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Effects of Dietary Choline, Folic acid and Vitamin B_{12} on Laying Hen Performance, Egg Components and Egg Phospholipid Composition

By

Pradeep Krishnan Rajalekshmy

A DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Animal Science

Under the Supervision of Professor Sheila E. Scheideler

Lincoln, Nebraska

November, 2010

Effects of Dietary Choline, Folic acid and Vitamin B_{12} on Laying Hen Performance, Egg Components and Egg Phospholipid Composition

Pradeep Krishnan, Ph.D.

University of Nebraska, 2010

Advisor: Sheila E. Scheideler

In trial 1, a corn-soy basal diet was formulated with three levels of supplemental choline (0, 500 and 1000 ppm) and three levels of supplemental folic acid (0, 2 and 4 ppm) in a 3 x 3 factorial arrangement. Folic acid at 2 ppm increased egg production ($p \leq 0.04$). Egg wt was higher at 500 ppm of choline ($p \leq 0.06$) and 0 ppm ($p \leq 0.01$) of folic acid supplementation. There were choline by folic acid interaction effects on feed intake ($p \leq 0.001$), albumen wt ($p \leq 0.005$) and yolk wt ($p \leq 0.03$). Plasma folate and egg folate showed an increase ($p \leq 0.0001$) with added levels of dietary folic acid. Phosphatidylcholine (PC) concentration showed a trend to increase with higher levels of choline and folic acid supplementation. In trial 2, a corn-soy basal diet was formulated with 2 levels supplemental choline (500 and 1000 ppm), 2 levels supplemental folic acid (2 and 4 ppm), and 2 levels supplemental vitamin B_{12} (0.01 and 0.02 ppm) in a 2 x 2 x 2 factorial arrangement along with a control (no supplementation) group. Yolk wt showed choline x vitamin B_{12} interaction ($p \leq 0.001$). Phosphatidylcholine (PC) showed an increase ($p \leq 0.0001$) with added levels of choline, folic acid or vitamin B_{12}. The average value of PC at 500 and 1000 ppm of choline was 152.61 and 164.53 mg/g, respectively. Similarly, the average values for PC at 2 and 4 ppm of folic acid and 0.01 and 0.02 ppm of vitamin B_{12} were 153.06, 164.07, 155.68 and 161.46 mg/g, respectively. Results indicate that choline, folic acid and vitamin B_{12} can be

supplemented in a synergistic combination to increase egg yolk phosphatidylcholine content by 20 to 25 % compared to no supplementation.

Key words: Choline, Folic acid, Vitamin $B_{12,}$ Egg phosphatidylcholine

Dedication

This dissertation is dedicated to my wonderful family, who have raised me to be the person I am today. Thank you for all the unconditional love, guidance, and support that you have always given me, helping me to succeed. I also thank my family members in educating me that even the greatest assignment can be accomplished if it is done one step at a time. Love you all.

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Most importantly, none of this would have been possible without the love and patience of my family. My immediate family, to whom this dissertation is dedicated to, has been a constant source of love, concern, support and strength all these years. I would like to express my heart-felt gratitude to my family.

Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.

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CHAPTER-1

1. INTRODUCTION AND LITERATURE REVIEW:

A. CHOLINE: Choline, which is trimethyl, β-hydroxy ethyl ammonium (Sheard and Zeisel, 1989), is an organic compound which is thick, colorless and strongly alkaline with a molecular weight of 121.18. Choline is considered as an essential water soluble nutrient and is usually grouped within the B-complex family. It is chemically unique as it has three methyl group attached to the structure and takes part in various functions in the body as a methyl group's donor. Choline is a quaternary ammonium compound found in lipids that make up cell membrane and in the neurotransmitter acetylcholine with the structure:

$$CH_3$$

$$|$$

$$CH_3 - N^+ - CH_2 - CH_2OH$$

$$|$$

$$CH_3$$

Figure **1.1** Structure of Choline

(McDowell, 1989)

Despite being considered as one of the B- complex vitamins, choline does not answer the classical definition of a vitamin. It is required in relatively high amounts and does not have any coenzyme function. In biological tissues, most of the choline is present as bound form and a small proportion exists as free choline (Vitamin Tolerance of Animals, 1987). The common forms include phosphorylcholine, phosphatidylcholine (lecithin) and acetylcholine. Choline is also metabolically connected with glycine, betaine, cystine, serine, methionine and many other methyl containing biological compounds (Rose et al., 1952). It is a dietary constituent essential for the normal functioning of the cells.

Choline has three essential metabolic functions (Chan, 1984) which cannot be substituted by other methyl donors in the system which include;

(1) Structural components of cell membranes as a constituent of phospholipids (phosphatidylcholine) and thereby playing an essential role in the building and maintenance of cell structure
(2) Lipotropic agent in fat metabolism in liver by utilizing and transporting fat and thereby preventing fatty liver
(3) Precursor for acetylcholine synthesis, the neurotransmitter agent for nerve impulses

Choline also plays a non essential metabolic role as a source of labile methyl groups.

1. HISTORY

Choline was first isolated from pig bile by Strecker in 1862. Strecker referred to the substance obtained from bile as choline. Later choline was isolated from brain tissue and further it was found that presence of choline is not only limited to liver and brain but is present in almost every cell. The nutritional importance of choline was not known until the 1930's, following the discovery of insulin by Banting and Best (1922) and is now a dietary supplement for both animals and humans. Hershey (1931) observed that fatty degeneration of the liver associated with insulin deprivation in dogs could be corrected by feeding not only raw pancreas, but also lecithin. The lipotropic effect of choline in rats fed added cholesterol was observed by Best et al. (1932) wherein crude and purified lecithin was shown to influence the accumulation of fat in the liver. It was later reported by the same authors that choline was the active component in lecithin.

Aoyama et al. (1971) showed that protein supplementation (casein) in rats also protected against fatty liver. During early 1940's, many studies were carried out wherein supplementation of homocysteine to diets deficient in choline was shown to have lipotropic effects and the lipotropic action was thought to be due to the transfer of methyl groups during the process of transmethylation. The biological synthesis of labile methyl groups was studied by Du Vigneaud et al. (1940), with labeled methyl groups in rats and showed the presence of labeled choline methyl groups derived from sulfur methyl groups. The methyl groups derived from sulfur in turn originated from methionine in the diet. They also showed the evidence of the use of labeled methyl groups for the formation of choline and other methyl containing compounds.

The nutritional requirements for choline in rats and chickens were established during the 1950's and it was shown that nutrients such as folic acid and vitamin B_{12} reduce the requirement of choline (Welch and Couch, 1955). Choline has sparing effects on other dietary methyl donors like methionine, folic acid and vitamin B_{12}. The requirement for choline in the diet depends on the availability of these nutrients. The relationship and the sparing effect of choline with these nutrients were studied in the past because of the supplementation cost of these nutrients (Schaefer and Knowles, 1951). It was concluded that the specific requirement for one of these nutrients cannot be established unless the level of other nutrients are taken into consideration.

2. CHOLINE FUNCTIONS:

The majority of choline in biological tissues is found in phospholipids, the most common of which is phosphatidylcholine (PC) or lecithin. Other important metabolites of choline include phosphatidylethanolamine (PE) and sphingomyelin (Zeisel, 1999).

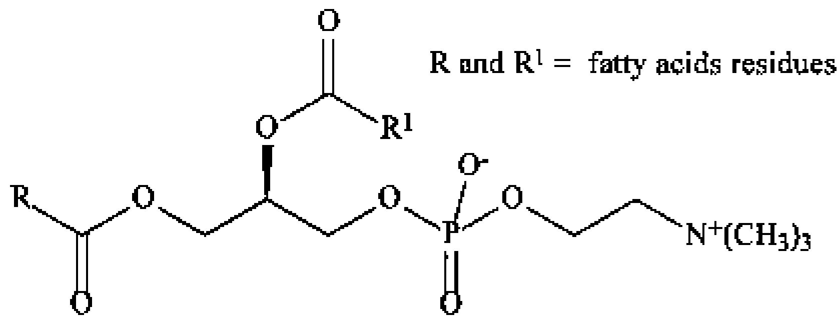

Figure **1.2** Phosphatidylcholine

Adapted from online source (mesotherapyworldwide.com)

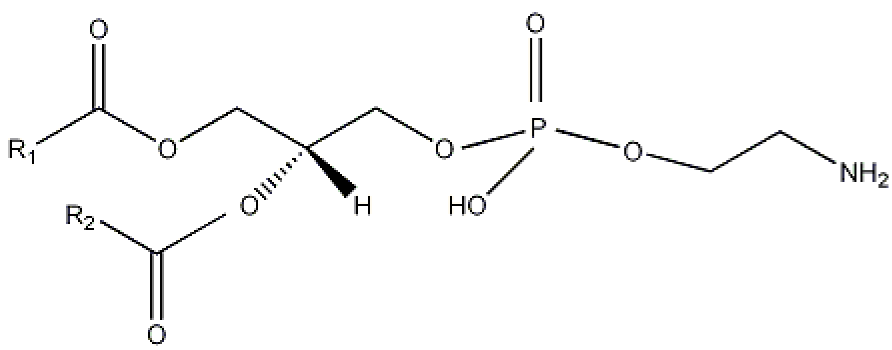

Figure **1.3** Phosphatidylethanolamine

Adapted from online source (http://www.lipidomicnet.org)

Choline and its metabolites play an important role in several biological functions and the four most important functions are as follows:

2.1 Component of Plasma Membranes

Choline is essential for building and maintaining the structure of the cell. Phospholipids are present in the cell membrane bilayers, and the primary function of these phospholipids is to regulate cell membrane integrity and porosity. Phospholipids also functions to hold protein and carbohydrate fractions to the cell membrane. The major portion (28-30%) of the egg lipids is made up of phospholipids (Privett et al., 1962). About 80% of the egg phospholipid is in the form of phosphatidylcholine (PC), and small amounts of phosphatidylethanolamine (PE), lysophosphatidyl ethanolamine and sphingomyelin are also present (Garland and Powrie, 1978). In the cell membrane, phosphatidylcholine and phosphatidylethanolamine account for nearly 35% of the phospholipid mass and approximately 20% exists as sphingomyelin and the rest as phosphatidylinositol (PI) and phosphatidylserine (PS) (Battaglia and Schimmel, 1997). There is a difference in the amount of PC, PE and sphingomyelin present in the cell membrane of different species, with the rat, pig and chicken having higher proportion of PC and PE as compared to sphingomyelin. In ruminant species (cattle and sheep), the proportion of sphingomyelin is higher compared to that in other species (Ansell et al., 1973). The phospholipids and total fatty acids present are affected by nutritional state and the type of fatty acids present in the diet (Field and Clandinin, 1984).

2.2 Fat metabolism

Dietary choline was found to prevent the incidence of fatty liver in depancreatized dogs (Hershey and Soskin, 1931) and later in rats, chicks and other species. The primary carrier of lipids in the blood is lipoprotein and phosphatidylcholine is an important component of these lipoproteins. A study carried out by Jacobs et al. (2004) showed higher concentration of triglyceride in the liver when a choline deficient diet was fed to mice. An increase in the triglyceride in liver reflected a decrease in circulating triglyceride and an increase in serum fatty acids. This signifies the importance of choline as a lipotropic agent in the transport of lipid components from the liver. The exact cause of fatty liver in choline deficiency is not well understood. It has been shown that accumulation of lipids in the liver of choline and methyl group deficient rats is due to the decreased formation of low density lipoproteins resulting from impairment in the synthesis of apolipoprotein or due to the prevention of the attachment of triglycerides to apolipoprotein. This will prevent the release of triglycerides into the circulation (Mookerjea, 1971).

2.3 Nerve impulse transmission

Choline functions as a precursor of the neurotransmitter acetylcholine (Blusztajn and Wurtman, 1983). Acetylcholine is synthesized by mitochondria at the presynaptic terminal of the neural synapse. The transmission of acetylcholine stimulates nerve impulse in the neuron. Acetylcholine is the most common neurotransmitter in the nervous system. The inability of the brain to synthesize adequate choline for neural function was shown by (Ansell and Spanner, 1971) and it is believed that circulating free choline is one of the sources of choline for the synthesis of acetylcholine (Freeman and Jenden, 1976).

2.4 Methyl donor

Another metabolic function of choline is as a methyl donor. These labile methyl groups play an important role in many biological functions.

2.4.1 Synthesis of Choline and Lecithin

Synthesis of choline, methionine and other methyl donors and their interconversion involves complex biochemical processes. Nutritional interrelationships between choline, betaine, serine, vitamin B_{12} and folic acid are not well understood. Lecithin can be synthesized in animal tissues primarily through two pathways. The supply of dietary choline and other methyl donors determine the relative significance of each of these pathways. All species of animals are capable of synthesizing choline in the liver. The first pathway begins with phosphatidylethanolamine as (PtdEtn) in (Figure 1.4), which receives methyl groups that are supplied by S-adenosylmethionine (AdoMet) to form lecithin (phosphatidylcholine).

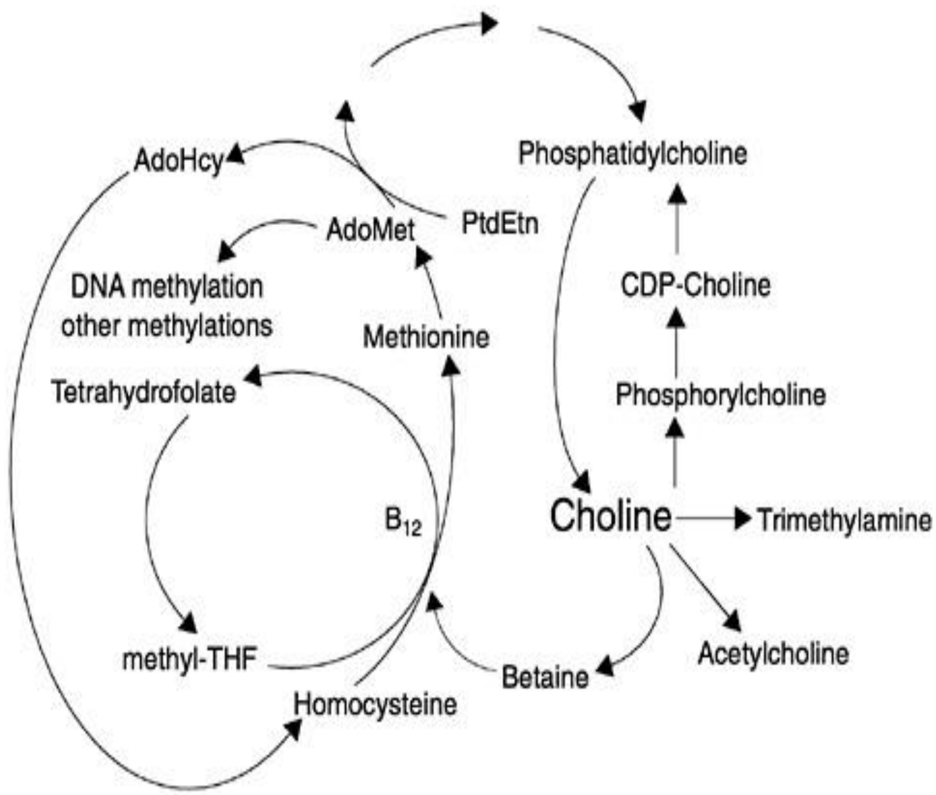

Figure **1.4** Interrelationship of Choline, folate and methionine metabolism

(Zeisel and Blusztajn, 1994)

In the second pathway, where dietary choline is abundant, choline is phosphorylated to form phosphorylcholine and then converted to cytidine diphosphocholine (CDP-Choline). This CDP-Choline combines with diacylglycerol to form phosphatidylcholine (Figure 1.5). In animals, the PE needed for the synthesis of choline is synthesized by decarboxylation of phosphatidylserine (Figure 1.6).

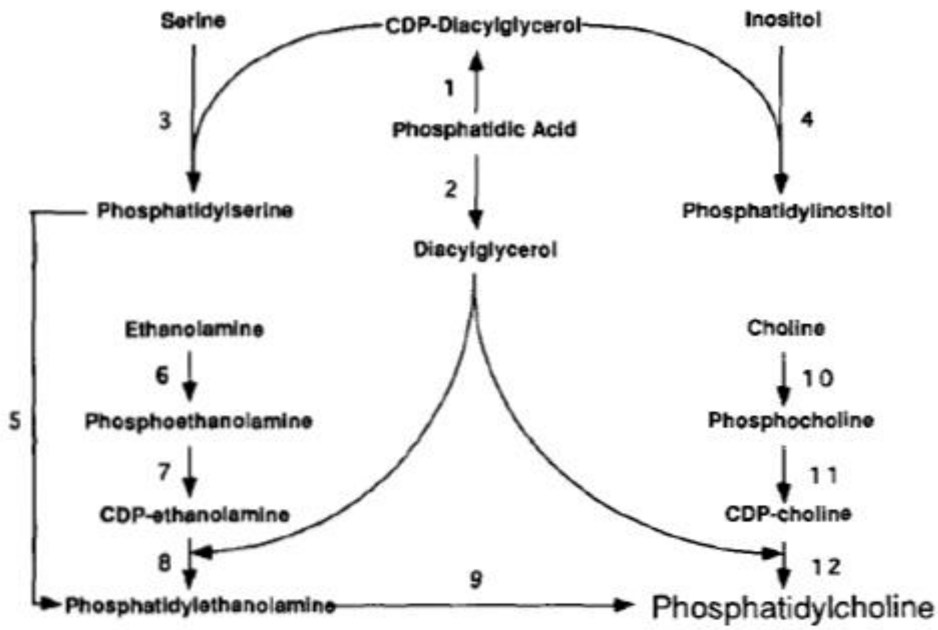

Figure **1.5** CDP choline pathway

(McMaster and Bell, 1994)

2.4.2 Transmethylation:

Choline is considered a potential source of labile methyl groups along with methionine and others. It is metabolically related to other methyl donors and is thus involved in the generation and use of active methyl groups. Choline is not directly involved in the methyl transfer reactions, and first must be oxidized to betaine by the enzyme choline oxidase (du Vigneaud et al., 1946). Transmethylation involves a series of reactions. At first methionine is formed by the transfer of a methyl group from betaine to homocysteine. Methionine is then adenylated to form Adenosylmethionine. The last step involves the transfer of methyl groups from adenosylmethionine to phosphatidylethanolamine to form phosphatidylcholine. This results in

Adenosylhomocysteine which is then converted to homocysteine for another methyl transfer reaction (Figure 1.6).

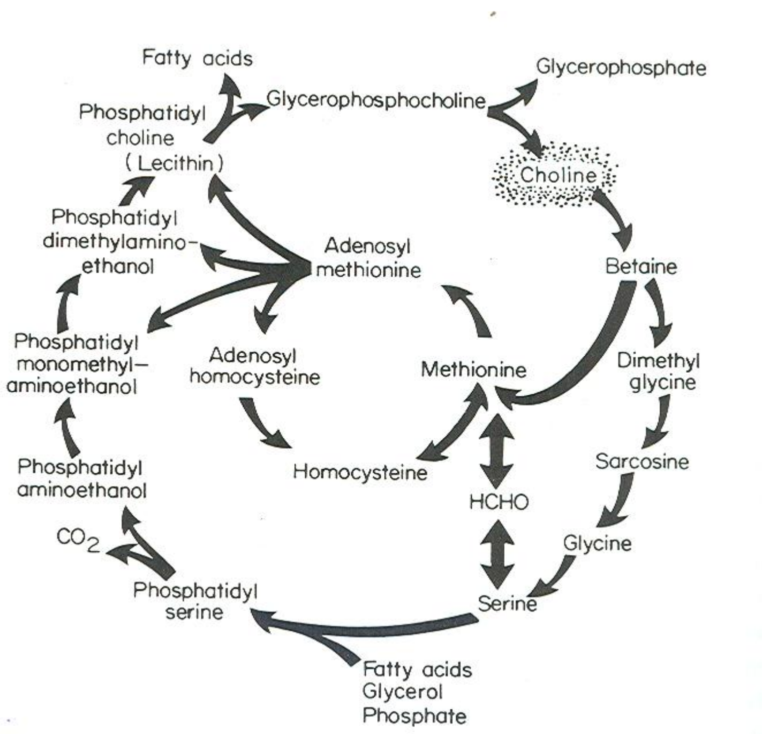

Figure **1.6** Methyl donor pathway for choline synthesis.

(Adapted from Scott et al., 1982)

2.4.3 Synthesis of Methyl group:

Transmethylation is the most common pathway in the biosynthesis of choline and methionine. Schaefer et al. (1950) reported that synthesis of choline and methionine can also occur if dietary methyl groups are available and if folic acid, vitamin B_{12} and homocysteine are also present. The methyl groups are derived from formate (HCHO), which is produced from demethylation of dimethylglycine, sarcosine and glycine (Figure 1.6). Methylene-tetrahydrafolate formed from formate carbon is reduced to form methyl tetrahydrofolate, which serves as the source of methyl groups for methionine synthesis from homocysteine (Niculescu and Zeisel, 2002). Vitamin B_{12} plays an important role in methyl group synthesis as an intermediate in the conversion of methyl tetrahydrofolate to methionine (Vitamin Tolerance of Animals, 1987). Kerwar et al. (1966) reported that methyl cobalamine may be a cofactor of the methyl transferase enzyme which is related to methionine synthesis from methyl tetrahydrofolate and homocysteine.

3. DEGRADATION OF CHOLINE:

Demethylation of choline is a process that cannot be turned around. Choline is degraded both in kidney and liver resulting in the end products of formate, ammonia and CO_2. Trimethylamine, which gives a fishy odor, is also a major end product of the dietary choline degradation (De La Huerga and Popper, 1952).

