THE EFFECTS OF CANNING ON B-VITAMIN RETENTION IN A MODEL CAT DIET WITH AN EMPHASIS ON THIAMINE

by

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Abstract

Water soluble B-vitamins play an integral role in normal metabolic function in cats. For example, thiamine deficiency results in anorexia, neurological impairment, and, in severe cases, death in a few weeks' time. However, little research has addressed how these vitamins are affected during cat food canning. Thiamine is the most susceptible to degradation during this process, with less known about how it affects the other B-vitamins. Therefore, our objectives were to determine the effects of modifying processing parameters on thiamine and other water-soluble B-vitamins in a model canned cat food.

In a series of five experiments, various processing parameters were adjusted: including cook (retort) time, batter moisture and temperature, pH, protein source, and the addition of sulfites. Pressure (172368.93 Pa) and temperature (121℃) within the retort remained the same for all treatments. As retort time increased, thiamine concentration decreased ($P \leq 0.05$). No loss of B-vitamin concentration was noted for thiamine, riboflavin, cobalamin, and pantothenic acid as batter moisture increased. Likewise, as batter temperature increased, concentration of riboflavin, niacin, pyridoxine, folic acid, and pantothenic acid remained constant ($P > 0.10$). When different types of thiamine were included for supplementation, thiamine mononitrate tended to have a greater retention of the vitamin than thiamine hydrochloride ($P = 0.12$). The protein sources selected for the experiment included chicken as a control, beef liver, chicken liver, pork liver, salmon, tuna, and whitefish. The salmon, tuna, and whitefish were grouped together for analysis. Beef liver, chicken liver, and pork liver were grouped together for analysis. The vitamin retention of each group was compared.

When compared to chicken or liver, thiamine retention was greatest in diets containing fish ($P \leq 0.05$). In addition, riboflavin, niacin, and cobalamin retentions were greatest ($P \leq 0.05$) in diets containing liver. The addition of sulfites came from dehydrated potatoes added to thediets in exchange for rice. Thiamine tended to decrease in those diets with sulfite containing dehydrated potatoes ($P = 0.07$) compared to diets containing rice. Pyridoxine and pantothenic acid retention decreased in diets containing dehydrated potatoes ($P \leq 0.05$) compared to diets containing rice.

The largest negative impact on thiamine retention was time in the retort; cobalamin, folic acid, and riboflavin were also negatively affected. Including sulfite-containing potatoes in the diet tended to decrease thiamine, pyridoxine, and pantothenic acid. It was expected that diets containing chicken would retain more thiamine than those formulated with fish and liver. However, diets containing fish retained more thiamine, pyridoxine, and pantothenic acid. Therefore, it appears that processing and diet composition can affect the B-vitamin content of canned cat foods and must be accounted for when producing commercial products.

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Chapter 1 - Introduction

Literature Review

Vitamins, by definition, are organic substances essential in minute quantities for the nutrition of most animals and some plants (McDowell, 2012). They act as coenzymes and precursors of coenzymes in the regulation of metabolic processes; but do not provide energy themselves. They are present in natural foodstuffs and/ or produced within the body through synthesis of precursors by intestinal tract bacteria (McDowell, 2012). These micro-nutrients distributed widely in foods are imperative for overall health. They are important for nervous system function, vision, healthy skin, and red blood cell formation (Bellows and Moore, 2012). The group of water-soluble vitamins, also known as B-vitamins, are not stored in the body and perform very specific and necessary functions for various body systems (Bellows and Moore, 2012). These water soluble vitamins are steadily lost through the urine, so it is imperative that they be supplied on a daily basis. B-vitamin deficiencies can lead to numerous issues with vital metabolic functions and debilitating conditions like anorexia, growth impairment, and unstable movements (NRC, 2006). Water soluble vitamins are potentially susceptible to losses in a canned diet because of their ability to dissolve in water, likely due to the bonding of hydrogen atoms to hydroxyl groups and amine tertiary structures, the near basic pH of canned cat diets, and exposure to high heat for extended periods. To overcome potential dietary deficiency issues, commercially processed foods are often over-formulated with surplus amounts of these essential nutrients to ensure that their loss in the production process are accounted for, and the final product contains the full amount the animal requires when fed. However, there is evidence to suggest this over supplementation does not always work. Between 2009-2013 there were five

separate recalls for wet and dry cat foods due to insufficient thiamine concentrations (FDA, 2014; Table 1.1).

Thiamine Absorption and Metabolism

Thiamine, or vitamin B1, was the first of the water soluble vitamins to be isolated. Characterization began in 1890 with the recognition that the disorder beriberi in humans was related to a lack of an essential food element. This was later determined to be a thiamine deficiency (Ellefson, 1985). Beriberi is characterized initially by loss of appetite and weight, and later signs of deficiency present as degeneration of the peripheral nerves and increased concentrations of pyruvate and lactate in the blood, which leads to polyneuritis (Ellefson, 1985). Thiamine was isolated in 1911, and is found naturally in whole grains and grain products, such as rice and wheat germ, as well as yeast and legumes, and meat organs, particularly the liver, heart, and kidney, (NRC, 2006). Digestion of thiamine primarily occurs in the small intestine. The quantity of thiamine in the diet dictates the amount and process of absorption: whether passive or active carrier transfer mechanisms are employed (Butterworth, 2006). The slower of the two transport processes, passive diffusion, occurs when there is an excessive amount of thiamine in the diet. If there is a scarcity in the diet, then active transport of thiamine occurs. Once absorbed, most of the thiamine is carried in red blood cells, and the remaining is bound to plasma proteins or is free in plasma (Talwar et al., 2000).

The most vital metabolic pathway in which thiamine is involved is the citric acid cycle or tricarboxylic acid cycle (TCA) cycle. Thiamine participates in the process of carbohydrate metabolism and the pentose phosphate pathway within the TCA cycle (Bräunlich and Zintzen, 1976). Signs of thiamine deficiency can be seen in animals primarily because of its essential role in carbohydrate metabolism (McDowell, 2000). The thiamine used in these integral processes is

phosphorylated as thiamine pyrophosphate (TPP), whose presence is essential in preventing buildup of pyruvate and lactate, two acids responsible for type B lactic acidosis (Fall and Szerlip, 2005). Thiamine pyrophosphate is the most common form of thiamine found in the circulation. Thiamine functions within the TCA cycle as a cofactor for the conversion of pyruvate to acetyl-CoA (Manzetti et al., 2014).

Thiamine is required in the diet for the intermediary metabolism of carbohydrates where thiamine pyrophosphate acts as a coenzyme in the breakdown of pyruvic acid to acetaldehyde (Davidson, 1992). Thiamine deficiency leads to an abundance of pyruvate and ischemic cell changes in the brain (Davidson, 1992). Cats fed a thiamine deficient diet for an extended period of time (two or more weeks) may develop neurological impairment resulting in head twitching and, ultimately, paralysis (Loew et al., 1970; Davidson, 1992). Ventroflexion, a flexion of the spine with movement of the head towards the chest, has been reported as a result of thiamine deficiency, and cats with the deficiency are reported to somersault when they jumped from a table to the floor (Jubb et al., 1956; Loew et al., 1970). It has also been reported that cats manifesting thiamine deficiency had a more difficult time learning how to complete tasks (Irle and Markowitsch, 1982). In this same study Irle and Markowitsch (1982) reported morphological brain damage, such as hemorrhages, scars or lesions, and a loss of neurons. Anorexia, vomiting, and weakness may also be observed within the first two weeks of deficiency (Jubb et al., 1956; Deady et al., 1981a,b). If the deficiency persists, death can occur within four weeks (Everett, 1944). Typically, a deficiency is reversible with a vitamin B-complex injection after symptoms are observed and diagnosed (Loew et al., 1970). A B-vitamin complex injection, which contains thiamine, is often a common treatment even if a deficiency is not fully diagnosed because a B-vitamin toxicity is unlikely to occur. A follow-up vitamin B complex injection

administered for 3 to 5 days, then followed by oral doses for 2 to 4 weeks is often prescribed (Plumb, 2011). Clearly, a thiamine deficiency is a health issue for cats and a potential economic problem for manufacturers of cat food because of its constant need by the animal and devastating results if left unaddressed. For this reason, it is important to assess the effect processing has on thiamine and the other water soluble B-vitamins to ascertain if they are impacted in a similar manner.

Thiamine Degradation

Thiamine is an essential part of the animal's diet, and unfortunately, is readily degraded during the canning process, a popular food form for cats. In 2011, wet and canned foods were approximately 7% ($1.52 billion) of a $21.7 billion North American market (Transparency Research, 2012). Despite thiamine degradation issues, there are currently very few published reports that address its retention in canned cat diets. Recurrent thiamine deficiency recalls have been one of many safety issues affecting the pet food industry over the past few years. Specifically, the only recalls in the last five years relating to water soluble vitamin shortage in cat foods have been due to thiamine deficiencies (FDA, 2014). Thiamine loss is an immediate health and economic concern, and a solution is needed. Unfortunately, published research regarding water soluble B-vitamins in a complex cat food matrix is lacking. Data is needed to assess how broadly cat foods are affected. The closest information available on this topic was recently reported by Markovich et al. (2014) who measured thiamine concentration in sub-samples of commercial canned cat foods secured from the market. The authors selected one product from each of 45 different brands. Most were fish-flavored, and one product contained no fish. The authors reported that more than 13% (12 of 90 cans) of products sampled were below the AAFCO (2014) minimum thiamine concentration of 5 mg/kg DM for cat foods, and 15% (14

of the 90) were below the NRC (2006) recommended allowance (1.40 mg/1000 kcal ME) for cats. Smaller companies who have annual global retail sales of less than $1 billion had lower thiamine concentrations than those of foods from larger companies with annual global retail sales greater than $2 billion. Despite this, the deficient thiamine levels were spread amongst both small and large companies. Based on the percentage of products with insufficient thiamine content, reported by Markovich (2014), more recalls associated with thiamine deficiency would be expected. One possibility is cat owners supplement their cats' wet food with a dry diet.

The primary aim of thermal processing food is to destroy spoilage and pathogenic microorganisms and their spores through the application of heat for an extended period through a time-temperature profile during food processing (Balso-Canto et al., 2007). This destruction of microorganisms helps ensure food safety. Common food production processes used to produce pet food that involve heat include expansion, extrusion, baking, pasteurization, and commercial sterilization in canning (Hendriks et al., 1999). Canning is a popular thermal process that offers a way to provide wet food to a cat or dog, a form they tend to prefer (Houpt and Smith, 1981). During the canning process, heat is applied. This heat can impart many benefits; the principle benefit of which is commercial sterility. This involves heating of a product over a specified period of time to aid in the destruction of pathogenic bacteria derived from the contents. In this case, we specifically focus on pathogenic bacteria potentially present in meat, the principal component of canned pet food (Hendriks et al., 1999). Canning also offers a means to extend shelf - life by providing an impermeable environment for cooked foods (Henry and Heppell, 2002). There are reports that thermal processing of pet foods may also increase digestibility and palatability (Hendriks et al., 1999). However, excessive heating during food processing can also decrease the digestibility of essential amino acids (in a rat model), suggesting this potential exists

for other species (Hendriks et al., 1999). Vitamins, such as water soluble vitamins like biotin, folic acid, pyridoxine, riboflavin and thiamine, have been shown to be unstable in the presence of excessive heat in various foods (Henry and Heppell, 2002). Some vitamins, such as thiamine, are more susceptible to thermal destruction than others. Even if vitamins are not affected by heat, they may be destroyed by other components of food processing, such as high moisture, exposure to light, or exposure to oxygen (Henry and Heppell, 2002).

One solution to compensate for losses from intense heat during processing is by super-fortifying diets with synthetic sources, a strategy that can be effective for many heat liable vitamins but is not always effective. Pet food manufacturers are warned that thiamine levels can drop by 90% due to canning (AAFCO, 2014), but in the last five years, there have still been several voluntary recalls due to its insufficiency (FDA, 2014; Table 1.1). Markovich et al., (2013) reported that recalls involved nine brands of cat foods and at least 23 clinically treated cats. Worse yet, these authors speculate that it is likely that clinical cases of thiamine deficiency are underreported, in part because the initial signs of thiamine deficiency are not unique to thiamine deficiency alone (Markovich et al., 2013). For example, thiamine deficiency shares signs with numerous other diseases resulting from disruptions to diet, changes in environment, or exposure to pathogens such as a drastic drop in weight or vomiting. Also, a constant supply of thiamine is essential for both cats and dogs because it is water soluble and subject to daily urinary excretion with no body storage mechanism (Garosi et al., 2003). This is particularly an issue in the cat, who has a larger demand for thiamine due to high metabolic activity for protein synthetic processes (NRC, 2006). Current thiamine requirements for cats are 5 mg/kg compared to 1 mg/kg in a conventional diet for dogs (AAFCO, 2014). Furthermore, the thiamine requirement for cats increases in more demanding stages of life, such as lactation and pregnancy.

For instance, there is a 12.5% increase in recommended intake of thiamine in cats during late gestation (6.3 mg/4,000 kcal diet) compared to those at maintenance (5.6 mg/4,000 kcal diet) (NRC, 2006). Due to this, any thiamine destruction could lead to pronounced and debilitating results in cats.

