Validation of animal and laboratory methods in digestibility research

by

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ABSTRACT

Experimentation in research explores new ideas and helps gain new knowledge which includes treatment observations, laboratory analyses, statistical analyses, and interpretation of the data. The use of appropriate methodology plays an important role in high quality research as it helps yield accurate, reliable and repeatable data. The same components can be analyzed by multiple methods due to the accumulation of approved methods. In this thesis two methodologies have evaluated to make suggestions based on time, budget, and resource availability. Currently there are several accepted methods for the drying of animal excreta; however some methods have been reported to negatively impact the nutrient composition more than other methods. Therefore, one objective of this thesis was to evaluate nutrient and energy losses due to drying method on feces, urine, and poultry excreta. Twelve individually penned growing pigs were fed one of three diets and 16 pens of 10 growing broilers were fed one of four diets that differed in nutrient composition. Feces, urine, and excreta that varied in nutrient composition were collected after 7 d of diet adaptation. Samples were dried using one of four methods: UD-undried, FD-freeze dried, OD55-oven dried at 55°C for 48 h, or OD100-oven dried at 100°C for 48 h, after which DM, GE, N, C, and S were determined. There were no differences among drying methods except FD excreta had a greater S concentration than OD ($P < 0.10$). Covariates are auxiliary variables used in the statistical covariance analysis to limit variation for accurately calculating the least squares means.

Digestibility research has shown limited use of covariates because the benefits are unknown. Therefore, the second objective of this thesis was to determine an appropriate covariate for digestibility experiments to limit variation that may affect treatment observations. Seventy two barrows were randomly assigned to one of three dietary treatments for Phase-1 (P1;

14 d). On d-14, pigs were randomly reassigned to one of the three diets within initial dietary treatment for Phase-2 (P2; 14 d). Fecal and blood samples were collected on d-10 or d-14 of P1 and d-14 P2. Fecal samples were dried and analyzed for C, ether extract, GE, N, NDF, P, and S. Plasma samples were analyzed for plasma urea nitrogen and triglycerides. When P1 criterion was used as a covariate for P2 data, it was significant for C, ether extract, GE, NDF, N, S, and plasma urea nitrogen ($P < 0.10$). In conclusion, the data indicate that if initial diets are known, one must balance subsequent treatments for the initial diet because of potential diet by diet interactions. If the initial diets are not known, then an initial criterion would be effective in reducing the variation associated with subsequently obtain data, and should be considered as a covariate in future nutrition research. Therefore, the methods utilized and processed are important for yielding accurate and precise data.

Key words: Methodology, drying methods, covariance analysis, digestibility, poultry, pigs

CHAPTER ONE

General Introduction

THESIS ORGANIZATION

The following thesis is organized into 5 chapters. Chapter 1 is a review of the importance of research, description of two methodologies used in swine and poultry nutrition research, and the problems associated with those methodologies. Chapter 2 is a summary of research conducted, which evaluated the impact of drying methods on fecal and urine samples of pigs, and poultry excreta. Chapter 3 is a summary of research conducted evaluating the ability of reducing experimental error in nutrition research with grow-finish pigs with the use of a covariate. Chapter 4 presents the general conclusions about the research conducted. Chapter 5 contains the acknowledgements.

Review of Literature

INTRODUCTION

Research explores new ideas and helps gain new knowledge through experimentation and reviews of the literature. In this context, experimentation includes treatment observations, laboratory analyses, statistical analyses, and interpretation of the data. The use of appropriate methodology plays an important role in high quality research as it helps yield accurate, reliable and repeatable data. Previous research and associated explanations contribute to, and therefore influence, the direction of today's research (Rajasekar et al., 2006).

In nutrition research, objectives are assigned which use proper methodologies and analysis of data to ensure accurate and precise results. According to Baker (1986), the use of correct procedures creates valid, credible, and accurate data. Thiex and Richardson (2003) reported a direct and negative influence on predicting performance when diet content has not

been properly analyzed. Baker (1986) reviewed and addressed problems and pitfalls in animal research due to inaccurate information being established for nutrient requirements; his paper emphasized that the use of appropriate experimental methodology enhances the quality of the data produced. Animal research methods in use at the present time have been improved over time and are currently being used to develop a better understanding of nutrition, physiology, meat quality, and genetics.

The same components can be analyzed by multiple methods. For example, moisture content of feed or feces can be measured many ways; Thiex and Richardson (2003) compared numerous methods and selected the 'Karl Fisher' procedure as the method which gave the most accurate results.

Nitrogen can also be assayed using various methods. The Kjeldahl procedure isolates nitrogen from other sample constituents by converting nitrogen to ammonia. The Dumas method decomposes organic material by combustion under pressure (AOAC, 1999). It is often the case that more than one approved method exists in the literature for measuring a particular parameter (for example digestibility), and several factors can influence methodology selection including budget, resource availability, research objectives, and the animal model.

Drying Methods

In nutrition and balance studies, feed, fecal, urine, excreta, and blood may be analyzed to evaluate the digestibility of feed (Just et al., 1982; van Kempen et al., 2003; Kerr, 2010). In order to evaluate digestibility, feed, fecal, excreta, and blood samples, will have to be preserved in some manner prior to the laboratory analysis. However, there are factors that may affect the sample prior to or during collection, preservation, or laboratory analysis; these may include temperature, collection vessel, time, and oxygen. Once the samples have been analyzed for the

appropriate components, digestibility coefficients can be calculated to accurately estimate digestibility.

These components within the sample may be negatively affected if proper handling, preserving, and analyzing have not been used. For example, the method utilized for drying samples may have a negative impact on the nitrogen and energy content in feces, urine, and excreta (Figure 1, Manoukas, 1964; Mahimairaja et al. 1990; Shannon and Brown, 1969). Currently, there are several methods that can be employed for the drying of animal excreta. Some methods appear to negatively impact nutrient composition data more than others. Many different methods are used for drying feces, urine, or excreta, incorporating varied combinations of temperature and drying time. For example, Knudsen and Hansen (1991) dried at 105°C, Pedersen et al. (2007) forced air dried samples at 60°C, and Kerr et al. (2009) dried at 70°C for 48 h. There is uncertainty if it is low or high temperatures which negatively affect the sample components; swine feces have been dried at a low (60ºC; Stein et al., 2006) and high 95ºC, Goebel and Stein, 2011) temperature prior to analysis with successful abilities to analyzing for nutrients accurately.

Cattle Manure

In cattle studies, Gallup and Hopps (1944) reported a 7% reduction in fecal nitrogen as a result of drying at 60°C and 100°C. Similarly, Colovos et al. (1957) reported an 11.9% protein loss due to convection oven drying at 65°C and Bratzler and Swift (1959) reported a 5.15% loss in nitrogen when fresh feces were dried in a forced air oven for 22 h at 65°C. In contrast, Favely and Woolley (1974) found oven drying feces at 60°C for 48 h resulted in the greatest nitrogen loss (20%) compared to oven drying at 75°C, 80°C, and 100°C for 24 h (8-20%). Relative to gross energy losses, Colovos et al. (1957) reported that gross energy losses in cattle feces was

approximately 14% if dried at 65°C compared to undried feces, while Bratzler and Swift (1959) reported no loss of gross energy due to drying at 65°C. Although temperature has been reported to affect nutrient losses, moisture losses may also differ between different drying methods, and as such, can affect subsequent analytical results. Lawrence (1971) reported a 2% decrease in dry matter when samples were oven dried at 50 and 100°C with a 5% nitrogen loss compared to freeze dried samples.

Pig Feces

The impact of drying on gross energy or nitrogen content of swine feces has been largely overlooked, with methods largely adapted from the cattle or poultry literature. In reviewing the literature, fecal composition has been shown to be negatively affected by drying method. Differences in fecal nitrogen and gross energy concentrations between the oven dried and freeze dried samples appear to be most pronounced. Lawrence (1971) found that oven drying (50°C and 100°C) increased nitrogen losses (5%) when compared to freeze dried samples which could lead to digestibility overestimations. In contrast, gross energy has been reported to be 16% higher in oven dried samples (50°C and 100°C) compared to freeze dried samples (Lawrence, 1971). Likewise, Mahimairaja et al. (1990) reported significant reductions in total nitrogen (12%) from pig slurry samples when oven dried at 105°C but freeze drying had no effect on the total nitrogen concentration when compared to undried manure.

Pig Urine

As previously stated, nutrients in animal excreta may be affected by the drying process; however in reviewing the literature, there seems to be no research that evaluates the impact of drying method on the components in the urine. This is important not only because nitrogen excretion routes change as diets differ in crude protein (Gatel and Grosjean, 1992; Kerr and

Easter, 1995) and fiber (Zervas and Zijlstra, 2002; Shriver et al., 2003), but also due to differing non-starch polysaccharide content in the diet (Canh et al., 1998). High dietary protein increases urinary nitrogen excretion which then increases energy excretion (Holmes et al., 1980; Newport, 1979). Morgan and Whittemore (1988) showed fiber to increase the urea which is secreted from the blood to the large intestine as ammonia and is excreted in the feces. Ammonia is released during the conversion of urea to ammonia due to microbial urease present in feces (Canh et al., 1997; van Kempen et al., 2003); therefore, nutrient balance experiments typically involve acidification of the urine to prevent volatilization of nitrogen (Pederson et al., 2007). Potential nitrogen losses can occur from the time of collection until the sample reaches the laboratory without addition of acid. Urine that has been contaminated with fecal material can result in a 13%, 26%, and 68%, urea nitrogen loss at room temperature (23°C), when exposed to air for 8 h, 12 h, and 24 h, respectively (van Kempen et al., 2003). It is clear that considerable variation exists in previous literature due to the differences in such nutrient losses.