4. FEED CHOLINE CONTENT:

Choline is found in the form of PC (lecithin) or sphingomyelin in most feedstuffs and food sources (Neill et al., 1978). In general, most cereal grains and fruits and vegetables with high starch content are low in choline content. Specifically, corn and corn by-products are very low in choline. Oil seeds such as peanuts, cottonseed and soybeans are good sources of choline (Engel, 1943) because of their relatively high phospholipid content.

5. DIETARY CHOLINE REQUIREMENT ON PERFORMANCE AND EGG QUALITY:

A number of factors may influence a hen’s requirement for choline, such as age, feed intake and dietary crude protein or methionine levels (Mookerjea, 1971). The first limiting amino acid in laying hens for egg production is methionine and, given the common function with choline in methyl group donation, interactions between the two nutrients may be anticipated.

Table 1.1 NRC requirements for choline

Species	**Diet**	**Requirement**
Broiler		
0-3 weeks	Cereal grain basal diet	1300 mg/kg of diet
3-6 weeks	Cereal grain basal diet	1000 mg/kg of diet
6-8 weeks	Cereal grain basal diet	750 mg/kg of diet
Laying Hen		
0-6 weeks	Cereal grain basal diet	1300 mg/kg of diet
6-12 weeks	Cereal grain basal diet	900 mg/kg of diet
12-18 weeks	Cereal grain basal diet	500 mg/kg of diet
Production cycle	Cereal grain basal diet	105 mg/hen/day
Turkey		
0-4 weeks	Cereal grain basal diet	1600 mg/kg of diet
4-8 weeks	Cereal grain basal diet	1400 mg/kg of diet
8-16 weeks	Cereal grain basal diet	1100 mg/kg of diet
16-20 weeks	Cereal grain basal diet	950 mg/kg of diet
20-24 weeks	Cereal grain basal diet	800 mg/kg of diet
Breeding	Cereal grain basal diet	800-1000 mg/kg of diet

The metabolic requirement for choline can be met in two ways; either by choline in the diet or by choline synthesis in the body through labile methyl groups. Clinical signs of deficiency occur when synthesis cannot take place at a rate to meet choline requirement for rapid growth/ production. Symptoms of choline deficiency include reduced growth, fatty liver and perosis in chicks (McDowell, 1989). Choline deficiency is also thought to be the factor responsible for fatty liver hemorrhagic syndrome (McMullin, 2004) in laying hens, because of consumption of excess energy. When laying hens are fed diets containing high levels of dietary energy the hens tend to deposit excess energy as fat deposits in their bodies, especially the liver. The condition is most often seen in birds that appear to be healthy and in a state of high egg production. Non-laying hens will not eat as much of the high-energy feed and therefore are not affected as much as high producing hens. Symptoms include fatty and hemorrhagic liver, reduced egg production and excessive abdominal fat.

Choline requirements (Table 1.1) have been recommended for different species of poultry at different stages of production (NRC, 1994). In general, the requirement is higher for young growing birds than for older replacement pullets or laying birds. Growth requirements are based primarily on dose response studies where a fixed level of methionine, folic acid and vitamin B_{12} are fed, and graded levels of choline are added to the diet (Schaefer et al., 1950). The current NRC recommendation (1994) for choline intake in laying hen is 1040 mg/kg at feed intake levels of 100 g/hen/day, which will provide approximately 105 mg of choline/hen/day. Requirements of choline have generally been determined through the use of purified diets and often do not take into account the bioavailability from feedstuffs or effects of other dietary factors.

The need for choline supplementation in corn-soybean meal based laying hen diets is open to controversy. The requirement for choline was shown to increase in chicks when soybean meal was included in the diet (Berry et al., 1943, Marvel et al., 1943 and Mishler et al., 1946). Soybeans contain relatively high amount of choline (>2500 mg/kg) and the biological availability of choline from soybean has been reported to be poor. The percentage bioavailable choline from soybean (77-95%) was less than 100% relative to choline from choline chloride (Emmert and Baker, 1997). The authors also carried out a study in chicks (10-22 days of age) using a choline deficient basal diet and showed a linear response in wt. gain to incremental addition of choline chloride up to 1115 mg/kg feed. Increasing choline chloride to 2000 ppm resulted in further wt. gain but to a lesser extent. Molitoris and Baker (1976) reported the availability of choline from soybean meal as between 57 % and 76%. They suggested that a reduced dietary choline requirement in purified diets versus corn-soybean meal diets was the result of reduced availability of naturally occurring choline in soybean meal versus the use of choline chloride in purified diets. Nesheim et al. (1971) suggested that choline supplementation is required in laying hens only when replacement pullets have been supplemented with choline after 8 weeks of age. In their experiments, no improvement in egg production was obtained with choline supplementation with the practical rearing or laying diets studied, even when the methionine content of the diet was marginal. Scott et al. (1982) also reported that the requirement for choline in laying hens is affected by the amount of choline present in the diet of growing pullets. It was observed that laying hens required supplemental choline for highest egg production when the diet of growing pullets was supplemented with choline. The effect of choline supplementation during the pullet growing phase on subsequent layer performance was studied by Tsaigbe et al. (1982). They did not observe any positive response for choline supplementation during the growing phase on performance of birds during their production cycle. Addition of 0.05 or 0.1% choline to a practical laying hen basal ration containing no supplemental methionine (0.525% TSAA) and 1041 mg choline/kg significantly increased egg production and egg size. Addition of 0.1% of supplemental methionine resulted in no response in laying hens to supplemental choline (Crawford et al., 1969). It was concluded that laying hens get accounted from supplemental choline when supplemental methionine is just adequate or below the requirements. They also found that supplementation of choline at levels of 4600 -7330 mg/kg of the diet in the form of choline HCl in laying hens for a period of 8 months produced a decrease in liver lipid with a significant increase in feed intake at 7330 mg/kg. They also demonstrated that increase in choline content of eggs and body tissues coincided with peak production, irrespective of choline intake.

The choline requirement in relationship to dietary source and level of methionine in broiler diets has also been researched. Pesti et al. (1981) suggested that the interrelationship between choline and methionine requirements could be determined by substituting choline and methionine at different ratios. They also suggested that the growth response observed by the addition of soybean meal was not just a function of choline availability. In most of the choline and methionine interaction studies, baseline methionine and choline concentrations in the feed were not taken into consideration. Studies with purified diets showed that methionine up to 0.7% over the dietary requirements had no effect on the requirements of choline in chicks (Molitoris and Baker, 1976). Ngo and Coon (1974) reported the dietary choline requirement of young chicks fed a corn-casein diet to be 834 ppm. Classen et al. (1965) observed a higher choline requirement (1434 ppm) when birds were fed a typical broiler diet for optimum performance. Miles and Harms (1983) demonstrated that in broiler diets, supplemental methionine could be completely replaced by adding 0.11% choline along with 0.1% sulfate but not in turkey poult diets (Harms and Miles 1984). Day-old broiler chicks fed semi purified diets supplemented with choline chloride at levels to 8800 ppm for four weeks showed maximum growth rates at 880 to 1760 ppm of choline chloride in the diet (Deeb and Thornton, 1959). Body wt and feed efficiency were shown to be negatively affected at levels higher than 2200 ppm. The tolerance level of choline supplementation in poultry was shown to be relatively high. Supplementation of choline in the form of choline dihydrogen citrate at levels of 500 to 2500 ppm of purified diet in day-old leghorn chickens showed no adverse effects on growth over a period of 21 days (Ketola and Nesheim, 1974).

Supplementation with methyl donors in the form of 0.23% choline or 0.23% betaine was equivalent to supplementation with 0.23% methionine in 21-day chick experiments, using basal diets containing 0.31% methionine and 0.43% cystine (Pesti et al., 1980). The optimum choline required in chick diets was between 1900 and 2130 ppm with levels of 0.32% methionine and 0.42% cystine in the diet. Supplementation of 700 to 800 ppm choline and low levels (0.025-0.05%) of methionine was shown to be more economical than supplementation of higher levels of methionine when the cost of choline was between 35 to 45 % of supplemental methionine. A study by Spires et al. (1982) in broiler chickens found that supplemental choline could replace up to two-thirds of the supplemental methionine from 0-47 days in diets containing basal levels of 0.30% methionine and 0.43% cystine in the starter phase and 0.25 and 0.42% methionine and cystine, respectively, in the finisher phase. The total sulfur amino acid and choline levels of 0.81 and 0.24%, 0.75 and 0.24% and 0.75 and 0.24% in the starter, grower and finisher phases showed optimal feed/gain responses (King and Spires, 1983).

Quillin et al. (1961) suggested that choline has a biological equivalence of 42% of that of methionine. The effect of supplementation of practical diets for laying hens with choline in the past has shown conflicting results. Gish et al. (1949), Skinner et al. (1951) and Johnson (1954) were unable to improve egg production by supplementing practical diets for laying hens with choline. However, Daghir et al. (1960) reported improved egg production when the choline content of a practical-type diet was increased from 400 to 700 mg per lb. Similarly, Saloma et al. (1965) also obtained a small improvement in egg production when a practical laying diet was supplemented with choline. Homes and Kramer (1965) reported that addition of choline and vitamin B_{12} improved egg production of hens fed a corn-soybean meal ration. Griffith et al. (1969) reported that choline or a combination of choline, B_{12} and methionine reduced liver fat levels in hens fed practical-type diets and also found that choline, B_{12} or methionine improved egg production and egg size when added to a diet low in methionine.

Welch and Couch (1955) did not find any improvement in egg production when hens were fed a basal diet deficient in methionine and 0.145% choline. However, significant responses in egg production were obtained when the basal diet was supplemented with combinations of choline and homocysteine. March (1981) found no beneficial effects on egg production or egg size when soybean meal or rapeseed meal rations containing 0.5% TSAA were supplemented with 1000 mg choline/kg ration. Schexnailder and Griffith (1973) observed an increase in egg production and egg weight with a decrease in liver fat when choline and vitamin B_{12} were added to low protein or adequate protein diets which were supplemented with amounts of methionine to meet the hen's requirement. Supplementation of choline as Choline HCl at levels of 3834 -5228 ppm in laying hens for up to 252 days was shown to produce eggs with fishy taint (March and MacMillan, 1979). Tsiagbe et al. (1988) observed an increase in phospholipids and PC content in egg yolk of 36-wk-old hens with addition of choline (1000 ppm) at peak egg production which would tend to suggest an active CDP-choline pathway for the synthesis of PC.

EGG CHOLINE:

The average intake of choline was below the adequate intake (AI) and 10% or less had usual intakes above the AI (Jensen et al., 2007). AI was estimated to be 550 mg/d for men and 425 mg/d for women. When adequate intake recommended for choline was provided to men in a study by Fischer et al. (2007), 20% of men developed fatty liver and muscle damage, which are signs of choline deficiency. Foods that are good sources of choline can be frequent contributors to the healthy way of eating. Two large eggs provide 252 mg of choline (all in yolk); a little less than half the recommended daily supply, and also contain 630 mg of phosphatidylcholine (Howe et al., 2004). Although most sources just report the free choline at 252 mg, phosphatidylcholine is the most common form in which choline is incorporated into the egg phospholipids.

B. FOLIC ACID:

Folic acid is also known as vitamin B_9. Folate is a collective term for a group of compounds with a pteroylglutamic acid backbone with different oxidation states; the primary function of which includes one carbon transfer reactions (Selhub and Rosenberg, 1996) thereby involving in purine and pyrimidine synthesis, interconversion of serine and glycine, histidine degradation and synthesis of methyl groups. Folic acid is a yellow to orange brown crystalline powder, which is odorless and readily soluble in alkali, hydroxides and carbonates. It is insoluble in alcohol, acetone, ether and chloroform (McDowell, 1989) with a molar mass of 441.4 g/mol (http://en.wikipedia.org/wiki/Folic_acid)

Folic acid, which is a B group vitamin, does not occur naturally, in appreciable amounts, in foods as free folacin. It is usually found in the tissues of plants and animals as conjugates. However, due to its stability and commercial availability, it is the form that is used in vitamin supplements, fortified foods and vitamin premixes. Folic acid was first isolated by Stokstad (1943) in yeast. The term folic acid and folate are often used interchangeably. Folic acid actually refers to the fully oxidized synthetic compound used in dietary supplements and in food fortification and folate refers to the various tetrahydrofolate derivatives naturally present in foods.

1. FOLIC ACID FUNCTIONS:

Folacin, as 5, 6, 7, 8- tetrahydrofolic aicd, is required in single carbon transfer reactions which include remethylation of homocysteine, glycine-serine interconversion and purine synthesis. Folacin is also necessary for the biosynthesis of labile methyl groups, the metabolism of which plays an important role for the body in the biosynthesis of methionine from homocysteine and of choline from ethanolamine.

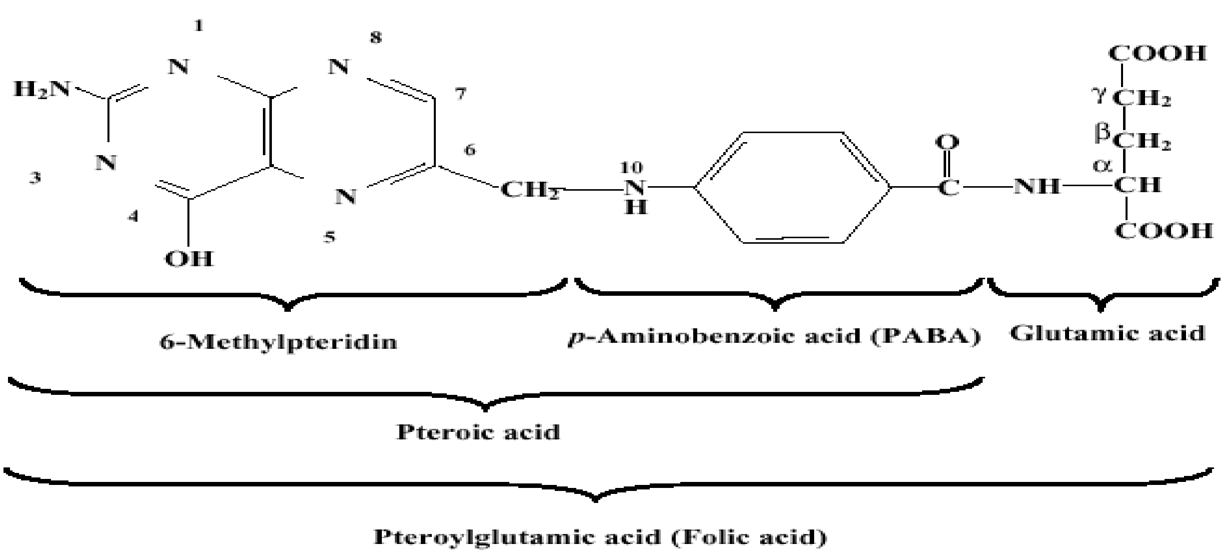

Figure **1**.7 Structure of folic acid

(Adapted from http://ethesis.helsinki.fi/julkaisut/maa/kotie/vk/polonen/fig3.gif)

2. DIETARY FOLIC ACID REQUIREMENT:

Dietary folic acid, is digested via hydrolysis and, after hydrolysis and absorption from the intestine, is transported in plasma as monoglutamate derivatives, predominantly as 5-methyltetrahydrofolate. Studies show that about 79-88% of labeled folacin is absorbed, and that absorption is rapid since serum concentrations usually peak about 2 hr after ingestion (Godwin and Rosenberg, 1975). The availability of folic acid from different food items was in the range of 37-72% (Babu and Srikantia, 1976). Folic acid requirement differ markedly in different species of animals. The requirement of folic acid for monogastric species is dependent to a lesser extent on intestinal folic acid synthesis and utilization by the animal; most species normally do not require dietary folic acid because of their ability to utilize intestinal microbial metabolites. A practical poultry diet, comprised of corn, soybean meal and other common feedstuffs should meet the folic acid requirement as it contains folic acid at levels of 1-1.5 mg/kg (Scott et al., 1982) and the requirements were shown to be higher when birds were fed diets high in protein. Folic acid requirement is also affected by other factors such as infection and type of medication. The form in which folic acid is fed to the birds also has an influence on its requirement. Folic acid is very sensitive to heat, light and moisture (Gadient, 1986) and presence of minerals in the feed mix also affects the requirement as it destroys the vitamin (Adams, 1982). The folic acid requirement increases if there is deficiency of choline, vitamin B_{12}, vitamin C or iron in the diet. Most folic acid in poultry feedstuffs is present in conjugated form, which the young chick is fully capable of using (Cropper and Scott, 1967). The requirement for folic acid (Table 1.2) is also largely affected by the stage of production and the extent of growth. At peak production and when growth rate is maximal, the requirement for folic acid also increases. According to the NRC (1984) the folic acid requirement in case of poultry for egg hatchability is comparatively higher than that for production. The amount of folic acid required for optimal egg production was reported to be 0.12 ppm (Taylor, 1947).

Table 1.2 NRC requirements for folic acid

Species	Diet	Requirement
Broiler		
0-3 weeks	Cereal grain basal diet	0.55 mg/kg of diet
3-6 weeks	Cereal grain basal diet	0.55 mg/kg of diet
6-8 weeks	Cereal grain basal diet	0.50 mg/kg of diet
Laying Hen		
0-6 weeks	Cereal grain basal diet	0.55 mg/kg of diet
6-12 weeks	Cereal grain basal diet	0.25 mg/kg of diet
12-18 weeks	Cereal grain basal diet	0.25 mg/kg of diet
Production cycle	Cereal grain basal diet	0.25 mg/kg of diet
Turkey		
0-4 weeks	Cereal grain basal diet	1 mg/kg of diet
4-8 weeks	Cereal grain basal diet	1 mg/kg of diet
8-16 weeks	Cereal grain basal diet	0.8 mg/kg of diet
16-20 weeks	Cereal grain basal diet	0.8 mg/kg of diet
20-24 weeks	Cereal grain basal diet	0.7 mg/kg of diet
Breeding	Cereal grain basal diet	0.7-1 mg/kg of diet

The current NRC (1994) requirement for folic acid for white egg laying hens and brown egg laying hens eating 100 g feed per day would be 0.25 and 0.23 ppm, respectively. The nutritional status of animals with respect to folic acid adequacy is determined by the plasma folate concentration. Growth rate have also been used as a measure of folate adequacy in animals.

3. FOLIC ACID ON PERFORMANCE AND EGG QUALITY:

The effect of folic acid on the performance and egg shell quality of 54-wk-old Babcock B300 hens was observed by Keshavarz (2003) who reported that there was no effect on egg production of folic acid supplementation at 0.5 to 0.7 mg/kg ration. They also found that reducing folic acid levels resulted in reduced egg weight in case of Babcock B300 hens. Similar results were also observed by Hebert et al. (2005) when two leghorn strains-Hyline W36 and Hyline W98 hens received a barley based ration containing 0, 2, 4, 8, 16, 32, 64 and 128 mg/kg of crystalline folic acid for 21 days. Supplementation of folic acid at lower (6 ppm) and higher (9 ppm) levels showed a significant difference with respect to egg weight as reported by Husseiny et al., (2008). They also reported that the egg quality parameters as represented by egg shell thickness, egg shell percentage, egg contents percentage (yolk% and albumen%), Haugh units and yolk index did not show any significant difference with folic acid supplementation at 6, 9 and 12 ppm in Bovan hens on a corn soy based diet. Similar results for egg shell thickness, percent egg content and Haugh units were observed by Hebert et al. (2004) with 4 ppm of folate supplementation in a barley based diet. Khalifah and Shahein (2006) showed no difference of folic acid supplementation (0-32 mg/kg diet) on egg weight or productivity on a corn soy based diet in the Baheij chicken strain. House et al. (2002) concluded that there was a difference in egg weight among folic acid levels; ie. birds consuming diets containing 8 and 16 mg folic acid /kg ration produced eggs weighing 53.3 and 52.6 g, respectively. Increasing folic acid levels in the diet affected egg shape index, egg shell thickness and yolk percentage; whereas albumen height, yolk index and shell and albumen percentages were not affected by dietary folic acid supplementation at 4 ppm.

A recent study by Bunchasak and Kachana (2009) showed the effect of feeding different levels of folic acid on performance and egg lipid composition. The study suggests that levels of folate (0.31-10.31 mg/kg diet) in a diet based on corn and soybeans did not affect egg production of older laying hens (64-72 weeks of age). Supplementation with folate did not significantly increase the total phospholipids content in serum or egg yolk, but there was a positive correlation between dietary folic acid supplementation and the serum total phospholipid content.

There is a tendency for elevated total serum phospholipid upon dietary supplementation with folate. Akesson et al. (1982) in humans and Singla et al. (2006) in weanling rats reported that phospholipid N-methylation in the liver was depressed during folate deficiency because of a decreased availability of S-adenosylmethionine caused by the low concentration of methylated folate in the liver. Methionine is metabolized to S-adenosylmethionine, which is a product of the folate dependent remethylation pathway and serves as a universal methyl donor during phospholipid synthesis (Kado et al., 2005). Three molecules of S-adenosylmethionine are required for the three methylations of phosphatidylcholine (Higdon, 2003). Folic acid supplementation may enhance phospholipid synthesis via these pathways. Because phospholipids are essential lipids for physiological and biochemical processes, increased levels of phospholipids through folic acid supplementation may be physiologically beneficial to laying hens.

4. EGG FOLATE:

Eggs contain folate at levels of approximately 22 µg per large egg (USDA, 2004) or roughly 6% of the adult RDA for folate. There has been growing awareness of the need for increased consumption of folate by humans. The folate content of eggs can be increased threefold by increasing the crystalline folic acid in the laying hen diet to 4 mg/kg (House et al., 2002). Hebert et al. (2005) also showed that enrichment of eggs with folate is possible when dietary folic acid levels are increased in laying hen diets.

