Shelf life and quality of minimally processed pet foods and pet food ingredients

by

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B.S., Kansas State University, 2012
M.S., Kansas State University, 2016

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
College of Agriculture

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Abstract

Pet food sales in the US have increased at a 5.1% annual rate since 2015 (Packaged Facts, 2020) to an estimated $54.62 billion in 2019. Much of this growth has been due to new food forms and ingredients. The fastest growing categories have been raw-frozen and freeze-dried foods. Further, new minimally processed animal proteins and plant proteins have been introduced. Raw-frozen and freeze-dried pet foods contain a large proportion of high fat meats which increases the chance for oxidation. Further, many of the new minimally processed protein sources and alternative ingredients such as legumes have not been evaluated for their nutritional contribution. The link is processing and how it influences the nutritional quality of these two foundations of essential nutrients for pets. The objective therefore is to determine the impact process has on oxidation of fats in these new food forms and the quality of protein in these novel ingredients.

Two experiments were conducted to determine the impact of increased storage time on raw-frozen and freeze-dried pet food patties based on chicken or lamb. Raw-frozen samples were stored at -20°C 0, 4, 8 or 16 weeks. Freeze-dried samples were stored frozen for 12, 24, and 36 weeks prior to freeze drying and then stored dried for an additional 4, 8, 16 weeks. Raw-frozen chicken and lamb patties had increased peroxide value (PV) as storage time increased ($P < 0.05$). Propanal content was higher in both raw-frozen chicken and lamb at 4 and 8 weeks of storage but at 16 weeks was not different from week 0 ($P < 0.05$). Freeze-dried samples had reduced PV during storage and increased free fatty acid and propanal concentration ($P < 0.05$). Among antioxidants, mixed tocopherols provided more protection against oxidation compared to other treatments ($P < 0.05$). Two 10-day chick growth assays were conducted to determine protein efficiency ratio (PER) of various proteins differing in process and source. Spray dried egg

(SDEG) was considered the reference in both experiments and resulted in the highest PER values ($P < 0.05$). Rendered protein meals, dehydrated chicken, and two spray dried chicken powders were evaluated in experiment one. The dehydrated chicken and the high protein chicken powder had similar PER values to SDEG. The rendered protein meals had lower PER values compared to the gently processed meats and SDEG ($P < 0.05$). Protein digestibility amino acids scores (PDCAAs) were determined in experiment one and were highly correlated to PER ($R=0.80$ for dog and $R=0.95$ for cat; $P<0.05$). In experiment two, the PER of all legume sources were lower than SDEG ($P < 0.05$). When legume sources were mixed with SDEG, there was an improvement in PER but not enough to match SDEG. Overall, these experiments provide supporting information regarding lipid and protein changes due to process and storage. Fat and protein are the two primary vehicles for the delivery of required nutrients to pet foods and the process can have a deleterious effect on their availability. Decreasing processing temperatures and providing preservative antioxidants may benefit nutrient retention in modern processed foods.

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Chapter 1 - Literature Review – Lipid Oxidation and Protein Quality in Pet Foods

Introduction

A pet can be described as any animal that is kept for companionship and not for consumption. Two-thirds of household in the United States own a pet with cats and dogs accounting for approximately 184 million of the total number of pets in the United States (APPA, 2017). Because of the large number of pets owned in the United States, there are many options available to feed these animals. Pet food sales in 2019 were $54.62 billion with annual growth of 5.1% since 2015 (Packaged Facts, 2020). Most of the products in the pet food market are dry extruded kibbles and canned foods, yet the fastest growth comes from new product forms and novel ingredients. Specifically, the fastest growing categories in the pet food industry are raw, frozen, and freeze-dried products. Between 2011 and 2014 freeze-dried sales increased from $22.7 million to $73.9 million (Lange, 2015). Raw pet food sales grew 196% and frozen grew 235% between 2012 and 2016 (Lange, 2017). These products often cover the gamut of marketing options, such as grain free and limited ingredient, that are also ingredient name options in standard kibble and canned food. An assumption of raw, frozen and freeze-dried products is that they, like their dry/can counterparts, are shelf stable when stored. In addition to this assumption that these products have a long shelf life, meat is also the main ingredient. Along with greater amounts of water, meat-based products also contain greater concentrations of fat than extruded kibbles. Fat content is of concern with extended storage times because it can oxidize and become rancid. This creates off odors and flavors and may reduce the nutritional value. Another concern,

22 especially with the raw-frozen foods are freeze-thaw cycles that may occur during transport and
23 storage.
24 Raw-frozen products may also be placed in frozen storage for extended periods of time. It
25 is often thought that freezing will stop all reactions that may take place at regular temperatures.
26 However, oxidation still occurs in meat products during frozen storage (freezer burn) for
27 extended periods of time (Belles et al., 2017; Soyer et al. 2010). In addition to extended freezing
28 time, the number of freeze-thaw cycles can also impact the quality of the meat. It has been
29 reported that increasing the number of freeze-thaw cycles increases the level of oxidation
30 products (Qi et al., 2012; Ali et al., 2015; Rahman et al., 2015; Chen et al. 2018). The addition of
31 antioxidants to these product categories might help slow or reduce the degree of fat oxidation.
32 For refrigerated meat products antioxidants, whether synthetic or natural, have been reported to
33 slow the progression of oxidation (Wilkinson et al, 2001; Botsoglou et al., 2003; Stika et al,
34 2007)
35 In addition to these new minimally processed food forms novel protein sources and
36 exploration of minimal processing applied to ingredients is being explored in pet foods. For
37 years, conventional pet foods were reliant on rendered meat and bone meal and grains like corn
38 and wheat. These traditional rendered protein meals are produced using high temperatures, 115
39 to 145°C (240 to 290 °F) as a method to separate and melt the fat from the solids (Meeker and
40 Hamilton, 2006). During this process, proteins can be damaged making them less digestible and
41 damage some amino acids (e.g. lysine). There has also been a push from consumers to use novel
42 protein alternatives, such as legumes. Both protein strategies are subject to less heat than
43 traditional rendered ingredients. Yet few of them have been evaluated for their protein quality as
44 part of a nutritionally complete diet. There are several methods in which one can evaluate the

45 quality of these protein sources. These include such assays as protein efficiency ratio studies,
46 evaluations of digestibility by in-vivo and in-vitro methods, nitrogen balance studies, and (or)
47 scores of utility such as protein digestibility amino acid score (PDCAAS) or digestible
48 indispensable amino acid score. Whether or not these new food forms and new ingredients are
49 justified, their quality and safety must be assured. Proteins and fats represent the two macro
50 elements which support the nutritional needs of the animal. Measures of lipid oxidation and how
51 it can be slowed are essential. There is little to no published research on raw-frozen and freeze-
52 dried pet food regarding stability, but one can use data from similar industries to form a
53 hypothesis as to what may be occurring in these types of pet food. In addition, how various
54 methods of processing affect protein quality and its evaluation are vital. With these methods an
55 understanding of the shortcomings of conventional processes and benefits of new protein sources
56 and lower input processes can be achieved. The goal is to characterize lipid and protein changes
57 during storage and processing in order to create better pet food formulations and ultimately
58 improve the nutrition of pets.

59 Lipid Oxidation

60 Lipid oxidation and its by-products

61 Lipid oxidation refers to the deterioration of fat, more so for unsaturated than saturated
62 fatty acids, as it reacts with prooxidants such as oxygen (Frankel, 2005; Schaich et al., 2013).
63 Lipid oxidation may be referred to as lipid peroxidation or autoxidation. The two types of lipids
64 are saturated and unsaturated. Saturated fatty acids contain no double bonds, meaning each
65 carbon is connected to two hydrogens. These fats are more difficult to oxidize as they have the
66 maximum number of attached hydrogens (Powar and Chatwal, 2007). Saturated fats are able to
67 tightly pack next to one another and are solids at room temperature (Powar and Chatwal, 2007).

68 Unsaturated fatty acids contain one or more double bonds. When this double bond occurs, it
69 causes a bend (change in the bond angle) in the molecule, exposing the double bond (Powar and
70 Chatwal, 2007). These double bonds react with other molecules, such as oxygen and metals,
71 during lipid oxidation. The more double bonds the more likely the fat will oxidize, and at an
72 increased rate (McClements and Decker, 2017).

73 Lipids, or fat, are comprised of a glycerol backbone with three fatty acids linked by ester
74 bonds. These ester-linkages impart a three-dimensional conformation; wherein, the fatty acids
75 intertwine with each other due to bends in unsaturated fatty acids. This may impart some
76 protection against oxidation as the double bonds become more difficult for oxygen to react.
77 Enzymes present in meat and offal are able to hydrolyze, or separate, the fatty acids from the
78 glycerol back bone. When this hydrolysis (Figure 1-1) occurs, free fatty acids and glycerol are
79 produced (Powar and Chatwal, 2007). The free fatty acids separated from their glycerol
80 backbone are more susceptible to react with radicals and oxidize more readily (Haard, 2000)
81 perhaps due to a loss of the steric hindrance and the conformational protection that was afforded
82 by the other fatty acids on glycerol.

83 The oxidation process involves production of primary and secondary oxidation products.
84 Primary oxidations products are referred to as hydroperoxides. The formation of primary
85 oxidation products is driven by singlet oxygen, lipoxygenases, and ionizing radiation. Figure 1-2
86 provides an example of the reaction occurring during the formation of hydroperoxides. In this
87 example, linoleic acid, an essential fatty acid for both dogs and cats, has a hydrogen abstracted
88 between the double bonds (this action will be explained below). When this occurs, an unpaired
89 electron remains and is referred to as a free radical. In the next step, oxygen (O_2) attaches to the
90 free radical creating a lipid peroxyl radical. This will also cause a rearrangement of the double

91 bonds. In order to stabilize the molecule, a hydrogen will be abstracted from a different fatty acid
92 to create a hydroperoxide. Hydroperoxide formation is self-perpetuating and thereby the process
93 is autocatalytic and often described as autoxidation.
94 Once hydroperoxides are formed, they can react further leading to breakdown products
95 such as aldehydes, ketones, alcohols, esters, hydrocarbons, and short chain fatty acids.
96 Aldehydes produced include nonenal, hexanal, propanal, and malondialdehyde depending on the
97 chain length and bonds of the degraded fatty acid. These can react further with other molecules
98 like proteins and DNA (Catalá, 2006). Malondialdehyde is a common oxidation product formed
99 through the scission process of fatty acids with at least three double bonds (Schaich et al., 2013).
100 The resulting products are considered secondary oxidation products and are responsible for the
101 off odors and flavors identified with rancid products (Frankel et al., 1985; Dehaan et al., 2004).
102 As an example, Figure 1-3 illustrates one pathway in which a hydroperoxide from
103 linoleic acid can be altered. This is just one of many possibilities as a lipid is oxidized into
104 secondary products. The formation of secondary oxidation begins when the hydrogen is
105 abstracted, leaving a free radical on the oxygen. The molecule then undergoes a scission process
106 that will break the molecule into two, one of which will possess the free radical. This free radical
107 can be stabilized by the addition of hydrogen.
108 It is important to understand what is happening as different products are forming in order
109 to slow their development and determine which analytical method is appropriate for analysis. A
110 sequence of transition events is depicted in Figure 1-4 for the products produced from the
111 oxidation process. Over time the primary oxidation products (labeled LO· products) slowly
112 increase at the beginning. The formation of hydroperoxides will begin to rapidly increase due in
113 part to autoxidation. As the formation rate of these products begins to plateau the substrate

114 available for primary oxidation declines and they begin to breakdown (via scission, redox, etc.)
115 and secondary oxidation products increase.

116 **Methods to characterize lipid oxidation**

117 There are many methods commonly used to measure primary and secondary oxidation
118 products in ingredients and pet food. Fats are often analyzed for their propensity to oxidize, the
119 level of primary oxidation products, and concentration of secondary oxidation products.
120 Hydroperoxides are primary oxidation products (Mehta et. al, 2015) that are commonly tested to
121 determine whether a product is becoming rancid. For secondary oxidation products, there is a
122 wide array of compounds produced which can be measured. However, most require a pure fat
123 sample. Most pet foods are complex in nature and contain fat along with proteins, minerals,
124 vitamins, and carbohydrates. Thus, the fat must be extracted from the ingredients or foodstuff
125 first. This can be accomplished using solvents, such as hexane or chloroform-methanol, to
126 separate the fat from the food followed by solvent evaporation utilizing a rotary evaporator to
127 yield a fat sample. Acid hydrolysis may also be needed to extract fat from a product, especially if
128 it is locked in a starch matrix such as an extruded kibble. Throughout the research literature,
129 there are assays that were used to evaluate a fat undergoing some sort of degradation or
130 oxidation. Propensity to oxidize is often measured as free fatty acid (FFA) concentrations and
131 iodine values (iV). Peroxide value is used to evaluate the concentration of hydroperoxides while
132 para-anisidine value (AV), thiobarbituric acid reactive substances (TBARS), and gas
133 chromatography coupled to mass spectrometry via headspace analysis (GC) are used to evaluate
134 secondary oxidation products.

135 **Free Fatty Acid Concentration**

136 This method that can help identify how susceptible a fat is to oxidation by the
137 measurement of free fatty acid concentration. Free fatty acids are produced by enzymatic
138 hydrolysis of triglycerides. When the fatty acids are de-esterified from the glycerol, they become
139 less protected against oxidation because their double bonds are more exposed to attack by free
140 radicals. With an increase in free fatty acid concentration, especially those that contain more
141 unsaturated fatty acids, there is a much greater chance that oxidation will occur (Kinsella et al.,
142 1978; Mistry and Min; 1987). Free fatty acids can act as prooxidants and it may occur at a faster
143 rate as more substrate is exposed as they attract metals (Waraho etl al., 2009; Wahaho et a.
144 2011).

145 The official method of the American Oil Chemist Society utilized a change in color to
146 determine free fatty acid concentration (AOCS, 1997). In short, fat is mixed with hot neutralized
147 alcohol and phenolphthalein indicator. A solution containing sodium hydroxide is then titrated
148 into the fat/alcohol mixture until a pink color is achieved and sustained for 30 seconds. Below
149 are equations used to determine free fatty acid concentration.

150 **Equation 1. Free fatty acid concentration as oleic acid.**

151 $$FFA\ as\ oleic, \% = \frac{ml\ of\ alkali\ \times N \times 28.2}{sample\ weight\ (g)}$$

152 **Equation 2. Free fatty acid concentration as lauric acid.**

153 $$FFA\ as\ lauric, \% = \frac{ml\ of\ alkali\ \times N \times 20.0}{sample\ weight\ (g)}$$

154 **Equation 3. Free fatty acid concentration as palmitic acid.**

155 $$FFA\ as\ palmitic, \% = \frac{ml\ of\ alkali\ \times N \times 25.6}{sample\ weight\ (g)}$$

156 **Iodine Value**

157 A gross method to characterize the total level of unsaturation in fats, and thereby their
158 vulnerability to oxidation is the iodine value (iV). This method is often used in the swine

159 industry as an indicator of fat firmness or quality (Hugo and Roodt, 2007; Nemechek et al.,
160 2015). In this application a higher value indicates more unsaturated fats which is less desirable
161 by the meat packing industry (McClelland et al.; 2012) because it leads to soft-bellies (bacon).
162 For food oxidation, a higher iV would be indicative of more unsaturation in the fat (Benz et al.,
163 2010; O'Keefe and Pike, 2010). Iodine value can be determined by dissolving fat in a solvent
164 and measuring the amount of iodine absorbed. It can also be calculated by determining the fatty
165 acid profile by gas chromatography which has been used in swine studies (Equation 4; AOCS,
166 1998; Nemechek, 2015).

167 **Equation 4. Calculating iodine value utilizing fatty acid analysis.**

168

169–170
$$IV = [C16{:}1] \times 0.95 + [C18{:}1] \times 0.86 + [C18{:}2] \times 1.732 + [C18{:}3] \times 2.616 + [C20{:}1] \times 0.785 + [C22{:}1] \times 0.723$$

171 **Peroxide Value**

172 Peroxide value is a measure of the primary oxidation products, or hydroperoxides, that
173 are produced during the initial steps of oxidation (Figure 1-2). Several methods have been
174 developed to determine the peroxide value in oil and fat samples, and include: AOAC (2000),
175 AOCS (Cd 8b-90; 1996), FOX (Shantha & Decker, 1994), and IDF (74A; 1991). However, each
176 of these assays provide a slightly different value according to Mehta et al. (2015). The
177 colorimetric methods (IDF and FOX) yield lower values than the iodometric methods (AOAC
178 and AOCS). It is also difficult to reproduce peroxide values due to the rapid creation and
179 destruction of hyrdoperoxides during oxidation (Guillan et al., 2002; Van de Voort et al. 1994).
180 Due to this rapid formation and destruction, it is important to understand which side of the
181 oxidative curve your product may be. Thus, it is best used as initial (iPV) only or to follow the
182 time course of PV formation rather than as a single point in time test. For, if the product were

183 fully oxidized it could potentially yield a low peroxide value having already degraded primary
184 into a secondary oxidation products.

185 **Anisidine Value**

186 Because of the need to understand whether a low peroxide value is due to decomposition
187 (i.e., hydroperoxides have reacted and created secondary products), several assays can be used to
188 measure the amount of secondary oxidation products. The anisidine value (AV) is a non-volatile
189 quantification of secondary oxidation products. It estimates the amount of carbonyl compounds
190 (α and β- unsaturated aldehydes) within the fat (Roozen and Linssen, 1992). Aldehydes are
191 generated when hydroperoxides (primary oxidation product) are broken down. To determine
192 anisidine value, isooctane is mixed with the fat sample and *p*-anisidine. The aldehydes react with
193 the *p*-anisidine which leads to a color formation which can be measured at 350 nm in a
194 spectrophotometer (AOCS, 1997).

195 **Thiobarbituric Reactive Substances**

196 Malonaldehyde (MDA), another secondary oxidation product, is measured as
197 thiobarbituric acid reactive substances (TBARS). There are two common methods to determine
198 the amount of malondialdehyde in a sample - one by a rapid, wet method (Buege and Aust, 1978;
199 Sinnhuber and Yu, 1958) and a second via distillation (Tarladig et al., 1960; Koniecko, 1979). In
200 the rapid wet method, the sample is mixed directly with thiobarbituric acid (TBA), boiled for 10
201 minutes, centrifuged, and the supernatant is collected. During the 10 minutes of incubation, TBA
202 reacts with carbonyls to form a red color that can be measured at 532 nm on a spectrophotometer
203 (McClements and Decker 2017). This method can have interference and development of a
204 yellow color when sugars are present (AMSA, 2012). This interference from sugars can be
205 overcome by the distillation method; wherein, a sample along with water, HCl, antifoaming

206 agent, and boiling beads are placed in a flask and attached to a distillation column and boiled.
207 Once 50 ml of distillate is collected, 5 ml of TBA is added to 5 ml of the distillate and boiled for
208 35 minutes, then cooled. A 3 ml sample is then pipetted into a cuvette and analyzed at 532 nm in
209 a spectrophotometer.

210 This method is not specific to malonaldehyde. Thiobarbituric acid can react with other
211 substances, including alkanals, acetaldehyde, sugars, and non-enzymatic browning products
212 (Tarladgis et al, 1962; Kosugi et al, 1987; Marcuse and Johansson, 1973; McClements and
213 Decker, 2017). These reactions have been reported to create a yellow or orange pigment in
214 freeze-dried samples and can be measured between 450-455 nm (Wilkinson et al., 2001). Due to
215 this interference, Kamarei and Karel (1984) created a method to measure malonaldehyde via
216 fluorescence, by crosslinking MDA with amino groups to create Schiff bases. Both the TBARS
217 and fluorescence methods have shown an increase in secondary products when beef and chicken
218 were stored for an extended amount of time (Wilkinson et al., 2001).

219 **Gas chromatography – Mass Spectrometry (GC-MS)**

220 Gas chromatography-mass spectrometry (via head space) can be used to analyze volatile
221 compounds created during oxidation. These compounds may include hexanal, propanal, pentane,
222 pentanal, nonanal, decanal, and 2,4-decadienal to name a few (McClements and Decker, 2017;
223 Goodridge et al, 2003; Ahn et al. 1998; Frankel, 1983; Frankel et al., 1981). Hexanal represents
224 the breakdown of linoleic and arachidonic acid (Frankel, 1980; Belitz et al., 2013), which are
225 essential fatty acids. Propanal is generated by the breakdown of linolenic acid (Frankel, 1980;
226 Belitz et al., 2013) a critical omega-3 fatty acid.

227 Gas chromatography is completed by placing a sample in a vial and heating it for a
228 specified amount of time to allow for compounds to become volatilized. These volatiles are

229 either trapped in the space above the sample (headspace) or collected in a trap (Qian et al. 2010).
230 Other methods to obtain volatiles from a sample are distillation extraction, solvent extraction,
231 and solid-phase extraction. Once the volatiles are collected, they are injected into the column for
232 separation. Columns can be packed or capillary. Compounds are then carried through the column
233 by a carrier gas (mobile phase), often nitrogen, then separated. The volatiles can be separated
234 based on polarity, size, boiling point, and charge (Ismail and Neilsen, 2010). Gas
235 chromatography is often coupled to mass spectrometry. Mass spectrometry involves adding a
236 charge to the compound for resolution based on a mass to charge ratio (Smith and Thakur, 2010).
237 The charged elements then travel through electrostatic fields before detection. Coupling these
238 two methods allows for the identification of elements as they elute form the GC column (Smith
239 and Thakur, 2010).

240 **Human Sensory Panel**

241 The degradation of fat into the secondary oxidation products produces off aromas and
242 flavors in a product (Frankel, 1987; Goodridge et al., 2003). These changes in aromas and
243 flavors can be described by human sensory panels (Chanadang et al., 2016; Nunez de Gonsalez
244 et al., 2008; Lee and Ahn, 2005; Kulkarni et al., 2011; Lee et al., 2006; Naceena et al., 2008;
245 Dwivedi et al., 2006). These panels can help determine whether a product has become oxidized
246 and is no longer acceptable to consumers. Trained and untrained panels may be used to examine
247 a product. Trained sensory panelists detect specific aromas that are present and give them a
248 numerical value based on freshly created reference samples while untrained panelists generally
249 will describe if they dislike or like the product.

250 **Antioxidants**

251 Antioxidants are commonly used in products at low concentrations to slow the oxidation
252 of lipids and proteins. Antioxidants are classified as primary or secondary antioxidants. Primary
253 antioxidants are those that donate a hydrogen or electron to free radical (Shahidi, 2015). This
254 donation by the antioxidant reduces propagation in the oxidation cycle (Masuda et al., 2001;
255 Saito et al., 2004). The secondary antioxidants reduce the impact of prooxidants, such as metal
256 ions. Secondary antioxidants include ethylenediaminetetraacetic acid (EDTA), chelators, and
257 beta-carotene (Shahidi, 2015).

