DETERMINATION OF NITROGEN CORRECTED TRUE METABOLIZABLE ENERGY BY NEAR INFRARED REFLECTANCE SPECTROSCOPY

by

LAUREN REID

(Under the Direction of ADAM DAVIS)

ABSTRACT

Accurate poultry diet formulation depends on accurate prediction of the nutritional values of feed ingredients. One of the more useful nutritional values to know is nitrogen corrected true metabolizable energy (TME_N). The animal bioassay used to determine TME_N of feed ingredients is costly, and in commercial feed mill settings it cannot be completed on delivered ingredients before they are utilized in diets. Near Infrared Reflectance Spectroscopy (NIRS) is a rapid analysis method that enables a Multi-Purpose Analyzer to be calibrated to predict nutritional component values after analyzing the reflectance of a feed ingredient in the near infrared spectrum. A calibration was made for TME_N (Kcal/kg) using 53 samples which resulted in prediction equation with a correlation coefficient of 0.96. This prediction equation was then validated with 48 new samples and the correlation coefficient between the NIRS and bioassay TME_N values was 0.92 indicating a highly successful NIRS calibration was achieved.

INDEX WORDS: true metabolizable energy, nitrogen, near infrared reflectance spectroscopy, diet formulation, gross energy

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LAUREN REID

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LAUREN REID

Major Professor:	Adam Davis
Committee:	Brian Fairchild
	Andrew Benson

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
May 2017

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CHAPTER 1

TRUE METABOLIZABLE ENERGY

Worldwide, meat and eggs produced by poultry are the largest source of animal protein produced and eaten by humans. Thus, it is vital to maintain and even increase the efficiency and productivity of poultry farming as the world population continues to grow. Feeding commercial poultry is the costliest aspect of their production. Over 70 percent of the cost of raising a modern broiler is associated with feeding it (Yegani and Korver, 2012). Therefore, it is imperative to match the nutrient requirements needed for maintenance, growth and production of poultry with the nutrients provided by poultry diets.

In general, poultry diets are composed of a cereal grain such as corn or wheat to provide energy, a concentrated source of protein such as soybean meal or animal by-product meal, and fat/oil to provide additional energy, increase palatability and reduce 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, in the United States poultry production has been based on utilizing corn and soybean meal as the main ingredients. Often some of the soybean meal would be replaced with meat and bone meal products produced from animal processing waste (Scott et al., 1982).

Over the past few years there has been an evolution in feeding commercial poultry in the United States. The growth of ethanol production as an alternative to fossil fuel has led to tremendous increase in the cost of poultry feed ingredients, especially corn. But more importantly, ethanol production produces a co-product called dried distiller's grains with

solubles (DDGS) which is now commonly used in poultry diets at the expense of corn and soybean meal (Lumpkins et al., 2004). A few consumer driven trends also influence poultry diet formulations. One of these is the avoidance of animal by-products in poultry diets so that the birds are consuming an all vegetable diet rather than being fed a diet containing ingredients derived from other animals. Similarly, the production of free range poultry, poultry fed diets that are antibiotic/pharmaceutical free or organic poultry that can only be fed diets containing organic feed ingredients has increased tremendously. Finally, poultry production companies are currently much more sensitive to providing diets that do not have nutrient excesses as these excesses can lead to fat accretion that is carcass waste and increased nitrogen and phosphorus excretion that cause environmental concerns (Bodin and Aubret, 2005; van Kempen and Simmons, 1997; Givens et al., 1997).

With these different pressures to find either cheaper feed ingredients to replace the dependency on ingredients whose prices have been inflated due to ethanol and biomass energy production, or to utilize organically produced feed ingredients and feed ingredients that may have nutraceutical properties, new feed ingredients for poultry production are being investigated and utilized. While these alternative feed ingredients are being introduced there is also a demand to accurately define the nutrient specifications of traditional ingredients to minimize the production of diets with nutrient deficiencies or excesses. The ultimate goal of feed analysis is to maximize ingredient value (Bastianelli, 2013), and when evaluating feed ingredients for poultry diets the most important nutrient specifications to have are the true metabolizable energy and digestible amino acid content of these ingredients. This is because it is the available energy and amino acids that are going to sustain and fuel the birds rapid growth from hatch to market

weight in the broiler industry and prolific egg production in the table egg industry (Scott et al., 1982). For the purpose of this thesis the focus will be on true metabolizable energy.

Energy Definitions

Energy is not a nutrient. Instead, it is a property of the energy yielding nutrients of a feed ingredient when these nutrients are metabolically oxidized by an animal. There are a multitude of ways to classify and determine energy content. Several measurable parameters can be used to describe energy content, but this information is only useful to nutritionists when it can be utilized in a manner that enables accurate diet formulation. That is, it is the most beneficial to find the energy value that most accurately reflects the energy of a consumed feed ingredient that can be used by an animal for growth, maintenance, and/or reproduction.

Gross energy is a value that is determined by measuring the heat produced when a feed ingredient is completely oxidized to carbon dioxide and water. It is also referred to as the heat of combustion and obtained experimentally utilizing a bomb calorimeter. However, the amount of gross energy an animal can utilize is limited by its digestive capability. That which cannot be absorbed through digestion is lost in the excreta as fecal energy. Therefore, the apparent digestible energy is the gross energy of the feed ingredient/feed consumed minus the gross energy of the resulting feces. Poultry excrete feces and urine together so apparent digestible values are rarely used in formulating poultry diets (Scott et al., 1982).

Because apparent digestible energy values are rarely available for poultry, a much more common measure is apparent metabolizable energy (AME) which is the gross energy of the feed ingredient/feed consumed minus the gross energy contained in the feces, urine and gaseous products of digestion. In poultry where gaseous products from digestion are typically negligible AME is a more logical measure because fecal and urine wastes are combined in poultry excreta

(Scott et al., 1982). Typically, a correction factor for nitrogen retained in the carcass of the animal is applied yielding AME_N.

True metabolizable energy (TME) is a further refinement of AME as it is the gross energy of the feed ingredient/feed consumed minus the gross energy of the excreta of feed origin. Excreta contain undigested feed, but it also contains intestinal cells and secretions (endogenous component) that are not of feed origin. The TME procedure corrects for the endogenous component of the feces so that the digestible energy component of the feed is not penalized for endogenous component of the feces (Scott et al., 1982). Like the AME, TME values are also corrected for nitrogen retained in the animal yielding a TME_N value.

The heat increment of feeding represents heat loss due to nutrient digestion, assimilation and nutrient interconversion. Metabolizable energy minus the heat increment yields net energy. Net energy is used by the animal to cover maintenance energy costs and any energy left over can be used for growth and/or production. Determining net energy is very difficult as it requires metabolic respiratory chambers and thus very few net energy values have been determined for feed ingredients (Scott et al., 1982). Nutritionists therefore tend to formulate diets based on AME_N or TME_N values.

Methods for TME Determination

In general, for the TME_N method, adult single comb white leghorn roosters are used. The roosters undergo a 24 hour feed withdrawal and then are precision fed a fixed amount (typically 30 to 35 grams) of a pure feed ingredient, and then excreta is collected for the next 48 hours after feeding. Typically, 8 to 10 roosters are fed per feed ingredient. Another 8 to 10 roosters that were fasted for 24 hours will not be fed, but feces will be collected from this group of roosters for 48 hours and they will serve as the endogenous control birds to calculate the

endogenous energy portion of the feces of the fed roosters (Sibbald and Morse, 1983). Because both sets of roosters will be in a negative nitrogen balance during this procedure it is essential for a nitrogen correction of the energy content of the feces from both the fed roosters and the endogenous controls to be performed (Parsons et al., 1982; Sibbald and Morse, 1983; Dale and Fuller, 1984). The degree of the negative nitrogen balance of the fed roosters will decrease as the protein content of the feed ingredient being fed increases. Without the nitrogen correction (called a TME calculation) the energy value for the ingredient is inflated, and thus a TME_N value is lower than a TME value for tested feed ingredients and are a better reflection of the true metabolizable energy of the ingredient (Dale and Fuller, 1984).

There are advantages to the TME_N procedure relative to the AME_N given that the birds are precision fed a know quantity of feed there is no feed refusal and only the tested ingredient has to be fed. The AME_N is dependent on presenting the tested ingredient as a portion of a complete palatable diet that the test birds will eat and then measuring the amount of feed that is consumed in a given 2 to 5 day period. The AME_N procedure is prone to errors with spilled/wasted feed and feed contamination of the feces being collected. The AME_N procedure is done with young growing birds which typically precludes them being used again for further AME_N testing (Scott et al., 1982). In contrast, a colony of adult leghorn roosters can be used multiple times in a given year to test different feed ingredients for TME_N determinations as their growth and metabolism and space and facility requirements are not changing like the growing chicks used in AME_N determinations.

The TME_N procedure does have some negative aspects. The amount of endogenous loss actually varies relative to the amount and physical characteristics of the feed ingredient passing through the gastrointestinal tract (Farrell, 1981; Tenesaca and Sell, 1981; Hatel, 1986; Farrell et

al., 1991). However, in the TME_N procedure the endogenous waste is constant as it is determined from unfed birds. Another criticism is that when one feed ingredient is fed alone it eliminates the possibility of synergistic or antagonistic effects between multiple ingredients found in a complete diet. For example, the presence of fatty acids in a diet can add to the energy derived from other ingredients (Young, 1961; Artman, 1964).

Equation estimates of TME_N

Despite the benefits of using TME_N as an accurate estimation of feed energy, it remains an extremely time consuming and expensive procedure. As a population of roosters are needed, a bomb calorimeter and nitrogen analyzer are needed for laboratory analyses of the ingredient and collected feces, and it often takes at least a week to get results for a feed ingredient. Thus, a more efficient method for determining TME_N would be extremely valuable to nutritionists and researchers alike.

