# EFFECTS OF VARYING LEVELS OF PHYTASE SUPPLEMENTATION ON NUTRIENT DIGESTIBILITY IN HORSES FED A COMMON TEXTURED RATION

By

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Bachelor of Science

Oklahoma State University

Stillwater, Oklahoma

1995

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirement for
the Degree of
MASTER OF SCIENCE
December, 2001

EFFECTS OF VARYING LEVELS OF PHYTASE

SUPPLEMENTATION ON NUTRIENT

DIGESTIBILITY IN HORSES FED

A COMMON TEXTURED

RATION

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ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to all the faculty, friends, and family for all the support and guidance for the past two years. A sincere thanks goes to my major adviser Dr. Steven Cooper for all of his help, advise, and for making me think. My sincere thanks also go to the rest of my committee; Dr. David Freeman and Dr. Robert Teeter. Special thanks goes to the BASF Corporation for supplying the product NATUPHOS 600® for the phytase study.

I would like to thank all of the employees of the OSU Equine Center whose time and effort were invaluable. Also to the undergraduate and graduate students who helped me see these projects through and for giving up their free time. To my friend Genny Hasty for giving up may hours of sleep to help me. All the lab technicians for answering my endless number of questions: Tim Bodine, Kathy Swenson, and Fifi Melouk.

Special thanks goes to my husband Paul, for all of his support and understanding through the entire process and for his words of encouragement at times of difficulty. Thanks also go to my parents for their support and instilling in me the ability to stick to my guns.

Finally I would like to thank the Animal Science Department and the Athletic Department for financial support during the past two years

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## NOMEMCLATURE

| | |
|---|---|
| Ca | Calcium |
| $CaCl_2$ | Calcium Chloride |
| Cr | Chromium |
| $Cr_2O_3$ | Chromic Oxide |
| $dH_2O$ | dionized water |
| DMD | dry matter digestibility |
| DMI | dry matter intake |
| FO | fecal output |
| ICAP | Inductively Coupled Argon Plasma Spectrometry |
| ICP | Inductively Coupled Plasma Spectrometry |
| $KBrO_3$ | Potassium Bromide |
| Mg | Magnesium |
| $MnSo_4$ | Manganese Sulfate |
| P | Phosphorus |
| q.s. | fill to volume |
| UV | ultra violet light |
| UO | urine output |

# CHAPTER I

## Introduction

Mineral nutrition is of considerable concern for equine producers as it plays a major role in healthy growth. Currently, much of the feed industry's focus has been in the area mineral requirements and their supplementation. This is especially true concerning phosphorus, as any excess phosphorus fed is excreted into the environment as a pollutant. Fortifying swine and poultry rations with the supplemental enzyme phytase has allowed producers to feed less phosphorus while still satisfying animal needs. As extensive research has been successfully conducted using phytase for swine and poultry, the possibility exists for its application in equine nutrition. Can phytase improve phosphorus utilization in the horse, and if calcium and phosphorus metabolism is related, will phytase affect calcium homeostasis?

Digestion trials are conducted to monitor the intake of nutrients in relation of the nutrients excreted in the feces by the animal. Fecal collections are required to conduct nutritional trials to answer these questions. It has been common practice in digestion trials to use an external indicator such as chromic oxide, to estimate fecal output and nutrient digestibility. However there has been evidence that suggests that chromic oxide may not be as reliable as total fecal collection for this purpose. Poor recovery of consumed chromium in feces would result in an underestimation of nutrient digestibility. Therefore as such total fecal collections would yield a more accurate estimation of fecal

output and subsequent nutrient digestibility.

The objective of this project was to: 1) to evaluate the differences in route of administration of chromic oxide and compare calculated fecal output values to a total fecal collection for the determination of dry matter digestibility, and 2) to evaluate the effects of phytase supplementation on nutrient digestibility in horses fed a common textured ration.

# CHAPTER II

## Review of Literature

## Experiment 1

### *Digestibility Markers*

Digestion trials require a record of nutrients consumed and nutrients excreted. It is essential to quantitatively evaluate the undigested feed residue in the feces. Total collections can be labor intensive, expensive, and may alter normal eating patterns yet it is the method that all indirect indicators must be compared to. External markers have long been used to estimate fecal output and dry matter digestibility when total fecal collections are not be done. An ideal marker should have the following characteristics 1) totally indigestible and unabsorbed, 2) pharmacologically inactive within the digestive tract, 3) pass through the tract at a uniform rate, 4) be readily determined chemically, and 5) preferably a substance naturally present in the feed (Maynard et al. 19790.

Markers can be divided into two types, internal and external. Internal markers are defined as a constituent of the ingested material and are indigestible and unabsorbed. Internal markers require little preparation because these markers are part of the cell wall fraction. Lignin and acid-insoluble ash, both examples of internal markers, are in higher quantities in roughage diets when compared to concentrate diets (Thonney et al., 1979).

External markers, such as chromic oxide or rare earth elements, are substances that must be added to the feed. External markers used in animal nutrition research include chromic oxide, ytterbium, lanthanum, cerium, and titanium dioxide (Pond and Houpt, 1986; Moore et al., 1992). These markers have been incorporated into the ration as, a pulse dosed, or in top-dressing. Chromic oxide is one of the most widely used digestion indicators. Various markers have been used in digestion trials since the early 1900's. In 1918, Edin was the first to propose chromic oxide as an indigestible substance suitable for use as an indicator. However, problems have been associated with the use of chromic oxide as incomplete recovery and difficulty in its analytical determination have been reported (Kotb and Luckey, 1972; McCarthy et al., 1974; Parr, 1977; Fenton and Fenton, 1979).

*Internal Markers*

*Lignin.* Lignin is a highly insoluble and biologically unavailable mixture of polymers of phenolic acids (Pond et al., 1995). Lignin is present in the bran portion of cereal grains and in the cell wall of plants. Lignin is regarded as being indigestible because there appears to be no known anaerobic microorganism or mammalian enzyme to degrade lignin (Fahey and Jung, 1983). However, there is conflicting evidence suggesting that lignin may be partially digestible. Louw (1941), Davis et al. (1947), and Bondi and Meyer (1948) observed that lignin might be degraded in the ruminal digestive tract while others have reported that lignin was indigestible (Ellis et al., 1946 and Kane et al., 1950).

Experiments were conducted to evaluate the extent of lignin degradation and the accuracy of lignin in the estimation of dry matter digestibility in cattle. Thonney et al. (1979) fed steers diets composed of 0, 20, 40, 60, 80% concentrate with the rest of the diet supplied by early or late cut grass hay. Maintenance level intake combined with a total fecal collection and permanganate lignin marker was used to determine dry matter digestibility. Dry matter digestibility was underestimated using the permanganate lignin (51%) compared to the total fecal collection (75%). These results suggest that lignin is degraded in the digestive tract since it failed to measure dry matter digestibility accurately. Porter and Singleton (1971) found that as much as 10.2% of dietary lignin is lost in the digestive tract of ruminants. They also concluded that the site of degradation is in the rumen because the concentration of lignin in the duodenum is similar to that of fecal lignin.

Another source of variability when using lignin as a marker is that analytical techniques for determining lignin are empirical at best and differ significantly in their estimation of plant lignin content (Fahey and Jung, 1983). Analytical methods used for determining lignin include 72% sulfuric acid detergent, permanganate, and acetyl bromide soluble lignin assays. The routine laboratory method is a 72% sulfuric acid technique which yields crude Klason lignin. Muntifering (1982) evaluated differences in apparent lignin digestibility due to roughage source and analytical method. Muntifering found that total tract lignin digestion was greatest for corn cobs, while tall fescue and cotton seed hulls did not differ. Muntifering also observed a marker x diet interaction and, of the three analytical methods, acetyl bromide soluble lignin overestimated the extent of ruminal organic matter digestion. Therefore, these results would demonstrate

that choice of analytical method and the extent of recovery may impact results of digesta flow measurements when using lignin.

The previous research suggests that lignin may in fact be degraded and solubilized in the digestive tract. As such, the use of this marker should be used with caution because an incomplete recovery of lignin will result in an underestimation of dry matter digestibility. Lignin should only be used as a marker if fecal recoveries of lignin are high (Fahey and Jung, 1983).

*Acid-Insoluble Ash.* Acid-insoluble ash is the fraction of feed which is insoluble in boiling hydrochloric acid (HCl). Acid-insoluble ash yields similar results to that of total fecal collection (McCarthy et al., 1974; Van Keulen and Young, 1977; Thonney et al., 1979; and Miraglia et al., 1999). Acid-insoluble ash has proven to be a reliable marker because there is little diurnal variation and analytical techniques are very precise. However, there are concerns of ingesting soil or bedding, which would contaminate analysis. Another problem arises when feeding grains and alfalfa, which contain low amounts of acid-insoluble ash. Therefore, sand, diatomaceous shells or silica are often added to the ration (Vankeulen and Young, 1977).

Van Keulen and Young (1977) evaluated three laboratory procedures with varying ashing sequences. The three methods were a concentrated HCl, 4N HCl, and 2N HCl. There was no significant difference in dry matter digestibility between the three analytical techniques, however the 4N HCl method did produce numerically higher values. Van Keulen and Young observed no diurnal variation in acid-insoluble ash in eight different rations. They concluded from their study that acid-insoluble ash is a good

marker method in determining dry matter digestibility when compared to a total fecal collection.