Chicken Excreta

In poultry, several sources (Manoukas et al.,1964; Shannon and Brown, 1969; and Mahimairaja et al.,1990) have reported that oven drying resulted in greater nitrogen and gross energy losses compared to freeze drying. In some cases, freeze drying was equal to the undried samples (Figure 1). Manoukas et al. (1964) demonstrated that oven drying poultry excreta at 65°C resulted in energy and nitrogen losses of 11.4% and 5.2%, respectively, compared to fresh, undried excreta. Relative to drying temperature, Shannon and Brown (1969) reported that energy losses were lowest (1.3%) for freeze dried excreta, while losses of energy decreased with increasing oven temperatures (5.5% at 60°C, 3.3% at 100°C, and 2.8% at 120°C) compared to undried excreta. For nitrogen, freeze drying or oven drying at 60°C resulted in a loss of

approximately 4.7%, while increasing the temperature to 100°C and 120°C increased losses to 7.8% and 10.5%, respectively. Ribeiro et al. (2001) reported that when compared to undried excreta, increasing oven temperature from 55°C to 100°C increased nitrogen losses from 8.3% to 13.2%, respectively. Wallis and Balnave (1983) reported greater energy and nitrogen losses when excreta were freeze dried compared to oven dried at either 60°C or 80°C. Blake and Potter (1987) reported that increasing drying temperatures (40-175°C) for 24 h resulted in decreased dry matter percentage, but when expressed on a fresh weight basis, there were no apparent effects on nitrogen or gross energy losses up to 100°C to 120°C. However the authors also reported that above 120°C (140°C and 175°C), both nitrogen and gross energy content were negatively affected by about 11% (Blake and Potter, 1987). Blake and Potter (1987) also found all water to be removed when poultry excreta was dried at 100°C or above and resulted in no significant nitrogen losses. Giddens and Rao (1975) indicated drying manure at a lower temperature (23ºC for 10 d) tended to cause a greater loss in nitrogen versus fast drying at a higher temperature (100ºC for 8 h; 47.6% and 23.6%, respectively). Pan et al. (2009) reported no impact on total nitrogen of acidifying poultry excreta compared to fresh excreta, indicating that once in the lab and properly stored, little nitrogen is lost during the course of laboratory procedures. In contrast, Ribeiro et al. (2001) reported a 10% nitrogen loss from broiler excreta when it was not acidified to pH 4.5 prior to oven drying at 55°C or 100°C for an unspecified amount of time. Hartfiel (1961) evaluated the impact of drying method, reporting that freeze drying and oven drying of urine had little to no effect on energy concentration. Dale et al. (1985) found no significant differences in the true metabolizable energy nitrogen corrected (TME_n) when poultry excreta samples were freeze dried or oven dried at 60°C. Sibbald (1979) reported no difference in TME_n between the freeze dried or oven dried excreta. However, Jorgensen et al.

(1984) reported that method of fecal preparation for nitrogen analysis (undried, freeze dried, or oven dried at 70°C) did not affect protein digestibility measurements.

Figure 1. Effects of drying method on nitrogen and energy losses in poultry

Loss compared to undried sample		
Nitrogen, %	**Energy, %**	**Reference**
4.7 (OD 60°C)	5.5 (OD 60°C)	Shannon and Brown, 1969
7.8 (OD 100°C)	3.3 (OD 100°C)	Shannon and Brown, 1969
8.3 (OD 55°C)	ND	Ribeiro et al., 2001
10.5 (OD 120°C)	1.2 (OD 120°C)	Shannon and Brown, 1969
11.4 (OD 65°C)	5.2 (OD 65°C)	Manoukas et al., 1964
13.2 (OD 100°C)	ND	Ribeiro et al., 2001
4.7 (FD)	1.3 (FD)	Shannon and Brown, 1969
OD = oven dried, FD = freeze dried		

Consequently, if the drying method has an effect on sample composition, the accuracy of nutrient balance is reduced, making interpretation of the results biased relative to the degree of nutrient loss. As there is no consensus regarding the impact of drying methods on energy and nitrogen losses, additional information is needed to improve the interpretation of data in future nutrient balance experiments.

Covariates

In addition to experimental design, the method chosen for statistical analysis of the data is a key component of successful research. In order to properly interpret data, the correct statistical model statement must be employed to achieve proper data analysis. An example of this would be the analysis of covariance procedure.

When analyzing data in nutrition studies, animal variation may result in inadvertent misinterpretation of results. Variation is inevitable in animal experimentation; for example,

analysis of covariance is often used to increase experiment precision in experiments evaluating body composition (Brown et al., 1985), feed intake (Smith et al., 2010), growth (Albuquerque and Meyer, 2001; Rikard-Bell et al., 2009), and meat quality (Lefaucheur et al., 2010). Others have reported using analysis of covariance to be an option for certain model traits in an animal's life such as age, weight, or genetics (Kirkpatrick et al., 1990 and Schaeffer and Dekkers, 1994).

The purpose of a covariate is to limit the degree of nuisance variation and the overparameterizing factors in order to accurately measure treatment effects (Meyer and Hill, 1997). The covariate chosen is usually an auxiliary variable (such as changes in body weight), which may influence the outcomes of interest but is not confounded by the experimental treatments being studied (Ramsey and Schafer, 2002). Analysis of covariance increases the precision and decreases the error resulting from animal-to-animal variation (Henderson Jr, 1982). Examples of covariates used in animal studies include on- and off- test body weights (Rikard-bell et al., 2009; Schwab et al., 2010), body weight and age to adjust organ weights in growing chicks (Brown et al., 1985), and birth and weaning weights for postweaning growth in cattle (Elzo and Wakeman, 1998).

As with growth, digestibility coefficients can be adjusted as long as an appropriate covariate has been selected (Lee et al., 1997). In general, analysis of covariance is not often utilized in nutritional studies; instead, animals are often blocked relative to initial body weight or previous dietary treatment. However, there is no reason why analysis of covariance should not be used in metabolism studies, provided the correct covariate can be selected. For example, Urriola and Stein (2010) utilized digestibility values from period-1 to correct for differences in digestibility among pigs to estimate digestibility coefficients in subsequent periods.

Conclusion

Proper research methodologies are crucial for obtaining accurate and dependable information. Everything in research uses some type of methodology to proceed to the next step. Therefore, the methods utilized are important for yielding accurate and precise data. In particular, two areas with limited information are excreta drying methods and the use of analysis of covariance in digestibility studies. A better understanding of commonly used drying methods is required to determine which method limits nutrient losses in collected animal excreta samples. In addition, further information is required on the use of covariates in digestibility studies.

Therefore, one objective of this thesis is to determine if drying procedures will influence the outcome of digestibility experiments through changes in energy, nitrogen and sulfur losses from pig feces, pig urine, and poultry excreta. The second objective of this thesis was to determine an appropriate covariate for use in digestibility experiments to better manage variation.

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CHAPTER TWO

Effects of drying methods on nitrogen and energy concentrations in pig feces and urine, and poultry excreta

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ABSTRACT

Accurate estimation of nutrient digestion and retention are critical in nutrient balance and feed evaluation studies because errors that occur are often additive. However, there is no standard universal method for drying feces, urine, or excreta prior to laboratory analysis. The objective of this study was to evaluate the impact of four different drying methods on nutrient concentrations in feces, urine, and excreta. Twelve individually penned growing pigs were fed one of three diets and 16 pens of 10 growing broilers were fed one of four diets that differed in NDF and CP. Feces, urine, and excreta that varied in nutrient composition were collected after 7 d of diet adaptation. Samples were dried using one of four methods: UD-undried, FD-freeze dried, OD55-oven dried at 55°C for 48 h, or OD100-oven dried at 100°C for 48 h, after which DM, GE, N, C, and S were determined. In swine feces, drying resulted in a loss of GE ($P < 0.10$) and S ($P < 0.05$) by 5% and 58%, respectively, compared to UD feces. There was no difference among drying method on DM, GE, N, C, or S concentrations. There were no differences in urinary GE due to drying or between drying methods, however urinary DM was greatest by FD compared to OD ($P < 0.05$) and higher for OD55 compared to OD100 ($P < 0.01$). In poultry excreta, GE ($P < 0.05$), N ($P < 0.10$), and S ($P < 0.01$) were reduced by drying by an average of

6%, 10%, and 66%, respectively. There were no differences among drying methods except FD excreta had a greater S concentration than OD ($P < 0.10$). Regardless of drying method, some GE and N loss appears to be inevitable, but there is no apparent advantage between freeze drying and oven drying. The apparent greater S losses warrant further investigation.

