EVALUATION OF OXIDIZED RENDERED PROTEIN MEALS IN PET FOODS

by

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Abstract

Rendered protein meal is an important source of dietary protein and fat in pet food. However, fats in rendered meals can oxidize rapidly if not protected. The most common measurement of oxidation is the peroxide value (PV), but the analysis is highly variable. Additionally, the incorporation of oxidized protein further shortens its shelf life. Therefore, our objectives were to evaluate methods to measure fat quality in rendered protein meals and to determine the effect of increasingly oxidized protein meals on the shelf life of extruded pet foods. In Experiment 1, samples of five chicken byproduct meals (CBPM) from each of three locations and five beef meat and bone meals (BMBM) from each of two locations were analyzed for PV, anisidine value (AV), and thiobarbituric acid reactive substances (TBARS). The PV varied by method and location ($P < 0.05$). The alternative oxidation analytical methods, AV and TBARS, were not strongly correlated to PV ($R^2 > 0.01$). In Experiment 2, one metric ton of each unpreserved CBPM and unpreserved BMBM were collected and left unpreserved (U) or preserved with either ethoxyquin (E) or mixed tocopherols (T). These were held at ambient conditions (25°C, 51% RH) and monitored for PV and AV until values plateaued (41 and 63 days for CBPM and BMBM, respectively). Each "aged" meal was then incorporated into a model extruded cat food diet (~30% protein). Samples of kibble for each treatment were collected and stored at an elevated temperature and humidity (40°C, 70% RH) for 18 weeks and an ambient temperature and humidity (~22°C, 45% RH) for 12 months. The initial reduction ($P < 0.05$) in PV of the U (highly oxidized) BMBM and CBPM after processing suggests oxidation levels were diluted by food production. The oxidized meal led to a shorter shelf life ($P < 0.05$) in the finished food by PV analysis; but, sensory analysis by quick assessment did not completely corroborate these findings. These results suggest that PV doesn't fully describe rendered protein

meal stability or have a direct impact on shelf life for consumers; but may have a negative impact on pets due to oxidized lipid consumption.

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Chapter 1 - Literature Review

In 2014, pet industry expenditures were an estimated $58 billion, with pet food purchases accounting for about $22 billion of that total and growing at a rate of 4% per year (APPA, 2015). With sales increasing each year, pet ownership has also been increasing. There are approximately 163 million dogs and cats and an estimated 148 million specialty pets, such as fish, rabbits, hamsters, gerbils, and exotic animals, in U.S. homes (APPA, 2015; Aldrich, 2006). Over the past few decades, more families are considering their pets to be a member of the family; thus, increasing their expectations of the food quality being fed to their pets. The pet food choices that are available in stores are almost limitless, while becoming more wholesome, fresh, and tracking well with human food trends (Aldrich, 2006).

With over two-thirds of homes in the U.S. owning a pet, there is a significant reliance and connectedness between the home and the pet food and rendering industries (Aldrich, 2006). It is estimated that the pet food industry consumes nearly 2.4 million tons of rendered products annually, which represents roughly 25 percent of the total U.S. production of rendered materials (Aldrich, 2006; Swisher, 2005). Rendered protein meals are a source of high quality protein, and the category includes meat and bone meal, lamb meal, poultry (by-product) meal, turkey (by-product) meal, and fish meal (Aldrich, 2006). Pet food diets are often formulated with a protein level between 18 and 26 percent, and rendered protein meals comprise a large amount of that protein (Aldrich, 2006). Much of the pet food research being conducted is focusing on providing pets with optimal nutrition, which includes supplementing a high quality protein source and specific amino acid ratios in their diet (Aldrich, 2006).

The protein meals that are commonly incorporated into these diets also carry with them a modest quantity of fat (10 to 25%), which is subject to oxidation if not properly preserved (Aldrich, 2006). Dietary oxidized fat has been associated with reduced weight gain and body fat content, decreased serum levels of vitamin E, alteration of some parameters of cellular immune function, and impaired rate of bone formation in canines (Turek et al., 2003). The data from Turek et al. (2003) implies that consuming oxidized fat has implications for both humans and companion animals, and also highlights the need to control oxidation of the products that are incorporated into foods to ensure proper growth and optimum health. Preserving rendered protein meals requires an effective use of very potent antioxidants, such as synthetic (BHT, BHA, ethoxyquin) and/or natural preservatives, like mixed tocopherols. Synthetic preservatives have proven very effective, but the pet food manufacturing companies are pressured to use the less effective and more costly natural-mixed tocopherols by consumers. Questions exist regarding the true efficacy of some forms of the natural antioxidant preservatives, whether topical application of the mixtures is effective, and if the methods used to detect the application or the resulting stability are accurate.

Rendering Process

The North American rendering industry recycles approximately 26 million metric tons of perishable material annually generated by the livestock and poultry, meat/poultry processing, food processing, supermarket, and restaurant industries (National Renderers Association, 2015). The products produced from rendering have been utilized by various industries with over 3,000 uses identified (Meeker and Hamilton, 2006). Meat and bone meal, meat meal, poultry meal, hydrolyzed feather meal, blood meal, fish meal, and animal fats are the primary products

resulting from the rendering process, and are valuable feed ingredients for livestock, poultry, aquaculture, and companion animals (Meeker and Hamilton, 2006).

The rendering process utilizes a variety of equipment and processes that involve a combination of both physical and chemical transformations that vary depending on the raw material composition. Rendering processes approved by the Rendering Code of Practice include the use of heat to inactivate potential biological hazards, such as bacteria, viruses, protozoa, and parasites, reduce moisture, and facilitate the separation of fat from meat and/or bone (Meeker and Hamilton, 2006). There are many different types of rendering systems, but each can generally fit into one of four categories: wet, dry batch, dry continuous, or continuous low-temperature rendering systems (Ockerman et al., 1999). Wet rendering utilizes an autoclave that injects steam and pressure to cook raw material. Similar to other rendering systems, dry batch and dry continuous systems both involve the collection and sanitary transport of raw material to a facility where it is ground to a similar particle size and heated in a cooking vessel (Meeker and Hamilton, 2006). Since the 1960s, continuous rendering systems have been installed to replace batch systems, resulting in only a few batch cooker plants remaining in operation in North America (Prokop, 1992; Lehmann, 2001). The continuous and batch rendering systems are similar, except that the continuous system utilizes a single, continuous cooker rather than several parallel batch cookers; but the continuous cookers typically cook material faster, resulting in a higher quality fat product (Prokop, 1992). No steam or hot water is added to the raw material in a dry batch or continuous rendering system, and raw materials are cooked using a steam jacketed, hollowed agitator (Ockerman et al., 1999). Continuous low-temperature systems cook raw material in a dry or wet (steam injection) cooker where most of the moisture is removed by mechanical methods that take advantage of the differing densities of fat, water, and solids

(Ockerman et al., 1999). Depending on the raw material composition and the type of system used, the cooking process is generally 40 to 90 minutes using steam at temperatures of 115°C to 145°C (Meeker and Hamilton, 2006). The high cooking temperature removes a large fraction of moisture and facilitates the separation of fat from the protein and bone (Meeker and Hamilton, 2006). The cooked material is then completely separated from the fat by a screw press, and the fat is stored or transported in tanks (Meeker and Hamilton, 2006). The remaining cooked material, now predominantly protein, minerals, and some residual fat, is then further processed by grinding and cooking to remove additional moisture and is then be transferred for storage or shipment as rendered protein meal (Meeker and Hamilton, 2006). The rendered protein meal can then be utilized in animal feed. The rendering process is regulated by the United States Food and Drug Administration (FDA) to ensure the production of safe and nutritious animal feed ingredients (Meeker and Hamilton, 2006).

Rendered Protein Meals in Pet Food

Rendered protein meals are a valuable and important ingredient in livestock, poultry, aquaculture, and companion animal diets (Meeker, 2009). Pet food companies require specific purchasing requirements for all ingredients, including rendered products, that usually follow or exceed specifications set by the American Association of Feed Control Officials (AAFCO; Aldrich, 2006). The majority of protein used in extruded pet foods is rendered protein meal, and may include a blend of meat and bone meal, lamb meal, poultry (by-product) meals, turkey (by-product) meal, and fish meal (Aldrich, 2006). All these rendered protein meals can all provide a diet with a concentrated source of protein and amino acids, and some can also be great sources of vitamins and essential minerals (Hamilton, 2003). However, the nutrient composition for each rendered protein meal can vary by type and source.

Different types of rendered protein meals can be directly compared by evaluating their protein quality, which is a measure of how well the amino acid profile and bioavailability matches the requirements of the animal being considered. Nutritionally, meat and bone meal has relatively consistent level of protein of about 50%, but it can also have up to 25% ash, which can present problems when formulating a diet (Parsons et al., 1997; Pearl, 2004; Aldrich, 2006). The 2014 AAFCO definition of meat and bone meal is "the rendered product from mammal tissues, including bone, exclusive of blood, hair, hoof, horn, hide trimmings, manure, stomach and rumen contents, except in such amounts as may occur unavoidably in good processing practices." It has been reported that the protein quality of meat and bone meal is roughly comparable to lamb meal, but the effects of incorporating lamb meal in pet food diets is lacking (Johnson and Parsons, 1997; Johnson et al., 1998; Aldrich, 2006). The protein quality of lamb meal is also reported to be comparable to about 75 percent of chicken by-product meal (Johnson and Parsons, 1997; Johnson et al., 1998). Poultry protein meals, such as chicken by-product meal, are commonly incorporated as a high quality protein source in premium pet foods, but there is large variation in their nutrient composition due to differences in raw material, rendering system utilized, and processing parameters during rendering (Locatelli et al., 2003; Aldrich, 2006). This inconsistency requires more effort by the pet food companies to assure a consistent finished product (Aldrich, 2006). The 2014 AAFCO definition of chicken by-product meal is that it "consists of the ground, rendered, clean parts of the carcass of slaughtered chicken, such as necks, feet, undeveloped eggs and intestines, exclusive of feathers, except in such amounts as might occur unavoidable in good processing practice."

Another rendered protein meal that is becoming more popular in pet foods, but is often labeled as poultry (by-product) meal, is turkey protein meal (Aldrich, 2006). Nutritionally,

turkey meal is considered to be greater than meat and bone meal because of its higher protein content (62 to 65% vs. 50% for turkey meal vs. meat and bone meal, respectively). However, turkey meal is slightly less favorable than pet food grade chicken meal due to higher level of ash (18 to 25% vs. 25% for turkey meal vs. meat and bone meal, respectively; Aldrich, 2006). Comparatively, fish meal has approximately 19% ash and a high level of easily digestible protein (Aldrich, 2006). Fish meal also contains 8 to 12% fat, which is rich in omega-3 fatty acids that have beneficial effects in the treatment of chronic inflammatory diseases, such as rheumatoid arthritis, psoriasis, and colitis, and heart disease (Aldrich, 2006; Blok et al., 1996; Herold and Kinsella, 1986; Glomset, 1985).

The protein requirements for dogs and cats vary, and are based on differences in the age of the animal, growth stage, breed, environment, health and so on (Case et al., 2011). Proteins have numerous functions in the body, such as serving as major structural components (e.g. hair, feathers, skin, nails, tendons, ligaments, cartilage), connective tissue formation, muscle regulation, nutrient digestion, transport/carrier functions, hormone precursors, and immune system maintenance (Case et al., 2011). The AAFCO Nutrient Profiles (2014) recommends that adult maintenance dog and cat foods contain at least 18 and 22.75% of metabolizable energy (ME) calories as protein, respectively. Further recommendations include a minimum of 22 and 26.25% protein ME for growth and reproduction of puppies and kittens, respectively (AAFCO, 2014). Protein levels increase during growth and reproduction in order for the accretion of new tissue (Case et al., 2011). Pet food diets are generally formulated with a protein level of 18 to 26%, but as the animals are monogastric in nature, their protein requirement actually hinges on amino acid requirements (Aldrich, 2006).

There are a total of 22 amino acids found in protein chains that dogs and cats need, and they each contain the amino group -NH_2 (Case et al., 2011). Of these 22 amino acids, 12 can be synthesized within the body if an adequate source of dietary nitrogen is supplied (Case et al., 2011). These amino acids are otherwise known as nonessential, and include alanine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, hydroxylysine, hydroxyproline, proline, serine, and tyrosine. The other ten or eleven amino acids are known as essential because they cannot be synthesized within the body and must be supplied in the diet, which include arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, and taurine (for cats only; Case et al., 2011). These amino acids are most effectively met with animal based proteins or rendered protein meals rather than vegetable protein sources. Neirinck et al. (1991) points out that the amino acid profile of vegetable and animal protein sources are comparable, but the availability and digestibility of the amino acids in vegetable proteins are lower due to the difficulty of degradation in the gut. Still, Brown (1989) suggested that animal protein sources can be blended with vegetable sources to increase the overall quality of the final protein. Brown (1989) also implied that some of the limiting amino acids in cereals like lysine and tryptophan can be overcome by feeding animal sources that are rich in these amino acids. The studies by Murray et al., (1997) and Cramer et al., (2007) confirm that animal by-products in pet food diets are highly digestible and good sources of nutrients.

In addition to the protein and fat benefits, animal-based proteins are a great source of minerals, such as calcium and phosphorus. Murray et al. (1997) reported that calcium and phosphorus levels in rendered protein meals were higher than those in raw animal by-products. Calcium and phosphorus are known as macrominerals that are required for metabolic reactions and the formation and maintenance of bone (Case et al., 2011). Meat and bone meal and chicken

by-product meal, as defined by AAFCO, must contain a calcium level of no more than 2.2 times the actual phosphorus level, and a minimum of 4% phosphorus (AAFCO, 2014; Meeker, 2009). Dietary calcium and phosphorus are formulated into diets in ratio to one another, with common ratios ranging from 1.2:1 to 1.4:1 used by most nutritionists (Kealy et al., 1996). More specifically, the AAFCO *Nutrient Profiles* for dogs and cats require a minimum level of 1.0% calcium and 0.8% phosphorus for growth and reproduction, and 0.6% calcium and 0.5% phosphorus for adult maintenance (AAFCO, 2014; Case et al., 2011).

To reach these concentrations, the availability of calcium and phosphorus in dog and cat diets should be taken into account because there are many factors that can affect their bioavailability (Case et al., 2011). The calcium and phosphorus levels in plant sources are less available than those from animal sources (Case et al., 2011). A deficiency in these minerals is unusual due to the typical successful production of well-formulated pet foods, but is more often observed in calcium rather than phosphorus due to its presence in many foods (Case et al., 2011). A deficiency in calcium is commonly developed when there are higher levels of phosphorus relative to calcium in a diet (Case et al., 2011). High levels of phosphorus can decrease the absorption of calcium and results in bone demineralization and subsequent loss of bone mass (Hintz et al., 1987; Case et al., 2011). High phosphorus concentrations are a particular concern in diets that contain high proportions of muscle and organ meats that can decrease the absorption of calcium (Case et al., 2011). According to Schoenmakers et al. (1999) an excess of calcium, with or without a proportional increase in phosphorous, decreases both calcium and phosphorous absorption in puppies, but fortunately not on a permanent basis.

While the mentioned rendered protein meals are only a glimpse into the many traditional and alternative protein sources available to the pet food industry, compositional protein quality,

palatability, amino acid, minerals, vitamins, essential fatty acids concentration, digestibility, and availability should be considered when choosing a protein source (Dust et al., 2005).

Dietary Fats and Oils

Depending on the supplier and the animal source, the quantity of crude fat in rendered protein meals may range from 10 to 25% (Aldrich, 2006). In a diet, fat provides a concentrated source of energy and essential fatty acids, facilitates fat-soluble vitamin absorption, and provides palatability benefits (Aldrich, 2006). Dietary fat is part of a diverse group of compounds known as lipids, which are important in foods and most biological systems (Case et al., 2011; Shahidi et al., 2009). Dietary fat is one of the major macronutrients, so it is required for most functions within living organisms, such as growth, reproduction and maintenance (Shahidi, et al., 2009).

Energy of Fat

The energy in a diet is often reported in kilojoules (kJ) or kilocalories (kcal), also known as calories, which are equivalent to 4.16 kJ (Wakshlag et al., 2014). Pet food labels often refer to the energy in the diet as metabolizable energy (ME), which accounts for the dietary energy remaining after energy loss in the urine, feces, and gases (Wakshalg et al., 2014). Fat is a highly digestible nutrient and is assigned a metabolizable energy (ME) value of 8.5 kcal/g, while protein and carbohydrates each contribute 3.5 kcal/g (Case et al., 2011). High-fat diets are normally fed to dogs and cats during growth, gestation, lactation, and prolonged periods of physical exercise to enhance their ability to use fatty acids for energy (Case et al., 2011). It has been reported by Reynolds et al. (1994) that consuming a high-fat diet increased the utilization of free fatty acids for sled dogs during training compared to a diet high in carbohydrates. High-fat diets bring with them a large amount of energy, which will be stored in adipose tissue in the body if not expended, often leading to obesity if not corrected (German, 2006). The solution is to provide

cats and dogs with a purpose-formulated diet that generally fluctuates the quantity of energy and fat, protein, and micronutrients depending on the energy needed for normal metabolic functions and expenditure (German, 2006).

Fatty Acids in Animal Fats

A majority of lipids have a chemical structure that consists of one unit of glycerol and three units of fatty acids, otherwise chemically known as a triglyceride (Figure 1.1, 1.2, and 1.3; Meeker, 2006). In each ingredient, there are different fatty acids bound to the glycerol backbone that give the respective fats their unique fatty acid profile and resulting function (Meeker and Hamilton, 2006; Aldrich, 2006). The nutritionally important fatty acids commonly found in animal protein sources include C_{16} and C_{18} species, such as palmitic, stearic, palmitoleic, oleic, linoleic, linolenic acids (Voet et al., 2013; AOCS, 2013). A large portion of the fatty acids in animal-based lipids, such as palmitoleic, oleic, linoleic, and linolenic acids, are unsaturated or polyunsaturated, which indicates that these fatty acids contain one or more double bonds (Voet et al, 2013). Lipids that do not have double bonds within their structures are identified as saturated fatty acids, and include palmitic and stearic acid (Meeker and Hamilton, 2006). The fatty acid composition of beef tallow contains about 46-55% saturated fatty acids and 45-51% unsaturated fatty acids; while poultry fat contains 25-31.5% saturated and 57-75% unsaturated fatty acids (AOCS, 2013). The breakdown of fatty acid compositions can be viewed in Figure 1.4 (AOCS, 2013). The fatty acid profile of these animal fats can also change depending on the cut or part of the animal that is being processed (AOCS, 2013). For example, Figure 1.5 outlines that beef kidney fat has a higher percentage of palmitic acid than the subcutaneous beef fat (AOCS, 2013).

Essential Fatty Acids

According to the National Research Council (2006), dogs and cats require both n-6 and n-3 essential fatty acids, which include linoleic acid (18:2n-6), arachidonic acid (20:4n-6), α-linolenic acid (18:3n-3), eicosapentaenoic acid (20:5n-3), docosapentaenoic acid (22:5n-3), and docosahexaenoic acid (22:6n-3). These fatty acids are deemed "essential" because they are required for normal metabolic function, but cannot be synthesized by the body in sufficient quantities and therefore need to be supplied in the diet. The most important essential fatty acid requirement for dogs and cats is the parent forms of n-6 and n-3 fatty acids, linoleic and α-linolenic acid, that are required in adult maintenance, reproduction, growth, and lactation (NRC, 2006; Case et al., 2011). The AAFCO *Nutrient Profiles* (2014) require a minimum level of 5% fat for adult dog maintenance, with a minimum of 1% of the food's dry weight as linoleic acid, and 8% fat for growth and reproduction in a food containing 3,500 kcal/kg. For cats, AAFCO *Nutrient Profiles* (2014) require a minimum level of 9% fat, with 0.5% linoleic acid and 0.02% arachidonic acid for all life stages in a diet containing 4,000 kcal/kg. Dogs are capable of synthesizing adequate quantities of essential fatty acids from linoleic acid, including gamma-linolenic acid and arachidonic acid, by alternating desaturation and elongation reactions (Case et al., 2011). Cats may be able to produce a sufficient quantity of arachidonic acid from linoleic acid during maintenance to meet their needs, but the fatty acid must be supplemented in the diet during pregnancy for normal neonatal development (Pawlosky et al., 1994; Bauer, 2006; Case et al., 2011). It has been confirmed by Pawlosky et al. (1994) that cats have limited Δ-6 desaturase activity in the liver and brain that is needed to break down linoleic acid into arachidonic acid that is essential for growth and neurological development. Some of the best sources of linoleic acid are from plant sources, such as corn, soybean, and safflower oils, while animal sources, such as poultry and pork fat, provide moderate quantities (Case et al., 2011). Arachidonic acid is only

found in animal-based fats like fish, pork, and poultry (Case et al., 2011). An excess of fat can cause loose stool or diarrhea in cats and dogs, while a deficiency in essential fatty acids can cause dermatological issues, reproduction difficulties, and limited growth or neurological development (MacDonald et al., 1984; Case et al., 2011). Well-formulated pet foods provide cats and dogs with adequate essential fatty acids if they are stored and fed correctly.

Fat Soluble Vitamin Absorption

Vitamins are organic dietary constituents that are essential for normal cell function, growth, and development (Case et al., 2011). They can be categorized into two classes: water-soluble and fat-soluble. Water-soluble vitamins are absorbed by the small intestine by passive diffusion, and the excess is excreted through urine (Evert et al., 2013). There are a total of nine water-soluble vitamins: vitamin C (ascorbic acid), B1 (thiamine), B2 (riboflavin), B3 (niacin), B5 (pantothenic acid), B6 (pyridoxine), B7 (biotin), B9 (folic acid), and B12 (cobalamin; Evert et al., 2013). Fat-soluble vitamins: A, D, E, and K, are also absorbed in the small intestine utilizing the same mechanisms as dietary fat (Case et al., 2011; Evert et al., 2013). If excess fat soluble vitamins are consumed, the excess is often stored in the liver (Case et al., 2011).

Vitamin A aids in the following functions: vision, bone growth, reproduction, and maintenance of epithelial tissue (Case et al., 2011). Retinol, a related chemical compound to vitamin A, is the most biologically-active form, and is a physiological requirement for all animals. Almost all animals are able to convert vitamin A precursors, such as beta-carotene, to retinol except for cats (Schweigert et al., 2002). Cats lack the dioxygenase enzyme that is needed to cleave beta-carotene to active vitamin A, thus requiring active vitamin A to be supplemented into cat diets (Gershoff et al., 1962). Beta-carotene is an enzyme that is found in carotenoids, which are synthesized by plant cells (Case et al., 2011). Animal-based products like fish liver

oils, milk, liver, and egg yolk do not contain carotenoids, but can be dietary sources of active vitamin A (Case et al., 2011; Evert et al., 2013). Deficiencies in vitamin A do not often occur due to the adequate amounts included in commercial pet foods, but signs of deficiency include impaired growth, reproductive failure, loss of epithelial integrity, and dermatoses (Case et al., 2011). Toxicity of vitamin A is observed in cats that are fed diets composed solely of liver or other organ meats because they cannot regulate the absorption of the active vitamin A (Case et al., 2011). Signs of toxicity in cats include a disorder called deforming cervical spondylosis that causes the formation of bony outgrowths on the cervical vertebrate (Case et al., 2011). The AAFCO *Nutrient Profiles* (2014) suggest that the minimum requirement for Vitamin A for dogs during maintenance, growth, and reproduction, is 5,000 IU/kg, with a maximum of 250,000 IU/kg. For cats, AAFCO *Nutrient Profiles* (2014) suggest a minimum requirement of 9,000 IU/kg during growth and reproduction, and 5,000 IU/kg during maintenance, with a maximum requirement of 750,000 IU/kg.