To that end, researchers have developed equations based on proximate analyses of feed ingredients to predict TME_N in classes of feed ingredients such as bakery meal, poultry by product meal and dried distillers grains plus solubles (Pesti et al., 1986; Dale et al., 1990; Dale, 1996; Batal and Dale, 2006). While these equations can provide a rough estimate and differentiate samples of an ingredient that vary greatly in energy quality, they typically do not have the predictive power (R^2 from 0.45 to 0.77) to reliably differentiate between most samples of an ingredient.

In summary, in order for production animals to achieve optimal growth, production and reproduction; the optimal balance of nutrients must be provided. These nutrients include: fats, carbohydrates, protein, vitamins, and minerals. Energy which is not a nutrient, but is derived from the combustion of nutrients by an animal, is the fuel that drives an animal's maintenance,

growth and production. The feeding value of a diet depends on the concentration and digestibility of the nutrients in the feed ingredients making up a diet, and these can be highly variable in different feed ingredients and different lots of the same feed ingredient. Poultry nutritionists utilize TME_N values to assess the energy quality of feed ingredients and to formulate diets that meet the energy needs of poultry.

CHAPTER 2

NEAR INFRARED REFLECTANCE SPECTROSCOPY

Introduction to NIRS

Near infrared reflectance spectroscopy (NIRS) was first described as potential technique for chemical analysis by Norris and Hart (1965). Subsequently, this technique was applied to forage quality analysis (Norris et al., 1976) and its use for this purpose has gained popularity and credibility ever since (Moughan et al., 2000). The technique involves the selective absorption of electromagnetic radiation in the region of 730 nm to 2500 nm that matches characteristic vibration frequencies of organic functional groups (Smith et al., 2001). However, because in the near infrared spectrum there are only a few areas where absorbance can only be due to one type of functional group, statistical tests are applied to identify secondary relationships between spectral data and determined reference values (Foley et al., 1998). It was quickly realized that reflectance spectroscopy and multiple regression techniques were the key to diminishing the effects of constituent scattering and interference (Norris, 1992). Thus, the rapid advancement of NIRS techniques was initially limited by a lack of computer processing power for handling large amounts of spectral data and statistical analysis.

Basic Principles

The underlying principles behind NIRS are most simply an integration of light spectroscopy, statistics, and computer science. In NIRS for feed analyses, a light source interacts with the finely ground feed sample and the reflected light is measured by detectors. Because feeds and feed ingredients are opaque, near infrared reflectance is typically measured rather than

transmittance. The reflected light from the sample indirectly indicates the amount of light energy absorbed by the sample. Specific bonds in feed ingredients such as O-H, N-H and C-H bonds will absorb light at specific wavelengths, and the remaining amount of projected light that was reflected is detected by photosensitive material and quantified as absorbed light. Because different molecules have different unique atom arrangements, each molecule (for example all riboflavin molecules) within a feed sample has its own infrared fingerprint that can be detected and quantified.

The molecules of a particular nutrient in a feed ingredient are not going to all be free molecules, instead they can be associated or even chemically bound to a host of other different molecules contained in the feed ingredient. Thus, feed constituents are distinguished from each other through their unique 'overtones' and 'combination bands' that are created as a chemical group is excited by light energy to a higher energy state (Moughan et al., 2000). The conversion of radiant energy to an electrical signal is mediated directly by the photosensitive materials in the instrument detectors. Incident photons directly affect the electrical state of this photosensitive material that is usually made of lead sulphide or silicon (Givens et al., 1997). Ultimately, any interference between constituents can be mitigated with mathematical treatment and other statistical procedures as will be discussed in a subsequent section.

Another issue that must be addressed with NIRS of samples is light scattering. Scattering can occur when the radiation transmitted through a sample is reflected, refracted or diffracted against other random particles in the sample. Scattering largely depends on the average particle size, moisture content, and temperature of the sample. Variation in sample particle sizes will affect the amount of reflectance and absorbance. In addition, the presence of excess water in a sample causes O-H bond stretch and O-H deformation bond vibrations in the 1850-2000 nm

region of the near infrared spectrum, thereby distorting the analysis (Baker et al., 1994). A change in sample temperatures will likewise distort the chemical bonds within a sample (Hansen et al., 2000). Unfortunately, there is no mathematical law capable of describing scattering in a medium with a heterogenous distribution of absorbing constituents that would be found for example in feed ingredients. Therefore, a machine must be calibrated to account for these different physical properties, simultaneously using samples that are processed and analyzed in a highly uniform manner as will be discussed shortly (Givens et al., 1997).

Once spectral data is obtained, the ultimate goal is to establish a calibration curve that relates spectral wavelength scans of a group of samples being analyzed for the amount of a substance and the known concentrations of this substance obtained through chemical or biological analyses of these samples. Once a calibration curve is obtained that has a high correlation between the known wet chemistry values and the spectral analyses, this calibration curve is used to predict the concentration of the substance of interest in other samples. In making the calibration curve the spectral analyses must be transformed to achieve the best fitting correlation between the spectral analyses and the known wet chemistry values of the samples. A variety of mathematical treatments such as: log, first, and second derivative transformations are employed. However, there is no ideal agreed upon transformation. Curve-smoothing techniques can also be employed to reduce overlapping bands and to eliminate as many confounding elements caused by light scatter and reflectance as possible (Givens et al., 1997). In addition, as the number of samples available with spectral wavelength scans decrease for a given calibration the more critical sample preparation uniformity becomes in constructing a useful calibration (Moughan et al., 2000).

Sample preparation for NIRS

Sample preparation and proper spectrometer use can have very influential effects on spectral data and calibration. Before analysis, a minimum of a 15 minute instrument warm up period is essential as spectral analysis of samples is very sensitive to the temperature of the instrument (Westerhaus, 1989a). A temperature gradient occurs within the instrument as the lamp and detector warm up, which will lead to different spectral readings (Workman, 2008). In addition, temperature variaiton in the samples must be mimimized as temperature variations will affect the bonding of organic elements within the sample and as a result cause changes in the spectral absorbance bands and overtones (Hansen et al., 2000).

The most important part of sample preparation is grinding of feed products down to a particle size that does not create unintentional interference when scanned by a spectrometer, and to ensure that the product being scanned is homogenous as a powder or liquid. It has been determined experimentally that uniform grinding increases the overall accuracy of a NIRS calibration (Fontaine et al., 2001). Ideally, NIRS analysis would take place as soon as possible after sample preparation (Abrams, 1989).

There are two general types of feed grinders: shearing and impacting. Shearing type mills, such as the Wiley mill, have knives that first reduce particle size before the sample is pushed through a sieve into the collection vessel. Impact-type grinders function by forcing the feed against a carborundum-impregnanted wall before it passes through the sieve. Bakalli et al. (2000), reported that there were significant differences in analysis outcome for whole soybeans ground with a Cyclotec (impact) versus a Wiley (shearing) grinder (2000). They also found that even though a Cyclotec grinder caused a 3% loss in sample moisture due to more air exposure, preparing samples with this instrument led to less experimental error. Different sized sieves or

screens are utilized throughout the literature. There is roughly an equal representation of 1 mm screens (Valdes et al., 1985; Bakalli et al., 2000; van Kempen and Bodin, 1998) and 0.5 mm screens (Fontaine et al., 2002; Bakalli et al., 2000; Smith et al., 2001) being used in current literature. It is important to note screen size in experimental procedures because an NIR spectrometer is very sensitive to the amount of sample surface presented for analysis (Foley et al., 1998). The mean particle size and distribution can both affect reflectance properties and can even account for up to 90% of spectral variance (Givens et al., 1997).

Smith et al. (2001) found that the accuracy of NIRS predictions for phytate content was partially dependent on the weight and depth of the sample used in a sample cup. The most accurate predictions were found for samples that weighed 12 to 18 grams and were packed into a NIRS sample cup at a depth of 7 to 10 mm. The depth of the sample in a cup was directly related to the height of the spectra because of the effect of particle size and packing density. The density of the sample can inadvertently cause a higher reflectance than expected which in turn results in lower absorbance values, and therefore lower proximate analysis predictions (Smith et al., 2001).

Given a lack of environmental consistency when analyzing samples and sample non-uniformity are often the major sources of noise in NIRS measurements above and beyond noise which is inherent to the analysis (Norris, 1989), it is imperetive that the samples to be analyzed are uniformly ground and scanned under the same environmental conditions. This is especially true for animal diet samples, because if they are not finely ground the different ingredients can have different bulk densities and interactions with one another which would have a direct impact on reflectance properties (Valdes et al., 1985).

NIRS calibration curve and validation

After sample preparation, the selection process of samples for integration into the calibration curve begins. For accurate calibration a wide number of samples must be obtained that reflect the variability in concentration expected of the parameter of interest in samples to be tested later using this curve (Fontaine et al., 2002). Then a spectra analysis must be completed on each of the obtained samples and correlated with known wet chemical analysis values of the parameter of interest in each of the samples to produce a regression curve called a calibration or prediction curve. If there are visible sample outliers, they should be reanalyzed to attempt to improve accuracy (Fontaine et al., 2002). In some cases, outlier samples are excluded from the calibration curve if the spectral and wet chemistry values indicate the sample may have atypical or poor qualities (van Kempen and Bodin, 1998; Fontaine et al., 2001).

Once the calibration curve is obtained, it has to be validated with a different set of samples in which the concentration of the parameter of interest in each sample is determined using NIRS analysis with the prediction curve and by wet chemistry analysis. For success, the concentrations of the parameter of interest and the physical characteristics of the samples used for the validation should be represented in the samples used to make the calibration equation (Westerhaus, 1989b).

There is not a widely agreed upon method for choosing samples for calibration versus validation, or for determining how many samples should be used to make a calibration. Tahir et al. (2012) suggested approximately 80% of the available samples should be used for calibration and the other 20% for validation of the calibration curve. Fontaine et al. (2001) recommend that a minimum of 30-50 samples be collected before the initial calibration is developed. After the samples used for the calibration and validation are analyzed, they should be stored in a freezer in

glass bottles (Valdes and Leeson, 1992b), polyethylene bottles (Fontaine et al., 2002), or plastic bags (Tahir et al., 2012; Smith et al., 2001), so that they could be used again if necessary.