C. VITAMIN B_{12}

As early as the 1920's, liver extracts and other products derived from animals were found to increase growth of rats, chicks and pigs and were termed animal protein factors. In 1948, crystalline vitamin B_{12} was isolated from liver and was found to contain high amounts of vitamin B_{12} (McDowell, 1989) and is the largest (molecular weight =1355.4) and the most complex of all the vitamins.

The requirement for vitamin B_{12} is lower compared to all other B-complex vitamins. Vitamin B_{12} is different from other vitamins as it is synthesized only in certain bacteria and therefore animal foods but not plant foods are considered to be the major dietary sources of vitamin B_{12} (Ball, 1998). Vitamin B_{12} is the common name for a group of compounds having B_{12} activity with very complex structures (Ellenbogen, 1984) containing near 4.5% cobalt. Vitamin B_{12} is the only metabolite that contains cobalt which gives this water soluble vitamin its red color. Vitamin B_{12} is crystalline in nature and is insoluble in organic solvents such as acetone, chloroform or ether (McDowell, 1989). It is soluble in water and is stored mainly in liver. B_{12} refers to a group of cobalt containing compounds known as cobalamines and the main cobalamines in humans and animals are hydroxycobalmine, adenosylcobalamine and methylcobalmine. The natural concentrations of this vitamin in feeds are generally low and the synthetic form of this vitamin is normally used as feed supplement.

1. VITAMIN B_{12} FUNCTIONS:

Vitamin B_{12} is an integral part of different enzyme systems with reactions involving the synthesis or transfer of single carbon units. Functions of this vitamin are metabolically related to other dietary nutrients such as choline, methionine and folic acid. Vitamin B_{12} functions as a cofactor in transmethylation reaction and also in the synthesis of labile methyl groups.

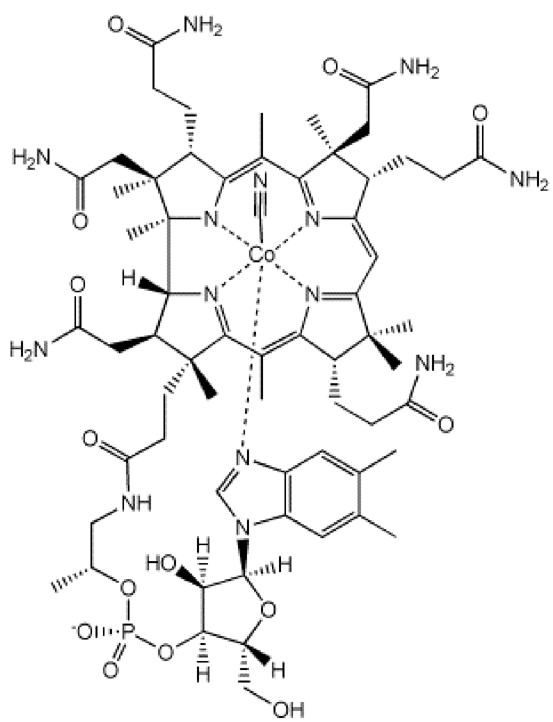

Figure **1.8** Structure of vitamin B_{12}

(Adapted from http://www.daviddarling.info/images/vitamin_B12.gif)

(1) DNA synthesis.

(2) Transfer of methyl group from methyl tetrahydrofolate to homocysteine.

(3) Synthesis of protein from amino acids esp serine, methionine and phenylalanine.

(4) Involved in carbohydrate metabolism as a part of methylmalonyl CoA isomerase which is a vitamin B_{12} requiring enzyme and also in fat metabolism through its effect on the thiol groups.

Vitamin B_{12} plays an important role in the reduction of single carbon compounds and thus is involved with folic acid in the biosynthesis of labile methyl groups. Vitamin B_{12} is also important for folic acid dependent reactions of intermediary metabolism wherein vitamin B_{12} moderates the relative amount of methyl to nonmethyl tetrahydrofolates. Vitamin B_{12} is also required as a carrier of methyltetrahydrofolate (McDowell, 1989).

2. DIETARY VITAMIN B_{12} REQUIREMENT:

Vitamin B_{12} requirements are relatively small and the recommended daily intake is only a few micrograms per kilogram of feed. Vitamin B_{12} requirements of chicken depend upon the level of several other nutrients in the diet. The B_{12} requirement depends on level of choline, methionine and folic acid in the diet. Small amounts of vitamin B_{12} are obtained by direct absorption of the vitamin produced by bacterial synthesis in the intestine in case of poultry (NRC, 1984). Yacowitz et al. (1952) using a microbial assay, estimated that 2.5 ng of vitamin B_{12}/g of yolk is needed to ensure hatchability. Peterson et al. (1953) established the requirement for breeding hens as between 3.3-4.4 µg/kg of diet using microbiological methods for vitamin analysis. The NRC (1994) requirement of vitamin B_{12} (Table 1.3) for white and brown egg laying hens eating 100 g feed per day is estimated to be 0.003 mg/kg diet. The trace mineral cobalt is not considered an essential mineral for chickens.

Table 1.3 NRC requirements for Vitamin B_{12}

Species	Diet	Requirement
Broiler		
0-3 weeks	Cereal grain basal diet	0.01 mg/kg of diet
3-6 weeks	Cereal grain basal diet	0.01 mg/kg of diet
6-8 weeks	Cereal grain basal diet	0.007 mg/kg of diet
Laying Hen		
0-6 weeks	Cereal grain basal diet	0.009 mg/kg of diet
6-12 weeks	Cereal grain basal diet	0.003 mg/kg of diet
12-18 weeks	Cereal grain basal diet	0.003 mg/kg of diet
Production cycle	Cereal grain basal diet	0.004 mg/kg of diet
Turkey		
0-4 weeks	Cereal grain basal diet	0.003 mg/kg of diet
4-8 weeks	Cereal grain basal diet	0.003 mg/kg of diet
8-16 weeks	Cereal grain basal diet	0.003 mg/kg of diet
16-20 weeks	Cereal grain basal diet	0.003 mg/kg of diet
20-24 weeks	Cereal grain basal diet	0.003 mg/kg of diet
Breeding	Cereal grain basal diet	0.003 mg/kg of diet

Rostagno et al. (2000) reported a need for 0.2 ppm of cobalt in the diet of laying hens. Birds synthesize vitamin B_{12} using cobalt inside the ceca, but the levels are below the requirements, and it must be supplemented (McDonald et al., 1975). Furthermore, there is no consensus about cobalt supplementation in chicken diets. In practice, many nutritionists supplement cobalt to trace mineral premix at 0.29 g of cobalt per ton of feed (Kato et al., 2003).

3. VITAMIN B_{12} ON PERFORMANCE AND EGG QUALITY:

The effect of supplementing vitamin B_{12} to a corn soy based diet at three different levels (0, 0.01 and 0.08 ppm) on the performance of laying hens as well as the lipid composition of serum, liver and egg yolk (64-72 wks of age) was reported by Bunchasak and Kachana (2009) who found no significant difference on egg output, hen-day production or feed intake as well as serum total phospholipids. Keshavarz (2003) and Leeson and Caston (2003) reported that egg production was not reduced by vitamin B_{12} deficiency as long as the feed intake was not significantly affected. Accordingly, data of Husseiny et al. (2008) also showed that feeding diets supplemented with 0.01-0.02 ppm of vitamin B_{12} did not have any significant effect on egg production. However, the authors observed a difference in egg wt with no difference in egg shell thickness, egg shell percentage, egg content percentage, Haugh units and yolk index at these inclusion levels of vitamin B_{12}. Vitamin B_{12} had no effect on egg production and egg loss, and the absence of vitamin B_{12} in the diet did not result in any clinical signs related to B_{12} deficiency. Squires and Naber (1992) reported a decrease in egg production only after 12 weeks of production when a diet without B_{12} was given. This decrease in egg production after 12 weeks might be explained by the fact that birds have hepatic storage of vitamin B_{12} and the reserves were not affected up to 12 weeks when the nutrient is not given in the diet (Scott et al., 1982).

Kato et al. (2003) reported that supplementation of vitamin B_{12} at 10μg/kg in a corn soy based diet in Lohmann laying hens during the second cycle of production significantly increased egg weight (69.8 g) compared to no supplementation (68.6 g); however, egg mass was not affected by vitamin B_{12} supplementation. These data was similar to those reported by Skinner et al. (1951) who demonstrated the importance of vitamin B_{12} supplementation. Kato et al. (2003) also reported that vitamin B_{12} supplementation decreased specific gravity of eggs compared to the treatment without vitamin B_{12}. This may be due to the fact that vitamin B_{12} increases egg size and consequently specific gravity was decreased. A smaller shell percentage was also observed with vitamin B_{12} supplementation. In the absence of vitamin B_{12}, egg shell thickness and the weight of the egg shell per unit of surface area were higher than the treatment with vitamin B_{12}. Haugh unit values did not show any significant difference with vitamin B_{12} supplementation.

4. VITAMIN B_{12} CONTENT OF EGG:

Vitamin B_{12} is found in animal products such as seafood, dairy products and eggs (Watanabe, 2007). Vitamin B_{12} content in the whole egg is about 0.9-1.4 µg/100g (USDA, 2004) and most of the vitamin is found in the egg yolk. Eggs potentially contribute 20-30% of daily intake of B_{12} (Song and Kerver, 2000). However, vitamin B_{12} in eggs is poorly absorbed (< 9%) relative to other animal food products (Watanabe, 2007).

CHAPTER-2

Effect of Dietary Choline and Folic acid on Laying Hen Performance, Egg Folate and Egg Phospholipid Composition

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ABSTRACT This study was designed to determine the effect of choline and folic acid on egg production, egg quality and the phospholipid composition of egg yolk. A corn-soy basal diet was formulated with three levels of supplemental choline (0, 500 and 1000 ppm) and three levels of supplemental folic acid (0, 2 and 4 ppm) in a 3 x 3 factorial arrangement. Folic acid at 2 ppm increased egg production ($p \leq 0.04$). Egg wt was higher at 500 ppm of choline ($p \leq 0.06$) and 0 ppm ($p \leq 0.01$) of folic acid supplementation. There were choline by folic acid interaction effects on feed intake ($p \leq 0.001$), albumen wt ($p \leq 0.005$) and yolk wt ($p \leq 0.03$). At lower levels of choline supplementation (0 and 500 ppm), 4 ppm folic acid inclusion increased feed intake and egg albumen compared to 0 ppm folic acid; while at the highest level of choline inclusion (1000 ppm) high folic acid (2 and 4 ppm) decreased feed intake and egg albumen. Yolk wt was highest at 1000 ppm of choline with 0 ppm of folic acid addition. Plasma folate and egg folate showed an increase ($p \leq 0.0001$) with added levels of dietary folic acid. Phosphatidylcholine (PC) concentration showed a trend to increase with higher levels of choline and folic acid supplementation.

Key words: Choline, Folic acid, Egg phosphatidylcholine

Introduction

Since its discovery in hog bile by Strecker (1862), choline has been recognized as an essential dietary nutrient for most animal species. It is considered a member of the B-complex vitamin group and an essential nutrient in laying hen diets for the formation of the phospholipid lecithin found in egg yolk and liver (Ewing, 1963). Choline is a key metabolite for building and maintaining cell structure and plays an essential role in fat metabolism in the liver. Choline is essential for the formation of acetylcholine and is a source of labile methyl groups for the formation of methionine from homocysteine. Egg phospholipids consist of phosphatidylcholine, phosphatidylethanolamine and sphingomyelin and contain long chain polyunsaturated fatty acids in a high bioavailable form. Phosphatidylcholine, which makes up the the majority of egg lipid is a good source of dietary choline for consumers.

There are essentially two pathways for phosphatidylcholine (PC) synthesis.

Phosphatidylcholine can be synthesized from preexisting choline molecules using the base exchange pathway or the cytidine diphosphate (CDP) choline pathway (Zeisel, 1981) and/or the sequential methylation of phosphatidylethanolamine (PE) using the methyl transferase pathway (Blusztajn et al., 1979). Synthesis of choline and related compounds require folic acid as a donor of methyl groups. Low levels of folic acid leads to inadequate production of S-adenosylmethionine (SAM), creating a condition of hypomethylation (Newmann, 1999).

In 1998, the National Academy of Sciences (NAS) issued a report identifying choline as an essential nutrient for humans and recommended adequate intake amounts. The recommended adequate intake (AI) for the adult human male is 550 mg/day and for adult human female is 425 mg/day. Phospholipids form 28-30% of egg lipids with phosphatidylcholine comprising about 80% of the total (Garland and Powrie, 1978). A standard large egg provides 125 mg of choline and also contains 630 mg of phosphatidylcholine and is considered a good dietary source of choline for humans (USDA, 2004).

The current recommendation for choline intake in laying hens is 105 mg/day at feed intake levels of 100 g/day (NRC, 1994) with a dietary choline concentration of approximately 1100 mg/kg diet. Several researchers in the past have studied the effect of choline supplementation (March, 1981; Nesheim et al., 1971; Griffith et al., 1969; and Crawford et al., 1969) on egg production and egg quality parameters. Crawford et al. (1969) demonstrated that significant increases in choline content of eggs and body tissues coincided with peak egg production.

The NRC recommendation for folic acid intake at feed intake levels of 100 g/day is 0.25 mg/kg diet. A standard large egg naturally contains approximately 22 µg of folate (USDA, 2004) or roughly 6% of the adult RDA for folate. Folate content of the egg can be increased threefold by increasing crystalline folic acid in the laying hen diet to 4 mg/kg (House et al., 2002). However, information on the changes in the phospholipid composition of the egg yolk in response to choline alone and in combination with folic acid is lacking.

The objective of the proposed study was to determine the effect of supplemental choline and folic acid on the phospholipid composition of egg yolk and thereby the potential of eggs to be fortified with choline. Our hypothesis was that supplementation of choline and folic acid may generate additional methyl groups and add to the active methyl pool. This may bring about a more active methyl transferase activity leading to an increase in PC synthesis.

Materials and Methods

The proposed study was conducted at the F-house Poultry Research Barn of the Department of Animal Science, University of Nebraska, for a period of eight weeks. Animal care approval was received from the Institutional Animal Care and Use Committee (IACUC) of University of Nebraska (Protocol # 06-10-044D).

One hundred and forty-four, 29-week-old Single Comb White Leghorn Bovan hens[1] were housed in layer cages (Farmer Automatic)[2] at a density of four birds per cage (400 cm^2/ bird). The birds had ad-libitum access to feed and water throughout the study and were subjected to a 16-h photoperiod.

A 3x3 factorial arrangement with three levels of supplemental choline (0, 500 and 1000 ppm) and three levels of supplemental folic acid (0, 2 and 4 ppm) were assigned in a randomized complete block design, resulting in a total of nine experimental treatments. Blocking was done in order to reduce the effect of temperature stratification in the cage unit. Each treatment was assigned to four replicate cages with four birds per cage for a total of thirty six cages. Hens were fed the dietary treatments for eight weeks.

A corn-soy basal diet (Table 2.1) was formulated to be isocaloric (2775 kcal ME/kg) and isonitrogenous (16.7 % CP) and to meet the NRC (1994) requirements for laying hens. The basal diet was formulated by removing choline (choline chloride, 60%)[3] and folic acid (folic acid, 10%)[4] from the vitamin premix and the appropriate levels of these nutrients were added to the diets according to the design of the experiment. The basal diet was calculated to contain

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[1] Kumm's Kustom Pullets, Nebraska, U.S.A
[2] Farmer Automatic, P. O. Box 39 Register, Georgia 30452, U.S.A
[3] International Nutrition, 7706 I Plaza, Omaha, NE 68127-1841, U.S.A
[4] International Nutrition, 7706 I Plaza, Omaha, NE 68127-1841, U.S.A

1089 mg/kg and 0.57 mg/kg of choline and folic acid, respectively. The analyzed values for choline and folic acid in the basal diet were 972 and 0.31 mg/kg, respectively. The calculated and analyzed values for total choline and folic acid in the experimental diets are shown in Table 2.2. Dietary samples were collected from each diet formulated, sieved through a 1mm screen, ground and stored for analysis of gross energy (GE), N, Ca, P, choline and folic acid. Gross energy was determined using a Parr adiabatic oxygen bomb calorimeter[5]. Dietary N was determined using the Kjeldahl method as established by the Association of Official Analytical Chemists (A.O.A.C, 1984). The N content in the diet was multiplied by 6.25 to determine protein content of the diet. Calcium and P were determined by procedures established by the A.O.A.C (1984). Choline content of the feed was analyzed by the Reineckate procedure as established by the A.O.A.C (1965). It involves the extraction of choline containing lipids, formation and separation of a water insoluble Reinecke salt, and then determination of absorption of the acetone soluble Reinecke solution by the colorimetric[6] measurement of the supernatant at 520 millimicrons. The amount of folic acid present in the feed was analyzed using the HPLC method as described by Poo-Prieto et al. (2006). The feed samples and the folic acid standard[7] (2 g) were homogenized in 10 mL extraction buffer (0.075 M phosphate buffer containing 1% ascorbate and 0.1% mercaptoethanol) and were heated for 15 min at 120°C, and then cooled in ice. The homogenate samples were treated with a trienzyme (α- amylase, rat plasma and protease)[8] and incubated at 37°C for 2 hrs. The samples were kept in a boiling water bath for 5 min, cooled in ice and centrifuged for 15 min at 36,000 x g. The supernatants were

[5] Parr Instrument Company, 211 Fifty third Street, Moline, Illinois, 61265-9984, U.S.A
[6] Shimadzu Scientific Instruments, 7102 Riverwood Drive, Columbia, MD 21046, U.S.A
[7] Sigma-Aldrich, 3050 Spruce St, St. Louis, MO 63103, U.S.A
[8] Fisher Scientific, U.S.A.

then filtered through a Millipore microfilter[9] with a pore size of 0.45 µm. The samples were analyzed using a Waters[10] 600 chromatograph equipped with a UV detector at 280 nm. The 20 µL of samples and standard were injected into a variant microsorb C18 column (250 x 4 mm, packed with 5 µm silica) and eluted with 10 % acetonitrile and 30 mM phosphate buffer (pH 2.3) at a flow rate of 1mL/min. The linear regression equation obtained from the standard curve was used for estimating the folic acid content of the feed samples.

Hens were weighed at the start and end of the trial and the average hen wt. by cage was calculated to determine body weight change. Feed consumption was recorded on a daily basis and the intake was calculated per hen per day. Eggs were collected daily and egg production (EP) was calculated on a hen/day basis. Egg mass (EM) was calculated as a factor of egg weight (EW) and egg production (g egg wt. x % egg production).

Egg weights were recorded every week on one day of egg production and the eggs were examined for shell quality by specific gravity weekly. Egg components (yolk, albumen and Haugh units) were measured weekly on two eggs per replicate pen. Haugh units (Haugh, 1937), which is an index of albumen quality, were calculated from egg weight and albumen height, which was measured in the middle of the thick albumen, equi-distant from the outer edge of the albumen and yolk.

$$HU = 100 \log (h-.01*5.6745(30w\^{}.37-100) +1.9)$$

where:

- HU = Haugh unit
- h = observed height of the albumen in millimeters

[9] Fisher Scientific, U.S.A.
[10] Waters Corporation, 34 Maple St, Milford, MA 01757, U.S.A

- w = weight of egg in grams

After 8 wks on the trial, four eggs per treatment pen were collected and used to determine egg phospholipid composition (Christie, 1985). Eggs were cracked and yolk was separated from albumen without rupturing the vitelline membrane. Yolks were rolled onto tissue paper in order to totally remove albumen and weighed. Yolks were individually broken, homogenized and lyophilized completely. Dry matter was determined and the dry samples were frozen at -20°C. The dry yolk was finely ground and sieved through a 1mm screen for a uniform sample. A sample of 0.5 g of egg yolk was weighed into a centrifuge tube and was extracted thrice with 5 mL of HPLC grade methanol[11] for maximum extraction (Fletcher et al., 1984). The sample was vortexed for 15 sec, and centrifuged at 3000 x g, for 15 min, filtered through Whatman filter paper no: 1 (110mm) into a 25 mL volumetric flask and made up to volume with HPLC grade methanol. The filtrate was again filtered for further purification of the sample through a 30 mm HPLC syringe filter made of regenerated cellulose with a pore size of 0.45 µm.

The following procedure of High Performance Liquid Chromatography (HPLC) was a modification of the gradient elution system developed by Christie (1985). Phospholipids were analyzed using a Hewlett Packard 1050 HPLC[12] system equipped with a Beckman 110B solvent delivery module[13] and a 20 µL sample loop. Separation was achieved using a microporasil column (10µm 125A 3.9x300mm column, Waters)[14] mounted to a HP 3395 Integrator[15] and absorbance was measured at 214 nm. The solvent system was HPLC grade methanol: acetonitrile (70:30 v/v) at a flow rate of 1.5 mL/min. Samples of 0.02 mL were injected for

[11] Fisher Scientific, U.S.A.
[12] Lab Extreme, Inc Kent City, MI 49330
[13] Lab Extreme, Inc Kent City, MI 49330
[14] Waters Corporation, 34 Maple Street, Milford, MA 01757
[15] Quantum Analytics Inc, 363 Vintage Park Drive, Foster City, CA 94404

analysis. Aliquots of sample and standard solutions (L-a-phoshphatidylcholine (500mg) and L-a-phosphatidylethanolamine (100mg)[16] dissolved in methanol/acetonitrile[17] (70:30) were injected into the column at ambient temperature. A 79853C Variable Wavelength UV detector (HP)[18] was used for quantitative determination of phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Linear regression modeling was used to plot peak area versus concentration for each phospholipid class. The egg yolk samples were also used to measure egg folate concentration as described by Vahteristo et al. (1997). Samples (0.5 g of egg yolk) were homogenized in 3 mL of extraction buffer (0.075 M phosphate buffer containing 1% ascorbate and 0.1% mercaptoethanol) and were kept in a boiling water bath for 10 min. The samples were then rapidly cooled in ice and centrifuged at 11,000 x g, for 30 min at 4°C. The residue was redissolved in 5mL extraction buffer and centrifuged for 10 min. The supernatant was made up to 10 mL with extraction buffer in a 10 mL volumetric flask. The pH of the extract was adjusted to 4.9 with acetic acid and 1 mL of the enzyme (rat plasma)[19] was added. The samples were then flushed with N_2 and were incubated in a microwave oven at 37°C for 2 hrs. The extract was kept in a boiling water bath for 5 min and then cooled in ice. The samples were purified using HPLC grade Millipore membrane filter[20] with a pore size of 0.45 µm. Samples were analyzed using a Waters[21] 600 chromatograph equipped with a fluorescence detector. The excitation and emission wavelengths were set at 290 nm and 360 nm, respectively. The 20 µL of samples and standard[22] were injected into a variant Microsorb C18 column (250 x 4 mm, packed with 5 µm silica) for elution with 10 % acetonitrile and 30 mM phosphate buffer (pH 2.3) at a flow rate of 1mL/min.