Key words: drying method, feces, poultry excreta, swine, urine

INTRODUCTION

Accurate nutrient estimation in urine, feces and excreta are critical for nutrient digestibility studies (Just et al., 1982; van Kempen et al., 2003; Kerr, 2010). Currently, no standard method of drying feces, urine, or excreta for determination of energy or N concentration has been accepted in the scientific community (Knudsen and Hansen, 1991; Pedersen et al., 2007; Kerr et al., 2009). In poultry, Manoukas et al. (1964) demonstrated that oven drying (**OD**) at 65°C resulted in energy and N losses of 11.4% and 5.2%, respectively, compared to fresh, undried **(UD)** excreta. Shannon and Brown (1969) reported energy losses were lowest (1.3%) with freeze drying (**FD**), while losses of energy decreased with increasing OD temperature (5.5% at 60°C, 3.3% at 100°C, and 2.8% at 120°C) compared to UD excreta. For N, FD or OD at 60°C resulted in a loss of approximately 4.7%, while increasing the temperature to 100°C or 120°C increased losses to 7.8 and 10.5%, respectively. Ribeiro et al. (2001) reported that increasing oven temperature from 55°C to 100°C increased N losses from 8.3% to 13.2%, respectively, relative to UD excreta. Wallis and Balnave (1983) reported greater energy and N losses when excreta were FD compared to OD at either 60°C or 80°C. Dale et al. (1985) found no significant differences in the TME_n when poultry excreta samples were either FD or OD at 60°C, while Jorgensen et al. (1984) reported that method of fecal preparation for N analysis (UD, FD, or OD 70°C) did not affect protein digestibility measurements. Because there is no consensus regarding

the impact of drying on energy and N losses, additional information is needed. The objective of this study was to determine how each of three drying methods (OD55, OD100, and FD) compared to UD on DM, GE, N, C, and S concentrations of swine feces and urine, and poultry excreta.

MATERIALS AND METHODS

The Institutional Animal Care and Use committee at Iowa State University and Auburn University approved all experimental protocols for the swine and poultry research, respectively.

Pig Sample Management

Experimental diets (Table 1) were mixed at the University of Kentucky (Lexington, KY), and were formulated to contain varying levels of CP (15.06 to 18.76%) and NDF (4.5 to 15.4%). Three blocks of four gilts (initial BW of 47.1 kg, Landrace × Large White × Duroc cross) were placed in individual pens (0.57 × 2.21 m) and randomly assigned to dietary treatments. Pigs were offered water and feed ad libitum and were weighed at the beginning and end of the experiment. Pigs were allowed 7 d for adaptation to the diet prior to urine and fecal collections. Following the adaptation period, urine and fecal grab samples from each pig were obtained twice daily, 0700 and 1800, for the subsequent 10 d to ensure adequate sample volumes for laboratory analysis. Fresh urine was collected daily in plastic containers without the addition of acid, while feces were collected in sealable plastic bags, and immediately stored at 10°C. Following the 10 d of collection, samples were pooled within pig and stored at -20°C until further analysis.

Feces were thawed, homogenized, and subsequently subdivided to accomplish the different drying methods after thorough mixing. Oven dried samples were placed in a forced air oven either at 55° or 100°C (±2°C) for 48 h (**OD55** or **OD100**, respectively). Lyophilization was achieved in a freeze drier at -80^{0}C for 24 h and then placed in vacuum tubes, where the samples

were cooled to -110^{0}C under a vacuum pressure of ≤ 100μ for 48 h. Urine samples were thawed in a refrigerator, mixed, and separated into 2 categories, acidified and unacidified. After thawing, acidification took place immediately by adding 1.5 mL of 6 M HCl to lower the initial pH to 2 to minimize the potential of bacterial growth while the unacidified samples were not treated. Urine was enclosed in plastic screw-top containers and refrigerated at all times prior to DM, GE, N, S, and C analyses. Drying method was a factor in urine GE determination, but due to equipment utilized in the current experiment (thermocombustion), N, S, and C were analyzed only as UD. For GE analysis, cellulose pellets were used to absorb and subsequently dry the urine for the OD methods, while cotton balls and small plastic bags (Jeb Plastics Inc., Wilmington, DE) were used to absorb urine in the crucible for the FD method. In both instances, samples were allowed to dry for 48 h. The oven varied by ±1°C during times of drying, and the freeze drier varied ±2°C.

Poultry Sample Management

Experimental diets used were formulated and mixed at Auburn University, AL as described in Table 2 that ranged in CP (17.85 to 26.95%) and NDF (7.62 to 15.78%). Broilers (160 Ross × Ross 708, 10 per pen; 5 males and 5 females) were fed a common diet ad libitum from d-1 to d-15 and then randomly assigned to one of four dietary treatments from d-15 to d-22 (four replicate pens per diet). Broilers were offered feed and water ad libitum. Excreta samples were collected on pans under the pens on d-23 and d-24, subsequently stored at -20°C for later analysis, after which they were thawed and homogenized prior to drying method and chemical analyses.

Chemical Analyses

Percentage DM was determined by measuring the weight of samples prior to, and subsequent to, the completion of each respective drying method. Dried fecal and excreta samples

were ground through a 1-mm screen in preparation for chemical composition determination. For UD urine GE concentrations, 1 mL of urine was added to 0.5 g of dried cellulose and then analyzed for GE. For urine dried at 55° and 100°C, 3 mL of urine was added to 0.5 g of dried cellulose and dried for 24 h under the respective drying temperature prior to determining GE. Freeze dried urine samples were analyzed by the addition of 2 mL of urine to a 0.15 g dried cotton ball and frozen for 24 h at -80°C, after which the samples were placed under the freeze drying vacuum conditions for 24 h. Gross energy of feces, urine, and excreta was determined by the use of an isoperibol bomb calorimeter (Model: 1281, Parr Instrument Co. Moline, IL), with benzoic acid in the samples for a standard. In all cases, the GE of the cellulose or plastic bag plus cotton ball was subtracted from the total energy (urine plus carrier) to determine urinary GE. To maintain a laboratory CV below 5% for these analyses, duplicate analyses were performed on all fecal and excreta samples from each pig or pen of broilers, whereas urine samples were analyzed in triplicate on cellulose or cotton balls. Nitrogen and C were analyzed by thermocombustion (VarioMax, Elementar Analysensysteme, Gmbh, Hanau, Germany) which uses catalytic tube combustion to volatilize the sample. Percentage DM, C, N, and S, and kcal GE were calculated for feces, urine, and excreta on an UD basis (Tables 3 through 5).

Statistics

Data were analyzed using Proc MIXED procedure (SAS Inst. Inc., Cary, NC) with the individual pig or the pen of broilers defining the experimental unit. The statistical model for feces and urine drying included method, diet, and pig as fixed effects; with group added as an additional fixed effect in analyzing the poultry data. Method- and diet-within pig were random effects for the swine data, and group-, chicken-within group, and method-within chicken were random effects for the poultry data. To test the effect of urinary acidification on DM, GE, N, C,

or S concentrations, acidification was added as a fixed effect, but because there was no effect, it was not included in the final model. Means are reported as LSMEANS. Data are discussed relative to an over model protected F-test, or an unprotected F-test using preplanned contrasts between the UD and dried samples, between freeze drying and oven drying, and between oven drying at 55 or at 100°C. The use of both statistical analyses provides an unbiased discussion of the data (Barnette and McLean, 1998).

RESULTS AND DISCUSSION

Some form of sample preparation is usually required prior to analysis due to the detection and measurement conditions of various types of laboratory equipment. Determining the moisture content of feeds can be accomplished by multiple methods (Thiex and Richardson, 2003) and in swine experiments they have been dried at both moderate (60ºC, Stein et al., 2006) and high (95ºC, Goebel and Stein, 2011) temperatures. However, obtaining a representative sample to adhere to the AOAC method 930.15 (AOAC, 2005) requiring only 2 g, may be difficult. Although this may be possible for feed samples that are relatively dry samples and have been ground, it would be difficult after a multiple day collection of growing-finishing pigs or poultry. The times and temperatures picked for this study are similar to that which we have utilized in the past (Kerr et al., 2009), and are not unlike that utilized by others (Yen et al., 2004; Stein et al., 2006; Lindemann et al., 2010).

Previous research indicate that analysis of animal excreta, currently utilized in nutrition and agricultural research, may be affected by drying method (Lawrence, 1971; Sistani et al., 2001). Reduced fecal N concentration has been reported due to drying cattle feces (Gallup and Hopps, 1944; Colovos et al., 1957; Bratzler and Swift, 1959), with Falvey and Woolley (1974)

reporting a decrease in N losses with increasing drying temperatures. A similar effect of increasing temperature on reducing N losses has also been reported in the drying of various poultry manure when combined with soil (Giddens and Rao, 1975) where it has been indicated that the ability of microorganisms to degrade urea or uric acid to ammonia is reduced under a more rapid-high temperature drying method compared to a slower-low temperature method. Relative to GE losses, Colovos et al. (1957) reported that GE losses in cattle feces was approximately 14% if dried at 65°C compared to UD feces, while Bratzler and Swift (1959) reported no loss of GE is due to drying cattle feces due to drying at 65°C.