The NIRS systems that are currently sold are equipped with powerful software capabilities that provide mathematical and statistical functions to improve calibration curves and validate the strength of the calibration. Mathematical treatment can be used to improve the correlation of the regression equation and decrease the sum of squared error. The main purpose of data treatments is to reduce noise, reduce variability and enable the relevant spectral information to be isolated for analysis (Westerhaus, 1989b). Variation within spectra is mainly the result of random radiation scatter at the surface of particles, varying radiation path length through a sample, and the chemical composition (Barnes et al., 1989).

Pre-processing techniques can be classified as scatter correction methods (Standard Normal Variate) or spectral derivatives (Rinnan et al., 2009). Standard Normal Variate (SNV) is a common technqiue applied for scatter correction. As discussed earlier, scattering can occur when the radiation transmitted through a sample is reflected, refracted or diffracted against other random particles in the sample. Scatter correction allows the elimination of undesirable scatter elements before the data is modeled through estimation of the correction coefficients (Rinnan et al., 2009). The correction coefficient is commonly defined by the original reference spectra value minus average of the reference spectra all divided by the standard deviation of the sample spectra. Spectral derivatives can remove additive and multiplicative effects, and in practice involve a smoothing step before the derivitive is calculated (Rinnan et al., 2009).

Other mathematical treatments allow the selection of the most ideal wavelengths (instead of a complete spectrum scan) and coefficients by stepwise multiple linear regression wherein the reflectance values serve as the independent and the known chemical values as the dependent

variables (Valdes et al., 1985). A modified partial least squares regression (MPLS) algorithm can reduce data points to terms based on spectral differences as well as the reference data values (Losada et al., 2009). A limit is usually set for these terms to avoid calculating a regression based on spectral noise (Fontaine et al., 2001). Scans can also be subject to a detrending scatter correction (van Kempen and Bodin, 1998). Detrending mainly serves to reduce the variation in curvilinearity within spectra by reducing the variation in spectral shapes (Barnes et al., 1989). Finally, the spectral data can be transformed according to the first and second derivatives. Transformation of spectral data will sometimes improve the fit of the data for a regression analysis.

There are a variety of statistical methods applied throughout the literature for determining the accuracy of calibration predictions. The simplest is an R-square value that indicates the degree of relationship between the wet chemistry and NIRS concentration values of the tested substance. A MPLS algorithm can be applied to first and second derivatives of the spectra (Losada et al., 2009). The fractions of explained variance of cross validation should be in agreement with the R-squared value of calibration for all samples. Furthermore, the standard error of the cross validation and standard error of the calibration should be similar and minimized (Fontaine et al., 2002; Valdes et al., 1985a). Dividing the standard deviation by the standard error of the cross validation or the standard error of prediction was also found to be a meaningful statistic for NIRS predictions. A ratio over 3 would indicate that the calibration equation is very useful while ratios under 2 indicate that there is limited applicability to the given equation (Fontaine et al., 2002; Losada et al., 2009).

The 1-variance ratio statistic can also be used to explain how much of the feed ingredient variation is explained by the calibration equation, in accordance with the R^2 value. A positive

value would indicate that NIRS predictions from cross validation are more useful than using the average of analyzed values to predict sample component values (Tahir et al., 2012). Finally, in order for NIRS predictions to have the same confidence level as the wet chemical method, the standard deviations would need to be at least three-times the standard error of cross validation (Smith et al., 2001).

NIRS Application in animal diets

Animal nutritionists face several issues related to ingredient resources when formulating diets for commercial animal production. Considerable variation exists in the nutrient composition of a given ingredient. For example, a plant derived ingredient can vary based on such things as the cultivar variety, the agronomic conditions during the production of the crop, harvesting conditions, storage protocol, and differences in further processing procedures (Bastianelli, 2013). Before using an alternative or novel raw ingredient in an animal diet its nutrient composition and nutrient availability must be determined. Wet chemistry and animal bioassays procedures exist and are used to evaluate feed ingredients. However, these procedures are costly, time consuming, and create waste products (Fontaine et al., 2001). Thus, using wet chemistry and animal bioassays, is not plausible for the evaluation of individual batches of feed ingredient despite the possibility for considerable variation in composition (Tahir et al., 2012). Because of the lack of known nutrient values on individual batches of ingredients, nutritionists formulate diets using best estimates of the nutrient values of ingredients established based on previous research. Additionally, to avoid a potential decrease in animal performance conservative estimates of the nutrients in feedstuffs are used. Therefore, the vast majority of formulated diets have an excess of nutrients needed to meet requirements which decreases

profitability and contributes to animal feces having higher levels of nutrients which can cause environmental concerns such as algal blooms and ground water contamination.

The use of NIRS has the potential to allow animal nutritionists to formulate diets based on known nutrient specifications of their ingredients and help reduce the need for over formulation (van Kempen and Simmins, 1997; Yegani and Korver, 2012). Fortunately, many components of feed absorb in the near infrared reflectance region of the electromagnetic spectrum (Valdes and Leeson, 1992a). The most useful region has been cited as being from 1200 to 2500 nm (Norris, 1989). Unlike other analysis techniques, with NIRS no reagents are used and no waste products are created apart from dust during sample preparation (Bakalli et al., 2000). Furthermore, NIRS does not require special laboratory technician training after calibrations have been developed (Moughan et al., 2000). Samples can be analyzed in a matter of minutes (Yegani and Korver, 2012), only 4 to 5 grams of sample are required for analysis, and this sample can be recovered easily and used in the diet after the analysis is performed. Additionally, a sample can undergo analysis for multiple nutrient components simultaneously (Fontaine et al., 2001). With NIRS, analysis of individual batches is entirely possible as they are delivered to a feed mill. Finally, after the initial cost of purchasing a NIRS spectrometer, the cost to perform a sample analysis is extremely low (Bastianelli, 2013).

Although NIRS analysis will not be able to reduce the variability in nutrient composition, it will be able to reduce the estimation error for the component value of a batch of ingredient (Bodin and Aubret, 2005). Knowing the true specifications of ingredients at the time of purchase can give the nutritionist more power over the purchasing contract and decrease the incidences of unfair transactions (Givens et al., 1997).

For NIRS to accurately predict a component value of a feed sample it must be calibrated to do so by analyzing ingredients with known chemical composition. Calibrations can be made for a single feed ingredient, mixed feeds, or using several different feed ingredients. Once the initial calibration and regression equation has been produced, an equivalent number of independent samples should be used to validate the prediction equation. NIRS has already proven very effective in predicting dry matter, crude protein and fat content in feed samples such as wheat (Garnsworthy et al., 2000). Correlation coefficients for protein calibration found in the literature have ranged from 0.91 to 0.99 (Valdes et al., 1985b; Aureli et al., 2017). When comparing NIRS techniques to nitrogen-based regression analysis to predict protein concentrations, it was found that NIRS calibrations were able to explain 21-58% more variation within a feedstuff category (van Kempen and Bodin, 1998). Kryeziu et al. (2007) also reported that there was no significant difference between standard wet chemical analysis results and standardized calibrations (correlations of 0.89-0.99).

While NIRS is proving very effective at predicting nutrient components of feed ingredients and diets as reviewed by Rahman et al. (2015), the use of NIRS to predict more complex parameters such as nutrient digestibility or metabolizable energy is not well developed. Obtaining digestible amino acid values and metabolizable energy values involve animal bioassays which are expensive and completed by relatively few research laboratories. Furthermore, the laboratories completing these analyses on a routine basis have not been involved in NIRS research. Thus, the production of reliable wet chemistry/bioassay values for nutrient digestibility and metabolizable energy has not been correlated with the production of accurate NIRS calibration curves.

Although there are a great number of advantages to NIRS for analyzing chemical composition of feed ingredients and feeds, there are a couple disadvantages. One is the cost of NIRS systems that are suitable for high quality feed analyses, as the cost exceeds $100,000 which precludes their wide spread distribution and use. Second is the significant problem that has been encountered in transferring calibrations and data from one NIRS system to another. Optic differences between instruments of different brands and even within brands, and laboratory conditions and procedures utilized for each individual NIRS system makes it very difficult to successfully electronically transfer calibrations from one machine to the next (Givens et al., 1997). Furthermore, calibrations based on components determined through in vivo analysis are extremely hard to transfer and reproduce between laboratories because so much of a calibration's accuracy depends on the in vivo reference provided (Bastianelli, 2013). At this point, calibration curves are typically not electronically transferred. Instead, what occurs is all of the calibration and validation samples used for a calibration in one NIRS system are saved and then all of these samples will be transported to a new NIRS system and scanned followed by the input of the corresponding wet chemistry values to create a new calibration and validation.

In summary, NIRS technology offers great promise in the accurate analysis of nutrients in feed ingredients and diets. NIRS spectral analyses are heavily dependent on sample preparation and storage. Thus, creating and validating a high quality NIRS prediction curve is dependent on the uniformity of NIRS procedures as well as the quality and uniformity of the corresponding wet chemical and/or animal bioassay analyses.

CHAPTER 3

STATEMENT OF PURPOSE

Formulating properly balanced poultry diets that best meet the nutrient needs of the birds is only possible with the most accurate information about the nutritive values of the feed ingredients used to make the diets. Improperly balanced diets lead to poor animal production, increased environmental waste, and economic inefficiency especially given that feed accounts for roughly 70% of poultry production costs (Yegani and Korver, 2012).

Although dietary energy is not a nutrient, it provides the fuel for maintenance, growth and production. One of the best measures of the available energy for feed ingredients and diets for poultry is the TME_N. Unfortunately, the amount of time and resources that are required to perform this analysis through an animal bioassay and subsequent laboratory wet chemistry analyses is significant. Turn-around times for TME_N analyses typically exceed a week. The cost of each TME_N analysis is significant due to the cost of housing animals, training staff, and the laboratory equipment needed for sample processing and analyses.