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[16] Indofine Chemical Company, 121 Strykerlane, Hillsborough, NJ 08844
[17] Fisher Scientific, U.S.A
[18] Lab Extreme Inc, Kent City, MI 49330
[19] Pel-Freez, Arkansas, LLC
[20] Fisher Scientific, U.S.A.
[21] Waters Corporation, 34 Maple St, Milford, MA 01757, U.S.A
[22] Sigma-Aldrich, 3050 Spruce St, St. Louis, MO 63103, U.S.A

Quantification was done using a standard curve in which the peak area was plotted against the concentration. The linear regression equation thus obtained was used for estimating egg folate content.

Blood samples were collected from two birds from each pen via wing venipuncture to determine plasma folate. Blood samples were collected with a 5 mL sterile syringe and were transferred to a 5 mL vacutainer tube with EDTA. The samples were cooled on ice and immediately centrifuged at 12,000 x g, for 5 min and were retained and stored at -80°C until analysis. Plasma folate was extracted using the method of Clifford et al. (1990). One mL of plasma was added to 3 mL of extraction buffer (0.075 M phosphate buffer containing 1% ascorbate and 0.1% mercaptoethanol) and the mixture was autoclaved for 10 min at 121°C. The samples were centrifuged at 5°C, 3000 x g, for 10 min. The supernatants were used for plasma folate determination. Plasma folate concentration was determined through the use of a competitive binding assay, SimulTRAC B_{12} [^{57}Co]/Folate[^{125}I] Radioassay kit purchased from ICN Pharmaceuticals[23] according to the manufacturer's recommended protocol.

Data were analyzed for repeated measures using the Mixed Model procedure of SAS software (Proc Mixed, 2001; SAS Institute, Inc., Cary, NC) for a randomized complete block design with a 3x3 factorial arrangement. Repeated measures were done to ensure treatment is effective over a specified period. Each cage of hens represented an experimental unit for analysis. Repeated measures analysis was used to measure the average treatment effect over time and the possible treatment by time interactions. Repeated measures analysis was also done to identify the possible covariance patterns in the repeated measurements and to determine the appropriate model to describe the time and measurement relationship. The appropriate

[23] ICN Pharmaceuticals, Diagnostic Division, 13 Mountain View Avenue, Orangeburg, New York 10962-1294

covariance pattern for model fit was selected for each measurement using the information criteria. Data were tested for the main effects of choline, folic acid, time and their interaction and significance was reported at $p \leq 0.05$. The model used for the data analysis was:

$$Y = \mu + C_i + F_j + L_k + T_l + CF_{ij} + E_{ijklm}$$

where μ is the overall mean; C_i is the effect of choline; F_j is the effect of folic acid; L_k is the effect of location; T_l is the effect of time; CF_{ij} is the interaction of choline and folic acid; E_{ijklm} is the residual error. The separation of means was done using LS means statement with pdiff option.

The data for body wt gain, plasma folate, egg folate and egg phospholipid were analyzed using the model

$$Y = \mu + C_i + F_j + L_k + CF_{ij} + E_{ijkl}$$

where μ is the overall mean; C_i is the effect of choline; F_j is the effect of folic acid; L_k is the effect of location; CF_{ij} is the interaction of choline and folic acid; E_{ijkl} is the residual error. Main effects of choline, folic acid, location and choline by folic acid interaction were evaluated. The separation of means was done using LS means statement with pdiff option. The data were also tested for the linear and quadratic effects of choline and folic acid

Results and Discussion

Feed intake of birds increased (107.05 g, 108.45 g and 108.13 g) at higher levels of folic acid inclusion compared to no (0 ppm) supplementation (Table 2.3). However, there was no difference ($p \geq 0.538$) in feed intake between 2 and 4 ppm of folic acid addition. Supplementation of choline at any level did not show any difference ($p \geq 0.868$) in feed intake. There was a choline by folic acid interaction effect ($p \leq 0.006$) on feed intake (Graph 2.1). At 0 ppm level of choline supplementation, folic acid supplementation at 2 ppm ($p \leq 0.005$) and 4 ppm increased ($p \leq 0.001$) feed intake compared to 0 ppm level of folic acid. At higher levels of choline supplementation (500 and 1000 ppm) there was no difference ($p \geq 0.05$) in feed intake at any level of folic acid supplementation. Similarly at the lowest level of folic acid supplementation (0 ppm) there was an increase ($p \leq 0.014$) in feed intake at highest level of choline supplementation (1000 ppm) compared to 0 ppm level of choline addition. There was a difference in feed intake between 0 and 500 ppm ($p \leq 0.049$) and 0 and 1000 ppm ($p \leq 0.02$) of choline supplementation at folic acid level of 4 ppm. Feed intake decreased at 4 ppm of folic acid inclusion when choline level was increased from 0 to 1000 ppm (109.4 g, 107.6 g and 107.3 g, respectively). The increase in feed intake observed in this study with added folic acid in the diet was in agreement with previous studies by Hebert et al. (2005) and House et al. (2002), wherein addition of supplemental folic acid to laying hen diets had a marginal, but significant effect on feed intake. Supplementation of choline to laying hen diets had conflicting results on feed intake. Supplementation of choline to the laying hen diet did not show any difference in feed intake in this study which was in agreement with data from Keshavarz and Austic (1985). This was expected for this study as diets were formulated to meet the methionine requirement to compensate for a potential sparing effect of choline for methionine.

Body weight gain did not show any difference (Table 2.3) with choline ($p \geq 0.172$) or folic acid supplementation ($p \geq 0.616$), which was in accordance with previous reports by Kahraman et al. (2008) and Griffith et al. (1969). Egg production (Table 2.3) increased ($p \leq 0.06$) with folic acid supplementation, at higher levels (2 and 4 ppm) compared to no (0 ppm) supplementation. Egg production at 0, 2 and 4 ppm of folic acid inclusion was 91.9 %, 95.4 %

and 93.2 % respectively. Egg production showed a quadratic effect ($p \leq 0.023$) with supplemental folic acid. The increase in egg production observed in this study with added folic acid is suggestive of improved energy utilization with the addition of this nutrient. This result is contrary to that observed by Bunchasak and Kachana (2009), wherein it was suggested that level of folic acid (0.31-10.31 mg/kg diet) added to a corn-soy based diet had no effect on egg production of older laying hens (64-72 weeks of age). However, the present study was carried out at the peak of egg production starting from 29 to 36 wks of age. It is the lack of demonstrable impacts on performance that has generally limited the inclusion of folic acid in layer diets, a fact supported by recent surveys of commercial feed mills (BASF, 2000). Percentage egg production was unaffected by choline supplementation, which is supported by the fact that benefits from supplemental choline in layer diets occurs mainly when the methionine and total sulfur amino acid requirements (TSAA) of the birds are not met. The TSAA and the methionine content of the diet formulated for this study were 0.75% and 0.46%, respectively, which is well above the NRC requirement for methionine (0.30%) and TSAA (0.58%) for birds consuming 100 g of feed per day. There was no choline by folic acid interaction effect on percentage egg production ($p \geq 0.174$).

Supplementation of choline showed an effect on egg wt. ($p \leq 0.06$). Egg wts at 0, 500 and 1000 ppm of choline supplementation were 59.3, 59.7 and 58.8 g, respectively (Table 2.4). This reduction in egg wt. at the highest level of choline supplementation (1000 ppm) was small and unexpected. Folic acid supplementation also affected ($p \leq 0.01$) egg wts at 0, 2 and 4 ppm of folic acid, being 59.90, 58.70 and 59.33 g, respectively. Egg wt showed a quadratic effect ($p \leq 0.002$) with added levels of folic acid. The highest egg wt. was observed at 0 ppm of folic acid supplementation compared to 2 and 4 ppm. The decrease in egg wt. at higher levels of folic acid supplementation in this study could be attributed to the corresponding increase in percentage egg production observed at these levels. No choline by folic acid interaction effects ($p \geq 0.198$) were observed for egg wt.

There was choline by folic acid interaction effect ($p \leq 0.04$) on egg mass (Graph 2.2), which was calculated as grams of egg produced per day. Egg mass increased numerically with increasing levels of folic acid at 0 and 500 ppm of choline supplementation. At 1000 ppm of choline supplementation folic acid addition at 4 ppm decreased egg mass (52.8 g) compared to 0 ppm (55.4 g) and 2 ppm (56.8 g). This decrease in egg mass corresponds to the decrease in egg wt observed with this treatment combination.

Yolk wt showed ($p \leq 0.03$) choline by folic acid interaction effects (Graph 2.3). At 1000 ppm of choline supplementation, yolk wt was higher ($p \leq 0.006$) at 0 ppm of folic acid supplementation (15.5 g.) compared to 2 ppm of folic acid (15 g.). This increase in yolk wt. corresponded to a similar treatment effect on egg wt. Perhaps the increase in yolk wt could be attributed to increased deposition of choline. Similar results were observed by Tsiagbe et al. (1988), where an increase in yolk wt was recorded at 1000 ppm of choline addition to a corn soy based ration compared to ration with no choline supplementation. There was no difference in yolk wt between 0 and 4 ppm ($p \geq 0.164$) and 2 and 4 ppm ($p \geq 0.171$) of folic acid at the highest level of choline inclusion.

Albumen wt. also showed ($p \leq 0.005$) choline by folic acid interaction (Graph 2.4). At the lowest level of folic acid (0 ppm) inclusion, albumen wt. was highest (36.63 g) at 1000 ppm of choline; and at the highest level of folic acid supplementation (4 ppm), albumen wt. was lowest (34.93 g) in combination with 1000 ppm of choline. This may be due to the synergistic effect of these nutrients on the amino acid utilization. Albumen wt. showed a quadratic effect ($p \leq 0.0005$) with folic acid supplementation.

The interaction effect for Haugh units (Table 2.4) was approaching significance ($p \leq 0.07$). At 500 ppm of choline supplementation, Haugh units increased ($p \leq 0.016$) between 2 ppm (88.52) and 4 ppm (91.17) of folic acid. Similarly at 4 ppm of folic acid inclusion, there was a difference in Haugh units ($p \leq 0.012$) between 500 ppm and 1000 ppm of choline addition with higher Haugh units observed at 500 ppm and 4 ppm of choline and folic acid, respectively (Graph 2.4). This increase in Haugh unit may be reflection of higher egg wt observed with this treatment combination as Haugh Unit is a measure of egg wt and albumen ht.

Plasma folate (ng/mL) and egg folate (µg/egg) showed a difference with added levels of folic acid (Table 2.5). Supplementing folic acid at 2 and 4 ppm in the diet increased plasma folate concentration ($p \leq 0.0001$); egg folate content also increased with 2 and 4 ppm folic acid supplementation ($P=0.0001$). Plasma folate and egg folate showed a quadratic effect ($p \leq 0.0001$) with added levels of folic acid. The plasma folate concentration at 0, 2 and 4 ppm of dietary folic acid supplementation were 18.8, 30.7 and 37.4 ng/mL, respectively. The egg folate content at similar dietary folic acid supplementation was 18.2, 31.2 and 37.6 µg/egg. The results of this present study agree with the findings of Hoey et al. (2009) that it is possible to feed synthetic folic acid at higher levels to produce animal foods enriched with natural folate. The increase in plasma and egg folate with added dietary folic acid indicates a positive correlation of dietary folic acid content with plasma folate concentration and egg folate content. This result supports the theory that laying hens have highly efficient conservation and delivery systems for folate (Sherwood et al. 1993). The study further enhances the evidence of the sensitivity of egg folate concentrations to dietary folic acid levels. By adding folic acid to cereal based laying hen diets, it is possible to increase the folate content of eggs two to three fold.

Total phospholipids expressed on a dry matter basis (mg/g) of egg yolk (Table 2.6) did not differ with choline ($p \geq 0.332$) and/or folic acid ($p \geq 0.703$) supplementation. Egg phospholipid composition in terms of phosphatidylcholine (PC) on an mg/g basis did not show any difference with added choline ($p \geq 0.406$) or folic acid ($p \geq 0.649$). Similarly phosphatidylethanolamine (PE) also did not show any difference with added choline ($p \geq 0.217$) or folic acid ($p \geq 0.846$). The PC content per egg also did not show any difference when choline ($p \geq 0.327$) and folic acid ($p \geq 0.873$) were supplemented to the basal diet. The highest concentration of PC per egg (1063.7 mg) was observed at 1000 ppm of choline and 4 ppm of folic acid supplementation which was comparatively higher than the basal diet (1010.4 mg) with no supplementation. The non-significance observed for the PC content may be attributed to the small sample size (n= 4 per treatment) used in this particular study. PC: PE ratio also did not show treatment effects with added choline ($p \geq 0.368$) or folic acid ($p \geq 0.456$). A strong positive correlation was observed between the PC content of the egg and PC: PE ratio ($p \leq 0.0001$) and a negative correlation ($p \leq 0.03$) were observed between egg PE and phospholipid ratio (Table 2.7).

Crawford et al. (1969) showed that varying the choline content of the diet from 595 to 5729 mg/kg had no effect on tissue or egg yolk choline. However, the present study was aimed at looking the PC content of the egg rather than free choline present in egg in the former study. An increase in egg wt. when laying hen diets were supplemented with choline was related to changes in the phospholipid composition of the egg yolk by Tsiagbe et al. (1988). The results in this study indicate that in spite of the additional dietary supply of methyl groups, the inability to utilize the excess methyl groups through methyl transfer pathway for methylating PE to PC resulted in no increase in egg PC.

A large part of methionine in liver is converted to S-adenosylmethionine (SAM) most of which is used for the methylation of PE to form PC. The transfer of methyl groups from folic acid metabolite to homocysteine in the single carbon metabolic pathway, forming methionine is catalyzed by methionine synthase, a vitamin B_{12} dependent enzyme. In this study it is most likely that the availability of excess methyl groups with a minimal amount of vitamin B_{12} in the diet might have been a limiting factor affecting the methyl transfer pathway for choline synthesis. From the previous studies, it appears that in the absence of sufficient amount of vitamin B_{12}, the activity of methionine synthase enzyme is affected. This in turn influences the potential to make use of dietary methyl groups resulting in a methyl trap, and the amount of methionine available for phospholipid methylation may therefore be reduced. Since vitamin B_{12} functions in the synthesis of labile methyl groups from more highly oxidized one-carbon-atom compounds (Arnstein, 1958), in the utilization of the α-carbon of glycine for the synthesis of ethanolamine (Stekol et al. 1952) and in the conversion of formate into serine (Arnstein, 1958), an association between vitamin B_{12} and phospholipid synthesis may be expected. The insufficient response of dietary choline to increase egg PC in this study also suggests that the activity of CDP choline pathway for PC synthesis was minimal. This would imply that at the peak of the production cycle, the methyltransferase pathway would be more active in *de novo* choline synthesis. However, the relative proportion of PC synthesized via the CDP choline pathway compared with the methyltransferase pathway at different stages of the production cycle needs further study.

In summary, in the present study, the addition of choline and folic acid did impact the performance of the birds as reflected by an interaction effect of choline and folic acid on feed intake and egg quality parameters such as egg mass, albumen wt and yolk wt. There was a marginal but significant effect of choline and folic acid on egg wt and folic acid alone on egg production. However, there was a lack of response to added choline and folic acid on egg PC content. To our knowledge there is no information in the literature regarding the effect of supplementing dietary choline and folic acid on egg phospholipid composition that can be used for comparative purpose with those reported here. The information obtained from this present study indicates that the potential exists for increasing egg PC content. Supplementing vitamin B_{12} to the diet with added choline and folic acid could possibly moderate the methyl trap and increase the methyl transfer activity leading to increased utilization of dietary methyl groups for enhanced PC synthesis.

Acknowledgment

We wish to acknowledge the technical assistance of Dr Susan Cuppett, Department of Food Science and Technology, University of Nebraska Lincoln with HPLC determination. We also wish to thank Kumm's Kustom Pullets for providing pullets used in this study.

Table 2.1 Diet Composition of basal diet

Ingredients	%
Yellow Corn	63.19
Soybean meal	25.00
Limestone	8.52
DCP	1.75
Fat blend	0.93
Salt	0.32
DL- Methionine	0.19
Vitamin premix[1]	0.20
Mineral premix[2]	0.10

Nutrient composition	Calculated	Analyzed
ME kcal/kg	2775	2973.20
Protein %	16.7	16.53
TSAA %	0.75	-
Methionine %	0.46	-
Lysine %	0.84	-
Ca %	3.50	3.80
Total P %	0.42	0.61
Sodium %	0.15	-
Choline mg/kg	1089	972
Folic acid mg/kg	0.57	0.31

[1]Vitamin premix provided per kg of basal diet: Vitamin A 3000 IU, Vitamin D_3 300 IU, Vitamin E 5 IU, Vitamin K 0.5 mg, B_{12} 0.004 mg, Biotin 0.10 mg, Niacin 10mg, Pantothenate 2mg, Pyridoxine 2.5 mg, Riboflavin 2.5mg, Thiamin 0.7 mg.

[2]Mineral premix provided per kg of basal diet: Iodine 0.035 mg, Iron 45 mg, Manganese 20 mg, Selenium 0.06 mg and Zinc 35 mg.

Table 2.2 Choline and folic acid content in the experimental diets:

Experimental Diets	Calculated (ppm)		Analyzed (ppm)	
	Choline	Folic acid	Choline	Folic acid
1	1089	0.57	972	0.31
2	1089	2.57	972	1.93
3	1089	4.57	860	3.64
4	1589	0.57	1340	0.40
5	1589	2.57	1418	2.06
6	1589	4.57	1362	4.38
7	2089	0.57	1908	0.34
8	2089	2.57	1740	1.88
9	2089	4.57	1864	3.90

Table 2.3 Effect of choline and folic acid on feed intake, body wt and egg production

Choline (ppm)	Folic acid (ppm)	Feed Intake (g/hen/d)	Body wt change (kg/hen)	Egg Production (%)
0	0	105.4	-0.024	90.6
0	2	108.7	-0.002	94.3
0	4	109.4	-0.070	94.4
500	0	107.3	0.046	93.0
500	2	109.0	0.075	94.3
500	4	107.6	0.018	94.1
1000	0	108.3	-0.082	92.2
1000	2	107.5	-0.016	97.7
1000	4	107.3	0.082	90.9
SEM[1]		0.650	0.050	1.73
Main effects				
Choline (ppm)				
0		107.8	-0.032	93.1
500		108.0	0.046	93.8
1000		107.7	-0.005	93.9
SEM		0.397	0.029	1.14
Folic acid (ppm)				
0		107.0[a]	-0.020	91.9[a]
2		108.4[b]	0.018	95.4[b]
4		108.1[b]	0.010	93.2[ab]
SEM		0.397	0.029	1.14
Statistical probabilities				
Choline		0.86	0.172	0.87
Folic acid		0.02	0.616	0.04
Choline x Folic acid		0.006	0.232	0.17

Means with no common superscripts differ significantly ($p \leq 0.05$)

[1]SEM- Standard Error of Mean

Table 2.4 Effect of choline and folic acid on egg quality parameters

Choline (ppm)	Folic acid (ppm)	Egg wt (g)	Egg mass (g/day)	Yolk wt (g)	Albumen wt (g)	Haugh Unit
0	0	59.6	54.2	15.1	35.3	89.4
0	2	58.5	55.2	15.3	34.8	90.1
0	4	59.8	56.4	14.9	36.0	90.2
500	0	59.9	55.7	15.1	35.4	89.5
500	2	59.3	56.0	15.2	34.8	88.5
500	4	59.9	56.3	15.1	35.9	91.1
1000	0	60.0	55.4	15.5	36.6	89.4
1000	2	58.1	56.8	15.0	35.1	90.2
1000	4	58.2	52.8	15.2	34.9	88.4
SEM[1]		0.55	0.97	0.15	0.47	0.77
Main effects						
Choline (ppm)						
0		59.3[ab]	55.3	15.1	35.4	89.9
500		59.7[a]	56.0	15.1	35.4	89.7
1000		58.8[b]	55.0	15.2	35.5	89.3
SEM		0.39	0.59	0.11	0.38	0.44
Folic acid (ppm)						
0		59.9[a]	55.1	15.2	35.8[a]	89.4
2		58.7[b]	56.0	15.2	34.9[b]	89.6
4		59.3[ab]	55.2	15.1	35.6[ac]	89.9
SEM		0.39	0.59	0.11	0.38	0.44
Statistical probabilities						
Choline		0.06	0.47	0.40	0.830	0.64
Folic acid		0.01	0.49	0.33	0.003	0.74
Choline x Folic acid		0.19	0.04	0.03	0.005	0.07

