

STABILITY OF ESSENTIAL NUTRIENTS IN PET FOOD MANUFACTURING AND
STORAGE

by

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Abstract

Processing pet food can be beneficial, but can also have adverse effects on shelf-life and nutrient survival. Most affected are supplemental vitamins and essential fatty acids (EFA). Pet food complicates this relative to human foods by combining all elements into the product before processing and requiring an extensive shelf-life (up to 2 years). The objective of this research was to determine the effects of processing, diet, and storage conditions on vitamin (vitamin A, vitamin D₃, vitamin E, folic acid and thiamine) and omega-3 fatty acid (with an emphasis on eicosapentaenoic acid; EPA 20:5n3, and docosahexaenoic acid; DHA; 22:6n3) retention. The research was conducted in two separate experiments. Each experimental diet was produced on a single-screw extruder and triple-pass dryer. Target nutrients were evaluated in premixes in tandem to extruded diets. The vitamin study was conducted as a 3 X 2 X 2 factorial arrangement of treatments with 3 levels of dietary crude protein (CP), 2 screw speeds in the extruder, and 2 levels of time X temperature combinations in the dryer. Vitamins were added at 10 times normal levels to aid in analysis. The EFA study was conducted as a 3 X 3 factorial arrangement of treatments with 3 levels of dietary protein and 3 different omega-3 sources: fish oil, fish meal, or purpose-grown algae rich in DHA. In the vitamin premix study, the quantity of vitamins declined by approximately 50% over 6 months storage in ambient conditions (AMB; 20C, 50%RH), and all except folic acid were lost to some degree in stressed shelf life testing (SSLT; 50C, 70% RH) over 6 weeks. In all cases, the concentration of vitamins in food exiting the extruder and dryer were lower than target levels. As CP increased, the retention was higher ($P \leq 0.05$) for vitamins A, E, and folic acid off the extruder (e.g. 225,352 vs. 219,184 and 206,249 IU/kg of vitamin A for high vs. medium and low CP, respectively), and vitamin D₃, E, and folic acid off the dryer (e.g. 9,047 vs. 7,473 and 6,945 IU/kg of vitamin D₃ for high vs. medium and low CP,

respectively). During storage of finished pet food in AMB, vitamins A and D₃ were lost ($P < 0.05$) to the greatest degree (49 and 22%, respectively). The total retention following both processing and AMB storage was 27, 68, 78% for vitamins A, D₃, and E, respectively, while folic acid and thiamine were relatively stable. In SSLT storage, all vitamins except vitamin E were depleted more than 60% ($P < 0.05$) by 24 weeks, whereas total retention following both processing and SSLT storage was 3, 59, 43, 33, and 7% for vitamins A, D₃, and E, folic acid, and thiamine, respectively. This would suggest that beyond processing losses, the vitamins are relatively stable in premixes and foods if stored in AMB conditions. In the study to evaluate fatty acid stability within a vitamin premix, EPA, DHA, and total omega-3 fatty acids were relatively stable during storage over 6 weeks with losses no greater than 12% in stressed shelf life testing (SSLT; 40C, 70% RH). While in ambient conditions (23C, 50% RH) over 3 months, there was a total loss of EPA, DHA and total fatty acids by 17, 9, and 11%, respectively. Exiting the extruder and dryer, EPA and DHA were not affected by CP level or Omega-3 source. As SSLT storage of finished pet food increased through 24 weeks, EPA, DHA, and total fatty acids declined slightly ($P < 0.05$; 125, 82 mg/kg for EPA and 77, 60 mg/kg for DHA, and 418, 476 mg/kg for total fatty acids at 0 vs. 24 wk. As time in ambient storage reached 24 months, EPA, DHA, and total fatty acids declined slightly ($P < 0.05$; 125 vs. 78 mg/kg for EPA and 77 vs. 50 mg/kg for DHA, and 387 vs. 373 for total fatty acids at 0 vs. 24 mo.) Algal-DHA appears to be a stable source of DHA when compared to fish oil and fishmeal. During processing retention of fat soluble vitamins was less than water soluble vitamins, and the omega-3 fatty acids were relatively unaffected. Whereas, vitamins appeared to be more sensitive to temperature during storage and the omega 3 fatty acids more affected by time.

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Chapter 1 - Introduction

More than half of all American households own at least one dog or cat (American Pet Products Association Inc., 2015). Ownership has continued to increase over the last several decades as companion animals play a significant role in our lives by providing physical and emotional benefits (Serpell, 1991). Approximately 97.3 million households own either a cat or a dog in 2015 (American Pet Products Association Inc., 2015). Nearly \$22.26 billion was spent on pet food alone in 2014, with estimates for growth of pet food sales to reach \$23.04 billion in 2015, according to the American Pet Products Association, Inc.

Extruded and baked products intended for human consumption differ from those for companion animals. Humans consume a variety of food sources providing a wide range of nutrients, where pet food is uniquely formulated to be nutritionally balanced (protein, lipids, carbohydrates, vitamins and minerals) and fed as a complete food item. With nutritional guidelines in place, like the National Research Council (2006) Nutrient Requirements of Dogs and Cats, the framework to provide balanced nourishment for pets to promote overall health and wellbeing is defined. The challenge is assuring their food is capable of delivering.

The raw ingredients sourced for pet food are often derived from the food industry as secondary or inedible by-products. After initial processing for the food industry, the excess material is collected and processed (e.g. drying or rendering) into valuable ingredients and made available to the pet food industry. The thermal processes in the food and feed industry provide substantial desirable benefits for digestibility and pathogen control, but can have adverse effects leading to oxidation and degradation of essential nutrients (Aldrich, 2012). The combination of essential nutrient sacrifice during raw storage, chemical interactions within a mash, and the

effects of thermal processing can result in the need for additional supplementation in order to support the animals' nutrient requirements.

Using current nutrient analytical information for finished products, nutritionists commonly over-formulate to offset vitamins sacrificed during thermal processing and food storage. While this seems an easy solution, it can increase the risk for nutrient imbalances or toxicities in companion animals if the target levels are not obtained. Thiamine alone in cat and dog food has led to numerous recalls of commercial foods in the pet food industry (Loew et al., 1970; Davidson, 1992; FDA, 2016).

Little research has been published on the magnitude of vitamin and essential fatty acid loss in pet food as a result of processing. It is imperative that we examine the effects processing and storage have on vitamin and essential fatty acid stability to aid in proper supplementation and compensate for the inevitable losses that are described in the original research herein. Besides nutrient losses there may also be changes to the sensory attributes of foods which has previously not been described (Appendix A). Therefore, the objectives of this work were to determine the effects of processing conditions and dietary protein on essential nutrients (vitamin A, vitamin D₃, vitamin E, folic acid, thiamine, and omega-3 fatty acid sources (fish oil, fish meal, and algal sources of DHA, DHAgold™ S17-B; DSM Nutritional Products) added to the diet by premixes following extrusion-drying processing and extended storage.

Chapter 2 - Literature Review

Vitamins as Essential Nutrients

Over the last century, the significant link between nutrition and health was discovered, in which dietary cures for numerous human diseases, such as beriberi, rickets, pellagra and scurvy, were found (Basu and Dickerson, 1996). These diseases plagued the human race until they were remedied by nutrient repletion. These essential organic elements initially described as vital amines, or, as we know them today, vitamins, are obtained from a broad cross section of foods or may be produced synthetically to support the vital functions of life. Supporting all the necessary vitamin requirements from ingredient sources alone is extremely difficult, and deficiencies can pose extreme risks to the animal. Because of this, nearly every commercial canine and feline food is supplemented to meet the animal's essential vitamin requirements.

The daily intake of vitamins is relatively small when compared to other nutrients. Vitamins are indispensable, performing specific functions as coenzymes for metabolic processes in order to maintain health and life (Adams, 1982). Without a sufficient amount of these micronutrients, there will be impairment to effective metabolism, growth, reproduction and health. Each vitamin has its own purpose(s), and each differs in chemical structure and composition. They are classified and grouped based on their solubility in fat or water, and differ greatly in functions.

Fat Soluble Vitamins

The fat soluble vitamins A, D, E, and K are absorbed intact or in a slightly modified physiologically form from foods such as eggs, milk and milk products, fruits, vegetables and oils. Daily consumption is not required because following absorption, they can be stored in the

liver and fatty depots of the body, and excesses can be expelled through metabolites in feces (Groff, 1995). Although less susceptible to acute deficiency due to storage capabilities physiologically, in foods the fat soluble vitamins degrade rapidly and are susceptible to light, oxidation, and alkali conditions (Albertini et al., 2010). All of which are common factors during commercial pet food manufacturing processes.

One of the first essential micronutrients to be recognized officially, ‘Vitamin A’ refers to a group of fat soluble compounds comprised of mostly retinoids (Basu and Dickerson, 1996). Retinoids are chemical derivatives of preformed vitamin A and pro-vitamin A carotenoids (Groff, 1995). Preformed vitamin A, or retinyl esters and retinol, are found in organ meats like liver, eggs, whole milk, butter and cheese. Pro-vitamin A carotenoids, or β -carotene, α -carotene and β -cryptoxanthin, are found in carrots, yellow and dark green leafy vegetables such as broccoli and spinach. The plants themselves do not produce vitamin A; rather many species are able to convert β -carotene into vitamin A through an enzymatic cleaving process by 15, 15'-dioxygenase at the intestinal mucosa (Wyss et al., 2000, 2001). Cats are the exception and lack this enzyme (Schweigert et al., 2002) and require preformed vitamin A in the diet. Adequate intake of vitamin A is vital in vision and ocular health, immune system development, and neurologic function.

Standard pet food formulations may contain β -carotene, and more likely, preformed vitamin A through macro-ingredients like fish oil, chicken meal and liver; however, the levels in the final ration for consumption may not be fully adequate to support the animals' nutritional requirements. Further, vitamin A is sensitive to physical and chemical processes, and the commercial pet food manufacturing process can be detrimental to vitamin stability (Tran et al., 2008). Therefore, fortification of foods is commonly considered to be necessary for complete

and balanced diets. But fortification is not always straightforward since losses can occur during food processing, transportation and storage (Dary & Mora, 2002).

The latest publication of the National Research Council changed the units used when expressing vitamin A requirements from International Units (IU) to retinol equivalents (NRC, 2006). This factors for the bioavailability of vitamin A from the various sources. For example, 1 mg of retinol equals 3,333 IU of vitamin A for cats. The National Research Council (2006) proposed a vitamin A requirement of 1,515 retinol equivalents (RE) per kg DM for adult dogs and 1,000µg retinol per kg DM for adult cats.

The terminology of vitamin A can refer to substances with a similar molecular structure, whether natural or manufactured commercially. Because vitamin A is susceptible to oxidation, most commercial manufacturer of vitamin A supplements encapsulate the vitamin A resin in protein-starch beadlet. This slows the degradation of vitamin A by adding a protective barrier from the external influences that accelerate degradation such as oxygen, UV light and chemical catalysts (Albertini et al., 2010). Simple storage in vitamin premixes can drop vitamin A levels from 3.9% to 20% per month and extrusion, and another 25% to 40% depending on extrusion parameters. Further, an additional 8% to 30% can be lost over time for product stored in the warehouse or on the retail shelf (Coelho, 2003).

Vitamin D is another generic descriptor for a group of fat-soluble secosteroids that were discovered as a function of research to cure the disease known as rickets. Beyond the deficiency disease, vitamin D is responsible for calcium and phosphorous homeostasis. Vitamin D is crucial because calcium and phosphorous are necessary for a vast array of physiological functions, muscle contraction, nerve conductivity, bone formation, immunology, and much more (NRC, 2006). Although vitamin D is often referred to as a singular item, it actually describes a group of

compounds where the two forms ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃) are most prominent.

Ergocalciferol is derived from the plant steroid ergosterol and is the less effective and investigated of the two forms. The more common and heavily researched form is cholecalciferol. To become active, it undergoes two hydroxylation steps in the body: one in the liver (25-hydroxyvitamin D₃) and a second in the mitochondria of the kidney proximal tubules (1,25-dihydroxyvitamin D₃; NRC, 2006). Once active, it functions by binding to steroid receptors homologous to those of estrogen and testosterone. In man and many animals, cholecalciferol is produced by UV radiation in the skin from 7-dehydrocholesterol (a cholesterol metabolite). Dogs and cats have much the same machinery in place to carry out this reaction, but because they also possess an abundance of the enzyme 7-dehydrocholesterol 7-reductase that converts 7-dehydrocholesterol to cholesterol, this critical intermediate is unavailable for conversion to cholecalciferol (Morris, 1999). In other words, there isn't enough of the starting material left to produce vitamin D₃. So for dogs and cats, we have to supply it in the diet.

Natural forms of Vitamin D₃ can be found in marine sources such as fish liver oils, sardines, herring, and salmon. Other sources for vitamin D include eggs, meat and milk, but due to processing and formulation, it is difficult to include enough of these raw materials to fulfill the animals' nutritional requirements. According to the NRC, 2006 the cholecalciferol recommended allowance is 13.8 µg per kg DM for adult dogs (4,000 kcal diet) and 7 µg per kg DM for adult cats. The most common commercial sources for vitamin D₃ are produced utilizing a partial synthesis technology starting with 7-dehydrocholesterol derived from a wool grease based cholesterol by various methods (Schlossman et al., 1978)

Like vitamin A, vitamin D is susceptible to oxidation and destruction during thermal processing. Most commercial manufacturers of vitamin D₃ supplements that are used in the pet food industry encapsulate the molecule via cross-linked beadlet technology in combination with Vitamin A. This slows the degradation of vitamin D₃ and protects it from external influences that accelerate degradation such as oxygen, UV light and chemical catalysts. Coelho (1991) reported that during extrusion and drying 25% to 65% is lost depending on extruder temperatures and shelf life can account for 15% loss as product is in storage within a warehouse or retail shelf.

Vitamin E is another general term that actually encompasses eight fat-soluble compounds found in nature. Half of the compounds are classified as tocopherols, while the other are identified as tocotrienols. Functionally, these compounds perform a major biological function as an antioxidant breaking the chain reaction associated with free-radicals. Free-radicals are unpaired electrons naturally found as a part of metabolic process and in the environment, and can be harmful if left unregulated.

A number of vitamin E compounds exist; structurally all forms are similar with a 6-chromanol ring and a 16-carbon side chain (isoprenoid) on one end. The fully methylated (-CH₃) and saturated side chain of the α -tocopherol results in the highest affinity for the RRR- α -tocopherol. It is commonly found in vegetable oil sources, but is more commonly produced synthetically (Chow, 2001). The production of synthetic vitamin E occurs by molecular distillation along with methylation and esterification resulting in an 'all-racemic' mix, which is relatively low in RRR-tocopherol actives. Additional enhancement must occur in order to modify the synthetic form to make it stable. This is accomplished through an acetylation reaction with acetate or succinate (Torres et al., 2008).

The all-rac- α -tocopherol form of vitamin E is widely used for the stabilization of raw ingredients, but these true levels are commonly discounted by nutritionist as the amount of vitamin E needed is accounted for by the vitamin premix. The NRC (2006) expresses the requirement of vitamin E in mg α -tocopherol, but this is not a universal standard. The vitamin E requirements for dogs and cats is 30 and 38 mg/kg DM, respectively. Unlike vitamin A and D, vitamin E is more stable when exposed to acid, heat and moisture (Coelho, 2003). However, significant destruction of vitamin E can occur when exposed to other conditions like oxidation, UV light, trace minerals like copper, manganese, zinc, iron and lead salts (Riaz, 2009). During extrusion and drying, almost 15% can be lost and an additional 2.9% each month thereafter (Coelho, 2003).

Water Soluble Vitamins

Water soluble vitamins (B-complex and vitamin C) are obtained by the ingestion of breads, cereals, fruits, vegetables and animal products. Mammals lack the ability to store these critical compounds so, they are required daily in the diet. They are passively absorbed in the small intestine, and excess is excreted in the urine. In general, the water soluble vitamins are more heat stable when compared to the fat soluble; however, water soluble vitamins are more sensitive to a change in pH or exposure to trace elements common in vitamin and trace mineral premixes (Coelho, 2003).

Thiamine, vitamin B₁, was the first of the water-soluble vitamins to be isolated from rice bran in the early 1900's and found to be the cure for the disease beriberi. Thiamine deficiencies in cat and dog food have led to recalls of commercial foods in the pet food industry (Loew et al., 1970; Davidson, 1992). An acute thiamine deficiency results in symptoms such as fatigue, insomnia, irritability and lack of concentration. Chronic cases can lead to anorexia, cognitive

impairment, convulsions and even death. Cats are often more susceptible to deficiency, perhaps because their requirement is five times greater than that of the dog.

The molecular structure of thiamine {3-[(4-amino-2-methyl-5-pyrimidinyl) methyl] 5-(2-hydroxyethyl)-4-methylthiazolium chloride} is unique and contains a complex chemistry allowing the molecule to engage in both oxidation and reduction reactions (Georghiou, 1977). Thiamine present in plants is found in a non-phosphorylated form, whereas thiamine in animal tissues is predominantly bound as thiamine pyrophosphate (TPP), monophosphate (TMP) and triphosphate (TTP; NRC, 2006). The primary functions of thiamine relate to the coenzyme role in the form of TPP. As the coenzyme form, it aids in the full activation of enzymes, which play a crucial role in the production of energy from carbohydrate metabolism. Another vital role of thiamine is the activation of the transketolase enzyme, which is responsible for the catabolism reactions in the pentose phosphate pathway. This pathway provides the basis for an array of prominent compounds such as adenosine triphosphate (ATP), guanosine triphosphate (GTP), nicotinamide adenine dinucleotide phosphate (NADPH) and the nucleic acids deoxyribonucleic acid (DNA) and ribonucleic acid (RNA; Tanphaichitr, 1999). Other functions for thiamine that do not involve coenzyme functions is the important role it plays in nerve and muscle tissue in the TTP form (Rindi, 1996).

In most natural ingredients, thiamine is found in small amounts. Dried brewer's yeast, some meat sources, such as pork and some species of fish, whole grain cereals, bran, pulses and nuts are rich sources of thiamine. These are common ingredients found in pet food formulations. Despite this, thiamine levels are generally insufficient to meet dietary requirements, so fortification is necessary. According to the NRC (2006), adult maintenance requirements for dogs and cats are 2.25 and 5.6 mg/kg DM, respectively (4,000 kcal diet). Two common

supplemental sources are thiamine mononitrate and thiamine hydrochloride. Thiamine mononitrate is the most preferred source in vitamin premixes because the hydrochloride form has hygroscopic tendencies and is more prone to instability (Adams, 1982).

Thiamine is well known for its lack of stability. Degradation occurs when thiamine is exposed to a number of common conditions. It is easily oxidized by UV light, gamma irradiation, and destroyed by elevated heat, high water activity, and sulfites (often utilized as a preservative), and can be sacrificed by natural thiaminase enzyme activity found in fish viscera (NRC, 2006; Tribble, 2015). An estimated 6-50% may be lost during the extrusion process and 1-39% for the dryer depending on processing parameters. Further, commercial pet food's sit for an extensive amount of time on the shelf which can reduce thiamine an additional 4-4.5% for each month following processing (BASF, 2000).

Vitamin B₉ is a member of the folate group which includes folic acid and other natural occurring folates. More commonly known as folic acid, vitamin B₉ is a synthetic form that is frequently used in supplemental vitamin premixes. The name was derived from the Latin word for "leaves", as it was first isolated from spinach. Naturally rich sources of folate are beef liver, dark leafy greens, like spinach and kale, beans, egg yolks, milk and dairy products (Subar et al., 1989)

Structurally, folic acid (pteroylmonoglutamic acid) consists of a pteridine ring system linked by a methylene group. Within the body, the active form of folate, tetrahydrofolate, acts as a coenzyme for a number of essential metabolic reactions such as the metabolism of amino acids. Folate is also heavily involved in other functions, such as the synthesis of nucleic acids and formation of blood cells. This process is essential for normal cell division and proper growth in order to prevent anemia and fetal development (Brody et al., 2001).

Unlike thiamine, folic acid is relatively stable when exposed to processing conditions. However, destruction of folic acid can occur when exposed oxidation and UV light (Riaz, 2009). Research has reported that during extrusion and drying, nearly 30% can be lost depending on the extruder temperatures and configuration and an additional 4.5% loss per month thereafter (Coelho, 2003). Because of this loss, pet food requires additional supplementation with folic acid to meet the NRC (2006) adult maintenance requirements for dogs and cats are 270 and 750 µg/kg DM, respectively (4,000 kcal/diet).

Little research has been published regarding the magnitude of vitamin losses through premix storage, extrusion manufacturing and storage of pet food. The most recent recommendations for fortification to overcome losses was provided by a technical bulletin from BASF authored by Coelho (2000). However, the research supporting their recommendations was never published so that researchers could better understand the context driving their conclusions. In this technical bulletin Coelho (2000) accounted for degradation in all cases. The magnitude of loss was greater for vitamin A, vitamin D₃, MSBC (synthetic Vitamin K₃), folic acid and thiamine when compared to riboflavin, vitamin B₁₂ and vitamin E. This work served as the foundation for their recommendations over the past 20 years, however the matrix of modern pet food has changed. Today, the ingredient matrix has become more consumer driven with formats such as grain-free and high protein formulations to name but a few. In addition, the vitamin premix manufacturers have made advancements in vitamin technology to increase stabilization (Albertini et al., 2010). Currently, nutritionists are compensating for estimated losses by over-formulating based on these previous assumptions. They may also utilize complementary ingredients as another strategy. To fill the voids in our knowledge, some companies will perform validation testing to compare theoretical versus actual vitamin levels in the finished kibble as a

final step. However, this can be too little too late and lead to great waste. There is a need for updated retention data to verify that the vitamin fortification in processed pet foods.

Essential Fatty Acids

Like vitamins, fats are also an important component of companion animal diets. They provide a source of energy for the body, over twice the calories of carbohydrate and protein while aiding in the absorption of other essential nutrients like vitamins A, D, E, and K. Fat can also aid manufacturing and enhance flavor and texture, while supplying essential fatty acids (EFA). Dietary fat has been recognized as essential in nature since the early 20th century, with later focus specifically on linoleic acid (LA; C18:2n-6) and α -linolenic acid (ALA; C18:3n-3; Spector, 2015).

Essential fatty acid requirements vary among species. Unlike plants, companion animals are unable to synthesize sufficient omega-3 and omega-6 fatty acids for maintenance. There are differences, as well among the species. For example, the domestic dog is able to synthesize arachidonic acid (ARA) from LA (Dunbar & Bauer, 2002), but it is a dietary requirement for the cat (Rivers et al., 1975). More recent research has led to an increased awareness about omega-3 fatty acids, such as ALA, eicosapentaenoic acid (EPA; 20:5n-3), and docosahexaenoic acid (DHA; 22:6n-3). Increased consumption of these fatty acids has been reported to decrease inflammation, improve skin and coat quality, boost immune system response, treat cancer and retinal degeneration, enhance development of the nervous system and improve overall cognition (Lenox, 2013).