NIRS is an analysis method with a wide array of uses in the feed industry. It has been previously used to create calibrations for determining parameters such as protein, fat, vitamin, mineral, amino acid and dry matter content in diets and feed ingredients. Once a NIRS calibration has been developed and validated, determinations of the parameter of interest in subsequent samples is fast, involves no chemicals and creates no waste. Thus, if a NIRS system is set up at a feed mill all incoming ingredients can be tested to establish its nutrient specifications and the formulation of more precise diets.

Currently there is not a NIRS calibration for TME_N. Therefore, the goal of this research is to create and validate a NIRS calibration for TME_N.

CHAPTER 4

MATERIALS AND METHODS

TME_N Bioassay

All animal procedures were approved by the University of Georgia Animal Care and Use Committee. Four hundred day of hatch Bovans White male chicks obtained from Centurion Poultry (Lexington, GA) were raised in floor pens (7.31 by 6.14 meters) equipped with 4 plastic pan feeders (0.14 m^2) and 2 water lines each containing 20 nipples. The cockerels were reared following breeder guidelines except that they were maintained on 8 hours of light (2 lux) from 2 to 20 weeks of age. Feed and water were provided ad libitum throughout rearing. All diets were formulated to meet or exceed NRC (1994) requirements.

When the birds reached 20 weeks of age, 192 roosters were transferred to individual wire battery cages which were 33.02 cm wide, 45.72 cm deep and 45.72 cm tall in the front and 40.64 cm tall in the back because of the sloped floor of the cages. Each battery contains 48 individual cages and there were two batteries in each of 2 rooms (each room had 96 roosters). Each room measures 7.31 by 6.14 meters. Each individual cage has a nipple drinker and access to a galvanized steel feeder trough. The birds had free access to water and a nutritionally complete diet when not being utilized for TME_N determinations.

For the TME_N procedure adult roosters between 22 and 80 weeks of age were utilized from the battery cages. For each feed ingredient or diet tested, 8 to 10 roosters were food fasted for 28 to 30 hours. Fasted roosters were then moved to another room (7.31 by 6.14 meters) which was equipped with 40 individual suspended wire cages that measured 30.48 cm wide,

45.72 cm long and 50.8 cm tall. Each cage was equipped with a nipple drinker to provide each rooster free access to water. Each cage was also equipped with a stainless steel feces collection pan. Each rooster was tube fed 35 grams of the ground test feed ingredient or diet. If the feed ingredient was fluffy and low density, such as rice hulls, less than 35 grams was fed to prevent crop impaction. After tube feeding the bird was then placed back in to a cage and excreta from each individual bird was then collect for the next 48 hours. An additional 10 un-fed roosters served as endogenous controls and had their individual feces collected for 48 hours as well. The fed and endogenous control roosters had free access to water, but had no access to feed during the 48 hour feces collection period. At the end of the feces collection period the roosters were returned to their original battery cages with free access of both feed and water. Feces were scraped from each individual collection pan and dried for subsequent analyses. Individual roosters are not utilized for the TME_N procedure more than once in a 3 week period. Once the roosters reach 80 weeks of age they will be euthanized and replaced in the battery cages by a new cohort of 20 week old roosters.

The rearing room, the 2 rooms containing the battery cages, and the TME_N feeding room, is each equipped with its own separate computerized controller that controls the temperature and ventilation of the room. Each room is equipped with a gas fired furnace and there is an evaporative cooling system for intake air that is brought in at the front of each room. Air is cleared from the end of each room by one 61 cm inch fan.

Chemical Reference Methods

Total nitrogen and gross energy were determined on all feed ingredients fed to the roosters and on the collected feces from the fed and endogenous control roosters. These analyses were completed by the University of Georgia Agricultural and Environmental Services

Laboratory (Athens,GA). A Parr 6400 Calorimeter was used for gross energy determination following the manufacturer's protocol (specifically, the ASTM D4868 and ISO 1928:2009 methods were employed). Total nitrogen was assessed using the Dumas method referred to by the AOAC (2006) as method number 968.06.

TME_N Calculation

Once the gross energy and nitrogen content of the feed ingredients and feces are known the values can be utilized to calculate the TME_N of the test ingredient using the following equation formulated by Sibbald (1976) and modified by Dale and Fuller (1984):

> {Gross energy of the feed on a gram basis multiplied by the grams fed – [(Gross energy of the excreta on a gram basis multiplied by the excreta weight in grams) – 8.73 multiplied by the (grams of excreta multiplied by the nitrogen content of the excreta) – (grams of feed multiplied by the nitrogen content in the feed)] – [(grams of endogenous control excreta multiplied by the endogenous control excreta gross energy on a gram basis – (grams of endogenous control excreta multiplied by the endogenous control nitrogen multiplied by 8.73] this is all divided by the grams of feed fed. Then this result is multiplied by 1000 to go from kilocalories energy per gram of diet to kilocalories energy per kilogram of diet.

The factor of 8.73 reflects the correction factor for voided nitrogen that is mostly uric acid (Titus et al., 1959).

NIRS Sample Selection

In addition to making a NIRS calibration curve for TME_N, 2 additional calibration curves were made for nitrogen and gross energy content since these components were measured in the wet chemistry procedures to calculate the TME_N values. Samples selected for inclusion in the

calibration curves were free of signs of molding and degradation. The reference values used for the calibration curves were based on an 'as is' basis because this is a better representation of the ingredients that nutritionists will have ready access to (Edney et al., 1994). The number of samples used for calibration differed between the nitrogen, gross energy, and TME_N curves due to the elimination of outliers. A variety of common and uncommon feed ingredients were included in each curve to represent the widest range in feed ingredient parameters possible. A description of the samples used in the nitrogen, gross energy and TME_N calibrations can be found in Tables 4.1, 4.2, and 4.3, respectively. A summary of the samples used for the validation of the nitrogen, gross energy, and TME_N calibrations are found in Tables 4.4, 4.5, and 4.6, respectively.

Table 4.1. Nitrogen Calibration Curve Sample Descriptions

Ingredient Type	Number of Samples	Mean Nitrogen Concentration (%)	Minimum Nitrogen Concentration (%)	Maximum Nitrogen Concentration (%)
DDGS	13	4.23	3.28	4.68
Corn	5	1.17	1.01	1.34
MBM	5	9.64	7.53	12.19
SBM	6	7.48	6.98	7.72
Wheat	1	2.18	---	---
Specialty[1]	20	4.26	0.74	9.35
Complete Diet[2]	5	3.01	2.79	3.28

[1] Specialty ingredients include: DDGS Novozymes A, DDGS Novozymes C, Mill Feed, Sorghum, Dried Grape Pomace, Sugarbeet Root Powder, Organic Wheat, Organic Sunflower Meal, Moringa Powder, Diet Novozymes A, Novozymes Diet Novozymes C, Expeller Pressed Canola, Cottonseed Meal, Organic SBM, Brazil Nut Powder, Rice Protein, Bakery meal

[2]Includes: 2 broiler withdrawal diets and 3 broiler finisher diets

Table 4.2. Gross Energy Calibration Curve Sample Descriptions

Ingredient	Number of Samples	Mean Gross Energy (Kcal/kg)	Minimum Gross Energy (Kcal/kg)	Maximum Gross Energy (Kcal/kg)
DDGS	13	4570	4394	4863
Corn	5	3816	3746	3892
SBM	6	4446	4180	4896
Wheat	1	3993	---	---
Specialty[1]	16	4339	3578	5215
MBM	4	4612	4093	5323
Complete Diet[2]	5	4115	3943	4229

[1]Specialty Ingredients Include: Sugarbeet Root Powder, Mill Feed, Sorghum, Moringa powder, Organic Wheat, Bakery Meal, Brazil Nut Powder, Organic SBM, Bakery Meal, Organic Sunflower Meal, Expeller Pressed Canola Oil, Rice Protein, DDGS Novozymes A, DDGS Novozymes C

[2]Includes: 3 broiler finisher diets and 2 broiler withdrawal diets

Table 4.3. TME_N Calibration Curve Sample Descriptions

Ingredient	Number of Samples	Mean TME_N (Kcal/kg)	Minimum TME_N (Kcal/kg)	Maximum TME_N (Kcal/kg)
DDGS	12	2506	2303	2717
Corn	5	3320	3284	3382
SBM	5	2909	2453	3267
Bakery Meal	5	3549	3350	3832
Specialty[1]	16	2421	1174	3443
MBM	5	3356	2673	4523
Complete Diet[2]	5	2896	2596	3218

[1] Specialty Ingredients Include: Alfalfa Meal, Mill Feed, Dried Grape Pomace, Cottonseed Meal, Spinach Powder Extract, DDGS Novozymes B, Canola, Sunflower Meal, DDGS Novozymes D, Diet Novozymes D, Diet Novozymes B, Sorghum

[2]Includes: 2 boiler finisher diets and 3 broiler withdrawal diets

Table 4.4. Nitrogen Validation Curve Sample Descriptions

Ingredient Type	Number of Samples	Mean Nitrogen Concentration (%)	Minimum Nitrogen Concentration (%)	Maximum Nitrogen Concentration (%)
DDGS	12	4.25	3.99	4.51
Corn	5	1.19	1.09	1.39
MBM	5	9.80	7.60	10.99
SBM	5	7.50	7.29	7.61
Wheat	1	2.57	---	---
Specialty[1]	16	3.46	0.33	6.91
Complete Diet[2]	5	2.91	2.70	3.10

[1] Specialty ingredients include: Ground Hulls, Novozymes DDGS D, Novozymes DDGS B, Organic Corn, Dried Bakery Meal, Alfalfa Meal, Organic Wheat Midds, Pea Meal, Sunflower Meal, Diet Novozymes B, Diet Novozymes D, Spinach Powder Extract, Canola, Black Soldier Fly Larva, Bakery Meal
[2]Includes: 3 broiler withdrawal diets and 2 broiler finisher diets