Means with no common superscripts differ significantly ($p \leq 0.05$)
[1]SEM- Standard Error of Mean

Table 2.5 Effect of choline and folic acid on plasma and egg folate content:

Choline (ppm)	Folic acid (ppm)	Plasma folate (ng/mL)	Egg folate (µg/egg)
0	0	18.5	19.2
0	2	30.0	31.1
0	4	36.8	36.9
500	0	19.6	18.5
500	2	30.3	30.9
500	4	38.1	38.5
1000	0	18.5	16.9
1000	2	31.9	31.6
1000	4	37.3	37.5
SEM[1]		0.81	0.69
Main effects			
Choline (ppm)			
0		28.5	29.1
500		29.3	29.3
1000		29.2	28.7
SEM		0.47	0.40
Folic acid (ppm)			
0		18.8[a]	18.2[a]
2		30.7[b]	31.2[b]
4		37.4[c]	37.6[c]
SEM		0.47	0.40
Statistical probabilities			
Choline		0.38	0.54
Folic acid		0.0001	0.0001
Choline x Folic acid		0.48	0.12

Means with no common superscripts differ significantly (p≤0.05)

[1]SEM- Standard Error of Mean

Table 2.6 Effect of choline and folic acid on egg phospholipid composition

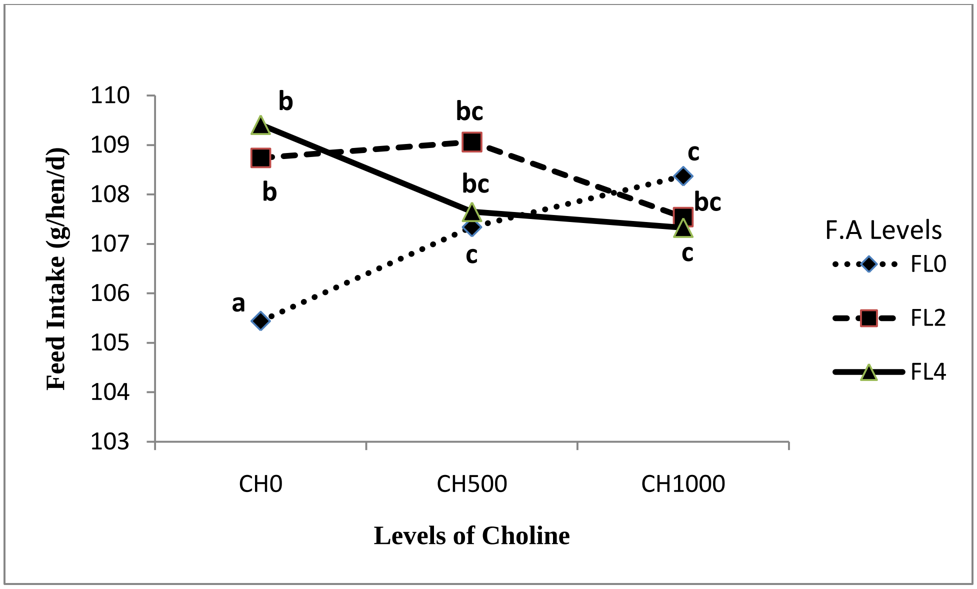
Choline (ppm)	Folic acid (ppm)	Total PL (mg/g)	PC (mg/g)	PE (mg/g)	PC: PE	PC/EGG (mg)	PE/EGG (mg)
0	0	183.2	141.5	32.6	4.4	1010.4	232.8
0	2	183.1	140.6	33.4	4.2	1015.8	241.3
0	4	178.4	136.9	31.5	4.4	963.6	221.5
500	0	177.4	134.8	32.4	4.2	958.6	230.5
500	2	182.5	142.5	30.8	4.6	1019.9	220.4
500	4	185.3	145.4	31.8	4.6	1034.4	226.7
1000	0	184.4	143.4	32.3	4.5	1046.7	235.8
1000	2	184.7	142.5	32.1	4.5	1006.7	227.0
1000	4	191.2	148.1	33.7	4.4	1063.7	242.5
SEM[1]		4.898	4.646	0.73	0.12	36.231	6.236
Main effects							
Choline (ppm)							
0		181.6	139.7	32.5	4.3	996.6	231.9
500		181.7	140.9	31.7	4.5	1004.2	225.9
1000		186.8	144.7	32.7	4.5	1039.0	235.1
SEM		2.968	2.682	0.42	0.007	20.917	3.60
Folic acid (ppm)							
0		181.7	139.9	32.4	4.3	1005.2	233.1
2		183.4	141.9	32.1	4.5	1014.1	229.6
4		185.0	143.4	32.3	4.5	1020.6	230.2
SEM		2.968	2.682	0.42	0.007	20.917	3.60
Statistical probabilities							
Choline		0.33	0.40	0.21	0.36	0.32	0.20
Folic acid		0.70	0.64	0.84	0.45	0.87	0.77
Choline x Folic acid		0.62	0.52	0.10	0.18	0.33	0.09

[1]SEM- Standard Error of Mean

Table 2.7 Correlations between added choline, folic acid, egg wt, yolk wt, PC, PE and PC: PE ratio of egg yolk

Variable	Choline	Folic acid	PC:PE	PE	PC	Yolk wt
Egg wt, g	-0.08	-0.09	0.007	-0.20	-0.13	0.42
	0.21	0.17	0.96	0.22	0.41	0.0001
Yolk wt,g	0.05	-0.07	-0.004	0.19	0.14	
	0.39	0.26	0.97	0.24	0.38	
PC, mg/g	0.23	0.16	0.71	0.41		
	0.17	0.33	0.0001	0.01		
PE, mg/g	0.05	-0.02	-0.34			
	0.74	0.89	0.03			
PC:PE	0.18	0.18				
	0.27	0.26				

Significance levels are shown below correlation values.

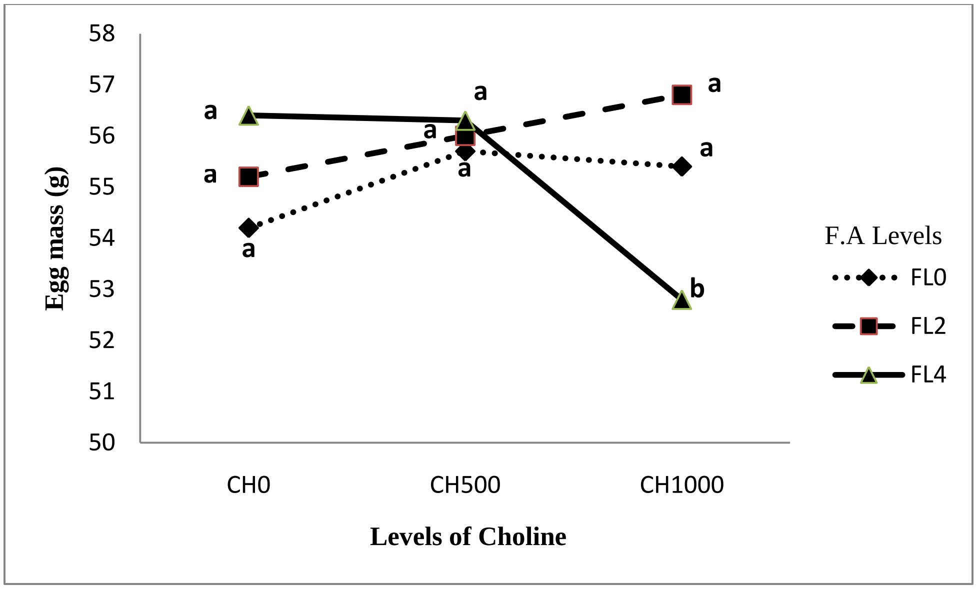


Graph 2.1 Choline and folic acid interaction effect on feed intake

Means with no common superscripts differ significantly ($p \leq 0.05$)

CH0, CH500, CH1000 represents 0,500 and 1000 ppm of choline

FL0, FL2, FL4 represents 0, 2 and 4 ppm of folic acid

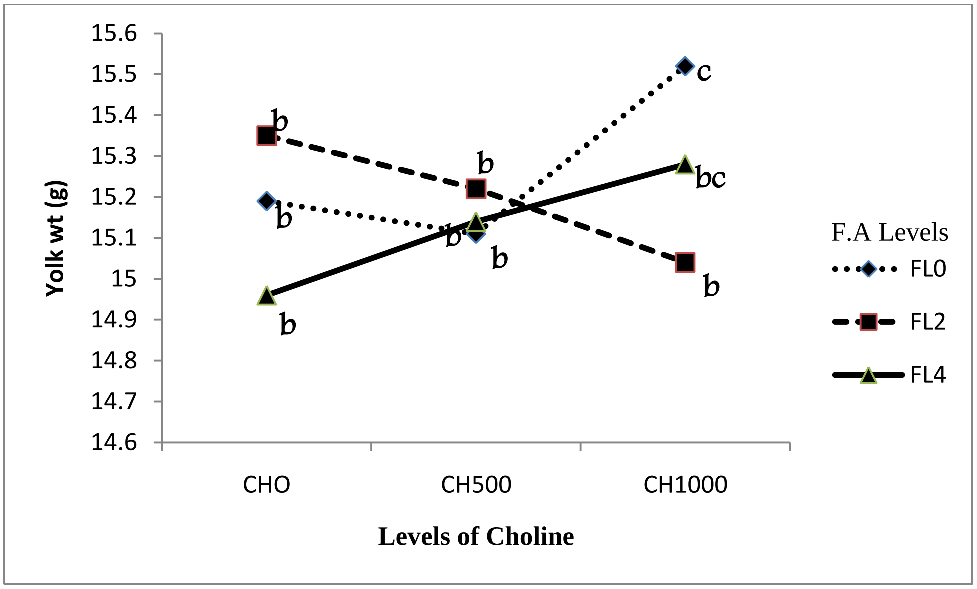


Graph 2.2 Choline and folic acid interaction effect on egg mass

Means with no common superscripts differ significantly ($p \leq 0.05$)

CH0, CH500, CH1000 represents 0,500 and 1000 ppm of choline

FL0, FL2, FL4 represents 0, 2 and 4 ppm of folic acid

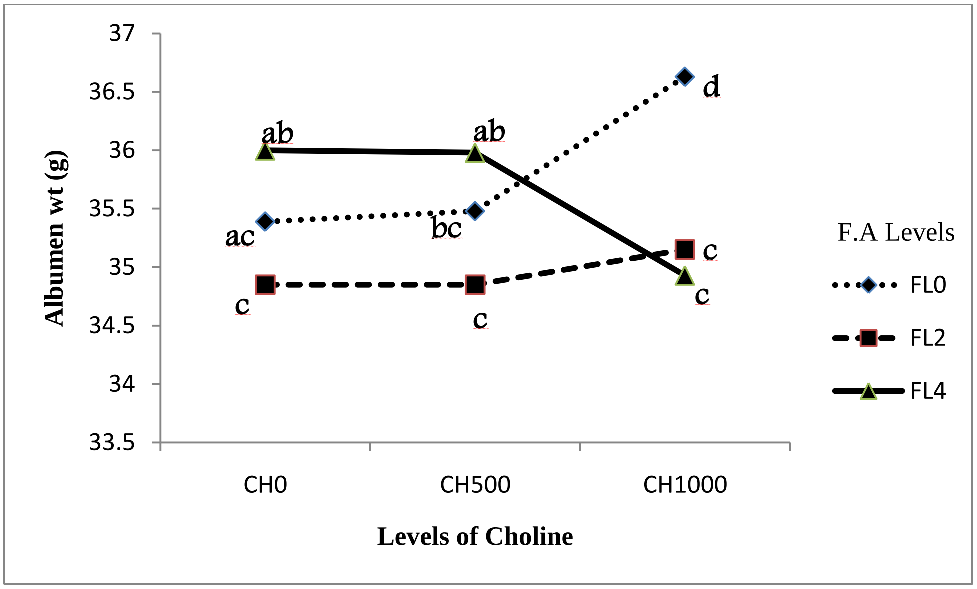


Graph 2.3 Choline and folic acid interaction effect on yolk wt

Means with no common superscripts differ significantly ($p \leq 0.05$)

CH0, CH500, CH1000 represents 0,500 and 1000 ppm of choline

FL0, FL2, FL4 represents 0, 2 and 4 ppm of folic acid



Graph 2.4 Choline and folic acid interaction effect on albumen wt

Means with no common superscripts differ significantly ($p \leq 0.05$)

CH0, CH500, CH1000 represents 0,500 and 1000 ppm of choline

FL0, FL2, FL4 represents 0, 2 and 4 ppm of folic acid

CHAPTER-3

Effect of Choline, Folic acid and Vitamin B_{12} on Egg Components and Egg Phospholipid Composition in Laying Hens

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ABSTRACT This study was designed to determine the effect of added choline, folic acid and vitamin B_{12} to a corn-soy basal diet on egg production, egg quality and egg yolk phospholipid composition. A corn-soy basal diet was formulated with 2 levels supplemental choline (500 and 1000 ppm), 2 levels supplemental folic acid (2 and 4 ppm), and 2 levels supplemental vitamin B_{12} (0.01 and 0.02 ppm) in a 2 x 2 x 2 factorial arrangement along with a control (no supplementation) group. Yolk wt showed choline x vitamin B_{12} interaction ($p \leq 0.001$). Plasma folate and vitamin B_{12} concentration increased with supplementation of folic acid ($p \leq 0.0001$) and vitamin B_{12} ($p \leq 0.0001$), respectively. Phosphatidylcholine (PC) showed an increase ($p \leq 0.0001$) with added levels of choline, folic acid or vitamin B_{12}. The average value of PC at 500 and 1000 ppm of choline was 152.61 and 164.53 mg/g, respectively. Similarly, the average values for PC at 2 and 4 ppm of folic acid and 0.01 and 0.02 ppm of vitamin B_{12} were 153.06, 164.07, 155.68 and 161.46 mg/g, respectively. Results indicate that choline, folic acid and vitamin B_{12} can be supplemented in a synergetic combination to increase egg yolk phosphatidylcholine content by 20 to 25 % compared to no supplementation.

Key words: Choline, Folic acid, Vitamin B_{12}, Egg phosphatidylcholine

Introduction

Phosphatidylcholine (PC) is the principal phosphorus containing lipid of animal tissues. The majority of choline in animal tissues is found in these specialized fat molecules. Most of the egg yolk consists of lipids in the form of lipoproteins. The similarity in the protein and lipid composition of plasma very low density lipoprotein (VLDL) and egg yolk VLDL provide the evidence that this component is transferred from plasma to yolk in a relatively unchanged form (Gornall and Kuksis, 1973). Phosphatidylcholine comprises a substantial proportion of the phospholipids of hen's plasma and egg yolk VLDL. The capacity of animal tissues to synthesize phospholipids could be concluded from the fact that phospholipids are not essential dietary constituents. Egg yolk is a good source of phosphatidylcholine. Choline is primarily utilized for the synthesis of vital lipid components of the cell membranes, phosphatidylcholine (Zeisel and Blusztajn, 1991).

The metabolic pathways of choline, methionine, methyl folate and vitamin B_{12} are interdependent. The pathways of choline and one carbon metabolism meet at the formation of methionine from homocysteine. In vitro studies indicate the laying hen to have efficient phosphatidylcholine (lecithin) synthesizing ability. Levels of cytidine diphosphate choline were high in hen liver mitochondria (Kennedy and Weiss, 1956) and hen liver microsomes were as efficient as rat liver microsomes in synthesizing lecithin from S-adenosyl methionine and phosphatidylethanolamine (Bremer and Greenberg, 1960). Several components of the diet are important for the supply and turnover of single carbon compounds in the body, such as methionine, choline, vitamin B_{12} and folic acid. Folic acid along with vitamin B_{12} in the diet may influence phospholipid levels (Blumenstein, 1964). High levels of dietary choline was required to maintain maximum egg weight in laying Japanese quail with added methionine, folic acid and vitamin B_{12} (Latshaw and Jensen, 1972). Tsiagbe et al. (1988) observed a relationship between phospholipid content and wt. of eggs when diets of laying hens were supplemented with choline. Phosphatidylcholine is the most widely distributed phospholipid and in many tissues makes up about half the total phospholipid fraction (Ansell and Hawthrone, 1964). Phospholipids form 28-30% of egg lipids with phosphatidylcholine comprising about 80% of the total phospholipids (Garland and Powrie, 1978).

The current recommendations for choline intake in laying hens is 105 mg/day at feed intake levels of 100 g/day (NRC, 1994), implying a dietary choline concentration of approximately 1100 mg/kg diet. Crawford et al. (1969) indicated that significant increases in choline content of eggs and body tissues coincided with peak egg production. The NRC recommendation for folic acid intake at feed intake levels of 100 g/day is 0.25 mg/kg diet. Folate content of eggs can be increased to threefold by increasing the crystalline folic acid in the laying hen diet to 4 mg/kg (House et al., 2002). The requirement for vitamin B_{12} at feed intake levels of 100 g/day in laying hens is 0.004 mg/kg diet. Even though many studies have been conducted to determine the effect of methyl group donors and their interaction on the performance of laying hens, information is lacking on the combined effects of these nutrients on egg phospholipid composition.

The results from the previous study indicate that in spite of the additional dietary supply of methyl groups, the inability to utilize the excess methyl groups through methyl transfer pathway for methylating PE to PC resulted in no increase in egg PC. Our hypothesis was that supplementation of choline and folic acid may generate additional methyl groups and add to the active methyl pool and addition of vitamin B_{12} to the diet would affect the metabolism of methyl groups. This may bring about a more active methyl transferase activity leading to an increase in

PC synthesis. The objective of this study was to determine the interactive effect of supplemental choline, folic acid and vitamin B_{12} on the phospholipid composition of the egg yolk and thereby the potential of eggs to be fortified with choline.

Materials and Methods

The study was carried out at the poultry research facility of the Department of Animal Science, University of Nebraska for a period of six weeks. Animal care approval was given by the Institutional Animal Care and Use Committee (IACUC) of University of Nebraska (Protocol # 06-10-044D).

Two hundred and sixteen, 29-week-old Single Comb White Leghorn Hy-Line® W-36 hens[24] were housed in layer cages (Chore-Time)[25] at a density of four birds per cage (400 cm^2/bird). The birds had ad-libitum access to feed and water during the study and were exposed to a 16 hour photoperiod.

A 2 x 2 x 2 factorial arrangement with two levels of supplemental choline (500 and 1000 ppm), two levels of supplemental folic acid (2 and 4 ppm), and two levels of supplemental vitamin B_{12} (0.01 and 0.02 ppm) along with a control were assigned in a randomized complete block design resulting in a total of nine experimental treatments. Blocking was done in order to minimize the effect of temperature stratification in the cage unit. Each treatment was assigned to six replicate cages with four birds per cage for a total of fifty four cages. Hens were fed the dietary treatments for six weeks.

[24] M.G.Waldbaum Co, 501 Main St, Wakefield, NE-68784
[25] Chore-TimeBrock, Inc. A Berkshire Hathaway Company, 611 North Higbee Street, P.O. Box 2000, Milford, IN 46542-2000,U.S.A.

A corn-soy basal diet (Table 3.1) was formulated to be isocaloric (2874 kcal ME/kg) and isonitrogenous (16 % CP) and to meet the NRC (1994) requirements for laying hens. Basal diet was formulated by taking out choline (choline chloride, 60%)[26] and folic acid (folic acid, 10%)[27] and folic acid from the vitamin premix and the required levels of these nutrients were added to the diets according to meet the experimental treatment levels. The basal diet was calculated to contain 1089 mg/kg of choline, 0.57 mg/kg folic acid, and 0.004 mg/kg vitamin B_{12} respectively. The analyzed values for choline, folic acid and vitamin B_{12} in the basal diet were 943, 0.38 and 0.002 mg/kg, respectively. The calculated and analyzed values for choline, folic acid and vitamin B_{12} of the experimental diets are shown in Table 3.2.

Dietary samples were saved from each diet. The samples were sieved through a 1 mm screen, ground and stored for analysis of gross energy (GE), N, Ca, P, choline, folic acid and vitamin B_{12}. Gross energy was determined using a Parr adiabatic oxygen bomb calorimeter[28]. Dietary N was determined using the Kjeldahl method as established by the Association of Official Analytical Chemists (A.O.A.C, 1984). The N content in the diet was multiplied by 6.25 to determine protein content of the diet. Calcium and phosphorus were determined by spectrophotometric procedure established by the A.O.A.C. (1984). Choline content of the feed was analyzed by the Reineckate procedure as established by A.O.A.C. (1965). It involved the extraction of choline containing lipids, formation and separation of a water insoluble Reinecke salt and then determination of absorption of the acetone soluble Reinecke solution by the colorimetric[29] measurement of the supernatant at 520 millimicrons.