In agreement with Van Keulen and Young , McCarthy et al. (1974), Thonney et al. (1979), Van Leeuwen et al. (1996), and Miraglia et al. (1999) all reported that dry matter digestibility was not different for acid-insoluble ash and total fecal collection. McCarthy et al. (1974) concluded that when feeding diets with high amounts of acid – insoluble ash the analysis for detecting acid-insoluble ash becomes more reliable. Thonney et al. (1979) concluded that acid-insoluble ash was similar to total fecal collections when evaluating different concentrate: roughage ratios. Miraglia et al. (1999) found no significant difference between total fecal collection and acid-insoluble ash in the digestibility coefficients for dry matter, organic matter, energy, crude fiber, crude protein, ADF, NDF, nitrogen free extract and ether extract. Van Leeuwen et al. (1996) reported that any variation of acid-insoluble ash among diets is due to the variation in acid-insoluble ash in the feedstuffs. He suggested that diatomaceous shells should be added to rations to guarantee the analysis of acid-insoluble ash.

*External markers*

*Rare Earth Elements*. Several rare earth markers, including cerium (Ce), ytterbium (Yb), and dysprosium (Dy), have been employed as markers for digestibility and rate of passage studies. Rare earth elements can be used at a low concentration and are characterized as having a high binding affinity to forage. Teeter et al. (1984) observed that feeds absorbed increasing amounts of Yb as its concentration in the suspending fluid was increased. Teeter et al. (1984) also observed a plateau effect as

binding sites became saturated. This can be supported by the fact that there was little change in Yb concentration between 48 and 72 hours suggesting that equilibrium between feed binding sites and free Yb in solution was reached at 48 hours. Krysl et al. (1985) labeled two types of forage with Yb and found that estimated fecal outputs from Yb did not differ significantly from the total fecal collection. Krysl and others had recovery rates of Yb between 100% and 103%. Prigge et al. (1981) observed that a single dose of Yb gave values for digestibility which did not differ from total fecal collection. However, when dosing twice daily, estimated fecal outputs differed significantly form the total collection values. Prigge et al. (1981) found significant diurnal variation in Yb excretion and concluded that this variation was due to incomplete binding to the forage or the formation of insoluable hydroxides. These hydroxides would move through the digestive tract at a different rate. It appears that Ytterbium can be a reliable marker for fecal output and dry matter digestibility.

Cerium, another rare earth element, has been used to determine dry matter digestibility. Cerium-144 has been used recently in digestibility trials as an external indicator. The radioisotope technique has been highly desirable because of accuracy and the time required for analytical procedures. Studies using cerium-144 have recovered up to 96.5% of the fed cerium-144 (Knapka et al., 1967 and Miller et al., 1967). Knapka et al. (1967) determined that cerium-144 is not absorbed in the gastro-intestinal tract because there was no trace of radioisotope in the blood or urine.

Cerium-144 is a highly desirable external indicator. However, cerium-144 causes radio-contamination not only in the livestock areas used but in the lab equipment as well. Also, the use of radioisotopes are highly regulated. Many times in large animal nutrition

radioisotopes are not desirable because food animals would be contaminated and disposal of these animals would be difficult.

*Polyethylene glycol.* Polyethylene glycol (PEG) is a water-soluble marker that is almost completely recovered in the feces. Polyethylene glycol was found to be an ideal marker by Sperber, et al. (1953). Polyethylene glycol is of high molecular weight (MW 4000) and has been reported to have no significant absorption, precipitation or uptake by the rumen. A study conducted by Hopson and McCroskey (1972) wanted to observe the effects of dosage level and molecular weight of PEG in determining fecal output. Recovery rates ranged from 77.6 to 99.1% with the lowest recovery rate from the 200 g/d dosage. The poor recovery from the larger dosage could be due to the PEG loss in the dosing process. Hopson and McCroskey (1972) concluded that PEG follows the liquid phase of the digesta and should be used with a uniform forage for best recoveries. Knapka et al. (1967) observed little diurnal variation in PEG and concluded that PEG passed through the digestive tract at a uniform rate.

*Titanium Oxide.* Several insoluble metal oxides have been used in digestibility studies. The most common of these oxides is chromic oxide. However, titanium oxide has received attention by ruminant nutritionists in recent years. Titanium oxide is white, almost odorless, tasteless and is water and acid soluble (Peddie et al., 1982). Lloyd et al. (1954) observed incomplete recovery of titanium oxide in the feces resulting in a lower digestibility coefficient than that of chromic oxide. Delayed excretion was related to possible accumulation of the marker in the gastro-intestinal tract,most likely in the

cecum. In contrast, other researchers have found high recovery rates of titanium oxide in fecal material. Jagger et al. (1992) observed recovery rates of 98.3 % and 96.9% with 1g/kg and 5g/kg of titanium oxide, respectively. Slower feed intakes can possibly explain the lower recovery rate for the higher level of titanium oxide. Peddie et al. (1982) observed a recovery rate of 97.5% at 5g/kg and did not observe any palatability problems. Peddie concluded that titanium oxide met the criteria for an ideal marker: 1) it is apparently non-toxic, 2) does not effect food intake or digestibility, 3) easily determined by chemical methods, and 4) it can be recovered from the feces.

*Chromic Oxide.* Chromic oxide is the most widely used external indicator for determining fecal output and subsequent dry matter digestibility. Chromic oxide was introduced as an external maker by Edin (1918). Many researchers have discussed the problems associated with chromic oxide use. These problems consist of incomplete recoveries and large diurnal variation in the marker. Chromic oxide is a dense, green powder that has a specific gravity of 5.1, which allows it to travel slowly through the digestive tract. Chromic oxide has been shown to lodge in the crevices fiber allowing it to move with the fibrous rather than the soluble portion of the feed (King and Moore, 1957). Consequently, chromic oxide is not very useful in determining digesta retention

The use of chromic oxide as an external indicator began in the early 1950's. Dansky and Hill (1952) observed an average recovery rate of 95-96% when chromic oxide was added at .2% and 1% of the diet, however recovery rates have been reported to be low as 76% in chickens (Dansky and Hill 1952). These researchers concluded that the incomplete recovery was caused by the failure to accurately quantify fecal output. This

would result in a lower digestibility coefficient than that from the total fecal collection. In contrast, Clawson et al. (1955) found no significant difference in digestibility values when comparing total fecal collection to the chromic oxide marker method. Results from the previous mentioned study indicate that the chromic oxide concentration in the feces reaches equilibrium with feed consumed between three to four days after the initial feeding.

Smith and Reid (1954) evaluated diurnal variation and observed the lowest recovery of fecal chromium at 2:00 pm and the highest at 12:00 am. Smith and Reid (1954) found no significant difference in recovery rates and the estimation of FO and DMD when $Cr_2O_3$ was administered via a bolus or mixed in the concentrate.

Research has reported conflicting results associated with chromium recovery rates. Recovery rates have been reported as low as 35% but average 85-95% (Haenlein et al., 1966; Knapka et al., 1967; Kotb and Luckey, 1972; McCarthy et al., 1974; Parr, 1977; Fenton and Fenton, 1979; Jagger et at., 1992;). However, research also shows that recoveries can average around 99-100% (Prigge et al., 1981; Saha and Gilbreath, 1993; Bakker and Jongbloed, 1994). Explanations for these differences are fineness of the powder, confinement causing a decrease in retention time and physical form of the diet (Barnicote, 1945; Haenlein et al., 1966; Knapka et al., 1967; Sauer et al., 1979; Bakker and Jongbloed, 1994). Therefore, caution must be used when using chromic oxide as an external indicator.

*Purpose*

Chromic Oxide is the most common marker used in animal nutrition. In most cases the chromic oxide is administer through a bolus or incorporated in the feed. There has not been research in the area of comparing administration routes in animals especially the horse. Therefore, the purpose of this study was to evaluate the differences in mixed and top-dressed administration of chromic oxide and compare calculated fecal output to a total fecal collection for the determination of dry matter digestibility.

# CHAPTER III

## Materials and Methods

## Experiment 1

### *Experimental Design*

Eight Quarter Horse yearlings (four fillies and four geldings) were used in a split plot design experiment to estimate fecal output and dry matter digestibility using the different methods of chromic oxide administration. Horses were blocked by weight and sex and then randomly assigned to one of two treatment groups. Horses were weighed prior to the start of the trial. Body weight ranged from 331.1 kg to 404.5 kg, with an average of 382.4 kg for the group. Feed intake was set at 2.5% of the average body weight (9.56 kg). Horses were fed at 0700 and 1900 daily. The experimental period consisted of a 7-d adjustment period followed by a 72-h collection period. Horses were housed in 3.7 m x 3.7 m stalls and allowed free exercise (3 h/d) during the adjustment period and were hand walked (15-20 min/d) during the collection period. All horses were dewormed and vaccinated prior to the trial.

### *Experimental Ration and Treatments*

The diets consisted of a 1.3 cm pelleted concentrate of corn, soybean meal and dehydrated alfalfa meal fed in a 60:40 ratio with native prairie grass hay (Table 1).

