MEASUREMENT OF TRUE ILEAL PHOSPHORUS DIGESTIBILITY IN FEED INGREDIENTS FOR POULTRY

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This Thesis is Dedicated to My Father (Mr. U.I. Mutucumarana), Mother (Late Mrs. A.L.Y. De Silva), and All the Teachers Who Led the Way and Expected Me to Achieve Such a Goal One Day……..!!!

Abstract

Global interest in improving the utilisation of phosphorus (P) by poultry has recently increased due to concerns over environmental pollution through excess P excretion, depletion of non-renewable inorganic phosphate deposits, and increasing price of inorganic phosphate supplements. Use of a sound criterion, preferably based on P digestibility, to assess P availability is needed to enable greater efficiency of utilisation of dietary P. No established methodology is currently available to measure the true digestible P contents in common feed ingredients for poultry.

The first experiment of this thesis (Chapter 3) investigated the effects of dietary calcium (Ca) concentrations (6, 9 and 12 g/kg) on the digestibility of P, Ca, nitrogen, fat and starch in different intestinal segments and on the apparent metabolisable energy of diets in young broiler chickens. The results showed that the digestion of P and Ca was completed by upper ileum and jejunum, respectively. The site of digestion of P and nitrogen was found to shift depending on the dietary Ca concentrations. The digestibility coefficients of P in low, normal and high Ca diets at the lower ileum were determined to be 0.417, 0.379 and 0.325, respectively. The overall data showed that increasing dietary Ca concentrations negatively influenced the digestion of P, nitrogen and fat, but had no effect on those of Ca, starch and apparent metabolisable energy.

The second experiment (Chapter 4) was conducted to determine endogenous losses of P and Ca in broiler chickens. The data showed that the ileal endogenous P losses in birds differed depending on the methodology employed. The ileal endogenous flow of P in birds fed P-free, gelatin-based and casein-based diets were 25, 104 and 438 mg/kg dry matter intake (DMI), respectively. Ileal endogenous flow of Ca in birds fed casein-based diet was estimated to be 321 mg/kg DMI.

The next three experiments (Chapters 5, 6 and 7) investigated the potential usefulness of regression method to evaluate true ileal P digestibility of seven feed ingredients. True ileal P digestibility coefficients of maize, canola meal, wheat, sorghum, soybean meal and maize-distiller's dried grains with solubles for broiler chickens were determined to be 0.676, 0.469, 0.464, 0.331, 0.798 and 0.727, respectively. For plant-based ingredients, the determined true digestible P values were consistently higher than corresponding non-phytate P values (Maize, 1.72 vs. 0.75; canola meal, 4.55 vs. 2.82; wheat, 1.49 vs. 1.11; sorghum, 0.78 vs. 0.55; soybean meal, 5.16 vs. 2.15; maize-distiller's dried grains with solubles, 5.94 vs. 4.36 g/kg, as fed

basis, respectively). Phytate P in maize (54.25%), soybean meal (69.7%) and maize-distiller's dried grains with solubles (41.5%) were well digested by broilers compared to canola meal (25.2%), wheat (18.1%) and sorghum (13.0%). True ileal P digestibility coefficients of three meat and bone meal (MBM) samples ranged from 0.420 to 0.693. Total and true digestible P contents of three MBM samples (MBM-1, MBM-2 and MBM-3) were determined to be 37.5 and 26.0; 60.2 and 36.6; and 59.8 and 25.1 g/kg, as fed basis, respectively, suggesting that P in MBM is not highly digestible. The overall data suggested that the use of regression approach to estimate true ileal P digestibility in feed ingredients has number of limitations. Overestimation as a result of using Ca- and P-deficient diets and the negative endogenous P losses observed for some ingredients (canola meal, sorghum and MBM-3) were main concerns. Negative ileal endogenous P losses were also shown to be associated with low true ileal P digestibility in these ingredients.

In the final experiment (Chapter 8), two regression-based methodologies were compared for the measurement of true ileal P digestibility in maize and soybean meal. The results showed that the methodology influenced P digestibility in maize and soybean meal. The use of assay diets containing a narrow Ca:total P ratio yielded higher P digestibility for both ingredients.

In this thesis research, the regression method was used to determine true ileal P digestibility of ingredients, but this approach suffers from several drawbacks. The data reported in this thesis also demonstrated that high dietary Ca concentrations were detrimental to the digestibility of nutrients, particularly of P, nitrogen and fat.

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Publications

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List of Abbreviations

%	Percent
˚C	Degree Celsius
$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3/calcitrol
$25\text{-}(OH)_2D_3$	25-hydroxycholecalciferol
^{91}Y	Yttrium
AAFCO	Association of American Feed Control Officials
ADP	Adenosine di-phosphate
AIDC	Apparent ileal digestibility coefficient
AME	Apparent metabolisable energy
AMP	Adenosine mono-phosphate
ANOVA	Analysis of variance
AOAC	Association of analytical chemists
ATP	Adenosine tri-phosphate
ATTRC	Apparent total tract retention coefficient
BW	Body weight
BWG	Body weight gain
Ca/Ca^{2+}	Calcium/Calcium ion
$Ca_{10}(PO_4)_6(OH)_2$	Calcium hydroxyapatites
$Ca_3(PO_4)_2$	Calcium orthophosphate
$CaCo_3$	Calcium carbonate
c-GMP	Cyclic guanine mono-phosphate
Co_3^{2-}	Carbonate ion
Corp.,	Corporation
CP	Crude protein
DL	Dextrorotatory and levorotatory
DDGS	Distiller’s dried grains with solubles

df	Degree of freedom
dl	Decilitre
DM	Dry matter
DMI	Dry matter intake
DNA	Deoxy-ribonucleic acid
EPL	Endogenous phosphorus loss
FGF	Fibroblast growth factor
FI	Feed intake
g	Gram
g/b/d	Grams per bird per day
GE	Gross energy
GLM	General linear model
GMD	Geometric mean diameter
GSD	Geometric standard deviation
h	Hours
h^2	Heritability
$H_2PO_4^-$	Dihydrogen phosphate ion
HCl	Hydrochloric acid
HDL	High density lipoproteins
HPO_4^{-2}	Hydrogen phosphate ion
IdP	Ileal digestible phosphorus
IP_6	Phytate/Myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate
IU	International units
K^+	Potassium ion
kg	Kilogram
KH_2PO_4	Potassium dihydrogen phosphate

L-tryptophan	Levorotatory form of tryptophan
LDL	Low density lipoproteins
M	Molar
MBM	Meat and bone meal
mg	Milligram
min	minute
MJ	Mega joule
mm	Millimetre
mM	millimolar
mRNA	Messenger ribonucleic acid
N	Nitrogen
Na/Na^+	Sodium/Sodium ion
Na_2HPO_4	Disodium hydrogen phosphate
nm	Nano metre
NPP	Non-phytate phosphorus
NRC	National Research Council
NS	Not significant
P	Phosphorus
P	*P*-value
P_D	Ileal phosphorus output
P_E	Excreta phosphorus output
P_I	Dietary phosphorus content
P_i	Inorganic phosphorus
PO-DMI	Total output of phosphorus per dry matter intake
PTH	Parathyroid hormone
Q	Quadratic
r^2	Coefficient of determination, simple

RNA	Ribonucleic acid
SAS	Statistical analysis software
SD	Standard deviation
SE	Standard error
SEM	Standard error of mean
SLC	Solute carrier family
Ti	Titanium
TPI	True phosphorus indigestibility
TPUC	True phosphorus utilisation coefficient
U	Phytase units
UV	Ultraviolet
VLDL	Very low density lipoproteins
WPSA	World Poultry Science Association
α	Alpha
µ	Microns
µmoles	Micromoles

CHAPTER 1

General introduction

Phosphorus (P) is a critical nutrient for animals and the inclusion of adequate amounts of P in the diet is necessary for the normal skeletal development, growth and health. It is the second most abundant mineral in animal body and about 80% of P is found in bones and teeth (Suttle, 2010). Phosphorus is involved in almost all metabolic reactions in the body, including genetic transmission and transcription, maintenance of cell integrity, fluidity and acid-base balance and cellular metabolism of carbohydrates, fat and protein (McDowell, 2003; Suttle, 2010).

In recent years, there are growing concerns about P excretion from intensive animal operations into the environment. The major reason for the excretion of P from intensive animal operations is the inability of monogastric animals to fully digest and utilise the P bound in phytic acid, which represents the major portion of P contained in feed ingredients of plant origin (Ravindran *et al*., 1995). The other important contributing factor is the practice in the feed industry to provide an additional amount of P in the diet as a safety margin. The provision of a safety margin is considered necessary due to the uncertainty about the (i) true P requirement for various classes of poultry, (ii) P concentrations in raw materials, and (iii) availability of P from various sources. More importantly, there is confusion regarding the terminology used to describe available P (e.g. available P, non-phytate P, retainable P) in feed ingredients (Angel *et al*., 2002).

Phosphorus needs in feed formulations are met primarily by the inclusion of inorganic phosphates. Gradual depletion of global feed phosphates deposits, however, is becoming a serious issue and the price of phosphates has increased in recent years making P the third most expensive nutrient in poultry diets. It is accepted that the use of a well-defined criterion for P availability will ensure greater efficiency of utilisation of dietary P and reduce the excretion of P into the environment. Of the various possibilities, measurement of digestible P may be the preferable method to assess P availability for poultry (Rodehutscord, 2009).

It has been shown by Manangi and Coon (2006) that increasing dietary concentrations of non-phytate P result in increased levels of plasma inorganic P and that this excess P is eliminated via the urine. Plasma inorganic P level is increased until the physiological threshold is reached and then remains constant. For broilers, this critical

threshold is reported to range for dietary non-phytate P appears to be between 2 to 3 g/kg (Manangi and Coon, 2006). Digestibility values of P in feed ingredients for pigs are usually determined over the total tract (Stein *et al*., 2008; Akinmusire and Adeola, 2009), because faecal samples can be collected without urine contamination. In poultry, however, total tract P digestibility measurements will yield misleading data especially if the dietary non-phytate P concentrations are above the physiological threshold and the digestibility measurements need to be made at the ileal level. Additionally, ileal P digestibility measurements minimises the errors encountered due to modifying effects of hindgut microflora (Rodehutscord, 2009).

Number of published reports are available on apparent or true digestibility values of P in common feed ingredients for pigs (Dilger and Adeola, 2006a, Fang *et al*., 2007a,b; Pedersen *et al*., 2007; Akinmusire and Adeola, 2009). In these assays, three approaches, namely regression analysis, direct method and substitution method have been used to estimate P digestibility. Corresponding data for poultry, however, are scant. Dilger and Adeola (2006b) estimated the true ileal P digestibility of soybean meal for broilers using the regression analysis technique where soybean meal was used as the only dietary source for calcium (Ca) and P. Wu *et al*. (2004), using the direct method, reported the apparent ileal digestibility of P in sorghum, wheat, maize and barley.

Based on pig digestibility studies, three major issues have been identified relating to the use of apparent P digestibility values in feed formulations (Fan *et al*., 2001). First, reported apparent P digestibility values are variable within the same feed ingredient. Second, apparent P digestibility values underestimate the true P utilisation and, finally, apparent P digestibility values measured in single ingredients are not always additive when used in diet formulations. Therefore, it is essential to determine the true P digestibility values of feed ingredients by correcting for intestinal endogenous losses. However, published data on endogenous losses of P in poultry are limited and available estimated values are shown to be affected by assay methodology, animal factors, and dietary factors such as Ca and non-phytate P levels (Al-Masri, 1995; Rodehutscord, 2009).

Phosphorus absorption in the gastrointestinal tract of poultry has been investigated extensively (Hurwitz and Bar, 1970; 1971; Hurwitz *et al*., 1979). Majority of these studies have been performed during the early 1970's using radio-labelled yttrium (^{91}Y) as a non-absorbed reference material and no recent studies, with modern strains of poultry, are available.

Of the number of factors, the dietary Ca concentration and the Ca:P ratio in poultry diets are considered as the major determinants of P availability. Studies to evaluate P absorption along the gastrointestinal tract and the specific region or regions in the digestive tract where Ca may affect the utilisation of P in chickens are limited.

The main issues that were addressed in this experimental research was; 'Whether dietary Ca concentrations alter digestion site of P in small intestine of broiler chickens, how endogenous P losses in broiler chickens can be estimated and whether the regression method can be used to measure true ileal P digestibility in common feed ingredients for poultry?' To answer these issues, a series of experiments were conducted.

This thesis consists of nine chapters. The first two chapters address the frame work for the experimental research, with Chapter 1 discussing the rationale for the focus of the research. Chapter 2 reviews the different terminologies used in nutritional studies to describe available P, factors affecting P digestion and dynamics of P digestion and absorption. Chapter 2 also provides an overview of techniques currently used in P evaluation systems. Chapter 3 through 8 present the experimental work in this thesis. Each chapter includes an abstract, introduction, materials and methods, results and discussion and conclusions. These experiments were conducted with broiler chickens to investigate,

1. The digestion of nutrients along the gastrointestinal tract as influenced by dietary Ca concentrations (Chapter 3)
2. The measurement of ileal endogenous losses of P (Chapter 4)
3. The measurement of true ileal P digestibility in common feed ingredients (Chapters 5, 6 and 7)
4. The influence of methodology to measure true P digestibility in maize and soybean meal (Chapter 8)

The final chapter (Chapter 9) is a general discussion of the experimental results generated in this thesis. This chapter highlights the major findings and addresses the practical implications and possible areas for future research.

CHAPTER 2

Literature review

2.1. Introduction

Phosphorus (P) is the twelfth most abundant element in the lithosphere and was first discovered and named in 1669 by a German chemist Henning Brandt (Gleason, 2007). It is a non-metallic element and is widely distributed in the form of phosphates in soils, rocks, in the ocean, in living cells and in most foods (Corbridge, 2013). In the periodic table, P belongs to Group 15, with the atomic number and atomic weight, 15 and 30.934, respectively (Corbridge, 2013). Phosphorus is one of the vital macro-elements required by animals to mediate major metabolic pathways of the body. The importance of P as a major structural component in animals is well known (McDowell, 2003; Suttle, 2010).

Uncertainty about P requirements has resulted in use of safety margins in poultry feed formulations (De Boever *et al*., 1994). Feeding above P requirements is one of the primary reasons for the excretion of excess P in manure. Confusion in the terminologies currently used to express P availability and lack of a precise P evaluation system also limits feeding poultry to match the requirement (Rodehutscord, 2001; Angel *et al*., 2002).

Rapid depletion of non-renewable inorganic feed phosphate resources, together with their rising cost, demand that there is an urgent need to establish a better criterion to express P availability and to develop a suitable method to measure proposed criteria in feed ingredients for poultry. To achieve these aims, an understanding of the dynamics of P digestion and absorption, determinants of P utilisation and criteria currently applied in different P evaluation systems are important. The present chapter provides an overview of these aspects. Published data on different methodologies for the measurements of P availability are also discussed. The review will focus on poultry, but comparative details from other animal species are also provided.

2.2. Phosphorus: Role in poultry nutrition

Phosphorus is a vital macro-mineral which contributes to approximately 10 g/kg of body weight in birds. The skeleton and muscle tissues comprise of 850 and 60 g/kg, respectively, of the total P in the body. Phosphorus supports the skeletal system by providing a strong mechanical support together with calcium (Ca) as phosphates

[$Ca_3(PO_4)_2$] and hydroxyapatites [$Ca_{10}(PO_4)_6(OH)_2$] (McDowell, 2003). Bones are therefore able to serve as a P source during periods of P deficiency. Non-skeletal P is a component of extracellular and intracellular fluids and is found in concentrations of 10 and 140 g/kg, respectively (Pond *et al*., 2005; Studziński *et al*., 2006).

Phosphorus is found in organic combinations in deoxy- and ribonucleic acids (DNA and RNA), and contributes to genetic transmission and genetic transcription. In cell membranes, P exists as phospholipids and contributes in the maintenance of cell fluidity and integrity (Suttle, 2010). Furthermore, P is involved in the myelination of nerves and neurones. Phosphorus is a vital component in buffer systems of body fluids and blood, thus contributing to maintain acid-base balance (McDowell, 2003; Suttle, 2010). Phosphorus actively participates in most vital body metabolic pathways and the utilisation of carbohydrates, protein and fat. Phosphorus actively participates in protein metabolism as an active component in nucleoproteins and phosphoproteins. Phosphorus is believed to be an essential component in muscle protein synthesis. The chemical reactions of phosphorylation and dephosphorylation control many of the cellular activities together with hormones and enzymes (McDowell, 2003).

In energy metabolism, P is an active participant in energy utilisation and transfer cascades via adenosine mono-phosphate (AMP), adenosine di-phosphate (ADP) and adenosine tri-phosphate (ATP). High energy phosphate bonds in the ATP release energy during catabolic reactions of the body. Phosphorus is present in hormonal second messengers cyclic AMP, cyclic guanine mono-phosphate (c-GMP) and inositol polyphosphates. Phosphorus is also an active component of 2,3-diphosphoglycerate, which controls the release of oxygen from haemoglobin. In fat metabolism, P actively participates in fatty acid transportation via formation of phospholipids (McDowell, 2003). Phosphorus plays a key role in the sodium (Na)-potassium ion pump and regulates transportation of metabolites through phospholipid bilayer.

In laying hens, P is required for egg formation. Phosphorus in egg shell mainly exists as phosphates of Ca and magnesium (Sugino *et al*., 1997). Phosphorus is a constituent of egg white proteins such as ovalbumin and flavoprotein and in egg yolk it is mainly aggregated in phosvitin and phospholipids (Vadehra *et al*., 1973; Sugino *et al*., 1997).

Phosphorus has also been known to control the appetite of animals and promote feed utilisation (Suttle, 2010). Deficiency of P has been known to cause poor bone mineralisation and rickets in poultry (Soares, 1995; Coon *et al*., 2002). The main

clinical symptoms of rickets include rubbery beaks, swollen joints and enlargement of epiphysis. In older birds, dietary deficiency of P leads to osteomalacia resulting weak bones (Coon *et al*., 2002). In laying birds, P deficiency has been shown to lower the rate of egg production and egg weight through appetite depression (Bar and Hurwitz, 1984). The literature describing the effects of excess dietary P on poultry are scant. However, increasing dietary P concentrations have been shown to increase the moisture content of excreta in layers (Smith *et al*., 2000).

2.3. Terminology

Different terms are used in nutritional studies to describe different forms of P and confusion exists among the terms currently used (Angel *et al*., 2002; Applegate and Angel, 2008).

2.3.1. Total phosphorus (total P)

Total phosphorus (total P) refers to the 'total amount of P which is determined by an atomic absorption spectrophotometry, a colorimetric method or inductively coupled plasma spectroscopy, following digestion of the given sample'. This fraction includes all forms of P.

2.3.2. Phytate phosphorus (phytate P)

The term phytate P is used to describe all organic forms of P present in a phosphorylated sugar alcohol called phytate (*myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate; IP_6). But the routine measurement of phytate P is not easy.

2.3.3. Non-phytate phosphorus (non-phytate P)

Non-phytate phosphorus (non-phytate P) is defined as the portion of P that is not bound with the phytin molecule. The content of non-phytate P in feed ingredients is calculated by subtracting analysed phytate P from analysed total P. This is the widely used term in poultry nutrition to express the P requirement in poultry (NRC, 1994).

2.3.4. Available phosphorus (available P)

Available P is another general criterion used to express the amount of P in feed ingredients that is available to animals and to express P requirements (Plumstead, 2007). Phosphorus availability can be defined as the amount of P in a feed ingredient that is biologically available to be absorbed and metabolically utilised by the animal

(Weremko *et al*., 1997) and is most often expressed as the relative bioavailability of P (Sands *et al*., 2003).

The term available P is often used interchangeably with non-phytate P, when expressing the P requirement of animals. The major difference between available P and non-phytate P is that the term available P includes all absorbed forms of P, both inorganic P (P_i) and organic P (including phytate P) forms. On the other hand, non-phytate P excludes any form of phytate P available to the animal (Angel *et al*., 2002). The slope-ratio assay technique is generally used to assess the available P content in feed ingredients where a low-P diet is supplemented with graded concentrations of P from a reference source (e.g. monosodium phosphate, monocalcium phosphate) and the responses (e.g. tibia ash, body weight gain, toe ash) are measured. The available P content of the test ingredient is then calculated by comparing the relative response obtained from the test ingredient with that of the reference material (Plumstead, 2007).

2.3.5. Retained phosphorus

Retained P refers to the P that is retained in the body and is calculated by subtracting excreta P output from dietary P intake. This retainable phosphorus system has been described in detail by van der Klis and Versteegh (1996) and is widely used in the Netherlands where it is popularised as the 'opneembare phosphor system'. Dietary retainable P values, however, are valid only at low dietary P concentrations. For broilers, this threshold is reported to be between 2 to 3 g/kg non-phytate P (Manangi and Coon, 2006).

2.3.6. Digestible phosphorus

The term apparent digestible (or absorbed) P is defined as the portion of dietary P intake that is digested and absorbed at the terminal ileal level. The resulting values are corrected for the endogenous P losses to calculate true digestible P values.

2.4. Dietary sources of P for poultry

The nutritive value of feed ingredients for poultry varies depending on the species, variety or cultivar, the season of the year, location, processing, storage conditions and the class of poultry being fed. Phosphorus concentrations in feed ingredients are similarly affected by the aforementioned factors. Phosphorus concentration is known to differ more widely than those of other macro-minerals which are naturally found in the same feed ingredient (Suttle, 2010).

Phosphorus in poultry feed formulations can be supplied from three different sources, namely plant-based feed ingredients, animal-based ingredients and inorganic mineral sources.

2.4.1. Plant-based ingredients

Cereals, milling by-products and oilseed meals are the major plant-based ingredients in poultry diets. Phosphorus in plant-based sources is primarily found in the form of phytate or Ca-magnesium salts of phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate; IP_6). Phytate is commonly found in all plant seeds and their by-products, and serves as the chief storage of P (Ravindran *et al*., 1995) and a source of P and cation for germination (Williams, 1970). Phytate P comprised 60 to 80% of total P in the seeds of legumes and cereals (O'Dell *et al*., 1972). It is therefore generally assumed that two-thirds of the total P in plant ingredients exists in this form and is poorly utilised by poultry and pigs (Ravindran *et al*., 1995; Viveros *et al*., 2000).

Of the plant-based sources, cereals constitute the bulk of poultry diets, but they are poor sources of P due to their proportionately high phytate P content. Total and phytate P concentrations in commonly used cereals vary widely between and within the same feed ingredient (Table 2.1). Oilseeds are comparatively rich sources of P than cereals, but possess high phytate P concentrations. Phytate phosphorus content is found to be the highest in sesame meal (De Boland *et al*., 1975).

Location and distribution of phytate P in cereals differ. In maize, nearly 90% of total phytate is aggregated in the germ (O'Dell *et al*., 1972). Small grains possess high phytate P contents in the aleurone layer, testa and pericarp which make up the bran (Kornegay, 2001). In wheat, nearly 87% of total phytate P is aggregated in the aleurone layer (O'Dell *et al*., 1972; Steiner *et al*., 2007). Phytic acid content in the wheat endosperm is negligible (Williams, 1970; O'Dell *et al*., 1972). In rice kernels, pericarp constitutes nearly 80% of total phytate P (O'Dell *et al*., 1972). Therefore cereal by-products are rich in phytate P compared to cereals (Table 2.1). In legume seeds, phytate is known to be accumulated in cotyledon (Kornegay, 2001). A high concentration of phytic acid in soybean meal is dispersed in seeds as sub-cellular inclusions or protein bodies called 'globoids' (Prattley and Stanley, 1982).

The availability of P among plant-based ingredients differs widely. Evidence from studies by Temperton and co-workers suggested that the availability of P in wheat is due partly to the endogenous phytase activity in these ingredients (Temperton and

Cassidy, 1964a,b). Phytase activity in wheat (1200 U/kg) and wheat bran (2957 U/kg) are markedly higher than that in maize (12 U/kg) and sorghum (24 U/kg) (Eeckhout and De Paepe, 1994; Godoy *et al*., 2005). Oilseeds such as soybean meal (8 U/kg), peanut meal (3 U/kg) and rapeseed meal (16 U/kg) contain little or no phytase activity (Eeckhout and De Paepe, 1994).

Table 2.1. Total and phytate P concentrations (g/kg) in common feed ingredients of plant origin

	Total P (g/kg)	Phytate P (g/kg)	Proportion of phytate P to total P (%)
Cereals			
Barley	3.21(2.73-3.70)[a]	1.96 (1.86-2.20)[a]	61.0 (59-68)[a]
Maize	2.62 (2.30-2.90)	1.88 (1.70-2.20)	71.6 (66-85)
Sorghum	3.01 (2.60-3.09)	2.18 (1.70-2.46)	72.6 (65-83)
Wheat	3.07 (2.90-4.09)	2.19 (1.80-2.89)	71.6 (55-79)
Cereal by-products			
Rice bran	17.82 (13.40-27.19)	14.17 (7.90-24.20)	79.5 (42-90)
Wheat bran	10.96 (8.02-13.71)	8.36 (7.00-9.60)	76.3 (50-87)
Oilseed meals			
Canola meal	9.72 (8.79-11.50)	6.45 (4.00-7.78)	66.4 (36-76)
Cottonseed meal	10.02 (6.40-11.36)	7.72 (4.9-9.11)	77.1 (70-80)
Soybean meal	6.49 (5.70-6.94)	3.88 (3.54-4.53)	59.9 (53-68)

Adopted from: Selle and Ravindran (2007).
[a]Range of values.

2.4.2 Animal by-products

Animal by-products are rich sources of P (Table 2.2) and the P availability is generally considered to be 100% (NRC, 1994). However, P availability varies according to the source, processing technique, seasonal changes and particle size of the P source (Orban and Roland, 1992) and range between 59 and 74% of total P in the major animal by-products (van der Klis and Versteegh, 1996). A wide variation has also been noted in the total P contents of animal by-products (NRC, 1994; van der Klis and Versteegh, 1996).

Table 2.2. Phosphorus concentrations (g/kg) in by-products of animal origin

Feed ingredient	Total P (g/kg)[1]
Bone meal	125
Fish meal (Herring-mechanically extracted)	17
Meat and bone meal	51
Meat meal	41
Poultry by-product meal	17

[1]Total P = Non-phytate P.
Source: NRC (1994).

2.4.3. Inorganic sources

Inorganic phosphates are usually used as mineral supplements in poultry diets, because of their high content (Table 2.3) and availability of P (Peeler, 1972; Waldroup, 1999). They occur naturally as rock phosphates which are widely distributed in Northern Europe, Africa, Asia, USA, China and Middle East (Mehmood *et al*., 2009; Van Kauwenbergh, 2010). Crude phosphates, which are of igneous and sedimentary origins, are obtained by surface (open cast or strip) mining or underground methods and are converted into orthophosphate after removing undesirable impurities such as cadmium, arsenic, chromium, zinc, copper, nickel and uranium (Mehmood *et al*., 2009; Van Kauwenbergh, 2010). Type of inorganic phosphate produced is mainly dependant on the manufacturing process. The major inorganic feed phosphates used in animal diets are different forms of calcium phosphates such as mono and dicalcium phosphates and defluorinated phosphates. Selection of an inorganic phosphate source for poultry diet formulations depends on number of factors, including biological availability, chemical composition, availability, cost, physical handling qualities, and free from harmful impurities.

Table 2.3. Phosphorus concentrations (g/kg) in inorganic feed phosphate sources

Feed phosphate type	Total P (g/kg)[1]
Phosphate (defluorinated)	180
Calcium phosphate (dibasic form)	187
Calcium phosphate (mono-dibasic)	210
Sodium phosphate (dibasic form)	208
Sodium phosphate (monobasic form)	218
Phosphate (rock curacao, ground)	140

[1]Total P = Non-phytate P.
Source: NRC (1994).

2.5. Digestion and absorption of P in poultry

2.5.1. Digestion of P in poultry

Digestion of feed P is primarily determined by the form of P in which it is naturally present in feed ingredients (Hill *et al*., 2008). However, P should be available in the inorganic form (P_i) to be absorbed in the gastrointestinal tract (Gropper *et al*., 2008; Hill *et al*., 2008). Therefore, dietary P in the organic form must be first hydrolysed into P_i by enzymes phytases, phospholipase C and alkaline phosphatase to release P from bound forms (Ravindran *et al*., 1995; Gropper *et al*., 2008). Dietary factors such as vitamin D_3, zinc, manganese and molybdenum are known to increase the activity of alkaline phosphatase while magnesium and Ca have a negative effect (McCuaig and Motzok, 1972). Increased alkaline phosphatase activity has been observed when chicken were fed low P diets (Davies *et al*., 1970; McCuaig and Motzok, 1972). Environment, age, breed and sex have also been identified as other factors influencing the phytase and alkaline phosphatase activity in chickens (Davies *et al*., 1970; McCuaig and Motzok, 1972).

2.5.2. Absorption of P in poultry

Inorganic P exists in two forms in the intestinal lumen: the divalent form, HPO_4^{-2}, and the monovalent form, $H_2PO_4^-$, and are the basic forms in which P is absorbed (Quamme, 1985). Phosphate can also be absorbed as a structural part of some organic compounds such as phospholipids (Borgström, 1976).

Absorption of P is reported to occur along the entire small intestine with the largest fraction being absorbed in the jejunum (Walling, 1977). In broilers, most of the P_i was shown to be absorbed in the upper jejunum with no net absorption in lower segments (Hurwitz and Bar, 1970). In turkeys, absorption is reported to take place in both the duodenum and jejunum (Hurwitz *et al*., 1979). Layers have been shown to absorb P throughout the intestine with the rate declining towards lower segments (Hurwitz and Bar, 1965). Thus any P released as a consequence of phytate hydrolysis by hindgut microflora is unabsorbed by poultry and is simply excreted (Leytem *et al*., 2007). However, in equine and porcine species, some P is absorbed from the large intestine (Barlet *et al*., 1995; Liu *et al*., 2000) whereas in ruminants P absorption takes place to some extent in the fore-stomach (Barlet *et al*., 1995).

Several factors have been shown to influence P absorption including dietary Ca concentration, Ca:P ratio, physical and chemical forms of P, dietary P concentration, passage rate of feed, digesta viscosity, chelating agents, pH of the gastrointestinal tract and interactions with other minerals (Wise, 1983; van der Klis, 1993; Coon *et al*., 2002).

2.5.2.1. Mechanisms of P absorption

Two distinct mechanisms have been suggested for the absorption of P in the small intestine: (i) active transport which is a saturable process occurring through Na-dependent phosphate co-transporters and (ii) passive transport, a concentration dependent diffusion process (Gropper *et al*., 2008; Sabbagh *et al*., 2011). Active transport occurs primarily in the proximal small intestine, while passive transport occurs in the jejunum and ileum (Danisi and Straub, 1980; Sutton *et al*., 2004; Leytem *et al*., 2007). In contrast, Walling (1977) reported that, in rats, active P_i absorption was highest in the jejunum and was mediated by vitamin D_3.

Active transport of P is linearly related to Na^+ concentration in the intestinal lumen, while passive transport is correlated with phosphate ion concentration. Therefore, a dominant active transport of P can be expected at low dietary intakes of P, whereas passive transport being the dominant mechanism at high dietary intakes (Danisi and Straub, 1980; Sutton *et al*., 2004).

Phosphorus transport across enterocytes involves three steps: (i) Entry of P_i across the brush-border membrane into the enterocyte; (ii) Transfer of P_i to the serosal side of the epithelial cell, and (iii) Transport of P_i from the epithelial cell into the extracellular space across the basolateral membrane (Matsumoto *et al*., 1980; Yan *et al*., 2007).

Trans-epithelial transport of P_i in the small intestine occurs as a unidirectional process involving Na^+ dependent symport systems and basolaterally localised anion exchange mechanisms (Murer *et al*., 1994). Active transport across the brush-border membrane is found to be the rate-limiting step (Quamme and Shapiro, 1987). In enterocytes, the apical entry of P_i is dependent on the presence of luminal Na^+ and this apical Na^+/P_i symport in the duodenum is similar to proximal tubular Na^+/P_i symport system which differs only in their pH dependency (Murer *et al*., 1994). Intestinal absorption of P_i is determined by several factors, including plasma Ca and P concentrations, dietary availability of P and calcitrol, the hormonal form of vitamin D

(1,25-$(OH)_2$ D_3) (Quamme and Shapiro, 1987). Calcitrol has been identified as an important modulator of intestinal P absorption. The effect of calcitrol on intestinal apical transport of P_i is characterised by an increase in the maximal transport rate and the synthesis of protein functional in P_i translocation (Fuchs and Peterlik, 1979; Murer *et al*., 1994). Dietary deprivation of P is found to increase the hormonal form of vitamin D as an intestinal adaptive response leading increased reabsorption of P_i (Murer *et al*., 1994). In a study conducted by Fuchs and Peterlik (1979) using everted gut sacs to determine P_i transportation in chicken jejunum, vitamin D and Na^+ were found to act in concert with regard to optimal absorption. This study also suggested that Na^+ activates P_i translocation by enhancing the binding of P ion to the carrier. Information on mechanisms of intracellular P_i transport is limited (Quamme and Shapiro, 1987). However, the existence of specific and vitamin D-dependent cytosolic P_i-carrier proteins is yet to be proven (Breves and Schröder, 1991). Measurement of intracellular P_i is complicated by metabolic conversion and or intracellular compartmentalisation of P_i (Breves and Schröder, 1991). Mechanisms of P_i transport across the basolateral membrane are not yet fully understood (Quamme and Shapiro, 1987; Breves and Schröder, 1991; Marks *et al*., 2010). Studies with rat jejunum suggest that it involves a Na^+-independent carrier mediated mechanism (Figure 2.1) which is driven by an electro-chemical gradient (Danisi *et al*., 1984; Quamme, 1985).

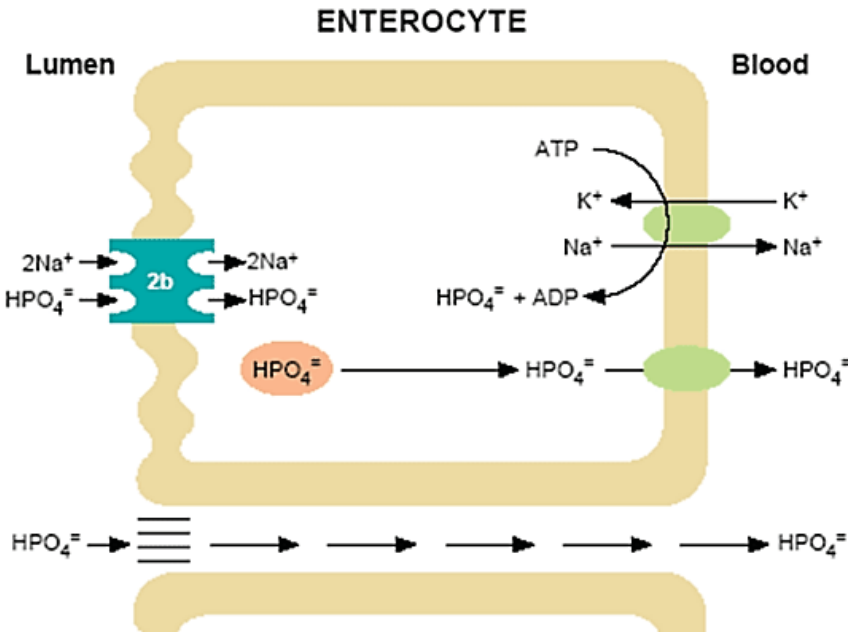


Figure 2.1. A model for the inorganic phosphorus transport in the intestine (Source: Carpenter and Drezner, 2007).

At the baso-lateral membrane, P diffuses into the blood and is transported in both organic and inorganic forms. The organic form of P primarily being transported as a constituent of phospholipids [e.g. chylomicrons, very low density lipoproteins, (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL)] and the inorganic forms are transported attaching to serum proteins and as an ionized element (Pond *et al*., 2005).

2.5.2.2. Phosphorus co-transporters and P homeostasis

Phosphorus homeostasis is primarily determined by the modulation of intestinal uptake of dietary P, renal P reabsorption and excretion and the exchange of P between extracellular and bone storage pools (Favus *et al*., 2006; Marks *et al*., 2010). It is believed that P balance is achieved mainly by control of P through renal reabsorption and that the intestinal absorption of dietary P plays only a limited role (Murer *et al*., 1994; Marks *et al*., 2010). However, recent studies have shown that intestinal absorption specifically occurring in the duodenum can result in acute changes in serum P_i concentration in response to dietary P load which stimulate the release of 'enteric phosphatonin' promoting renal P excretion (Marks *et al*., 2010).

Plasma Ca and P concentrations, dietary availability of P and calcitrol mediate intestinal absorption of dietary P, whereas renal conservation is mediated through the availability of Ca, P (intrinsic adaptation) and acid-base balance (hydrogen ions), and these controls alter Na-dependent P transport across the brush-border membrane (Quamme and Shapiro, 1987). Studies in mammals show that up to 80% of filtered P_i is reabsorbed in the proximal tubule with the highest rate occurs in the proximal convoluted tubules (Murer *et al*., 1994). Similarly, the highest rate of enteric P_i reabsorption in mammals occurs in the proximal small intestine (Murer *et al*., 1994). However, the efficiency of P_i absorption in the small intestine of chickens is comparatively poor and the apparent ileal absorption is reported to range between 40 to 50% in broilers fed diets containing recommended concentrations of dietary Ca and P (Ravindran *et al*., 2000; Tamim *et al*., 2004; Yan *et al*., 2007).

The molecular identity of Na-dependent co-transport system has been extensively studied using rats and mice as model animals and it was demonstrated that the transport of P_i by brush-border membrane vesicles and the gene expression of co-transporters are age-related and diminish with the age (Yan *et al*., 2007). Some attempts

have also been made to understand and characterisation of the molecular basis of co-transport homologues in chickens (Yan *et al*., 2007).

Phosphorus homeostasis is thought to be dependent on the function of Na-dependent phosphate transporters; members of the solute carrier family SLC34, which comprise of three type II transporters, NaPi-IIa (SLC34A1), NaPi-IIb (SLC34A2) and NaPi-IIc (SLC34A3) (Marks *et al*., 2010; Forster *et al*., 2011). Expression of these transporters at the brush-border membrane makes the rate limiting step for the trans-epithelial uptake of P (Marks *et al*., 2010). The basic mechanism of P_i transport across the brush-border membrane in proximal tubules and enterocytes are similar except the involvement of tissue specific proteins, NaPi-IIa and NaPi-IIb, respectively (Marks *et al*., 2010). However, it is evident that the members of type III transporters of SLC20 family are also involved in P transport (Marks *et al*., 2010; Forster *et al*., 2011). Recent studies indicate that type III transporters, specifically PiT1 and PiT2, are involved in P transport across the brush-border membrane of the enterocytes and renal epithelia, respectively (Marks *et al*., 2010).

Type II and type III transporters differ in their preference for P (Marks *et al*., 2010). NaPi-IIa and NaPi-IIb transport P with a stoichiometry of 3:1 Na^+:HPO_4^{2-} whereas NaPi-IIc transports P with a stoichiometry of 2:1 Na^+:HPO_4^{2-}. In contrast, type III transporters prefer monovalent P ($H_2PO_4^-$) and reported with a stoichiometry of 2:1 Na^+:$H_2PO_4^-$ (Virkki *et al*., 2007).

Species differences have been noted in the expression of NaPi-IIb protein in small intestine. In mice, highest concentrations of NaPi-IIb mRNA and protein have been found in the ileum, which is associated with the highest rate of absorption (Marks *et al*., 2010). In contrast, maximum P_i absorption in rats occurs in jejunum with the highest expression of NaPi-IIb (Marks *et al*., 2010). In chickens, NaPi-IIb co-transporter is almost exclusively expressed in the small intestine with the highest expression in the duodenum followed by the jejunum (Yan *et al*., 2007). Based on molecular studies, it has been revealed that the mRNA of type NaPi-IIb co-transporter could be detected in crypts and villi of the jejunum of broilers (Yan *et al*., 2007). However, these transporters dominate only under low dietary P concentrations or fasting conditions and, therefore, Na-independent transport is believed to have a more important role in overall P absorption along the small intestine (Marks *et al*., 2010).

Using rats as model animals, it has been shown that PiT1 protein is localised in the brush-border membrane of enterocytes in the duodenum and jejunum with the

highest concentration being found in the jejunum, while PiT1 mRNA is present throughout the small intestine with highest concentrations found in the ileum (Giral *et al*., 2009; Marks *et al*., 2010). But it has been reported that gene expression of this receptor is unaffected by changes in dietary P concentrations (Giral *et al*., 2009; Marks *et al*., 2010). In contrast, mRNA concentrations of PiT2 are low in intestinal segments and these could be increased by 1,25-$(OH)_2D_3$ (Katai *et al*., 1999; Marks *et al*., 2010).

Dietary P and 1,25-$(OH)_2D_3$ are thought to be the most important physiological regulators of intestinal P absorption (Hattenhauer *et al*., 1999; Murer *et al*., 2004; Marks *et al*., 2006) and no direct effect of parathyroid hormone (PTH) on NaPi-IIb expression has been detected except its stimulatory effect on 1,25-$(OH)_2D_3$ synthesis (Marks *et al*., 2010). Parathyroid hormone is considered to be a major physiological regulator of renal P absorption and high circulation PTH concentrations stimulate rapid endocytosis of NaPi-IIa from the proximal tubule brush-border membrane (Traebert *et al*., 2000; Bacic *et al*., 2006). Intestinal gene expression for PiT2 and NaPi-IIb has been found to be age-dependent and therefore age-dependent decrease in intestinal P absorption has been speculated (Arima *et al*., 2002; Xu *et al*., 2002; Kirchner *et al*., 2008; Marks *et al*., 2010).

2.5.2.3. Interactions between P and Ca homeostasis

Phosphorus and Ca homeostasis have been extensively studied in avian species. The mode of regulation is similar to that of mammalian species with minor differences (Norris, 2007). Birds possess four parathyroid glands which resemble those of mammals and have ultimobranchial glands which resemble the C-cells in mammalian thyroid gland (Norris, 2007).