258 Antioxidants can also be classified as synthetic or natural. Synthetic antioxidants are
259 those that are chemically manufactured and include butylated hydroxyanisole (BHA), butylated
260 hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), and propyl gallate (Biswas et al., 2004;
261 Formanek et al., 2001; Jayathilakan et al. 2007; Shahidi, 2015). These are also considered
262 primary antioxidants that are able to donate an electron to free radicals (Shahidi, 2015). Due to
263 public influence, many food producers are shifting to the use of natural antioxidants. Sources that
264 have antioxidant activity in a food system and are considered natural when there is need to label
265 a product as containing no artificial flavor, color, chemical preservative, or do not contain any
266 synthetic ingredients (Kumar et al., 2015; USDA, 2005). Natural antioxidants can be found in
267 spices and herbs (e.g., rosemary, oregano, etc.), and fruits (e.g., cranberries, grapes, and plums,
268 etc.) due to their high levels of phenolic compounds (McClements and Decker, 2017). Sources
269 containing phenolic compounds have the ability to donate a hydrogen or electron, which also
270 classifies them as primary antioxidants (Muchuweti et al., 2007; Shahidi, 2015).

271 **Primary Antioxidants**

272 As previously stated, primary antioxidants are those that are able to donate either a
273 hydrogen or an electron to a free radical, such as peroxyl radical, for stabilization and the
274 prevention of further oxidation. These types of antioxidants may also be referred to as free
275 radical scavengers. During this process the antioxidant donates an electron/hydrogen to the free
276 radical, stabilizing it from further oxidation reactions. The antioxidant itself then becomes a
277 radical allowing for further interaction with other radicals. The antioxidant radical can then bind
278 with a peroxyl radical in a termination reaction. Figure 1-5 provides an example of how primary
279 antioxidants neutralize a free radical. In this example, the antioxidant donates a hydrogen to the
280 peroxyl radical, stabilizing it. This antioxidant undergoes rearrangement and now has an
281 unpaired electron and becomes a radical itself. This allows the antioxidant radical to react a
282 second time with another antioxidant radical or another peroxyl radical. In Figure 1-5, the
283 antioxidant radical bonds to a peroxyl radical which stabilized both molecules. This is considered
284 a termination reaction.

285 **Secondary Antioxidants**

286 Prooxidants can enhance the rate at which oxidation takes place in a food product. These
287 include transition metals, singlet oxygen, and enzymes (McClements and Decker, 2017; Schaich,
288 2013). Chelators are used to stabilize metals, such as iron and copper, by binding to sites with a
289 free electron. Iron and copper are considered the most catalytic metals, but manganese, nickel,
290 and chromium are also of concern (USDA, 2012; Punniyamurthy et al, 2005).
291 Ethylenediaminetetraacetic acid (EDTA) can be used to reduce ability of metals to participate in
292 oxidation reactions. When EDTA is bound with iron, only one active site remains, reducing
293 radical formation (Aust et al., 1985). When EDTA is bound with copper, it becomes stable and it

294 can’t react to create radicals (Allen, 2015). Other examples of metal chelators include sodium
295 tripolyphosphate, citric acid, and flavonoids.

296 **Lipid oxidation in pet food and meat**

297 Currently there is little academic published research regarding lipid oxidation related to
298 raw, frozen, and freeze-dried pet foods. The research that is published has been completed with
299 protein meals, kibble, and sensory evaluation (Chanadang et al., 2016; Gray, 2015; Di
300 Donfrancesco et al., 2012). However, there is a great deal of research related to oxidation and
301 storage of raw, frozen, and freeze-dried products from the meat industry which can be used to
302 create a hypothesis regarding what might occur in pet food products of a similar nature. One
303 must remember though, that raw pet foods are nutritionally complete, which unlike meat, also
304 contains additives such as minerals and vitamins which may impact oxidation.

305 Rendered animal protein meals are commonly used in extruded pet food products. These
306 meals provide higher quantity of quality protein more economically than raw meat. During the
307 rendering process, fat is separated from the cooked material. However, some fat remains in the
308 protein meal which can oxidize during storage if not protected. For rendered protein use in food
309 that is stored for long periods of time, such as pet food, it is important to understand how these
310 food products oxidize before and after processing, and which antioxidants are effective at
311 controlling oxidation. Gray (2015) examined the oxidation of chicken by-product meal (CBPM)
312 and beef meat and bone meal (BMBM) treated with no antioxidant, mixed tocopherols (MT), or
313 ethoxyquin and measured PV and AV over time. Peroxide value and AV in the CBPM and
314 BMBM not treated with an antioxidant increased during storage. When treated with MT, there
315 was an increase in AV for both CBPM and BMBM, while PV only increased in BMBM.
316 Ethoxyquin kept both PV and AV stable for during storage for CBPM and BMBM. The CBPM

317 and BMBM were then used to create kibble for a second shelf-life study. Peroxide value for
318 mixed tocopherols increased while ethoxyquin treatments remained stable over 18 weeks, while
319 AV increased for all treatments. Volatile compounds (were measured before and after extrusion
320 and were reduced by the extrusion process. It is likely these volatile compounds were removed
321 due to high heat of the extrusion process.

322 Not only are there analytical tests to determine if a pet food product is acceptable, but
323 sensory analysis can be useful by leveraging a trained sensory panel to describe a product based
324 on smell, taste, texture, and appearance. A lexicon of attributes related to dry pet food was
325 determined using a trained panel and 21 commercially available pet foods with 70 attributes
326 being described (Di Donfrancesco et al, 2012). This list can be useful for identifying what
327 humans can detect related to lipid oxidation. Chanadang et al. (2016) utilized a trained sensory
328 panel to describe differences in kibble made from CBPM and BMBM that were stored for 0, 3, 6,
329 9, or 12 months. There were no differences for the oxidized oil or rancid aromas in the BMBM.
330 However, by 12 months of storage the panel was able to identify differences for oxidized oil and
331 rancid aromas in the CBPM. The kibble used in this study were from the study of Gray (2015).
332 This suggests that human panels may be able to detect a rancid product that will be fed to their
333 pet. If these odors are present in a newly purchased bag (i.e. the bag has been on the store shelf
334 for an extended period of time) the owner may decide to purchase a different brand.

335 The more important factor to consider with oxidized pet food are the health implications
336 and the impact they may have on the animal consuming the diet. Puppies (2 months of age) fed
337 an oxidized diet had reduced weight gain, serum vitamin E, reduced linoleic acid (in the diet,
338 serum and bone), and slower bone formation (Turek et al., 2003). Linoleic acid is an essential
339 fatty acid containing 2 double bonds that can be altered during oxidation. Plus, as reported in this

340 study linoleic acid also played an important role in bone development in these puppies. There is
341 no information published on the effects of feeding an oxidized diet to dogs at different ages nor
342 how cats can be impacted.

343 Pet owners may freeze raw products for extended periods of time, and this has the
344 possibility to expose the food to intermittent freeze-thaw cycles before consumption. There is
345 limited research related to pet food products in this regard, so one must extrapolate from human
346 food research about what might be happening. For example, there is a great deal of research
347 examining lipid oxidation in meats that are frozen and stored for extended periods of time and
348 which have undergone several freeze-thaw cycles. Coombs et. al. (2018) reported thiobarbituric
349 reactive substances increased in lamb that was stored for 52 weeks. Holman et al. (2018) stored
350 beef samples for 12 months and observed an increase in TBARS.

351 Packaging systems can also have an impact on shelf life. For example, Bellés et al.
352 (2017) observed an increase in TBARS when lamb was stored under modified atmospheric
353 packaging while lamb in vacuum skin packaging remained stable over 28 days. Chicken leg and
354 breast meat stored at -18 °C for up to 6 months had an increase in peroxide value 2 and 3
355 months, respectively, before the peroxide value declined and TBARS increased over 6 months of
356 storage for chicken leg and breast meat (Soyer et al., 2010).

357 The number of freeze-thaw cycles can also impact the level of lipid oxidation in meat
358 products. Chen et al. (2018) completed 7 freeze-thaw cycles (frozen -20°C thawed at 4°C) and
359 observed an increase in both peroxide value and TBARS in beef. In a similar study with three
360 freeze-thaw cycles, PV, FFA and TBARS increased in beef samples (Rahman et al. 2015).
361 Chicken breast exposed to six freeze-thaw cycles resulted in increased TBARS (Ali et al. 2015).
362 Lamb that was subjected to 15 freeze-thaw cycles (-20°C and 4°C) had TBARS values that

363 doubled by the 15th cycle and FFA concentration increased as well (Qi et al. 2012). Raw-frozen
364 pet food may experience multiple freeze-thaw cycles as it is being transported, purchased, and
365 stored, leading to the potential for product oxidation.

366 In addition to the number of freeze-thaw cycles and the length of storage time, the type of
367 antioxidant used will also affect the amount of oxidation that occurs within a product. Wilkinson
368 et al. (2001) determined the effects of sodium erythorbate (ERY), tocopherols (TOC), and
369 tertiary butylhyrdroquinone (TBHQ) on TBARS values in freeze dried beef and freeze-dried
370 chicken. The freeze-dried beef (stored at 49°C for 30 days) had lower levels of malondialdehyde
371 with ERY and TBHQ while the freeze-dried chicken had lower values for all antioxidant
372 treatments when compared to a control containing no antioxidants. Beef steaks treated with an
373 antioxidant or a combination of the antioxidant and beefy flavoring resulted in lower TBARS in
374 both raw and cooked forms compared to a control (no antioxidant or flavoring) at six months of
375 storage (Stika et al. 2007). Lamb treated with 400 mg rosemary extract kept total aldehydes, total
376 ketones, total alcohols, and total furans lower for up to 11 days relative to lamb loin that was
377 untreated with antioxidant (Ortuño et al., 2016; $P < 0.05$). Cooked mutton had reduced TBARS
378 and total carbonyls when treated with different combinations of potential antioxidants, such as
379 ascorbic acid or spices over 6 months of storage (Jayathiliakan et al., 2007). Taken together these
380 reports help make the case that an antioxidant, whether natural or synthetic, can help retard the
381 onset and amount of oxidation that might occur in meats. Whether this translates to raw-frozen
382 and freeze-dried pet foods remains to be verified.

383 Protein Quality

384 Digestibility and amino acid profile are two main factors when considering protein
385 quality. Protein, or amino acids, are essential for life as they constitute a large portion of the

386 body in the form of muscle and organs. Proteins are important in digestion and metabolism.
387 Determining protein quality of standard or conventional vs new or novel ingredients is often
388 evaluated for use in pet foods in order to provide a score and concentration of the essential
389 nutrients that can be met or must be augmented within the complex food. Protein quality can be
390 impacted by the manufacturing process of the protein, such as rendering. Rendering is a high
391 heat process in which reactions, such as browning reactions, take place that lead to unavailable
392 amino acids for the animal. In addition to processing, the source of the protein can alter quality.
393 Deboned chicken breast will contain more connective tissue compared to a chicken breast; which
394 reduces its nutritional quality. Following are several methods that can be used to determine
395 protein quality of ingredients that might be incorporated into pet foods.

396 **Determining Protein Quality**

397 There are several methods that can be used to evaluate the quality of a given protein
398 source. Some include use of an animal model (*in-vivo*) while others are benchtop (in-vitro)
399 laboratory methods. Animal models are the most beneficial in order to directly evaluate an
400 ingredient by the animal for which it is intended (De Godoy et al., 2009; Cramer et al., 2007;
401 Dust et al., 2005; Donadelli et al., 2019). The drawback is this approach can take extensive
402 amounts of time, is laborious, and expensive. Using a benchtop method can improve the
403 turnaround time and reduce the cost in evaluating a protein source, but it may not accurately
404 mimic the digestive process of the animal. If one were to replicate animal digestion the strength
405 of enzymes can be challenging to match. The enzymes released by the animal in response to food
406 may differ from that used in an *in-vitro* system. Enzymes released in the digestive tract may also
407 require activation, a facet that can be difficult to simulate in an in vitro system. Further, if there
408 are any enzyme inhibitors present (i.e. trypsin inhibitor) this can prevent enzyme activation and

409 alter the results between the two assay types (Nosworthy et al., 2018b). Enzymes in the *in-vitro*
410 system are already active; whereas in vivo they may require activation. To further describe this
411 concept an overview of several methods that have been used to evaluate protein quality and their
412 benefits or drawbacks will follow.

413 **In-vivo Digestibility and Growth Assays**

414 It is common when evaluating any new ingredient to conduct a digestibility or growth
415 assay. There are several methods that can be used including a simple digestibility where
416 disappearance between food intake and fecal excretion is used to determine protein
417 degradability. More complex assays such as ileal amino acid digestibility by dogs or pigs, the
418 precision fed cecectomized rooster assay, or precision fed broiler chick assays can also be used
419 to determine the protein and amino acid digestibility (Wang et al., 2017; Le et al., 2017; Hill et
420 al., 1996; Hill et al, 200; De Godoy et al., 2009; Johnson et al., 1998). In addition, growth studies
421 which determine average daily gain and feed efficiency can also be used to examine whether a
422 protein source could be considered as a replacement to a standard protein source such as soybean
423 meal. A growth study can also be conducted to determine the protein efficiency ratio of a protein
424 source (Cramer et al., 2007; Donadelli et al., 2019). This can be compared to a standard or
425 reference, such as casein or spray dried egg. Growth assays to determine protein quality are not
426 commonly considered with companion animals because of time (companion animals grow at a
427 much slower rate than birds), cost, and public perception.

428 ***Digestibility***

429 Apparent total tract digestibility of foods has been utilized for decades to determine
430 nutrient disappearance of various feed stuffs and foods. The rationale is that if a nutrient from the
431 food has diminished or disappeared from the feces then it will have been utilized by the animal.

432 The standard methodology would collect all feces and compute the disappearance. However, this
433 is not always practical and the use of indigestible markers such as chromic oxide or titanium
434 dioxide can aid in the estimation of nutrient utilization. The use of markers allows for
435 identification of protein sources that are more digestible (greater utilization) in dogs and cats
436 without the use of cannulas. The difference in both the nutrient content and marker content in the
437 food and feces is applied according to Equation 5 which calculates nutrient digestibility when
438 chromic oxide as a marker was used (Alvarenga and Aldrich, 2018).

439 **Equation 5. Nutrient Digestibility using chromic oxide.**

440
$$Nutrient\ Digestibility = \frac{[1 - (\%\,Cr\ in\ food\ \times \%\,nutrient\ in\ feces)]\ \times 100}{(\%\,Cr\ in\ feces\ \times \%\,nutrient\ in\ feces)}$$

441 Chromic oxide (Cr_2O_3) has been used to determine digestibility in pigs (Lærke et al.,
442 2012; Favero et al., 2014; Brestenský et al., 2017; Van Leewen et al, 1996; Want et al, 2018),
443 broilers (Leytem et al., 2008), and dogs (Alvarenga et al, 2019; Hill et al. 1996; Carciofi et al.,
444 2007). However, in recent years there has been a shift away from the use of chromic oxide as a
445 marker due to its potential carcinogenic effects (Peddie et al, 1982;). Titanium dioxide may be a
446 reasonable alternative marker to replace chromic oxide (Alvarenga et al., 2019).

447 Titanium dioxide is approved for use in foods as a coloring agent as long as it is below
448 1% of the weight of the food (Code of Federal Regulations, 2019). Similar to chromic oxide,
449 titanium dioxide has been used to determine digestibility in dogs (Alvarenga et al. 2019), pigs
450 (Favero et al., 2014; Want et al., 2018; Kiarie et al., 2016; Jang et al., 2017), broiler chicks
451 (Morgan et al., 2014, Smeets et al., 2015), and in cattle (Titgemeyer et al., 2001). Because
452 titanium dioxide has been considered as a replacement for chromic oxide it is important to make
453 sure the two markers provide similar digestibility results. Alvarenga and Aldrich (2019)
454 determined the correlation between chromic oxide and titanium dioxide in dogs to be 0.914 (P <

455 0.001), which is in agreement with previous work in which no difference was observed between
456 the two markers (Kavanagh et al., 2001; Wang et al., 2018).

457 The drawback to apparent total tract digestibility is that it doesn't account for sloughing
458 intestinal cells, fermentation of material by bacteria in the colon, and in the case of dogs or cats,
459 hair in the feces. Animal models that characterize the digesta prior to entry into the large
460 intestine or cecal-colonic fermentation include cecectomized roosters and ileal cannulation. For
461 the cecectomized rooster the ceca of the birds are removed, preventing microbial fermentation
462 that would lead to changes in the amino acid content of the excreta have been used to determine
463 amino acid digestibility without the complication by bacteria (De Godoy et al., 2009; Johnson et
464 al., 1998; Rojas et al., 2013; Deng et al., 2016). For this assay, a precise amount of the ingredient
465 is then fed to the rooster and all the excreta is collected for amino acid and energy analysis to
466 determine disappearance.

467 The second method to determine nutrient digestibility with minimal interference from
468 colonic fermentation is to collect samples at the terminal ileum from a cannulated animal. A
469 cannula is surgically placed at the distal end of the ileum which allows for a sample of the
470 digesta to be collected prior to entry into the large intestine and by difference to estimate small
471 intestinal nutrient digestibility. This is often referred to as the true ileal digestibility. Again, this
472 helps reduce the interference that may occur from the fermentation by colonic microbes, microbe
473 death, and sloughing of cells in the large intestine that are faced with a total tract digestibility
474 estimate. Like the other digestibility methods, the difference in the nutritional content of the feed
475 and the material collected at the canula in the ileum allows for the determination of intestinal
476 disappearance of a given nutrient. This method has been extensively used in pig digestibility
477 studies (Yáñez et al., 2011; Zhou et al., 2015; Liu et al., 2016; Wang et al., 2017; Le et al., 2017)

478 and was used previously with dogs (Hill et al., 1996; Hill et al, 2001; Spears et al., 2005; Murray
479 et al., 1998; Johnson et al., 1998) but due to activist pressures is not as common a practice in
480 today's research with companion animals.

481 ***Protein Efficiency Ratio Assay***

482 A growth assay can also provide insight into nutritional quality of an ingredient. One of
483 the more elegant procedures is a protein efficiency ratio (PER) which ranks growth based on
484 limitations of a protein (Schaafsma, 2005). This assay is often conducted with rats and broiler
485 chicks (De Godoy et al., 2009; Cramer et al., 2007; Dust et al., 2005; Hevia and Clifford, 1977;
486 Johnson and Parsons, 1997; Mesomya et al. 2005; Morrison and Campbell, 1960; Donadelli et
487 al., 2019). Both animals are genetically uniform and grow rapidly. For this type of study, the
488 animals are fed a diet that contains all essential nutritional requirements from fatty acids,
489 minerals, and vitamins. The only nutrient that is insufficient for growth is protein, or more
490 specifically availability of a limiting amino acid. These diets typically contain 9-10% crude
491 protein from a single test protein. The sample protein is also analyzed for the level of each amino
492 acid. The diet is then fed for a period of time, generally 10 days, and the amount of weight
493 gained, and feed consumed is recorded. This is then used to calculate the protein efficiency ratio
494 (Equation 6). In addition to PER, net protein ratio, which accounts for protein maintenance costs,
495 can be determined (Equation 7).

496 **Equation 6. Protein efficiency ratio.**

497 $$PER = \frac{BWG}{CPI}$$

498 **Equation 7. Net protein ratio.**

499 $$NPR = \frac{(BWG - GNfree)}{CPI}$$

500 This method has value for protein quality determination for several reasons. The first is
501 that it is more rapid than a digestibility study. It also does not require a surgical procedure for the
502 animal such as the cecectomized rooster assay or ileal cannulation. This assay also allows for
503 small differences in the amino acid level and availability between different sources to be
504 determined. Using the amino acid profile of each source, one can explain why one protein source
505 may have performed better than another. This assay also highlights differences between the
506 manner in which a source was produced, i.e. the heat imparted on the ingredient during
507 production which may have bound or destroyed a limiting amino acid. For example, by
508 determining the amount of available lysine differences in PER between ingredients might help
509 explain the results and can be used to provide rationale for differences in growth and in amino
510 acid levels.

511 **In-vitro Digestibility**

512 *In-vitro* digestibility assays are often conducted to achieve quicker and less costly results
513 than an *in vivo* animal experiment. The general idea behind an *in vitro* assay is to mimic the
514 conditions found in the animal, in this case the stomach and small intestinal digestive processes.
515 Commercial enzymes are available for purchase in order to simulate what occurs throughout the
516 digestive tract of the desired species. For example, protein digestibility can be determined by
517 utilizing pepsin and pancreatin enzymes (Barrón-Hoyos et al., 2013; Akeson and Stahmann,
518 1964; Almeida et al., 2015; Toomer et al., 2015). This method involves a hydrochloric acid
519 solution containing pepsin, simulating the stomach of a monogastric animal. The pepsin begins
520 to cleave the polypeptide chains into smaller segments before they reach the small intestine. The
521 second step involves a phosphate buffer and pancreatic enzymes. When the digesta reaches the
522 small intestine, the pH increases and pancreatin enzymes are released to further break down the

523 protein into single amino acids, di-peptides, and tri-peptides. The sample is then incubated for an
524 additional 18 hours. The nitrogen content of the remaining sample can then be determined with
525 either a LECO or by using the Kjheldal method.

526 A more recent version for determining *in-vitro* protein digestibility involves a pH change
527 after 10 minutes (Nosworthy, Franczyk, Zimoch-Korzycka, et al., 2017; Nosworthy, Franczyk,
528 Medina et al., 2017; Nosworthy et al., 2018a). The test sample is mixed with a solution
529 containing trypsin, chymotrypsin, and protease and incubated at 37°C for 10 minutes. During
530 this time the drop in pH is recorded and used to calculate protein digestibility (Equation 8). This
531 method differs from the previous as it does not mimic the time and conditions involved
532 throughout the gastrointestinal tract of an animal and in most cases results in slightly lower
533 estimate compared to digestibility determined utilizing rats.

534 **Equation 8. In-vitro protein digestibility**

535 $$In - vitro\ protein\ dig. = 65.66 + 18.10\Delta p\mathrm{H}_{10min}$$

536 Advantages to conducting an *in-vitro* protein digestibility include lower cost, less time to
537 conduct the assay, and the ability to evaluate a single protein source. Compared to a 10-day PER
538 study, the *in-vitro* assay can yield results in 2-3 days, saving time and ultimately cost (birds,
539 feed, battery, facility, etc.). A limitation of this assay is that it does not always analyze a
540 complete diet or feed. The ability to evaluate a single protein source may also be a limitation as
541 interference from other ingredients in a complete diet may reduce the quality when fed to the dog
542 or cat (i.e. an overestimation of quality). In addition, the *in-vitro* assay avoids any issues that
543 would be present in the animal related to trypsin inhibitors. The enzymes used in the *in-vitro* are
544 already activated, removing this activation step that takes place in the animal. This assay also
545 uses a small amount of sample (less than 1g) leading to potential increased variation in results.