Table 1. Composition of Diet, As Fed

| Ingredient (%) | Mixed Diet | Top-Dressed Diet |
|---|---|---|
| Corn | 16.9 | 17.0 |
| Wheat | 17.0 | 17.0 |
| Alfalfa Meal | 15.0 | 15.0 |
| Soybean Meal | 10.1 | 10.1 |
| Limestone | 0.15 | 0.15 |
| Trace Mineral Salt | 0.3 | 0.3 |
| Dicalcium Phosphate | 0.45 | 0.45 |
| Chromic oxide | 0.1 | -- |
| Prairie Grass Hay | 40 | 40 |
| **Nutrient Analysis**[a] | | |
| DE (Mcal/kg) | 2.5 | 2.5 |
| ADF % | 9.2 | 9.2 |
| NDF % | 17.4 | 17.4 |
| Crude Protein % | 14.9 | 14.9 |
| Calcium % | 0.57 | 0.57 |
| Phosphorus % | 0.47 | 0.47 |

[a]Calculated Values

Rations were formulated to meet or exceed current NRC (1989) requirements for yearling horses. The two methods of chromic oxide application were 1) mixed with the concentrate during pelleting (mixed diet) and 2) top-dressed on the concentrate prior to feeding (top-dressed diet). The chromic oxide was mixed with corn sugar syrup and poured onto the pelleted ration prior to feeding for the top-dressed treatment. Feed intake was calculated so that each horse would receive 12 g of chromic oxide (8 g of chromium) per day.

*Fecal Collection*

Total fecal output was collected throughout the 72-h collection period. Horses were tied on rubber mats on a wooden floor in box stalls during the collection to minimize stepping into the feces. Floors were swept regularly to reduce any contamination from urine. To measure total fecal output, feces were collected off the floor every two hours during each 24-h interval. Diurnal fecal samples were also collected at 0600, 1200, 1800, and 2400 to measure the variation in chromium excretion. Total feces were weighed at the end of each 24-h interval and aliquots were taken and frozen for subsequent analysis. Frozen samples were stored in freezer type plastic bags to ensure minimal moisture and microbial contamination. Fecal samples were then dried for 72 h at 50°C for dry matter determination.

*Feed Collection*

A single batch of feed was used for each treatment throughout this experiment. The feed was sampled by taking a sample from every fifth bag over the total batch of

feed (approximately eight samples total). The aliquots were taken from the top, middle, and bottom of each bag sampled.

*Laboratory Procedures*

*Chromic Oxide Analysis*. Feed and fecal samples were ground and then ashed at 500°C for 6 h. Phosphoric acid was added to dissolve and hydrolyze the ashed sample. Magnesium sulfate and potassium bromide was also added to the sample. The sample was placed on a hot plate until a color change was observed (purple or gray). After color change, the sample was cooled and dissolved with deionized water and calcium chloride. A full laboratory procedure can be seen in appendix A. Chromium analysis of the feed and fecal samples was completed using inductively coupled plasma spectroscopy (ICAP 61, Spectro Analytical Instruments, Fitchburg, MA).

A spike curve was generated to validate the ICAP machine for the level of chromium used. Ten random samples were ashed and digested according to the procedure in appendix A. Samples were spiked with 10 ppm and 20 ppm of chromium standard and were read on the ICAP (Appendix B) for chromium concentration. Results from this procedure found that there was no error associated with the chromium determination due to laboratory procedures.

*Statistical Analysis*

Data were analyzed using the general linear model procedure of SAS (SAS Inst. Inc., Cory, NC) with horse, treatment, and day as main effects. Least squares means

were calculated and differences between treatments were detected using the p-diff procedure of SAS (significance at $P<0.05$).

# CHAPTER IV

## Results and Discussion

## Experiment 1

### *Application Analysis*

Data for dry matter intake and the effect of chromic oxide application method on fecal output (FO) and dry matter digestibility (DMD) are reported in table 2. Dry matter intake did not differ significantly between treatments. Calculated FO was higher ($p<0.05$) in the mixed diet (4190g/d) versus the top-dressed (3338g/d). As a result, DMD was lower ($p<0.05$) in the mixed diet (56%) versus the top-dressed (64%). This disagrees with Smith and Reid (1954) who found no significant difference in the estimation of FO and DMD when $Cr_2O_3$ was administered via a bolus or mixed in the concentrate. In the present study, however, measured FO and subsequent DMD did not differ significantly between treatments. The difference observed in calculated values would indicate a lower recovery of chromium in the mixed diet versus the top-dressed. In the present study, the recovery rates of fecal Cr were 60% and 81% for the mixed and top-dressed diets, respectively. In support of these findings, Knapka et al. (1967) and Jagger et al. (1992) reported low recovery rates of Cr when using $Cr_2O_3$ as an inert marker. Research has also indicated that $Cr_2O_3$ produced significantly lower estimates for fecal output, which has been attributed to the fineness of the particle leading to retention by the

Table 2. Effect of Chromic Oxide Application on Intake, Fecal Output, and Dry Matter Digestibility[a]

| | Treatment | | |
|---|---|---|---|
| | **Mixed** | **Top-dressed** | **S.E.M.** |
| Intake, g | 9421 | 9442 | 24.15 |
| Fecal Output, g | | | |
| Measured | 2530[d] | 2693[d] | 97.71 |
| Calculated | 4190[be] | 3338[ce] | 61.63 |
| Dry Matter Digestibility, % | | | |
| Measured | 73[d] | 71[d] | 2.3 |
| Calculated | 56[be] | 64[ce] | 0.64 |

[a]Values are least square means.

[bc]Means within a row with different superscripts differ ($P<0.05$).

[de]Means within a column with different superscripts differ ($P<0.05$).

gastro-intestinal tract (Barnicote, 1945; Morre, 1959). The yearlings in the present study were on pasture prior to the start of the trial, and then confined to stalls immediately prior and during the trial. Knapka et al. (1967) suggests that confinement could alter gut motility, thereby restricting $Cr_2O_3$ movement and resulting in low recoveries.

Since there was a significant difference in FO and DMD between application method, all subsequent data will be reported within each treatment. Within the mixed diet, calculated FO (4190g/d) was higher ($P<0.05$) than the measured (2530g/d). Subsequently, DMD was lower ($P<0.05$) when using calculated FO values (56%) as compared to the measured values (73%). Concerning the top-dressed diet, calculated FO (3338g/d) was significantly higher than measured (2693g/d). As well, calculated DMD (64%) was significantly lower when compared to the measured (71%). These data suggest an overestimation of FO and subsequent underestimation in DMD of calculated values compared to the measured values.

*Feed Analysis*

The variation of chromium concentration in the feed is illustrated in figure 1. There was no significant variation in chromium concentration between the eight samples. This demonstrates uniformity throughout the batch of feed.

*Diurnal Analysis*

Changes in diurnal fecal chromium excretion can be seen in figure 2. There was no significant diurnal variation in fecal chromium excretion within each treatment. The present study suggests that two doses of 6g of $Cr_2O_3$ 12 h apart was sufficient to keep

diurnal variation to a minimum. Knapka et al. (1967) and Jagger et al. (1992) also found no significant variation in diurnal chromium excretion rates. Contrary to these findings, Haenlein et al. (1966) and Van Leeuwen et al. (1996) observed significant changes in diurnal chromium excretion in horses and pigs. The present study however does show that fecal excretion of Cr was significantly higher in the top-dressed diet compared to the mixed at all measured intervals. This further supports the previous data, which demonstrates a higher calculated FO in the mixed diet versus the top-dressed.

Daily chromium fecal excretion can be seen in figure 3. Within the mixed treatment, there was a significant difference in Cr fecal excretion between day 1 and day 3 ($P<0.05$). Within the top-dressed treatment, Cr fecal excretion was significantly less for day 1 when compared to days 2 and 3. This suggests that a 7-d adjustment period may not be long enough to achieve saturation of chromium in the equine gastro-intestinal tract. If saturation did not occur, then values for fecal output would be greater and dry matter digestibility would be less than values obtained from a total fecal collection.