Hormonal regulation of P and Ca homeostasis is characterised by common mechanisms and this is mainly achieved by three hormones: (i) PTH, (ii) calcitonin and (iii) 1,25-$(OH)_2D_3$ (Breves and Schröder, 1991; McDowell, 2003). There is emerging evidence that phosphatonins (e.g. FGF-23) may also be involved in P homeostasis (Favus *et al*., 2006; Berndt and Kumar, 2009). The regulation of plasma concentrations of 1,25-$(OH)_2D_3$ depends on both plasma P_i and Ca concentrations (Breves and Schröder, 1991). The regulatory effects of Ca on plasma 1,25-$(OH)_2D_3$ are mediated by (i) direct stimulation and/or (ii) PTH which has been shown to stimulate the activity of renal 25-hydroxycholecalciferol-1-hydroxylase (Breves and Schröder, 1991) (Figure 2.2). However, direct stimulation of 25-hydroxycholecalciferol-1-hydroxylase by low

serum P_i has been detected only in monogastrics and parathyroidectomised animals, and does not depend on the presence of PTH (Breves and Schröder, 1991; Berndt and Kumar, 2009).

At high dietary intakes of P, the body reaches a state of hyperphosphatemia which triggers parathyroid gland to secrete PTH (Bergwitz and Jüppner, 2010). High PTH concentration in turn will stimulate renal excretion of P to normalise plasma P_i concentration (Breves and Schröder, 1991). On the other hand, hyperphosphatemia directly suppresses renal 1-α hydroxylation to lower calcitrol production (Breves and Schröder, 1991; Bergwitz and Jüppner, 2010). Low calcitrol production suppresses intestinal P_i and Ca absorption and bone resorption to normalise plasma P_i concentration (Breves and Schröder, 1991). In the hypophosphatemic state, renal calcitrol production is stimulated and the hormone is released to the circulation which results increased P_i absorption from the small intestine (Schröder *et al.*, 1996). In addition, calcitrol is involved in the simultaneous mobilisation of P from soft tissues and bones resulting in hypercalcemia (Schröder *et al.*, 1996). Both hypophosphatemia and hypercalcemia suppress PTH release from the parathyroid gland minimising urinary P losses and normalise P_i concentrations in the plasma (Schröder *et al.*, 1996; Berndt and Kumar, 2009). Thus P homeostasis occurs making the gastrointestinal tract and kidneys as main routes for excretion of P in monogastric animals (Figure 2.2).

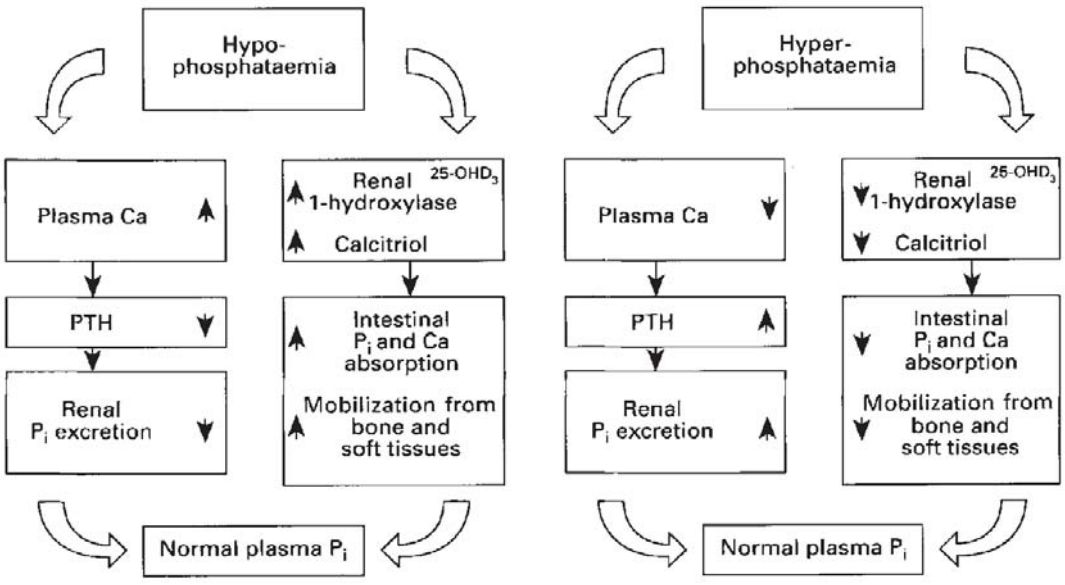


Figure 2.2. Adaptational mechanisms to hypo- and hyperphosphataemia. P_i, inorganic phosphate; 25-OHD_3-1-hydroxylase, 25-hydroxycholecalciferol-1-hydroxylase; PTH, parathyroid hormone; Calcitrol, 1,25-dihydroxycholecalciferol (Source: Breves and Schröder, 1991).

2.5.3. Factors affecting phosphorus absorption and utilisation

2.5.3.1. Phytic acid content in the feed

Proportion of P in the form of phytic acid is a major dietary factor determining P availability in feed ingredients for poultry. Phytic acid is poorly utilised by poultry due to insufficient phytase enzyme activity to hydrolyse phytates in the poultry gastrointestinal tract (Maddaiah *et al*., 1964; Godoy *et al*., 2005; Marounek *et al*., 2010). Therefore utilisation of phytate P or phytate P hydrolysis is dependent upon the availability of extrinsic or intrinsic phytase enzyme to the monogastric animals. Phytate-bound P should be hydrolysed into inorganic phosphates and inositol to make P available for poultry (Figure 2.3). In ruminants, the microflora in the rumen and reticulum play a major role in the hydrolysis of phytate. However, the hydrolysis of phytate in ruminants can be negatively affected by high dietary phytate concentrations (Godoy *et al*., 2005).

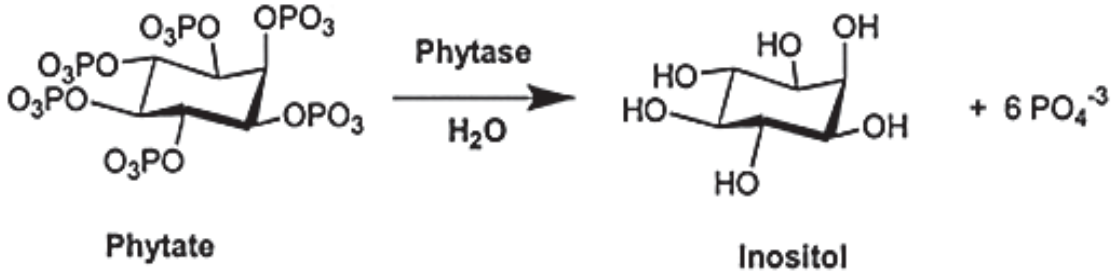


Figure 2.3. Mechanism of phytate hydrolysis by phytase (Garrett *et al*., 2004).

The hydrolysis of phytate phosphorus and P absorption by poultry and pigs are influenced by number of factors, including dietary Ca and P concentrations, vitamin D_3 and vitamin D_3 metabolites, age, genotype, enzymes, type of dietary ingredients, source of dietary phytate, feed processing and particle size (Mohammed *et al*., 1991; Ravindran *et al*., 1995; Angel *et al*., 2002; Nahm, 2007).

2.5.3.2. Dietary Ca

The most critical factor affecting phytate P availability is the Ca ion concentration in the upper gut where it forms a precipitate (as reviewed by Selle *et al*., 2009). Although Ca does not exhibit the strongest affinity to chelate with phytate as other inorganic minerals, it readily forms an insoluble complex with phytate due to high dietary inclusions in poultry diets (Angel *et al*., 2002). High concentrations of dietary Ca, in the form of limestone, have been found to increase the pH of the digesta with negative effects on phytate P hydrolysis (Shafey *et al*., 1991; Angel *et al*., 2002). Additionally, high concentrations of dietary Ca increases the size of the IP_6-mineral complex reducing the surface area to be attacked during hydrolysis by the phytase (Angel *et al*., 2002). Therefore, the dietary Ca concentration, and the Ca:P ratio, in poultry diets are critical determinants of P availability. Past studies have confirmed the negative relationship between phytate P hydrolysis and dietary Ca concentrations. Ballam *et al*. (1984) demonstrated that chicks fed diets with 10 g/kg Ca hydrolysed less phytate than those fed diets containing 8.5 g/kg Ca. A similar finding was reported by Mohammed *et al*. (1991) who noted that the utilisation of phytate P can be increased by 15% when the dietary Ca content was reduced from 10 to 5 g/kg. Nelson and Kirby (1987) also reported improved dietary phytate P hydrolysis from 5.6 to 55.0% when dietary Ca concentration was reduced from 5.2 to 1.2 g/kg in broiler diets. The low dietary Ca concentration, however, resulted in poor performance and bone mineralisation. NRC (1994) recommends a Ca:non-phytate P ratio of about 2:1 (weight to weight basis) for broilers. For laying hens, it has been suggested that Ca:P ratio does not have any practical significance because of the higher Ca requirement for egg shell formation. A much wider Ca:non-phytate P ratio of 12:1 is used in layer diets (McDowell, 2003).

High Ca concentration also lowers P bioavailability by reducing the activity of intestinal phytase. McCuaig *et al*. (1972) found that the activity of intestinal phytase decreased from 2.56 to 0.64 μmoles of P released per minute per gram of mucosa from 3-week old broilers when the dietary Ca concentration was increased from 6 to 15 g/kg. Similar results were observed by Applegate *et al*. (2003), who reported that the intestinal phytase activity of broilers was reduced at normal dietary Ca concentration (9 g/kg) as compared to that at low Ca concentrations (4 g/kg). In contrast, in a study conducted to determine the activity of duodenal and jejunal phytase in 18-day old ducklings fed diets with Ca concentrations of 7.4 and 11.1 g/kg, Rush *et al*. (2005)

reported that the specific intestinal phytase activity and brush-border vesicle Ca concentration were similar among treatments.

It has also been shown that the dietary Ca:P ratio influences the efficacy of exogenous microbial phytase. Qian *et al*. (1997) observed that the efficacy of microbial phytase in maize-soy broiler diets was markedly reduced at a wider Ca:P ratio. Widening the Ca:total P ratio from 1.4:1 to 2.0:1 lowered the efficacy by 13.4 and 14.9%, respectively, and this negative effect is more pronounced at lower concentrations of phytase and available P. Similar effects have been reported in studies with pigs, turkeys, broilers and layers (van der Klis *et al*., 1997; Liu *et al*., 2000; Zyla *et al*., 2000; Aksakal and Bilal, 2002; Selle *et al*., 2009). The observed effect of wider Ca:P ratio may be due to the influence on phytate P utilisation, less accessibility of phytase on Ca-phytate insoluble complex and/or the ability of Ca to suppress phytase activity by competing for active sites of the enzyme (Qian *et al*.,1997). Reaction of Ca with dietary P_i leading to massive precipitation of calcium orthophosphate [$Ca_3(PO_4)_2$] may also lower the absorption of Ca and P (Hurwitz and Bar, 1971; Plumstead *et al*., 2008; Selle *et al*., 2009).

2.5.3.3. Inorganic P

Inclusion of high dietary concentrations of P_i has been found to proportionately reduce phytate P hydrolysis (Ballam *et al*., 1984) and lower the digestibility of P in poultry (Ravindran *et al*., 2000). These effects were attributed to the inhibition of phytase activity by P_i, the end product of phytate hydrolysis (as reviewed by Selle *et al*., 2009). Inhibitory effect of P_i (Na_2HPO_4) on rapeseed phytase activity has also been reported by Mahajan and Dua (1997). Increased activity of intestinal alkaline phosphatase (Davies *et al*., 1970; McCuaig and Motzok, 1972) and phytase (Davies *et al*., 1970) in chicks fed low P_i diets has also been observed. This negative effect is greater when dietary Ca and P_i concentrations are both increased which results increased precipitation of IP_6-mineral complexes due to lowered solubility of these minerals and phytate P (Angel *et al*., 2002). Similarly, reaction of P_i with Ca lead to the flocculent precipitation of $Ca_3(PO_4)_2$ can also make P less available for absorption (Hurwitz and Bar, 1971; Selle *et al*., 2009).

2.5.3.4. Age and genotype

Older birds efficiently utilise phytate P since more phytase activity is found in the gastrointestinal tract. Phytate P retention in 16 to 21 day and 42 to 46 day old broilers were 6.8 and 17.3% respectively (Matyka *et al*., 1990). However, no difference in phytate P retention (0 vs. 3%) was observed by Nelson (1976) in 4 and 9 week old broilers.

Edwards (1983) stated that layer type birds utilise phytate P more efficiently than meat type birds. Maddaiah *et al*. (1964), however, observed that the intestinal phytase activity in layers was lower than those in chicks and rats. Similarly, no differences were found in the specific activity of brush-border phytase of broilers and layers in a study conducted by Maenz and Classen (1998).

Differences in phytate P utilisation have also been observed between different strains of broilers (Edwards, 1983). In contrast, no strain effect was noted for phytate P hydrolysis between Ross 308 and Hubbard x Peterson broilers. The apparent ileal phytate P hydrolysis in 22-day old Ross x Ross broilers and Hubbard x Peterson broilers were found to be 22.0 and 24.1% of the total phytate P at the dietary Ca concentration of 9 g/kg (Applegate *et al*., 2003). Similarly, using 37-week old ISA Brown laying hens and 31-week old Ross 308 broiler breeder hens fed wheat-maize-soybean based diet, Marounek *et al*. (2010) showed that the ileal digestibility of phytate P (20 and 18%, respectively) were not greatly different.

2.5.3.5. Vitamin D_3 and metabolites

Numerous studies have demonstrated that vitamin D_3 or its metabolites (1,25-dihydroxycholecalciferol and 1α-hydroxycholecalciferol) improved P utilisation in broilers (Mohammed *et al*., 1991; Edwards, 1993; Biehl *et al*., 1995; Mitchell and Edwards, 1996; Biehl and Baker, 1997; Qian *et al*., 1997; Applegate *et al*., 2003; Snow *et al*., 2004; Rama Rao *et al*., 2007) and layers (Carlos and Edwards, 1998) fed low P diets. The observed improvement in P utilisation may be due to increased (i) synthesis or activity of intestinal phytase and/or alkaline phosphatase (Davies *et al*., 1970, Biehl *et al*., 1995; Mitchell and Edwards, 1996), (ii) P absorption (Wasserman and Taylor, 1973), (iii) phytate P hydrolysis (Mohammed *et al*., 1991), (iv) resorption of P in the kidney (Veum, 2010) and/or (v) accumulation of P in bones (Veum, 2010). Vitamin D_3 has been shown to modify P absorption in the jejunum of chickens while enhancing Ca absorption in duodenum which was independent from P absorption (Hurwitz and Bar,

1972). The enhancing effect of vitamin D_3 on P absorption may be the result of primary action of vitamin D and/or secondary to its effect on Ca absorption (Hurwitz and Bar, 1972). Low P and low Ca diets are found to have a stimulatory effect on 25-hydroxyvitamin D_3-1α-hydroxylase in chickens (Baxter and DeLuca, 1976), which in turns triggers renal calcitrol (1,25-$(OH)_2D_3$) production (Veum, 2010). Cholecalciferol (vitamin D_3) is hydroxylated in the liver and converted into 25-hydroxycholecalceferol (25-$(OH)D_3$) which in turn converted in the kidney to 1,25-$(OH)_2D_3$ in the presence of 25-hydroxyvitamin D_3-1α-hydroxylase (Borle, 1974). Parathyroid hormone is found to have a stimulatory effect on the conversion of vitamin D_3 to its active hormonal form of 1,25-$(OH)_2D_3$ (Borle, 1974; Veum, 2010). Calcitrol regulates Ca absorption by regulating the synthesis of calbindin-D_{28k}, a specialised Ca binding protein found in the intestine and kidneys of chickens, which is similar to calbindin-D_{9k} found in mammals (Veum, 2010). Receptors for 1,25-$(OH)_2D_3$ have been found on the basolateral membrane of chicken intestinal epithelium and the numbers are higher in young birds (Veum, 2010).

2.5.3.6. Phytase enzyme

Phytases (*myo*-inositol hexaphosphate hydrolases) are widely distributed in plants, animals and microorganisms, and are capable of hydrolysing one or more phosphate groups from IP_6 yielding P_i and a series of lower phosphoric esters (Angel *et al*., 2002). Two classes of phytase enzyme are currently in use, namely 3-phytase and 6-phytase which initiate dephosphorylation of IP_6 at the 3 and 6 positions, respectively, to yield lower phosphoric esters (Selle and Ravindran, 2007).

The ability of plant phytase to hydrolyse phytate has been known for more than a century when the phytase activity was first detected in rice bran (Suzuki *et al*., 1907). However, the phytase activity in majority of feed ingredients has been found to be minor although significant phytase activity has been found in some cereals (e.g. wheat, triticale, rye, barley) and their by-products (Weremko *et al*., 1997; Selle and Ravindran, 2007). Plant phytases have very narrow pH spectrum of activity, and most work optimally at pH near five (Eeckhout and De Paepe, 1991; Wodzinski and Ullah, 1996). Plant phytases are liable to be inactivated by steam pelleting (Jongbloed and Kemme, 1990b; Wodzinski and Ullah, 1996), by acidic pH in the upper gastrointestinal tract (Phillippy, 1999) and by the action of pepsin, a protease in gastric secretions (Phillippy, 1999). Nevertheless, plant phytases have been shown to improve P utilisation of

broilers, layers and pigs in some studies (Pointillart *et al*., 1987; Oloffs *et al*., 2000) whereas no improvements were noted in others (Leytem *et al*., 2008).

Mucosal phytase activity in the gastrointestinal tract of poultry is of minor importance. The presence of an intrinsic phytate degrading enzyme was first reported by Patwardhan (1937) in the gastrointestinal tract of rats. Since then numbers of studies have shown the ability of mucosal phytase to utilise phytate P in poultry (Maddaiah *et al*., 1964; Maenz and Classen, 1998; Tamim and Angel, 2003; Tamim *et al*., 2004; Marounek *et al*., 2010) and pigs (Hu *et al*., 1996). Phytase activity has been detected in the crop and all segments of the small intestine of poultry (Maenz and Classen, 1998; Marounek *et al*., 2010) with the highest activity found in the duodenum (Maenz and Classen, 1998). The activity of mucosal phytase is significantly affected by high dietary Ca concentrations (Tamim and Angel, 2003; Tamim *et al*., 2004). This effect of high dietary Ca is due largely to insoluble Ca-phytate complex formation (Wise, 1983). An adaptive capacity to enhance mucosal phytase and phosphatase activity has been observed (McCuaig *et al*., 1972; McCuaig and Motzok, 1972) when the broilers were fed P deficient diets. Mucosal phytase activity is also affected by factors such as age (Matyka *et al*., 1990) and genetics (Maddaiah *et al*. 1964; Maenz and Classen, 1998). Zhang *et al*. (2003) reported the capacity of chicks to hydrolyse phytate P is found to be a heritable trait ($h^2 = 0.10$).

The ability of gut microflora of ruminants to hydrolyse phytate P is well documented (Nelson *et al*., 1976; Godoy and Mechy, 2001), but studies on the effects of microfloral phytase in pigs (Sandberg *et al*., 1993) and poultry (Kerr *et al*., 2000; Marounek *et al*., 2010) are limited. A study conducted by Marounek *et al*. (2010) noted a considerably high phytase activity in the caeca of laying hens fed wheat-maize-soybean diet without a phytase supplement. This study also found a higher total tract phytate P digestibility than the ileal digestibility in laying hens and broiler breeders fed phytase un-supplemented diets (33 and 35% vs. 20 and 18% respectively).

Exhaustive reviews are available on the impact of microbial phytase on poultry (Kornegay, 2001; Selle and Ravindran, 2007) and pig (Selle and Ravindran, 2008) nutrition. The development of phytase as an exogenous source of phytate P degradation was initiated in 1962 (Wodzinski and Ullah, 1996), but exogenous phytases were not commercially available until 1991 (Selle and Ravindran, 2008). Currently used commercial phytases are derived from a number of micro-organisms such as, bacteria, yeasts and fungi. Of these, phytases derived from *Aspergillus niger*, which is a 3-

phytase, and *Peniophora lycii* and *Escherichia coli*, which are 6-phytases are widely used in the poultry industry (Selle and Ravindran, 2007).

Inclusion of microbial phytase in poultry diets has been found to improve utilisation of phytate bound P, performance and skeletal strength in broilers (Selle and Ravindran, 2007). However, in laying hens (22 to 61-week old) fed P deficient-normal Ca diets, microbial phytase supplementation has found to be less effective in improving laying performance. Improvement was observed only for tibia mineral composition and feed conversion efficiency in layers (Liebert *et al*., 2005). Similarly it has been observed that neither the production characteristics nor feed conversion ratio of layers could be improved by elevated microbial phytase doses (van der Klis *et al*., 1997).

2.5.3.7. Type of feed ingredient

Availability of P varies among feed ingredients. Phosphorus in feed phosphates and animal by-products is more available than that in plant-based feed ingredients. A considerable variation in phytate P contents is observed between plant-based ingredients. The source of phytate P has been found to influence on phytate P availability. This may be due to the difference in solubility of phytate P in different feed ingredients at acidic pH (Nahm, 2007; Diarra *et al*., 2010). Soluble phytates are more readily hydrolysed by phytase enzyme (Nahm, 2007; Diarra *et al*., 2010). Inclusion of cereals such as wheat, rye and barley rich in intrinsic phytase activity has been shown to have a positive effect in phytate P hydrolysis and P utilisation by animals (Selle and Ravindran, 2007). Therefore, plant breeding for low phytate, soluble phytate and high intrinsic phytase is an area of great interest to increase P utilisation by animals (Diarra *et al*., 2010).

2.6. Phosphorus availability; techniques and criteria of evaluation

The term ‘P availability’ can be defined as the portion of P in a particular feed ingredient that can be absorbed and fully utilised by an animal. However, the definition of the term ‘available P’ is known to be affected by different response criteria used in different P evaluation systems (Shastak and Rodehutscord, 2013). Methodologies employed in the evaluation of P availability in feed ingredients are discussed below under three headings: (i) Qualitative measurements of P availability (ii) Quantitative measurements of P availability, and (iii) *In vitro* (solubility) tests (Shastak and Rodehutscord, 2013).

2.6.1. Qualitative measurements of P availability: blood, bone and growth assays

The history of traditional biological value assays goes back to 1945 and assay procedures have gradually progressed to date. The objective of majority of these studies (Bird *et al*., 1945; Gillis *et al*., 1954; Motzok *et al*., 1956; Dilworth and Day, 1964; Day *et al*., 1973; Huyghebaert *et al*., 1980; Potter, 1988; Potter *et al*., 1995) was to evaluate bioavailability of feed phosphates to poultry. In these assays, the biological value of a test P source is determined by 'slope ratio assay' where chicken are fed a P-deficient basal diet and test diets with different concentrations of the test phosphate during a 2 to 3 week experimental period and the response criteria (e.g. feed conversion, weight gain, bone ash, P content of bone, bone strength, bone densitometry, plasma or blood P concentration, alkaline phosphatase concentration *etc*.) are compared with those fed a standard phosphate assumed to be having 100% availability (Coon *et al*., 2002). Multiple response criteria have been used by some authors when computing the relative biological value (Sullivan, 1966) and biological index (Soares *et al*., 1978). As described by Nelson and Peeler (1961), validation of the selected qualitative measurements of P availability can be achieved by (i) feeding P-deficient diet to the animal, (ii) maintaining the concentration of added P below the animal's requirement, and (iii) using an appropriate standard source to compare with the inorganic P source tested. The most widely used P bioavailability assay was developed by Gillis *et al*. (1954) by using reagent grade beta-tricalcium phosphate and percent bone ash as the response criteria. The data obtained from these studies are relative, primarily applicable for inorganic phosphates and do not reflect the absolute P availability for animals.

2.6.2. Quantitative measurements of P availability

2.6.2.1. Balance studies

Balance experiments are based on direct quantitative estimation of P availability in feed ingredients. The data generated are expressed as either total tract digestibility or retention of P. Total tract digestibility or retention of P can be calculated by the following formula.

$$\text{Phosphorus retention (\%)} = \frac{(\text{Total P ingested} - \text{total P excreted})}{\text{Total P ingested}} \text{ x } 100$$

The digestibility value thus obtained is 'apparent' and must be corrected for endogenous P losses to obtain the 'true' value. In pigs, digestibility value of P in feed ingredients is usually determined over the total tract (Petersen and Stein, 2006; Fang *et al*., 2007b; Pedersen *et al*., 2007; Yang *et al*., 2007; Stein *et al*., 2008; Akinmusire and Adeola, 2009) and this approach is workable because faecal samples can be collected without urine contamination. In poultry, measurements of total tract digestibility are practically unsound because of the collective voiding of urine and faeces. Due to this reason, calculations based on poultry excreta thus reflect retainable P values, rather than digestible P, of a particular feed ingredient (van der Klis and Versteegh, 1996). Plasma P_i concentration in poultry is increased until the physiological threshold is reached. When dietary non-phytate P exceeds the non-phytate P for physiological threshold, the excess P is excreted as urinary P confounding the data at excreta level. Studies by Manangi and Coon (2006) suggest that, for broilers, this critical threshold for dietary non-phytate P is between 2 to 3 g/kg (Figure 2.4). Therefore, in poultry, total tract P digestibility measurements will yield acceptable values only if the dietary non-phytate P concentrations are below this physiological threshold.

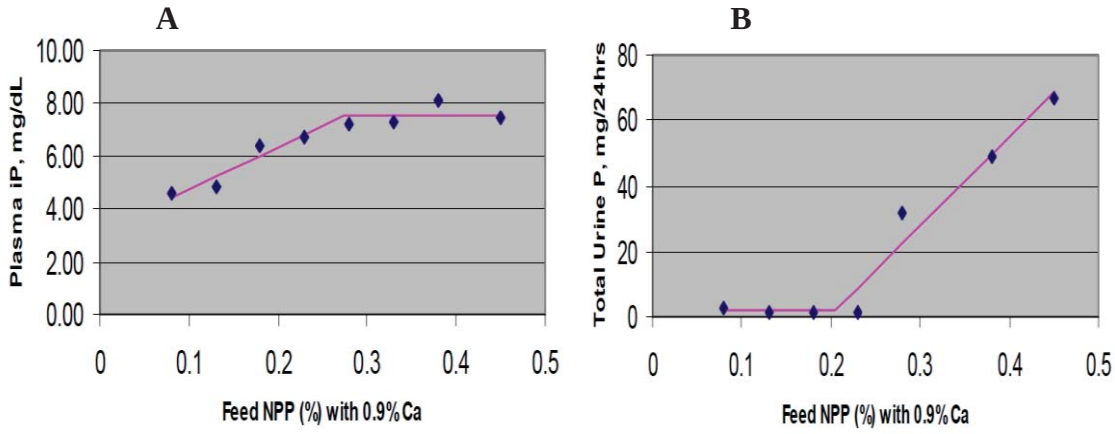


Figure 2.4. The effect of different dietary non-phytate P (NPP) concentrations on plasma inorganic P (iP) (A) and total urinary P (B) in a five-day bioassay of 50-day old male broilers (Source: Manangi and Coon, 2006).

Although measuring retainable P in feed ingredients is simpler and could be carried out without sacrificing birds, the accuracy of the value depends on dietary P intake.

2.6.2.2. Digestibility studies

Ileal assays have been widely used to determine the amino acid digestibility of feed ingredients for poultry (Ravindran *et al*., 1999; Ravindran *et al*., 2005a,b). Studies investigating P absorption in the gastrointestinal tract of poultry in early 1970's were performed using radio-labelled yttrium (^{91}Y) as a non-absorbed reference material (Hurwitz and Bar, 1970; 1971; Hurwitz *et al*., 1979). Some studies have evaluated the ileal digestibility of inorganic phosphate sources for broilers (Ketels and De Groote, 1988; Shastak *et al*., 2012) and young turkeys (Grimbergen *et al*., 1985; Kornegay *et al*., 1996).

Ileal digestibility assays are preferred over P retention assays as the values determined are unaffected by hindgut microbial activity and the exclusion of urinary P contribution (Shastak and Rodehutscord, 2013). Recently it has been shown that the response in ileal P digestibility to increasing dietary P remains linear than the corresponding response in P retention over wider range of dietary P (Rodehutscord *et al*., 2012). But both total tract retention and ileal digestibility approaches yielded similar results for inorganic P sources (Shastak *et al*., 2012).

2.6.2.3. Comparative whole body analysis

Whole body analysis provides an estimate of P that retained in the animal's body. Phosphorus contents of the whole body are determined at the beginning and the end of a balance trial to obtain the information of P retention (Hemme *et al*., 2005). However, this method is less popular due to (i) its labour intensive nature (ii) the difficulty in obtaining homogenous representative samples from the whole body, and (iii) practical difficulties arise in sample preparation and processing such as grinding and lyophilisation (Shastak and Rodehutscord, 2013).

2.6.3. *In vitro* (solubility) tests

Evaluation of biological availability of phosphate sources by animal trials are expensive and, time consuming and labour intensive. Therefore attempts have been made to investigate the possibility of using *in vitro* solubility tests to evaluate biological value of feed phosphates (Gillis *et al*., 1948; Day *et al*., 1973; Coffey *et al*., 1994). However the results have been contradictory and the success of solubility tests to evaluate the bioavailability of phosphates is questionable (Waldroup, 1999).

2.7. Phosphorus digestibility measurements in feed ingredients

Currently feed formulations for poultry are based on NRC (1994) recommendations which use non-phytate P as the requirement criteria. It is, however, being increasingly recognised that non-phytate P is not totally available to the birds and that phytate P is not totally unavailable and the extent of utilisation of phytate P varies among feed ingredients (Leske and Coon, 2002). Improved knowledge of P digestibility in feed ingredients will enable the formulation of diets closer to the requirement, improve P utilisation and minimise the excretion of P into the environment.

A large volume of data on apparent or true digestibility values of P in feed ingredients for pigs are now available (Fan *et al*., 2001; Shen *et al*., 2002; Bohlke *et al*., 2005; Petersen and Stein, 2006; Fang *et al*., 2007b; Pedersen *et al*., 2007; Yang *et al*., 2007; Stein *et al*., 2008; Akinmusire and Adeola, 2009). Corresponding data for poultry are limited with no precise methodology established to measure digestibility of P in feed ingredients.

Although the term 'digestibility' is frequently used synonymously with the term 'availability', these are two distinct terms. To be available, the particular nutrient must be in a form that can be digested, absorbed and utilised by the animal. Therefore digestibility does not itself confirm 100% availability of that particular nutrient to the animal. Digestibility assays are categorised into two main categories: (i) excreta digestibility and (ii) ileal digestibility

2.7.1. Excreta digestibility in poultry

Digestibility can be defined as the fraction of nutrient ingested that is not excreted in the faeces (Lemme *et al*., 2004). Determination of excreta (or total tract) digestibility was the most common method used in nutrient digestibility research in the past. For P, however, excreta digestibility measurements in poultry have two main drawbacks. First, the excreta of poultry contain P from both faeces and urine. Therefore, calculations based on poultry excreta are reflective of P retention and it is not accurate to refer this measurement as digestibility. Since urinary excretion is the major pathway of excreting excess P, measurements can be greatly affected. Second, measuring digestibility at the excreta level includes the possible utilisation of P by hindgut microbes (Marounek *et al*., 2010). However, studies by Vasan *et al*. (2008), using intact and caecectomised birds, have shown that the caeca of birds has no effect on P utilisation. The effect of renal excretion of P can be overcome by using colostomised birds (Manangi *et al*., 2007).

Total tract digestibility or retention of P in poultry can be calculated by measuring dietary P input and excreta P output as shown in Section 2.6.2.1.

2.7.2. Ileal digestibility in poultry

The effect of urinary P and possible hindgut modification can be overcome by determining the digestibility at the ileal level. The indicator method is used in ileal digestibility studies. An indigestible indicator which does not alter nutrient digestibility and which has a high recovery rate of almost 100% is added to the test diet. Titanium dioxide, chromic oxide and acid insoluble ash are the widely used dietary indicators in P digestibility studies. The ratio of P and indicator in the test diet and ileal digesta are used to calculate the apparent P digestibility as shown below.

$$\text{Apparent P Digestibility } (\%) = \frac{[\,(P/I)d - (P/I)i]}{(P/I)d} \text{ x } 100$$

Where, $(P/I)_d$ = ratio of P and indicator in the diet, and

$(P/I)_i$ = ratio of P and indicator in the ileal digesta

2.7.3. Methodologies to measure digestibility in feed ingredients

In pig digestibility studies, three approaches, namely regression analysis (Fan *et al*., 2001; Shen *et al*., 2002; Yang *et al*., 2007; Akinmusire and Adeola, 2009), direct method (Bohlke *et al*., 2005; Petersen and Stein, 2006; Almeida and Stein, 2010) and substitution method (Fang *et al*., 2007b) have been used to estimate P digestibility in feed ingredients. Only limited studies have been conducted to date to measure the digestible P content in feed ingredients for poultry. Dilger and Adeola (2006b) estimated the true ileal P digestibility of soybean meal for broilers using the regression analysis technique where soybean meal was used as the sole dietary source of Ca and P. Recently Iyayi *et al*. (2013) and Liu *et al*. (2013) reported studies conducted to estimate the true P digestibility of black-eyed pea and peanut flour without or with phytase supplementation and to assess the effect of different Ca:P ratios in estimation of true P digestibility of soybean meal, respectively. Wu *et al*. (2004), using the direct method, determined the apparent ileal digestibility of P in sorghum, wheat, maize and barley.

In the direct method, the test ingredient serves as the sole source of P in the test diet. Calculation of the apparent ileal digestibility coefficient (AIDC) of P in the diet is

assumed to represent the P digestibility of the test ingredient. However, as described for amino acids and proteins (Ravindran and Bryden, 1999; Lemme *et al*., 2004), when the direct method is used to determine the P digestibility in low-P ingredients (e.g. cereals), the AIDC of P can be underestimated due to the relatively greater proportion of endogenous P in the digesta or excreta (Figure 2.5). Therefore, small differences in the dietary P content of low-P ingredients will result in large changes in P digestibility values between lower and upper limits. The digestibility coefficient of P calculated by the direct method is 'apparent' and this limitation can be overcome by correcting the estimate for endogenous P losses.

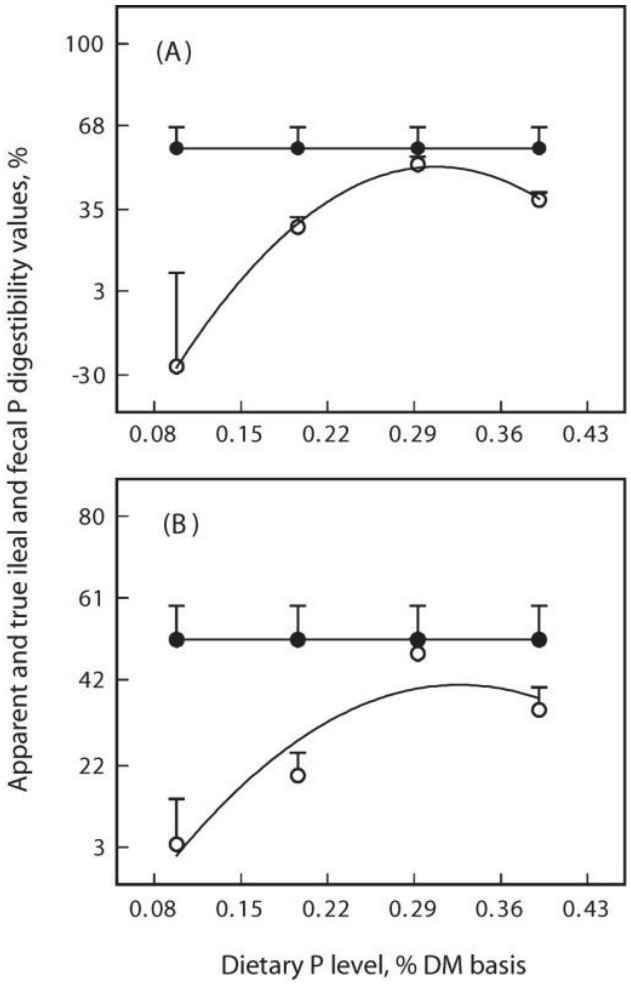


Figure 2.5. Effects of dietary P concentrations (percentage, on DM basis, mean ± SEM), on apparent (○) and true (●) ileal and faecal P digestibility in growing pigs fed soybean meal-based diets. A) Ileal level, B) faecal level (Source: Ajakaiye *et al*., 2003).

In the substitution method, AIDC of P in test ingredients is evaluated by using two diets (a reference diet and a test diet). The reference diet may consist of two or more common feed ingredients (e.g. maize-soybean meal), whereas the test diet consists of a mixture (e.g. 70:30) of predetermined ratios of the reference and the test feed ingredient. The digestibility of P in the test ingredient is determined by using the equation described by Zhou *et al*. (2004). The substitution method assumes that there is no interaction between the reference diet and the test ingredient, and that the AIDC values are additive (Lemme *et al*., 2004). A modified method for substitution method has been suggested for P digestibility measurements by Fang *et al*. (2007b), where a series of test diets are formulated from the test ingredient to contain graded concentrations of P and the true digestibility of P at adjacent dietary P concentrations is calculated, one at a time, and averaged.

In the regression method, a series of semi-purified diets are formulated from the test ingredient to contain graded concentrations of P where the test ingredient is used as the only dietary source for P. The total output of P per dry matter intake (PO-DMI) in the ileal digesta/excreta (g/kg DMI) is plotted against to dietary P contents (P_I) on a dry matter (DM) basis. True P indigestibility and endogenous P losses are the slope and intercept, respectively, of the linear regression of PO-DMI on P_I. True P indigestibility is an indirect measure of the inefficiency at which dietary P is extracted. True P digestibility is calculated by subtracting % true P indigestibility by 100 (Dilger and Adeola, 2006b). Although endogenous P loss and true P digestibility coefficient of the particular feed ingredient are simultaneously determined, the technical complexity of the regression method is a drawback for not gaining wider acceptance in nutritional research. Some regression studies with broilers have reported negative endogenous P losses, resulting in true P digestibility estimates being lower than its corresponding apparent P digestibility coefficients (Iyayi *et al*., 2013; Liu *et al*., 2013).

2.7.4. Apparent vs. true digestibility

All P present in the digesta or excreta does not originate from the diet. Some are of endogenous origin such as P derived from salivary, gastric and pancreatic juices, biliary secretions and sloughed mucosal cells (Fan *et al*., 2001). The digestibility determined therefore is ‘apparent’ and does not account for endogenous P losses.

Fan *et al*. (2001) identified three major issues relating to the use of apparent P digestibility values in feed formulations. First, reported apparent P digestibility values

are highly variable within the same feed ingredient. Second, apparent P digestibility values underestimate the true P utilisation (Figure 2.5) and, third, apparent P digestibility values measured in single ingredients are not always additive when used in diet formulations.

2.7.5. Endogenous P losses

There is a continuous secretion of endogenous P and Ca into the lumen of the intestinal tract of poultry. These endogenous P mix with dietary P, and are partially digested and absorbed. The unabsorbed fraction left beyond the lower ileum is considered as a loss to the animal. Measurement of these endogenous losses is a primary requirement for the estimation of true digestibility of P. Endogenous P losses have been quantified in pigs (Petersen and Stein, 2006; Pettey *et al*., 2006; Almeida and Stein, 2010). However, published data on endogenous losses of P in poultry are scant and the values can be affected by assay methodology, animal factors and dietary Ca and non-phytate P concentrations (Al-Masri, 1995; Rodehutscord, 2009).

In pigs, regression analysis and P-free diets have been used to determine endogenous P losses (Pettey *et al*., 2006; Almeida and Stein, 2010). There appears to be no differences between faecal and ileal endogenous P losses (Ajakaiye *et al*., 2003). Measurement of excreta endogenous P losses, rather than ileal losses, in poultry will result in erroneous results due to the contribution of endogenous urinary P. Minimal P diets (Rutherfurd *et al*., 2002; 2004), regression analysis (Dilger and Adeola, 2006b) and radioisotope-dilution technique (Al-Masri, 1995) have been thus far used to estimate the endogenous P losses in poultry. But the estimates generated for ileal endogenous P are highly variable, ranging from 145 to 446 mg/kg DMI (Rutherfurd *et al*., 2004; Dilger and Adeola, 2006b), with some negative estimates, ranging from -290 to -864 mg/kg DMI (Iyayi *et al*., 2013; Liu *et al*., 2013).

Bile is the primary source of endogenous P in poultry. About 90% of the mammalian bile lipids are composed of phospholipids (Cross *et al*., 1987). Bile phospholipids are found both as vesicles and as mixed micelles conjugated with bile salts (Sklan and Budowski, 1978; Coleman, 1987). Phosphatidylcholine represents approximately 80-95% of these biliary phospholipids and possess different phospholipid profile than cell membranes (Coleman, 1987).

Bile composition in different animal species (Table 2.4) has been investigated (Haslewood, 1978; Coleman *et al*., 1979), but studies on the composition of chicken

bile are scant. Several studies have reported the composition of bile salt and phospholipid composition in chicken bile (Anderson *et al*., 1957; Webling and Holdsworth, 1965; Sklan and Budowski, 1978; Razdan *et al*., 1997; Alvaro *et al*., 1986). According to Alvaro *et al*. (1986), the molar percent of phospholipids in chicken gall bladder bile is mainly composed of phosphatidylcholine (70.8%) while phosphatidylethanolamine represents the rest (29.5%). Phosphatidylcholine in bile is hydrolysed into lysophosphatidylcholine in the duodenum while the remainder is hydrolysed and absorbed progressively in the jejunum of chickens (Sklan and Budowski, 1978). The presence of minute amounts of inorganic P in human bile, reported by some authors, might have been derived from phospholipid hydrolysis (Reinhold *et al*., 1937). Phosphorus content in bile has been found to be increased by calcitonin (Yamaguchi and Katayama, 1982), but negatively affected by dietary Ca concentrations (Díaz-Castro *et al*., 2013).

Table 2.4. Composition of human bile (g/dl)

Constituent	Range	Mean
Total solids	3.82-28.60	16.21
Total lipids	3.06-24.05	15.06
Bilirubin	0.07-14.13	0.89
Total bile acids	0.71-9.01	5.87
Phospholipids	0.55-6.75	3.40
Cholesterol	0.14-2.18	1.08
Free fatty acids	0.00-0.38	0.05
Monoglycerides	0.00-7.71	0.49
Diglycerides	0.00-0.09	0.02
Ca^{2+}	0.01-0.05	0.03
Na^{+}	0.36-0.62	0.47
K^{+}	0.03-0.09	0.06

Source: Haslewood (1978).