Vitamin D is important in pet foods because it plays a role in calcium and phosphorus homeostasis and metabolism, particularly on intestinal absorption, retention, and bone deposition of calcium (Zoran, 2002). Most animals are able to meet their vitamin D needs by absorbing ultraviolet radiation through their skin, which involves synthesizing vitamin D_3 (cholecalciferol) from 7-dehydrocholesterol (Case et al., 2011). It has been suggested by How et al. (1994) that dogs and cats have a limited ability to convert 7-dehydrocholesterol to vitamin D_3, and that vitamin D must be supplemented in the diet. The quantity of vitamin D that is supplemented in the diet is often dependent on the corresponding calcium and phosphorus levels (Cline, 2012). The AAFCO *Nutrient Profiles* (2014) suggest that the minimum requirement for vitamin D for dogs at maintenance, growth, and reproduction is 500 IU/kg, with a maximum requirement of

5,000 IU/kg. For cats, AAFCO *Nutrient Profiles* (2014) suggest a minimum requirement of 750 IU/kg for growth and reproduction, and 500 IU/kg for maintenance, with a maximum requirement of 10,000 IU/kg. Pet food ingredients with high concentrations of vitamin D include the liver and fatty tissues of animals, suggesting that in past times, cats and dogs likely met their vitamin D requirements through the consumption of prey (Zoran, 2002). A deficiency in vitamin D is rare in pets that consume appropriate diets, but can develop into rickets due to insufficient deposition of calcium and phosphorus (Case et al., 2011). An excess of vitamin D can cause hypercalcemia and calcification of soft tissues and is often a result of accidental cholecalciferol rodenticide poisoning or excessive vitamin D levels in commercial pet food (Case et al., 2011).

According to the NRC (2006), vitamin E is found in blood plasma, erythrocytes, and tissues where it has antioxidant properties. The vitamin functions as a neutralizer of free radicals and prevents oxidative damage of lipids within cellular membranes. Vitamin E is also involved in several immune functions, such as cell signaling, regulation of gene expression, and other metabolic processes (NIH, 2013). Vitamin E requirements increase as the dietary levels of polyunsaturated fatty acids increase, or as the number of double bonds increase, because the vitamin protects unsaturated dietary fats from destructive oxidation by donating hydrogen's to free radicals (NRC, 2006). The AAFCO *Nutrient Profiles* (2014) recommend a minimum level of 50 IU/kg on a dry matter basis for dogs during growth, reproduction, and maintenance, with a maximum level of 1,000 IU/kg. For cats, AAFCO *Nutrient Profiles* (2014) recommend a minimum level of 30 IU/kg on a dry matter basis during growth, reproduction, and maintenance, with no maximum level. In addition, AAFCO *Nutrient Profiles* (2014) also suggests that 10 IU/kg of vitamin E per gram of fish oil be added above the minimum level for cat diets due to its high level of unsaturated fatty acids that increase the likelihood of oxidation (Hendriks et al.,

2002). Some of the best sources of vitamin E are available in nuts, seeds, vegetable oils, green leafy vegetables, and fortified cereals (NIH, 2013). A deficiency in vitamin E in dogs and cats is not common, but can be caused by consuming a diet that has been poorly formulated, inadequately stored, or supplemented with high levels of polyunsaturated fatty acids (Hayes et al., 1969; Van-Vleet, 1975). Deficiency in vitamin E can cause skeletal muscle and retinal degeneration, decreased reproductive performance, and impaired immunological response (Hayes et al., 1970; Riis et al., 1981). According to the NRC (2006), vitamin E in excessive doses has been shown to be relatively nontoxic.

Vitamin K aids in normal blood coagulation and several other clotting factors (Case et al., 2011). This vitamin is needed at very low levels, relative to other fat soluble vitamins, for dogs and cats because of its ability to be synthesized by bacteria in the intestine (Case et al., 2011). The NRC (2006) advises that the vitamin K requirement of a non-fish containing cat diet may be met by microbial synthesis in the intestine. The AAFCO *Nutrient Profiles* (2014) does not have a minimum or maximum level of vitamin K for dogs at any life stage, but has a minimum requirement for cats of 0.1 mg/kg on a dry matter basis. Although, as a precaution, the NRC (2006) has suggested a recommended allowance of 1.40 mg for dogs and 1.0 mg for cats at maintenance. Deficiencies in naturally-occurring vitamin K have only been reported for cats when high levels of fish are in the diet, suggesting that cat foods containing fish interfere with vitamin K synthesis or absorption (Case et al., 2011). An interference with the absorption of vitamin K can cause a deficiency and initiate symptoms of hemorrhage and decreased levels of clotting factors in the blood (Case et al., 2011). Many rat and mouse poisons can cause a deficiency in vitamin K if accidentally ingested because of their ability to bind vitamin K (NRC, 2006).

Palatability of Fat

There are many ways to enhance the palatability of pet food, either by mixing it in with the food or applying it on the surface (Ockerman et al., 1999). The incorporation of dietary fat can increase the palatability of a pet food diet by altering flavor and texture without limit in dogs and up to a certain point for cats (Zaghini et al., 2005; Aldrich, 2006). The studies by Houpt et al., (1978) and Lohse (1974) suggest that dogs prefer meat with higher concentrations of fat compared to a lean meat in a two-choice preference test or in an operant conditioning apparatus. In addition to increased palatability, Ahlstrom et al. (1998) suggests that dogs have a greater digestibility of fat than protein or carbohydrates in the diet. Kendall et al. (1982) demonstrated a hyperbolic relationship between apparent fat digestibility and fat intake for dogs and cats fed contrasting types of pet foods.

Lipid Oxidation Process

As mentioned previously, rendered protein meals carry with them a modest quantity of fat that is susceptible to oxidation (Aldrich, 2006). Oxidation denatures lipids, as well as nucleic acids, proteins, and structural carbohydrates (Sies et al., 1985). The effects of dietary oxidized lipids on canines have been demonstrated by Turek et al. (2003), where the main effects included decreased weight gain, body fat composition, vitamin E serum levels, immune function, and bone formation. Turek et al. (2003) emphasized the importance of controlling oxidation products in pet food so that pets have the opportunity to grow properly and maximize health.

The most common process leading to oxidative deterioration of lipids is its spontaneous reaction with oxygen, otherwise known as autoxidation (Gordon et al., 2001). Autoxidation can be initiated or catalyzed by several factors other than oxygen during processing, refining, and storage, which include metals, free fatty acids, light, heat, and lipoxygenases (Chow et al., 2008).

Transition metals, such as iron or copper, aid in the oxidation process by facilitating the transfer of electrons leading to increased formation of free radicals (Ladikos et al., 1990; Gordon et al., 2001; Chow et al., 2008). Free fatty acids react with lipids to cause oxidative damage and are also known as prooxidants (Chow et al., 2008). Ultraviolet and near-ultraviolet light provides the oxidation process with energy to begin the deterioration of lipids (Chow et al., 2008; Sherwin, 1978; Patterson, 1989). When heat over 60°C is applied, the rate of oxidation nearly doubles for every additional 15°C (Chow et al., 2008; Sherwin, 1978). Lipoxygenases are naturally-occurring enzymes that are present in both plant and animal tissues that form positive flavors during mastication, but also negatively contribute to oxidative deterioration (Gordon et al., 2001; Chow et al., 2008). The types of fats that are more susceptible to the autoxidation process are unsaturated or polyunsaturated fatty acids (Voet et al., 2013; Frankel, 1998). For each additional double bond in these fatty acids, the rate of oxidation doubles (Voet et al., 2013; Frankel, 1998). For example, poultry by-product meal contains more unsaturated fats than beef meat and bone meal which theoretically makes poultry by-product meal more susceptible to oxidation relative to beef meat and bone meal (Leeson et al., 2005),.

The autoxidation process of these fats can be divided into three stages: initiation, propagation, and termination (Figure 1.6; Catala, 2006). The initiation stage of autoxidation consists of a relatively slow reaction where a hydrogen atom is abstracted from the α-methylenic carbon in the unsaturated fatty acid group of the fat molecule, which results in a free radical (Catala, 2010; Akoh et al. 2008). The free radical is then "free" to react with oxygen to form a peroxyl radical (Catala, 2010; Akoh et al. 2008). The free radicals are strong initiators of oxidation and become autocatalytic in this process (Sherwin, 1978). The double bonds within unsaturated and polyunsaturated fats weaken the single carbon-hydrogen bonds from the carbon

atom nearby, thus aiding in the hydrogen abstraction from the carbon (Catala, 2010). Next, the propagation phase begins with lipid-lipid interactions where one lipid radical is converted to a different lipid radical, which occur several times before the termination phase (Akoh et al., 2008; Steele et al., 2004). More specifically, a lipid peroxyl radical slowly subtracts a hydrogen from an adjacent molecule and forms a lipid-free radical and lipid hydroperoxide (Akoh et al., 2008; Denisov et al., 2005). The concentration of the hydroperoxides increases with time and starts to form a sigmoid-shaped kinetic curve (Denisov et al., 2005). Lipid hydroperoxides are primary products of autoxidation, but are tasteless and odorless and will therefore not deter food quality (Shahidi, 2005; Chow et al., 2008). The fatty acid oleate decomposes during autoxidation to form nonanal and octanal, and produces dec-2-enal during photoxidation (Antolovich, et al., 2002). The fatty acid linoleic breaks down during autoxidation into hexanal, pentane, and deca-2,4-dienal and forms hept-2-enal and hexanal during photoxidation (Antolovich et al., 2002). Linolenic acid breaks down during autoxidation into hepta-2, 4-dienal and ethane and breaks down into propanal and but-2-enal during photoxidation (Antolovich et al., 2002). However, it is the secondary products or the further degradation of the primary oxidation products that are responsible for off-flavors and odors (Akoh et al., 2008). The radicals continue to breakdown and undergo carbon-carbon cleavage that form secondary oxidation products that include aldehydes, ketones, alcohols, hydrocarbons, volatile organic acids, and epoxy compounds (Kanner et al., 1992; Shahidi et al., 2005). These secondary oxidation products are actually responsible for the flavor deterioration of foods and odors that are often associated with rancidity (Sherwin, 1978; Frankel, 1987; Patterson, 1989).

Methods for Measuring Lipid Oxidation

There are numerous analytical methods that are commonly used to measure lipid oxidation in foods, but no single method can detect all oxidation products within food systems (Shahidi et al., 2002). Therefore, selecting the proper method that matches the particular application is important. Methods can be accurately selected based on what they are measuring, such as the loss of initial substrates, absorption of oxygen, formation of free radicals, or formation of primary and secondary oxidation products (Shahidi et al., 2002; Dobarganes et al., 2002). Many of the methods utilized to measure lipid oxidation involve the use of physical and chemical tests, including instrumental analyses (Shahidi et al., 2002). Shahidi et al., (2005) outlined these tests and analyses, which include "the active oxygen method and weight-gain methods for oxygen absorption; chromatographic analysis for changes in reactants; iodometric titration, ferric ion complexes, and Fourier transform infrared (FTIR) methods for peroxide value; spectrometry for conjugated dienes and trienes, 2-thiobarbituric acid (TBA) value, *p*-anisidine value (AV), and carbonyl value for secondary oxidation products; Rancimat and Oxidative Stability Instrument (OSI) method for oil stability index; and electron spin resonance (ESR) spectrometric assay for free-radical types and concentration." The feed and pet food industries commonly measure primary and secondary oxidation of rendered protein meals using peroxide value (PV), oxygen bomb, thiobarbituric acid reactive substances (TBARS), p-anisidine value, gas chromatography and sensory analysis.

Peroxide Value (PV)

The peroxide value (PV) of a fat or oil is an analytical method that measures hydroperoxide concentration (Shahidi et al., 2005). Hydroperoxides are formed continuously in the initial stages of lipid oxidation, and are therefore referred to as primary oxidation products

(Shahidi et al., 2005). The basis of a current standard method for measuring PV is the iodometric titration method, which is based on the oxidation of the iodide ion (I^-) by hydroperoxides (ROOH; Antolovich et al., 2002). The American Oil Chemists' Society (AOCS) has two approved PV chemical methods, Cd 8b-90 and Cd 8-53, applicable to normal fats and oils that use iodometric titration. These two methods only differ by the type of saturated solution that is used to dissolve the sample, either acetic acid-isooctane or acetic acid-chloroform (Li, et al., 2000). The method Cd 8b-90 requires between 0.3 to 5.0 g of sample depending on the 'expected' peroxide value, while method Cd 8-53 requires a large 5 g sample regardless of expected PV (AOCS, 1998). Both AOCS methods add a saturated potassium iodide solution to the oil sample to initiate a reaction with the hydroperoxides (AOCS, 1998; Shahidi et al., 2005). The iodine is then liberated, titrated with a standardized solution of sodium thiosulfate, and starch is added as an endpoint indicator (Shahidi et al., 2005; AOCS, 1998). The PV is then expressed as meq/kg or milliequivalents of oxygen per kilogram of sample (Antolovich et al., 2002). A low PV may signify either the beginning or advanced oxidation, and can be distinguished based on the PV over time or by measuring secondary oxidation products (Min et al., 2010). A high quality, fresh fat will have a PV of zero, with very poor quality fats resulting in a PV $\geq$ 20 meq/kg (Min et al., 2010). The iodometric titration method has many disadvantages, which includes lack of sensitivity, difficulty in determining the titration endpoint, labor-intensiveness, and the generation of large quantities of reagent and solvent waste (Li et al., 2000; Antolovich et al., 2002; Eymard et al., 2003; Osawa et al., 2007; Osawa et al., 2008). Shahidi et al. (2002) and Antolovich et al. (2002) suggested that other disadvantages affecting PV results may be attributed to iodine being absorbed by unsaturated bonds, iodide may be further oxidized by dissolved oxygen during analysis, and there may be variations in reactivity of different

peroxides. Guillen et al. (2002) hypothesized that it is difficult to obtain reproducible PV values due to the high hydroperoxide generation and degradation rates, which has been found by other authors such as Van de Voort et al. (1994). Due to the numerous disadvantages of the iodometric titration method, other methods have been developed based on the same reaction with other techniques (Shahidi et al., 2005; Antolovich et al., 2002).

Using fast methods for conventional routine analysis is very convenient, especially since many of the pet food manufacturers do not have a laboratory equipped for these types of analyses (Osawa et al., 2008). For example, MP Biomedicals (2014; Solon, OH) has developed a rapid method (SafTest: PeroxySafe™) for analyzing peroxide value for many types of oil, food and animal matrixes using micro-analytical and membrane separation principles. The rapid method correlates well with the AOCS Official method Cd 8-53 (certification #030501) with a correlation coefficient of 0.99 for meals, animal fats, and oils (Osawa et al., 2008; MP Biomedicals, 2014). "The PeroxySafe™ quantifies PV by transferring a free electron to a metal-chromogen complex whose visible spectrum then changes and is read using the SafTest II Analyzer" (MP Biomedicals, 2014; Solon, OH). The PeroxySafe™ kit yields an estimate of the 'within laboratory precision' of 6% when analyzing rendered protein meals and 5% for animal fats and oils (MP Biomedicals, 2014). Sample preparation, such as heating, mechanical disruption, and membrane separation is required to analyze animal-based meals (MP Biomedicals, 2014). Advantages to using the rapid method include that it provides reproducible results, is simple and quick to use, sensitive (0.02 to 50 meq/kg), and requires a small amount of sample and reagents (Osawa et al., 2008). Osawa et al., (2008) recommends the use of kits for shelf-life studies of samples that require lipid extraction prior to analysis because they are precise, quick, involve significantly less sample and reagents, require little space/equipment in

the lab, and subjects the analyst to less risks. Disadvantages of the kit include that it has a limit of qualification of up to 50 meq/kg and is costly.

Again, the AOCS official iodometric titration methods are for 'normal fats and oils,' referring to fats and oils that have already been processed and extracted from their source. There is no official method for the extraction of the residual fat from rendered protein meals, which leads to various extraction methods being used. It has been suggested that various extraction solvents and methods will affect PV results, but there is no published research to support this claim. A solvent for fat extraction should be chosen based on a low boiling point, high solubility with lipids, ability to evaporate readily and leave no residue, low affinity for moisture, and low cost (Min et al., 2010). Unfortunately, meeting all of these requirements can be quite difficult. "The type of solvent and the actual method of lipid extraction depend on both the chemical nature of the sample and the type of lipid extract desired" (Akoh et al., 2002). The organic solvents that are commonly used to extract lipids are ethyl ether, petroleum ether, pentane, hexane, and their combinations (Min et al., 2010). Triglycerides in lipids are soluble in nonpolar solvents, such as hexane and petroleum ether (Min et al., 2010). Research regarding solvents for fat extraction of rendered protein meals and the impact they have on PV results needs to be further explored.

Oxygen Bomb

As lipid oxidation occurs, a sample absorbs oxygen from the surrounding environment (O' Keefe et al., 2010). A method that allows you to measure the time it takes for a sample to oxidize and absorb oxygen is with the use of an oxygen bomb. An oxygen bomb is a closed system that measures the time required for the rapid disappearance of oxygen that the sample uptakes; therefore, it can be used to determine the oxidative stability of a sample (O' Keefe et al.,

2010). The sample is placed in a heavy-walled container that has a pressure recorder attached, pressurized to 689.5 kPa with oxygen, and placed in a boiling water bath (O' Keefe et al., 2010). The time is measured until there is a sharp drop in pressure, which corresponds with the rapid absorption of oxygen by the sample (O' Keefe et al., 2010). Youmans et al. (1955) demonstrated that the oxygen bomb method corresponds well with rancidity shelf life tests. This method also has an advantage compared to the oil stability index and active oxygen method because it may be used with intact foodstuffs instead of extracted lipids (Stauffer, 1996). Thus, the stability of rendered protein meals may be measured without prior extraction. A disadvantage of the oxygen bomb method is the challenge of maintaining the high oxygen pressure and high temperature for the undetermined amount of time it may take to measure the oxidative stability of a well-preserved sample (Warner et al., 1995).

Thiobarbituric Acid Reactive Substances (TBARS)

The thiobarbituric acid reactive substances (TBARS) analysis, also referred to as thiobarbituric acid (TBA), measures malonaldehydes, or secondary lipid oxidation products. It is commonly used in meat products (O'Keefe et al., 2010). A sample of known weight is mixed with distilled water and adjusted to a pH of 1.2 prior to being transferred to a distillation flask (O' Keefe et al., 2010). Addition of an antioxidant to the mixture at this step will hinder further decomposition of labile hydroperoxides and keep the TBA values from rising (Shahidi et al., 1991). Antifoam reagent and boiling beads are added to the distillation flask, and the sample is then distilled rapidly (O' Keefe et al., 2010). The first 50 mL of sample is collected, and an aliquot of the distillate is mixed with TBA reagent and heated for 35 minutes in boiling water (O' Keefe et al., 2010). The reaction of malonaldehydes with TBA yields a pink-colored compound that is measured spectrophotometrically at 530 nm against a standard curve, and the

absorbance reading is converted to milligrams of malonaldehyde per kilogram of sample (Du et al., 1992; O' Keefe et al., 2010; Papastergiadis et al., 2012).

The basis for measuring malonaldehydes is the 2009 AOCS official method (Cd 19-90), but it is a direct method for analysis of fats and oils and does not require the extraction steps of malonaldehydes in food matrices. The TBA can react directly with a food sample so the TBA-MDA solution can be extracted prior to the analysis, but many interaction products can give a positive reaction. This colorimetric method is not specific for malonaldehydes, and may be reading other oxidation products (Frankel et al., 1983; Papastergiadis et al., 2012). Components of food matrices, such as sugar degradation, protein degradation, and browning reaction products contribute to the formation of the TBA color complex (Du et al,. 1992; Guillen-Sans et al., 1998; Papastergiadis et al., 2012). Tarladgis et al. (1960) and Vyncke (1975) suggest either distillation or solvent extraction of malonaldehydes from food matrices may help render a more accurate reading. These suggestions minimize the interferences with TBA, but the long analysis time and heating of the sample during distillation may initiate further oxidation of the sample. A higher malonaldehyde level may result, thus serving as a basis for the addition of antioxidant during the distillation step (Papastergiadis et al., 2012). Papastergiadis et al. (2012) revealed that the TBARS test is reliable for the determination of malonaldehydes in vegetable oils, and unprocessed meat or fish products, but malonaldehydes are overestimated in processed beef, pork, fish, dry nuts, cheese, and potato crisps due to interference from other compounds. Other substances beyond aldehydes, including malondialdehydes, can react with TBA and are referred to as TBARS and include, ketones, ketosteroids, acids, esters, sugars, imides and amides, amino acids, oxidized proteins, pyridines, and pyrimidines (Guillen-Sans et al., 1998). Guillen-Sans et al. (1998) also states that "substances formed especially in meats and meat derivatives are

usually designated as TBARS." Other analytical techniques for the determination of malonaldehydes have been published, such as the use of other extraction methods, ultraviolet spectrophotometry, high-performance liquid chromatography (HPLC), and gas chromatography/mass spectroscopy (GC-MS; Fernandez et al., 1997).

p-Anisidine Value (AV)

The AOCS official method (Cd 18-90) for p-anisidine value (AV) measures secondary oxidation products in fats and oils, such as α- and β- unsaturated aldehydes or more specifically, 2-alkenals and 2,4-dienals (Crapiste et al., 1999; O'Keefe et al., 2010). To a limited degree, this method also measures saturated aldehydes depending on the aldehyde type (Dubois et al., 1996). The method is relatively simple, but it requires substantial analytical time, precision, and uses toxic reagents (Dubois et al., 1996). The analysis requires about 1 g of fat diluted in 100 mL isooctane, with the absorbance measured at 350 nm against an isooctane blank. Precisely 5 mL of fat solution and five mL of isooctane were both allowed to react with 1 mL of p-anisidine for exactly ten minutes, at which an absorbance measurement of the fat solution must be read against the isooctane/p-anisidine blank. The spectrophotometer measures a chromogen that is formed from the reaction between the aldehydes and the p-anisidine (O'Keefe et al., 2010). The results of the reaction are defined as 100 times the absorbance at 350 nm of the fat solution after ten minutes of reaction time (O'Keefe et al., 2010). The disadvantage to this laboratory technique is the large amount of potential variation involved. Osawa et al. (2008) reported a coefficient of variation of 6.5 to 900 percent using the AOCS official method on lipid contents of pet food samples. This significantly affects the reproducibility within laboratories.

In the initial stages of oxidation, AV remains constant and rapidly increases following the decomposition of peroxides as demonstrated by Crapiste et al. (1999). Guillen et al. (2002),

suggested that the high rate of hydroperoxide generation doesn't always involve the high rate of generation of secondary oxidation products in various oils. More specifically, Guillen et al. (2002) shared olive and rapeseed oil generated secondary oxidation products almost simultaneously with the generation of hydroperoxides, but in sunflower and safflower oil the degradation of hydroperoxides began when the concentration of these compounds were significant (Guillen et al., 2002). Therefore, it is not known if PV and AV work simultaneously depending on the oil or fat source and the level of preservatives in samples. This would suggest a combination of different analytical techniques is required to monitor lipid oxidation because there is not one method that can detect all oxidative changes in food (Crapiste et al., 1999). The AV is also often used in conjunction with peroxide value in the industry to calculate total oxidation, which is commonly referred to as the totox value (Akoh et al., 2002; O'Keefe et al., 2010). Calculating the totox value is often considered to provide the past history of an oil or fat, but it "does not have any sound scientific basis because it combines variables with different dimensions" (Akoh et al., 2002).

Other methods of measuring AV have been published, such as the Fourier transform infrared (FTIR) transmission-based spectroscopic method (Voort et al., 1994; Dubois et al., 1996; Man et al., 1999), and gas chromatography/mass spectroscopy (Luo et al., 1995).

Gas Chromatography-Mass Spectrometry (GC-MS)

When hydroperoxides breakdown, they produce volatiles and short-chain compounds that contribute to the formation of nonvolatile molecules (Boskou et al., 2010). Gas chromatography is used for the quantitative analysis of the volatile compounds, and can be utilized for a large number of sample forms, such as liquid, semiliquid, and solid samples (Frankel et al., 1999). The volatile compounds that are produced include aldehydes, ketones, alcohols, acids, esters, sulfur

compounds, pyrazines, furans, alkanes, benzene derivatives, and terpenes (Boskou et al., 2010). The aldehydes are the most important decomposition products because they are the most abundant and contribute significantly to the flavor and odor of foods (Frankel, 1985; Boskou et al., 2010). The major volatile aldehydes formed from oxidation of edible oils have between 3 and 10 carbon units, which include propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal, and decanal (Man et al., 1999). Propanal is thought to be the primary breakdown product of linolenic acid; whereas, butanal is formed from side reaction pathways; pentanal from linoleic acid; hexanal from linoleic acid; heptanal from linoleic and oleic acid; and octanal, nonanal and decanal from oleic acid (Belitz et al., 2013). Among the various methods that measure volatile aldehydes, those involving gas chromatography/mass spectrometry offer the advantage of selectivity and sensitivity (Luo et al., 1995; Liu et al., 1997). Luo et al. (1995) and Liu et al. (1997) conclude that the determination of aldehydes and other lipid peroxidation products in biological samples by gas chromatography/mass spectrometry is important for a better understanding of the biochemical consequences of lipid peroxidation.

