.99 ± 0.474	3.59	4.74
Other ingredients[4]	35	1.25 ± 1.235	0.02	6.53	37	1.62 ± 1.182	0.33	4.17
Peanut meal	4	4.95 ± 0.321	4.55	5.30	6	5.11 ± 0.206	4.76	5.36
Soybean meal	14	2.97 ± 0.560	2.00	3.79	116	3.15 ± 0.472	1.55	4.87
Sorghum	3	0.34 ± 0.016	0.32	0.35	5	0.32 ± 0.007	0.31	0.33
Wheat	0	-	-	-	7	0.50 ± 0.056	0.45	0.62
Wheat middlings	2	0.89 ± 0.117	0.81	0.98	5	0.85 ± 0.122	0.72	1.00
Aggregated	148	2.42 ± 1.486	0.02	6.53	880	2.66 ± 1.131	0.25	5.36

[1] Amino acid manufacturing by-product.

[2] Animal by-product- includes unidentified animal by-product meal, blood meal, dissolved air flotation skimmings, dried porcine mucosa, feather meal, fish meal, hatchery waste, meat and bone meal, offal and waste, poultry by-product meal, and poultry tankage.

[3] Dried distillers' grains with solubles.

[4] Other ingredients/ all products with 6 or less total samples- includes alfalfa meal, algae, almond shells, arrow root leaves, barley, Brazil nut powder, black soldier fly larvae, calliandra leaves, carinata meal, cassava leaves, corn germ meal, corn gluten feed, cottonseed meal, flaxseed meal, hemp leaves by-product, hemp seed, hominy, isolated DDGS products, jatropha meal, millet, moringa powder, pea meal, pongamia meal, rice bran, rice hulls, rice protein, rye, soy hulls, sprouted seeds, sugar beet root powder, sunflower meal, supermarket waste, sweet potato leaves, wheat DDGS, and yeast products.

Table 4.3. Mathematical evaluation of the near infrared reflectance spectroscopy (NIRS) validation curves for digestible arginine, histidine, and isoleucine when using the corresponding calibration prediction curves for all poultry feed ingredients (Experiment 1a, 1b, 1c).

OPUS® validation parameters	Arginine		Histidine		Isoleucine	
Samples in validation	880		879		878	
R- squared	0.93		0.96		0.93	
RMSEP[1]	0.29		0.10		0.19	
RPD[2]	3.85		5.18		3.85	
Bias[3]	-0.014		0.005		0.023	
Ratio of predicted to true[4]	Count	Percentage	Count	Percentage	Count	Percentage
<90.00	135	15	162	18	182	21
90.00- 92.49	35	4	49	6	62	7
92.50- 94.99	68	8	85	10	80	9
95.00- 97.49	90	10	86	10	88	10
97.50- 99.99	93	11	74	8	62	7
100.00- 102.49	90	10	58	7	79	9
102.50- 104.99	94	11	76	9	68	8
105.00- 107.49	69	8	61	7	60	7
107.50- 110.00	53	6	55	6	42	5
>110	153	17	173	20	155	18
Absolute percentage deviation[5]	Count	Percentage	Count	Percentage	Count	Percentage
<2.5%	183	21	130	15	142	16
<5.0%	367	42	293	33	297	34
<10.0%	591	67	544	62	541	62
<15.0%	696	79	691	79	686	78
<20.0%	749	85	780	89	749	85

[1] Root mean square error of the prediction.

[2] Residual predictive deviation.

[3] Bias- systematically averaged deviation between the data set of the true and the predicted values.

[4] Equation for determining the ratio of the predicted value to the true value is |(predicted value/true value)*100|.

[5] Equation for absolute percentage deviation from true value is [(|true value-NIRS predicted value|)/true value]*100.

Table 4.4. Digestible histidine content of ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 1b).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n	%			n	%		
AA man.by-product[1]	2	1.00 ± 0.461	0.68	1.33	9	1.38 ± 0.512	0.95	2.65
Animal by-product[2]	75	1.08 ± 0.979	0.45	6.62	485	0.91 ± 0.573	0.39	6.43
Bakery meal	3	0.23 ± 0.052	0.17	0.27	17	0.22 ± 0.045	0.15	0.32
Canola	1	0.86	0.86	0.86	18	0.79 ± 0.102	0.63	1.00
Corn	5	0.21 ± 0.031	0.16	0.24	42	0.19 ± 0.022	0.15	0.25
DDGS[3]	10	0.96 ± 0.310	0.45	1.25	98	0.69 ± 0.246	0.32	1.35
Fermented product	1	1.37	1.37	1.37	17	1.37 ± 0.228	0.72	1.64
Isolated soy	4	1.54 ± 0.200	1.35	1.72	7	1.37 ± 0.159	1.22	1.71
Other ingredients[4]	22	0.47 ± 0.402	0.00	1.55	49	0.55 ± 0.327	0.06	1.13
Peanut meal	3	0.98 ± 0.029	0.95	1.00	7	0.96 ± 0.040	0.90	1.02
Soybean meal	14	1.15 ± 0.266	0.65	1.60	116	1.09 ± 0.162	0.54	1.69
Sorghum	3	0.16 ± 0.008	0.15	0.16	5	0.17 ± 0.027	0.15	0.22
Wheat	1	0.20	0.20	0.20	6	0.26 ± 0.044	0.19	0.32
Wheat middlings	4	0.30 ± 0.032	0.26	0.33	3	0.36 ± 0.033	0.33	0.38
Aggregated	148	0.91 ± 0.796	0.00	6.62	879	0.84 ± 0.512	0.06	6.43

[1] Amino acid manufacturing by-product.

[2] Animal by-product- includes unidentified animal by-product meal, blood meal, dissolved air flotation skimmings, dried porcine mucosa, feather meal, fish meal, hatchery waste, meat and bone meal, offal and waste, poultry by-product meal, and poultry tankage.

[3] Dried distillers' grains with solubles.

[4] Other ingredients/ all products with 6 or less total samples- includes alfalfa meal, algae, almond shells, arrow root leaves, barley, Brazil nut powder, black soldier fly larvae, calliandra leaves, carinata meal, cassava leaves, corn germ meal, corn gluten feed, cottonseed meal, flaxseed meal, hemp leaves by-product, hemp seed, hominy, isolated DDGS products, jatropha meal, millet, moringa powder, pea meal, pongamia meal, rice bran, rice hulls, rice protein, rye, soy hulls, sprouted seeds, sugar beet root powder, sunflower meal, supermarket waste, sweet potato leaves, wheat DDGS, and yeast products.

Table 4.5. Digestible isoleucine content of ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 1c).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n	%			n	%		
AA man.by-product[1]	1	2.48	2.48	2.48	10	3.22 ± 0.442	2.32	3.60
Animal by-product[2]	78	1.89 ± 0.780	0.37	3.97	482	1.59 ± 0.559	0.26	3.89
Bakery meal	4	0.33 ± 0.049	0.26	0.38	16	0.37 ± 0.059	0.26	0.49
Canola	2	1.29 ± 0.031	1.26	1.31	17	1.14 ± 0.190	0.77	1.49
Corn	6	0.24 ± 0.036	0.19	0.30	41	0.23 ± 0.036	0.17	0.33
DDGS[3]	9	1.11 ± 0.504	0.62	1.87	99	1.06 ± 0.428	0.57	2.18
Fermented product	4	2.62 ± 0.480	2.21	3.31	14	2.75 ± 0.684	0.83	3.34
Isolated soy	1	2.43	2.43	2.43	10	2.65 ± 0.346	2.30	3.16
Other ingredients[4]	15	0.75 ± 0.582	0.04	1.75	56	0.94 ± 0.633	0.11	2.39
Peanut meal	3	1.56 ± 0.119	1.43	1.66	7	1.49 ± 0.092	1.32	1.59
Soybean meal	20	1.91 ± 0.465	1.16	3.32	110	1.99 ± 0.343	0.87	3.16
Sorghum	2	0.35 ± 0.014	0.34	0.36	6	0.36 ± 0.055	0.31	0.44
Wheat	2	0.44 ± 0.041	0.41	0.47	5	0.36 ± 0.050	0.31	0.40
Wheat middlings	2	0.39 ± 0.041	0.36	0.42	5	0.35 ± 0.041	0.31	0.42
Aggregated	149	1.58 ± 0.871	0.04	3.97	878	1.47 ± 0.720	0.11	3.89

[1] Amino acid manufacturing by-product.

[2] Animal by-product- includes unidentified animal by-product meal, blood meal, dissolved air flotation skimmings, dried porcine mucosa, feather meal, fish meal, hatchery waste, meat and bone meal, offal and waste, poultry by-product meal, and poultry tankage.

[3] Dried distillers' grains with solubles.

[4] Other ingredients/ all products with 6 or less total samples- includes alfalfa meal, algae, almond shells, arrow root leaves, barley, Brazil nut powder, black soldier fly larvae, calliandra leaves, carinata meal, cassava leaves, corn germ meal, corn gluten feed, cottonseed meal, flaxseed meal, hemp leaves by-product, hemp seed, hominy, isolated DDGS products, jatropha meal, millet, moringa powder, pea meal, pongamia meal, rice bran, rice hulls, rice protein, rye, soy hulls, sprouted seeds, sugar beet root powder, sunflower meal, supermarket waste, sweet potato leaves, wheat DDGS, and yeast products.