Vitamin Losses due to Diet and Processing

Reports of vitamin losses due to processing are limited and primarily focused on thiamine in pet foods. Not only are manufactured foods produced with elevated temperatures during processing, but other factors prior to and associated with processing can deplete or render thiamine inactive. The diet itself has components that may result in detrimental effects to the retention of thiamine prior to any processing. These include moisture, pH, and enzymes found in some ingredients that have detrimental effects on thiamine (Henry and Heppell, 2002; Hanes et al., 2007). The typical pH of a wet canned cat food is around 6.0 (Henry and Heppell, 2002). Thiamine is most stable when pH is below 5.0 (NRC, 2006). Thiamine can also be destroyed by sulfites that are used to preserve meats and other foods. These sulfites are known to sever thiamine at the methylene bridge (Singh et al., 2005).

The chemical structure of thiamine (Figure 1.1) aids in the understanding of thiamine's instability through the canning process. The methylene bridge connecting the pyrimidine and thiazole moiety of the structure can be easily broken by sulfites. The thiazole moiety is less stable than the pyrimidine structure, and is easily broken by hydrolysis. The methylene bridge and sulfur ion within thiamine contribute to its instability in thermal processing.

Thiamine can also be inactivated by a broad class of thiaminase enzymes found mostly in shellfish and fish viscera (Loew et al., 1970). Thiaminase is destroyed by heat processing. However, if the diet contains raw meats that are not properly cooked, destruction of thiamine

could result (Marks et al., 2011). McCleary and Chick (1977) reported that the optimum temperature range for most thiaminase enzymes is between 20 - 80℃. They found the temperature for 50% denaturation to be between 60 - 65℃. The pH of a pet food diet can also have an effect on the stability of thiamine. Thiaminase is stable at a wide range of pH levels (3.0 -12.0; McCleary and Chick, 1977), but its optimal pH is 6.0. Because of all these factors, over-formulation or adding extra vitamins to compensate for processing and diet sources of degradation is a common practice to ensure thiamine adequacy in cat diets when they are consumed (AAFCO, 2014). Over-formulation is not an economic issue as thiamine is relatively inexpensive as an ingredient at $2.98/kg or $1.34/lb. However, over-formulation does not necessarily mean adequate retention of thiamine and could lead to an expensive recall.

Thiamine Deficiency in Other Species

Thiamine nutrition issues extend beyond household pets. The most prominent is an acute thiamine deficiency, which occurs in cattle reared in high intensity production practices. However, the issue is not a dietary shortage per se. Rather, the bacteria found in the rumen of sheep and cattle generally produce adequate thiamine to support the animal's needs. However, some rumen bacteria also produce thiaminase enzymes. As previously described, these enzymes render thiamine inactive. If these degradative enzymes are produced at a rate greater than thiamine is consumed, then a deficiency may occur. According to Roberts and Allen (2012), thiamine deficiency reduces energy availability to the brain, which leads to brain degeneration called polioencephalomalacia (PEM). For cattle and sheep fed in feedlots, where the diet is high in soluble carbohydrates and low in functional fiber, thiaminase producing bacteria can proliferate in the rumen, leading to thiaminase over-expression, resulting in a thiamine deficiency. The death rate in the feedlot is generally higher than seen for grazing cattle when

affected by the same fermentation disruption. Clinical signs of the disease include agitation, muscle twitching, blindness, recumbency, and seizures. Unlike cats, who take weeks to succumb from the disease, PEM in cattle causes death in 24-48 hours.

Analytical Methods

The official technique to measure thiamine is a fluormetric method accepted by the Association of Analytical Chemists (AOAC) (2012). The method, AOAC 942.23, describes the extraction of free thiamine from a 10 g sample in dilute acid after autoclaving. This resulting solution is incubated with a buffered enzyme to release the bound thiamine. An ion-exchange column is used to further purify the solution, after which an aliquot of the purified solution is converted to thiochrome through a reaction with potassium ferricyanide. This thiochrome is extracted in isobutyl alcohol and measured on a fluorometer against a known standard. The fluorometer's input filter is set at a wavelength of 365 nm with a wavelength of 435 nm for the output filter.

Thiamine analytical methods using high performance liquid chromatography (HPLC) have also been reported. They are quick and sensitive, but not an officially accepted method at this time. The HPLC can be used to measure numerous compounds, depending upon their excitation and emission wavelength, as well as the mobile phase and column used in the designated procedure. For example, the method reported in the Analyst (2000) describes measurement of both thiamine and riboflavin in pet foods and animal feeds. The AOAC and Analyst methods have some similarities to each other. Each depends on an extraction of free thiamine during a boiling step and is incubated with a buffered enzyme to release the bound thiamine.

Other Water Soluble B-vitamins

Thiamine is the B-vitamin most associated with recalls. However, it was important to look at other water soluble B-vitamins because of the lack of information available on their retention in a complex canned cat diet and to determine if there was a relationship between processing and the retention of the B-vitamins as a whole class of vitamins.

Riboflavin, or vitamin B2, is the precursor of flavin adenine dinuncloeotide (FAD) and flavin mononucleotide (FMN;Higdon et al., 2008). These coenzymes are important for energy production and metabolic pathways where they serve as electron carriers for numerous oxidation-reduction reactions (Higdonet al., 2008). A deficiency of this vitamin can contribute to decreased availability of other vitamins by interrupting products of enzymes involved in their metabolism (NRC, 2006). The vitamins dependent upon riboflavin include folic acid, pyridoxine, niacin, and fat soluble vitamins K and D (NRC, 2006). Acute riboflavin deficiency in cats presents as anorexia, periauricular alopecia, and epidermal atrophy (Gershoff et al., 1959). Chronic deficiency can lead to cataracts and fatty livers.

Niacin, or vitamin B3, is important to the production of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), coenzymes imperative for normal metabolic functions. The reduced form of NAD, NADH, plays an essential role in energy expenditure reactions, and NADPH, the reduced form of NADP, is needed in numerous synthetic reactions involving energy storage (NRC, 2006). Most animals synthesize niacin as an end product of the metabolism of the essential amino acid tryptophan. However, niacin cannot be synthesized from tryptophan by cats because a competitive enzyme, picolinic carboxylase, converts tryptophan to acetyl coenzyme A rather than to NAD. Thus, cats must

receive their entire requirement of niacin from their diet (Case et al., 2011). Heath et al. (1940) reported anorexia, elevated body temperature, a fiery red tongue with ulceration, and congestion in a clinical case of niacin deficiency in cats. Carvalho da Silva et al. (1952) observed young cats fed diets devoid of niacin ceased growing after 10 to 15 days, lost body weight, and died in 15 to 50 days. Niacin can be found in meats, legumes, and grains, although the niacin found in plant and grain sources may be stored in the bound form (niacinogen) and is therefore not available to the animal (Ghosh et al., 1963). Conversely, the primary form of niacin in meat sources is in the unbound, biologically available form (Baker, 1995).

Pantothenic Acid, once called vitamin B5, is an integral vitamin at all stages of life. The vitamin is found in all body tissues and in all forms of living tissue (Case et al., 2011). Pantothenic acid is an integral component of Coenzyme A, where it is required for the primary regulatory step of its synthesis (Plesofsky, 2001). Because pantothenic acid is found in numerous types of foods, practical deficiencies are rare. In experimentally- induced deficiencies, Gershoff and Gottlieb (1964) reported that cats failed to grow, histological changes and lesions were found in the small bowel consisting of giant blunted villi in the jejunum and upper ileum.

Pyridoxine, Vitamin B6, is associated with three compounds: pyridoxine, pyridoxal, and pyridoxamine. Pyridoxal, which is part of the coenzyme pyridoxal 5'-phosphate (PLP), is the biologically active form of pyridoxine (McDowell, 2000). It has been reported that PLP is involved in more than 100 enzymatic reactions, nearly half being transamination reactions (Sauberlich, 1985). This coenzyme is required for numerous reactions associated with amino acid metabolism and is to a lesser degree required for the metabolism of glucose and fatty acids. Other types of reactions associated with PLP include decarboxylation, oxidative deamination, and side-chain elimination (NRC, 2006). It is important in physiological processes such as

gluconeogenesis, nervous system function, immune response, and gene expression (Leklem, 2001). Pyridoxine is found in a variety of foods including fish, organ meats, potatoes, and non-citrus fruits (Mackey et al., 2006). Naturally occurring deficiencies of this vitamin have not been reported in dogs and cats (Case et al., 2011), but experimentally induced deficiencies exist. Carvalho da Silva et al. (1959) reported growth depression, convulsive seizures, and kidney lesions in cats fed a vitamin B6 deficient diet.

Folic acid, vitamin B9, is active in the body as tetrahydrofolic acid (Case et al., 2011). This vitamin plays an important role in the body because of its involvement in the synthesis of thymidine, a component of deoxyribonucleic acid (DNA). Clinically, a deficiency presents as a decrease in red blood cells (anemia) and white blood cells (leukopenia;Afonsky, 1954). Long-term deficiencies can lead to a decrease in growth rate (Carvalho da Silva et al., 1955) and bone marrow lesions (Yu et al., 1999). Folic acid can be found in leafy green vegetables and organ meats. It is synthesized by the bacteria in the large intestine of cats, dogs, and other species (Case et al., 2011).

Cobalamin, vitamin B12, is unique as the only vitamin that contains a trace element, Cobalt, and is the only vitamin synthesized solely by microorganisms (Case et al., 2011). Most sources of colabamin in a cat's diet are derived from animal proteins (NRC, 2006). Cobalamin is involved in fat and carbohydrate metabolism and is necessary for the production of myelin, a membrane that serves as an electrical insulator around certain nerve fibers (Tymoczko et al., 2010). A deficiency of this vitamin leads to anemia and impaired neurological function (Case et al., 2011). Simpson et al. (2001) reported abnormally low concentrations of cobalamin in the plasma of cats exhibiting weight loss, diarrhea, vomiting, anorexia, or thickened intestines.

Conclusions

Thiamine retention in pet foods is widely variable (Markovich et al., 2014), and dietary thiamine deficiencies must be addressed because they remain a health issue for cats (FDA, 2014). Deficiencies or deaths caused by insufficient thiamine in the diet may go under reported because clinical signs mirror many illnesses. At present, there are no reported techniques to assure thiamine adequacy in processed foods beyond over-formulating the diet. An alternative to this super-fortifying needs to be developed in order to ensure nutritionally sufficient diets that are acceptable to the animal, consumer, and producer. There is a need for research exploring techniques to understand the factors associated with thiamine degradation in commercially-produced cat foods. Since a deficiency can ultimately result in death, a solution is imperative.

While thiamine stability in canned cat diets is a problem, even less is known regarding the effects of processing on the other B-vitamins in a pet diet. They are generally assumed to be stable, but perhaps thiamine is not the only vitamin that is readily degraded by processing. The degree these other vitamins must be supplemented beyond baseline to ensure their adequacy in the diet is not reported. It is important to understand the specific characteristics of a diet and processing have on water soluble vitamin retention. With a better understanding of new technologies and more economical vitamin inclusions, fewer chances for recalls and animal deficiencies would likely occur.

Given the lack of published data in the area of B-vitamins degradation in canned pet foods, more research into the facets of their degradation should be explored. Therefore, it was our objective to determine the effects of processing parameters on the retention of water soluble B-vitamins, with an emphasis on thiamine. It was our hypothesis that thiamine and other B-

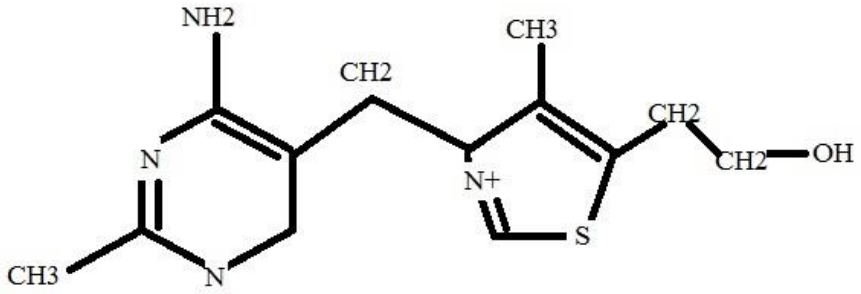
vitamins would be readily degraded during the canning process of a conventional cat food and that it was primarily a function of the combination of time and temperature.

Tables and Figures

Table 1.1 FDA Recalls for thiamine deficiency in cat foods (2009-2014)

Date of Recall	Company Associated	Food Type
October 20, 2009	Diamond Pet Food®	Dry Kibble
June 9, 2010	P&G Pet Care®	Canned Food
February 28, 2011	Wellness Pet®	Canned Food
May 11, 2012	Nestle Purina®	Canned Food
March 10, 2013	Diamond Pet Food®	Dry Kibble

Figure 1.1 Chemical structure of thiamine (Gregory, 1997)



Chapter 2 - Effects of Batter Moisture, Batter Temperature, and Retort Time on Water Soluble B-Vitamins

Introduction

Pet food recalls involving thiamine have increased in the last five years (FDA, 2014). It is plausible that the high heat and pressure associated with thermal processing are responsible for the decreased thiamine retention. Thiamine is known to be a heat- labile B-vitamin (Henry and Heppell, 2002). This is especially important to the cat, who requires the vitamin at nearly five times that of the dog due to high protein metabolism. If not supplied in adequate quantities in the diet, thiamine deficiency can lead to paralysis and death (NRC, 2006).