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[26] International Nutrition, 7706 I Plaza, Omaha, NE 68127-1841, U.S.A
[27] International Nutrition, 7706 I Plaza, Omaha, NE 68127-1841, U.S.A
[28] Parr Instrument Company, 211 Fifty third Street, Moline, Illinois, 61265-9984
[29] Shimadzu Scientific Instruments, 7102 Riverwood Drive, Columbia, MD, 21046 U.S.A

The amount of folic acid present in the feed was analyzed using HPLC method as described by Poo-Prieto et al. (2006). The feed samples and the folic acid standard[30] (2 g) were homogenized in 10 mL extraction buffer (0.075 M phosphate buffer containing 1% ascorbate and 0.1% mercaptoethanol) and were heated for 15 min at 120°C, and cooled in ice. The homogenate samples were treated with trienzyme (α- amylase, rat plasma and protease)[31] and incubated at 37°C for 2 hrs. The samples were then kept in a boiling water bath for 5 min, cooled in ice and centrifuged for 15 min at 36,000 x g. The supernatants were then filtered through a Millipore microfilter[32] with a pore size of 0.45 µm. Samples were analyzed using a Waters[33] 600 chromatograph equipped with a UV detector at 280 nm. The 20 µL of samples and standard were injected into a variant Microsorb C18 column (250 x 4 mm, packed with 5 µm silica) by elution with 10 % acetonitrile and 30 mM phosphate buffer (pH 2.3) at a flow rate of 1 mL/min. The linear regression equation obtained from the standard curve was used for estimating the folic acid content of the feed samples. Vitamin B_{12} in the feed was determined by the procedure as established by Heudi et al. (2006). 1 g of the feed sample was weighed into a 250 mL Erlenmeyer flask. Vitamin B_{12} was extracted using 60 mL of 50 mM sodium acetate buffer pH 4. The samples were treated with 1 mL of 1% sodium cyanide, α- amylase[34] and pepsin[35] and incubated at 42°C for 3 hrs. The solution was then heated in a boiling water bath for 30 min, cooled and was filtered through Whatman filter paper # 1 (110mm) to a 100 mL volumetric flask and the volume was made up with distilled water. The purified samples were then analyzed using liquid chromatography using a C18 column (250 x 4 mm, packed with 5 µm

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[30] Sigma-Aldrich, 3050 Spruce St, St. Louis, MO, 63103 U.S.A
[31] Fisher Scientific, U.S.A.
[32] Fisher Scientific, U.S.A.
[33] Waters Corporation, 34 Maple St, Milford, MA 01757, U.S.A
[34] Fisher Scientific, U.S.A.
[35] Fisher Scientific, U.S.A.

silica) with elution with water and 0.025 % trifluoroacetic acid and acetonitrile at a flow rate of 0.2 mL/min. The absorbance was monitored using a UV detector at 361 nm. The vitamin B_{12} content of the samples was quantified using an external standard curve.

Hens were weighed at the start and end of the trial and the average hen wt. by cage was calculated to determine body weight change. Feed consumption was recorded on a daily basis and the intake was calculated per hen per day. Eggs were collected daily and percentage egg production (EP) was calculated on a hen/d basis.

Egg weights were recorded every week on one day of egg production. Egg components (yolk, albumen and shell) were measured on a weekly basis on two eggs per replicate pen. After 6 wks on trial, six eggs per treatment were collected and used to determine the egg phospholipid composition (Christie, 1985). Eggs were cracked and yolk was separated from albumen without rupturing the vitelline membrane. Yolks were rolled onto tissue paper (in order to totally remove albumen) and weighed. Yolks were individually broken, homogenized and lyophilized completely. Dry matter was determined and the dry samples were frozen at -20°C. The dry yolk was finely ground and sieved through a 1mm screen for a uniform sample. A sample of 0.5 g of egg yolk was weighed into a centrifuge tube and was extracted thrice for maximum extraction using HPLC grade methanol (Fletcher et al., 1984). The sample was vortexed for 15 sec, the mixture was centrifuged (3000 x g for 15 min), the supernatant was filtered through Whatman filter paper # 1 (110mm) into a 25 mL volumetric flask and made up to volume with HPLC grade methanol. The filtrate was again filtered for further purification of the sample through a 30 mm HPLC syringe filter made of regenerated cellulose with a pore size of 0.45 μm.

The phospholipid composition was determined by high performance liquid chromatography. The following procedure was a modification of the gradient elution system developed by Christie (1985). Phospholipids were analyzed using a Hewlett Packard 1050 HPLC[36] system equipped with a Beckman 110B solvent delivery module[37] and a 20 µL sample loop. Separation was achieved using a Microporasil column (10µm 125A 3.9x300mm column, Waters)[38] mounted to a HP 3395 Integrator[39] and absorbance was measured at 214 nm. The solvent system was HPLC grade methanol: acetonitrile (70:30 v/v) at a flow rate of 1.5 mL/min. Samples of 0.02 mL were injected for analysis. Aliquots of sample and standard solutions (L-a-phoshphatidylcholine (500mg) and L-a-phosphatidylethanolamine (100mg)[40] dissolved in methanol/acetonitrile (70:30) were injected in the column at ambient temperature. A 79853C Variable Wavelength UV detector (HP)[41] was used for quantitative determination. Linear regression modeling was used to plot peak area versus concentration for each phospholipid class. The linear equations thus obtained were used for the quantitative measurements of phosphatidylcholine (PC) and phosphatidylethanolamine (PE).

Blood samples were collected from two birds per replicate pen via wing venipuncture to determine plasma folate and vitamin B_{12} levels. Blood samples were collected with a 5 mL sterile syringe and were transferred to a 5 mL vacutainer tube with EDTA. The samples were cooled on ice and immediately centrifuged at 12,000 x g for 5 min and retained and stored at -80°C until analysis. Plasma folate and B_{12} concentrations were determined using a competitive

[36] Lab Extreme, Inc Kent City, MI 49330
[37] Lab Extreme, Inc Kent City, MI 49330
[38] Waters Corporation, 34 Maple Street, Milford, MA 01757
[39] Quantum Analytics, Inc. 363 Vintage Park Drive, Foster City, CA 94404
[40] Indofine Chemical Company, 121 Strykerlane, Hillsborough, NJ 08844
[41] Lab Extreme, Inc Kent City, MI 49330

binding assay, SimulTRAC B_{12} [^{57}Co]/Folate[^{125}I] radioassay kit purchased from ICN Pharmaceuticals[42].

Data were analyzed for repeated measures by ANOVA using the Mixed Model procedure of SAS software (Proc Mixed, 2001; SAS Institute, Inc., Cary, NC). Each cage of hens represented an experimental unit for analysis. Repeated measures were done to ensure treatment is effective over a specified period. Repeated measures analysis was used to measure the average treatment effect over time and the possible treatment by time interactions. Repeated measures analysis was also done to identify the possible covariance patterns in the repeated measurements and to determine the appropriate model to describe the time and measurement relationship. The appropriate covariance pattern for model fit was selected for each measurement using the information criteria. The data were tested for the main effects of choline, folic acid, vitamin B_{12} and their interactions. Significance was reported at $p<0.05$. The model used for the data analysis was

$$Y = \mu + C_i + F_j + V_K + L_l + T_m + CF_{ij} + CV_{ik} + FV_{jk} + CFV_{ijk} + E_{ijklmn}$$

where μ is the overall mean; C_i is the effect of choline; F_j is the effect of folic acid; V_k is the effect of vitamin B_{12}; L_l is the effect of location; T_m is the effect of time; CF_{ij} is the interaction of choline and folic acid; CV_{ik} is the interaction of choline and vitamin B_{12}; FV_{jk} is the interaction of folic acid and vitamin B_{12}; CFV_{ijk} is the interaction of choline, folic acid and vitamin B_{12}; E_{ijklmn} is the residual error. Main effects of choline, folic acid, vitamin B_{12}, location, time and choline by folic acid interaction, choline by vitamin B_{12}, folic acid by vitamin B_{12} and choline by

[42] ICN Pharmaceuticals, Diagnostic Division, 13 Mountain View Avenue, Orangeburg, New York 10962-1294

folic acid by vitamin B_{12} interactions were evaluated. The separation of means was done using LS means statement with pdiff option.

The one time measurements of the data for body wt gain, plasma folate and vitamin B_{12} concentration and for egg phospholipid were analyzed using the model

$$Y = \mu + C_i + F_j + V_K + L_l + CF_{ij} + CV_{ik} + FV_{jk} + CFV_{ijk} + E_{ijklm}$$

where μ is the overall mean; C_i is the effect of choline; F_j is the effect of folic acid; V_k is the effect of vitamin B_{12}; L_l is the effect of location; CF_{ij} is the interaction of choline and folic acid; CV_{ik} is the interaction of choline and vitamin B_{12}; FV_{jk} is the interaction of folic acid and vitamin B_{12}; CFV_{ijk} is the interaction of choline folic acid and vitamin B_{12}; E_{ijklm} is the residual error. Main effects of choline, folic acid, vitamin B_{12}, location, time and choline by folic acid interaction, choline by vitamin B_{12}, folic acid by vitamin B_{12} and choline by folic acid by vitamin B_{12} interactions were evaluated. The separation of means was done using LS means statement with pdiff option. Data were also tested for comparing control with the experimental treatments using Dunnett test.

Results and Discussion

The calculated and analyzed values for choline, folic acid and vitamin B_{12} in the experimental diets are presented in Table 3.2. The calculated value for choline in the experimental diets was 1589 and 2089 ppm at supplemental levels of 500 and 1000 ppm, respectively. The analyzed values for choline in the diet varied from 1306 to 1420 ppm when 500 ppm of choline was supplemented in the diet. The values ranged from 1764 to 1936 ppm at 1000 ppm of choline addition. Folic acid was supplemented at levels of 2 and 4 ppm to the experimental diet and the overall calculated value for folic acid in the diet was 2.57 and 4.57

ppm. The analyzed values for folic acid in the experimental diets ranged from 1.78 to 2.03 ppm and 3.66 to 4.10 ppm at 2 and 4 ppm of folic acid supplementation respectively. Vitamin B_{12} was supplemented at 0.01 and 0.02 ppm to have 0.014 and 0.024 ppm of overall vitamin B_{12} in the experimental diets. However, the analyzed values for vitamin B_{12} at 0.01 ppm supplemental level were in the range of 0.007 to 0.009 ppm. At 0.02 ppm of vitamin B_{12} supplementation the analyzed value of B_{12} was in the range of 0.013 to 0.016 ppm. The analyzed values for choline, folic acid and vitamin B_{12} were lower than the calculated values. The percentage variations in the analyzed vitamin B_{12} between diets when supplemented at 0.01 and 0.02 ppm were 42.5, 44.3, 39.3, 34.3% and 44.6, 32.5, 44.2 and 40.9%, respectively. As per the established guidelines for analytical variations associated with the analysis of the nutrient content of the feed or ingredients by Association of American Feed Control Official (AAFCO, 2009), which are designed to serve as a reference point for determining acceptability of feeds and ingredients based on laboratory results, the acceptable Analytical Variation (AV %) based on AAFCO check sample program for vitamin B_{12} is 45%. In the present study, addition of choline, folic acid and vitamin B_{12} to the laying hen diets did not impact the performance of the hens such as feed intake, body wt gain or percent egg production (Table 3.3). This was likely due to the relatively short experimental period (6 weeks). The present experiment was not primarily designed with the intent to measure the impact of these nutrients on productivity. Although increasing dietary choline did not affect egg production, it could be concluded that the basal diet met the choline requirement (1040 mg/kg) and was sufficient to maintain egg production. The amount of nutrients available was adequate to satisfy the needs of the hens under the conditions of this experiment which reflected in no difference. The failure of supplemental choline to improve egg production of birds fed basal diet formulated to contain 16% protein indicate that these basal diets had enough TSAA to satisfy birds need for egg production.

There was no difference in egg wt. with added levels of choline, folic acid or vitamin B_{12} (Table 3.4). There was a choline x vitamin B_{12} interaction effect ($p \leq 0.001$) on yolk wt. At 500 ppm of choline supplementation, yolk wt was higher when vitamin B_{12} was added at 0.01 ppm and at 1000 ppm of choline supplementation; yolk wt was higher when vitamin B_{12} was added at 0.02 ppm (Graph 3.1). The yolk wt was greater ($p \leq 0.0291$) in all the experimental treatments supplemented with choline, folic acid and vitamin B_{12} compared to the control group. The increase in yolk wt observed in the experimental treatments compared to control may be explained by the increased phospholipid deposition in the egg yolk in the experimental treatments. A strong positive correlation ($p \leq 0.0001$) was observed between egg wt and yolk wt (Table 3.7).

Albumen wt. showed choline x folic acid x vitamin B_{12} interaction effects ($p \leq 0.057$). At 500 ppm of choline, addition of vitamin B_{12} (0.01 ppm) to 2 and 4 ppm folic acid levels did not affect albumen wt. However, at this level of choline supplementation, albumen wt increased at 4 ppm of folic acid level when vitamin B_{12} content was increased to 0.02 ppm (Graph 3.2). At 1000 ppm of choline, vitamin B_{12} addition at 0.01 ppm to folic acid levels increased albumen wt whereas vitamin B_{12} addition at 0.02 ppm to folic acid levels decreased albumen wt (Graph 3.3). It could be concluded that factors affecting egg wt. do not operate in the same way on the yolk and on the white and that the liver and the magnum of the oviduct respond differently to, or have different affinities or priorities for the nutrients provided through circulation. The difference in albumen wt. observed with the treatment combinations was not following any pattern and could be most likely due to the physiological differences associated with each hen in depositing the amount of albumen stored in the albumen secreting portion of the oviduct.

Shell wt. showed folic acid x vitamin B_{12} interaction effects ($p \leq 0.0329$). Shell wt decreased at higher levels of folic acid (4 ppm) when vitamin B_{12} was added at 0.01 ppm with no difference in shell wt when vitamin B_{12} was added at 0.02 ppm to supplemental folic acid levels (2 and 4 ppm) (Graph 3.4). Shell wt. was lower in the experimental treatments compared to the control ($p \leq 0.024$). The decrease in shell wt. observed with experimental treatments could be more likely a function of increase in the yolk content in this treatment groups with subsequent reduction in the amount of shell.

Plasma folate concentration showed a significant increase ($p \leq 0.0001$) with increasing levels of folic acid in the diet (Table 3.5). Plasma folate level increased from 18.7 ng/mL for birds consuming the basal diet with no folic acid supplementation to 37.4 ng/mL for birds consuming diet with 4 ppm of folic acid. Plasma folate is a sensitive indicator to measure folate adequacy (Jacques et al. 1993) and it was observed in this study that dietary folic acid content correlated positively with plasma folate concentration. Supplementation of vitamin B_{12} in the diet significantly increased ($p \leq 0.0001$) plasma vitamin B_{12} concentration (Table 3.5). The plasma vitamin B_{12} concentration at 0, 0.01 and 0.02 ppm of vitamin B_{12} supplementation was 40.2, 41.4 and 44.1 ng/mL, respectively. The plasma vitamin B_{12} concentration was increased by almost 10% with dietary supplementation of vitamin B_{12} at 0.02 ppm. This result was in agreement with that of Bunchasak and Kachana (2009) wherein the serum vitamin B_{12} was shown to increase with dietary vitamin B_{12} supplementation.

The egg phospholipid composition expressed in terms of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (mg/g of yolk) showed main and interaction effects ($p \leq 0.0001$) (Table 3.6). The PC content of egg yolk (mg/g) in the control diet was 139.15 mg; however when dietary choline was supplemented at levels of 500 ppm and 1000 ppm the PC content of the egg yolk was 152.61 and 164.53 mg/g, respectively ($p \leq 0.0001$). Similarly, at levels of 2 and 4 ppm of folic acid supplementation, the PC content of egg yolk was 153.06 and 164.07 mg/g ($p \leq 0.0001$) and at 0.01 and 0.02 ppm levels of vitamin B_{12} addition, the PC content of egg yolk was 155.68 and 161.46 mg/g, respectively ($p \leq 0.0001$). Overall, the highest amount (176.29 mg/g) was measured in the diet with the most vitamin supplementation (1000 ppm choline, 4 ppm folic acid and 0.02 ppm of vitamin B_{12}). The PE content (mg/g) of egg yolk also showed main and interaction effect ($p \leq 0.0001$) with added levels of choline, folic acid or vitamin B_{12}. The PE content of the egg yolk in the control diet was 39.46 mg/g. PE showed a decrease with added levels of choline at 500 and 1000 ppm ($p \leq 0.0001$) and the values being 34.72 and 33.77 mg/g, respectively. Similarly with dietary supplementation of folic acid (2 and 4 ppm) and vitamin B_{12} (0.01 and 0.02 ppm) also the PE concentration of the egg yolk was reduced ($p \leq 0.0001$) to 35.19, 33.30, 35.62 and 32.82 mg/g, respectively.

PC content calculated on an egg basis (mg/egg) showed main ($p \leq 0.0001$) and interaction effects ($p \leq 0.03$) with added levels of choline, folic acid and vitamin B_{12} (Table 3.6). PC content of control group with no supplementation of choline, folic acid or vitamin B_{12} was 1111.8 mg/egg. PC content of egg supplemented with 500 and 1000 ppm of dietary choline was 1234.8 and 1299.1 mg/egg, respectively. Supplementation of folic acid (2 and 4 ppm) and vitamin B_{12} (0.01 and 0.02 ppm) also increased the phosphatidylcholine content of the egg to 1233.5, 1300.1, 1217.3 and 1316.6 mg/egg, respectively. The interaction effect (Graph 3.5) show at 500 ppm of choline supplementation, vitamin B_{12} at 0.01 and 0.02 ppm increased PC at all levels of folic acid inclusion. At 1000 ppm of choline supplementation, vitamin B_{12} addition at 0.01 ppm to folic acid levels did not increase PC content whereas addition of vitamin B_{12} at 0.02 ppm increased PC with levels of folic acid (Graph 3.6). PC: PE ratio showed two way interaction effects between choline and folic acid ($p \leq 0.0001$), choline and vitamin B_{12} ($p \leq 0.01$) and folic acid and vitamin B_{12} ($p \leq 0.0001$). PC: PE ratio was 3.52 for the group fed the basal diet with no supplementation (Table 3.6). When the basal diet was supplemented with 500 and 1000 ppm of choline egg there was significant increase in PC and decrease in PE content which reflected a higher PC: PE ratio of 4.41 and 4.90, respectively. An increase in PC: PE ratio resulted from folic acid and vitamin B_{12} addition and the values being 4.35, 4.95, 4.37 and 4.94 for 2 and 4 ppm of folic acid and 0.01 and 0.02 ppm of vitamin B_{12}, respectively. At 500 and 1000 ppm of choline addition increasing the levels of folic acid in the diet increased phospholipid ratio (Graph 3.7). PC: PE content also increased at 500 and 1000 ppm of choline supplementation when vitamin B_{12} content was increased in the diet (Graph 3.8). Similarly, there was an increase in the egg PC: PE ratio at 2 and 4 ppm folic acid addition with an increase in the vitamin B_{12} in the diet (Graph 3.9).

The increase in PC and subsequent reduction in PE with choline addition would suggest that base exchange of choline for ethanolamine in PE increased with choline supplementation. The addition of 1000 ppm of choline resulted in a 25 mg increase in PC and a 6 mg decrease in PE on an mg/g basis would tend to suggest an active CDP choline pathway for the synthesis of phosphatidylcholine. Supplementation of folic acid and vitamin B_{12} also increased the phosphatidylcholine content of the egg yolk which suggests a more active methyltransferase activity in de novo choline synthesis. Serum phospholipids have been previously reported to be elevated with dietary supplementation of folic acid (Bunchasak and Kachana, 2009). Supplementation of folic acid and vitamin B_{12} should increase the concentration of methylated folate in the liver. An increase in the concentration of methylated folate reflects in an increase in the availability of S-adenosylmethionine (SAM). This intermediary metabolite in the single carbon metabolism serves as a universal methyl donor for phospholipid synthesis.

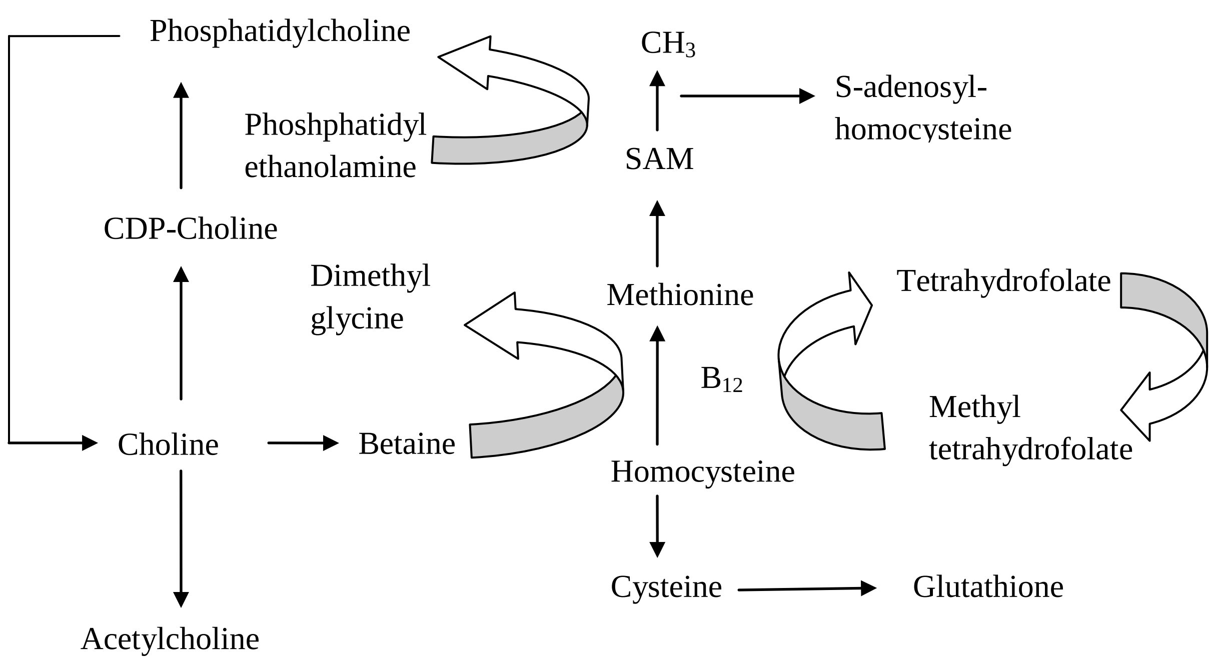

Figure 3.1 Metabolic pathways for choline and its relationship with folic acid and vitamin B_{12} as methyl carrier for methylation pathways

A strong positive correlation was observed between added levels of choline, folic acid and vitamin B_{12} on egg PC content (Table 3.7). Similarly a negative correlation was observed between folic acid ($p \leq 0.0003$) and vitamin B_{12} ($p \leq 0.0001$) on egg PE. This would suggest that with added levels of folic acid and vitamin B_{12} more PC was formed from PE through the methyl transfer pathway.