546 **Protein Digestibility Corrected Amino Acid Score**

547 Protein digestibility corrected amino acid score has been used widely by the World
548 Health Organization to determine protein quality of foods and food ingredients for humans
549 (Schaafsma, 2000). This score can be determined by obtaining the protein digestibility and
550 essential amino acid profile of the test protein. The digestibility of the protein can be determined
551 either *in-vivo* (Nosworthy, Franczyk, Zimoch-Korzycka, et al., 2017; Nosworthy et al., 2018;
552 Hughes et al., 2011; Sarwar, 1997) or *in-vitro* (Dong et al., 2014; Tavono et al., 2016)
553 The amino acid profile is used to create an amino acid score and the lowest value is used
554 for PDCAAS calculation (Equation 9; Dong et al., 2014). The limiting amino acid is determined
555 by using a reference value. In human research the WHO/FAO/UNU essential amino acid scoring
556 pattern for 1-2 year old children is used. However, for pets a more appropriate reference might
557 be the recommended values for dog maintenance provided in the NRC or AAFCO. Once
558 digestibility and the limiting amino acid has been determined based on the amino acids scores,
559 protein digestibility corrected amino acid score can be calculated (Equation 9; Equation 10;
560 Schaafsma, 2000).

561 **Equation 9. Amino acid score.**

562 $$AAS = \frac{Amino\ Acid\ in\ test\ protein}{Reference\ Protein}$$

563 **Equation 10. Protein digestibility corrected amino acid score.**

564 $$PDCAAS = (LAA * Digestibility) * 100$$

565 There are several drawbacks to the PDCAAS method. Protein quality of ingredients that
566 contain antinutritional factors may be overestimated (Schaafsma, 2012). For example, Sarwar
567 (1997) determined that proteins (raw black beans and mustard flower, raw soybean meal) that
568 contained antinutritional factors had lower PER values compared to PDCAAS. In addition, the
569 bioavailability of the amino acids is not taken into account when determining PDCAAS. A lower

570 bioavailability is not accounted for in the PDCAAS method as the amino acid profile used is on
571 an as is basis. An example of determining what is biologically available would be lysine vs
572 available lysine. However, this method still aids in the understanding of overall quality of protein
573 included in foods.

574 **Protein Quality Assessment of Ingredients Used in Pet Food**

575 There have been many authors who have evaluated the protein quality of ingredients that
576 are used in pet food. Johnson and Parsons (1997) evaluated lamb meal, poultry by-product meal,
577 and meat and bone meal with varying ash levels and processing temperature using the chick PER
578 assay. This work demonstrated no difference in PER based on ash content, but did show the
579 higher processing temperature in the meat and bone meal resulted in lower PER. These
580 ingredients were then used in a digestibility study utilizing cecectomized roosters and illeally-
581 cannulated dogs (Johnson et al., 1998). Higher processing temperature resulted in lower amino
582 acid digestibility in the precision fed cecectomized rooster. This difference was not seen with the
583 ileal-cannulated dogs. Shirley and Parsons (2000) also observed reduced digestibility of amino
584 acids as processing temperature increased in the precision fed cecectomized rooster model.

585 Dust et al. (2005) examined several chicken and blood protein sources in a chick protein
586 efficiency ratio assay. Ingredients from blood sources (plasma, blood cells, etc.) resulted in lower
587 values compared to protein sources from muscle tissue. One potential reason for this is that the
588 level of lysine was approximately double arginine, which is known to have antagonistic effects
589 with absorption (Allen and Baker, 1972; D'Mello and Lewis, 1970; O'Dell and Savage, 1966).
590 Spray dried material had higher PER than the rendered proteins (Dust et al., 2005). These spray
591 dried ingredients are not exposed to the harsh conditions that rendered proteins are, leading to

592 potential reductions in protein quality. This was also observed by Cramer et al. (2007) when
593 rendered products had lower PER values than freeze-dried whole animal parts.
594 Donadelli et al. (2019) evaluated traditional pet food protein sources that were processed
595 in different manners, along with several protein sources that are more novel. In their work spray
596 dried chicken protein had higher PER values compared to those that were subjected to harsher
597 processing conditions. Novel protein sources, such as rice protein concentrate, pea protein
598 isolate, and soy protein isolate, resulted in lower PER values compared to the control of spray
599 dried egg. These vegetable proteins had lower levels of methionine, potentially leading to the
600 lower PER values.
601 With an increase in co-products from the ethanol industry and the incorporation of more
602 plant proteins, research has increased in this area. Distillers dried grains with solubles resulted in
603 lower PER values compared to soybean meal (De Godoy et al., 2014; Smith, 2018) in a chick per
604 assay. Distillers dried grains with solubles also was reported to have lower total amino acid
605 digestibility in cecectomized roosters (De Godoy et al, 2014).
606 Soybean meal has produced consistent PER among several studies (De Godoy et al,
607 2014; Smith, 2018; Donadelli et al., 2019). It is a popular source of protein in the livestock
608 industry and has been used in pet food as well. Due to negative consumer perception regarding
609 soy, the use of other plant proteins has become more common in pet foods. Several studies have
610 evaluated protein quality of a variety of legumes via *in-vivo* and *in-vitro* digestibility studies
611 (Nosworthy, Franczyk, Zimoch-Korzycka, et al., 2017; Nosworthy, Franczyk, Medina et al.,
612 2017; Nosworthy et al., 2018a; Nosworthy et al., 2018b). In this series of studies, rats were used
613 to determine the PER and digestibility was determined using rats and an *in-vitro* assay.
614 Compared to casein, all PER and digestibility values for legumes were lower. In an experiment

615 examining several legume sources chickpeas resulted in the highest PER and split green peas the
616 lowest (Nosworthy, Neufeld et al., 2017). These legumes were also used to calculate PDCAAS
617 and navy beans resulted in the highest value.

618 Conclusion

619 Fat and protein are two macronutrients that are important for animal health. The quality
620 and stability of these can be altered during production and storage. Both can be evaluated in a
621 number of ways and it is important to understand each assay and any limitations it may have in
622 order to properly evaluate the complete food or ingredient. As lipids oxidize, they are broken
623 down into products that are not nutritionally beneficial to the animal. This can lead to reduced
624 growth and bone formation when oxidized fat is consumed by dogs (Turek et al., 2003). It has
625 also been shown that minimally processed foods, raw-frozen and freeze-dried, will oxidize if a
626 preservative system is not used. The addition of antioxidants can slow this process and prolong
627 the shelf life of the material.

628 Protein content of a food could be high, however the amino acids could be nonessential
629 or not biologically available to the animal. This would reduce the digestibility of the protein
630 sources and reduce the uptake of amino acids that are needed by the animal for normal metabolic
631 functions. Throughout the literature it is clear that processing conditions in which the protein
632 source was exposed to high temperatures during production impacts the overall quality of the
633 ingredient. Specifically, high heat processing tends to result in reduced protein quality.
634 Currently there are several key findings missing in pet food research related to lipid oxidation in
635 raw-freeze dried foods and the used of new protein sources. Gaps in lipid oxidation knowledge
636 include the lack of information on a complete diet. Much work related to raw-frozen and freeze-
637 dried products are related to single ingredients – like meats. Meat used in frozen and freeze-dried

638 products are also “leftovers” from the food industry. Before they are incorporated into pet foods,
639 they may go through several freeze-thaw cycles. There is also little research determining the
640 shelf-life of raw-frozen and freeze-dried pet foods before they are no longer acceptable to the pet
641 parent or nutritionally to the pet. Protein quality research is lacking in the evaluation of new
642 proteins sources that are being incorporated in to pet diets. Gently processed material is being
643 incorporated by companies to differentiate themselves with little knowledge regarding the
644 benefits or pitfalls. Legumes also fall into the category of minimally researched ingredients that
645 are included in pet diets.

646 Thus, the objective of this dissertation is to identify changes to lipids in raw-frozen and
647 freeze-dried foods when they are stored for an extended period of time. In order to complete this,
648 two experiments were conducted using four different antioxidants, 2 meats, and various freezing
649 and freeze-dried storage times. The second objective is to identify differences in protein quality
650 of protein sources due to processing method or protein source. Two chick growth assays were
651 completed in addition to the development of a pepsin-pancreatin in-vitro digestibility for the
652 calculation of protein digestibility corrected amino acid scores. The outcome of this research will
653 help to improve formulation strategies to deliver better nutrition to pets.

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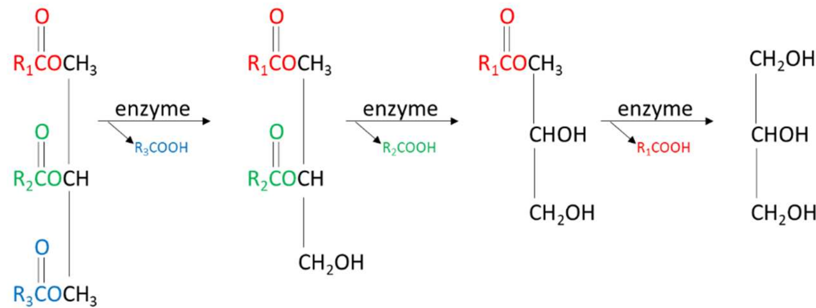
1011 # Tables and Figures



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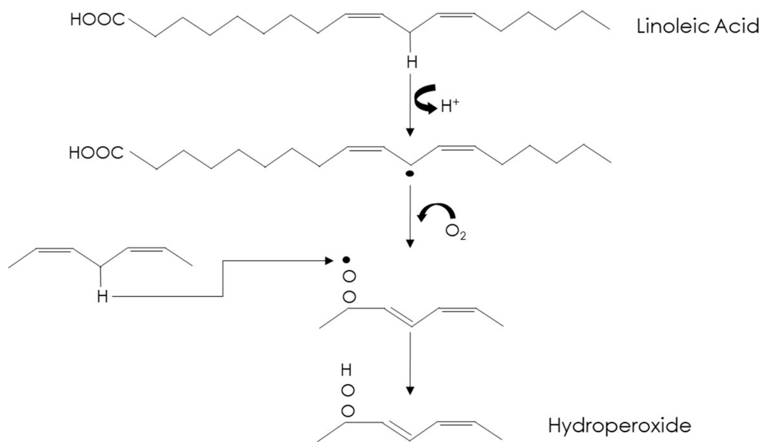
1013 **Figure 1-1. Enzymatic hydrolysis of a triglyceride (Adapted from Powar and Chatwal,**
1014 **2007).**



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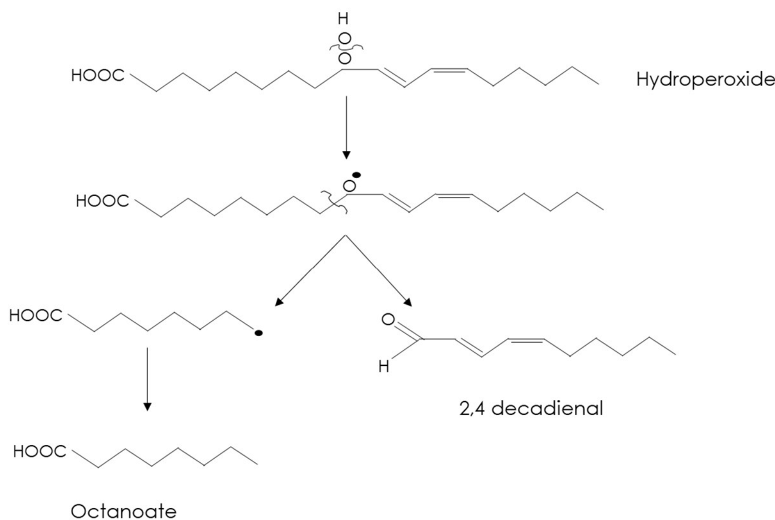
1016 **Figure 1-2. Oxidation of linoleic acid to form hydroperoxide (McClements and Decker,**
1017 **2017).**



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1019 **Figure 1-3. Breakdown of a hydroperoxide into secondary oxidation products (Frankel,**
1020 **2005).**

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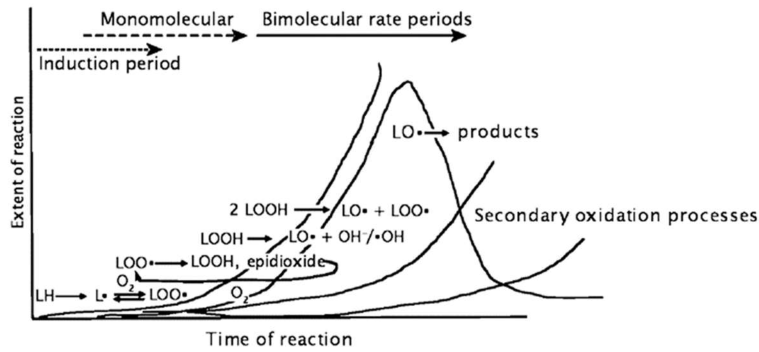

1022 **Figure 1-4. Oxidation curve of produced when lipids are broken down (Schaich, 2005).**

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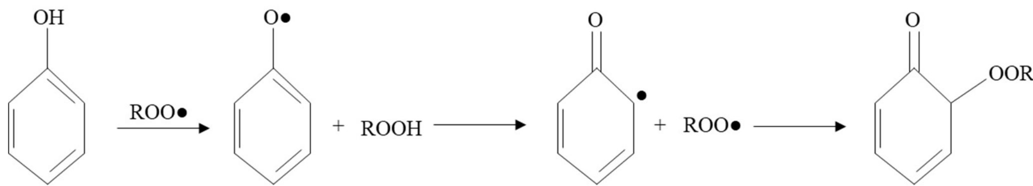

1026 **Figure 1-5. Termination reaction of a primary antioxidant with a peroxyl radical**
1027 **(Clements and Decker, 2017).**

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1035 # Chapter 2 - Evaluation of frozen and freeze-dried chicken patties
1036 treated with different antioxidants and stored for various periods of
1037 time

1038 ## Abstract

1039 Much of the growth in the pet food industry has hinged on product differentiation due to new
1040 protein sources and new food forms. Raw-frozen and freeze-dried products have been some of
1041 the fastest growing categories in the pet food industry. These products contain high levels of raw
1042 meat, which can also contribute increased amounts of fat compared to dry kibble. There is
1043 currently little research on the stability of the fat in these products. Thus, the objective of this
1044 study was to determine the effect of storage time and antioxidant type in raw-frozen and freeze-
1045 dried pet food products on measures of oxidation. The study was conducted as a 4 × 4 factorial
1046 arrangement of treatments with main effects of antioxidant and storage time. A mixture
1047 containing 50% chicken and 12.5% each of sweet potato, pumpkin, apple, and rice was produced
1048 and made into individual patties of approximately 100 grams. Each set of patties was treated with
1049 either butylated hydroxyanisole (BHA), mixed tocopherols (MT), mixed tocopherols and green
1050 tea (MT + GT) or left untreated (control) and then frozen. These were stored in freezers (-20°C)
1051 for 4, 8, or 16 weeks before analysis. For the freeze-dried evaluation, patties were stored frozen
1052 for 12, 24, or 36 weeks then freeze-dried. The dried patties were then stored in an incubation
1053 chamber for 4, 8, or 16 weeks. For frozen patties the peroxide value (PV) increased by week 16
1054 in all treatments except MT ($P < 0.05$). Hexanal concentration did not change during the 16
1055 weeks of storage, but propanal increased for the first 4 weeks of storage before decreasing to
1056 week 16 ($P < 0.05$). Freeze-dried patties PV declined through 16 weeks storage ($P < 0.05$). Free

1057 fatty acid and propanal concentrations increased compared to week 0 ($P < 0.05$). Among
1058 antioxidant treatments, MT was able to retard oxidation in products better than the other
1059 treatments. Hexanal, propanal and thiobarbituric reactive substances for the control and BHA
1060 treatments did not differ from one another ($P > 0.05$). Overall, both the raw-frozen and freeze-
1061 dried chicken patties resulted in oxidation during extended storage. The treatment with mixed
1062 tocopherols slowed oxidation relative to the control. This suggests the need for preservation to
1063 prevent rancidity in raw (frozen) and freeze-dried pet food products.

Introduction

Pet food sales in the U.S. exceeds $30 Billion with an estimated 4% annual growth (APPA, 2019). Extruded dry food accounts for roughly 70% of the market; however, much of the growth in the pet food market is from other categories; namely raw-frozen and freeze-dried options. Between 2012 and 2016, raw food forms accounted for in growth from $43M to $101M in sales for a of 235% growth during the period (Lange, 2017). Freeze-dried product sales increased from $22.7M to $73.9M between 2011 and 2014 (APPA, 2019). These products feed consumer demands by providing natural, limited ingredient, grain free, and preservative free options. They are predominately meat based. Which means they may possess a higher level of fat than traditional foods. Consumers presume that raw-frozen and freeze-dried products have an extensive shelf-life; however, they may undergo many freeze-thaw cycles and might not be as stable.

There are very few studies examining the impact of feeding oxidized diets to pets. Turek et al. (2003) fed oxidized diets to puppies and reported reduced weight gain, slower bone formation, and reduced antioxidant capacity in growing puppies. Consumption of oxidized oil has been shown to impact gut health, growth performance, and vitamin E levels in pigs (Boler et al., 2012; Huang et al., 2016). There are also studies in chickens in which growth performance was reduce and increased cell turnover in the gastrointestinal tract and liver (Dibner et al., 1996). Reduced protein digestibility has been observed in lactating cattle when fed and oxidized diets (Vázquez-Añón and Jenkins, 2007).

No studies have been reported in which raw-frozen or freeze-dried pet foods have been stored for an extended time period. From meat industry research it has been reported that there is an increase in the amount of oxidation products with short and long-term frozen storage of meat

1087 (Bellés et al., 2017; Coombs et al., 2018; Soyer et a., 2010). Freeze-thaw cycles have been
1088 reported to increase oxidation with increasing number of cycles (Chen et al., 2018; Qi et al.,
1089 2012). Further, increased storage time for freeze-dried products has also led to increased
1090 oxidation product concentration (Wilkinson et al., 2001).

1091 Even though research from human food applications can help identify potential
1092 mechanisms in pet food products, they are often single ingredient applications (e.g. beef or
1093 chicken) and differ from pet food which is a complex blend of ingredients and nutritional
1094 supplements. The shelf-life of these product is also not as extensive as those of pet foods (which
1095 can exceed 1 year). Thus, to provide information in this gap, the objective of this study was to
1096 determine the effects of storage time and antioxidant use in raw-frozen and freeze-dried pet
1097 foods.

1098 # Materials and Methods

1099 ## Sample Preparation

1100 Meat patties were produced to formula with 50% mechanically separated chicken (CJ
1101 Foods, Bern, KS), and 12.5% of each sweet potato (Eastside Market, Manhattan, KS), pumpkin
1102 (Britt's Farm, Manhattan, KS), apple (Eastside Market, Manhattan,KS), and rice (Walmart,
1103 Manhattan,KS). The rice was cooked according to the instructions on the package (1 part rice per
1104 2 parts water) until soft, then rice was cooled prior to addition to the mix. Each ingredient was
1105 then ground in a meat grinder (Weston 10-3201-W #32; Independence, OH) through a 7 mm
1106 plate to achieve a consistent size. Each ingredient was subsampled for proximate analysis (Table
1107 2-1).

1108 Two large master batch (22.67 kg) of the ingredients was produced. An aliquot (appx
1109 0.904 kg) of the chicken was retained for future use. The master batch was mixed in a planetary

1110 mixer (Hobart H600T; Troy, OH) for 5 minutes. The master batch was then split into four equal
1111 parts (approximately 5.44 kg) for the addition of antioxidant treatments. Antioxidant treatments
1112 included a control (no antioxidant), butylated hydroxyanisole (BHA; 1% of mix; Camlin Fine
1113 Sciences), mixed tocopherols (MT; 0.30% of mix; Camlin Fine Sciences), and mixed
1114 tocopherols + green tea (MT + GT; 0.20% + 0.10% of mix, respectively; Camlin Fine Sciences).
1115 The chicken that was set aside was split into four equal parts (0.226 kg) and was mixed with the
1116 appropriate treatment in a food processor for 1 minute to facilitate incorporation of antioxidants
1117 into the treatment batches. For the control the meat was simply mixed in the food processor
1118 before being added to the mixture. This was then blended in smaller (9 kg hand powered mixers
1119 (Cabellas IK-541001, Sidney, NE) until it was incorporated evenly. From these, a subsample of
1120 100 g was formed into patties. These were stacked two to a bag with parchment separating the
1121 patties which were then placed into plastic storage bags (Ziploc) and frozen at -20°C. Raw frozen
1122 samples were stored in the freezer for 4, 8, and 16 week periods before analysis. Samples for
1123 freeze-dried evaluation were stored frozen for 12, 24 and 36 weeks then removed and dried
1124 under vacuum (25-1,000 Millitorr) at a commercial facility with a 36 hour cycle time (Chasing
1125 Our Tails, Inc., Hudson, NH). Once dried samples were placed in an environmental chamber at
1126 35°C (humidity was allowed to fluctuate) for 4, 8, and 16 weeks. At the end of each period, a
1127 new set of samples (2 per storage bag) were removed for analysis.

1128 **Sample Analysis**

1129 **Fat Extraction**

1130 Fat extraction from the meat matrix was conducted in four steps: mix, filter, evaporate,
1131 and store. Specifically, one 100 g meat patty was placed in a 500 ml beaker with 250 ml of
1132 hexane and stirred by hand for 15 minutes. Hexane was removed by filtration through filter paper

1133 (110 mm diameter, Grade 1; Whatman, GE Healthcare Life Sciences Marlborough, MA) using a
1134 vacuum pump. A second extraction with hexane was conducted on the meat mixture (250 ml)
1135 and stirred for an additional 15 minutes and filtered. The supernatant was then placed in a round
1136 bottom flask and rotary evaporator (Rotovap Büchi R-114; Brinkmann Instruments, Inc.;
1137 Riverview, FL) with a water bath (Büchi B-490; Brinkmann Instruments, Inc.; Riverview, FL)
1138 set at 40°C to remove hexane. The remaining fat sample was placed in conical bottom test-tubes
1139 (50 ml Falcon tubes, Corning Life Sciences, Corning, NY) and frozen until oxidation measures
1140 could be completed.

1141 **Gas Chromatography**

1142 Gas chromatography was carried out on a Shimadzu GC-17A fitted with Supelco column
1143 (SP-2560; 100 m × 0.25 mm × 0.2 µm thickness). The carrier gas was nitrogen at 20 ml/min. The
1144 initial temperature started at 130°. Temperature increased at a rate of 3.5°C/min until a final
1145 temperature of 210°C was reached. Fatty acids were identified based on the internal standard and
1146 were calculated following the equation identified by Sukhija and Palmquist (1988).