Experiment 1d. The NIRS calibration curve for digestible leucine contained 150 ingredient samples with a digestible leucine content range of 0.03% to 11.79% (Table 4.6). The digestible leucine validation curve had an RPD value above 4.0, and 77% of samples had a predicted value that deviated 10% or less from its actual value (Table 4.7).

Experiment 1e. The 150 sample NIRS calibration curve for digestible lysine had a calibration range from 0.03% to 7.74% (Table 4.8). In its corresponding validation curve containing 876 samples, only 60% of the samples had predicted values that deviated from their true value by less than 10% (Table 4.7).

Experiment 1f. The calibration curve for digestible methionine content contained 150 samples (Table 4.9). The validation curve for digestible methionine only had an R^2 and RPD of 0.86 and 2.69, respectively (Table 4.7).

Experiment 1g. The calibration curve for digestible phenylalanine used 150 samples and had a calibration range of 0.05% to 6.28% (Table 4.10). The validation curve for digestible phenylalanine had an R^2 of 0.96 and an RPD of 4.88 and 74% of the samples had predicted values that deviated less than 10% from their true value (Table 4.11).

Experiment 1h. The calibration curve for digestible threonine used 150 feed ingredient samples (Table 4.12). The threonine calibration curve was validated with 878 samples and 60% of those samples had predicted values that deviated 10% or less from their true value (Table 4.11).

Table 4.6. Digestible leucine content of ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 1d).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n		%		n		%	
AA man.by-product[1]	4	5.57 ± 1.274	3.69	6.48	7	5.70 ± 0.958	4.39	6.84
Animal by-product[2]	67	3.80 ± 1.921	1.41	11.79	493	3.19 ± 1.103	1.80	11.77
Bakery meal	3	0.76 ± 0.088	0.67	0.84	17	0.76 ± 0.133	0.56	1.03
Canola	4	2.18 ± 0.158	1.99	2.36	15	2.00 ± 0.291	1.54	2.62
Corn	3	0.49 ± 0.497	0.17	1.07	44	0.80 ± 0.096	0.65	1.12
DDGS[3]	13	3.43 ± 1.348	2.05	6.22	95	3.27 ± 1.252	1.95	6.86
Fermented product	2	5.39 ± 0.067	5.35	5.44	16	4.37 ± 0.825	2.70	5.44
Isolated soy	3	4.23 ± 0.606	3.88	4.93	8	4.13 ± 0.500	3.73	4.94
Other ingredients[4]	30	2.19 ± 1.777	0.03	9.10	43	1.51 ± 1.260	0.07	5.49
Peanut meal	1	3.10	3.10	3.10	9	2.84 ± 0.210	2.45	3.10
Soybean meal	16	3.19 ± 0.647	1.72	3.95	114	3.21 ± 0.567	1.37	5.23
Sorghum	1	1.11	1.11	1.11	7	1.29 ± 0.173	1.08	1.59
Wheat	1	0.76	0.76	0.76	6	0.73 ± 0.074	0.65	0.85
Wheat middlings	2	0.74 ± 0.090	0.68	0.81	5	0.72 ± 0.066	0.66	0.83
Aggregated	150	3.20 ± 1.879	0.03	11.79	879	2.93 ± 1.294	0.07	11.77

[1] Amino acid manufacturing by-product.

[2] Animal by-product- includes unidentified animal by-product meal, blood meal, dissolved air flotation skimmings, dried porcine mucosa, feather meal, fish meal, hatchery waste, meat and bone meal, offal and waste, poultry by-product meal, and poultry tankage.

[3] Dried distillers' grains with solubles.

[4] Other ingredients/ all products with 6 or less total samples- includes alfalfa meal, algae, almond shells, arrow root leaves, barley, Brazil nut powder, black soldier fly larvae, calliandra leaves, carinata meal, cassava leaves, corn germ meal, corn gluten feed, cottonseed meal, flaxseed meal, hemp leaves by-product, hemp seed, hominy, isolated DDGS products, jatropha meal, millet, moringa powder, pea meal, pongamia meal, rice bran, rice hulls, rice protein, rye, soy hulls, sprouted seeds, sugar beet root powder, sunflower meal, supermarket waste, sweet potato leaves, wheat DDGS, and yeast products.

Table 4.7. Mathematical evaluation of the near infrared reflectance spectroscopy (NIRS) validation curves for digestible leucine, lysine, and methionine when using the corresponding calibration prediction curves for all poultry feed ingredients (Experiment 1d, 1e, 1f).

OPUS® validation parameters	Leucine		Lysine		Methionine	
Samples in validation	879		876		875	
R- squared	0.95		0.94		0.86	
RMSEP[1]	0.29		0.28		0.10	
RPD[2]	4.41		4.01		2.69	
Bias[3]	0.019		-0.027		-0.014	
Ratio of predicted to true[4]	Count	Percentage	Count	Percentage	Count	Percentage
<90.00	97	11	136	16	178	20
90.00- 92.49	63	7	32	4	41	5
92.50- 94.99	88	10	50	6	49	6
95.00- 97.49	91	10	68	8	43	5
97.50- 99.99	120	14	88	10	66	8
100.00- 102.49	107	12	90	10	54	6
102.50- 104.99	94	11	76	9	62	7
105.00- 107.49	66	8	60	7	57	7
107.50- 110.00	41	5	58	7	55	6
>110	112	13	218	25	270	31
Absolute percentage deviation[5]	Count	Percentage	Count	Percentage	Count	Percentage
<2.5%	227	26	177	20	121	14
<5.0%	413	47	320	37	225	26
<10.0%	673	77	522	60	427	49
<15.0%	758	86	637	73	580	66
<20.0%	804	91	706	81	678	77

[1] Root mean square error of the prediction.

[2] Residual predictive deviation.

[3] Bias- systematically averaged deviation between the data set of the true and the predicted values.

[4] Equation for determining the ratio of the predicted value to the true value is |(predicted value/true value)*100|.

[5] Equation for absolute percentage deviation from true value is [(|true value-NIRS predicted value|)/true value]*100.

Table 4.8. Digestible lysine content of ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 1e).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n	%			n	%		
AA man.by-product[1]	3	5.23 ± 1.454	3.64	6.50	5	5.54 ± 1.075	3.73	6.57
Animal by-product[2]	59	2.81 ± 1.522	1.20	7.74	501	2.36 ± 0.861	0.94	7.55
Bakery meal	6	0.25 ± 0.044	0.20	0.32	14	0.25 ± 0.075	0.13	0.39
Canola	4	1.24 ± 0.538	0.51	1.81	15	1.31 ± 0.563	0.29	2.07
Corn	12	0.21 ± 0.019	0.19	0.25	35	0.21 ± 0.035	0.15	0.34
DDGS[3]	20	0.69 ± 0.447	0.07	1.73	88	0.78 ± 0.431	0.05	1.74
Fermented product	1	0.70	0.70	0.70	17	3.60 ± 0.663	2.47	4.40
Isolated soy	2	2.55 ± 0.075	2.50	2.60	9	3.36 ± 0.607	2.70	4.17
Other ingredients[4]	24	0.93 ± 0.740	0.03	2.82	49	0.94 ± 0.730	0.09	2.84
Peanut meal	0	-	-	-	10	1.35 ± 0.161	0.97	1.53
Soybean meal	14	2.84 ± 0.556	1.68	3.97	116	2.62 ± 0.358	1.48	4.13
Sorghum	3	0.20 ± 0.021	0.18	0.22	5	0.18 ± 0.024	0.16	0.22
Wheat	2	0.34 ± 0.005	0.34	0.35	5	0.29 ± 0.032	0.26	0.34
Wheat middlings	0	-	-	-	7	0.50 ± 0.067	0.41	0.60
Aggregated	150	1.82 ± 1.582	0.03	7.74	876	2.02 ± 1.111	0.05	7.55

[1] Amino acid manufacturing by-product.

[2] Animal by-product- includes unidentified animal by-product meal, blood meal, dissolved air flotation skimmings, dried porcine mucosa, feather meal, fish meal, hatchery waste, meat and bone meal, offal and waste, poultry by-product meal, and poultry tankage.

[3] Dried distillers' grains with solubles.

[4] Other ingredients/ all products with 6 or less total samples- includes alfalfa meal, algae, almond shells, arrow root leaves, barley, Brazil nut powder, black soldier fly larvae, calliandra leaves, carinata meal, cassava leaves, corn germ meal, corn gluten feed, cottonseed meal, flaxseed meal, hemp leaves by-product, hemp seed, hominy, isolated DDGS products, jatropha meal, millet, moringa powder, pea meal, pongamia meal, rice bran, rice hulls, rice protein, rye, soy hulls, sprouted seeds, sugar beet root powder, sunflower meal, supermarket waste, sweet potato leaves, wheat DDGS, and yeast products.