Consequently, if the drying method has an effect on sample composition, the accuracy of nutrient balance is reduced, making interpretation of the results biased relative to the degree of nutrient loss. In the current experiment, we chose to feed diets ranging in CP (14.27 to 18.76%) and NDF (4.5 to 15.4%) to generate feces and urine of varying DM, GE, N, S, and C composition (Kerr and Easter, 1995; Cahn et al., 1997).

Drying Method - Pig Feces

The current experiment measured the impact of four commonly used drying methods on N and GE concentrations. Additionally, C and S were evaluated due to the impact of animal production on the release of these chemicals into the environment. When utilizing a protected F-test, there was no effect of drying method on DM, GE, N, or C concentrations in pig feces (Table 3). However, utilizing preplanned contrasts comparing UD to dried samples (OD55, OD100, and FD) there was a loss in GE ($P < 0.10$) of approximately 5%. However, there was no difference between FD and OD or between the two oven drying temperatures ($P > 0.10$; Table 3) for all analytes measured. Drying resulted in a loss of S of approximately 58% (model $P < 0.01$; UD vs. dry contrast, $P < 0.01$).

The impact of drying on GE or N content of feces in swine has been largely overlooked, with methods largely adapted from the cattle or poultry literature. Moisture losses may differ between different drying methods (Lawrence, 1971; Blake and Potter, 1987), and as such, can affect subsequent analytical results. Fecal composition has been shown to be affected by drying methods, where differences in N and GE concentrations have been reported between OD and FD (Lawrence, 1971). Likewise, Mahimairaja et al. (1990) reported significant reductions in total N from pig slurry after samples were OD at 105°C while FD had no effect on the total N concentration compared to UD manure. In contrast, Giddens and Rao (1975) indicated drying manure at a lower temperature (23ºC for 10 d) tended to cause a greater loss in N versus fast drying at a high temperature (100ºC for 8 h), but this was not observed in the current trial. We have no explanation for the relatively large loss of S compared to that noted for GE or N, but also have no other data from which to compare with.

Drying Method - Pig Urine

Urine is analyzed in metabolism studies to measure patterns in metabolizable constituents, with GE and N being the most common components evaluated. This is important not only because N excretion routes change as diets differ in CP (Gatel and Grosjean, 1992; Kerr and Easter, 1995) and fiber (Zervas and Zijlstra, 2002; Shriver et al., 2003), but also due to compounds that can acidify the urine (Canh et al., 1998). In addition, ammonia is released during the conversion of urinary urea to ammonia due to microbial urease present in feces (Canh et al., 1997; van Kempen et al., 2003). Therefore, nutrient balance experiments typically involve acidification of the urine to prevent loss of N due to microbial growth (Pederson et al., 2007). Consequently, we chose to measure the impact of urine acidification along with drying method on urine composition. In the current study, the addition of 1.5 mL of 6 M HCl to 50 mL of urine

to lower the pH to 2.0 had no impact on urinary DM, GE, N, C, or S concentrations (data not shown), indicating that losses of these components were not affected by diet acidification during sample processing. These data do not, however, reflect the potential losses that would occur from the time of collection until they reach the laboratory (van Kempen et al., 2003). Our data are supported by Pan et al. (2009) who reported no impact of acidifying poultry excreta on total N compared to fresh excreta, indicating that once in the lab and properly stored, little N is lost during the course of laboratory procedures. In contrast, Ribeiro et al. (2001) reported a 10% loss in the N content of broiler excreta when it was not acidified to pH 4.5 prior to OD at 55°C or 100°C. In the current experiment, there were no differences in urinary GE due to drying methods (Table 4). Because the equipment utilized in our lab analyzed urine by an UD process only, no comparison due to drying methods are possible for N, C, or S. The apparent differences in DM between FD and OD ($P < 0.05$) and between OD55 and OD100 ($P < 0.01$) indicates that FD was more efficient in dehydrating the samples compared to the two OD methods, and that OD55 was more efficient than OD100. We cannot explain these differences, but speculate that it may involve volatile compound losses under the OD conditions. Only one scientific report could be found which evaluated the impact of drying method on urine energy; and in that instance, similar to the current experiment, FD or OD of urine had little to no effect on urinary energy concentration (Hartfiel, 1961).

Drying Method - Chicken Excreta

For poultry excreta, the use of an overall model F test indicated there were no differences among drying methods for DM, GE, N, C, and S concentrations ($P > 0.10$; Table 5). However, the preplanned contrasts indicate that GE ($P < 0.05$), N ($P < 0.10$), and S ($P < 0.01$) were reduced by drying (FD, OD55, and OD100) by 6%, 10%, and 66%, respectively, compared with

UD. There was no difference between FD and OD for GE or N concentration ($P > 0.10$). However, OD resulted in a lower concentration of S compared to FD ($P < 0.10$).

Manoukas et al. (1964), Shannon and Brown (1969), and Mahimairaja et al. (1990) reported that OD resulted in greater N and GE losses compared to FD, and in some cases, FD was equal to UD. Relative to drying temperature, Shannon and Brown (1969) reported that increasing the OD temperature decreased GE losses, but increased N losses. Ribeiro et al. (2001) also reported that increasing oven temperature increased N losses. In contrast, Wallis and Balnave (1983) reported that FD had a greater impact than OD on the losses N or GE. Blake and Potter (1987) reported that increasing drying temperature increased DM percentage, but when data were converted to a fresh weight basis, there was no apparent effect on N or GE losses up to 100 to 120°C. However, above 120°C, both N and GE content were affected. Sibbald (1979) and Dale et al. (1985) reported no difference in TME between FD or OD excreta. Finally, data reported by Sistani et al. (2001) supports our findings in that FD, OD65, and OD105 increased N losses compared to UD, but there were no differences among any of the drying methods.

In the current experiment, drying method had no impact on C losses, but the high level of S losses warrants further investigation as this could have dramatic impacts on interpreting manure composition and gas emission data. The current data, along with results from published literature, indicate that regardless of drying method, some loss of GE and N appears to be inevitable. However, there appear to be no distinct advantage between freeze drying and oven drying relative to GE or N losses.

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Table 1. Composition of Swine diets, as-fed basis

Ingredient	Dehulled, degermed corn	Corn-soybean meal	Dried distillers grains with solubles
Corn	--	78.40	56.70
Soybean meal	18.00	18.00	15.00
Dehulled, degermed corn	78.19	--	--
Dried distillers grains with solubles	--	--	25.00
Soybean oil	0.50	0.50	0.50
L-Lysine•HCl	0.11	--	--
Dicalcium phosphate	0.85	0.70	0.15
Limestone	0.70	0.75	1.00
Sodium chloried	0.50	0.50	0.50
Vitamin premix[1]	0.05	0.05	0.05
Trace mineral premix[2]	0.05	0.05	0.05
Titanium dioxide	0.50	0.50	0.50
Clay[3]	0.50	0.50	0.50
Antibiotic[4]	0.05	0.05	0.05
Calculated composition			
ME, kcal/kg	3,293	3,332	3,193
CP, %	14.27	15.06	18.76
NDF, %	4.50	9.10	15.40
Crude fat, %	1.20	4.10	5.30
Phosphorus, %	0.34	0.47	0.48

[1] Supplied per kilogram of diet: vitamin A, 6,600 IU; vitamin D_3, 880 IU; vitamin E, 44 IU; vitamin K (menadione sodium bisulfate complex), 6.4 mg; thiamin, 4.0 mg; riboflavin, 8.8 mg; pyridoxine, 4.4 mg; vitamin B_{12}, 33 μg; folic acid, 1.3 mg; niacin, 44 mg; pantothenic acid, 22 mg; D-biotin, 0.22 mg.

[2] Supplied per kilogram of diet: Zn, 131 mg as ZnO; Fe, 131 mg as $FeSO_4 \cdot H_2O$; Mn 45 mg, as MnO; Cu, 13 mg as $CuSO_4 \cdot 5H_2O$; I, 1.5 mg as CaI_2O_6; Co, 0.23 mg as $CoCO_3$; Se, 0.28 mg as $NaSeO_3$

[3] AB-20, Prince Agriproducts, Quincy, IL.

[4] Tylan-40 supplied at 44mg/kg of diet, Elanco, Greenfield, IN.