1147 **Peroxide Value**

1148 Peroxide value was determined using the IDF standard method (IDF, 1991) with
1149 modifications. Barium Chloride Di-hydrate ($BaCl_2 \cdot 2H_2O$) solution was mix by adding 0.32 g of
1150 barium chloride di-hydrate with 10 mL of 0.4 M hydrochloric acid solution. Iron II sulfate
1151 heptahydrate ($FeSO_4 \cdot 7H_2O$) solution was mixed using 0.40 g of iron II sulfate heptahydrate
1152 ($FeSO_4 \cdot 7H_2O$) with 10 mL of HPLC graded water. These two solutions were both added to a 50
1153 ml centrifuge tube and were placed in a centrifuge for 15 min. at 4,000 rpm. Ammonium
1154 Thiocyanate solution was created by adding 3.0 g of ammonium thiocyanate in 10 mL with HPLC
1155 graded water. A sonicator was then used to help dissolve the ammonium thiocyanate. Next, 200

1156 μl of the barium iron solution and 200 μl of ammonium thiocynate were placed in a glass tube and
1157 mixed on a vortex for approximately 15 seconds.

1158 Samples were prepared by adding 2 g of sample to a 15 ml centrifuge tube and isooctane
1159 (10 ml) was added then the sample was vortexed for 1 minute. Test tubes were then placed on a
1160 rocker for an additional 10 minutes before being vortexed for an additional minute. The samples
1161 were then centrifuged at 4,000 rpm for 15 minutes. In a new glass tube, 200 μl of supernatant was
1162 mixed, using a vortex, with 30 μl of the ammonium thiocynate:iron II solution for 30 seconds and
1163 incubated at room temperature for 20 minutes. After the incubation, read the samples at 510 nm,
1164 using isooctane to blank the spectrophotometer.

1165 Peroxide value was calculated as

1166 $$PV\left(\frac{meq}{kg}\right) = \frac{[(As - Ab)\ \times m]}{W(Vanalyte/Vtotal)}$$

1167 where, As is the absorbance of the sample, Ab is the absorbance of the blank, m is the
1168 slope of the calibration curve, W is the mass of the sample in grams, V is the volume.

1169 **Thiobarbituric Reactive Substances**

1170 Thiobarbituric reactive substances quantification was conducted following the method
1171 outlined by Tarladgis et al. (1960). In short, 10 g of sample was placed in a flask with 97 ml of
1172 water, 1 ml of sulfanilamide solution and 2 ml of HCl solution. The sample was placed on heat
1173 and distillate was collected and stopped when 50 ml was produced. Next 5 ml of distillate and 5
1174 ml of TBA reagent were added to test tubes in duplicated. A blank containing 5 ml of distilled
1175 water and 5 ml of TBA was also created. The test tubes were placed in a boiling water bath for
1176 35 minutes for color development and allowed to cool in water for 10 minutes. In the final step, 3
1177 ml of sample was placed in a cuvette (in duplicate) and absorbance via spectrophotometer was
1178 determined at 532 nm. Thiobarbituric acid value was determine with the following equation:

1179 $$TBA\left(mg\frac{malonaldehyde}{100gm}of\ sample\right) = O.D.(Absorbance\ 532)\ \times 7.8$$

1180 **Free Fatty Acids**

1181 Free fatty acid content of fat samples was measured according to AOCS official method
1182 Ca 5a-40. Approximately 1 g of fat (extracted using hexane) was weighted into an Erlenmeyer
1183 flask with 50 ml ethanol, that had been neutralized, and 1 ml of phenolphthalein indicator. This
1184 solution was then titrated with 0.1N sodium hydroxide until a faint pink color was achieved. Free
1185 fatty acid content was calculated as:

1186 $$FFA\ as\ oleic, \% = \frac{ml\ of\ alkali\ \times N \times 28.2}{sample\ weight\ (g)}$$

1187 **GC - Headspace Analysis**

1188 The gas chromatography (GC)-headspace volatiles analysis was performed with an
1189 Agilent Technologies (Wilmington, DE) model 7890A gas chromatograph system equipped with
1190 an Agilent Technologies model 7697A headspace sampler. Parameters for the extraction of
1191 volatiles using headspace sampler were as follows: oven temperature, 80ºC; vial equilibration
1192 time, 60 min; pressure equilibrium time 0.5min; fill pressure 15 psi; injection time, 1.2 min; and
1193 GC cycle time 40 min. The sample loop and transfer line temperatures were set at 20 and 40ºC
1194 higher than the oven temperature, respectively. Vials (containing 2 g frozen or freeze-dried
1195 ground samples) were equilibrated and pressurized with carrier gas before injection into Agilent
1196 J&W DB-5ms Ultra Inert (30 m × 250 µm x 1 µm) GC column. Injector temperature was set at
1197 250ºC. Helium at 1.0 mL/ min, constant flow mode with average velocity of 36.623 cm/sec was
1198 the carrier gas. The flame-ionization detector (FID) temperature was set at 300ºC. Volatile
1199 compounds were identified by comparison of retention times with those of authentic reference
1200 compounds: propanal, hexanal, and 2,4-decadienal (Sigma, St. Louis, MO). Peak areas for

1201 individual total volatiles were integrated. Propanal, hexanal and 2,4-decadienal (Sigma, St.
1202 Louis, MO) were used as external standards. Determination of the amount of each identified
1203 headspace volatile was performed by semi-quantitation in terms of the relative amounts derived
1204 from the ratios of the peak areas of the volatiles and internal standard, 5-methyl-2-hexanone
1205 added into the samples. The software used to process peak areas was OpenLAB CDS
1206 ChemStation Edition for GC System (Agilent Technologies, Wilmington, DE). Each sample
1207 was analyzed in duplicate.

1208 **Statistical Analysis**

1209 Experimental design for raw-frozen chicken patties was a 4 × 4 factorial with main
1210 effects of storage time (0, 4, 8, and 16 weeks) and antioxidant (control, BHA, MT, and MT +
1211 GT). For freeze-dried samples, the experiment was set up as a 3 ×3×4 factorial design with main
1212 effects of frozen storage (12, 24, and 36 weeks), freeze-dried storage (4, 8, and 16 weeks), and
1213 antioxidant (control, BHA, MT, and MT + GT). All data was analyzed for unequal variances.
1214 Due to the unequal variances detected, log transformation was completed. Data were analyzed
1215 using software for mixed models (GLIMMIX procedure, SAS (v9.4, SAS Institute, Cary, NC).
1216 Means were separated using Bonferroni adjustment with significance at $\alpha = 0.05$ and are reported
1217 in log form.

1218 **Results**

1219 **Ingredient Composition**

1220 The proximate composition of the ingredients used in the study contained an appreciable
1221 quantity of moisture (78.84% on average; Table 2-1Table 2-1). The chicken used to produce
1222 experimental treatments contained 11.4% fat, which would provide approximately 5.7% of the
1223 total fat in the final mix; whereas the other ingredients contribute approximately 1.25% fat of the

1224 weighted average of calculated fat (6.95%). The chicken also provided the greatest concentration
1225 of protein (27.8% on dry matter basis).

1226 **Raw-Frozen Chicken Patties**

1227 The interaction between storage time and antioxidant was not significant for free fatty
1228 acid, hexanal, propanal and TBARS concentration so only main effects were reported. However,
1229 the interaction effect was significant for peroxide value. The peroxide value increased over time
1230 for the control, BHA and MT + GT. The control was greater ($P<0.05$) than all other treatments
1231 for 0, 4 and 8 weeks. The BHA treated chicken patties were similar to MT + GT patties at all
1232 time points. The chicken patties treated with MT remained stable throughout the 16 weeks
1233 storage and were maintained at a level similar to WK 0 ($P < 0.05$).

1234 The raw chicken patties were frozen for 0, 4, 8 or 16 weeks before analyses. Peroxide
1235 value increased from 2.91 meq/kg to 15.73 meq/kg from 0 to 16 weeks of frozen storage ($P <$
1236 0.05; Table 2-2). Free fatty acid content decreased over time while hexanal and thiobarbituric
1237 reactive substances did not change over 16 weeks of storage. Propanal content increased from
1238 week 0 to week 4 then decreased at 8 weeks and again at 16 weeks.

1239 The peroxide value was greatest ($P < 0.05$; 23.82 meq/kg) in the control chicken patties
1240 (Table 2-3) and lowest in the MT treatment with BHA and MT+GT patties intermediate. Free
1241 fatty acid content did not differ due to antioxidant. Hexanal concentration was highest for both
1242 the control and BHA treatments, while MT and MT + GT treatments had lower ($P < 0.05$)
1243 hexanal concentrations. The control chicken patties had the highest ($P < 0.05$) Propanal
1244 concentration, and MT and MT + GT treatments were the lowest, with the BHA treated patties
1245 intermediate. Likewise, the thiobarbituric reactive substances were lowest for the treatments

1246 containing MT and MT + GT (1.90 and 1.69, respectively), and BHA treated patties were

1247 similar to the control ($P < 0.05$).

1248 **Freeze-Dried Chicken Patties**

1249 For freeze dried patties the peroxide value, FFA, hexanal, propanal and TBARS the

1250 chicken patties treated with MT were lowest (Table 2-4; $P < 0.05$). Conversely, the control and

1251 BHA treatments had the highest values for peroxide value, hexanal, and propanal. Free fatty acid

1252 concentration was the highest ($P < 0.05$) for control freeze dried chicken patties, with BHA and

1253 MT + GT treatments intermediate between the extremes. The TBARS concentration was not

1254 different among the control, BHA, and MT + GT ($P > 0.05$) treatments.

1255 Freeze dried chicken patties treated with MT resulted in the lowest PV over time ($P <$

1256 0.05) regardless of whether they were stored frozen for 12, 24 or 36 weeks prior (Table 2-4). For

1257 each storage time PV increased (Figure 2-2; $P < 0.05$), yet within the dried product storage time

1258 there was a general reduction (Table 2-5; $P < 0.05$) in PV from 4 to 16 weeks. Among the 3

1259 frozen storage times (12 through 36 weeks) the free fatty acid concentration was highest ($P <$

1260 0.05) when chicken patties were not treated (control) or treated with BHA (Table 2-4). The

1261 MG+GT treatment provided an intermediate level of protection, and the MT treated patties had

1262 the lowest FFA across each frozen and dry storage times ($P < 0.05$). Interestingly, the FFA

1263 increased ($P < 0.05$) as product was stored in freeze-dried form (Table 2-5).

1264 The change in secondary oxidation products (hexanal, propanal and TBARS) was less

1265 variable. For the secondary oxidation products, the MT treated chicken patties were the lowest (P

1266 < 0.05) at most time points. Hexanal content and TBARS had little to no change over 16 weeks

1267 of frozen storage. Propanal content increased for all treatments, especially when frozen for 36

1268 weeks and stored in incubation for 16 weeks. Overall, propanal content for all treatments from 4

1269 – 16 weeks storage increased ($P < 0.05$).

1270 ## Discussion

1271 Throughout the course of this study, the measured level of primary oxidation products

1272 (e.g. peroxide value) in the raw-frozen chicken mixture increased. Peroxide measures the level of

1273 hydroperoxides present in samples. These oxidation products are first produced as fat begins to

1274 undergo oxidation. Soyer et al. (2010) demonstrated that breast and leg meat peroxide value

1275 increased during the first 2-3 months of frozen storage. After this, there was a decline in peroxide

1276 value which coincided with an increase in TBARS. These are secondary oxidation products that

1277 form as a result of hydroperoxide reacting further which creates new compounds. There was no

1278 evidence of a decline in peroxide value or an increase in secondary oxidation products, such as

1279 hexanal and propanal when chicken patties were frozen for 16 weeks. This is similar to results

1280 reported by Chipault and Hawkins (1971) who stored freeze-dried chicken at 60°C and observed

1281 an increase in peroxide value, but no change in secondary oxidation products measured by

1282 TBARS. This may indicate that the samples were in the early stages of oxidation, wherein

1283 secondary products remained below detection levels while hydroperoxides were being formed.

1284 The time it takes to completely freeze an item may also have an impact on the level of

1285 oxidation a product will undergo. The current study samples were frozen in a -20°C freezer

1286 instead of being subjected to a more rapid freeze that can be obtained with a blast chiller or

1287 liquid nitrogen. Kim et al. (2017) froze chicken in a freezer at -30°C and with liquid nitrogen (-

1288 70°C). Samples that were frozen at -30 °C had a higher level of peroxides and thiobarbituric

1289 reactive substances compared to those frozen with liquid nitrogen. If a different type of freezing,

1290 such as liquid nitrogen, had been used in the current study, it may have slowed the formation of
1291 hydroperoxides in the control, BHA, and MT + GT treatments.
1292 The freeze-dried patties, similar to the raw-frozen, resulted in signs of oxidation during
1293 storage. As the peroxide value in freeze-dried patties declined, there was an increase in propanal
1294 concentration while TBARS remained unchanged. Wilkinson et al. (2001) did not see an
1295 increased level of secondary oxidation products when TBARS were measured at 532 nm.
1296 However, Wilkinson et al. (2001) evaluated secondary products by measuring TBARS at 450 nm
1297 and by fluorescence. This was done because thiobarbituric reactive substances are not selective
1298 for just malonaldehyde, but can react with other substrates such as alkanals, acetaldehyde, and
1299 alkenals which produce a yellow/orange pigment that can be absorbed at 450-455 nm (Tarladgis
1300 et al, 1962; Kosugi et al, 1987; Marcuse and Johansson, 1973). This yellow/orange pigment has
1301 been observed when analyzing freeze-dried samples and can be absorbed at 453 nm (Täufel and
1302 Zimmermann, 1961; Wilkinson et al., 2001). When TBARS were measured at 450 nm Wilkinson
1303 et al. (2001) observed an increase. In addition, fluorescence was deployed to measure the Shiff
1304 bases that were created when an amino group is linked with malonaldehyde (Kamarei and Karel
1305 1984). Fluorescence detection in the freeze-dried chicken and beef also increased fluorescence
1306 units during storage (Wilkinson et al., 2001). Both of these methods may have provided more
1307 insight into secondary oxidation products present in freeze-dried samples and may be useful in
1308 future research.
1309 Antioxidant type has also had an impact on the level of oxidation that occurred in the
1310 raw-frozen chicken patties. Mixed tocopherols were able to retard peroxide value relative to the
1311 other treatments. There are several experiments which have been published that reported birds
1312 fed diets containing tocopherols led to an enriched concentration in the tissues before the meat

1313 was stored for an extended period of time (Smet et al. 2008; Botsoglou et al., 2003). Botsoglou et
1314 al. (2003) observed lower TBARS levels in both breast and thigh meat from tocopherol fed birds.
1315 Smet et al. (2008) reported lower TBARS values for tocopherols compared to the other
1316 treatments. This study also contained a combination treatment of tocopherols and green tea. This
1317 combination resulted in higher levels of oxidation compared to the tocopherol treatment alone.
1318 The current study had similar results in which the combination of mixed tocopherols and green
1319 tea resulted in higher levels of oxidation compared to mixed tocopherols. These results suggest
1320 that the use of green tea in place of a portion of the mixed tocopherols results in a reduction in
1321 antioxidant efficacy. It may be possible that green tea might not be an appropriate antioxidant
1322 system for raw foods or those containing chicken.

1323 Efficacy of the antioxidant can be impacted by the fat it is used to treat. The
1324 concentration of saturated (no double bonds) vs unsaturated (1 or more double bonds) can alter
1325 the rate at which fats oxidize. The more double bonds the more likely the fat will oxidize, and at
1326 an increased rate (McClements and Decker, 2017). Chicken fat contains approximately 65%
1327 unsaturated fatty acids in its total fat (NRC, 2012). Compared to other fats such as beef tallow
1328 (44.2% of total fat), this is much higher (NRC, 2012). These differences in unsaturation can lead
1329 to differences in the usefulness of an antioxidant.

1330 One shortcoming of the current work is the lack of data regarding the history for the raw
1331 materials and how many freeze-thaw cycles they may have been exposed to. The number of
1332 freeze-thaw cycles a raw-frozen pet food has experienced is of concern because a pet owner may
1333 partially thaw the product to make smaller portions before re-freezing for future use. Ali et al.
1334 (2015) observed an increase in TBARS in chicken breasts as the number of freeze-thaw cycles

1335 increased. This has also been observed in beef where peroxide value, TBARS, and acid value all
1336 increased with increasing freeze-thaw cycles (Chen et al., 2018).

1337 Other short comings include differences in analytical procedures and the impact on
1338 animal health. For analytical procedures, analyzing thiobarbituric reactive substances at 532 nm
1339 and 450-455 nm may enhance the understanding of oxidation in freeze-dried products. Other
1340 oxidation products, such as alkanals, can react with thiobarbituric acid and aren't measured at
1341 532 nm. Since it has been reported to provide information on oxidation in previous work
1342 (Wilkinson et al., 2001), it would provide useful information in future work related to freeze-
1343 dried meat products. Measuring fluorescence may also provide a better indication of
1344 malonaldehyde content in products as it measures the Schiff bases created by direct reaction with
1345 malonaldehyde.

1346 Currently, there is very little research on the impact oxidized fat has on the overall health
1347 of the animal. Turek et al. (2003) reported reduction in growth, bone formation, and vitamin E
1348 levels in puppies. Research is still needed on the long-term impacts of dog and cat health related
1349 to the level of oxidation in fat they consume.

1350 Conclusion

1351 Overall, both the raw-frozen and freeze-dried patties resulted in signs of fat oxidation
1352 throughout the extended storage time. Peroxide value increased in the raw-frozen chicken patties.
1353 Once freeze-dried the level of free fatty acids increased, indicating an increase in enzyme
1354 activity. The stored freeze-dried patties resulted in a decline in peroxide value, while propanal
1355 was increased. Antioxidant type played a vital role in the reduction of oxidation within samples.
1356 Mixed tocopherols provided the most effective defense against oxidation, followed by the

1357 combination of mixed tocopherols + green tea. In most instances, BHA did not prove to be as
1358 effective and was similar to that of samples that had no antioxidant.

1359 The changes observed in this study may help to fill-in the gap that is currently present in
1360 raw-frozen and freeze-dried pet food research. More research is needed to examine the impact of
1361 a complete diet, containing fortifying vitamins and mineral and how those might impact the
1362 degree of oxidation. Different meat types (i.e. beef, lamb, etc.) should be examined regarding the
1363 antioxidant which might be most effective as their fatty acid profiles will be slightly different.
1364 Finally, it would benefit a final validation if these diets were fed to the target animal species to
1365 determine acceptability and how oxidation may affect nutrient utilization and health.
1366

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1421 # Tables and Figures

1422 **Table 2-1. Proximate analysis of ingredients used to create chicken patties.[1]**

Ingredient	Moisture, %	Protein, %	Fat, %	Ash, %	Dry Matter, %
Chicken	73.00	15.00	11.40	1.28	27.00
Sweet Potato	78.14	0.93	1.10	1.18	21.86
Rice	69.10	3.10	1.76	0.17	30.90
Apple	85.48	0.28	0.58	0.38	14.52
Pumpkin	88.52	1.78	1.56	1.20	11.48

1423 [1]As is basis.

1424 **Table 2-2. Main effects of storage time on oxidation measures in frozen chicken patties.[1]**

Storage time, weeks	PV[2], meq/kg	FFA[2], ppm	Hex[2], ppm	Pro[2], ppm	TBARS[2], mg MDA/100g
0	2.91^{b}	0.10^{a}	28.73	5.12^{b}	2.37
4	10.18^{ba}	0.07^{ba}	39.52	11.04^{a}	2.09
8	7.44^{b}	0.07^{ba}	42.44	5.48^{b}	2.87
16	15.75^{a}	0.05^{b}	37.57	3.69^{c}	n.d.*
SE[3]	1.73	0.00	5.31	0.32	0.22

1425 [1]Mean for all treatments.

1426 [2] PV = peroxide value; FFA = free fatty acid concentration; Hex = hexanal concentration; Pro = propanal concentration; TBARS =
1427 thiobarbituric reactive substances

1428 [3]SE = standard error

1429 [a-c] Values within a column with unlike superscripts differ ($P < 0.05$)

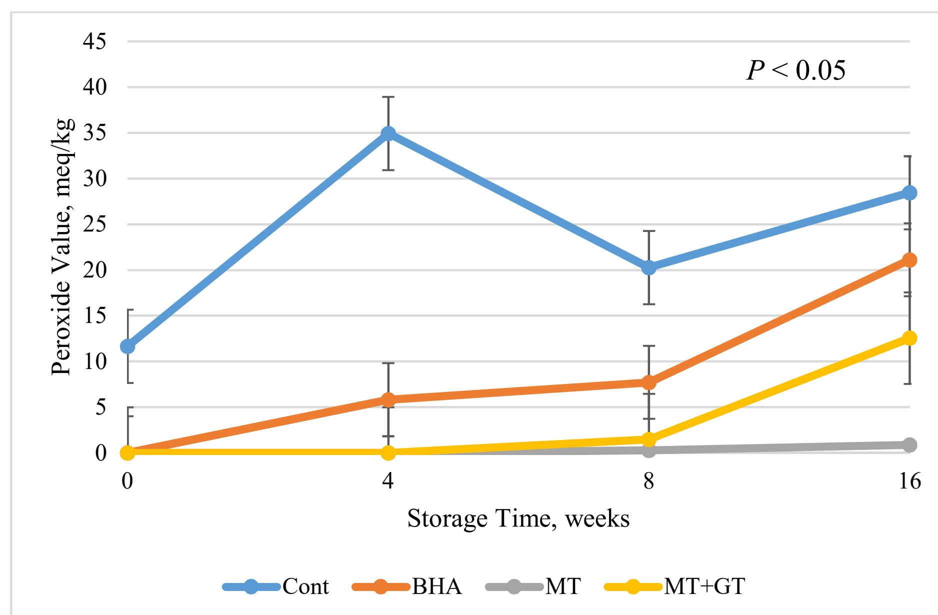
1430 [*]n.d.; not determined. Sample was lost due to equipment breakdown.