Until the last two decades, dog and cat diets were formulated to meet these essential needs with the knowledge that omega-3 fatty acids were beneficial, but little consideration was

given to the relationship of omega-3 fatty acids to omega-6 fatty acids (Connor, 1988). Omega-3 fatty acid sources like fish, fish oil, and flax seed can be more expensive and less stable than the more conventional fat sources like tallow, pork fat or poultry fat. These latter sources are predominately enriched with omega-6 fatty acids and contribute very little omega-3 fatty acids to the diet. Diets depleted of omega-3 fatty acids resulted in less health benefits when compared to a high omega-3 to omega-6 fatty acid ratio (Nesbitt et al., 2003).

Fatty Acid Metabolism

Fatty acids can be classified in a number of ways based on their hydrocarbon chain characteristics. The more common approach utilizes the descriptive methods based on chain length and the number of carbons within the fatty acid chain. Omega-3 and omega-6 fatty acids are classified as polyunsaturated fatty acids with the first double bond located between the third and fourth carbon and sixth and seventh carbon atom when counting from the methyl end of the molecule (Lenox, 2015).

Linoleic acid (LA) is metabolized through a series of elongation and desaturation steps to form arachidonic acid, a pro-inflammatory intermediate in the production of the eicosanoids: 4-series leukotrienes (LTB₄), 2-series prostaglandins (PGE₂), and 2-series thromboxanes (TX₂) and IL-1 and IL-6. Alpha-linolenic acid (ALA) is elongated to EPA and docosapentaneic acid (DPA; Bibus et al., 1998) and elongation and desaturation resulting in eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). EPA and DHA are considered anti-inflammatory intermediates in the production of the eicosanoids: 5-series leukotrienes (LTB₅), 3-series prostaglandins (PGE₃), and 3-series thromboxanes (TX₃; Calder, 2012). The two pathways depend upon the same delta-5-desaturase enzyme for the production of their respective pro-inflammatory or anti-inflammatory end products. Changes in the activity of this enzyme or

competition for the enzyme can have profound effects on inflammatory responses (Calder, 2012).

Omega-3 Fatty Acids

Omega-3 fatty acids made their debut within the commercial pet food markets through “derm” diets for dogs’ skin and coat. According to Scott et al., (1997), atopy (allergic skin reactions) is the most common allergic dermatitis in the dog, and second only to flea-bite hypersensitivity. These conditions, along with dermatitis, pruritus (localized itching) and erythema (skin redness and swelling) have been related to immune and inflammation responses that have been shown to be elevated and an improved skin and coat scoring through dietary supplementation (Reese et al., 2001).

Research results have been mixed with some studies showing significant improvements when supplemented with omega-3 and omega-6 fatty acids and others have failed to show benefit. For example, Scott et al. (1997) fed a commercial lamb and rice diet enriched with fish oil as a single-blinded, self-controlled clinical trial. It was reported 8 of 18 (44%) dogs that were previously unresponsive atopic mixed-breed dogs responded to an omega-6: omega-3 ratio of 5.5:1 within 21 days. In another study, Nesbitt et al., (2003) determined the effect of various doses of omega-3 fatty acids at various omega-6:omega:3 fatty acid ratios (1:1, 3:1, 6:1, and 27:1) on dogs (n=58) with pruritus for 56 days resulting in improved total clinical scores across all treatment groups. Abba et al. (2004) reported that different-stage atopic dogs fed essential fatty acid (EFA) supplementation resulted in an overall improvement in clinical evaluations for all dogs, however dogs with early stage atopy responded greater than the later stage. Rees et al. (2001) reported that dogs fed kibbles coated with crushed flaxseed or sunflower seed (source of omega-6s) for 84 days had improved hair coat scores for the first 28 days, regardless of

treatment. In this study the dog's skin condition scores improved during days 14 to 28 of the 84-day trial, but progress became stagnant following the initial 28 days. Whereas, the condition of dogs fed crushed sunflower seed-coated kibbles did not improve or deteriorate. Plasma triglyceride omega-3 concentrations were analyzed and reported greater on 84-day trial for dogs fed the kibble with crushed flaxseed. On the contrary, there were no fluctuations in omega-6 concentrations between the treatments throughout the study (Bauer et al., 1998).

Supplementation of omega-3 fatty acids hold promise for benefits against chronic disease, trauma, infection, and the effects of aging. Ontsouka et al. (2012) reviewed treatment of canine chronic enteropathies with a diet enriched with omega-3 PUFA and concluded the duodenal fatty acid uptake was potentially altered by the enriched diet. The uptake of PUFA could benefit treatment of canine inflammatory bowel disease. Mooney et al. (1998) reported diets containing omega-3s were found to be supportive to wound healing. Beagles (n=30) were fed diets enriched with various combinations of fish oil (menhaden), flax, and sunflower oil to achieve omega-6:omega-3 fatty acid ratios of 5:1, 10:1, 25:1, 50:1, and 100:1. Over the 12 week study skin biopsies contained increasing ratios of pro-inflammatory fatty acids and eicosanoids as the omega-6: omega-3 fatty acid ratio increased. These results support that omega-3 fatty acids modify inflammation and improve wound healing.

Companionship between pets and humans has evolved from the outdoor barnyard cat or dog to an indoor member of the family. This evolution has a direct relationship to life span of the companion animal and their quality of life. This has been in part due to increased owner education, more nutritious foods, and receiving better health care. With older animals, there is a growing interest in the nutritional needs of the geriatric dog and cat. Much like humans, dogs and cats show external signs of aging with slowing activity levels and changes to internal physiology

from declining enzyme activities and the immune system begins to decline in its response magnitude (Pati et al., 2015). Several studies with geriatric canines have reported an intimate link between the immune system and inflammation. Kearns et al. (1999) studied the effects of omega-6: omega-3 fatty acid ratios on immune response and oxidative status. In their work, supplementation of fish oil and ground flaxseed omega-3 fatty acids were fed in an experiment with both adult and geriatric Labrador retrievers (n = 18) and fox terriers (n = 18). They reported that in an 8 week switch-back feeding trial, diets enriched with omega-3s supported an immune response in old dogs without an effect on oxidative status. Hall et al. (2003) published a study which supported the lack of oxidative response by reporting an optimum dose of vitamin E of 101 mg/kg food for dogs on a high omega-6:omega-3 fatty acid ratio diet (40:1). The vitamin E supplementation, regardless of dose (17, 101, or 447 mg/kg food), did not substantially impact humoral or cell mediated immune responses in female geriatric dogs (n=32) that were fed a low, fish oil source omega-6:omega-3 fatty acid ratio diet (1.4:1). In contrast, Wander et al. (1997) also investigated the ratio of omega-6 to omega-3 fatty acid with 31:1, 5.4:1, and 1.4:1 ratios. Healthy geriatric female beagles (n=20) were fed diets supplemented with fish oil and corn oil to determine the effects on immune response and lipid peroxidation. The dogs fed an omega-6:omega-3 ratio of 1.4:1 was found to reduce immune response, decrease inflammatory eicosanoids (PGE₂), and oxidative status.

As dogs age, the prevalence of renal failure is increased from 1% to 10% in dogs over 15 years of age (Brown et. at 1998). For a number of years, this was thought to be nutritionally related to high protein diets or diets with elevated salts. However, the cause of the disease remains elusive as the link to protein level has been summarily dismissed (Brown et al., 1991). An active effort using a variety of strategies has been attempted to decrease the severity and

rapidity of this disease onset. One has been to alter the inflammatory effects on the nephron, lowering systemic arterial pressure, and altering plasma lipid concentrations to preserve renal function by adjusting the dietary fatty acids in the dogs' circulation. Brown et al. (2000) tested this hypothesis that omega-3 and omega-6 PUFA would reduce the magnitude of glomerular capillary hypertension by feeding adult mixed-breed dogs that were 11/12th nephrectomized (a model for renal failure) diets supplemented with menhaden fish oil (omega-3 enriched), safflower oil (super-rich omega-6 source), or beef tallow (saturated fat). Supplementation with safflower oil increased pro-inflammatory intermediates (PGE₂) and glomerular hypertension and hypertrophy got worse. Dogs fed beef tallow experienced a progressive decline in renal function, but at a slower rate. Omega-3 supplementation with menhaden fish oil appeared to be renal-protective by lowering inflammatory response in early stages of the disease for dogs.

Most of the research regarding the benefits of fatty acids on circulation and heart disease have been reported in man. Some of these studies have used the dog as a model and may be revealing about the activity of omega-3s. The known anti-inflammatory properties of consuming omega-3 fatty acids stemmed from research on the potential benefits for cardiac disease in dogs. Elevated cytokine concentrations have been correlated with heart failure in man, but it wasn't until Freeman et al. (1998) reported that when dogs (n=8) with chronic heart failure were supplemented with fish oil (1 g/d), they had improved cachexia scores and lower AA, EPA and DHA baselines for dogs with heart failure when compared to the control dogs. A reduction of interleukin-1 beta (IL-1; a pro-inflammatory cytokine) was also found and was correlated with predicted survival in this study. In another study, Billman et al. (1999) explored the effects of consuming fish oil on fatal ventricular arrhythmias. Dogs were surgically fitted with an inflatable hydraulic cuff placed around the left circumflex coronary artery and were intravenously dosed

with ALA, DHA, and EPA. The study concluded that each pure free fatty acid was as effective as the fish oil emulsion in preventing ischemia-induced ventricular fibrillation.

Cancer is an emotionally sensitive disease and does not discriminate amongst species. Approximately 4.2 million dogs and 1.66 million humans are annually diagnosed with cancer in the United States (Siegel et al., 2015, Schiffman and Breen, 2015). Advances in comparative oncology have allowed researchers from human and veterinary medicine to work across species to advance both human and animal oncology discoveries (Schiffman & Breen, 2015). However, little work has been reported on dietary approaches to enhance cancer therapy or survival in the dog or cat. Previous research has indicated that omega-3 fatty acids could aid in inhibiting metastasis. Ogilvie et al. (2000), conducted a double-blind randomized clinical feeding trial in which thirty-two dogs with lymphoma were supplemented with fish oil (menhaden) and arginine or soybean oil (control) diets before and after doxorubicin chemotherapy. It was reported that circulating DHA and EPA were greater in dogs with lymphoma which were fed diets containing fish oil. The elevated levels were associated with longer disease free intervals and survival times than for the control.

In other applications, certain breeds of dogs are prone to an inborn error of metabolism that leads to complete blindness, progressive rod-cone degeneration (PRCD), which parallels retinitis pigmentosa in humans. Aguirre et al. (1997) reported the disease could be associated with reduced uptake and synthesis of DHA. In one study, Anderson et al. (1991) supplemented poodles and Irish setters affected with PRCD with linseed oil (high in linolenic acid) and reported that circulating DHA did not increase as expected, but declined. Results were consistent with a defect in the desaturase activity for the poodles affected with PRCD when compared to the control. Therefore, it is likely that pre-formed DHA should be provided to dogs with this

condition. However, no reports in the literature were found in which DHA from marine oil sources were supplemented to animals exhibiting PRCD as a test of this hypothesis.

Clearly there is a benefit to omega-3 support in the diet. However, assuring that these very labile ingredients remain stable can be a challenge. In part because of the severe thermal process and overall nature by which pet foods are distributed and stored, some pet food companies opt to use fish meal rather than the oil as their source of omega-3s. Marine omega-3 oils are highly prone to oxidation. The oxidation process could potentially alter biological activity, which could render them ineffective (Albert et al., 2013). Fish meal provides a high quality protein and in a dry product seems to be easier to stabilize with current antioxidant technology when compared to fish oil, which is susceptible to oxidation. Oceanic sources of fish meal are derived from Menhaden, Whiting, Capelin, Herring, Pollock, and Salmon to name a few. Meal from catfish, due to their grain-based feeding practices, has a fatty acid profile more similar to chicken fat than that of ocean derived fish.

The importance of omega-3 fatty acids has become increasingly evident for brain and retinal development within recent years. Fish oil usually contains both EPA and DHA, and algal oils are predominately DHA. Research has shown that in neonate formulas PUFAs are essential for proper neural and retinal development in several mammalian species. Heinemann et al. (2005) fed varying amounts of vegetable and marine fatty acid to dogs during gestation and lactation (n=12) and reported improved visual performances, rod responses, rod sensitivities in the puppies fed the highest amounts of omega-3 PUFA's when compared to the other diet groups. Overall, the study concluded that preformed omega-3 fatty acids resulted in a more effective response during perinatal development and improved visual performance than ALA in enriching plasma DHA.

Ingredient Sources

Sources of omega-3 fatty acid supplementation commonly include flax seed, fish oil, and fish meal. More recently, purpose-grown algae has been shown to have potential as a supplement because it provides high levels of docosahexaenoic acid. Purpose-grown algae have been shown to be safe (Fedorova-Dahms, 2011, Hammond et al., 2001) and stable (Abuzaytoun and Shahidi, 2006). Recently there was evidence to suggest a better transfer rate of the DHA into circulation of puppies by supplementation with high levels of fish oil rather than flaxseed oil (Heinemann et al., 2005). Fish oil usually contains both EPA and DHA, whereas algal oil is predominantly DHA-rich and may provide a more targeted fatty acid solution.

With this targeted delivery of DHA in algal form in dog and cat diets, there exist questions regarding handling characteristics, stability through the pet food production processes, interactions with other ingredients, and potential impact on long-term shelf life of the fatty acid. Therefore our objectives were to determine the effect of processing on Omega-3 fatty stability of fish and algal source of DHA, (DHAgold™ S17-B; DSM Nutritional Products) added to the diet in a premix, the impact on extrusion and forced air drying processes, and extended storage time.

Commercial Manufacturing of Pet Food

The demand for companion animal commercial food goes beyond the fundamental requirements of nutritional adequacy as increased emphasis has been placed on the overall safety of the product. This is where the concept of thermal processing, also known as cooking, can be applied in a number of ways to preserve and extend the shelf-life of foods, enhance sanitation, and ensure food safety. Thermal processing is able to provide a number of benefits such as improving digestibility, enhancing flavor and texture, improving consistency, adding convenience, and controlling pathogens. Extensive thermal processing utilizing moisture, heat,

time and temperature can also have detrimental effects, including degradation of essential nutrients and oxidation of lipids. Most commercial pet food products can be grouped into three broad manufacturing processes: canned, baked, or extruded. Each of these methods is also commonly used in the manufacturing process of human foods.

Extrusion

Basic extrusion has been used in a vast array of industrial applications like rubber, plastic, metal. It was first applied to food in the 1930's (Bruin et al., 1978). Extrusion technology has gained widespread use in the food manufacturing today as a multifaceted process that combines several unit operations including: mixing, cooking, kneading, shearing, sterilization, shaping and forming, all within a single piece of equipment. Extrusion is considered one of the most aggressive food cooking processes because the dominant effects result from high pressure (400-1,000 PSI), heat (100-150°C), and humidity (30% moisture). The highly versatile equipment is able to produce a wide variety of products by allowing flexibility in the combination of ingredients, processing conditions, screw profile, and different die sizes and shapes. The time-temperature conditions in modern food cooking extrusion can be comparable to other high-temperature, short-time (HTST) processes; however, this style of processing offers a number of additional unique features. The basic component features of an extruder include a feeding or delivery system, preconditioner, extruder barrel and knife cutter. However, a number of additional independent variables and more complex systems can also be considered when reviewing the art of extrusion cooking (Bruin et al., 1978).

Feeding/Delivery Systems and Preconditioner The dry ingredients are first held and introduced into the extrusion process by a delivery system. Volumetric and gravimetric systems are the two main types; their name describe the style of delivery system. The material is fed into

the next portion of the extrusion system known as the preconditioner. This portion is not necessary to all applications, but is beneficial to pet food production as it preconditions the mix by hydrating ingredients while increasing retention time for the moisture to penetrate the starch matrix. Overall, the preconditioner allows for an increase in thermal energy input to the product.

Extruder Barrel The extruder barrel is where the work of the system occurs. It is comprised of screws, sleeves, barrel heads and dies (Figure 3.1). If one shaft, then it is designated as a single screw, and if two shafts, a twin-screw. This has implications on energy and mixing. The shaft and screw section of the extrusion system is responsible for the mixing and cooking of the ingredients where high pressure and severe shear transforms the ingredient matrix. It is this location that the additional independent variables may come into play such as injecting water or steam into the extruder, reconfiguring the screw profile to incorporate different flights, different shear or steam locks or adjusting the rotations per minute of the shaft. As the ingredients flow down the extruder barrel, a motor driving the screw shafts exerts mechanical energy onto the product in the form of friction (Riaz, 2000).

Knife Cutter/Dryer The final portion of the extrusion system is located at the end of the extruder barrel. This is where the die and knife cutter assembly are located. Their main purpose is to shape, form, and then cut the product to the desired length and shape. Additional independent variables can be selected at this point. The number of openings, number knife blades, and speed of rotation. An additional element upstream from the die-knife assembly is the back pressure valve that will allow the operator to restrict the flow of material and thereby increase the pressure on the mass immediately before it passes through the die opening. Mixing small water droplets throughout the dough allows for uniform vaporization that results in expansion of the product and cell formation as the water turns to gas during the exit from the die

opening. Pet food kibble is generally within the 20-30% wet basis moisture and 100°C as it leaves the extruder (Riaz, 2003). It is essential to convey or pneumatically transport the kibble to an additional step in order to dry the material. The drying apparatus in the pet food manufacturing process are often a convection ovens comprised of multiple perforated conveyor beds, heating elements, and air/heat-exchange systems. Procedurally the wet kibble is conveyed into shallow beds on the top belt as hot air (typically between 100-175°C) is forced through the belt and kibble bed. Having multiple conveyor levels allows for an increase in time for drying and improves the uniformity as the wet kibble has a more shallow bed depth to prevent clumping. As the kibble dries, the bed depth can be increased to accommodate for necessary retention time. Common drying principles apply during this application as an increase in temperature results in faster drying rates due to the heat transfer to the kibble (Petfood Technology, 2003).

Effects of Thermal Processing on Product Quality and Food Safety

Thermal processing kills pathogenic microorganisms within the food product and helps ensure food safety and reduce food-borne illnesses. The same thermal processing parameters that contribute to food safety can also impact product quality. A topic that has been extensively reviewed in human foods (Cheftel, 1986; Murray et al., 1999, Harper, 1978). There are a number of thermal processing production practices utilized to manufacture pet foods, such as extrusion, expansion, baking, canning, and pasteurization. These processes provide a number of benefits, primarily the destruction of pathogenic microorganisms to assure food safety, but they also impact nutritionally important chemical bonds (Björck, 1983). As an example, pet food manufacturing improves the utilization of starch through gelatinization as a result of extrusion cooking. This gelatinization process alters the starch and increases its digestibility (Murray et al.,

2001; Kienzle, 1994). However, in the extreme thermal processing negative effects to utilization and digestibility of essential amino acids and vitamins can occur (Hendriks et al., 1999).

All vitamins appear to be affected to some degree at every step of the process from production through shelf-storage. The loss in fat-soluble vitamins (A, D, E, and K) is the most significant in extruded products with losses of more than 50% occurring before the kibble goes into the bag (Coelho, 2000). In wet foods, the water-soluble B-vitamin thiamine can be almost completely lost due to its reactivity with heat, moisture, sulfites, elevated pH and the thiaminase enzymes found in fish and organ meats (Loew et al., 1970).

Pet food manufacturing is unique in that once the ingredients are exposed to the severe extrusion parameters and formed into a kibble, they exit the die relatively wet and must be dried to prevent mold growth. The typical pet food dryer uses a large volume, approximately 62.3 cubic meters per minute of super-heated air to remove moisture, much like a personal hairdryer and then an additional 39.6 cubic meters per minute on the cooler belt. Under these extremes in temperature and retention time, Tran (2008) reported in his thesis that lysine bioavailability may be reduced and linoleic acid lost. This was most probably due to oxidation. Further the additional hot-air drying time and temperature reduced kibble durability and texture. The alterations in kibble durability and texture could impact product quality and negatively affect merchandising and overall palatability for the animal.

There are many effects of thermal processing that have yet to be described. The main focus of most published reviews on processing changes has been restricted to dietary ingredients, livestock feed, and the effects processing variables during extrusion have when compared to the companion animal foods (Colheo, 1991; Dust et al., 2004; Hendriks et al., 1999). A number of difficulties are prevalent when summarizing published data for the extrusion process. Such as,

the variety of processing parameters, variation in equipment or type of extruder used among the trials, isolating single factors that influence the results, and interrelations between processing conditions. While some process conditions are nutritionally beneficial to animals, in several areas, formulators compensate with over fortification to offset processing losses for essential nutrients like vitamins, amino acids and fatty acids. Although this has been an effective method, occasional toxicities and deficiencies result in recalls (FDA, 2016). This would suggest that there is still need for more comprehensive evaluation of the nutritional effects of thermal processing of pet foods with better models to support fortification needs. Therefore, it was our objectives to determine the effect of processing conditions and dietary protein levels on essential fatty acid and vitamin retention (vitamin A, vitamin D₃, vitamin E, folic acid and thiamine) during extrusion and drying and subsequent effects during storage at ambient and elevated temperatures and humidity.

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Chapter 3 - Effects of processing conditions and dietary protein content on vitamin retention during extrusion, drying and storage at elevated and ambient temperatures in pet food.

Abstract

Little research has been published regarding the magnitude of vitamin losses through extrusion manufacturing and storage of pet food. The matrix of pet food has changed dramatically within the past twenty years since vitamin degradation was initially published. Therefore, the objective of our research was to determine the effects of thermal processing conditions and dietary protein content on vitamin retention (vitamin A, vitamin D₃, vitamin E, folic acid and thiamine) during extrusion and drying and the subsequent effect during storage at elevated temperatures. Three diets differing in protein (21.7, 25, and 30% CP; Low, Medium, and High, respectively) were produced. Diets were extruded at 350 and 450 rpm screw speed on a Wenger X-20 single screw extruder (Wenger Mfg, Sabetha, KS) and dried at 104°C for 6 min at each pass or 127°C for 10 min at each pass in a Wenger (Wenger Mfg, Sabetha, KS) triple pass dryer. Samples from each treatment were analyzed immediately following production. Without current established criterion for stressed shelf life studies of pet food, conditions consistent with industry laboratories were used; 50°C and 75% relative humidity for 3, 6, 12, 18 and 24 weeks. Additional samples were stored in ambient conditions (20°C and <50% relative humidity) and sampled at 3, 6, 12, and 18 months. Vitamin retention was not affected ($P > 0.05$) by extruder screw speed or dryer conditions. As time in stressed storage increased through 3, 6, 12, 18 and 24 weeks vitamin A ($P < 0.05$; 172,442, 108,192, 71,033, 24,687, 7,633.2, and 2,743.4 IU/kg, respectively), vitamin D₃ ($P < 0.05$; 7,821.8, 5,692.5, 4,001.4, 2,124.8, 1,656.1, and 3,059 IU/kg, respectively), vitamin E ($P < 0.05$; 960.6, 1092.6, 930.4, 943.6, 904.9, and

856.7 mg/kg, respectively), folic acid ($P < 0.05$; 1.97, 2.03, 4.43, 1.64, 1.42, and 0.67 mg/kg, respectively), and thiamine ($P < 0.05$; 25.7, 22.1, 18.2, 8.2, 3.7, and 1.8 mg/kg, respectively) concentrations decreased. These results suggest that the processing parameters in this study had little effect on vitamin losses, but elevated temperature during storage for 24 weeks could reduce vitamin content from initial by 98.4, 78.8, 10.8, 66, and 93% for vitamin A, vitamin D₃, vitamin E, folic acid and thiamine, respectively. As time in ambient storage (20°C) increased through 3, 6, 12, 18, and 24 months vitamin A ($P < 0.05$; 172,442, 119,939, 133,721, 85,734, 58,920, and 87,658 IU/kg, respectively) concentrations decreased nearly 50%. Whereas the trend-line for vitamin D₃ reflected a 30% sacrifice, but vitamin E, folic acid and thiamine appear to be relatively stable over 24 months. Vitamin fortification of extruded pet diets must take into account these changes to avoid deficiency diseases.