Table 2. Composition of broiler diets, as-fed basis

Ingredient, %	Dextrose	Corn gluten meal	Dried distillers grains with solubles	Corn germ meal
Corn	52.99	52.99	52.99	52.99
Soybean meal	27.95	27.95	27.95	27.95
Dextrose	15.00	--	--	--
Corn germ meal	--	--	--	15.00
Corn gluten meal	--	15.00	--	--
Dried distillers grains with solubles	--	--	15.00	--
Dicalcium phosphate	1.46	1.46	1.46	1.46
Limestone	0.96	0.96	0.96	0.96
DL-methionine	0.24	0.24	0.24	0.24
Sodium chloride	0.44	0.44	0.44	0.44
Vitamin premix[1]	0.21	0.21	0.21	0.21
Mineral premix[2]	0.21	0.21	0.21	0.21
Coccidostat[3]	0.04	0.04	0.04	0.04
Titanium dioxide	0.50	0.50	0.50	0.50
Calculated composition				
ME, kcal/kg	3,020	2,910	2,833	2,740
CP, %	17.85	26.95	21.87	21.02
NDF, %	7.62	9.30	13.05	15.78
Crude fat, %	2.92	3.11	4.40	3.24
Phosphorus, %	0.21	0.30	0.33	0.30

[1] Supplied per kg of diet: vitamin A, 8,000 IU; vitamin D, 2,000 IU; vitamin E, 8 IU; vitamin K (menadione sodium bisulfate complex), 2 mg; vitamin B_{12}, 0.02 mg; folic acid, 0.5 mg: D-pantothenic acid, 15 mg; riboflavin, 5.4 mg; niacin, 45 mg; thiamin, 1 mg; biotin, 0.05 mg; pyridoxine, 2.2 mg; choline, 500 mg.

[2] Supplied per kg of diet: Mn 65 mg as MnO; Zn 55 mg as ZnO; Fe 55 mg as $FeSO_4$; Cu 6 mg as $CuSO_4 \cdot 5H_2O$, I 1 mg CaI_2O_6; Se 0.3 mg as $NaSeO_3$.

[3] Bio-Cox, salinomycin sodium, 133g per kg of diet.

Table 3. The effect of drying method on the composition of pig feces, as is basis[1]

	Drying method[2]				Model[3]		Contrasts[4]		
Analyses	UD	FD	OD55	OD100	SEM	*P* value	UD vs dry	FD vs OD	OD55 vs OD100
DM, %	--	31.04	31.17	32.46	3.01	0.93	--	0.86	0.76
GE, cal/g	1,374	1,297	1,315	1,293	33	0.28	0.06	0.86	0.64
N, %	1.31	1.24	1.23	1.29	0.10	0.93	0.63	0.86	0.66
C, %	13.10	12.25	12.42	13.34	0.96	0.83	0.70	0.59	0.51
S, %	4.68	1.45	2.17	2.22	0.65	0.01	0.01	0.36	0.96

[1] Fresh fecal matter collected from growing pigs with 12 observations per drying method.

[2] Drying methods consisted of undried (UD), freeze drying (FD), and oven drying at 55°C (OD55) or 100°C (OD100).

[3] Model statistics.

[4] Preplanned contrast statements.

Table 4. The effect of drying method on the composition of pig urine, as-is basis[1]

	Drying method[2]				Model[3]		Contrasts[4]		
Analyses	UD	FD	OD55	OD100	SEM	*P* value	UD vs dry	FD vs OD	OD55 vs OD100
DM, %	--	4.50	4.32	3.60	0.55	0.01	--	0.03	0.01
GE, cal/g	103.1	103.7	117.7	93.3	10.8	0.46	0.89	0.89	0.12
N, %	0.80	--	--	--	-	--	--	--	--
C, %	0.98	--	--	--	-	--	--	--	--
S, %	0.78	--	--	--	--	--	--	--	--

[1] Fresh urine collected from growing pigs with 12 observations per drying method.

[2] Drying methods consisted of undried (UD), freeze drying (FD), and oven drying at 55°C (OD55) or 100°C (OD100).

[3] Model statistics.

[4] Preplanned contrast statements.

Table 5. The effect of drying method on the composition of poultry excreta, as is basis[1]

	Drying method[2]				Model[3]		Contrasts[4]		
Analyses	UD	FD	OD55	OD100	SEM	*P* value	UD vs dry	FD vs OD	OD55 vs OD100
DM, %	--	20.95	20.83	20.78	0.61	0.98	--	0.87	0.96
GE, cal/g	854	809	796	812	20	0.22	0.05	0.86	0.59
N, %	1.16	1.07	1.05	1.01	0.05	0.25	0.07	0.50	0.58
C, %	8.45	8.33	8.37	8.24	0.36	0.98	0.74	0.95	0.81
S, %	2.94	1.53	0.55	0.88	0.38	0.09	0.01	0.09	0.54

[1] Fresh excreta collected from growing broilers with 32 observations per drying method.

[2] Drying methods consisted of undried (UD), freeze drying (FD), and oven drying at 55°C (OD55) or 100°C (OD100).

[3] Model statistics.

[4] Preplanned contrast statements.

CHAPTER THREE

The use of a covariate reduces experimental error in nutrient digestion studies in growing pigs

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ABSTRACT

Covariance analysis limits error, the degree of nuisance variation, and overparameterizing factors to accurately measure treatment effects. Data dealing with growth, carcass composition, and genetics often utilize covariates in data analysis. In contrast, nutritional studies typically do not. The objectives of this study were to: 1) determine the effect of feeding diets containing dehulled degermed corn, corn-soybean meal, or distillers dried grains with solubles on nutrient digestibility coefficients, 2) evaluate potential interactive effects between initial and final treatment diets on the final treatment diet effects, and 3) determine if initial criterion (digestibility or physiological values) would effectively correct for variation among pigs that could thereby affect final treatment diet digestibility coefficients. Seventy two crossbred barrows (Yorkshire × Landrace × Duroc × Chester White) were randomly assigned to one of three dietary treatments for Phase-1 (P1; 14 d). On d-14, pigs were randomly reassigned to one of the three diets within initial dietary treatment for Phase-2 (P2; 14 d). Fecal and blood samples were collected on d-14 of P1 and d-14 of P2. Fecal samples were dried and analyzed for C, ether extract, GE, N, NDF, P, and S. Plasma samples were analyzed for plasma urea nitrogen and

triglycerides. Dietary treatments differed widely in CP, NDF, and P, resulting in an overall decrease in C, GE, NDF, N, P, S, plasma urea nitrogen, and triglycerides digestibility as dietary fiber increased in P1 and P2 ($P < 0.10$). There were no differences in P2 criteria due to blocking for the P1 diet. There tended ($P = 0.10$ to 0.20) to be a significant P1×P2 interactions for NDF and S, indicating that the response of pigs to the P2 diet may depend upon the P1 diet. In contrast, when the P1 parameter was used as a covariate for P2 data, it was significant for GE, NDF, N, S, and plasma urea nitrogen ($P < 0.10$), and C and ether extract showed tendencies, but not for P digestibility or plasma triglycerides and only. In conclusion, if initial diets are known, one should balance subsequent treatments for the initial diet because of potential diet by diet interactions. If the initial diets are not known, then an initial criterion would be effective in reducing the variation associated with subsequently obtained data, and should be considered as a covariate in future grow-finish swine digestibility research.

Key words: covariance, digestibility, fiber, pig

INTRODUCTION

Variation is inevitable in animal research with experiments evaluating body composition (Brown et al., 1985), feed intake (Smith et al., 2011), growth (Rikard-Bell et al., 2009), AA (Waguespack et al., 2012), and meat quality (Lefaucheur et al., 2010) often utilizing covariates in data analysis. Others (Kirkpatrick et al., 1990; Schaeffer and Dekkers, 1994) have reported covariance to be an alternative repeated record for certain model traits in an animal's life such as age, weight, or genetic line. The purpose of a covariate is to limit error, the degree of nuisance variation, and overparameterizing factors in order to accurately measure treatment effects (Meyer and Hill, 1997), where the variable does not directly impact the study (Ramsey and Schafer,

2002). In general, nutrition studies do not utilize covariates, but instead utilize blocking relative to initial BW or previous treatment. Recently, Urriola and Stein (2010) utilized digestibility values from period-1 to correct for differences among pigs for subsequent digestibility coefficients, but did not indicate whether the covariate was significant. As with data dealing with growth, genetics, or meat quality, digestibility coefficients obtained in nutritional studies may be needed to adjust for differences among animals to reduce experimental error as long as an appropriate covariate has been selected (Lee et al., 1997). The objectives of this study were to: 1-determine the effect of feeding diets containing dehulled degermed corn (**DDC**), corn-soybean meal (**CSBM**), or distillers dried grains with soluble (**DDGS**) on digestibility coefficients, 2-evaluate potential interactive effects between pre-treatment and post-treatment diets on post-treatment diet effects, and 3-determine if initial criterion (digestibility or physiological values) would be effective in reducing the error due to variation among pigs, thereby improving the precision of digestibility coefficients for the dietary treatments.

MATERIALS AND METHODS

The experiment was conducted under protocols approved by the University of Kentucky Institutional Animal Care and Use Committee.

Feeding Management

Experimental diets (Table 6) were mixed at the University of Kentucky (Lexington, KY) and formulated to contain varying levels of CP, NDF and P through the utilization of DDC, CSBM, and DDGS. Diets were formulated to meet requirements relative to NRC (1998) recommendations. The same diets were fed in each of two phases, with Phase-1 (**P1**) diets utilizing chromic oxide and Phase-2 diets (**P2**) utilizing titanium dioxide, each added at 0.5% of

the diet, to determine digestibility coefficients. Pigs were provided ad libitum access to feed and water throughout the experiment.