Table 4.5. Gross Energy Validation Curve Sample Descriptions

Ingredient	Number of Samples	Mean Gross Energy (Kcal/kg)	Minimum Gross Energy (Kcal/kg)	Maximum Gross Energy (Kcal/kg)
DDGS	10	4531	4337	4641
Corn	5	3799	3760	3927
SBM	5	4401	4104	4868
Wheat	1	3959	---	---
Specialty[1]	17	4360	3483	5938
MBM	4	4915	4488	5353
Complete Diets[2]	5	3999	3809	4202

[1] Specialty Ingredients include: Ground Rice Hulls, Organic Corn, Pea Meal, Organic Wheat Midds, Alfalfa Meal, Cottonseed Meal, Bakery Meal, DDGS Novozymes D, DDGS Novozymes B, Canola, Sunflower Meal, Diet Novozymes B, Diet Novozymes D, Black Soldier Fly Larva

[2]Includes: 2 broiler finisher diets and 3 broiler withdraw diets

Table 4.6. TME_N Validation Curve Sample Descriptions

Ingredient	Number of Samples	Mean TME_N (Kcal/kg)	Minimum TME_N (Kcal/kg)	Maximum TME_N (Kcal/kg)
DDGS	11	2573	2414	2887
Corn	5	3323	3292	3392
SBM	4	2614	2423	2991
Bakery Meal	6	3481	3255	3659
Specialty[1]	12	2689	730	4726
MBM	4	3157	2944	3472
Complete Diet[2]	5	2921	2789	3396

[1] Specialty Ingredients Include: Ground Hulls, Moringa Powder, DDGS Novozymes C, DDGS Novozymes A, Brazil Nut Powder, Canola, Wheat, Pea Meal, Diet Novozymes C, Diet Novozymes A, Black Soldier Fly Larva
[2]Includes: 3 broiler finisher diets and 2 broiler withdrawal diets

NIRS Sample Preparation

Samples were prepared by grinding at 18,000 RPM with a Retsch Type ZM200 grinder (Haan, Germany) with a 1 mm sieve. The large surface area of the collection tray allows heat production to be minimized during grinding. After grinding each sample the grinder was cleaned thoroughly using a professional wet/dry vacuum (Rigid, model WD 14500, St Elyria, OH) and brushes and absorbent towels (Kimwipes, Kimberly-Clark, Roswell, GA) between each sample. Feed ingredients were passed through the grinder twice when the feed ingredient type was changed to ensure proper flushing. Ground samples were then labeled and put into freezer proof quart Ziploc bags (SC Johnson and Sons, Inc., Racine WI). Ingredient samples that had already been finely ground were not ground further to avoid excessive sample loss. The final fineness of the ground feed ingredients was less than 40 micrometers.

Before scanning, samples were allowed to equilibrate to room temperature. Each sample bag was mixed and shook vigorously before being placed into sample cups for analysis to ensure a homogenous sample. The NIRS sample cup was filled so that no light could be seen from the bottom. Samples were not packed into the cup, and were not recovered after the analysis was complete.

Bruker Multi-Purpose Analyzer

A Bruker MPA: FT-NIR Spectrometer (Bruker Optics, Billerica, MA) equipped with sample rotator to ensure homogenous mixing of the sample while it is scanned was used to perform the NIRS analysis for each sample. A background scan was done before the scan of the feed ingredient/diet occurred. The MPA system included OPUS Version 7.5 software which was used for constructing the calibration curves and validating each curve.

Samples used for calibration and validation were chosen from the same pool, but were completely independent from each other. Spectra for each curve were manually assigned to either the calibration set or the validation test set. When possible, a similar number of spectra for each type of feed ingredient were included in the calibration and validation sets. For example, if there were a total of 8 DDGS samples; 4 would be assigned to calibration and 4 to the validation set. Optimization software included with OPUS allowed the determination of the best data pre-processing treatments to use for each curve as well as the most appropriate wavelength regions to analyze.

Statistical Analysis

The accuracy of the NIRS calibrations was evaluated according to several statistical parameters. The R^2 value and the Root Mean Square Error of Prediction were initially used to evaluate the strength of the test set validation and the calibration curve. As a second measure of accuracy, the standard deviation of the reference values was divided by the Standard Error of Prediction. A value over 3 is considered a sign of a robust calibration (Fontaine et al., 2001; Bodin and Aubret, 2005). Finally, the standard error of calibration (SEC) for each equation was compare to the standard error of the reference methods. Separate statistical analyses using the OPUS software were completed for each of the three parameter curves.

CHAPTER 5

RESULTS AND DISCUSSION

Three separate curves were calibrated for gross energy, nitrogen, and nitrogen corrected true metabolizable energy. A test set of samples was used for validation of the calibration curves. Of the total number of samples roughly half of the samples were used for calibration and the other half for validation.

Nitrogen

For the nitrogen prediction curve, 55 samples were used for the calibration and 49 for the validation test set. The frequency region that would provide the most accurate prediction for the nitrogen spectra set was determined by the OPUS software to be 7513.9 to 4235.2 nm (Table 5.1). The OPUS software analysis also determined it was unnecessary to pre-process the spectral data with any mathematical treatment, so none was applied. The correlation coefficient between the nitrogen concentration values determined by wet chemistry and the corresponding NIRS predicted nitrogen content for the calibration curve was R^2= 0.9344 (Table 5.1). The calibration curve had a root-mean-standard error of the estimate (RMSEE) value of 0.77, and a standard error of the estimate (SEE) of 0.69 (Table 5.1). The RMSEE is an indication of the magnitude of the residuals between the spectra used for the calibration set and the initial calibration line that was fitted to them by OPUS. The SEE is determined by calculating the standard deviation of the differences between wet chemical values for samples in the calibration set and the corresponding NIRS predictions. Low SEE values indicate that the calibration equation was able to fit the data more effectively.

Table 5.1. Descriptive statistics of the NIRS calibrations for nitrogen gross energy and TME_N.

	Nitrogen (%)		Gross Energy (Kcal/kg)		TME_N (Kcal/kg)	
Parameter	Determined	NIRS	Determined	NIRS	Determined	NIRS
Number of samples (n)	55	55	50	50	53	53
Mean	4.66	4.66	4350	4350	2810	2811
Minimum Value	0.74	-0.01	3578	3651	1174	1335
Maximum Value	12.19	11.24	5323	5375	4523	4422
SD	2.78	2.68	417	410	615	604
SEE[1]	0.69		74.00		118.00	
R^2	0.93		0.97		0.96	
RMSEE[2]	0.77		82.60		131.00	
Frequency Regions (nm)[3]	7513.9 to 4235.2		7506.2 to 4242.9		6102.1 to 4242.9	
Data Pre-processing[4]	None		First Derivative		Vector Normalization (SNV)	

[1]Standard Error of the Estimate. [2]Root Mean Standard Error of the Estimate. [3]The NIR frequency regions of the spectra deemed most useful for predictions by the OPUS software. [4]The pre-processing technique applied to the spectra by the OPUS software before a calibration equation was made.

For the validation of the calibration curve, a correlation coefficient between the wet chemistry values of the test set and the corresponding NIRS predicted values was R^2= 0.94 (Figure 5.1) with a standard error of prediction (SEP) of 0.63 (Table 5.2). Like the SEE, the SEP is determined by calculating the standard deviation of the differences between the wet chemical values for the test set and the corresponding NIRS predictions. The residual predictive difference (RPD) between the wet chemistry values and the NIRS prediction equation was 4.14%, and the systematic error (bias) was determined to be 0.09 (Figure 5.1). Bias is a measure of constant systematic variation between the test set wet chemical values and values predicted by NIR. A large amount of bias would cause the majority of the values to be high or low by a constant amount.

Previous researchers have reported NIRS calibration curves for percent nitrogen content of poultry excreta with an R^2= 0.92; SEP = 0.91 (Smith et al., 1999) and an R^2 = 0.99; SEP = 0.19 (Smith et al., 2001). Dealdana et al. (1995) reported a R^2 value of 0.97 and a SEP of 0.71 when calibrating total nitrogen content of grassland samples. Bastianelli et al. (2010) reported a correlation coefficient of R^2= 0.95 with a SEP of 0.31 for a calibration curve of percent nitrogen content of poultry excreta. The nitrogen calibration R^2 of 0.94 obtained in the current research is in line of the values of the previous research. However, the value is a bit lower than some of the values form the previous research and this indicates that further improvements in the current calibration could be made by adding more samples. It should also be pointed out the feed ingredients and diets used in the current research were very varied in composition and type (plant and animal products) while the samples from the previous research were much more homogenous in nature. It is generally recommended that a robust NIRS calibration regression equation should have a correlation coefficient of at least 0.9 (Windham et al., 1989). A common