1431

1432 **Table 2-3. Main effects of antioxidant on oxidation measures in frozen chicken patties.[1]**

Antioxidant	PV[2], meq/kg	FFA[2], ppm	Hex[2], ppm	Pro[2], ppm	TBARS[2], mg MDA/100g
No (Control)	23.82[a]	0.077	61.76[a]	7.75[a]	3.20[a]
BHA	8.66[b]	0.065	51.30[a]	6.78[ba]	2.99[ba]
MT	0.29[c]	0.080	10.83[b]	5.19[c]	1.90[bc]
MT + GT	3.50[cb]	0.089	24.37[b]	5.63[bc]	1.69[c]
SE[3]	2.00	0.008	5.31	0.32	0.25

1433 [1]Mean ± Standard error for all storage times.
1434 [2] PV = peroxide value; FFA = free fatty acid concentration; Hex = hexanal concentration; Pro = propanal concentration; TBARS =
1435 thiobarbituric reactive substances
1436 [3]SE = standard error
1437 [a-c] Values within column with unlike superscripts differ ($P < 0.05$)
1438



1439
1440 **Figure 2-1. Interaction effect of storage time and antioxidant on peroxide value in frozen chicken patties.**

1441

1442 **Table 2-4. Main effects of antioxidant on oxidation measures in freeze-dried chicken patties.[1]**

Antioxidant	PV[2], meq/kg	FFA[2], ppm	Hex[2], ppm	Pro[2], ppm	TBARS[2], mg MDA/100g
None	5.02[a]	14.07[a]	5.35[a]	3.66[a]	1.70[a]
BHA	4.90[a]	12.96[b]	5.33[a]	3.59[a]	1.67[a]
MT	2.54[c]	8.76[d]	3.24[c]	2.92[c]	0.57[b]
MT+GT	4.31[b]	10.66[c]	4.65[b]	3.22[b]	1.44[a]
SE[3]	0.091	0.238	0.070	0.053	0.091

1443 [1]Mean for all storage times.
1444 [2]PV = peroxide value; FFA = free fatty acid; Hex = hexanal; Pro = propanal; TBARS = thiobarbituric reactive substances.
1445 [3]SE = standard error.
1446 [a-c] Values within column with unlike superscripts differ ($P < 0.05$)

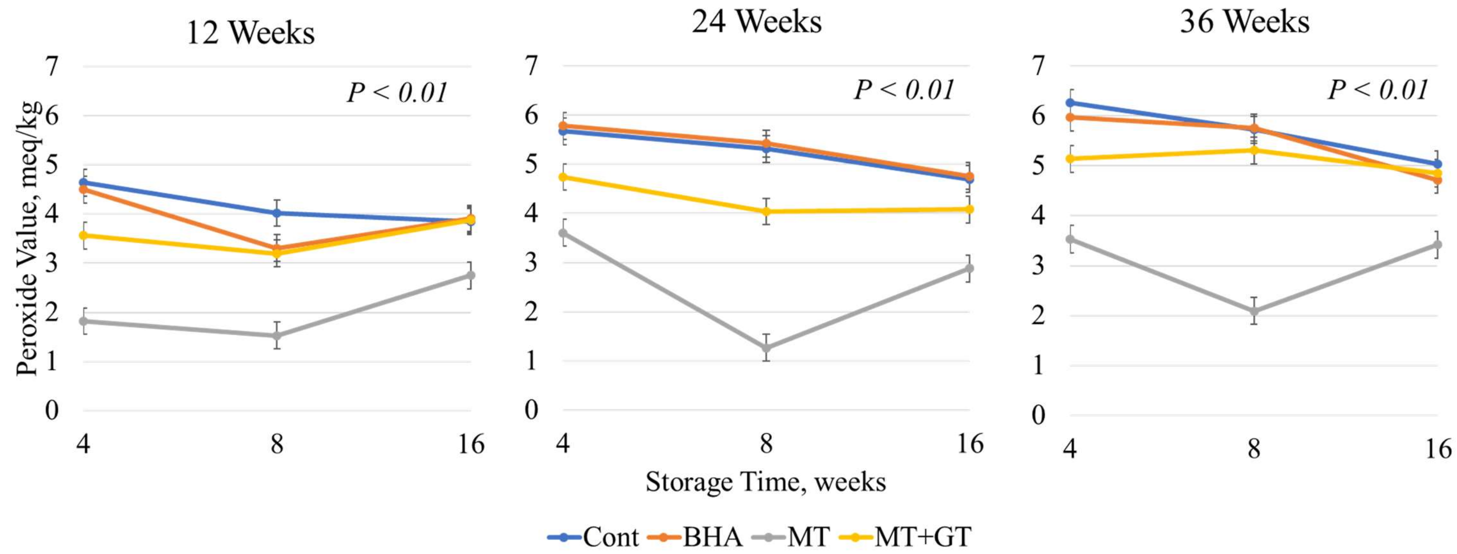
1447

1448 **Table 2-5. Main effects of dried storage time on oxidation measures in freeze-dried chicken patties.[1]**

Dried storage time, weeks	PV[2], meq/kg	FFA[2], ppm	Hex[2], ppm	Pro[2], ppm	TBARS[2], mg MDA/100g
4	4.60[a]	9.58[c]	4.78	2.91[c]	1.39
8	3.91[b]	11.51[b]	4.57	3.70[a]	1.37
16	4.07[b]	13.74[a]	4.58	3.44[b]	1.29
SE[3]	0.078	0.206	0.061	0.046	0.079

1449 [1]Mean for treatments.
1450 [2]PV = peroxide value; FFA = free fatty acid; Hex = hexanal; Pro = propanal; TBARS = thiobarbituric reactive substances.
1451 [3]SE = standard error.
1452 [a-c] Values within column with unlike superscripts differ ($P < 0.05$)

1453



1454

1455 **Figure 2-2. Effect of storage time and antioxidant on peroxide value in freeze-dried chicken patties frozen for different times.**

1456

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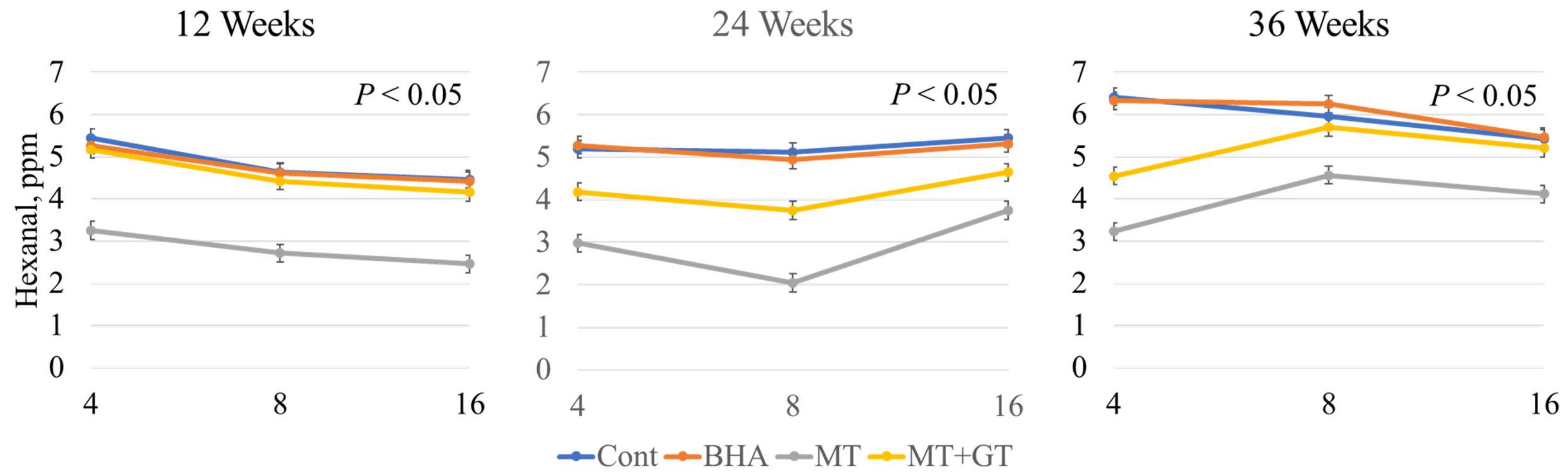

1458

1459 **Figure 2-3. Effect of storage time and antioxidant on hexanal concentration in freeze-dried chicken patties frozen for different**
1460 **times.**

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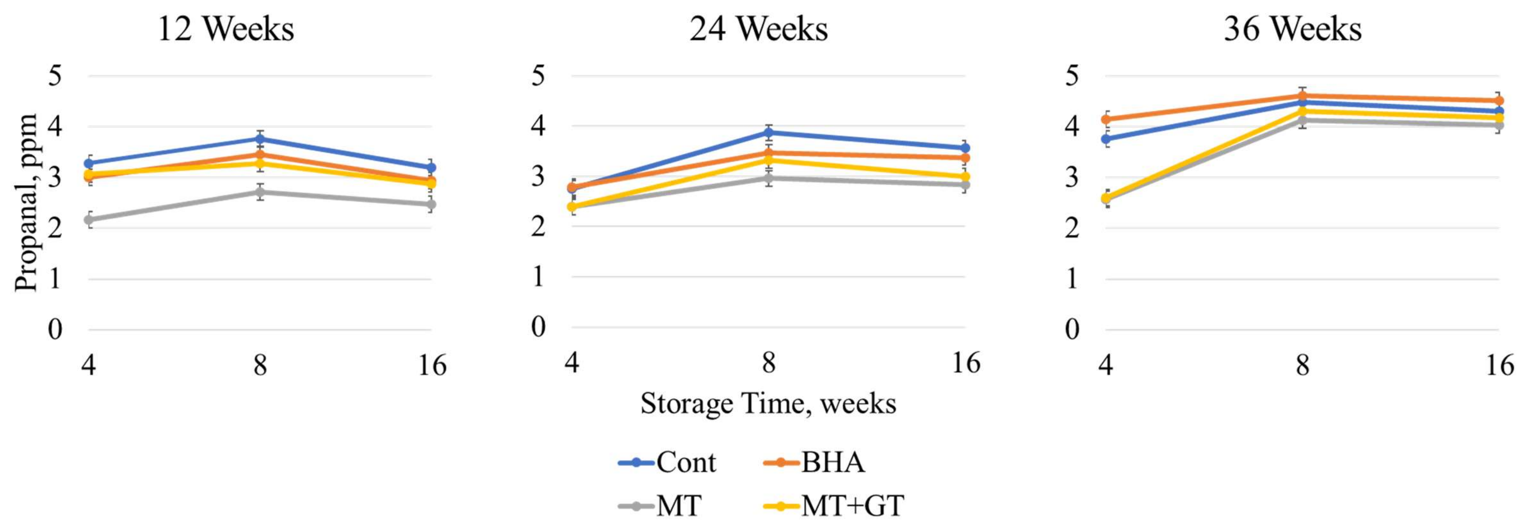

1462
1463 **Figure 2-4. Effect of storage time and antioxidant on propanal concentration in freeze-dried chicken patties frozen for different**
1464 **times (P < 0.05).**

1465

1466 # Chapter 3 - Evaluation of frozen and freeze-dried lamb patties
1467 # treated with different antioxidants and stored for various periods of
1468 # time

1469 ## Abstract

1470 Raw-frozen and freeze-dried pet foods are the fastest growing food forms being offered
1471 to pet parents and are helping to fuel continued growth in the pet food market. These foods
1472 contain high levels of protein and fat from raw meat. There is a lack of published research
1473 regarding the oxidative stability of these products. The objective of this experiment was to
1474 determine the impact of storage time and antioxidant addition on oxidation measurements in
1475 raw-frozen and freeze-dried lamb patties. Patties containing 50% lamb, and 12.5% each of rice,
1476 sweet potato, pumpkin, and apple were produced. Ingredients were mixed and made into 100g
1477 patties before being stored in plastic bags. Samples were frozen for 0, 4, 8, or 16 weeks before
1478 being removed for analysis. Freeze-dried samples were frozen for 12, 24, or 36 weeks before
1479 freeze-drying and further storage for 4, 8, or 16 weeks before analysis. The control with no
1480 antioxidant treatment resulted in higher levels of peroxide value (PV), hexanal, and propanal
1481 concentrations. Freeze-dried lamb patties treated with no antioxidant had higher peroxide value,
1482 hexanal concentration, propanal concentration, and thiobarbituric reactive substance ($P < 0.05$).
1483 Increasing the frozen storage time before freeze-drying resulted in a reduction of PV and
1484 increases in hexanal and propanal concentrations ($P < 0.05$). Increasing storage time post freeze-
1485 drying resulted in increased free fatty acid and propanal concentration. Increased storage time for
1486 both raw-frozen and freeze-dried meat patties indicated lipid oxidation was occurring. The use of

1487 antioxidants, specifically mixed tocopherols and BHA, reduced the level of oxidation that

1488 occurred.

1489

1490

1491 # Introduction

1492 Pet food sales have increased 5.1% since 2015 and sales were over $30 billion in 2019
1493 (Package Facts, 2020). This growth can be attributed to the use of new and novel protein sources
1494 and the growth in new food forms. Kibble comprises approximately 70% of pet food sold.
1495 However, the fastest growing food forms are raw-frozen and freeze-dried foods. Raw pet food
1496 sales increased by 235% between 2012 and 2016 while freeze-dried products increased by 325%
1497 between 2011 and 2014 (Lange, 2017; APPA, 2019). Raw-frozen and freeze-dried products
1498 contain high quantities of fresh meats compared to a kibble. The use of fresh whole meat
1499 inherently adds additional levels of fat. This elevated level of fat may become a concern as
1500 products are stored for extended periods of time. Specifically of concern is oxidation

1501 Little research has been published on the stability of raw-frozen and freeze-dried pet
1502 foods. These differ from ground and whole cuts of meat due to the addition of starches, fibers,
1503 vitamins and minerals to make them nutritionally complete. However, they are predominately
1504 meat, and in lieu of any research specific to these diet forms one must resort to comparison to the
1505 closest food type where research has been published – specifically in meats like beef, lamb and
1506 pork. Complicating the topic, the meat used in raw-frozen and freeze-dried pet foods may be
1507 stored for an extensive period of time before production of the complete diet. It has been
1508 reported that increased frozen storage time leads to increased levels of oxidation in chicken, beef,
1509 pork and lamb (Holman et al., 2018; Stika et al., 2007; Pikul et al. 1984; Botsoglou et al., 2003;
1510 Coombs et al. 2018; Cheng et al., 2019). Meat used in raw-frozen and freeze-dried products may
1511 also go through several freeze-thaw cycles before the product has been produced. Raw-frozen
1512 foods may also experience additional freeze-thaw cycles as it is transported to retail, home, and
1513 prepared for consumption. Meats that have undergone an increased amount of freeze-thaw cycles

1514 have been reported to result in increased oxidation (Rahman et al., 2015; Qi et al., 2012; Chen et
1515 al., 2018).

1516 This goes beyond concerns regarding rancidity and how it might affect consumer appeal
1517 or dog/cat acceptability. There is evidence that consumption of oxidized oil can impact the
1518 growth or performance of an animal (Dibner et al., 1996; Vazque and Jenkins, 2007; Boler et al,
1519 2012; Huang et al., 2016). There is limited research published regarding the impact on pet health
1520 when oxidized fat is consumed. However, one study examined the impact of feeding oxidized
1521 chicken fat to growing puppies and resulted in reduced weight gain, slower bone formation, and
1522 reduced antioxidant capacity in 12 weeks (Turek et al, 2003).

1523 The goal of this research is to begin filling-in gaps that are currently present in our
1524 knowledge about the shelf-stability of raw-frozen and freeze-dried pet foods. The objectives of
1525 this study were to a) evaluate lipid oxidation in raw-frozen and freeze-dried meat patties stored
1526 for an increased period of time and b) whether antioxidant preservatives could slow the
1527 degradation.

1528 Materials and Methods

1529 Sample Preparation

1530 Meat patties were produced to formula with lamb (50%; CJ Foods, Bern, KS), and
1531 pumpkin (Britt's Farm, Manhattan, KS), apple (Eastside Market, Manhattan,KS), rice (Walmart,
1532 Manhattan,KS), and sweet potato (Eastside Market, Manhattan, KS) at 12.5% each. Rice was
1533 cooked according to the instructions on the package (1part rice per 2 parts water) until soft, then
1534 cooled to room temperature (~22°C). Each ingredient was then ground in a meat grinder (Weston
1535 10-3201-W #32; Independence, OH) through a 7 mm plate to achieve similar size across
1536 ingredients. Each ingredient was subsampled for later proximate analysis (Table 3-1).

1537 A large master batch (22.67 kg) each of the formula was produced. A portion (appx 0.904
1538 kg) of the lamb was retained for future use. The master batch was mixed in a planetary mixer
1539 (Hobart H600T; Troy, OH) for 5 minutes. This batch was then split into four equal parts
1540 (approximately 5.44 kg) for the antioxidant addition. Antioxidant treatments included a control
1541 (no antioxidant), butylated hydroxyanisole (BHA; 1% of mix; Camlin Fine Sciences), mixed
1542 tocopherols (MT; 0.30% of mix; Camlin Fine Sciences), and mixed tocopherols + green tea (MT
1543 + GT; 0.20% + 0.10% of mix, respectively; Camlin Fine Sciences). The sub-sampled lamb was
1544 split into four equal parts (0.226 kg) and was mixed with the appropriate antioxidant treatment in
1545 a food processor for 1 minute. For the control, the meat was mixed in the food processor for 1
1546 minute before being added to the mixture. This smaller batch was then blended in smaller (9 kg)
1547 with hand powered mixers (Cabela's IK-541001, Sidney, NE) until it was incorporated evenly.
1548 From this mix, 100 g patties were formed and stored two to a bag with parchment separating
1549 patties and placed within plastic storage bags (Ziploc, Company, city state) and frozen at -20°C.
1550 Raw frozen samples were stored in the freezer for 4, 8, and 16 week periods before analysis.
1551 Samples for freeze-dried evaluation were stored frozen for 12, 24 and 36 weeks then removed
1552 and dried under vacuum (250-1,000 Millitorr) at a commercial facility with a 36 hour cycle time
1553 (Chasing Our Tails, Inc., Hudson, NH). Once dried, samples were placed in an environmental
1554 chamber at 35°C (without humidity control) for 4, 8, and 16 weeks. At the end of each period
1555 samples were removed for analysis.

1556 **Sample Analysis**

1557 **Fat Extraction**

1558 For fat extraction, one 100 g meat patty was placed in a 500 ml beaker. To the beaker 250
1559 ml of hexane was added and the mixture was stirred, by hand, for 15 minutes. The liquid portion

1560 of the sample was filtered through Whatman grade 1 filter papers (110 mm) utilizing a vacuum
1561 pump. Hexane was added to the meat mixture a second time (250 ml) and stirred for an
1562 additional 15 minutes and was again filtered. The hexane fraction collected was then placed in a
1563 round bottom Buchner flask and attached to a Rotovap (Büchi R-114; Brinkmann Instruments,
1564 Inc.; Riverview, FL) with a water bath (Büchi B-490; Brinkmann Instruments, Inc.; Riverview,
1565 FL) to separate the fat and hexane. The remaining fat sample was placed in falcon tubes (50 ml)
1566 and were frozen until evaluation of oxidation measures were completed.

1567 **Gas Chromatography**

1568 Gas chromatography was carried out on a Shimadzu (GC-17A) fitted with Supelco
1569 column (SP-2560; 100 m × 0.25 mm × 0.2 μm thickness). The carrier gas was nitrogen at 20
1570 ml/min. The initial temperature started at 130°. Temperature increased at a rate of 3.5°C/min
1571 until a final temperature of 210°C was reached. Fatty acids were identified based on the internal
1572 standard and were calculated following the equation identified by Sukhija and Palmquist (1988).

1573 **Peroxide Value**

1574 Peroxide value was determined using the IDF standard method (International Dairy
1575 Federation, 1991) with modifications. Barium Chloride Di-hydrate ($BaCl_2 \cdot 2H_2O$) solution was mix
1576 by adding 0.32 g of barium chloride di-hydrate with 10 mL of 0.4 M hydrochloric acid solution.
1577 Iron II sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) solution was mixed using 0.40 g of iron II sulfate
1578 heptahydrate ($FeSO_4 \cdot 7H_2O$) with 10 mL of HPLC graded water. These two solutions were both
1579 added to a 50 ml centrifuge tube and were placed in a centrifuge for 15 min. at 4000 rpm.
1580 Ammonium Thiocyanate solution was created by adding 3.0 g of ammonium thiocyanate in 10
1581 mL with HPLC graded water. A sonicator was then used to help dissolve the ammonium

1582 thiocyanate. Next, 200 µl of the barium iron solution and 200 µl of ammonium thiocynate were

1583 placed in a glass tube and mixed on a vortex for approximately 15 seconds.

1584 Sample preparation was conducted by adding 2 g of sample to a 15 ml centrifuge tube

1585 Isoocatane (10 ml) was added and the sample was vortexed for 1 minute. Test tubes were placed

1586 on a rocker for an additional 10 minutes before being vortexed for an additional minute. The

1587 samples were centrifuged at 4,000 rpm for 15 minutes. In a new glass tube, 200 µl of supernatant

1588 was mixed, using a vortex, with 30 µl of the ammonium thiocynate:iron II solution for 30 seconds

1589 and incubated at room temperature for 20 minutes. After incubation, samples were read at 510 nm,

1590 using isooctance to blank the spectrophotometer.

1591 Peroxide value was calculated as:

1592
$$PV\left(\frac{meq}{kg}\right) = \frac{[(As - Ab)\ \times m]}{W(Vanalyte/Vtotal)}$$

1593 where, As is the absorbance of the sample, Ab is the absorbance of the blank, m is the slope of

1594 the calibration curve, W is the mass of the sample in grams, V is the volume.

1595 **Thiobarbituric Reactive Substances**

1596 Thiobarbituric reactive substances were measured following the method outlined by

1597 Tarladgis et al. (1960). In short, 10 g of sample was placed in a flask with 97 ml of water, 1 ml of

1598 sulfanilamide solution and 2 ml of HCl solution. The sample was placed on heat, brought to a

1599 boil, and allowed to distill until 50 ml of distillate was collected. Next, 5 ml of distillate and 5 ml

1600 of thiobarbituric acid (TBA) reagent were added to test tubes in duplicated. A blank containing 5

1601 ml of distilled water and 5 ml of TBA was also created. The test tubes were placed in a boiling

1602 water bath for 35 minutes to allow color development and then allowed to cool in water for 10

1603 minutes. In the final step, 3 ml of sample was placed in a cuvette (in duplicate) and absorbance,

1604 via spectrophotometer, was determined at 532 nm. Thiobarbituric acid value was determine with

1605 the following equation:

1606 $$TBA\left(mg\frac{malonaldehyde}{100gm}of\ sample\right) = O.D.(Absorbance\ 532)\ \times 7.8$$

1607 **Free Fatty Acids**

1608 Free fatty acid content of fat samples was measured according to AOCS official method

1609 Ca 5a-40. Approximately 1 g of fat (extracted using hexane) was added to an Erlenmeyer flask

1610 with 50 ml neutralized ethanol and 1 ml of phenolphthalein indicator. This solution was titrated

1611 with 0.1 N sodium hydroxide until a faint pink color was achieved. Free fatty acid content was

1612 calculated as:

1613 $$FFA\ as\ oleic, \% = \frac{ml\ of\ alkali\ \times N \times 28.2}{sample\ weight\ (g)}$$

1614 **Headspace Analysis**

1615 The GC-Headspace volatile analyses was performed with an Agilent Technologies

1616 (Wilmington, DE) model 7890A gas chromatograph system equipped with an Agilent

1617 Technologies model 7697A headspace sampler. Parameters for the extraction of volatiles using

1618 headspace sampler were as follows: oven temperature, 80ºC; vial equilibration time, 60 min;

1619 pressure equilibrium time 0.5min; fill pressure 15 psi; injection time, 1.2 min; and GC cycle time

1620 40 min. The sample loop and transfer line temperatures were set at 20 and 40ºC higher than the

1621 oven temperature, respectively. Vials (containing 2 g frozen or freeze-dried ground samples)

1622 were equilibrated and pressurized with carrier gas before injection into Agilent J&W DB-5ms

1623 Ultra Inert (30 m × 250 µm x 1 µm) GC column. Injector temperature was set at 250ºC. Helium

1624 at 1.0 mL/ min, constant flow mode with average velocity of 36.623 cm/sec was the carrier gas.