Table 4.9. Digestible methionine content of ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 1f).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n	%			n	%		
AA man.by-product[1]	4	1.81 ± 0.220	1.50	2.00	7	1.54 ± 0.380	0.85	1.86
Animal by-product[2]	77	0.80 ± 0.324	0.45	1.67	482	0.68 ± 0.203	0.35	1.92
Bakery meal	5	0.21 ± 0.053	0.15	0.27	15	0.15 ± 0.031	0.07	0.20
Canola	4	0.56 ± 0.055	0.49	0.61	15	0.62 ± 0.057	0.55	0.75
Corn	9	0.14 ± 0.015	0.11	0.15	38	0.14 ± 0.024	0.08	0.19
DDGS[3]	11	0.71 ± 0.395	0.20	1.25	97	0.56 ± 0.219	0.22	1.12
Fermented product	6	1.40 ± 0.549	0.66	1.84	12	1.16 ± 0.574	0.43	1.80
Isolated soy	3	0.73 ± 0.108	0.67	0.86	8	0.72 ± 0.090	0.64	0.87
Other ingredients[4]	19	0.56 ± 0.471	0.03	1.58	51	0.40 ± 0.306	0.05	1.39
Peanut meal	0	-	-	-	10	0.46 ± 0.038	0.40	0.53
Soybean meal	10	0.53 ± 0.197	0.27	0.87	120	0.57 ± 0.083	0.30	0.89
Sorghum	1	0.13	0.13	0.13	7	0.16 ± 0.018	0.13	0.18
Wheat	0	-	-	-	7	0.17 ± 0.022	0.14	0.20
Wheat middlings	1	0.17	0.17	0.17	6	0.17 ± 0.012	0.16	0.19
Aggregated	150	0.72 ± 0.446	0.03	2.00	875	0.60 ± 0.274	0.05	1.92

[1] Amino acid manufacturing by-product.

[2] Animal by-product- includes unidentified animal by-product meal, blood meal, dissolved air flotation skimmings, dried porcine mucosa, feather meal, fish meal, hatchery waste, meat and bone meal, offal and waste, poultry by-product meal, and poultry tankage.

[3] Dried distillers' grains with solubles.

[4] Other ingredients/ all products with 6 or less total samples- includes alfalfa meal, algae, almond shells, arrow root leaves, barley, Brazil nut powder, black soldier fly larvae, calliandra leaves, carinata meal, cassava leaves, corn germ meal, corn gluten feed, cottonseed meal, flaxseed meal, hemp leaves by-product, hemp seed, hominy, isolated DDGS products, jatropha meal, millet, moringa powder, pea meal, pongamia meal, rice bran, rice hulls, rice protein, rye, soy hulls, sprouted seeds, sugar beet root powder, sunflower meal, supermarket waste, sweet potato leaves, wheat DDGS, and yeast products.

Table 4.10. Digestible phenylalanine content of ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 1g).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n	%			n	%		
AA man.by-product[1]	2	3.21 ± 0.157	3.10	3.32	9	2.90 ± 0.596	1.99	3.92
Animal by-product[2]	67	2.33 ± 1.086	0.86	6.28	493	1.85 ± 0.637	1.06	5.72
Bakery meal	3	0.49 ± 0.135	0.36	0.63	17	0.54 ± 0.086	0.36	0.69
Canola	4	1.08 ± 0.125	0.93	1.23	15	1.23 ± 0.145	0.99	1.51
Corn	4	0.34 ± 0.037	0.31	0.39	43	0.32 ± 0.052	0.25	0.51
DDGS[3]	8	1.74 ± 0.799	0.91	2.99	100	1.39 ± 0.508	0.84	2.76
Fermented product	7	2.83 ± 0.445	2.18	3.25	11	2.77 ± 0.646	0.98	3.31
Isolated soy	3	3.31 ± 0.025	3.29	3.34	8	2.63 ± 0.087	2.52	2.79
Other ingredients[4]	23	1.10 ± 0.844	0.05	3.52	47	1.13 ± 0.752	0.15	2.73
Peanut meal	5	2.39 ± 0.250	1.99	2.63	5	2.26 ± 0.098	2.17	2.42
Soybean meal	21	2.09 ± 0.609	1.21	3.61	109	2.23 ± 0.338	0.94	3.49
Sorghum	2	0.51 ± 0.006	0.51	0.52	6	0.51 ± 0.050	0.46	0.59
Wheat	0	-	-	-	7	0.52 ± 0.060	0.45	0.63
Wheat middlings	1	0.54	0.54	0.54	6	0.47 ± 0.043	0.43	0.55
Aggregated	150	1.97 ± 1.084	0.05	6.28	876	1.70 ± 0.761	0.15	5.72

[1] Amino acid manufacturing by-product.

[2] Animal by-product- includes unidentified animal by-product meal, blood meal, dissolved air flotation skimmings, dried porcine mucosa, feather meal, fish meal, hatchery waste, meat and bone meal, offal and waste, poultry by-product meal, and poultry tankage.

[3] Dried distillers' grains with solubles.

[4] Other ingredients/ all products with 6 or less total samples- includes alfalfa meal, algae, almond shells, arrow root leaves, barley, Brazil nut powder, black soldier fly larvae, calliandra leaves, carinata meal, cassava leaves, corn germ meal, corn gluten feed, cottonseed meal, flaxseed meal, hemp leaves by-product, hemp seed, hominy, isolated DDGS products, jatropha meal, millet, moringa powder, pea meal, pongamia meal, rice bran, rice hulls, rice protein, rye, soy hulls, sprouted seeds, sugar beet root powder, sunflower meal, supermarket waste, sweet potato leaves, wheat DDGS, and yeast products.

Table 4.11. Mathematical evaluation of the near infrared reflectance spectroscopy (NIRS) validation curves for digestible phenylalanine, threonine, and tryptophan when using the corresponding calibration prediction curves for all poultry feed ingredients (Experiment 1g, 1h, 1i).

OPUS® validation parameters	Phenylalanine		Threonine		Tryptophan	
Samples in validation	876		878		866	
R- squared	0.96		0.91		0.91	
RMSEP[1]	0.16		0.21		0.07	
RPD[2]	4.88		3.41		3.33	
Bias[3]	-0.011		-0.004		0.001	
Ratio of predicted to true[4]	Count	Percentage	Count	Percentage	Count	Percentage
<90.00	71	8	183	21	221	26
90.00- 92.49	49	6	33	4	48	6
92.50- 94.99	66	8	55	6	52	6
95.00- 97.49	113	13	73	8	76	9
97.50- 99.99	110	13	67	8	59	7
100.00- 102.49	107	12	79	9	57	7
102.50- 104.99	85	10	86	10	48	6
105.00- 107.49	62	7	74	8	38	4
107.50- 110.00	56	6	57	6	33	4
>110	157	18	171	19	234	27
Absolute percentage deviation[5]	Count	Percentage	Count	Percentage	Count	Percentage
<2.5%	217	25	146	17	117	14
<5.0%	414	47	305	35	241	28
<10.0%	648	74	525	60	411	47
<15.0%	746	85	645	73	553	64
<20.0%	805	92	722	82	647	75

[1] Root mean square error of the prediction.

[2] Residual predictive deviation.

[3] Bias- systematically averaged deviation between the data set of the true and the predicted values.

[4] Equation for determining the ratio of the predicted value to the true value is |(predicted value/true value)*100|.

[5] Equation for absolute percentage deviation from true value is [(|true value-NIRS predicted value|)/true value]*100.

Table 4.12. Digestible threonine content of ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 1h).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n	%			n	%		
AA man.by-product[1]	4	4.54 ± 0.862	3.25	5.08	7	3.97 ± 1.245	2.21	5.21
Animal by-product[2]	67	1.69 ± 0.725	0.73	4.20	493	1.62 ± 0.536	0.84	4.19
Bakery meal	4	0.36 ± 0.036	0.31	0.40	16	0.29 ± 0.044	0.21	0.36
Canola	8	1.12 ± 0.195	0.88	1.43	11	1.16 ± 0.146	0.86	1.31
Corn	1	0.24	0.24	0.24	46	0.23 ± 0.034	0.17	0.34
DDGS[3]	17	0.96 ± 0.415	0.50	1.79	91	0.94 ± 0.362	0.50	1.88
Fermented product	5	2.00 ± 0.983	0.81	3.09	13	2.55 ± 0.577	1.79	3.14
Isolated soy	3	2.06 ± 0.269	1.89	2.37	8	1.99 ± 0.245	1.81	2.40
Other ingredients[4]	22	0.84 ± 0.561	0.05	1.99	50	0.73 ± 0.531	0.05	2.01
Peanut meal	3	1.25 ± 0.057	1.19	1.31	7	1.08 ± 0.069	0.96	1.15
Soybean meal	11	1.66 ± 0.303	1.28	2.43	119	1.54 ± 0.265	0.73	2.42
Sorghum	1	0.28	0.28	0.28	7	0.26 ± 0.017	0.24	0.29
Wheat	0	-	-	-	7	0.29 ± 0.034	0.25	0.35
Wheat middlings	4	0.34 ± 0.034	0.30	0.38	3	0.39 ± 0.062	0.32	0.43
Aggregated	150	1.45 ± 0.906	0.05	5.08	878	1.39 ± 0.698	0.05	5.21

[1] Amino acid manufacturing by-product.

[2] Animal by-product- includes unidentified animal by-product meal, blood meal, dissolved air flotation skimmings, dried porcine mucosa, feather meal, fish meal, hatchery waste, meat and bone meal, offal and waste, poultry by-product meal, and poultry tankage.

[3] Dried distillers' grains with solubles.

[4] Other ingredients/ all products with 6 or less total samples- includes alfalfa meal, algae, almond shells, arrow root leaves, barley, Brazil nut powder, black soldier fly larvae, calliandra leaves, carinata meal, cassava leaves, corn germ meal, corn gluten feed, cottonseed meal, flaxseed meal, hemp leaves by-product, hemp seed, hominy, isolated DDGS products, jatropha meal, millet, moringa powder, pea meal, pongamia meal, rice bran, rice hulls, rice protein, rye, soy hulls, sprouted seeds, sugar beet root powder, sunflower meal, supermarket waste, sweet potato leaves, wheat DDGS, and yeast products.