Analysis by GC-MS is routinely coupled with solid-phase microextraction (SPME), which is a very simple, efficient, and solventless sample preparation method (Vas et al., 2004). "All of the steps relating to the conventional liquid-liquid extraction such as extraction, concentration, derivatization, and transfer to the chromatograph are integrated into one step and one device in SPME, considerably simplifying the sample preparation procedure" (Vas et al., 2004). The method uses a fused-silica fiber that is coated with an appropriate stationary phase around the outside that directly extracts volatiles from the headspace of the sample and then transfers the volatiles to the chromatograph (Vas et al., 2004). This method allows for the analysis of complex food matrices, such as rendered protein meals and pet food, without

extensive sample preparation. Dry dog foods were analyzed using this method with GC-MS in the study with Koppel et al., (2013), who found that the most abundant group of volatiles in dry dog foods were the overall aldehydes, and these were also associated with sensory analysis descriptors, such as rancid aromatics. The SPME decreases purchase and disposal costs of solvents and improves detection limits of volatiles (Vas et al., 2004), but the initial cost of equipment may be the ultimate cost factor. Jacobsen (1999), suggested that GC-MS be employed with sensory analysis to better understand the impact of lipid oxidation on sensory and the oxidation mechanisms in complex food systems.

Sensory Analysis

Lipid oxidation is one of the many factors that affect the shelf life of various complex food products, including pet food (Jacobsen, 1999). Objectionable off-flavors and odors are created during the shelf life of a product when the formation of secondary oxidation products increases (Jacobsen, 1999). Pet food acceptability is often determined by sensory characteristics that include appearance, aroma, texture, and flavor (Koppel et al., 2014). Sensory analysis of pet foods can be measured by pets and focuses on choice, consumption, or behavioral characteristics prior to, during, or after consumption (Koppel et al., 2014). Humans can also evaluate sensory analysis of pet foods by describing their sensory properties, acceptance or preference testing by pet owners and food, or attitude-related testing through questionnaires (Koppel et al., 2014). It is difficult to draw a parallel between human sensory analysis and pets, as they do not have the same taste and flavor perception (Chaudhari et al., 2010; Thorne et al., 1992). Still, drawing conclusions from human sensory analysis has benefits, such as understanding pet food acceptance or rejection by both humans and pets and relaying that information to pet food manufacturers and animal scientists to develop future products (Koppel et al., 2014). Human

sensory analysis has been used to detect off-flavors and odors in various products in the study with Jacobsen (1996), who found that the sensory properties that correlate with oxidation depends entirely on the product in question. This variation in sensory properties may also extend to pet foods that have been formulated with various ingredients and inclusion levels. In recent years, a lexicon was developed by Di Donfrancesco et al. (2012) that describes the appearance, texture, aroma and flavor characteristics of dry dog food. The study focused on evaluating a wide range of dry dog food formulas that would provide a reasonable representation of various categories (Di Donfrancesco et al., 2012). All of the chosen samples or types of dog food varied by category such as, size, age, grain-free, oral care, weight control, and sensitive skin (Di Donfrancesco et al., 2012). Pickering (2008, 2009) demonstrated dry and canned cat foods were evaluated using a human sensory panel, but the descriptive analysis techniques employed were traditionally used for human food. The development of human sensory analysis and pet food will benefit product development in the pet food industry and help increase palatability in pet products; therefore, providing pets and their owners with quality food.

Antioxidants

To hinder lipid oxidation and the development of off-odors and flavors in pet foods, antioxidants are used to preserve the food by increasing shelf life and ensuring palatability (Tucker, 2004). The addition of antioxidants prior to cooking has proven to increase the stability of rendered products throughout the cooking process (Dugan et al., 1954). Dugan et al. (1954) also suggested that antioxidants be added after rendering to avoid loss of antioxidants by steam distillation during rendering. Antioxidants function to reduce the rate of oxidation by neutralizing free radicals through donation of a hydrogen or electron, binding co-factors that are needed for the formation of free-radicals such as iron, or functioning in enzyme systems within

cells (Hilton, 1989; Tucker, 2004). These functions can be accomplished by the use of natural preservatives such as, vitamin E and ascorbic acid, or synthetic preservatives such as, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and ethoxyquin.

Consumers often hold the quality of pet food to the same standards as human food, and prefer natural to synthetic ingredients (Tucker, 2004). Natural preservatives are not as stable as the synthetic form and have poor "carry through," meaning their function is reduced when the pet food is processed and subjected to heat, pressure, and moisture common during extrusion (Hilton, 1989). Synthetic ingredients, unlike natural preservatives have carry through potential, are efficient and cost effective, but usage rates are restricted and tightly controlled because of concerns of their use in foods (Hilton, 1989). The permitted usage rate of ethoxyquin is 0.015%, or 150 ppm, in animal feed, fish food, and canned pet food, while BHA and BHT have an upper limit of 0.02% in fat or oil content of food (FDA, 2014).

Summary

In conclusion, the pet food industry utilizes a significant amount of rendered protein meals produced in the US annually. Oxidative stability of these protein meals is vital. Unfortunately, there is limited peer-reviewed published data available regarding the stability of these protein meals intended for pet food applications; wherein, the ingredient would be incorporated into a processed food intended to be shelf-stable for up to 18 months. It has been identified that consumption of rancid foods by pets has a negative impact on the long-term health and wellbeing. Thus, there is need to evaluate these ingredients in a manner consistent with their use in pet foods. Therefore, the first objective of this thesis was to identity physical and compositional factors that might influence the consistency of lipid oxidation and to determine the conventional methods for quantification of this process in rendered protein meals relative to the

efficacy of rapid methods (e.g. SafTest). The second objective was to evaluate the level of oxidation in rendered protein meals on shelf life and acceptability in extruded pet foods.

Figure 1.1 Glycerol compound example (Voet et al., 2013).

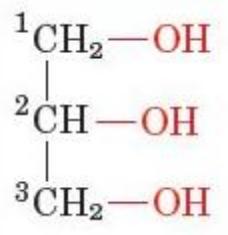


Figure 1.2 Triglyceride example (Voet et al., 2013).

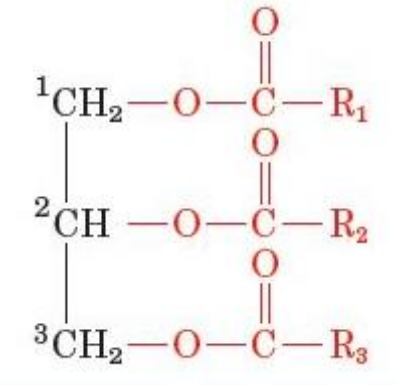


Figure 1.3 Glycerol with three fatty acid chains example (Voet et al., 2013).

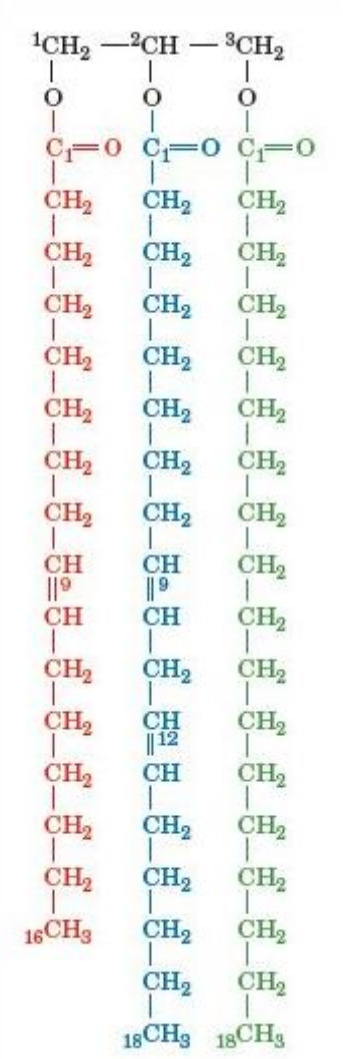


Figure 1.4 Fatty acid composition of different animal fats (AOCS, 2013).

Table 5: Properties of different animal fats

	C atoms	Beef tallow	Pork lard	Poultry fat
Melting point °C		40-50	34-44	23-40
Iodine value		25-45	45-75	65-75
Fatty acid composition				
Myristic acid	2-3	1-1.5	1-1.5	
Palmitic Acid	16	24-28	24-28	20-24
Stearic Acid	18	20-24	13-14	4-6
Saturated Fatty Acids %		46-55	38-43.5	25-31.5
Palmitoleic Acid	16	2-3	2-3	5-9
Oleic Acid	18	40-43	43-47	33-44
Linoleic Acid	18	2-4	8-11	18-20
Linolenic Acid	18	<1	<1	1-2
Unsaturated Fatty Acids %		45-51	54-62	57-75

Figure 1.5 Fatty acid profile of beef and pork fats (AOCS, 2013).

Table 6: Fatty acid profile of beef and pork fats

Species - Type of Fat (Location)	Fatty Acids [weight %]			
	16:0	18:0	18:1	18:2
Beef				
- Subcutaneous	24.6	11.1	46.6	1.7
- Intramuscular	24.7	18.3	42.4	1.9
- Kidney fat (suet)	25.0	29.2	33.5	1.5
Pork				
- Back fat	24.4	16.6	44.1	8.9
- Belly fat	25.5	16.8	41.3	8.2
- Intramuscular	26.0	15.4	43.0	7.3
- Leaf fat	28.5	20.1	37.2	7.3

Figure 1.6 Lipid oxidation process example (Akoh et al., 2008).

Initiation: $In^{\bullet} + RH \rightarrow InH + R^{\bullet}$

Propagation: $R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$

$ROO^{\bullet} + RH \rightarrow R^{\bullet} + ROOH$

Termination: $2ROO^{\bullet} \rightarrow O_2 + RO_2R$

$ROO^{\bullet} + R^{\bullet} \rightarrow RO_2R$

Chapter 2 - Evaluation of Methods Used to Analyze Oxidation of Rendered Protein Meals

Introduction

The pet food industry utilizes a significant quantity of the rendered protein meals produced in the Unites States annually. As a result, rendered protein meals often account for the majority of the protein used in extruded pet foods and provide high quality protein with a desirable balance of amino acids (Aldrich, 2006). Rendered protein meals have crude fat concentrations ranging from 10-25%, which may be subjected to oxidation (Aldrich 2006). It has been identified that consumption of rancid foods by pets, unlike production animals, has a negative impact on their long-term health and wellbeing (Turek 2003). Therefore, there is a need to evaluate the oxidative stability of these ingredients. Pets, unlike livestock, are fed for health and longevity, and their foods are commonly consumed months, rather than days, after production. Thus, extrapolation of permissible levels of oxidative rancidity from livestock feed research is not wholly applicable. Therefore, the objectives of this experiment were to determine the conventional methods for quantification of lipid oxidation in rendered protein meals relative to the efficacy of rapid methods (e.g. SafTest) and to identity physical and compositional factors that might influence their consistency.

Materials and Methods

Samples

Five samples of beef meat and bone meal (BMBM) were received from each of two locations and five samples of chicken byproduct meal (CBPM) were received from each of three locations. Each set of five samples were produced on different dates. Samples arrived in sealed 19 L buckets and plastic bags. They were then placed in freezers and held at 0°C until each

respective protein meal was analyzed. Due to freezer storage restrictions, the samples were transferred to plastic storage bags until analysis could be completed.

Sample Analysis

Samples were analyzed for proximate analysis, including moisture (AOAC Official Method 934.01), crude protein (AOAC Official method 990.03), crude fat (AOAC Official method 920.39), crude fiber (AOAC Official Method 978.10), and ash (AOAC Official Method 942.05), as well as p-anisidine value (AOCS Method Cd 18-90), thiobarbituric acid reactive substances (TBARS), peroxide value (AOCS Method Cd 8-53) via titration by two laboratories. Peroxide values were also measured via rapid method using the PeroxySafe™ Standard kit (MP Biomedical; Solon, OH). The original samples were stored in a freezer at 0°F for approximately five months and were reanalyzed for peroxide value (PV) and p-anisidine value (AV) prior to additional analysis of secondary oxidation products using the AldeSafe™ and AlkalSafe™ Standard kits (MP Biomedical; Solon, OH) . Due to lack of rendered protein meal from two of the three chicken byproduct meal locations, these samples were not included in the additional analysis of secondary oxidation products.

Fat Extraction from Rendered Protein Meals

Since there is no official method for extraction of oils from rendered protein meals for the determination of PV or AV, the procedure was modified from that described by Williams and Hron Sr. (1996). Modifications included approximately 200 g of the rendered protein meal which was weighed into a 1,000 mL beaker on a digital scale (Explorer: E1RW60, OHAUS, Parsippany, NJ) and to this 200 mL of hexane was added. The meal and hexane were allowed to mix for five minutes using a magnetic stir rod and a stir plate. Vacuum filtration then separated the meal from the oil and hexane. The filtration apparatus consisted of a standard laboratory

vacuum pump (DOA-V120-AE: Gast Manufacturing, Inc., Benton Harbor, Michigan), a vacuum hose, liquid trap, Büchner funnel, Erlenmeyer flask with hose adapter, and Whatman filter paper (grade: 41; Pittsburgh, PA). Once the hexane and oil were isolated, the mixture was transferred to a 1,000 mL roundbottom flask and attached to a rotating evaporator (Rotavap Büchi R-114: Brinkmann Instruments, Inc.) while partially submerged in a water bath (Büchi B-490: Brinkmann Instruments, Inc) at 50°C. The rotavap was used to gently evaporate the hexane from the oil. Approximately fifteen minutes later, the isolated oil was transferred to a 50 mL conical tube (BD Biosciences, San Jose, CA) and centrifuged (Sorvall Legend X1R: Thermo Fisher Scientific, Waltham, MA) at 5,000 rpm for fifteen minutes at 35°C. The isolated oil was then analyzed for PV and AV value.

Oxygen Absorption Test

Ten sub-samples of BMBM, five sub-samples of CBPM, and one sub-sample of turkey meal were analyzed for oxidative stability using the oxygen absorption method (AOCS Official Method Cd 12-57). The samples were sent to an external cooperating, commercial laboratory (Kemin Industries, Customer Laboratory Services, Des Moines, IA) in an insulated foam cooler on dry ice to hinder oxidation during shipment. The method measures the loss in oxygen pressure of a canister charged with a set amount (typically 3 bars) of oxygen that is suspended in an oil bath at 100°C. Transducers measure the change in pressure over a twenty-four hour period.

Volatile Compounds Measurement

Extraction Procedure of Volatile Compounds

Volatiles in dry dog foods were determined by headspace-solid phase microextraction (HS-SPME) as described by Koppel et al. (2013). Five sub-samples of BMBM received from each of two locations, five sub-samples of CBPM received from each of three locations, and one

sub-sample of turkey meal were analyzed in triplicate. Samples were ground through a 1 mm screen using a Wiley mill (Model 4: Thomas Scientific; Swedesboro, New Jersey), then 0.5 g of each was weighed into a 10 mL screw-cap vial with a polytetrafluoroethylene and silicone septum. To each vial, 0.98 mL distilled water was added to the ground sample. An internal standard was added consisting of 0.02 mL 1,3-dichlorobenzene (98%, Sigma Aldrich, St. Louis, MO, USA) dissolved in hexane (mixture of isomers, optima grade, Fisher Scientific; Pittsburgh, PA, USA), with final concentration in the sample of 0.2 mg/kg. The vials were equilibrated for 10 min at 40 °C in an autosampler (Pal system, model CombiPal, CTC Analytics, Zwingen, Switzerland) and agitated at 250 rpm. After reaching equilibrium, a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane fiber was exposed to the sample headspace for 30 min at 40 °C. The fiber method provides high capacity for trapping volatile compounds in food products (Ceva-Antunes 2006). After sampling, the analytes were desorbed from the SPME fiber coating prior to GC injection at 270 °C for 3 min in splitless mode.

Chromatographic Analyses

The isolation, identification, and semi-quantification of the volatile compounds were performed on a gas chromatograph (Varian GC CP3800; Varian Inc., Walnut Creek, CA, USA), coupled with a mass spectrometer (MS) detector (Varian: Saturn 2000). The GC-MS system was equipped with an RTX-5MS (Crossbond® 5% diphenyl/95% dimethyl polysiloxane) column (Restek, U.S., Bellefonte, PA, USA; 30 m x 0.25 mm x 0.25 μm film thickness). The initial temperature of the column was 40 °C held for 4 min; the temperature was then increased by 5 °C per min to 260 °C, and held at this temperature for 7 min. All samples were analyzed in triplicates. The quantities of volatile compounds were calculated against the internal standard peaks.

Most of the compounds were identified using two different analytical methods: 1) mass spectra (> 80%), and 2) Kovats indices (NIST/EPA/NIH Mass Spectral Library, Version 2.0, 2005). Identification was considered tentative when it was based on only mass spectral data. The retention times for a C7-C40 saturated alkane mix (Supelco Analytical, Bellefonte, PA, USA) was used to determine experimental Kovats indices for the volatile compounds detected.

Shelf-Life Challenge

Five sub-samples of BMBM from each of two locations, five sub-samples of CBPM received from one location, and one sub-sample of turkey meal were monitored for oxidation in shelf-life simulation. Wherein, approximately 400 grams of each protein meal, excluding samples of CBPM from each of two locations due to limited material, were weighed and subsampled into two small, brown, Kraft paper bags each. Samples were withdrawn at specified time increments (0, 2, 4, 8, 12, 18, 24, 32, 40 and 52 weeks) and were evaluated for PV and AV. For each sample, there were two brown paper bags per time. One bag was placed in ambient storage (~23°C, ~25% RH) and the other at an elevated temperature (36°C) and humidity (~52% RH). The samples kept at the elevated temperature were stored in an incubator (Thermo Scientific: Precision 818. Waltham, MA).

Particle Size Distribution

Particle size analysis was evaluated by standard Ro-Tap (Testing Sieve Shaker Model B: Combustion Engineering, Inc., Mentor, OH) according to the ASAE S319.4 method without a flow agent. Each sieve was cleaned, weighed, and recorded before the material was added. A 100 g sample of rendered protein meal was transferred onto the top sieve and the lid to the sieve stack was replaced. The sieve stack was then securely placed into the Rotap and allowed to operate for fifteen minutes. Each sieve had two agitators, a small rubber ball and a brush to aid particle

separation. After the allotted time, each sieve was weighed and the difference was determined from the original weight of the sieve. Data were evaluated as a plot of particle size relative to the weight accumulated. A determination was made regarding the average particle size, particles per gram, and surface area.

Bulk Density

From the original materials, ten BMBM, five CBPM, and one turkey meal sub-samples were analyzed in triplicate. The samples were removed from freezer storage and allowed to equilibrate to room temperature prior to analysis. The bulk density was determined using a standard Winchester cup setup (ASTM D6683-14) with a hopper, funnel, and leveling ruler (Seedburo Equipment Co., Chicago, Illinois). The technique used can be found in the 2005 version of Feed Manufacturing Technology V (American Feed Industry Association).

Angle of Repose

Ten sub-samples of BMBM, five sub-samples of CBPM, and one sub-sample of turkey meal were analyzed in triplicate. The samples were removed from freezer storage and allowed to equilibrate to room temperature prior to analysis. Approximately 300 g of each sample was used and new material was evaluated for each replicate. The angle of repose analysis used the same apparatus setup as the bulk density analysis, except a sheet of plexiglass replaced the Winchester cup. The plexiglass was placed horizontally and approximately ten centimeters below the funnel opening. A metal scoop was used to transfer each sample to a metal funnel that was suspended on a metal stand. Once the sample was measured and scooped into the funnel, the funnel door was opened allowing the sample to form a conical mound on the plexiglass. When the sample stopped flowing, the height (centimeters) of the conical mound and the diameter (centimeters) of the base of the pile (x- and y-axis) were measured using a ruler and recorded. The average

diameter was then calculated. The angle of repose was calculated by the arctangent of the height divided by the average diameter of the conical pile. After the measurements were recorded, the sample was placed aside and the funnel and plexiglass were wiped clean with a paper towel for the next test.

Tapped Density

Ten sub-samples of BMBM, five sub-samples of CBPM, and one sub-sample of turkey meal were analyzed in triplicate for density. The samples were removed from freezer storage and allowed to equilibrate to room temperature prior to analysis. The tapped density measurement was determined using an autotap (Quantachrome Instruments, Boynton Beach, FL). The analyzer requires the use of a 100 mL volumetric glass cylinder, which was cleaned and adequately dried prior to use. The top circumference of the cylinder was lined with transparent tape so that the final height of the tapped sample could be properly marked with a pen. The empty cylinder was weighed using a digital scale and recorded. The cylinder was then filled to the top with sample using a metal scoop. Excess sample from the top of the cylinder was cleared away with a ruler and secured on the autotap stand. The analyzer was then turned on and set to tap 750 times. After tapping, a pen was used to mark the final sample height around the circumference of the cylinder. The final weight of the sample and cylinder was recorded. The sample was then set aside and the cylinder was then wiped clean using a paper towel prior to the next replicate. This process was repeated for each replicate leaving a total of three pen lines on the transparent tape. When the replicates were finished, the cylinder was washed with soap and water. The cylinder was then filled with distilled water using a disposable pipette to the level of the pen line for each replicate. The weight of the cylinder and the distilled water were recorded. After the replicates for each sample were weighed, the distilled water was removed from the cylinder and the

cylinder was adequately dried. The used tape was removed and replaced around the circumference of the cylinder for the next sample.

Flow Property Measurement

One sample from each location was analyzed in triplicate for flow properties using a FT4 Powder Rheometer (Freeman Technology; Welland, UK). The flow properties measured were compressibility, stability and variable flow rate, shear, and wall friction. All flow property methods were performed as illustrated by Freeman Technology (Welland, UK).

Basic Flowability Energy (BFE)

The basic flowability energy (BFE) is defined as the energy that is required to establish a precise flow pattern for a specific volume of particulate materials (Bian et al., 2015).This test is used to measure the effect of size and distribution, shape, texture, density, etc on flow of a material. The BFE is calculated from the work done by moving the blade during a downward traverse through the material.

Stability Index (SI)

The stability test is a combination of conditioning and test cycles that are designed to evaluate if the material is going to change as a result of being made to flow. If there is any change in the flow energy during the test, it is a result of a change in the materials flow properties. The magnitude of this change quantifies the instability of powder. A 50 mm by 85 mL split vessel and 48.0 mm blade were used to measure the stability and variable flow rate for all samples.

Flow Rate Index (FRI)

The rate at which rendered protein meals are handled and moved within production, processing, and loading can vary greatly. Bulk material is sensitive to changes in flow rate, because it depends on properties that include particle size, cohesion, aeration, etc. Understanding the flow rate of material will help dismantle the process of conveying of rendered protein meals during handling and processing. The flow rate index is defined as the factor by which the flow energy is changed when the flow rate or tip speed is reduced by a factor of 10 (Freeman, 2005).

Compressibility

Compressibility measures how density changes in a material due to applied normal stress, which can be influenced by many factors such as particle size distribution, shape, texture, etc. This can also relate to many process environments, such as, handling, storage, and transportation. A 50 mm by 85 mL split vessel, 48.0mm blade, and vented piston were used to measure the compressibility of all samples.

Scanning Electron Microscope

A sub-sample of each CBPM and BMBM were analyzed using a scanning electron microscope (SEM) (S-3500N: Hitachi Science Systems, Ltd., Tokyo, Japan). A small quantity of material was mounted on an aluminum stub using a Pella Brand double-sided adhesive carbon tab (Redding, CA). The material was placed at one edge of the tab and then the stub was tilted and tapped to allow the sample to spread across the surface of the adhesive carbon tab. The excess sample was removed with a short burst of compressed air from a "duster" can. The samples were then sputter coated with an alloy of 60% gold and 40% palladium to a thickness of approximately four nanometers using a Desk II Sputter/Etch Unit (Denton Vacuum, LLC). This process is required to make non-metal material conductive, which allows the SEM to use

electrons to form an image of the sample. The images of BMBM and CBPM were magnified to 100 and 200 μm, respectively to achieve a resolution sufficient to make visual observations.