1625 The flame-ionization detector (FID) temperature was set at 300ºC. Volatile compounds were

1626 identified by comparison of retention times with those of authentic reference compounds:
1627 propanal, hexanal, and 2,4-decadienal (Sigma, St. Louis, MO). Peak areas for individual total
1628 volatiles were integrated. Propanal, hexanal and 2,4-decadienal (Sigma, St. Louis, MO) were
1629 used as external standards. Determination of the amount of each identified headspace volatile
1630 was performed by semi-quantitation in terms of the relative amounts derived from the ratios of
1631 the peak areas of the volatiles and internal standard, 5-methyl-2-hexanone added into the
1632 samples. The software used to process peak areas was OpenLAB CDS ChemStation Edition for
1633 GC System (Agilent Technologies, Wilmington, DE). Each sample was analyzed in duplicate.

1634 **Statistical Analysis**

1635 The experimental design for the raw-frozen lamb patties evaluation was a 4 × 4 factorial
1636 arrangement of treatments with the main effects of storage time (0, 4, 8, and 16 weeks) and
1637 antioxidant type (control, BHA, MT, and MT + GT). For freeze-dried samples, the experiment
1638 was set up as a 3 × 3 × 4 factorial arrangement of treatments with main effects of frozen storage
1639 (12, 24, and 36 weeks), freeze-dried storage (4, 8, and 16 weeks), and antioxidant (control, BHA,
1640 MT, and MT + GT). All data was analyzed for unequal variances and was transformed to log
1641 form. Data were analyzed using software for mixed models (GLIMMIX procedure, SAS v9.4,
1642 SAS Institute, Cary, NC). Means were separated using Bonferroni adjustment with significance
1643 at $\alpha = 0.05$ and data are reported as log transformations.

1644 **Results**

1645 **Raw-Frozen Lamb Patties**

1646 All ingredients contained a substantial amount of moisture (Table 3-1; 75.30% average).
1647 Lamb contained the greatest quantity of fat protein compared to all other ingredients. The total
1648 calculated fat for the lamb patties was 15.45%. Thus, as a proportion of the fat to the diet lamb

1649 contributed was 92.6% of the final formulation; whereas, rice contributed 2.88%, sweet potato
1650 1.80%, apple 0.94%, and pumpkin 2.55%. These ingredients provide 1.25% fat to the mixture.
1651 Effect of storage time on oxidation measurements are presented in Table 3-2. Peroxide
1652 value increased during 16 weeks of frozen storage ($P < 0.05$). Free fatty acid concentration and
1653 hexanal concentration were not impacted by storage time. Propanal content increased from week
1654 0 to week 4, remained stable between weeks 4 and 8, then declined at week 16 ($P < 0.05$).
1655 Thiobarbituric reactive substances increased over the first 8 weeks of storage. Samples for week
1656 16 were lost due to freezer malfunction.
1657 Effects of antioxidant on oxidation measurements in located in Table 3-3. Peroxide value
1658 was highest for treatments containing no antioxidant, followed by the MT + GT treatment.
1659 Mixed tocopherols and BHA patties had the lowest peroxide values ($P < 0.05$). Hexanal and
1660 propanal concentrations were highest for treatments containing no antioxidant. Mixed
1661 tocopherols + GT treatment had the lowest concentrations while BHA and MT patties were
1662 intermediate ($P < 0.05$). Thiobarbituic reactive substances were not impacted by antioxidant
1663 treatment.
1664 Hexanal concentration was highest for BHA treated patties at time 0 (Figure 3-2). At
1665 week 16, the control patties had the highest hexanal concentration and were higher than week 0.
1666 At week 16 BHA treated patties had the lowest hexanal concentration. Mixed tocopherols and
1667 MT + GT treatments were not different from one another. Propanal content was highest for
1668 treatments containing no antioxidant (Figure 3-3), all other treatments were similar to one
1669 another at week 16. Propanal content also increased for treatments from week 0 to week 4,
1670 remained stable until week 8, and then decline to week 16.

1671 **Freeze-Dried Lamb Patties**

1672 Impacts of frozen storage, freeze-dried storage and antioxidant treatments are presented
1673 in Table 3-4 to Table 3-6. Frozen storage time resulted in increased hexanal and propanal
1674 concentration during 36 weeks of storage ($P < 0.05$). Peroxide value, FFA and TBARS were
1675 reduced during frozen storage. Storage after freeze-drying resulted in no differences for PV,
1676 hexanal concentration, or TBARS ($P < 0.05$). Free fatty acid concentration and propanal
1677 concentration increased during 16 weeks of storage ($P < 0.05$).

1678 Treatments containing no antioxidant resulted in the highest PV, while MT and BHA had
1679 the lowest PV. Free fatty acid concentration was highest for BHA and MT while the treatments
1680 with no antioxidant were the lowest ($P < 0.05$). Hexanal concentration, propanal concentration,
1681 and TBARS were highest for the control patties while all other treatments were lower and similar
1682 to one another ($P < 0.05$).

1683 Interaction effects of frozen storage, dried storage, and antioxidant were not significant
1684 for FFA and TBARS in the freeze-dried patties. Between 12 and 24 weeks of frozen storage
1685 there was a drop in starting value for PV (Figure 3-4). At 12 weeks frozen storage, there was a
1686 clear separation in the treatments as the control patties had the highest peroxide value followed
1687 by the MT + GT treatment. Mixed tocopherols and BHA treatments had the lowest PV and
1688 similar to one another ($P < 0.05$). At 24 weeks the MT + GT treatment had the highest PV
1689 ($P<0.05$), while all other treatments were similar. At 36 weeks, the control and MT + GT patties
1690 had the highest PV.

1691 Patties treated with MT had the lowest hexanal concentration at 24 weeks and the highest
1692 at 36 weeks (Figure 3-5). Propanal content was highest in the control at 12 weeks frozen storage

1693 and similar to BHA and MTGT treatments at 24 weeks frozen storage and 16 weeks freeze-dried

1694 storage.

1695 Discussion

1696 Several authors have evaluated the lipid oxidation in frozen lamb (Coombs et al. 2018;

1697 Muela et al. 2015; Bellés et al. 2017). Given there is a deficiency of work in the area of raw meat

1698 based pet foods, we have to extrapolate from research related to fat oxidation from the meat

1699 research discipline. In that trade TBARS are often used to assess the level of oxidation. Coombs

1700 et al. (2018) examined chilled storage time and frozen storage duration in lamb meat. Frozen

1701 storage time, up to 52 weeks, did not consistently impact product oxidation as it was elevated for

1702 0 and 6 weeks of chilled storage but not at 2, 4, and 8 weeks (Coombs et al., 2018). The authors

1703 stated that different loins (samples) were used and the variation between samples led to

1704 inconsistencies. The current study resulted in increased TBARS as frozen storage time increased.

1705 Which agrees with results from Muela et al. (2015) who observed an increase in TBARS for

1706 lamb meat that was stored up to 21 months. In their study differences were present at 1 month of

1707 frozen storage. Increased TBARS have also been observed during time in the display case when

1708 different methods of freezing and frozen storage were applied to samples (Muela et al., 2010;

1709 Bueno et al., 2013). Bueno et al. (2013) also observed an increase in hexanal content when liquid

1710 nitrogen was used as a method of freezing, but not when a home freezer was used for 10 months

1711 of storage. Bellés et al. (2017) also observed increased TBARS values of lamb during storage.

1712 Other red meats, such as beef, have also been reported to have increased levels of TBARS during

1713 storage and display (Resconi et al. 2018). Turgut et al. (2017) reported an increase in peroxide

1714 value and TBARS in meatballs during 6 months of storage.

1715 In the current study, PV, hexanal concentration and TBARS were not affected as storage
1716 time reached 16 weeks in freeze-dried samples. Chipault and Hawkins (1971) observed increased
1717 peroxide value in freeze-dried chicken and beef with increased storage time. An increase in
1718 TBARS in freeze-dried meats has also been reported with longer storage times (Sun et al. 2001;
1719 Wilkinson et al., 2001). Thiobarbituric reactive substances have also been measured at 450-455
1720 nm as thiobarbituric acid and found that it is not selective for malonaldehyde, but also reacts
1721 with other compounds such as alkanals and acetaldehyde (Tarladgis et al, 1962; Kosugi et al,
1722 1987; Marcuse and Johansson, 1973). These reactions create a yellow/orange pigment that can't
1723 be measured at 523nm. A fluorescence method was developed for the determination
1724 malonaldehyde (MDA) concentration in freeze-dried samples utilizing Schiff bases that are
1725 created by reacting MDA with amino groups (Kamarei and Karel 1984). This method has been
1726 used in freeze-dried chicken and beef and resulted in an increase in fluorescence units as storage
1727 time was increased (Wilkinson et al., 2001). The application of this methodology in future
1728 research might provide a more accurate picture of malonaldehyde development during oxidation
1729 than the thiobarbituric reactive substances methods. Goodridge et al. (2003) reported increased
1730 hexanal content with increased freeze-dried storage. This was not observed in the current study.
1731 One factor that could cause the difference in the Goodridge et al. (2003) study was that they
1732 stored their samples at 50°C, while samples in the current work were stored at 35°C. Heat is a
1733 driving factor in the lipid oxidation process and may have led to no substantial differences in our
1734 results as the temperature applied was not as severe.

1735 The antioxidant system to retard oxidation can also have an impact. In the current study,
1736 BHA and MT provided better protection against oxidation than the control and MT + GT in raw-
1737 frozen and freeze-dried lamb patties. One possible reason for MT + GT not working as well is

1738 the dilution of MT with GT. When green tea was mixed with MT, the level of MT decreased by
1739 0.1%. This also occurred in previous work in which TBARS was higher for the combination of
1740 MT and GT in chicken breast meat (Smet et al., 2008). Carballo et al. (2018) reported similar
1741 TBARS values for lamb patties that were frozen and lacked protection with an antioxidant
1742 relative to one treated with astaxanthin (a plant-based antioxidant carotenoid). This same study
1743 observed increased TBARS for treatments containing sodium metabisulphite and reduced
1744 TBARS when sodium ascorbate was used. In the current study, BHA and MT provided similar
1745 results for TBARS in freeze-dried lamb patties that were stored for 16 weeks. Aksu et al. (2005)
1746 reported similar results after 300 days of storage between tocopherols and BHA when included at
1747 50 mg/kg in cooked meat.

1748 The type of fat in which the antioxidant is used to treat can also impact whether it works
1749 successfully. Chicken fat contains 28.7% saturated and 64.8% unsaturated fatty acids in total fat
1750 (NRC, 2012). Lamb contains a much higher level of saturated fats (46.6%) and a lower level of
1751 unsaturated fats (53.4%; Coombs et al., 2018). Saturated fats are not as easily oxidized compared
1752 to unsaturated fats. An antioxidant that works well in lamb fat might provide a lesser degree of
1753 protection due to the increased level of unsaturated fatty acids.

1754 Two aspects of raw-frozen and freeze-dried food that was not examined in the current
1755 study is the effect of freeze-thaw cycles on oxidation and changes in sensory attributes. Qi et al.
1756 (2012) reported an increase in TBARS values with increased freeze-thaw cycles. An increase in
1757 TBARS has also been reported in chicken and beef with increased freeze-thaw cycles (Chen et
1758 al., 2018; Rahman et al., 2015). Peroxide value, acid value, and free fatty acid content have also
1759 been reported to increase with more freeze-thaw cycles (Chen et al., 2018; Rahman et al., 2015).

1760 Sensory attributes can also be impacted by freezing time. Drip loss and cooking loss both
1761 increased when lamb was stored for 12 months (Daszkiewicz et al., 2018). Choi et al. (2018) also
1762 reported increased drip loss and lower water holding capacity as frozen storage time increased.
1763 Other sensory attributes, such as hardness and cohesiveness have been reduced by increasing
1764 frozen storage (Qi et al., 2012; Stika et al., 2007). These changes have also been reported in beef
1765 when storage time increased (Rahman et al. 2015). Increased storage time can also impact the
1766 color of the meat as reduced redness and an increase in yellow pigment has been reported (Bellés
1767 et al., 2017; Stika et al, 2007). Trained panelists have been used to evaluate meat as storage time
1768 was increased. For example, Muela et al. (2016) reported reduced juiciness and overall
1769 acceptability of lamb as it was stored for longer periods of time.

1770 Another shortcoming of this work is the lack of information on these samples as they
1771 relate to animal health. Turek et al. (2003) reported reduced weight gain and bone development
1772 in puppies fed oxidized fats. There is little work describing the health implications of raw-frozen
1773 and freeze-dried products as they oxidize. Impact on health and growth could be evaluated in a
1774 growth study utilizing chicks. Dibner et al. (1996) reported reduced growth in birds fed diets
1775 containing oxidized fat. However, birds don't have a long-life span and impacts on longevity
1776 would not attainable.

1777 **Conclusion**

1778 Raw-frozen lamb patties resulted in increased PV and TBARS as storage time increased.
1779 Samples containing no antioxidant had higher peroxide value, hexanal concentration and
1780 propanal concentration. Freeze-dried lamb patties had increased free fatty acid and propanal
1781 concentration with increased storage time. Mixed tocopherols and BHA provided the best
1782 defense against oxidation in freeze-dried lamb patties. This data suggests that freezing as a

1783 preservation system will not slow oxidation. The used of antioxidants reduced the level of
1784 oxidation that occurred.

1785 Due to little published research on raw-frozen and freeze-dried pet foods, meat industry
1786 data can be used to hypothesize what might happen. However, these are often simple and contain
1787 the meat only. Pet food is very complex and contains many ingredients as well as additional
1788 vitamins and minerals in the form of pre-mixes. The current research did not contain any
1789 additional vitamins and minerals, thus eliminating reactions that may occur in a complete diet.
1790 Raw-frozen and freeze-dried formulations that are nutritionally balanced for the intended species
1791 and age need to be evaluated to determine the role that vitamins and minerals play in the
1792 oxidation of pet foods.

1793 More research is needed to determine which antioxidant forms and appropriate dose
1794 works best for lamb compared to other meats. The impact of freeze-thaw cycles is also needed to
1795 determine the degree of oxidation occurring before the product is consumed. Health implications
1796 of consumption of oxidized oil is lacking pet food and is needed to improve diets for our pets.
1797 This research provides a start to filling gaps that currently exist in raw-frozen and freeze-dried
1798 pet food and confirms that raw-frozen and freeze-dried foods oxidize when no antioxidant is
1799 used.

1800

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Tables and Figures

Table 3-1. Proximate analysis of ingredients used to create lamb patties.[1]

Ingredient	Moisture, %	Protein, %	Fat, %	Ash, %	Dry Matter, %
Lamb	55.30	14.30	28.40	3.52	44.70
Sweet Potato	78.14	0.93	1.10	1.18	21.86
Rice	69.10	3.10	1.76	0.17	30.90
Apple	85.48	0.28	0.58	0.38	14.52
Pumpkin	88.52	1.78	1.56	1.20	11.48

[1]As is basis.

Table 3-2. Impact of frozen storage time on oxidation measures in raw-frozen lamb patties.[1]

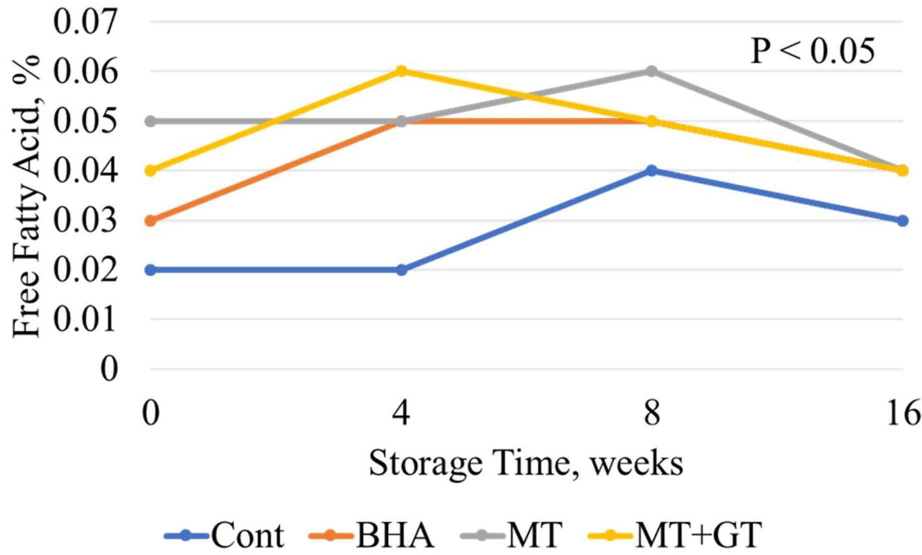
Storage time, weeks	PV[2], meq/kg	FFA[2], ppm	Hex[2], ppm	Pro[2], ppm	TBARS[2], mg MDA/100g
0	0.59^{c}	0.04^{b}	2.87	5.16^{b}	-0.36^{b}
4	1.22^{ba}	0.04^{a}	1.88	8.24^{a}	-0.51^{b}
8	0.81^{bc}	0.05^{a}	2.44	8.55^{a}	0.25^{a}
16	1.54^{a}	0.04^{b}	1.71	4.70^{b}	n.d.*
SE[3]	0.10	0.001	0.22	0.22	0.12

[1]Means for all treatments.
[2]PV = peroxide value; FFA = free fatty acid; Hex = hexanal; Pro = propanal; TBARS = thiobarbituric reactive substances.
[3]SE = standard error.
[a-c] Values within column differ ($P < 0.05$).
*n.d. = not determined. Sample lost due to equipment failure.

Table 3-3. Antioxidant effect on oxidation measures in raw-frozen lamb patties.[1]

Antioxidant	PV[2], meq/kg	FFA[2], ppm	Hex[2], ppm	Pro[2], ppm	TBARS[2], mg MDA/100g
Cont[2]	1.87^{a}	0.03^{c}	4.88^{a}	$10. 50^{a}$	0.18
BHA[2]	0.53^{c}	0.04^{b}	2.30^{b}	5.06^{b*}	-0.33
MT[2]	0.59^{c}	0.05^{a}	1.05^{b}	5.52^{b}	-0.28
MT + GT[2]	1.17^{b}	0.04^{ba}	0.67^{c}	5.58^{b}	-0.39
SE[3]	0.10	0.001	0.06	0.13	0.14

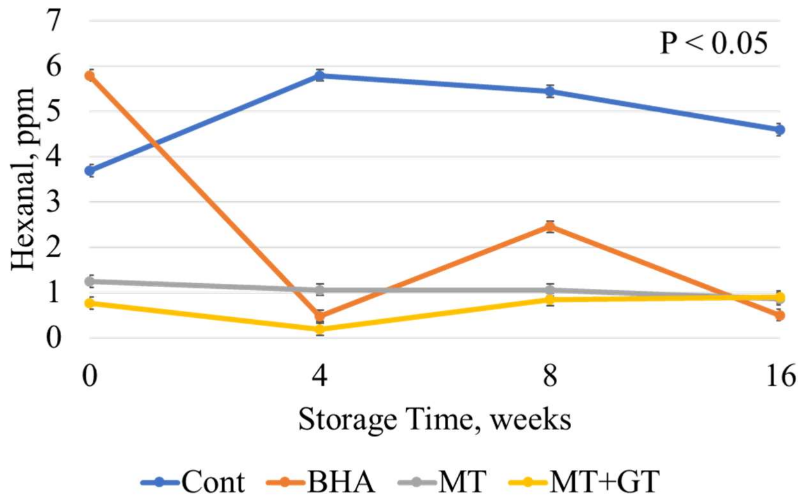
[1]Means for all treatments.
[2]PV = peroxide value; FFA = free fatty acid; Hex = hexanal; Pro = propanal; TBARS = thiobarbituric reactive substances; Cont = control; BHA = butylated hydroxyanisole; MT = mixed tocopherols; MT + GT = mixed tocopherols + green tea.
[3]SE = standard error.
[a-c] Values within column with unlike superscripts differ ($P < 0.05$).
*SE = 0.37



1922

1923 **Figure 3-1. Effect of storage time and antioxidant on free fatty acid concentration in frozen**
1924 **lamb patties.**

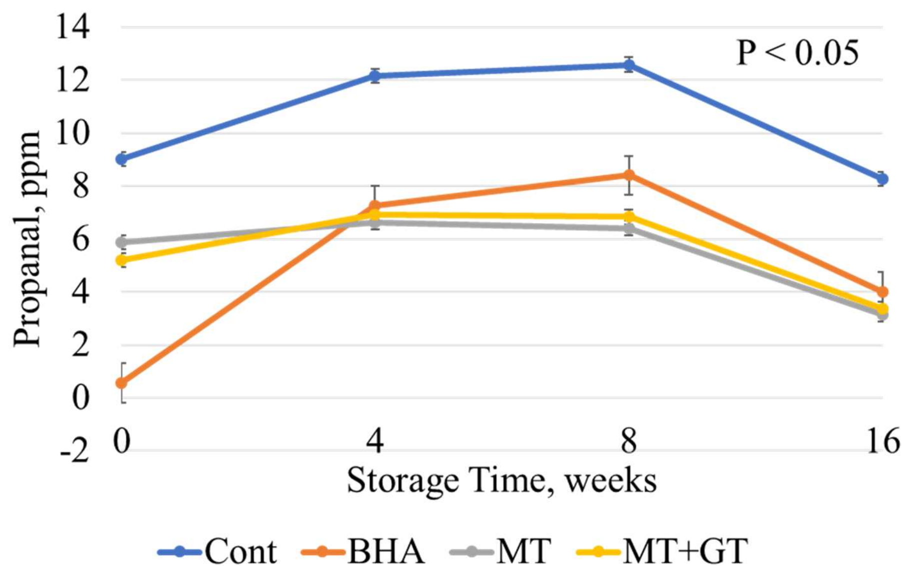
1925



1926

1927 **Figure 3-2. Effect of frozen storage time and antioxidant on hexanal concentration in raw-**
1928 **frozen lamb patties.**

1929



1930 **Figure 3-3. Effect of frozen storage and antioxidant on propanal concentration in raw-**
1931 **frozen lamb patties.**

1932

1933 **Table 3-4. Effect of frozen storage time on oxidation measurements in freeze-dried lamb**
1934 **patties.[1]**