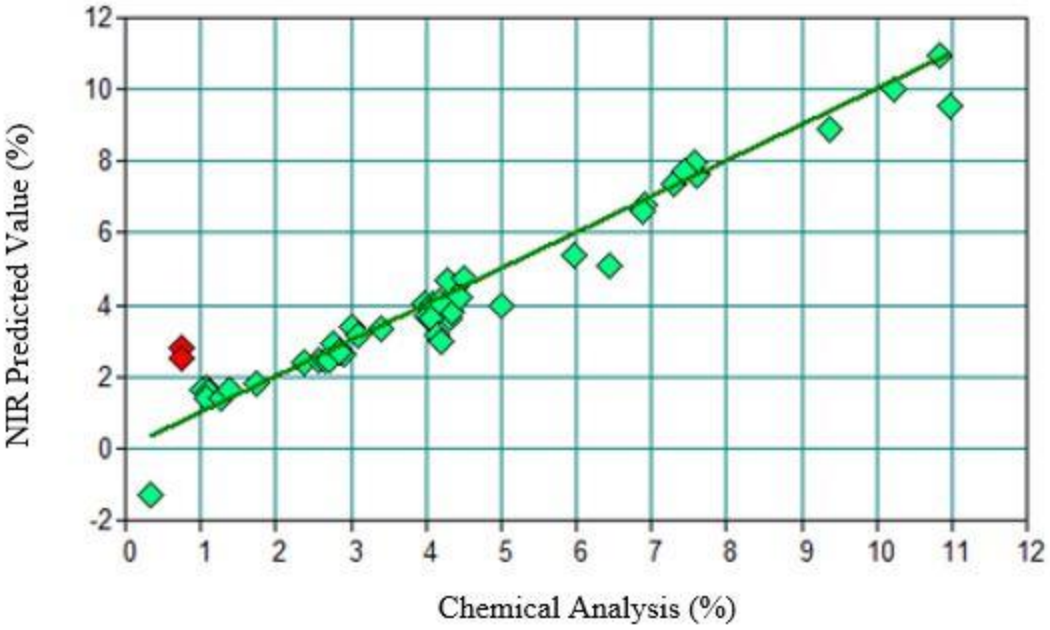

Figure 5.1. Validation Curve for Nitrogen (%). R^2= 0.94; RMSEP (Root mean standard error of prediction) = 0.66; RPD (residual predictive deviation) =4.14; Bias = 0.09; Green dots = values within bounds; Red dots = outliers

Table 5.2: Descriptive statistics of the NIRS validations for nitrogen gross energy and TME_N.

	Nitrogen (%)		Gross Energy (Kcal/kg)		TME_N (Kcal/kg)	
Parameter	Determined	NIRS	Determined	NIRS	Determined	NIRS
Number of samples (n)	49	49	47	47	48	48
Mean	4.41	4.32	4341	4334	2901	2863
Minimum Value	0.33	-1.28	3483	3604	730	590
Maximum Value	10.99	10.92	5938	6052	4726	4547
SD	2.74	2.61	524	517	601	584
SEP[1]	0.65		102.76		162.00	
R^2	0.94		0.96		0.92	
RMSEP[2]	0.66		103.00		166.00	
SD/SEP[3]	4.22		5.10		3.71	

[1]Standard Error of the Prediction. [2]Root Mean Standard Error of the Prediction. [3]The standard deviation of the wet chemical values divided by the standard error of the prediction. Values ≥ 3 are considered meaningful.

calculation used to evaluate the strength of a prediction equation is dividing the standard deviation of the reference values by the standard error of the prediction curve (Fontaine et al., 2001; Bodin and Aubret, 2005). For the nitrogen calibration curve, the calculated value for the SD/SEP was 4.22 (Table 5.2). A value of 3 or above is demonstrative of a meaningful calibration (Fontaine et al., 2001).

Gross Energy

For gross energy, 50 samples were used to establish the calibration curve and 47 were used for the validation test set. The frequency region that would provide the most accurate prediction for the gross energy spectra set was determined by the OPUS software to be 7506.2 to 4242.9 nm. The OPUS software recommended that a first derivative mathematical data pre-processing be implemented to improve the accuracy of the predictions and this was done. Previous research has also found that the first derivative mathematical treatment was the most accurate for this type of prediction (Valdes and Leeson, 1994). After pre-processing, the correlation coefficient between the calibration wet chemical values and the corresponding NIRS prediction values was R^2= 0.97 (Table 5.1). The RMSEE value for the calibration was 82.60 Kcal/kg (Table 5.1). Valdes and Leeson (1994) reported an SEE of 80.00 Kcal/kg when calibrating a gross energy curve for feed grade fats, which is higher than that of the currently calculated SEE of 74.00 Kcal/kg (Table 5.1). The RPD (residual predictive deviation) between test set chemical values and NIRS predictions was calculated to be 5.03 with a bias of 7.05 (Figure 5.2). The RPD represents the ratio of the reference value standard deviation and the mean error of the prediction. A value over 3 is indicative of a strong calibration (Williams and Sobering, 1995). The sample values used for the validation test set had a range of 3483 to 5939

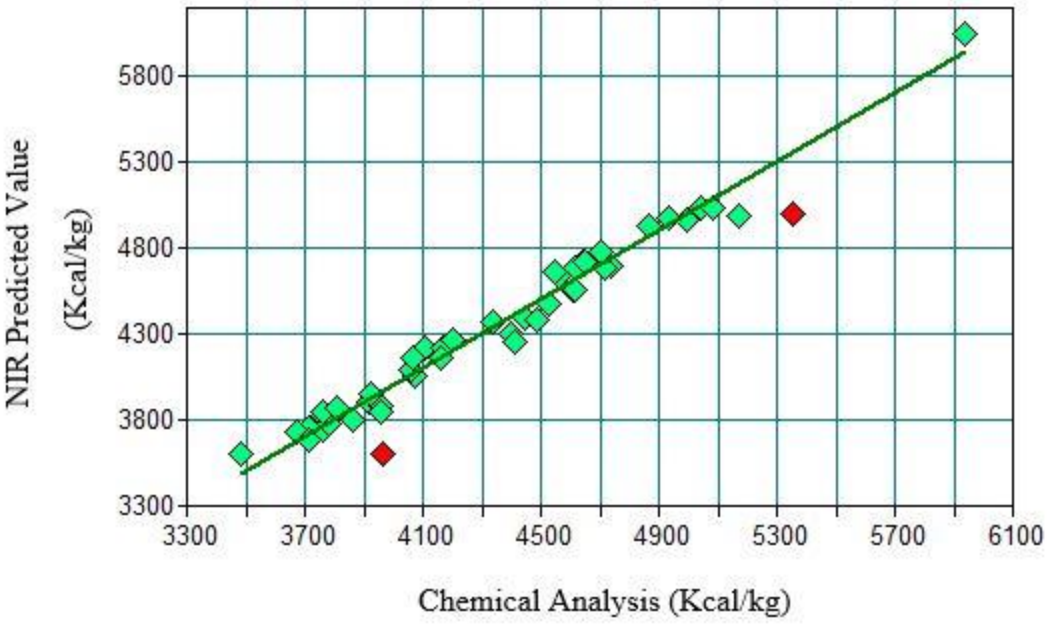

Figure 5.2: Validation Curve for Gross Energy (Kcal/kg). $R^2 = 0.96$; RMSEP (Root mean standard error of prediction) = 103; RPD (residual predictive difference) = 5.03; Bias = 7.05; Green dots = values within bounds; Red dots = outliers

Kcal/kg, and first derivative pre-processing was utilized once again. A correlation coefficient of R^2= 0.96 between the wet chemistry values for the validation set and the NIRS predicted values was achieved (Table 5.2) with a RMSEP of 103 Kcal/kg (SEP = 104 Kcal/kg). The RPD between the test set chemical values and the NIRS prediction values was calculated to be 5.03 with a bias of 7.05 (Figure 5.2).

Smith et al. reported a gross energy NIRS calibration curves for poultry excreta with an R^2 of 0.92; a SEP of 126 Kcal/kg (1999) and an R^2 of 0.88 and a SEP of 75 Kcal (2001). Bastianelli et al. also reported NIRS calibration curves for gross energy using poultry excreta samples and obtained the following values R^2= 0.97; SEP = 63.81 Kcal/kg (2007) and R^2= 0.99; standard error of cross validation (SECV, equivalent to SEP) = 0.14 (2010). Analysis of the gross energy content of compound rabbit food using NIRS resulted in a correlation coefficient of R^2= 0.92 and a SEP of 62.10 Kcal/kg (Xiccato et al., 1999). Losada et al. reported a correlation coefficient of R^2= 0.98 and a SECV of 61.70 Kcal/kg for a NIRS calibration of various grains commonly used in poultry feed (2009). Another NIRS gross energy calibration involving cereal food products reported an R^2= 0.99 with a SEP of 49.00 Kcal/kg (Kays and Barton, II, 2002). The R^2 and SEP values obtained in the current research were comparable to those obtained in the previous research and the fact that the SD/SEP value was 5.10 indicates that the NIRS prediction equation for gross energy was very meaningful (Table 5.2).

Nitrogen Corrected TME

For the NIRS analysis of TME_N, 53 samples were used for the calibration while 48 were used for the validation test set. The OPUS software determined that the most accurate spectral analysis should occur in the 6102.1 to 4242.9 nm frequency region, and that the standard vector normalization (SNV) data pre-treatment should be applied. With this mathematical

transformation the variation in slope at each wavelength is removed for each sample, thereby reducing scattering due to nonhomogenous particle sizes within a sample (Barnes et al., 1989). After this pretreatment, the correlation coefficient between the wet chemical values of the calibration set and the NIRS prediction values was R^2=0.96, with a RMSEE value of 131.00 Kcal/kg (Table 5.1). The same SNV data preprocessing was applied to the validation spectra. The correlation coefficient between the wet chemical values for the test set and NIRS values was R^2= 0.92, with a SEP value of 160.21 Kcal/kg (Table 5.2). The calculated RPD for the TME_N test set validation was 3.68 (Figure 5.3).