*Implications*

The results from this trial reveal a significant difference in fecal output for calculated values when compared to a total fecal collection. This would result in an overestimation in the calculated fecal output and consequently an underestimation in dry matter digestibility. In addition, there was an observable effect in the application method. Therefore, this trial suggests that chromic oxide may not be a reliable marker for the determination of total fecal output and subsequent dry matter digestibility in the horse

**Figure 1. Variation in Chromium Concentration within the Mixed Diet**

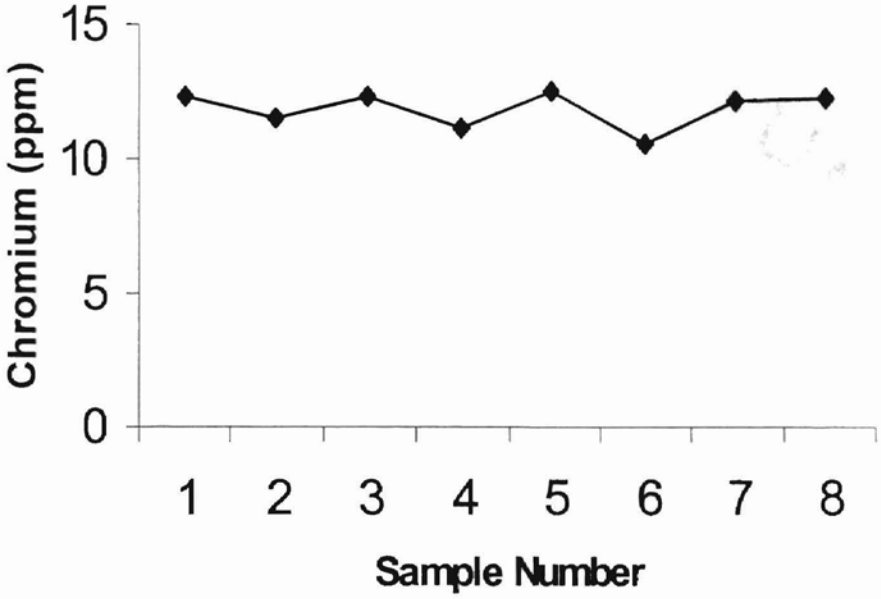
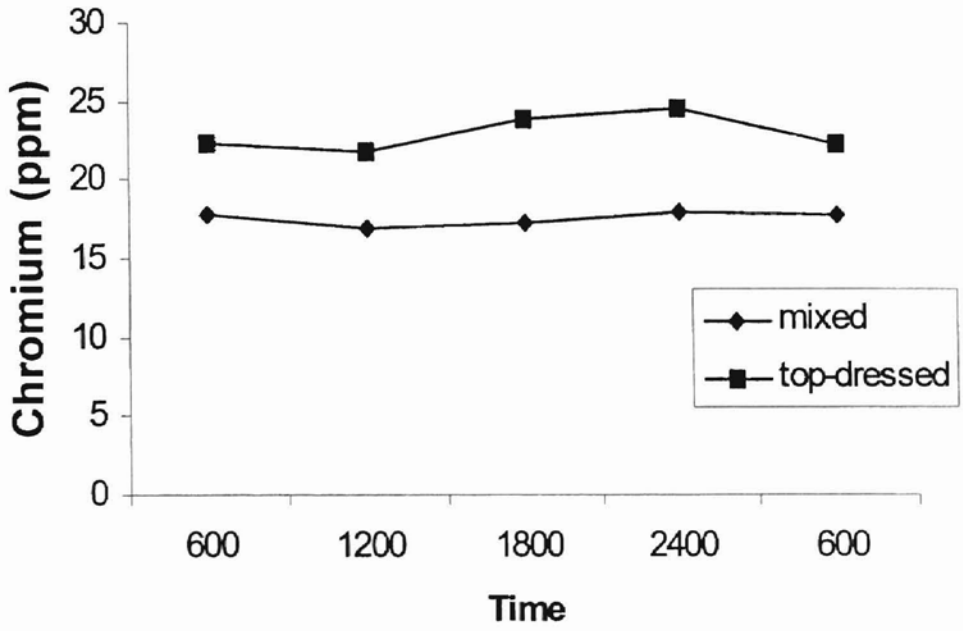
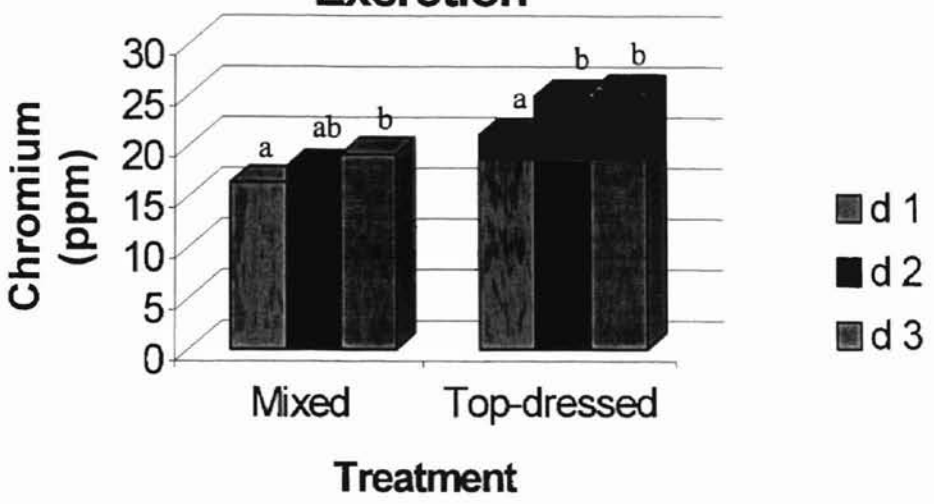



**Figure 2. Effect of Chromic Oxide Application on Fecal Chromium Concentration over a 24 Hour Period**



## Figure 3. Daily Fecal Chromium Excretion




[a,b] Days are different ($P<0.05$).

# CHAPTER V

## Review of Literature

## Experiment 2

### *Phytase and Phosphorus Availability*

*Phosphorus*. Phosphorus (P) is an essential nutrient in animal. Meaning the body can metabolize the element phosphorus. Phosphorus has many functions (Swenson and Reece, 1993; Pond et al., 1995) including:

1. Bone formation and maintenance
2. Teeth development
3. Muscle tissue and egg formation
4. Feed utilization
5. Maintenance of osmotic and acid-base balance
6. Component of nucleic acids which are important in genetic transmission and control of cellular metabolism
7. Energy utilization (phosphorylated carbohydrates and phosphate compounds such as adenosine triphosphate and creatine triphosphate
    a. Phospholipid formation
    b. Amino acid metabolism and protein formation
    c. Component of and activator of many enzyme systems.

Phosphorus is absorbed from both the small and large intestine, but in the horse phosphorus is primarily absorbed in the large intestine. In other animals, such as swine, dietary phosphorus is absorbed primarily in the small intestine, particularly the duodenum. Regardless of site, the amount of phosphorus absorbed is dependent upon the source, calcium: phosphorus ratio, intestinal pH and dietary levels of calcium, phosphorus, vitamin D, iron, aluminum, manganese and fat (Swenson and Reece, 1993). Excessive levels of calcium and other major minerals can tie up phosphate in precipitate form. Generally, the greater the dietary intake of phosphorus then the more phosphorus that is absorbed (Schryver et al., 1971). Absorption of phosphorus from the gastro-intestinal tract is facilitated by either active transport or passive diffusion across the intestinal lumen and can be stimulated by the presence of vitamin D (Swenson and Reece, 1993; Pond et al., 1995).

Once phosphorus is absorbed, it passes into the portal vein and either travels through the body or it can be incorporated into phospholipids in the cellular lining. Once in the portal vein, phosphorus is circulated throughout the body where it can be used for metabolic processes, especially bone development during periods of growth. Calcium and phosphorus are present in the bone primarily as apatite salt and as calcium phosphate and calcium carbonate (Schryver and Hintz, 1972; Jordan et al., 1975; Swenson and Reece, 1993). Bone is the reservoir for calcium and phosphorus in the body. In time of deficiency, the body will mobilize these reserves to maintain normal or constant levels in the blood and soft tissues. The plasma level of phosphorus is inversely related to the blood calcium level (Swenson and Reece, 1993).

Calcitonin and parathyroid hormone have similar functions concerning phosphorus, they both inhibit the reabsorption of phosphate in the tubules of the kidney (Swenson and Reece, 1993). The kidney, an important regulatory mechanism, excretes excess dietary calcium and phosphorus that is absorbed from the gastro-intestinal tract. Unabsorbed dietary calcium and phosphorus are excreted in the feces (Schryver et al., 1971).

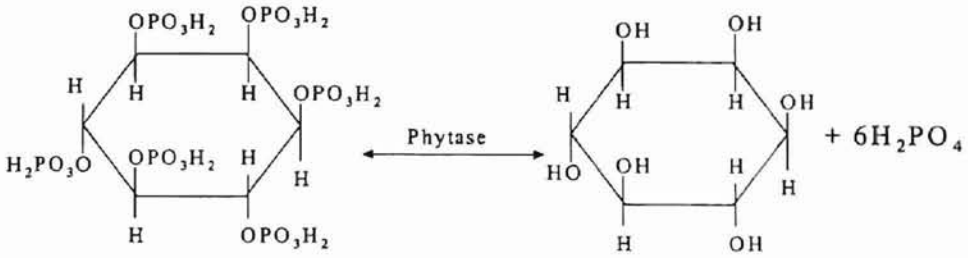
*Phytate Phosphorus*. Phytate (mycoinositol hexakiphosphate) is an effective chelating agent in that it can bind minerals and proteins in animal feedstuffs, and is considered a problem in mineral nutrition because of it's binding capabilities to calcium and phosphorus. Phytate can bind phosphorus, calcium, zinc, iron, and protein. Most of the phosphorus bound to phytate or phytin is not available to monogastric animals (Hintz et al., 1973). There has been some questions as to whether or not phytate is hydrolyzed in the intestine of nonruminants by the enzyme phytase. Researchers have reported phytase activity in the brush border of the small intestine of rats, rabbits, pigs and chickens (Cooper and Gowing, 1983; and Pointillart et al.,1984). If phytase is present in nonruminants, it is apparently not present in large enough quantities to notice significant differences in phosphorus digestibility (Wodzinski and Ullah, 1996).