Phosphorus in digestive juices also contributes to endogenous P fraction. In humans, a normal adult in P balance secretes approximately 210 mg of P in digestive juices per day (Levi and Popovtzer, 1999). Published data on the composition of pancreatic juices are available for many species (Ball, 1930; Partridge *et al*., 1982; Zebrowska *et al*., 1983), but no information is available for chickens. As described by Zebrowska *et al*. (1983), about 40% of the endogenous minerals in the duodenal digesta

are secreted by the pancreas. Pancreatic secretions from pigs were found to contain Na and potassium as the major cations, with low concentrations of P, Ca and magnesium (Partridge *et al*., 1982; Zebrowska *et al*., 1983). The diet composition, specifically the nature of protein and dietary fibre, has a marked influence on the volume and enzyme concentration of pancreatic juice (Partridge *et al*., 1982; Valette *et al*., 1992).

Part of endogenous P in animals may be derived from sloughed mucosal cells (Fan *et al*., 2001). Cell membrane lipids are mainly composed of phospholipids and shedding of mucosal cells therefore increases endogenous P. The major phospholipids in cell membranes of animals include phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin whereas phosphatidylinositol is present in minor quantities (Cooper, 2000). The dietary protein and fibre has confounding effects on mucosal cell slough off (Snook and Meyer, 1964; Nyachoti *et al*., 1997).

Similar to P, endogenous Ca mainly originates from biliary secretions, digestive juices and shed mucosa (Davies *et al*., 2004; Namgung and Tsang, 2011). Calcium can also be secreted into the digestive tract through paracellular route when intra-luminal Ca concentration is well below the serum ionised Ca concentration (Ghishan *et al*., 1980).

Calcium in bile is mainly found being conjugated with bile acids. In human, free ionized Ca in hepatic bile and gallbladder bile represent approximately 20 to 30% and 10 to 15% of total Ca, respectively, whereas the rest (70 to 90%) being in conjugated form (Gleeson *et al*., 1990). Of the conjugated form of Ca, up to 80% of Ca in hepatic bile and 40% of Ca in gallbladder bile exists as bile acid micelles (Williamson and Percy-Robb, 1980). Depending on the free ionised Ca in bile, Ca can react with Ca-sensitive anions such as carbonate, bilirubinate, phosphate and palmitate to form insoluble salts (Moore, 1984; Gleeson *et al*., 1990). Biliary Ca secretion is primarily dependent on plasma Ca concentrations and the secretion of Ca could occur in the absence of PTH and calcitonin (Limlomwongse *et al*., 1988). Biliary Ca is mainly secreted via transcellular pathway and via paracellular pathway to a lesser extent (Limlomwongse *et al*., 1988). Dietary Ca has been identified as one of the determinant of biliary Ca concentration (Jacyna, 1990). In humans, endogenous Ca excretion is known to be affected by body size and P intake (Davies *et al*., 2004).

The quantity of Ca in pancreatic juice is considerably low and is much lower than in the serum. A large increment in serum Ca contributes only a slight increase of Ca in the pancreatic secretions (Ball, 1930).

2.8 Summary

Digestible P is currently considered the most suitable criterion to express P availability in feed ingredients for poultry. Understanding the mechanism of digestion and absorption of P is therefore important to optimise the efficiency of P utilisation. This chapter presents an overview of digestion and absorption of P including endogenous P losses. Moreover, the factors influencing the efficiency of P utilisation in poultry are also discussed. Different techniques and criteria to measure P availability in poultry, with special emphasis on methodologies to measure digestible P and their limitations, are discussed.

CHAPTER 3

Influence of dietary calcium concentration on the digestion and absorption of nutrients along the intestinal tract of broiler chickens

3.1. Abstract

The effects of dietary calcium (Ca) concentration on the digestion and absorption of Ca, phosphorus (P), nitrogen (N), fat and starch along the intestinal tract of broiler chickens were assessed. Three-week old broilers were fed maize-soy diets containing 6, 9 or 12 g/kg of Ca (Ca: total P ratios of 1:1, 1.4:1 and 2:1, respectively) for six days and digesta were collected from the duodenum, jejunum, upper ileum and lower ileum. Apparent digestibility coefficients of P, Ca, N, fat and starch in different intestinal segments and apparent metabolisable energy (AME) were calculated using titanium marker ratios. Apparent digestibility coefficients of P and Ca were determined to be highly negative in the duodenum. Apparent P digestibility was negatively affected ($P < 0.05$) by increasing dietary Ca concentrations, but there was a Ca x intestinal site interaction ($P < 0.05$). Jejunum was the major site of P absorption in birds fed low Ca and normal Ca diets, but both jejunum and upper ileum were involved in birds fed high Ca diet. Dietary Ca concentration had no effect ($P > 0.05$) on the apparent Ca digestibility. Calcium was absorbed predominantly in the jejunum. Digestibility of N and fat was reduced ($P < 0.05$) by increasing dietary Ca concentrations. A significant ($P < 0.05$) dietary Ca x site interaction was observed for N. In birds fed low Ca and normal Ca diets, N was primarily digested by the end of jejunum, but in birds fed high Ca diet both jejunum and upper ileum were involved. At all dietary Ca concentrations, fat was digested mainly in the jejunum and upper ileum, but digestion continued in the lower ileum. Apparent starch digestibility and AME were unaffected ($P > 0.05$) by dietary Ca concentrations. Most of the starch digestion was completed by the end of jejunum. The present data suggest that the site of digestion of P and N shifts depending on dietary Ca concentrations. Increasing dietary Ca concentrations negatively influenced the digestion and absorption of P, N and fat, but had no effect on those of Ca and starch.

3.2. Introduction

The digestion and absorption of calcium (Ca) and phosphorus (P) in poultry have been generally measured over the total digestive tract (Tyler and Willcox, 1942; Common *et al*., 1948). Such evaluations, however, do not provide information on the site(s) of

intestinal absorption. Identification of the sites of Ca and P absorption is critical to understand the dynamics of digestion. Only limited studies have been conducted to investigate the absorption of Ca and P in broilers (Hurwitz and Bar, 1970; 1971; 1972). Results from these studies, using yttrium 91 (^{91}Y) as a non-absorbable reference material to calculate the absorption of P, showed that most of the dietary P and Ca were absorbed in the proximal segments of the intestine. The upper jejunum was identified as the major site of P absorption, with most of the dietary Ca being absorbed between the duodenum and lower jejunum (Hurwitz and Bar, 1970).

The inter-relationship that exists between dietary Ca and P concentrations in poultry nutrition and metabolism has long been known (Suttle, 2010). The negative effects of high dietary Ca on P absorption in different species are well documented (Young *et al*., 1966; Kaup *et al*., 1990; Liu *et al*., 2000). Generally a Ca:non-phytate P ratio of 2.2:1 is recommended for the optimum performance of broilers (NRC, 1994). A wider Ca:P ratio negatively influences the utilisation of both Ca and P, whereas a positive impact on retention can be achieved through more narrower ratios (Mohammed *et al*., 1991: Qian *et al*., 1997; Tamim *et al*., 2004; Santos *et al*., 2008). Because of this critical relationship, studies to identify the specific region(s) in the intestinal tract where Ca may alter the absorption of P are of interest.

Some studies have examined the specific regions in the digestive tract where Ca alters the utilisation of P in ruminants (Care, 1994) and pigs (Liu *et al*., 2000). Studies have also been conducted to examine the effect of dietary Ca concentrations on the absorption of P and Ca in layers (Hurwitz and Bar, 1965) and turkeys (Hurwitz *et al*., 1979). A suppressive effect of high dietary Ca concentrations on P absorption was evident in layers with no influence on Ca absorption (Hurwitz and Bar, 1965). In turkeys, the efficiency of P absorption was found to be slightly affected by dietary P concentrations, whereas Ca absorption was negatively influenced by the dietary Ca concentration (Hurwitz *et al*., 1979). Corresponding studies with broilers, however, are scanty.

Some reports suggest that high dietary Ca can adversely affect the utilisation of fat (Sibbald and Price, 1977), nitrogen and metabolisable energy (Shafey and McDonald, 1991a) in broilers. The absorption of protein, fatty acids and starch along the digestive tract of turkeys (Hurwitz *et al*., 1979; Sklan and Hurwitz, 1980) and chickens (Renner, 1965; Hurwitz *et al*., 1972; Bielorai *et al*., 1973; Sklan and Hurwitz, 1980; Weurding *et al*., 2001; Tancharoenrat *et al*., 2014) has been investigated.

However, none have examined the specific intestinal segments where Ca may alter the utilisation of these nutrients.

The aim of the current study was to examine the effects of dietary Ca concentration on the digestion and absorption of Ca, P, nitrogen (N), fat and starch along the intestinal tract of broiler chickens. The hypothesis that high dietary Ca may reduce the utilisation of nutrients and may alter the site of digestion was tested.

3.3. Materials and methods

The experimental procedures were approved by the Massey University Ethics Committee and complied with the New Zealand Code of Practice for the Care and Use of Animals for Scientific Purposes.

3.3.1. Birds

Day-old male broilers (Ross 308) were obtained from a commercial hatchery and, raised in floor pens and fed a commercial broiler starter diet. On day 14, birds were transferred to grower cages. On day 21 posthatch, birds were individually weighed and, 72 birds of uniform weight were selected and assigned to 12 grower cages of 6 birds each so that the cage average weight was similar**.** The floor pen and grower cages were housed in an environmentally controlled room. Room temperature was maintained at 32 ± 1^{o}C during the first week and gradually reduced to 21 ± 1^{o}C by the end of the third week. A lighting schedule of 20 h light per day was provided. Feed was given *ad libitum* and water was freely available throughout the 6-day experimental period.

3.3.2. Dietary treatments

A maize-soy basal diet (normal Ca) was formulated to contain recommended Ca concentrations and Ca:P ratio for Ross 308 broiler growers (Ross, 2007) (Table 3.1). Two other test diets (low Ca and high Ca) were formulated to contain similar nutrient profiles, except for dietary Ca (Table 3.1). The Ca concentrations in low, normal and high Ca diets were 6, 9 or 12 g/kg, respectively (corresponding to Ca: total P ratios of 1:1, 1.4:1 and 2:1, respectively). Titanium dioxide was added to all diets as an inert marker at the concentration of 3 g/kg.

Table 3.1. Ingredient composition (g/kg, as fed) of test diets

	Low Ca[1]	Normal Ca[2]	High Ca[3]
Maize	565.7	561.9	557.7
Soybean meal (480 g/kg CP)	377.0	375.0	372.0
Soybean oil	25.9	25.0	25.0
Limestone	7.1	14.9	23.0
Dicalcium phosphate	12.0	12.2	12.0
DL Methionine	2.0	1.6	1.0
Salt	2.0	2.0	2.0
Sodium bicarbonate	2.0	1.1	1.0
Titanium dioxide	3.0	3.0	3.0
Trace mineral-premix[4]	2.5	2.5	2.5
Vitamin premix[5]	0.8	0.8	0.8
Calculated composition[6]			
Metabolisable energy, MJ/kg	12.9	12.8	12.7
Crude protein, g/kg	230	229	227
Lysine, g/kg	13.3	13.2	13.1
Methionine, g/kg	5.8	5.4	4.8
Methionine + Cysteine, g/kg	9.4	9.0	8.3
Threonine, g/kg	9.7	9.7	9.6
Ca, g/kg	6.0	9.0	12.0
Total P, g/kg	6.3	6.3	6.3
Non-phytate P, g/kg	4.5	4.5	4.5
Ca:total P ratio	1:1	1.4:1	2:1
Analysed values			
Crude protein, g/kg	224.5	231.4	225.9
Crude fat, g/kg	39.5	36.7	38.2
Starch, g/kg	372.7	357.0	364.6
Ca, g/kg	7.9	10.6	12.3
Total P, g/kg	6.4	6.3	6.3
Ca:total P ratio	1.2:1	1.7:1	2:1

[1]Low Ca = Low Ca diet. [2]Normal Ca = Normal Ca diet. [3]High Ca = High Ca diet.

[4]Supplied per kg of diet: Co, 0.3 mg, I, 1.5 mg, Mo, 0.3 mg, Se, 0.3 mg, Mn, 100 mg, Cu, 10 mg, Zn, 80 mg, Fe, 60 mg, anti-oxidant, 100 mg.

[5]Supplied per kg of diet: vitamin A, 12,000 IU, vitamin D_3, 4,000 IU, thiamine, 3 mg, riboflavin, 9 mg, pyridoxine, 10 mg, folic acid, 3 mg, biotin, 0.25 mg, vitamin B_{12}, 0.02 mg, vitamin E, 80 mg, choline chloride, 0.6 g, nicotinic acid, 60 mg, Ca pantothenate, 15 mg, menadione, 4 mg.

[6]Calculated based on NRC (1994) values.

3.3.3. Digesta and excreta collection

Between days 25 and 27 posthatch, grab samples of fresh excreta were collected. Daily collections were pooled within a cage and, representative samples were taken and lyophilised (Model 0610, Cuddon Engineering, Blenheim, New Zealand). Excreta samples were then ground to pass through 0.5-mm sieve and stored in air-tight plastic containers at -4˚C till analysis for dry matter (DM), gross energy (GE) and titanium.

On day 27, the birds were euthanised by intravenous injection of sodium pentobarbitone and the digesta from duodenum, jejunum, upper ileum and lower ileum were collected. The duodenum was defined as the portion of the small intestine extending from pyloric junction to the distal-most point of insertion of the duodenal mesentery, whereas the jejunum was classified as the portion that descending down to Meckel's diverticulum (Amerah *et al*., 2009). The ileum was defined as the section of the small intestine starting from Meckel's diverticulum to a point approximately 40 mm anterior to the ileocaecal junction (Ravindran *et al*., 1999). The ileum was divided into two halves and identified as upper and lower ileum. Digesta from different segments were flushed out with reverse-osmosis water, pooled within a cage and lyophilised (Model 0610, Cuddon Engineering, Blenheim, New Zealand). Diet and digesta samples were ground to pass through 0.5-mm sieve and stored in air-tight plastic containers till analysis for DM, Ca, P, N, fat, starch and titanium.

3.3.4. Chemical analysis

Dry matter was determined by drying samples at 105°C for 16 h in a pre-weighed dried crucible in a convection oven (AOAC International, 2005; method no: 930.15). Samples were ashed and P was determined colorimetrically (UV mini 1240 Shimadzu Corp., Kyoto, Japan) at 680 nm (AOAC International, 2005; method no: 968.08D) (Appendix A). Calcium was determined by colorimetric assay (Flexor E, Vital Scientific NV, Spankeren/Dieren, the Netherlands) following digestion with 6M HCl to release Ca (AOAC International, 2005; method no: 968.08D) (Appendix B). Titanium was determined by the colorimetric method (UV mini 1240 Shimadzu Corp., Kyoto, Japan) at 410 nm as described by Short *et al*. (1996). Gross energy was determined by adiabatic bomb calorimetry (Gallenkamp Autobomb, London, UK) standardised with benzoic acid. Nitrogen was determined by total combustion (AOAC International, 2005; method no: 968.06,) using a CNS-2000 auto analyser (LECO Corporation, St. Joseph, MI). Fat was determined by Soxhlet extraction (AOAC International, 2005; method no:

991.36). Starch was measured using the alpha-amylase method (AOAC International, 2005; method no: 996.11).

3.3.5. Calculations

Apparent digestibility coefficients of P, Ca, N, fat and starch in different segments of the intestinal tract and energy utilisation coefficients were calculated by the following formula using the indigestible marker ratios.

Apparent digestibility coefficient of nutrient = $[(Nt/Ti)_d - (Nt/Ti)_i]/(Nt/Ti)_d$

Where, $(Nt/Ti)_d$ = ratio of nutrient and titanium in diet, and $(Nt/Ti)_I$ = ratio of nutrient and titanium in digesta or excreta.

Apparent metabolisable energy was calculated by the following formula.

AME (MJ/kg DM) = Energy utilisation coefficient x GE of the diet

3.3.6. Statistical analysis

Data were analysed using a repeated measure analysis using the General Linear Models procedures of SAS (2004) to assess the effects of dietary Ca concentration and Ca x intestinal site interactions. Data on duodenal values were not included in the statistical analysis, because of the negative digestibility coefficients determined in this segment. Cage served as the experimental unit. Differences were considered significant at $P < 0.05$ and, when a significant F-test was detected, means were separated using the least significant difference test.

3.4. Results

Analysed DM, Ca and P contents of test diets are shown in Table 3.1. Analysed values were in good agreement with calculated values, except for Ca. The analysed contents of Ca in the diets were 0.3 to 1.9 g/kg higher than calculated, but the expected differences in Ca between the diets were broadly achieved. Therefore it was assumed that these differences will not affect the interpretation of the results of the study.

3.4.1. Performance

All birds were healthy during the 6-day experimental period and no mortalities were recorded. Dietary Ca concentrations had no effect ($P > 0.05$) on the feed intake (Table 3.2).

Table 3.2. Effect of dietary Ca concentration on the feed intake and Ca intake (g/b/d) of broilers, day 21-27 posthatch[1]

	Feed intake (g/b/d)	Ca intake (g/b/d)[2]
Low Ca diet	131	1.035[a]
Normal Ca diet	133	1.413[b]
High Ca diet	134	1.638[c]
Pooled SEM[3]	3.60	0.0375
Probability	NS	***

NS, not significant; ***$P < 0.001$.
[1]Each value represents the mean of four replicates.
[2]Feed intake x analysed Ca concentration.
[3]Pooled standard error of mean.
[a,b,c]Means in a column not sharing a common superscript are significantly different ($P < 0.05$).

3.4.2. Digestibility of P and Ca

The influence of dietary Ca concentration on the apparent digestibility of P and Ca in different intestinal segments is presented in Table 3.3. A significant interaction ($P < 0.01$) between Ca concentration and intestinal site was observed for P digestibility. This interaction was due primarily to a lower P digestibility at the jejunum and a greater change in P digestibility between jejunum and upper ileum of birds fed the high Ca diet, compared to those fed low Ca and normal Ca diets.

Apparent digestibility of P was determined to be highly negative in the duodenum. In birds fed low Ca and normal Ca diets, P was rapidly digested and absorbed in the jejunum, but the digestion continued in the upper ileum. On the other hand, in birds fed the high Ca diet, digestibility was lower ($P < 0.05$) in the jejunum and the digestion shifted to the upper ileum. At all three dietary Ca concentrations, there were no differences ($P > 0.05$) between the digestibility of P determined at lower and upper ileal levels.

Dietary Ca concentration influenced ($P < 0.05$) the apparent P digestibility. In all intestinal segments, increasing concentrations of dietary Ca reduced ($P < 0.05$) P digestion. The negative effect was particularly evident in the duodenum of birds fed the high Ca diet. The digestibility coefficients of P in low, normal and high Ca diets at the lower ileum were 0.417, 0.379 and 0.325, respectively.

Apparent digestibility of Ca was negative in the duodenum (Table 3.3). Calcium digestibility values determined at jejunum, upper ileum and lower ileum were similar (*P*

> 0.05), with digestion of Ca being completed in the jejunum. Calcium digestibility was not influenced ($P > 0.05$) by dietary Ca concentrations.

Table 3.3. Influence of dietary Ca concentration on the apparent digestibility coefficients of phosphorus (P) and calcium (Ca) along the intestinal tract of broilers[1]

Diet[2]	Site	Apparent digestibility coefficient	
		P	Ca
Low Ca	Duodenum	-2.15	-0.453
	Jejunum	0.325[bc]	0.387
	Upper Ileum	0.387[ef]	0.415
	Lower Ileum	0.417[f]	0.364
Normal Ca	Duodenum	-1.11	-0.129
	Jejunum	0.292[b]	0.388
	Upper Ileum	0.349[cd]	0.359
	Lower Ileum	0.379[de]	0.375
High Ca	Duodenum	-1.93	-0.397
	Jejunum	0.141[a]	0.335
	Upper Ileum	0.292[b]	0.337
	Lower Ileum	0.325[bc]	0.334
SEM[3]		0.0139	0.0333
Main Effects			
Dietary Ca concentration			
Low Ca		0.376	0.389
Normal Ca		0.340	0.374
High Ca		0.252	0.335
SEM[3]		0.0267	0.0320
Site			
Duodenum		-1.73	-0.326
Jejunum		0.253	0.370
Upper Ileum		0.342	0.371
Lower Ileum		0.374	0.358
SEM[3]		0.0080	0.0192
Probability ($P \leq$)			
Dietary Ca concentration		*	NS
Site		***	NS
Dietary Ca concentration x Site		**	NS

NS, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
[1]Each value represents the mean of four replicates.
[2]Low Ca, low Ca diet; Normal Ca, normal Ca diet; High Ca, high Ca diet.
[3]Pooled standard error of mean (excluding the duodenal values).
[a,-f] Means in a column not sharing a common superscript are significantly different. ($P < 0.05$). Data were analysed excluding the duodenal values.

As shown in Figure 3.1, the site of absorption of P shifted with increasing dietary Ca concentrations. Phosphorus was absorbed primarily in the jejunum in birds fed low Ca and normal Ca diets. In birds fed the high Ca diet, both the jejunum and upper ileum were involved in P absorption. For Ca, digestion and absorption was completed by the jejunum (Figure 3.1).

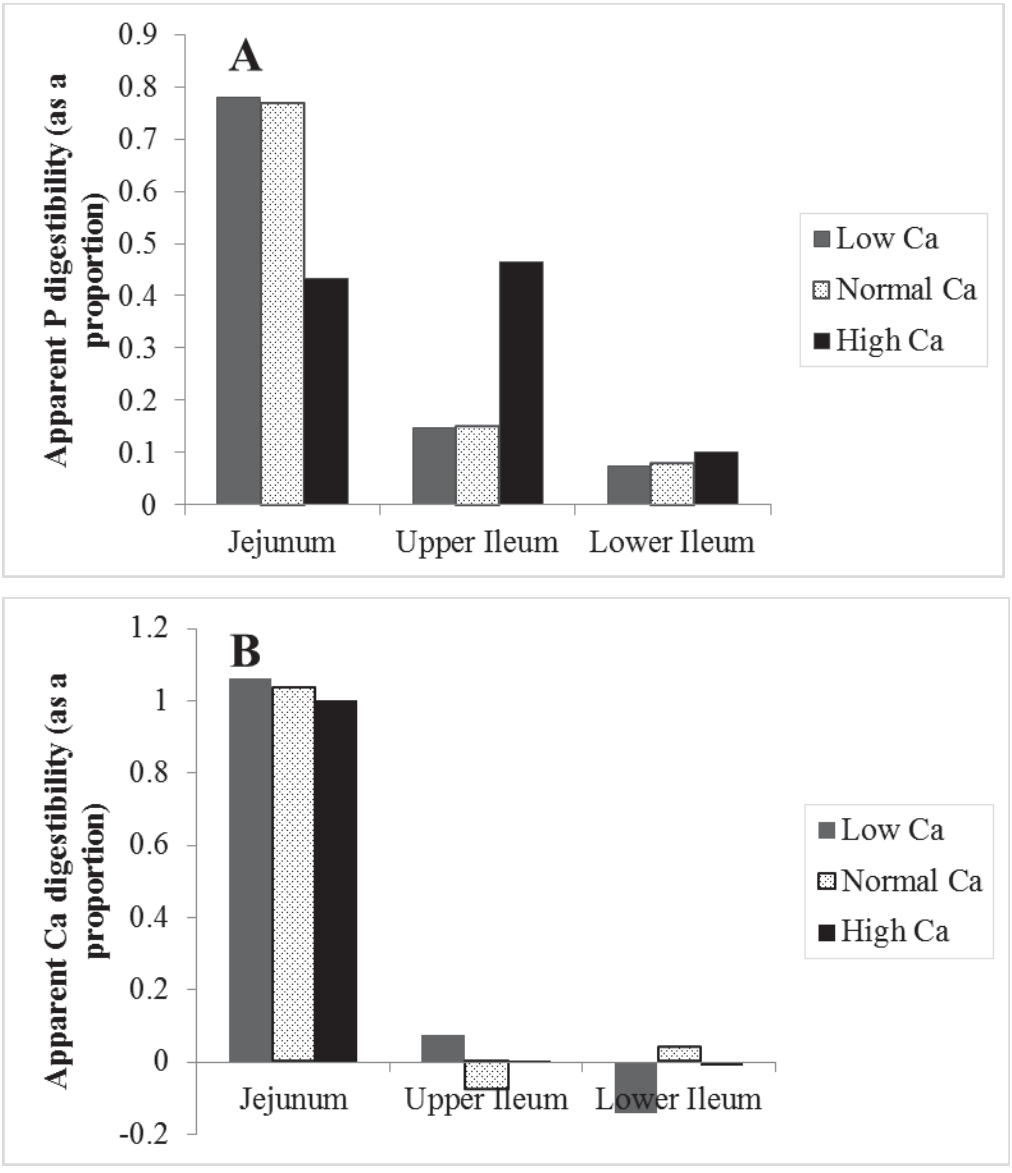


Figure 3.1. Digestion (as proportion of total digestion determined at lower ileum) of P (A) and Ca (B) along the small intestine of broilers fed diets containing different concentrations of Ca.

3.4.3. Digestibility of N, fat and starch

The influence of dietary Ca concentration on the apparent digestibility of N, fat and starch in different intestinal segments is presented in Table 3.4. Duodenal contents were not analysed for N, fat and starch due to insufficient digesta samples and duodenal data are therefore not presented. A significant interaction ($P < 0.05$) between Ca concentration and intestinal site was observed for N digestibility. This interaction was due primarily to a lower N digestibility at the jejunum and a greater change in N digestion between the jejunum and upper ileum of birds fed the high Ca diet, compared to those fed low Ca and normal Ca diets. In birds fed low Ca and normal Ca diets, N was primarily digested in the jejunum, and the digestion continued in the upper ileum (Figure 3.2). On the other hand, in birds fed the high Ca diet, both jejunum and upper ileum were involved in N digestion. At all three Ca concentrations, N digestion continued in the lower ileum and the digestibility coefficients determined at lower ileum and upper ileum differed ($P < 0.05$). However, the digestibility coefficients measured at the lower ileum was not different ($P > 0.05$) between the three diets.

Dietary Ca concentration had a significant effect ($P < 0.05$) on the apparent N digestibility, but there was also a significant Ca x intestinal site interaction. Nitrogen digestibility was reduced by increasing concentrations of dietary Ca in the jejunum and upper ileum, but was unaffected ($P > 0.05$) in the lower ileum. The digestibility coefficients determined for low, normal and high Ca diets at the lower ileum were 0.829, 0.833 and 0.812, respectively.

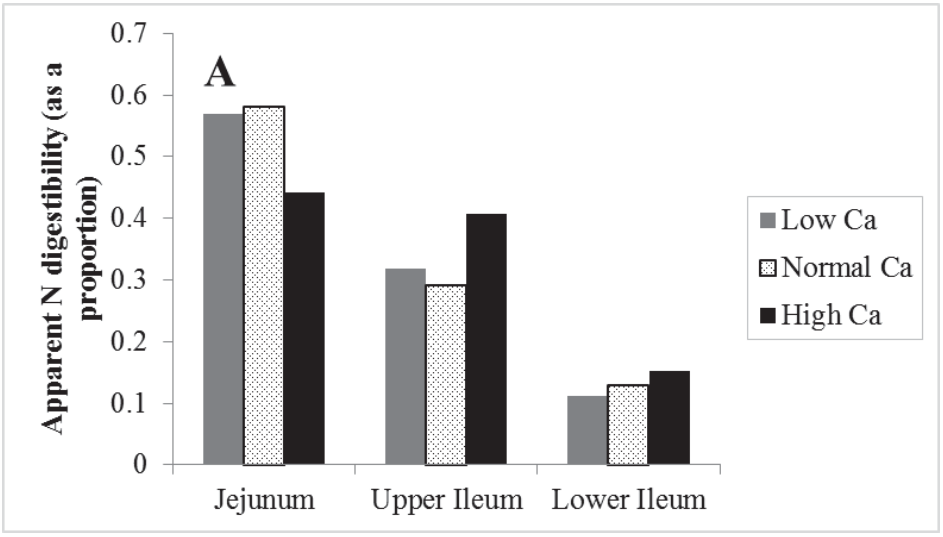
Dietary Ca concentration had a significant effect ($P < 0.05$) on the apparent fat digestibility. Fat digestibility was reduced by increasing Ca concentrations in all intestinal segments, as reflected by the lack of interaction ($P > 0.05$) between Ca concentration and intestinal site. As shown in Figure 3.2, in birds fed low Ca, normal Ca and high Ca diets, fractional fat digestion was highest by the end of jejunum and the upper ileum, but continued in the lower ileum Digestibility coefficients of fat determined in low, normal and high Ca diets at the lower ileum were 0.898, 0.890 and 0.856, respectively.

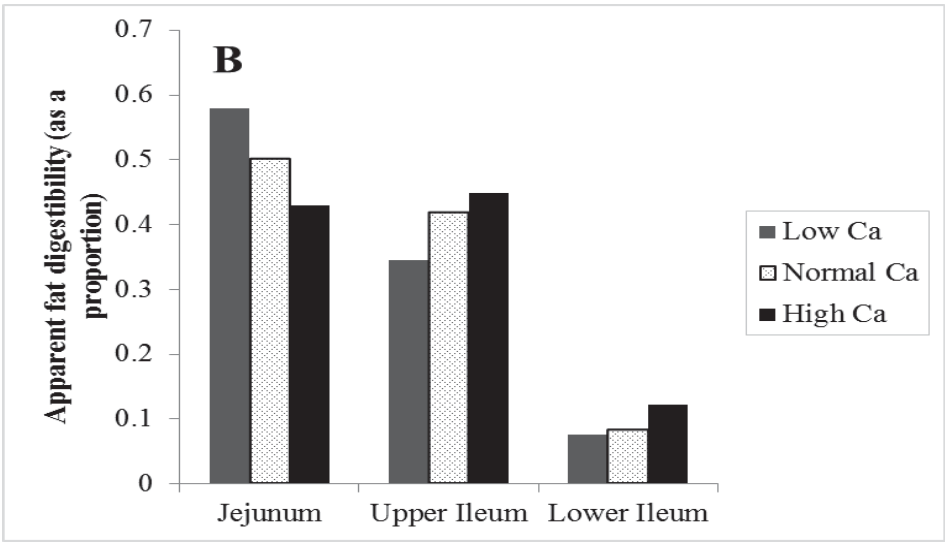
Table 3.4. Influence of dietary Ca concentration on the apparent digestibility coefficients of nitrogen (N), fat and starch along the intestinal tract of broilers[1]

Diet[2]	Site	Apparent digestibility coefficient		
		N	Fat	Starch
Low Ca	Jejunum	0.472[b]	0.520	0.841
	Upper Ileum	0.736[d]	0.830	0.958
	Lower Ileum	0.829[e]	0.898	0.979
Normal Ca	Jejunum	0.483[b]	0.445	0.840
	Upper Ileum	0.726[cd]	0.817	0.956
	Lower Ileum	0.833[e]	0.890	0.974
High Ca	Jejunum	0.359[a]	0.367	0.844
	Upper Ileum	0.689[c]	0.752	0.950
	Lower Ileum	0.812[e]	0.856	0.975
SEM[3]		0.0147	0.0206	0.0139
Main Effects				
Dietary Ca concentration				
Low Ca		0.679	0.749[a]	0.926
Normal Ca		0.680	0.717[a]	0.924
High Ca		0.620	0.658[b]	0.923
SEM[3]		0.0155	0.0162	0.0100
Site				
Jejunum		0.438	0.444[a]	0.842[a]
Upper Ileum		0.717	0.799[b]	0.955[b]
Lower Ileum		0.825	0.881[c]	0.976[b]
SEM[3]		0.0085	0.0119	0.0080
Probability ($P \leq$)				
Dietary Ca concentration		*	*	NS
Site		***	***	***
Dietary Ca concentration x Site		*	NS	NS

NS, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
[1]Each value represents the mean of four replicates.
[2]Low Ca, low Ca diet; Normal Ca, normal Ca diet; High Ca, high Ca diet.
[3]Pooled standard error of mean.
[a-e]Means in a column not sharing a common superscript are significantly different. ($P < 0.05$).

Apparent starch digestibility was not influenced ($P > 0.05$) by dietary Ca concentrations (Table 3.4) and there was no interaction ($P > 0.05$) between Ca concentration and intestinal site. Irrespective of dietary Ca concentrations, jejunum was the predominant site of starch digestion (Figure 3.2). The digestibility coefficients of starch in low, normal and high Ca diets at the lower ileum were 0.979, 0.974 and 0.975, respectively.





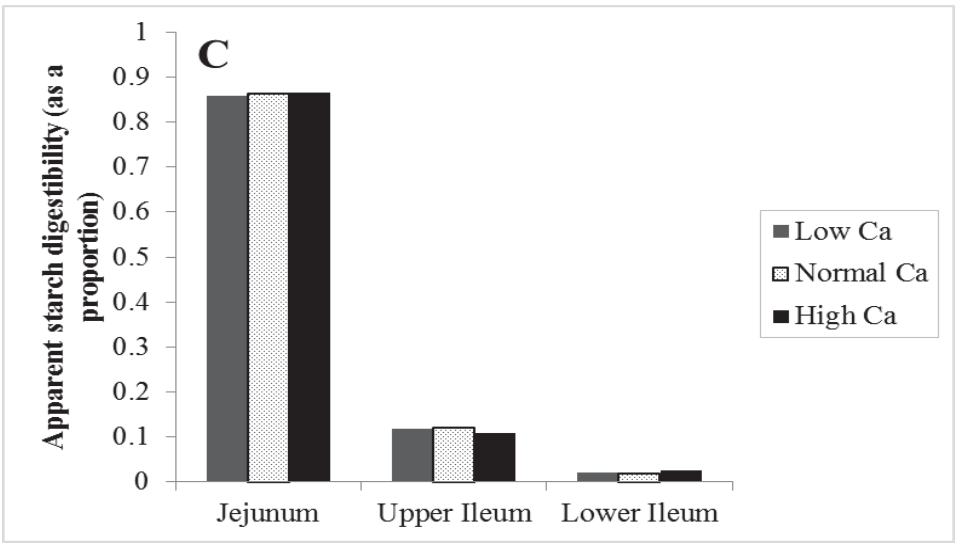


Figure 3.2. Digestion (as proportion of total digestion determined at lower ileum) of N (A), fat (B) and starch (C) along the small intestine of broilers fed diets containing different concentrations of Ca.

Apparent metabolisable energy was not affected ($P > 0.05$) by dietary Ca concentrations (Table 3.5). The AME of low, normal and high Ca diets were calculated to be 14.5, 14.3 and 14.2 MJ/kg DM, respectively.

Table 3.5. Influence of dietary Ca concentration on the apparent metabolisable energy contents (AME) of diets for broilers[1]

Diet[2]	AME (MJ/kg DM)
Low Ca	14.5
Normal Ca	14.3
High Ca	14.2
SEM[3]	0.08
Probability ($P \leq$)	
Dietary Ca concentration	NS

NS, not significant.
[1]Each value represents the mean of four replicates.
[2]Low Ca, low Ca diet; Normal Ca, normal Ca diet; High Ca, high Ca diet.
[3] Pooled standard error of mean.

3.5. Discussion

All three diets contained more Ca than calculated. This extra Ca probably came from soybean meal, since limestone is often added to soybean meal at the end of processing to prevent caking of the warm meal (Edwards, 1993). Calcium values used for diet formulation in the present study were obtained from table values (NRC, 1994). Therefore, analysed values were used in calculations of daily Ca intake of birds fed test diets.

Feed intake over the 6-day experimental period was not affected by dietary Ca concentrations, which is in an agreement with the previous reports (Atteh and Leeson, 1983; 1984; 1985; Shafey and McDonald, 1991a). Variability in feed intake by poultry is generally ascribed to differences in dietary energy contents (Atteh and Leeson, 1985). According to Roland *et al*. (1985) and Roland and Bryant (1994), the variability in intake responses to dietary Ca is dependent on the degree of excess or deficiency and whether the dietary energy was maintained as the Ca concentration was altered. The lack of Ca effect on feed intake is therefore to be expected as the diets used in the present study were formulated to be iso-caloric. However, feeding extremely high Ca concentrations (26 g/kg) to broilers was found to be detrimental to feed intake (Bryden and Balnave, 1983).

The highly negative apparent digestibility of P and Ca in the duodenum is indicative of marked net secretion of these minerals into this segment. This finding is in agreement with that of Hurwitz and Bar (1970) who also observed a net flow of Ca into the duodenum in 3-week old broilers fed diets containing 10.8 g/kg Ca and 7.7 g/kg P. A heavy endogenous secretion of P in duodenum has also been previously reported in layers (Hurwitz and Bar, 1965) and broilers (van der Klis, 1993) but not for turkeys (Hurwitz *et al*., 1979). This net flow of P may be explained by the secretion of copius input of digestive juices from pancreas and minute glands in the duodenal wall (Hurwitz *et al*., 1979). Phospholipids in the bile (van Berge Henegouwen *et al*., 1987) are other major contributors to the endogenous P flow. The major source of endogenous Ca may also be the bile in which the Ca exist as bile acid conjugates, free ionised Ca or insoluble Ca salts such as calcium bilirubinate, calcium carbonate ($CaCO_3$), calcium orthophosphate [$Ca_3(PO_4)_2$] and calcium palmitate (Moore, 1984; Gleeson *et al*., 1990). The existence of rapid, continuous duodenal-gizzard refluxes in chicken could also account, in part, for the observed negative digestibility estimates. Digesta, digestive enzymes and bile are known to be shuttled between the gizzard and duodenum to optimise nutrient digestion by prolonging the retention time (Duke, 1982) and such reverse passage may be expected to increase the net concentration of P and Ca lipids in the duodenum.

In the present study, P absorption predominantly occurred in the jejunum in broilers fed low and normal Ca diets. According to Hurwitz and Bar (1972), jejunum is the major site of vitamin D_3 action on P absorption. The absorption of P, however, continued in the upper ileum. An interesting finding of the current work was that both jejunum and upper ileum were the major sites of P digestion in birds fed diets with high Ca concentrations. Reasons for the observed shift in high Ca diet are not clear. Additional Ca as limestone has the potential to increase the precipitation of phytate P and phosphates by increasing both gut pH and Ca:phytate molar ratios (Selle *et al*., 2009) and it is tempting to speculate that this effect may have slowed phytate P hydrolysis and P absorption. However, at all Ca concentrations, digestion of P was completed by the upper ileum, and the digestibility coefficients determined at the upper and lower ileal sites were similar.

The results of the current study show that, irrespective of dietary Ca concentrations, Ca digestion was completed in the jejunum. In contrast, Hurwitz and Bar (1970) reported that Ca absorption was highest between the duodenum and lower

jejunum, and continued in the upper ileum of broilers fed diets containing 10.8 g/kg Ca. Swaminathan *et al.* (1978), on the other hand, found that the increase in Ca absorption was more pronounced in the ileum than in the duodenum and jejunum of birds as dietary Ca concentrations were reduced.

In all intestinal segments, apparent digestibility of P was progressively lowered with increasing concentrations of dietary Ca. These findings are in agreement with previous research (Hurwitz and Bar, 1965; Plumstead *et al.,* 2008). Several reasons may be provided for the negative effects of wider Ca:total P ratio on P digestion: i) High dietary Ca concentrations decrease the utilisation of phytate P by the formation of insoluble Ca-phytate complex (Qian *et al.,* 1997; Liu *et al.,* 1998; Plumstead *et al.,* 2008) making the P less available (Angel *et al.,* 2002). (ii) The ability of Ca to react with dietary inorganic P to form insoluble calcium orthophosphate (Hurwitz and Bar, 1971; Plumstead *et al*., 2008; Selle *et al*., 2009) may also make inorganic P less available for absorption at high Ca intakes. iii) Calcium is thought to be a key factor influencing the activity of mucosal phytase in the small intestine of poultry (Wise, 1983). Several studies have shown the negative effects of high dietary Ca concentrations on the intestinal activity of phytase (McCuaig *et al*., 1972; Angel *et al*., 2002; Applegate *et al*., 2003) and alkaline phosphatases (McCuaig *et al*., 1972) by competing for active sites of the enzyme.

Dietary Ca, at the concentrations used in the present study, had no effect on the apparent digestibility of Ca, which is in an agreement with the findings of Hurwitz and Bar (1965). In contrast, studies in several species including poultry have shown that the efficiency of Ca absorption is increased in low Ca diets (Swaminathan *et al.*, 1978), which was attributed as a response of animals to Ca restriction via increased production of 1,25-dihydroxycholecalciferol in the intestine (Edelstein *et al.*, 1975) which in turn stimulates the expression of Ca binding protein (DeLuca and Schnoes, 1976). At the dietary Ca concentration of 15 g/kg, maximum suppression of Ca binding protein has been observed (Hurwitz *et al*., 1995). On the other hand, some authors have reported a positive correlation between dietary Ca concentration and Ca absorption (Tamim and Angel, 2003; Tamim *et al.*, 2004).

In the present study, most of the digestion of N was found to have taken place by the end of jejunum in broilers fed low Ca and normal Ca diets. Similar to P digestion, both jejunum and upper ileum were involved in the digestion of N in birds fed the high Ca diet. However, apparent digestibility coefficients of N in birds fed low, normal and

high Ca diets were similar at the lower ileum and ranged between 0.812 to 0.833. These values are comparable with those expected for maize-soy diets. The major site of N digestion reported in this study is in an agreement with the findings of Bielorai *et al*. (1973) and Sklan and Hurwitz (1980). In both these studies, jejunum was found to be the major site of protein digestion with heavy N secretion into the duodenum.

In all intestinal segments, apparent digestibility of N was lowered at the highest concentration of dietary Ca. The negative effect of high dietary Ca on N digestion in poultry while in agreement with the findings Shafey and McDonald (1991a,b) and Wilkinson *et al*. (2014), is not readily explainable. It may be speculated that the increased pH in the gastric phase created by the provision of high levels of Ca as limestone (Ca carbonate), a source with extremely high acid-binding capacity (Lawlor *et al*., 2005), may be partly responsible. This increase in gastric digesta pH, in turn, may reduce the action of pepsin (Walk *et al*., 2012) and lower protein digestion. Another possibility is that the carbonate ion (CO_3^{2-}), one of the strongest known kosmotropes (Zhang *et al*., 2005), may play some role by lowering protein solubility. Shafey and McDonald (1991b) speculated that high Ca diets slow the digesta transit time as well as increase the intestinal microbial counts causing mucosal irritation and impairing absorption, but no supporting evidence was provided.