Experiment 1i. The digestible tryptophan calibration curve had the smallest range of values with a range of 0.02% to 1.61% (Table 4.13). The validation curve had an R^2 of 0.91 and an RPD of 3.33 (Table 4.11).

Experiment 1j. The calibration curve for digestible valine had a calibration range of 0.05% to 7.44% (Table 4.14) and was validated with 878 samples and only 58% of the validation samples had predicted values that deviated less than 10% from their true value (Table 4.15).

Experiment 2- Common animal by-products calibration

Experiment 2a- 2c. The calibration curves for digestible arginine (Table 4.16), histidine (Table 4.17), and isoleucine (Table 4.18) for commonly used animal by-products consisted of 150, 150, and 148 samples, respectively. The corresponding validation curves predicted 98%, 80%, and 79% of the validation samples with a 10% or less deviation from their bioassay determined values, respectively (Table 4.19).

Experiment 2d- 2f. Calibration curves for digestible leucine (Table 4.20), lysine (Table 4.21), and methionine (Table 4.22) content for commonly used animal by-products had a calibration range of 1.80%-6.61%, 0.96%-5.12%, and 0.35%-1.92%, respectively. The common animal products validation curves for leucine, lysine, and methionine predicted 95%, 81%, and 83% of the validation samples respectively, with a 10% or less deviation from their actual determined values (Table 4.23).

Table 4.13. Digestible tryptophan content of ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 1i).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n		%		n		%	
AA man.by-product[1]	2	0.85 ± 0.147	0.75	0.96	9	0.90 ± 0.176	0.55	1.07
Animal by-product[2]	66	0.50 ± 0.358	0.20	1.61	494	0.36 ± 0.173	0.02	1.58
Bakery meal	8	0.10 ± 0.021	0.08	0.14	12	0.11 ± 0.025	0.08	0.15
Canola	6	0.39 ± 0.071	0.29	0.47	13	0.39 ± 0.086	0.26	0.49
Corn	5	0.05 ± 0.004	0.05	0.06	42	0.06 ± 0.060	0.04	0.39
DDGS[3]	11	0.22 ± 0.107	0.05	0.35	87	0.21 ± 0.084	0.06	0.42
Fermented product	6	1.19 ± 0.252	0.70	1.35	12	0.85 ± 0.311	0.17	1.20
Isolated soy	0	-	-	-	11	0.75 ± 0.080	0.67	0.88
Other ingredients[4]	21	0.27 ± 0.231	0.02	0.81	48	0.30 ± 0.216	0.05	0.86
Peanut meal	2	0.39 ± 0.019	0.38	0.40	8	0.43 ± 0.031	0.37	0.46
Soybean meal	18	0.56 ± 0.179	0.24	0.94	112	0.60 ± 0.116	0.28	0.95
Sorghum	3	0.05 ± 0.043	0.02	0.10	5	0.07 ± 0.032	0.02	0.10
Wheat	1	0.12	0.12	0.12	6	0.14 ± 0.029	0.11	0.19
Wheat middlings	0	-	-	-	7	0.16 ± 0.021	0.12	0.18
Aggregated	149	0.43 ± 0.350	0.02	1.61	866	0.37 ± 0.220	0.02	1.58

[1] Amino acid manufacturing by-product.

[2] Animal by-product- includes unidentified animal by-product meal, blood meal, dissolved air flotation skimmings, dried porcine mucosa, feather meal, fish meal, hatchery waste, meat and bone meal, offal and waste, poultry by-product meal, and poultry tankage.

[3] Dried distillers' grains with solubles.

[4] Other ingredients/ all products with 6 or less total samples- includes alfalfa meal, algae, almond shells, arrow root leaves, barley, Brazil nut powder, black soldier fly larvae, calliandra leaves, carinata meal, cassava leaves, corn germ meal, corn gluten feed, cottonseed meal, flaxseed meal, hemp leaves by-product, hemp seed, hominy, isolated DDGS products, jatropha meal, millet, moringa powder, pea meal, pongamia meal, rice bran, rice hulls, rice protein, rye, soy hulls, sprouted seeds, sugar beet root powder, sunflower meal, supermarket waste, sweet potato leaves, wheat DDGS, and yeast products.

Table 4.14. Digestible valine content of ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 1j).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n		%		n		%	
AA man.by-product[1]	3	4.06 ± 0.194	3.90	4.27	8	3.94 ± 0.356	3.16	4.26
Animal by-product[2]	74	2.98 ± 1.544	1.06	7.44	486	2.17 ± 0.863	1.20	6.73
Bakery meal	1	0.54	0.54	0.54	19	0.43 ± 0.084	0.29	0.60
Canola	8	1.49 ± 0.231	1.17	1.89	11	1.26 ± 0.425	0.45	1.75
Corn	8	0.35 ± 0.054	0.31	0.46	39	0.29 ± 0.041	0.23	0.41
DDGS[3]	8	1.60 ± 0.671	0.81	2.36	100	1.30 ± 0.537	0.71	2.67
Fermented product	6	3.82 ± 0.691	2.45	4.32	12	3.03 ± 1.063	1.11	4.38
Isolated soy	1	3.23	3.23	3.23	10	2.64 ± 0.331	2.22	3.25
Other ingredients[4]	18	1.14 ± 0.903	0.05	2.82	54	1.15 ± 0.852	0.16	2.92
Peanut meal	2	1.78 ± 0.004	1.78	1.79	8	1.75 ± 0.102	1.57	1.88
Soybean meal	17	1.64 ± 0.488	0.82	2.39	113	2.02 ± 0.351	1.04	3.31
Sorghum	2	0.41 ± 0.003	0.41	0.41	6	0.45 ± 0.031	0.42	0.50
Wheat	1	0.39	0.39	0.39	6	0.47 ± 0.037	0.43	0.53
Wheat middlings	1	0.60	0.60	0.60	6	0.51 ± 0.065	0.44	0.61
Aggregated	150	2.27 ± 1.520	0.05	7.44	878	1.85 ± 0.953	0.16	6.73

[1] Amino acid manufacturing by-product.

[2] Animal by-product- includes unidentified animal by-product meal, blood meal, dissolved air flotation skimmings, dried porcine mucosa, feather meal, fish meal, hatchery waste, meat and bone meal, offal and waste, poultry by-product meal, and poultry tankage.

[3] Dried distillers' grains with solubles.

[4] Other ingredients/ all products with 6 or less total samples- includes alfalfa meal, algae, almond shells, arrow root leaves, barley, Brazil nut powder, black soldier fly larvae, calliandra leaves, carinata meal, cassava leaves, corn germ meal, corn gluten feed, cottonseed meal, flaxseed meal, hemp leaves by-product, hemp seed, hominy, isolated DDGS products, jatropha meal, millet, moringa powder, pea meal, pongamia meal, rice bran, rice hulls, rice protein, rye, soy hulls, sprouted seeds, sugar beet root powder, sunflower meal, supermarket waste, sweet potato leaves, wheat DDGS, and yeast products.

Table 4.15. Mathematical evaluation of the near infrared reflectance spectroscopy (NIRS) validation curve for valine when using the corresponding calibration prediction curve for all poultry feed ingredients (Experiment 1j).

OPUS® validation parameters	Valine	
Samples in validation	878	
R- squared	0.92	
RMSEP[1]	0.27	
RPD[2]	3.54	
Bias[3]	-0.015	
Ratio of predicted to true[4]	Count	Percentage
<90.00	175	20
90.00- 92.49	52	6
92.50- 94.99	56	6
95.00- 97.49	75	9
97.50- 99.99	71	8
100.00- 102.49	73	8
102.50- 104.99	83	9
105.00- 107.49	45	5
107.50- 110.00	57	6
>110	191	22
Absolute percentage deviation[5]	Count	Percentage
<2.5%	143	16
<5.0%	303	35
<10.0%	513	58
<15.0%	646	74
<20.0%	714	81

[1] Root mean square error of the prediction.

[2] Residual predictive deviation.

[3] Bias- systematically averaged deviation between the data set of the true and the predicted values.

[4] Equation for determining the ratio of the predicted value to the true value is |(predicted value/true value)*100|.

[5] Equation for absolute percentage deviation from true value is [(|true value-NIRS predicted value|)/true value]*100.

Table 4.16. Digestible arginine content of common animal by-product ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 2a).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n	%			n	%		
Unidentified meal[1]	6	3.14 ± 0.129	2.96	3.34	13	3.04 ± 0.261	2.59	3.48
Feather meal	6	5.01 ± 0.374	4.51	5.49	23	4.80 ± 0.571	2.88	5.24
Fish meal	0	-	-	-	6	3.64 ± 0.262	3.34	3.93
MBM[2]	99	3.00 ± 0.413	2.13	4.66	256	3.01 ± 0.340	2.28	4.04
PBM[3]	28	3.29 ± 0.595	1.91	4.53	70	3.38 ± 0.464	2.54	4.63
Poultry tankage	11	2.86 ± 0.125	2.67	3.06	15	2.82 ± 0.212	2.31	3.21
Aggregated	150	3.13 ± 0.588	1.91	5.49	383	3.19 ± 0.577	2.28	5.24

[1] These unidentified animal by-product meal ingredient samples were not further defined by tissue type or species of origin.

[2] Meat and bone meal samples of bovine and porcine origin.

[3] Poultry by-product meal.