Introduction

Vitamin retention during food processing is contingent on a number of factors. It can depend on the molecular structure and chemical characteristics of the vitamin, the heat and duration during processing, moisture levels, substrate pH, inorganic material composition, UV light, and oxygen (Coelho, 1991). These factors are common elements for vitamins in pet food production: from how they are initially stored in raw “straight” forms, then blended together with a carrier to create a premix, and then mixed with the remainder of the food macro-ingredients into a total ration. Each of these steps, coupled with other conditions during production, present a challenge when ensuring the food retains its target vitamin levels.

Combining natural sacrifice of essential nutrients during storage, with chemical interactions with the food matrix, and the effects of food processing increases the need for additional supplementation in order to meet animal requirements. Using current information about vitamin sacrifice nutritionists over-formulate to offset the losses due to thermal processing (Aldrich, 2016). While over-fortification may address some issues, this is not a complete solution and may even increase the risk for imbalances or toxicity in companion animals if production errors occur. Little research has been published regarding the magnitude of vitamins lost during storage in a premix or through pet food extrusion cooking steps and during product storage.

Advances in vitamin manufacturing technology has decreased susceptibility to losses and maintained bioavailability to the user in certain circumstances. For example, creating an external structure through an insoluble cross-linkage between a sugar and gelatin, “beadlet coating” technology surrounds the vitamin and provides physical protection. More specifically in this process, starch is applied externally as a spray-dried coating, which creates an external physical barrier preventing environmental chemical reactions with the vitamin molecule. Then, a protein-based coating is applied and heated to bind the coating and taking advantage of malliard

reactions to create the beadlet. This technology is not exempt from the harsh conditions of a process like that of a hammermill and it may be more expensive, but the reduction in sacrifice compared to the raw counterpart outweighs the additional cost of the gelatin beadlet (Diguet et al., 2012).

Several of the vitamin companies conducted retention and (or) loss studies in the 1990's and provided tables to formulators with estimates regarding fortification of supplements (BASF, 2000). However, the specific details of these studies were never published for review or deliberation. Additionally, modern pet food and some processing parameters have changed since the previous guidelines were produced. Thus, it was our objective to validate the previous the assumptions regarding vitamin stability in extruded foods and to determine the effects different levels of dietary protein, extruder processing conditions, and dryer conditions have on key vitamin retention in a pet food.

Materials and Methods

Dietary Treatments

The experiment was organized as a 3 X 2 X 2 factorial arrangement of treatments with three dietary protein levels, two extruder screw speeds, and two oven-dryer conditions. The three dietary treatments were designed to meet target crude protein levels; low (21%), medium (25%) and high (30%; Table 3.1). The formulations were intended to mimic the crude protein content of commercial maintenance canine, performance canine and feline diets, respectively. The inclusion of vitamins were increased 10 fold the recommended levels to aid in reducing error and analytical variance inherent with micronutrients.

Brewers rice, corn, wheat, beet pulp, chicken by-product meal, corn gluten meal, calcium carbonate, potassium chloride, salt, dicalcium phosphate, choline chloride, dry natural antioxidant preservative based on mixed tocopherols, trace mineral premix were supplied as a blend by a local mill (Lortscher Animal Nutrition Inc; Bern, KS., U.S.A.). The chicken fat was dosed with natural antioxidant liquid preservative (ADF; Springfield, Mo), and a dry flavor for dogs (AFB International; O'Fallon, MO., U.S.A.) were coated topically on the kibbles. The vitamin premixes (DSM Nutritional Products; Ames, IA., U.S.A.) were added to the ration prior to extrusion and were targeted to be included at 10 times the normal target level.

The dry ingredients were blended in a twin-shaft double ribbon mixer (Scott Equipment, New Prague, MN) and the particle size of the final batch was reduced in a hammermill (Bliss 4460; Lortscher Animal Nutrition; Bern, KS) to meet the requirements of 90% passing through a US# 14 sieve. A pre-weighed quantity of each base-mix was blended with vitamin premix in a double ribbon mixer (Scott Equipment, New Prague, MN) for 5 minutes and representative samples were obtained for analysis.

Extrusion

Dietary treatments were produced on a pilot-scale single screw extruder (Model X-20, Wenger Manufacturing Inc., Sabetha, KS, U.S.A.). Diets were initially conditioned with steam and water in a differential diameter cylinder (DDC) pre-conditioner (Wenger Manufacturing Inc., Sabetha, KS, U.S.A.) fed by a volumetric feeding system with feeder screw speeds at 20.4 rpm and a constant feed rate of 200 kg/hr. The pre-conditioner shaft speed was held to a constant 400 rpm to attain discharge temperatures between 96-99°C. Samples were taken at timed intervals to obtain a representative composite and then split for analysis.

The extruder screw configuration had three heating zones set to temperatures of 60, 75 and 90°C from the feed entry to discharge end of the extruder. The extruder profile (Figure 3.1) consisted of single flight screws and transitioning to double flight half pitch screws with shear locks increasing in size between the screw elements. The die plate consisted of a one circular insert of 5 mm in diameter and a face-mounted rotary knife equipped with six blades. The knife speed was kept constant at 1,730 rpm.

The three dietary treatments were processed at two extruder screw speeds of 350 and 450 rpm to evaluate different processing effects due to the residence time and exertion of mechanical energy. Upon exit from the extruder the extrudate was pneumatically conveyed to a double pass dryer/cooler (Series 4800, Wenger Manufacturing Co. Sabetha, KS, U.S.A.). Two levels of thermal energy (low and high) were obtained by varying the dryer temperature and retention times. Samples were taken at timed intervals to obtain a representative composite and then split for analysis. For the low setting, product was dried at 104°C and adjusted for 20 minute retention time (10 minutes each for the top and bottom belts) and then cooled on the third belt with room temperature air for 10 minutes until the target end product moisture of 6% was met.

For the high setting, product was dried at 126.7°C and adjusted for 12 minute retention time (6 minutes each for the top and bottom belts) and then cooled on the third belt with room temperature air for 6 minutes until the target end product moisture of 6% was met. Samples were taken at timed intervals to obtain a representative composite and then split for analysis. The pre-weighed quantities of finished product were enrobed in chicken fat and dry digest flavor in a double ribbon mixer for 5 minutes. Samples were taken at timed intervals to obtain a representative composite and then split for analysis.

Tests for specific mechanical energy (SME) were done in per unit mass of extrudate and calculated as follows:

$$SME \left(\frac{kJ}{kg} \right) = \frac{\left(\frac{T}{100} \right) \left(\frac{N}{N_{rated}} \right) P_{rated}}{\dot{m}}$$

Where T= net motor load percentage, N = screw speed (rpm), N_{rated} = rated screw speed (507 rpm), P_{rated} = rated power (37.3 kW), and \dot{m} = net mass flow rate (kg/s).

Nutrient Analysis

The proximate composition of finished product were analyzed according to AOAC International official methods (University Missouri Analytical Lab, Columbia, MO). Moisture was determined at 135°C for 2h (AOAC 930.15), crude protein by nitrogen via combustion (N X 6.25; AOAC 990.03), crude fat by acid hydrolyzed petroleum ether extract (AOAC 920.39), crude fiber (AOCS, Ba 6a-05), and ash by muffle furnace at 600°C for 2 h (AOAC 942.05; Table 3.2).

The vitamin A analysis was performed by enzymatic digestion at an alkaline pH followed by saponification, ether extraction and injection into a normal phase HPLC; detection

wavelength was 325 nm (AOAC 974.29). The vitamin D₃ analysis was a modified version of AOAC method 2011.12; wherein, a sample was mixed with D₂ as internal standard solution, ethanol, and potassium hydroxide. The mixture was saponified, cooled and extracted with n-heptane. The analysis was completed with Liquid Chromatography Mass Spectrometry (LC-MS/MS; AOAC 2011.12). Vitamin E analyzed by dispersing in deionized water and 3A alcohol and extracted with petroleum ether. The extract was analyzed by a normal phase HPLC system using fluorescence detection by a modified version of AOAC 971.3. Folic Acid was analyzed on a stand-alone Biacore Q, biosensor-based instrument. A specific concentration of antibody was injected at a set flow rate into a known volume of sample. The antibody binds to folic acid. The unbound antibody was measured when binding occurred. Antigen (or purified form of the vitamin) was then immobilized onto the surface. Thiamine was analyzed by autoclaving the thiamine sample in dilute acid to extract thiamine. The resulting solution was incubated with buffered enzyme solution to release bound thiamine. This solution was purified on an ion-exchange column and an aliquot was taken and reacted with potassium ferricyanide to convert thiamine to thiochrome. Thiochrome was extracted into isobutyl alcohol and determined on a fluorometer (AOAC 942.23, 953.17, 957.17).

Shelf Life Evaluation

The vitamin premix was stored in 4.0 mm thick whirl pack, low density virgin polyethylene bags in quantities of 200 g. Two storage conditions were used: ambient (23°C and <70% relative humidity) and stressed (55°C and 75% relative humidity) conditions. Samples stored in ambient conditions were analyzed at 0 months initially and 1, 2, 3 and 6 month

increments thereafter. Samples stored in stressed shelf life conditions (SSLT) were sampled initially and at 1, 2, 3 and 6 weeks following.

The dry mash, wet extrudate, and dry finished product were analyzed immediately following production for determination of processing effects. Finished product samples were stored in whirl packs and partitioned into groups representative of date and treatment for analysis. Two storage conditions were used in which treatments were placed in ambient (23°C and <70% relative humidity) and stressed (55°C and 75% relative humidity) conditions. Treatment samples stored in ambient conditions were analyzed initially (0 month) and at 3, 6, 12, 18 and 24 month increments, while the samples in the environmental chamber exposed to stressed conditions were sampled initially (0 month) and at 3, 6, 12, 18 and 24 weeks following production.

Statistical Analysis

Experimental treatments were organized as 3 X 2 X 2 a factorial arrangement with three levels of protein, two extruder shaft rotations speeds, and two drier time and temperature settings. Main effects from processing and shelf life stability studies were analyzed by two-way ANOVA using PROC GLIMMIX procedure (SAS version 9.2 SAS Institute Inc. Cary, North Carolina, U.S.A). Means separation was conducted using the LSMEANS statement. Differences were considered significant if $P < 0.05$ and trends $0.05 < P < 0.10$. Percent retained standard error of the means were reported from initial values.

Results

Chemical Composition

The experimental diets nutrient values were compared to the predicted levels to ensure formulated targets were met (Table 3.2). Each treatment met and exceeded the target value for crude protein for low, medium and high protein diets by 1.98, 2.24 and 4.37%, respectively (Table 3.2). The target moisture for each of the treatments was approximately 10% where the actual values were lower, averaging 4.79%. For each of the treatments, the targeted values for crude fiber, ash and crude fat were within 0.3% units when compared to the actual values.

Effects of Storage Conditions on Vitamin Retention within a Vitamin Premix

The retention of vitamins within a vitamin premix over a 6 week shelf life at stressed shelf life conditions of 50°C and 75% relative humidity were analyzed and reported in Figure 3.2. Vitamin A, vitamin D₃, vitamin E, and folic acid were relatively stable through week two, but thiamine decreased 24% from initial. From week two to week three there was a sacrifice of an additional 47, 62, 26, 70 and 6% for vitamin A, vitamin D₃, vitamin E, folic acid and thiamine, respectively. But, these values were not consistent with the final week of stressed storage conditions as overall sacrifice resulted in 36, 7, 14 and 35% for vitamin A, vitamin D₃, vitamin E and thiamine, respectively, and folic acid did not appear to decline over the 6 weeks (Figure 3.2).

The vitamin premix stored within ambient shelf life conditions (20°C and 50% relative humidity) over a period of six months (Figure 3.3) and vitamin A declined steadily from initial to month 6 by an average of 24% resulting in over half of the vitamin depleted. Vitamin D₃ decreased substantially from month two to month three (61%) with an overall sacrifice from initial of 67%. Like vitamin D₃, Vitamin E resulted in a greater sacrifice between months two and

three and by month six 56% of vitamin E was retained. Folic acid and Thiamine appeared relatively stable from initial through month three, and by month six retention was 65 and 40% for folic acid and thiamine, respectively.

Effects of Experimental Crude Protein Level on Vitamin Retention through Processing

The effects of experimental protein level on vitamin retention as the pet food ration exited the extruder appear to result in a lower vitamin concentration when compared to the target for all the protein levels (Table 3.3). The specific mechanical energy (SME) was higher for the low protein treatments when compared to the medium and high (130.1 vs. 114.1 and 119.5 kJ/kg). The same was true for the in barrel moisture as the low CP treatments were higher when compared to the medium and high (20.5 vs. 19.8 and 20.0%). Where the bulk density was greater for the high when compared to the medium and low CP treatments (392.3 vs. 320.8 vs. 310.5 g/L). Vitamin A retention was greatest ($P < 0.05$) for the high protein diet relative to the medium and low diets (225,352 vs. 206,249 and 219,184 IU/kg, respectively). Vitamin D₃ was not different among the treatments (average 8,139 IU/kg). The same relationship as seen for vitamin A occurred with vitamin E and folic acid. Thiamine retention was not affected by dietary protein level (average 23.13 mg/kg).

The effects of protein level on vitamin retention for product exiting the dryer were lower again when compared to the target (Table 3.4). Vitamin A levels do not appear to be affected by protein levels at the end of the production process (average 172,441.7 IU/kg). The vitamin D₃ levels were higher ($P < 0.05$) for the high protein diet compared to the medium and low (9,047.07 vs. 7,472.59 IU/kg and 6945.7 IU/kg, respectively). The same relationship occurred

with vitamin E; wherein, the high protein diet retained more vitamin E than the medium or low (1,102.3 vs. 884.8 and 894.7 IU/kg). Folic acid was also greater ($P < 0.05$) for High relative to Medium and Low protein (2.2 vs. 1.9 and 1.8 mg/kg, respectively). Thiamine did not differ among the dietary treatments (average 25.73 mg/kg).

Effects of Processing on Vitamin Retention

The formulated target concentration for vitamin A in the dry flour was 240,000 IU/kg and the actual 237,135 IU/kg on average (Figure 3.4). There was an effect on vitamin retention from the overall extrusion and drying process. However screw speeds during extrusion and dryer temperatures did not have an effect on vitamin retention. Given the lack of extruder screw speed and dryer temperature effects the remainder of the treatment data were pooled (Figure 3.5). Among the processing steps a greater quantity of vitamin A, vitamin D₃ and vitamin E were retained in the dry flour when compared to their initial targeted values. Following the initial processing through the extruder, vitamin A declined ($P = 0.24$) 29.5% and an additional ($P=0.02$) 19.3% following exit from the drier. Vitamin D₃ followed a similar trend ($P = 0.89$) with a 22.7% reduction through the extrusion process, but remained steady ($P = 0.1$) through the drier with a 2.6% loss. Vitamin E had the greatest sacrifice ($P = 0.72$) through the extrusion process (62%), but no additional degradation ($P = 0.88$) occurred from drying. Folic Acid and thiamine appeared to be relatively stable ($P = 0.74, 0.58$) through the manufacturing process.

Effects of Storage Conditions on Vitamin Retention

Samples from the various processing treatments were pooled and stored for a 24 week shelf life test at conditions of 50°C and 75% relative humidity. Over the 24 weeks, vitamin A decreased asymptotically ($P < 0.05$) with losses of 37.3, 21.6, 26.9, 9.8, and 2.8%, over 3, 6, 12, 18, and 24 weeks, respectively (Figure 3.6). Vitamin A was nearly depleted with a loss of 98.41% by 24 weeks. Vitamin D₃ shared a similar fate with losses ($P < 0.05$) of 27.2, 21.6, 24.0, and 6% over 18 weeks and a gain, perhaps due to sampling variation, of 17.94% at week 24 of the study, respectively. Overall, nearly 61% of the vitamin D₃ was sacrificed through the elevated temperature storage conditions. Like vitamin A and vitamin D₃, thiamine decreased steadily ($P <$

0.05) until near depletion over the 24 weeks with losses of 14.1, 15.1, 39.1, 17.2, and 7.5%, respectively. Folic acid and vitamin E displayed a different trend; wherein folic acid appeared to rise at week six, but then return to the trend line for 12, 18, and 24 weeks and result in a nearly 60% depletion ($P < 0.05$) by the 24 week end. Vitamin E decreased ($P < 0.05$) but appears to be relatively stable for the entire duration of the 24 weeks when compared to the other vitamins.

A matching set of samples of the extruded pet foods were stored in ambient conditions (20°C and 50% relative humidity) for 24 months (Figure 3.7). Among the samples vitamin A decreased steadily ($P < 0.05$) at each time point following initial production until 18 months then rose slightly at 24 months to result in a nearly 50% overall reduction. Vitamin D₃ results were more erratic; wherein, at three, 12, and 24 months there appeared to be a gain by analysis. However, this may be a function of sampling or analytical error with the retention declining ($P < 0.05$) by nearly 30% at 24 months versus the initial. Vitamin E, folic acid and thiamine appear to be relatively stable over a 24 month study if one considers final values relative to the initial concentrations.

Discussion

Proximate analysis was performed to ensure all products were close to the expected values for moisture, protein, fat, fiber and ash. The moisture content for each of the treatments were consistently less than the formulated value; however, 5% was targeted during production and confirmed by the analysis. Crude protein was consistently greater than the minimum, which was expected as the treatments were slightly over-formulated. Crude fiber and ash were very close to the target values as expected. However, the crude fat content was slightly less (~0.5%) than the formulated target for the medium and high crude protein treatments. During the extrusion process, a lipid-amylose complex forms and traditional methods of measuring fat are less effective compared to the recommended AOAC method 954.02 for acid hydrolysis. This method utilizes hydrochloric acid to break the trapped lipids and free the fat to be measured. The difference between the medium and high crude protein treatments versus the low crude protein may be attributed to the variability in raw materials.

Initially, it was anticipated that there would be excess of each vitamin in the premix when compared to the target as this is a common practice by premix manufacturers to assure customers always get slightly more than they specify, and to off-set storage degradation of various vitamins during transport and storage (Aldrich, 2016). Currently nutritionist compensate for vitamin sacrifice through storage from upwards of 4-10 times the recommended levels. The premix used in this study was enhanced an additional 10 times from the levels of compensation for loss. Since these levels are much higher than recommended, there was concern that the higher levels may influence survival, but also aid in reducing sampling and analytical error. The analysis of the vitamin premix was limited to single point analysis. In our study, all vitamins had some sacrifice in the vitamin premix stored in SSLT, e.g. vitamin A sacrifice exceeded 35%. However, the

losses were not as severe for those samples stored in ambient temperatures e.g. vitamin A sacrifice exceeded 50%. This was unexpected for vitamin A and does not agree with previous research by Christian (1983) who demonstrated the negative effects of environmental conditions on vitamin A stability within a premix. Wherein, vitamin A retention was 88% of initial when exposed to low temperature and humidity for three months, and decreased slightly to 86% when temperatures were increased, then bottomed-out (to only 2% retention) when they were exposed to high temperatures and humidity. His study concluded that vitamin A was susceptible to temperature, and adding humidity amplified the impact. In a study by Kuong et al. (2016) it was also observed that an increase in vitamin loss, e.g. loss of 93% of vitamin A for a coated premix occurred at higher temperatures and humidity. Similar to vitamin A, thiamine concentration in our study decreased during ambient storage, declining to nearly 35% by the sixth month. For all vitamins, sacrifice exceeded 30% at time point (six months). These levels of sacrifice are more severe when compared to the BASF technical bulletins that most nutritionists use to fortify their diets. This is also more severe than previous research by Coelho (1991); wherein, no less than 90% of vitamin A, vitamin D₃, vitamin E, thiamine, and folic acid were retained during ambient storage. Li et al. (2011) also observed folic acid to be stable during ambient storage (nine months), retaining 75-95% within a reconstituted rice product. This would suggest that vitamins are relatively stable if we can store the vitamins in controlled environments.

This study was performed on a small scale extruder and dryer vs. the larger more representative manufacturing equipment. The treatments were all produced in a single run where the run order was designed to help reduce the impact of sequencing and start up effects from the extruder. The degradation of vitamins during the manufacturing process was expected however, the lack of effects within the process changes was not. As the screw speed and dryer temperature

and retention time increased no differences were observed (Figure 3.2). The screw speed is directly related to the residence time for the material within the extruder. As the screw speed increases, the residence time decreases causing less contact between the extruder screw, barrel, and food materials. The decreased exposure to shear in the extruder and increased retention time would potentially decrease the amount of sacrifice through processing. The effects of changing screw speed has not been researched extensively in pet food applications. A few research studies have examined the effect of screw speed on physical parameters. In a study with a rice based extrudate it was observed that there was little to no impact on the physicochemical properties and sensory characteristics of the extrudate with varying screw speeds (Guha et al., 1997; Ding et al., 2004). This was consistent with the minimal effects observed by Fallahi et al. (2013) with similar extrusion parameters on a DDGS-based extrudate. Yanjik et al. (2010) observed the effects of thermal and mechanical energy on vitamin retention and concluded the predominant effects of degradation were caused by the mechanical input. Thermal and mechanical input energies are the mechanisms that speed starch gelatinization and may create amylose-lipid complexes (Gibson, 2015). This may explain why our lack of changes due to screw speed on vitamin retention were less affected by minor processing changes than by big differences between processes.