Organic phosphorus is found in most feedstuffs, especially grains such as wheat bran, oats and corn. Phosphorus can also be found in the inorganic form in substances such as dicalcium and monosodium phosphate. However, a majority of the phosphorus found in cereal grains is in the form of phytate phosphorus, which is largely unavailable to the animal (Nelson, 1967; Hintz et al., 1973; Pointillart et al., 1984). According to

Nelson et al. (1968) phytate phosphorus can be found at relatively high concentrations in common feed grains such as corn (0.6%), hulled oats (1.2%), and soybean meal (1.4%). Phytate is the storage form of phosphorus in seeds and is the mixed salt of myo-inositol hexaphosphoric acid. Monogastric or nonruminant animals lack the ability to cleave the o-phosphate groups from the phytate molecule (Cromwell et al., 1993; Wodzinzki and Ullah, 1996). Monogastrics can utilize phosphorus from phytate only after the compound has been hydrolyzed to inorganic phosphorus. However, if there is not enough phytase enzyme present, an exogenous source of phosphorus must be supplied to meet phosphorus requirements.

*Phytase.* Phytate can be hydrolyzed by an enzyme called phytase. Phytase was described and extracted from rice bran by Suzuki et al. (1907). Phytase is present in some feed ingredients, can be produced by microorganisms and can occur in the intestine of some animals. Ruminants have been reported to possess the enzyme (Wodzinzki and Ullah, 1996). Shieh and Ware (1968) isolated an organism from the soil in a flowerpot that produced large amounts of phytase. This organism would later be identified as *Aspergillus niger*. Shieh et al. (1969) developed the strain by optimizing the media and the conditions for the production of the enzyme. The strain produced two types of phytases, phytase A and B. The phytase A had two pH optima one at pH 2.5 and at pH 5.5. The phytase B had a pH optima of pH 2.0. The International Union of Biochemistry in 1979 lists two phytases: 3-phytase, EC 3.1.3.8, which hydrolyzes the ester bound at the 3 position and the 6-phytase, EC3.1.3.26, which first hydrolyzes the ester bond in the 6-position and then subsequent ester bonds in the substrate are hydrolyzed at different rates.

The 6-phytase dephosphorylates phytic acid completely, whereas the 3-phytase does not (Wodzinzki and Ullah, 1996). The hydrolysis of phytic acid to inositol and phosphoric acid can be seen below:




Phytic Acid Inositol

Adapted from: Anderson, R.J., 1914

Phytase can come from three different sources: plant, bacterial and fungal. Suzuki et al. (1907) was the first to detect phytase activity in rice and wheat bran. Since then, phytase activity in germinating plants has been reviewed and has been isolated in cereals such as triticale, wheat, corn, barley, rice and several different beans (Reddy et al., 1982). Eeckhout and De Paepe (1994) measured phytase activity in several different cereals. Rye (85 nKat.[1]/g), triticale (28 nKat/g), wheat (20 nKat/g) and barley (10 nKat/g) were rich in phytase activity. As well, many wheat by-products contained phytase. Phytase activity has been observed in several varieties of bacteria; however, bacteria do not produce high yields of the enzyme. Howson and Davis (1983) surveyed

[1] Kat= katal which is unit of measurement to express activities of all catalysts being the amount of a catalyst that catalyzes a reaction of 1 mole of substrate per second.

84 fungi from 25 species for phytase production, most of which came from the soil. The highest yield was observed from *Aspergillus niger* which yielded 110 nKat/ml (Shieh and Ware, 1968). *Aspergillus niger* produces two different phytases, one with an optima at pH 5.5 and pH 2.5 and the other with optima pH of 2.0 (Shieh and Ware, 1968; Simons et al., 1990). Through recent DNA technology, strains of *Aspergillus niger* have been developed to economically produce large quanities of phytase for refinement and use in the feed industry.

Nelson was among the first researchers to investigate the phytate content in plants. Nelson et al. (1968) assayed for total phosphorus and isolated phytate phosphorus in several common feed grains. Nelson et al. (1968) found that there was a significant amount of phosphorus unavailable to the chick because of the chick's inability to utilize phytate phosphorus (Nelson, 1967). The phytic acid content of some commonly fed grains are as follows: soybean meal (1.3 %), corn (0.6 %), rolled oats (0.7 %), alfalfa meal (0.0 %), and wheat midds (1.2 %). These values can be useful when formulating diets for adequate available phosphorus. Bone ash is an effective way to evaluate hydrolysis of phytate in chickens. Bone ash is more practical for evaluating the availability of dietary phosphorus than body weight as it more accurately reflects changes in bone mineral content.

Nelson continued his research by adding the phytase enzyme to chicken diets. Nelson et al. (1971) conducted a trial to observe the effect of adding a mold phytase on the utilization of phytate phosphorus by chickens. Phytase was added to the diet at levels ranging from 1 to 8 g/kg of the diet. One gram of the supplemental phytase hydrolyzed 950 mg of inorganic phosphate from calcium phytate in vitro in one hour under standard

conditions. The addition of phytase increased the percentage of bone ash indicating hydrolysis of phytate by the enzyme. Total hydrolysis occurred at the level of 3g/kg of supplemental phytase. Chickens can utilize hydrolyzed phytate phosphorus as well as supplemental inorganic phosphorus.

BASF and other biotechnology companies claim that phytase is able to increase phosphorus, calcium, magnesium, zinc, and protein digestibility. Phytase can also decrease the amount of phosphorus needed to meet dietary requirements thus lowering fecal phosphorus content. Recent research has evaluated the effects and limitations of phytase on mineral digestibility and fecal phosphorus.

Cromwell et al. (1993) conducted a trial to evaluate the efficacy of dietary phytase as means to improve phosphorus availability and possibly eliminate the need for inorganic phosphorus. This trial fed 225 pigs different levels of phosphorus (adequate and inadequate) and then supplemented half the diets with 500 units/g of phytase. They observed a decrease in growth performance and bone strength in the pigs with inadequate phosphorus; however this was less noticeable when the pigs were fed phytase. Bone strength was increased as the level of supplemental phytase was added, although this improvement was not seen in growth performance. The addition of phytase increased the availability of phosphorus from 25% to 58% in the soybean meal basal ration. In addition, the absorption of phosphorus increased in the pigs supplemented with phytase. Therefore they concluded that phytase improved the bioavailability of phytate phosphorus.

Simons et al. (1990) conducted three experiments with pigs to evaluate the improvement of phosphorus availability by microbial phytase. In the first experiment,

phytase was added to ground maize and soybean meal in an in vitro study. Phytase showed maximal activity at pH 5.5 and at pH 2.5. The soybean meal contained 12.1 g of phytic acid/kg DM and after complete hydrolysis 110 mmol of free phosphate/kg was formed. The maize contained 6.94 g of phytic acid/kg and after complete hydrolysis 63 mmol of free phosphate/kg was formed. This suggests that phytase can degrade phytic acid completely and liberate most of the phytate phosphorus in maize and soybean meal diets.

The second experiment with broilers was conducted to evaluate nutrient availability with the addition of phytase to a basic poultry ration. The apparent availability of phosphorus was significantly increased by adding different levels of phytase. As well, the availability of calcium was also improved. Growth rate and feed conversion on the low phosphorus diet with added phytase was significantly improved.

The objective in the third experiment, was to evaluate the effects of supplemental phytase in pig diets, similar to the previous chicken trial. Phytase did not affect dry matter digestibility. The addition of phytase did significantly improve phosphorus digestibility. As a result, the fecal concentration of phosphorus decreased by about 35% and the apparent absorption of phosphorus increased.

Hans Fisher (1992) evaluated the effects of low calcium and high amounts of cholecalciferol on low phosphorus diets. He observed that chickens on low phosphorus diets had a decrease in body weight and decrease in bone ash when compared to chickens fed adequate phosphorus. This reduction was caused by the decrease in phosphorus retention. The phytate phosphorus in the diets was held constant at 0.24% of the diet. Phytate phosphorus digestibility was improved by 50% with the combination of the

reduced calcium intake and the increased cholecalciferol intake. Fisher also observed that low levels of inorganic phosphorus increased circulating levels of cholecalciferol. Fisher concluded in chickens that the negative effects of phytate phosphorus in a low phosphorus diet could be reversed by reducing dietary calcium and increasing cholecalciferol.

Pointillart et al. (1984, 1985, and 1989) conducted a series of experiments to evaluate phytate phosphorus and phosphorus utilization in relation to various diets and calcium levels. In the first experiment, large white pigs were used to evaluate phytate and phosphorus utilization on a corn and wheat diet. Utilization of phosphorus was the highest for pigs on the wheat diet when compared to the corn diet. Wheat has a higher amount of plant phytase. Pointillart et al. (1984) also observed a stimulation of vitamin D metabolism to counteract the phosphorus deficiency in the corn diet.

The second experiment, also using pigs, evaluated intestinal phosphatase activity on diets with high amounts of phytate phosphorus. Pointillart et al. (1985) observed that mucosal phytase and intestinal alkaline phosphatase activities were not affected by phytate phosphorus content in the diet or if the animal is phosphorus deficient. The difference in mineral utilization of a high and low phytate diet depends on two factors: total phosphorus intake and the unavailable calcium-phytate formation.