Table 4.17. Digestible histidine content of common animal by-product ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 2b).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n	%			n	%		
Unidentified meal[1]	6	0.72 ± 0.168	0.58	0.99	13	0.67 ± 0.096	0.48	0.83
Feather meal	5	0.64 ± 0.283	0.39	1.10	24	0.69 ± 0.290	0.42	1.19
Fish meal	5	1.33 ± 0.335	0.90	1.81	1	1.14	1.14	1.14
MBM[2]	105	0.87 ± 0.298	0.44	2.38	250	0.78 ± 0.242	0.47	2.22
PBM[3]	27	0.90 ± 0.199	0.52	1.38	71	0.97 ± 0.237	0.52	1.60
Poultry tankage	2	0.78 ± 0.052	0.74	0.81	24	0.75 ± 0.070	0.59	0.89
Aggregated	150	0.88 ± 0.292	0.39	2.38	383	0.81 ± 0.248	0.42	2.22

[1] These unidentified animal by-product meal ingredient samples were not further defined by tissue type or species of origin.

[2] Meat and bone meal samples of bovine and porcine origin.

[3] Poultry by-product meal.

Table 4.18. Digestible isoleucine content of common animal by-product ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 2c).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n	%			n	%		
Unidentified meal[1]	5	1.49 ± 0.029	1.45	1.53	14	1.63 ± 0.180	1.34	1.90
Feather meal	10	3.60 ± 0.179	3.38	3.97	19	3.34 ± 0.572	1.66	3.89
Fish meal	3	2.60 ± 0.330	2.25	2.90	3	2.60 ± 0.253	2.32	2.82
MBM[2]	93	1.48 ± 0.386	0.77	2.90	262	1.38 ± 0.331	0.80	2.26
PBM[3]	20	1.74 ± 0.429	1.01	2.70	78	1.80 ± 0.331	1.13	2.83
Poultry tankage	17	1.62 ± 0.117	1.34	1.85	9	1.68 ± 0.092	1.51	1.79
Aggregated	148	1.70 ± 0.645	0.77	3.97	385	1.58 ± 0.561	0.80	3.89

[1] These unidentified animal by-product meal ingredient samples were not further defined by tissue type or species of origin.

[2] Meat and bone meal samples of bovine and porcine origin.

[3] Poultry by-product meal.

Table 4.19. Mathematical evaluation of the near infrared reflectance spectroscopy (NIRS) validation curves for arginine, histidine, and isoleucine when using the corresponding calibration prediction curves for common animal by-product feed ingredients (Experiment 2a, 2b, 2c).

OPUS® validation parameters	Arginine		Histidine		Isoleucine	
Samples in validation	383		383		385	
R- squared	0.94		0.94		0.95	
RMSEP[1]	0.14		0.06		0.13	
RPD[2]	4.05		3.96		4.54	
Bias[3]	0.001		-0.007		0.020	
Ratio of predicted to true[4]	Count	Percentage	Count	Percentage	Count	Percentage
<90.00	3	1	25	7	44	11
90.00- 92.49	12	3	20	5	25	6
92.50- 94.99	35	9	28	7	40	10
95.00- 97.49	71	19	42	11	53	14
97.50- 99.99	65	17	49	13	48	12
100.00- 102.49	74	19	53	14	57	15
102.50- 104.99	64	17	56	15	32	8
105.00- 107.49	41	11	42	11	22	6
107.50- 110.00	14	4	16	4	25	6
>110	4	1	52	14	38	10
Absolute percentage deviation[5]	Count	Percentage	Count	Percentage	Count	Percentage
<2.5%	139	36	102	27	105	27
<5.0%	273	71	201	52	193	50
<10.0%	376	98	306	80	303	79
<15.0%	383	100	350	91	358	93
<20.0%	383	100	370	97	378	98

[1] Root mean square error of the prediction.

[2] Residual predictive deviation.

[3] Bias- systematically averaged deviation between the data set of the true and the predicted values.

[4] Equation for determining the ratio of the predicted value to the true value is |(predicted value/true value)*100|.

[5] Equation for absolute percentage deviation from true value is [(|true value-NIRS predicted value|)/true value]*100.

Table 4.20. Digestible leucine content of common animal by-product ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 2d).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n	%			n	%		
Unidentified meal[1]	8	2.93 ± 0.239	2.63	3.45	11	2.98 ± 0.307	2.48	3.56
Feather meal	7	5.90 ± 0.359	5.51	6.61	22	5.61 ± 0.757	3.31	6.45
Fish meal	1	3.80	3.80	3.80	5	4.40 ± 0.392	3.79	4.77
MBM[2]	101	3.02 ± 0.719	1.80	5.29	254	2.77 ± 0.505	1.82	4.71
PBM[3]	26	3.33 ± 0.602	1.88	4.58	72	3.22 ± 0.524	2.06	5.26
Poultry tankage	5	2.96 ± 0.203	2.69	3.22	21	2.98 ± 0.214	2.36	3.30
Aggregated	148	3.21 ± 0.897	1.80	6.61	385	3.06 ± 0.844	1.82	6.45

[1] These unidentified animal by-product meal ingredient samples were not further defined by tissue type or species of origin.

[2] Meat and bone meal samples of bovine and porcine origin.

[3] Poultry by-product meal.

Table 4.21. Digestible lysine content of common animal by-product ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 2e).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n	%			n	%		
Unidentified meal[1]	9	2.01 ± 0.366	1.40	2.63	10	1.99 ± 0.165	1.73	2.26
Feather meal	11	1.44 ± 0.463	0.96	2.49	18	1.77 ± 0.580	0.94	3.06
Fish meal	2	4.96 ± 0.222	4.80	5.12	4	4.00 ± 1.255	2.20	5.06
MBM[2]	87	2.28 ± 0.656	1.43	5.12	268	2.15 ± 0.425	1.38	4.18
PBM[3]	35	2.67 ± 0.616	1.22	4.16	63	2.75 ± 0.694	1.84	4.87
Poultry tankage	4	2.22 ± 0.073	2.12	2.28	22	2.19 ± 0.139	1.89	2.47
Aggregated	148	2.33 ± 0.742	0.96	5.12	385	2.25 ± 0.570	0.94	5.06

[1] These unidentified animal by-product meal ingredient samples were not further defined by tissue type or species of origin.

[2] Meat and bone meal samples of bovine and porcine origin.

[3] Poultry by-product meal.

Table 4.22. Digestible methionine content of common animal by-product ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 2f).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n	%			n	%		
Unidentified meal[1]	8	0.62 ± 0.132	0.51	0.88	11	0.60 ± 0.079	0.47	0.71
Feather meal	7	0.50 ± 0.124	0.35	0.66	21	0.49 ± 0.067	0.40	0.64
Fish meal	3	1.76 ± 0.139	1.65	1.92	3	1.42 ± 0.556	0.81	1.90
MBM[2]	89	0.66 ± 0.180	0.42	1.58	266	0.62 ± 0.130	0.42	1.18
PBM[3]	30	0.92 ± 0.231	0.61	1.47	68	0.92 ± 0.215	0.46	1.62
Poultry tankage	7	0.66 ± 0.021	0.63	0.69	19	0.68 ± 0.044	0.59	0.75
Aggregated	144	0.73 ± 0.263	0.35	1.92	388	0.68 ± 0.200	0.40	1.90

[1] These unidentified animal by-product meal ingredient samples were not further defined by tissue type or species of origin.

[2] Meat and bone meal samples of bovine and porcine origin.

[3] Poultry by-product meal.

Table 4.23. Mathematical evaluation of the near infrared reflectance spectroscopy (NIRS) validation curves for leucine, lysine, and methionine when using the corresponding calibration prediction curves for common animal by-product feed ingredients (Experiment 2d, 2e, 2f).

OPUS® validation parameters	Leucine		Lysine		Methionine	
Samples in validation	385		385		388	
R- squared	0.97		0.90		0.93	
RMSEP[1]	0.14		0.18		0.05	
RPD[2]	5.89		3.19		3.74	
Bias[3]	-0.011		0.003		-0.001	
Ratio of predicted to true[4]	Count	Percentage	Count	Percentage	Count	Percentage
<90.00	8	2	36	9	32	8
90.00- 92.49	5	1	26	7	17	4
92.50- 94.99	31	8	38	10	28	7
95.00- 97.49	53	14	40	10	48	12
97.50- 99.99	79	21	49	13	61	16
100.00- 102.49	82	21	49	13	53	14
102.50- 104.99	64	17	51	13	45	12
105.00- 107.49	32	8	30	8	45	12
107.50- 110.00	20	5	27	7	26	7
>110	11	3	39	10	33	9
Absolute percentage deviation[5]	Count	Percentage	Count	Percentage	Count	Percentage
<2.5%	161	42	98	25	113	29
<5.0%	279	72	189	49	206	53
<10.0%	366	95	310	81	323	83
<15.0%	383	99	363	94	363	94
<20.0%	384	100	377	98	376	97

[1] Root mean square error of the prediction.

[2] Residual predictive deviation.

[3] Bias- systematically averaged deviation between the data set of the true and the predicted values.

[4] Equation for determining the ratio of the predicted value to the true value is |(predicted value/true value)*100|.

[5] Equation for absolute percentage deviation from true value is [(|true value-NIRS predicted value|)/true value]*100.

Experiment 2g-2j. The calibration curve for digestible phenylalanine (Table 4.24), threonine (Table 4.25), tryptophan (Table 4.26), and valine (Table 4.27) for commonly used animal by-products consisted of 146, 142, 150, and 148 samples, respectively. For the corresponding validation curves, 89%, 90%, 68%, and 87% of the samples, respectively, had digestible phenylalanine, threonine, tryptophan, and valine values that deviated less than 10% from their determined values (Tables 4.28- 4.29).