The results of the present study indicated that the fat digestion predominantly occurred in the jejunum and upper ileum, a finding that corresponds to that of Tancharoenrat *et al*. (2014). At all Ca concentrations, digestion continued in the ileum. The predominant role of jejunum may be partly explained by the fact that the concentration of fatty acid-binding protein is highest in the anterior segments of chickens and decreases progressively towards the distal segments (Katongole and March, 1979).

In general, apparent digestibility of fat was reduced with increasing concentrations of dietary Ca. This finding is in an agreement with that of Atteh and Leeson (1984) who found lower fat retention in broilers fed high Ca diets but the effect was dependant on the source of fat. During fat digestion, complete hydrolysis of triglycerides produces both glycerol and free fatty acids which are the absorbable units of fat (Mu and Høy, 2004). These free fatty acids have the potential to form insoluble salts with minerals, especially with Ca and magnesium, rendering both the fatty acids and minerals are unavailable to the animal (Atteh and Leeson, 1984). These insoluble soaps once formed will be excreted and it has been shown that the formation and

excretion of insoluble soaps are greater with fats containing high levels of saturated fatty acids as compared to those containing high levels of unsaturated fatty acids. Reduced fat retention was observed in both saturated and unsaturated fat, but the effect was more pronounced in the presence of saturated fat (Atteh and Leeson, 1983). Soybean oil, rich in unsaturated fatty acids, was included as the supplemental fat source in the present study. Observed negative effect of high dietary Ca concentration on fat digestion in the present study is suggestive of soap formation between Ca and unsaturated fatty acids. Similarly, Yacowitz *et al*. (1967) found a positive correlation between lipid excretion and dietary Ca concentration in rats fed maize oil, a source of high proportion of unsaturated fatty acids.

Dietary Ca, at the concentrations used in the present study, had no effect on the apparent digestibility of starch. In general, most of the starch digestion was completed by the end of jejunum, which accounted for 86 to 87% of total starch digestion. As stated by Weurding *et al*. (2001), nearly 90% of digested starch in cereal grains was completely digested prior to the ileum and 98% prior to the lower ileum.

Apparent metabolisable energy was unaffected by dietary Ca concentrations. Starch is the major energy source in cereal-based broiler diets and the observed absence of effect on the AME may be explained by the lack of effect of Ca on starch digestion. Suppressive effects of Ca on the AME have been reported to depend on dietary Ca concentration and the AME was not affected when dietary Ca concentrations were maintained at or below 12 g/kg (Atteh and Leeson, 1983; 1984). Feeding diets with over 12 g/kg of dietary Ca resulted significant decrease in the AME (Atteh and Leeson, 1984; Shafey and McDonald, 1991a). All diets in the current experiment contained Ca concentrations at or below 12 g/kg and this may explain the present findings.

3.6. Conclusions

In conclusion, the present study demonstrated that there was net secretion of P and Ca into the duodenum of broilers and that the digestion of P and Ca was essentially completed by upper ileum and jejunum, respectively. Increasing concentrations of dietary Ca supressed the digestion of P, N and fat in all intestinal segments except for N digestibility coefficients measured at the lower ileum. At low or normal dietary Ca concentrations, most of the absorption of P, N in broilers is found to take place by the jejunum. In birds fed high Ca diet, both jejunum and upper ileum involve in P and N digestion. At all three dietary Ca concentrations, fat was digested mainly in the jejunum

and upper ileum. Digestion of Ca and starch was completed primarily by end of jejunum and were unaffected by dietary Ca concentrations. Overall, the present data showed that increasing dietary Ca concentrations negatively influenced the digestion of P, N and fat, but had no effect on those of Ca and starch.

CHAPTER 4

Measurement of ileal and excreta endogenous losses of phosphorus in broiler chickens

4.1. Abstract

An experiment was conducted to estimate the ileal and excreta endogenous phosphorus (P) losses in broiler chickens. Three purified diets, namely a P-free diet and a gelatine-based diet containing negligible amount of P and a casein-based diet having 100% available P, were developed. Test diets were offered *ad libitum* from day 25 to 28 posthatch and ileal digesta were collected. Excreta samples were collected to estimate total tract endogenous losses. Endogenous flow of calcium (Ca) was estimated in birds fed the casein-based diet. The ileal endogenous flow of P in birds fed P-free, gelatin-based and casein-based diets were 25, 104 and 438, mg/kg dry matter intake (DMI), respectively. The corresponding values estimated at the excreta level were 830, 560 and 372 mg/kg DMI, respectively. Ileal and excreta endogenous flow of P in birds fed casein-based diet were similar ($P > 0.05$), but ileal flows were lower ($P < 0.05$) than excreta values in birds fed P-free and gelatin-based diets. Ileal and excreta endogenous flow of Ca in birds fed casein-based diet was estimated to be 321 and 527 mg/kg DMI, respectively, and were significantly different ($P < 0.05$). In conclusion, the present data showed that values determined for endogenous P losses in broiler chickens widely varied depending on the methodology employed.

4.2. Introduction

The main function of the gastrointestinal tract is the digestion and absorption of nutrients in the food, but there is also a significant amount of endogenous nutrients secreted into the gut. It is recognised that the amount of nutrients leaving the ileum represents the net balance between dietary nutrient intake and nutrient secretion minus the absorption of dietary nutrient and reabsorption of endogenous nutrient. Accurate measurement of and correction for these inevitable losses is necessary for the estimation of true ileal digestibility and to predict the net nutrient requirement for maintenance based on dry matter intake (Boisen and Moughan, 1996, Moughan and Fuller, 2003).

Estimates of endogenous P losses have been reported for pigs (Lopes *et al*., 1999a; Lopes *et al*., 1999b), ruminants (Salviano and Vitti, 1998) and equine (Furtado *et al*., 2000). But there have been no systematic studies conducted on ileal endogenous

phosphorus (P) losses in poultry. The primary sources of endogenous P are bile, enzyme secretions and sloughed epithelial cells. Although not strictly endogenous, gut microbes are normally considered as components of endogenous materials. Different approaches have been employed to measure endogenous P flow in animals and include regression method (Fan *et al*., 2001; Shen *et al*., 2002), feeding P-free diets (Petersen and Stein, 2004) or diets with minimal P content (Rutherfurd *et al*., 2002; 2004) and radio-isotope dilution technique (Al-Masri, 1995; Furtado *et al*., 2000). The aim of the present study was to determine the ileal endogenous losses of P using three different methodologies. Broiler chickens were fed purified diets containing no P, negligible P (gelatin-based diet) or 100% available P (casein-based diet) and endogenous losses were estimated at the ileal and excreta levels. Endogenous loss of calcium (Ca) was also estimated in birds fed casein-based diets.

4.3. Materials and methods

The experimental procedures were approved by the Massey University Ethics Committee and in accordance with the New Zealand Code of Practice for the Care and Use of Animals for Scientific Purposes.

Day-old male broilers (Ross 308) were raised in floor pens and fed a commercial broiler starter diet (12.9 MJ/kg metabolisable energy, 262 g/kg crude protein, 11.0 g/kg Ca, 6.7 g/kg total P). On day 14, birds were transferred to grower cages and were maintained on the same diet until the introduction of test diets on day 25 posthatch. The temperature was maintained at 31˚C on day 1 and reduced to 20˚C by day 25. The lighting schedule was as described in Chapter 3, Section 3.3.1. Feed was provided *ad libitum* and water was available at all times.

4.3.1. Dietary treatments

Three purified diets were developed (Table 4.1). The first diet was a protein and P-free diet. The second diet was based on gelatin, which is known to contain Ca (5 g/kg, NRC, 1994) and almost no P (NRC, 1994; Dänner *et al*., 2006). The third diet was a diet based on casein, which had negligible Ca and P contents. Phosphorus and Ca in casein were assumed to be 100% (NRC, 1994) and highly (Guéguen and Pointillart, 2000) available, respectively. All diets contained 3 g/kg titanium dioxide as an indigestible marker.

Table 4.1. Ingredient composition (g/kg as fed) of the purified diets

	Phosphorus-free diet	Gelatin-based diet	Casein-based diet
Dextrose	856.0	655.8	658.0
Casein	-	-	200.0
Gelatin[1]	-	200.0	-
Soybean oil	50.0	50.0	50.0
Cellulose[2]	50.0	50.0	50.0
L-tryptophan	-	0.2	-
Sodium bicarbonate	30.0	30.0	30.0
Magnesium oxide	2.0	2.0	2.0
Titanium dioxide	3.0	3.0	3.0
Sodium chloride	4.0	4.0	2.0
Trace mineral-premix[3]	2.5	2.5	2.5
Vitamin premix[4]	2.5	2.5	2.5
Calculated composition			
Metabolisable energy, MJ/kg	15.3	14.1	15.6
Crude protein	-	176	174
Lysine	-	7.4	15.9
Methionine	-	1.36	5.3
Methionine + Cysteine	-	1.54	6.0
Threonine	-	2.6	8.6
Ca	-	1.0	1.2
Total P	-	-	1.6
Non-phytate P	-	-	1.6
Analysed values			
Ca, g/kg as fed	0.5	0.8	0.5
Total P, g/kg as fed	< 0.09	< 0.09	1.6

[1]Davis Food Ingredients, Petone, New Zealand.
[2]Asahi Kasei Chemicals Corporation, Chiyoda-Ku, Tokyo, Japan.
[3]Supplied per kg of diet: Co, 0.3 mg, I, 1.5 mg, Mo, 0.3 mg, Se, 0.3 mg, Mn, 100 mg, Cu, 10 mg, Zn, 80 mg, Fe, 60 mg, anti-oxidant, 100 mg.
[4]Supplied per kg of diet: vitamin A, 37,500 IU, vitamin D_3, 12,500 IU, thiamine, 9 mg, riboflavin, 28 mg, pyridoxine, 31mg, folic acid, 9 mg, biotin, 0.78mg, vitamin B_{12}, 0.06 mg, vitamin E, 250 mg, choline chloride, 1.88 g, nicotinic acid, 187.5 mg, Ca pantothenate, 47 mg, menadione, 12.5 mg.

4.3.2. Birds

On day 25, birds were individually weighed and 72 birds (average weight ± SD, 1265 ± 12g) were assigned to 12 cages of 6 birds each. After four-hours of feed withdrawal, the test diets were introduced, and offered *ad libitum.* Water was available at all times. Feed intake during the test period was recorded.

4.3.3. Digesta and excreta collection

On day 26, collection trays were introduced and grab samples of fresh excreta were collected for two days and pooled within a cage. On day 28, birds were euthanised by intravenous injection of sodium pentobarbitone and the contents from the lower ileum were collected and processed as described in Chapter 3, Section 3.3.3. Daily excreta collections were processed as described in Chapter 3, Section 3.3.3. Samples of diets, digesta and excreta were ground to pass through 0.5-mm seive and stored in air-tight plastic containers till analysis for dry matter (DM), Ca, P and titanium (Ti).

4.3.4. Chemical analysis

Representative samples of diets, digesta and excreta were analysed for DM, Ca, total P and Ti as described in Chapter 3, Section 3.3.4.

4.3.5. Calculations

The flows of P and Ca were calculated, as milligrams lost per ingestion of kilogram of feed DM, by using the following formula (Moughan *et al*., 1992).

Endogenous phosphorus flow (mg/kg)

$$= \text{P or Ca}_{\text{(Digesta or excreta)}}\,\text{(mg/kg)} \times \frac{\text{Titanium}_{\text{(Diet)}}\,\text{(mg/kg)}}{\text{Titanium}_{\text{(Digesta or excreta)}}\,\text{(mg/kg)}}$$

4.3.6. Data analysis

Data were subjected to ANOVA procedures using the statistical software package SAS (2004). Means of endogenous flow of P in the ileal digesta and excreta between diets were separated using the least significant difference test. The differences between ileal and excreta endogenous P flow for each diet and Ca flow for casein-based diet were compared by paired t-test. Differences were considered significant at $P < 0.05$.

4.4. Results

Analysed Ca and P contents of three test diets and feed intakes of birds during the experimental period are shown in Tables 4.1 and 4.2, respectively. Daily feed intake of birds fed P-free, gelatin-based and casein-based was 76.6, 53.7 and 86.7 g/bird, respectively (Table 4.2).

Table 4.2. Feed intake (g/b/d) of broilers fed P free, gelatin-based and casein-based diets, day 25-28 posthatch[1]

Diet	Feed intake (g/b/d)
Phosphorus-free	76.6[b]±2.86
Gelatin-based	53.7[a]±0.67
Casein-based	86.7[c]±4.27
Pooled SEM[2]	2.99
Probability	***

[1]Each value represents the mean of four replicates (6 birds per replicate) ± standard error.
[2]Pooled standard error of mean.
****P* < 0.001
[a,c]Means in a column not sharing a common superscript are significantly different at *P* < 0.05.

Ileal endogenous flow of P in birds fed, P-free, gelatin-based and casein-based diets were estimated to be 25.1, 104 and 438 mg/kg DMI, respectively (Table 4.3). Ileal endogenous P flow in birds fed casein-based diet was higher (*P* < 0.05) than those in birds fed P-free and gelatin-based diets.

Table 4.3. Comparison of ileal and excreta endogenous P flow (mg/kg dry matter intake) in broiler chickens[1]

Diet	Endogenous P flow (mg/kg DMI)		Significance[2]
	Ileal	Excreta	
Phosphorus-free	25.1[a] ± 11.1	830[b] ± 139.1	*P* < 0.01
Gelatin-based	104[a] ± 41.3	560[ab] ± 49.2	*P* < 0.01
Casein-based	438[b] ± 67.1	372[a] ± 40.7	NS[3]
Pooled SEM[4]	45.9	88.4	

[1]Each value represents the mean of four replicates (6 birds per replicate) ± standard error.
[2]Endogenous ileal P flow vs. excreta P flow for respective diet.
[3]Not significant.
[4]Pooled standard error of mean.
[a,b]Means in a column not sharing a common superscript are significantly different at *P* < 0.05.

The endogenous flow of P in the excreta of birds fed P-free, gelatin-based and casein-based diets was 830, 560 and 372 mg/kg DMI, respectively (Table 4.3). Excreta endogenous P flows in birds fed casein-based and gelatin-based diets were similar ($P > 0.05$) but excreta endogenous P flow in birds fed casein-based diet was significantly lower than that of P-free diet. The endogenous flows were associated with high standard errors, due to the high variability between replicates.

Ileal and excreta endogenous flows of P in birds fed casein-based diet were similar ($P > 0.05$), but ileal flow was lower ($P < 0.01$) than excreta flows of P in birds fed P-free and gelatin-based and diets (Table 4.3).

Ileal and excreta endogenous flows of Ca in birds fed casein-based diet were calculated to be 321 and 527 mg/kg DMI, respectively, and were significantly different ($P < 0.05$).

4.5. Discussion

Previous studies conducted to estimate ileal endogenous P losses in poultry have used feeding of a minimal P diet (Rutherfurd *et al.,* 2002; 2004), an isotope-dilution technique (Al-Masri, 1995) or regression method (Dänner *et al.,* 2006; Dilger and Adeola, 2006b). These investigations have generated widely variable data, ranging from -864 to 446 mg of endogenous P/kg DMI (Table 4.4), depending on the methodology.

The present results demonstrate that the ileal endogenous flow of P is diet-dependant. Endogenous P losses in birds fed gelatin-based and casein-based diets were found to be higher than in those fed the P-free diet. Phosphorus-free diet was devoid of protein and the absence of protein will reduce enzyme secretions which in turn lowers the endogenous P secretion into the gut lumen. In gelatin-based and casein-based diets, the presence of protein is expected to increase the secretion of proteolytic enzymes and may explain, at least in part, the higher endogenous P flow estimated in birds fed these diets. Endogenous P losses determined in birds fed the casein-based diet yielded the highest estimate and were 4- and 14-fold greater than those estimated for gelatine-based and P-free diets, respectively. This calculation was based on the assumption that the casein-P is 100% available (NRC, 1994), but it is possible that casein-P may not be 100% digestible and the values generated with casein diet may have been overestimated. But the ileal endogenous P flow determined with the casein-based diet in the present study (438 mg/kg DMI) was in close agreement with the finding (446 mg/kg DMI) of Rutherfurd *et al*. (2004) reported using a minimal P diet.

Gastric, biliary and pancreatic secretions together with sloughed enterocytes are the main contributors of endogenous P (Fan *et al*., 2001). Bile is the primary source of endogenous P in poultry. In mammals, about 90% of bile lipids are typically composed of phospholipids (Cross *et al*., 1987). According to Alvaro *et al*. (1986), phosphatidylcholine and phosphatidylethanolamine are the major phospholipids in chicken bile. No published data are available on the mineral content of pancreatic secretions in chickens. Zebrowska *et al.* (1983) reported that about 40% of the endogenous minerals in the duodenal contents of pigs are secreted by the pancreas. But pancreatic secretions in pigs contain only low concentrations of P and Ca (Partridge *et al*., 1982; Zebrowska *et al*., 1983). It is known that the secretion of pancreatic proteolytic enzymes is sensitive to the nature of the protein source ingested (Snook and Meyer, 1964; Valette *et al*., 1992). The presence of protein has been found to increase cell slough-off and mucous secretion (Snook and Meyer, 1964). Casein is a phosphoserine rich protein with a higher buffering capacity and in order to attain optimum pH for enzyme activity, the pancreas secrete more bicarbonate with the casein-based diets leading to increased water secretion and thereby the volume of pancreatic secretions (Valette *et al*., 1992). According to Snook and Meyer (1964), dietary proteins with high biological value, such as casein, are potent stimulators of the synthesis and secretion of pancreatic enzymes.

Microbes in the gastrointestinal tract also contribute to endogenous nutrient losses (Cotton, 1972). Diet is a key factor influencing the composition and counts of microflora in the gastrointestinal tract (Barnes, 1972). Microbial cell walls are composed of phospholipids (Cotton, 1972) and high microbial turnover may have contributed to the higher ileal endogenous P losses determined in birds fed casein- and gelatin-based diets.

The comparison of ileal and excreta endogenous flows provides interesting insight into the P homeostasis in poultry. Markedly higher endogenous P in the excreta of birds fed P-free diet suggests an increase P output *via* urine when diets contain little or no Ca. A study by Liu *et al*. (2013) has shown that Ca-deficient diets lead to lower P retention in broilers. As described by Mundy and Guise (1999), a drop in ionised blood plasma Ca concentration is immediately sensed by the parathyroid gland which in turn responds with an increase in parathyroid hormone secretion. A rise in parathyroid hormone attempts to normalise serum Ca concentration by (i) increasing bone resorption and releasing Ca and P from bones to the extra cellular fluid, (ii) promoting

reabsorption of Ca while inhibiting P reabsorption at renal tubules, and (iii) increasing the absorption of Ca and P *via* stimulating synthesis of 1,25-dihydroxyvitamin D [$1,25(OH)_2D_3$] (Mundy and Guise, 1999). Since the secretion of parathyroid hormone depends on the serum ionised Ca concentration (Mundy and Guise, 1999), it can be assumed that P excretion in urine will be negatively correlated with dietary Ca concentrations.

Studies using the radioisotope dilution (Al-Masri, 1995) have estimated endogenous P losses in the excreta of broilers fed cereal-soybean meal diets to be 135, 109, 31, and 30 mg/d/bird at dietary Ca:P ratios of at 1:1, 1.5:1, 2:1 and 2.5:1, respectively. However, this technique can overestimate the endogenous losses due to the distribution of radio-active substance amongst various body components (Dilger and Adeola, 2006b). Another limitation is that the estimation of endogenous P losses using conventional diets as in the study of Al-Masri (1995) might be affected by dietary P levels as well as by the phytate-P in feed ingredients. The presence of phytate is known to cause hyper-secretion of digestive enzymes (Selle and Ravindran, 2007).

As presented in Tables 4.4 and 4.5, published data on endogenous losses of P in poultry are not only limited, but also highly variable. These data are difficult to interpret as researchers have rarely employed similar methodology which would allow for direct comparisons of data. The discrepancy among published reports is due to a number of confounding factors, including differences in assay methodology and, animal and dietary factors. Although regression method allows theoretical estimation of endogenous P losses when dietary P output is regressed against dietary P intake, in some studies endogenous P losses have been determined to be negative (Iyayi *et al*., 2013; Liu *et al*., 2013) reflecting an inherent limitation of the regression method. Such negative values will result in true digestibility being lower than its corresponding apparent digestibility values.

The excreta endogenous P losses determined in birds fed casein-based diet was numerically lower than those in birds fed gelatin-based diet (372 vs. 560 mg/kg DMI). But this difference was not statistically significant because of high variability between replicates.

Table 4.4. Comparison of published data on ileal endogenous phosphorus flow in broilers

Reference	Method	Diet	Endogenous P flow, mg/kg DMI
Present study	P-free diet	Dextrose-based	25.1
Present study	no/minimal P	Gelatin-based	104
Present study	100% available P	Casein-based	438
Iyayi *et al*. (2013)	Regression	Black-eyed pea	-843
Iyayi *et al*. (2013)	Regression	Peanut flour	-290
Liu *et al*. (2013)	Regression	Soybean meal	-448 to -864
Dilger and Adeola (2006b)	Regression	Conventional soybean meal	209
Dilger and Adeola (2006b)	Regression	Low-phytate soybean meal	145
Rutherfurd *et al*. (2004)	minimal P	Synthetic amino acids	446
Rutherfurd *et al*. (2002)	minimal P	Synthetic amino acids	272

Table 4.5. Comparison of published data on excreta endogenous phosphorus flow in broilers

Reference	Method	Diet	Endogenous P flow, mg/kg DMI
Present study	P-free diet	Dextrose-based	830
Present study	no/minimal P	Gelatin-based	560
Present study	100% available P	Casein-based	372
Iyayi *et al*. (2013)	Regression	Black-eyed pea	-377
Iyayi *et al*. (2013)	Regression	Peanut flour	1104
Liu *et al*. (2013)	Regression	Soybean meal	-726 to -803
Dänner *et al*.(2006)[1]	Regression	Wheat semolina-gelatin	<10 mg/kg BW
Dilger and Adeola (2006b)	Regression	Conventional soybean meal	191
Dilger and Adeola (2006b)	Regression	Low-phytate soybean meal	396
Al-Masri (1995)	Radioisotope-dilution	Cereal-soybean meal based diet	30-135 mg P/day/bird

[1]Turkeys.

Estimation of inevitable losses of Ca has received less attention than P in nutritional research and no comparable data on ileal endogenous Ca losses in poultry are available. Casein-based diet used in the present experiment was analysed to contain negligible levels of Ca (0.5 g/kg diet). Also the bioavailability of Ca in casein is high (Guéguen and Pointillart, 2000).

Excreta endogenous Ca flow determined in birds fed the casein-based diet in the present study was significantly higher than the ileal Ca flow, indicative urinary excretion of Ca. As mentioned earlier, Ca-deficient diets can induce the secretion of parathyroid hormone to stimulate bone resorption to normalise serum ionised Ca concentration. In this regulatory process, excess Ca derived from bone resorption can be excreted *via* urine if not reabsorbed from renal tubules.

4.6. Conclusions

In conclusion, values obtained for endogenous P losses showed a wide variability depending on the methodology employed. Endogenous flow estimated with the P-free diet may be considered as being representative of 'basal' losses, which are related to the dry matter intake and are independent of the raw material or diet composition. Perhaps this can be used in the calculation of true P digestibility of ingredients for poultry. In contrast, the estimates from casein- and gelatin-based diets also include specific losses which are influenced by the presence of protein that stimulates endogenous secretions. To the author's knowledge, the current work is the first study comparing different methodologies to determine endogenous P flow in poultry. Clearly further research is warranted to confirm the present findings.

CHAPTER 5

Measurement of true ileal digestibility and total tract retention of phosphorus in maize and canola meal for broiler chickens

5.1. Abstract

The study reported herein was conducted to determine and compare the non-phytate phosphorus (P), digestible P and retainable P contents of maize and canola meal for broiler chickens. Four semi-purified diets were formulated from each of ingredient to contain graded concentrations of non-phytate P. The experiment was conducted as a randomised complete block design with four weight blocks of eight cages each (6 birds per cage). A total of 192 broilers (Ross 308), 21-day old, were assigned to the eight test diets. Ileal digestibility and total tract retention coefficients of P were determined by the indicator and total collection methods, respectively, and linear regression method was used to determine the true P digestibility and true P retention coefficients. The apparent ileal digestibility of P in maize was influenced (quadratic, $P < 0.05$) by increasing dietary non-phytate concentrations, whereas P retention was unaffected ($P > 0.05$). The apparent ileal P digestibility in broilers fed diets based on canola meal was similar ($P > 0.05$) at different P concentrations. Phosphorus retention in broilers fed diets based on canola meal (linear, $P < 0.01$) decreased with increasing P concentrations. True ileal P digestibility and true P retention coefficients of maize were 0.676 and 0.632, respectively. The corresponding values for canola meal were 0.469 and 0.486, respectively. In both ingredients, the determined true ileal digestibility and total tract retention coefficients were not different ($P > 0.05$). Total P, non-phytate P, true digestible P and true retainable P contents of maize were determined to be 2.5, 0.8, 1.7 and 1.6 g/kg (as fed), respectively. The corresponding values for canola meal were 9.7, 2.8, 4.6 and 4.7 g/kg (as fed), respectively. The present data demonstrated that the regression method can be used to measure true P digestibility of low and high P feed ingredients and that both true ileal digestibility and retention coefficients are suitable to assess P availability in broilers.

5.2. Introduction

Phosphorus (P) is a critical nutrient for animals. In recent years, there is increasing interest in improving the utilisation dietary P for animals due to concerns over environmental pollution through excess P excretion, depletion of non-renewable global

inorganic phosphate deposits, and unpredictable prices of inorganic phosphate supplements. Use of a well-defined criterion for P availability is therefore necessary to ensure greater efficiency of utilisation of dietary P and reduce the excretion of P into the environment. There is, however considerable confusion regarding the current terminology used to describe available P (available P, non-phytate P, retainable P) in feed ingredients. Of the various possibilities, measurement of digestible P may be the preferable method to assess P availability for poultry (Rodehutscord, 2009; WPSA, 2013).

Published data on apparent or true digestibility values of P in common feed ingredients for pigs are available (Fan *et al*., 2001; Bohlke *et al*., 2005; Fang *et al*., 2007b). In these evaluations, three approaches, namely regression analysis, direct method and substitution method, have been used to estimate P digestibility. Corresponding data for poultry, however, are scant. Dilger and Adeola (2006b) estimated the true P digestibility of soybean meal for broilers using the regression method where soybean meal was used as the only dietary source for calcium (Ca) and P. Wu *et al*. (2004), using the direct method, determined the apparent ileal digestibility of P in sorghum, wheat and maize. Leytem *et al*. (2008) using the direct method measured the apparent ileal and total tract digestibility of P in maize, barley and oat where the test ingredient was used as the sole source of dietary P for broilers.

Presently, available P in feedstuffs is generally referred to as non-phytate P (NRC, 1994), which is defined as the portion of P that is not bound to the phytate molecule. These two terms (available P and non-phytate P) are used interchangeably, although studies have clearly demonstrated that non-phytate P is not totally available and phytate P is not totally unavailable to the animal (Angel *et al*., 2002; Coon *et al*., 2002). Retainable P, on the other hand refers to the P that is retained in the body and this term is used in the Netherlands as a measure of P availability in feed ingredients. It is, however, known that increasing dietary concentrations of non-phytate P result in increased levels of plasma inorganic P and, once a physiological threshold is reached, the excess P is eliminated via the urine. Studies by Manangi and Coon (2006) suggest that, for 40 to 50 day-old broilers, the critical threshold range for dietary non-phytate P appears to be between 2 to 3 g/kg. Digestibility values of P for pigs are usually determined over the total tract and this approach is workable because faecal samples can be collected without urine contamination (Fang *et al*., 2007b; Stein *et al*., 2008; Akinmusire and Adeola, 2009). In poultry, however, total tract measurements will yield

misleading data if dietary non-phytate P concentrations are above the physiological threshold and the measurements therefore should be made at the ileal level.

No published data, that compare the different measurements of P availability in feed ingredients for broilers, are currently available. The objective of the study described in this chapter was to determine and compare different measurements of P availability (non-phytate P, digestible P, and retainable P) of maize and canola meal for broilers.

5.3. Materials and methods

The experimental procedures were approved by the Massey University Ethics Committee and in accordance with the New Zealand Code of Practice for the Care and Use of Animals for Scientific Purposes.

5.3.1. Ingredients

Maize and canola meal were purchased from local commercial sources and were chosen to represent ingredients with low and high P contents, respectively. Representative samples were obtained and analysed in triplicate for DM, total P, phytate P and Ca.

5.3.2. Birds

Day-old male broilers (Ross 308) were raised in floor pens and fed a commercial broiler starter diet (262 g/kg crude protein, 11.0 g/kg Ca, 6.7 g/kg total P) until the introduction of test diets. Feed and water were available at all times. On day 21, birds were individually weighed and a total of 192 birds were assigned to four blocks based on body weight. Each block had eight cages (6 birds per cage) and the eight test diets were assigned to a cage within each block. Housing conditions were described in Chapter 3, Section 3.3.1.Group body weights and feed intakes were recorded on days 21 and 28 posthatch. Mortality was recorded daily.

5.3.3. Diets

Four semi-purified diets based on maize (236.5, 473, 709.5 and 946 g/kg diet) were formulated to contain graded concentrations of total P (0.60, 1.20, 1.80 and 2.40 g/kg) corresponding to 0.17, 0.35, 0.53 and 0.70 g/kg non-phytate P, respectively; Table 5.1). Similarly, four semi-purified diets were formulated from canola meal (135, 270, 405 and 540 g/kg diet) to contain graded concentrations of total P (1.31, 2.62, 3.92 and 5.24 g/kg; corresponding to 0.38, 0.76, 1.14 and 1.52 g/kg non-phytate P, respectively). In each set of

diets, maize and canola meal were used as the only dietary source for P. In maize-based diets, Ca: non-phytate P ratio was maintained at 2:1 by the addition of limestone. All diets contained 3 g/kg titanium dioxide (Merck KGaA, Darmstadt, Germany) as an indigestible marker. The diets, in mash form, were offered *ad libitum* and the birds had free access to water.

5.3.4. Sample collection and processing

Between days 24 and 28 posthatch, feed intake and excreta output were measured quantitatively per cage for four consecutive days. Daily collections were lyophilised, pooled within a cage, representative samples were taken, and processed as described in Chapter 3, Section 3.3.3.

On day 28 posthatch, birds were euthanised by intravenous injection of sodium pentobarbitone and the digesta from the lower half of the ileum were collected (Ravindran *et al*., 2005b), and processed as described in Chapter 3, Section 3.3.3. for chemical analysis.

5.3.5. Chemical analysis

Representative samples of test diets, excreta and digesta were analysed for DM, Ca, total P and titanium (Ti) as described in Chapter 3, Section 3.3.4. Phytate P in test ingredients was analysed by using Megazyme kit (K-PHYT, Megazyme International Ireland, Bray, Ireland) as described in Appendix C.

5.3.6. Calculations

The true ileal digestibility and total tract retention coefficients were calculated according to the procedure outlined by Dilger and Adeola (2006b). The apparent ileal digestibility coefficients (AIDC) of P of the test diets (at each level of inclusion) were calculated using the indicator ratio (Equation 1).

$$AIDC = 1 - [(T_I/T_o) \times (P_o/P_I)] \qquad [1]$$

Where, AIDC is the apparent ileal digestibility coefficient of P (calculated for ileal digesta), T_I is the Ti concentration in the diet, T_o is the Ti concentration in the ileal digesta, P_O is the P concentration in ileal digesta, and P_I is the P concentration in the diet. All analysed values were expressed as grams per kilogram of DM.

Apparent total tract retention coefficient (ATTRC) in the test diets (at each level of inclusion) were calculated using the following equation (Adeola, 2001).

$$ATTRC = (\text{Total P ingested} - \text{total P excreted})/(\text{Total P ingested})$$

Table 5.1. Ingredient composition (g/kg, as fed) of maize- and canola meal-based diets

Ingredient	Maize-based diet				Canola meal-based diet			
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 1	Diet 2	Diet 3	Diet 4
Maize	236.5	473.0	709.5	946.0	-	-	-	-
Canola meal	-	-	-	-	135.0	270.0	405.0	540.0
Soybean oil	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Dextrose	712.3	474.9	237.6	0	814.7	679.7	544.7	409.7
Limestone	0.88	1.75	2.65	3.7	-	-	-	-
Salt	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Sodium bicarbonate	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Titanium dioxide	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Vitamin premix[1]	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Trace mineral-premix[2]	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Calculated Analysis								
Metabolisable energy, MJ/kg	15.23	14.82	14.41	13.99	14.66	13.66	12.67	11.68
Crude protein, g/kg	20.1	40.2	60.3	80.4	51.3	102.6	153.9	205.2
Ca[3], g/kg	0.38	0.75	1.14	1.58	1.03	2.05	3.07	4.10
Total P[3], g/kg	0.60	1.20	1.80	2.40	1.31	2.62	3.92	5.24
Non-phytate P[3], g/kg	0.17	0.35	0.53	0.70	0.38	0.76	1.14	1.52
Ca:non-phytate P ratio	2.1:1	2.1:1	2.1:1	2.2:1	2.7:1	2.7:1	2.7:1	2.7:1
Analysed values								
Ca, g/kg	0.60	0.95	1.17	2.12	1.24	2.08	2.51	3.69
Total P, g/kg	0.62	1.32	1.69	2.41	1.36	2.60	3.90	5.29

[1]Supplied per kg of diet: vitamin A, 12,000 IU, vitamin D_3, 4,000 IU, thiamine, 3 mg, riboflavin, 9 mg, pyridoxine, 10 mg, folic acid, 3 mg, biotin, 0.25 mg, vitamin B_{12}, 0.02 mg, vitamin E, 80 mg, choline chloride, 0.6 g, nicotinic acid, 60 mg, Ca pantothenate, 15 mg, menadione, 4 mg.
[2]Supplied per kg of diet: Co, 0.3 mg, I, 1.5 mg, Mo, 0.3 mg, Se, 0.3 mg, Mn, 100 mg, Cu, 10 mg, Zn, 80 mg, Fe, 60 mg, anti-oxidant, 100 mg.
[3]Calculated based on values determined for individual ingredient.

Total output of P in the ileal digesta expressed as g/kg dry matter intake (DMI) was calculated from the following equation.

$$P_{O\text{-}DMI}\,(g/kg) = P_{O\text{-}DMO} \times (T_I/T_O) \qquad [2]$$

Where $P_{O\text{-}DMI}$ and $P_{O\text{-}DMO}$ represent the P output (as analysed in digesta) on DMI and DM output bases, respectively, and T_I is the Ti concentration in the diet (g/kg DM) and T_O is the Ti concentration in ileal digesta (g/kg DM digesta). This study was designed as a randomised complete block, with each of the eight diets (four maize diets and four canola meal diets) was fed once within a block of eight cages. In this manner, P outputs were regressed against dietary P contents per block of four cages for maize and canola meal using the following statistical model.

$$P_{O\text{-}DMI}\,(g/kg) = (TPI \times P_I) + EPL \qquad [3]$$

Where, $P_{O\text{-}DMI}$ represents the P output concentration on DMI basis (dependent variable); P_I represents dietary P content on a DM basis (independent variable), TPI represents true P indigestibility and EPL represents mean endogenous P in ileal digesta or excreta per maize or canola meal on DMI basis. In this equation, TPI and EPL are the slope and intercept, respectively, of a simple linear regression of $P_{O\text{-}DMI}$ on P_I. True P indigestibility is an indirect measure of the inefficiency at which dietary P is extracted by the bird; therefore, true P utilisation coefficient (TPUC) was calculated as,

$$TPUC = 1 - (TPI) \qquad [4]$$

Where, TPUC and TPI represent true P utilisation and true P indigestibility estimates, respectively.

5.3.7. Statistical analysis

Data were analysed as a randomised complete block design using the GLM procedure of SAS (2004). Cage was served as the experimental unit for all statistical analyses, and differences were considered significant at an alpha level of 0.05. The model for this analysis included block (3 df), and maize or canola meal inclusion level (3 df). Orthogonal polynomial contrasts were used to determine the effects of graded P intake on different parameters tested. Mean true P utilisation coefficient and endogenous P loss (g/kg DMI) from estimates were obtained by regressing P output (g/kg DMI) against dietary P content (g/kg DM) from samples pooled per cage. Therefore, standard errors for these regression coefficients were based on total of 16 observations for each feed ingredient. Regression coefficients (slopes) were compared between sampling sites (ileal digesta and excreta samples) for maize and canola meal using a Student's t-test.

5.4. Results

Analysed composition of the diets and test ingredients are presented in Tables 5.1 and 5.2, respectively. The analysed P concentrations of maize-based diets were 0.01 to 0.12 g/kg higher than calculated values. In canola meal-based diets, analysed P concentrations in all four diets were closer to the calculated values. Dietary concentrations of P increased with increasing inclusion levels of maize and canola meal in the diets. However, the analysed concentrations for Ca in three of the four maize diets were between 0.20 and 0.54 g/kg higher than expected. In two of the four canola meal diets, analysed Ca values were 0.41 to 0.56 g/kg lower than expected.

Birds remained healthy during the 7-day experimental period, and no mortality or leg problems were recorded. Feed intake and body weight gain of chickens fed diets containing graded concentrations of dietary P from maize and canola meal are summarised in Table 5.3. Weight loss was observed in birds fed the diet with the lowest dietary inclusion (236.5 g/kg) of maize. Feeding increasing concentrations of maize increased weight gain (linear, $P < 0.001$) and feed intake (linear, $P < 0.001$) of birds. Weight gain and feed intake increased (quadratic, $P < 0.01$) as the concentration of canola meal in the diet was increased.

Table 5.2. Analysed composition of maize and canola meal (g/kg, as fed basis)

	Maize	Canola meal
Total P	2.54	9.70
Phytate P	1.79	6.88
Non-phytate P[1]	0.75	2.82
Ca	0.19	7.60

[1]Calculated as the difference between total and phytate P.

Dietary P contents and P outputs in birds fed maize and canola meal-based diets are presented in Table 5.3. Increasing concentrations of dietary P linearly increased ($P < 0.001$) ileal and excreta P outputs in birds fed both diets. In birds fed maize-based diets, excreta P output was higher than ileal P output by an average of 0.14 g/kg. Interestingly, in contrast, in birds fed canola meal-based diets, excreta P output was lower than ileal P output by 0.09 g/kg on average.

Table 5.3. Growth performance (days 21-28 posthatch) and, dietary P content and total P output (days 24-28 posthatch) in birds fed diets containing graded concentrations of P from maize and canola meal for broilers[1]

Measurement	Maize-based diets							Canola meal-based diet						
	Diet 1	Diet 2	Diet 3	Diet 4	SEM	Probability		Diet 1	Diet 2	Diet 3	Diet 4	SEM	Probability	
						L^2	Q^2						L^2	Q^2
BWG[3], g/b/d	-5.46	3.69	18.65	34.77	2.28	***	NS	6.11	29.74	45.33	53.92	1.84	***	**
FI[4], g/b/d	72.68	82.67	97.05	107.32	5.25	***	NS	86.55	105.13	111.96	108.73	3.38	***	**
P_I[5], g/kg DM	0.69	1.49	1.93	2.75	-	-	-	1.53	2.92	4.36	5.89	-	-	-
P_D[6], g/kg DMI	0.27	0.44	0.66	0.92	0.04	***	NS	0.54	0.94	1.59	2.88	0.19	***	*
P_E[7], g/kg DMI	0.38	0.48	0.88	1.09	0.07	***	NS	0.46	0.87	1.55	2.72	0.11	***	**

NS, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

[1]Each value represents the mean of four replicates (6 birds/ replicate).

[2]L = linear effect; Q = quadratic effect.

[3]BWG = body weight gain.

[4]FI = feed intake.

[5]P_I = Dietary P content; DM = Dry matter.

[6]P_D = Ileal P output; DMI = Dry matter intake.

[7]P_E = Excreta P output.

The apparent P utilisation of birds fed maize-based diets ranged from 0.605 to 0.704 at the ileal level and 0.451 to 0.675 over the total tract (Table 5.4). The apparent ileal digestibility of P from maize was affected (quadratic, $P < 0.05$) by increasing dietary P concentrations, while P retention was unaffected ($P > 0.05$). In contrast, the apparent P digestibility of canola meal was similar ($P > 0.05$) at different dietary P concentrations. Phosphorus retention in broilers fed canola meal diets linearly ($P < 0.01$) decreased with increasing P concentrations.

Strong linear relationships were observed between digesta and excreta outputs and dietary P content for both maize and canola meal (Figures 5.1 and 5.2). True P digestibility of maize and canola meal and the estimated endogenous P losses are presented in Table 5.5. True ileal P digestibility and retention coefficients of maize were determined to be 0.676 and 0.632, respectively. The corresponding values for the canola meal were 0.469 and 0.486, respectively. No differences ($P > 0.05$) were found between true ileal digestibility and true retention coefficients of P determined for maize and canola meal (Table 5.5). Endogenous P losses estimated for maize at the ileal and excreta levels were 0.020 and 0.077 g/kg DMI respectively. In birds fed canola meal based diets, the endogenous P losses were determined to be negative at both ileal and excreta levels, with values of -0.464 and -0.487 g/kg DMI, respectively.

Table 5.4. Apparent ileal digestibility and total tract retention of phosphorus (P) in birds fed diets containing graded concentrations of P from maize and canola meal for broilers[1]

	Maize-based diets		Canola meal-based diets	
	Ileal P digestibility coefficient	Phosphorus retention coefficient	Ileal P digestibility coefficient	Phosphorus retention coefficient
Diet 1	0.605[a]	0.451	0.648	0.697[b]
Diet 2	0.704[b]	0.675	0.679	0.701[b]
Diet 3	0.656[ab]	0.545	0.635	0.643[b]
Diet 4	0.664[b]	0.605	0.512	0.539[a]
Pooled SEM	0.017	0.051	0.061	0.032
Probability	*	NS	NS	*
L[2]	NS	NS	NS	**
Q[2]	*	NS	NS	NS

NS, not significant; *$P < 0.05$; **$P < 0.01$.

[1]Each value represents the mean of four replicates (6 birds/ replicate).

[2]L = linear effect; Q = quadratic effect.

[a-b]Means in a column not sharing a common superscript are significantly different at $P < 0.05$.