In the third experiment Pointillart et al. (1989) wanted to evaluate the effects of calcium level on phytate utilization. In comparing low and high calcium diets, the high calcium diet caused the pigs to be phosphorus deficient. This was evidenced by a decrease in phosphorus bone content and severe hypophosphatemia (Pointillart et al., 1989).

There is a close relationship between calcium, phosphorus and the availability of phytate phosphorus. The calcium:phosphorus ratio may be important when using microbial phytase in pig diets. A high dietary level of calcium may decrease phytate phosphorus bioavailability by forming a complex with phytic acid that is less available for degradation by phytase (Wise, 1983 and Fisher, 1992). Furthermore, a high dietary level of calcium may directly reduce the activity of the phytase enzyme and limit phytase efficacy (Lei et al., 1994).

Qian et al. (1996) evaluated the effect of calcium: phosphorus ratios on phytase efficacy in pigs. This study was conducted with pigs using three Ca:P ratios (1.2, 1.6, and 2.0:1), two available phosphorus levels (0.07 and 0.16%), and two levels of phytase (700 and 1050 units/kg of diet). They observed a linear decrease in average daily gain and apparent digestibility of calcium and phosphorus as the calcium: total phosphorus ratio became wider. The 1050 units/kg of phytase resulted in greater digestibility of phosphorus, but did not affect calcium or dry matter digestibility. Fecal phosphorus excretion was decreased with increasing levels of phytase and was not affected by the Ca:P ratio. Shear force, stress energy and percent bone ash of the metacarpal and $10^{th}$ rib linearly decreased as the calcium:total phosphorus became wider. An interaction of available phosphorus and phytase for the $10^{th}$ rib indicated a response to the diet containing the higher level of available phosphorus level.

Qian et al. (1996) also observed a decrease in phytase efficiency do to wider calcium:phosphorus ratios. At the Ca:P ratios utilized in this study, the decrease in performance, bone characteristics, phosphorus digestibility and phosphorus excretion could be attributed to a reduction in phytase activity that was adversely affected by the

wide calcium:phosphorus ratio. Therefore, they concluded that supplementation of phytase in pig diets should be used with a narrow calcium:total phosphorus ratio (1.2:1).

Swine and poultry nutrition has established that phytase can increase phosphorus utilization, decrease the amount of phosphorus fed and decrease phosphorus fecal excretion. Therefore, research has turned toward the efficacy of phytase in equine diets. Two studies have been conducted with horses evaluating the use of phytase. Hainze et al. (2001) conducted an experiment using yearling geldings to evaluate fiber utilization with and without microbial phytase. These researchers found no significant difference in fiber digestion with the supplementation of phytase. Morris-Stoker et al. (2001) conducted an experiment using mature geldings to evaluate the effects of phytase on nutrient digestibility. These horses were fed a common textured diet with 200 units/g of supplemental phytase. There was no significant increase in digestibility for phosphorus, calcium, magnesium or dry matter.

*Purpose*

Equine studies have been inadequate in determining the efficacy of supplemental phytase in equine nutrition. Phosphorus pollution in ground water has been an environmental concern for both the swine and poultry industry for several years. This may soon be an issue in the equine industry with the growing interest in horses. Also, calcium and phosphorus digestibility is a major concern in growing horses in the promotion of healthy bone growth. Therefore, the purpose of this study was to evaluate the effects of varying levels of phytase on nutrient digestibility in a common textured ration.

# CHAPTER VI

## Materials and Methods

## Experiment 2

### *Experimental Design*

Four mature sedentary Quarter Horse gelding were used in a 4 x 4 Latin square design experiment to study the effects of Phytase (NATUPHOS® 600[2]) on phosphorus availability and mineral balance in mature horses fed varying levels of Phytase. Each horse was on each treatment diet only once (Table 3). Horses were weighed prior to the start of the trial. Body weight ranged from 569.7 kg to 615.2 kg with an average of 594.7 kg for the group. Feed intake was set at 2% of the group's average body weight (11.82 kg) to ensure equal intake of the enzyme. During each of the four experimental periods, horses were fed one of four experimental diets during an 11-d adjustment period followed by a 72-h collection period. Horses were allowed 5 h to eat and were then turned out into a dry lot for exercise. Routine deworming were completed throughout the trial.

### *Experimental Ration*

Diets consisted of a textured concentrate of rolled corn, oats and soybean meal fed in a 50:50 ratio with native prairie grass hay (Table 4). Rations were formulated to meet

Table 3
Latin Square Design

| | Horse 1 | Horse 2 | Horse 3 | Horse 4 |
|---|---|---|---|---|
| Period 1 | 0 | 300 | 600 | 900 |
| Period 2 | 300 | 600 | 900 | 0 |
| Period 3 | 600 | 900 | 0 | 300 |
| Period 4 | 900 | 0 | 300 | 600 |

0 → Control
300 → 300 FTU/kg of Natuphos® 600[2]
600 → 600 FTU/kg of Natuphos® 600[2]
900 → 900 FTU/kg of Natuphos® 600[2]

[2] The NATUPHOS 600 product was donated courtesy of the BASF Corporation Wyandotte, Wisconsin.

Table 4
Composition of Diet, As Fed Basis

| | Treatment | | | |
|---|---|---|---|---|
| **Ingredient %** | **0** | **300** | **600** | **900** |
| Rolled corn | 19.3 | 19.3 | 19.3 | 19.3 |
| Whole oats | 22.5 | 22.5 | 22.5 | 22.5 |
| Soybean meal | 4.7 | 4.7 | 4.7 | 4.7 |
| Molasses | 2.5 | 2.5 | 2.5 | 2.5 |
| Limestone | 0.75 | 0.75 | 0.75 | 0.75 |
| Salt | 0.25 | 0.25 | 0.25 | 0.25 |
| Vitamin A 30 | 0.04 | 0.04 | 0.04 | 0.04 |
| **Natuphos® 600** | **--** | **0.025** | **0.05** | **0.075** |
| Prairie Hay | 50 | 50 | 50 | 50 |
| Diet Analysis, DM Basis | | | | |
| **Nutrient %** | | | | |
| DE (Mcal/kg)[a] | 2.5 | 2.5 | 2.5 | 2.5 |
| CP | 9.23 | 6.68 | 9.68 | 9.78 |
| Ca | 0.55 | 0.64 | 0.85 | 0.62 |
| P | 0.30 | 0.28 | 0.27 | 0.25 |
| Mg | 0.22 | 0.21 | 0.21 | 0.20 |

current NRC (1989) requirements for mature sedentary geldings and contained equivalent amounts of energy, protein, calcium, and phosphorus (Table 5). The treatments were as follows: 1) control (0) with no Phytase added to basal ration, 2) 300 FTU/kg added to basal ration, 3) 600 FTU/kg added to basal ration, and 4) 900 FTU /kg added to basal ration. There was a significant difference in calcium concentration for the 0 and 600 diets. This difference may have been the result of an unintentional mixing error or variability in feed ingredients. When concentrate samples were ashed a greater amount of ash was detected in the 600 diet The Phytase enzyme was added in powder form to the rations. Horses were fed at 0700 and 1600 and were allowed 5 h to eat. Fresh clean water was available at all times.

*Mineral Balance*

During the 72-h collections, total urine and feces were collected. Urine production was collected via a urine harness. Urine was collected every 2 h and measured for volume. An aliquot sample representing 4% of total volume was then composited and frozen for future analysis. Horses were tied on rubber mats for total fecal collection. Feces were collected off a concrete floor every 2 h and composited. At the end of each 24-h period, feces were weighed and aliquots were taken and frozen. Frozen samples were stored in freezer type plastic bags to ensure minimal moisture and microbial contamination. The three sub-samples were consolidated for each period and dried for 72 h at 50°C for dry matter digestibility and mineral analysis.

*Laboratory Procedures*

*Urine Mineral Analysis.* Urine mineral values for phosphorus ($P^{+}$), calcium ($Ca^{++}$) and magnesium ($Mg^{++}$) were analyzed using the composited frozen urine samples. Samples were thawed and analyzed by the Clinical Pathology division of Oklahoma State University Veterinary Teaching Hospital (Appendix D). Urinary $Ca^{++}$, $P^{+}$, and $Mg^{++}$ were analyzed using a Vitros 250 analyzer (Johnson & Johnson Clinical Diagnostics, Inc., Rochester, NY).

*Fecal and Feed Mineral Analysis.* Fecal mineral concentrations were determined by Dairy One Forage Testing Laboratory, in Ithaca NY (Appendix C). Duplicate samples were ashed in a muffle furnace at 500°C for four hours. Three ml of 6N HCl are added to the ashed residue and evaporated to dryness on a 100°C hot plate. Minerals were extracted with an acid solution of 1.5 $NHNO_3$ + .5N HCl, and determined using an Inductively Coupled Plasma Spectrometery (ICAP-61 Thermo Jarrell Ash Corporation, Franklin MA.)

*Statistical Analysis*

Data were analyzed using general liner model procedure of SAS (SAS Inst. Inc., Cory NC) with horse treatment and period as main effects. Least squares means were calculated and differences between treatments were detected using the p-diff procedure of SAS (significance at $P<0.05$).