Experiment 3- DDGS products calibration

Experiment 3a-3j. The calibration curves for the digestible, essential amino acid content in DDGS were calibrated with as few as 32 samples and as many as 39 samples from the total of 108 DDGS samples (Table 4.30). The individual validation curves for each essential amino acid all had R^2 values above 0.93 (Tables 4.31-4.34). The percent of validation samples in which arginine, histidine, isoleucine, leucine, and lysine content were predicted from their corresponding calibration curves with less than 10% deviation from the determined values was 89%, 90%, 85%, 100%, and 62%, respectively (Tables 4.31- 4.32). Likewise for methionine, phenylalanine, threonine, tryptophan, and valine, the percentage of predicted DDGS samples with a 10% or less error rate was 84%, 99%, 97%, 63%, and 88%, respectively (Tables 4.32- 4.34).

Table 4.24. Digestible phenylalanine content of common animal by-product ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 2g).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n	%			n	%		
Unidentified meal[1]	4	1.73 ± 0.197	1.59	2.02	15	1.76 ± 0.141	1.43	2.02
Feather meal	13	3.52 ± 0.531	1.93	4.04	16	3.50 ± 0.429	2.09	3.92
Fish meal	1	2.59	2.59	2.59	5	2.35 ± 0.177	2.09	2.57
MBM[2]	92	1.75 ± 0.358	1.06	3.03	263	1.62 ± 0.293	1.06	2.78
PBM[3]	30	1.94 ± 0.301	1.25	2.55	68	1.86 ± 0.283	1.10	2.73
Poultry tankage	6	1.65 ± 0.191	1.37	1.89	20	1.74 ± 0.081	1.59	1.89
Aggregated	146	1.95 ± 0.615	1.06	4.04	387	1.76 ± 0.474	1.06	3.92

[1] These unidentified animal by-product meal ingredient samples were not further defined by tissue type or species of origin.

[2] Meat and bone meal samples of bovine and porcine origin.

[3] Poultry by-product meal.

Table 4.25. Digestible threonine content of common animal by-product ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 2h).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n	%			n	%		
Unidentified meal[1]	4	1.46 ± 0.113	1.31	1.55	15	1.51 ± 0.220	1.16	2.07
Feather meal	8	2.92 ± 0.516	1.95	3.40	21	2.86 ± 0.368	1.54	3.25
Fish meal	1	2.42	2.42	2.42	5	2.33 ± 0.306	1.94	2.66
MBM[2]	91	1.51 ± 0.380	0.90	2.90	264	1.39 ± 0.253	0.91	2.28
PBM[3]	32	1.66 ± 0.373	0.84	2.60	66	1.64 ± 0.311	1.14	2.87
Poultry tankage	6	1.56 ± 0.031	1.51	1.59	20	1.59 ± 0.131	1.24	1.79
Aggregated	142	1.63 ± 0.495	0.84	3.40	391	1.54 ± 0.435	0.91	3.25

[1] These unidentified animal by-product meal ingredient samples were not further defined by tissue type or species of origin.

[2] Meat and bone meal samples of bovine and porcine origin.

[3] Poultry by-product meal.

Table 4.26. Digestible tryptophan content of common animal by-product ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 2i).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n	%			n	%		
Unidentified meal[1]	6	0.24 ± 0.015	0.23	0.26	13	0.27 ± 0.035	0.21	0.34
Feather meal	10	0.38 ± 0.106	0.26	0.60	19	0.39 ± 0.080	0.26	0.54
Fish meal	2	0.69 ± 0.094	0.62	0.76	4	0.59 ± 0.147	0.40	0.72
MBM[2]	90	0.31 ± 0.080	0.16	0.58	261	0.31 ± 0.076	0.17	0.54
PBM[3]	37	0.42 ± 0.120	0.20	0.71	60	0.42 ± 0.106	0.26	0.71
Poultry tankage	5	0.34 ± 0.047	0.28	0.40	21	0.34 ± 0.034	0.27	0.41
Aggregated	150	0.35 ± 0.111	0.16	0.76	378	0.33 ± 0.095	0.17	0.72

[1] These unidentified animal by-product meal ingredient samples were not further defined by tissue type or species of origin.

[2] Meat and bone meal samples of bovine and porcine origin.

[3] Poultry by-product meal.

Table 4.27. Digestible valine content of common animal by-product ingredients used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 2j).

Ingredient type	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n	%			n	%		
Unidentified meal[1]	8	2.13 ± 0.268	1.74	2.60	11	2.14 ± 0.229	1.72	2.51
Feather meal	9	5.24 ± 1.052	2.65	6.10	20	5.41 ± 0.788	2.37	6.07
Fish meal	0	-	-	-	6	3.03 ± 0.292	2.55	3.40
MBM[2]	99	1.98 ± 0.471	1.28	3.39	256	1.92 ± 0.429	1.20	3.40
PBM[3]	26	2.16 ± 0.480	1.18	3.41	72	2.12 ± 0.372	1.36	3.22
Poultry tankage	6	2.20 ± 0.159	2.02	2.47	20	2.20 ± 0.212	1.65	2.61
Aggregated	148	2.23 ± 0.923	1.18	6.10	385	2.18 ± 0.885	1.20	6.07

[1] These unidentified animal by-product meal ingredient samples were not further defined by tissue type or species of origin.

[2] Meat and bone meal samples of bovine and porcine origin.

[3] Poultry by-product meal.

Table 4.28. Mathematical evaluation of the near infrared reflectance spectroscopy (NIRS) validation curves for phenylalanine, threonine, and tryptophan when using the corresponding calibration prediction curves for common animal by-product feed ingredients (Experiment 2g, 2h, 2i).

OPUS® validation parameters	Phenylalanine		Threonine		Tryptophan	
Samples in validation	387		391		378	
R- squared	0.95		0.95		0.87	
RMSEP[1]	0.11		0.10		0.03	
RPD[2]	4.35		4.53		2.77	
Bias[3]	-0.013		-0.020		0.001	
Ratio of predicted to true[4]	Count	Percentage	Count	Percentage	Count	Percentage
<90.00	15	4	9	2	56	15
90.00- 92.49	17	4	12	3	24	6
92.50- 94.99	38	10	23	6	19	5
95.00- 97.49	46	12	43	11	43	11
97.50- 99.99	57	15	68	17	40	11
100.00- 102.49	61	16	67	17	40	11
102.50- 104.99	54	14	54	14	44	12
105.00- 107.49	42	11	51	13	19	5
107.50- 110.00	29	7	34	9	29	8
>110	28	7	30	8	64	17
Absolute percentage deviation[5]	Count	Percentage	Count	Percentage	Count	Percentage
<2.5%	118	30	134	34	80	21
<5.0%	218	56	232	59	166	44
<10.0%	344	89	351	90	258	68
<15.0%	378	98	385	98	323	85
<20.0%	385	99	388	99	351	93

[1] Root mean square error of the prediction.

[2] Residual predictive deviation.

[3] Bias- systematically averaged deviation between the data set of the true and the predicted values.

[4] Equation for determining the ratio of the predicted value to the true value is |(predicted value/true value)*100|.

[5] Equation for absolute percentage deviation from true value is [(|true value-NIRS predicted value|)/true value]*100.

Table 4.29. Mathematical evaluation of the near infrared reflectance spectroscopy (NIRS) validation curve for valine using the corresponding calibration prediction curve for common animal by-product feed ingredients (Experiment 2j).

OPUS® validation parameters	Valine	
Samples in validation	385	
R- squared	0.97	
RMSEP[1]	0.14	
RPD[2]	6.18	
Bias[3]	0.004	
Ratio of predicted to true[4]	Count	Percentage
<90.00	19	5
90.00- 92.49	23	6
92.50- 94.99	40	10
95.00- 97.49	58	15
97.50- 99.99	63	16
100.00- 102.49	48	12
102.50- 104.99	43	11
105.00- 107.49	38	10
107.50- 110.00	22	6
>110	31	8
Absolute percentage deviation[5]	Count	Percentage
<2.5%	111	29
<5.0%	212	55
<10.0%	335	87
<15.0%	372	97
<20.0%	381	99

[1] Root mean square error of the prediction.

[2] Residual predictive deviation.

[3] Bias- systematically averaged deviation between the data set of the true and the predicted values.

[4] Equation for determining the ratio of the predicted value to the true value is |(predicted value/true value)*100|.

[5] Equation for absolute percentage deviation from true value is [(|true value-NIRS predicted value|)/true value]*100.

Table 4.30. Digestible essential amino acid concentrations of dried distillers' grains with solubles ingredient samples used in the near infrared reflectance spectroscopy (NIRS) calibration and validation curves (Experiment 3a-3j).

Amino acid	Calibration				Validation			
	Sample count	Mean	Minimum	Maximum	Sample count	Mean	Minimum	Maximum
	n		%		n		%	
Arginine	33	1.28 ± 0.538	0.53	2.38	75	1.23 ± 0.441	0.55	2.21
Histidine	37	0.71 ± 0.274	0.32	1.35	71	0.72 ± 0.258	0.34	1.32
Isoleucine	32	1.06 ± 0.442	0.57	2.18	76	1.06 ± 0.432	0.62	2.05
Leucine	35	3.46 ± 1.462	1.95	6.86	73	3.20 ± 1.150	1.98	6.41
Lysine	39	0.75 ± 0.461	0.05	1.74	69	0.78 ± 0.420	0.07	1.73
Methionine	35	0.60 ± 0.267	0.20	1.25	73	0.57 ± 0.234	0.22	1.20
Phenylalanine	35	1.48 ± 0.604	0.84	2.99	71	1.38 ± 0.510	0.88	2.76
Threonine	34	0.88 ± 0.360	0.50	1.88	74	0.98 ± 0.372	0.53	1.79
Tryptophan	32	0.21 ± 0.090	0.05	0.42	65	0.20 ± 0.085	0.06	0.38
Valine	36	1.37 ± 0.574	0.71	2.67	72	1.30 ± 0.540	0.76	2.54

Table 4.31. Mathematical evaluation of the near infrared reflectance spectroscopy (NIRS) validation curves for arginine, histidine, and isoleucine when using the corresponding calibration prediction curves for dried distillers' grains with solubles (Experiment 3a, 3b, 3c).