Statistical Analysis

Oxidative rancidity and stability

Results were summarized according to production facility and protein type among the 26 samples submitted. Variation among means was determined by product type. The data were analyzed as a completely randomized design and the means were separated by significant F values with $\alpha = 0.05$ of the Generalized Linear Model (GLM) procedure with the aid of statistical analysis software (SAS Institute, Inc., Cary, NC). As described in physical samples, turkey meal was excluded from statistical analysis, but was still reported for informational purposes. For the relationship between accelerated and ambient shelf-life evaluations, regression analysis was performed using statistical analysis software (SAS Institute, Inc. Cary, NC).

Particle Size, Bulk Density, Angle of Repose, and Tapped Density

All sample analysis was conducted in triplicate. Data were analyzed as a completely randomized design and means were separated by significant F values with $\alpha = 0.05$. Statistical analysis of experimental results was performed using the GLM procedure with the aid of SAS. Due to lack of replicate samples for turkey meal, this data were excluded from statistical analysis and simply included for informational purposes.

Results

Sample Analysis

Beef meat and bone meal (BMBM) sample B2 was slightly higher in protein than B1 (51.0% and 46.8%; $P < 0.0001$), but both BMBM samples were lower in protein than the

chicken by-product meal (CBPM) samples, respectively (table 2.1). The CBPM sample C1 was the highest in protein, but did not differ from C3 (70.7% vs. 68.4%; $P > 0.05$), while C2 was the lowest in protein, respectively (61.2%; $P < 0.0001$). The moisture of BMBM sample B1 did not differ from B2 (2.8% vs. 2.9%; $P > 0.05$) and was similar to CBPM samples C1 and C3, respectively (3.8% vs. 2.4%; $P > 0.05$). The CBPM sample C2 had the highest moisture than the other samples (4.0%; $P < 0.0001$), but did not differ from C1, respectively (13.2%; $P > 0.05$). The BMBM sample B1 was higher in fat than B2, respectively (11.9% vs. 8.4%; $P < 0.0001$). The CBPM sample C1 was the lowest in fat (13.2%; $P < 0.0001$) with C2 being intermediate (15.1%; $P < 0.0001$) and C3 being the highest, respectively (16.6%; $P < 0.0001$). The BMBM sample B1 was higher in fiber than B2, respectively (2.9% and 1.6%; $P < 0.0001$). The CBPM sample C1 was the lowest in fiber (0.3%; $P < 0.0001$), while C2 and C3 did not differ (1.1% vs. 1.0%; $P > 0.05$). The BMBM sample B1 was highest in ash than B2 (37.4% vs. 33.4%; $P < 0.0001$). The CBPM sample C1 and C3 did not differ in ash (10.5% vs. 10.5%; $P > 0.05$), but C2 was highest (15.4%; $P < 0.0001$). Due to limited resources and lack of replication for TM, this sample was included for informational purposes only.

The comparison of oxidation measures for the samples received can be found in Tables 2.2 and 2.3. In Table 2.2, the titration PV of material received from B1 did not differ from B2 (16.85 and 4.0 meq/kg; $P > 0.05$). Whereas, the titration PV of the CBPM C1 was greater (169.35 meq/kg; $P < 0.0001$) than either C2 or C3, but C2 and C3 did not differ from each other (2.66 and 2.22 meq/kg; $P > 0.05$). The TM had a titration PV of 6.7 mEq/kg, which was numerically the greatest PV recorded for turkey meal among the three PV methods. The lab 2 PV for location B1 was greater than that of location B2 (5.25 and 4.05 meq/kg; $P < 0.0001$) and the lab 2 PV for C1 was less (2.12 meq/kg; $P < 0.0001$) than either C2 or C3, but C2 and C3 did not

differ from each other (3.39 and 3.52 meq/kg; $P > 0.05$). The lab 2 PV for TM was 3.4 mEq/kg, which was different numerically from the other two PV methods. The SafTest PV for location B1 did not differ from B2 (21.64 and 1.39 meq/kg; $P > 0.05$). For CBPM, the SafTest PV for location C1 was greater (127.93 meq/kg; $P < 0.0001$) than either C2 or C3, but C2 and C3 did not differ from each other (0.49 and 0.47 meq/kg; $P > 0.05$). The SafTest PV for TM was 0.7 mEq/kg, which was numerically the lowest recorded PV for TM among the three PV methods. The AV of material received from B1 did not differ from B2 (3.57 and 0.57 g/g; $P > 0.05$). Whereas, the AV of C2 was less (0.30 g/g; $P < 0.0001$) than either C1 or C3, but C1 and C3 did not differ from each other (5.92 and 4.47 g/g; $P > 0.05$). The AV for TM was 0.64 g/g. The TBARS of material from location B1 differed from that of B2 (0.04 and 0.04 mg MDA eq/g oil; $P < 0.0001$). For CBPM, the TBARS of material from C1, C2, and C3 differed from each other (0.05, 0.07, and 0.07 mg MDA eq/g oil; $P < 0.0001$). The TBARS of TM was 0.1 mg MDA eq/g oil.

Lab 1 PV was well correlated with the SafTest PV method (R = 0.98), but was not correlated with lab 2 PV (R = 0.46; Table 2.3). The SafTest method was not correlated with lab 2 PV (R = 0.37). The AV method did not have a strong correlation with lab 1 PV (R = 0.61), SafTest PV (R = 0.63), or lab 2 PV (R = 0.22). The TBARS were not well correlated with lab 1 PV (R = 0.09), SafTest PV (R = 0.13), or lab 2 PV (R = 0.48). TBARS and AV were not correlated (R = 0.01).

Due to limited samples of rendered protein meal from sources C1 and C2, these samples were not included in the additional analysis of secondary oxidation products in Table 2.4. Comparing the initial analyzed samples (Table 2.2) to the frozen, five month old samples (Table 2.4), the PV and AV of source B1 increased, whereas source B2 decreased in PV and increased

in AV. The opposite effect occurred for source C3 as the PV increased and the AV decreased. In Table 2.4, the PV of B1 did not differ from B2 (43.4 and 3.5 meq/kg; $P > 0.05$), which also did not differ from the CBPM sample C3 (5.3 meq/kg; $P > 0.05$). The BMBM sample B1 AV was greater than B2 (9.5 and 2.9 g/g; $P < 0.0001$), but the CBPM sample C3 did not differ from the BMBM samples (4.1 g/g; $P > 0.05$). The BMBM sample B1 AldeSafe did not differ from B2 (0.7 and 0.5 μmol/mL) and the CBPM sample C3 did not differ from the BMBM samples (0.5 μmol/mL; $P > 0.05$). The BMBM sample B1 AlkalSafe was greater than B2 (1.8 and 0.3 nmol/mL; $P < 0.0001$), but B2 did not differ from the CBPM sample C3 (0.3 nmol/mL; $P > 0.05$).

The correlation coefficients for the relationships between oxidation measures for the meal samples are presented in table 2.5 for the second set of analysis. Initial data from Table 2.3 suggested that there was a weak correlation among PV and AV (R = 0.61). In this series, the PV and AV's have a strong correlation (R = 0.97). There were also strong correlations between PV and AldeSafe (R = 0.902); AlkalSafe (R = 0.89). The AV method was correlated with the AldeSafe method (R = 0.948) and the AlkalSafe method (R = 0.94). The AldeSafe method and the AlkalSafe method also had a strong correlation (R = 0.97).

Oxygen Absorption Test

In Figure 2.1, the induction period of the oxygen bomb results for the CBPM sample C3 absorbed the greatest amount of oxygen within the first hour of analysis with a decrease of 0.78 psi. The instability of sample C3 suggests that it may have been in the termination stage of oxidation resulting in a low PV and a high AV value (Table 2.2). Sample C3 also had the greatest overall pressure drop with a psi of 28.58, which coincides with the degree of unsaturation and percent of crude fat (Table 2.1) relative to the BMBM samples. The BMBM

sample B1 was highly oxidized and absorbed a great deal of oxygen within the first hour of analysis with a decrease of 5.31 kPa, which coincides with the high PV results in table 2.2. The TM sample had the third highest oxygen uptake with a decrease of 4.01 kPa within in the first hour, but had a total pressure drop of only 107.70 kPa. Turkey meal is similar in composition and unsaturation to CBPM and even had a higher percent of crude fat (20.7%; Table 2.1), but TM did not absorb as much oxygen; thus, suggesting that TM was stabilized better relative to CBPM. The PV and AV of TM also coincide with the oxygen bomb test (Table 2.2). The BMBM sample B2 absorbed the least amount of oxygen within the first hour (3.31 kPa) and overall (79.78 kPa), which coincides with the low PV and AV in Table 2.2.

Gas Chromatography/Mass Spectrometry

The hexanal concentration of material received from B1 did not differ from B2 (3,758.01 and 1,325.01 μg/kg; $P > 0.05$; Table 2.6). Whereas, the hexanal concentration of CBPM C1 was greater (9,201.95 μg/kg; $P < 0.0001$) than either C2 or C3, but C2 and C3 did not differ from each other (2,850.89 and 766.58 μg/kg; $P > 0.05$).The heptanal concentration of B1 was greater than B2 (981.34 and 313.90 μg/kg; $P < 0.0001$), but B1 did not differ from C1 and C3 (876.15 and 916.28 μg/kg; $P > 0.05$). The material from C1, C2 and C3 did not differ from each other (876.15 and 387.21 and 916.28 μg/kg; $P > 0.05$). The octanal concentration of B1 was greater than B2 (2,004.69 and 203.60 μg/kg; $P < 0.0001$), but B1 did not differ from C1 (1,084.33 μg/kg; $P > 0.05$). Whereas, the octanal concentration of material received from B2 did not differ from C1, C2 or C3 (203.60 and 1,084.33 and 256.09 and 53.83 μg/kg; $P > 0.05$) and the CBPM samples did not differ from each other ($P > 0.05$). The nonanal concentration of B1 did not differ from B2 or C1 (1,262.31 and 359.59 and 2026.40 μg/kg; $P > 0.05$), but B2 and C1 differed from each other ($P < 0.0001$). The B2 nonanal concentration of 359.59 μg/kg did not differ from C2 or

C3 (502.75 and 67.96 μg/kg; $P > 0.05$). The decanal concentration of B1 (11.43 μg/kg) was less than B2 (11.43 and 36.60 μg/kg; $P < 0.0001$), but did not differ from C1 (10.52μg/kg; $P > 0.05$). The decanal concentration of B2 did not differ from C2 (36.60 and 36.29 μg/kg; $P > 0.05$), but B2 was smaller than C3 (36.60 and 117.99 μg/kg; $P < 0.0001$).

Shelf Life Challenge

The PV of BMBM sample B1 decreased from time 0 to 52 weeks (43.42 to 6.21 meq/kg, respectively; Table 2.7) while held at an ambient temperature and relative humidity (23°C, 25% RH). The BMBM sample B2 increased from 3.54 to 7.96 meq/kg from 0 to 52 weeks. The PV of CBPM sample C3 decreased over the shelf life study from 5.30 to 3.54 meq/kg, respectively. The TM sample T1 increased in PV from 2.22 to 4.43 meq/kg, respectively. In table 2.8, the AV of BMBM from location B1 decreased from time 0 to 52 weeks (8.33 to 7.02 meq/kg, respectively), but the AV increased for B2, C3, and T1 over time (2.90 to 6.35 g/g; 4.13 to 4.79 g/g; and 5.94 to 7.62 g/g, respectively). These results agree with the oxygen bomb test in figure 2.1 in that samples B1, C3 and T1 were the least stable and either have a high PV and AV or a low PV and high AV; whereas sample B2did not high PV and AV until 32 weeks.

The PV of BMBM sample B1 decreased from time 0 to 52 weeks (43.42 to 4.88 meq/kg, respectively; Table 2.9) while held at an accelerated temperature and relative humidity (35°C, 52% RH). The PV of BMBM sample B2 increased during the 52 week shelf life from 3.54 to 21.72 meq/kg. The PV of CBPM sample C3 decreased from 5.30 to 4.88 meq/kg from time 0 to 52 weeks. The PV of TM sample T1 increased from 2.22 to 6.65 meq/kg during the shelf life study. In table 2.10, the AV of BMBM sample B1 decreased from 9.46 to 8.65 g/g during the shelf life, respectively. The AV of BMBM sample B2, CBPM sample C3, and TM sample T1 increased from time 0 to 52 weeks (2.90 to 15.02 g/g; 4.13 to 10.44 g/g; 5.94 to 11.39 g/g,

respectively. These results agree with the oxygen bomb test in figure 2.1 in that samples B1 and C3 were the least stable and either have a high PV and AV or a low PV and high AV; whereas sample B2 did not have high PV and AV until 18 weeks. Interestingly, the sample T1 had a high PV at 32 weeks and a high AV at 52 weeks, which does not agree with the stability of T1 in the oxygen bomb test in Figure 2.1 that was more equivalent in stability to sample B1.

Particle Size Distribution

The particle size of material received from B1 did not differ from B2 (522.40 and 462.20; $P > 0.05$; Table 2.11). Whereas, the CBPM particle size from C1 was smaller (458.20; $P < 0.0001$) than either C2 or C3 but, C2 and C3 did not differ from each other (532.80 and 544.80; $P > 0.05$). The particles per gram for location B1 was less than B2 (28,634.00 and 46,285.00; $P \leq 0.05$) and particles per gram for location C1 differed from C2 and C3 (115.52 and 96.32 and 94.56; $P \leq 0.05$), but C2 and C3 did not differ from each other ($P > 0.05$). The surface area for B1 was less than that of B2 (105.12 and 120.14 cm^2/g; $P \leq 0.05$). Among the CBPM, the surface area for C1 was greater than that of C2 and C3 (115.52 and 96.32 and 94.56 cm^2/g; $P < 0.0001$), but C2 and C3 did not differ from each other ($P > 0.05$). Due to limited resources, the CBPM from locations C1 and C2 were excluded from the bulk density, tapped density, and angle of repose analysis. The bulk density for B1 was less than B2 (631.48 and 659.22 kg/m^3; $P < 0.0001$) and C3 had the lowest bulk density (515.40 kg/m^3; $P < 0.0001$). The tapped density for B1 did not differ from B2 (318.48 and 325.68 kg/m^3; $P > 0.05$) and the CBPM sample C3 had the lowest tapped density (262.53 kg/m^3; $P < 0.0001$). The angle of repose for B1 did not differ from B2 (32.38 and 33.87°; $P > 0.05$), but the CBPM sample C3 had the highest angle (38.39°; $P < 0.0001$).

Flow Property Measurements

The basic flowable energy was greatest for the BMBM samples compared to the CBPM samples (Table 2.12). The stability index varied slightly between the BMBM samples, but were similar between both the BMBM and CBPM samples (Table 2.12). The compressibility percentage was greatest for CBPM compared to the BMBM samples (Figure 2.2).

Discussion

Sample Analysis

Proximate analysis varied within samples and, as expected, between rendered protein meal types (Table 2.1). The variability within each type of rendered protein meal suggests that there was a difference in rendering methods, processing technology, or conditions like temperature, time, or pressure. This variability is consistent with previous studies by Johnson et al. (1997) and Johnson et al. (1998), where processing conditions had an effect on nutrient compositions. The crude protein values for the BMBM sample B2 was consistent with the industry standard of about 50%, but B1 was lower than the standard. The variability within CBPM samples for crude protein was likely due to the grade of CBPM received; Dozier et al., (2005) found that pet food-grade CBPM generally has higher crude protein of about 66.6% than that of feed-grade CBPM of approximately 60.0%. All CBPM samples were in accordance with and higher in crude protein than the industry standard of 58% (National Renderers Association, 2006). The moisture content of the BMBM samples are consistent with the moisture content of meat and bone meal in the study with Johnson et al., (1997) of 2 to 4%. Although, the BMBM samples were significantly lower in moisture than the industry standard of 7.5% (National Renderers Association, 2006). The moisture content of the CBPM samples varied numerically, but they were all less than the samples analyzed in the study by Johnson et al., (1997) with 4%

moisture content. The CBPM samples were also lower in moisture than the industry standard of 6.5% (National Renderers Association, 2006). The crude fat of BMBM sample B1 was above the industry standard of 10%, and was comparable to the average crude fat content of 12% of twelve samples in the study by Adedokun et al., (2005), but sample B2 was slightly lower (National Renderers Association, 2006). The crude fat of the CBPM samples were higher than the industry standard of 12%, but were agreement with the CBPM samples analyzed in the studies by Dozier et al., (2005), Johnson et al., (1997), and Cramer et al., (2007; National Renderers Association, 2006). The crude fiber content of the BMBM samples was lower than the industry standard of 3% (National Renderers Association, 2006; Auvemann et al., 2004). The CBPM samples were also lower in crude fiber than the industry standard of 2.1% (National Renderers Association, 2006). The ash content of the BMBM samples was higher than the industry standard of 28.8% and would be classified as a "high ash" product in the study with Johnson et al., (1997; National Renderers Association, 2006). The CBPM samples were lower than the industry standard of 18% (National Renderers Association, 2006). Although, the CBPM samples C1 and C3 would be classified as a "regular" ash product in the study with Cramer et al., (2007) and C2 may be classified as a "high" ash product in the study with Johnson et al., (1997).

The results of the oxidation measurements of rendered protein meals in table 2.2 varied within samples, between rendered protein meal types, and between methods. The goal was to demonstrate this variability of oxidation measurements between methods, samples, and laboratories. Laboratory 1 and 2 measured the peroxide values of all rendered protein meal samples using the same AOCS Official Method (Cd 8-53) that is commonly used in food testing laboratories (MP Biomedical, 2014), but the results were quite different. This research supports the claims of inconsistencies between laboratories of peroxide value analysis of rendered protein

meals as it is a common dilemma between the pet food and rendering industries. A large portion of the published research compares the AOCS official PV method (Cd 8-53) to alternative methods for measuring PV, but research is lacking in comparing how variable the official method is between laboratories. The official method states that it "is highly empirical and that any variation in the test procedures may result in variation of results," which suggests that variation in results is bound to take place between laboratories (AOCS, 1998). This would appear to be true based on our results. Although in the study by Shantha et al., (1994), it is suggested that the official method is reliable, reproducible, and gives low coefficients of variation, which may be true within a laboratory, but may differ compared to another. In regards to variation, Guillen et al., (2002) found that the variations of PV determinations were small when the samples had low PV values, but the variation grew as PV increased. The coefficient of variation within the official method has been found to be as high as 10 and 9% (Guillen et al., 2002; Van de Voort et al., 1994) and as low as 1.1% (Miller et al., 1988) within laboratories.

The SafTest™ had a similar peroxide value and variation to that of laboratory 1, but did not have a similar PV to that of laboratory 2. Again, this supports the inconsistencies in peroxide values reported by quality control laboratories in the industry. The SafTest™ (MP Biomedical; Solon, OH) method correlates well with the AOCS method (R = 0.97 to 0.99) cited by MP Biomedicals and in the study with Yildiz et al., (2003) with soybean oil. The SafTest™ (MP Biomedical; Solon, OH) method also states the coefficient of variation is about 6% for meals, which is lower than the high coefficient of correlation of 9-10% in the study with Guillen et al. (2002) and Van de Voort et al. (1994). Even though the SafTest™ (MP Biomedical; Solon, OH) method has a high correlation with the AOCS official method (Cd 8-53) there is still a slight source of error which contributes to the numerous sources of errors in the official method. These

errors account for the overall variability between results of peroxide value measurements between laboratories, which could result in the acceptance or rejection of a source of rendered protein meal by the pet food company.

The AV is often utilized in conjunction with PV to determine the total oxidation, because a single test is not necessarily conclusive (Guillen et al., 2002). This would suggest a positive relationship between PV and AV. We found the correlation coefficient of AV versus laboratory 1 and 2 and the SafTest™ were not as closely correlated as originally thought. In the study with Gotoh et al., (2007), AV and PV was found to have a coefficient of correlation of R = 0.79 when analyzing the oxidation of fats and oils in instant noodles. This lack of correlation may be linked to how empirical the AOCS official methods for AV (Cd 18-90) and PV (Cd 8-53) are. In the study with Osawa et al., (2008), it is suggested that an interference with water in the reagents can give a skewed result even after the purification of all reagents prior to analysis. Osawa et al., (2008) also reported a coefficient of variation of 6.5 to 900 percent using the AOCS official method on lipid contents of pet food samples. This significantly affects the reproducibility within laboratories.

The TBA or TBARS method is commonly used in the meat industry due to its speed and simplicity of use (Gomes et al., 2003). This assay analyzes for secondary oxidation products such as, malondialdehydes (Pikul et al., 1983, 1989; Salih et al., 1987; Gomes et al., 2003, Juntachote et al., 2006). In the review by Fernandez et al., (1997), it was concluded from research by others that various factors affect the extent and amount of malondialdehydes that are formed by the oxidation of polyunsaturated fatty acids such as, the degree of unsaturation of the fatty acid, the presence of metals, pH, and the temperature and duration of heating during the TBA assay (Dahle et al., 1962; Gray, 1978). Other substances besides aldehydes, including

malondialdehydes, can react with TBA and are referred to as TBARS and include, ketones, ketosteroids, acids, esters, sugars, imides and amides, amino acids, oxidized proteins, pyridines, and pyrimidines (Guillen-Sans et al., 1998). This suggests that the TBARS assay is not as specific as some of other methods that have been deployed in determining TBA by ultraviolet spectrophotometry (Kwon et al,. 1963), HPLC (Bird et al., 1983; Csallany et al., 1984), and gas chromatography-mass spectrometry (Luo et al., 1995; Liu et al., 1997). Liu et al., (1997) states that the TBA assay is useful, because of its simplicity and sensitivity, but only if the analyst clearly understands it non-specificity. The TBA assay has been directly compared to the peroxide value in the study with Dahle (1962) where the two analyses correlated well when the peroxides were only produced from fatty acids containing three or more double bonds. This would suggest that the malondialdehydes produced depends on the type of fat or the type of fat in the food being analyzed. This may explain the poor correlation of TBARS with the results of the other analyses (Table 2.3).

MP Biomedical designed other kits to substitute for the official methods of determining AV (AlkalSafe™) and TBA (AldeSafe™) values with quick, easy procedures that required less toxic solvents and compact equipment that could be utilized in small areas, such as a quality control laboratory. In Table 2.4 and 2.5, the AV correlated well with the AlkalSafe™ test, which was higher than the correlation coefficient of R = 0.74 in the study with Osawa et al., (2005) whom evaluated the rapid method with frying oils. There was a stronger AV correlation with the AldeSafe™ test than the AlkalSafe™ in Table 2.5 even though the tests are supposed to be analyzing for different oxidation products, α- and β- unsaturated aldehydes and malondialdehydes; although, malondialdehydes are classified as a type of aldehyde, which may have resulted in an overlap of analysis. This correlation may also suggest that either the

AlkalSafe™ or AldeSafe™ method may suffer from non-specificity as does the TBA assay. The AldeSafe™ method had a strong correlation with TBA in the study with Foo et al., (2006) of R = 0.92 when analyzing frying fats. Osawa et al., (2008) and Foo et al., (2006) would recommend the utilization of the rapid methods, AlkalSafe™ and AldeSafe™, due to their strong correlation with the AOCS official methods (Cd 18-90 and Cd 19-90).

Oxygen Absorption Test

The oxygen bomb test measures the stability of oils and fats and the efficacy of antioxidants as in the study with Barlow et al. (1989), whom evaluated the use of various levels of antioxidants that were applied to fish meal and their stability. They found that the fish meal that was not adequately preserved absorbed more oxygen and had a higher total pressure drop during the analysis relative to fish meal that had been properly preserved. In the study with Yagi (1970), the peroxide value of safflower oil was measured and as it increased, the time required for consumption of oxygen decreased.