The degradation of the vitamin A, vitamin D₃, vitamin E and thiamine were expected considering previous research and the combination of the harsh conditions during pet food manufacturing. In conjunction with a number of environmental factors, the chemical confirmation also increases their susceptibility to degradation. In order to function properly, vitamins must be reactive to serve their purpose for normal physiology; however, this reactivity increases susceptibility to decay from external sources i.e. oxidation, heat, light, and radiation. As an example, vitamin E functions as an antioxidant protecting the body from damage but in

doing so sacrifices itself. Similar circumstances occur for vitamin A, beta-carotene, etc. (Coelho, 1991; BASF, 2000; Killeit, 1994). Some vitamins may be less reactive in the whole. Li et al. (2011) demonstrated the stability of folic acid within a reconstituted rice product under high temperature and humidity (40°C and 60% RH). Under similar extrusion temperature ranges to our study (91-100°C), the level of vitamin sacrifice were less (Coelho, 1991). He reported a reduction of only 12, 5, 6, 8, and 9% for vitamin A, vitamin D₃, vitamin E, folic acid and thiamine, respectively. Thiamine degradation has been extensively researched. For example, Guzman-Tello et al. (2007) reviewed thiamine degradation as an indicator for processing severity and concluded that under first order kinetics within their model. These processing data suggest that the initial production has profound impact on the vitamin survival, but changing the parameters within the process do not seem to have as large an impact. Further, the variability in degradation among these studies may indicate that diet composition has a big part in the outcome on how vitamins behave within a complex pet food matrix.

Following extrusion, there was a positive effect of experimental protein level on vitamin retention. Previous research has shown that as crude protein levels increase there is an offsetting starch decrease which can increase product bulk density. Increased bulk density was observed in this study as the CP level increased. This results in a decreased expansion ratio as extruded kibble expands the starch to create the cellular structure during expansion (Zhu et al., 2010). The cross-sectional images of the cellular structure reveals a decrease in cellular size for products with an increased strength of the cell walls as the crude protein levels increase (Allen et al., 2007). Thus, one might surmise that more vitamin gets trapped in the starch-matrix and exposed to the external environment with higher starch: lower protein foods. Said another way, the lower the expansion the less surface area exposed externally to air and oxidation and their associated

damage. The hardened external shell of the kibble, decreased cellular size, or increased protein entrapping the vitamins may provide a solution to a greater vitamin A retention if the excess air, light, and heat is unable to penetrate the kibble. To corroborate this hypothesis, the high protein diet had the highest retention for vitamin E and folic acid. It doesn't appear to affect all vitamins the same as vitamin D₃ and Thiamine were not affected by crude protein level (Table 3.3).

Shipping and storage of pet foods often requires transport across the equator and throughout very humid and hot environments where temperatures can exceed 40 to 50°C. Thus the experimental conditions in our study were intended to mimic these real conditions. Additional loss was expected as sacrifice due to heat has already been observed, however the elevated temperatures over a 24 week period depleted vitamin levels to nearly non-existent for vitamin A and thiamine and upwards of 70% sacrifice for vitamin D₃ and folic acid (Figure 3.6). This is consistent with the knowledge about vitamin A and thiamine sensitivity to heat and humidity (Coelho, 2002). Pet food can be stored in environmentally controlled warehouses with temperatures in the 20 to 22°C range. In these cases we observed only slight losses of vitamin A, which was consistent with Kuong et al. (2016) who observed a loss of 30% in hot extruded rice and upwards of 77% for a coated rice premix. So too, this was expected based on Coelho (1991) and BASF (2000). However, in our study, even during ambient storage conditions nearly half of the vitamin A was depleted by 24 months, and vitamin D₃ was a bit less at nearly 30%. However, vitamin E appeared to be relatively stable over the 24 month study. This might be expected as vitamin E is a known antioxidant and stabilizing agent in feed and food (Riaz, 2009). As well, folic acid and thiamine appear to be relatively stable in ambient conditions (Figure 3.7).

Conclusion

There was vitamin degradation when premixes were stored within extreme (increased humidity and temperature) and ambient conditions. The collective extrusion and drying process resulted in vitamin losses; but, there were minimal effects from different screw speeds during extrusion or from changing the dryer conditions. Diets with higher protein content retained more vitamins than medium and low protein diets. Elevated temperature during storage for 24 weeks could reduce vitamin content from initial by 98.44, 60.89, 10.81, 65.54, and 92.86% for vitamin A, vitamin D₃, vitamin E, folic acid and thiamine. Where, ambient storage conditions for 24 months could reduce vitamin A content from initial by 50%, vitamin D₃ by nearly 30%, where vitamin E, folic acid and thiamine appear relatively stable. The total retention following both processing and AMB storage was 27, 68, 78% for vitamins A, D₃, and E, respectively while folic acid and thiamine were relatively stable. Therefore, storage conditions over a products entire shelf life play a substantial role when determining the level of vitamin sacrifice during storage.

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Table 3.1: The ingredient composition of experimental diets evaluated in extruded pet food tests for determining shelf life of vitamin retention.

Ingredient, % as-is basis	Crude Protein Level		
	Low	Medium	High
Brewer's Rice	20.18	17.50	13.02
Corn	20.18	17.50	13.02
Wheat	20.18	17.50	13.02
Beet Pulp	4.00	4.00	4.00
Chicken By-Product Meal	21.99	26.09	34.05
Corn Gluten Meal, 60%	2.50	5.00	7.50
Calcium Carbonate	0.25	0.25	0.25
Potassium Chloride	0.39	0.38	0.37
Salt	0.40	0.40	0.40
Dicalcium Phosphate	1.01	0.80	0.46
Choline Chloride, 60%	0.20	0.20	0.20
Natural Preservative	0.06	0.06	0.06
Chicken Fat ^a	7.57	9.20	12.55
Digest-Dry Dog ^a	0.50	0.50	0.50
Vitamin Premix^b	0.50	0.50	0.50
Trace Mineral Premix ^c	0.10	0.10	0.10

^aChicken Fat and Digest topically applied.

^bVitamin Premix: Calcium Carbonate, Roughage Products, Vitamin E Supplement, Niacin Supplement, Vitamin B12 Supplement, Mineral Oil, D-Calcium Pantothenate, Vitamin A Supplement, Thiamine Mononitrate, Pyridoxine Hydrochloride, Riboflavin Supplement, Vitamin D₃ Supplement, Biotin, And Folic Acid.

^cTrace Mineral Premix: Calcium Carbonate, Zinc Sulfate, Ferrous Sulfate, Copper Sulfate, Mineral Oil, Manganous Oxide, Sodium Selenite, Calcium Iodate

Table 3.2: Nutrient Composition of Experimental diets for Vitamin Stability

Nutrient Composition, % as-is basis					
Treatments	Crude Protein*	Moisture	Crude Fiber	Ash	Crude Fat* *
Low Protein – Target	21.00	10.00	2.35	6.26	12.00
Low Protein – Actual	23.75	5.03	2.42	6.27	12.12
Medium Protein – Target	25.00	10.00	2.36	6.60	14.00
Medium Protein – Actual	27.24	4.34	2.09	6.17	13.84
High Protein – Target	30.00	10.00	2.35	6.27	18.00
High Protein – Actual	34.37	5.01	2.55	6.90	17.10

* *Percentage N X 6.25*

** Crude Fat by Acid Hydrolysis

Table 3.3: The effects of experimental protein level (low, medium and high) on vitamin retention exiting the extruder, as-is basis.

Item	Unit	Target*	Protein Level			SEM	P=
			Low	Medium	High		
Vitamin A	IU/kg	241,014.0	206,249.0 ^b	219,184.0 ^a	225,352.0 ^a	3,791.4	0.020
Vitamin D ₃	IU/kg	10,205.0	7,836.0	8,204.0	8,377.0	215.8	0.260
Vitamin E	IU/kg	1,269.0	755.0 ^c	801.0 ^b	893.0 ^a	32.0	0.050
Folic Acid	mg/kg	2.3	1.4 ^b	1.6 ^b	1.8 ^a	0.05	0.004
Thiamine	mg/kg	28.2	22.5	23.3	23.6	0.36	0.200

^{ab} Means in a row with unlike superscripts differ ($P < 0.05$).

*Target was not evaluated in statistical analysis and is provided as a form of reference for the target amount intended based on vitamins in premix.

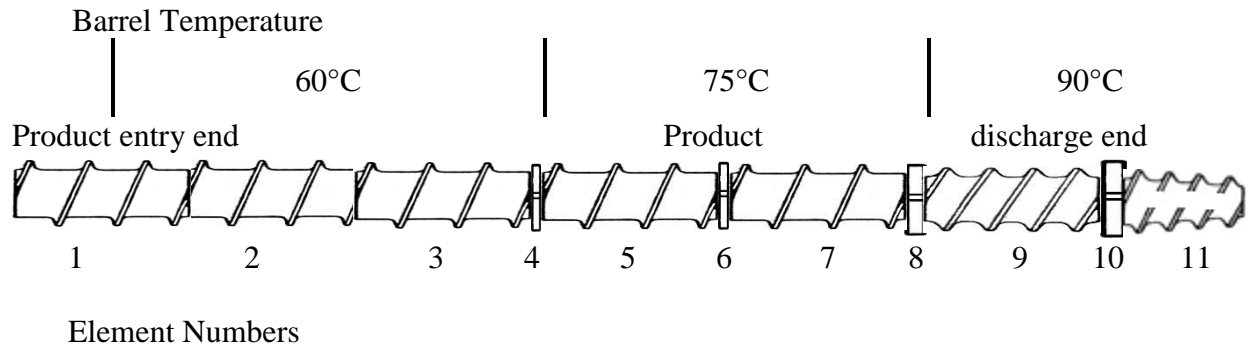
Table 3.4: The effects of experimental crude protein level on vitamin retention as kibble exits the drier.

Item	Unit	Target*	Protein Level			SEM	P=
			Low	Medium	High		
Vitamin A	IU/kg	241,014.0	173,022.0	173,234.0	171,069.0	3,521.9	0.80
Vitamin D ₃	IU/kg	10,205.3	6,945.7 ^b	7,472.6.0 ^b	9,047.1 ^a	458.1	0.006
Vitamin E	IU/kg	1,269.1	894.7 ^b	884.8 ^b	1,102.3 ^a	26.6	0.001
Folic Acid	mg/kg	2.3	1.8 ^b	1.9 ^b	2.2 ^a	0.03	0.0006
Thiamine	mg/kg	28.2	25.5	25.1	26.6	0.6	0.14

^{ab} Means in a row with unlike superscripts differ ($P < 0.05$).

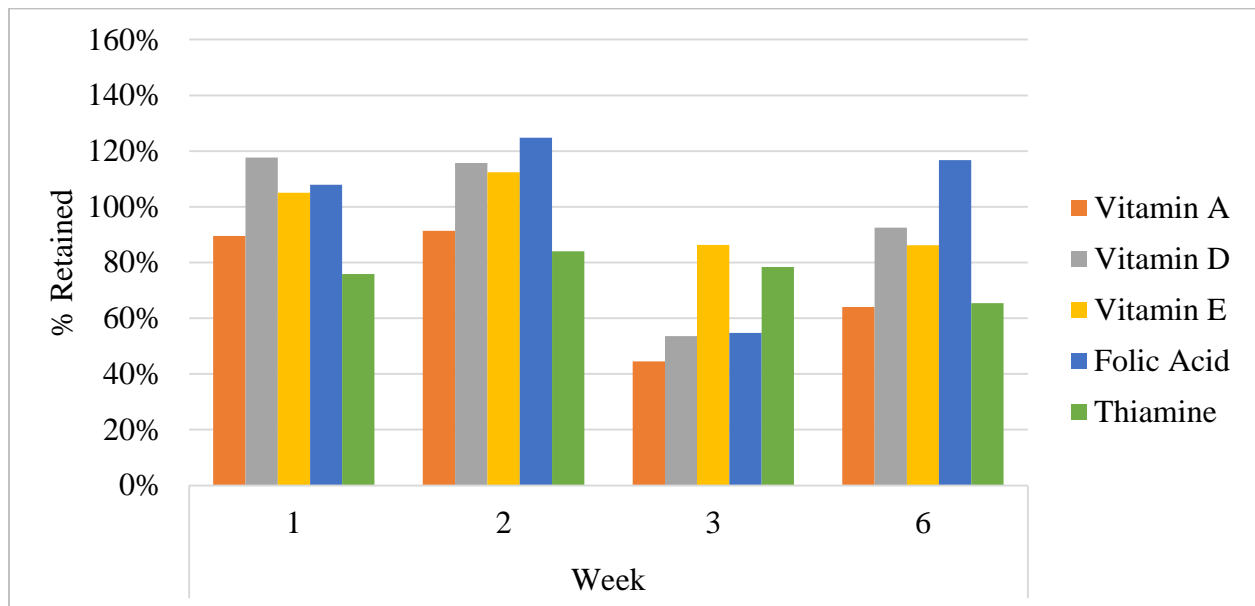
*Target was not evaluated in statistical analysis and is provided as a form of reference for the target amount intended based on vitamins in premix.

Figure 3.1: Schematic showing pilot scale single screw extruder profile and barrel.



Extruder screw element numbers with screw types. 1-3 =single flight screws; 4=small steamlock; 5=single flight screw; 6=small steamlock; 7=single flight screw; 8= medium steamlock; 9=half pitch, double flight screw; 10=large steamlock; and 11=half pitch, double flight, cut cone.

Figure 3.2: The retention of vitamins in a vitamin premix over six week's shelf life (stressed shelf life testing; 50°C 75%RH)



**Figure 3.3: The retention of vitamins in a vitamin premix over a six month shelf life
(ambient conditions; 20°C and 50%RH)**

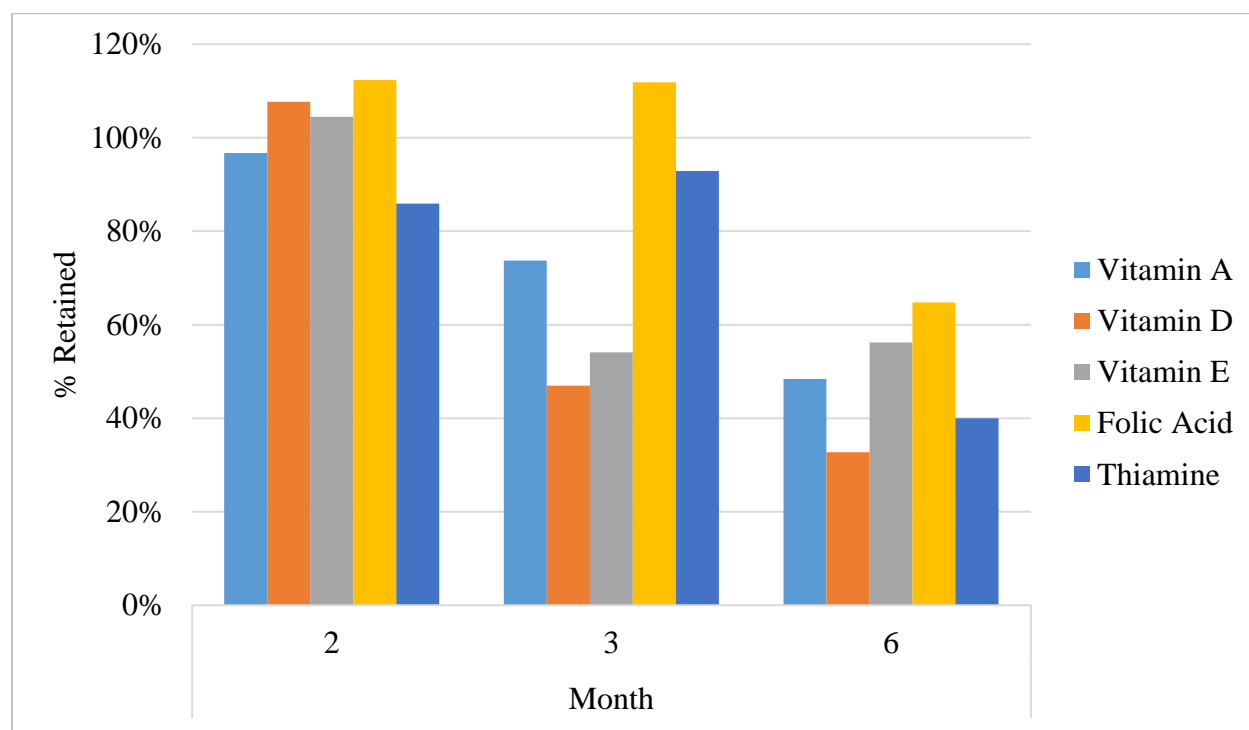


Figure 3.4: The effects of retention time on vitamin A levels during processing.

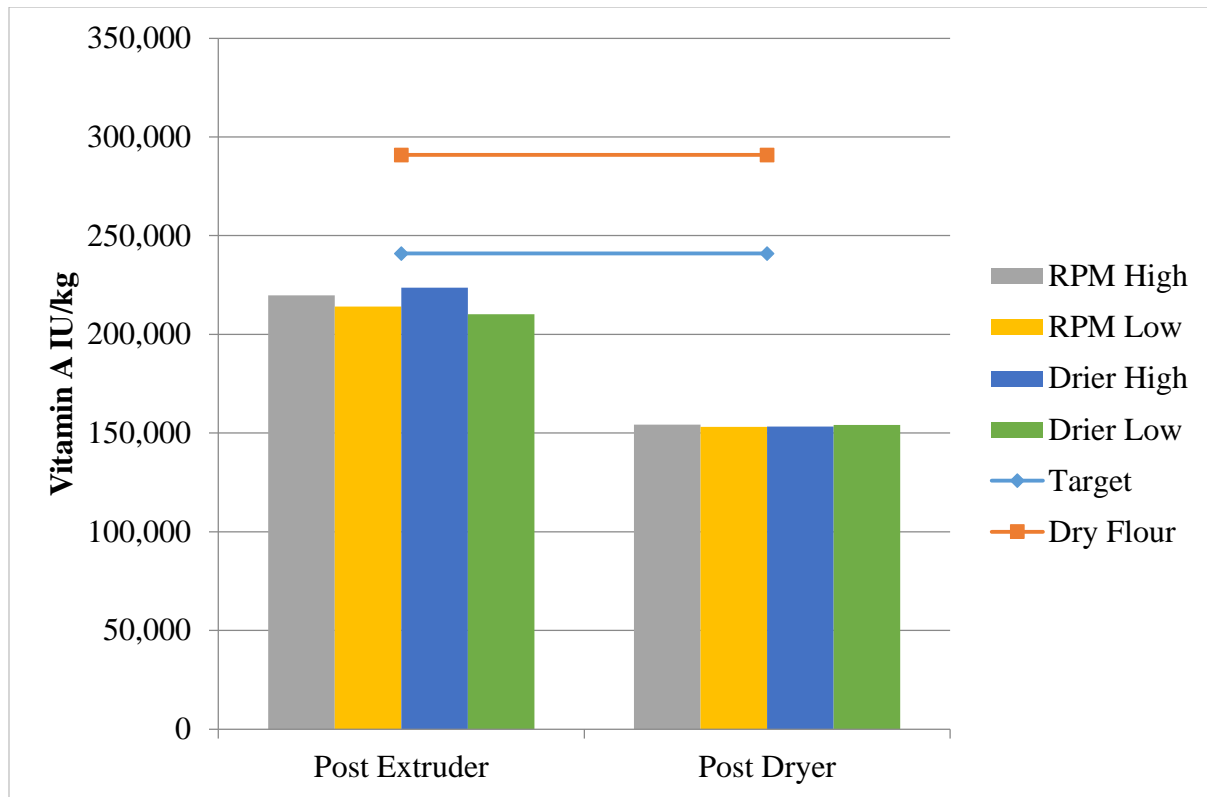


Figure 3.5: The main effects of processing on vitamin retention in pet food diets.

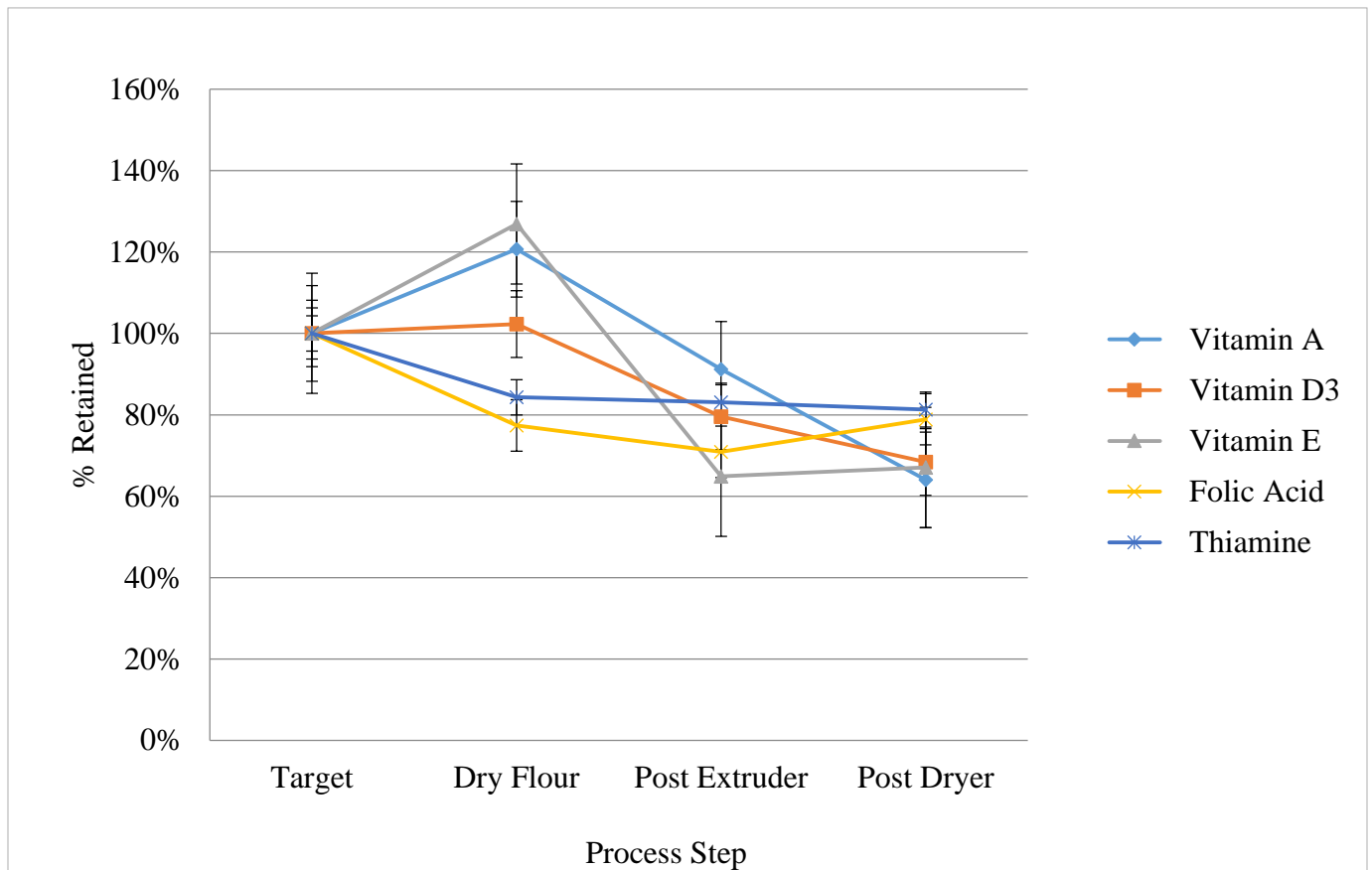


Figure 3.6: The retention of vitamins in extruded pet foods over 24 week's shelf life (stressed shelf life testing; 50°C 75%RH).