In this particular study with Hy-Line® hens, feed intake was comparatively lower than in the previous study with Bovan hens. Similar results in Hy-line® hens was reported by Harms and Russell (1999) wherein hens were shown to consume less energy per g of egg content compared to Bovan hens. This strain difference indicates a genetic difference in dietary feed consumption and supporting the fact that efficiency of energy utilization is an inherited characteristic. Similarly, egg wt and albumen wt were shown to be higher in Bovan hens compared to Hy-Line® hens. It appears that genetic selection for increased feed consumption and larger egg size in the Bovan strain hens resulted in more egg albumen and less yolk lipid compared to Hy-Line® hens. Sensitivity and responsiveness to increasing dietary choline, folic acid and vitamin B_{12} in terms of increasing egg PC content was higher in this study with Hy-Line® hens. It appears from the previous studies that separate enzymes are responsible for the formation of PC and PE. The higher increase in egg PC content seen in the current study with Hy-Line® hens suggests the presence of a more active methyl transferase enzyme system in this strain of White Leghorns compared to Bovan. However, conclusions can only be drawn with future research directed towards the enzyme activity associated with PC synthesizing pathways. Considering egg PC concentrations, a strain-specific requirement for folic acid and vitamin B_{12} could be deduced as the higher egg producing strain benefited from increased folic acid and vitamin B_{12} through an increase in egg PC.

Increasing choline, folic acid and vitamin B_{12} of the laying hen diet increased the egg PC content, thus offering the potential to improve the nutritional image of the egg. Choline makes up 13 % of the PC by wt; thus with a PC content of 1433 mg per egg would thus provide 186 mg of choline. One choline enriched egg could provide approximately one third of the AI for choline (550 mg/day in adult male and 450 mg/day in adult female). Vitamin costs are typically less than 1.5 % of the total feed cost (Ward, 1993). The additional cost per ton of feed with supplementation of choline[43], folic acid[44] and vitamin B_{12}[45] at 1000, 4 and 0.02 ppm to the basal diet in this study was $2.33. At 90% production and an average 92 g of feed intake per hen per day in this study, this equates to an additional cost of 0.29 cents per dozen of eggs produced compared to the basal diet. Even though common table eggs can be crafted into extraordinary source of natural choline, if the investment to produce choline enriched egg is economically favorable needs further consideration.

In summary, supplementing choline, folic acid and vitamin B_{12} to a corn-soy basal diet showed improvement in terms of yolk wt. in the experimental treatments compared to the control. The phospholipid composition was also affected with an increase in PC content of egg and subsequent reduction in the egg PE concentration. The results of this study thus offer an optimistic view of the role of dietary methyl groups on egg phospholipid composition.

Acknowledgement

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[43] International Nutrition, Choline chloride, 60% - 59 cents/lb
[44] International Nutrition, Folic acid, 10% - $1.56/lb
[45] International Nutrition, Vitamin B_{12} 1% - $8.25/lb

Table 3.1 Diet Composition of basal diet

Ingredients	%	
Yellow Corn	65.90	
Soybean meal	20.53	
Limestone	8.98	
DCP	1.97	
Fat blend	1.81	
Salt	0.37	
DL- Methionine	0.20	
Vitamin premix[1]	0.20	
Mineral premix[2]	0.10	
Nutrient composition	Calculated	Analyzed
ME kcal/kg	2874	2746
Protein %	16.00	16.30
TSAA %	0.72	-
Methionine %	0.45	-
Lysine %	0.88	-
Ca %	3.93	3.57
Total P %	0.48	0.66
Sodium %	0.17	-
Choline mg/kg	1089	943
Folic acid mg/kg	0.57	0.38
Vitamin B_{12} mg/kg	0.004	0.002

[1]Vitamin premix provided per kg of basal diet: Vitamin A 3000 IU, Vitamin D_3 300 IU, Vitamin E 5 IU, Vitamin K 0.5 mg, B_{12} 0.004 mg, Biotin 0.10 mg, Niacin 10mg, Pantothenate 2mg, Pyridoxine 2.5 mg, Riboflavin 2.5mg, Thiamin 0.7 mg.

[2]Mineral premix provided per kg of basal diet: Iodine 0.035 mg, Iron 45 mg, Manganese 20 mg, Selenium 0.06 mg and Zinc 35 mg.

Table 3.2 Choline, folic acid and vitamin B_{12} content in the experimental diets:

Experimental Diets	Calculated (ppm)			Analyzed (ppm)		
	Choline	Folic acid	B_{12}	Choline	Folic acid	B_{12}
1	1089	0.57	0.004	943	0.38	0.002
2	1589	2.57	0.014	1420	1.87	0.008
3	1589	2.57	0.024	1347	2.03	0.013
4	1589	4.57	0.014	1306	3.66	0.007
5	1589	4.57	0.024	1382	3.89	0.016
6	2089	2.57	0.014	1860	1.78	0.008
7	2089	2.57	0.024	1936	1.90	0.013
8	2089	4.57	0.014	1764	4.10	0.009
9	2089	4.57	0.024	1912	3.96	0.014

Table 3.3 Choline, folic acid and vitamin B_{12} on feed intake, body wt and egg production

Choline	Folic acid (ppm)	B_{12}	Feed Intake (g/hen/d)	Body wt gain (kg/hen)	Egg Production (%)
0	0	0	93.3	0.098	93.4
500	2	0.01	93.0	-0.017	92.1
500	2	0.02	93.0	-0.013	92.0
500	4	0.01	91.6	-0.197	91.0
500	4	0.02	93.2	0.008	90.9
1000	2	0.01	93.2	0.038	92.9
1000	2	0.02	93.0	-0.025	89.9
1000	4	0.01	92.1	0.023	90.7
1000	4	0.02	92.5	0.031	91.4
SEM			1.20	0.083	1.08
Main effect					
Choline (ppm)					
500			92.7	-0.056	91.5
1000			92.8	0.017	91.4
SEM[1]			1.34	0.065	0.54
Folic acid (ppm)					
3			93.1	-0.004	91.8
4			92.3	- 0.034	91.1
SEM			1.34	0.065	0.54
Vitamin B_{12} (ppm)					
0.01			92.4	-0.039	91.7
0.02			93.0	0.0004	91.3
SEM			1.34	0.065	0.54
Statistical probabilities					
Ctrl vs Trt			0.70	0.11	0.36
Choline			0.86	0.13	0.93
Folic acid			0.19	0.53	0.32
Vitamin B_{12}			0.36	0.41	0.59
Choline x Folic acid			0.93	0.29	0.72
Choline x Vitamin B_{12}			0.57	0.17	0.72
Folic acid x Vitamin B_{12}			0.35	0.15	0.28
Choline x Folic acid x Vitamin B_{12}			0.61	0.48	0.26

[1]SEM- Standard Error of Mean

Table 3.4 Effect of choline, folic acid and vitamin B_{12} on egg quality parameters

Choline (ppm)	Folic acid (ppm)	B_{12} (ppm)	Egg wt (g)	Yolk wt (g)	Albumen wt (g)	Shell wt (g)
0	0	0	57.0	14.7	33.8	7.7
500	2	0.01	57.3	15.5	33.9	7.8
500	2	0.02	56.4	15.3	33.4	7.6
500	4	0.01	56.6	15.6	33.9	7.5
500	4	0.02	56.6	15.1	33.8	7.6
1000	2	0.01	56.4	15.4	33.4	7.4
1000	2	0.02	56.9	15.5	34.0	7.3
1000	4	0.01	57.4	15.3	34.6	7.3
1000	4	0.02	56.5	15.5	33.8	7.4
SEM[1]			0.40	0.11	0.30	0.09
Main effects						
Choline (ppm)						
500			56.7	15.4	33.7	7.6
1000			56.8	15.4	33.9	7.4
SEM			0.22	0.05	0.17	0.07
Folic acid (ppm)						
2			56.7	15.4	33.7	7.5
4			56.8	15.3	34.0	7.5
SEM			0.22	0.05	0.17	0.07
Vitamin B_{12} (ppm)						
0.01			56.9	15.5	33.9	7.5
0.02			56.6	15.3	33.7	7.5
SEM			0.22	0.05	0.17	0.07
Statistical probabilities						
Ctrl vs Trt			0.55	0.0001	0.99	0.02
Choline			0.88	0.49	0.31	0.0001
Folic acid			0.90	0.35	0.10	0.19
Vitamin B_{12}			0.27	0.07	0.41	0.90
Choline x Folic acid			0.38	0.85	0.53	0.09
Choline x Vitamin B_{12}			0.60	0.001	0.73	0.80
Folic acid x Vitamin B_{12}			0.73	0.53	0.37	0.03
Choline x Folic acid x Vitamin B_{12}			0.06	0.24	0.057	0.57

Means with no common superscripts differ significantly ($p \leq 0.05$)

[1]SEM- Standard Error of Mean

Table 3.5 Effect of choline, folic acid and vitamin B_{12} on plasma folate and B_{12} concentration:

Choline	Folic acid (ppm)	B_{12}	Plasma folate (ng/mL)	Plasma B_{12} (ng/mL)
0	0	0	18.7	40.2
500	2	0.01	29.8	40.7
500	2	0.02	30.5	43.5
500	4	0.01	36.5	41.5
500	4	0.02	36.9	44.4
1000	2	0.01	31.4	41.4
1000	2	0.02	31.5	44.5
1000	4	0.01	36.3	42.0
1000	4	0.02	37.4	44.1
SEM			0.53	0.59
Main effects				
Choline (ppm)				
500			33.4	42.5
1000			34.1	42.9
SEM[1]			0.27	0.34
Folic acid (ppm)				
2			30.8[a]	42.5
4			36.7[b]	43.0
SEM			0.27	0.34
Vitamin B_{12} (ppm)				
0.01			33.5	41.4[a]
0.02			34.1	44.1[b]
SEM			0.27	0.34
Statistical probabilities				
Ctrl vs Trt			0.0001	0.0001
Choline			0.07	0.26
Folic acid			0.0001	0.28
Vitamin B_{12}			0.14	0.0001
Choline x Folic acid			0.18	0.33
Choline x Vitamin B_{12}			0.97	0.72
Folic acid x Vitamin B_{12}			0.67	0.53
Choline x Folic acid x Vitamin B_{12}			0.40	0.54

Means with no common superscripts differ significantly ($p \leq 0.05$)

[1]SEM- Standard Error of Mean

Table 3.6 Effect of choline, folic acid and vitamin B_{12} on egg phospholipid composition

Choline	Folic acid (ppm)	B_{12}	PC (mg/g)	PE (mg/g)	PC/EGG (mg)	PC:PE
0	0	0	139.1	39.4	1111.8	3.5
500	2	0.01	145.9	36.1	1152.9	4.0
500	2	0.02	150.6	34.3	1242.3	4.3
500	4	0.01	154.4	35.9	1274.4	4.2
500	4	0.02	159.3	32.4	1269.6	4.9
1000	2	0.01	156.1	36.4	1217.9	4.2
1000	2	0.02	159.5	33.8	1320.8	4.7
1000	4	0.01	166.1	34.1	1224.1	4.8
1000	4	0.02	176.2	30.6	1433.5	5.7
SEM			0.529	0.31	36.646	0.04
Main effects						
Choline (ppm)						
500			152.6[a]	34.7[a]	1234.8[a]	4.4[a]
1000			164.5[b]	33.7[b]	1299.1[b]	4.9[b]
SEM[1]			0.289	0.15	16.376	0.02
Folic acid (ppm)						
2			153.0[a]	35.1[a]	1233.5[a]	4.3[a]
4			164.0[b]	33.3[b]	1300.1[b]	4.9[b]
SEM			0.289	0.15	16.376	0.02
Vitamin B_{12} (ppm)						
0.01			155.6[a]	35.6[a]	1217.3[a]	4.3[a]
0.02			161.4[b]	32.8[b]	1316.6[b]	4.9[b]
SEM			0.289	0.15	16.376	0.02
Statistical probabilities						
Crtl vs Trt			0.0001	0.0001	0.0026	0.0001
Choline			0.0001	0.0001	0.0088	0.0001
Folic acid			0.0001	0.0001	0.0066	0.0001
Vitamin B_{12}			0.0001	0.0001	0.0001	0.0001
Choline x Folic acid			0.0001	0.0004	0.7485	0.0001
Choline x Vitamin B_{12}			0.0050	0.4267	0.0191	0.01
Folic acid x Vitamin B_{12}			0.0001	0.0062	0.8941	0.0001
Choline x Folic acid x Vitamin B_{12}			0.0001	0.4095	0.0373	0.1274

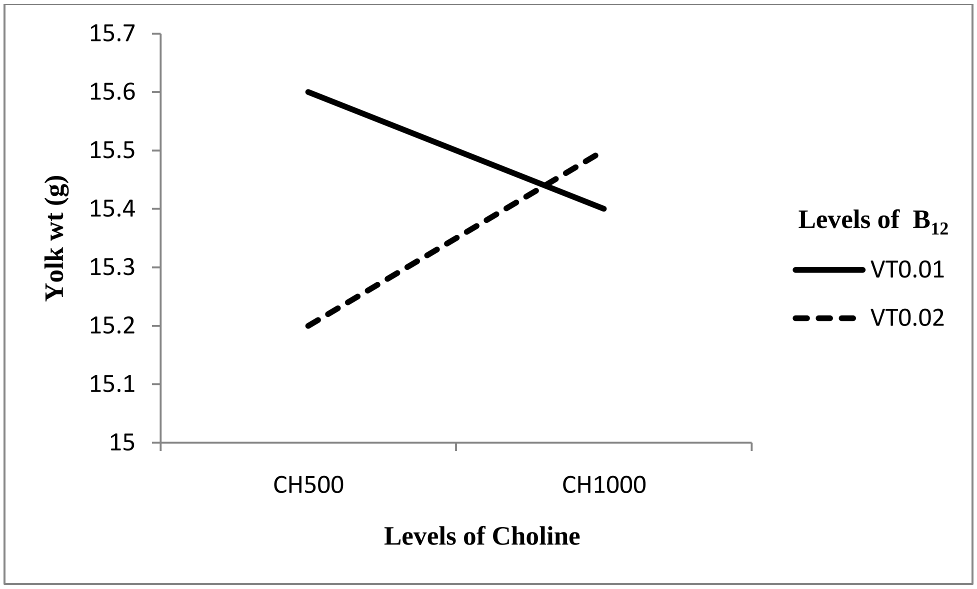
Means with no common superscripts differ significantly ($p \leq 0.05$)

[1]SEM- Standard Error of Mean

Table 3.7 Correlations between added choline, folic acid, vitamin B_{12}, egg wt, yolk wt, PC,PE and PC:PE ratio of egg yolk

Variable	Choline	Folic acid	Vitamin B_{12}	PC:PE	PE	PC	Yolk wt
Egg wt, g	0.006	0.009	-0.067	-0.08	0.08	-0.08	0.61
	0.91	0.87	0.25	0.54	0.56	0.55	0.0001
Yolk wt,g	0.03	-0.05	-0.09	-0.03	0.04	-0.03	
	0.52	0.38	0.10	0.80	0.77	0.83	
PC, mg/g	0.67	0.63	0.32	0.93	-0.73		
	0.0001	0.0001	0.02	0.0001	0.0001		
PE, mg/g	-0.22	-0.50	-0.71	-0.92			
	0.12	0.0003	0.0001	0.0001			
PC:PE	0.48	0.58	0.55				
	0.0001	0.0001	0.0001				

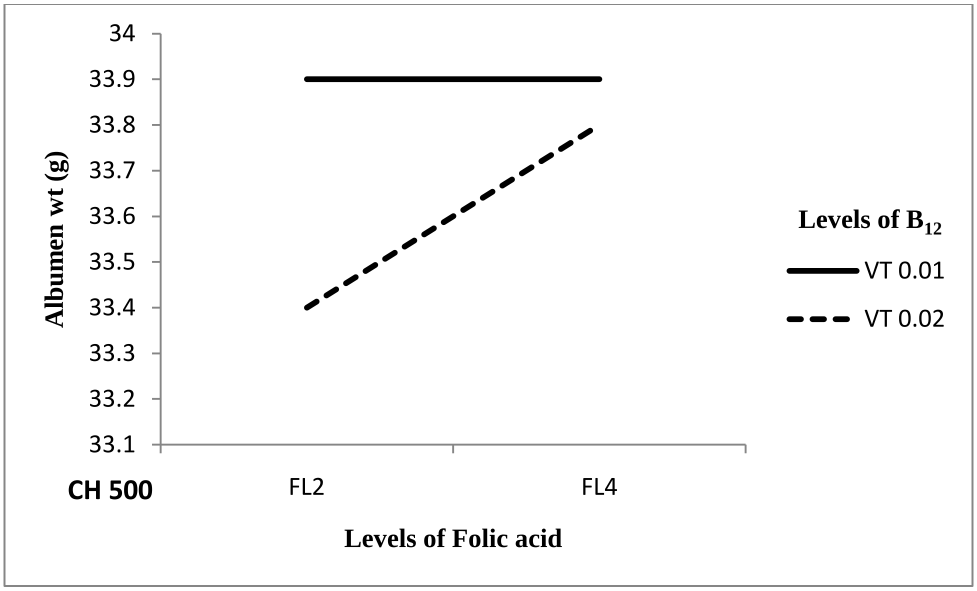
Significance levels are shown below correlation values.



Graph 3.1 Choline and vitamin B_{12} interaction effect on yolk wt

CH 500, CH1000 represents 500 and 1000 ppm of choline

VT 0.01, VT 0.02 represents 0.01 and 0.02 ppm of vitamin B_{12}

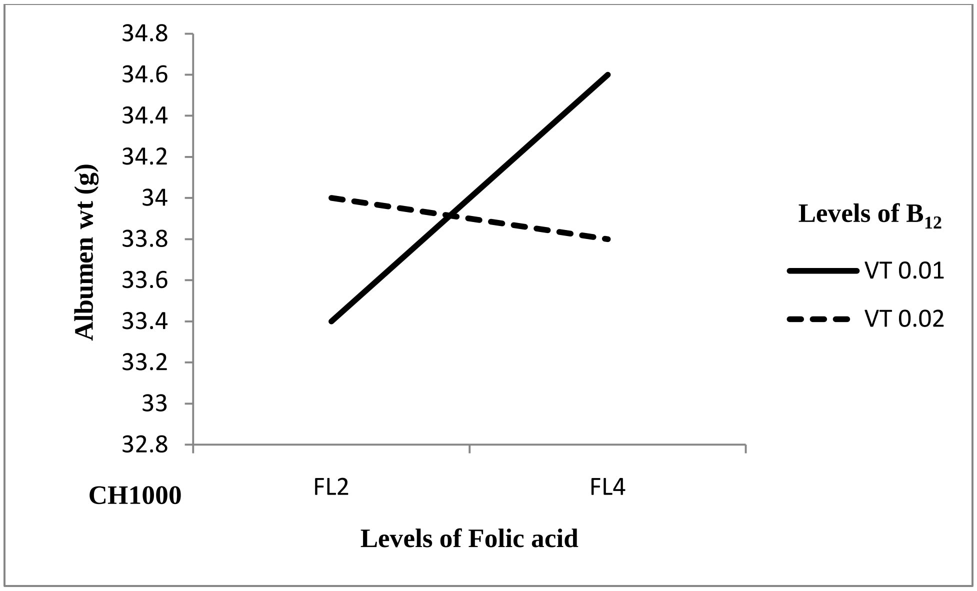


Graph 3.2 Choline, folic acid and vitamin B_{12} interaction effect on albumen wt

CH500 represents 500 ppm of choline

FL2, FL4 represents 2 and 4 ppm of folic acid

VT 0.01, VT 0.02 represents 0.01 and 0.02 ppm of vitamin B_{12}

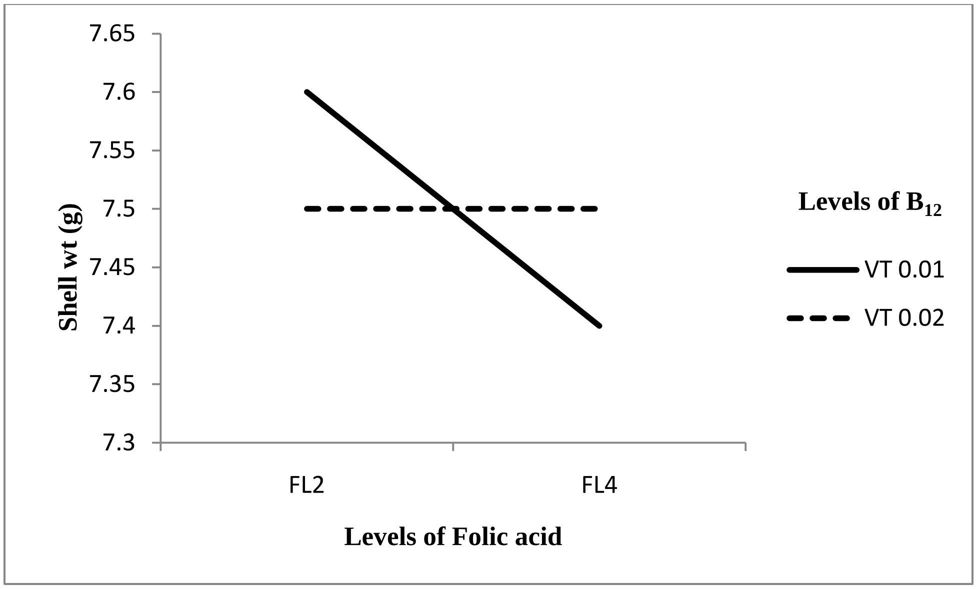


Graph 3.3 Choline, folic acid and vitamin B_{12} interaction effect on albumen wt