Gas Chromatography/Mass Spectrometry

The aldehydes that were measured using GC-MS ranged in the number of carbon atoms from 6 to 10, such as hexanal, heptanal, octanal, nonanal, and decanal, because they are important contributors to rancid and unpleasant flavors and odors in oxidized oils (Frankel et al., 1985; DeHaan et al., 2004). It was found in the study with DeHaan et al. (2004) that hexanal, heptanal, octanal, and nonanal compounds were consistently detected in animal fats, but decanal was not always found. This would support the low values of decanal that are found in the BMBM and CBPM samples in Table 3.4 relative to the other volatile compounds. In two studies by Greenberg (1981), the volatile aldehydes of meat and bone meal and poultry by-product meal were identified in order of decreasing abundance as hexanal, heptanal, octanal, nonanal, and

decanal; although, decanal was not identified in the poultry by-product meal. Hexanal was identified as the aldehyde in the most abundance, representing about 43% of the overall relative concentration in meat and bone meal and 40% in poultry by-product meal (Greenberg, 1981). These aldehydes have been identified as contributors to the flavor of cooked beef and are more concentrated in beef and chicken meat that are uncured versus cured (Ramarathnam et al., 1993; Elmore et al., 1999). It was also suggested by Ramarathnam et al. (1993) that several factors, both pre-slaughter and post-mortem, have an effect on the compounds formed in meat products, such as the type of feed, storage and sanitation conditions, and processing methods.

Tompkins et al. (1999), the hexanal and heptanal concentrations found in soybean frying oil had a high coefficient of correlation with *p*-anisidine value (R = 0.81, $P = 0.0001$ and R = 0.66, $P = 0.0009$), but *p*-anisidine value was not significantly correlated with nonanal (R = 0.33, $P = 0.1299$). Hexanal and heptanal are breakdown products of linoleic acid and linolenic acid; whereas nonanal is only a breakdown product of oleic acid. Additional analysis of the poor correlation between nonanal and *p*-anisidine should be further studied. Similar to the *p*-anisidine value and peroxide value relationship, the hexanal concentration of a food could be utilized in conjunction with peroxide value to identify both primary and secondary oxidation products.

Shelf Life Challenge

The rate of hydroperoxides formed in the initiation and propagation phase of oxidation forms a sigmoidal curve as in the study with Juntachote et al., (2006) whom analyzed the PV of cooked ground pork treated with various antioxidants during a 14 day shelf life study. This result is similar to the data in Tables 2.7 and 2.9 in that the PV of sample B2 illustrates the initiation and propagation stage of oxidation. The sample B1 in table's 2.7 and 2.9 was in the termination stage of oxidation during analysis; thus, the decreasing PV's and high AV's. The termination

stage is further demonstrated by Zlatkevich, (2002). The CBPM sample C3 may have been considerably past the termination phase of oxidation; hence, the low PV's and the high AV's in the Tables 2.7 through 2.10. The TM sample T1 PV and AV results in Table 2.7 through 2.10 do not agree with the oxygen bomb test that proved the instability of the sample, yet oxidized thereafter B2 in the accelerated storage (table 2.9 and 2.10).

Particle Size Distribution

The particle size of 18 samples of meat and bone meal in the study with Garcia et al., (2006), had a geometric mean diameter range of 256 to 800 μm (Table 2.11). The CBPM samples also fell within this range. The differences between the types of rendered protein meals, BMBM and CBPM, and their respective particle size analyses are attributed to the type of rendering system and the processing parameters used, so differences between these analyses are inevitable. Identifying the particle size, particles per gram, and the surface area of the material being produced at a rendering facility may aid in the antioxidant techniques used to stabilize these materials. The bulk density of the meat and bone meal samples in Table 2.11 were greater than the average meat and bone meal samples in the study with Garcia et al. (2006) of 500 kg/m^3, but the CBPM sample displayed a comparable measurement. The high bulk density measurements of the BMBM may be due to the high levels of ash compared to the CBPM sample (Mendez et al., 1998). The angle of repose of the BMBM samples was smaller than the CBPM sample which correlates with particle size and density.

Flow Property Measurements

The BFE value of BMBM was not significantly different than the CBPM sample, but the average value of CBPM indicated that the flow properties would be better than that of the BMBM (Table 2.12). Both the BMBM and CBPM samples had average flow rate sensitivities

because the FRI value was less than 1.5, which signifies that rendered protein meals are not extremely cohesive (Leturia et al., 2014; Bian et al., 2015). The SI was slightly over 1 within both BMBM and CBPM samples, but they are considered normal for a stable material; thus, an SI above or below this range would be considered as an unstable material (Bian et al., 2015). The compressibility testing results reveal that the percentage change in volume increased with normal stress applied for BMBM and CBPM samples (figure 2.2). Finer particles within the CBPM sample could have resulted in the higher compressibility (Roopwani et al., 2011). The CBPM compressibility increased at a higher rate than the BMBM samples, which may indicate that higher consolidation pressures will lead to potential flow problems in CBPM (Onwulata et al., 1996). The flow properties of the rendered protein meals may give more insight in how to properly preserve these ingredients.

Scanning Electron Microscope

The corresponding scanning electron micrographs of BMBM and CBPM are pictured in Figures 2.3 and 2.4, respectively. The scanning electron micrographs are revealing the non-uniform topography of the particulate in the rendered protein meals. One can discern various components such as bone and upon very close inspection can see “streams” of solidified fat. It also points out that there are a multitude of fissures and crevices where preservatives (and antimicrobials) might be challenged to penetrate. This doesn’t provide resolution to the challenges itself, but may inspire more creative ways to approach the problem than has been deployed in the past.

Summary

The beef meat and bone meal samples were slightly lower in protein and fat when compared to the chicken by-product meal, which is likely due to the higher than anticipated

levels of ash. When these meals were evaluated for peroxide value the data recorded for the three methods were inconsistent, which confirms the inconsistencies reported between quality control laboratories in the industry. The peroxide value from laboratory 1 was well correlated with the SafTest™ peroxide value method, but neither of these methods correlated well with the peroxide value method from laboratory 2. There is a need for additional evaluation of the methods used to determine oxidative stability of rendered protein meals. The oxygen bomb and GC/MS may also be used to better understand the extent of lipid oxidation, but these methods are not often used in small quality control laboratories, because they are not quick methods of analysis. The particle size, flow properties, and SEM images may provide a better understanding of how to preserve this ingredient.

Table 2.1 Proximate analysis of beef meat and bone meal (BMBM), chicken by-product meal (CBPM), and turkey meal (TM) samples received for comparative oxidative composition.

	BMBM		CBPM				TM
Supplier/Source	B1	B2	C1	C2	C3		T1
N=	5	5	5	5	5	**SEM**	1
Crude Protein, %	46.8^{d}	51.0^{c}	70.7^{a}	61.2^{b}	68.4^{a}	0.973	61.8
Moisture, %	2.8^{bc}	2.9^{bc}	3.8^{ab}	4.0^{a}	2.4^{c}	0.357	1.9
Crude Fat, %	11.9^{d}	8.4^{e}	13.2^{c}	15.1^{b}	16.6^{a}	0.442	20.7
Crude Fiber, %	2.9^{a}	1.6^{b}	0.3^{d}	1.1^{c}	1.0^{c}	0.143	1.0
Ash, %	37.4^{a}	33.4^{b}	10.5^{d}	15.4^{c}	10.5^{d}	0.804	14.2

^{abcd} Means within a row that lack a common superscript differ $P \leq 0.05$.

Table 2.2 Comparison of oxidation measures in various samples of beef meat and bone meal (BMBM), chicken by-product meal (CBPM), and turkey meal (TM).

	BMBM		CBPM					TM
Supplier/Source	B1	B2	C1	C2	C3			T1
N=	5	5	5	5	5	**SEM**	*P*-Value	1
Lab 1 Peroxide Value (mEq/kg)	16.85^{b}	4.0^{b}	169.35^{a}	2.66^{b}	2.22^{b}	33.957	0.0100	6.65
Lab 2 Peroxide Value (mEq/kg)	5.25^{a}	4.05^{b}	2.12^{d}	3.39^{c}	3.52^{c}	0.078	<0.0001	3.40
SafTest Peroxide Value (mEq/kg)	21.64^{b}	1.39^{b}	127.93^{a}	0.49^{b}	0.47^{b}	32.580	0.0500	0.70
p-Anisidine Value (g/g)	3.57^{ab}	0.57^{bc}	5.92^{a}	0.30^{c}	4.47^{a}	1.057	0.0100	0.64
TBARS (mg MDA eq/g oil)	0.04^{e}	0.04^{d}	0.05^{c}	0.07^{b}	0.07^{a}	0.0023	<0.0001	0.06

^{abcde} Means within a row that lack a common superscript differ $P \leq 0.05$.

TBARS – 2-Thiobarbituric Acid Reactive Substances

Table 2.3 Coefficient of correlation analysis (Pearson) of methods used to determine oxidative stability of rendered protein meals, respectively.

	Lab 1 Peroxide Value	SafTest Peroxide Value	Lab 2 Peroxide Value	p-Anisidine	TBARS
Lab 1 Peroxide Value (meq/kg)	-	0.98*	0.46*	0.61*	0.09
SafTest Peroxide Value (mEq/kg)		-	0.37*	0.63*	0.13
Lab 2 Peroxide Value (mEq/kg)			-	0.22*	0.48*
p-Anisidine (g/g)				-	0.01
TBARS (mg MDA eq/g oil)					-

* Coefficient of correlation significant at $P < 0.05$.

TBARS – 2-Thiobarbituric Acid Reactive Substances

Table 2.4 Comparison of oxidation measures of various samples of beef meat and bone meal (BMBM) and chicken by-product meal (CBPM).

	BMBM		CBPM		
Supplier/Source	B1	B2	C3		
N=	5	5	5	**SEM**	***P*-Value**
Peroxide Value (mEq/kg)	43.4	3.5	5.3	13.693	0.107
p-Anisidine Value (g/g)	9.5	2.9	4.1	2.000	0.085
AldeSafe (µmol/mL)	0.7	0.5	0.5	0.080	0.158
AlkalSafe (nmol/mL)	1.8[a]	0.3[b]	0.3[b]	0.365	0.021

[ab] Means within a row that lack a common superscript differ $P \leq 0.05$.

Table 2.5 Correlation of oxidation measures of various samples of beef meat and bone meal (BMBM) and chicken by-product meal (CBPM).

	Peroxide Value	p-Anisidine	AldeSafe	AlkalSafe
Peroxide Value (meq/kg)	1.000	0.978	0.902	0.893
p-Anisidine (g/g)	-	1.000	0.948	0.940
AldeSafe (µmol/mL)	-	-	1.000	0.965
AlkalSafe (nmol/mL)	-	-	-	1.000

* Coefficient of correlation significant at $P < 0.05$.

Figure 2.1 Oxygen absorption test of beef meat and bone meal (BMBM) from locations B1 and B2, chicken by-product meal (CBPM) from location C3, and turkey meal (TM) from one location, respectively.

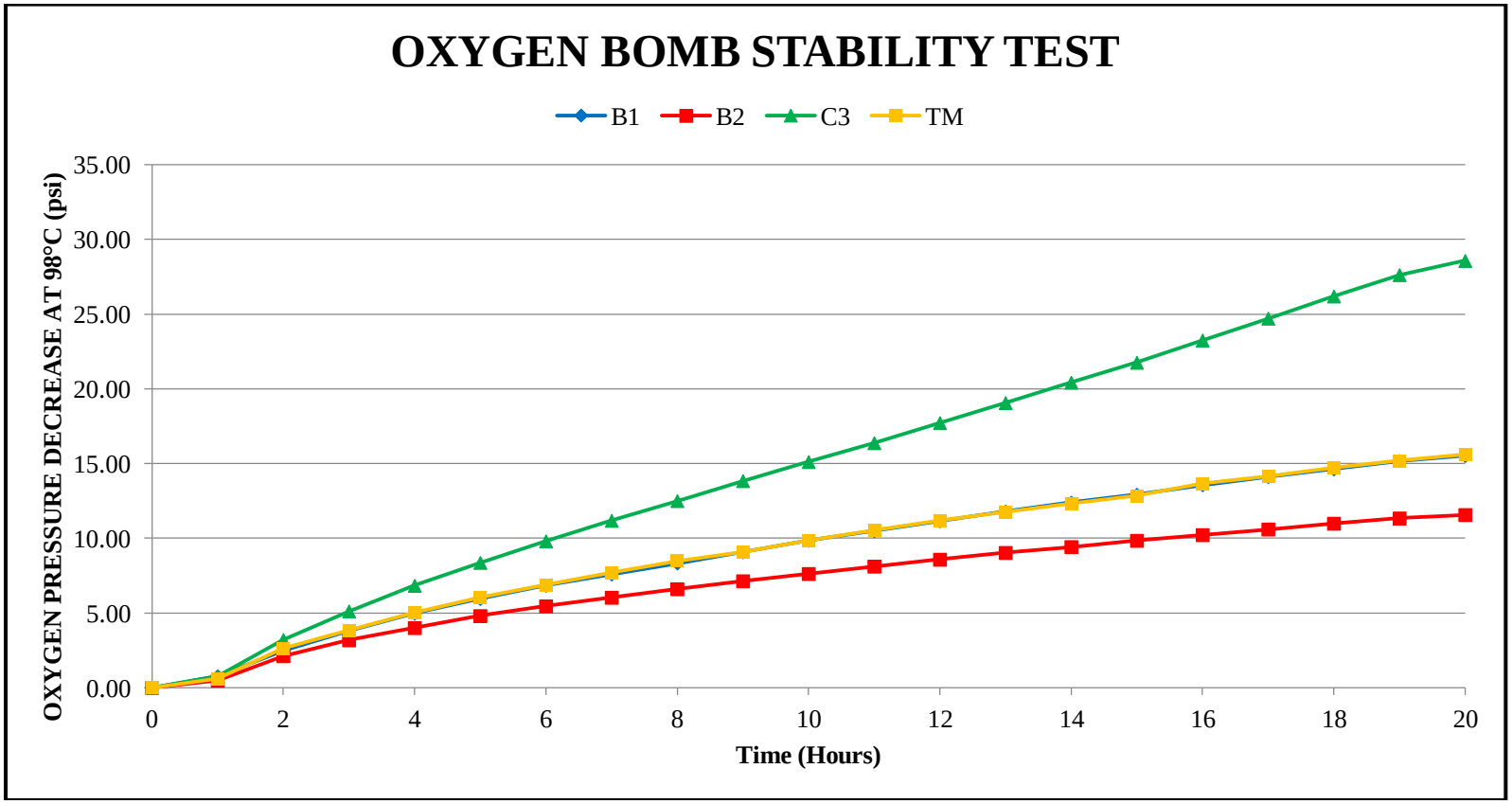


Table 2.6 Concentration of volatile compounds via gas chromatography (GC) analysis of beef meat and bone meal (BMBM) and chicken by-product meal (CBPM) from 2 and 3 locations, respectively.

	BMBM		CBPM				
Supplier/Source	B1	B2	C1	C2	C3		
# of Samples Received	5	5	5	5	5	SEM	*P*-Value
Hexanal (µg/kg)	3,758.01^{b}	1,325.03^{b}	9,201.95^{a}	2,850.89^{b}	766.58^{b}	1,258.508	0.0010
Heptanal (µg/kg)	981.34^{a}	313.90^{c}	876.15^{abc}	387.21^{bc}	916.28^{ab}	196.525	0.0662
Octanal (µg/kg)	2,004.69^{a}	203.60^{b}	1,084.33^{ab}	256.09^{b}	53.83^{b}	434.989	0.0234
Nonanal (µg/kg)	1,262.31^{ab}	359.59^{bc}	2,026.40^{a}	502.75^{bc}	67.96^{c}	395.568	0.0147
Decanal (µg/kg)	11.43^{c}	36.60^{b}	10.52^{c}	36.29^{b}	117.99^{a}	5.623	<0.0001

^{abc} Means within a row that lack a common superscript differ $P \leq 0.05$.

Table 2.7 Analysis of primary oxidation measures (mean ± Sd) on ambient (23°C, 25% RH) storage of rendered protein meal samples beef meat and bone meal (BMBM), chicken by-product meal (CBPM), and turkey meal (TM).

	Time (Weeks)	BMBM		CBPM	TM*
Supplier/Source		B1	B2	C3	T1
# of Samples Received		5	5	5	1
Peroxide Value (meq/kg)	0	43.42 ± 49.974	3.54 ±1.133	5.30 ± 1.168	2.22
	2	39.49 ± 38.327	3.56 ±1.146	4.01 ± 2.230	4.45
	4	40.78± 38.566	4.44 ± 0.015	4.44 ± 1.477	4.44
	8	36.80 ± 33.529	3.53 ± 1.131	5.32 ± 1.869	2.22
	12	35.99 ± 31.433	4.88 ± 0.933	3.99 ± 0.933	4.43
	18	34.96 ± 29.917	5.31 ± 1.867	4.43 ± 2.081	2.23
	24	26.60 ± 20.057	5.32 ± 1.147	5.33 ± 1.874	2.22
	32	13.95 ± 11.120	13.76 ± 20.845	3.55 ± 1.720	4.44
	40	8.42 ± 6.334	9.76 ± 12.425	3.55 ± 1.874	2.21
	52	6.21 ± 2.293	7.96 ± 7.445	3.54 ± 1.140	4.43

* Due to limited resources and replicate samples, turkey meal is included for informational purposes only.

Table 2.8 Analysis of secondary oxidation measures (mean ± Sd) on ambient (23°C, 25% RH) storage of rendered protein meal samples beef meat and bone meal (BMBM), chicken by-product meal (CBPM), and turkey meal (TM).

	Time (Weeks)	BMBM		CBPM	TM*
Supplier/Source		B1	B2	C3	T1
# of Samples Received		5	5	5	1
p-Anisidine Value (g/g)	0	8.33 ± 7.859	2.90 ± 0.808	4.13 ± 0.378	5.94
	2	7.80 ± 5.959	2.70 ± 1.244	3.60 ± 1.064	2.95
	4	6.38 ± 5.214	2.77 ± 1.271	3.23 ± 0.640	1.24
	8	6.74 ± 4.217	2.22 ± 0.371	3.06 ± 0.989	0.01
	12	3.62 ± 2.563	1.04 ± 0.390	2.89 ± 1.289	-
	18	10.54 ± 5.988	3.37 ± 0.527	5.34 ± 0.303	7.32
	24	7.80 ± 4.990	3.31 ± 0.673	3.45 ± 0.360	4.01
	32	7.87 ± 5.201	5.25 ± 2.193	5.02 ± 0.498	8.28
	40	4.44 ± 2.243	5.25 ± 1.509	6.88 ± 3.680	-
	52	7.02 ± 3.178	6.35 ± 1.761	4.79 ± 0.242	7.62

* Due to lack of replicate samples, turkey meal is included for informational purposes only.
- Unreadable sample.

Table 2.9 Analysis of primary oxidation measures (mean ± Sd) on accelerated (35°C, 52% RH) storage of rendered protein meal samples beef meat and bone meal (BMBM), chicken by-product meal (CBPM), and turkey meal (TM).

	Time (Weeks)	BMBM		CBPM	TM*
Supplier/Source		B1	B2	C3	T1
# of Samples Received		5	5	5	1
Peroxide Value (meq/kg)	0	43.42 ± 49.422	3.54 ± 1.133	5.30 ± 1.168	2.22
	2	25.76 ± 22.974	4.89 ± 1.753	4.89 ± 2.738	2.22
	4	22.27 ± 18.818	3.55 ±1.140	4.44 ± 2.095	6.67
	8	15.98 ± 13.665	3.55 ± 1.150	4.87 ± 0.933	4.44
	12	12.42 ± 9.801	4.44 ± 1.480	3.55 ± 1.142	4.43
	18	10.65 ± 5.814	11.97 ± 3.832	4.43 ± 0.012	6.64
	24	8.00 ± 4.356	35.99 ± 11.628	3.99 ± 1.745	11.13
	32	6.20 ± 4.020	39.08 ± 28.225	5.76 ± 1.140	17.69
	40	6.20 ± 1.750	31.00 ± 18.256	3.98 ± 0.930	11.08
	52	4.88 ± 0.938	21.72 ± 13.825	4.88 ± 2.734	6.65

* Due to lack of replicate samples, turkey meal is included for informational purposes only.

Table 2.10 Analysis of secondary oxidation measures (mean ± Sd) on accelerated (35°C, 52% RH) storage of rendered protein meal samples beef meat and bone meal (BMBM), chicken by-product meal (CBPM), and turkey meal (TM).

	Time (Weeks)	BMBM		CBPM	TM*
Supplier/Source		B1	B2	C3	T1
# of Samples Received		5	5	5	1
p-AnisidineValue (g/g)	0	9.46 ± 7.231	2.90 ± 0.808	4.13 ± 0.378	5.94
	2	8.11 ± 5.080	3.87 ± 0.634	4.55 ±0.506	5.82
	4	8.32 ± 5.224	3.02 ± 0.429	3.99 ±0.337	5.79
	8	6.92 ± 4.970	2.70 ±0.700	3.77 ± 0.943	5.08
	12	8.05 ± 5.308	4.26 ± 0.793	4.37 ± 0.835	7.55
	18	8.14 ± 4.372	5.81 ± 2.345	3.63 ± 0.012	5.85
	24	6.51 ± 3.370	8.77 ± 2.747	4.10 ± 0.664	3.98
	32	4.81 ± 2.612	8.65 ± 2.416	3.46 ± 1.428	3.16
	40	4.64 ± 2.441	8.60 ± 2.729	1.69 ± 0.789	6.30
	52	8.65 ± 3.803	15.02 ± 4.574	10.44 ± 7.284	11.39

* Due to lack of replicate samples, turkey meal is included for informational purposes only.

Table 2.11 Particle size analysis (mean ± Sd) of beef meat and bone meal (BMBM) and chicken by-product meal (CBPM) from 2 and 3 locations, respectively.

	BMBM		CBPM				
Supplier/Source	B1	B2	C1	C2	C3		
N=	5	5	5	5	5	**SEM**	***P*-Value**
Particle Size, Dgw[1]	522.40^{ab}	462.20^{bc}	458.20^{c}	532.80^{a}	544.80^{a}	20.517	0.0158
Particle Size, Sgw[2]	1.84	1.88	1.69	1.60	1.63	.	.
Particles / Gram	2,8634.00^{b}	4,6285.00^{a}	3,1660.00^{b}	1,4445.00^{c}	1,4557.00^{c}	3,106.572	< 0.0001
Surface Area, cm^2/gram	105.12^{bc}	120.14^{a}	115.52^{ab}	96.32^{c}	94.56^{c}	4.577	0.0021
Bulk Density (kg/m^3)	631.48^{b}	659.22^{a}	-	-	515.40^{c}	7.428	< 0.0001
Tapped Density (kg/m^3)	318.48^{a}	325.68^{a}	-	-	262.53^{b}	3.405	< 0.0001
Angle of Repose (Ɵ)	32.38^{b}	33.87^{b}	-	-	38.39^{a}	0.569	< 0.0001

^{abc} Means within a row that lack a common superscript differ $P \leq 0.05$.
[1]Geometric mean diameter.
[2]Geometric standard deviation.
- Due to limited resources, CBPM from locations C1 and C2 were excluded from bulk density, tapped density, and angle of repose analysis.

Table 2.12 Dynamic flow property measurements (mean ± Sd) of beef meat and bone meal (BMBM) and chicken by-product meal (CBPM) from 2 and 1 locations, respectively.

	BMBM		CBPM
Supplier/Source	B1	B2	C3
N=	3	3	3
BFE, mJ	1,258.0 ± 131.39	1,234.2 ± 54.03	1,139.6 ± 5.30
SI	1.1 ± 0.16	1.0 ± 0.02	1.1 ± 0.11
FRI	0.9 ±0.04	1.0 ± 0.11	0.9 ± 0.01

BFE – basic flowability energy; SI – stability index; FRI – flow rate index.

Figure 2.2 Graphical representation of compressibility of beef meat and bone meal (BMBM) and chicken by-product meal (CBPM) from two and one locations, respectively.