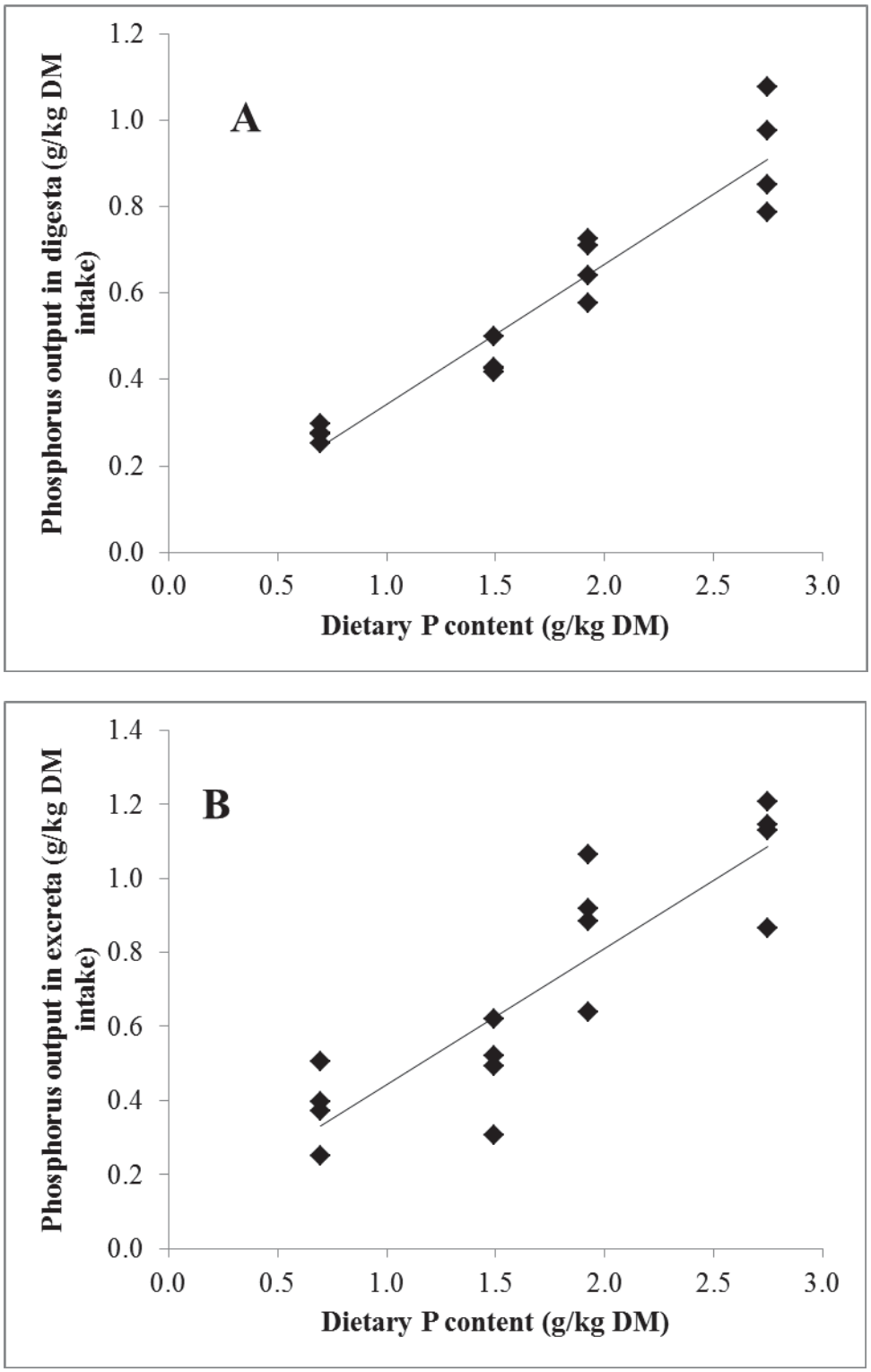


Figure 5.1. Linear relationship between total phosphorus (P) outputs (Y: g/kg DM diet intake) in ileal digesta (A) and excreta (B) and dietary P content (X: g/kg DM) in 21-day old broilers fed maize-based diets containing graded P concentrations.

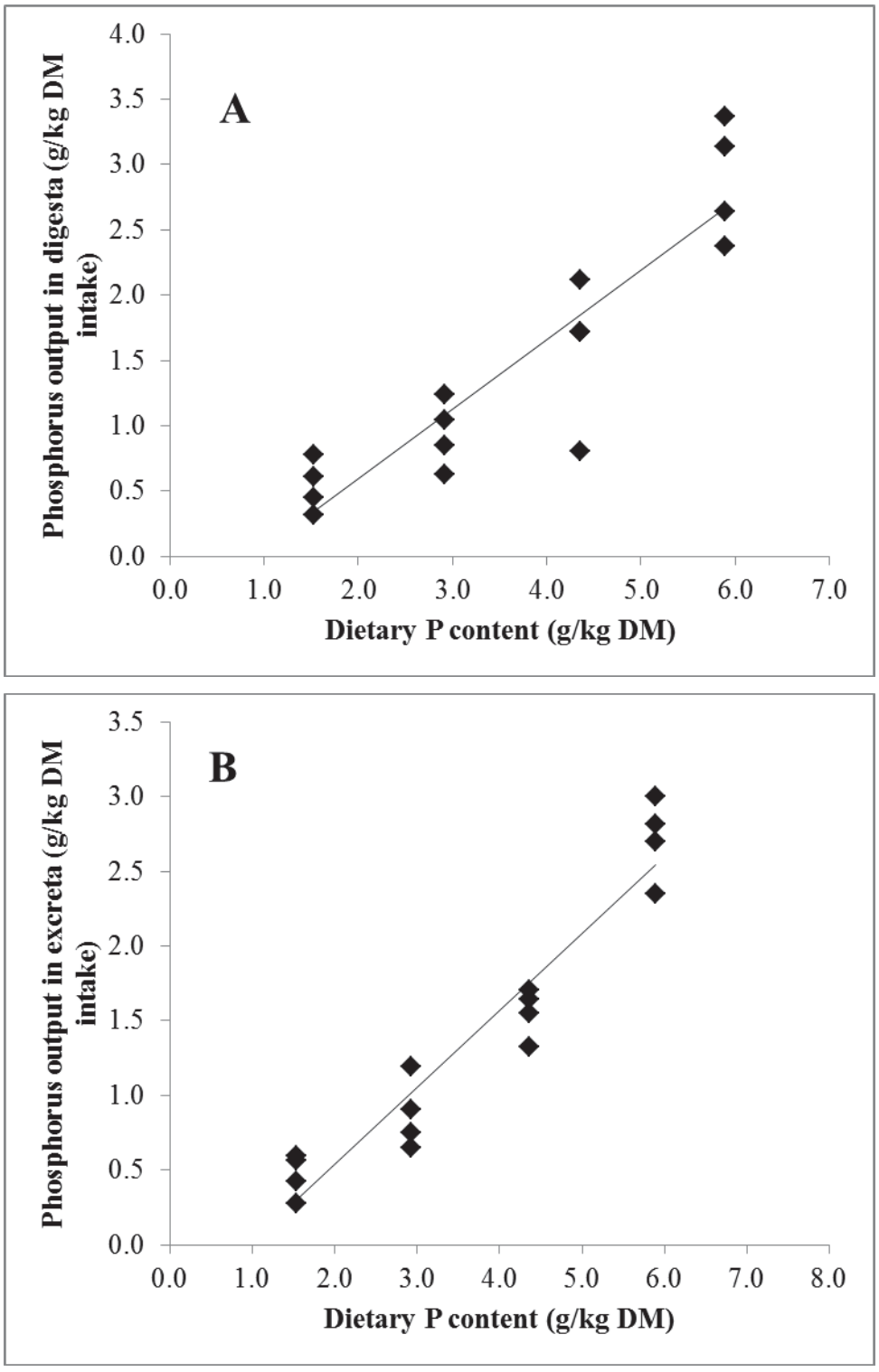


Figure 5.2. Linear relationship between total phosphorus (P) outputs (Y: g/kg DM diet intake) in ileal digesta (A) and excreta (B) and dietary P content (X: g/kg DM) in 21-day old broilers fed canola meal-based diets containing graded P concentrations.

Table 5.5. Linear relationship between ileal or excreta P outputs (g/kg DMI) vs. dietary P content (g/kg DM) of maize and canola meal fed to broilers[1]

	Regression equation[2]	SE of the slope[3]	SE of the intercept[3]	r^2	Endogenous P loss (g/kg DMI)[4]	Digestibility/ retention coefficient[5]
Maize						
True ileal P digestibility	Y = 0.324X + 0.020	0.03	0.05	0.91	0.020	0.676
True P retention	Y = 0.368X + 0.077	0.05	0.10	0.76	0.077	0.632
Canola meal						
True ileal P digestibility	Y = 0.531X - 0.464	0.07	0.26	0.82	- 0.464	0.469
True P retention	Y = 0.514X - 0.487	0.04	0.17	0.92	- 0.487	0.486

[1]Each value represents the mean of four replicates (6 birds/ replicate).
[2]Regression of ileal digesta or excreta P output (g/kg DMI) against dietary P content (g/kg DM) as determined by feeding broilers with diets containing graded levels of either maize or canola meal. The slope represents true P indigestibility and the intercept represents the endogenous P loss (g/kg DMI).
[3]Standard error of regression.
[4] Calculated as described in Dilger and Adeola (2006b).
[5] Within each ingredient, digestibility and retention coefficients were similar ($P > 0.05$).

5.5. Discussion

The analysed dietary P concentrations were closer to calculated concentrations and therefore used for the calculation of P digestibility and retention coefficients in the current study. The analysed concentrations of Ca, however, differed from expected in most diets and the interpretation of these data is difficult as all diets were formulated using the same batch of ingredients. It was assumed that these do not reflect true differences between formulated and analysed values, and hence would not influence the P utilisation calculations. Interestingly, a similar trend between analysed and calculated Ca values at low dietary Ca concentrations has been previously reported (Driver *et al*., 2005).

No mortality or leg problems were observed during the 7-day experimental period. However, bone deformities and poor survival rates (33%) were reported in a study conducted by Hayes *et al*. (1979) when day-old chicks were fed up to 13 days of age on a maize based semi-purified diet with no added P source. In the present study, birds responded linearly in weight gain and feed intake when fed maize and canola meal-based diets containing increasing concentrations of P. However, weight loss was evident when the birds were fed with the lowest dietary inclusion (236.5 g/kg diet) of maize. Weight gain was highest in birds fed the highest inclusion of canola meal which contained the highest contents of P and crude protein.

Phosphorus output in the excreta is the sum of undigested dietary and endogenous P, P utilised by the hindgut microflora and P excreted via urine. The higher excreta P output observed in birds fed maize-based diets, relative to ileal P output, in the present study is most likely to be due to urinary excretion of P. Exact reasons for the increased urinary P excretion are unclear, as the dietary non-phytate P concentrations in maize-based diets are below the critical physiological threshold of 2 g/kg reported for 40 to 50-day old broilers (Manangi and Coon, 2006) and the Ca: non-phytate P ratios were within the accepted range. It is, however, possible that this critical threshold may be age-dependent and may not be applicable to the 3-week old broilers used in the current work. In contrast, the lower excreta P output, relative to ileal output, in birds fed canola meal- based diets is indicative of postileal disappearance of P. This could happen due to microbial degradation of phytate P and absorption of released P in the hindgut, but the absorption of P in the hindgut of poultry is generally considered to be negligible (Dilger and Adeola, 2006b). It is possible that the observed shifts in ileal and excreta P outputs might be ingredient-specific rather than being influenced by dietary non-phytate P intake.

Apparent ileal P digestibility values determined at different inclusion levels of maize were different. In contrast, the apparent digestibility values for canola meal were not influenced by ingredient inclusion level. These results suggest that the direct method is not a suitable method to estimate apparent ileal digestibility of cereal grains with low P contents. This finding may be explained by the fact that the contribution of endogenous P is relatively high at low dietary P intake. Lack of influence of inclusion level on the apparent ileal P digestibility of canola meal may suggest that the direct method can be employed to estimate apparent ileal P digestibility of ingredients with high P contents. The apparent ileal P digestibility coefficients determined for maize in the present study ranged from 0.605 to 0.704. These findings are in a close agreement with those of Wu *et al*. (2004) who reported a value of 0.70, but lower than the value of 0.86 obtained by Leytem *et al*. (2008). In both these studies, direct method was employed where maize was used as the sole source of dietary P and Ca.

Apparent total tract retention coefficients determined at different dietary inclusion levels of maize were similar. However, the values determined in the present study (0.451 to 0.675) were higher than the retention coefficients of 0.25 and 0.35 reported by Leytem *et al*. (2008) and, Leske and Coon (1999), respectively. The higher P retention coefficients in the present study may be explained by the addition of limestone in maize-based diets, which may have reduced Ca mobilization from bones and therefore reduced P excretion via urine. In contrast, apparent P retention for canola meal was influenced by inclusion levels, with retention linearly decreasing from 0.70 to 0.54 with increasing dietary P contents. These values were higher than the retention coefficient of 0.39 observed by Leske and Coon (1999) for canola meal.

Strong linear relationships between digesta and excreta P outputs and dietary P intake were observed for both maize and canola meal which is a primary requirement for the application of regression technique (Dilger and Adeola, 2006b). This relationship permits the determination of diet-independent theoretical estimate of endogenous P losses (g/kg DMI) and simultaneous measurement of true P digestibility of a particular feed ingredient (Fan *et al*., 2001; Dilger and Adeola, 2006b). True ileal P digestibility coefficients of maize and canola meal were determined to be 0.676 and 0.469, respectively. The corresponding retention coefficients were 0.632 and 0.486, respectively. No differences were found between the true ileal digestibility and true retention coefficients of P in both maize and canola meal. This is to be expected since all diets were formulated below the P requirement.

True ileal P digestibility in maize has not been previously reported, but the true ileal digestibility of P in canola meal determined in the present work was considerably lower than the value of 0.66 reported by Adeola and Applegate (2010) (Table 5.6). However, true retention coefficient of P determined for canola meal in the present study was in agreement with the value (0.39) reported by these researchers. Endogenous P losses estimated for maize at the ileal and excreta levels were 0.020 and 0.077 g/kg DMI, respectively. In the birds fed canola meal based diets, endogenous P losses were determined to be negative at both ileal and excreta levels, with values of -0.464 and -0.487 g/kg DMI, respectively. These negative estimates for endogenous P losses are clearly an anomaly reflecting an inherent limitation of the regression method, where the first or last data points could markedly change the intercept and slope. It is well known that mathematical error is inherent in any extrapolation beyond the data recorded (Moughan *et al*., 1998). Such negative estimates for endogenous P losses, with the regression method, have also been previously reported in growing male turkeys (Dänner *et al*., 2006) and broilers (Rodehutscord *et al*., 2012; Shastak *et al*., 2012; Iyayi *et al*., 2013; Liu *et al*., 2013).

Table 5.6. Comparison of present data with published data of true (TD) and apparent digestibility (AD) coefficients of phosphorus in maize and canola meal for broilers

References	Method		TD	AD
Maize				
Present study	Regression	Digesta	0.676	-
Present study	Regression	Excreta	0.632	-
Present study	Direct	Digesta	-	0.605-0.704
Present study	Direct	Excreta	-	0.451-0.675
Wu *et al*. (2004)	Direct	Digesta	-	0.70
Leytem *et al*. (2008)	Direct	Digesta	-	0.86
Leytem *et al*. (2008)	Direct	Excreta	-	0.25
Canola meal				
Present study	Regression	Digesta	0.469	-
Present study	Regression	Excreta	0.486	-
Present study	Direct	Digesta	-	0.512-0.679
Present study	Direct	Excreta	-	0.539-0.701
Adeola (unpublished)[1]	Regression	Digesta	0.66	-
Adeola (unpublished)[1]	Regression	Excreta	0.39	-

[1]Cited by Adeola and Applegate (2010).

Analysed phytate P contents of maize and canola meal were within the range reported in the literature (Selle and Ravindran, 2007). The data, summarised in Table 5.7, show that the P evaluation system based on non-phytate P is not reflective of P availability in feed ingredients.

Table 5.7. Comparison of total P, phytate P, non-phytate P, true digestible P and true retainable P contents of maize and canola meal (g/kg, as fed)

	Maize	**Canola meal**
Total P	2.54	9.70
Phytate P	1.79	6.88
Non-phytate P[1]	0.75	2.82
True digestible P	1.72	4.55
True retainable P	1.61	4.72
As % of total P		
Phytate P	70.5	70.9
Non-phytate P	29.5	29.1
True digestible P	67.7	46.9
True retainable P	63.4	48.6

[1]Calculated as the difference between total P and phytate P.

True digestible P and retainable P contents were considerably higher than the non-phytate P contents in both maize and canola meals, suggesting that a portion of phytate-bound P is being utilised by broiler chickens. If we assume that non-phytate P in maize is 100% digestible, then it can be calculated that 54.2% of phytate-bound P was digested and absorbed. The corresponding value for phytate P hydrolysis canola meal was 25.2%. However, it is known that the utilisation of phytate P by chickens can vary widely, ranging from 0 to as high as 75%, and that dietary factors including the level of Ca, non-phytate P, P and vitamin D_3 may influence phytate P hydrolysis in the digestive tract (Angel *et al*., 2002). Of these factors, dietary Ca level is probably the major factor influencing phytate P hydrolysis (Ballam *et al*., 1984; Mohammed *et al*., 1991; Tamim and Angel, 2003). It is therefore likely the higher true P utilisation estimates determined in our study may be due, at least in part, to the low dietary Ca concentrations in the test diets. The recommended dietary requirement for Ca for growing broilers is 9.0 g/kg (Ross, 2007) and all the test diets contained much lower concentrations of Ca. Ballam *et al*. (1984) observed that chicks fed diets containing 8.5 g/kg Ca hydrolysed more phytate P than those fed diets with 10 g/kg Ca (0.10 vs. 0.23).

Mohammed *et al*. (1991) also found that phytate P utilisation was increased by 15% when dietary Ca concentration was reduced from 10.0 to 5.0 g/kg. Similarly, Tamim and Angel (2003) reported that, reducing the dietary Ca concentration from 6.8 to 1.8 g/kg increased the apparent phytate P disappearance from 0.21 to 0.69.

5.6. Conclusions

In conclusion, the present data demonstrated that the direct method is not a suitable method to estimate apparent ileal digestibility of cereal grains with low P contents, but can be applied for the estimation of apparent ileal P digestibility of feed ingredients with high total P contents. Regression method can be used to measure true P digestibility of low and high P ingredients. The negative endogenous P losses determined in the current work, however, call the validity of regression method into question. Under the conditions of the present study, P digestibility and retention coefficients in maize and canola meal were not different, indicating that retention values were not influenced by the urinary excretion of P when birds were fed below the P requirement. Overall, these data suggest that P evaluation based on digestible P may provide a better assessment of P availability than that based on non-phytate P. Further studies are, however, needed before definite conclusions are made.

CHAPTER 6

Measurement of true ileal digestibility of phosphorus in some common feed ingredients for broiler chickens

6.1. Abstract

An experiment was conducted to estimate the true ileal digestibility of phosphorus (P) in wheat, sorghum, soybean meal and maize-distiller's dried grains with solubles (maize-DDGS) in broiler chickens. Four semi-purified diets were formulated from each ingredient to contain graded concentrations of non-phytate P. The experiment was conducted as a randomised complete block design with four weight blocks of 16 cages each (5 birds per cage). A total of 320, 21-day old broilers (Ross 308), were assigned to the 16 test diets with four replicates per diet. Apparent ileal digestibility coefficients of P were determined by the indicator method and the linear regression method was used to determine the true P digestibility coefficients. The apparent ileal P digestibility coefficients of wheat-based diets were not influenced ($P > 0.05$) by increasing dietary P concentrations, whereas those of diets based on sorghum, soybean meal and maize-DDGS differed ($P < 0.05$) at different P concentrations. The apparent ileal P digestibility in broilers fed diets with soybean meal and maize-DDGS linearly ($P < 0.001$) increased with increasing P concentrations. True ileal P digestibility coefficients of wheat, sorghum, soybean meal and maize-DDGS were determined to be 0.464, 0.331, 0.798, and 0.727, respectively. Ileal endogenous P losses in birds fed diets with wheat, soybean meal and maize-DDGS were estimated to be 0.080, 0.609 and 0.418 g/kg dry matter intake (DMI), respectively. In birds fed sorghum-based diets, endogenous P losses were estimated to be negative (-0.087 g/kg DMI). True digestible P contents of wheat, sorghum, soybean meal and maize-DDGS were determined to be 1.49, 0.78, 5.16 and 5.94 g/kg, respectively. The corresponding non-phytate P contents in wheat, sorghum, soybean meal and maize-DDGS were 1.11, 0.55, 2.15 and 4.36 g/kg, respectively. These differences between digestible P and non-phytate P contents may be suggestive, at least in part, of overestimation of P digestibility under the calcium-deficient conditions employed in the regression method.

6.2. Introduction

Use of a well-defined criterion for phosphorus (P) availability is expected to ensure greater efficiency of utilisation of dietary P in poultry. It is recognised that the current

use of different terminologies, namely available P, non-phytate P and retainable P, to describe available P in feed ingredients leads to the overestimation of true P requirements for poultry resulting in excess P being excreted (WPSA, 2013). Measurement of digestible P has been suggested as the preferable approach to assess P availability in poultry to minimise the P excretion into the environment (WPSA, 2013).

A number of reports are available on the apparent or true digestibility values of P in common feed ingredients for pigs (Fan *et al*., 2001; Bohlke *et al*., 2005; Fang *et al*., 2007b; Stein *et al*., 2008; Rojas *et al*., 2013). In these studies, three approaches, namely regression analysis, the direct method and the substitution method, have been used to estimate P digestibility. Only limited attempts have been made to determine the digestible P content in feed ingredients for poultry and two approaches, namely the direct method (Wu *et al*., 2004; Leytem *et al*., 2008) and regression method (Dilger and Adeola, 2006b) have been used. These experiments were performed using test ingredient as the sole dietary source of P and calcium (Ca). In the study reported in Chapter 5 of the present thesis, non-phytate P, true retainable P and true digestible P contents of maize and canola meal for broilers were measured using the regression approach. The present study is an expansion of the previous study to determine the true ileal digestibility of P in four other common ingredients (wheat, sorghum, soybean meal and maize-DDGS) for broilers.

6.3 Materials and methods

The experiment procedures were approved by the Massey University Ethics Committee and complied with the New Zealand Code of Practice for the Care and Use of Animals for Scientific Purposes.

6.3.1. Ingredients

Wheat, sorghum, soybean meal and maize-DDGS samples were purchased from local commercial sources and representative samples were obtained and analysed in triplicate for dry matter (DM), total P, phytate P and Ca. Phytase activity in wheat and maize-DDGS were also determined. Non-phytate P contents were calculated as the difference between total P and phytate P.

6.3.2. Birds

Day-old male broilers (Ross 308) from a local hatchery were raised on floor pens and fed a commercial broiler starter diet (12.9 MJ/kg metabolisable energy, 262 g/kg crude

protein, 11.0 g/kg Ca, 6.7 g/kg total P). On day 14, birds were transferred to grower cages. On day 21, the birds were individually weighed and a total of 320 birds were assigned to four blocks based on body weight. Each block had 16 cages (5 birds per cage) and the 16 test diets were assigned to a cage within each block. The floor pens and grower cages were housed in environmentally controlled rooms. Housing conditions have been described in Chapter 3, Section 3.3.1.

6.3.3. Dietary treatments

Four semi-purified diets were formulated from each of the four test ingredients (Tables 6.1 and 6.2) using the nutrient compositional values presented in NRC (1994, 2012). In each set of diets, the test ingredient was used as the only dietary source for P. Ca:non-phytate ratio was maintained in all diets at 2:1 by the addition of limestone. All diets contained 3 g/kg titanium dioxide (Merck KGaA, Darmstadt, Germany) as an indigestible marker. The diets, in mash form, were offered *ad libitum* from day 21 to 28 and water was freely available.

6.3.4. Sample collection and processing

Group body weights and feed intake were recorded on days 21 and 28. On day 28 posthatch, birds were euthanised by intravenous injection of sodium pentobarbitone and the contents of the lower half of the ileum were collected (Ravindran *et al*., 2005b) and processed as described in Chapter 3, Section 3.3.3. for chemical analysis. Representative samples of test diets and digesta were analysed for DM, Ca, total P and Ti.

6.3.5. Chemical analysis

Representative samples of test ingredients, test diets and digesta were analysed according to the procedures outlined in Chapter 3, Section 3.3.4. Phytate P in test ingredients was analysed as described in Chapter 5, Section 5.3.5. The analysis of phytase activity in wheat and maize-DDGS was based on the method described by Engelen *et al*. (1994), where the samples were incubated with sodium phytate to liberate inorganic phosphates from the substrate. One phytase Unit is defined as the amount of enzyme which liberates 1 μmol inorganic P/min from 5.1 mM-sodium phytate at pH 5.5 and 37˚C.

Table 6.1. Ingredient composition (g/kg, as fed) of wheat- and sorghum-based diets

Ingredient	Wheat-based diet (g/kg)				Sorghum-based diet (g/kg)			
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 1	Diet 2	Diet 3	Diet 4
Wheat	236.5	473.0	709.5	946.0	-	-	-	-
Sorghum	-	-	-	-	236.5	473.0	709.5	946.0
Soybean oil	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Dextrose	713.5	475.7	237.9	0	712.5	475.0	237.5	0
Limestone	1.34	2.65	3.95	5.3	1.0	2.0	3.0	4.0
Salt	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Sodium bicarbonate	18.4	18.4	18.4	18.4	19.7	19.7	19.7	19.7
Titanium dioxide	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Vitamin premix[1]	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Trace mineral premix[2]	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Calculated composition								
Metabolisable energy , MJ/kg	14.8	13.9	13.1	12.2	15.2	14.7	14.2	13.7
Crude protein, g/kg	33.3	66.7	100.0	133.4	20.8	41.6	62.4	83.2
Ca, g/kg	0.63	1.24	1.86	2.49	0.47	0.95	1.42	1.89
Total P, g/kg	0.88	1.75	2.63	3.50	0.71	1.42	2.13	2.84
Non-phytate P, g/kg	0.31	0.62	0.92	1.23	0.24	0.47	0.71	0.95
Ca:non-phytate P ratio	2.0:1	2.0:1	2.0:1	2.0:1	2.0:1	2.0:1	2.0:1	2.0:1
Analysed values								
Ca, g/kg	1.01	1.29	1.88	2.40	0.91	1.12	1.75	2.58
Total P, g/kg	0.85	1.48	2.24	3.08	0.57	1.21	1.80	2.31

[1]Supplied per kg of diet: vitamin A, 12,000 IU, vitamin D_3, 4,000 IU, thiamine, 3 mg, riboflavin, 9 mg, pyridoxine, 10 mg, folic acid, 3 mg, biotin, 0.25 mg, vitamin B_{12}, 0.02 mg, vitamin E, 80 mg, choline chloride, 0.6 g, nicotinic acid, 60 mg, Ca pantothenate, 15 mg, menadione, 4 mg.
[2]Supplied per kg of diet: Co, 0.3 mg, I, 1.5 mg, Mo, 0.3 mg, Se, 0.3 mg, Mn, 100 mg, Cu, 10 mg, Zn, 80 mg, Fe, 60 mg, anti-oxidant, 100 mg.

Table 6.2. Ingredient composition (g/kg, as fed) of soybean meal-and maize-distiller's dried grans with solubles (DDGS)-based diets

Ingredient	Soybean meal-based diet (g/kg)				DDGS-based diet (g/kg)			
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 1	Diet 2	Diet 3	Diet 4
Soybean meal (480 g/kg CP)	135.0	270.0	405.0	540.0	-	-	-	-
DDGS	-	-	-	-	135.0	270.0	405.0	540.0
Soybean oil	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Dextrose	814.1	678.5	542.9	407.3	812.5	675.3	538.1	400.9
Limestone	0.63	1.25	1.82	2.43	2.2	4.4	6.6	8.8
Salt	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Sodium bicarbonate	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Titanium dioxide	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Vitamin premix[1]	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Trace mineral premix[2]	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Calculated composition								
Metabolisable energy, MJ/kg	14.7	14.1	13.4	12.6	14.9	14.1	13.4	12.6
Crude protein, g/kg	65.5	130.9	196.4	261.9	36.9	73.9	110.9	147.9
Ca, g/kg	0.60	1.20	1.79	2.38	1.07	2.13	3.19	4.26
Total P, g/kg	0.84	1.67	2.51	3.35	0.97	1.94	2.92	3.89
Non-phytate P, g/kg	0.29	0.59	0.89	1.19	0.53	1.05	1.58	2.11
Ca:non-phytate P ratio	2.0:1	2.0:1	2.0:1	2.0:1	2.0:1	2.0:1	2.0:1	2.0:1
Analysed values								
Ca, g/kg	0.96	1.56	2.12	2.84	1.09	2.12	2.73	3.46
Total P, g/kg	0.92	1.91	2.67	3.52	1.15	2.16	3.33	4.51

[1]Supplied per kg of diet: vitamin A, 12,000 IU, vitamin D_3, 4,000 IU, thiamine, 3 mg, riboflavin, 9 mg, pyridoxine, 10 mg, folic acid, 3 mg, biotin, 0.25 mg, vitamin B_{12}, 0.02 mg, vitamin E, 80 mg, choline chloride, 0.6 g, nicotinic acid, 60 mg, Ca pantothenate, 15 mg, menadione, 4 mg.
[2]Supplied per kg of diet: Co, 0.3 mg, I, 1.5 mg, Mo, 0.3 mg, Se, 0.3 mg, Mn, 100 mg, Cu, 10 mg, Zn, 80 mg, Fe, 60 mg, anti-oxidant, 100 mg.

6.3.6. Calculations

The apparent and true digestibility of P in each test ingredient was calculated according to the equations 1 to 4 outlined in Chapter 5, Section 5.3.6. This study was designed as a completely randomised block design, with each of the 16 test diets fed once within 64 cages. In this manner, P outputs were regressed against dietary P contents per 16 cages for each of the test ingredient.

6.3.7. Statistical analysis

Data were analysed as a randomised complete block design using the GLM procedure of SAS (2004) as described in Chapter 5, Section 5.3.7.

6.4. Results

The calculated and analysed nutrient composition of the test diets are presented in Tables 6.1 and 6.2, respectively. Analysed P, Ca and phytate P contents of test ingredients, along with assumed contents used in formulations, are summarised in Table 6.3. The analysed P concentrations of most diets were in close agreement with calculated values. Dietary concentrations of P and Ca increased with increasing inclusion levels of each ingredient. Phytase activity determined for wheat and maize-DDGS were 421 and 666 U/kg, respectively.

Table 6.3. Analysed composition of test ingredients (g/kg, as fed basis)

	Wheat	Sorghum	Soybean meal	DDGS
Total P	3.22 (3.7)[1]	2.37(3.0)[1]	6.46 (6.2)[1]	8.17 (7.2)[1]
Phytate P	2.11	1.82	4.31	3.82
Non-phytate P[2]	1.11(1.3)[1]	0.55(1.0)[3]	2.15 (2.2)[1]	4.36 (3.9)[1]
Ca	0.55 (0.5)[1]	1.03(0.4)[1]	2.84 (2.7)[1]	0.46 (1.7)[1]
Phytase activity, U/kg	421	-	-	666

[1]Values in parenthesis refer to assumed values, from NRC (1994), used in diet formulations.
[2]Calculated as the difference between total and phytate P.
[3]Value in parenthesis refer to assumed value, from NRC (2012), used in diet formulations.

Birds remained healthy during the 7-day experimental period and no leg problems were recorded. Mortality was negligible and only 5 birds out of the 320 birds died. Feed intake and body weight gain of birds fed diets containing graded

concentrations of dietary P from wheat, sorghum, soybean meal and maize-DDGS are summarised in Table 6.4. Weight loss was observed in birds fed the lowest dietary inclusion (236.5 g/kg) of wheat. Feeding diets with increasing inclusions of wheat increased the weight gain (linear, $P < 0.001$; quadratic, $P < 0.05$) and feed intake (linear, $P < 0.001$). Weight loss was also observed in birds fed the first two inclusions levels of sorghum (236.5 and 473 g/kg). Feeding graded concentrations of sorghum linearly increased ($P < 0.001$) the body weight gain of birds, but the feed intake was not influenced ($P > 0.05$). Increasing dietary inclusions of soybean meal increased (linear, $P < 0.001$) the body weight gain and feed intake of birds. Weight loss was observed in birds fed the diet with the lowest inclusion (135 g/kg) of soybean meal. Surprisingly, all dietary levels of maize-DDGS resulted in weight loss of birds. However, the body weight changes and feed intake of birds fed maize-DDGS diets were not affected ($P > 0.05$) by dietary inclusion level.

Dietary P contents and ileal P outputs in birds fed diets containing graded levels of wheat, sorghum, soybean meal and maize-DDGS are presented in Table 6.4. In all ingredients, increasing dietary concentrations of P linearly ($P < 0.001$) increased ileal P outputs.

The AIDC of P from wheat was not affected ($P > 0.05$) by increasing dietary P concentrations (Table 6.5). The AIDC of P from sorghum was affected (quadratic, $P < 0.001$) by increasing dietary P concentrations. Dietary inclusion of sorghum at 946 g/kg lowered ($P < 0.05$) the AIDC of P compared to inclusion levels of 473 and 709.5 g/kg. The AIDC of P from soybean meal (linear, $P < 0.001$; quadratic, $P < 0.001$) and maize-DDGS (linear, $P < 0.001$) were also affected by ingredient inclusion level. In birds fed soybean meal and maize-DDGS-based diets, the AIDC of P was found to be the highest between inclusion levels 2 and 4 (Table 6.5).

Strong linear relationships were observed between digesta P outputs and dietary P contents for all four test ingredients (Figures 6.1 and 6.2). True ileal P digestibility of the test ingredients and estimated endogenous P losses are presented in Table 6.6. True ileal P digestibility coefficients of wheat, sorghum, soybean meal and maize-DDGS were determined to be 0.464, 0.331, 0.798 and 0.727, respectively. Ileal endogenous P losses estimated for wheat, soybean meal and maize-DDGS were 0.080, 0.609 and 0.418 g/kg DMI, respectively. In birds fed sorghum-based diets, the endogenous P losses were determined to be negative (-0.087 g/kg DMI).

Table 6.4. Growth performance (day 21-28 posthatch) and, dietary P content, total ileal P output in birds fed diets containing graded concentrations of P from wheat, sorghum, soybean meal and maize-DDGS for broilers[1]

	Measurement	Diet 1	Diet 2	Diet 3	Diet 4	SEM	Probability L[2]	Q[2]
Wheat	BWG[3], g/b/d	-8.46	3.88	9.41	13.2	1.86	***	*
	FI[4], g/b/d	71.0	83.7	99.0	111.9	4.65	***	NS
	P_I[5], g/kg DM	0.95	1.67	2.54	3.48	-	-	-
	P_D[6], g/kg DMI	0.54	0.98	1.58	1.86	0.16	***	NS
Sorghum	BWG[3], g/b/d	-12.83	-4.67	1.38	2.37	2.26	***	NS
	FI[4], g/b/d	68.5	76.9	80.5	67.1	1.90	NS	***
	P_I[5], g/kg DM	0.64	1.36	2.02	2.58	-	-	-
	P_D[6], g/kg DMI	0.49	0.65	1.11	1.81	0.06	***	**
Soybean meal	BWG[3], g/b/d	-14.0	1.29	20.3	42.2	2.20	***	NS
	FI[4], g/b/d	60.3	72.1	85.9	106.1	2.91	***	NS
	P_I[5], g/kg DM	1.03	2.14	3.00	3.97	-	-	-
	P_D[6], g/kg DMI	0.89	0.96	1.16	1.48	0.06	***	NS
Maize-DDGS	BWG[3], g/b/d	-7.38	-5.17	-6.96	-9.44	1.85	NS	NS
	FI[4], g/b/d	70.4	78.8	76.0	70.0	3.56	NS	NS
	P_I[5], g/kg DM	1.29	2.44	3.76	5.10	-	-	-
	P_D[6], g/kg DMI	0.78	1.05	1.48	1.80	0.09	***	NS

NS, not significant; *P < 0.05; **P < 0.01; ***P < 0.001.
[1]Each value represents the mean of four replicates (5 birds/ replicate).
[2]L = linear effect; Q = quadratic effect.
[3]BWG = body weight gain.
[4]FI = feed intake.
[5]P_I = Dietary P content; DM = Dry matter.
[6]P_D = Ileal P output; DMI = Dry matter intake.

Table 6.5. Apparent ileal phosphorus (P) digestibility coefficients of diets containing graded concentrations of P from wheat, sorghum, soybean meal and maize-DDGS for broilers[1]

	Wheat	Sorghum	Soybean meal	Maize-DDGS
Diet 1	0.438	0.233[a]	0.140[a]	0.395[a]
Diet 2	0.413	0.518[b]	0.552[b]	0.571[b]
Diet 3	0.379	0.450[b]	0.615[b]	0.606[b]
Diet 4	0.466	0.298[a]	0.627[b]	0.648[b]
Pooled SEM	0.071	0.030	0.035	0.034
Probability	NS	***	***	**
Linear effect	NS	NS	***	***
Quadratic effect	NS	***	***	NS

NS, not significant; **P < 0.01; ***P < 0.001.
[1]Each value represents the mean of four replicates (5 birds/ replicate).
[a-b]Means in a column not sharing a common superscript are significantly different at P < 0.05.

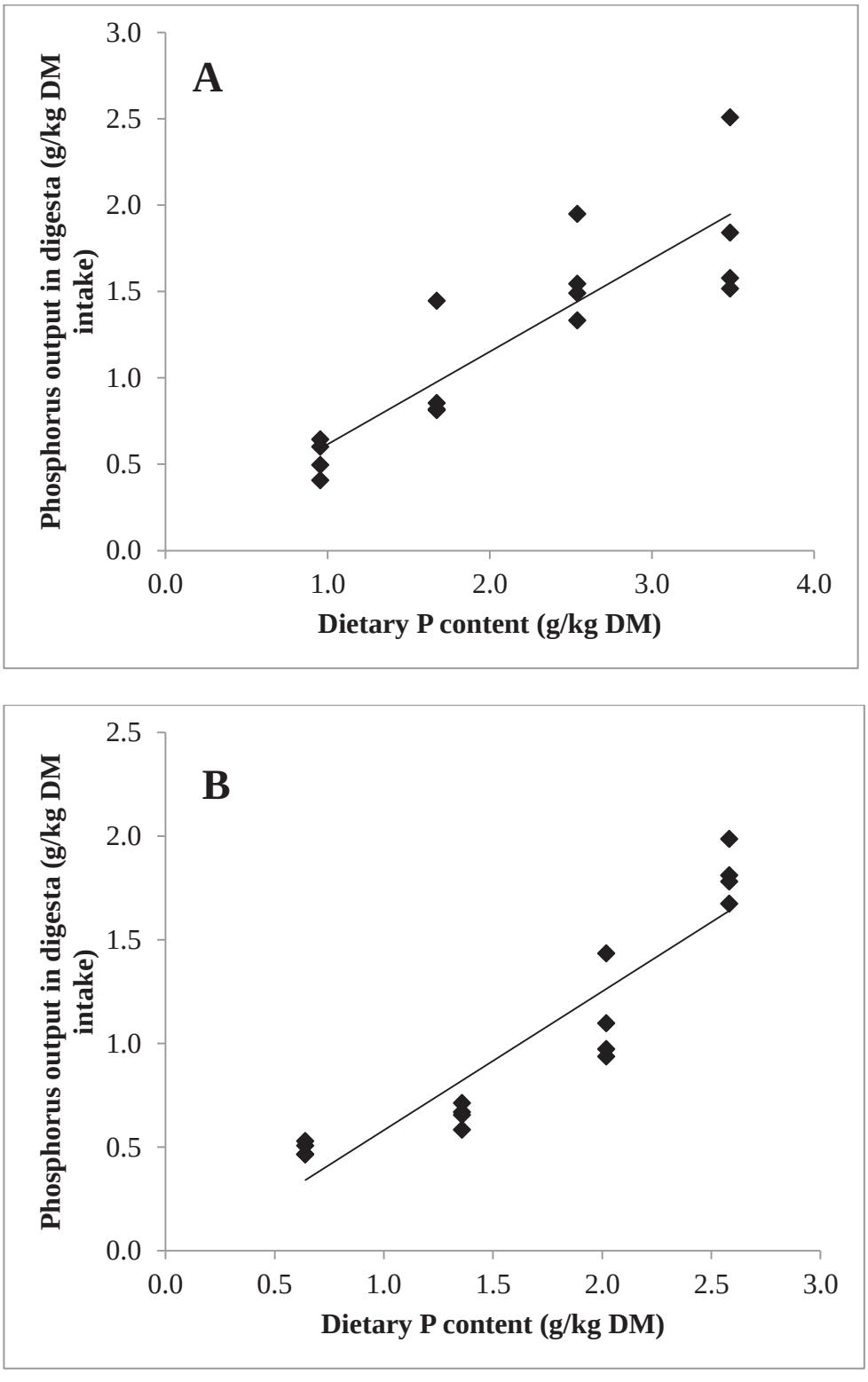
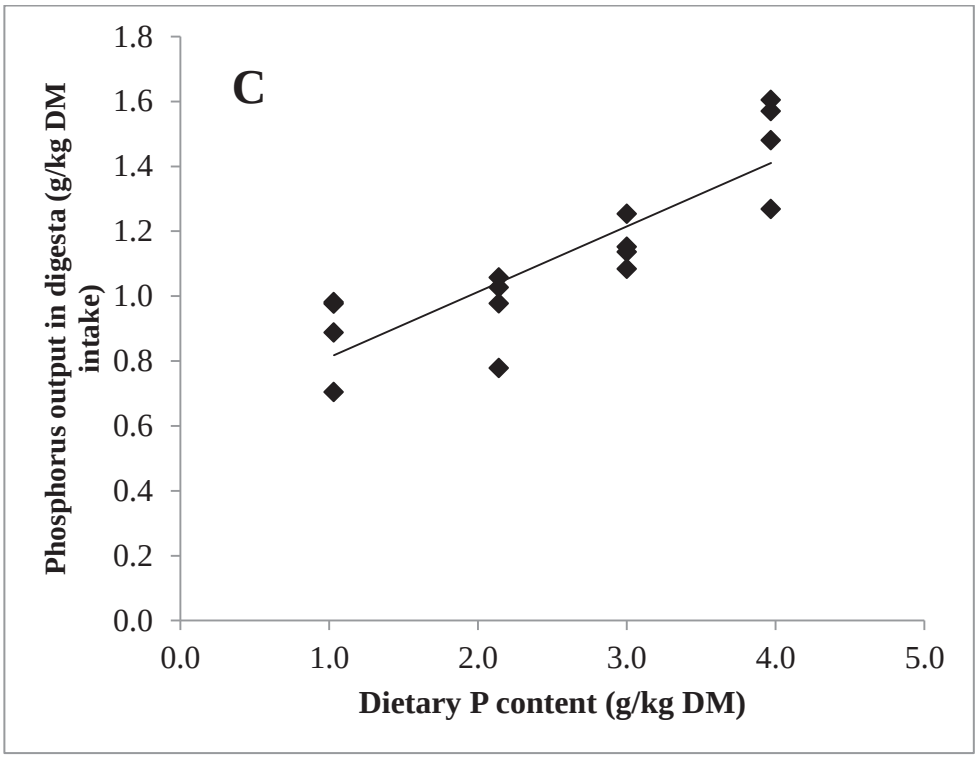


Figure 6.1. Linear relationship between total phosphorus (P) outputs (Y: g/kg DM diet intake) in ileal digesta and dietary P content (X: g/kg DM) in 21-day old broilers fed wheat-based diets (A) and sorghum-based diets (B) containing graded P concentrations.