While very little is understood about how readily thiamine is degraded through the canned cat food process, there is even less research available describing the effects of high heat and pressure on the other B-vitamins, such as riboflavin, niacin, pantothenic acid, folic acid, pyridoxine, and cobalamin. Because literature examining this topic is lacking in a complex canned cat food matrix, it is important to evaluate these other B-vitamins to determine if they are prone to some of the same degradation challenges.

Therefore, the objectives of these experiments were to evaluate the effects of processing parameters, specifically time, temperature, and batter moisture, on retention of B-vitamins in a canned cat food. Our hypothesis was that an increase in each processing parameter would result in B-vitamin degradation.

Materials and Methods

The project was conducted in two sequential experiments, each arranged in a 3x3 factorial design. In experiment 1, the retort times were 45, 60, and 90 minutes and batter moisture was evaluated at 65, 75, or 85%. In the second experiment, retort time was again evaluated at 45, 60, and 90 minutes with batter temperature evaluated at 50, 60, or 70° C. Industry standard retort times, batter moistures, and batter temperatures are 60 minutes, 75%, and 60° C, respectively.

Model Canned Cat Diet

A model canned cat diet was formulated to emulate a commercial cat food. The intent was to produce the same formula throughout all trials. The base diet was formulated to a moisture level of 65% in order to allow for adjustments throughout the experiment. Ingredients and inclusion levels are found in Table 2.1.

The mechanically separated chicken was acquired as frozen blocks from CJ Foods (Bern, KS) and is consistent with that common to pet food producers. Meat was kept frozen until three days before canning experiments. The meat was tempered in a refrigerator at 4° C until pliable for use. The brown rice was purchased at a local grocery store (Great Value, Manhattan, KS). The egg used for the experiments was in spray-dried form (IsoNova, Springfield, MO). Carrageenan (Carrageenan T-40) and Guar gums (Guar 80) were supplied from DuPont Animal Health (St .Louis, MO). The vitamin premix was supplied by DSM Nutritional products (Parsippany, NJ) and the trace mineral premix and macro minerals were obtained from Lortscher Animal Nutrition, Inc. (Bern, KS).

The initial B-vitamin content for the vitamin premix was determined prior to experimental treatment preparation. Thiamine, riboflavin, niacin, pyridoxine, folic acid,

cobalamin, and pantothenic acid content were all determined to be 42.59 12.78, 191.65, 15.08, 2.13, 0.057, and 30.17 mg/kg on a dry matter basis, respectively (Table 2.2).

Canning

Experiment 1: The individual ingredients for the diet were weighed and added to the cooking container and warmed on direct heat from an electric burner. Carrageenan and guar gum were added as gelling agents as the batter started to warm to approximately 40˚C. The batter was heated on the burner until it reached 60˚C. In order to increase the moisture content for the experimental treatments, 1.8 kg of batter was replaced with water for each 10% increase in moisture. For each target moisture 1.8 kg batter was divided into four individual aliquots and dispensed into cans (300 x 407 cans, Freund Container & Supply, Lisle, IL). Lids (300, EZO, Freund Container & Supply, Lisle, IL) were placed on cans and sealed onto the base with a mechanical seamer (Dixie Seamer, 91118, Athens, Georgia) without vacuum. This was repeated sequentially for each successive retort cooking time. Given the retort process cannot be stopped to remove product without disrupting the process, the food was produced in three separate cooking cycles: 45, 60, and 90 minutes (Dixie Retort, 100-43, Athens, GA). After each cooking cycle, samples were removed from the retort and allowed to equilibrate to room temperature then stored in these conditions until further analysis.

Experiment 2: The batter was again cooked in a large container over an electric burner until the target temperatures were reached. Batter temperature was determined by placing a thermometer in the middle of the batter while constantly stirring the contents. At each batter temperature, four aliquots were collected and placed into cans. This was repeated three times, resulting in a total of 12 cans per each treatment. Three sets of 12 cans were cooked in the retort

for 45, 60, and 90 minutes, respectively. Following completion of cooking in the retort, samples were allowed to reach room temperature and stored until further analysis.

Samples were analyzed for moisture content (AOAC 950.46) and pH (OakTon pH meter, # 289107,Vernon Hills, IL), thiamine (AOAC 942.23), riboflavin (AOAC 944.33), niacin (AOAC 944.13), pantothenic acid (AOAC 945.74) pyridoxine (AOAC 961.15), folic acid (AOAC 992.05), and cobalamin (AOAC 952.20). Blinded B-vitamin analyses were performed at Covance Laboratory, Madison, WI.

Statistics

Experimental treatments were arranged in a 3x3 factorial arrangement (Ott and Longnecker, 2004) with main effects of batter moisture, batter temperature, and retort time. Means were separated by significant F tests, using a procedure for mixed models with the aid of statistical analysis software (GLIMMIX; SAS Institute Cary, NC). Due to limited replication for the treatments, only the main effect means are reported. Means were considered different at a $P < 0.05$ and trends are noted at $P < 0.10$.

Results

Experiment 1: Retort Time and Batter Moisture

The target and recorded moistures and pH after processing from experiment 1 are reported in Table 2.3. The pH of all samples averaged 6.8 ± 0.2 and was consistent among the treatments with the exception of 85% moisture and 45 minutes.

The effect of retort time and batter moisture (45, 60, and 90 minutes) in experiment 1 on B-vitamins is reported in table 2.4. Thiamine concentration was diminished due to the increased heat with each increase of time ($P < 0.05$; 5.11, 4.30, and 2.96 mg/kg for 45, 60, and 90 minutes, respectively). Other B-vitamin concentrations were unaffected by retort time ($P > 0.10$).

As batter moisture increased from 65 to 85%, retention of all B-vitamins, with the exception of Niacin and Folic Acid, increased (Table 2.4). Thiamine retention was lowest ($P < 0.05$) for the 65%, compared to 75 or 85% batter moisture (3.57, 3.67, and 5.12 mg/kg, respectively). Riboflavin levels were lowest ($P < 0.05$) for 65 and 75 than 85% batter moisture (10.41, 9.89, vs 13.00 mg/kg, respectively). Niacin retention did not differ among treatments ($P > 0.10$). Pyridoxine retention tended to be lower ($P < 0.10$) for 75% versus the 85% moisture

with 65% being unexplainably intermediate (11.46, 15.70, and 11.95 mg/kg, respectively). Folic acid retention was not different among batter moisture treatments ($P > 0.10$). Cobalamin retention was greatest ($P < 0.05$) for 65 and 75% moisture compared to 85% batter moisture (0.022, 0.018, and 0.040 mg/kg, respectively). Pantothenic acid retention was greatest ($P<0.05$) for 85% batter moisture compared to 65 and 75% (67.31, vs 46.32, and 49.52mg/kg, respectively).

Experiment 2: Retort Time and Batter Temperature

Moisture and pH results after processing (experiment 2) are reported in Table 2.5. The moisture and pH remained relatively constant among treatments at 68.3% ± 3.3 and 6.73 ± 0.09, respectively

The main effect means for retort time and batter temperature are reported in Table 2.6. Thiamine retention again decreased ($P < 0.05$) with each increment of retort time from 5.11, to 3.86, and 2.53 mg/kg for 45, 60, and 90 minutes, respectively. Riboflavin retention also declined with retort time, but only differed ($P < 0.05$) between 45 and 90 minutes; (10.44, 10.35, vs 9.54 mg/kg for 45, 60, and 90 minutes, respectively). Niacin retention declined ($P < 0.05$) at the first time increment, but did not change thereafter (228.51 vs 200.51, and 202.60 mg/kg for 45, 60, and 90 minutes, respectively). Pyridoxine, folic acid, and cobalamin retention did not differ due to retort time ($P > 0.10$). Pantothenic acid retention was similar for 45 and 90 minutes, and greater ($P < 0.05$) for 60 minutes (44.52, 44.55, vs 46.57 mg/kg, respectively).

The main effect means for batter temperature, with the exception of cobalamin, resulted in a vitamin retention that increased as batter temperature increased. The changes were relatively small. For example, thiamine level was 2.8% greater ($P < 0.05$) for 70° C than the batter temperature 50° C. Thiamine retention was 3.1% greater for the batter temperature of 50° C

compared to the batter temperature of 60° C (3.92 and 3.54mg/kg, respectively). Riboflavin and niacin increased ($P < 0.05$) by 14.1 and 13.7%, respectively from 50 to 70° C batter temperature. Pyridoxine had the largest increase in retention due to batter temperature ($P < 0.05$) from 10.40 to 14.14 mg/kg for 50 and 70° C batter temperature, respectively. Folic acid and pantothenic acid both increased 20 and 18.5%, respectively for batter temperature increases from 50 to 70° C. Cobalamin was not affected by batter temperature ($P > 0.10$).

Discussion

It was anticipated that the analyzed value of thiamine within the vitamin premix would be closer to target (Table 2.2). Losses within the vitamin premix would appear to be attributed to storage. This was not expected when the study began and therefore was not taken into account upon initial determination of premix addition to the diet. We concede that the initial B-vitamin content of each ingredient was not analyzed due to budget constraints. However, this work warrants further investigation into the degradation of B-vitamins through the process and reassessing the approach is worthwhile.

The moisture and pH of the experimental treatments met the target. The results affirm this was achieved; wherein, each parameter was within 2 percentage units from the target and there was sufficient separation between treatments to have an impact due to moisture content prior to retort. The pH of the treatment at 85% moisture and processed at 45 minutes had a pH 6.0. This may be associated with pH stability due to a decrease in cook time.

It was expected that thiamine would be degraded due to an increase in retort time. The magnitude of degradation was great, but did not wholly agree with the literature (AAFCO, 2014; Gerber et al., 2009; Ryley and Kajda, 1994). Previous research on thiamine retention in canned meats and vegetables (Briozzo, et al., 1987) and cooking of various cuts of meats (Gerber et al.,

2009) showed significant losses up to 100% thiamine. It is interesting that not all B-vitamins were affected in the same way by the processing parameters. The more than 40% reduction in retention of cobalamin was not anticipated, and further emphasizes how little is known about how B-vitamins behave in a complex canned cat food.

In addition, cobalamin remained unchanged by modifications to batter moisture. When B-vitamins were influenced by batter moisture, the direction of influence was surprising. The effect expected was a decrease in overall B-vitamin retention as batter moisture increased because of the vitamins' solubility and reported fragility in water. One potential reason for an increase in retention as the moisture increased may have been due to the sedimentation through removal of the batter. Or, as removal of batter took place from one experimental treatment to the next, there were not sufficient concentrations of gums to keep solids suspended. In order to remedy this, it would be worthwhile to produce cans of cat food in a different, more random assembly sequence. For example, producing diets in a different order, or being sure to keep food suspended while changing the moisture content would be beneficial. Because the results associated with a change in batter moisture were ambiguous, the need for corroborating research is warranted. A follow-up experiment is needed to evaluate a change to the order the treatments were prepared, which would allow for a comparison of vitamin degradation prior to processing. Keeping a record of the time cans were allowed to rest prior to processing may also allow for a better understanding of the effect the rest period has on B-vitamin retention.

The results for the B-vitamins retention in the second experiment followed a similar pattern to experiment 1 relative to losses associated with time in the retort. This confirms that increasing the amount of time thiamine is exposed to heat will decrease B-vitamin retention. The 70% reduction of thiamine justifies the recommendations made in AAFCO (2014) reported in

the unsubstantiated claims regarding the impact of processing on thiamine retention (NRC, 2006).

Batter temperature did not seem to have a substantial effect on B-vitamin retention. While the effect was significant in respect to niacin, the magnitude of degradation was not physiologically relevant. Pantothenic acid retention was not affected, which makes sense because it has been reported that pantothenic acid is not a very heat labile vitamin (Hamm and Lund, 1978). Further, ingredients, such as chicken, brown rice, and egg used in the diet preparation may contribute to pantothenic acid for these experiments (Higdon et al., 2008). Even though some of the increase in the quantity of remaining vitamins increased due to batter moisture adjustments, the amount was small and we pose no reasonable mechanistic theory to explain the results. While thiamine has traditionally been considered the most reactive vitamin, perhaps it is not the only vitamin that needs to be over-formulated in canned pet diets.

Conclusion

Thermal processing had a negative effect on B-vitamin concentrations in a canned cat food. The vitamins most affected by increasing retort time were thiamine and cobalamin with losses of 70 and 40%, respectively. Adjustments to B-vitamins in a canned cat food diet may now be better quantified to assure proper final fortification.

Tables and Figures

Table 2.1 Model canned cat food ingredient composition and expected nutrient composition.