# CHAPTER VII

## Results and Discussion

## Experiment 2

### *Dry Matter Analysis*

Data for daily dry matter intake (DMI), fecal output (FO), dry matter digestibility (DMD) and urine output (UO) are shown in table 6. There was no significant difference ($P>.05$) in DMI, FO, DMD, and UO across all diets. Dry matter digestibility ranged from 59% to 61%. Dry matter digestibility values observed in the present study differ from Baker et al. (1998) who reported values ranging from 48% to 52% in mature horses. These higher DMD values may be the result of taking a total fecal collection in the present study compared to using the chromic oxide marker method in the previous study. Results of experiment 1 demonstrated that lower DMD values were obtained from using chromic oxide when compared to values obtained from a total fecal collection. Phytase did not affect DMD at any level. This is in agreement with others who have observed no effect of phytase on DMD in rations fed to pigs and horses (Kornegay and Qian, 1996; Yi et al., 1996; Harper et al., 1997; Hainze et al., 2001; Morris-Stoker et al., 2001).

Table 5

Effect of Phytase on Intake, Fecal Output, Urine Volume, and Dry Matter Digestibility[ab]

| | Treatment | | | | |
|---|---|---|---|---|---|
| **Item** | **0** | **300** | **600** | **900** | SEM |
| Concentrate Intake g/d | 5902 | 5902 | 5902 | 5902 | -- |
| Forage Intake g/d | 5419 | 5334 | 5391 | 5618 | -- |
| Total DM Intake g/d | 11321 | 11236 | 11293 | 11520 | 308.83 |
| Feces g/d | 4678.0 | 4446.3 | 4587.8 | 4487.3 | 495.68 |
| DM Digestibility % | 59.0 | 60.5 | 59.5 | 61.0 | 3.72 |
| Urine ml/d | 4091.5 | 5428.3 | 5258.5 | 5358.3 | 463.74 |

[a]Values are least squares means.

[b]No significant difference was observed between treatment means.

*Mineral Analysis*

*Phosphorus.* Data for the effects of phytase on phosphorus balance and digestibility are shown in table 7. Phosphorus intake decreased significantly between the 0 and the 900 diets. These differences observed in intake are probably due to the low standard error (0.246) among horses across treatments. The NRC recommends an absolute phosphorus intake of 14 g/d for mature geldings. The phosphorus intake in the present study ranged form 28 g/d to 33 g/d, this exceeds their requirement. Fecal and urinary excretion of phosphorus was not significantly different across treatments. This contradicts findings from others in pigs (Qian et al., 1996; Harper et al., 1997) who have reported up to a 21% reduction in phosphorus excretion. Phosphorus balance and digestibility were not significantly improved with increasing amounts of phytase supplemented to the diet. This agrees with Morris-Stoker et al. (2001) who also observed no significant increase in phosphorus digestibility with addition of phytase to horse diets.

Phosphorus digestibility in the present study ranged from 11 to 17%, which is low, compared to NRC for mature horses (30 to 50%). However these values are in agreement with others (Hintz et al., 1973; Baker et al., 1998; Morris-Stoker et al., 2001) who also reported low phosphorus digestibilities. Phytase has been shown to increase phosphorus digestibility (Nelson et al., 1971; Cromwell et al., 1993; Qian et al., 1996; Harper et al., 1997) in pigs and chickens. However, these previous diets contained phosphorus below NRC recommended levels and supplemental phytase improved the digestibility of phosphorus to the level of the basal ration. Furthermore, the improvement in phosphorus digestibility with supplemental phytase did not exceed the basal ration with zero supplementation. Phosphorus intake for horses in the present study exceeded

Table 6

Effect of Phytase on Mineral Balance and Digestibility[a]

| | Treatment | | | | |
|---|---|---|---|---|---|
| **Mineral g/d** | **0** | **300** | **600** | **900** | **SEM** |
| Phosphorus | | | | | |
| Intake | 32.83[b] | 29.21[c] | 29.93[c] | 28.22[d] | 0.246 |
| Urine | 0.11 | 0.08 | 0.10 | 0.07 | 0.014 |
| Fecal | 27.23 | 25.57 | 26.38 | 25.04 | 1.575 |
| Balance | 5.51 | 3.56 | 3.45 | 3.10 | 1.682 |
| Digestibility % | 17.04 | 12.06 | 11.34 | 11.09 | 5.96 |
| Calcium | | | | | |
| Intake | 61.14[b] | 70.79[c] | 95.43[d] | 70.85[c] | 0.653 |
| Urine | 12.38 | 16.28 | 16.13 | 16.78 | 1.534 |
| Fecal | 28.74 | 36.90 | 36.60 | 35.43 | 3.209 |
| Balance | 20.01[b] | 17.63[b] | 42.69[c] | 18.66[b] | 4.165 |
| Digestibility % | 53.08 | 47.29 | 61.51 | 49.73 | 4.75 |
| Magnesium | | | | | |
| Intake | 23.78[b] | 22.47[c] | 23.15[bd] | 21.89[ce] | 0.183 |
| Urine | 6.38 | 7.73 | 8.18 | 7.93 | 0.808 |
| Fecal | 13.62 | 12.08 | 12.95 | 11.58 | 0.699 |
| Balance | 3.89 | 2.67 | 2.04 | 2.38 | 0.963 |
| Digestibility % | 42.96 | 46.05 | 43.71 | 47.02 | 3.43 |

[a]Values are least squares means

[bcde]Means within a row with different superscripts differ $p<0.05$

the recommended level set forth by the NRC. Therefore, any possible improvements in phosphorus digestibility may not have been observed due to the high levels of phosphorus in the diets.

*Calcium.* Data for the effects of phytase on calcium balance and digestibility are shown in table 7. Horses consuming the 0 diet had lower ($P<0.05$) calcium intake as compared to diets 300, 600 and 900. This difference may be explained by the fact that horse had a large refusal for diet 0 during the first rotation. The higher intake for calcium on the 600 diet is due to an unintentional mixing error when adding limestone to the diet. As preciously mentioned calcium levels in the 600 diet were 25 to 30% higher than the other diets. The NRC recommends an absolute calcium intake of 20 g/d for mature geldings. The calcium intake for the present study ranged from 61 g/d to 95 g/d which greatly exceeds the calcium requirement. There was no significant difference in fecal or urinary calcium excretion across all diets. The 600 diet had a higher ($P<0.05$) calcium balance (42.69 g/d) when compared to the 0, 300, and 900 diets (20.01, 17.63, and 18.66 g/d respectively). This increase in balance can also be explained by the higher calcium intake for that diet. There was no significant difference in calcium digestibility across all treatments, however, there was a trend ($P = .07$) for the 600 diet to be higher (61.51%) when compared to the 300 diet (47.29). Increases in calcium digestibility with addition of phytase have been observed in pigs and chickens (Qian et al., 1996;Gordan and Roland, 1998)

Qian et al. (1996) evaluated the effects of calcium:phosphorus ratio on the efficacy of phytase. These researchers observed an increase in phosphorus and calcium

digestibility with the addition of phytase. However, as the calcium:phosphorus ratio widened, the improvement in calcium and phosphorus digestibility decreased. The degree of reduction in phytase efficacy for increasing 0.1 unit of Ca:total P was approximately 2%. The actual Ca:P ratios in the present study ranged from 1.8:1 to 3.1:1. This exceeds the recommendation of Qian et al., (1996) of 1.2: 1. This low ratio for calcium:phosphorus is currently not recommended in most equine diets. The NRC states that a ratio below 1:1 may be detrimental to calcium absorption. If the phosphorus content exceeds calcium in a diet this will lead to interference of calcium absorption. If the animal's demand for calcium is not met through the diet then the body will begin to pull calcium from the bone. This can result in weak bones, laminitis, and enlarged facial bones (Swenson and Reece, 1993). Formulating diets with a Ca:P ratio of 1:1contains an inherent risks, especially if mixing errors occur. Furthermore, the modern equine feed industry will probably not formulate diets with a ratio less than 2:1 due to the fact that feedstuffs can possess variability in nutrient concentration. Horses can tolerate calcium: phosphorus as high 6: 1 without adverse effects on phosphorus (Jordan et al., 1975). Therefore it is safer to formulate diets with a wide calcium:phosphorus ratio.

*Magnesium.* Data for the effects of phytase on magnesium balance and digestibility are shown in table 7. Magnesium intakes were significantly different ($P<0.05$) between treatments. These statistical differences observed in intake were due to the low standard error (0.183) among horses across treatments. The NRC recommends a magnesium intake of 7.5 g/d for mature geldings. The magnesium intake for the geldings in the present study ranged from 22 g/d to 24 g/d, which exceeds the suggested

magnesium intake. Fecal and urinary excretion of magnesium was not significantly different for all treatments. Magnesium excretion values are similar to values reported by Baker et al., (1998) in mature sedentary horses. Magnesium balance and digestibility were not significantly improved with increasing levels of phytase. These findings are in agreement with Morris-Stoker et al. (2001), who observed no significant difference in magnesium digestibility with the addition of supplemental phytase in horses. Although this contradicts findings by Rimbach et al. (1995) in which supplemental phytase improved the apparent absorption of magnesium in rats.