Pig Management and Collections

Seventy two crossbred barrows [Yorkshire × Duroc; Duroc × Chester White] were individual penned and randomly assigned to one of three dietary treatments (Table 6). All pigs were initially separated into three groups of 24 pens and fed P1 diets for 14 d and then randomly reassigned within P1 dietary treatment into the three P2 dietary treatments and fed an additional 14 d resulting in nine groups of eight pigs (Figure 2). On d-14 (P1) and d-28 (P2) fresh fecal samples were collected into 16 ounce plastic containers and placed into a -20°C freezer until analyzed. On each collection day, samples were collected from 0700 to 1200 to ensure adequate sample size for subsequent analysis. Pigs were also weighed on d-14 and d-28 of the experiment. In addition, on d-10 (P1) and d-28 (P2), blood samples were collected via venipuncture from 1000 to 1200 into 50 mL centrifuge tubes containing sodium heparin (14.3 USP units/mL), centrifuged at 900 × g for 18 min at 4°C. The resultant plasma was harvested and stored at -20°C until analyzed.

Chemical Analysis

Prior to analysis, fecal samples were thawed, homogenized, and dried in a forced-air oven at 70 °C for 48 h prior to grinding. Feed and fecal samples were ground through a 1-mm screen before composition was determined. Gross energy was determined with isoperibol bomb calorimeter (Model: 1281, Parr Instrument Co. Moline, IL), with benzoic acid as a standard. Duplicate analyses were performed on all fecal samples for C, N, and S by thermocombustion (VarioMax, Elementar Analysensysteme, Gmbh, Hanau, Germany). Ether extract (**EE**) was determined using petroleum ether as described by Luthria et al. (2004) using an ASE 350

(Dionex Corporation, Sunnyvale, CA). Neutral detergent fiber was determined using a fiber analyzer (Ankom 200, Macedon, NY; Van Soest and Roberston, 1979). Phosphorus and chromic oxide (P1) were analyzed at a commercial laboratory (SDK Labs, Hutchinson, KS), chromic oxide by inductively coupled plasma spectroscopy (Ultima 2, Horiba Jobin-Yvon Inc., Edison, NJ) according to standard method 3120B (American Public Health Association, 1992), and P by colorimetric determination (Technicon Auto Analyzer II, Technicon, Tarrytown, NY) using AOAC method 976.06 (AOAC, 1995). Titanium dioxide (P2) was analyzed by digesting the samples in sulfuric acid and hydrogen peroxide and subsequent absorbance was measured using a UV spectrophotometer (AOAC method 988.05, AOAC, 1973).

Plasma urea nitrogen (**PUN**) concentrations were determined using a method described by Kerr et al. (2004) where the samples were analyzed colorimetrically using a kit (Kit B755'-'10, Point Scientific Inc., Lincoln Park, MI) followed by UV absorbance (Varian Cary 50 Spectrophotometer Varian Analytical Instruments, Walnut Creek, CA). Plasma triglycerides were quantified using an enzymatic kit (GPO, Pointe Scientific Inc., Lincoln Park, MI) wherein lipase converts the triglycerides to glycerol and FFA. Glycerol kinase and glycerophosphate oxidase was then used to derive H_2O_2 after which the absorbance was measured at 540 nm.

Calculations and Statistical Analysis

Feed and fecal Cr and Ti concentrations were used to estimate apparent C, EE, GE, N, NDF, P, and S total tract digestibility by indirect marker methodology. Digestible C, EE, GE, N, NDF, P, and S were calculated as $[1 - (\text{marker}_{\text{feed}} \times \text{digestibility criterion}_{\text{feces}})/(\text{marker}_{\text{feces}} \times \text{digestibility criterion}_{\text{feed}})] \times 100$. Feed and fecal samples were analyzed in duplicates for C, EE, GE, N, P, and S, while NDF was measured in triplicate. Post-experiment evaluation of the treatment digestibility data identified statistical outliers (greater than three standard deviations

above/below the mean) and a total of six data points were removed from the data analysis. Two pigs were fed DDGS and one was fed DDC during P1, then two of the three were fed CSBM during P2 while the other was fed DDC.

Data were analyzed using the Proc MIXED procedure (SAS Inst. Inc., Cary, NC) to evaluate digestibility coefficients or plasma criterion among dietary treatments. The initial model for P1 and P2 included dietary treatment only. A second model utilized P1, P2, and their interaction as model parameters, along with initial BW as a covariate. A third model utilized initial criterion and initial body weight as covariates. Initial criterion and initial BW were used as fixed effects when the covariance analysis was performed and were reported as least square means. The individual pig was the experimental unit for all parameters. Data with $P \leq 0.10$ were considered to be significant due to liberality, $P = 0.10$ to 0.20 were tendencies.

RESULTS AND DISCUSSION

Diet Effects within Phase

Nutritional studies are crucial for providing knowledge to support the use of ingredients, feed additives, and medications in swine diets. Furthermore, understanding of nutrient and energy digestibility is important in characterizing the impact of dietary treatments on subsequent nutrient utilization. One pig was removed from the data analysis because of a rectal prolapse; therefore data from 71 pigs were used. In the current study, P1 dietary treatments differed widely in CP, NDF, and P resulting in an overall decrease in C, GE, and N digestibility as dietary fiber increased (Table 2). Apparent total tract digestibility of EE, NDF, and S were higher in pigs fed DDC compared to pigs fed CSBM and DDGS, but did not differ between pigs fed CSBM and DDGS. Phosphorus digestibility was highest for pigs fed DDG and lowest for pigs fed the

CSBM diet. Overall, the nutrient and GE digestibility decreased significantly ($P < 0.10$) as dietary fiber increased. The current data are supported by Kuan et al. (1983) and Stanogias and Pearce (1985) who reported a similar response to increasing dietary fiber, where apparent total tract digestibility of CP, DM, and EE decreased due to increasing fiber. Also, fiber type has been reported to decrease digestibility of the cell wall, NDF, DM, CP (Ehle et al., 1982), AA (Pedersen et al., 2007), and TDF (Urriola et al., 2010). Plasma urea N and triglycerides increased ($P < 0.01$) as dietary fiber increased,

The impact of P2 diets on P2 fecal digestibility and blood parameters, with no consideration of carry-over effects from the previous phase diet, is shown in Table 3. As expected, pigs fed P2 diets showed similar digestibility differences among dietary treatments in comparison to data reported for P1, where increasing dietary fiber levels resulted in an overall decrease in nutrient and GE digestibility ($P <0.10$). Urriola and Stein (2010) also reported that inclusion of 30% DDGS reduced total and hindgut GE digestibility in grower pigs by 6% and 9%, respectively, largely due to the fiber contained in DDGS. Distillers dried grains with solubles may also reduce fermentation in the hindgut due to viscosity and rate of passage, resulting in lower digestibility (Urriola and Stein, 2010). In the current experiment, nutrient digestibility was affected ($P < 0.10$) by diet. The P level in the experimental diets increased with increasing amount of fiber (DDC versus CSBM and DDGS). However, P digestibility decreased ($P <0.10$). In contrast to the current experiment, Pettey et al. (2006) reported a linear increase in total tract P digestibility as dietary P increased, but they utilized a purified diet with monosodium P and their increase in dietary P was much greater than in the current experiment. Similar to P1 data, PUN and triglyceride concentrations were significantly affected ($P < 0.10$) by the P2 diet. The observed increase in PUN concentration as CP increased is in agreement with previous

literature (Zervas and Zijlstra, 2002) and agrees with others (Brown and Cline, 1974; Fuller et al., 1979; Coma et al., 1995) who have reported that N metabolism, and consequently PUN, can be affected by dietary AA concentrations.

Potential Impact of P1 Diet on P2

Although digestibility and physiological data were expected to differ among diets as described above, the real focus in the current experiment was whether the composition of the P1 diet may have affected the data obtained during P2 of the experiment. Because literature suggests that age or body weight may affect digestibility (Noblet and van Milgen, 2004) and may be used as a covariate to adjust for variation in body weight within treatment (Boddicker et al., 2011; Young et al., 2011), we elected to include initial BW as a covariate in this and subsequent analyses. Table 4 presents the results of the evaluation of the data when previous diet is known, along with potential interactive effects between P1 and P2 diets, and the use of initial BW as a covariate.

Day-14 BW as a covariate significantly impacted GE, N, and S digestibility, $P = 0.10$, 0.01, and 0.04, respectively, and tended to affect P ($P = 0.17$), PUN ($P = 0.12$), and triglyceride ($P = 0.20$) values. We elected, however, to retain BW as a covariate in this (Table 4) and subsequent (Table 5) analyses even though it may not have been significant. Although there were no significant interactions between P1 and P2 diet on P2 treatment means, NDF and S tended to have a P1 × P2 interaction, $P = 0.17$, and 0.14, respectively (Table 4), suggesting that P1 diet may affect P2 data. Likewise, there was no impact of P1 diet on any of the P2 criteria measured. The lack of an interaction between P1 and P2 or an overall lack of P1 on P2 data was partially expected as increasing the length of feeding or adaptation to a diet (Gargallo & Zimmerman, 1981) before samples are collected likely limits the impact of a P1 diet on subsequent treatment

means. Even with using BW as a covariate and some tendencies for P1 × P2 interactions utilized in the analysis, most fecal digestibility coefficients and plasma parameters differed due to P2 dietary treatment in a manner similar to that described above. However, the data shown in Table 4 suggests it is important to account for the initial diet fed and potential interactions between the initial and final diet in animal nutrition research to reduce nuisance variation. It also suggests that longer adaptation time to subsequent dietary treatments may be needed to reduce or prevent previous diet effects on subsequent treatment means.