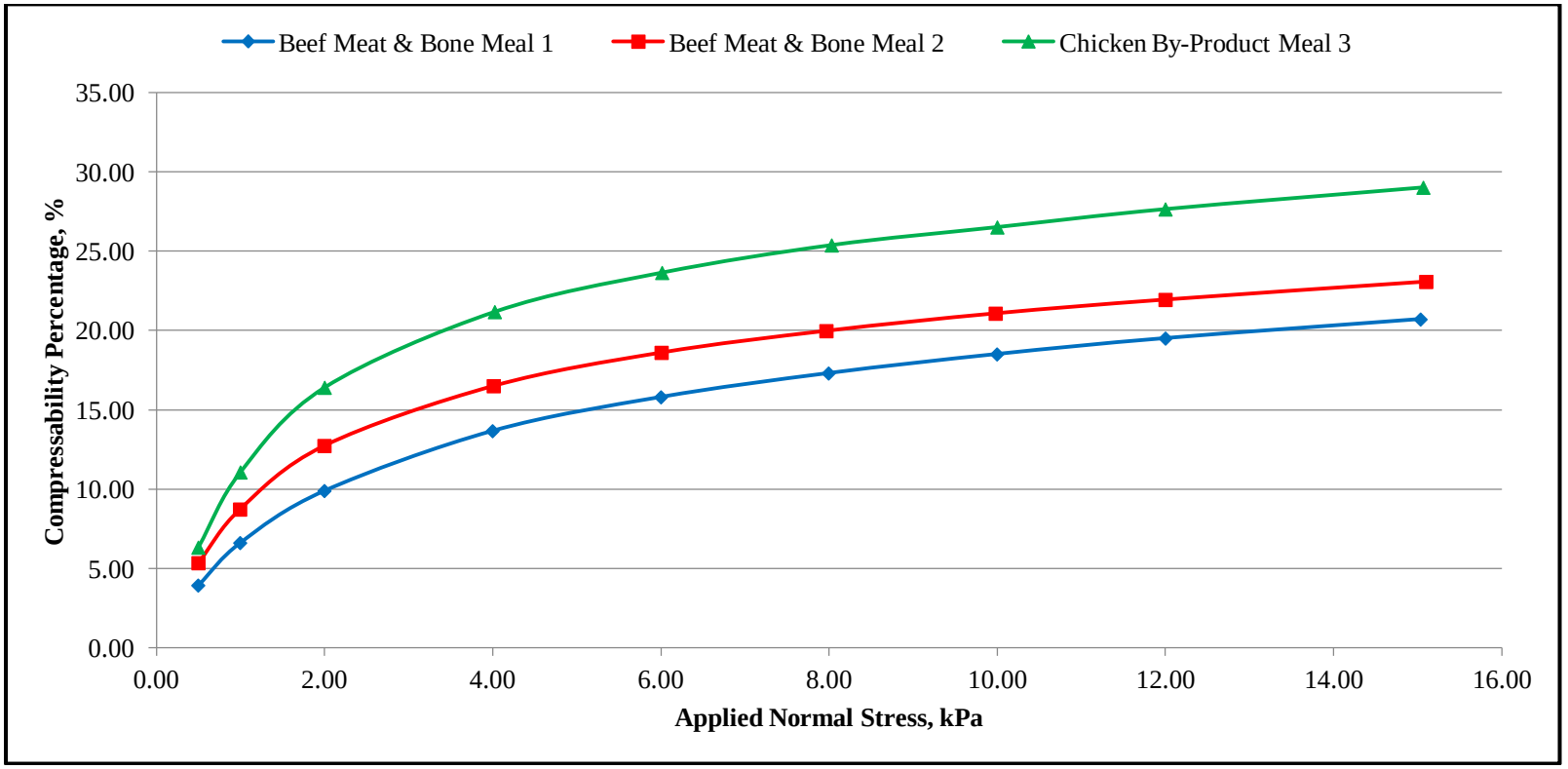


Figure 2.3 Scanning electron micrographs of a representative sample of beef meat and bone meal (BMBM) magnified to 100 µm, respectively.

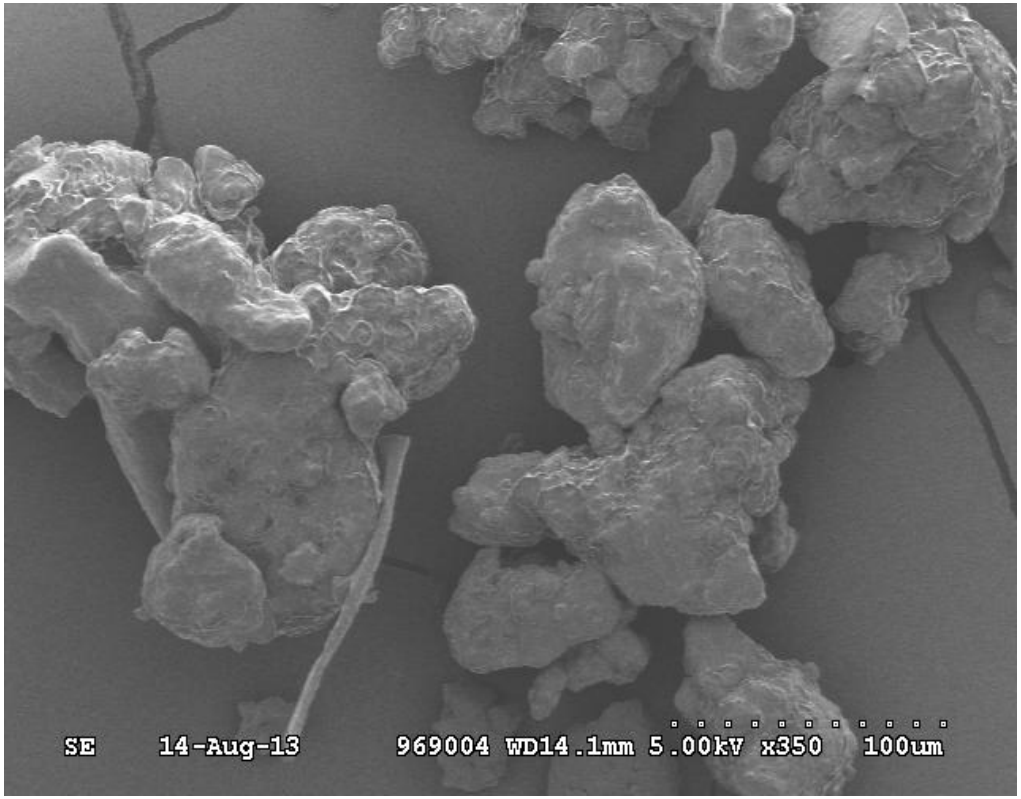
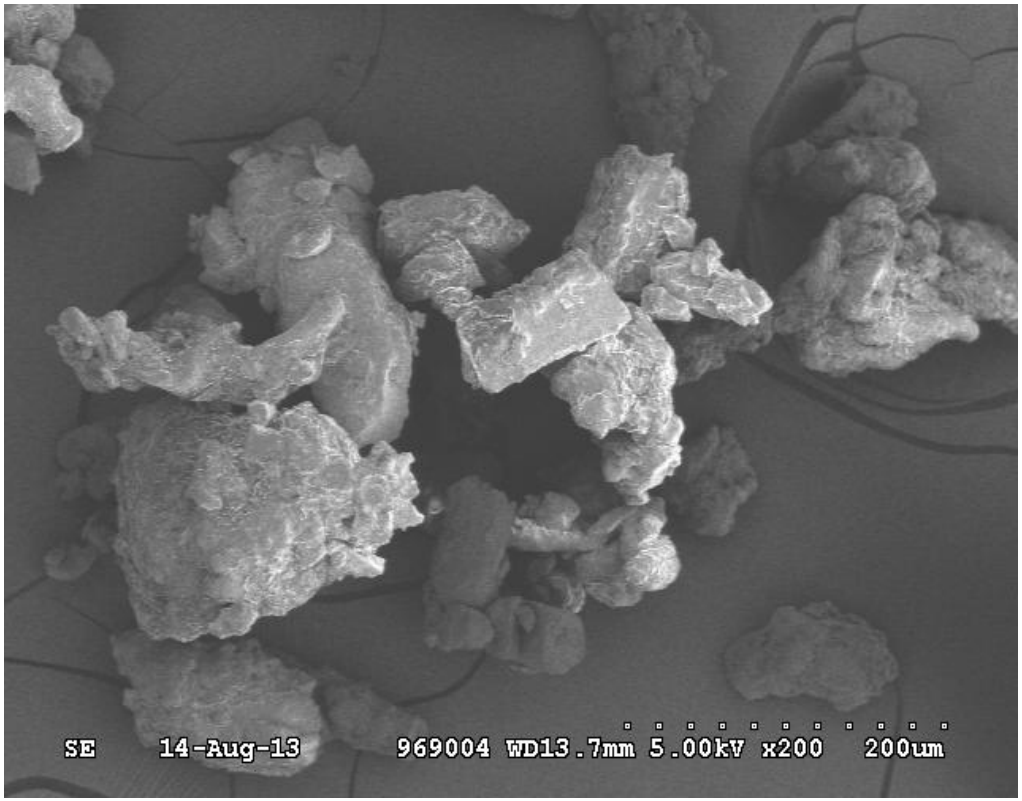


Figure 2.4 Scanning electron micrographs of a representative sample of chicken by-product meal (CBPM) magnified to 200 µm, respectively.



Chapter 3 - The Impact of Rendered Protein Meal Level of Oxidation on Shelf Life and Acceptability in Extruded Pet Foods

Introduction

Pet foods are a significant user of rendered protein meals. There is increasing pressure by pet food companies to produce foods from naturally preserved ingredients and to guarantee the shelf-life of these foods for 12 months or more. It is assumed that erosion of shelf-life starts with oxidative rancidity of the raw ingredients. Thus, increasing pressure has been put on ingredient suppliers to assure a low measure of oxidation – commonly a low peroxide value. The acceptable ranges for these measures have been somewhat arbitrarily set. Further, a majority of pet foods are extruded and dried, which is a heat intensive process that often causes volatile compounds to be vaporized. The products of oxidation are just such volatile compounds. Thus, the questions that arise are whether the standards currently being used for purchasing specifications of rendered protein meals are relevant to the finished products shelf-life or whether producing foods with previously oxidized protein meals will shorten the shelf-life of the foods and decrease their acceptability. Thus, an experiment has been proposed in which rendered protein meals will be secured (meat and bone meal and chicken by-product meal) that have been preserved with ethoxyquin, mixed tocopherols, or left unpreserved. These meals were allowed to oxidize in ambient (summer) conditions over a period of 5 to 9 weeks and then extruded into a “high protein” pet food. Samples of the pet food were analyzed for stability for 1 year to determine the influence of the protein meal on their relative stability. From this data regression analysis was used to determine a target maximal level of oxidation that is acceptable for the production of a year-long pet food shelf-life. The hypothesis of this study was that extrusion of protein meals during pet food manufacturing drives off lipid volatiles, thereby resetting the oxidation clock. To

test the hypothesis, the objective of this experiment was to evaluate the level of oxidation in rendered protein meals on shelf life and acceptability in extruded pet foods.

Materials and Methods

Rendered Protein Meal

Approximately one metric ton of unpreserved BMBM and 735 kilograms of CBPM were acquired from cooperating rendering plants. The meals were split into six equal subsamples of about 133 kg into separate 64-L pails. The first two 133 kg sub-samples of BMBM and CBPM were left untreated and labeled BMBM-01, BMBM-02, CBPM-01, and CBPM-02. Each untreated 133 kg sub-sample was placed inside a fiber drum that was lined with a plastic bag. The second set of 133 kg sub-samples of BMBM were weighed into a horizontal "counterpoise" paddle batch mixer (133 kg, Hayes and Stolz; Fort Worth, TX). A hand-pump pressurized sprayer was used to apply a 1:10 dilution of Naturox™ (Kemin Industries, Des Moines, IA) and canola oil, and mixed for five minutes. Approximately 1,200 ppm of Naturox™ (Kemin Industries, Des Moines, IA) was applied to each sub-sample and meal. Each sub-sample drum was labeled: BMBM-MT1, BMBM-MT2, CBPM-MT1, and CBPM-MT2. After mixing, each 133 kg sub-sample was placed inside a fiber drum that was lined with a plastic bag and a lid was secured on top of each drum. Between the two similar treatments of the samples, for example BMBM-MT1 and BMBM-MT2, an air hose and brushes were used to remove any remaining residue from the equipment. Using the equipment and procedures described above, the third set of 133 kg sub-samples from each meal had a 1:10 dilution of ethoxyquin (Kemin Industries, Des Moines, IA) while applied to them the contents were mixing. The rendered protein meal was allowed to mix for five minutes after all of the antioxidant had been applied. The pressurized sprayer sat on a digital scale to visually allow a reading of how much antioxidant/canola oil was

being applied. To each corresponding subsample and meal, 150 ppm of ethoxyquin (Rendox™ , Kemin Industries, Des Moines, IA) was applied. Each subsample drum was labeled: CBPM-ET1, CBPM-ET2, BMBM-ET1, and BMBM-ET2. After mixing, the samples were stored as described above. The mixer was sanitized prior to experimental procedures and in-between each treatment using a five percent bleach solution and allowed to thoroughly dry. All twelve drums were kept at room temperature (25°C, 51% RH). The BMBM was stored for 63 days and the CBPM for 41 days prior to extrusion. Each drum was allowed to oxidize and was analyzed for PV and AV approximately every five days during storage (table 3.1 and 3.2). Prior to processing, each treatment was analyzed for volatile compounds using gas chromatography.

Diets

All ingredients were individually weighed prior to mixing in a Wenger double ribbon mixer for three minutes before the micro ingredients were added and allowed to mix an additional three minutes. After mixing, the diets were then bagged in 22.7 kilogram paper bags in preparation for extrusion. Experimental pet food diets similar to a typical cat food (~30% protein) were produced (table 3.3; BIVAP Extrusion Laboratory; Kansas State University; Manhattan, KS).

Processing

Treatment Sequence

Experimental diet production required sequential days. The sequence of treatment production was: start-up material (a blend of chicken by-product meal, brewers rice, corn, wheat, and beet pulp), CBPM-01, CBPM-02, BMBM-01, BMBM-02, CBPM-MT1, CBPM-MT2, CBPM-ET1, and CBPM-ET2. Prior to the second day of extrusion, the pre-conditioner was

thoroughly cleaned. The treatment sequence for the second day of extrusion was: BMBM-MT1, BMBM-MT2, BMBM-ET1, and BMBM-ET2.

Extruder Parameters

Each diet was mixed as an individual replicate and extruded accordingly. The raw mixed ingredients were extruded on a single screw extruder (Wenger X-20, Wenger Manufacturing; Sabetha, Kansas) using a typical pet food screw profile. The extruder screw profile included 1-Inlet screw, 2-Single flight full-pitch screw, 3-Small shear lock, 4-Singleflight full-pitch screw, 5-Small shear lock, 6-Single flight screw, 7-Medium shear lock, 8-Doubleflight single pitch screw, 9-Large shear lock, 10-Double flight cut cone screw (Figure 3.1). The extruder barrel jacket temperature for zone one was 30°C, zone two was 70°C, and zone three was 90°C. The extruder die shape and size was a five millimeter circle with a knife setup of six solid blades.

Target Extruder Conditions

The target extruder conditions for moisture were 27-30% and a dry feed rate of 180 kg/h. The steam was added at a rate of two-thirds the water in the pre-conditioner while the remainder of the moisture was added to the extruder barrel and recalculated to achieve the processing moisture goal. The target bulk density was 350 g/L. The bulk density measurement was duplicated for each treatment and an average bulk density was calculated. The target extruder screw speed was 340 to 450 rpm based on the control diets performance. Once the parameters for the control diet were set, they were held constant for the remainder of the experimental treatment processing.

Dryer Conditions

The extruded kibble exiting the extruder was pneumatically conveyed to a dual pass dryer/single pass cooler (Wenger 4800 Series, Wenger Manufacturing; Sabetha, Kansas). The dryer was set at 99°C and ten minutes per pass and ten minutes through the cooler. The dryer conditions were adjusted to achieve a target final moisture of 7.5%. To confirm the pet food achieved the final moisture level, the kibbles were analyzed (AOAC 930.15). The pet food was not coated with flavors or fats upon exiting the dryer to eliminate confounding factors.

Shelf-Life

Three kg of kibble per treatment were placed in freezer storage bags, each bag was punctured with a pin sized hole to facilitate air exchange. Each bag was labeled with their respective treatment and storage duration. Samples for ambient storage (~22°C and 45% relative humidity) were prepared for 0, 3, 6, 9, 12, and >12 months. These were stored in a covered plastic tote. Samples for "accelerated" conditions were prepared for 0, 3, 6, 12, and 18 weeks. They were held at 40°C and 70% relative humidity in an environmental chamber (Cincinnati Sub-Zero Stability Temperature/Humidity Chamber; Cincinnati, Ohio). Samples for each time point in accelerated storage were arranged vertically throughout the environmental chamber.

Sample Analysis

The rendered protein meals and samples collected for the shelf-life evaluation were evaluated for peroxide (AOCS Official Method Cd 8-53) and anisidine value (AOCS Official Method Cd 18-90). The rendered protein meals prior to extrusion and the kibble samples for the shelf-life evaluation were analyzed for volatile compounds via gas chromatography (GC) head space analysis for hexanal and other 10-carbon or smaller aldehydes.

Fat Extraction from Pet Food

Since there is no official method for extraction of oils from pet food for the determination of peroxide value (PV) or anisidine value (AV), the technique was modified from the procedures of Williams and Hron (1996) in the following manner: 1 kg of pet food was ground through a 1 mm screen in a Wiley mill (Model 4: Thomas Scientific; Swedesboro, New Jersey). Subsamples of each respective CBPM treatment (400 g) and BMBM treatment (900 g) sample were required. The BMBM pet food was split into two equal 450 g subsamples to aid extraction. Each sample was weighed into a 1,000 mL beaker to which an equal amount of hexane was added. The ground pet food and hexane were allowed to mix for five minutes using a magnetic stir plate. Separation of oil and hexane for the pet food was done by vacuum filtration that consisted of a standard laboratory vacuum pump, a vacuum hose, liquid trap, Büchner funnel, Erlenmeyer flask with hose adapter, and Whatman Grade 41 filter paper (GE Healthcare Life Sciences; Pittsburgh, Pennsylvania). Once the hexane and oil were separated, the mixture was transferred to a 1,000 mL roundbottom flask attached to a rotating evaporator (Rotavap Büchi R-114: Brinkmann Instruments, Inc.) that was partially submerged in a water bath (Büchi B-490: Brinkmann Instruments, Inc) at 50°C. The rotating evaporator was used to gently separate the hexane from the oil (15 minutes). The isolated oil was transferred to a 50 mL conical tube (BD Biosciences) and centrifuged (Sorvall Legend X1R: Thermo Fisher Scientific) at a 5,000 rpm for fifteen minutes at 35°C. The isolated oil was then analyzed for PV (AOCS Official Methods Cd 8-53) and AV (AOCS Official Methods Cd 18-90).

Volatile Compounds Measurement

Extraction Procedure of Volatile Compounds

Volatiles in dry dog foods were evaluated by headspace-solid phase microextraction (HS-SPME) as described by Koppel (2013). Five sub-samples of BMBM received from each of two locations, five sub-samples of CBPM received from each of three locations, and the one sub-sample of turkey meal were analyzed in triplicate. The samples were ground to a particle size of 1 mm using a Wiley mill (Model 4: Thomas Scientific; Swedesboro, New Jersey), then 0.5 g of each sample was weighed into a 10 mL screw-cap vial with a polytetrafluoroethylene/silicone septum. To this, 0.98 mL distilled water was added to the ground sample in the vial along with an internal standard of 0.02 mL 1,3-dichlorobenzene (98%, Sigma Aldrich, St. Louis, MO, USA) dissolved in hexane (mixture of isomers, optima grade, Fisher Scientific; Pittsburgh, PA, USA). The final concentration was 0.2 mg/kg. The vials were equilibrated for 10 min at 40 °C in the autosampler (Pal system, model CombiPal, CTC Analytic;, Zwingen, Switzerland) and agitated at 250 rpm. After the equilibrium, a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane fiber was exposed to the sample headspace for 30 min at 40 °C. The fiber method was chosen for its high volatile capacity in food products (Ceva-Antunes et al., 2006). After sampling, the analytes were desorbed from the SPME fiber coating prior in the GC injection port at 270 °C for 3 min in the splitless mode.

Chromatographic Analyses

The isolation, identification, and semi-quantification of the volatile compounds were performed on a gas chromatograph (Varian GC CP3800; Varian Inc.; Walnut Creek, California), coupled with a Varian mass spectrometer (MS) detector (Saturn 2000). The GC-MS system was equipped with an RTX-5MS (Crossbond® 5% diphenyl/95% dimethyl polysiloxane) column (Restek, U.S.; Bellefonte, Pennsylvania; 30 m x 0.25 mm x 0.25 μm film thickness). The initial temperature of the column was 40 °C held for 4 min; the temperature was then increased by 5 °C

per min to 260 °C, and held at this temperature for 7 min. All samples were analyzed in triplicates. The quantities of volatile compounds were calculated against the internal standard peaks.

The compounds were identified using two different analytical methods: 1) mass spectra (> 80%) and 2) Kovats indices (NIST/EPA/NIH Mass Spectral Library, Version 2.0, 2005). Identification was considered tentative when it was based on only mass spectral data. The retention times for a C7-C40 saturated alkane mix (Supelco Analytical; Bellefonte, Pennsylvania) was used to determine experimental Kovats indices for the volatile compounds detected.

Human Rancidity Panel

At each time point in the shelf-life study, a team of ten untrained volunteers were enlisted to analyze each sample for their aroma. Each panelist was given a score sheet to rank samples based on their perception of the products relative level of rancidity. Scores were one to five, with a higher score for more rancid notes. The average rancidity score for each sample was recorded at each time point. All sample jars were washed thoroughly and dried in an air oven at 100°C between each sensory evaluation period. The samples were randomly assigned to a jar number and labeled one to twelve to correspond with the twelve dietary treatments. Approximately 50 kibbles of each sample were placed in the four ounce jars and covered with a lid. All samples were stored in a freezer at -18°C until the sensory analysis by the trained panelists is arranged.

Statistical Analysis

Oxidative rancidity and stability

Results were summarized according to protein type, treatment, and time of sample analysis from rendered protein meal arrival to processing. Variation among means was

determined by treatment and time of sample analysis. The data were analyzed as a completely randomized design and the means were separated by significant F values with $\alpha = 0.05$ of the GLM procedure using statistical software (SAS Institute, Inc.; Cary, North Carolina).

The data were analyzed as a completely randomized design and the means were separated by significant F values with $\alpha = 0.05$ of the GLM procedure using statistical software (SAS Institute, Inc.; Cary, North Carolina). For the relationship between accelerated and ambient shelf-life evaluations, regression anlaysis was performed. Results were summarized according to protein type, treatment, and time of sample analysis of kibble. Variation among means was determined by protein type, treatment, and time of sample analysis of kibble.

Chromatographic analyses

For the relationship between accelerated and ambient shelf-life evaluations, linear regression was performed by regression analysis. Results were summarized according to protein type, treatment, and time of sample analysis of kibble. Variation among means was determined by protein type, treatment, and time of sample analysis of kibble. The data were analyzed as a completely randomized design and the means were separated by significant F values with $\alpha = 0.05$ of the GLM procedure of SAS statistical software (SAS Institute, Inc.; Cary, North Carolina).

Human rancidity panel

The data were analyzed as described previous section.

Results

Processing

Actual Extruder Conditions

The feeder screw speed averaged 18.5 RPM with a standard deviation of 0.085 between all treatments. The discharge temperature from the preconditioner to the extruder averaged 95.58°C between all treatments with a standard deviation of 1.240. The shaft speed of the extruder averaged 595 RPM with a standard deviation of 1.6 between all treatments. The percent moisture added to the treatments during processing averaged 28.7%, with a standard deviation of 0.24. The die temperature averaged 115.6°C, with a standard deviation of 4.38 between all treatments. The throughput of the BMBM treatments averaged 220 kg/hr, with a standard deviation of 4.8 and the CBPM throughput averaged 204 kg/hr, with a standard deviation of 7.7.