Ingredient	%	Nutrient	Unit	As-Is
Water	20.00	Crude Protein	%	13.33
Chicken	71.40	Crude Fat	%	15.24
Rice, Brown	6.50	Crude Fiber	%	1.31
Eggs	0.50	Thiamine	mg/kg	1.87
Guar Gum	0.50	Riboflavin	mg/kg	2.12
Carrageenan	0.50	Pantothenic Acid	mg/kg	5.42
Vitamin premix	0.05	Niacin	mg/kg	24.66
Trace mineral premix	0.05	Pyridoxine	mg/kg	2.66
		Folic Acid	mg/kg	0.27
		Cobalamin	mg/kg	0.0078

Table 2.2 The vitamin content of the experimental premix used to evaluate canned cat food diet processing losses

B-Vitamin	Target (mg/kg DM)	Analyzed value (mg/kg DM)
Thiamine	5,208.35	3,747.39
Riboflavin	3,754.70	4,238.00
Niacin	56,251.57	55,114.82
Pyridoxine	4,427.56	5,323.59
Folic Acid	626.93	541.75
Cobalamin	16.53	15.55
Pantothenic Acid	8,855.11	10,855.95

Table 2.3 Moisture and pH of canned cat foods in which cook time in the retort (45, 60, and 90 minutes) and final moisture target (65, 75, and 85%) were evaluated

Cook Time, min	Target Moisture, %	Measured Moisture, %	pH
45	65	69	6.97
45	75	75	6.75
45	85	85	6.00
60	65	68	6.69
60	75	75	6.75
60	85	84	6.78
90	65	66	6.70
90	75	72	6.70
90	85	86	6.79

Table 2.4 Main effect means for the vitamin content (mg/kg) affected by retort time and batter moisture.

	Retort Time, minutes			Batter Moisture, %				*P* =	
	45	60	90	65	75	85		Retort	Batter
N=	3	3	3	3	3	3	SEM	Time	Moisture
Thiamine	5.11[a]	4.30[b]	2.96[c]	3.57[a]	3.67[b]	5.12[b]	0.19	0.0031	0.0072
Riboflavin	11.74	11.09	10.47	10.41[a]	9.89[a]	13.00[b]	0.55	0.36	0.032
Niacin	186.19	214.75	199.33	175.66	188.45	236.15	23.16	0.71	0.26
Pyridoxine	14.10	12.07	12.94	11.95[ab]	11.46[a]	15.70[b]	1.05	0.46	0.084
Folic Acid	1.04	0.96	0.92	0.91	0.85	1.16	0.087	0.68	0.14
Cobalamin	0.026	0.027	0.027	0.022[a]	0.018[a]	0.04[b]	0.0012	0.72	0.0004
Pantothenic Acid	53.23	55.01	54.91	46.32[a]	49.52[a]	67.31[b]	3.40	0.92	0.024

[abc] Means within a row and main effect with unlike superscripts differ ($P < 0.05$)

[xyz] Means within a row with unlike superscripts differ ($0.05 < P < 0.10$)

Table 2.5 Moisture and pH for experiment 2 in which cook time in the retort (45, 60, and 90 minutes) and batter temperature (50, 60, and 70˚C) were modified to evaluate vitamin degradation in a canned cat food

Cook Time, min	Target Moisture, %	Measured Moisture, %	pH
60	65	69	6.71
60	65	67	6.75
60	65	65	6.82
60	65	70	6.71
60	65	67	6.75
60	65	66	6.71
60	65	71	6.67
60	65	70	6.74
60	65	70	6.73

Table 2.6 Main effect means for the vitamin content in canned cat food due to retort time and batter temperature

	Retort Time, min			Batter Temp., °C				*P* =	
	45	60	90	45	60	90		Retort	Batter
N =	3	3	3	3	3	3	SEM	Time	Temp.
Thiamine	5.11[a]	3.86[b]	2.53[c]	3.92[ba]	3.54[a]	4.03[b]	0.11	0.0002	0.0700
Riboflavin	10.44[a]	10.35[ab]	9.54[b]	9.59[a]	9.80[a]	10.94[b]	0.22	0.0850	0.0260
Niacin	228.51[a]	200.51[b]	202.6[b]	199.67[a]	205.00[a]	226.94[b]	2.30	0.0017	0.0023
Pyridoxine	11.39	12.93	12.53	10.40[a]	12.31[ab]	14.14[b]	0.52	0.2100	0.0180
Folic Acid	0.88	0.93	0.89	0.85[a]	0.83[a]	1.02[b]	0.034	0.6300	0.0330
Cobalamin	0.0195	0.0198	0.023	0.022	0.019	0.021	0.002	0.5400	0.4900
Pantothenic Acid	44.52[a]	46.57[b]	44.55[a]	41.79[a]	44.33[b]	49.51[c]	0.40	0.0360	0.0004

[a,b,c] Means within a main effect and row with unlike superscripts differ ($P<0.05$).

Chapter 3 - Effect of pH, thiamine type, and acidulant on thiamine retention

Introduction

Previous work indicated that thermal stability of thiamine in model systems improved when pH declined from near neutral to acidic conditions (Farrer, 1955, 1945; Mauri et al., 1986; Briozzo et al., 1987). However, none have examined the effect of adjusting pH on a complex food system, such as a canned cat diet. It has been reported that pH, inorganic bases, metal complexes, and oxidation-reduction systems contribute to thiamine degradation (Dwivedi and Arnold, 1972). Based on this, it was important to not only examine the effects of pH adjustment on thiamine retention in a complex food system but to determine if there was a difference in thiamine retention if a diet's pH was adjusted with two separate acidulants. Work has been published related to thiamine type and thiamine stability in chick diets (Waibel et al., 1953). The authors reported that thiamine mononitrate was somewhat more stable in the diets than thiamine hydrochloride. Currently, there is no published work examining the effect of thiamine type (hydrochloride or mononitrate) selected on the retention of thiamine within a canned cat food. Therefore, it was our objective to determine the effects of pH, thiamine type (hydrochloride or mononitrate), and acidulant type (hydrochloric acid and sodium bisulfate) on thiamine retention in a canned cat diet.

Materials and Methods

Model Canned Cat Diet

A model canned cat diet was formulated to emulate a commercial cat food diet. The intent was to produce consistent diets throughout all trials. This diet was formulated to a moisture level of 78% (Table 3.1).

The chicken meat was acquired as frozen blocks from CJ Foods (Bern, KS) and is consistent with that common to pet food producers. The meat was kept frozen until three days before canning experiments. The meat was tempered in a refrigerator (40° C) until pliable for use. The brown rice was purchased at a local grocery store (Wal-Mart, Manhattan, KS). The egg used for the experiments was in the spray-dried form (IsoNova, Springfield, MO). Carrageenan (Carrageenan T-40) and Guar (Guar 80) gums were supplied from DuPont Animal Health (St .Louis, MO). The vitamin premix was supplied by DSM (Parsippany, NJ) and the trace mineral premix and macro- minerals were obtained from Lortscher Animal Nutrition, Inc. (Bern, KS).

The initial B-vitamin content for the vitamin premix was determined before formulation. Contents of thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, folic acid, and cobalamin were 840.87 mg/kg, 296.78 mg/kg, 280.52 mg/kg, 826.74 mg/kg, 248.73 mg/kg, 38.44 mg/kg, and 1.07 mg/kg DM basis, respectively.

It was necessary to determine the actual concentration of thiamine in each thiamine type (hydrochloride and mononitrate) because both types of thiamine contained different percentages of nitrate. Determining this would allow for determination of thiamine added to the diet. This can be determined as follows:

Thiamine Mononitrate: There was 2.4 g of thiamine mononitrate (197.6 g dextrose) used for the diets and the concentration of nitrate was 97%. Therefore, there was 2.33 (2.4 x 0.97) g or 2,330 mg of thiamine mononitrate in the 200 g carrier and vitamin placed in the diet.

2330 mg ÷ 0.20 kg = 11,650 mg/kg

0.068 kg of vitamin in a 13.608 kg diet

11,650 mg/kg x (0.068 kg ÷13.608 kg)

58.20 mg/kg ÷ (1-moisture of diet)

58.20 mg/kg ÷ (1-0.78) =264.55 mg/kg Thiamine mononitrate

Thiamine Hydrochloride: There was 2.4 g of thiamine hydrochloride (197.6 g dextrose) used for the diets and the concentration of thiamine hydrochloride was 98%. So, there was 2.35 (2.4 x 0.98) g or 2,352 mg of thiamine hydrochloride in the 200 g of carrier and vitamin placed in the diet.

2,352 mg ÷ 0.20 kg= 11,760 mg/kg

0.068 kg of vitamin in a 13.608 kg diet

11,760 mg/kg x (0.068 kg÷13.608 kg)

58.80 mg/kg ÷ (1-moisture of diet)

58.80 mg/kg ÷ (1-0.78) =267.27 mg/kg Thiamine hydrochloride

Experimental Treatments

Experiment 1: The objective of the first experiment was to determine the effect of thiamine type (thiamine hydrochloride or thiamine mononitrate, DSM Parsippany, NJ) and pH (range of 2.0 to 8.0 in 0.5 deviations) on the retention of thiamine in canned pet food. Treatments were arranged in a 2 x 13 factorial. Hydrochloric acid (HCl) and

sodium hydroxide (NaOH) reagents were used to adjust the pH. Dextrose was used to ensure a uniform dispersion of the vitamin in the diet; wherein, 2.4 g of each thiamine type was placed into a small mixing bowl along with 197.6 g of dextrose and mixed with a spoon for one minute. From this mixture, 68 g was added to two separate stock pots containing a standard canned cat diet. Table 3.1 contains the diet used for this experiment.

Experiment 2: The objective of the second experiment was to determine the effect of two different acidulants (hydrochloric acid or sodium bisulfate; range of 2.0 to 6.0 in 0.5 deviations) on B-vitamin retention. Treatments were arranged in a 2 x 9 factorial arrangement. The acidulant mixes were produced in a similar manner as experiment 1. A total of 258 g Sodium Bisulfate (SBS, Jones-Hamilton, Walbridge, OH) was added to distilled water and brought to a total volume of 500 mL. A vitamin premix containing all seven B-vitamins, similar to the first pH experiment was added (68 g) to the model canned cat diet.

Canning

Experiment 1: Four separate 13.6 kg batches of batter were prepared. Two of the batches contained thiamine mononitrate and two contained thiamine hydrochloride. Batter in each batch was heated to 60˚C then separated into six different 2.27 kg subsamples. The experiment consisted of 26 treatments and four replicates per treatment each. Batter pH was adjusted by dropping 4M HCl or 4M NaOH until the target pH was obtained. An OakTon pH meter (Vernon Hills, IL #289107) was used to determine the pH. Once the desired pH was reached, the volume of each reagent was recorded. Treatments were placed into 300 x 407 cans (Freund Container & Supply, Lisle, IL) and

seamed (Dixie Seamer, 91118, Athens, Georgia). Once all cans were seamed, they were placed into a retort (Dixie Retort, 100-43, Athens, Georgia). The retort was operated at 121˚C and at a pressure of 172368.93 Pa for one hour.

Following cooking in the retort and a rest period of 48 hours, samples were analyzed for moisture content (AOAC 950.46) and pH (OakTon, Vernon Hills, IL) to confirm containment of targets. Samples were analyzed for thiamine (AOAC 942.23) and data were reported on a DM basis.

Experiment 2: Three separate 13.6 kg batches were prepared. Once a single batch reached 60˚C it was separated into six different subsamples of 2.27 kg. One 13.61kg batch was sufficient for six treatments for a total of 18 treatments. Each subsample (2.27 kg) was acidified with either 4M hydrochloric acid or 4M sodium bisulfate and the volume required to obtain the desired pH was recorded. The pH was measured on an OakTon (Vernon Hills, IL) pH meter. Once all treatments met the desired pH they were placed into 300 x 407 cans and seamed and placed into the retort. The retort was operated at 121˚C and 172368.93 Pa for one hour.

Cans were allowed to rest for 48 hours following retort before analysis for moisture content (AOAC 950.46) and pH (OakTon, Vernon Hills, IL). Samples were analyzed for thiamine (AOAC 942.23), riboflavin (AOAC 940.33), niacin (AOAC 944.13), pantothenic acid (AOAC 945.74), pyridoxine (AOAC 961.15), folic acid (AOAC 992.05), and cobalamin (AOAC 952.20) and were reported on a DM basis. B-vitamin analyses were performed at Covance Laboratory, Madison, WI.

Statistics

Experimental treatments were arranged in a factorial arrangement (Ott and Longnecker, 2004) with main effects of batter moisture, batter temperature, and retort time. Means were separated by significant F tests, using a procedure for mixed models with the aid of statistical analysis software (GLIMMIX; SAS Institute Cary, NC). Due to limited replication for the treatments, only the main effect means are reported. Means were considered different at a $P < 0.05$ and trends are noted at $P < 0.10$.

Results

Experiment 1: Thiamine hydrochloride and thiamine mononitrate

Initial batter pH, moisture of the processed product, pH post-processing and volume of reagent required to reach the desired pH pre-processing were recorded for each treatment (Table 3.2). The initial pH for the batter used to produce all the treatments was 6.44 ± 0.1 and the moisture of each batch was 78.3% ± 4.9%. The pH post-processing for the samples containing thiamine hydrochloride from target 2.0 to 8.0 increased with each unit change, but differed from the initial target. Likewise the pH for samples containing thiamine mononitrate were similar to those of the thiamine hydrochloride. The volume required to obtain the desired pH did not differ between the batches of food containing thiamine hydrochloride and those containing thiamine mononitrate.

Thiamine concentration was analyzed after a resting period of at least 48 hours post-retort. Thiamine hydrochloride and thiamine mononitrate did not differ in their retention throughout the canning process ($P = 0.12$; Table 3.2). The general trend of the two types of thiamine was that as the pH increased from 2.0 to 8.0, the percent of thiamine retained decreased.