Previous research using NIRS to predict metabolizable energy has been very limited. Valdes and Leeson (1992) reported a NIRS calibration for AME_N in poultry diets with an R^2 of 0.92 and a SEE of 59.00 Kcal/kg. Losada et al. reported calibration statistics for AME_N of cereal grains of R^2= 0.82; SECV= 180 Kcal/kg (2009) and R^2= 0.95; SECV= 165.87 Kcal/kg (2010). Although nitrogen corrected TME NIRS prediction equations have not been completed in poultry previously there are two reports with TME NIRS calibrations. Edney et al., (1994) analyzed barley and obtained a NIRS calibration with R^2= 0.90 and a SECV of 50 Kcal/kg. Analysis of wheat for TME using NIRS using either young or adult birds for the bioassay resulted in an average calibration correlation of R^2= 0.63 and an average SECV of 56.13 Kcal/kg (Garnsworthy et al., 2000). In the curent research, even while utilizing a variety of ingredients instead of just one as in the prevous research and with adding the nitrogen correction which added more variation in the wet chemistry values given it is another analyis, the obtianed R^2 is better than the previous non nitrogen corrected TME calibrations. In addition the SD/SEP value of 3.71 for the current calibration indicates that it is highly meaningful and robust (Table 5.2). However, a close analysis of all the results from the samples used in the TME_N validation indicates that the

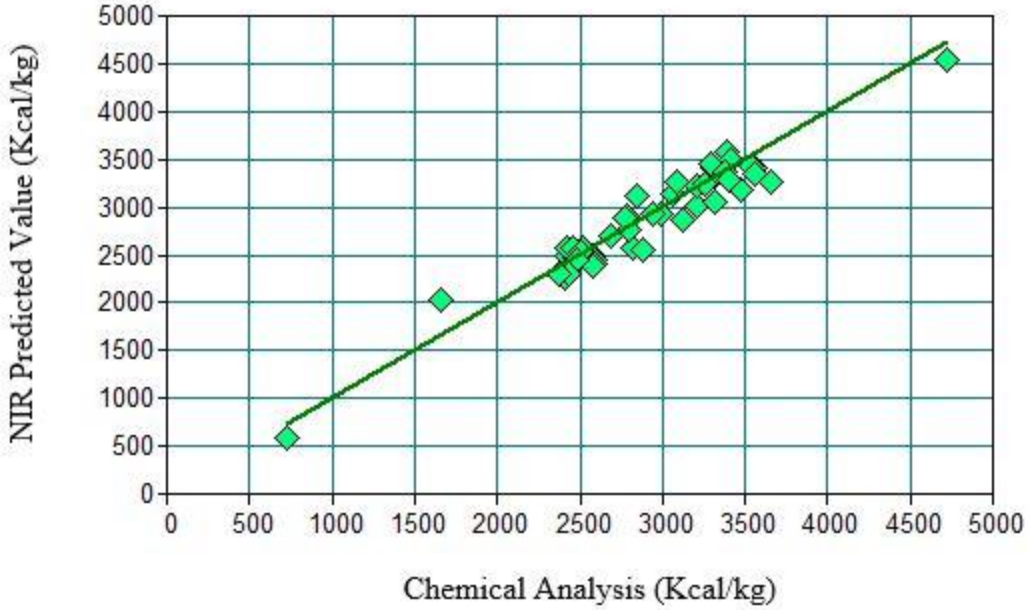

Figure 5.3: Validation Curve for TME_N (Kcal/kg). $R^2 = 0.92$; RMSEP (Root mean standard error of prediction) = 166; RPD (residual predictive difference) = 3.68; Bias = 37.6; Green dots = values within bounds

NIRS prediction underestimated all the TME_N values for bakery meal, while over estimating all the values for corn relative to the determined values (Table 5.3). This may suggest that making NIRS TME_N calibration curves specific for a given ingredient may improve the predictive strength of the calibration relative to wet chemistry determined values.

Table 5.3. Complete list of samples used for validation curves.

	Nitrogen (%)		Gross Energy (Kcal/kg)		TME_N (Kcal/kg)	
Sample Name, (Identification #)	Determined[1]	NIRS	Determined[2]	NIRS	Determined[3]	NIRS
Alfalfa Meal Organic (16.018)	2.38	2.38	3965	3608	1173	1335
Bakery Meal (17.024)	---	---	---	---	3255	3248
Bakery Meal (17.028)	---	---	---	---	3313	3046
Bakery Meal (17.030)	---	---	---	---	3557	3401
Bakery Meal (17.032)	---	---	---	---	3541	3432
Bakery Meal (17.034)	---	---	---	---	3559	3351
Bakery Meal Feed Commodities (16.001)	1.74	1.81	4401	4297	3659	3268
Black Soldier Fly Larva (16.012)	6.45	5.97	5938	6052	4727	4547
Brazil Nut Powder (16.022)	---	---	---	---	2466	2473
Canola Perdue (17.045)	5.98	5.40	4937	4981	---	---
Canola Perdue (17.046)	---	---	---	---	2769	2883
Corn ADM Composite (15.001)	1.10	1.55	3762	3819	3319	3384
Corn ADM Composite (15.003)	1.09	1.37	3784	3777	3300	3417
Corn Ayden Carolina Ag (15.015)	1.27	1.38	3760	3850	3292	3460
Corn Ayden William Warren (15.018)	1.39	1.66	3927	3950	3392	3582
Corn Organic (16.024)	1.01	1.61	3778	3803	---	---
Corn Univ. of Arkansas (16.010)	1.09	1.71	3764	3739	3314	3325
Cottonseed Meal (15.032)	---	---	4075	4061	---	---

[1] Total nitrogen was assessed using the Dumas method referred to by the AOAC (2006) as method number 968.06. [2] A Parr 6400 Calorimeter was used for gross energy determination following the manufacturer's protocol (specifically, the ASTM D4868 and ISO 1928:2009 methods were employed). [3] TME_N values of the test ingredient were calculated using the equation formulated by Sibbald (1976) and modified by Dale and Fuller (1984).

Table 5.3. Continued

	Nitrogen (%)		Gross Energy (Kcal/kg)		TME_N (Kcal/kg)	
Sample Name, (Identification #)	Determined[1]	NIRS	Determined[2]	NIRS	Determined[3]	NIRS
DDGS Andersons Greenville (16.090)	---	---	---	---	2481	2449
DDGS Bahl Control (15.062)	4.40	4.20	4548	4662	2514	2572
DDGS Cardinal Ethanol (16.069)	---	---	---	---	2581	2392
DDGS Direvo 1 (16.003)	---	---	---	---	2454	2579
DDGS Direvo 2 (16.004)	4.51	4.74	4610	4560	---	---
DDGS Direvo Control (14.023)	4.08	4.03	---	---	2514	2572
DDGS Direvo Treated (14.014)	4.34	3.80	4449	4397	---	---
DDGS Direvo Post Trial 3 (14.013)	---	---	---	---	2586	2401
DDGS Direvo Untreated (14.015)	---	---	---	---	2594	2439
DDGS Evonik at NC State (16.007)	3.99	4.01	4613	4684	3060	3131
DDGS Evonik 1 (15.045)	4.06	3.60	---	---	---	---
DDGS Evonik 2 (15.046)	---	---	---	---	2887	2558
DDGS Evonik 4 (15.047)	4.29	4.68	4337	4373	---	---
DDGS Mount Vernon (16.094)	4.3104	3.624	---	---	2503	2541
DDGS Novozymes A (16.030)	---	---	---	---	2473	2562
DDGS Novozymes B (16.031)	0.75	2.78	4738	4703	---	---

[1] Total nitrogen was assessed using the Dumas method referred to by the AOAC (2006) as method number 968.06. [2] A Parr 6400 Calorimeter was used for gross energy determination following the manufacturer's protocol (specifically, the ASTM D4868 and ISO 1928:2009 methods were employed). [3] TME_N values of the test ingredient were calculated using the equation formulated by Sibbald (1976) and modified by Dale and Fuller (1984).

Table 5.3. Continued

	Nitrogen (%)		Gross Energy (Kcal/kg)		TME_N (Kcal/kg)	
Sample Name, (Identification #)	Determined[1]	NIRS	Determined[2]	NIRS	Determined[3]	NIRS
DDGS Novozymes C (16.032)	---	---	---	---	2417	2568
DDGS Novozymes D (16.033)	0.75	2.50	4714	4688	---	---
DDGS Pilgrims Commonwealth (16.068)	4.46	4.24	4615	4559	2481	2449
DDGS Pilgrims Council Bluffs (16.091)	4.38	4.15	4558	4641	3659	3268
DDGS Pilgrims Flint Hills (16.072)	4.18	4.10	4530	4474	---	---
DDGS Pilgrims Guntersville (16.094)	4.31	3.62	4641	4720	3203	3006
DDGS Pilgrims Poet Alexandria (16.043)	---	---	---	---	2414	2261
DDGS Poet Fostoria (16.070)	---	---	---	---	2461	2375
DDGS Purdue Green Plains (16.042)	---	---	---	---	2826	2573
DDGS Purdue Poet Nutrition (16.043)	4.02	3.68	4412	4253	---	---
Diet Novozymes A (16.110)	---	---	---	---	3412	3487
Diet Novozymes B (16.111)	4.16	3.14	5039	5038	---	---
Diet Novozymes C (16.112)	---	---	---	---	3382	3378
Diet Novozymes D (16.113)	4.21	3.00	5083	5038	---	---
Diet Evonik Broiler Finisher 1 (16.100)	---	---	---	---	2789	2898
Diet Evonik Broiler Finisher 2 (16.101)	3.02	3.38	4202	4269	---	---

[1] Total nitrogen was assessed using the Dumas method referred to by the AOAC (2006) as method number 968.06. [2] A Parr 6400 Calorimeter was used for gross energy determination following the manufacturer's protocol (specifically, the ASTM D4868 and ISO 1928:2009 methods were employed). [3] TME_N values of the test ingredient were calculated using the equation formulated by Sibbald (1976) and modified by Dale and Fuller (1984).