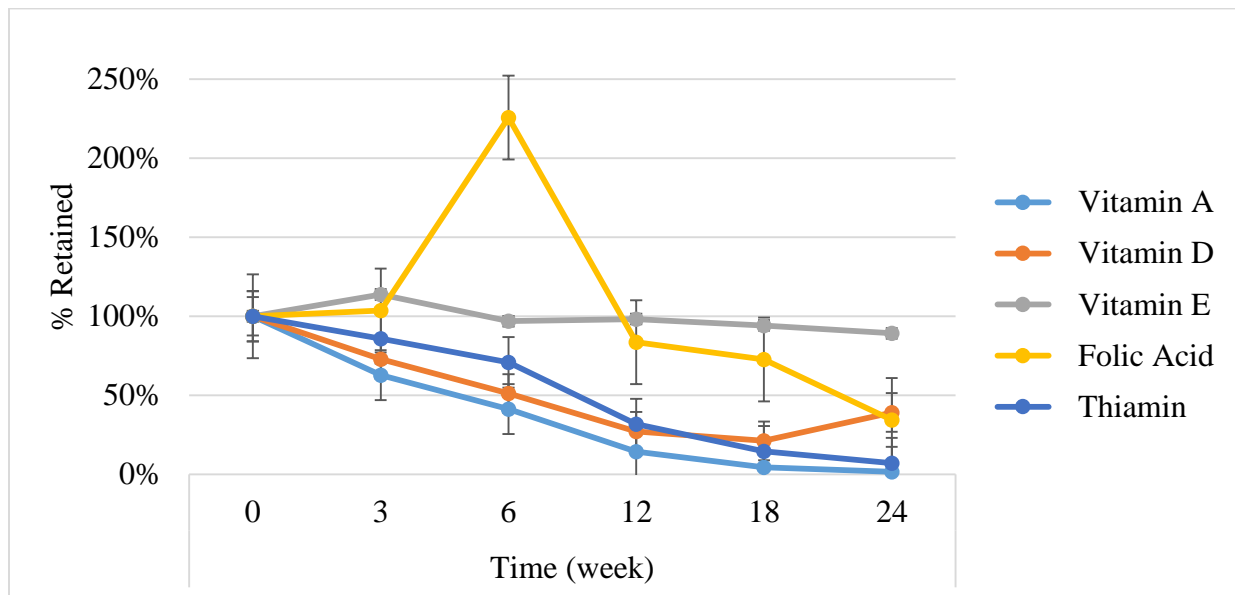
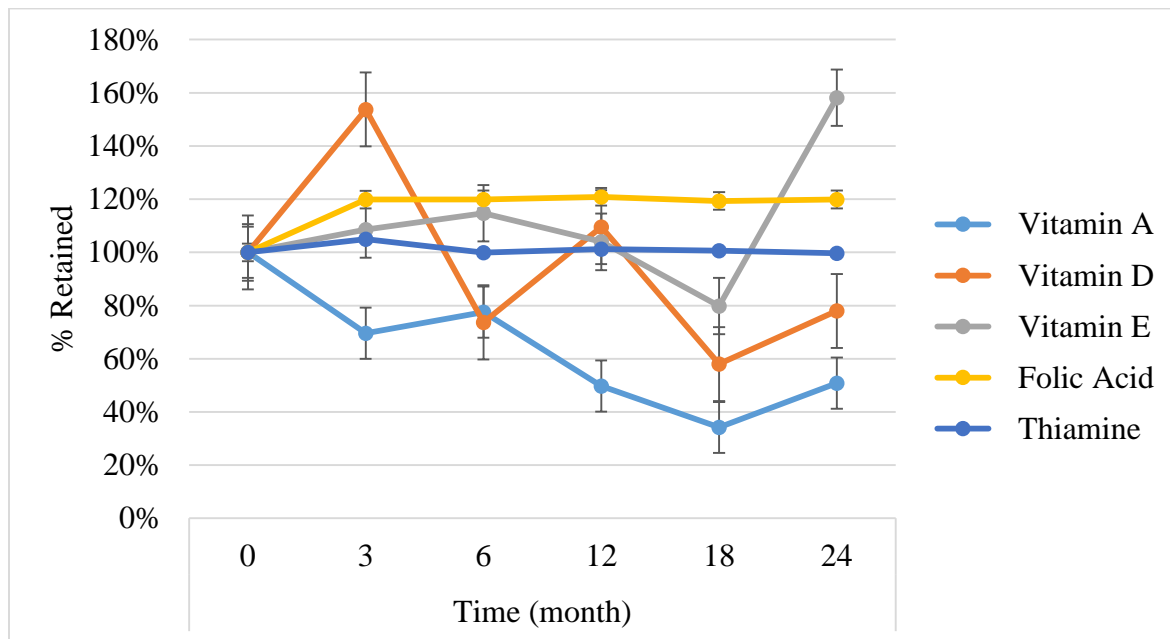


Figure 3.7: The retention of vitamins in extruded pet foods over 24 month shelf life (ambient conditions; 20°C and 50%RH).



Chapter 4 - Effects of processing conditions and dietary protein content on DHA, EPA and total omega-3 fatty acid retention during extrusion, drying and during storage at elevated and ambient temperatures in pet foods.

Abstract

Essential fatty acid research has shown that omega-3 fatty acids, such as eicosahexaenoic acid (EPA) and docosahexaenoic acid (DHA; 22:6n3) may help maintain normal body structure, function and aid in long-term health and wellbeing. Common sources of omega-3 fatty acids include flax seed, fish oil, fishmeal, and more recently purpose-grown algae. This commercially-produced source of omega-3 fatty acids has been evaluated as supplements to animal diets and the impact on metabolism; however, questions regarding the effect of process and storage in pet foods are unanswered. The objective was to determine the effect of processing on stability of an algal source of DHA, (DHAGold™ S17-B; DSM Nutritional Products) added to the diet by premix, extrusion-drying processing, and extended storage. Three nutritionally complete pet diets at protein levels 21.0, 25, and 30% CP (Low, Medium and High, respectively) were produced with equal levels of DHA supplied by Algal-DHA S17-B, fishmeal and fish oil. Diets were produced on a Wenger X-20 single screw extruder (Wenger Mfg, Sabetha, KS) and dried at 104°C for 10 min at each pass in a triple pass dryer (Wenger Mfg, Sabetha, KS). Samples from each treatment were analyzed immediately following production for moisture and fatty acids. Shelf-life samples were collected in whirlpaks with a pin-hole and stored at 40°C and 75% relative humidity for analysis at 3, 6, 12, 18 and 24 weeks following production. Retention of EPA and DHA at production time was not affected by CP level ($P > 0.05$), but was impacted by

DHA source ($P < 0.05$). The total omega-3 fatty acids were affected by DHA source and CP level ($P < 0.05$). As time in storage progressed through 0, 3, 6, 12, 18 and 24 weeks EPA ($P < 0.05$; 125.27, 104.45, 101.85, 88.9, 83.8, and 82 mg/kg, respectively) and DHA ($P < 0.05$; 77.4, 69.4, 72.3, 61.1, 64.0, 60.1 mg/kg, respectively) declined slightly; but, total omega-3 fatty acids ($P < 0.05$; 373.9, 476.7, 478.5, 444.6, 433.1, 418.0 mg/kg, respectively) were greater at all times than the start. These results suggest that elevated temperatures during storage for 24 weeks could result in slight EPA and DHA sacrifice. As time in storage progressed through 0, 3, 6, 12, 18, and 24 months EPA ($P < 0.05$; 125.27, 103.9, 105.0, 96.3, 86.5, and 78.3 mg/kg, respectively) and DHA ($P < 0.05$; 77.4, 70.56, 71.1, 67.9, 59.8, and 50.1 mg/kg, respectively) declined slightly; but, total omega-3 fatty acids ($P < 0.05$; 373.9, 452.5, 471.7, 488.0, 425.8, and 387.1mg/kg, respectively) were greater at all times than the start. Algal-DHA S17-B appears to be a stable source of DHA when compared to fish oil and fish meal.

Introduction

Nutrition research has demonstrated that omega-3 fatty acids such as α -linolenic acid (ALA; 18:3n3), eicosapentaenoic acid (EPA; 20:5n3) and docosahexaenoic acid (DHA; 22:6n3) may help maintain normal body structure and (or) function and aid in long-term health and wellbeing (Larsen, 2011). Common ingredient sources of omega-3 fatty acids in commercially produced companion animal formulations include flax seed, fish oil, fish meal, and more recently purpose-grown algae (Palmquist, 2009). This commercially produced source of omega-3s has been evaluated as a supplement to animal diets and for its impact on metabolism; however, questions regarding the effect of process and storage in pet foods remain unanswered. Therefore our objectives were to determine the effect of processing on omega-3 fatty acid stability of fish and algal sources (DHAgold™ S17-B; DSM Nutritional Products) of DHA that were added to the diet by a premix, followed by extrusion and drying processes, and extended storage.

Materials and Methods

Dietary Treatments

Three dietary treatments were prepared based on CP levels; low (21.0%), medium (25%) and high (30%; Table 4.1). The formulations were intended to simulate a crude protein content similar to commercial maintenance canine, performance canine, and feline diets, respectively. The diets were formulated to have similar DHA levels to that of the algal source by replacing a portion of the diet with either Menhaden fish meal or Menhaden fish oil.

The ingredients (brewers rice, corn, wheat, beet pulp, chicken by-product meal, fish meal, corn gluten meal, fish oil, calcium carbonate, potassium chloride, salt, dicalcium phosphate, choline chloride, dry natural antioxidant, trace mineral premix, and vitamin premix) were ground and mixed into a basemix by a local mill (Lortscher Animal Nutrition Inc; Bern, KS., U.S.A.). The chicken fat was dosed with natural antioxidant liquid (ADF; Springfield, Mo) and applied topically to kibbles after drying along with a dry flavor for dogs (AFB International; O'Fallon, MO., U.S.A.). The vitamin premixes with the algal-DHA (DHAGold™ DSM Nutritional Products, Ames, IA., U.S.A.) was mixed prior to extrusion.

The dry ingredients were blended in a twin-shaft double ribbon mixer (Scott Equipment, New Prague, MN) and the particle size reduced in a hammermill (Bliss 4460; Lortscher Animal Nutrition; Bern, KS) such that 90% would pass through a US#14 sieve. Pre-weighed quantities of each base-mix were blended with vitamin premix in a double ribbon (Scott Equipment, New Prague, MN) for 5 minutes and representative samples were obtained for analysis.

Extrusion

Dietary treatments were produced on a pilot-scale single screw extruder (Model X-20; Wenger Manufacturing Inc., Sabetha, KS) Diets were initially conditioned with steam and water

in a differential diameter cylinder (DDC) pre-conditioner (Wenger Manufacturing Inc., Sabetha, KS) fed by a volumetric feeding system with feeder screw speeds at 20.4 rpm and a constant feed rate of 200 kg/hr. The pre-conditioner shaft speed was held constant at 400rpm to attain discharge temperatures between 88-93°C. Samples were taken at timed intervals to obtain a representative composite and then split for analysis.

The extruder screw configuration commonly used for pet production had three heating zones set to temperatures of 60, 75 and 90°C from the feed entry to discharge end of the extruder. The extruder profile consisted of single flight screws and transitioning to double flight half pitch screws with shear locks increasing in size between the screw elements (Figure 4.4). The die plate consisted of a single circular 5 mm diameter insert and a face-mounted rotary knife equipped with six blades. The knife speed was kept constant at 1,730 rpm.

The three dietary treatments were processed at an extruder screw speed of 350 rpm. Samples were taken at timed intervals to obtain a representative composite and then split for analysis. Extrudate leaving the die was pneumatically conveyed to a double pass dryer/cooler (Series 4800, Wenger Manufacturing Co. Sabetha, KS). Product was dried at 104°C and adjusted for 20 minute retention time (10 minutes each for the top and bottom belts) and then cooled on the third belt with room temperature air for 10 minutes until the target end product moisture of 6% was met. Samples were taken at timed intervals to obtain a representative composite and then split for analysis. The pre-weighed quantities of finished product were coated with chicken fat and dry dog flavor in a double ribbon mixer for 5 minutes. Samples were taken at timed intervals to obtain a representative composite and then split for analysis.

Tests for specific mechanical energy (SME) were done in per unit mass of extrudate and were calculated as follows:

$$SME \left(\frac{kJ}{kg} \right) = \frac{\left(\frac{T}{100} \right) \left(\frac{N}{N_{rated}} \right) P_{rated}}{\dot{m}}$$

Where T= net motor load percentage, N = screw speed (rpm), N_{rated} = rated screw speed (507 rpm), P_{rated} = rated power (37.3 kW), and \dot{m} = net mass flow rate (kg/s).

Nutrient Analysis

The proximate composition of finished product were analyzed (University Missouri Analytical Lab, Columbia, MO) according to AOAC official methods. Moisture was determined at 135°C for 2 h (AOAC 930.15), CP by nitrogen via combustion (N X 6.25; AOAC 990.03), crude fat by acid hydrolyzed petroleum ether extract (AOAC 920.39), crude fiber (AOCS, Ba 6a-05), and ash by muffle furnace at 600°C for 2h (AOAC 942.05).

Shelf Life Evaluation

The vitamin premix was stored in 4.0 mm thick whirl pack low density virgin polyethylene bags. Two storage conditions were used: ambient (23°C and <50% relative humidity) and stressed (40°C and 75% relative humidity) conditions. Samples stored in ambient conditions were analyzed initially at 0 months then 1, 2, and 3 month increments thereafter. Samples stored in stressed conditions were sampled initially and at 1, 2, 3 and 6 weeks.

The dry mash, wet extrudate, and dry finished product were analyzed immediately following production for determination of effects of processing. Finished product stored whirl packs. Two storage conditions were used; where treatment samples were placed in ambient (23°C and <70% relative humidity) and stressed (40°C and 75% relative humidity) conditions. Each treatment samples were stored in ambient conditions were analyzed at 0, 3, 6, 12, 18 and 24 month increments while the samples in the environmental chamber exposed to stressed conditions were sampled initially and at 3, 6, 12, 18 and 24 weeks following production.

Fatty Acid Analysis

Omega-3 fatty acid analysis utilized the One Step Extraction (OSE) method. Samples freeze dried, then requiring 40-70 mg total fat in the weight of the sample to be extracted, combining acid digestion, solvent extraction and trans-esterification of food matrix in a single reaction tube. This results in the methyl ester form of the omega-3 fatty acid(s), which is measured using GC-FID to quantify eicosapentenoic acid (EPA), docosahexaenoic acid (DHA) and other Omega-3's.

Statistical Analysis

Treatments were organized in a factorial arrangement with three levels of protein and three sources of DHA. Main effects from processing and shelf life stability studies were analyzed by two-way ANOVA using PROC GLIMMIX procedure (SAS version 9.2 SAS Institute Inc. Cary, North Carolina, U.S.A). Means separation was conducted using the LSMEANS statement. Differences were considered significant if $P < 0.05$ and trends $0.05 < P < 0.10$.

Results

Chemical Composition

The dietary nutrient values were compared to the theoretical to ensure formulated targets were met among the three omega-3 sources. Each treatment met and exceeded the target value for crude protein for low, medium and high protein diets by an average of 2.2%, respectively (Table 4.2). The target for moisture for each of the treatments was less than 10% and results were with an average of 3.45% (Table 4.2). For each of the treatments, the targeted values for crude fiber and ash were within 0.5% tolerance when compared to the actual values obtained (Table 4.2). Targeted crude fat amongst the treatments ranged from 12, 14, and 18% for low, medium, and high protein diets. Low and medium CP diets were within an average of 1.1% when analyzed by acid-hydrolysis for crude fat within the treatments. However the omega-3 source with fish meal formulated at high CP was elevated when compared to the other omega-3 sources (20.3 vs. 16.0 and 15.5% for algal source and fish oil (Table 4.2).

Effects of Storage Conditions on Omega-3 Fatty Acid Retention within a Vitamin Premix

The retention of DHA, EPA and total omega-3 fatty acids within a vitamin premix over a six week shelf life at SSLT of 40°C and 75% relative humidity were analyzed and reported in figure 4.2. DHA, EPA and total omega-3 fatty acids were relatively stable during this time period.

The vitamin premix was also stored within ambient shelf life conditions (23°C and 50% relative humidity) over a period of three months (Figure 4.2). EPA dropped from initial to month 2 by 16%, but remained steady through the rest of the study. DHA and total omega-3 fatty acids

followed a similar trend dropping an initial 6 and 7% and remaining stable through the end of the shelf life test (Figure 4.2).

Effects of Experimental Crude Protein Level on Omega-3 Retention through Processing

The main effect means of dietary CP levels on fatty acid concentrations were obtained at various steps in the extrusion process (Table 4.3). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) retention did not differ amongst treatments ($P > 0.05$) across the crude protein levels, however the total omega-3 fatty acids were lowest ($P < 0.05$) for the low and medium protein diets compared to the high protein (3,348 and 3,650 vs. 4,220 mg/kg for low and medium vs. high CP). Following processing through the preconditioner, EPA and DHA retention did not differ amongst treatments ($P > 0.05$) across the crude protein levels, however the total omega-3 fatty acids were lowest ($P < 0.05$) for the low protein diet when compared to the medium and high protein (2,545 vs. 2,791 and 2,915 mg/kg). Retention following extrusion did not differ amongst crude protein for all three EPA, DHA, and total omega-3 fatty acids. In the finished kibble, EPA and DHA again did not differ ~774 and 1,252 mg/kg for EPA and DHA, respectively, amongst the dietary treatments, but the total omega-3 fatty acids retention was greater ($P < 0.05$) for the higher crude protein treatment than medium and low (4,220 vs. 3,650 and 3,348 mg/kg, respectively).). The specific mechanical energy (SME) was higher for the low protein treatments when compared to the medium and high (127.2 vs. 90.8 and 91.8 kJ/kg). The in barrel moisture was highest for the high CP treatment when compared to the medium and low (19.5 vs. 18.3 and 18.3%). Where the bulk density was greater for the high when compared to the medium and low CP treatments (452.3 vs. 384.3 vs. 356.3 g/L). Overall the main effect means of dietary crude protein level on the fatty acid (mg/kg) concentrations

resulted the higher ($P < 0.05$) crude protein levels, the higher total omega-3 fatty acid concentrations. However, eicosapentenoic acid and docosahexaenoic acid specifically were not affected ($P > 0.05$) by dietary protein concentration.

Effects of Processing on DHA Retention

The effects of processing on EPA reflect fluctuations at various stages of the process. The diets formulated with DHA as an omega source did not consider EPA as a factor for the algae treatment, however when analyzed incidental levels were recovered. The EPA levels decreased ($P < 0.05$) at 195, 101, and 91 mg/kg in the dry flour, off preconditioner, and off extruder, respectively (Figure, 4.1-A). Diets formulated with fish meal as an omega-3 source were expected to provide 1,200 mg/kg EPA, but during the manufacturing process this decreased ($P < 0.05$) from 1,069, in the dry mash to 950, and 802 mg/kg when measured off preconditioner, and off the extruder, respectively. The diets formulated with fish oil were expected to contain 930 mg/kg EPA and when analyzed during processing resulted in 877, 695, and 1,000 mg/kg in the dry flour, off the preconditioner, and off the extruder, respectively. Across all the treatments finished kibble exceeded the expected EPA values of 0, 1,200, and 930mg/kg with results of 92, 1251, and 979 mg/kg.

All treatments were formulated to meet a targeted amount of 670 mg/kg of DHA (Figure 4.1-B). Formulas supplemented with the algae source of DHA exceeded the target by 1,657 mg/kg when measured in the dry flour. However, once processed in the preconditioner DHA levels decreased by greater than 50% to 1,001 mg/kg and remained near these levels through the extruder and dryer. The diets formulated with fish meal and fish oil were closer to the target value (1,132 and 861 mg/kg, respectively). Like the algae source, fish meal and fish oil levels of

DHA decreased ($P < 0.05$) by 11% and 20%, following the preconditioner but remained relatively stable through the additional processing.

The total omega-3 fatty acids encompass both DHA and EPA, as well as some other omega-3 fatty acids like linolenic acid (4.1-C). The overall effects of processing on the total omega-3 fatty acid retention were similar to the results observed for EPA and DHA. Wherein, the dry mash for each omega-3 fatty acid treatment exceeded the target by greater than 2,245, 1,676, and 1,636 mg/kg for algae, fish meal and fish oil sources, respectively. Following processing in the preconditioner, each treatment omega-3 fatty acid level decreased ($P < 0.05$; 1,400, 1,905, and 1,988 mg/kg, for algae, fish meal and fish oil sources, respectively). Following preconditioning the algae and fish meal omega-3 fatty acid levels remained stable with a slight increase in the finished kibble; however, the fish oil gained omega-3 fatty acids following extrusion (3,650 mg/kg vs. 3,847 mg/kg in the finished kibble).

Effects of Storage Conditions on Omega-3 Retention within a Vitamin Premix

The retention of omega-3 fatty acids within a premix were analyzed over a six week shelf life under stressed conditions of 50°C and 75% relative humidity (Figure 4.2). The retention of omega-3 fatty acids appears to be stable over time with a reduction of 12, 12, and gain of 10% for EPA, DHA and total omega-3 fatty acid, respectively. The retention of vitamins within a premix, over a three month shelf life under the ambient conditions of 20°C and 50% relative humidity decreased the first month by 6, 16 and 7% for EPA, DHA and total omega-3 fatty acid, respectively. After this initial loss, the omega-3 fatty acids appear to be relatively stable from month two to month three with a total overall loss of 9, 17, and 11% for EPA, DHA and total omega-3 fatty acid, respectively.