Thiamine hydrochloride retention (Figure 3.1) remained consistent for pH from 3.0 - 5.0 with an average retention of 52% ± 2%. The maximum retention (64%) occurred at a pH of 2.0. Beyond pH 5.0, the retention of thiamine continually decreased (R^2=0.94). Total thiamine retentions (and retention%) for the thiamine hydrochloride source (DM basis) were 166.86 mg/kg (64%), 131.38 mg/kg (61%), 140.37 mg/kg (53%), 164.12 mg/kg (55%), 166.07 mg/kg (54%), 150.23 mg/kg (52%), 131.73 mg/kg (53%), 141.62 mg/kg (49%), 126.63 mg/kg (48%), 122.35 mg/kg (43%), 92.56 mg/kg (39%), 70.77 mg/kg (30%), and 64.10 mg/kg (23%) for pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0, respectively.

Thiamine mononitrate retention (Figure 3.2), with the exception of a pH 4.0, was greatest from pH 2.0 to 6.0. After pH 6.0 the retention declined linearly to pH 8.0 (R^2=0.98). The maximum retention of thiamine occurred at pH 2.0 (70%). Total retention (and retention %) for the thiamine mononitrate source (DM basis) were 161.90 mg/kg (70%), 162. 49 mg.kg (62%), 173.14 mg/kg (60%), 154.31 mg/kg (56%), 124.39 mg/kg (48%), 147.21 mg/kg (58%), 150.24 mg/kg (55%), 120.28 mg/kg (52%), 136.21 mg/kg (55%), 153.70 mg/kg (48%), 129.77 mg/kg (41%), 121.69 mg/kg (35%), and 93.52 mg/kg (32%) at pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0, respectively.

Experiment 2: Acidulant effect on thiamine retention

Initial batter pH, can moisture, post-processing pH, and volume required to reach the desired pH pre-processing were recorded for each treatment (Table 3.3). Water soluble B-vitamins were analyzed after a minimum resting period of 48 hours post-retort.

The initial pH of each batter was 6.4 ± 0.2 and average moisture content for each batter was 78.00 ± 0.06% with the exception of the two batters whose pH were acidified to pH 2.0 by 4M hydrochloric acid and 4M sodium bisulfate, which were 67% and 50%, respectively.

The volume required to achieve the desired pH prior to retort was recorded for each individual acidulant which allowed for the evaluation of acidulant efficiency. The two acidulants were close in volume needed to obtain pH 6.0, 5.5, 5.0, 4.5 and 4.0. The volume required to change the pH increased more drastically with SBS than HCl; wherein 2.0 mL, 6.0 mL, 10.0 mL, 12.0 mL, 17.0 mL, 37.0 mL, 53.0 mL, 50.0 mL, and 105.0 mL of SBS met the pH 6.0, 5.5, 5.0, 4.5, 4.0, 3.5, 3.0, 2.5, and 2.0, respectively. The inconsistent volume of acidulant required for pH 3.0 and 2.5 was not expected as we do not expect any unusual isoelectric focusing. The required amounts of HCl for pH changes were 2.0 mL, 5.0 mL, 7.0 mL, 9.0 mL, 13.0 mL, 19.0 mL, 23.0 mL, 45.0 mL, and 60.0 mL for pH 6.0, 5.5, 5.0, 4.5, 4.0, 3.5, 3.0, 2.5, and 2.0, respectively.

The pH observed post-processing differed from the pH that was recorded prior to the retort. The measured pH post-processing for batches acidified with SBS were 2.55, 4.40, 4.50, 4.98, 5.40, 5.53, 5.68, 6.07 and 6.31 for target pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0, respectively. As the target pH increased, the difference between the pH before and after processing decreased. This trend was also observed for the batches acidified with HCl. The measured pH after retort for the batches acidified with HCl were 4.04, 4.16, 4.69, 4.88, 5.29, 5.5, 5.74, 6.02, and 6.19 for target pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0, respectively.

Thiamine retention was greater ($P < 0.05$) for thiamine mononitrate (145.30 mg/kg) when SBS was the acidulant (P=0.037) (Table 3.4). The average riboflavin, niacin, pantothenic acid, pyridoxine, and cobalamin retention all followed a similar pattern to thiamine (Table 3.4). Riboflavin retention was not affected by acidulant (P=0.101). Niacin retention was greater (P<0.05); 288.46 mg/kg and 248.22 mg/kg when HCl was the acidifier than when SBS was the acidifier (P=0.014). Pantothenic acid retention was greater (P=0.007) when acidified with HCl and SBS (237.09 mg/kg vs. 196.38 mg/kg, respectively). Pyridoxine retention was greater (P=0.005) at 118.90 mg/kg when HCl was the acidifier versus 103.35 mg/kg when SBS was the acidifier used. Folic acid retention was greater (P=0.29) with SBS (average retention of 11.42 mg/kg) relative to diets acidified with HCl (average of 10.42 mg/kg). Cobalamin retention was not different due to acidulant (P>0.05); average 0.36 mg/kg).

Discussion

In both experiments, an adjustment to pH changed the thiamine retention. Specifically, the retention of thiamine was greatest in more acidic conditions, particularly from 2.0 to 4.0 pH. Previous work has been reported that the thermal stability of thiamine greatly increased when pH was reduced (Farrer, 1955, 1945; Mauri, 1984; Mauri et al., 1986). The most rapid decline in retention occurred greater than pH 6.0. Work done by Dwivedi and Arnold (1972, 1973) described breakdown products from thiamine at pH values greater than 6.0. This research concluded that research on complex food systems was needed to better evaluate potential flavor and toxicological significance of thiamine breakdown. Research completed by Briozzo et al. (1987) described vegetable puree (pea and corn) and meat puree (pork and beef liver) in which thiamine retention improved in

canned low-acid foods with a mild reduction of pH (about 1.0 pH unit) compared to baseline (pH = 5.0). The purees were adjusted between a pH of 5.0 to 6.9. This large expected improvement was not observed, potentially because flavor, color, or water binding characteristics may have been adversely affected according to the authors. In their work, thiamine retention in the vegetable products plateaued at pH values below 5.2. The meat purees had increased thiamine retention at lower pH values. In a similar manner, canned cat diets contain multiple ingredients that may buffer pH during their cook time. The results of previous research and our current results suggest that there is a benefit in thiamine retention with a decreased pH.

Experiment 1: Thiamine mononitrate retained a greater percentage of thiamine than thiamine hydrochloride. This was expected as thiamine hydrochloride is more soluble in water compared to thiamine mononitrate (100 g soluble in 100 mL and 30 g soluble in 100 mL, respectively; EFSA, 2011).

Experiment 2: The average retention of thiamine was greater when HCl was used as an acidulant rather than SBS. The average retention of riboflavin, niacin, pantothenic acid, pyridoxine, and cobalamin all followed a similar retention pattern. Folic acid was the only B-vitamin that did not follow this pattern and had a greater average retention when SBS was used. The findings of this study suggest that the chemical structure of the two acidifying agents is what ultimately led to these results. More research is necessary to address this.

Conclusion

Overall, a diet with lower pH retained more thiamine than cat foods with a pH greater than 6.0. However, there are practical implications to this finding because diets

with a pH drastically below 6.0 could hinder the palatability and processing capabilities of a canned cat diet. The type of acidulant (SBS vs. HCl) used to adjust pH of the batches of food affected the retention of some B-vitamins. More research to substantiate the results from these findings is required to confirm the changes in overall B-vitamin retention as pH was manipulated.

Tables and Figures

Table 3.1 Model canned cat food and predicted nutrient composition for diets formulated with Thiamine Hydrochloride and Thiamine Mononitrate

Ingredient	Diet, %	Nutrient	Unit	As-Is
Water	52.45	Crude Protein	%	7.84
Chicken	40.60	Crude Fat	%	9.06
Rice	4.40	Crude Fiber	%	0.85
Soybean Oil	0.50	Thiamine Hydrochloride	mg/kg	58.80
Egg	0.50	Thiamine Mononitrate	mg/kg	58.20
Vitamin Premix	0.50			
Guar Gum	0.50			
Carrageenan	0.50			

Table 3.2 Main effect means of thiamine retention due to type of thiamine (mg/kg, DM)

	Thiamine Type			
	Mononitrate	Hydrochloride		
N=	13	13	SEM	*P*=
Thiamine	140.68	128.37	5.27	0.12

Table 3.3 Moisture, pH and volume of acid used for Thiamine Hydrochloride and Thiamine Mononitrate

Target pH	Thiamine Type	Recorded Moisture, %	Initial Batter pH	Volume required, mL and eluent used	pH post processing
2.0	Hydrochloride	0.77	6.42	44.0 HCl	3.51
2.5	Hydrochloride	0.73	6.48	35.0 HCl	3.77
3.0	Hydrochloride	0.78	6.42	30.0 HCl	4.10
3.5	Hydrochloride	0.80	6.46	22.0 HCl	4.52
4.0	Hydrochloride	0.81	6.45	16.0 HCl	4.94
4.5	Hydrochloride	0.79	6.41	12.0 HCl	5.29
5.0	Hydrochloride	0.77	6.48	8.0 HCl	5.63
5.5	Hydrochloride	0.80	6.47	6.0 HCl	5.85
6.0	Hydrochloride	0.78	6.45	3.0 HCl	6.19
6.5	Hydrochloride	0.79	6.54	0	6.53
7.0	Hydrochloride	0.75	6.52	2.0 NaOH	6.67
7.5	Hydrochloride	0.75	6.51	5.0 NaOH	7.01
8.0	Hydrochloride	0.79	6.51	7.0 NaOH	7.17
2.0	Mononitrate	0.75	6.43	42.0 HCl	3.18
2.5	Mononitrate	0.78	6.42	35.0 HCl	3.63
3.0	Mononitrate	0.80	6.39	30.0 HCl	4.04
3.5	Mononitrate	0.79	6.37	17.0 HCl	4.84
4.0	Mononitrate	0.78	6.40	17.0 HCl	4.93
4.5	Mononitrate	0.77	6.39	11.0 HCl	5.45
5.0	Mononitrate	0.79	6.46	8.0 HCl	5.63
5.5	Mononitrate	0.75	6.24	6.0 HCl	5.83
6.0	Mononitrate	0.77	6.43	3.5 HCl	6.14
6.5	Mononitrate	0.82	6.40	0	6.50
7.0	Mononitrate	0.82	6.42	2.0 NaOH	6.74
7.5	Mononitrate	0.83	6.45	3.5 NaOH	6.96
8.0	Mononitrate	0.80	6.43	4.0 NaOH	6.99

Table 3.4 Main effect means for B-vitamin retention as a result of using Hydrochloric acid (HCl) or Sodium Bisulfate (SBS) to acidify a canned cat food.

	HCl	SBS		
N=	9	9	SEM	*P*=
Thiamine	158.81[a]	145.30[b]	5.4031	0.0369
Riboflavin	127.36	114.32	7.0282	0.1008
Niacin	288.46[a]	248.22[b]	12.90	0.0142
Pantothenic Acid	237.09[a]	196.38[b]	11.32	0.0070
Pyridoxine	118.90[a]	103.35[b]	4.0048	0.0047
Folic Acid	10.42	11.42	0.8885	0.2900
Cobalamin	0.38	0.34	0.0963	0.1019

[abc] Means within a row and main effect with unlike superscripts differ (P<0.05)

Figure 3.1 The retention (%) of Thiamine when thiamine hydrochloride was the source and pH was modified in the range of 2.0-8.0