Storage Time, wks	PV[2], meq/kg	FFA[2], ppm	Hex[2], ppm	Pro[2], ppm	TBARS[2], mg MDA/100g
12	2.43[a]	2.26[b]	2.30[b]	3.10[b]	2.24[a]
24	1.897[b]	2.73[a]	2.04[c]	2.96[b]	1.04[c]
36	1.93[b]	2.05[c]	2.69[a]	3.77[a]	1.79[b]
SE[3]	0.045	0.015	0.041	0.040	0.106

1935 [1]Means for all treatments.
1936 [2]PV = peroxide value; FFA = free fatty acid; Hex = hexanal; Pro = propanal; TBARS =
1937 thiobarbituric reactive substances.
1938 [3]SE = standard error.
1939 [a-c]Means within a column with unlike superscripts differ ($P < 0.05$)

1940

1941

1942

1943

1944 **Table 3-5. Effect of freeze-dried storage time on oxidation measurements in freeze-dried**
1945 **lamb patties.[1]**

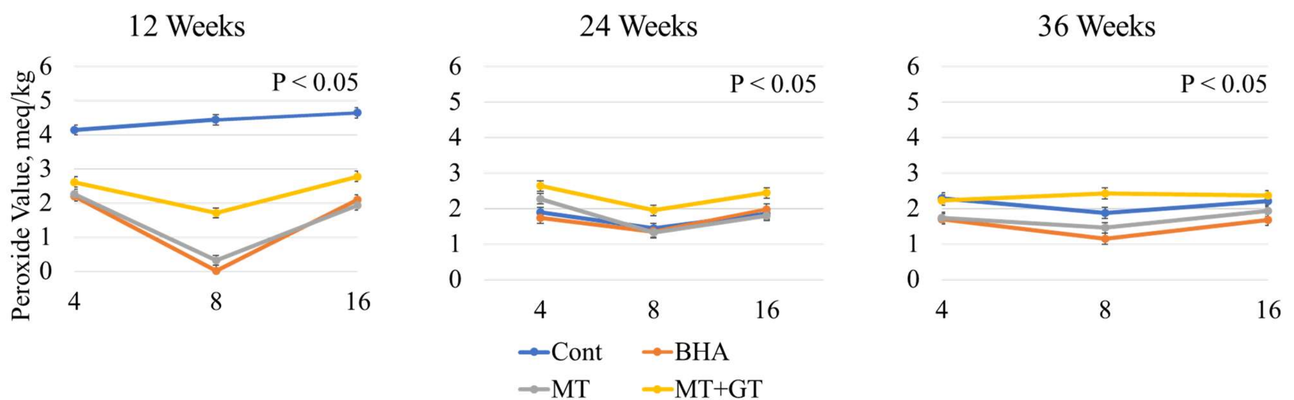
Storage Time	PV[2], meq/kg	FFA[2], ppm	Hex[2], ppm	Pro[2], ppm	TBARS[2], mg MDA/100g
4	2.31[a]	2.14[c]	2.49[a]	2.81[b]	1.72[a]
8	1.62[b]	2.38[b]	1.97[b]	3.45[a]	1.67[a]
16	2.31[a]	2.53[a]	2.58[a]	3.56[a]	1.68[a]
SE[3]	0.045	0.015	0.041	0.040	0.106

1946 [1]Means for all treatments.
1947 [2]PV = peroxide value; FFA = free fatty acid; Hex = hexanal; Pro = propanal; TBARS =
1948 thiobarbituric reactive substances.
1949 [3]SE = standard error.
1950 [a-c]Means within a column with unlike superscripts differ ($P < 0.05$)
1951

1952 **Table 3-6. Effect of antioxidant treatment on oxidation measurements in freeze-dried lamb**
1953 **patties.[1]**

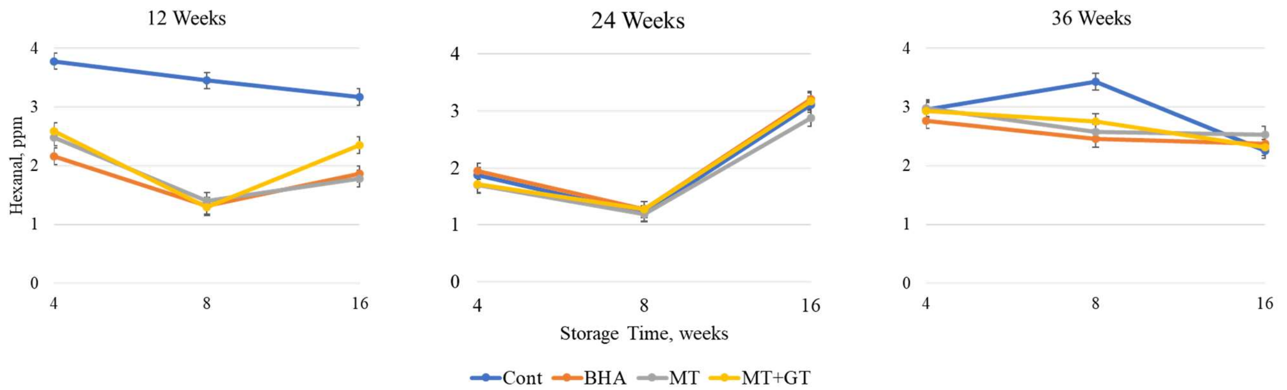
Antioxidant	PV[2], meq/kg	FFA[2], ppm	Hex[2], ppm	Pro[2], ppm	TBARS[2], mg MDA/100g
No	2.76[a]	2.29[b]	2.80[a]	3.49[a]	2.23[a]
BHA	1.55[c]	2.37[a]	2.15[b]	3.18[b]	1.47[b]
MT	1.68[c]	2.38[a]	2.17[b]	3.21[b]	1.46[b]
MT + GT	2.35[b]	2.34[ba]	2.26[b]	3.22[b]	1.61[b]
SE[3]	0.052	0.018	0.048	0.046	0.122

1954 [1]Means for all treatments.
1955 [2]PV = peroxide value; FFA = free fatty acid; Hex = hexanal; Pro = propanal; TBARS =
1956 thiobarbituric reactive substances.
1957 [3]SE = standard error.
1958 [a-c]Means within a column with unlike superscripts differ ($P < 0.05$)
1959
1960



1961
1962 **Figure 3-4. Effect of storage time and antioxidant on peroxide value in freeze-dried lamb**
1963 **patties frozen for different period of time.**

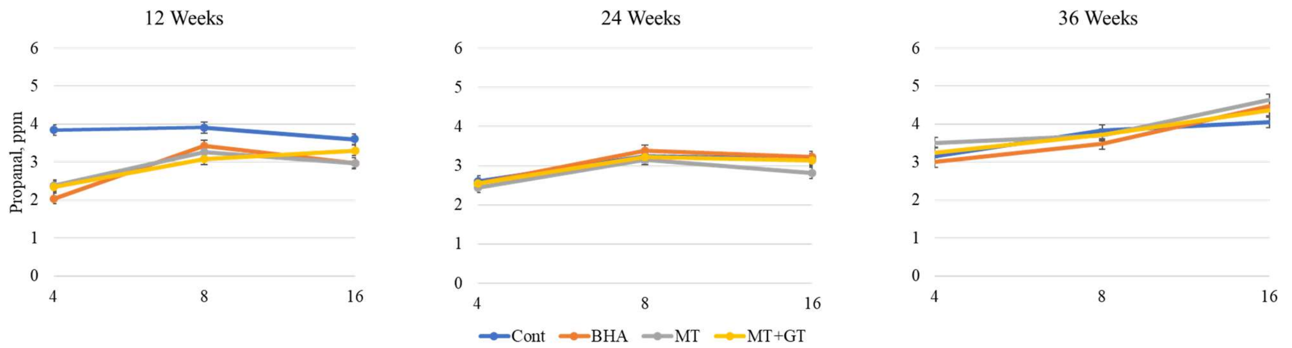
1964



1965

1966 **Figure 3-5. Effect storage time and antioxidant on hexanal concentration in freeze-dried**
1967 **lamb patties frozen for different periods of time (interaction P < 0.05).**

1968



1969

1970 **Figure 3-6. Effect of storage times and antioxidant on propanal concentration in freeze-**
1971 **dried lamb patties frozen for 12, 24, or 36 weeks (interaction P < 0.05).**

1972

Chapter 4 - Evaluation of Protein Quality of Chicken Proteins Intended for Pet Food

Abstract

The pet food industry is constantly incorporating new sources of protein into diets to differentiate from what is available in the market. Thus, there is a need for the evaluation of these ingredients. The objective of this study was to determine the protein quality of chicken-based ingredients processed under different conditions. A 10-day chick growth assay was conducted to determine the protein efficiency ratio (PER) of each protein source. An *in-vitro* protein digestibility assay was conducted and protein digestibility corrected amino acid scores (PDCAAS) were computed for each ingredient to determine their relationship to the PER. Spray dried egg (SDEG) had the highest PER value (4.94) and was similar to high protein chicken powder and dehydrated chicken breast (4.71 and 4.44, respectively). High fat chicken powder PER (4.26) was similar to the high protein chicken powder and dehydrated chicken breast but resulted in a lower ($P<0.05$) PER compared to SDEG. The rendered products (chicken meal, chicken by-product meal, and poultry by-product meal) had significantly lower PER ($P<0.05$; 3.34, 3.25 and 2.55, respectively) than the dried meats or egg. Corn gluten meal had the lowest PER (0.19; $P<0.05$). The *in-vitro* protein digestibility was highly correlated ($R=0.91$) to PER. Maintenance recommendations for dog and cat (NRC, 2006) were used to calculate PDCAAS, which resulted in similar ranking to the chick PER results ($R=0.80$ for dog and $R=0.95$ for cat; $P<0.05$). These results would suggest that the *in-vitro* digestibility and PDCAAS could be used as an alternative method to evaluate protein quality of ingredients in the production of pet foods.

1994 # Introduction

1995 Pet food is a $30 billion-dollar industry in the US that continues to grow by 4% each year
1996 (1). Much of this growth is due to new types of foods based on alternative processes and/or novel
1997 concentrated protein sources from plant or animal origin. Commonly used protein sources, such
1998 dried egg or rendered meat products, have been evaluated extensively as protein sources for
1999 livestock and pets (2-6). However, many of the new minimally processed protein sources lack
2000 published research to describe their nutritional value. Thus, there is a need for ingredient
2001 evaluation to determine nutrient quality and utilization. Evaluation by the target species (e.g. the
2002 dog or cat) would be ideal. But this can be expensive, time consuming, and has social and
2003 welfare implications for most pet food companies. Another approach is to utilize a model animal,
2004 such as the chick in a protein efficiency ratio (PER) assay (2). Chicks can be sensitive to subtle
2005 changes in diet composition and process. A more rapid option that does not rely on an animal
2006 model, is an *in-vitro* (bench-top) assay. The goal is to mimic the digestion conditions occurring
2007 in the animal to quantify nutrient disappearance. This protein digestibility can be used to
2008 calculate a protein digestibility corrected amino acid score (PDCAAS; 7). This method has been
2009 evaluated in human nutrition for decades and is an accepted method by the World Health
2010 Organization (7). This computed value is based on the amount of the first limiting essential
2011 amino acid compared to a reference protein. The objective of this experiment was to determine
2012 the effect of process (minimally processed vs. high heat process) on protein efficiency ratio of
2013 various protein sources relative to an *in-vitro* protein digestibility and calculated PDCAAS. It
2014 was our hypothesis that the PDCAAS method would be correlated to the chick results and
2015 provide an alternative to evaluate protein on a more rapid basis.

2016 ## Materials and Methods

2017 ### Ingredients

2018 Individual protein ingredients including spray dried egg (SDEG; International
2019 Dehydrated Foods, Springfield, MO), high fat chicken powder (HFCP; Humankind, Dresher, PA
2020 ; International Dehydrated Foods, Springfield, MO), high protein chicken powder (HPCP;
2021 Humankind, Dresher, PA ; International Dehydrated Foods, Springfield, MO), dehydrated
2022 chicken breast (DCB; Humankind, Dresher, PA), chicken meal (CM; Tri-Star LLC Pet food,
2023 Kansas City, KS) chicken by-product meal (CBPM; Tri-Star LLC Pet Food, Kansas City, KS),
2024 poultry by-product meal (PBPM; Farmers Union Industries, LLC, Redwood Falls, MN), and
2025 corn gluten meal (CGM; ADM, Chicago, IL) were obtained from the study sponsor. Vitamin and
2026 mineral premixes (Harlan Teklad, Madison, WI) were sourced immediately prior to the
2027 production of experimental chick diets. Ingredients were analyzed for moisture, crude protein,
2028 crude fat, crude fiber, and ash (Error! Reference source not found.; AOAC 930.15, AOAC
2029 990.03, AOAC 945.16, AOCS Ba 6a-05, and AOAC 942.05, respectively). Test ingredients were
2030 also analyzed for their amino acid composition (AOAC 982.30E (a,b,c), 2006).

2031 ### Diets

2032 The N-free diet served as a negative control (2). The experimental diets consisted of test
2033 ingredients added to the N-free diet, replacing equal proportions of corn starch and dextrose in a
2034 manner similar to Cramer et al. (6). Each experimental diet was formulated to contain 10% crude
2035 protein solely from the test ingredient. The SDEG served as the positive control. Each diet
2036 contained soybean oil (source of essential fatty acids), minerals, vitamins, and choline chloride to
2037 meet the chicks daily requirements (8).

2038 **Chick Protein Efficiency Ratio Assay**

2039 One day old male broilers (Cobb*Cobb) were obtained from Cobb Vantress (Siloam
2040 Springs, AR) and placed on a starter diet (23% CP) for 6 days. On day 7, following an 8 hour
2041 fast, chicks were weighed individually and sorted by weight. Chicks were assigned to pen by
2042 weight to achieve similar starting weights across treatments. Experimental diets were randomly
2043 assigned to pen and battery in a completely randomized experimental design. Water was
2044 provided *ad libitum* throughout the experiment. After 10 days on feed, birds were fasted for 8
2045 hours and then weighed by pen. Feed disappearance was recorded to determine crude protein
2046 intake and gain:feed. Protein efficiency ratio (PER) and net protein ratio (NPR) were calculated
2047 as follows:

2048
$$PER = \frac{BWG}{CPI}$$

2049
$$NPR = \frac{(BWG - GNfree)}{CPI}$$

2050 where BWG is body weight gain (g), CPI is crude protein intake (g), GNfree is weight gain
2051 (loss) of the chicks from the nitrogen free diet (g). The net protein ratio accounts for the
2052 maintenance protein requirement.

2053 **In-vitro protein digestibility**

2054 To determine *in-vitro* protein digestibility, 1 gram of sample was weighed into 50 ml
2055 centrifuge tubes. To this, 15 ml of 0.1N HCL-pepsin (porcine; Merck Millipore 516360-2.5GM)
2056 solution was added to each tube and placed in a shaking water bath for 3 hours at 37°C. After
2057 pepsin incubation, the tubes were removed and 7.5 ml of 0.5N NaOH was added to each to
2058 neutralize the sample and stop pepsin hydrolysis. To this, porcine pancreatin (4 mg; Sigma
2059 Aldrich P1750-100G) was added to 7.5 ml of phosphate buffer (pH 8), followed by 1 ml of

2060 sodium azide (for microbial control). Tubes were then placed in the shaking water bath for 18

2061 additional hours at 37°C.

2062 After 18 hours of incubation, 1 ml of 10% TCA was added to each tube. The samples

2063 were then centrifuged at 20,000×g for 5 minutes. The supernatant was removed, and the samples

2064 were washed with distilled water and centrifuged again. This process was repeated 3 times.

2065 Samples were then filtered using dried filter paper (Whatman 541) and then dried over night at

2066 105°C. The residual sample was then analyzed for nitrogen utilizing the modified Kjeldahl

2067 procedure described by Bremner et al. (9).

2068 Protein digestibility was determined using the following equations:

2069 $$Crude\ protein\ (CP) = N\% * 6.25$$

2070 $$Residue\ CP = residue\ weight * CP$$

2071 $$Protein\ Digestibility = \left(\frac{Sample\ CP\ (g) - Residue\ CP(g)}{Sample\ CP\ (g)}\right) \times 100$$

2072 Protein corrected amino acid scores (PDCAAS) were determined using the following

2073 equations:

2074 $$Limiting\ Amino\ Acid\ (LAA) = \frac{Amino\ Acid\ in\ test\ protein}{Reference\ Protein}$$

2075 $$PDCAAS = (LAA * Digestibility) * 100$$

2076 **Statistical Analysis**

2077 Chick data were analyzed as a completely randomized design using the GLM procedure

2078 of a commercial statistical analysis software (SAS v9.4, SAS Corporation, Carry, N.C.).

2079 Treatment means were separated by significant F with $\alpha = 0.05$. The relationship between protein

2080 efficiency ratio, *in-vitro* protein digestibility, and protein digestibility corrected amino acid score

2081 were analyzed using the Pearson correlation coefficient (SAS v9.4, SAS Corporation, Carry,
2082 N.C.).

2083 **Results**

2084 Moisture content was highest for DCB (18.69%) and CGM (12.14%). All other test ingredients
2085 had moisture levels below 6%. Crude protein content was highest for the HPCP, followed by
2086 CGM, the rendered protein meals (CM, CBPM, PBPM), and SDEG. The lowest crude protein
2087 values were observed with HFCP and DCB (Table 4-1). Fat content was highest for SDEG and
2088 HFCP. The rendered protein meals had fat contents ranging from 12.5-15%. Dehydrated chicken
2089 breast and CGM had the lowest total crude fat, 8.40% and 2.79%, respectively. Fiber was not
2090 detected in SDEG, HFCP, and HPCP. All other test proteins had fiber content below 1.50%.
2091 Poultry by-product meal had the highest amount of ash (25.15), followed by CM and CBPM
2092 (13.2% and 13.5%, respectively).

2093 The essential amino acid profile along with total non-essential amino acids and the level
2094 of hydroxyproline as a percent of total amino acids, are reported in Error! Reference source not
2095 found.. The available lysine content for SDEG was 7.38% (Table 4-2). High fat chicken powder,
2096 HPCP, and DCB all had available lysine values between 8-8.5%. Available lysine for CM was
2097 5.63%, CBPM 5.65%, and PBPM 5.19%. Corn gluten meal had the lowest available lysine level
2098 at 1.73%. Spray dried egg had the highest methionine level at 3.28%., followed by HPCP
2099 (2.73%), HFCP (2.49%), DCB (2.39%), and CGM. Methionine values were below 2% for CM,
2100 CBPM, PBPM. Arginine content for CGM was much lower compared to all other ingredients.
2101 Total non-essential amino acids were higher for HPCP, CM, CBPM, PBPM, and CGM.
2102 Dehydrated chicken breast had the lowest total non-essential amino acids. Hydroxyproline, an
2103 indicator of connective tissue, was greater in the rendered products (CM, CBPM, and PBPM).

Hydroxyproline was not detected in SDEG and was below 0.30% for DCB and CGM. Both of the chicken powders had levels of hydroxyproline between 1-1.65%.

Body weight gain was greatest for HPCP, DCB, and SDEG ($P < 0.05$;Table 4-3). High fat chicken powder was lower than the HPCP but not different from SDEG or DCB. The poorest weight gain was observed in chicks fed CGM as the protein source. Feed intake was reduced when diets contained PBPM or CGM compared to SDEG ($P < 0.05$). Feed intake was highest for the minimally processed protein sources (SDEG, HFCP, HPCP, and DCB) compared to the rendered protein meals (CM, CBPM, PBPM) and CGM ($P < 0.05$). Corn gluten meal had the poorest feed intake ($P < 0.05$).

Spray dried egg had the highest PER at 4.94 g gain/g protein intake. The PER of HPCP and DCB were similar to SDEG (4.71 and 4.44, respectively), while the PER for HFCP was lower (4.26; $P < 0.05$) than SDEG but was not different from the HPCP or DCB ($P < 0.05$). The next distinct group was the CM and CBPM. Chicken meal had a PER of 3.35 and CBPM had a PER of 3.25. Both ingredients had lower ($P < 0.05$) PER compared to SDEG, HPCP, HFCP, and DCB. Poultry by product meal had a PER of 2.55, which was lower ($P < 0.05$) than the other two rendered protein meals. The lowest PER value was observed for chicks fed the diet containing CGM (0.19; $P < 0.05$). Net protein ratio was also calculated to account for protein maintenance requirements and these values were ranked similarly to the PER.

In-vitro digestibility was above 95% for SDEG, HFCP, HPCP, and DCB (Table 4-4). Digestibility of CM was 68%, CBPM was 70%, and PBPM was 74%. The lowest digestibility was observed for CGM at 53%. For all test proteins, tryptophan was the limiting amino acids for dogs (maintenance). Protein corrected amino acid scores were strongly correlated with PER values ($R = 0.80$; $P < 0.05$). Poultry by-product meal and CGM had the lowest PDCAAS and

lowest PER. High protein chicken powder had the highest PDCAAS (23.26). To calculate PDCAAS for cats at maintenance, phenylalanine + tyrosine and taurine were used (Table 4-4). For cats, the PDCAAS were correlated to the PER ($R = 0.79$; $P < 0.05$). Spray dried egg had the highest PDCAAS followed by HPCP (66.77 and 61.73, respectively). Chicken meal and CBPM were similar (44.94 and 45.62, respectively) and PBPM was lower (37.59). The lowest PDCAAS was observed with CGM (19.76).

Discussion

Body weight gain for chicks fed spray dried egg, chicken by-product meal, and poultry by-product meal were similar to those reported by Dust et al. (10). For chicks fed the chicken meal treatment, weight gain was higher than that reported by Donadelli et al. (2). This difference could in part be the result of bird genetics over time, the composition, and processing of the protein meals. In this case it may be due to the lower available lysine in these two test ingredients. Lysine availability for CM in this experiment was 88.8%, whereas, the availability reported by Donadelli et al. (2) was 78%. The reduced lysine availability is a potential indication of greater heat or residence time during processing. Wang and Parsons observed that high processing temperature reduced weight gain of chicks fed experimental diets containing meat and bone meal (11).