Table 5.3. Continued

	Nitrogen (%)		Gross Energy (Kcal/kg)		TME_N (Kcal/kg)	
Sample Name, (Identification #)	Determined[1]	NIRS	Determined[2]	NIRS	Determined[3]	NIRS
Diet Evonik Broiler Finisher 3 (16.102)	---	---	---	---	2794	2753
Diet Evonik Broiler Finisher 4 (16.104)	3.10	3.18	3809	3874	---	---
Diet Evonik Broiler Finisher 5 (16.105)	---	---	---	---	3396	3276
Diet Evonik Broiler Withdraw (16.106)	2.70	2.47	3861	3801	---	---
Diet Evonik Broiler Withdraw (16.107)	---	---	---	---	2844	3115
Diet Evonik Broiler Withdraw (16.108)	2.89	2.66	4058	4093	---	---
Diet Evonik Broiler Withdraw (16.109)	---	---	---	---	3209	3223
Diet Evonik Broiler Withdraw (16.110)	2.84	2.70	4066	4160	---	---
Ground Rice Hulls Wayne Farms (16.028)	0.33	-1.28	3483	3604	---	---
MBM Darpro Kansas City (16.051)	10.24	10.03	---	---	3122	2860
MBM Darpro Omaha (16.053)	9.36	8.88	4647	4723	2944	2923
Moringa Powder (14.016)	---	---	---	---	1653	2036
Pea Meal (14.012)	2.80	2.91	3916	3906	2693	2707
Poultry By Product Jackson Chicken (15.044)	7.60	7.64	4488	4389	3091	3265

[1] Total nitrogen was assessed using the Dumas method referred to by the AOAC (2006) as method number 968.06. [2] A Parr 6400 Calorimeter was used for gross energy determination following the manufacturer's protocol (specifically, the ASTM D4868 and ISO 1928:2009 methods were employed). [3] TME_N values of the test ingredient were calculated using the equation formulated by Sibbald (1976) and modified by Dale and Fuller (1984).

Table 5.3. Continued

	Nitrogen (%)		Gross Energy (Kcal/kg)		TME_N (Kcal/kg)	
Sample Name, (Identification #)	Determined[1]	NIRS	Determined[2]	NIRS	Determined[3]	NIRS
Poultry Composite Simmons C-Viscera (14.018)	10.99	9.51	5353	4997	2944	2923
Poultry Composite Simmons PBM (14.020)	10.83	10.92	5170	4989	3472	3182
SBM Bahl (15.065)	7.29	7.38	4168	4215	730	590
SBM Perdue Salisbury (16.041)	7.57	7.96	4160	4157	2578	2498
SBM Perdue Williamette Biomass (16.039)	7.44	7.73	---	---	---	---
SBM Perdue XP Bergeweif (16.044)	7.57	7.93	4704	4783	2991	2933
SBM Univ. of Arkansas (16.011)	7.61	7.69	4104	4218	2423	2490
Sunflower Meal Organic (16.019)	3.41	3.32	4996	4965	---	---
Spinach Powder Extract (16.021)	5.00	4.00	---	---	---	---
Unknown Novozymes Treatment A (16.114)	---	---	3711	3759	---	---
Unknown Novozymes Treatment B (16.115)	---	---	---	---	2438	2303
Unknown Novozymes Treatment C (16.116)	6.91	6.78	3672	3734	---	---
Unknown Novozymes Treatment D (16.117)	6.910	6.78	---	---	2372	2304

[1] Total nitrogen was assessed using the Dumas method referred to by the AOAC (2006) as method number 968.06. [2] A Parr 6400 Calorimeter was used for gross energy determination following the manufacturer's protocol (specifically, the ASTM D4868 and ISO 1928:2009 methods were employed). [3] TME_N values of the test ingredient were calculated using the equation formulated by Sibbald (1976) and modified by Dale and Fuller (1984).

Table 5.3. Continued.

	Nitrogen (%)		Gross Energy (Kcal/kg)		TME_N (Kcal/kg)	
Sample Name, (Identification #)	Determined[1]	NIRS	Determined[2]	NIRS	Determined[5]	NIRS
Unkown Novozymes Treatment E (16.118)	6.88	6.60	3713	3684	---	---
Wheat Midds Organic 1 (16.017)	2.57	2.44	3959	3887	---	---
Wheat Midds Organic 2 (16.026)	2.65	2.46	3955	3848	---	---

[1] Total nitrogen was assessed using the Dumas method referred to by the AOAC (2006) as method number 968.06. [2] A Parr 6400 Calorimeter was used for gross energy determination following the manufacturer's protocol (specifically, the ASTM D4868 and ISO 1928:2009 methods were employed). [3] TME_N values of the test ingredient were calculated using the equation formulated by Sibbald (1976) and modified by Dale and Fuller (1984).

CHAPTER 6

CONCLUSION

The purpose of this research was to develop an accurate NIRS calibration curve for TME_N to reduce the need for animal bioassays in determining TME_N for feed ingredients and diets. Given that the TME_N value of a diet or feed ingredient is not a direct concentration value like other nutrient values such as the concentration of a particular amino acid or mineral, and is instead a reflection of the digestibility of multiple nutrients and an endogenous loss correction, creating a successful NIRS calibration seemed unlikely. Therefore, NIRS calibration curves for nitrogen and gross energy were also created for the analyzed feed ingredients and diets. If the nitrogen and gross energy of the feed ingredient/diet and that from the corresponding dried collected feces from the rooster bioassay could be determined accurately by NIRS, then at least the TME_N value of the feed ingredient or diet could still be calculated from NIRS scans of the feed ingredient/diet and feces. Using NIRS values for gross energy and nitrogen to calculate the TME_N would be easier than having time consuming and costly wet chemical analyses of the ingredient and feces completed. However, after analysis of the R^2, SEE, SEP and SD/SEP values of the TME_N calibration and validation, it was clear that the focus moving forward with this research could be on just improving the TME_N calibration which already had a robust capability.

Future Improvements

As research moves forward with the further development of the NIRS TME_N calibration there are a couple avenues to pursue to improve the calibration. The first objective is to at least

double the number of samples used in the calibration. A cause of innate error in a calibration, are the inherent errors that are dependent on the reference method (Yegani and Korver, 2012). The TME_N calibration is dependent on reference values that have variation due to rooster differences in the bioassay as well as potential analytical errors in the bomb calorimeter determination of gross energy and in the chemical determination of nitrogen content of the feed ingredients and feces. Simply increasing the number of samples analyzed will help decrease the influence of this innate error. Furthermore, in the current research the ingredients were only scanned once, and any potential analytical errors in the spectral scans would thus be more prominent (Fontaine et al. 2002). Therefore, in improving the calibration, each sample could be scanned multiple times and the spectral values averaged. At the very least, this rescanning approach needs to be completed with samples that deviate the most from the predicted calibration regression line.

The TME_N calibration and validation would also likely be improved by testing more low and high energy ingredients as most of the commonly used feed ingredients and formulated poultry diets fall in the 2300 to 3800 Kcal/kg range (Figure 5.3). More samples of rice hulls and oat hulls could be used to obtain TME_N values less than 1000 Kcal/kg and more samples of alfalfa meal, cottonseed meal and wheat bran could be used to obtain TME_N values in the 1000 to 2000 Kcal/kg range. Cereal grains such as corn and wheat and bakery meals typically have TME_N values above 3400 Kcal/kg, but lower than 3800 Kcal/kg. The cereal grains provide most of the energy in poultry diets and any extra energy is provided by vegetable oils or animal fats which have TME_N values typically in the 6000 to 9000 Kcal/kg range. Regretfully, oils and fats cannot be precision fed directly to roosters in the TME_N bioassay so they have to be mixed with corn (sample fed consists of 15% oil/fat and 85% ground corn) and the TME_N value has to be

calculated by difference from a pure corn sample. Because calculating the TME_N by difference introduces more error in the reference values and because oils as a liquid cannot be screened by NIRS in the same procedure as non liquid feed ingredients, they were not included in the current NIRS TME_N calibration or validation. Thus, the highest energy ingredient tested in the current research was ground black soldier fly larva meal. Black soldier fly larva is being investigated as a potential animal feed ingredient and it has a TME_N values above 4000 Kcal/kg. It is expected that we will have several more independent samples of black soldier fly larva to add to the TME_N calibration and validation.

In future research, the accuracy of the TME_N NIRS calibration may be improved by having multiple independent calibrations. Because of feed ingredient prices, the formulation of organic diets, and the use of feed ingredients for nutraceutical purposes many alternative feed ingredients are being test for potential use in poultry diets. For alternative feed ingredients that vary widely in their nutrient specifications, a TME_N calibration as constructed for the current research that includes a wide spectrum of TME_N values is most appropriate. However, for ingredients such as corn, soybean meal, dried distillers grains, and meat and bone meal the accuracy of the TME_N calibration might be better if it was ingredient specific, and this needs to be investigated in future research.

Implications

One of the most obvious implications for these results is that in the future the need for expensive and time consuming animal bioassays for determining TME_N could be reduced. One of the best measures of the available energy for feed ingredients and diets for poultry is the TME_N, currently poultry nutritionists formulate diets based on book values for TME_N. This is despite the fact that they know that the TME_N value of a plant derived ingredient can vary based

on such things as the cultivar variety, the agronomic conditions during the production of the crop, harvesting conditions, storage protocol and differences in further processing procedures. For feed ingredients such as animal by products, bakery by products and ethanol production by products, there is also considerable variation based on the starting materials used and what is blended together for the final product. To combat these known variations nutritionists use conservative nutrient values for ingredients, and thus diets typically have unnecessary nutrient excesses. In the future, a NIRS system installed at a feed mill equipped with a validated TME_N calibration would allow the TME_N to be determined on every batch of an ingredient coming in prior to feed formulation and feed mixing.

In addition to knowing the TME_N of feed ingredients, another critical piece of information that poultry nutritionists want for the ingredients they are using to formulate diets is the digestible amino acid levels. Similar to the TME_N determination, determining digestible amino acid levels involves an animal bioassay and correction for endogenous losses. Because the procedures are so similar the success of the NIRS TME_N calibration suggests that a NIRS calibration for digestible amino acid levels is also plausible.

In summary, despite the TME_N determination of feed ingredients involving an animal bioassay and multiple chemical analyses, the advancements in NIRS technology allowed a suitable NIRS calibration for TME_N determinations to be developed. Although the created NIRS TME_N calibration and validation were robust in their predictive power, there is potential for further improvement with subsequent research.

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