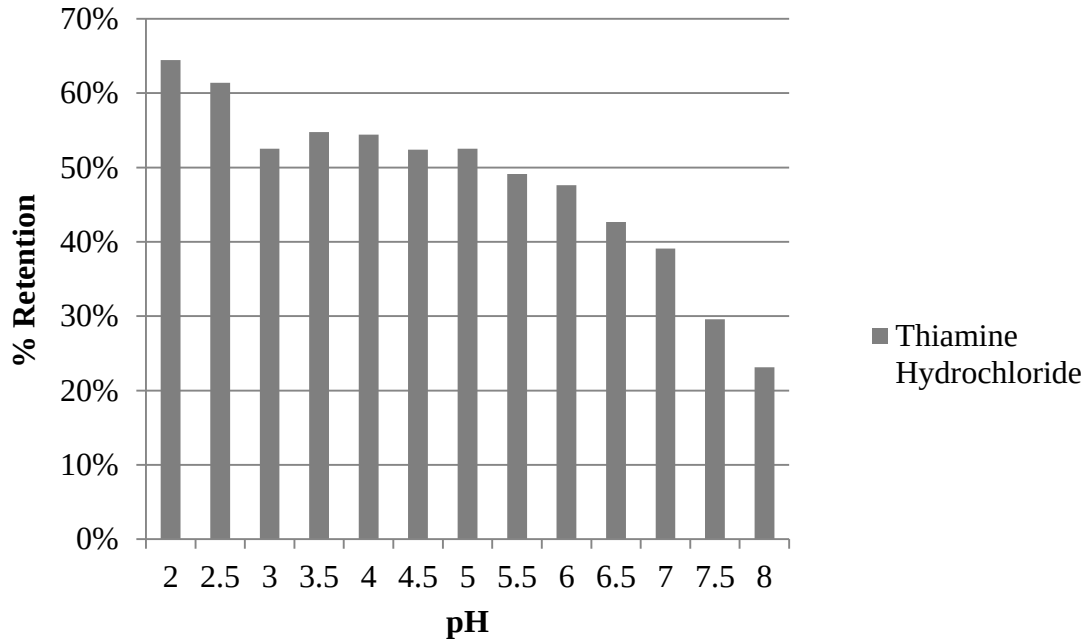
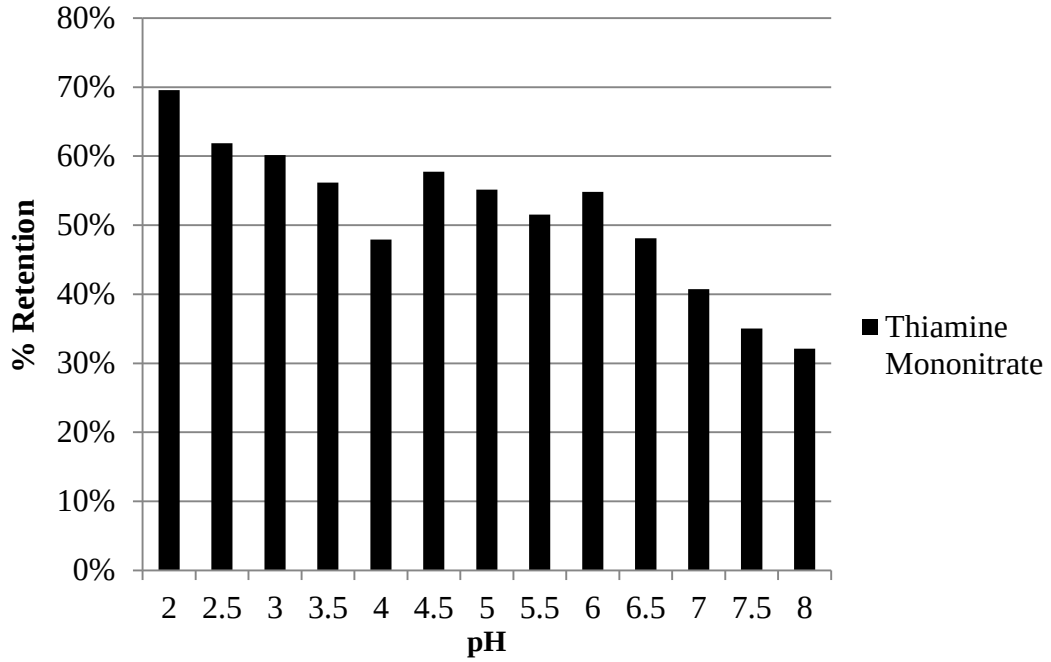


Figure 3.2 The retention (%) of thiamine when thiamine mononitrate was the source in a pH range from 2.0-8.0.



Chapter 4 - Effect of Protein Source and Indirect Sulfite inclusion on B-Vitamin Retention

Introduction

Thiamine is susceptible to destruction in pet foods; especially in canned foods. Many things degrade thiamine including heat, high pH, and moisture. But, they don't account for all of the observed losses; thiaminase enzymes and sulfites could be a significant contributor as well (Wedekind et al., 2010). Sulfite inclusion in meats has presumably been discontinued (21CFR182) and it is possible that enzymes are denatured in food processing.

In a previous report, Singh, et al. (2005) studied a group of dogs all fed sulfite-preserved meat. They reported that the dogs presented with clinical signs of thiamine deficiency (i.e. vomiting, ataxia, and hyperextension). While some dogs recovered with a 50 mg vitamin B1 shot, others deteriorated neurologically and were euthanized. Steel (1997) reported a case study on one 11 year old cat. This cat was fed a raw vacuum-packed kangaroo diet. About a month after the cat began the diet, the cat presented with ventroflexion of the head. Shortly after this symptom was observed, the cat died. Upon necropsy, it was determined that the cat had suffered from thiamine deficiency. The remaining food was analyzed and sulfur dioxide concentrations of at least 275 mg/kg were reported.

Thiaminases are enzymes that render thiamine inactive and are common in fish (Markovich et al., 2013). Types of fish differ in the amounts of thiaminase activity, as do

parts within a single fish (Wedekind, et al., 2010). Viscera of fish contain more thiaminase than the muscles or skeleton (Houston and Hulland, 1988). While processing fish presents little risk of thiaminase activity because the enzyme is heat labile, the amount of time the enzyme is in contact with foods containing thiamine prior to processing plays a role in the amount of thiamine that is destroyed (NRC, 2006). One aim of our research was to determine if meats not previously described as providing thiaminase enzyme activity might, by indirect evaluation, have an impact on B-vitamin retention in a canned cat diet.

Therefore, the objectives of this study were to determine the effect of ingredients that might contain sulfites or functional enzymes from protein source on the retention of B-vitamins.

Materials and Methods

Model Canned Cat Diet

A model canned cat diet was formulated to emulate a very basic cat food. This helped ensure that the diets were consistent throughout all trials. The different meats were included in the same quantity for each respective treatment. Diets were arranged in a 2 x 3 factorial design with two starch sources: low sulfite = rice and high sulfite = dehydrated potatoes and three meat sources: mechanically-separated chicken, fish (salmon, tuna, and whitefish), and liver (beef, chicken, and pork; Table 4.1). The dietary treatments were formulated to a target moisture level of 78% to ensure a consistent nutrient composition throughout the experiments.

Ingredients for the diets were acquired from various suppliers in the geographic region. All meat protein sources were purchased as frozen blocks from a regional pet food company (CJ Foods Bern, KS). The brown rice was purchased at a local grocery store (Great Value, Manhattan, KS) and dehydrated potatoes were purchased from ACJ International, LLC (Fargo, ND). Spray-dried egg was supplied by IsoNova (Springfield, MO), and the gums carrageenan (Carrageenan T-40) and guar gums (Guar 80) were provided by DuPont Animal Health (St .Louis, MO). The vitamin and mineral premixes were purchased from Lortscher Animal Nutrition, Inc. (Bern, KS).

Prior to processing, B-vitamin contents of the vitamin premix were determined to be: 840.87 mg/kg thiamine, 296.78 mg/kg riboflavin, 280.52 mg/kg niacin, 826.74 mg/kg pantothenic acid, 248.73 mg/kg pyridoxine, 38.44 mg/kg folic acid, and 1.07 mg/kg cobalamin, respectively on a DM basis (Chapter 3).

The Canning Process

Prior to treatment processing all frozen meat sources were placed in a refrigerator (4˚C) for a minimum of 24 hours to temper. Once softened to a pliable consistency the meats were weighed according to protocol and placed into a stockpot. The stockpot with meat proteins was placed on a stove top along with half of the required water (2.38kg for a 9.07kg batch) and the remaining dry ingredients (excluding the gelling agents) and brought up to temperature (40˚C). After which gelling agents were added slowly to the batch. These meat batches were split into two separate 4.54 kg batches then the starches, brown rice or potatoes, were added. This mixture was stirred and heated to 40˚C before adding the remaining half of the water. As temperatures increased to 60˚C the batter thickened. At which point it was removed and sub-samples were ladled into 300 x407

cans (~0.45 kg) lids were placed on the filled cans and seamed (Dixie Seamer, 91118, Athens, Georgia) then placed into the retort (Dixie Retort, 100-43, Athens, Georgia) until all samples were completed. Once all treatments were produced they cooked for 1 hour at 121˚C and 172368.93 pascals. Cans were removed from the retort and allowed to cool for a minimum of 48 hours before being handled for analyses.

Experimental dietary treatments were analyzed for moisture content (AOAC 950.46) and pH (OakTon pH meter, serial number 289107, Vernon Hills, IL). Once validated, samples were sent to an external lab for B-vitamin analysis. Data were reported on a DM basis.

Statistical Analysis

The dietary treatments were evaluated as a completely randomized design. Means were separated by Fishers LSD, with contrast for chicken as the control (CH) vs liver (L) vs fish (FI). Data were analyzed with the aid of statistical analysis software (SAS Institute, Cary, NC) using the GLM procedures. Differences were considered significant with a P value ≤ 0.05.

Results

Experiment 1: Change in Protein Source

Thiamine concentration (Table 4.2) in diets containing fish was greater than liver with chicken being intermediate between them ($P < 0.05$; 202.26 vs 131.19 and 140.72 mg/kg, respectively). Riboflavin content was greater in liver (200.83 mg/kg; $P < 0.05$) than in fish and chicken (153.46 mg/kg and 113.66 mg/kg, respectively). Niacin content was greater in liver (444.64 mg/kg; $P < 0.05$) than fish and chicken (327.90 and 238.42mg/kg, respectively). Pyridoxine content was greater for fish than chicken, with liver being intermediate (135.89 vs. 100.59 and 113.34 mg/kg, respectively; $P < 0.05$). No differences were noted among treatments for folic or pantothenic acid ($P > 0.05$). Cobalamin was greater in the liver containing dietary treatments than chicken, and fish was intermediate between them (1.53 vs. 0.45 and 0.62 mg/kg, respectively; $P < 0.05$). No differences in pantothenic acid were observed among treatments (average 284.00 mg/kg).

Experiment 2: Indirect Sulfite Addition

There was a trend ($0.05 < P < 0.10$) for thiamine and riboflavin reduction due to the inclusion of sulfite containing potatoes in the diet. Niacin, folic acid, and cobalamin retention were not affected by the presence of sulfite containing potatoes ($P > 0.10$). Pyridoxine and pantothenic acid retention were lower ($P < 0.05$) due to the addition of sulfite - containing potatoes.

Discussion

Thiaminase is an enzyme that renders thiamine unavailable to the animal. It is present in organ meats and fish (McCleary and Chick, 1976), which may impact the quantity of thiamine available to the animal. The greater thiamine retention in fish found in this experiment, which may imply there was more enzyme activity in the liver from other meat sources (beef, chicken, and pork). Alternatively, the thiaminase-rich viscera of the fish was not amply present in the fish used. Thiaminase is rendered inactive when cooked. It is possible that this enzyme acted on thiamine prior to cooking.

Thiaminase enzyme activity is a complicated assay that requires a radiometric technique (Zajicek et al., 2005). It was our intent to determine if various meats, not previously described as providing thiaminase enzyme activity might, by indirect evaluation, have an impact on thiamine and other B-vitamin retention in pet foods. The expectation was there might be a change in thiamine retention due to thiaminase activity in different meat sources. Because liver and fish meats are both commonly used in wet pet food, it was important to evaluate the impact of different species meats.

The diets containing fish all had lower retention of B-vitamins by at least 40% aside from niacin. Cobalamin and niacin were both retained at greater levels in the diets containing liver by at least 40% compared to diets containing chicken Folic acid and riboflavin had lower retentions by 26.04% and 32.33%, respectfully in diets containing liver compared to their starting amounts. The remaining B-vitamin retention was 50% or less in the diets containing liver. Thiamine was the vitamin most substantially lost (some 80%), and niacin was the vitamin least affected by varying the protein source. All B-vitamins, aside from niacin, had a decreased retention of at least 50% in the chicken diets.

From this it becomes apparent that diets prepared with chicken had the greatest decline in vitamin retention. This was not expected. It was thought that diets containing liver had the potential for elevated [thiaminase] enzyme activity and might be detrimental to the retention of multiple B-vitamins. Whereas, diets containing fish had the greatest thiamine retention. This observation was not expected because fish had the potential to contain appreciable thiaminase activity.

The results for B-vitamins lost due to the addition of sulfite containing potatoes are found in Figure 4.2. Generally, all B-vitamins were lost at a greater percentage in diets with added sulfite containing potatoes than for the rice diet. Thiamine and pantothenic acid were the two vitamins with the greatest percentage lost in diets with sulfite potatoes relative to those without. Thiamine had an average loss of 83.61% in the diets containing sulfites and an average of 77.62% loss in diets with no added sulfites ($0.05 < P < 0.10$). Pantothenic acid lost 71.60% of its original content in diets containing sulfites and 55.42% in those diets with no added sulfites ($P < 0.05$). Riboflavin, pyridoxine, and folic acid were not as affected. Pyridoxine, riboflavin and folic acid losses for the diets including sulfites were 56.57%, 50.17%, and 45.71%, respectfully (45.99%, 36.57%, and 36.65% for the diets not containing sulfites. Cobalmin's losses were small when compared to the other B-vitamins. In diets with potatoes laden with sulfites cobalamin levels decreased by 13.41% and in diets with no sulfite addition, cobalamin declined by only 3.17%.

Sulfite use for preserving meats is no longer acceptable in the pet food industry (21CFR182). However, it is still used to help preserve dried vegetables. The addition of a sulfite-preserved vegetable (dehydrated potato) source was examined compared to a

control batter with no addition of this sulfite source. In the presence of sulfites, thiamine is cleaved into its inactive, or biologically unavailable form, pyrimidine and thiazole (Studdert, 1991). Sulfites can destroy thiamine in the diet, in a vitamin premix, or basal ingredients when sulfite-preserved ingredients are added to the diet. Little research has been published regarding the effects of sulfites on B-vitamin survival. Our work evaluating the effects of sulfite addition to the diet was intended to explore thiamine and the other B-vitamins losses and any potential detrimental effects.

Other research has shown that the addition of sulfites, as a preservative in meats, can have a detrimental effect on the health of cats (Singh, et al. 2005, Steel, 1997, Studdert, 1991). Foods not recognized as a source of thiamine may be preserved with sulfites as long as they are not labeled as raw or fresh (21CFR182).