OPUS® validation parameters	Arginine		Histidine		Isoleucine	
Samples in validation	75		71		76	
R- squared	0.98		0.98		0.98	
RMSEP[1]	0.07		0.04		0.06	
RPD[2]	6.54		6.59		7.45	
Bias[3]	-0.012		-0.005		0.009	
Ratio of predicted to true[4]	Count	Percentage	Count	Percentage	Count	Percentage
<90.00	0	-	0	-	5	7
90.00- 92.49	3	4	5	7	3	4
92.50- 94.99	7	9	6	8	7	9
95.00- 97.49	13	17	8	11	13	17
97.50- 99.99	10	13	11	15	20	26
100.00- 102.49	15	20	14	20	12	16
102.50- 104.99	9	12	14	20	5	7
105.00- 107.49	6	8	4	6	3	4
107.50- 110.00	4	5	2	3	2	3
>110	8	11	7	10	6	8
Absolute percentage deviation[5]	Count	Percentage	Count	Percentage	Count	Percentage
<2.5%	25	33	25	35	32	42
<5.0%	47	63	47	66	50	66
<10.0%	67	89	64	90	65	86
<15.0%	72	96	71	100	74	97
<20.0%	73	97	71	100	76	100

[1] Root mean square error of the prediction.

[2] Residual predictive deviation.

[3] Bias- systematically averaged deviation between the data set of the true and the predicted values.

[4] Equation for determining the ratio of the predicted value to the true value is |(predicted value/true value)*100|.

[5] Equation for absolute percentage deviation from true value is [(|true value-NIRS predicted value|)/true value]*100.

Table 4.32. Mathematical evaluation of the near infrared reflectance spectroscopy (NIRS) validation curves for leucine, lysine, and methionine when using the corresponding calibration prediction curves for dried distillers' grains with solubles (Experiment 3d, 3e, 3f).

OPUS® validation parameters	Leucine		Lysine		Methionine	
Samples in validation	73		69		73	
R- squared	0.99		0.96		0.97	
RMSEP[1]	0.11		0.09		0.04	
RPD[2]	10.10		4.85		6.20	
Bias[3]	-0.002		0.007		0.002	
Ratio of predicted to true[4]	Count	Percentage	Count	Percentage	Count	Percentage
<90.00	0	-	15	22	5	7
90.00- 92.49	2	3	5	7	5	7
92.50- 94.99	3	4	6	9	5	7
95.00- 97.49	6	8	3	4	8	11
97.50- 99.99	23	32	3	4	15	21
100.00- 102.49	22	30	6	9	10	14
102.50- 104.99	12	16	9	13	7	10
105.00- 107.49	3	4	8	12	6	8
107.50- 110.00	2	3	3	4	4	5
>110	0	-	11	16	8	11
Absolute percentage deviation[5]	Count	Percentage	Count	Percentage	Count	Percentage
<2.5%	45	62	9	13	25	34
<5.0%	63	86	21	30	40	55
<10.0%	73	100	43	62	61	84
<15.0%	73	100	50	72	70	96
<20.0%	73	100	56	81	73	100

[1] Root mean square error of the prediction.

[2] Residual predictive deviation.

[3] Bias- systematically averaged deviation between the data set of the true and the predicted values.

[4] Equation for determining the ratio of the predicted value to the true value is |(predicted value/true value)*100|.

[5] Equation for absolute percentage deviation from true value is [(|true value-NIRS predicted value|)/true value]*100.

Table 4.33. Mathematical evaluation of the near infrared reflectance spectroscopy (NIRS) validation curves for phenylalanine, threonine, and tryptophan when using the corresponding calibration prediction curves for dried distillers' grains with solubles (Experiment 3g, 3h, 3i).

OPUS® validation parameters	Phenylalanine		Threonine		Tryptophan	
Samples in validation	71		74		65	
R- squared	0.99		0.99		0.93	
RMSEP[1]	0.06		0.04		0.02	
RPD[2]	8.60		10.10		3.72	
Bias[3]	0.009		-0.002		-0.002	
Ratio of predicted to true[4]	Count	Percentage	Count	Percentage	Count	Percentage
<90.00	1	1	1	1	9	14
90.00- 92.49	2	3	0	-	1	2
92.50- 94.99	10	14	6	8	4	6
95.00- 97.49	9	13	12	16	9	14
97.50- 99.99	19	27	13	18	8	12
100.00- 102.49	12	17	22	30	7	11
102.50- 104.99	10	14	11	15	3	5
105.00- 107.49	6	8	4	5	6	9
107.50- 110.00	2	3	4	5	3	5
>110	0	-	1	1	15	23
Absolute percentage deviation[5]	Count	Percentage	Count	Percentage	Count	Percentage
<2.5%	31	44	35	47	15	23
<5.0%	49	69	58	78	27	42
<10.0%	70	99	72	97	41	63
<15.0%	71	100	74	100	55	85
<20.0%	71	100	74	100	59	91

[1] Root mean square error of the prediction.

[2] Residual predictive deviation.

[3] Bias- systematically averaged deviation between the data set of the true and the predicted values.

[4] Equation for determining the ratio of the predicted value to the true value is |(predicted value/true value)*100|.

[5] Equation for absolute percentage deviation from true value is [(|true value-NIRS predicted value|)/true value]*100.

Table 4.34. Mathematical evaluation of the near infrared reflectance spectroscopy (NIRS) validation curve for valine when using the corresponding calibration prediction curve for dried distillers' grains with solubles (Experiment 3j).

OPUS® validation parameters	Valine	
Samples in validation	72	
R- squared	0.98	
RMSEP[1]	0.07	
RPD[2]	7.30	
Bias[3]	0.008	
Ratio of predicted to true[4]	Count	Percentage
<90.00	3	4
90.00- 92.49	6	8
92.50- 94.99	6	8
95.00- 97.49	13	18
97.50- 99.99	13	18
100.00- 102.49	11	15
102.50- 104.99	2	3
105.00- 107.49	6	8
107.50- 110.00	6	8
>110	6	8
Absolute percentage deviation[5]	Count	Percentage
<2.5%	23	32
<5.0%	40	56
<10.0%	63	88
<15.0%	71	99
<20.0%	72	100

[1] Root mean square error of the prediction.

[2] Residual predictive deviation.

[3] Bias- systematically averaged deviation between the data set of the true and the predicted values.

[4] Equation for determining the ratio of the predicted value to the true value is |(predicted value/true value)*100|.

[5] Equation for absolute percentage deviation from true value is [(|true value-NIRS predicted value|)/true value]*100.

CHAPTER 5

DISCUSSION

The current research indicates that the NIRS calibrations created do not meet the accuracy standards set forth by the hypothesis of this research for the quantification of digestible amino acid content of feed ingredients used in poultry diets. It was hypothesized that the NIRS calibration curves created for the detection of digestible, essential amino acid content in feed ingredients would predict the values with less than 5% deviation from the actual determined values for 95% or more of samples tested. Using the universal ingredient calibrations, no digestible, essential amino acid calibration predicted more than 47% of samples with less than 5% deviation from the determined digestible amino acid content for any of the 10 essential digestible amino acids. The individual ingredient calibration for DDGS did show accuracy in predicting digestible leucine and threonine with 86% and 78% of samples, respectively, being predicted with a 5% or less deviation from their animal bioassay true value. These results demonstrate the potential for improved accuracy of individual ingredient calibrations over that of the corresponding universal calibrations. Similarly, Cope (2021) reported that using individual ingredient NIRS calibrations for the detection of nitrogen corrected true metabolizable energy (TME_N) significant increased the predictability of the calibration relative to a universal ingredient calibration.

For feed manufacturers developing new feed ingredients, the universal feed ingredient calibration will need further development. When new feed ingredients are being developed, it will not be feasible to develop ingredient specific calibrations given the expense and number of samples needed. However, these manufacturers simply need a good estimate of amino acid digestibility to decide if a product is worth further development. The current universal ingredient calibrations for digestible, essential amino acid content predicted 75-92% of samples with a deviation of 20% or less from their actual bioassay value. If the calibration curves can be improved so that 100% of samples are predicted with a deviation of less than 20% from their true value, they would be sufficient for feed ingredient development.

The current research with digestible amino acid determination by NIRS agreed with the previous research of Cope (2021) and the NIRS prediction of TME_N, that the traditional statistical evaluation of validation curves using such things as R^2 and RPD values do not provide a robust enough evaluation of a calibration curves predictability for poultry diet formulation. For example, the DDGS validation curves for digestible leucine, phenylalanine, and threonine all had R^2 values of 0.99, but these validations only predicted 86%, 69%, and 78% of samples within a 5% deviation from their true value, respectively. Most NIRS research publications do not publish the predicted values and the true nutrient values, nor the practical accuracy value itself, as reported by this research, which makes a practical evaluation of their developed NIRS calibrations difficult.