Utilization of Covariate Analysis

Similar to that described for 'diet effects within phase' (Table 3) and 'potential impact of P1 diet on P2' (Table 4), digestibility and plasma parameters were expected to differ among diets. However, the critical question was whether or not appropriate covariate(s) could be used to enhance the accuracy of measuring the effects of diet on digestibility and plasma parameters. Previously, Brown et al. (1985) utilized body weight and Dean et al. (2005) utilized initial PUN concentration as covariates to adjust for variation in particular criteria of interest. Only recently has this been utilized in nutritional studies where period-1 digestibility values have been used to adjust subsequent digestibility values (Urriola and Stein, 2010). Along with utilizing initial BW as a covariate for P2 data, we evaluated P1 criteria as a potential covariate to impact P2 dietary outcomes. As shown in Table 5, using P1 criterion as a covariate for P2 was significant for apparent total tract digestibility of GE, NDF, N, S, and PUN ($P \leq 0.05$) and tended to be significant for C and EE ($P = 0.12$ and 0.11, respectively), but was not significant for P digestibility ($P = 0.37$) or plasma triglycerides ($P = 0.64$). The significance of these covariates, and in several cases the slight numerical reductions in the standard error of the treatment mean,

suggests that when a previous digestibility criterion is known, the use of a P1 criterion as a covariate improves our ability to identify P2 dietary treatment effects.

There were no differences in P2 criteria due to blocking by P1 diet (Table 4), but in considering this 'blocking' factor we are considering the use of 24 pigs as the 'block'. However when considering the P1 × P2 interaction, which in essence considers the use of 8 pigs as a 'blocking' factor, there were 2 significant effects indicating that the response of pigs to the P2 diet was dependent upon the P1 diet. In contrast, when the individual pig was utilized as a covariate (a blocking factor of 1), 5 parameters were significant out of the 9 parameters measured; GE, NDF, N, S, and plasma urea nitrogen. Overall this suggests that controlling experimental variation improved our ability to more accurately compare treatment means. In the current experiment, the group of pigs which were selected did not vary substantially in age, genetics, sex, and body weight, and as such, may have decreased our ability to detect the effects of BW, initial diet, or initial criterion on subsequent treatment means. In addition, our diets differed widely in nutrient composition, such that in no case did the difference in data analysis affect the comparisons of individual treatment means. Our data are supported by Waguespack et al. (2012) who reported that in 9 of 14 experiments summarized, baseline PUN was significant for final PUN, but in only one experiment did the significance level change and in only 3 experiments did the individual treatment mean comparisons change. We would speculate, however, that with a greater variation in the source of pigs or with smaller differences expected between dietary treatments, balancing subsequent treatments for the initial diet because of potential diet by diet interactions would be helpful in data interpretation. Furthermore, if the previous diet fed to pigs is not known, then the use of an initial digestibility coefficient or plasma

analysis would be effective in reducing the variation associated with subsequently obtained data, and should be considered as a covariate in future nutrition research.

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Table 6. Composition of Phase-1 and Phase-2 experimental diets used in a grow-finish pig digestibility study, as-fed basis

Ingredient	DDC[1]	CSBM[1]	DDGS[1]
Corn	--	78.40	56.70
Soybean meal, 48% CP	18.00	18.00	15.00
Dehulled, degermed corn	78.19	--	--
Dried distillers grains with solubles	--	--	25.00
Soybean oil	0.50	0.50	0.50
L-Lysine•HCl	0.11	--	--
Dicalcium phosphate	0.85	0.70	0.15
Limestone	0.70	0.75	1.00
Sodium chloride	0.50	0.50	0.50
Vitamin premix[2]	0.05	0.05	0.05
Trace mineral premix[3]	0.05	0.05	0.05
Marker[4]	0.50	0.50	0.50
Clay[5]	0.50	0.50	0.50
Antibiotic[6]	0.05	0.05	0.05
Calculated composition			
Crude fat, %	1.20	4.10	5.30
CP, %	14.27	15.06	18.76
ME, kcal/kg	3,293	3,332	3,193
NDF, %	4.50	9.10	15.40
Phosphorus, %	0.34	0.47	0.48
Sulfur, %	0.10	0.18	0.21
Analyzed composition[7]			
Crude fat, %	1.28	3.72	4.77
CP, %	13.25	16.00	19.75
GE, kcal/kg	3,770	3,973	4,131
NDF, %	2.51	7.25	12.50
Phosphorus, %	0.28	0.46	0.49
Sulfur, %	0.17	0.20	0.32

[1] Abbreviations: DDC = dehulled, degermed corn; CSBM = corn, soybean meal; DDGS = distillers dried grains with solubles.

[2] Supplied per kilogram of diet: vitamin A, 6,600 IU; vitamin D_3, 880 IU; vitamin E, 44 IU; vitamin K (menadione sodium bisulfate complex), 6.4 mg; thiamin, 4.0 mg; riboflavin, 8.8 mg; pyridoxine, 4.4 mg; vitamin B_{12}, 33 μg; folic acid, 1.3 mg; niacin, 44 mg; pantothenic acid, 22 mg; D-biotin, 0.22 mg.

[3] Supplied per kilogram of diet: Zn, 131 mg as ZnO; Fe, 131 mg as $FeSO_4 \cdot H_2O$; Mn 45 mg, as MnO; Cu, 13 mg as $CuSO_4 \cdot 5H_2O$; I, 1.5 mg as CaI_2O_6; Co, 0.23 mg as $CoCO_3$; Se, 0.28 mg as Na_2O_3Se

[4] The addition of 0.5%, Cr_2O_3 (≥ 98% purity, Elementis Chromium LP, Corpus Christi, TX) represents an addition of 3.35 mg chromium/g diet, analyzed content equaled 2.76 mg chromium/kg diet (P1).The addition of 0.5% TiO2 (99% purity, Tronox Pigments GmBH, Krefield, Germany) represents an addition of 2.97 mg titanium/g diet, analyzed content equaled 2.89 mg titanium/kg diet (P2).

[5] AB-20, Prince Agriproducts, Quincy, IL.

[6] Tylan-40 supplied 44mg/kg of diet, Elanco, Greenfield, IN.

[7] Diets analyzed in laboratory for composition, Ames, IA, except for P which was analyzed by SDK Labs, Hutchison, KS.

Table 7. Day 14 apparent total tract digestibility and plasma analysis of grow-finish pigs fed diets differing in nutrient composition (Phase-1)

	Phase-1 diet[1]			Statistics	
Criterion	DDC	CSBM	DDGS	SEM	*P*-value
Fecal, %[2]					
Carbon	95.5[a]	85.0[b]	81.5[c]	0.38	0.01
Ether extract	57.2[a]	52.2[b]	52.5[b]	1.02	0.01
Gross energy	95.0[a]	86.2[b]	80.0[c]	0.38	0.01
Neutral detergent fiber	66.0[a]	58.3[b]	58.0[b]	2.63	0.05
Nitrogen	89.0[a]	82.9[b]	80.4[c]	0.53	0.01
Phosphorus	55.7[a]	45.0[c]	47.9[b]	1.00	0.01
Sulfur	82.3[a]	81.0[b]	80.0[b]	0.43	0.01
Plasma, mg/dL[2]					
Urea nitrogen	15.3[b]	16.3[b]	20.1[a]	0.73	0.01
Triglycerides	27.8[b]	47.5[a]	55.3[a]	4.16	0.01

[1] Abbreviations: DDC = dehulled, degermed corn; CSBM = corn, soybean meal; DDGS = distillers dried grains with solubles. Average BW of 59.2 kg and 71.0 kg for d 0 and d 14, respectively.

[2] Pigs previously fed a common diet. Mean digestibility coefficients calculated based upon fecal grab samples collected on d 14 from 24 pigs per diet. Blood was collected from pigs on d 10.

[a,b,c] Means in the same row not sharing a common superscript differ ($P < 0.10$).

Table 8. Day 28 apparent total tract digestibility and plasma analysis of grow-finish pigs, fed diets differing in nutrient composition (Phase-2)

	Phase-2 diet[1]			Statistics	
Criterion	DDC	CSBM	DDGS	SEM	*P*-value
Fecal, %[2]					
Carbon	95.6[a]	88.1[b]	84.1[c]	0.25	0.01
Ether extract	56.1[a]	41.1[c]	51.9[b]	0.93	0.01
Gross energy	95.1[a]	87.0[b]	83.6[c]	0.28	0.01
Neutral detergent fiber	70.6[a]	59.0[b]	57.1[b]	1.93	0.01
Nitrogen	89.3[a]	84.0[b]	83.2[b]	0.39	0.01
Phosphorus	47.4[a]	42.9[b]	43.0[b]	1.34	0.04
Sulfur	81.1[b]	75.8[c]	82.9[a]	0.37	0.01
Plasma, mg/dL[2]					
Urea nitrogen	15.1[b]	15.7[b]	19.1[a]	0.64	0.01
Triglycerides	40.1[b]	52.6[a]	50.9[ab]	4.33	0.11

[1] Abbreviations: DDC = dehulled, degermed corn; CSBM = corn, soybean meal; DDGS = distillers dried grains with solubles. Average BW on d 14 and d 28 were 71.0 kg and 88.6 kg, respectively.