Final Product

The average bulk density of the kibble for the BMBM treatments out of the extruder was 418.2 g/L (±30.13) and the CBPM treatments averaged 466.5 g/L (±22.87). The kibble out of the extruder for the BMBM treatments averaged a radial expansion of 9.2 mm (±0.39) and the CBPM treatments averaged 8.4 mm (±0.53). The average bulk density of the kibble out of the dryer for the BMBM treatments was 401.8 g/L (±28.25) and the CBPM treatments averaged 459.7 g/L (±36.41). The average radial expansion of the kibble out of the dryer for the BMBM treatments was 9.11mm (±0.236) and the CBPM treatments averaged 8.46 mm (±0.197). The average moisture of the kibble for the BMBM treatments was 3.79% (±0.266) and the CBPM treatments averaged 5.22% (± 1.061) moisture.

Rendered Protein Meals

The PV and AV of the unpreserved BMBM increased from 3.33 meq/kg and 6.91 g/g at day 2 to 87.99 meq/kg and 14.30 g/g by day 59 (Table 3.1). The BMBM preserved with mixed tocopherols had a PV and AV of 2.22 meq/kg and 0.00 g/g at day 2 and increased to 8.14 meq/kg

and 7.76 g/g by day 59. The BMBM preserved with ethoxyquin had a constant PV and AV from day 2 (2.22 meq/kg and 0.00 g/g) till day 59 (2.22 meq/kg and 0.00 g/g).

The PV and AV of the unpreserved CBPM increased from 4.43 meq/kg and 0.15 g/g at day 2 to 81.20 meq/kg and 3.02 g/g by day 38 (Table 3.2). The CBPM preserved with mixed tocopherols had a PV and AV of 1.11 meq/kg and 0.00 g/g at day 2 and increased to 4.43 meq/kg and 1.21 g/g by day 38. The CBPM preserved with ethoxyquin had an initial PV of 1.12 meq/kg at day 2 and increased to 2.22 meq/kg from day 7 to 38. The AV held constant at 0.00 g/g from day 2 to 38.

Gas Chromatography/Mass Spectroscopy – Rendered Protein Meals

The hexanal concentration of the BMBM treatment with mixed tocopherols was greater (P <0.0001) than the unpreserved and ethoxyquin treatments, which were also different from each other (P < 0.0001; 15,355.82 μg/kg; 5,515.74 μg/kg; and 1,233.18 μg/kg, respectively; Table 3.4). The hexanal concentration of the unpreserved CBPM was greater than the ethoxyquin CBPM treatment (5,762.33 and 1,550.73 μg/kg; P < 0.0001), but did not differ from the CBPM mixed tocopherols (3,128.79 μg/kg; P > 0.05). The hexanal concentration of the unpreserved BMBM treatment (5515.74μg/kg) did not differ from the CBPM unpreserved (5,762.33 μg/kg) and mixed tocopherols (3,128.79 μg/kg) treatments (P > 0.05). The hexanal concentration of the BMBM ethoxyquin treatment did not differ from the CBPM ethoxyquin treatment (1233.18 and 1550.73 μg/kg) (P > 0.05).

The heptanal concentration of the unpreserved BMBM treatment was greater than the BMBM mixed tocopherols and ethoxyquin treatments (4,642.83 and 3,081.80 and 1108.29 μg/kg; P < 0.0001). The unpreserved CBPM treatment did not differ from the CBPM mixed tocopherols and ethoxyquin treatments (631.62 and 233.47 and 269.79 μg/kg; P > 0.05). The

heptanal concentration of the BMBM ethoxyquin treatment did not differ from the unpreserved CBPM treatment (1108.29 and 631.62 μg/kg; $P > 0.05$).

The octanal concentration of the unpreserved BMBM was greater than the BMBM mixed tocopherols and ethoxyquin treatments (7,212.00 and 4,733.21 and 691.75 μg/kg; $P < 0.0001$). The octanal concentration of the unpreserved CBPM did not differ from the CBPM mixed tocopherols and ethoxyquin treatments (462.19 and 251.86 and 154.69 μg/kg; $P > 0.05$). The octanal concentration of the BMBM ethoxyquin treatment did not differ from the CBPM unpreserved, mixed tocopherols, and ethoxyquin treatments (462.19 and 251.86 and 154.69 μg/kg; $P > 0.05$).

The BMBM unpreserved nonanal concentration was greater than the BMBM ethoxyquin treatment (4,515.96 and 767.47 μg/kg; $P < 0.05$), but the unpreserved treatment did not differ from the BMBM mixed tocopherols treatment (4395.62 μg/kg; $P > 0.05$). The nonanal concentration of the CBPM unpreserved, mixed tocopherol, and ethoxyquin treatments did not differ from each other (525.92 and 260.47 and 179.75 μg/kg; $P > 0.05$). The BMBM ethoxyquin nonanal concentration did not differ from the CBPM unpreserved, mixed tocopherols, and ethoxyquin treatments (767.47 and 525.92 and 260.47 and 179.75; $P > 0.05$).

The decanal concentration for the BMBM and CBPM treatments did not differ from each other ($P > 0.05$).

Shelf Life

The PV of the unpreserved BMBM treatment decreased from 10.07 meq/kg at 0 weeks to 6.66 meq/kg by 18 weeks; whereas, the AV for this treatment increased from 10.08 g/g at 0 weeks to 17.52 g/g by 18 weeks (Table 3.5). The PV of the mixed tocopherols and ethoxyquin treatments increased from 0 weeks to 18 weeks (2.22 to 15.48 meq/kg and 2.22 to 3.33 meq/kg).

The AV of the BMBM mixed tocopherols and ethoxyquin treatments increased from 9.62 to 15.98 g/g and 3.03 to 6.12 g/g from 0 to 18 weeks. The PV of the unpreserved CBPM treatment increased from 14.41 meq/kg at 0 weeks to 53.15 meq/kg by 18 weeks. The AV of the unpreserved CBPM treatment also increased during the 0 to 18 week shelf life (15.56 to 33.41 g/g). The PV and AV of the CBPM mixed tocopherols treatment increased from 2.78 meq/kg and 3.85 g/g at 0 weeks to 23.21 meq/kg and 15.45 g/g by 18 weeks. The PV and AV of the CBPM ethoxyquin treatment also increased from 2.22 meq/kg and 1.79 g/g at 0 weeks to 4.44 meq/kg and 7.53 g/g by 18 weeks.

Gas Chromatography/Mass Spectroscopy – Kibble

The hexanal concentration of the unpreserved BMBM treatment increased from 1,418.86 μg/kg at 0 weeks to its greatest concentration of 1,986.35 μg/kg at 6 weeks and decreased to 1,462.56 μg/kg by 18 weeks (Table 3.6). The BMBM mixed tocopherols hexanal concentration decreased from 7,861.76 μg/kg at 0 weeks to 2,221.49 μg/kg at 12 weeks, but increased to 3,557.83 μg/kg at 18 weeks. The BMBM ethoxyquin treatment increased from 295.08 μg/kg at 0 weeks to its greatest concentration of 1,779.82 μg/kg at 18 weeks. The CBPM unpreserved treatment increased from 1,062.29 μg/kg at 0 weeks to 4,954.59 μg/kg by 18 weeks. The CBPM mixed tocopherols treatment decreased from 723.93 μg/kg at 0 weeks to 580.50 μg/kg at 3 weeks, but increased to 2,501.54 μg/kg by 18 weeks. The CBPM ethoxyquin treatment increased from 255.50 μg/kg at 0 weeks to 1,263.36 μg/kg by 18 weeks.

The heptanal concentration of the unpreserved BMBM treatment increased and decreased over the 18 weeks shelf life (842.44 μg/kg at 0 weeks; 747.26 μg/kg at 3 weeks; 1,592.52 μg/kg at 6 weeks; 1,004.65 μg/kg at 12 weeks; and 1,008.85 μg/kg at 18 weeks). The concentration of the BMBM mixed tocopherols treatment decreased from 521.51 μg/kg at 0 weeks to 290.67

μg/kg at 3 weeks, but increased to 774.46 μg/kg by 18 weeks. The concentration of the ethoxyquin treatment increased from 97.77 μg/kg at 0 weeks to 520.84 μg/kg by 18 weeks. The CBPM unpreserved treatment increased from 133.24 μg/kg at 0 weeks to 980.11 μg/kg by 18 weeks. The CBPM mixed tocopherols and ethoxyquin followed the same trend as the unpreserved treatment and increased from 0 weeks to 18 weeks (43.46 to 672.14 μg/kg and 28.75 to 234.28 μg/kg).

The octanal concentration of the BMBM unpreserved treatment fluctuated during the 18 week shelf life (1,857.98 μg/kg at 0 weeks; 1,839.88 μg/kg at 3 weeks; 2,068.78 μg/kg at 6 weeks; 1,816.19 μg/kg at 12 weeks; and 23,78.81 μg/kg at 18 weeks). The BMBM mixed tocopherols treatment decreased from 812.89 μg/kg at 0 weeks to 515.64 μg/kg at 6 weeks, but then increased to 996.08 μg/kg at 18 weeks. The BMBM ethoxyquin treatment increased from 197.17 μg/kg at 0 weeks to 236.74 at 3 weeks, decreased to 146.76 μg/kg at 6 weeks, and increased to 327.26 μg/kg at 18 weeks. The CBPM unpreserved treatment increased from 217.55 μg/kg at 0 weeks to 904.95 μg/kg by 18 weeks. The CBPM mixed tocopherols and ethoxyquin treatments also had an increase in octanal concentration over the 18 week shelf life (75.08 to 339.17 μg/kg and 27.71 to 129.28 μg/kg).

The nonanal concentration of the BMBM unpreserved treatment increased and decreased over the course of the 18 week shelf life (1,696.82 μg/kg at 0 weeks; 1,768.01 μg/kg at 3 weeks; 951.42 μg/kg at 6 weeks; 1,106.41 μg/kg at 12 weeks; and 830.94 μg/kg at 18 weeks). The BMBM mixed tocopherols treatment decreased from 1256.11 μg/kg at 0 weeks to 321.21 at 12 weeks, but increased to 607.18 at 18 weeks. The BMBM ethoxyquin treatment increased from 85.70 μg/kg at 0 weeks to 384.07 μg/kg at 3 weeks, decreased to 146.50 μg/kg at 6 weeks, and increased to 268.72 μg/kg at 18 weeks. The CBPM unpreserved treatment increased from 296.32

μg/kg at 0 weeks to 500.55 μg/kg at 3 weeks, decreased to 284.78 at 6 weeks, and increased to 554.74 at 18 weeks. The CBPM mixed tocopherols treatment decreased from 137.99 μg/kg at 0 weeks to 90.54 μg/kg at 3 weeks, but increased to 181.12 by 18 weeks. The CBPM ethoxyquin treatment increased from 59.63 μg/kg at 0 weeks to 84.45 μg/kg at 3 weeks, decreased to 68.01 μg/kg at 6 weeks, and increased to 94.78 μg/kg at 18 weeks.

The decanal concentration of BMBM unpreserved treatment fluctuated throughout the 18 week shelf life (213.08 μg/kg at 0 weeks; 324.06 μg/kg at 3 weeks; 55.45 μg/kg at 6 weeks; 36.52 μg/kg at 12 weeks; and 38.45 μg/kg at 18 weeks). The BMBM mixed tocopherols treatment decreased and increased throughout the course of the 18 week shelf life (42.70 μg/kg at 0 weeks; 7.59 μg/kg at 3 weeks; 20.34 μg/kg at 6 weeks; 17.34 μg/kg at 12 weeks; and 17.91 μg/kg at 18 weeks). The BMBM ethoxyquin treatment increased from 35.97 μg/kg at 0 weeks to 38.53 μg/kg at 3 weeks, decreased to 10.08 by 6 weeks, and increased to 15.25 μg/kg by 18 weeks. The CBPM unpreserved treatment increased from 7.41 μg/kg at 0 weeks to 10.57 μg/kg at 3 weeks, decreased to 5.54 μg/kg by 12 weeks, but increased to 13.57 μg/kg by 18 weeks. The CBPM mixed tocopherols treatment increased from 3.87 μg/kg at 0 weeks to 7.25 μg/kg at 3 weeks, decreased to 2.83 μg/kg at 6 weeks, but increased to 3.85 μg/kg by 18 weeks. The CBPM ethoxyquin treatment increased from 1.80 μg/kg at 0 weeks to 2.30 at 6 weeks, decreased to 1.97 μg/kg at 12 weeks, but increased to 2.32 μg/kg by 18 weeks.

Human Rancidity Panel

The BMBM unpreserved treatment had the lowest numeric rancidity score compared to the other BMBM treatments at 0 weeks (2.00), but the treatments did not differ ($P > 0.05$; Table 3.7). The unpreserved treatment had the highest score at 18 weeks (3.65) shelf life compared to all the BMBM and CBPM treatments ($P < 0.05$). The BMBM mixed tocopherols treatment

increased from a score of 2.05 at 0 weeks to 2.45 at 18 weeks, which had, numerically, the lowest score at 18 weeks among the BMBM treatments, but did not differ from the BMBM ethoxyquin treatment ($P > 0.05$). The BMBM ethoxyquin treatment, numerically) had the highest score at 0 weeks (2.10) and had an intermediate score by 18 weeks (2.55), but did not differ from the other BMBM treatment at 0 weeks and the BMBM mixed tocopherols treatment at 18 weeks ($P > 0.05$) . The CBPM unpreserved treatment, numerically) had the highest rancidity score compared to the other CBPM treatments at 0 weeks (2.25), but did not differ from the ethoxyquin treatment ($P > 0.05$). The CBPM unpreserved treatment remained the highest among the CBPM treatments at 18 weeks (2.65), but did not differ from the other CBPM treatments ($P > 0.05$). The CBPM mixed tocopherols treatment had the lowest score at week 0 (1.30; $P < 0.05$), but did not differ from the other treatments at 18 weeks (2.05; $P > 0.05$). The CBPM ethoxyquin treatment had an intermediate score at time 0 (2.10) and 18 weeks (2.20), but did not differ from the CBPM unpreserved treatment at 0 weeks and either CBPM treatments at 18 weeks. Among the BMBM and CBPM at 18 weeks, the unpreserved treatments numerically had the highest rancidity score (3.65 and 2.65), the mixed tocopherols treatments had the lowest rancidity score (2.45 and 2.05), and the ethoxyquin treatments had intermediate scores (2.55 and 2.20).

Discussion

Rendered Protein Meals

The PV of unpreserved BMBM and CBPM provide a vivid representation for the stages of lipid oxidation: initiation, propagation, and termination (Figures 3.1 and 3.2). Both rendered protein meals behaved similarly to sunflower oil over a shelf life of 90 days (Crapiste et al., 1999), unrefined Pollock oil over a shelf life of 12 weeks (Sathivel et al., 2008), and instant

noodles over a shelf life over several intervals (Gotoh et al., 2007). The unpreserved BMBM and CBPM were expected to oxidize rapidly and were projected to be extruded into a model cat food diet at the peak of propagation; although, the unpreserved BMBM was beginning to enter the termination phase prior to processing. This may have contributed to the decrease in PV during the 18 week shelf life of this treatment (Table 3.5). The mixed tocopherols treatments slightly oxidized during storage; whereas, the ethoxyquin treatment remained stable. This supports the conclusion of Hilton (1989) and Frankel (1996) that mixed tocopherols are not as stable as the synthetic form of antioxidants. The AV of the unpreserved and mixed tocopherols treatments continuously rise in the same overall direction as in the study with Crapiste et al. (1999) and Gotoh, et al. (2007). The ethoxyquin treatments did not form aldehydes prior to processing; again, agreeing with Hilton (1989) that the synthetic antioxidant is more stable than the natural form.

Gas Chromatography/Mass Spectroscopy – Rendered Protein Meals

The aldehydes that were measured using GC-MS ranged in several carbons in length, but we chose to report on number of carbon atoms from 6 to 10, such as hexanal, heptanal, octanal, nonanal, and decanal, because they are important contributors to rancid and unpleasant flavors and odors in oxidized oils (Frankel et al., 1985; DeHaan et al., 2004). It was found in the study with DeHaan et al., (2004) that hexanal, heptanal, octanal, and nonanal compounds were consistently detected in animal fats, but decanal was not always found. This would support the low values of decanal that are found in the BMBM and CBPM samples relative to the other volatile compounds (table 3.4). In two studies by Greenberg (1981), the volatile aldehydes of meat and bone meal and poultry by-product meal were identified in order of decreasing abundance as hexanal, heptanal, octanal, nonanal, and decanal. Hexanal was identified as the

aldehyde in the most abundance, representing about 43% of the overall relative concentration in meat and bone meal and 40% in poultry by-product meal (Greenberg, 1981). Shahidi et al., (1994) found that during a three week shelf life study of meat, hexanal concentration increased rapidly during the early stages of storage and dramatically decreased after six days; thus, mimicking the similar phases of hydroperoxide formation during lipid oxidation. Shahidi et al. (1994) suggests that caution should be given when using hexanal as a marker of lipid oxidation as it could correspond with two different points of oxidation. This may explain the higher level of hexanal in the BMBM mixed tocopherols treatment than the BMBM unpreserved treatment and the difference in phases of oxidation (Table 3.4). The aldehydes listed above have been identified as contributors to the flavor of cooked beef and are more concentrated in beef and chicken meat that are uncured versus cured (Elmore et al., 1999, Ramarathnam et al., 1993). It is also suggested by Ramarathnam et al. (1993) that several factors, both pre-slaughter and post-mortem, have an effect on the compounds formed in meat products, such as the type of feed, storage and sanitation conditions, and processing methods.

Tompkins et al., (1999), found the hexanal and heptanal concentrations in soybean frying oil had a high coefficient of correlation with *p*-anisidine value (R = 0.81, $P = 0.0001$ and R = 0.66, $P = 0.0009$), but *p*-anisidine value was not significantly correlated with nonanal (R = 0.33, $P = 0.1299$). Hexanal and heptanal are breakdown products of linoleic acid and linolenic acid; whereas nonanal is only a breakdown product of oleic acid. Additional analysis of the poor correlation between nonanal and *p*-anisidine should be further studied. Similar to the *p*-anisidine value and peroxide value relationship, the headspace analysis of a food could be utilized in conjunction with peroxide value to identify both primary and secondary oxidation products (Elizalde et al., 1991).

Processing – Final Product

The lower average bulk density of the BMBM out of the extruder agrees with the higher radial expansion relative to the CBPM treatments due to the higher surface area of the BMBM treatments. The lower bulk densities of the BMBM and CBPM treatments out of the dryer would be consistent with the moisture lost during drying of the kibble. The higher radial expansion and lower density of the BMBM treatments may have had an effect on the lower percent moisture of the kibble after drying. The goal was to keep the radial expansion of all treatments consistent as it has been suggested that kibble with a larger surface area are subject to increased oxidation relative to the more dense kibble (Labuza et al., 1971; Nawar et al., 1985; Rao et al., 1989). The cell structures within the kibble of the BMBM and CBPM treatments would have been interesting to compare as it has been suggested that an increase in radial expansion increases the cells or "air pockets" within kibble; which, may also suggest an increase in subjection to oxygen of the kibble with larger "air pockets" (Camire et al., 1990).

Diets

The experimental diet was similar to that produced for a typical cat food (table 3.3), which was representative of a higher inclusion of rendered protein meal (30 to 40% of the formula). The higher inclusion would allow for a better measurement of the oxidation of the rendered protein meal without too much dilution from other ingredients.

Treatment Sequence

The process sequence was organized so as to decrease the possibility of residual transmission of antioxidants between treatments; wherein, the diets with unpreserved BMBM

and CBPM were extruded first. During processing of the unpreserved BMBM, large pieces of bone from the BMBM blocked the die; therefore, the remaining BMBM treatments needed to be ground to a 3/64 grind to remove any large pieces of bone. After the diets with the unpreserved treatments were extruded, the diets with CBPM preserved with mixed tocopherols and ethoxyquin were completed in the same day. Prior to extrusion the following day, the preconditioner was scraped and cleaned to remove any residual antioxidants due to the fact that ethoxyquin has good "carry through" during processing (Hilton, 1989). The remaining diets with BMBM preserved with mixed tocopherols and ethoxyquin were then extruded.

Shelf Life Challenge

The oxidation of the unpreserved treatments was rapid and extensive, but oxidation occurred regardless of treatment (Table 3.5). The unpreserved treatments were expected to have a shorter shelf life than the other preserved treatments, but the preserved treatments had a shorter shelf life than expected; hence, the high values of AV in the preserved treatments towards the end of the shelf life. In the study with Lin et al., (1998), the effect of fat type (beef tallow and poultry fat) and fat content on lipid oxidation of extruded kibble were measured by the TBARS assay. Their control diet without added fat oxidized quicker than the diet coated with poultry fat or beef tallow. This was assumed to be due to the added preservatives in the fat that contributed to the decrease in oxidation of the poultry fat and beef tallow (Lin et al., 1998). This compares favorably to our data in that the unpreserved BMBM and CBPM treatments oxidized faster than the preserved treatments (Table 3.5). In the 14 month shelf life study with Lin et al., (1998), the kibble with the added poultry fat oxidized quicker than the kibble with the added beef tallow presumably due to the increase of polyunsaturated fatty acid profile of poultry. This fact also agrees with our results in that the CBPM preserved with mixed tocopherols and ethoxyquin had a

higher PV and comparable AV to the BMBM mixed tocopherols and ethoxyquin treatments at the end of the 18 week shelf life (Table 3.5).

The PV at zero weeks (Table 3.5) for the BMBM and CBPM unpreserved and mixed tocopherol treatments were less than the PV of the raw rendered protein meal prior to processing. This decrease in PV after processing may have been attributed to the dilution of the other ingredients within the kibble formulation. The PV for all treatments, excluding the BMBM unpreserved treatment, continued to increase over time. The BMBM unpreserved treatment continued into the termination phase as demonstrated prior to processing (Table 3.1). The secondary oxidation products, or AV, increased for all treatments, but not as dramatically for the unpreserved BMBM as the CBPM (Table 3.5). The AV of the BMBM unpreserved treatment prior to processing was considerably higher for the unpreserved treatments when compared to the mixed tocopherols and ethoxuquin treatments. Processing may have broken down the aldehydes into smaller compounds, such as organic acids, that the AV assay could not measure after processing. Alternatively, oxidation of the other ingredients within the kibble may have contributed to the increase in AV during the 18 week shelf life.

Gas Chromatography/Mass Spectroscopy Shelf Life - Kibble

The aldehydes that were measured using GC-MS ranged in several carbons in length, but we chose to report on number of carbon atoms 6 to 10, such as hexanal, heptanal, octanal, nonanal, and decanal, because they are considered important contributors to rancid and unpleasant flavors and (or) odors in oxidized oils (Frankel et al., 1985; DeHaan et al., 2004). It was found in the studies with DeHaan et al (2004) and Koppel et al., (2013), that hexanal, heptanal, octanal, and nonanal compounds were consistently detected in animal fats and dry kibble, but decanal was not always found. This would support the low values of decanal that

were found in the BMBM and CBPM treatments relative to the to the other volatile compounds (Table 3.6). In the study of Koppel et al. (2013), the total aldehydes contributed more than 50% of the total compounds identified in grain and grain-free dry kibble samples. In two studies by Greenberg (1981), the volatile aldehydes of meat and bone meal and poultry by-product meal were identified in order of decreasing abundance as hexanal, heptanal, octanal, nonanal, and decanal. Hexanal was identified as the aldehyde in the most abundance, representing about 43% of the relative concentration in meat and bone meal and 40% in poultry by-product meal (Greenberg, 1981). Shahidi et al. (1994) found that during a three week shelf life study of meat, hexanal concentration increased rapidly during the early stages of storage and dramatically decreased after six days; thus, mimicking the similar phases of hydroperoxide formation during lipid oxidation. Shahidi et al., (1994) suggests that one should be cautious when using hexanal as a marker of lipid oxidation as it may correspond to two different points in the oxidation process. This increase and decrease was observed in the BMBM unpreserved and mixed tocopherols treatments for all volatile compounds within these treatments. The initial increase of hexanal and other volatiles was observed in the BMBM ethoxyquin and CBPM treatments (Table 3.6). The aldehydes listed above have been identified as contributors to the flavor of cooked beef and are more concentrated in beef and chicken meat that are uncured versus cured (Elmore et al., 1999, Ramarathnam et al., 1993). This increase in concentration is evident in the unpreserved CBPM treatment relative to the preserved treatments (Table 3.6). It is also suggested by Ramarathnam et al. (1993) that several factors, both pre-slaughter and post-mortem, have an effect on the compounds formed in meat products, such as the type of feed, storage and sanitation conditions, and processing methods.