Diet formulation

When considering diet formulation for poultry, the 5% deviation threshold was established to ensure that 95% of ingredient samples analyzed using NIRS would have a maximum of 5% deviation from their bioassay determined value, equivalent to the error associated with the cecectomized rooster bioassay. With this low error, feed formulation with NIRS could be as precise as using data from the animal bioassay for obtaining nutrient content of ingredients. This method would be more accurate than applying correction factors to book values to account for variation in the growing, processing, and storage conditions of ingredients. This 5% value is smaller than the 7.5% value for formulation of mixed ration diets suggested by Van Kempen and Simmins (1997). The use of NIRS for prediction of digestible amino acid content would allow nutrient values used in formulation to better reflect the range of all possible nutrient content values of feed ingredients rather than using a single average value intended to represent all possible ranges of that nutrient. The use of NIRS in diet formulation has been shown to improve diet formulation in poultry. Van Kempen and Simmins (1997) suggested that diet over formulation could be reduced from 7.5% to 3% using the NIRS calibrations created in that research. The R^2 values presented in this research are higher than the published R^2 values ranging from 0.70-0.90 for multiple products by Van Kempen and Simmins (1997), suggesting that based on R^2 values, this research could further reduce digestible amino acid excess in poultry diets. Steed et al. (2020) demonstrated that specific diets formulated using NIRS strategies matched performance of the control diet and improved production profitability over the control diet on a per kg of feed basis and a per kg of whole chicken without giblets (WOG) basis.

Equations for determining digestible amino acid content have been developed but require laboratory analysis input values such as moisture, ash, crude protein, crude fiber, ether extract, total phenols, and non- starch polysaccharides for the calculation of digestible amino acids. For example, using the artificial neural network (ANN) algorithm and proximate analysis values, multiple studies have achieved R^2 values above 0.90 for many of the essential amino acids (Roush and Cravener, 1997; Cravener and Roush, 1999; Cravener and Roush, 2001; Ebadi et al., 2011; Sedghi et al., 2013). While the R^2 values are sufficiently high, these studies do not provide a comparison of the predicted values to the true values, so practical accuracy for independent samples may be less than the current NIRS research. Furthermore, these equations still require the use of proximate analysis which, as previously stated, takes time, and becomes prohibitively expensive on a commercial level. It is also simply not possible for poultry companies to store vast amounts of feed ingredients for days or weeks while laboratory analyses are completed. The NIRS procedure offers accuracy that is equal to or exceeds the accuracy of other methods, based on R^2 values, but can be completed quickly, so that all ingredients can be evaluated when arriving at the mill and results will be available in real time for use in diet formulation.

Ingredient specific calibrations

Future research is needed to determine if the creation of individual product calibrations with a sufficient number of samples for each ingredient and a sufficient range of nutrient content will improve predictability of DDGS products. Similar to the common animal products calibration, the DDGS calibration included standard protein DDGS, hi protein DDGS, high oil content DDGS, low oil content DDGS, and DDGS processed through different cooking methods, but all produced from corn. Other DDGS products including DDGS from beverage production (as opposed to DDGS from ethanol production), dried distillers' grain samples without solubles, DDGS made from other grains like sorghum and wheat, and further processed DDGS products with fiber removed may require individual calibrations be created for the prediction of digestible amino acid content in the more specialized product groups (Corredor et al., 2006; Parsons et al., 2006; Martinez Amezcua et al., 2007; Tres et al., 2014). These products have unique nutrient profiles that differ from standard corn DDGS samples. In the current research, a sample of wheat DDGS was available and evaluated using the DDGS calibration, yielding mixed results, with 5 amino acids predicted with 43% deviation from the true value or higher, and only 3 amino acids were predicted with less than 10% deviation from true (data not shown). Given the accuracy of the DDGS calibration in predicting corn DDGS samples, the inaccuracy of prediction of the wheat DDGS sample demonstrates that alternative DDGS samples will need individual calibrations created for accurate determination of digestible amino acids.

The presence of moisture can saturate the absorption spectra in the 950-1075nm, 1340-1450, and 1810-2000nm regions and impact predictability of nutrient content with utilizing these regions for analysis (Baker et al., 1994). The presence of moisture may be partially mitigated by the exclusion of these regions from the calibration, as done in this research, but drying the samples would allow use of these regions for prediction of digestible amino acid content, enhancing the ability of NIRS to evaluate samples. However, the animal bioassay values are determined on an “as is” basis, and the feed ingredients are fed on an “as is” basis. Evaluating feed ingredient samples on a dry matter basis is not feasible for the commercial poultry industry as time is required for drying each sample. Poultry nutritionists need NIRS as a rapid evaluation tool for evaluating feed ingredients upon arrival at the mill, without having to wait for the sample to be dried. Similar to wet chemistry analysis, the drying process would delay the integration of ingredients into diets or not be completed before the diets are milled, limiting the usefulness of the NIRS data in diet formulation.

Amino acid reactivity

The individual amino acids present in proteins are linked by peptide bonds. During digestion, the peptide bonds are cleaved by an array of proteases (Neurath, 1964), liberating the individual amino acids for absorption. However, some essential amino acids such as lysine, methionine, and threonine have reactive groups that allow the formation of additional bonds with other molecules such as carbohydrates, polar molecules, and other proteins (Alexander and Hill, 1952; Smith and Lewis, 1966; Shemer and Perkins, 1975; Cowieson et al., 2004; Rutherfurd and Moughan, 2012), in addition to forming peptide bonds. Often when these reactive amino acids make these additional bonds, they can no longer be liberated during digestion and become undigestible. Thus, during an NIRS scan of a feed ingredient, the NIRS system not only has to detect and quantitate the total amount of the amino acid, but also whether it is binding with other molecules that will prevent its digestion, and with amino acids containing reactive groups allowing multiple bond formations, this is even more difficult.

Lysine predictability was especially low in the DDGS calibration. Though the R^2 of this validation was 0.96, only 62% of samples were predicted with 10% or less deviation from their true value, the lowest of all essential amino acids in that experiment. Lysine has an additional reactive amino group that can form bonds with carbohydrates (Hurrell and Carpenter, 1974). The heat processing of DDGS, especially in the presence of moisture, enhances the bond formation between lysine and carbohydrates, and this process is known as a Maillard reaction. Maillard reactions can occur in poorly processed DDGS, and can decreases the digestibility of lysine, which significantly increases the variability of digestible lysine content in DDGS samples (Almeida et al., 2013).

Compared to other essential amino acids, methionine possesses a reactive sulphur group and threonine has a reactive hydroxyl group. Thus, proteins containing these amino acids can bond with other molecules, which can make them undigestible as reviewed by (Rutherfurd and Moughan, 2012). Future research will need to determine if incorporating more feed ingredient samples that contain these amino acids in oxidized form or complexed with other molecules due to poor processing conditions will allow the NIRS software to better differentiate between undigestible forms of these amino acids, which will improve the predictability of their NIRS calibrations.

Analysis of amino acids of low concentrations

In the current research, the NIRS prediction of digestible tryptophan concentrations in feed ingredients was among the poorest of all essential amino acids. Variation in tryptophan amino acid content analysis have been reported, with coefficients of variation ranging from 3.9-16.1% (Allred and MacDonald, 1988). Llames and Fontaine (1994) demonstrated that amino analysis repeatability within a single laboratory yielded coefficients of variation from 1.1-12.7% across all amino acids in all ingredients analyzed, while reproducibility of the analysis procedures, or measures of accuracy when performed in different laboratories, increased the variation to 3.5-28.2% across all amino acids and all ingredients. To illustrate the variation in tryptophan analysis in the current research, 8 random feed samples and 8 random feces samples were chosen, and laboratory analysis was performed in duplicate for each of the 16 total samples. The tryptophan concentration for each sample was determined by high performance liquid chromatography (HPLC) (University of Missouri Agricultural Experiment Station Chemical Laboratory, Columbia, MO). The determined tryptophan concentrations ranged from 0.06%-0.32%, but more importantly, the average coefficient of variation between the duplicate samples was 18.5% (data not shown). With this degree of variability in tryptophan concentration determinations, it is not surprising that the validation of the digestible tryptophan calibrations was also poor, as the predictive capacity of NIRS calibrations is dependent in large part to the quality of determined values used to build the calibration (Conzen, 2014). Total tryptophan concentrations in feed ingredients and in the dried feces resulting from the cecectomized rooster assay is naturally low and routinely below 0.25% (data not shown). Although the published detection limit for amino acid analysis by HPLC is 0.01% for most amino acids (AOAC, 2023), the low concentrations of tryptophan in samples can contribute to increased variation in tryptophan detection in duplicate samples. Tryptophan is also subject to degradation during hydrolysis when being prepared for analysis (Kambhampati et al., 2019), which can also add variation between duplicate preparations of the same sample.

Summary

In summary, the NIRS calibration models created in this research for the detection of digestible amino acids in poultry feed ingredients were not considered accurate enough for feed formulation using the threshold of samples having 5% or less deviation from their true values. These models did show efficacy for initial evaluation of poultry feed ingredients and showed high predictability when allowing samples to have 10% or less deviation from their true value, especially when evaluating ingredients using ingredient specific models. The use of high accuracy NIRS models will allow nutritionists to evaluate ingredients immediately upon arrival to the mill and will allow use of that real time data in diet formulation. Further improvements to this work may include expanding the ingredient specific models through more samples or through breaking them into more specific ingredient groups.

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