Effects of Storage Conditions on Omega-3 Retention

The samples produced at different protein levels were pooled for the shelf-life evaluation for retention of omega-3 fatty acids. As stressed shelf life storage of finished pet food increased through 24 weeks EPA, DHA, and total fatty acids declined slightly ($P < 0.05$; 125, 82 mg/kg for EPA and 77, 60 mg/kg for DHA, and 418, 476 mg/kg for total fatty acids at 0 vs. 24 wk) with an overall retention greater than 90% (Figure 4.3). As time in ambient storage reached 24 months EPA, DHA, and total fatty acids declined slightly ($P < 0.05$; 125 vs. 78 mg/kg for EPA and 77 vs. 50 mg/kg for DHA, and 387 vs. 373 for total fatty acids at 0 vs. 24 mo.) with an overall retention greater than 80% (Figure 4.3)

Discussion

Proximate analysis was performed to ensure all products were close to the expected values for moisture, protein, fat, fiber and ash. The moisture content for each of the treatments were consistently less than the formulated value, however 5-6% was targeted during the trials. Products met the intended level of dryness. Crude protein was consistently greater than the minimum formulated value, which was expected as the raw materials aim to meet a target versus a minimum value. Crude fiber and ash were very close to the target values as expected. There was a discrepancy for the high crude protein fish meal treatment at 20.28% when compared to the fish oil and algal treatments of 15.54 and 16.04%. During experimental design, the focus was to formulate comparable levels of DHA amongst the treatments of 670 mg/kg, however this was more difficult to achieve during formulation than anticipated. During the extrusion process, a lipid-amylose complex may form making traditional methods of measuring fat less effective compared to the recommended AOAC method 954.02. This method utilizes hydrochloric acid to break the trapped lipid and allow the total fat to be measured. The difference between the high crude protein fish meal treatment and the others is attributed to the variability in raw materials.

The algal source for DHA was mixed within the vitamin premix and single analysis was obtained for this research. When analyzed prior to manufacturing, 85-95% of the targeted value for each EPA, DHA and total omega-3 fatty acids was present. Following the initial production and sampling the premix was stored in elevated temperature and ambient storage. Abuzaytoun and Shahidi (2006) observed the stability of algal oils at room temperature without protection from light and concluded that minor oil constituents play a major role in stability. Similar to our study, we anticipated stability and retention of the omega-3 fatty acids when exposed to room temperatures. We observed an initial drop for EPA, DHA, and total omega-3 fatty acids of 16, 6,

and 7% from the first month to the second, respectively. Following the initial loss, the fatty acids appeared to be relatively stable through the remainder of the study.

The degradation of total omega-3 fatty acids was anticipated as the severe thermal process and overall nature by which pet foods are manufactured, it is common for pet food companies opt to use fish meal rather than the oil as their source of omega-3s. Marine omega-3 oils are highly prone to oxidation and other secondary oxidation products. The oxidation process could potentially alter biological activity, which could deem them ineffective (Albert et al., 2013). Fish meal provides a high quality protein and in a dry product and seems to be easier to stabilize with current antioxidant technology when compared to fish oil (which is susceptible to oxidation). The dry nature of the fish meal contains an inherent form of oil where the added fish oil could impact extrusion. Fish oil usually contains both EPA and DHA and algal oils are predominately DHA, so the treatments formulated with DHA as an omega-3 source did not consider EPA as a factor for the treatment. However when analyzed incidental levels were recovered during each step in the manufacturing process for this treatment. The EPA recorded in the dietary treatments containing fish meal and fish oil remained stable following a drop, e.g. 182 mg/kg for the fish oil treatment in the pre-conditioner step. DHA and total-omega-3 fatty acids followed the similar trend as EPA taking higher levels in the dry flour and remaining stable following a decrease in the pre-conditioning step of the manufacturing process. Nielsen (2015) reported the complexity determining stability in complex products and antioxidant mechanisms. He concluded extrinsic environmental factors e.g. exposure to light, heat, and oxygen can increase oxidation, all of which are present during pet food manufacturing. Because of the complexity in the products and inherent variability, he suggested each product be evaluated to

determine optimal composition, processing conditions, and efficacy of antioxidants within the omega-3 enriched products.

Each dietary treatment was also formulated to determine if there were any effects of crude protein level on the stability of omega-3 fatty acids. There were minimal effects of crude protein when examining EPA and DHA during each step of the manufacturing process, however the total omega-3 fatty acids did differ ($P < 0.05$) among the dietary treatments for crude protein for all steps in the process except when exposed to extrusion only. Previous research has shown that as crude protein levels increase, there is an offsetting starch decrease, which can increase product bulk density. This results in a decreased expansion ratio as extruded kibble expands the starch to create the cellular structure during expansion (Zhu et al., 2010). The cross-sectional images of the cellular structure reveals a decrease in cellular size for products with an increased strength of the cell walls as the crude protein levels increase (Allen et al., 2007). Thus, one might surmise that more omega-3 fatty acids are trapped in the starch-matrix and exposed to the external environment with higher starch: lower protein foods. Said another way, the less the expansion, the less the surface area internal and external to be exposed to air and oxidation. The hardened external shell of the kibble and decreased cellular size may provide clues to how a greater total omega-3 fatty acid retention could occur if the excess air, light, and heat is unable to penetrate the kibble.

Shipping and storage of pet foods often requires transport across the equator and throughout very humid and hot environments where temperatures can exceed 40-50°C. Thus, the experimental conditions in our study were intended to mimic these real conditions. Since our treatments contained a high level of polyunsaturated fatty acids, we expected a decrease in omega-3 fatty acids when exposed to shelf life studies and observe volatile oxidation compounds

to increase. Adios et al. (2002) observed an increase in volatile oxidation compounds in herring oil when placed within a closed vessel, exposed to oxygen, and stored in SSLT at 50°C for up to 53 hours. Consistent with our study, samples were stored at extreme temperatures of 40°C, but samples were stored for and analyzed over 24 weeks. Within the 24 week SSLT EPA, DHA, and total omega-3 fatty acid concentrations declined slightly ($P < 0.05$) however retention was greater than 90%. Adios et al. (2002) also reviewed the stability of volatile oxidation compounds for herring oil when placed in lower storage temperatures consistent with ambient at 20°C. They observed a direct correlation between the oxidation of products over time and temperature dependency as secondary oxidation was observed for all treatments. The environmental conditions were similar to our study with ambient 23°C and 50% relative humidity. Within 24 months EPA, DHA, and total omega-3 fatty acid concentrations declined slightly ($P < 0.05$) with a retention greater than 80%. In comparison to Adios et al. (2002), the volatile oxidation compounds were analyzed for each SSLT and ambient conditions, and it was observed that attributes known for oxidation in marine lipid sources were present (Appendix A).

Conclusion

DHAgold® S17 B retained stability when stored in ambient and stressed conditions within a vitamin premix. During the manufacturing processing, a loss occurred for EPA, DHA (DHA gold® S17 B), and the total omega-3 fatty acids during the preconditioning step. Further research is needed to determine if sacrifice occurred in result of the preconditioning conditions or if there may be an over-estimate of omega-3 fatty acids in the dry ration. Overall fluctuations occurred at various stages in the process, but final concentrations in the finished foods was not affected by the process. Fluctuations occurred in the higher crude protein levels leading to higher total omega-3 fatty acid concentrations, however the target values were met and excess was not reported. EPA and DHA were not affected by the dietary protein concentration. More explorative studies need to be completed to determine the effects of the preconditioner step of the manufacturing process has on omega-3 fatty acid stability and the overall molecular breakdown and bio-availability of the fatty acids.

EPA, DHA and the total omega-3 concentrations were not affected by stressed (24 weeks) or ambient (24 month) storage conditions. DHAgold® S17 B appears to be stable in processed pet foods and comparable to the more traditional omega-3 fatty acid sources.

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Table 4.1: The ingredient composition of experimental diets processed through extrusion to evaluate the effects of processing on DHA retention and shelf life.

Ingredient, % as-is basis	Omega-3 Source								
	Algal-DHA			Fish Meal			Fish Oil		
	Crude Protein Level			Crude Protein Level			Crude Protein Level		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
Fish Meal	0.00	0.00	0.00	11.00	11.00	11.000	0.000	0.000	0.000
Fish Oil	0.00	0.00	0.00	0.00	0.00	0.000	0.665	0.665	0.665
Algal-DHA + Vit	0.50	0.50	0.50	0.00	0.00	0.000	0.000	0.000	0.000
Chicken By-Product Meal	22.00	26.09	34.05	11.01	16.80	22.94	21.92	27.80	33.95
Rice, Brewers	20.17	17.50	13.01	20.33	17.21	13.18	20.34	17.21	13.20
Corn	20.17	17.50	13.01	20.33	17.21	13.18	20.34	17.21	13.20
Wheat	20.17	17.50	13.01	20.33	17.21	13.18	20.34	17.21	13.20
Chicken Fat	7.57	9.20	12.54	8.13	9.54	13.10	6.89	8.30	11.87
Beet Pulp	4.00	4.00	4.000	4.00	4.00	4.00	4.00	4.00	4.00
Corn Gluten Meal, 60%	2.50	5.00	7.50	2.50	5.00	7.50	2.50	5.00	7.50
Digest - Dry Dog	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Micro Nutrients ^{a,b,c}	2.41	2.19	1.84	1.85	1.49	1.39	2.46	2.09	1.89

^a**Micro Nutrients:** Calcium Carbonate, Potassium Chloride, Salt, Dicalcium Phosphate, Choline Chloride 60% dry, Natural Antioxidant

^b**Vitamin Premix:** Calcium Carbonate, Roughage Products, Vitamin E Supplement, Niacin Supplement, Vitamin B12 Supplement, Mineral Oil, D-Calcium Pantothenate, Vitamin A Supplement, Thiamine Mononitrate, Pyridoxine Hydrochloride, Riboflavin Supplement, Vitamin D₃ Supplement, Biotin, And Folic Acid.

^c**Trace Mineral Premix:** Calcium Carbonate, Zinc Sulfate, Ferrous Sulfate, Copper Sulfate, Mineral Oil, Manganous Oxide, Sodium Selenite, Calcium Iodate

Table 4.2: The nutrient composition of experimental diets*processed through extrusion to evaluate the effects of processing on DHA retention and shelf life.

Analysis %	Omega-3 Source								
	Algal-DHA			Fish Meal			Fish Oil		
	Crude Protein Level			Crude Protein Level			Crude Protein Level		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
Crude Protein**	23.70	27.43	32.34	23.38	27.68	31.64	23.62	27.32	31.24
Moisture	5.62	6.74	6.10	6.78	6.41	7.40	6.36	6.32	7.19
Crude Fiber	2.09	1.72	1.66	1.71	1.67	1.85	1.66	1.59	1.78
Ash	6.04	6.34	7.15	6.08	6.92	6.99	6.01	6.56	6.66
Crude Fat	10.6	12.25	16.04	11.36	12.15	20.28	10.87	12.51	15.54

*Results are expressed on an "as is" basis unless otherwise indicated.

**Percentage N X 6.25

Table 4.3: Main effect means of dietary crude protein level on the fatty acid (mg/kg) concentrations at various processing steps as kibble is being produced through extrusion.

CP Level	Low	Medium	High	SEM	P-Value
Mash					
EPA ^c	703	707	730	24.4	0.06
DHA ^d	1,850	1,415	1,055	10.7	0.56
Total O ₃ ^e	4,245 ^b	4,207 ^b	5,202 ^a	73.5	0.01
Preconditioner Mash					
EPA	552	597	596	17.9	0.24
DHA	853	921	911	30.4	0.33
Total O ₃	2,545 ^b	2,791 ^a	2,915 ^a	58	0.03
Off Extruder (undried)					
EPA	652	624	618	16	0.37
DHA	991	985	1,029	37.9	0.70
Total O ₃	2,984	2,994	3,050	112	0.91
Off Drier (finished kibble)					
EPA	721	799	802	18.7	0.19
DHA	1,127	1,200	1,430	88.1	0.20
Total O ₃	3,348 ^b	3,650 ^b	4,220 ^a	65.3	0.01

^{ab} Means in a row with unlike superscripts differ ($P < 0.05$).

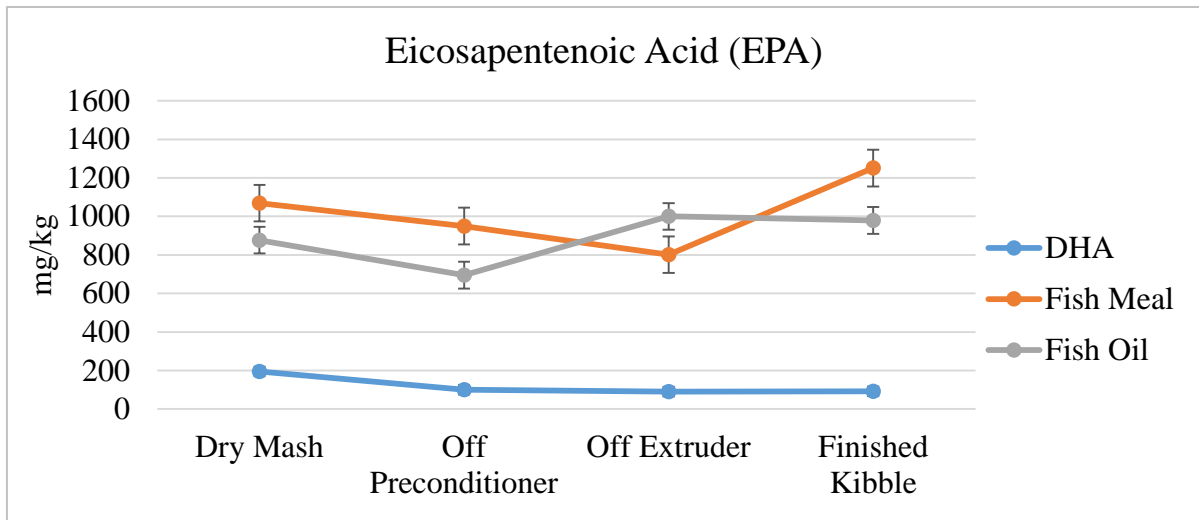
^c EPA - eicosapentenoic acid

^d DHA - docosahexaenoic acid

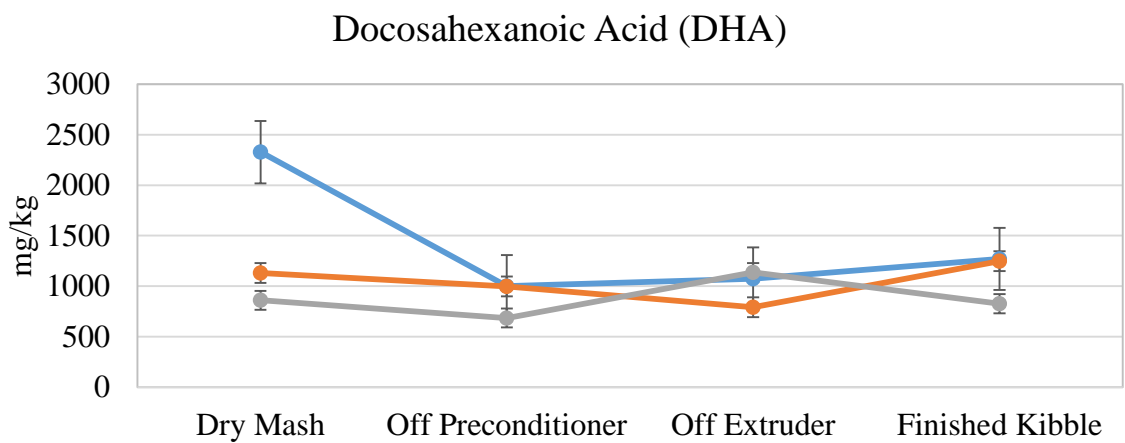
^e Total O₃ - Octadecatrienoic acid (ALA), octadecatetraenoic acid (SDA), eicosatetraenoic acid (ETA), eicosapentenoic acid (EPA), heneicosapentaenoic acid (HPA), docosapentaneic acid (DPA) and docosahexaenoic acid (DHA)

Figure 4.1:(A-C): The effect of fatty acid source (mg/kg) on resulting concentration at various processing points during kibble production.

A.



B.



C.

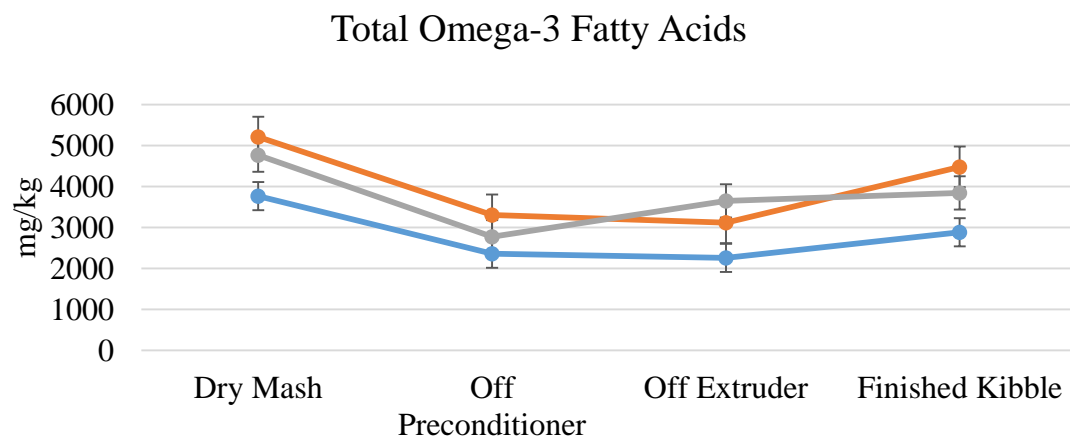


Figure 4.2: Effect of omega-3 source on fatty acid stability in premix over time (Stressed Shelf Life Testing; 40°C 75%RH and Ambient 23°C and 50%RH)

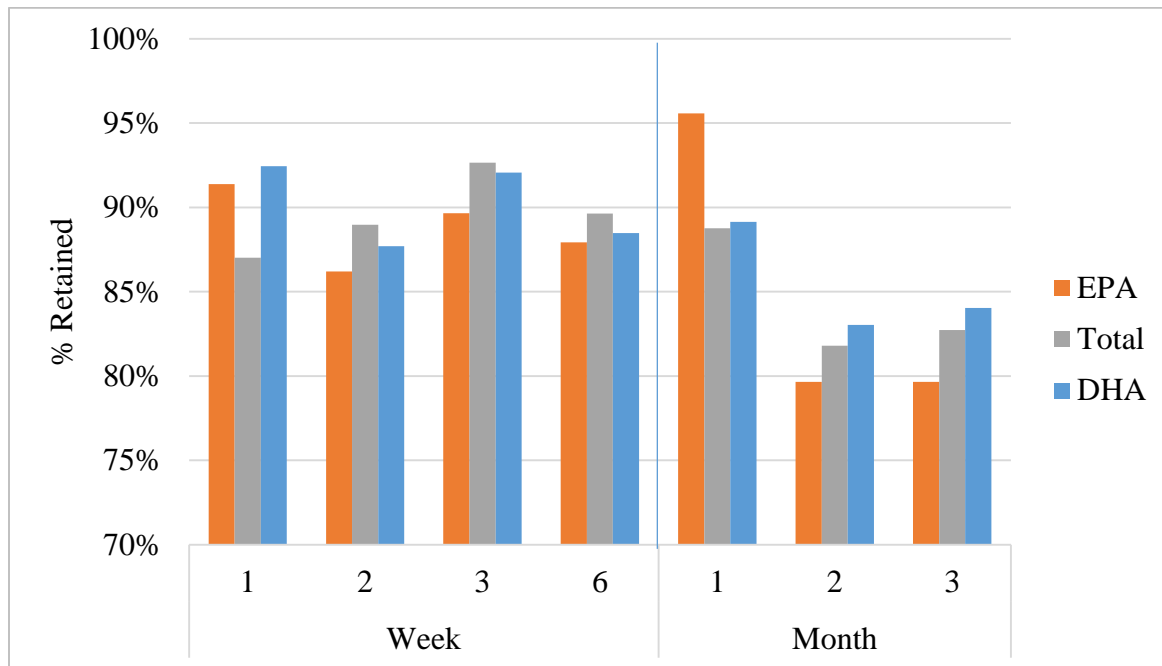


Figure 4.3: Effect of omega-3 source on fatty acid stability in kibble over time (Stressed Shelf Life Testing; 40°C 75%RH and Ambient 23°C and 50%RH).