Human Rancidity Panel

The goal for using an untrained panel was to explore in a preliminary study circumstances that would mimic an at-home consumer based analysis of the pet food and to determine what the consumer may identify as "rancid." The term "rancid" is often used to designate the off-flavors and odors caused by lipid oxidation in foods in addition to several other sensory attributes (Jacobsen, 1999). Although, the meaning of the term "rancid" may depend on the food product in question. What is "rancid" for one particular food may differ for another (Jacobsen, 1999). The sensory attributes of various foods can be identified with a trained sensory panel equipped to breakdown the components (Koppel et al., (2013), or a lexicon can be developed as in the study with Di Donfrancesco et al., (2012). This analysis provided a basis for a complimentary sensory analysis that will be completed with trained panelists at a later date.

Interestingly, the untrained panelists identified the BMBM ethoxyquin treatment to be more "rancid" compared to the other BMBM treatments in the beginning of the shelf life until 18 weeks. But this treatment had the lowest PV and AV throughout the shelf life. The high sensory scores of the of the BMBM ethoxyquin treatment may be attributed to the strong odor of ethoxyquin itself or some other unidentified olfactory note. In the study with Thompkins et al. (1999), AV correlated ($R = 0.82$) well with the development of off-odors as the frying/heating time of oil samples increased. This agrees with our results for the AV of the BMBM unpreserved treatment at 18 weeks; wherein, the untrained panelist identified this treatment as the most "rancid" treatment. But the high sensory scores did not agree with the low AV of the other two BMBM treatments at the beginning of the shelf life. The untrained panelist identified the CBPM unpreserved treatment as the most "rancid" treatment throughout most of the shelf life study. These results correspond to high AV of this treatment as well.

Summary

The model used to create oxidized meals was effective. The foods produced with oxidized meals had some “re-set” to the oxidation levels following extrusion. Oxidation of the kibble occurred regardless of treatment, but was rapid and extensive in meals without preservative. The ingredient oxidation levels were diluted by food production and their oxidation may not completely account for later food product deterioration. Rendered protein meal stability is essential to the shelf life of extruded pet foods.

Table 3.1 Analysis of oxidation measures on beef meat and bone meal (BMBM) prior to processing (mean ± SD).

		BMBM		
	Time (Days)	Unpreserved	Mixed Tocopherols	Ethoxyquin
Peroxide Value (meq/kg)	2	3.33 ± 1.282	2.22 ± 0.000	2.22 ± 0.000
	7	34.44 ± 32.054	2.22 ± 0.006	2.23 ± 0.006
	12	70.95 ± 10.242	3.33 ± 1.282	2.22 ± 0.000
	17	95.53 ±10.260	2.22 ± 0.000	2.22 ± 0.006
	23	109.51 ± 1.819	2.22 ± 0.000	2.23 ± 0.006
	28	102.29 ± 2.448	4.43 ± 0.000	2.22 ± 0.000
	33	106.75 ± 5.260	5.55 ± 1.287	2.23 ± 0.006
	40	104.52 ± 2.690	5.55 ± 1.299	2.22 ± 0.000
	48	96.53 ± 1.155	5.55 ± 1.299	2.23 ± 0.006
	59	87.99 ± 2.448	8.14 ± 1.144	2.22 ± 0.006
p-Anisidine Value (g/g)	2	6.91 ± 0.000	0.00 ± 0.000	0.00 ± 0.000
	7	7.33 ± 6.443	1.91 ± 0.635	0.00 ± 0.000
	12	17.23 ± 2.806	5.28 ± 0.352	0.00 ± 0.000
	17	9.92 ± 1.542	1.61 ± 1.137	0.00 ± 0.000
	23	12.11 ± 0.00	2.11 ± 0.000	0.00 ± 0.000
	28	6.75 ± 2.465	1.48 ± 0.508	0.00 ± 0.000
	33	15.19 ± 4.284	5.23 ± 1.628	0.00 ± 0.000
	40	18.70 ± 0.375	7.47 ± 1.645	0.00 ± 0.000
	48	17.72 ± 1.801	5.68 ± 2.448	0.00 ± 0.000
	59	14.30 ± 3.299	7.76 ± 0.966	0.00 ± 0.000

Table 3.2 Analysis of oxidation measures on chicken by-product meal (CBPM) prior to processing (mean ± SD).

		CBPM		
	Time (Days)	Unpreserved	Mixed Tocopherols	Ethoxyquin
Peroxide Value (meq/kg)	2	4.43 ± 5.110	1.11 ± 1.282	1.12 ± 1.287
	7	15.58 ± 5.138	3.34 ± 1.287	2.22 ± 0.000
	12	35.55 ± 7.700	3.33 ± 1.276	2.23 ± 0.006
	19	45.58 ± 14.070	3.33 ± 1.282	2.22 ± 0.000
	27	67.64 ± 1.305	3.34 ± 1.287	2.22 ± 0.006
	38	81.20 ± 11.367	4.43 ± 0.010	2.22 ± 0.006
p-Anisidine Value (g/g)	2	0.15 ± 0.173	0.00 ± 0.000	0.00 ± 0.000
	7	1.54 ± 0.745	0.37 ± 0.156	0.00 ± 0.000
	12	3.62 ± 2.177	1.80 ± 1.397	0.00 ± 0.000
	19	4.32 ± 3.285	1.96 ± 1.189	0.00 ± 0.000
	27	4.46 ± 2.211	1.50 ± 0.421	0.00 ± 0.000
	38	3.02 ± 3.006	1.21 ± 1.072	0.00 ± 0.000

Figure 3.1 Oxidation measures on beef meat and bone meal (BMBM) prior to pet food processing (average of ±2 days).

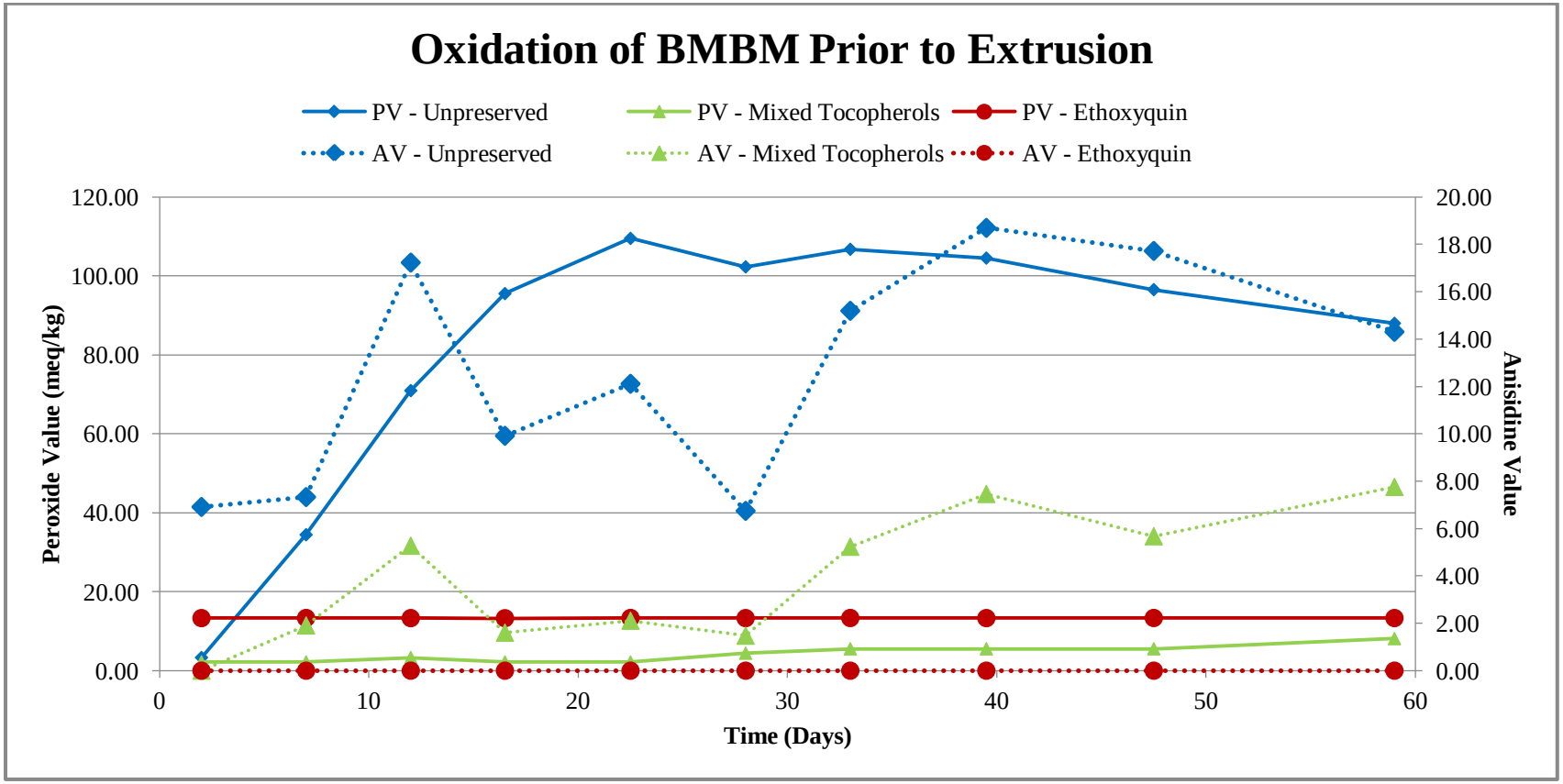


Figure 3.2 Oxidation measures on chicken by-product meal (CBPM) prior to pet food processing (average of ±2 days).

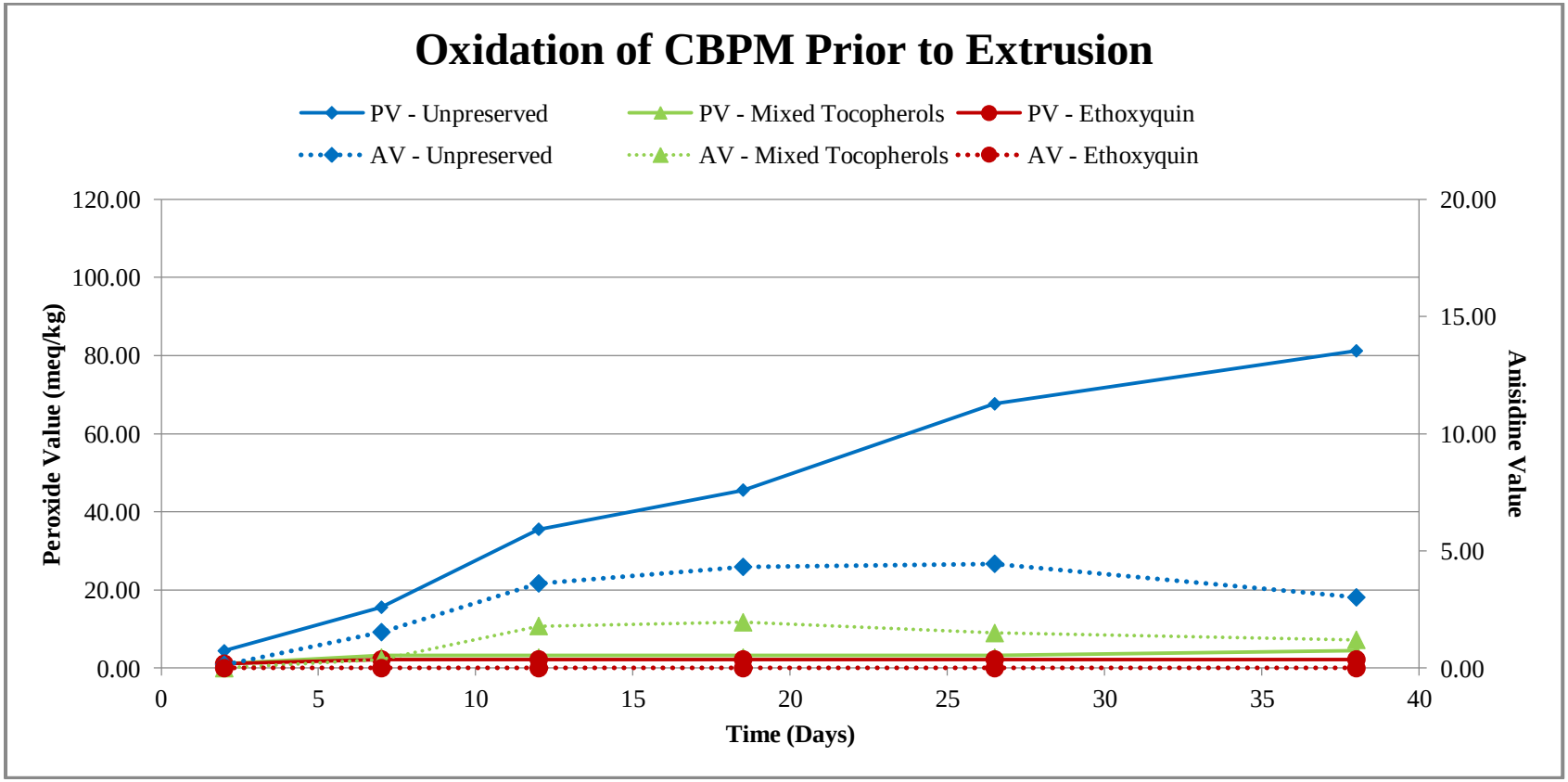


Table 3.3 Pet food diet produced with oxidized chicken by-product meal (CBPM) and beef meat and bone meal (BMBM) inclusion.

Ingredient	Diet, %	Diet %
Chicken By-Product Meal	37.80	-
Meat and Bone Meal	-	51.37
Rice, Brewers	18.92	14.38
Corn	18.92	14.38
Wheat	18.92	14.38
Beet Pulp	4.00	4.00
Potassium Chloride	0.40	0.40
Monosodium Phosphate	-	0.25
Salt	0.25	0.25
Choline Chloride, 60% Dry	0.20	0.20
Vitamin Premix (Kansas)	0.15	0.15
Trace Mineral Premix (Kansas)	0.10	0.10
DL Methionine	0.10	-
Taurine	-	0.05
Ingredient Total	**100.00**	**100.00**

Figure 3.3. Extruder Screw Profile

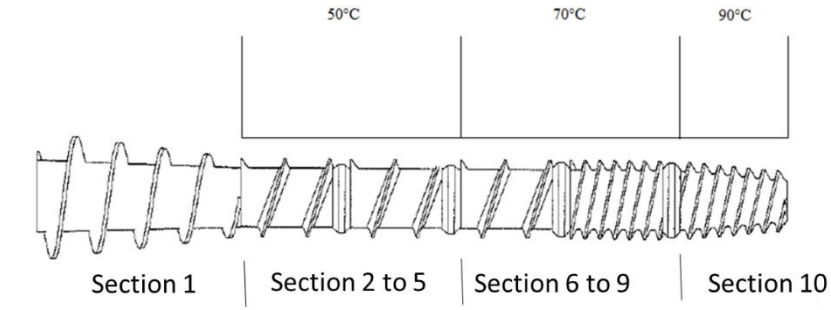


Table 3.4 Analysis of volatile compounds of rendered protein meals prior to extrusion via gas chromatography.

Compound	BMBM			CBPM			SEM	*P*-Value
	Unpreserved	Mixed Tocopherols	Ethoxyquin	Unpreserved	Mixed Tocopherols	Ethoxyquin		
Hexanal (µg/kg)	5,515.74^{b}	15,355.82^{a}	1,233.18^{c}	5,762.33^{b}	3,128.79^{bc}	1550.73^{c}	818.615	0.0004
Heptanal (µg/kg)	4,642.83^{a}	3,081.80^{b}	1,108.29^{c}	631.62^{cd}	233.47^{d}	269.79^{d}	233.253	0.0014
Octanal (µg/kg)	7,212.00^{a}	4,733.21^{b}	691.75^{c}	462.19^{c}	251.86^{c}	154.69^{c}	435.510	0.0011
Nonanal (µg/kg)	4,515.96^{a}	4,395.62^{a}	767.47^{b}	525.92^{b}	260.47^{b}	179.75^{b}	292.107	0.0014
Decanal (µg/kg)	182.48	657.80	233.32	16.32	9.83	8.54	194.503	0.4480

^{abcd} Means within a row that lack a common superscript differ $P \leq 0.05$.

Table 3.5 Analysis of oxidation measures (mean ± Sd) under accelerated shelf life conditions (40°C, 70% RH) of kibble produced from oxidized rendered protein meals.

		BMBM			CBPM		
	Time (Weeks)	Unpreserved	Mixed Tocopherols	Ethoxyquin	Unpreserved	Mixed Tocopherols	Ethoxyquin
Peroxide Value (meq/kg)	0	10.07 ± 1.345	2.22 ± 0.006	2.22 ± 0.006	14.41 ± 1.259	2.78 ± 0.641	2.22 ± 0.000
	3	8.33 ± 0.652	6.10 ± 0.641	2.77 ±0.629	14.98 ± 1.917	3.33 ± 1.282	2.18 ± 2.140
	6	9.85 ± 2.275	10.79 ± 1.166	2.46 ± 0.566	36.25 ± 5.618	4.91 ± 1.132	1.96 ± 1.960
	12	8.86 ± 0.000	13.29 ± 0.023	2.22 ± 0.006	50.96 ± 20.490	14.40 ± 1.328	2.22 ± 1.700
	18	6.66 ± 0.006	15.48 ± 0.006	3.33 ± 1.287	53.15 ± 12.754	23.21 ± 3.834	4.44 ± 1.800
p-Anisidine Value (g/g)	0	10.08 ± 2.794	9.62 ± 0.930	3.03 ± 0.370	15.56 ± 0.393	3.85 ± 1.166	1.79 ± 1.200
	3	10.38 ± 1.016	7.79 ± 0.941	0.88 ± 0.803	6.47 ± 0.410	2.46 ± 0.629	1.29 ± 0.930
	6	13.86 ± 0.306	11.12 ± 1.068	3.95 ± 0.647	11.28 ± 1.039	4.34 ± 0.266	2.66 ± 2.570
	12	18.77 ± 1.651	15.52 ± 0.675	6.57 ± 0.485	22.04 ±5.433	10.72 ± 0.416	7.02 ± 6.660
	18	17.52 ± 0.156	15.98 ± 2.026	6.12 ± 0.704	33.41 ± 5.987	15.45 ± 1.744	7.53 ± 7.400

[abcdefghijklmno] Means within a row and column that lack a common superscript differ $P \leq 0.05$.

Table 3.6 Concentration of volatile compounds of accelerated (40°C, 70% RH) kibble via gas chromatography.

Compound		BMBM			CBPM			SEM	*P*-Value
	Time (Weeks)	Unpreserved	Mixed Tocopherols	Ethoxyquin	Unpreserved	Mixed Tocopherols	Ethoxyquin		
Hexanal (µg/kg)	0	1,418.86^{ijklm}	7,861.76^{a}	295.08^{op}	1,062.29^{jklmno}	723.93^{mnop}	255.50^{p}	274.812	<0.0001
	3	1,545.81^{hijkl}	3,516.53^{c}	388.69^{op}	1,813.94^{fghij}	580.50^{nop}	331.92^{op}		
	6	1,986.35^{efghi}	2,624.21^{de}	361.84^{op}	2,575.39^{def}	941.55^{klmnop}	743.61^{mnop}		
	12	1,574.72^{hijkl}	2,221.49^{efgh}	588.73^{nop}	3,113.67^{cd}	1,637.76^{hijk}	811.59^{lmnop}		
	18	1,462.56^{hijklm}	3,557.83^{c}	1779.82^{ghij}	4,954.59^{b}	2,501.54^{defg}	1,263.36^{ijklmn}		
Heptanal (µg/kg)	0	842.44^{bc}	521.51^{cdef}	97.77^{ghi}	133.24^{ghi}	43.46^{hi}	28.75^{i}	123.664	<0.0001
	3	747.26^{bcd}	290.67^{fghi}	114.27^{ghi}	139.40^{ghi}	65.99^{hi}	30.07^{hi}		
	6	1,592.52^{a}	386.55^{efgh}	118.94^{ghi}	354.25^{efghi}	97.45^{ghi}	92.72^{ghi}		
	12	1,004.65^{b}	672.35^{bcde}	210.57^{fghi}	426.41^{defg}	332.00^{efghi}	170.63^{fghi}		
	18	1,008.85^{b}	774.46^{bcd}	520.84^{cdef}	980.11^{b}	672.14^{bcde}	234.28^{fghi}		
Octanal (µg/kg)	0	1,857.98^{b}	812.89^{cde}	197.17^{hijk}	217.55^{ghijk}	75.08^{jk}	27.71^{k}	136.938	<0.0001
	3	1,839.88^{b}	546.15^{defgh}	236.74^{ghijk}	336.02^{fghijk}	90.20^{jk}	46.25^{k}		
	6	2,068.78^{ab}	515.64^{defghi}	146.76^{ijk}	462.84^{efghij}	111.57^{jk}	64.61^{k}		
	12	1,816.19^{b}	724.24^{cdef}	181.13^{hijk}	607.95^{cdefg}	253.97^{ghijk}	109.88^{jk}		
	18	2,378.81^{a}	996.08^{c}	327.26^{ghijk}	904.95^{cd}	339.17^{fghijk}	129.28^{ijk}		
Nonanal (µg/kg)	0	1,696.82^{a}	1256.11^{b}	85.70^{lm}	296.32^{hijkl}	137.99^{klm}	59.63^{m}	80.500	<0.0001
	3	1,768.01^{a}	767.97^{def}	384.07^{ghij}	500.55^{gh}	90.54^{klm}	84.45^{lm}		
	6	951.42^{cd}	408.06^{ghi}	146.50^{klm}	284.78^{hijklm}	104.06^{klm}	68.01^{lm}		
	12	1,106.41^{bc}	321.21^{hijk}	159.45^{jklm}	408.64^{ghi}	143.49	83.25^{lm}		
	18	830.94^{de}	607.18^{efg}	268.72^{hijklm}	554.74^{fg}	181.12^{ijklm}	94.78^{klm}		
Decanal (µg/kg)	0	213.08^{b}	42.70^{cd}	35.97^{cdef}	7.41^{g}	3.87^{g}	1.80^{g}	9.293	<0.0001
	3	324.06^{a}	7.59^{g}	38.53^{cde}	10.57^{fg}	7.25^{g}	2.04^{g}		
	6	55.45^{c}	20.34^{defg}	10.08^{fg}	5.73^{g}	2.83^{g}	2.30^{g}		
	12	36.52^{cdef}	17.34^{defg}	14.79^{efg}	5.54^{g}	3.61^{g}	1.97^{g}		
	18	38.45^{cde}	17.91^{defg}	15.25^{efg}	13.57^{efg}	3.85^{g}	2.32^{g}		

^{abcdefghijklmnop} Means within a row and column that lack a common superscript differ $P \leq 0.05$.

Table 3.7 Sensory (rancidity) characteristics on kibble samples (40°C, 70% RH) kibble samples.*

		BMBM			CBPM				
	Time (Weeks)	Unpreserved	Mixed Tocopherols	Ethoxyquin	Unpreserved	Mixed Tocopherols	Ethoxyquin	SEM	*P*-Value
Human Rancidity Score	0	2.00^{efghij}	2.05^{efghij}	$2.10^{defghij}$	$2.25^{cdefghij}$	1.30^{k}	$2.10^{defghij}$	0.236	0.0067
	3	$2.10^{defghij}$	1.85^{hijk}	2.60^{bcdef}	2.05^{efghij}	1.95^{fghijk}	1.85^{hijk}		
	6	1.65^{jk}	2.65^{bcde}	2.90^{bc}	2.05^{efghij}	$2.10^{defghij}$	1.90^{ghijk}		
	12	$2.50^{bcdefgh}$	$2.45^{bcdefghi}$	3.05^{ab}	2.75^{bcd}	1.80^{ijk}	1.90^{ghijk}		
	18	3.65^{a}	$2.45^{bcdefghi}$	2.55^{bcdefg}	2.65^{bcde}	2.05^{efghij}	$2.20^{defghij}$		

^{abcdefghijk} Means within a row and column that lack a common superscript differ $P \leq 0.05$.

* Based on a scale of 1 to 5 (5 = very rancid) utilizing an untrained panel.

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