*Implications*

The results of the present study show that the addition of phytase to a textured horse ration did not improve dry matter, phosphorus, calcium, or magnesium digestibility in mature horses fed up to 900 FTU/kg of NATUPHOS 600. Furthermore, there was no decrease in fecal phosphorus excretion. Possible reasons for the lack of effect of phytase could be due to the wide calcium:phophorus ratio in the diet, the a high phosphorus content in the grain, or the level of supplemental phytase added to the diet. Further research needs to be conducted to characterize the phosphatase enzymes present in the equine intestine and further evaluate the effects of phytase on nutrient digestibility utilizing different rations.

# CAHPTER VII

## Summary and Implications

Experiment 1

Experiment 1 indicates a significant difference in fecal output for calculated values when compared to total measured. This resulted in an overestimation in the calculated fecal output and consequently an underestimation in dry matter digestibility. In addition, there was an observable effect in the application method. Therefore, this trial suggests that chromic oxide may not be a reliable marker for the determination of total fecal output and subsequent dry matter digestibility in the horse. In which case, a total fecal collection was performed in experiment 2.

Experiment 2

The results of experiment 2 indicate no significant improvement in nutrient digestibility at any level of supplemental phytase. There was no improvement in dry matter, phosphorus, calcium, and magnesium digestibility with the addition of phytase. As well as there was no decrease in fecal phosphorus excretion, which has been observed in swine and poultry.

To improve the results of experiment 2 some directions should be taken with equine studies. First, formulate diets with phosphorus concentrations at NRC levels or slightly lower, and maintain a narrow calcium:phosphorus ratio. Second, use a level of

phytase higher than 900 FTU/kg. Finally, conduct an in vitro study to characterize the phosphatase, including phytase, enzymes present in the equine intestine. This would allow for further understanding of the action and efficacy of phytase in equine nutrition.

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# Appendix

## Appendix A

*Chromium Analysis*

The following is the procedure for fecal chromium analysis adapted form Tim Bodine Ruminant Nutrition Laboratory Technician at Oklahoma State University.

1. Ash 1 g (! 0.0010 g) of sample in 50 ml glass beaker
2. Put 12.5 ml of $CaCl_2$ (using Repipet or repeating pipettor) into labled 100 ml volumetric flasks, cover and set aside
3. Add 3 ml of Solution A (using Repipet or repeating pipettor) to 50 ml beakers with ashed sample
4. Add 4 ml of $KBrO_3$ (using Repipet or repeating pipettor) to 50 ml beakers with ashed sample and Solution A and mix thoroughly
5. Place on pre-heated hot plate and cover with watch glass
6. Allow to digest until bubbles stop forming (may turn purple or gray)
7. Remove from heat and allow to cool
8. Dissolve with $dH_2O$ (hot works best) and transfer into the 100 ml volumetric flask
9. Cover top of 100 ml volumetric flask with Parafilm and mix thoroughly by inversion
10. Allow to stand for at least 1 h (will be stabel for at least 24 h) prioe to reading on ICP
11. If reading on AA allow to stand for at least 1 h, pippet off 1 ml from top of 100 ml volumetric into 20 ml scintillation vial and dilute with $ddH_2O$ (1:6 for fecal)

Solutions:

Solution A – 1030 ml (approximately 170 samples in duplicate)

- Mix 1 L 85% phosphoric acid and 30 ml 1% $MnSO_4$ (7.5786 g $MnSO_4$ 1 $H_2O$ dissolved in $dH_2O$ in 100 ml volumetric )

$MnSO_4$, 1% -- 100 ml (approximately 560 samples in duplicate)

- 7.5786 g $MnSO_4$ 1 $H_2O$ in a 100 ml volumetric, q.s. to 100 ml with $dH_2O$

$KBrO_3$, 4.5% -- (approximately 12 samples in duplicate/ 120 samples in duplicate)

- 4.5 g $KBrO_3$ in a 100 ml volumetric, q.s. to 100 ml with $dH_2O$ (45 g $KBrO_3$ in a 1L volumetric, q.s. to 100 ml with $dH_2O$

$CaCl_2$, 4000ppm – (approximately 40 samples in duplicate)

- 11.1 g $CaCl_2$ 2 $H_2O$ in a volumetric, q.s. to 100ml with $dH_2O$

## Appendix B

*ICAP*

The ICP is used for the determination of major and trace metallic elements in solution. An ICAP (Inductively Coupled Argon Plasma) can perform multielement analysis over a wide concentration range. The plasma is a gas in which a significant fraction of its atoms or molecules are ionized. Interactions between plasma and argon gas form a magnetic field with charged particles. Plasma is formed by placing a quartz torch inside an induction coil. The induction coil injects free electrons into argon gas, then generates an oscillating magnetic field that causes electrons to form currents. The sample is injected into the center of the plasma. Atoms from the sample absorb energy and become excited. These atoms are unstable and emit energy in the form of photons of light. The energy from the atoms corresponds to wavelengths in the UV spectrum. Each energy level results in a different wavelength. The light energy is sent to a channel card, which integrates and produces a voltage proportional to the electrical energy. The ICAP can read UV light wavelengths ranging 200 to170 nm and the computer selects the most appropriate line for each element. A computer completely operates the spectrometer system. The computer calibrates, standardizes, analyzes, and displays the data.

# Appendix C

*Mineral Analysis*

*Fecal.* This mineral procedure is adapted from the protocol followed by the Dairy One Forage Lab in Ithaca, NY. This procedure for mineral analysis used in determining mineral concentrations of the fecal, grain, and forage samples in experiment 2. Elements including Ca, P, Mg, Na, Fe, ZN, Cu, Mn, Mo, and Co analyzed using a Thermo Jarrell Ash IRIS Advantage Inductively Coupled Plasma (ICP) Radial Spectrometer.

1. General feeds and forage types- Samples were ashed in muffle furnace at 500°C for 4 h. Three ml of 6N HCl are added to ash residue and evaporated to dryness on a 100°-120°C hot plate. Mineral extract with acid solution (1.5N $HNO_3$ + 0.5N HCl) and determined using an IRIS Advantage.
2. Grain and Mineral Mixes – High Organic Matter (OM) mixes ashed 2 h at 500°C. Low OM samples not ashed. Ten ml Mineral Mix extracting solution (1.8N HCl + 0.3 $HNO_3$) added to sample and digested on 100° - 120°C hot plate. Filtered through Whatman 4 filter paper into volumetric flasks using 1.5N $HNO_3$ + 0.5N HCl and minerals determined using an IRIS Advantage

## Appendix D

*Urine.* This mineral procedure is adapted from the protocol used by the Oklahoma State University Veterinary Clinical Pathology Laboratory. This procedure is used to analyze urine for mineral concentration. Oklahoma State University Veterinary Clinical Pathology Laboratory uses the Vitros 250 analyzer (Johnson & Johnson Clinical Diagnostics, Inc., Rochester, NY) for such minerals as calcium phosphorus and magnesium.

Sample Preparation:

1. Thaw frozen sample to room temperature.
2. Mix samples well (re-suspend crystals to a homogenous state).
3. Divide sample into two portions.
4. Centrifuge (2800 RPM for 5 min) sample intended for Mg analysis in order to remove cellular and crystalline particles.
5. Aliquot Mg sample onto the Vitros 250 and analyze.
6. Pre-treat second portion with HCl at the rate of 3 drops per 5 ml of urine (releases Ca and P from the crystals).
7. Centrifuge (2800 RPM for 5 min) sample intended for Ca and P analysis in order to remove any remaining cellular and crystalline particles.
8. Aliquot sample onto Vitros 250 and analyze.

*Vitros 250 analyzer*

*Phosphorus.* The Vitros P slide is a dry, multilayered analytical element coated on a polyester support. The analysis is based on the reaction of inorganic phosphate wih ammonium molybdate to form an ammonium phosphomolybdate complex at acidic pH (Fiske and Subbarow, 1925). p-Methylaminophenol sulfate reduces the complex to form a stable heteropolymolybdenum blue chromophore. A 10 *u*L of sample is deposited on the slide and is evenly distributed by the spreading layer. Phosphorus in the sample forms a complex with ammonium molybdate. This complex reduced by p-methylaminophenol sulfate to give a blue complex the concentration of P in the sample is determined by measuring blue complex by reflectance spectrophotometry. The sample is read on the wavelength 670 nm for approximately 5 min at 37° C.

*Calcium.* The Vitros Ca slide is a dry, multilayered, analytical element coated on a polyester support. A 10 *u*L drop of sample is deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. The bound calcium is dissociated from the binding proteins, allowing the calcium to penetrate through the spreading layer into the underlying reagent layer. There Ca forms a complex with Arsenazo III dye, causing a shift in the absorption maximum. The reflection density of the colored complex is measured spectrophotometrically and is proportional to the Ca concentration in the sample. The sample is read using 680 nm wavelength for approximately 5 min at 37°C.

*Magnesium.* The Vitros Mg slide is a dry, multilayered, analytical element coated on a polyester support. A 10 *u*L drop of sample is deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. Magnesium from the sample

then reacts with the formazan dye derivative in the reagent layer. The resulting Mg dye complex causes a shift in the dye absorption maximum from 540 nm to 630 nm. The amount of sye complex formed is proportional to the Mg concentration present in the sample and is measure by reflection density. . The sample is read using 630 nm wavelength for approximately 5 min at 37°C.

# VITA

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Master of Science

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