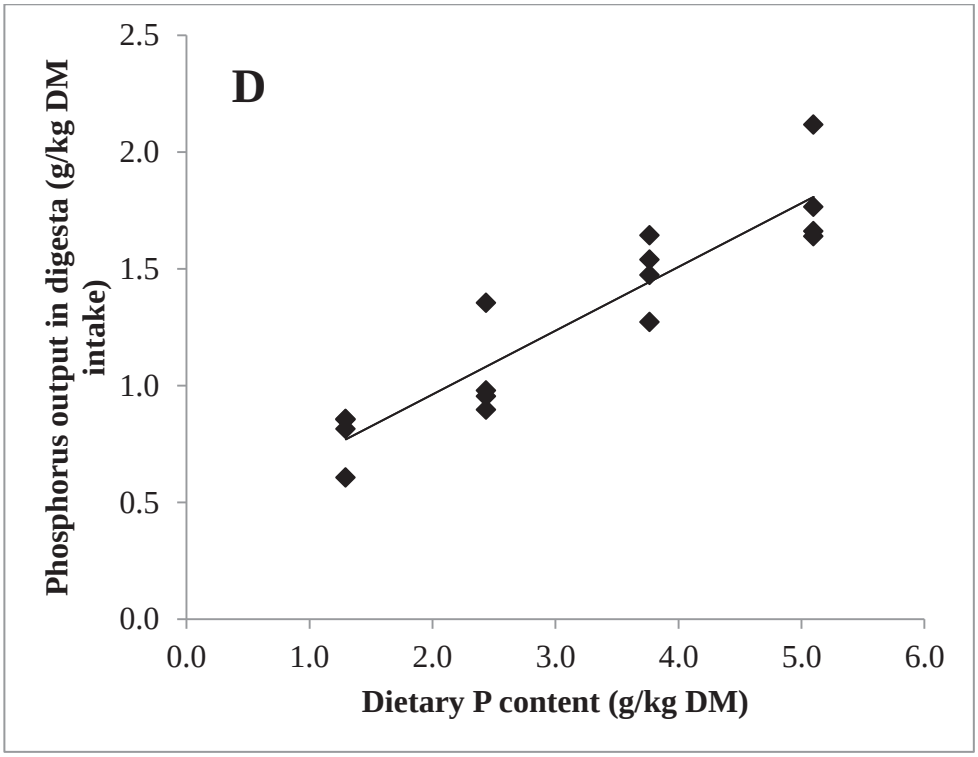


Figure 6.2. Linear relationship between total phosphorus (P) outputs (Y: g/kg DM diet intake) in ileal digesta and dietary P content (X: g/kg DM) in 21-day old broilers fed soybean meal-based diets (C) and maize-DDGS-based diets (D) containing graded P concentrations.

Table 6.6. Linear relationship between ileal P outputs (g/kg DMI) vs. dietary P content (g/kg DM) of wheat, sorghum, soybean meal and maize-DDGS fed to broilers[1]

True ileal P digestibility	Regression equation[2]	SE of the slope[3]	SE of the intercept[3]	r^2	Endogenous P loss (g/kg DMI)[4]	Digestibility coefficient
Wheat	Y = 0.536X + 0.080	0.08	0.19	0.77	0.080	0.464
Sorghum	Y = 0.669X - 0.087	0.07	0.13	0.86	-0.087	0.331
Soybean meal	Y = 0.202X + 0.609	0.03	0.09	0.75	0.609	0.798
Maize-DDGS	Y = 0.273X + 0.418	0.03	0.10	0.86	0.418	0.727

[1]Each value represents the mean of four replicates (5 birds/ replicate).

[2]Regression of ileal digesta P output (g/kg DMI) against dietary P content (g/kg DM) as determined by feeding broilers with diets containing graded levels of either wheat, sorghum, soybean meal or maize-DDGS. The slope represents true P indigestibility and the intercept represents the endogenous P loss (g/kg DMI).

[3]Standard error of regression.

[4] Calculated as described in Dilger and Adeola (2006b).

6.5. Discussion

Analysed P and phytate-P contents of wheat, sorghum, soybean meal (Selle *et al*., 2003; Selle and Ravindran, 2007) and maize-DDGS (Min *et al*., 2008) were within the range reported in the literature. The analysed dietary P concentrations in test diets were close to calculated values and were used for the calculation of P digestibility coefficients.

Birds fed diets containing wheat, sorghum and soybean meal responded linearly in body weight gains with increasing concentrations of P. However, weight loss was observed in birds fed with the lowest dietary inclusion level of these ingredients which may be explained on the basis of protein deficiency. Body weight gain was highest in birds fed the diet with highest inclusion of soybean meal, which had the highest dietary protein content. Feeding diets containing all levels of maize-DDGS resulted in weight loss. This observation was unexpected, since maize-DDGS is a good source of protein (Świątkiewicz and Koreleski, 2008). The observed weight losses cannot be attributed to protein deficiency, but may relate, at least in part, to the digestibility of amino acids. In particular, low lysine digestibility is known to be a problem in over-processed DDGS (Stein and Shurson, 2009). Excessive heat application during the DDGS manufacturing process favours Maillard reaction to occur between lysine and carbohydrates moieties with the resultant reduction in lysine digestibility. Studies have shown that DDGS can be safely included in broiler diets from 120 to 150 g/kg during both grower and finisher phases and inclusion above this level resulted poor growth performance (Lumpkins *et al*., 2004; Świątkiewicz and Koreleski, 2008). All the maize-DDGS-based diets used in the present study contained maize-DDGS above the recommended level for growers, but still does not explain the observed weight losses.

Apparent ileal P digestibility coefficients determined at different inclusion levels of wheat ranged from 0.379 to 0.466, but were not significantly different. The AIDC of P (0.466) determined at the highest inclusion level of wheat (946 g/kg) in the present study compares well with the value (0.51) reported by Wu *et al*. (2004). In the latter study, AIDC of P was measured using the direct method at the dietary inclusion of 990 g/kg where wheat was used as the sole source of dietary P and Ca. It is known that the wheat possesses a high endogenous phytase activity (Eeckhout and De Paepe, 1994). However, the relatively low AIDC of P determined for wheat in the present study is not reflective of 421 U/kg phytase activity determined in this sample. It is known that wheat endogenous phytase activity is less effective than microbial phytase in the gut because

of a narrower pH spectrum of activity (Sandberg *et al*., 1996). Furthermore, very low pH may destroy wheat phytase and it is more susceptible to proteolytic digestion than microbial phytase (Phillippy, 1999). It is also plausible that the presence of non-starch polysaccharides, especially soluble arabinoxylans, may be partly responsible as the non-starch polysaccharides in wheat can increase digesta viscosity and reduce nutrient digestion (Choct and Annison, 1992).

Apparent ileal P digestibility coefficients at different inclusion levels of sorghum differed, with dietary inclusion at 946 g/kg markedly reducing the digestibility. However, apparent ileal P digestibility coefficient of sorghum for broilers determined by Wu *et al*. (2004) using the direct method (0.36) is in close agreement with that (0.298) found in the present study at its highest inclusion level. The reasons for the low P digestibility in sorghum are not clear. Presence of tannins in sorghum is reported to form complexes with P rendering it unavailable (Waghorn *et al*., 1994; Mansoori and Acamovic, 1996; Medugu *et al*., 2012) and increase endogenous P losses (Mansoori and Acamovic, 1996). Another possible reason may be the Ca:P ratios in sorghum-based diets. In the present study, as noted earlier, the diets were formulated to achieve dietary Ca:non-phytate P ratio of 2:1.based on assumed values (NRC, 1994, 2012), but subsequent analysis showed that the resulting Ca:non-phytate P ratio was 4.8:1 A high Ca:non-phytate P ratio has been shown to have a suppressive effect on the absorption and digestibility of inorganic soluble forms of P due to formation of insoluble Ca:P complexes in the digestive tract of birds (Hurwitz and Bar, 1971; Plumstead *et al*., 2008).

Increasing dietary inclusion of soybean meal linearly increased the AIDC of P from 0.140 to 0.627, which are lower than those reported by Dilger and Adeola (2006b) for conventional soybean meal (0.712 to 0.888) and low-phytate soybean meal (0.754 to 0.889). These researchers used soybean meal as the sole dietary source of P and Ca, and low dietary Ca in their test diets may have increased hydrolysis of phytate P in soybean meal. Liu *et al*. (2013) determined the P digestibility of soybean meal for broilers at different dietary Ca:P ratios (0.8, 1.2, 1.6 and 2.0) and found that the apparent ileal P digestibility ranged from 0.64 to 0.90. However, the test diets used by these researchers had casein as a baseline protein supplement. Casein also contained P, the digestibility of which was much higher than that of soybean meal (Liu *et al*., 2013) and this could have confounded the results. The AIDC of P reported for soybean meal in pigs are much

lower than the current estimate and ranged from -0.248 to 0.371 (Fan *et al*., 2001), -0.267 to 0.527 (Ajakaiye *et al*., 2003), and 0.372 (Bohlke *et al*., 2005).

The AIDC of P for DDGS determined in the present study ranged from 0.395 to 0.648. No previous published data are available for the P digestibility in maize-DDGS for poultry. However, in bioavailability studies with poultry a range of relative P bioavailability coefficients from 0.54 to 1.02 (Martinez Amezcua *et al*., 2004; Lumpkins and Batal, 2005; Martinez Amezcua and Parsons, 2007) has been reported. In these studies, P bioavailability was estimated using potassium dihydrogen phosphate (KH_2PO_4) as the standard. Apparent total tract digestibility coefficient of P for maize-DDGS in pigs has been reported to range from 0.501 (Pedersen *et al*., 2007) and 0.686 (Almeida and Stein, 2010).

Strong linear relationships were observed between digesta P outputs and dietary P intake for all test ingredients which is a primary requirement for application of regression technique. This relationship permits the theoretical estimation of diet independent endogenous P loss (g/kg DMI) and the simultaneous measurement of true P digestibility (Fan *et al*., 2001; Dilger and Adeola, 2006b). True ileal P digestibility of wheat, sorghum, soybean meal and maize-DDGS were determined to be 0.464, 0.331, 0.798 and 0.727, respectively. To the author's knowledge, true ileal P digestibility in wheat, sorghum and maize-DDGS for poultry have not been previously reported. Using the direct method, standardised total tract digestibility of P in maize-DDGS for pigs has been determined in several studies, and values ranging from 0.63 to 0.73 have been reported (Widmer *et al*., 2007; Almeida and Stein, 2010; Baker *et al*., 2013).

The true ileal digestibility of P in soybean meal estimated in the present work for broilers (0.798) was considerably lower than those reported by Dilger and Adeola (2006b) for conventional soybean meal (0.939) and low-phytate soybean meal (0.938). On the other hand, the present estimate was considerably higher than the values (0.458 to 0.553) reported by Liu *et al*. (2013), when measured at dietary Ca:total P ratios ranging from 1.2 to 2.0. However, our estimate compared closely with the value of 0.708 reported by Liu *et al*. (2013) at the dietary Ca:total P ratio of 0.8. In the present study, a dietary Ca:total P ratio of 0.7 was used. It has been previously demonstrated that the utilisation of dietary P was improved at narrower Ca:P ratios (Qian *et al*., 1997).

Ileal endogenous P losses estimated for wheat, soybean meal and maize-DDGS were 0.080, 0.609 and 0.418 g/kg DMI, respectively. In birds fed sorghum-based diets, endogenous P losses were estimated to be negative (-0.087). As discussed in Chapter 5,

the negative estimate for endogenous P losses is an anomaly reflecting the inherent limitation of the regression method. Such negative estimates for endogenous P losses, with the regression method, have also been previously reported for broilers (Iyayi *et al*., 2013; Liu *et al*., 2013).

The data summarised in Table 6.7 show that the P evaluation system based on non-phytate P is not reflective of digestible P contents in feed ingredients. True digestible P contents were considerably higher than the non-phytate P contents in all ingredients, suggesting that a portion of phytate-bound P is being utilised by birds. If we assume that non-phytate P in these ingredients are totally digestible, then it can be calculated that 18.1, 13.0, 69.7 and 41.5% of phytate-bound P from wheat, sorghum, soybean meal and maize-DDGS, respectively, was digested by broiler chickens. It is recognised that the ability of birds to utilise phytate-bound P in feed ingredients can vary widely, ranging from 0 to as high as 75%, and a number of factors including the dietary concentrations of Ca, P, vitamin D_3 and fibre, solubility and location of phytate, feed processing and age of the bird may influence phytate P hydrolysis (Ravindran *et al*., 1995; Angel *et al*., 2002). Of these, dietary Ca is probably the most important factor. It is well documented that the utilisation of phytate-bound P in broilers is increased when dietary Ca concentrations were maintained below the requirement (Ballam *et al*., 1984; Mohammed *et al*., 1991; Tamim and Angel., 2003). Davies *et al*. (1970) observed that the intestinal phytase activity is more than three-fold greater in chicks fed a P-deficient diet compared with those fed a P-adequate control diet. The broilers appear to have an innate ability to regulate intestinal phytase activity as an adaptive response to P deficient environment and, therefore, capable of utilising more P from phytate-bound P at low dietary P levels. All diets used in the present experiment were P- and Ca-deficient and, therefore, it is possible that the P digestibility may be over-estimated under these conditions. The observed differences in true P digestibility between the cereals (wheat and sorghum) and soybean meal in the current work is of interest. It is possible that the low P digestibility in cereals may be due partly to the form as well as the location (aleurone or fiber) of the phytate, whereas phytate in soybeans is in more soluble form and evenly distributed (Selle and Ravindran, 2007). Bohn *et al*. (2007) noted that the phytate in wheat was present as phytate protein globoids and resistant to degradation by phytase.

Table 6.7. Comparison of total P, phytate-P, non-phytate P, and true digestible P contents of wheat, sorghum, soybean meal and maize-DDGS (g/kg, as fed)

	Wheat	Sorghum	Soybean meal	Maize-DDGS
Total P	3.22	2.37	6.46	8.17
Phytate P	2.11	1.82	4.31	3.82
Non-phytate P[1]	1.11	0.55	2.15	4.36
True digestible P	1.49	0.78	5.16	5.94
As % of total P				
Phytate P	65.5	76.9	66.7	46.7
Non-phytate P	34.5	23.1	33.3	53.3
True digestible P	46.4	33.1	79.8	72.7

[1]Calculated as the difference between total P and phytate P.

6.6. Conclusions

The present data suggest that the regression method can be used to measure true P digestibility of feed ingredients widely ranging in P concentrations. The true ileal P digestibility coefficients in wheat, sorghum, soybean meal and maize-DDGS were determined to be 0.464, 0.331, 0.798 and 0.727, respectively. The determined digestible P contents were markedly higher than the corresponding non-phytate P contents and this may be suggestive, at least in part, of overestimation of P digestibility under the Ca- and P-deficient conditions employed in the regression method. The validity of measuring P digestibility under such deficient conditions requires further investigation and discussion.

CHAPTER 7

Measurement of true ileal phosphorus digestibility in meat and bone meal for broiler chickens

7.1. Abstract

An experiment was conducted to estimate true ileal phosphorus (P) digestibility of three meat and bone meal (MBM-1, MBM-2 and MBM-3) samples for broiler chickens. Four semi-purified diets were formulated from each sample to contain graded concentrations of P. The experiment was conducted as a completely randomised design with six replicates (6 birds per replicate) per dietary treatment. A total of 432, 21-day old broilers (Ross 308), were assigned to the 12 test diets. Apparent ileal digestibility coefficient of P was determined by the indicator method and the linear regression method was used to determine the true P digestibility coefficient. The apparent ileal digestibility coefficient of P in birds fed diets containing MBM-1 and MBM-2 was unaffected ($P > 0.05$) by increasing dietary concentrations of P. The apparent ileal digestibility coefficient of P in birds fed MBM-3 diets decreased (linear, $P < 0.001$; quadratic, $P < 0.01$) with increasing P concentrations. In birds fed diets with MBM-1 and MBM-2, ileal endogenous P losses were estimated to be 0.049 and 0.142 g/kg dry matter intake (DMI), respectively. In birds fed MBM-3 diets, endogenous P loss was estimated to be negative (-0.370 g/kg DMI). True ileal P digestibility of MBM-1, MBM-2 and MBM-3 were determined to be 0.693, 0.608 and 0.420, respectively. True ileal P digestibility coefficients determined for MBM-1 and MBM-2 were similar ($P > 0.05$) and higher ($P < 0.05$) than that for MBM-3. Total P and true digestible P contents of MBM-1, MBM-2 and MBM-3 were determined to be 37.5 and 26.0; 60.2 and 36.6; and 59.8 and 25.1 g/kg, as fed basis.

7.2. Introduction

Meat and bone meal (MBM) is commonly used in poultry diets in many countries, including Australasia, as a source of amino acids, calcium (Ca) and phosphorus (P). It is, however, a highly variable product in terms of amino acid, Ca and P contents (Hendriks *et al*., 2002; Hendriks *et al*., 2004; Adedokun and Adeola, 2005; Robbins and Firman, 2005; Sulabo and Stein, 2013). The Ca and P contents of MBM are reported to vary from 40 to 150 g/kg and 18 to 70 g/kg, respectively (Waldroup, 1999).

The relative bioavailability of P in MBM has been assessed for pigs (Burnell *et al*., 1988; Burnell *et al*., 1989; Coffey and Cromwell, 1993; Traylor *et al*., 2005a,b) and poultry (Waldroup and Adams, 1994; Sell and Jeffrey, 1996; Coffey and Cromwell, 1995; Traylor *et al*., 2000) using the slope ratio technique. The data obtained from these studies are relative and do not reflect the actual P availability in MBM for animals.

Currently, the measurement of digestible P is considered as the preferred method to assess P availability of pigs and poultry (Sulabo and Stein, 2013; WPSA, 2013). Some data on the apparent and true digestibility of P for MBM in pigs are available (Poulsen, 1995; Sulabo and Stein, 2013), but there is no corresponding information for poultry.

In Chapters 5 and 6 of this thesis, the regression method was used to determine the true P digestibility in plant-based feed ingredients for poultry. The objective of the present study was to determine the true P digestibility of three MBM samples for broilers.

7.3. Materials and methods

The experimental procedures were approved by the Massey University Ethics Committee and in accordance with the New Zealand Code of Practice for the Care and Use of Animals for Scientific Purposes.

7.3.1. Ingredients

Three meat and bone meal (MBM-1, MBM-2 and MBM-3) samples were obtained from different rendering plants. Representative samples were obtained and analysed in triplicate for dry matter (DM), total P, Ca, crude protein, ash and crude fat. Particle size distribution of the meals was also determined and the geometric mean diameter (GMD) and geometric standard deviation (GSD) were calculated.

7.3.2. Birds

Day-old male broilers (Ross 308) were raised in floor pens and fed a commercial broiler starter diet. Feed and water were available at all times. On day 14, birds were transferred to grower cages and were maintained on the same diet until the introduction of test diets on day 21. On day 21, birds were individually weighed and a total of 432 birds of uniform weight were selected and assigned to 72 cages (6 birds per cage). Each of the 12 dietary treatments was then assigned to six cages. Housing conditions have been described in Chapter 3, Section 3.3.1. Group body weights and feed intake were recorded on days 21 and 24 posthatch. Mortality was recorded daily.

7.3.3. Diets

Four semi-purified diets (20, 40, 60, and 80 MBM g/kg diet) were formulated, on the basis of analysed P concentrations to contain graded concentrations of total P (Table 7.1). Inclusion levels for MBM were chosen to maintain the dietary P contents below the requirement, which is a prerequisite for the linear regression function. In the test diets, MBM served as the only source for P. Calcium:non-phytate P ratio in all diets was maintained at 2:1. The diets contained 3 g/kg titanium dioxide (Merck KGaA, Darmstadt, Germany) as an indigestible marker. The diets, in mash form, were offered *ad libitum* and the birds had free access to water.

7.3.4. Sample collection and processing

On day 24 posthatch, birds were euthanised by intravenous injection of sodium pentobarbitone and the digesta from the lower half ileum were collected (Ravindran *et al*., 2005b) and processed as described in Chapter 3, Section 3.3.3.

7.3.5. Chemical analysis

Representative samples of MBM were analysed for DM, crude protein (nitrogen x 6.25), crude fat, total P and Ca, according to the procedures outlined in Chapter 3, Section 3.3.4. Ash content in MBM samples was determined using standard procedures (AOAC International, 2005; method no: 942.05). Representative samples of test diets and digesta were analysed for DM, Ca, total P and titanium (Ti) as described in Chapter 3, Section 3.3.4.

7.3.6. Particle size distribution of MBM

Representative MBM samples were tested in duplicate to determine the particle size distribution. A set of sieves (Endecotts, London, UK) sized 0.075, 0.125, 0.212, 0.5, 1 and 2 mm and a Endecotts test sieve shaker (Model: E.F.L. 2000) were used to separate the particles into different fractions. The samples were passed through a sieve stack on the shaker for 10 minutes, the amount of particles retained on each sieve was weighed and, the GMD and GSD of MBM samples were calculated using the following formula as described in Baker and Herrman (2002). These calculations were based on the assumption that the weight distribution of samples is logarithmically normal (Martin, 1985).

$d_i = (d_u \times d_o)^{0.5}$

$GMD = \log^{-1}[\Sigma (W_i \log d_i / \Sigma W_i]$

$GSD = \log^{-1}[\Sigma W_i (\log d_i - \log GMD)^2 / \Sigma W_i]^{0.5}$

Where,

d_i = diameter of i^{th} sieve in the stack,

d_u = diameter opening through which particles will pass (sieve proceeding i^{th}),

d_o = diameter opening through which particles will not pass (i^{th} sieve),

W_i = weight fraction on i^{th} seive.

7.3.7. Calculations

The true P digestibility in MBM was calculated according to equations 1 to 4 outlined in Chapter 5, Section 5.3.6. Phosphorus outputs were regressed against dietary P contents per 24 cages for each of the MBM sample.

7.3.8. Statistical analysis

Data were analysed using the GLM procedure of SAS (2004). Cage was served as the experimental unit for all statistical analyses and differences were considered significant at an alpha level of 0.05. Data from the four inclusion levels of each MBM sample were analysed as completely randomised design. Orthogonal polynomial contrasts, true P utilisation coefficients and endogenous P losses were estimated as described in Chapter 5, Section 5.3.7. Standard errors for true ileal P coefficients were based on total of 24 observations for each MBM sample. Regression coefficients between three MBM samples were compared using the covariance analysis. The model for covariance analysis included the dietary P content (1 df), MBM sample (1 df) and the interaction between the MBM sample and dietary P content (1 df).

Table 7.1. Ingredient composition and analysis (g/kg, as fed) of meat and bone meal (MBM)-based diets

	MBM-1				MBM-2				MBM-3			
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 1	Diet 2	Diet 3	Diet 4	Diet 1	Diet 2	Diet 3	Diet 4
Meat and bone meal	20.0	40.0	60.0	80.0	20.0	40.0	60.0	80.0	20.0	40.0	60.0	80.0
Soybean oil	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Dextrose	717.2	701.6	686.1	670.5	717.2	701.6	686.1	670.5	717.2	701.6	686.1	670.5
Maize starch	179.3	175.4	171.5	167.6	179.3	175.4	171.5	167.6	179.3	175.4	171.5	167.6
Cellulose	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Potassium chloride	3.1	2.6	2.0	1.5	3.1	2.6	2.0	1.5	3.1	2.6	2.0	1.5
Salt	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Titanium dioxide	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Trace mineral-vitamin premix[1]	3.4	3.4	3.4	3.4	3.4	3.4	3.4	3.4	3.4	3.4	3.4	3.4
Calculated analysis												
Metabolisable energy, MJ/kg	15.17	15.04	14.91	14.78	15.17	15.04	14.91	14.78	15.17	15.04	14.91	14.78
Crude protein[2], g/kg	10.72	21.44	32.16	42.88	9.76	19.52	29.28	39.04	9.48	18.96	28.44	37.92
Ca[2], g/kg	1.43	2.86	4.30	5.73	2.36	4.72	7.09	9.45	2.29	4.58	6.88	9.17
Total P[2], g/kg	0.75	1.50	2.25	3.00	1.20	2.41	3.61	4.81	1.20	2.39	3.59	4.78
Non-phytate P[2], g/kg	0.75	1.50	2.25	3.00	1.20	2.41	3.61	4.81	1.20	2.39	3.59	4.78
Ca:non-phytate P ratio	1.91:1	1.91:1	1.91:1	1.91:1	1.96:1	1.96:1	1.96:1	1.96:1	1.92:1	1.92:1	1.92:1	1.91:1
Analysed values												
Ca, g/kg	1.40	2.36	4.05	5.72	2.19	4.58	6.95	8.53	1.91	4.04	5.89	8.73
Total P, g/kg	1.11	1.68	2.36	3.59	1.35	2.75	4.22	5.15	1.42	2.32	3.78	4.85

[1]Supplied per kg of diet: Co, 0.3 mg, I, 1.5 mg, Mo, 0.3 mg, Se, 0.3 mg, Mn, 100 mg, Cu, 10 mg, Zn, 80 mg, Fe, 60 mg, anti-oxidant, 100 mg.vitamin A, 12,000 IU, vitamin D_3, 4,000 IU, thiamine, 3 mg, riboflavin, 9 mg, pyridoxine, 10 mg, folic acid, 3 mg, biotin, 0.25 mg, vitamin B_{12}, 0.02 mg, vitamin E, 80 mg, choline chloride, 0.6 g, nicotinic acid, 60 mg, Ca pantothenate, 15 mg, menadione, 4 mg.
[2]Calculated based on values determined for individual MBM sample.

7.4. Results

Analysed Ca and P contents of the diets are presented in Table 7.1. Dietary concentrations of P and Ca increased with increasing inclusion of MBM. Most diets contained 0.07 to 0.61 g/kg P more than calculated. The dietary Ca concentrations were 0.01 to 0.92 g/kg lower than the calculated values.

The nutrient composition differed among MBM samples (Table 7.2). In general, the composition of MBM-2 and MBM-3 was similar and both contained more P, Ca and ash than MBM-1. The protein and fat contents were higher in the MBM-1 than those of the other two samples.

Table 7.2. Analysed composition of meat and bone meal (MBM) samples (g/kg, as fed basis)

	MBM-1	MBM-2	MBM-3
Crude protein (nitrogen x 6.25)	536	488	474
Crude fat	114	93	88
Ash	237	357	362
Total P	37.54	60.17	59.8
Ca	71.59	118.1	114.6

The particle size distribution of the three MBM samples are presented in Figure 7.1, which shows that the relative proportion of particles > 1.0 mm (coarse) and 0.5 to 1.0 mm (medium) were higher in MBM-1 (32 and 63%, respectively) and MBM-3 (35 and 60%, respectively). MBM-2 contained a lower proportion of coarse and medium-sized particles (26 and 34%, respectively). The GMD of MBM-1, MBM-2 and MBM-3 were calculated to be 866, 622, and 875μ, respectively. The corresponding GSD were 1.53, 1.95 and 1.51, respectively.

Broilers remained healthy during 3-day experimental period and no mortality or leg problems were recorded. Feed intake and body weight gain of birds fed diets containing graded concentrations of dietary P from the three MBM samples are presented in Table 7.3. Inclusion of MBM at all four dietary levels resulted weight loss in birds. In birds fed MBM-2 diets, weight losses were lowered (linear, $P < 0.05$) with increasing MBM. In birds fed MBM-3 diets feed intake linearly increased with increasing dietary P concentrations (linear, $P < 0.01$). Feed intake was not affected ($P > 0.05$) in birds fed MBM-1 and MBM-2 diets.

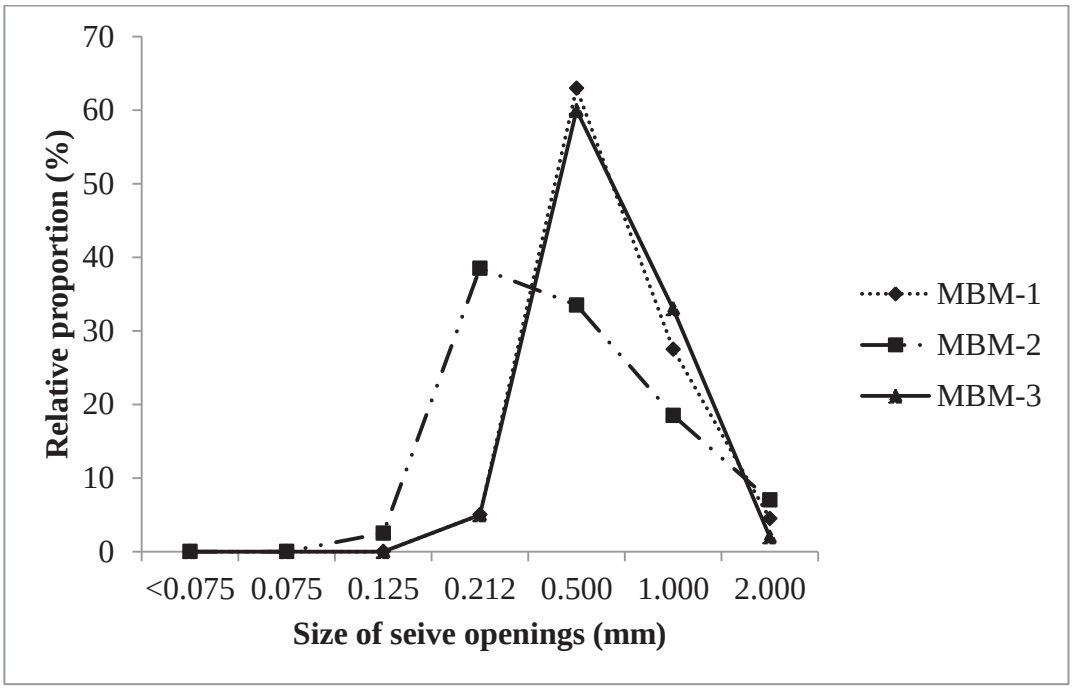


Figure 7.1. Particle size distribution of the three meat and bone meal (MBM) samples.

Table 7.3. Growth performance (day 21-24 posthatch), dietary P content, and ileal P output in birds fed diets containing graded concentrations of P from meat and bone meal (MBM) for broilers[1]

	Measurement	Diet 1	Diet 2	Diet 3	Diet 4	SEM	Probability L[2]	Q[2]
MBM-1	BWG[3], g/b/d	-48.3	-45.9	-46.5	-40.6	2.77	NS	NS
	FI[4], g/b/d	74.2	71.9	76.4	81.9	3.70	NS	NS
	P_I[5], g/kg DM	1.21	1.83	2.57	3.92	-	-	-
	P_D[6], g/kg DMI	0.419	0.562	0.920	1.22	0.045	***	NS
MBM-2	BWG[3], g/b/d	-46.3	-47.2	-43.3	-40.0	2.10	*	NS
	FI[4], g/b/d	78.1	79.0	79.1	82.7	2.93	NS	NS
	P_I[5], g/kg DM	1.47	3.00	4.61	5.61	-	-	-
	P_D[6], g/kg DMI	0.743	1.22	2.10	2.26	0.115	***	NS
MBM-3	BWG[3], g/b/d	-48.7	-44.3	-47.2	-39.6	5.66	NS	NS
	FI[4], g/b/d	74.2	74.2	82.5	83.8	2.70	**	NS
	P_I[5], g/kg DM	1.56	2.54	4.13	5.28	-	-	-
	P_D[6], g/kg DMI	0.478	1.15	2.09	2.63	0.089	***	NS

NS, not significant; *P < 0.05; **P < 0.01; ***P < 0.001.
[1]Each value represents the mean of six replicates (6 birds/replicate).
[2]L = linear effect; Q = quadratic effect.
[3]BWG = body weight gain.
[4]FI = feed intake.
[5]P_I = Dietary P content; DM = Dry matter.
[6]P_D = Ileal P output; DMI = Dry matter intake.

In all three MBM, increasing dietary concentrations of P linearly increased ($P < 0.001$) ileal P outputs (Table 7.3).

The AIDC of P in birds fed MBM-1 and MBM-2 diets was not affected ($P > 0.05$) by increasing dietary concentrations of P and ranged from 0.643 to 0.694, and 0.494 to 0.597, respectively (Table 7.4). The AIDC of P in birds fed the MBM-3 diets decreased (linear, $P < 0.001$; quadratic, P < 0.01) with increasing dietary P concentrations and ranged from 0.494 to 0.693.

Table 7.4. Apparent ileal phosphorus (P) digestibility coefficients of diets containing graded concentrations of P from meat and bone meal (MBM) for broilers[1]

	MBM-1	MBM-2	MBM-3
Diet 1	0.655	0.494	0.693[b]
Diet 2	0.694	0.593	0.548[a]
Diet 3	0.643	0.545	0.494[a]
Diet 4	0.688	0.597	0.501[a]
Pooled SEM	0.024	0.032	0.024
Probability	NS	NS	***
Linear effect	NS	NS	***
Quadratic effect	NS	NS	**

NS, not significant; **P <0.01; ***P < 0.001.
[1]Each value represents the mean of six replicates (6 birds/ replicate).
[a-b]Means in a column not sharing a common superscript are significantly different at $P < 0.05$.

Strong linear relationships were observed between ileal P output and dietary P content of all MBM samples (Figure 7.2). True ileal P digestibility coefficients of MBM-1, MBM-2, and MBM-3 were determined to be 0.693, 0.608, and 0.420, respectively (Table 7.5). The corresponding ileal endogenous P losses estimated were 0.049, 0.142 and -0.370 g/kg DMI, respectively. True ileal P digestibility coefficients determined for MBM-1 and MBM-2 were similar ($P > 0.05$) but were higher ($P < 0.05$) than that of MBM-3.

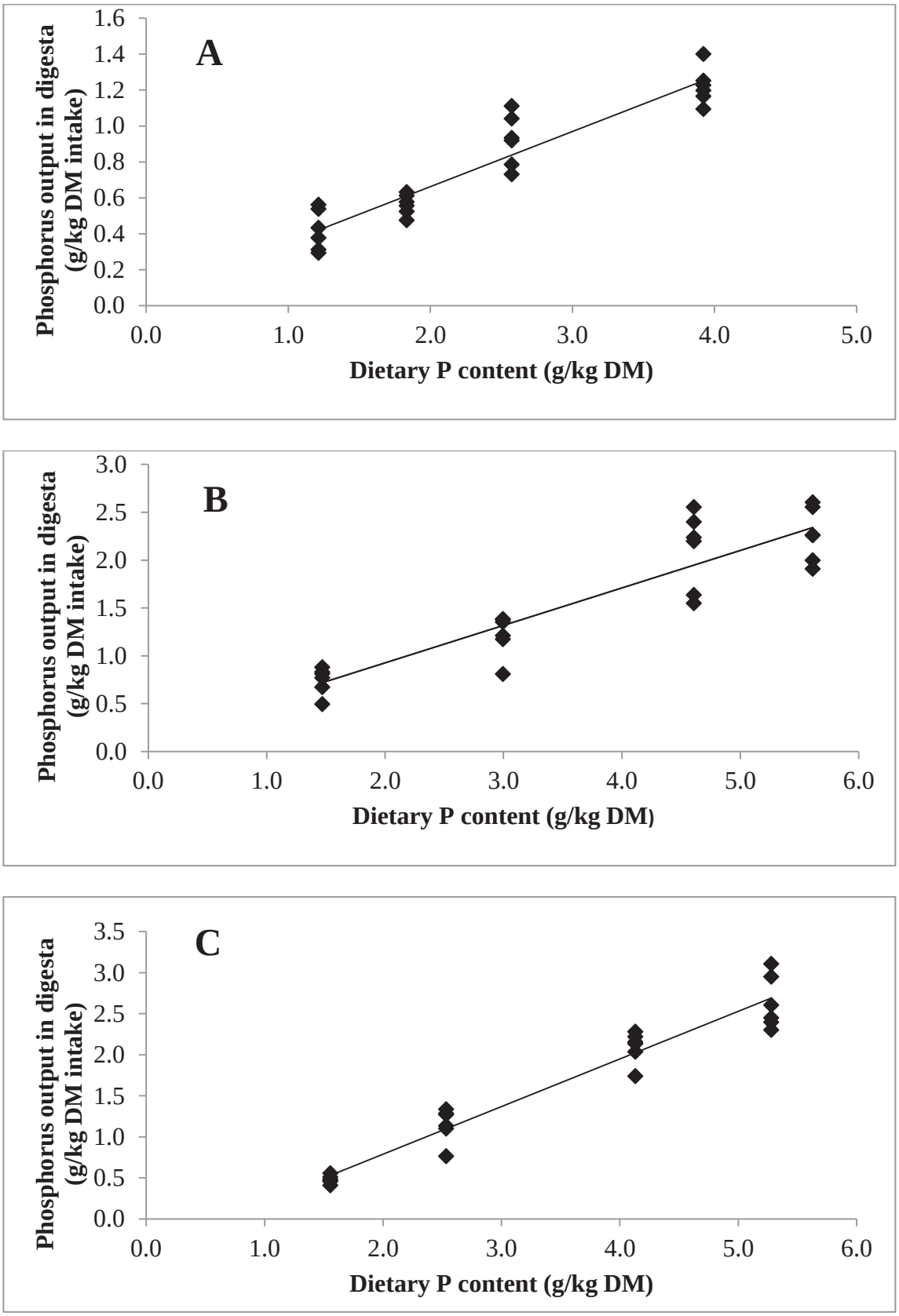


Figure 7.2. Linear relationship between total phosphorus (P) outputs (Y: g/kg DM diet intake) in ileal digesta and dietary P content (X: g/kg DM) in 21-day old broilers fed MBM-1 (A),MBM-2 (B) and MBM-3 (C) containing graded P concentrations.

Table 7.5. Linear relationship between ileal P output (g/kg DMI) vs. dietary P content (g/kg DM) of the three meat and bone meal (MBM) samples fed to broilers[1]

	Regression equation[2]	SE of the slope[3]	SE of the intercept[3]	r^2	Endogenous P loss (g/kg DMI)[4]	True ileal P digestibility coefficient
MBM-1	Y = 0.307X + 0.049	0.02	0.06	0.89	0.049	0.693[b]
MBM-2	Y = 0.392X + 0.142	0.04	0.15	0.84	0.142	0.608[b]
MBM-3	Y = 0.580X - 0.370	0.03	0.11	0.94	-0.370	0.420[a]

[1]Each value represents the mean of six replicates (6 birds/ replicate).
[2]Regression of ileal digesta P output (g/kg DMI) against dietary P content (g/kg DM) as determined by feeding broilers with diets containing graded levels of MBM-1, MBM-2 or MBM-3. The slope represents true P indigestibility and the intercept represents the endogenous P loss (g/kg DMI).
[3]Standard error of regression.
[4] Calculated as described in Dilger and Adeola (2006b).
[a-b]Means in a column not sharing a common superscript are significantly different at $P < 0.05$.

7.5. Discussion

Meat and bone meal is defined as a rendered product derived from mammalian tissues, including bone, but exclusive of any blood, hair, hoof, horn, hide trimmings, manure, stomach and rumen contents, except in such amounts as may occur unavoidably in good processing practices (AAFCO, 2000). Based on the proportion of bone to soft tissue used in the rendering process, the end product is termed as meat meal (containing more than 550 g/kg crude protein and less than 44 g/kg P) or meat and bone meal (containing less than 550 g/kg crude protein and more than 44 g/kg P) (Ravindran *et al*., 2005a). Of three samples assessed in the present study, MBM-1 falls into the category of meat meal, but all samples were considered as MBM for the purpose of this research. The variation observed in the composition among MBM samples in the current work is consistent with previous published data (Drewyor and Waldroup, 2000; Hendriks *et al*., 2002; Ravindran *et al*., 2005a; Traylor *et al*., 2005b; Hendriks *et al*., 2006; Sulabo and Stein, 2013). Such variation is due largely to differences in the type of raw material used and bone to soft tissue ratio (Kondos and McClymont, 1972; Skurray, 1974).

Weight loss was observed at all four dietary levels of MBM. The observed effects could be explained on the basis of protein deficiency. All MBM diets used in the current work were protein-deficient and calculated to contain 9.48 to 42.88 g/kg crude protein, much below the recommendations of 210 to 230 g/kg (Ross, 2007).

To the author's knowledge, apparent ileal P digestibility of MBM for broiler chickens has not been previously reported. Apparent total tract digestibility of P in MBM has been determined for pigs (Jongbloed and Kemme, 1990a; Poulsen, 1995; Sulabo and Stein, 2013) and fish (Zhou *et al*., 2004). Using the difference method, the apparent total tract digestibility of P in MBM for juvenile cobia (*Rachycentron canadum*) was determined to be 0.624 (Zhou *et al*., 2004). Sulabo and Stein (2013) reported the apparent total tract digestibility of P of eight MBM samples in pigs to range from 0.521 to 0.801. The results of the current study demonstrated that, depending on the inclusion level, the AIDC of P in birds fed MBM-1, MBM-2 and MBM-3 ranged from 0.643 to 0.694, 0.494 to 0.597, and 0.494 to 0.693, respectively. The AIDC of P in birds fed MBM-1 and MBM-2 were unaffected by increasing concentrations of dietary P. But the AIDC of P in birds fed diets containing MBM-3 quadratically decreased with increasing dietary P concentrations. Jongbloed and Kemme (1990a), estimated the apparent total tract digestibility of P in bone meal, MBM and meat meal to be 0.68,

0.80, and 0.85, respectively, which suggested that P in bone tissues are less digestible than P in soft tissues.