[2] P2 data repeats mean of 8 pigs previously fed each P1 diet as described in Figure 1. Mean digestibility coefficients calculated based upon fecal grab samples collected on d 28 from 24 pigs per diet. Blood collected from pigs on d 28.

[a,b,c] Means in the same row not sharing a common superscript differ ($P < 0.10$).

Table 9. Day 28 apparent total tract digestibility and plasma analysis in grow-finish pigs fed diets differing in nutrient composition, Phase-2, as affected by the initial diet (Phase-1) and its potential interaction with the Phase-2 diet

	Phase-2 diet[1]				Statistics[2]			
Criterion	DDC	CSBM	DDGS	SEM	P1	P2	P1×P2	P1 BW
Fecal[3]								
Carbon	95.6[a]	88.1[b]	84.3[c]	0.25	0.84	0.01	0.35	0.41
Ether extract	56.2[a]	41.1[c]	51.9[b]	1.32	0.98	0.01	0.31	0.28
Gross energy	95.0[a]	87.0[b]	83.6[c]	0.28	0.59	0.01	0.34	0.10
Neutral detergent fiber	70.8[a]	58.8[b]	57.1[b]	1.91	0.30	0.01	0.17	0.54
Nitrogen	89.2[a]	84.0[b]	83.3[b]	0.37	0.83	0.01	0.26	0.01
Phosphorus	47.4[a]	43.0[b]	42.9[b]	1.34	0.81	0.04	0.53	0.17
Sulfur	81.1[b]	75.8[c]	82.9[a]	0.35	0.63	0.01	0.14	0.04
Plasma[2]								
Urea nitrogen	15.1[b]	15.7[b]	19.1[a]	0.63	0.75	0.01	0.64	0.12
Triglycerides	40.3[b]	52.6[a]	51.2[ab]	4.35	0.27	0.11	0.70	0.20

[1] Abbreviations: DDC = dehulled, degermed corn; CSBM = corn, soybean meal; DDGS = distillers dried grains with solubles. Average BW on d-14 and d-28 were 71.0 kg and 88.6 kg, respectively.

[2] P1 = impact of Phase-1 diet (P1) on Phase-2 (P2) digestibility; P2 = Phase-2 diet effect; P1×P2 = interaction between P1 and P2 diet on P2 diet means; P1 BW = P1 BW (d-14) as a covariate for P2. P1BW as a covariate was significant for GE, NDF, N, and S; respective b-values (slope) 0.05, -0.13, 0.10, and 0.08.

[3] P2 data repeats mean of 8 pigs previously fed each P1 diet as described in Figure 1.Mean digestibility coefficients calculated based upon fecal grab samples collected on d-28 from 24 pigs per diet.

[a,b,c] Means in the same row not sharing a common superscript differ ($P < 0.10$).

Table 10. Day 28 apparent total tract digestibility for Phase-2 diets by using Phase-1 individual criterion as a covariate

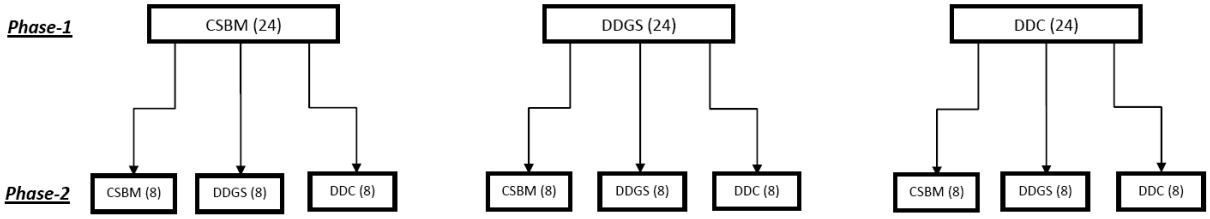
	Diet[1]			Statistics[2]			
Criterion	DDC	CSBM	DDGS	SEM	P2 diet	P1 criterion	P1 BW
Fecal[3]							
Carbon	95.5[a]	88.1[b]	84.3[c]	0.25	0.01	0.12	0.31
Ether extract	56.2[a]	41.1[c]	51.7[b]	0.92	0.01	0.11	0.49
Gross energy	95.0[a]	87.0[b]	84.0[c]	0.27	0.01	0.04	0.07
Neutral detergent fiber	70.0[a]	59.7[b]	57.3[b]	1.84	0.01	0.01	0.62
Nitrogen	89.2[a]	84.0[b]	83.2[b]	0.36	0.01	0.02	0.01
Phosphorus	47.4[a]	43.0[b]	42.9[b]	1.34	0.03	0.37	0.16
Sulfur	81.0[b]	75.9[c]	82.9[a]	0.33	0.01	0.01	0.01
Plasma[2]							
Urea nitrogen	15.1[b]	15.7[b]	19.1[a]	0.61	0.01	0.03	0.32
Triglycerides	39.4[b]	53.0[a]	51.2[ab]	4.38	0.08	0.64	0.20

[1] Abbreviations: DDC = dehulled, degermed corn; CSBM = corn, soybean meal; DDGS = distillers dried grains with solubles. Digestibility of Phase-2 diets. Average BW on d-14 and d-28 were 71.0 kg and 88.6 kg, respectively.

[2] P2 data repeats mean of 8 pigs previously fed each P1 diet as described in Figure 1. P2 diet = Phase-2 diet effect; P1 criterion = use of Phase-1 criterion as a covariate for P2; P1 BW = P1 BW as a covariate for P2. Mean digestibility coefficients calculated based upon fecal grab and blood samples collected on d-28 from 24 pigs per diet. P1 criterion as a covariate was significant for GE, NDF, N, S, and PUN; respective b-values (slope) 0.05, 0.30, 0.11, 0.31, and 0.20 and respective P1BW 0.05, -0.10, 0.10, 0.09 and 0.07.

[a,b,c] Means in the same row not sharing a common superscript differ ($P < 0.10$).

Figure 2. Allotment of a total of 72 crossbred barrows into Phase-1 and Phase-2 diets. Numbers in parentheses represent the number of pigs per treatment.



CHAPTER FOUR

GENERAL CONCLUSIONS

The research presented in this thesis was to evaluate two specific methodologies used in animal research, which included animal excreta drying method and the use of a covariate for digestibility experiments. It is quite clear that drying method and digestibility covariates have been researched very little. However, the limited literature available leaves no suggestions for drying animal excreta without the negative impact from the heat and time which affect nitrogen and energy concentrations or to use covariates in digestibility. In addition, there have been leads towards lowering the negative impacts due to drying methods and the beneficial use of covariates in digestibility.

In reviewing the drying method literature, it became clear that there was an underlying fundamental question that needed to be addressed: "What is the proper method for drying animal excreta with the least amount of impact on nitrogen and energy loss?" The considerable amount of variation that exists among the published values in the literature appears to be somewhat related to this fundamental question.

The variation among drying methods has led nutritionists to overestimate the digestible nitrogen and energy values. Chapter 2 provides information about animal excreta nutrient and energy concentrations when freeze dried and oven dried at 55 and 100°C. There appears to be no distinct advantage between freeze drying and oven drying relative to gross energy or nitrogen losses. Similar results were discovered when urine samples were acidified to pH 2.0; there were no nutrient or energy losses during the sampling and acidifying process. This reflects the potential losses that could occur during the time of collection to laboratory analysis. Comparing the results from published literature, indicates that regardless of drying method, some losses of

gross energy (5%) and nitrogen (4%) appears to be inevitable. In the current experiment, drying method had no impact on carbon losses, but the high level of sulfur losses warrants further investigation.

Although there is limited information on the use of covariates in digestibility studies previous research have utilized them without explaining the benefits. The unknown benefits of the covariate in digestibility studies have led to the question of "Does the use of a digestibility covariate have significance on accurately measuring treatment differences?" In Chapter 3 of previous digestibility covariates were used significantly useful by adjusting for previous treatments. There are circumstances to the use of covariates, such as when initial diets are known, one must balance subsequent treatments for the initial diet because of potential diet by diet interactions. However, if the initial diets are not known, then an initial criterion would be effective in reducing the variation associated with subsequently obtain data, and should be considered as a covariate in future nutrition research. This thesis confidently provides information on the use covariates in digestibility studies to accurately measure treatment estimate without nuisance animal variation during experimentation.

Everything in research uses some type of methodology to proceed to the next step. Animal excreta drying method and digestibility covariates are two of many methodologies used in research to achieve accurate and precise data. Properly utilizing methods create confident and credible research, which expands researchers' knowledge.

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