CH1000 represents 1000 ppm of choline

FL2, FL4 represents 2 and 4 ppm of folic acid

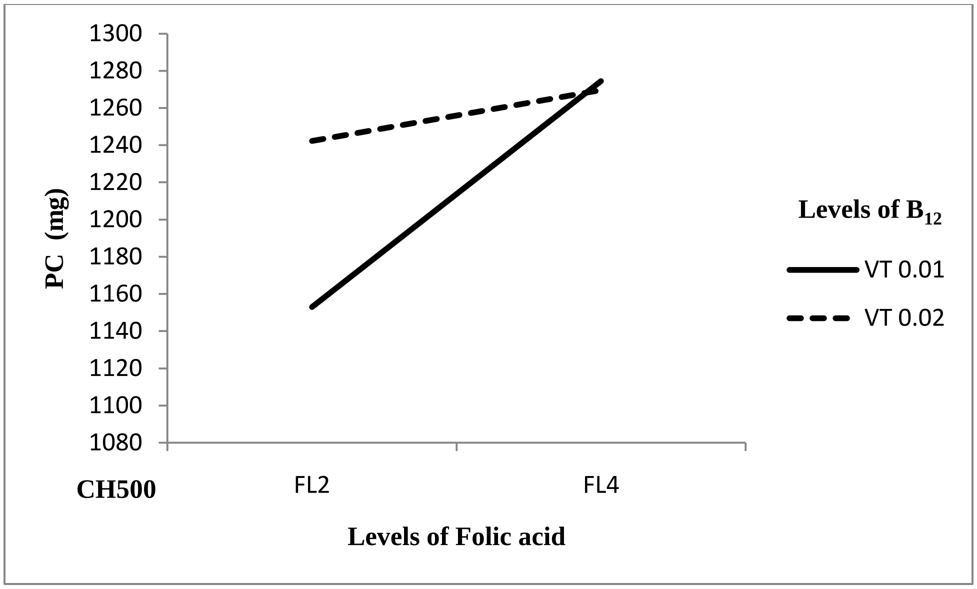
VT 0.01, VT 0.02 represents 0.01 and 0.02 ppm of vitamin B_{12}



Graph 3.4 Folic acid and vitamin B_{12} interaction effect on shell wt

FL2, FL4 represents 2 and 4 ppm of folic acid

VT 0.01, VT 0.02 represents 0.01 and 0.02 ppm of vitamin B_{12}

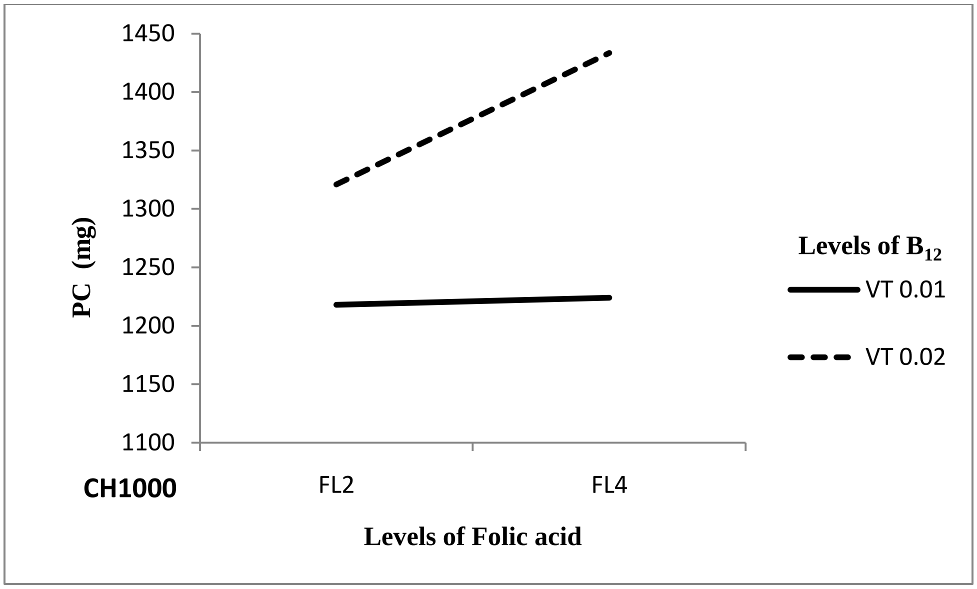


Graph 3.5 Choline, folic acid and vitamin B_{12} interaction effect on egg PC

CH500 represents 500 ppm of choline

FL2, FL4 represents 2 and 4 ppm of folic acid

VT 0.01, VT 0.02 represents 0.01 and 0.02 ppm of vitamin B_{12}

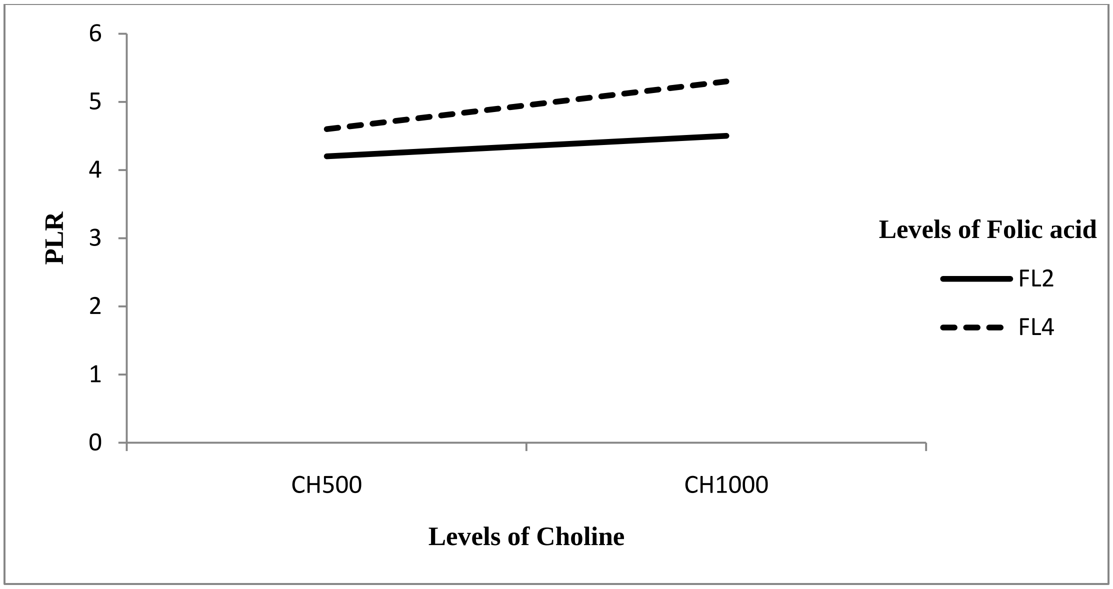


Graph 3.6 Choline, folic acid and vitamin B_{12} interaction effect on egg PC

CH1000 represents 1000 ppm of choline

FL2, FL4 represents 2 and 4 ppm of folic acid

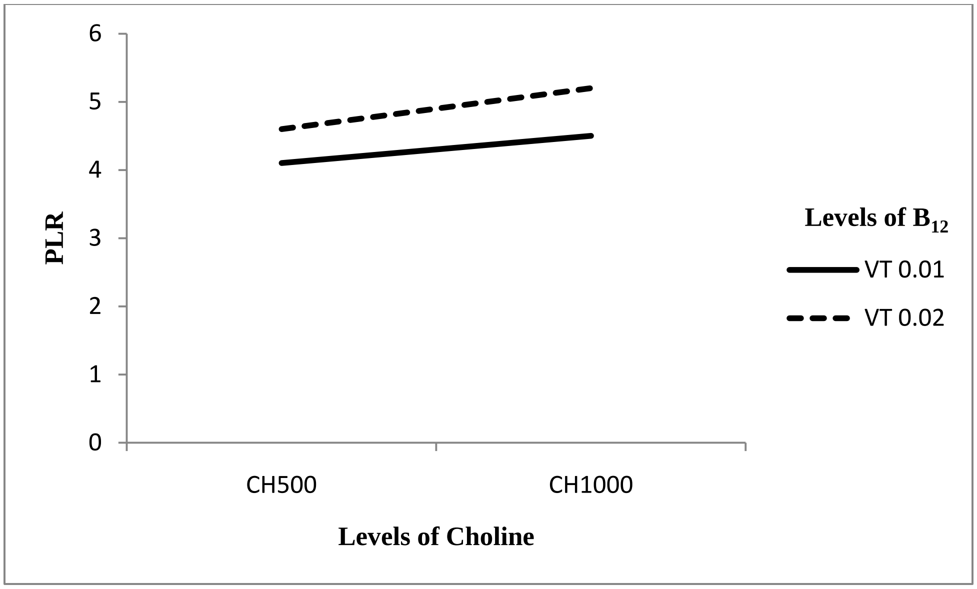
VT 0.01, VT 0.02 represents 0.01 and 0.02 ppm of vitamin B_{12}



Graph 3.7 Choline and folic acid interaction effect on Phospholipid Ratio

CH500, CH1000 represents 500 and 1000 ppm of choline

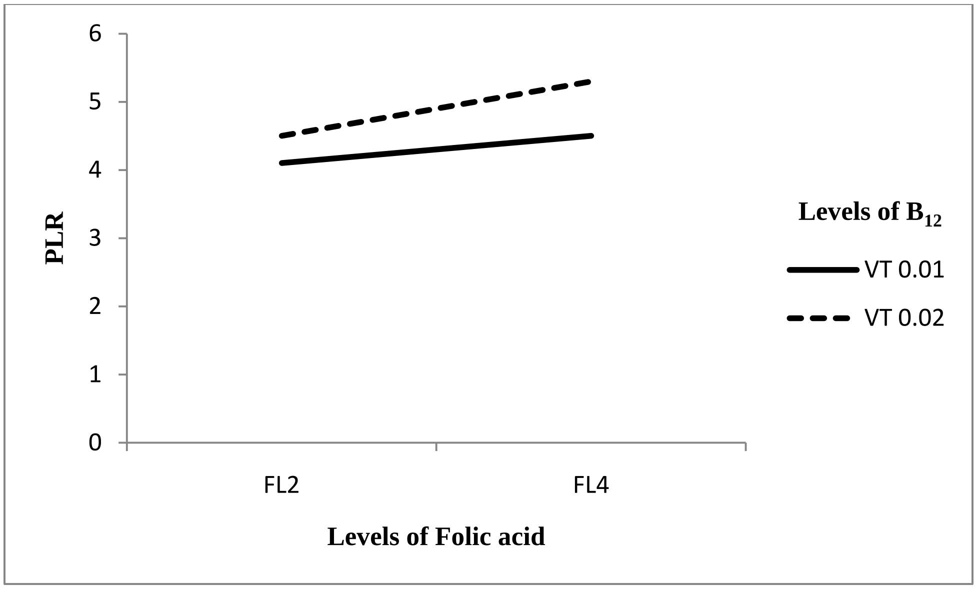
FL2, FL4 represents 2 and 4 ppm of folic acid



Graph 3.8 Choline and vitamin B_{12} interaction effect on Phospholipid Ratio

CH500, CH1000 represents 500 and 1000 ppm of choline

VT 0.01, VT 0.02 represents 0.01 and 0.02 ppm of folic acid



Graph 3.9 Folic acid and vitamin B_{12} interaction effect on Phospholipid Ratio

FL2, FL4 represents 2 and 4 ppm of folic acid

VT 0.01, VT 0.02 represents 0.01 and 0.02 ppm of vitamin B_{12}

Conclusion

The focus of this research has been to improve the phospholipid profile of egg yolk through dietary manipulation in laying hens. The study addressed the impact of supplementing dietary nutrients choline, folic acid and vitamin B_{12} in combination on the phospholipid composition of the egg yolk. The hypothesis of the study was that elevating the metabolic generation and use of active methyl groups in the hen would improve the phosphatidylcholine content, the major phospholipid of egg yolk, through activation of phosphatidylcholine synthesizing pathways. To evaluate this hypothesis, nutrient which can donate methyl groups like choline and folic acid were supplemented in the laying hen diet. Vitamin B_{12} was also enriched in the diet to improve the methyl group utilization. The methodology was designed with different combination levels of these nutrients followed by the quantitative analysis of the collected data. The results of the study demonstrated that supplementing choline along with folic acid improved the phosphatidylcholine content of the egg and the maximum deposition resulted from added levels of vitamin B_{12} along with choline and folic acid. It has been implied that the deposition of phosphatidylcholine could be increased by the collective activation of all the phosphatidylcholine synthesizing pathways. It has also been argued that maximal deposition of phosphatidylcholine with added levels of vitamin B_{12} was due to increased activity of the methyl transfer pathway. It can be concluded that 1000 ppm choline, 4 ppm folic acid and 0.02 ppm vitamin B_{12} supplementation to a corn-soy basal diet, can increase the phosphatidylcholine content of egg by 20 to 25 %. Therefore, the hypothesis of this study was supported: metabolic generation and use of active methyl groups have indeed improved the phospholipid profile of egg yolk.

Considerations beyond the traditional measures of productivity and egg quality are needed to reflect the growing awareness and concern that consumers have regarding the nutritional quality of foods they are selecting. Per capita annual egg consumption in the U.S. declined from a high of 31.6 dozen in 1950 to a low of 20.7 dozen in 2009. This reduction was due to numerous factors, chief among them was the fact that eggs represent a significant source of dietary cholesterol. The perceived link between eggs, dietary cholesterol, serum cholesterol, and the risk for cardiovascular disease has been fostered over the last three decades. More recent studies have provided strong evidence that the impact of dietary cholesterol on serum low-density lipoprotein cholesterol is not as strong as originally stated. More specifically for eggs, it has been shown that, in healthy, non diabetic subjects, the risk for the development of cardiovascular disease was not significantly different between those consuming less than 1 egg/wk versus those consuming greater than 1 egg/d. The availability of specialty or designer eggs was conducive in leveling off of decline in egg consumption. However, there is an increasing need for further enhancements to the nutritional image of egg to maintain/increase egg consumption in the future.

Understanding the factors that influence the level of choline in eggs is critical for ensuring the delivery of a product to consumers that has a consistent level of this key nutrient. The potential to improve the nutritional image of eggs by increasing the choline content of the egg offers the egg as an important vehicle to increase choline intake by humans. Increased awareness of the need to increase choline intake in an effort to reduce the risk of cardiovascular and hepatic diseases as well as its use in preventing memory disorders and cancer presents an opportunity to specifically market choline enriched eggs to this target population. Early work with human subjects revealed intestinal problems with bacterial degradation of free choline to trimethylamine in the small intestine. However, choline in the form of PC was well tolerated at rates up to 100 g/d. These attributes will position phosphatidylcholine enriched egg as a good vehicle for the delivery of choline into the human diet.

Future Research

The role of choline in nutrition has been investigated continuously since it was found to reduce the accumulation of fat in the liver. Subsequently, other functions of choline in animals and humans have been discovered. Research in human nutrition has focused on the potential for choline to alleviate various disease conditions. Future research in laying hens should be directed towards the activity of enzymes involved in choline synthesizing pathways at different stages of production cycle and also additional research is required to assess production factors, such as level of production, age of flock and dietary factors that are likely to influence egg choline content in order to determine the optimal strategies for the production of choline-enriched eggs. Attachment to binding proteins within hepatocytes and transport via the systemic circulation is the putative mechanism whereby choline is transported to the developing yolk. It would be of interest to focus on studies to elucidate the key control points that regulate the choline transport and deposition in an avian model system.

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Appendices

Appendix A. Gross Energy Determination of feed

Approximately 1 g of the sample was pelleted in Parr pellet press. The combustion capsule was weighed accurately, and with a forceps, the pellet was placed in the capsule and was weighed. The bomb calorimeter was charged with the sample and a 10 cm long fuse (nickel alloy wire) was attached to the electrodes and the wire loop was allowed to touch the surface of the charge containing the capsule. 1ml of distilled water was added to the bottom of the bomb which functions as a sequestering agent and absorbent. The bomb was carefully closed and was filled with oxygen (30 atmospheric pressure). The calorimetric bucket was filled with 2 liters of distilled water. The bucket was set in the calorimeter and the lifting handles were attached to the two holes in the side of the screw cap and the bomb was lowered into the water with its feet spanning the circular boss in the bottom of the bucket. Calorimeter cover was closed and the thermometer bracket was lowered. The power switch was turned on and the calorimeter was run for 5 min while the controller brings the jacket temperature to equilibrium with the bucket. After the temperature was equalized, the initial temperature was recorded. The bomb was then fired and the final temperature was recorded once the temperature reaches a stable maximum and remains constant for at least two minutes. Bomb was then removed and interior of bomb was washed with distilled water. The unburned piece of fuse wire was measured. The bomb washings were then titrated with standardized sodium carbonate solution using methyl red indicator till the solution changes from red to yellow. The volume and the normality of sodium carbonate solution used were recorded. The gross energy of combustion was calculated from the equation

$$GE = tW-e_1-e_2/m;$$

t is the temperature difference (final-initial)

W is the energy equivalent of calorimeter

e_1 is the correction in calories for heat of formation of nitric acid

e_2 is the correction in calories for heat of combustion of fuse wire

m is the mass of the sample in grams.

e_1 = Normality of actual Na_2CO_3/0.0725 x Nomality of ideal Na_2CO_3 X C_1

e_2= 2.3 x C_2

C_1 = ml of standard Na_2CO_3 used in acid titration

C_2 = net length of fuse wire burned, cm.

Appendix B. Nitrogen Determination of feed

The amino nitrogen is oxidized by sulfuric acid in the presence of a catalyst to ammonium sulfate. Sodium hydroxide is added, converting the ammonium ion to NH_3, which is collected by distillation. The NH_3 is then quantitatively titrated.

Reagents

1. 50 % Sodium Hydroxide
2. 0.1% Bromocresol Green
3. 0.1% Methyl Red
4. 4% Boric acid witn indicators
5. 0.15 N HCl for titration

Digestion: 0.7 g of sample was weighted into Kjeltec digestion tube along with two blank tubes and two kjeltabs (catalyst) were added to the tubes. 15 ml of concentrated H_2SO_4 was added and swirled gently to wet the sample. The samples were digested in a block digestor set at 420°C for 60 min. The digestor was then allowed to cool for 5 min and 80 ml of water was added, shaking it to dissolve any crystals that may form.

Distillation: The digestion tube was placed in the distillation apparatus and receiver flasks were filled with 25 ml of red boric acid solution. The distillation apparatus adds alkali and distill the ammonia over into the receiver flask. After distillation the solution in the receiver flask turned green.

Titration: The solution in the receiver flask was titrated against 0.15 N HCl until a purple-rose endpoint is reached and the volume of HCl added was recorded.

The percentage protein in the sample was calculated by:

% protein = (ml HCl – ml blank) x normality of HCl x 14.007 x 6.25/g of sample x 10;

where 6.25 is the constant for calculating % crude protein from % nitrogen.

Appendix C. Calcium Determination of feed

4 g of dried feed samples in a crucible was placed in muffle furnace. Ashed for 6h at 600°C. The samples were then cooled and 10 ml of 3 M HCl was added to the ash in the crucible. The samples were boiled for 10 min, cooled and washed into a 100 ml volumetric flask. Samples were filtered into a plastic tube and were used for calcium assay.

Reagents:

1. 0.3 N HCl
2. 15 g strontium chloride in 100 ml water. This will contain 50 mg Sr/ml
3. Calcium standard solutions
4. Blank (water)
5. Diluted samples (1:50000)

The standards were prepared by pipetting 0.1, 0.2, 0.3, 0.4 and 0.5 ml of stock Ca (1000 ppm) into five different 100 ml volumetric flasks. To each of these flasks add 10 ml water, 4 ml 50 mg/ml strontium solution and made up the volume with 0.3 N HCl. Blank was prepapred by pipetting 10 ml water and 4 ml 50 mg/ml strontium solution and made up the volume with 0.3 N HCl.

The samples and standards were allowed to stand for 1 hr and were analyzed using Atomic Absorption Spectrophotometer under set conditions.

% Ca = Absorbance x dilution factor/10^6 x sample wt. X 100

Appendix D. Phosphorus Determination of feed

4g of dried feed samples in a crucible was placed in muffle furnace. Ash for 6h at 600°C. The samples were then cooled and 10 ml of 3 M HCl was added to the ash in the crucible. The samples were boiled for 10 min, cooled and washed into a 100 ml volumetric flask. Samples were filtered into a plastic tube and were used for phosphorus assay.

Reagents:

1. 2 L of molybdovanadate
2. Phosphorus standard (2mg P/ml). Dissolve 8.788 g potassium orthophosphate, dihydrogen (KH_2PO_4) in water and dilute to 1 liter. Working standards contain 10, 20, 30, 40 and 50 µg P/ml)
3. Diluted sample solution (1:1000).
4. Blank (4 ml molybdovanadate to 1ml water)

4ml of molybdovanadate reagent was added to 1ml of each standard and sample and mixed well. Allowed to stand for 10 min and the absorbance was read at 400 nm on UV-VIS Shimadzu spectrophotometer.

% P = Absorbance x dilution factor/10^6 x sample wt. X 100