True ileal P digestibility of MBM-1, MBM-2 and MBM-3 were determined to be 0.693, 0.608 and 0.420, respectively. No previous work has determined the true ileal digestibility coefficient of P in MBM for poultry. Sulabo and Stein (2013), using the direct method, estimated the standardised total tract digestibility coefficients of P in eight MBM samples range from 0.548 to 0.844. The reason for the observed variation in true ileal P digestibility among the three samples in the present study is not clear. The digestibility of P in MBM is reported to decrease with increasing ash concentrations (Sulabo and Stein, 2013). In contrast, however, Traylor *et al*. (2005b) found that P in high-ash MBM was highly available to pigs compared to that P from low-ash origin. In the present study, ash content may explain the differences in P digestibility of MBM-1 and MBM-3, but not the similarity between MBM-1 and MBM-2 nor the differences between MBM-2 and MBM-3.

Differences in particle size distribution may be another factor influencing the P digestibility of MBM. A study by Orban and Roland (1992) showed that the texture of bone meal may influence on P utilisation by broilers. Based on *in vitro* tests, these researchers found that the solubility of coarse (3.3 mm), granular (2.2 mm) and fine (0.8 mm) chicken bone meals were 13, 53 and 32%, respectively. In the present study, the GMD of MBM-1, MBM-2 and MBM-3 were calculated to be 866, 622 and 875µ, respectively. In MBM-1 and MBM-3 samples, particle size distribution was similar. However, the differences in GMD in MBM samples did not explain the observed differences in P digestibility. A study by Sell and Jeffrey (1996) reported that the particle size of MBM had no effect on P utilisation by turkey poults. Similarly, Traylor *et al*. (2005b) reported that particle sizes of 470 to 635µ in MBM had no influence on P bioavailability in pigs.

Ileal endogenous P losses estimated for MBM-1 and MBM-2 samples were 0.049 and 0.142 g/kg DMI, respectively. In birds fed MBM-3-based diets endogenous P loss was estimated to be negative (-0.370 g/kg DMI). The differences in nutrient composition of MBM samples could not be explained on the basis of observed variation in endogenous P estimates. As discussed in Chapters 5 and 6, the negative estimate for endogenous P losses is not physiologically possible and an anomaly reflecting the limitation of the regression method. Such negative estimates for endogenous P losses, with the regression method, have been previously reported for broilers (Iyayi *et al*.,

2013; Liu *et al*., 2013). In the regression method, the slope of linear regression represents the true P indigestibility and the intercept represents the endogenous losses. Although the endogenous loss estimates are not used for the calculation of true digestibility, the low true P digestibility coefficient (0.420) observed in the current study for MBM-3 was associated with a negative endogenous P estimate. It appears that the low P digestibility estimated for MBM-3 is reflective of the inherent weakness of the regression method, rather than the actual digestibility of the sample.

Relative bioavailability studies in turkey poults (Sell and Jeffrey, 1996) and pigs (Traylor *et al*., 2005b) have shown that P in MBM is equally or highly available as dicalcium or monocalcium phosphate. It is generally assumed that P in MBM is 100% available to poultry, but present data (Table 7.6) show that is not the case.

Table 7.6. Comparison of total P and true digestible P contents of the three MBM samples (g/kg, as fed)

	MBM-1	MBM-2	MBM-3
Total P	37.5	60.2	59.8
True digestible P	26.0	36.6	25.1
As % of total P			
True digestible P	69.3	60.8	42.0

7.6. Conclusions

True ileal digestibility coefficients of P differed among the three MBM samples assayed in the present study. True ileal digestibility coefficients of P determined for MBM-1, MBM-2 and MBM-3 samples were 0.693, 0.608 and 0.420, respectively. Overall, the present data suggest that the general assumption that P in MBM is highly digestible is not valid.

CHAPTER 8

Measurement of true ileal phosphorus digestibility in maize and soybean meal for broiler chickens: comparison of two methodologies

8.1. Abstract

An experiment was conducted to determine the true ileal phosphorus (P) digestibility of maize and soybean meal for broiler chickens using two methodologies based on the regression method. The two methods differed in dietary protein content and dietary Ca: P ratios. In Method 1, the test ingredient was used as the sole dietary source of protein and P and a dietary Ca:non-phytate P ratio of around 2:1 was maintained by the addition of limestone. In Method 2, dried egg albumen was used as additional protein supplement, disodium phosphate as P supplement for maize and a dietary Ca:total P ratio of 1.3:1 was maintained by the addition of limestone. Two sets of sequential semi-purified diets containing 200, 460 and 720 g/kg of maize or 400, 510 and 620 g/kg of soybean meal were formulated. Each set was evaluated as a 2 x 3 factorial arrangement of treatments, which included two methods and three inclusion levels. The experiment was conducted as a randomised complete block design with four weight blocks of 12 cages each (6 birds per cage). A total of 288 of 21-day old male broilers (Ross 308) were assigned to the twelve test diets. Apparent ileal digestibility coefficient of P was determined by the indicator method and linear regression was used to determine the true ileal P digestibility. The ileal P output and ileal digestible P increased linearly with increasing inclusion levels of maize ($P < 0.01$) and soybean meal ($P < 0.001$). The apparent ileal P digestibility coefficients of P determined for maize were higher ($P < 0.001$) in Method 2 compared to Method 1 whereas the opposite effect was observed for soybean meal. True ileal P digestibility coefficients determined for maize in Methods 1 and 2 were 0.728 and 0.426, respectively, and were different ($P < 0.01$). True ileal P digestibility coefficients determined for soybean meal in Methods 1 and 2 were 0.740 and 0.523, respectively, and were different ($P < 0.01$). The present findings showed that estimation of true ileal P digestibility in maize and soybean meal is influenced by the methodology.

8.2. Introduction

Measurement of digestible phosphorus (P) has been identified as the preferable criteria to express P availability in feed ingredients for poultry (WPSA, 2013). In Chapters 5, 6 and 7 of this thesis, a linear regression method proposed by Dilger and Adeola (2006b) was used to determine the true ileal P digestibility of seven common feed ingredients for broiler chickens. In this method, the birds were fed semi-purified diets containing graded levels of the ingredient, which served as the sole source of dietary protein and P. In the test diets, calcium (Ca):non-phytate P ratio was maintained at 2:1 by the addition of limestone. This model has been used in several studies to determine the true P digestibility of feed ingredients (Dilger and Adeola, 2006b; Iyayi *et al*., 2013) and to assess the effects of dietary Ca to P ratios (Liu *et al*., 2013) and phytase supplementation (Iyayi *et al*., 2013) on true P digestibility.

Recently, a different methodology has been proposed by the Working Group No 2 (Nutrition) of the European Federation of Branches of World's Poultry Science Association (WPSA) for the determination of true ileal P digestibility in ingredients for broilers (WPSA, 2013). This method also proposes a regression approach, but differs in the composition of test diets. In this method, birds are fed diets containing graded concentrations of the feed ingredient. Dried egg albumen is also included to maintain normal dietary protein levels and in low-P ingredients such as cereals, mono-sodium phosphate is included to maintain a dietary Ca:total P ratio of 1.3 to 1.4.

These two methods, outlined above, differed in dietary protein content and dietary Ca:total P ratios. The effect of wider dietary Ca:total P ratios in lowering the apparent (Applegate *et al*., 2003; Tamim *et al*., 2004) and true ileal digestibility (Liu *et al*., 2013) and retention of P (Mohammed *et al*., 1991; Qian *et al*., 1997) in broilers is well known. No published data are available on the effects of protein deficiency on P digestibility. In one study, low dietary protein was found to have no effect on P retention of broilers (Jacob *et al*., 2000).

The objective of this present study is to compare these two methodologies for the measurement of true ileal P digestibility in maize and soybean meal for broiler chickens. The hypothesis that the differences in diet composition between the two methods will influence P digestibility measurements was tested.

8.3. Materials and methods

The experimental procedures were approved by the Massey University Ethics Committee and were in accordance with the New Zealand Code of Practice for the Care and Use of Animals for Scientific Purposes.

8.3.1. Ingredients

Maize and soybean meal were purchased from local commercial sources and were chosen to represent ingredients with low and high P contents. Upon receipt, representative samples were obtained and analysed in triplicate for DM, total P, Ca and phytate P.

8.3.2. Birds

Day-old male broilers (Ross 308) were obtained from a commercial hatchery and raised in floor pens and fed a commercial broiler starter diet. On day 14, birds were transferred to grower cages and were maintained on the same diet until the introduction of test diets on day 21. On day 21, the birds were individually weighed, blocked by weight, and 288 birds were assigned to four blocks based on body weight. Each block had 12 cages (6 birds per cage) and 12 test diets were assigned to a cage within each block. The floor pens and grower cages were housed in an environmentally controlled room. Housing conditions have been described in Chapter 3, Section 3.3.1.

Group body weights and feed intake were recorded on days 21 and 28. Mortality was recorded daily.

8.3.3. Diets

A total of 12 semi-purified diets were formulated using the analysed values for Ca and P in maize and soybean meal (Table 8.1).

In Method 1, three test diets were formulated from each ingredient and the ingredient served as the sole dietary source of protein and P. Dietary Ca:non-phytate P ratios in the three maize-based and three soybean meal-based diets were maintained at 2:1 and 2.2:1, respectively (corresponding to Ca:total P ratio of 0.59:1 and 0.66:1, respectively) by the addition of limestone. Maize-based diets (200, 460, and 720 g/kg) were formulated to contain graded concentrations of total P (0.52, 1.20, and 1.88 g/kg, respectively). Soybean meal diets (400, 510, and 620 g/kg) were formulated to contain graded concentrations of total P (2.69, 3.43, and 4.16 g/kg, respectively).

In the diets used in Method 2, dried egg albumen (crude protein, 830 g/kg, total P, 1.25 g/kg and Ca, 0.42 g/kg) was added as a supplementary protein and Ca:total P ratio was maintained between 1.3 and 1.4. Maize-based diets (200, 460, and 720 g/kg) were formulated to contain graded concentrations of total P (2.36, 3.04, 3.72 g/kg, respectively) by the addition of disodium phosphate (P, 208 g/kg; NRC, 1994) which was assumed to be 100% available. Soybean meal diets (400, 510, and 620 g/kg) were formulated to contain graded concentrations of total P (2.71, 3.45, and 4.19 g/kg, respectively). All diets contained 3 g/kg titanium dioxide as an indigestible marker. The diets were offered in mash form. Feed and water were given *ad libitum* throughout the seven-day experimental period.

8.3.4. Sample collection and processing

On day 28, all birds were euthanised by intravenous injection of sodium pentobarbitone and, contents of the lower half ileum were collected and processed as described in Chapter 3, Section 3.3.3. for chemical analysis.

8.3.5. Chemical analysis

Representative samples of maize and soybean meal were analysed for DM, total P and Ca as described in Chapter 3, Section 3.3.4. Representative samples of test diets and digesta were analysed for DM, Ca, total P and titanium (Ti) as described in Chapter 3, Section 3.3.4. Phytate P in the test ingredients was analysed as described in Chapter 5, Section 5.3.5.

8.3.6. Calculations

Calculations described for Method 1 are based on indigestible P (Dilger and Adeola, 2006b) and those in Method 2 are based on digestible P (WPSA, 2013) in ileal digesta, but both provide similar true P digestibility estimations. Both calculation procedures also provide similar estimates for endogenous P losses, resulting positive and negative estimates when based on indigestible P (Dilger and Adeola, 2006b) and digestible P (WPSA, 2013), respectively. True ileal P digestibility of maize and soybean meal in Methods 1 and 2 were calculated using both procedures. The calculation procedures are briefly outlined below.

Table 8.1. Composition of maize-based and soybean meal-based diets, as fed basis

	Maize-based diets (g/kg)						Soybean meal-based diets (g/kg)					
	Method 1[1]			Method 2[2]			Method 1[1]			Method 2[2]		
Ingredient	200	460	720	200	460	720	400	510	620	400	510	620
Maize	200.0	460.0	720.0	200.0	460.0	720.0	-	-	-	-	-	-
Soybean meal, 480 g/kg CP	-	-	-	-	-	-	400.0	510.0	620.0	400.0	510.0	620.0
Soybean oil	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Dried egg albumen, 830 g/kg CP	-	-	-	225.0	225.0	225.0	-	-	-	20.0	20.0	20.0
Dextrose	749.0	488.0	227.0	524.2	262.2	-	547.9	437.4	326.9	523.9	411.9	299.9
Limestone, 380 g/kg Ca	0.75	1.7	2.7	8.0	10.0	12.2	1.8	2.3	2.8	5.8	7.8	9.8
Disodium phosphate	-	-	-	7.5	7.5	7.5	-	-	-	-	-	-
Salt	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Sodium bicarbonate	20.0	20.0	20.0	5.0	5.0	5.0	20.0	20.0	20	20.0	20.0	20.0
Titanium dioxide	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Vitamin premix[3]	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Trace mineral premix[4]	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Calculated composition												
Metabolisable energy (MJ/kg)	15.29	14.84	14.39	15.30	14.83	14.36	13.41	12.80	12.19	13.35	12.72	12.08
Crude protein, g/kg	17.0	39.1	61.2	203.8	225.9	248.0	194	247.4	300.7	210.6	264.0	317.3
Ca[5], g/kg	0.31	0.70	1.12	3.16	3.95	4.82	1.77	2.26	2.75	3.30	4.36	5.42
Total P[5], g/kg	0.52	1.20	1.88	2.36	3.04	3.72	2.69	3.43	4.16	2.71	3.45	4.19
Non-phytate P[5], g/kg	0.15	0.35	0.55	1.99	2.19	2.39	0.81	1.03	1.25	0.83	1.05	1.28
Ca:total P ratio	0.59	0.59	0.59	1.34	1.30	1.29	0.66	0.66	0.66	1.22	1.26	1.29
Ca:non-phytate P ratio	2.03	2.00	2.03	1.58	1.80	2.02	2.20	2.20	2.20	3.97	4.14	4.25
Analysed values												
Ca, g/kg	0.49	0.64	0.94	3.13	3.26	4.78	1.92	2.60	2.99	3.48	4.48	5.60
Total P, g/kg	0.56	1.16	1.87	2.30	2.89	3.81	2.77	3.84	4.82	2.89	4.05	4.52

[1]Dilger and Adeola (2006b) method. Ingredient supplied all the protein.
[2]WPSA (2013) method. Additional protein (dried egg albumen) and disodium phosphate was provided.
[3]Supplied per kg of diet: vitamin A, 12,000 IU, vitamin D_3, 4,000 IU, thiamine, 3 mg, riboflavin, 9 mg, pyridoxine, 10 mg, folic acid, 3 mg, biotin, 0.25 mg, vitamin B_{12}, 0.02 mg, vitamin E, 80 mg, choline chloride, 0.6 g, nicotinic acid, 60 mg, Ca pantothenate, 15 mg, menadione, 4 mg.
[4]Supplied per kg of diet: Co, 0.3 mg, I, 1.5 mg, Mo, 0.3 mg, Se, 0.3 mg, Mn, 100 mg, Cu, 10 mg, Zn, 80 mg, Fe, 60 mg, anti-oxidant, 100 mg.
[5]Calculated based on values determined for individual ingredient.

8.3.6.1. Indigestible P in ileal digesta

In Methods 1 and 2, the true ileal P digestibility in maize and soybean meal were calculated according to equations 1 to 4 outlined in Chapter 5, Section 5.3.6. Phosphorus outputs were regressed against dietary P contents for maize and soybean meal.

8.3.6.2. Digestible phosphorus in ileal digesta

In Methods 1 and 2, the apparent ileal digestibility coefficients (AIDC) of P of the test diets (at each level of inclusion) were first calculated using the indicator ratio.

$$AIDC = 1-[(T_I \times P_o)/(T_o \times P_I)] \text{……..Equation (1)}$$

Where, AIDC is the apparent ileal digestibility coefficient of P, T_I is the Ti concentration in the diet, T_o is the Ti concentration in the ileal digesta, P_o is the P concentration in ileal digesta, and PI is the P concentration in the diet. All analysed values were expressed as grams per kilogram of DM.

The ileal digestible P (IdP) in ileal digesta expressed as g/kg dry matter intake (DMI) was calculated as,

$$IdP\ (g/kg) = AIDC \times P_{Diet} \text{ ……Equation (2)}$$

Ileal digestible P (IdP) (expressed in g/kg DMI) obtained in each experiments were plotted against total P concentration (in g/kg DM) as a linear regression. True P utilisation coefficient (TPUC) was calculated as the slope of linear regression.

8.3.7. Statistical Analysis

A randomised complete block design with a 2 x 3 factorial arrangement of treatments was used to investigate the response of broiler chickens to two methods of estimating P digestibility with three graded levels of maize or soybean meal. Data from each ingredient were analysed separately, since the objective of the current work was to compare the two methods. Data were analysed using the GLM procedure of SAS (2004). Cage served as the experimental unit and differences were considered significant at an alpha level of 0.05. The model for this analysis included block (3 df), methodology (1df) and ingredient inclusion level (2 df) and the interaction between methodology and ingredient inclusion level (2 df). Within each method, orthogonal polynomial contrasts were used to determine the effects of graded P intake on tested parameters (n = 12). Mean true P utilisation coefficient of each test ingredient was obtained by regressing P output (g/kg DMI) in digesta against dietary P content (g/kg

DM) and by regressing IdP (g/kg DMI) against dietary P content (g/kg DM). Regression coefficients between methods were compared using covariance analysis. The model for covariance analysis included dietary P content (1 df), methodology (1 df) and the interaction between the methodology and dietary P content (1 df).

8.4. Results

The calculated and analysed Ca and P contents of maize- and soybean meal-based test diets are presented in Table 8.1. The analysed P concentrations in all diets were similar to the calculated values being only 0.01 to 0.16 g/kg higher or lower than expected. Dietary concentrations of P and Ca increased with increasing inclusion levels of maize and soybean meal. The analysed concentrations for Ca in five of the six maize diets were between 0.03 and 0.70 g/kg lower than expected. In all six soybean meal diets, analysed Ca values were 0.12 to 0.34 g/kg higher than expected. Analysed total P, Ca and phytate P contents of the test ingredients are summarised in Table 8.2.

Table 8.2. Analysed composition of maize and soybean meal (g/kg, as fed basis)

	Maize	Soybean meal
Total P	2.61	6.72
Phytate P	1.85	4.70
Non-phytate P[1]	0.76	2.02
Ca	0.13	2.72

[1]Calculated as the difference between total and phytate-P.

8.4.1. Maize

Birds remained healthy during the 7-day experimental period and no leg problems were recorded. Mortality was negligible and only 3 birds out of 144 birds died. Feed intake and body weight gain of birds fed diets containing graded concentrations of maize in Methods 1 and 2 are presented in Table 8.3. In Method 1, weight loss was observed in birds fed two of the three diets and loss was highest in birds fed the diet with the lowest inclusion (200 g/kg) of maize. Body weight increased only at the highest inclusion level (720 g/kg) of maize. In Method 2, there was a linear increase ($P < 0.05$) in body weight gain in birds fed diets containing graded concentrations of maize. Linear increase ($P < 0.01$) in feed intake of birds fed diets for Method 1 was observed. For Method 2, feed intake was quadratically ($P < 0.05$) affected.

Table 8.3. Growth performance (day 21-28 posthatch) and, dietary P content, total ileal P output and input in birds fed diets containing graded concentrations of P from maize, Methods 1 and 2[1]

	Method 1			Method 2				*P*-value						
											Method 1		Method 2	
Inclusion level	200	460	720	200	460	720	Pooled SEM	Method	Maize Inclusion level	Method x inclusion level	L^2	Q^2	L^2	Q^2
BWG[3], g/b/d	-18.27	-3.09	5.56	49.43	65.03	64.03	2.95	***	***	NS	***	NS	*	NS
FI[4], g/b/d	83.2	98.3	100.8	100.4	116.6	107.6	3.65	***	**	NS	**	NS	NS	*
P_I[5], g/kg DM	0.63	1.30	2.12	2.51	3.20	4.27	-	-	-	-	-	-	-	-
P_D[6], g/kg DMI	0.38	0.77	0.79	0.45	0.79	1.45	0.058	***	***	***	**	*	***	*
IdP[7], g/kg DMI	0.25	0.54	1.33	2.06	2.41	2.82	0.058	***	***	*	***	*	***	NS

NS, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

[1]Each value represents the mean of four replicates (6 birds/ replicate).

[2]L = Linear effect; Q = Quadratic effect.

[3]BWG = Body weight gain.

[4]FI = Feed intake.

[5]P_I = Dietary P content; DM = Dry matter.

[6]P_D = Ileal P output; DMI = Dry matter intake.

[7]IdP = Ileal digestible P.

Dietary P contents, P outputs and ileal digestible P contents in birds fed maize-based diets are summarised in Table 8.3. Ileal P output increased with increasing maize inclusion levels in Method 1 (linear, $P < 0.01$) and Method 2 (linear, $P < 0.001$; quadratic, $P < 0.05$). Ileal P output was influenced by methodology and higher ($P < 0.001$) in Method 2, but a significant ($P < 0.001$) interaction was observed between the ingredient inclusion level and the method. This interaction was due to a greater increase in ileal P output with increasing maize inclusion levels in Method 2, relative to Method 1.

Ileal digestible P content increased (linear, $P < 0.001$) with increasing maize inclusion levels in both methods. Ileal digestible P content was higher ($P < 0.001$) in Method 2. However, an interaction ($P < 0.05$) was also found between the method and maize inclusion level, which was due to a greater magnitude of increase in ileal digestible P with increasing maize inclusion levels in Method 1, relative to Method 2.

The apparent ileal P digestibility coefficients of maize determined by Methods 1 and 2 ranged from 0.403 to 0.625 and 0.661 to 0.822, respectively (Table 8.4). An interaction ($P < 0.001$) between the method and maize inclusion level was observed. In Method 1, apparent ileal P digestibility was linearly ($P < 0.05$) increased with increasing maize inclusion levels, while values linearly ($P < 0.001$) decreased in Method 2.

In both methods, strong linear relationships were observed between ileal P outputs and dietary P contents for maize (Table 8.5). Similarly, strong linear relationships were observed between digestible and dietary P contents for maize (Table 8.5; Figure 8.1). True P digestibility coefficients calculated according to Dilger and Adeola (2006b) and WPSA (2013) for individual methods yielded the same results. True ileal P digestibility coefficient (0.728) determined for maize by Method 1 was significantly higher ($P < 0.01$) than its corresponding value (0.426) obtained by Method 2 (Table 8.5).

Table 8.4. Apparent ileal digestibility coefficient (AIDC) of phosphorus (P) in birds fed diets containing graded concentrations of P from maize, Methods 1and 2[1]

	Maize inclusion level	AIDC
Method 1	200	0.403[a]
	460	0.412[a]
	720	0.625[b]
Method 2	200	0.822[d]
	460	0.754[cd]
	720	0.661[bc]
Pooled SEM		0.0407
Probability		
Method		***
Inclusion level		NS
Method x inclusion level		***
Method 1		
Linear effect		*
Quadratic effect		NS
Method 2		
Linear effect		***
Quadratic effect		NS

NS, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

[1]Each value represents the mean of four replicates (6 birds/ replicate).

[a-d]Means in a column not sharing a common superscript are significantly different at $P < 0.05$.

Table 8.5. Linear relationship between ileal P outputs (g/kg DMI) vs. dietary P content (g/kg DM) and ileal digestible P (g/kg DMI) vs. dietary P content (g/kg DM) of maize fed to broilers, Methods 1 and 2[1]

	Regression equation	SE of the slope[2]	SE of the intercept[2]	r^2	Endogenous P loss (g/kg DMI)	True ileal P digestibility coefficient
Ileal P output vs. dietary P[3]						
Method 1						
True ileal P digestibility	Y = 0.272X + 0.277	0.076	0.113	0.56	0.277	0.728[b]
Method 2						
True ileal P digestibility	Y = 0.574X - 1.016	0.069	0.234	0.87	-1.016	0.426[a]
Digestible P vs. dietary P[4]						
Method 1						
True ileal P digestibility	Y = 0.728X - 0.277	0.076	0.113	0.90	-0.277	0.728[b]
Method 2						
True ileal P digestibility	Y = 0.426X + 1.016	0.069	0.234	0.79	1.016	0.426[a]

[1]Each value represents the mean of four replicates (6 birds/ replicate).
[2]Standard error of regression.
[3]Regression of ileal digesta P output (g/kg DMI) against dietary P content (g/kg DM) as determined by feeding diets containing graded levels of maize by Method 1 or 2. The slope represents true P indigestibility and the intercept represents the endogenous P loss (g/kg DMI) (Dilger and Adeola, 2006b).
[4]Regression of ileal digestible P (g/kg DMI) against dietary P content (g/kg DM) as determined by feeding diets containing graded levels of maize by Method 1 or 2. The slope represents true P digestibility and the intercept represents the endogenous P loss (g/kg DMI) (WPSA, 2013).
[a-b] True ileal P digestibility coefficients within a calculation without a common superscript are different at $P < 0.05$.

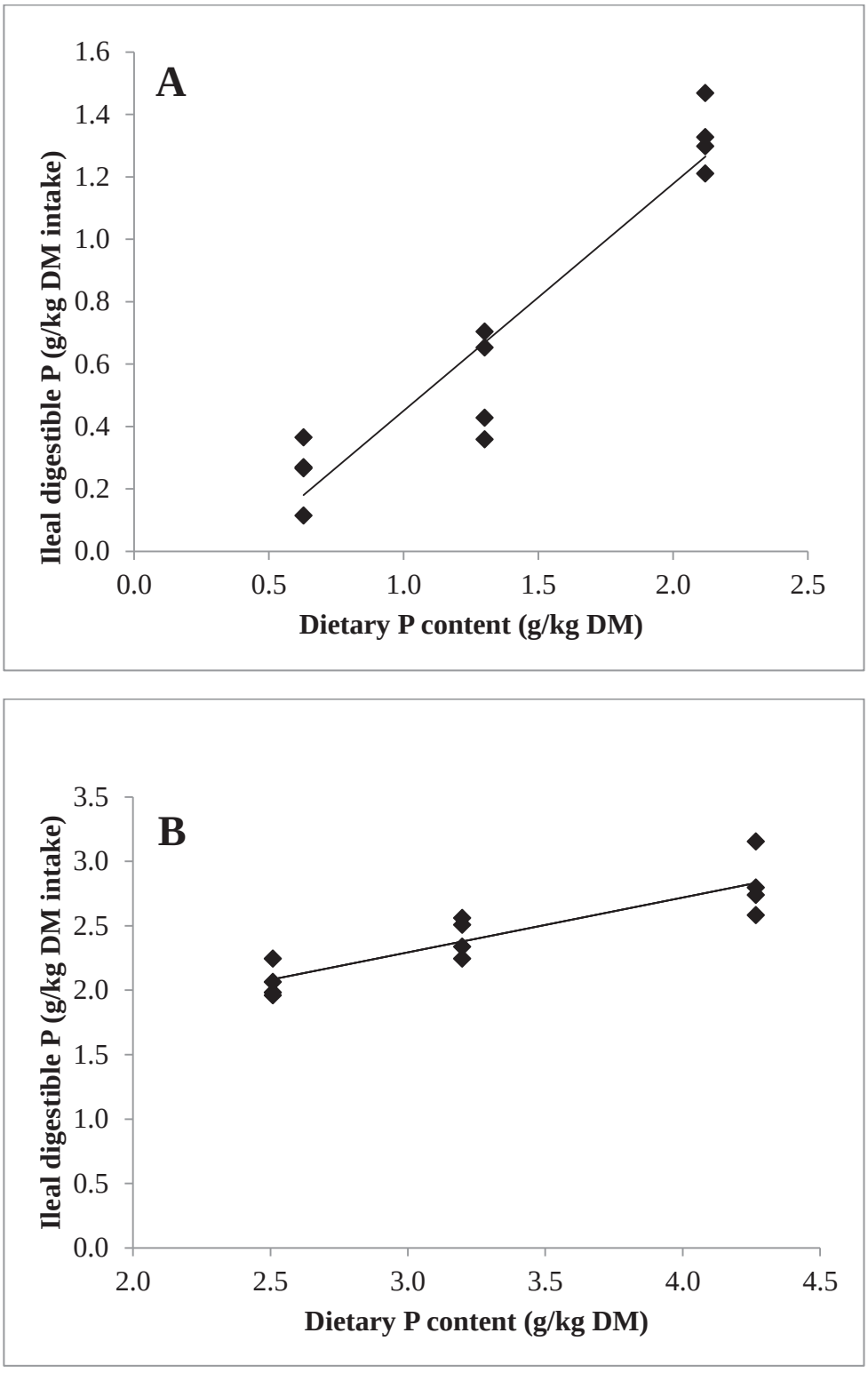


Figure 8.1. Linear relationship between digestible P (Y: g/kg DMI) in ileal digesta and dietary P content (X: g/kg DM) in 21-day old broilers fed maize-based diets containing graded P concentrations, in Methods 1 (A) and 2 (B).

8.4.2. Soybean meal

Birds remained healthy during 7-day experimental period and no mortality or leg problems were recorded. Feed intake and body weight gain of birds fed diets containing graded concentrations of soybean meal in Methods 1 and 2 are presented in Table 8.6. In Method 1, weight gain increased linearly ($P < 0.05$) and the gain was highest in birds fed the highest inclusion of soybean meal (620 g/kg). Weight gain was not influenced by increasing inclusion levels in Method 2. Weight gains were higher ($P < 0.05$) in Method 2, compared to Method 1. Feed intake of birds was not influenced ($P > 0.05$) by soybean meal inclusion level or the method.

Dietary P contents, P outputs and ileal digestible P contents in birds fed soybean meal-based diets for Methods 1 and 2 are summarised in Table 8.6. Ileal P output increased with increasing soybean meal inclusion levels in Method 1 (linear, $P < 0.001$; quadratic, $P < 0.01$) and Method 2 (linear, $P < 0.001$), but a significant ($P < 0.01$) interaction was observed between the soybean meal inclusion level and the method. This interaction was due to a greater increase in ileal P output between two soybean meal inclusion levels (510 and 620 g/kg) in Method 2, relative to Method 1. However, ileal P outputs were higher ($P < 0.001$) in Method 2.

Ileal digestible P content increased with increasing soybean meal inclusion levels in Method 1 (linear, $P < 0.001$) and Method 2 (linear, $P < 0.001$; quadratic, $P < 0.01$). Ileal digestible P content was higher ($P < 0.001$) in Method 1. However, an interaction ($P < 0.001$) was also found between the method and soybean meal inclusion level, which was due to reduced magnitude of increase in ileal digestible P with increasing soybean meal inclusion levels (between 510 and 620 g/kg) in Method 2, relative to Method 1.

The apparent ileal P digestibility coefficients of soybean meal in Methods 1 and 2 ranged from 0.761 to 0.807 and 0.613 to 0.676, respectively (Table 8.7). In Methods 1 (linear, $P < 0.01$; quadratic, $P < 0.01$) and 2 (linear, $P < 0.05$), the apparent ileal P digestibility decreased with increasing soybean meal inclusion levels. An interaction ($P < 0.05$) between the method and soybean meal inclusion level was also observed which was due mainly to the differences in magnitude of decrease in apparent ileal P digestibility between 510 and 620 g/kg inclusion levels in Method 2, relative to method 1.

Table 8.6. Growth performance (day 21-28 posthatch) and, dietary P content, total P output and input in birds fed diets containing graded concentrations of P from soybean meal for broilers, Methods 1 and 2[1]

	Method 1			Method 2				*P*-value						
											Method 1		Method 2	
Inclusion level	400	510	620	400	510	620	Pooled SEM	Method	Soybean meal inclusion level	Method x inclusion level	L[2]	Q[2]	L[2]	Q[2]
BWG[3], g/b/d	47.0	60.3	68.7	61.2	65.9	76.4	5.16	*	**	NS	*	NS	NS	NS
FI[4], g/b/d	122.4	129.8	132.4	127.6	124.6	130.9	5.03	NS	NS	NS	NS	NS	NS	NS
P_I[5], g/kg DM	3.13	4.35	5.43	3.25	4.49	5.03	-	-	-	-	-	-	-	-
P_D[6], g/kg DMI	0.60	1.04	1.19	1.05	1.50	1.95	0.045	***	***	**	***	**	***	NS
IdP[7], g/kg DMI	2.53	3.31	4.23	2.20	2.99	3.08	0.045	***	***	***	***	NS	***	**

NS, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
[1]Each value represents the mean of four replicates (6 birds/ replicate).
[2]L = Linear effect; Q = Quadratic effect.
[3]BWG = Body weight gain.
[4]FI = Feed intake.
[5]P_I = Dietary P content; DM = Dry matter.
[6]P_D = Ileal P output; DMI = Dry matter intake.
[7]IdP = Ileal digestible P.

Table 8.7. Apparent ileal digestibility coefficient (AIDC) of phosphorus (P) in birds fed diets containing graded concentrations of P from soybean meal, Methods 1and 2[1]

	Soybean meal inclusion level	AIDC
Method 1	400	0.807[d]
	510	0.761[c]
	620	0.780[cd]
Method 2	400	0.676[b]
	510	0.665[b]
	620	0.613[a]
Pooled SEM		0.0112
Probability		
Method		***
Inclusion level		**
Method x inclusion level		*
Method 1		
Linear effect		**
Quadratic effect		**
Method 2		
Linear effect		*
Quadratic effect		NS

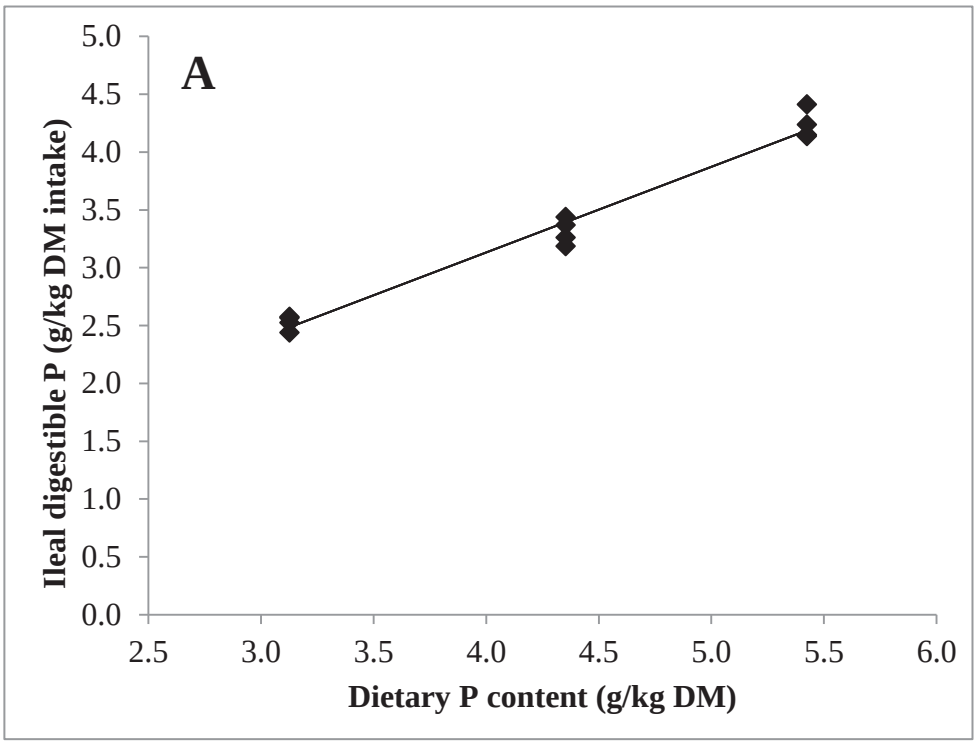
NS, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
[1]Each value represents the mean of four replicates (6 birds/ replicate).
[a-d]Means in a column not sharing a common superscript are significantly different at $P < 0.05$.

True P digestibility coefficients of soybean meal and the respective endogenous P losses are presented in Table 8.8. In both methods, strong linear relationships were observed between ileal digesta P and dietary P contents with increasing soybean meal inclusion levels. Similar linear relationships were observed for ileal digestible P (Table 8.8; Figure 8.2). However, true digestibility coefficients calculated based on these two regression criteria (P output vs. dietary P content or digestible vs. dietary P content) yielded the same results. True ileal P digestibility coefficients of soybean meal determined by Methods 1 and 2 were 0.740 and 0.523, respectively. True ileal P digestibility coeffients determined for soybean meal were lower ($P < 0.01$) in Method 2.

Table 8.8. Linear relationship between ileal P outputs (g/kg DMI) vs. dietary P content (g/kg DM) and ileal digestible P (g/kg DMI) vs. dietary P content (g/kg DM) of soybean meal fed to broilers, Methods 1 and 2[1]

	Regression equation	SE of the slope[2]	SE of the intercept[2]	r^2	Endogenous P loss (g/kg DMI)	True ileal P digestibility coefficient
Ileal P output vs. dietary P[3]						
Method 1						
True ileal P digestibility	Y = 0.260X - 0.171	0.04	0.16	0.84	-0.171	0.740[b]
Method 2						
True ileal P digestibility	Y = 0.477X - 0.530	0.05	0.22	0.89	-0.530	0.523[a]
Digestible P vs. dietary P[4]						
Method 1						
True ileal P digestibility	Y = 0.740X + 0.171	0.04	0.16	0.98	0.171	0.740[b]
Method 2						
True ileal P digestibility	Y = 0.523X + 0.530	0.05	0.22	0.91	0.530	0.523[a]

[1]Each value represents the mean of four replicates (6 birds/ replicate).
[2]Standard error of regression.
[3]Regression of ileal digesta P output (g/kg DMI) against dietary P content (g/kg DM) as determined by feeding diets containing graded levels of soybean meal by Method 1 or 2. The slope represents true P indigestibility and the intercept represents the endogenous P loss (g/kg DMI) (Dilger and Adeola, 2006b).
[4]Regression of ileal digestible P (g/kg DMI) against dietary P content (g/kg DM) as determined by feeding diets containing graded levels of soybean meal by Method 1 or 2. The slope represents true P digestibility and the intercept represents the endogenous P loss (g/kg DMI) (WPSA, 2013).
[a-b] True ileal P digestibility coefficients within a calculation without a common superscript are different at $P < 0.05$.



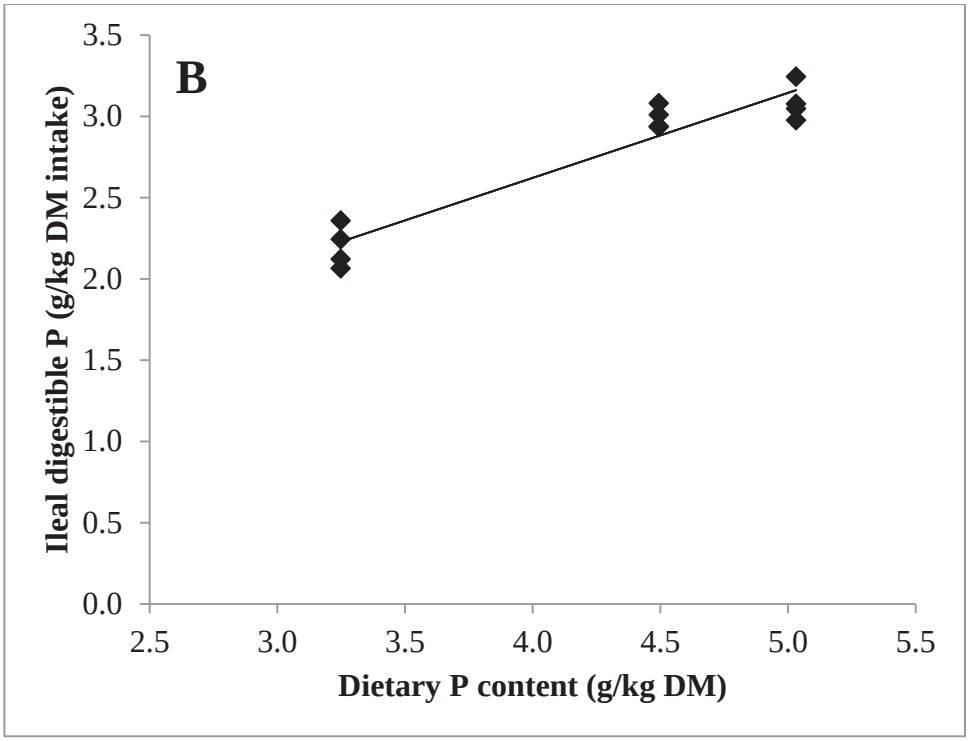


Figure 8.2. Linear relationship between digestible P (Y: g/kg DMI) in ileal digesta and dietary P content (X: g/kg DM) in 21-day old broilers fed soybean meal-based diets containing graded P concentrations in Methods 1(A) and 2 (B).

8.5. Discussion

The study reported herein compared two regression-based methodologies, which differed in dietary protein content and Ca:total P ratios, to the estimate the true P digestibility of maize and soybean meal.

Phytate P content of maize (1.85 g/kg) and soybean meal (4.70 g/kg) used in the present study were within the range reported in the literature (Weremko *et al*., 1997; Selle and Ravindran, 2007). The analysed dietary P concentrations for maize and soybean meal were closer to calculated concentrations and therefore used for the calculation of P digestibility. In all diets, the analysed concentrations of Ca, however, differed slightly from expected.

In Method 1, weight loss was observed when the birds fed diets with low dietary inclusions (200 and 460 g/kg diet) of maize. This is in agreement with our previous findings, which showed that the low dietary inclusions of maize resulted weight loss in 21-day old broilers (Chapter 5). These diets were low in protein, which explains the observed weight loss. Low dietary P concentrations could also contribute to poor growth rates and feed intakes. Weight gain and feed intake were highest in the birds fed the highest inclusion of soybean meal which contained the highest contents of P and crude protein. Addition of dried egg albumen, as an additional protein source in Method 2, improved the body weight gain of birds fed both maize and soybean meal-based diets.