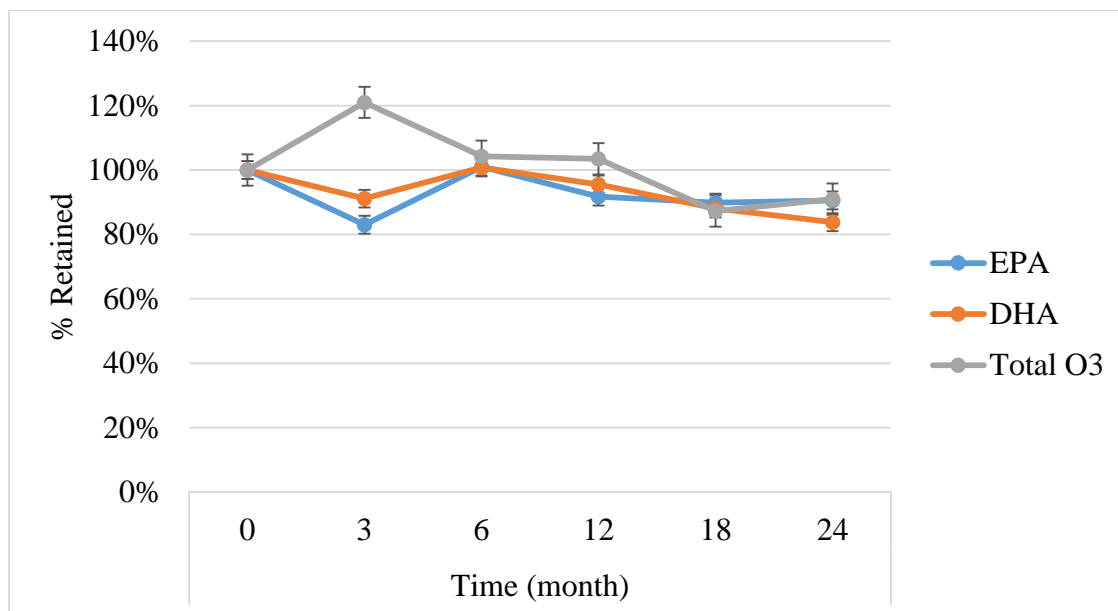
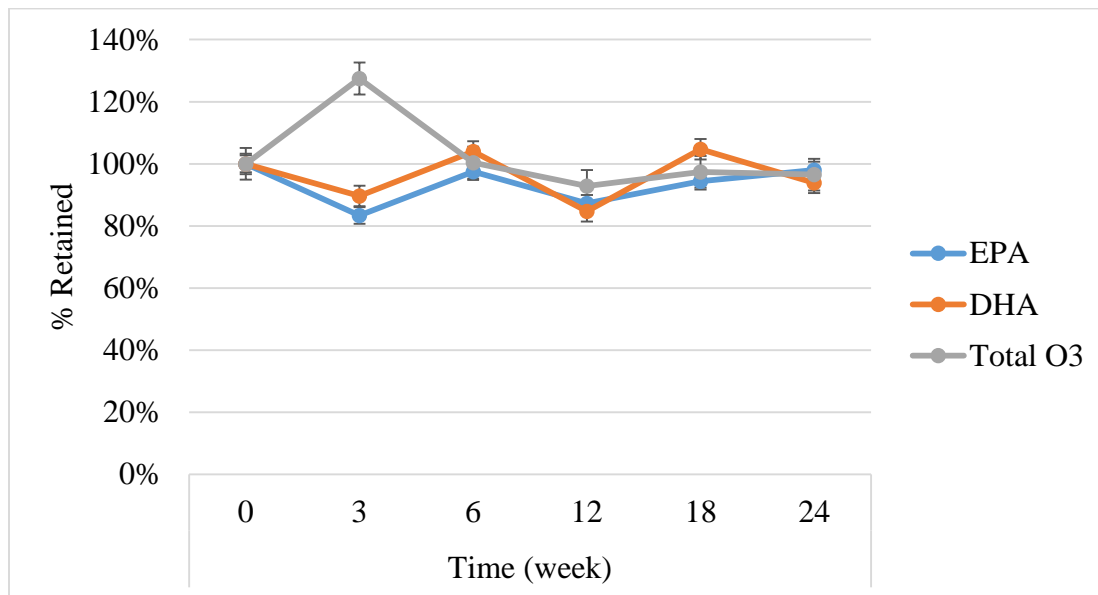
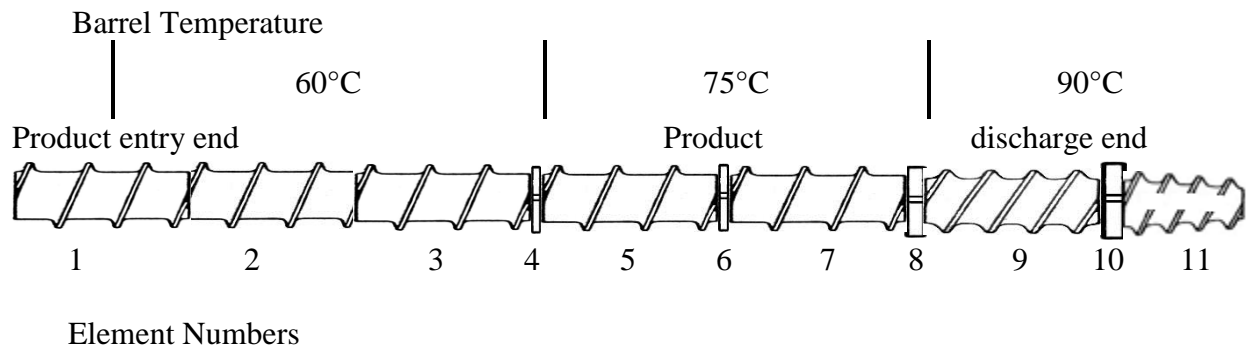


Figure 4.4: Schematic showing pilot scale single screw extruder profile and barrel



1-3=single flight screws; 4=small steamlock; 5=single flight screw; 6=small steamlock; 7=single flight screw; 8= medium steamlock; 9=half pitch, double flight screw; 10=large steamlock; and 11=half pitch, double flight, cut cone.

Appendix A - The effects of storage conditions on sensory and volatiles of extruded pet food supplemented with fish or algal source of omega-3 fatty acids.

Introduction

Research has shown that long-chain omega-3 polyunsaturated fatty acids (PUFA's) such as eicosapentaenoic acid (EPA: 20:5n3) and docosahexaenoic acid (DHA; 22:6n3) have numerous health benefits such as maintaining normal body structure, lowering blood pressure, and aid in long-term heart health (Morris et al., 1993; Mozafarrian 2008). Unfortunately, these long-chain omega-3 PUFA sources like fish oil and the oil in fish meal are exceptionally prone to oxidation when compared to the monounsaturated fatty acids in many popular vegetable oils. The oxidation of these PUFA's results in off-flavors and odors (Cho et al., 1987). These off-flavors and odors have been shown to greatly affect the acceptability and has limited the application of DHA in food and feed products (Stansby, 1971). Therefore, our objectives were to determine the effects of storage conditions on sensory and volatiles of extruded pet food supplemented with fish and algal sources of omega-3 fatty acids.

Materials and Methods

Dietary Treatments

Three dietary treatments were prepared based on crude protein levels; low (21.7%), medium (25%) and high (30%; Table 4.1). The formulations were intended to simulate a crude protein content similar to commercial maintenance canine, performance canine, and feline diets, respectively. The diets were formulated to have similar DHA levels to that of the algal source by replacing a portion of the diet with either Menhaden fish meal or Menhaden fish oil.

The ingredients (brewers rice, corn, wheat, beet pulp, chicken by-product meal, fish meal, corn gluten meal, fish oil, calcium carbonate, potassium chloride, salt, dicalcium phosphate, choline chloride, dry natural antioxidant and trace mineral premix) were ground and mixed into a basemix by a local mill (Lortscher Animal Nutrition Inc; Bern, KS., U.S.A.). The chicken fat was dosed with natural antioxidant liquid (ADF; Springfield, Mo) and applied topically to kibbles after drying along with a dry dog flavor (AFB International; O'Fallon, MO., U.S.A.). The vitamin premixes with the algal-DHA (DHAGold™ DSM Nutritional Products, Ames, IA., U.S.A.) was mixed prior to extrusion.

The dry ingredients were blended in a twin-shaft double ribbon mixer (Scott Equipment, New Prague, MN) and the particle size reduced in a hammermill (Bliss 4460; Lortscher Animal Nutrition; Bern, KS) such that 90% would pass through a US #14 sieve. Pre-weighed quantities of each base-mix were blended with vitamin premix in a double ribbon (Scott Equipment, New Prague, MN) for five minutes.

Extrusion

Dietary treatments were produced on a pilot-scale single screw extruder (Model X-20; Wenger Manufacturing Inc., Sabetha, KS). Diets were initially conditioned with steam and water

in a differential diameter cylinder (DDC) pre-conditioner (Wenger Manufacturing Inc., Sabetha, KS) fed by a volumetric feeding system with feeder screw speeds at 20.4 rpm and a constant feed rate of 200kg/hr. The pre-conditioner shaft speed was held constant at 400rpm to attain discharge temperatures between 88-93°C.

The extruder screw configuration commonly used for pet production had three heating zones set to temperatures of 60, 75 and 90°C from the feed entry to discharge end of the extruder. The extruder profile consisted of single flight screws and transitioning to double flight half pitch screws with shear locks increasing in size between the screw elements (Figure 4.3). The die plate consisted of a single circular 5 mm diameter insert and a face-mounted rotary knife equipped with six blades. The knife speed was kept constant at 1,730 rpm.

The three dietary treatments were processed at an extruder screw speed of 350 rpm. Extrudate leaving the die was pneumatically conveyed to a double pass dryer/cooler (Series 4800, Wenger Manufacturing Co. Sabetha, KS, U.S.A.). Product was dried at 104°C and adjusted for 20 minute retention time (10 minutes each for the top and bottom belts) and then cooled on the third belt with room temperature air for 10 minutes until the target end product moisture of 6% was met. The pre-weighed quantities of finished product were coated with chicken fat and dry dog flavor in a double ribbon mixer for five minutes.

Tests for specific mechanical energy (SME) were done in per unit mass of extrudate and were calculated as follows:

$$SME \left(\frac{kJ}{kg} \right) = \frac{\left(\frac{T}{100} \right) \left(\frac{N}{N_{rated}} \right) P_{rated}}{\dot{m}}$$

Where T= net motor load percentage, N = screw speed (rpm), N_{rated} = rated screw speed (507 rpm), P_{rated} = rated power (37.3 kW), and \dot{m} = net mass flow rate (kg/s).

Nutrient Analysis

The proximate composition of finished product were analyzed (University Missouri Analytical Lab, Columbia, MO) according to AOAC official methods. Moisture was determined at 135°C for 2 h (AOAC 930.15), crude protein by nitrogen via combustion (N X 6.25; AOAC 990.03), crude fat by acid hydrolyzed petroleum ether extract (AOAC 920.39), crude fiber (AOCS, Ba 6a-05), and ash by muffle furnace at 600°C for 2h (AOAC 942.05).

Shelf Life Evaluation

Finished product was stored in 35 pound poly-lined, kraft paper bags. Two storage conditions were used; where samples were placed in ambient (23°C and <70% relative humidity) and stressed (35°C and 75% relative humidity) conditions. Samples stored in ambient conditions were analyzed at 0, 3, 6, 12, 18 and 24 month increments while the samples in the environmental chamber exposed to stressed conditions were sampled initially and at 0, 1, 2, 3, 4, 5, 6, 7, and 8 weeks following production. One sample bag exposed to the stressed conditions was left open to be exposed to the environment and the additional bags remained closed until sampled.

Fatty Acid Analysis

Omega-3 fatty acid analysis utilized the One Step Extraction (OSE) method, requiring 40-70 mg total fat in the weight of the sample to be extracted, combining acid digestion, solvent extraction and trans-esterification of food matrix in a single reaction tube. This results in the methyl ester form of the omega-3 fatty acid(s), which is measured using GC-FID to quantify eicosapentenoic acid (EPA), docosahexaenoic acid (DHA) and other Omega-3's.

Statistical Analysis

Treatments were organized in a factorial arrangement with three levels of protein and three sources of DHA. Sensory analysis was performed by a difference from control (DFC) test comparing the control with each of the treatments and analyzed by using the Dunnett statistical test at an alpha of 0.05.

Sensory and Volatile Analysis

The sensory analysis was conducted using a difference from control (DFC) test which compared the control to each of the three dietary treatments at the initial sample collection (time 0) and 3, 6, 12, 18, and 24 months for ambient and 0, 1, 2, 3, 4, 5, 6, 7 and 8 weeks for stressed, thereafter. Samples were presented with a labelled and masked control, to ensure panelist accuracy, along with coded dietary treatments. The panelists were pre-screened. At a minimum 10-16 panelist were qualified for their ability to recognize fishy/rancid attributes within pet food. These qualified individuals were then engaged to test the samples. Data were obtained on ballots and summarized. Individual sensory parameter differences were compared against the control. A Dunnett statistical test of α of 0.05 was used in which the average value of a sample compared against a control to determine if a significant difference occurred.

Samples were also prepared by homogenizing kibble with mortar and pestle, then assessed for headspace volatile analysis by a 2D Gas-Filled Time-Of-Flight (GC-GF-TOF) with Solid phase micro-extraction (SPME).

Results

Sensory analysis was performed by a difference from control (DFC) test, comparing a control to each of the treatments when exposed to ambient conditions (23°C and <70% relative humidity). Amongst the low protein diets, the treatment containing fish meal resulted in significant differences when compared to the control sample. These samples were described as extremely fishy, marine and some slight metallic and painty notes. These same attributes were noted in the fish oil however. In contrast, the fish oil and algae source were also both slightly higher than the control except for month 12 and 24, but were not statistically different (Figure B-1). The same was true for the medium protein treatment containing fish meal as it resulted in significant differences when compared to the control sample. These samples were described as extremely fishy, marine and some slight metallic and painty notes. In contrast, the fish oil and algae source were also both slightly higher than the control except for month 6, 1 and 24, but were not statistically different (Figure B.2). The high protein treatment containing fish meal again resulted in significant differences ($P < 0.05$) when compared to the control except for month 24. The treatments containing fish oil and algae sources of omegas were found to have greater fishy, marine and some slight metallic and painty notes. Most results were greater than the control except analysis for month 6 and 24 for fish oil and month 18 for algae but were not statistically different (Figure B.3).

The samples stored in ambient conditions (23°C and <70% relative humidity) were analyzed volatiles in the headspace and peaks for for 1-Octen-3-one, 1-Penten-3-one (CAS), 2, 4-Heptadienal II, 2, 4-Decadienal, (E,E)-(CAS), 2-Decenal, (E)-(CAS), 3-Octen-2-one (CAS), 4-Heptenal, (Z), Heptanal (CAS), and Trans-2-CIS-6-Nonadienal, identified, respectively. These volatiles are known oxidation indicators and the dietary treatments were compared against the control. The low protein treatments formulated with the algae source were different ($P < 0.05$)

from the control for 1-Octen-3-one, 2, 4-Heptadienal II, 3-Octen-2-one (CAS), 4-Heptenal, (Z), Heptanal (CAS; Figure B.4). Treatments formulated with the fish oil were also analyzed for key volatile indicators and were different ($P < 0.05$) from the control for 1-Octen-3-one, 1-Penten-3-one (CAS), 2, 4-Heptadienal II, 2,4-Decenal, (E)-(CAS), 3-Octen-2-one (CAS), 4-Heptenal, (Z), Heptanal (CAS), and Trans-2-CIS-6-Nonadienal (Figure B.4). Like the treatments with fish oil, the treatments containing fish meal were also different ($P < 0.05$) from the control in 1-Octen-3-one, 1-Penten-3-one (CAS), 2, 4-Heptadienal II, 3-Octen-2-one (CAS), 4-Heptenal, (Z), and Trans-2-CIS-6-Nonadienal (Figure B.4). The medium protein treatments formulated with the algae source was significantly different from the control when analyzed for 1-Octen-3-one, 2, 4-Heptadienal II, 2, 4-Decadienal, (E,E)-(CAS), 2-Decenal, (E)-(CAS) and 4-Heptenal, (Z) (Figure B-5). Treatments formulated with the fish oil were also analyzed for key volatile indicators and were different ($P < 0.05$) from the control for 1-Octen-3-one, 2, 4-Heptadienal II, 2, 4-Decadienal, (E, E)-(CAS), 2-Decenal, (E)-(CAS), 4-Heptenal, (Z), and Trans-2-CIS-6-Nonadienal (Figure B.5). Like the treatments for fish oil, the treatments for fish meal also differed ($P < 0.05$) from the control in 2, 4-Heptadienal II, 2-Decenal, (E)-(CAS), 4-Heptenal, (Z), and Trans-2-CIS-6-Nonadienal (Figure B.5). The high protein treatments formulated with the algae source was significantly different from the control when analyzed for 2, 4-Heptadienal II, 4-Heptenal, (Z), Heptanal (CAS), and Trans-2-CIS-6-Nonadienal (Figure B-6). Treatments formulated with the fish oil were also analyzed for key volatile indicators and were significantly different ($P < 0.05$) from the control for 2, 4-Heptadienal II, 2, 4-Decadienal, (E, E)-(CAS), 2-Decenal, (E)-(CAS), 4-Heptenal, (Z), Heptanal (CAS), and Trans-2-CIS-6-Nonadienal (Figure B-6). Like the treatments for fish oil, the treatments for fish meal also differed ($P < 0.05$) from

the control in 2, 4-Heptadienal II, 2, 4-Decadienal, (E, E)-(CAS), 2-Decenal, (E)-(CAS), 4-Heptenal, (Z), Heptanal (CAS), and Trans-2-CIS-6-Nonadienal (Figure B.6).

Sensory analysis was performed by a difference from control (DFC) test, comparing a control to each of the treatments within an open bag when exposed to stressed environmental conditions (35°C and 75% relative humidity) for 8 weeks. Amongst the low protein diets, the treatment containing fish meal resulted in differences when compared to the control sample. These samples were described as very fish and marine, with metallic and painty notes as the duration of the study progressed. These same attributes were noted in the fish oil however, in contrast, the fish oil and algae source were also both higher than the control except for week eight for the algae source, but were not different (Figure B-7). The same was true for the medium protein treatment containing fish meal as it resulted in significant differences ($P < 0.05$) when compared to the control sample. These samples again, were described as extremely fishy, marine and some slight metallic and painty notes. In contrast, the fish oil and algae source were also both higher than the control, but were not statistically different (Figure B.8). The high protein treatment containing fish meal again resulted in differences ($P < 0.05$) when compared to the control. The treatments containing fish oil were recorded to have greater fishy, marine and some slight metallic and painty notes and results were greater than the control. Both fish oil and algae meal treatments were not statistically different from the control (Figure B.9).

Sensory analysis was performed by a difference from control (DFC) test, comparing a control to each of the treatments within a closed bag when exposed to stressed environmental conditions (35°C and 75% relative humidity) for 24 weeks. Amongst the low protein diets, the treatment containing fish meal resulted in significant differences when compared to the control sample. These samples were described as very fish and marine, with metallic and painty notes as

the duration of the study progressed. These same attributes were noted in the fish oil however, in contrast, the fish oil was higher than the control except for week 24, but were not different ($P < 0.05$; Figure B-10). The treatments containing algae source of omegas were lower than those reported in the control, with the exception of the initial and when analyzed in week six. The same was true for the medium protein treatment containing fish meal as it resulted in significant differences ($P < 0.05$) when compared to the control sample. These samples again, were described as extremely fishy, marine and some slight metallic and painty notes. In contrast, the fish oil was both higher than the control, with the exception of week 13, but was not statistically different. The treatment containing algae meal was the least different when compared to the control (Figure B.11). The high protein treatment containing fish meal resulted in significant differences ($P < 0.05$), except for week 18 when compared to the control. The treatments containing fish oil were recorded to have greater fishy, marine and some slight metallic and painty notes and results were greater than the control with a significant difference at week 18. The treatments containing algae meal were not statistically different from the control (Figure B.12).

Discussion

In the 24 month sensory analysis, all cases except the dietary treatment with high protein at 24 months were not different between the three treatments (Figure B-3). This corresponds to the headspace results wherein all three treatments are different from the control (Figure B.6). All treatments containing fish meal were defined as very fishy, marine, slight metallic and painty notes. The treatments containing fish oil were also described as having slightly fishy attributes where these characteristics were not mentioned for the treatment containing algae meal.

Across the volatiles, a few displayed significant differences amongst the dietary treatments when compared to the control, as notated with asterisks in Figure B.4. For example, 4-Heptenal is a well-known odor active compound that is associated with oxidation and displayed differences between the dietary treatments. Although each treatment displayed a statistically elevated level when compared to the control, DHAGold also showed a significantly lower level when compared to the fish meal and fish oil. This same pattern occurred for other volatile compounds when compared; for example, 2, 3-heptadienal and 2, 6-nonadienal (Figure B.4, B.5, B.6). In the 24 month analysis, only the dietary treatment with high protein resulted in no significant difference between the three treatments, correlating with the headspace results where all three treatments are significantly different from the control (Figure B.6).

Over an eight week open-bag shelf and sensory analysis study, the results indicate that within each of the dietary treatments, the fish meal gave the worst sensory analysis and consistently was significantly different from the control sample. Attributes common with rancidity and fishy notes were described in the fish meal and fish oil treatments, however the fish oil and algae source treatments had similar sensory properties when compared to the control.

Over a 24 week, closed-bag shelf life and sensory analysis study, the results indicate that within each of the dietary treatments, the fish meal gave the worst sensory analysis and

consistently was significantly different from the control sample. Attributes common with rancidity and fishy notes were described in the fish meal and fish oil treatments, however the fish oil and algae source treatments had similar sensory properties when compared to the control. As the samples aged across the study, the difference amongst the samples began to diminish. This is contributed to the decline in quality of the control sample as reports of rancid attributes were noted.

Conclusion

During the course of the shelf-life sensory analysis, the results indicate that within each of the dietary treatments, the fish meal resulted in the worst sensory scores and consistently was different from the control sample. The sensory results were in agreement with the headspace analysis for samples stored in ambient conditions over the month 24 shelf life analysis -- several oxidation indicators were higher in the fish meal and fish oil samples when compared to DHAGold and the control. When consumers feed their pet's algal sources of DHA, the decreased oxidation indicators may provide a more pleasant experience than the volatiles found from the fish sources.

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Figure A.1. The effects of storage over 24 months in ambient conditions (23°C, <70%RH) on sensory scores for low protein dietary treatment. Results with (*) denote samples were significantly different at $\alpha = 0.05$ from the control at the same time period.

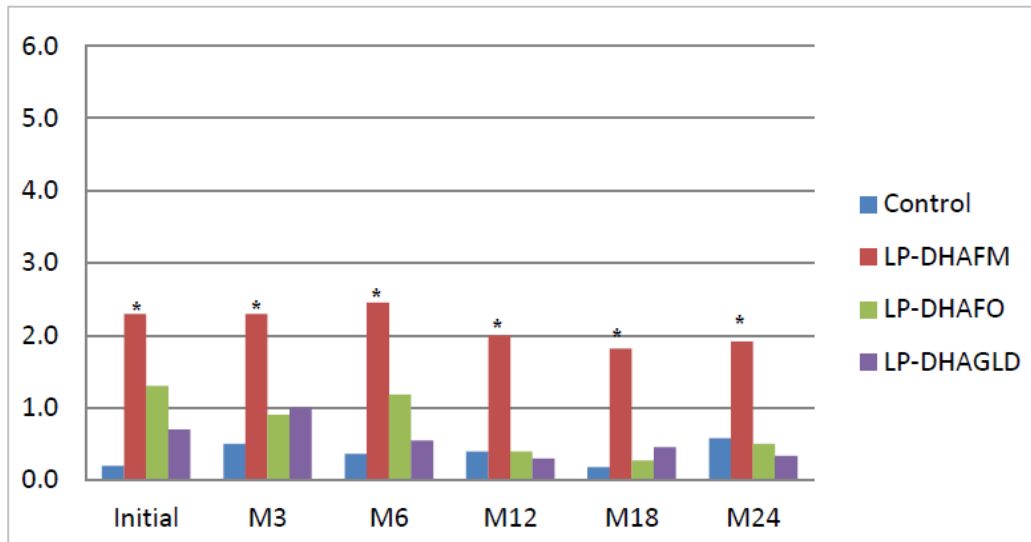


Figure A.2. The effects of storage over 24 months in ambient conditions (23°C, <70%RH) on sensory scores for medium protein dietary treatments. Results with (*) denote samples were significantly different at $\alpha = 0.05$ from the control at the same time period.

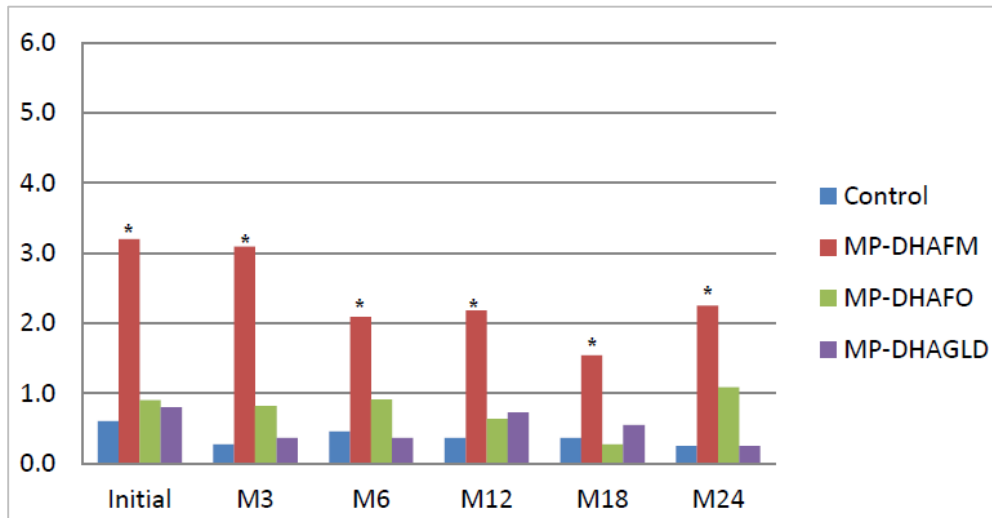


Figure A.3. The effects of storage over 24 months in ambient conditions (23°C, <70%RH) on sensory scores for high protein dietary treatments. Results with (*) denote samples were significantly different at $\alpha = 0.05$ from the control at the same time period.