Thiaminase enzyme in fish tissues was reported to degrade thiamine in canned foods (Fitzsimmons, et al. 2005, Tillitt, et al. 2005, Zajicek, et al. 2005, and Zajicek et al. 2009). Fish is an important protein source for many canned cat food diets. However, no work has been published in which a canned cat diet was evaluated for B-vitamin changes due to protein sources which potentially contain thiaminase enzyme activity and (or) due to the addition of sulfites. In this experiment, thiamine retention declined in diets containing sulfites as expected. Whereas, it was not expected that diets containing fish would retain a greater amount of thiamine than those containing meats or liver. This effect may have been linked to an inactivation of thiaminase due to heat or pH or some other protective effect and should be explored in more detail.

Conclusion

Use of liver, which potentially contains degradative enzymes, could have rendered the thiamine inactive. Interestingly, the diets with the most thiamine retained were the diets which contained fish. This was not expected based previously published work. The other B-vitamin retention in the diets containing the sulfite laden potatoes was less than the retention of the B-vitamins in diets containing rice. The findings of this experiment further emphasize the need for more research to the effects of processing on vitamin retention in a more complex canned cat food.

Tables and Figures

Table 4.1 Ingredient composition (%) of experimental canned cat foods.

* Fish (Salmon, Tuna, Whitefish), Liver (Beef, Chicken, Pork)

	Rice			Potato		
Ingredients	Chicken	Fish*	Liver*	Chicken	Fish*	Liver*
Water	52.45	52.45	52.45	52.45	52.45	52.45
Chicken	40.60	-	-	40.60	-	-
Fish	-	40.60	-	-	40.60	-
Liver	-	-	40.60	-	-	40.60
Rice, Brown	4.40	4.40	4.40	-	-	-
Dehydrated Potato	-	-	-	4.40	4.40	4.40
Eggs	0.50	0.50	0.50	0.50	0.50	0.50
Soybean Oil	0.50	0.50	0.50	0.50	0.50	0.50
Guar Gum	0.50	0.50	0.50	0.50	0.50	0.50
Carrageenan	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix	0.50	0.50	0.50	0.50	0.50	0.50
Trace mineral Premix	0.05	0.05	0.05	0.05	0.05	0.05

Table 4.2 Main effect means for the vitamin content (mg/kg DM) affected by protein source

	Chicken*	Fish*	Liver*		
N=	2	6	6	MSE	*P*=
Thiamine	140.72[ab]	202.26[a]	131.19[b]	1906.21	0.0422
Riboflavin	113.66[b]	153.46[b]	200.83[a]	1144.77	0.0177
Niacin	238.42[b]	327.90[b]	444.64[a]	3171.54	0.0015
Pyridoxine	100.59[b]	135.89[ab]	113.34[a]	359.43	0.0667
Folic Acid	14.13	19.62	28.43	89.58	0.1531
Cobalamin	0.45[ab]	0.62[b]	1.53[a]	0.39	0.0460
Pantothenic Acid	236.60	336.71	278.70	12,946.14	0.5063

[abc] Means within a row and main effect with unlike superscripts differ (P<0.05)

*Chicken (Control), Fish (Salmon, Tuna, Whitefish), Liver (Beef, Chicken, Pork)

Table 4.3 Main effect means for the vitamin content (mg/kg DM) affected by sulfites

	Control	Potato		
N=	7	7	MSE	*P*=
Thiamine	188.22	137.80	2365.08	0.0700
Riboflavin	188.25	147.89	1710.79	0.0900
Niacin	380.75	349.56	9153.53	0.5500
Pyridoxine	134.34[a]	108.02[b]	336.96	0.0200
Folic Acid	24.35	20.87	111.98	0.5500
Cobalamin	1.04	0.93	0.62	0.8000
Pantothenic Acid	360.29[a]	234.80[b]	8837.53	0.0300

[abc] Means within a row and main effect with unlike superscripts differ (P<0.05)

Figure 4.1 The B-vitamins lost due to protein source as a proportion (%) to starting levels.

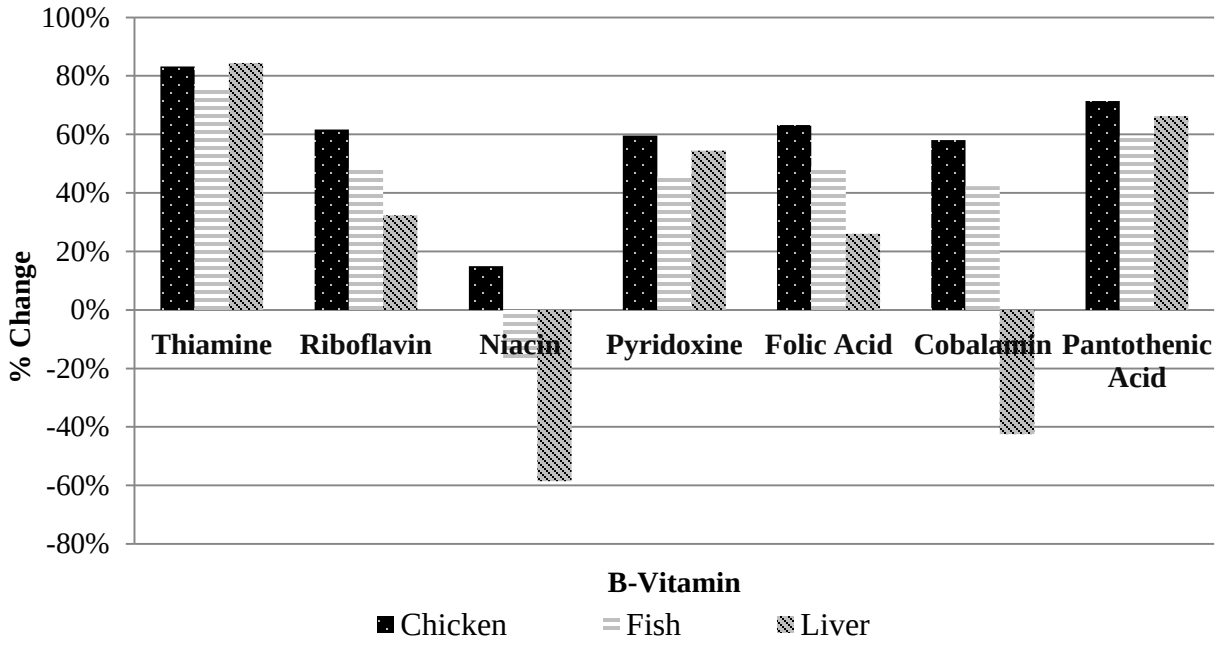
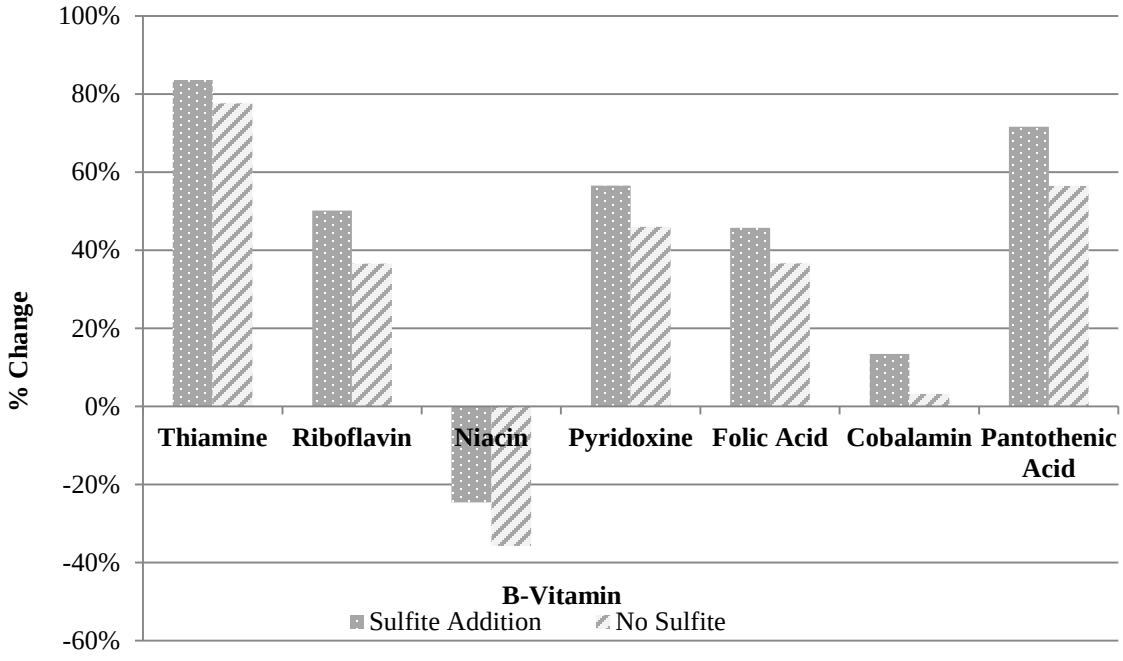


Figure 4.2 The levels of B-vitamin lost due to addition of sulfite laden dried potatoes as a proportion of starting levels.



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Appendix A - Applying HPLC methodology to Thiamine determination

Introduction

High performance liquid chromatography (HPLC) is an effective way for separating and quantifying compounds that are soluble in a liquid. It offers speed and versatility over low pressure column liquid chromatography (Reuhs and Rounds, 2010). These advantages led to the selection of an HPLC method for thiamine analysis. *Determination of thiamine and riboflavin in pet foods and animal feedingstuffs* was published in 2000 by the Analytical Methods Committee. The methods outlined in this paper were used to establish a method in our lab for determining thiamine content of canned cat food samples. The method was ideally suited because it was explicitly based on pet food analysis in a canned food application. The development of a method for a thiamine analysis in our own laboratory would allow for more timely and direct determination of thiamine retention in canned foods.

Current official methodology for thiamine (vitamin B1) determination was established as an official method by the *Association of Official Analytical Chemists* (AOAC, 942.23) for fluorometric determination. Wavelength sensitivity was established at 365 nm – 435 nm on a fluorescence detector. High Performance Liquid Chromatography (HPLC) provided more efficiency in sample preparation and thiamine determination. It was the objective of this project to analyze thiamine by HPLC in a canned cat diet from 0 to 100 mg/kg level and compare it with the fluorometric determined thiamine content.

Method Selection

An HPLC method for thiamine and riboflavin in pet foods published in Analyst, (2000) was chosen because it had been validated by multiple labs to quantify accuracy and precision in a pet food application. Three separate, collaborative experiments were conducted to determine the most accurate and repeatable results.

Procedure

Thiamine determination follows two main steps: thiamine extraction and tagging with a chromogen for detection thiochrome derivatization. Thiochrome is an oxidative product of thiamine that fluoresces. The sample extraction required proper pH, enzymatic degradation of the food matrix, and proper glassware. Thiamine can be affected by UV light so care was taken to keep samples from direct light was vital.

Reagents:

- 0.1 M Hydrochloric Acid: Measure 1,000 mL of distilled water into graduated cylinder. Place 300mL into a 1,000 mL bottle. Pipette 8.212 mL Hydrochloric Acid (Sigma-Aldrich-320331-2.5L) into glass bottle. Add remaining distilled water from graduated cylinder to glass bottle.
- 2.5M Sodium acetate: Weigh 51.3 ± 0.2 g anhydrous sodium acetate (Fisher Scientifi-S210-500) into a 250 mL volumetric flask. Dissolve and dilute to volume with deionized water.
- Clara –Diastase (Sigma-Aldrich-86959-10G , ≥ 35 U/mg)

- 50%w/v trichloroacetic acid in de-ionized water: Weigh 125 ±0 .2 g trichloroacetic acid (Fisher Scientific- A324-500) into a 250mL volumetric flask. Dissolve and dilute to volume with de-ionized water.
- Alkaline ferricyanide solution: Prepare 15% sodium hydroxide solution by weighing 15 g sodium hydroxide (Fisher Scientific-S318-500) and dissolving it into 100 mL of distilled water. Dilute 1 ± 0.05 g potassium ferricyanide (Avantor -6932-04) to 100 mL with 15% sodium hydroxide solution prepared immediately before use
- Thiamine hydrochloride standard solution: Accurately weigh 0.025 g thiamine hydrochloride (Sigma-Aldrich T4625-100G, ≥ 99%) to at least four decimal places and pour into a 250 mL amber volumetric flask and dilute to volume with 0.1M HCl. Further dilute the solution 5 mL to 100 mL with 0.1M HCl (Solution A). This solution is approximately 5 µg/mL. Pipette 5 mL of solution A into a 50 mL amber volumetric flask and dilute to volume with 0.1M HCl to give a working standard of 0.5 µg/mL thiamine hydrochloride.
- Water saturated isobutanol: Add deionized water to isobutanol (Alfa Aesar-22908-1L, HPLC Grade, > 99%). Shake vigorously and allow to stand until two distinct layers appear. Remove bottom layer into waste.
- Chloroform: HPLC Grade (Fisher Scientific-43685)
- Methanol: HPLC Grade (Fisher Scientific-22909)

Sample Preparation

1. Weigh 15 g of wet pet food into a 150 mL amber glass bottle

2. Add 60 mL of 0.1M HCl to amber glass bottle and heat in a boiling water bath for 1 hour swirling occasionally
3. Allow to cool and adjust pH to 4.3 to 4.7 by adding 2.5M sodium acetate
4. Add 0.2 g clara-diastase, swirl the solution and incubate at 37˚C (water bath used) for not less than 16 h and no more than 17 h
5. Add 2 mL 50% trichloroacetic acid and heat on a steam bath for 10 minutes (water bath at 60˚C used)
6. Cool to room temperature and transfer quantitatively to a 100 mL volumetric flask and dilute to volume with de-ionized water
7. Filter through a GFA filter paper into a 150 mL polythene bottle (brown used to reduce light exposure) Solution can be frozen at this stage

Thiochrome

Thiochrome will need to be derivitized after the sample preparation. The steps are as follow:

1. If sample is frozen, allow to thaw for approximately 1 hour at room temperature
2. Pipette 5.0 mL of the sample solution and the thiamine hydrochloride working standard into separate 25 mL amber stoppered test tubes
3. Add 5.0 mL water-saturated isobutanol to each tube and mix for 5 seconds
4. Add 3.0 mL 1% potassium ferricyanide in 15% sodium hydroxide and immediately shake vigorously for 15 seconds to extract the thiochrome into the isobutanol

5. Allow the phases to separate and transfer the upper isobutanol layer to an amber HPLC vial.
6. Inject solution into HPLC for thiamine assay

HPLC Specifications

The column was a C18 (Waters Spherisorb® 5µm NH2 4.6x250mm, Milford, MA), with a mobile phase which consisted of 80% chloroform to 20% methanol. This mobile phase was modified from the base method (*Analyst;* 90% chloroform, 10% methanol). However, this seemed to yield the best results with the equipment and column for thiamine concentration. The flow rate remained the same (2.0 mL/minute) and the injection volume and excitation wavelength (20 µl and 365 nm, respectfully) remained the same.