8.5.1. Apparent ileal P digestibility coefficients

Maize

Apparent ileal P digestibility coefficients determined at different inclusion levels of maize in Method 1 were found to be different, which is in agreement with previous findings (Chapter 5). The apparent ileal P digestibility coefficients determined for maize in Method 1 ranged from 0.403 to 0.625. These values were considerably lower than the values of 0.70 and 0.86 reported by Wu *et al*. (2004) and Leytem *et al*. (2008), respectively. In both of these studies, direct method was used where ingredient served as the sole dietary source of P and Ca. The AIDC of P determined for maize by Method 2 also differed depending on ingredient inclusion level. These results suggest that the direct method may not be an appropriate method to determine AIDC of P for ingredients with low P contents.

In Method 1, maize served as the sole dietary source of P in diets, whereas, the diets used in Method 2 were supplemented with disodium phosphate and dried egg albumen. Inclusion of these highly digestible P sources would have increased the P intake of birds resulting in higher apparent digestibility coefficients.

Soybean meal

Apparent ileal P digestibility coefficients determined at different inclusion levels of soybean meal by Method 1 differed which is in agreement with our previous findings (Chapter 6). The AIDC of P determined for soybean meal in Method 1 ranged from 0.761 to 0.807 and higher than those reported in Chapter 6 (0.140 to 0.627). However, the values reported in the current work are in agreement with the values (0.814 to 0.891) reported by Liu *et al*. (2013) for soybean meal measured at a dietary Ca:total P ratio of 0.8.

Similarly, AIDC of P determined by Method 2 for soybean meal-based diets were different at different inclusion levels. These values (0.613 to 0.676) were lower than the values reported by Liu *et al*. (2013) for soybean meal (0.694 to 0.810) measured at dietary Ca:total P ratio of 1.2.

8.5.2. True ileal P digestibility coefficients

Maize

In this study, true ileal P digestibility coefficients determined for maize in Methods 1 and 2 were 0.728 and 0.426, respectively. True ileal P digestibility of maize determined by Method 1 was comparable to our previous finding (0.676; Chapter 5), but the digestibility coefficient determined by Method 2 was considerably lower. These findings can be explained by the differences in dietary Ca content, Ca:total P ratio or both. No published data are available on the influence of dietary protein content on P digestion. Because of the confounding effects of Ca, no definite conclusion can be drawn based on the present data. It is, however, possible that part of these differences may arise from the formation of unavailable protein-phytate complexes (Mothes *et al*., 1990) and this possibility needs to be explored in future research.

Soybean meal

True ileal P digestibility coefficients estimated for soybean meal in Methods 1 and 2, were 0.740 and 0.523, respectively. The lower digestibility determined by Method 2 is in agreement with the observation by Liu *et al*. (2013) who reported that true ileal

digestibility coeffients of P is influenced by dietary Ca:total P ratios. According to these researchers, true ileal P digestibility was decreased from 0.71 to 0.46, when dietary Ca:total P ratios were increased from 0.8 to 2.0, respectively. True ileal P digestibility coefficients reported in the present study were considerably lower than the values reported by Dilger and Adeola (2006b) for conventional (0.939; dietary Ca:total P ratio, 0.56 to 0.81) and low-phytate (0.938; dietary Ca:total P ratio, 0.79 to 1.01) soybean meals.

8.5.3. Non-phytate P vs. true digestible P

The data summarised in Table 8.9 shows that the true digestible P contents evaluated by both methods were considerably higher than the respective non-phytate P contents in maize and soybean meal, suggesting that a portion of phytate-bound P is being utilised by broiler chickens but this may be an adaptive response to low P diets used in this experimental model. As discussed in Chapter 6, poultry have an innate ability to regulate intestinal phytase activity as an adaptive response to P deficient environment and, therefore capable of utilising more P from phytate bound P when P deficient diets were fed. All diets used in the present experiment were P- and Ca-deficient and, it is possible that the P digestibility may have been over-estimated.

Table 8.9. Comparison of total P, phytate-P, non-phytate P, and true digestible P contents of maize and soybean meal (g/kg, as fed)

	Maize	Soybean meal
Total P	2.61	6.72
Phytate P	1.85	4.70
Non-phytate P[1]	0.76	2.02
True digestible P (Method 1)[2]	1.90	4.97
True digestible P (Method 2)[3]	1.11	3.51
As % of total P		
Phytate P	70.8	70.0
Non-phytate P	29.2	30.0
True digestible P (Method 1)[2]	72.8	74.0
True digestible P (Method 2)[3]	42.6	52.3

[1]Calculated as the difference between total P and phytate P.
[2]Ingredient supplied all the protein; Ca:non-phytate P ratio was around 2:1.
[3]Additional protein (dried egg albumen) and P (disodium phosphate) was provided; Ca:total P ratio was 1.3.

If it is assumed that the non-phytate P in maize is 100% digestible, then it can be calculated that 61.5% of phytate-bound P was digested and absorbed when diets were not supplemented with additional source of protein and inorganic P (Method 1). Similarly, it was previously observed that the broilers are capable of utilising 54.2% of phytate-bound P in maize when fed P-deficient diets (Chapter 5). In contrast, when supplemented with additional dietary sources of protein and inorganic P (Method 2), only 18.8% of the phytate P was utilised. The inherent ability of phytate-bound P to chelate proteins via formation of binary protein-phytate complexes below the isoelectric point of the protein is known (Mothes *et al*., 1990) and this ability is influenced by the type of protein, pH and dietary salts (Yu *et al*., 2012; Bye *et al*., 2013). The relatively low phytate P utilisation observed in birds fed maize-based diets in Method 2 may be attributed, partly, to the formation of protein-phytate complexes.

If the non-phytate P in soybean meal is assumed to be 100% digestible, then it can be calculated that 62.9% of phytate-bound P is being utilised by birds when soybean meal-based diets were not supplemented with additional dietary protein source (Method 1). In agreement, it was previously observed that 69.7% of phytate-bound P in soybean meal can be digested by broiler chickens when soybean meal was used in diets as the sole source of dietary protein and P (Chapter 6). In contrast, when supplement with additional dietary source of protein (Method 2) only 31.8% of the phytate P was utilised.

As discussed in Section 8.5.1., high dietary Ca concentration and wider Ca:P ratio can negatively influence on phytate P hydrolysis. The estimated higher true P digestibility and higher phytate P utilisation observed for both ingredients in Method 1, as compared to Method 2, may be due partly to the relatively lower dietary Ca concentrations in the test diets. The recommended dietary requirement for Ca for growing broilers is 9.0 g/kg (Ross, 2007) and all the diets used in current experiment were formulated to contain sub-optimal concentrations of Ca.

8.6. Conclusions

The present data demonstrated that the measurement of true ileal P digestibility in maize and soybean meal was influenced by the methodology. On the basis of current findings, however, it was not possible to make a definite conclusion on which measurement is truly reflective of the actual P digestibility. The findings also suggest that the magnitude of phytate-bound P utilisation differed depending on the dietary Ca concentration.

CHAPTER 9

General discussion

9.1. Introduction

Phosphorus (P) is a vital macro-mineral for poultry. Provision of adequate amounts of P in poultry diets is therefore considered essential for optimum biological functions. However, uncertainty about true P requirements for poultry has led to the inclusion of inorganic P supplements with safety margins in feed formulations and feeding poultry above P requirements which contribute to the excretion of excess P from intensive operations into the environment. In addition, confusion about current terminologies used to express the P availability (available P, non-phytate P and retainable P) of feed ingredients restricts feeding poultry to the optimum requirement (Rodehutscord, 2001; Angel *et al*., 2002). The depletion of non-renewable inorganic phosphates deposits is another global issue making P the third most expensive nutrient in poultry diets. Therefore, there is growing interest in exploring ways to define a sound criterion to express the P availability for poultry. Of the various criteria proposed to express P availability, measurement of digestible P is currently considered as the preferable method to assess P availability for poultry (WPSA, 2013).

Published data on apparent or true digestibility values of P in common feed ingredients for pigs are available. In these pig assays, three approaches, namely the regression analysis, the direct method and the substitution method have been used to estimate P digestibility (Table 9.1). However, corresponding data for poultry are limited. The main focus of the studies reported in this thesis was to investigate the potential usefulness of regression method, which provides a direct estimation of true P digestibility, to measure P digestibility of a range of common feed ingredients for broiler chickens.

9.2. Dynamics of Ca and P digestion in poultry

Identification of the intestinal sites of Ca and P absorption is critical to understand the dynamics of P digestion and this was examined in the first experiment (Chapter 3).

Table 9.1. Summary of phosphorus (P) digestibility measurement studies of feed ingredients for pigs and poultry

Reference	Ingredient	Method
Pigs		
Bohlke *et al*. (2005)	Low-phytate maize, maize and soybean meal	Direct
Petersen and Stein (2006)	Dicalcium phosphate, monocalcium phosphate, monosodium phosphate	Direct
Stein *et al*. (2006)	Field peas (*Pisum sativum L.*)	Direct
Widmer *et al*. (2007)	High protein distillers dried grains and maize germ	Direct
Almeida and Stein (2010)	Maize, soybean meal and DDGS[1]	Direct
Baker *et al*. (2013)	Dicalcium phosphate and DDGS[1]	Direct
Rojas *et al*. (2013)	Maize, maize coproducts and bakery meal	Direct
Sulabo and Stein (2013)	Meat and bone meal	Direct
Fang *et al*. (2007a)	Soybean meal, peas, faba beans, maize, oats, broken /rough rice meal, buckwheat and sorghum	Substitution
Fang *et al*. (2007b)	Soybean meal and wheat middling meal	Substitution
Pedersen *et al*. (2007)	DDGS[1]	Substitution
Stein *et al*. (2008)	Monocalcium phosphate	Substitution
Fan *et al*. (2001)	Soybean meal	Regression
Shen *et al*. (2002)	Maize	Regression
Ajakaiye *et al*. (2003)	Soybean meal	Regression
Dilger and Adeola (2006a)	Conventional and low-phytate soybean meal	Regression
Fang *et al*. (2007b)	Soybean meal and wheat middling meal	Regression
Yang *et al*. (2007)	Brown rice	Regression
Akinmusire and Adeola (2009)	Canola meal and soybean meal	Regression
Poultry		
Wu *et al*. (2004)	Sorghum, maize, barley and wheat	Direct
Leytem *et al*. (2008)	Maize, normal barley, low-phytate barley and high fat low-lignin oat	Direct
Dilger and Adeola (2006b)	Conventional and low-phytate soybean meal	Regression
Shastak *et al*. (2012)	Monosodium phosphate and dibasic calcium phosphate	Regression
Liu *et al*. (2013)	Soybean meal	Regression
Iyayi *et al*. (2013)	Black-eyed pea and peanut flour	Regression

[1]DDGS = distiller's dried grains with solubles.

This experiment investigated the effects of dietary Ca concentration on the digestion of P, Ca, nitrogen, fat and starch along the gastrointestinal tract of young broilers fed maize-soy diets containing 6, 9, or 12 g/kg Ca. The results showed that P absorption predominantly occurred in the jejunum when birds were fed diets with low (6 g/kg) and normal Ca (9 g/kg) concentrations, but shifted to both the jejunum and upper ileum when high Ca diet (12 g/kg) was fed. At all three dietary Ca concentrations, digestion of P was completed by the upper ileum and the apparent digestibility of P determined at the upper and lower ileal sites were similar. The data suggested that, regardless of dietary Ca concentrations, digesta samples can be collected at the lower ileum in P digestibility studies.

Calcium was absorbed predominantly by the jejunum. Dietary Ca concentration influenced P digestibility, with digestibility being higher in the low Ca diet. Increasing dietary Ca concentrations, however, had no effect on those Ca and starch digestibility and apparent metabolisable energy (AME) of diets.

A noteworthy observation in this study was the anti-nutritive effects of high dietary Ca. Increasing dietary Ca concentrations not only lowered the digestibility of P, but also of nitrogen and lipids. These negative effects are not widely recognised and have important practical implications in poultry feed formulations. Based on these findings, it is suggested that the Ca concentration in broiler diets should be maintained low as realistically as possible to maximise the utilisation of other nutrients. However, the information on minimum Ca requirement for optimal skeletal health is scant and further research is warranted.

9.3. Endogenous losses of P

The major function of the gastrointestinal tract is to digest and absorb the nutrients in the food, but a significant amount of endogenous nutrients is also secreted into the gut. Only a portion of these endogenous nutrients is digested and reabsorbed. The estimation of these net endogenous nutrient losses is of practical importance due to their confounding effects on nutrient digestibility. Accurate measurement of and correction for these endogenous nutrient losses are necessary for the estimation of true ileal digestibility and the maintenance requirements of animals (Nyachoti *et al*., 1997). Estimates for endogenous P losses of pigs [106 to 211 mg/kg dry matter intake (DMI)] have been published (Petersen and Stein, 2006; Stein *et al*., 2006; Widmer *et al*., 2007;

Almeida and Stein, 2010; Stein, 2011; Baker *et al*., 2013; Sulabo and Stein, 2013), but there have been no systematic studies to determine endogenous P losses in poultry.

Previous studies to estimate endogenous P losses in poultry have used different techniques including feeding of a minimal P diet (Rutherfurd *et al*., 2002; 2004), regression method (Dänner *et al*., 2006; Dilger and Adeola, 2006b) or an isotope-dilution technique (Al-Masri, 1995), resulting in wide variability in reported values (Tables 4.4 and 4.5). Data presented in Chapter 4 showed that the ileal endogenous flow of P determined using P-free, gelatin-based and casein-based diets were 25, 104 and 438, mg/kg DMI, respectively. Endogenous P flow estimated with the P-free diet may be reflective of basal losses which is independent of the raw material or diet composition. As suggested in Chapter 4, this estimate may be used in the calculation of true P digestibility of feed ingredients for poultry. Higher endogenous P flows derived from casein- and gelatin-based diets may be representative of specific losses driven by dietary protein. These data imply that the endogenous P losses are method sensitive and diet dependent. To the author's knowledge, this is the first study comparing different methodologies to determine endogenous P losses in poultry. However, since only limited published data are available, further research is warranted to confirm the present findings.

9.4. Measurement of true ileal P digestibility in poultry feed ingredients

Only limited published data are available on the P digestibility of feed ingredients for poultry (Dilger and Adeola, 2006b; Iyayi *et al*., 2013; Liu *et al*., 2013). The study described by Dilger and Adeola (2006b) used the regression model where the test ingredient was used as the sole dietary source of P, Ca and protein. This model was also used in the studies reported in Chapters 5, 6 and 7 with the modification that the dietary Ca:non-phytate P ratio was maintained at 2:1 by the addition of limestone.

The major outcome of the current work is to provide a direct comparison of the widely used non-phytate P content with the true digestible P content of seven poultry feed ingredients (Table 9.2). Such a comparison has not been previously reported.

Surprisingly, for all plant-based ingredients, the determined digestible P contents were found to be consistently higher than the corresponding non-phytate P contents. For maize (Chapter 5), soybean meal (Chapter 6) and maize-DDGS (Chapter 6), the differences were considerable.

Table 9.2. Phytate P, non-phytate P and true digestible P contents of feed ingredients (as a percentage of total P)

Chapter	Ingredient	% of total P		
		Phytate P	Non-phytate P[1]	True digestible P
5	Maize	70.5	29.5	67.7
	Canola meal	70.9	29.1	46.9
6	Wheat	65.5	34.5	46.4
	Sorghum	76.9	23.1	33.1
	Soybean meal	66.7	33.3	79.8
	Maize-DDGS[2]	46.7	53.3	72.7
7	Meat and bone meal[3]			
	MBM-1	-	100	69.3
	MBM-2	-	100	60.8
	MBM-3	-	100	42.0

[1]Calculated as the difference between total P and phytate P.
[2]Maize-DDGS = maize-distiller's dried grains with solubles.
[3]Three meat and bone meal samples (MBM-1, MBM-2 and MBM-3).

These findings are suggestive of the ability of broilers to utilise a portion of phytate-bound P in feed ingredients, but the amount of phytate P digested is clearly ingredient specific (maize, 54.2; canola meal, 25.2; wheat, 18.1; sorghum, 13.0; soybean meal, 69.7 and maize-DDGS, 41.5%). The location, size of globoid phytin crystals, form (Adeola and Sands, 2003) and solubility of phytin (Selle and Ravindran, 2007) vary among grains and legume seeds, and could be responsible for the observed difference in the utilisation of phytate P. It is likely that these factors also contribute, in part, to the differences in true ileal P digestibility between feed ingredients. Dietary Ca concentration, presence of anti-nutrients and endogenous phytase activity are other potential contributing factors.

It is generally assumed that the P in meat and bone meal (MBM) is highly digestible and high availability values of approximately 80% are generally used by feed formulators. The data reported in Chapter 7 suggest that this assumption is not correct. True P digestibility in the three MBM samples assessed ranged between 42 and 69%. Differences in raw material composition and processing techniques may explain the observed variability. Such variability is a major concern that needs to be considered in feed formulations.

True P digestibility of feed ingredients evaluated in the present work and the published data with pigs and broilers are presented in Table 9.3. It can be seen that the estimates varied widely depending on the species and method of estimation.

Table 9.3. Comparison of the present data with published data for true digestibility coefficients of P in feed ingredients for broilers and pigs

Ingredient	Reference	Species	Method	Site	True P digestibility coefficient
Maize	Chapter 5	broilers	regression	Ileal	0.676
	Chapter 5	broilers	regression	Total tract	0.632
	Shen *et al.* (2002)	pigs	regression	Ileal	0.539
	Shen *et al.* (2002)	pigs	regression	Total tract	0.598
	Fang *et al.* (2007a)	pigs	substitution	Total tract	0.403
	Almeida and Stein (2010)	pigs	direct	Total tract	0.264
	Rojas *et al.* (2013)	pigs	direct	Total tract	0.425
Canola meal	Chapter 5	broilers	regression	Ileal	0.469
	Chapter 5	broilers	regression	Total tract	0.486
	Adeola (unpublished)[1]	broilers	regression	Ileal	0.66
	Adeola (unpublished)[1]	broilers	regression	Total tract	0.39
	Akinmusire and Adeola (2009)	pigs	regression	Total tract	0.343
Wheat	Chapter 6	broilers	regression	Ileal	0.464
Sorghum	Chapter 6	broilers	regression	Ileal	0.331
	Fang *et al.* (2007a)	pigs	substitution	Total tract	0.423

Table 9.3. *Cont.….*

Ingredient	Reference	Species	Method	Site	True P digestibility coefficient
Soybean meal	Chapter 6	broilers	regression	Ileal	0.798
	Dilger and Adeola (2006b)	broilers	regression	Ileal	0.939 and 0.938
	Dilger and Adeola (2006b)	broilers	regression	Total tract	0.598 and 0.769
	Liu *et al*. (2013)	broilers	regression	Ileal	0.458 to 0.708
	Liu *et al*. (2013)	broilers	regression	Total tract	0.526 to 0.583
	Fan *et al*. (2001)	pigs	regression	Ileal	0.507
	Fan *et al*. (2001)	pigs	regression	Total tract	0.485
	Ajakaiye *et al*. (2003)	pigs	regression	Ileal	0.590
	Ajakaiye *et al*. (2003)	pigs	regression	Total tract	0.513
	Dilger and Adeola (2006a)	pigs	regression	Ileal	0.438 and 0.632
	Dilger and Adeola (2006a)	pigs	regression	Total tract	0.452 and 0.621
	Fang *et al*. (2007b)	pigs	regression	Total tract	0.494
	Fang *et al*. (2007b)	pigs	substitution	Total tract	0.506
	Akinmusire and Adeola (2009)	pigs	regression	Total tract	0.409
	Almeida and Stein (2010)	pigs	direct	Total tract	0.483
DDGS[2]	Chapter 6	broilers	regression	Ileal	0.727
	Almeida and Stein (2010)	pigs	direct	Total tract	0.729
	Rojas *et al*. (2013)	pigs	direct	Total tract	0.765
	Baker *et al*. (2013)	pigs	direct	Total tract	0.631
MBM[3]	Chapter 7	broilers	regression	Ileal	0.420 to 0.693
	Sulabo and Stein (2013)	pigs	direct	Total tract	0.548 to 0.844

[1]Cited by Adeola and Applegate (2010).
[2]DDGS = distiller's dried grains with solubles.
[3]MBM = meat and bone meal.

Differences between true ileal digestibility and retention of P may occur as a result of either urinary P excretion or postileal absorption and secretion of P (Shastak *et al*., 2012). It has been reported that the physiological threshold for urinary excretion for dietary non-phytate P is 2 to 3 g/kg in 40 to 50-day old broilers (Manangi and Coon, 2006) and that dietary P levels above this threshold resulted an increased urinary P excretion. The data from Chapter 5 showed that both true P retention and digestibility estimates were similar for maize and canola meal and that, within the dietary P levels tested, urinary P excretion was not affected by homeostatic mechanisms.

9.5. Comparison of methodologies to measure true ileal P digestibility

The experiment reported in Chapter 8 compared true ileal P digestibility of maize and soybean meal determined using two methodologies based on the regression method. The two methods differed in dietary protein content and dietary Ca:total P ratios. Data showed that the method influenced the estimation of true ileal P digestibility in maize and soybean meal (Table 9.4). A wider Ca:total P ratio reduced true ileal P digestibility of both ingredients.

Table 9.4. Comparison of total P, phytate-P, non-phytate P, and true digestible P contents of maize and soybean meal as percentage (%) of total P

As % of total P	Maize	Soybean meal
Phytate P	70.8	70.0
Non-phytate P[1]	29.2	30.0
True digestible P (Method 1)[2]	72.8	74.0
True digestible P (Method 2)[3]	42.6	52.3

[1]Calculated as the difference between total P and phytate P.
[2]Ingredient supplied all the protein; Ca:non-phytate P ratio was around 2:1.
[3]Additional protein (dried egg albumen) and P (disodium phosphate) was provided; Ca:total P ratio was 1.3 (WPSA, 2013).

The higher true P digestibility estimated in Method 1 may be explained partly by the comparatively low Ca concentrations and narrow Ca:total P ratio of the test diets. A recent study by Liu *et al*. (2013) also reported that the wider Ca:total P ratios (0.8 vs. 1.2, 1.6 and 2.0) negatively influenced true P digestibility estimates in soybean meal.

The true digestible P contents determined by both methods were considerably higher than the respective non-phytate P contents in maize and soybean meal. But the utilisation of phytate-bound P was higher in Method 1 than in Method 2 (maize, 61.5 vs.

18.8%; soybean meal, 62.9 vs. 31.8%), suggesting that the degree of phytate P utilisation depends on the Ca:P ratio.

No published data are available on the influence of dietary protein content on P digestibility. Because of the confounding effects of Ca, no definite conclusion can be drawn from the current work regarding the possible effects of dietary protein content on P digestibility estimates. It is, however, possible that the formation of unavailable protein-phytate complexes (Mothes *et al*., 1990) may be responsible for part of the differences between the methodologies and this possibility needs to be explored in future studies.

9.6. Limitations of the study

When this thesis project was initiated in 2011, the only published methodology available for the measurement of true P digestibility was that of Dilger and Adeola (2006b) and hence this approach formed the basis of studies reported herein. As the project progressed, it became evident that this model has several limitations. First, all the diets used in the experiments (Chapters 5, 6, 7 and 8) were P- and Ca-deficient, leading to possible overestimation of P digestibility. It is widely accepted that the broilers utilised phytate bound P more efficiently when Ca- and P- deficient diets are fed. Such increases in P digestibility as a result of low dietary Ca (Tamim and Angel, 2003) may potentially inverse the ratio of digestible Ca to digestible P (Angel *et al*., 2014). The validity of measuring P digestibility in Ca-deficient environments therefore needs to be investigated further if progress is made towards a digestible P system.

Second, the stated advantage of the regression method, over the direct method, is to provide a direct estimation of true P indigestibility coefficient and endogenous P losses from the slope and the intercept of the regression equation, respectively. However, in the current work, the endogenous P losses for some ingredients were determined to be negative (Table 9.5). It may be argued that these negative estimates do not have any implications on P digestibility estimates as these values are not used in the calculation of true P digestibility. Although the intercept is not directly used in the calculation of true P digestibility, the present findings clearly show that it has an impact on the slope of the regression. As shown in Table 9.5, these negative estimates for endogenous P were always associated with low true P digestibility and should be considered as a possible limitation of the regression method.

Table 9.5. Endogenous phosphorus (P) losses and true ileal digestibility coefficients of P in feed ingredients determined by regression method

Chapter	Ingredient	Endogenous P losses (g/kg DMI)[1]	True ileal P digestibility coefficient[1]
5	Maize	0.020	0.676
	Canola meal	-0.464	0.469
6	Wheat	0.080	0.464
	Sorghum	-0.087	0.331
	Soybean meal	0.609	0.798
	Maize-DDGS[2]	0.418	0.727
7	Meat and bone meal[3]		
	MBM-1	0.049	0.693
	MBM-2	0.142	0.608
	MBM-3	-0.370	0.420

[1]Calculated as described in Dilger and Adeola (2006b).
[2]Maize-DDGS = maize-distiller's dried grains with solubles.
[3]Three meat and bone meal samples (MBM-1, MBM-2 and MBM-3).

A major practical limitation of the regression model is that it needs at least three to four ingredient inclusion levels with four to six replicates for each inclusion. Thus the regression method is costly, labour-intensive and time consuming compared to the direct method where only one inclusion level is tested. The regression method also limits the number of feed ingredients tested at a given time.

The analysed dietary concentrations of P in the studies described in Chapters 5, 6, 7 and 8 were in an agreement with the calculated values, but dietary Ca concentrations differed from expected in most diets especially at low dietary Ca concentrations. These differences, and their possible influence on P utilisation, may be another practical issue in P digestibility assays.

9.7. Suggestions for future research

As discussed above, the use of regression method suffers from a number of drawbacks. The use of direct method to estimate P digestibility therefore needs to be investigated. Direct method will not require the investment, in terms number of treatments and birds, as for the regression method. Given the considerable variation that exists in P digestibility within a given ingredient, across different ingredients or introduced by factors such as species, age, feed processing, additives, nutrient density and ratios *etc*., the method that gives opportunity for relatively high throughput will be valuable. Compared with the undigested dietary P fraction, the fraction of endogenous P losses

that contribute to the total P output on DMI basis is negligible. Thus it may be argued the correction of basal endogenous P losses to calculate true P digestibility may not be required and that apparent digestibility values can be directly considered as P digestibility estimates.

True P digestibility values are preferable over apparent values as they are unaffected by dietary P content (Ajakaiye *et al*., 2003). The additivity of true or apparent P digestibility values of feed ingredients in pig diets has been evaluated (Fang *et al*., 2007a). Future research is warranted to determine the additive effect of true digestible P contents determined for individual feed ingredients in compound poultry diets.

Determination of the true P digestibility of inorganic phosphates is urgently needed, since they supply significant amounts of P in poultry diets. Studies evaluating the true ileal P digestibility of mineral supplements in broiler chickens are limited (Shastak *et al*., 2012). A suitable methodology needs to be established for inorganic phosphates through appropriate modifications to the composition of assay diets.

The various factors that may influence true ileal P digestibility in broilers were not considered in the current work. The ability of poultry to digest and absorb nutrients is known to be influenced by age (Batal and Parsons, 2002; Huang *et al*., 2005). Studies investigating the effect of age on phytate P retention for broiler chickens are also available (Nelson, 1976; Matyka *et al*., 1990), but the results are contradictory. The effect of age on ileal P digestibility of feed ingredients for broilers has not been examined thus far. Research has shown that the development and the function of digestive tract increase with advancing age (Batal and Parsons, 2002). Since the P requirement is age dependent, it is of interest to study how true ileal P digestibility of individual feed ingredients change with advancing age.

The effect of bird type (layer vs. broilers) on the true ileal P digestibility also warrants investigation. Edwards (1983) reported that the layer type birds utilise phytate P more efficiently than meat type birds.

It is well known that microbial phytase supplementation improves the apparent ileal P digestibility in feed mixtures for broilers (Ravindran *et al*., 2000; Rutherfurd *et al*., 2004). Given that the addition of microbial phytase is currently routine in poultry diets and since the effect of phytase is diet-dependent, measurement of true P digestibility with a background of microbial phytase is of practical relevance. Only one study has investigated the effect of microbial phytase supplementation on true ileal P

digestibility of feed ingredients thus far (Iyayi *et al*., 2013). Using the regression approach, these researchers found that dietary phytase supplementation (1,000 U/kg) improved the true P digestibility in black-eyed pea and peanut flour.

The effect of feed form (mash vs. pellet) on apparent nutrient utilisation depends on the type of feed ingredient and the nutrient concerned (Abdollahi *et al*., 2013). Studies investigated the effect of feed form on P utilisation in cereal-based diets for broilers are limited and the results are contradictory (Edwards *et al*., 1999; Kilburn and Edwards, 2001; Abdollahi *et al*., 2013). As shown by Abdollahi *et al*. (2013), pelleting increased the apparent ileal P digestibility in broiler starters fed maize-based diets, but not the wheat-based diets. No studies thus far have explored the effect of feed form on true ileal P digestibly of feed ingredients for broilers.

Measurement of retainable P as an alternative to digestible P in poultry feed ingredients need to be explored as the values can be obtained without scarifying the birds. But the urinary excretion must be considered. If the birds are fed below the non-phytate P requirement, then the urinary P excretion will be negligible and retainable P values will be expected to be similar to ileal digestibility estimates. The data reported in Chapter 5 demonstrated that both true P retention and true ileal P digestibility can be used for evaluating feed ingredients in broilers.

9.8. Summary and main conclusions

The main focus of the work presented in this thesis was to evaluate the regression approach to determine the true ileal P digestibility of some common feed ingredients for broiler chickens. True ileal P digestibility of seven ingredients were estimated and, within each ingredient, the determined true digestible P content was compared with the widely-used non-phytate P content. In addition, digestion of P and Ca along the intestinal tract of young broilers and the effects of dietary Ca concentration on the digestibility of various nutrients were investigated. Preliminary data were also obtained on the ileal endogenous P losses in broiler chickens.

The data showed that the dietary Ca concentration influenced the digestion of P, nitrogen and fat, but had no impact on the digestibility of Ca, starch and dietary AME. It is suggested that the utilisation of those nutrients affected by high dietary Ca concentrations can be optimised by maintaining the dietary Ca concentration as low as realistically as possible. Jejunum was the major site of nutrient digestion in broiler chickens. Increasing dietary Ca concentration, however, was observed to shift P and N

digestion to both the jejunum and upper ileum. Digestion of P was completed by the upper ileum, suggesting that in P digestibility assays, lower ileum can be used as the site of sample collection regardless of the dietary Ca concentration.

Endogenous P losses in broiler chickens were found to be influenced by the composition of the assay diet. Extremely low dietary Ca concentrations, and the presence or absence of dietary protein impacted the estimates. Presence of protein resulted in higher ileal endogenous P losses.

In the present work, the regression method was used to measure the true ileal P digestibility of several feed ingredients. However, the data highlighted several limitations of the regression method, including possible over-estimation of P digestibility due to the low Ca and P contents in the assay diets and negative endogenous P losses observed for some feed ingredients. These negative endogenous losses were associated with low P digestibilities.

Assay conditions in regression methodology was found to influence the estimates of true ileal P digestibility in maize and soybean meal, with lower dietary Ca contents and narrower Ca:total P ratios increasing the digestibility. Whether macronutrients such as protein and fat will influence P digestibility is not known and this needs to be investigated in future studies.

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APPENDICES

Appendix A. Determination of P

Phosphorus contents of diets, digesta and excreta samples were determined as follows. Samples were prepared for P determination as described in AOAC international (2005; method no: 968.08D).

1. Sample preparation:

1. One g of sample was accurately weighed into a beaker (tarred for beaker weight) in triplicate.
2. The beakers are placed in a convection oven at 105˚C overnight (16 hours).
3. The beakers were removed from the oven and cooled in a desiccator.
4. The weights of the dry sample + beaker were recorded.
5. The samples and beakers were placed in a muffle furnace at 550˚C over night.
6. Samples were removed from the muffle furnace and cooled in a desiccator.
7. The beakers with ash were weighed to record the weight.
8. Ten millilitres of 6M hydrochloric acid (HCl) was added to each beaker, covered with a glass plate and digested for 20 minutes in a heating block pre-heated to 250˚C.
9. The beakers were removed from the heating block followed by cooling.
10. The digested samples were washed into a 25 ml volumetric flask with Milli-Q water.
11. The volume was made up to 25 ml, with Milli-Q water.

2. Colourimetric step:

1. Ten millilitres of P stock standard solution (0.1 mg/ml P) was pipetted into a 100 ml volumetric flask and made the volume up to 100 ml with Milli-Q water.
2. This solution was used to prepare a series of solutions to be used in standard curve as follows.

Standard solution (ml)	Milli-Q water (ml)
0.00	10.75
0.50	10.25
1.25	9.5
2.5	8.25
3.75	7.00
5.00	5.75
10.00	0.75

3. From the samples prepared for colourimetric step, 0.25 ml from each was pipetted into a labled test tube. To each test tube 10.5 ml of Milli-Q water was added.
4. To each sample and standard solutions in test tubes, 1.25 ml of ammonium molybdate solution was pipetted followed by pipetting of 0.5 ml of amino-napthol sulphonic acid reagent.
5. Test tubes were capped, mixed in vortex and stand left for 20 minutes.
6. The sample solutions were poured into cuvettes (2 ml) and the absorbance of each was measured at 680 nm by a spectrophotometer.

3. Calculation:

Standard curve was drawn and the P concentration in the measured solution was calculated by linear regression. Percentage of P was calculated as:

% P = [C x V x DF)/(W x 10)

Where,

C = concentration P in measured solution (mg/l)

V = volume of solution (l)

DF = dilution factor

W = weight of the sample (g), and

10 = factor to convert g/kg to %.

Appendix B. Determination of Ca

Calcium contents of diets, digesta and excreta samples were determined as follows. Samples were prepared for Ca determination as described in AOAC international (2005; method no: 968.08D) and FAO (2011) and the Ca contents were determined by colorimetric assay using o-cresolpthalein complexone (CPC) to develop colour (Ravindran, 2009).

1. Sample preparation:

1. One g of sample was accurately weighed into a beaker (tarred for beaker weight).
2. The beakers were placed in a muffle furnace at 550˚C over night (16 hours).
3. Samples were removed from the muffle furnace and cooled in a desiccator.
4. Ten millilitres of 6M HCl was added to each beaker, covered with a glass plate and digested for 20 minutes in a heating block pre-heated to 250˚C.
5. The beakers were removed from the heating block followed by cooling.
6. The digested samples were washed into a 25 ml volumetric flask with Milli-Q water.
7. The volume was made up to 25 ml, with Milli-Q water.

2. **Colourimetric step:**

1. Calcium carbonate ($CaCo_3$) 5 mmol standard solution (0.5mg/ml) was prepared and worked out to produce a series of dilutions of 5.0, 4.0, 3.0, 2.0 and 1.0 mmol/l standards.
2. To 20µl of each sample, blank and standards, 0.65 ml of ethanolamine buffer (1 mol/l, pH 10.6) at room temperature was pipetted and mixed for 25 seconds.
3. To this, 0.250 ml of Chromogen, containing o-cresolpthalein complexone (0.3 mmol/l)/l;8-hydroxyquinoline (13.8 mmol/l) and HCl (122 mmol/l) was pipetted, mixed by vortex and left atleast for 2 minutes.
4. The absorbance was read at 578 nm, against the blank.
5. Milligrams of Ca^{2+}/100 ml of each standard solution were calculated by below formula.

$$\text{mg } Ca^{2+}/100 \text{ ml} = [C \times 40.08 \times 1000]/ 100.1$$

Where,

C = weight of $CaCo_3$ in grams

40.08 = atomic mass of Ca

100.1 = formula mass of $CaCo_3$

Therefore, for the highest standard solution the Ca^{2+} concentration should be 20 mg/100ml.

6. Plotted the standard curve using absorbance (nm) vs. Ca^{2+}concentration (mg/l) of series of standard solutions.

3. Calculation:

The Ca^{2+} concentration of the samples were calculated by the following formula:

% Ca = [C x V x DF)/(W x 10)

Where,

C = concentration Ca in measured solution (mg/l)

V = volume of solution (l)

DF = dilution factor

W = weight of the sample (g), and

10 = factor to convert g/kg to %.

Appendix C. Determination of phytate P content in feed ingredients

Phytate P contents were determined using Megazyme kit (K-PHYT, Megazyme, Bray, Ireland).

1. The standard assay procedure:

1. One g of sample was accurately weighed into a 75 ml glass beaker in triplicate.
2. Each sample was extracted with 20 ml of HCl (0.66 M), covered the beaker with foil and left constantly stirred overnight.
3. An aliquot (1 ml) of extract was transferred to a 1.5 ml microfuge tube and centrifuged at 13,000 rpm for 10 minutes.
4. The resulting extract supernatant (0.5 ml) was immediately transferred to fresh 1.5 ml microfuge tube and was neutralised by adding 0.5 ml of sodium hydroxide solution (0.75 M).
5. To 0.05 ml of the neutralised extract, 0.60 ml distilled water was added followed by addition of 0.20 ml of buffer (pH 5.5) and 0.02 ml of phytase suspension (12,000 U/ml).
6. The mixture was mixed by vortex and incubated in a water bath set at 40 ˚C for 10 minutes.
7. To the incubated mixture, 0.20 ml of buffer (pH 10.4) and 0.02 ml of alkaline phosphate suspension (80 U/ml) were added.
8. The mixture was mixed by vortex and incubated in a water bath set at 40˚C for 15 minutes and the reaction was terminated by the addition of 0.3 ml of Trichloroacetic acid (50% w/v).
9. The contents were centrifuged at 13,000 rpm for 10 minutes and to 1 ml of the carefully pipetted supernatant, 0.5 ml of colour reagent [5 parts of ascorbic acid (10% w/v) in 1M sulphuric acid, and 1 part of 5% w/v ammonium molybdate) was added, mixed by vortex and incubated in a water bath set at 40˚C for 1 hour.
10. After 1 hour, mixed by vortex and transferred 1 ml to a semi-microcuvette and read the absorbance at 655 nm within 3 hours against to a series of P standard solutions prepared (ranging from 0 µg to 7.5 µg/ml P) from the P standard solution provided (50 µg/ml) to determine the total P content.
11. Simultaneously, the free P was determined by mixing 0.05 ml of neutralised extract with 0.20 ml of buffer (pH 5.5) and 0.62 ml of distilled water, mixed by vortex and followed by incubating at 40˚C for 10 minutes.

12. After 10 minutes, the next reaction was started by addition of 0.02 ml distilled water and 0.20 ml of buffer (pH 10.4).
13. The mixture was mixed by vortex and incubated in a water bath set at 40˚C for 15 minutes followed by the addition of 0.30 ml Trichloroacetic acid (50% w/v) to terminate the reaction.
14. Centrifuged the content at 13,000 rpm for 10 minutes and carefully pipetted the resulted 1 ml of supernatant, to which 0.5 ml of colour reagent was added and the absorbance was measured at 655 nm.
15. With parallel measuring absorbance of total P and free P, the absorbance for the standard was recorded using the standard solution supplied along with the kit.

2. Calculation:

1. The absorbance (A_{655}) of each P standard (STD 1 to 4) was determined.
2. The absorbance of STD 1 to 4 ($\Delta A_{phosphorus}$) was determined by subtracting the absorbance of STD 0 from the absorbance of other standards (STD 1 to 4).
3. For each standard (STD 1 to 4) M was calculated as follows.

$$M = [P\ (\mu g)/\Delta A_{phosphorus}] \qquad [\mu g/\Delta A_{phosphorus}]$$

4. The mean M was calculated by the formula mentioned below and resulted "Mean M" was used to calculate the P content of the test samples.

$$\text{Mean M} = [(M_{STD1} + M_{STD2} + M_{STD3} + M_{STD4})/4] \qquad [\mu g/\Delta A_{phosphorus}]$$

5. The absorbance (A655) was determined for both the "Free P" and "Total P" samples and the absorbance of the "free P"' sample was subtracted from the absorbance of the "Total P" sample to obtain ($\Delta A_{phosphorus}$).

6. The concentration of phytate P can be calculated as follows:

$$c = [(\text{mean M} \times 20 \times F)/(10{,}000 \times 1.0 \times v)] \times \Delta A_{phosphorus} \qquad [g/100\ g]$$

Where,

Mean M = mean value of P standards [$\mu g/\Delta A_{phosphorus}$]

20 = original sample extract volume [ml]

F = dilution factor (55.6)

$\Delta A_{phosphorus}$ = absorbance change of sample

10,000 = conversion from μg/g to g/100 g

1.0 = weight of original sample material [g]

v = sample volume used in the colourimetric determination step (1 ml)

3. Modified sample extraction:

a) Samples with A_{655} above that of STD 4, the standard assay procedures were repeated using 1 g of sample material in 100 ml of HCl (0.66 M). In this occasion the original sample extract volume (ml) became 100 ml.

$c = [\text{mean M} \times 100 \times 55.6/10{,}000 \times 1.0 \times 1.0] \times \Delta A_{phosphorus}$ [g/100g]

b) Samples with A_{655} below 0.100 absorbance unit, the standard assay procedures were repeated using the appropriate amount of sample material (2.5 g if A_{655} generated phytic acid content is 0.05 to 0.10 g/L).

$c = [\text{mean M} \times 20 \times 55.6/10{,}000 \times 2.5 \times 1.0] \times \Delta A_{phosphorus}$ [g/100g]

Appendix D. Statement of contribution to doctoral thesis containing publications

DRC 16

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Ruvini Kamalika Mutucumarana

Name/Title of Principal Supervisor: Professor V. Ravindran

Name of Published Research Output and full reference:

Mutucumarana R., Ravindran V., Ravindran G., Cowieson A. (2014) Measurement of true ileal digestibility of phosphorus in some feed ingredients for broiler chickens. Journal of Animal Science 92:5520-5529.

In which Chapter is the Published Work: Chapter 6

Please indicate either:

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Mutucumarana R., Ravindran V., Ravindran G., Cowieson A. (2014) Influence of dietary calcium concentration on the digestion of nutrients along the intestinal tract of broiler chickens. Journal of Poultry Science 51:392-401.

In which Chapter is the Published Work: Chapter 3

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We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Ruvini Kamalika Mutucumarana

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Name of Published Research Output and full reference:

Mutucumarana R., Ravindran V., Ravindran G., Cowieson A. (2014) Measurement of true ileal digestibility and total tract retention of phosphorus in corn and canola meal for broiler chickens. Poultry Science 93:412-419.

In which Chapter is the Published Work: Chapter 5

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