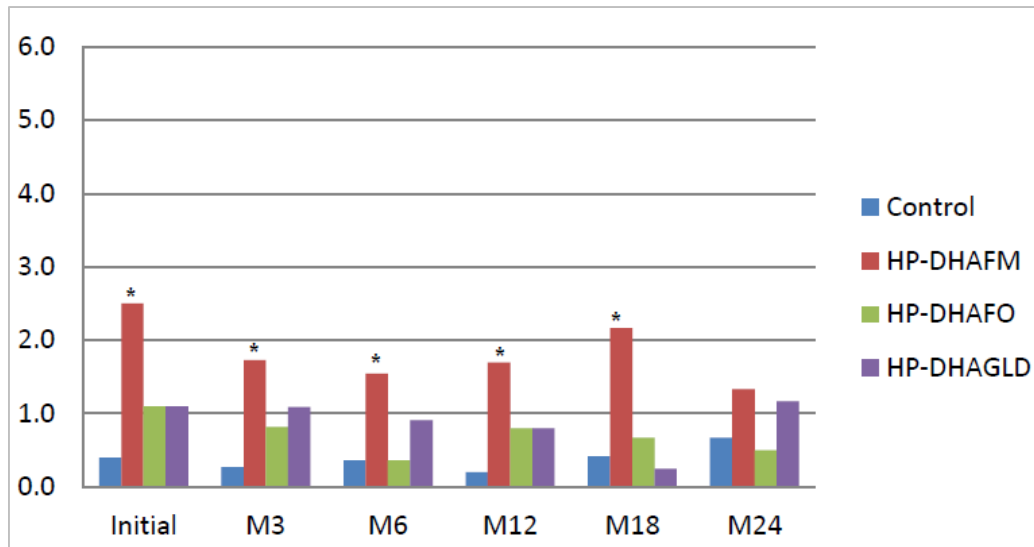


Figure A.4. The effects of storage over 24 months in ambient conditions (23°C, <70%RH) on volatile indicators (ppb) for low protein dietary treatments. Results with (*) denote samples were significantly different at $\alpha = 0.05$ from the control at the same time period. (a) Inset showing zoomed region for 4-Heptenal.

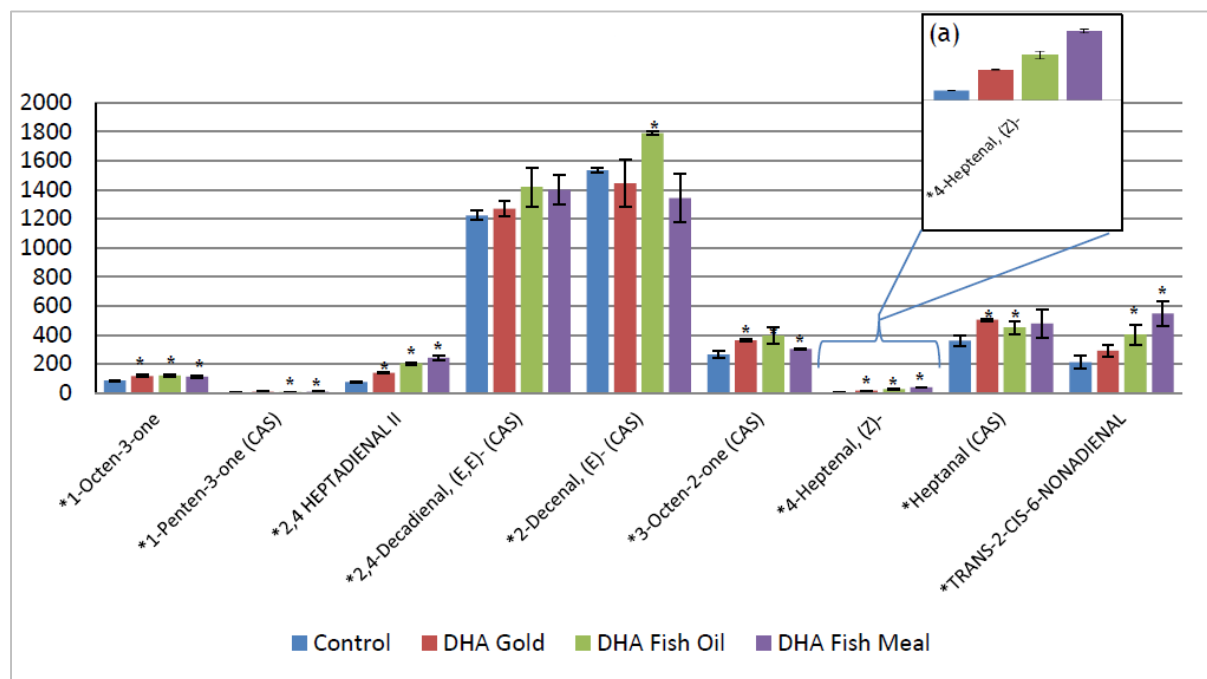


Figure A.5. The effects of storage over 24 months in ambient conditions (23°C, <70%RH) on volatile indicators (ppb) for medium protein dietary treatments. Results with (*) denote samples were significantly different at $\alpha = 0.05$ from the control at the same time period. (a) Inset showing zoomed region for 4-Heptenal.

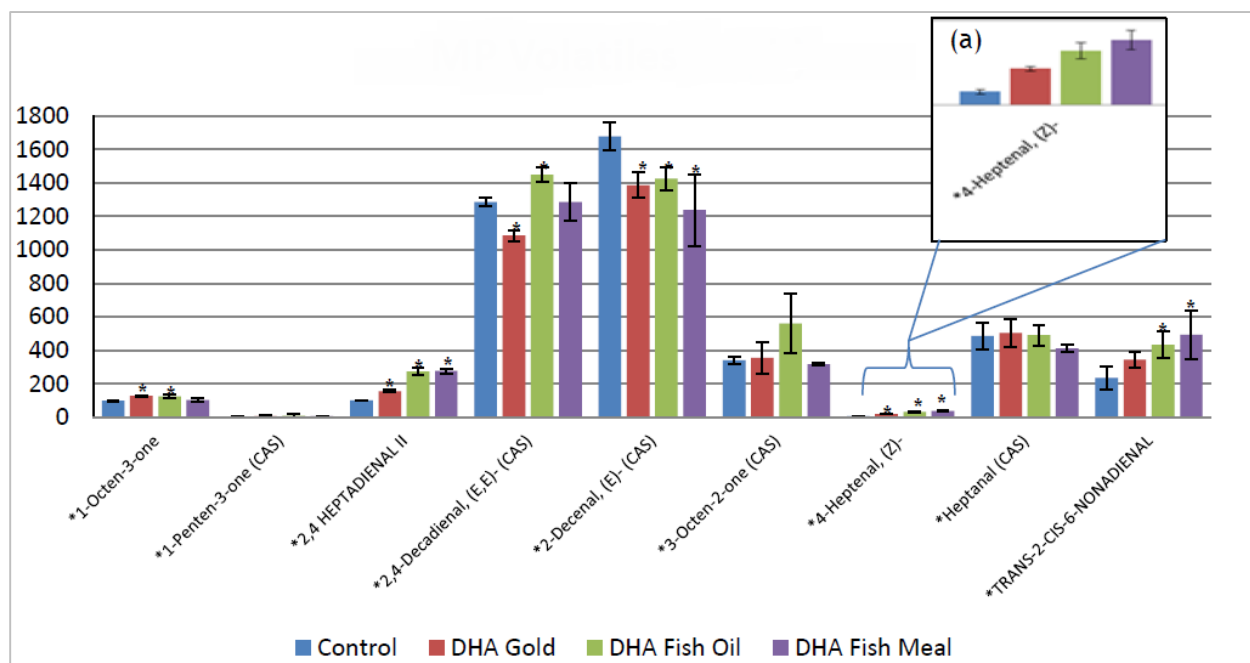


Figure A.6. The effects of storage over 24 months in ambient conditions (23°C, <70%RH) on volatile indicators (ppb) for high protein dietary treatments. Results with (*) denote samples were significantly different at $\alpha = 0.05$ from the control at the same time period. (a) Inset showing zoomed region for 4-Heptenal.

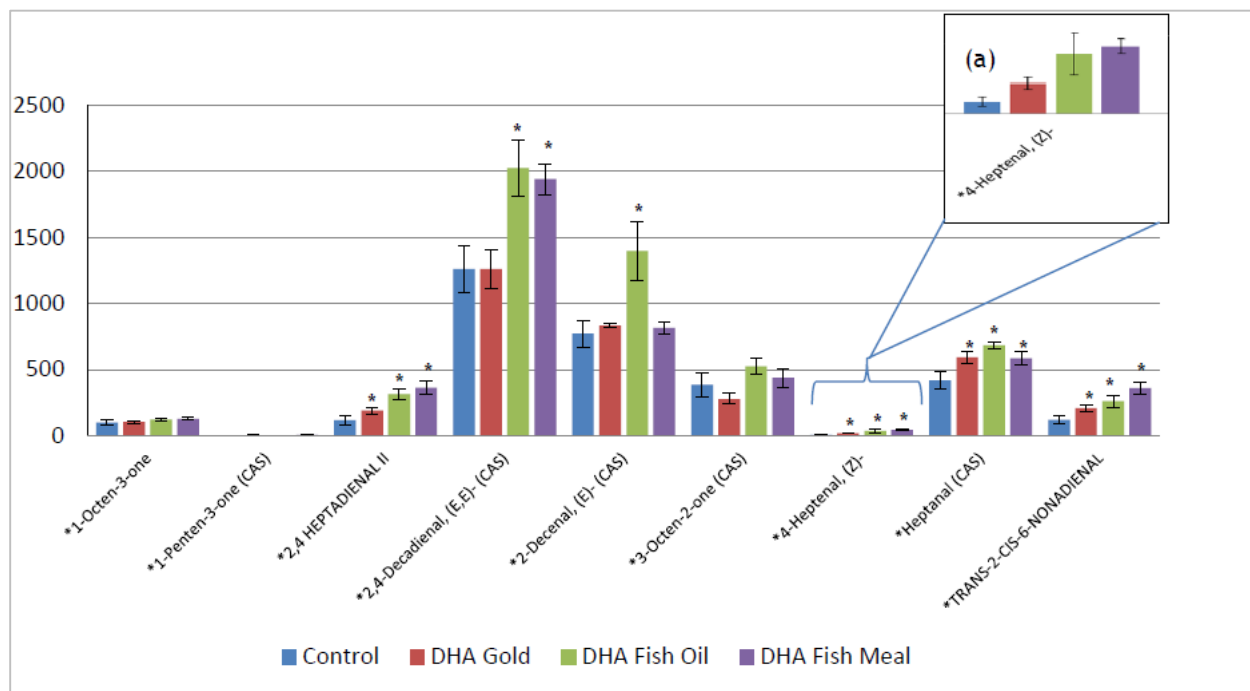


Figure A.7. The effects of elevated storage over 8 weeks in stressed conditions (35°C, <75%RH) on volatile indicators (ppb) for low protein dietary treatments within an open bag. Results with (*) denote samples were significantly different at $\alpha = 0.05$ from the control at the same time period.

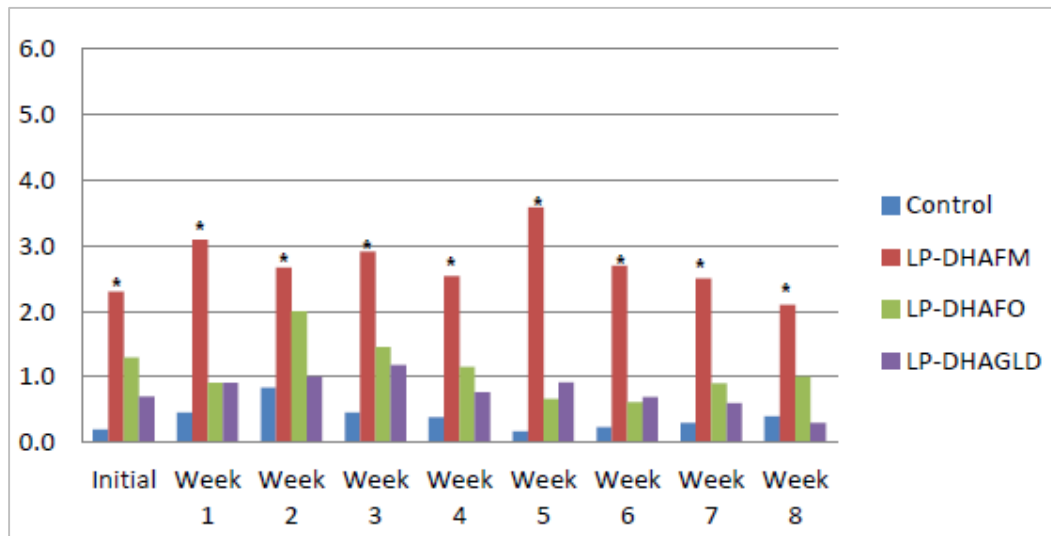


Figure A.8. The effects of elevated storage over 8 weeks in stressed conditions (35°C, <75%RH) on volatile indicators (ppb) for medium protein dietary treatments within an open bag. Results with (*) denote samples were significantly different at $\alpha = 0.05$ from the control at the same time period.

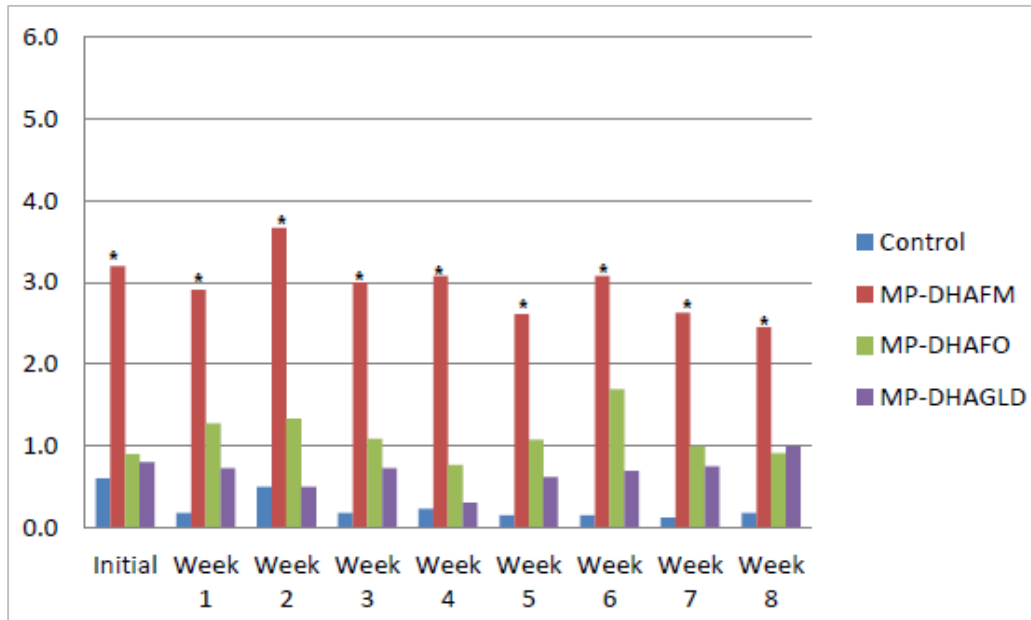


Figure A.9. The effects of elevated storage over 8 weeks in stressed conditions (35°C, <75%RH) on volatile indicators (ppb) for high protein dietary treatments within an open bag. Results with (*) denote samples were significantly different at $\alpha = 0.05$ from the control at the same time period.

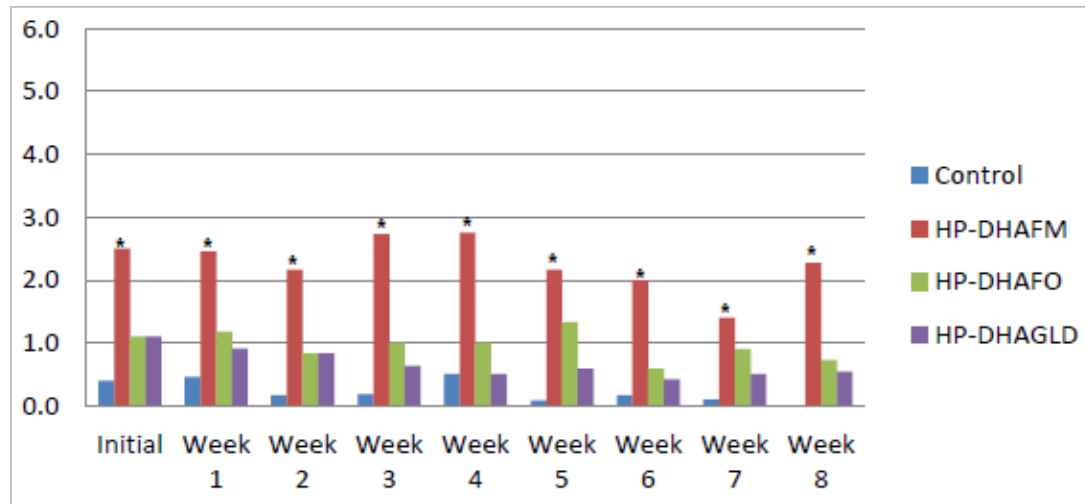


Figure A.10. The effects of elevated storage over 24 weeks in stressed conditions (35°C, <75%RH) on volatile indicators (ppb) for low protein dietary treatments within a closed bag. Results with (*) denote samples were significantly different at $\alpha = 0.05$ from the control at the same time period.

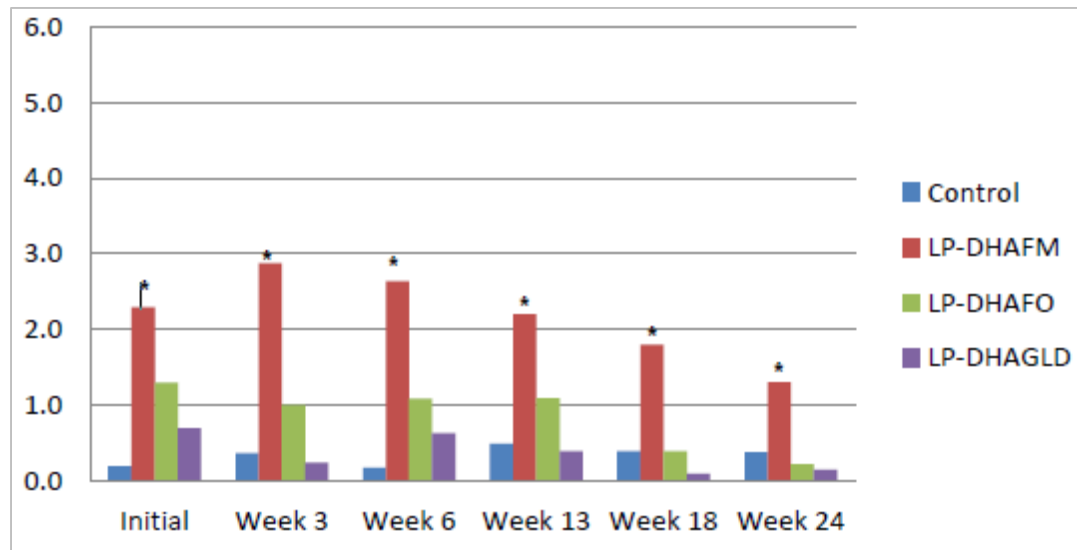


Figure A.11. The effects of elevated storage over 24 weeks in stressed conditions (35°C, <75%RH) on volatile indicators (ppb) for medium protein dietary treatments within a closed bag. Results with (*) denote samples were significantly different at $\alpha = 0.05$ from the control at the same time period.

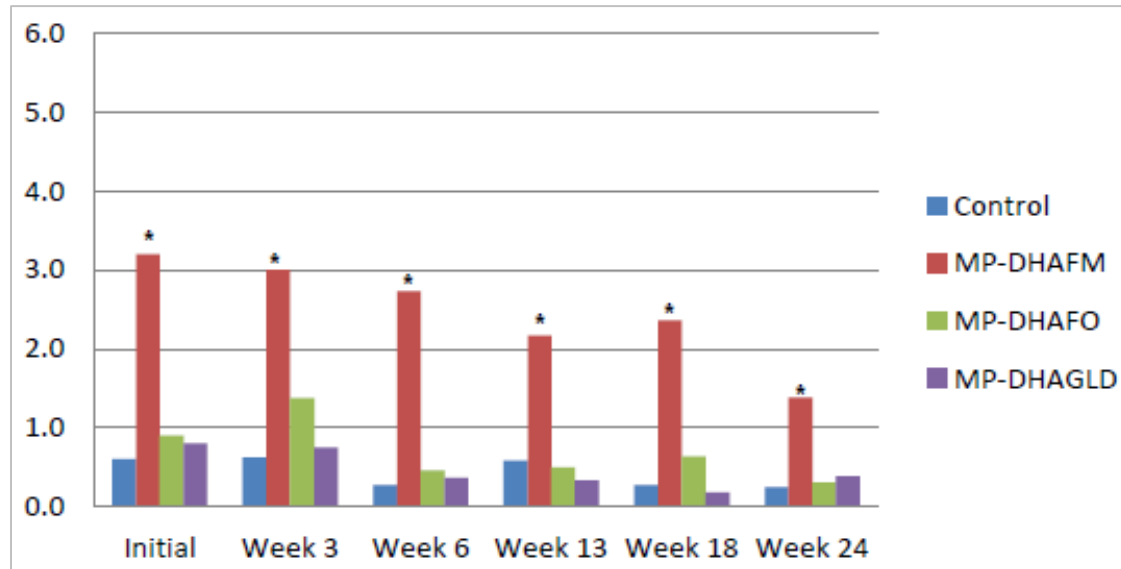


Figure A.12. The effects of elevated storage over 24 weeks in stressed conditions (35°C, <75%RH) on volatile indicators (ppb) for high protein dietary treatments within a closed bag. Results with (*) denotes samples were significantly different at $\alpha = 0.05$ from the control at the same time period.