The HPLC apparatus was a Hewlett Packard (HP) 1100 series (Agilent Technologies, Santa Clara, CA). Software for the HPLC was HP ChemStation software (Agilent Technologies, Santa Clara, CA).

Tables and Figures

Figure A.1 Report readout from a thiamine standard containing 0.5 ug/ml run on the HPLC

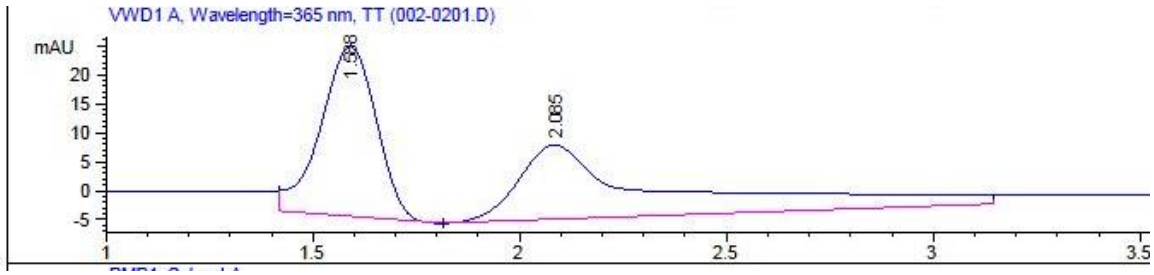
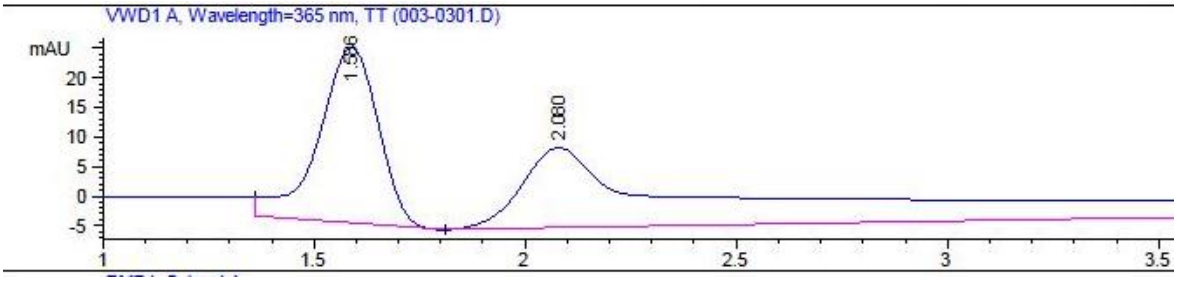


Figure A.2 Report readout from a thiamine standard containing 1.0 ug/ml run on the HPLC



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Appendix B - Initial trials for pH effect on B-vitamins

Introduction

Previous work described the effect of pH on thiamine stability in food products (Farrer 1945, Feliciotti and Esselen 1957, and Mulley et al 1975). The researchers found thiamine is less likely to be destroyed at such a rapid rate at more a more acidic pH. Research performed by Briozzo et al. (1987), examined the effect of pH (range of 5.0 to 6.9) on thiamine retention in pea, corn, pork, and beef liver puree and found that even a mild reduction in pH could increase the retention of thiamine. The thiamine reduction was different for meats and vegetables; wherein, pork retained the most thiamine and corn lost the most (50% and 20% retention at pH 6.8, respectively). However, there has been no work published on the effect pH has on B-vitamins retention in a complex canned cat diet. Therefore, it was important to examine the pH effect on B-vitamin retention in a complex food matrix. For these experiments, a pH range of 2.0-8.0 in 0.5 pH increments was conducted.

Because this is novel research, preliminary trials were necessary to ensure cans capable of shipment were produced. From these initial tests, it was clear that as the amount of acid in a can increases, the integrity of the can and seal were compromised to a point the lids no longer contained the contents. It was necessary to repeat the experiment by altering pH in a stepwise fashion to ensure a complete batch could be produced. Initial results from separate pH results are reported below. No statistics were conducted on these experiments.

Materials and Methods

In the first experiment, the strength of the different reagents were both 1M. A 500 mL 1M solution of HCl requires 41.059 mL of HCl (Sigma-Aldrich) into 125 mL of distilled water. To this, the bottle was filled to 500 mL. A 500 mL 1M solution of NaOH required 26.403 g of NaOH into 125 mL of distilled water to achieve uniformity and the aforementioned molarity. The bottle was filled to 500 mL with distilled water. Because this creates an exothermic reaction, care was taken when adding the sodium hydroxide to the distilled water. The results from this trial were unreliable because the volume of acidulant and alkaline solution required to obtain the desired pH (read on an OakTon pH meter) was not recorded and a 100 mL graduated cylinder was used to measure the amount of acidulant or buffer used. In experiments 4 and 5, a biuret was used to reduce error. The strength of the acidifier and buffer were both intensified to 4M. This was done by placing 394.16 mL of HCl into 500 mL of distilled water and then adding the remaining 105.84 mL of distilled water into a 1000 mL bottle. The 4M SBS solution was made by adding 105.61 g of NaOH to 500 mL of distilled water and mixing the solution on a stir plate. The beginning trials used a batch system for modifying the pH. One 13.6 kg batch was set aside to be adjusted with HCl and one 13.6 kg batch was used for a NaOH adjustment. Once one set of five cans was removed from the batch, more acidifier or base was added to the batch to further adjust to the next pH requirement. This method led to the possibility of major differences of pH within a set of cans because the pH was taken from the middle of the stock pot containing the 13.6 kg batch. Once the batch was at 60˚C, enough of the batch for 4 cans was removed and placed in a separate bowl. This sample of the diet was adjusted with either HCl or NaOH to reach the desired pH.

Because there was no analysis other than pH and moisture on some of the preliminary trials, no ranges for thiamine retention were possible.

Moisture

Moisture analysis was conducted based on the AOAC 950.46 official method. This requires a sample of wet pet food obtained by mixing the contents within the can and removing a 2.0 g composite sample to obtain moisture content. The sample is placed on a moisture dish and placed in a forced air oven at 101˚C-103˚C. This sample is left in the oven for 16-18 hours then weighed.

pH

The pH was recorded on an OakTon pH meter (Model: 289107, Vernon Hills, IL). Before each experiment was performed, the pH meter was calibrated. The batch of food to be measured was mixed thoroughly (~1 minute) and then the meter was placed in the middle of the bowl containing the cat food.

Results

The first trial was done to determine what volume of acidulant or buffer would be required to reach each desired pH (range of 2.0 to 8.0 at 0.5 increments). In this trial, 1M of HCl was used to obtain pH 2.0 to 6.0 and 1M NaOH was used to attain pH 6.5 to 8.0. Two separate 9.07 kg batches of cat food were prepared. After a measured pH 6.4 in batch one, the volumes of HCl required to reach pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0 were 270, 200, 211, 190, 147, 130, 130, 90, and 59 mL, respectively. After a starting pH of 6.2 was measured in the second batch, the volume of NaOH to reach pH 6.5, 7.0, 7.5, and 8.0 was 65, 20, 60, and 60 mL, respectively.

The second trial aimed to determine how much of each reagent would be needed to manipulate the pH as treatments were serially removed. The diet from this experiment was not used for sub-samples so no data other than quantity required to change the pH was recorded. The previous parameters for the first trial remained the same wherein; HCl and NaOH solutions were both prepared at strength 1M. Three 9.07 kg batches were prepared for this trial and six aliquots of each treatment were taken as the corresponding pH was reached. The initial pH of the first batch was 6.54, so there was no manipulation to reach a pH of 6.5. The volume needed to reach pH 4.5, 5.0, 5.5, and 6.0 was 42, 20, 60, and 50 mL, respectively. The second batch of 9.07 kg contained treatments whose pH was 4.0, 3.5, 3.0, 2.5, and 2.0 (required 120, 130, 165, 230, and 620 mL of HCl, respectively). The final batch contained those treatments allocated to pH 7.0, 7.5, and 8.0 (20, 50, and 40 mL of NaOH required, respectively). The third trial did not produce enough cans for proper analysis. The fourth trial of this experiment was manipulated with HCl and NaOH that were both made up to 4M. The volume required to change the pH of those diets containing thiamine hydrochloride of HCl was 44, 35, 30, 22, 16, 12, 8, 6, and 3 mL, (pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0, respectively). The sodium hydroxide was used to manipulate to pH from 6.5 to 7.0, 7.5, and 8.0 (0, 2, 5, and 7, respectively). The volume required to change the pH of the diets containing thiamine mononitrate of HCl was 42, 35, 30, 17, 17, 11, 8, 6, and 3.5 mL (pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0, respectively). The sodium hydroxide was used to manipulate to pH from 6.5 to 7.0, 7.5, and 8.0 (0, 2, 3.5, and 4, respectively). The general trend of all of the trials that had pH and moisture recorded were that pH would increase after the cook process. The difference between pH pre-cook and post-cook decreased as the target pH

increased. Moisture appeared to decrease at low target pH (2.0) to lower than 50%. This may have occurred because of how the acidulant evaporates during oven time.

Tables and Figures

Table B.1 Target pH, eluent used, batter pH, and volume required to change the pH of a wet cat food

Target pH	Eluent Used	Initial Batter pH	Volume required, mL
2.0	HCl	6.4	270.0
2.5	HCl	6.4	200.0
3.0	HCl	6.4	211.0
3.5	HCl	6.4	190.0
4.0	HCl	6.4	147.0
4.5	HCl	6.4	130.0
5.0	HCl	6.4	130.0
5.5	HCl	6.4	90.0
6.0	HCl	6.4	59.0
6.5	NaOH	6.2	65.0
7.0	NaOH	6.2	20.0
7.5	NaOH	6.2	60.0
8.0	NaOH	6.2	60.0

Table B.2 Target pH, eluent used, batter pH, and volume required to change the pH of a wet cat food

Target pH	Eluent Used	Initial Batter pH	Volume required, mL
2.0	HCl	6.54	620.0
2.5	HCl	6.54	230.0
3.0	HCl	6.54	165.0
3.5	HCl	6.54	130.0
4.0	HCl	6.54	120.0
4.5	HCl	6.54	42.0
5.0	HCl	6.54	20.0
5.5	HCl	6.54	60.0
6.0	HCl	6.54	50.0
6.5	NaOH	6.54	0.0
7.0	NaOH	6.54	20.0
7.5	NaOH	6.54	50.0
8.0	NaOH	6.54	40.0

Table B.3 Target pH, type of thiamine used, and volume of eluent required to manipulate pH of a wet canned cat food.

Target pH	Thiamine Type	Volume required, mL and eluent used
2.0	Hydrochloride	44.0 HCl
2.5	Hydrochloride	35.0 HCl
3.0	Hydrochloride	30.0 HCl
3.5	Hydrochloride	22.0 HCl
4.0	Hydrochloride	16.0 HCl
4.5	Hydrochloride	12.0 HCl
5.0	Hydrochloride	8.0 HCl
5.5	Hydrochloride	6.0 HCl
6.0	Hydrochloride	3.0 HCl
6.5	Hydrochloride	0.0
7.0	Hydrochloride	2.0 NaOH
7.5	Hydrochloride	5.0 NaOH
8.0	Hydrochloride	7.0 NaOH
2.0	Mononitrate	42.0 HCl
2.5	Mononitrate	35.0 HCl
3.0	Mononitrate	30.0 HCl
3.5	Mononitrate	17.0 HCl
4.0	Mononitrate	17.0 HCl
4.5	Mononitrate	11.0 HCl
5.0	Mononitrate	8.0 HCl
5.5	Mononitrate	6.0 HCl
6.0	Mononitrate	3.5 HCl
6.5	Mononitrate	0
7.0	Mononitrate	2.0 NaOH
7.5	Mononitrate	3.5 NaOH
8.0	Mononitrate	4.0 NaOH

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