In this experiment, corn gluten meal resulted in the lowest body weight gain for chicks and is consistent with previous reports (2, 14). Examining the amino acid profile of corn gluten meal, the lysine content was substantially lower than the other test ingredients. Peter et al. completed a deletion assay to determine the limiting amino acid for corn gluten meal when fed to young chicks and reported that lysine was the most limiting for growth (13). The second and third limiting amino acids observed by Peter et al. (2000) were tryptophan and arginine (13). The

2150 amino acid data for this current research presents much lower proportions of these two amino
2151 acids compared to the other protein sources evaluated in this study. In addition to the low lysine
2152 and arginine levels in corn gluten meal, it is also important to consider the ratio of these two
2153 amino acids to each another. It has been reported that when there is more arginine in the diet
2154 compared to lysine, growth of birds will be depressed (14-15). A possible reason for this
2155 antagonistic relationship between lysine and arginine is the manner in which they are absorbed in
2156 the gastrointestinal tract, where both arginine and lysine are absorbed by the same transporter,
2157 leading to competition for uptake (16).
2158 Protein efficiency ratio is a measure of protein quality that can identify small differences
2159 in amino acid profile and allows for the evaluation of a single ingredient. Spray dried egg is often
2160 used as the ideal protein source when conducting PER assays, thus it typically results in the
2161 greatest value. In the current experiment, PER for spray dried egg was the highest at 4.94. Other
2162 authors have reported that spray dried egg was the highest value in their studies (2, 10). Poultry
2163 by-product meal resulted in a lower PER score, most likely because it contains lower amounts of
2164 cysteine, methionine, and tryptophan compared to spray dried egg. Dust et al. reported a similar
2165 amino acid profile for their poultry by-product source and their resulting PER results (10).
2166 Protein efficiency ratio was lower compared to spray dried egg and the methionine and
2167 tryptophan levels followed. Johnson and Parson also observed a similar PER value for poultry
2168 by-product meal to that of the current experiment (17).
2169 Chicken meal in this experiment had a higher PER value than what was reported by
2170 Donadelli et al. (2). The chicken meal sources in the current experiment had a cysteine level of
2171 1.24 as a percent of total amino acids. The availability of lysine in this ingredient was 88.8%.
2172 The chicken meal sourced in the previous experiment had a slightly lower level of cysteine and

2173 the availability of lysine was 78%. These lower values may explain why the chicken meal in the
2174 current experiment resulted in a higher PER.
2175 In this experiment chicken by-product meal did not differ from chicken meal. This was
2176 not the case in the experiment conducted by Donadelli et al. as the chicken by-product meal had
2177 a higher PER (2). This may be due to higher cystine concentration and lysine availability of the
2178 chicken by-product meal. In the current experiment these differences were not present.
2179 Poultry by-product meal had a lower PER value compared to the chicken meal and
2180 chicken by-product meal. Comparing these two ingredients, the level of cystine and lysine were
2181 lower than the chicken meal or the chicken by-product meal. Dust et al. reported similar results
2182 in which birds fed the poultry by-product meal treatment had lower performance compared to
2183 birds fed an experimental diet with chicken by-product meal (10). However, in the case of these
2184 two ingredients, Dust et al. reported a lower arginine level. The level of cystine was not reported
2185 for thier study (10). Johnson and Parsons also examined poultry by-product meal and reported
2186 PER to be slightly higher at 9 days (17). This specific poultry meal was used in a second study in
2187 which digestibility was determined in cecectomized roosters and illealy cannulated dogs (18).
2188 The amino acid profile reported in the second experiment had a higher level of lysine,
2189 methionine, and cystine which might explain the higher PER obtained in the study.
2190 In addition to an *in-vivo* study (PER), protein quality can be measured via *in-vitro*
2191 laboratory assay. One such method is the *in-vitro* pepsin-pancreatin digestibility assay (19).
2192 Utilizing the pepsin-pancreatin *in-vitro* method, digestibility values were obtained for the test
2193 proteins. Spray dried egg, the control protein, had a digestibility of 99%. Norberg et al. reported
2194 the digestibility of amino acids utilizing ducks as the model with an average of 92.2% among the
2195 indispensable amino acids (3). The dehydrated chicken breast had a digestibility of 95%. Oba et

2196 al. reported an average of 88.4% for indispensable amino acids in raw chicken (20). The
2197 anatomical location of the raw chicken, i.e. breast or leg, was not described. However, the amino
2198 acid profile was reported. The level of lysine on a dry matter basis for the raw chicken was 2.94,
2199 while the dehydrated chicken breast had a level of 3.89. This difference and the unknown
2200 anatomical locations of the chicken source are potential reasons why these differences occurred.
2201 The digestibility of the raw chicken was also determined using a cecectomized rooster and led to
2202 potential differences in enzyme strength between the *in-vivo* and *in-vitro* methods (20).
2203 Rojas and Stein reported that the apparent ileal digestibility of chicken meal when fed to
2204 weanling pigs was 57.5% which is lower than the 68% for the current chicken meal source (21).
2205 Compared to the current chicken meal, there were few differences in the amino acid profile but
2206 lysine was lower at 3.49 vs 3.93. The percent available lysine was also not reported as a lower
2207 available lysine could lead to the reduced digestibility in the chicken meal fed to pigs. A lower
2208 lysine availability could be an indication of damage due to heat during processing. This damage
2209 could lead to a lower protein digestibility as the amino acids are no longer available for digestion
2210 by enzymes. Poultry by-product meal was also fed to weanling pigs in the same study and
2211 resulted in a digestibility of 62.9% where the poultry by-product meal utilized for the *in-vitro*
2212 assay resulted in a digestibility of 74%. All of the amino acids for the both poultry by-product
2213 meal sources are fairly similar to one another. However, available lysine was not reported (21).
2214 The lysine availability could indicate whether the protein source was damaged by heat during
2215 production. There are also biological differences when comparing an *in-vivo* model to an *in-vitro*
2216 model. The *in-vivo* model evaluating foods often contains a variety of different ingredients to
2217 meet the animals nutrient requirements which could interfere with digestibility. The *in-vitro*
2218 model used in the current study evaluated the protein source alone to determine digestibility.

2219 The cecectomized rooster is often reported as a model to determine the digestibility of an
2220 ingredient (12, 18, 21-22). In this assay the ceca is removed to eliminate protein from
2221 fermentation by microbes in the excreta. This allows for small intestinal digestibility to be
2222 determined. Corn gluten meal resulted in our lowest *in-vitro* protein digestibility (53%). This
2223 was much lower compared to the *in-vivo* (cecectomized rooster assay) of Kim et al. and de
2224 Godoy et al. who reported digestibility for indispensable amino acids of 93% and 94%,
2225 respectively (12, 24). This difference is quite large between the *in-vivo* and the *in-vitro* study. A
2226 comparison of protein disappearance with an incubation time of 6 vs 24 hours in an *in-vitro*
2227 model demonstrated that at 6 hours of incubation crude protein disappearance was 49.3% (12).
2228 Thus, nearly half of the protein was digested. When the sample was incubated for 24 hours, the
2229 disappearance increased to 94.1% which compares favorably with the cecectomized rooster
2230 results. However, for the *in-vitro* protein digestibility assay technique 6 hours of HCl-pepsin
2231 incubation is very common (12, 25). There are also several reports for 3 hours incubation (26-
2232 28). Preliminary work conducted in our lab resulted in no difference between 3 and 6 hours.
2233 Thus, 3 hour HCl-pepsin digestion was used for this study. This lower digestibility also agrees
2234 better with the values that were obtained for the current PER, in which both the PER and
2235 digestibility estimates were lower for the corn gluten meal source.

2236 The protein digestibility obtained from the *in-vitro* assay was used to calculate a
2237 PDCAAS. Protein digestibility corrected amino acid score would typically use a digestibility
2238 score obtained from rats (29). However, there are reports in which the an *in-vitro* digestibility
2239 estimate was used with good results (12). This PDCAAS method considers the limiting amino
2240 acid of each ingredient and provides a numerical value that can be used to rank or score proteins
2241 on their quality. The goal of this research was to calculate these scores and determine if they

2242 were correlated to the chick PER. For these calculations, the recommended allowance for both
2243 dog and cat at maintenance were used (8). The recommended allowance was chosen instead of
2244 the minimum in part due to the lack of information for amino acid requirements in the current
2245 NRC for cat maintenance at the minimum levels. In addition, when formulating a complete diet,
2246 a formulator will opt to use the recommended level instead of the minimum requirement.

2247 The limiting amino acid for PDCAAS using dog maintenance as the reference
2248 was tryptophan while the limiting for the cats was phenylalanine + tyrosine. The PDCAAS was
2249 calculated for each essential amino acid in order to determine these limiting amino acids. In
2250 addition to the limiting amino acids, lysine, available lysine, and methionine + cystine were also
2251 considered. However, the lowest amino acids scores for dogs and cats observed were tryptophan,
2252 and phenylalanine + cystine, and taurine, respectively. Using available lysine as a measure
2253 allows for consideration of any protein damage from heat during ingredient manufacturing.
2254 These limiting amino acids were used to calculate the PDCAAS (Table 4-4). In the current work
2255 the calculated PDCAAS were compared to the PER values obtained from the growth assay. The
2256 PDCAAS for cats had a correlation of $R = 0.79$ ($P < 0.05$) and the dogs was $R = 0.80$ ($P < 0.05$)
2257 relative to the PER. The goal of this study was to determine if the two methods would rank the
2258 proteins in a similar manner. Sarwar reported both PER and PDCAAS values for different
2259 protein sources fed to rats but did not report a correlation value between the two (30). Using their
2260 data, a Pearson correlation was calculated to be $R = 0.71$. This is lower than the values observed
2261 in the current study, but still sufficiently high enough to suggest some agreement. Sarwar also
2262 stated that there was a potential drawback to PDCAAS since it can overestimate the protein
2263 quality of an ingredient (30). Sarwar suggested that PDCAAS determined total digestibility and
2264 does not take into account differences in bioavailability of specific amino acids (30). The prime

example from their study was mustard flour with a PER score of 0 versus a PDCAAS score of 84. However, even if there was an overestimation, it was possible protein sources would have been ranked in a similar manner to an animal PER study if a test for correlation had been conducted. The outcome from our work could potentially reduce the need to use animals for routine assessment of protein quality and reduce the time necessary for evaluating an ingredient. This might provide an opportunity for ingredient testing in a production setting and allow for adjustments to address shortcomings in a timelier fashion.

Conclusions

Overall, utilizing the chick protein efficiency ratio assay, we were able to rank the protein sources based on their protein quality of the ingredients. The gently processed proteins (high fat chicken powder, high protein chicken powder, and dehydrated chicken breast) had significantly greater protein quality than the rendered protein meals. This is reflected in their higher PER and digestibility results. *In-vitro* protein digestibility was correlated to the PER scores ($R = 0.91$; $P < 0.05$). The PDCAAS ranked samples in a similar manner to PER ($R = 0.80$ and $R = 0.95$ for dog and cat; $P < 0.05$). From this data, an *in-vitro* digestibility and computed PDCAAS value for ingredients may be an option to assess protein quality in a more ethical and timely basis.

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2369 overestimates quality of proteins containing antinutritional factors and of poorly
2370 digestible protein supplemented with limiting amino acids in rats.

2371

2372

Tables

2373 **Table 4-1. Proximate analysis of experimental protein sources.**

	Percentage[1]				
Ingredient	Moisture	CP	Fat	Fiber	Ash
Spray dried egg	5.90	57.6	35.6	n.d.[2]	4.46
High fat chicken powder	3.18	50.0	33.7	n.d.[2]	5.00
High protein chicken powder	4.27	81.1	17.9	n.d.[2]	3.67
Chicken breast dehydrated	18.69	49.8	8.40	1.00	6.86
Chicken meal	4.95	69.2	14.7	0.21	13.2
Chicken by-product meal	5.41	69.4	12.4	0.32	13.5
Poultry by-product meal	3.46	61.0	12.6	0.26	25.1
Corn gluten meal	12.14	75.0	2.79	1.48	2.14

2374 [1]Dry matter basis
2375 [2]Not detected = n.d.
2376

Table 4-2. Essential amino acid (AA) composition of protein sources, available lysine, sum of total non-essential amino acids, available lysine, sum of total non-essential amino acid, and hydroxyproline, expressed as a percentage of total amino acids.

	Percentage[1]														
Ingredient	Arg	Cys	His	Ile	Leu	Lys	Avail Lys.	Met	Phe	Thr	Trp	Tyr	Val	Total NE[2]	HyPro[3]
Spray dried egg	6.10	2.40	2.48	5.41	8.47	7.56	7.38	3.28	5.48	4.57	1.71	4.04	6.90	22.84	0.00
High fat chicken powder	6.71	1.15	3.15	4.62	7.58	8.48	8.31	2.49	4.13	4.35	1.36	4.22	5.03	21.94	1.64
High protein chicken powder	6.96	1.17	2.87	4.98	8.12	8.86	8.51	2.73	4.41	4.54	1.50	3.94	5.36	34.14	1.02
Chicken breast dehydrated	6.57	1.12	3.56	5.12	8.18	8.23	8.00	2.39	4.18	4.41	1.48	5.17	5.43	17.01	0.23
Chicken meal	7.07	1.24	2.06	4.07	7.05	6.34	5.63	1.94	4.08	3.87	1.08	3.57	4.97	32.65	3.47
Chicken by-product meal	7.19	1.15	2.09	4.02	6.98	6.41	5.65	1.98	4.06	3.81	1.07	3.54	4.84	32.58	3.67
Poultry by-product meal	7.31	0.92	1.88	3.46	6.15	5.85	5.19	1.77	3.63	3.44	0.83	2.95	4.20	33.15	5.30
Corn gluten meal	3.20	1.73	1.86	4.01	15.27	1.80	1.73	2.22	5.99	3.21	0.69	5.00	4.37	35.15	0.07

[1]As is basis.

[2]Total non-essential (NE) = ala, asp, glu, gly, hylys, hypro, lan, orn, pro, ser.

[3]Hypro = hydroxyproline

Table 4-3. Weight gain (BWG), feed intake, crude protein intake (CP), protein efficiency ratio (PER), net protein ratio (NPR), and PER and NPR as a proportion of egg for various protein sources fed to week-old chicks for 10 days.[1]

Treatment	BWG (g)	Feed Intake (g)	CP intake (g/bird)	PER[2]	NPR[3]	PER % SDEG	NPR % SDEG
Spray dried egg	123.29[ba]	249.54[ba]	24.95[ba]	4.94[a]	5.81[a]	100.00[a]	100.00[a]
High fat chicken powder	101.91[bc]	232.95[bac]	23.29[bac]	4.26[b]	5.27[b]	86.40[b]	90.68[b]
High protein chicken powder	127.95[a]	271.75[a]	27.17[a]	4.71[ba]	5.50[ba]	95.30[ba]	94.74[ba]
Chicken breast dehydrated	121.12[ba]	270.00[a]	27.00[a]	4.44[ba]	5.26[b]	89.94[ba]	90.55[b]
Chicken meal	89.91[dc]	267.95[a]	26.79[a]	3.35[c]	4.16[c]	67.97[c]	71.71[c]
Chicken by-product meal	73.04[de]	225.20[bc]	22.52[bc]	3.25[c]	4.22[c]	65.94[c]	72.71[c]
Poultry by-product meal	52.12[e]	204.00[c]	20.40[c]	2.55[d]	3.61[d]	51.65[d]	62.21[d]
Corn gluten meal	3.16[f]	121.91[d]	12.19[d]	0.19[e]	2.00[e]	3.89[e]	34.40[e]
N-free basal diet	-21.63[g]	107.96[d]	-	-	-	-	-

[1]means in a column with unlike superscript differ ($p < 0.05$)

[2] bird gain (g) per protein intake (g)

[3] bird gain (g) – (treatment 1 loss, g) per unit protein intake (g)

Table 4-4. Pepsin-pancreatin in-vitro digestibility, limiting amino acid (LAA), amino acid score (AAS), and protein digestibility corrected amino acid score (PDCAAS) of select protein sources intended for pet food.

		Dog			Cat		
Ingredient	Digestibility, %	LAA	AAS[1]	PDCAAS	LAA	AAS[1]	PDCAAS[2]
Spray dried egg	99	Trp	0.19	18.73	Phe + Tyr	0.67	66.77
High fat chicken powder	99	Trp	0.13	13.30	Phe + Tyr	0.53	52.30
High protein chicken powder	97	Trp	0.24	23.26	Phe + Tyr	0.85	82.92
Chicken breast dehydrated	95	Trp	0.12	11.67	Phe + Tyr	0.50	47.22
Chicken meal	68	Trp	0.15	9.91	Phe + Tyr	0.66	44.94
Chicken by-product meal	70	Trp	0.14	10.03	Phe + Tyr	0.65	45.62
Poultry by-product meal	74	Trp	0.10	7.70	Phe + Tyr	0.51	37.59
Corn gluten meal	53	Trp	0.09	4.95	Arg	0.55	29.19
Pearson correlation (vs PER)[3]	0.91			0.80			0.79
Probability	< 0.05			< 0.05			< 0.05

[1]Amino Acid Score (AAS) = amino acid content of test protein/ref AA. Reference AA dog or cat maintenance recommended values (NRC, 2006).

[2] Protein corrected amino acid score (PDCAAS) = (AAS*protein digestibility)*100.

3 PER values reported in Table 3.

Chapter 5 - An evaluation of amino acid profile and protein quality of various legumes for use in companion animal diets

Abstract

In recent years, there has been steady interest regarding the use of new and/or novel protein sources in pet foods. This has led to the incorporation of many different types of protein sources being used including legumes and lentils. Because these are new ingredients in pet food, there is little information on their nutritional benefits in scientific literature. The objective of this experiment was to determine the protein quality of various legumes utilizing the chick protein efficiency ratio (PER) assay. Two 10-day chick growth assays were conducted with experimental diets containing 10% crude protein from a single protein source. A diet containing no protein was used as a negative control and spray dried egg was considered the positive control. Weight gain and feed intake were recorded for the calculation of PER. In experiment 1, spray dried egg resulted in the highest PER (5.18; $P < 0.05$). Ground chickpea PER was lower (3.18) than spray dried egg but not different from sunflower meal ($P < 0.05$). All other treatments had PER values below 1.6 and were lower ($P<0.05$) than spray dried egg, spray dried whole egg, sunflower meal, and ground chickpea. In experiment 2, pea protein concentrate and pea protein isolate were fed as sole protein sources and in combination with spray dried egg to a predicted level of MET to meet the chicks requirements. Birds fed pea protein concentrate and pea protein isolate had the lowest ($P<0.05$) PER values (1.69 and 1.62, respectively). Protein efficiency ratio was greatest for SDEG and was similar to 40:60 SDEG:pea protein concentrate (5.35 and 4.85, respectively; $P < 0.05$). The other combination treatments had improved PER values relative to pea protein concentrate or pea protein isolate on their own ($P < 0.05$). However, all treatments, except 40:60 SDEG:pea protein concentrate, were lower than spray dried egg ($P < 0.05$). Legumes in both

24 studies underperformed compared to spray dried egg. However, combining legumes with an

25 ingredient that had higher levels of methionine and cysteine increased the performance of birds

26 fed those treatments.

27

28 ## Introduction

29 Approximately two thirds of U.S. homes own a pet, resulting in a pet food industry worth
30 more than $30 billion (APPA, 2016; Packaged Facts, 2016). Humanization has been a driving
31 factor behind today's market trends, which is reflected in pet owners purchasing decisions.
32 Today's owners are seeking pet foods similar to their own dinner plates and are more likely to
33 shop for and purchase foods with higher meat quantity and quality (Okin, 2017). Although
34 companion animal consumption of animal proteins only accounts for a small percentage of
35 global protein use some of these sources are also potential food for humans. This results in
36 increased competition and, consequently, less animal proteins available to pet food companies
37 without a major increase in cost (Fiacco et al., 2018). This has led to selection away from more
38 traditional ingredients, such as meat or plant by-products. These are not utilized in the human
39 food stream, and therefore many quality nutritional co-products are being diverted to waste
40 (Swanson et al., 2013).

41 In order to increase sustainability, it may be necessary to shift towards plant-based or
42 other novel proteins that can readily expand the ingredient base for both pet and human food
43 systems. These sources could potentially include crops like beans, lentils, peas, byproducts like
44 sunflower meal, and alternative insect proteins such as fly larvae. Due to the newness of these
45 sources to the pet marketplace, there is a need to evaluate their protein quality to understand their
46 value as potential replacements for traditional ingredients like rendered protein meals. There are
47 several methods that can be deployed to evaluate these sorts of ingredients. They include direct
48 nutrient analysis, measures of digestibility studies, and growth assays like the protein efficiency
49 ratio (PER). The PER assay is often utilized to evaluate protein sources for pet foods because it
50 can quickly rank the protein with a fast-growing genetically uniform animal (chicks or rats) and

51 provide repeatable results. These assays are more rapid than what can obtained with pets due to
52 their longer time to maturity. Protein efficiency ratio assay also allows for identification of
53 shortcomings in amino acid profile as protein intake is limited. The objective of this experiment
54 was to determine the protein quality of legumes fed to week old broiler chicks for 10 days.

55 ## Materials and Methods

56 ### Ingredients

57 Test ingredients included spray dried egg (SDEG), ground faba bean, ground chickpea,
58 ground pinto bean, ground red lentil, ground green pea, ground navy bean, pea protein isolate,
59 and pea protein concentrate. Each test ingredient was supplied by the study sponsor (IsoNova,
60 Springfield, MO). The vitamin premix and mineral premix (Harlan Teklad, Madison, WI) were
61 obtained immediately prior to the production of experimental chick diets. Individual ingredients
62 were analyzed for moisture, crude protein, crude fat, crude fiber, and ash (Table 5-3 and Table
63 5-4; AOAC 930.15, AOAC 990.03, AOAC 945.16, AOCS Ba 6a-05, and AOAC 942.05,
64 respectively). Essential and non-essential amino acid profile as well as available lysine were also
65 determined for each protein source (Table 5-5; Table 5-6; Table 5-7; Table 5-8; AOAC 982.30E
66 (a,b,c), 2006; AOAC 975.44).

67 ### Diets for Experiment 1 and 2

68 The N-free diet was used as a negative control in a similar manner to Donadelli et al.
69 (2019). Test proteins were added to the N-free diet in replacement of corn starch and dextrose in
70 equal proportions until 10% crude protein was achieved (Cramer et al. 2007). Spray dried egg was
71 used as a positive control. To meet the chicks daily requirements soybean oil (source of essential
72 fatty acids), minerals, vitamins, and choline chloride were added to each diet (NRC, 1994).

73 **Chick Protein Efficiency Ratio Assay**

74 One day old male broilers (Cobb*Cobb) were obtained from Cobb Vantress (Siloam
75 Springs, AR) and placed on a starter diet, containing 23% CP, for six days. On day seven, chicks
76 were fasted for eight hours then weighed individually and sorted by weight. Chicks were
77 assigned to pen by weight to achieve similar starting weights across treatments. Experimental
78 treatments were assigned to pen and battery in a completely randomized blocked design. Both
79 water and food were provided *ad libitum* for the length of the experiment. After 10 days of food
80 consumption, birds were fasted for eight hours before obtaining final pen weight. To determine
81 feed efficiency and crude protein intake, feed consumption was recorded. Weight gain and crude
82 protein intake were used for the calculation of protein efficiency ratio (PER) and net protein ratio
83 (NPR). Both were calculated as follows:

84 $$PER = \frac{BWG}{CPI}$$

85 $$NPR = \frac{(BWG - GNfree)}{CPI}$$

86 where BWG is body weight gain (g), CPI is crude protein intake (g), GNfree is weight gain (or
87 loss) of the chicks from the nitrogen free diet (g).

88 **Statistical Analysis**

89 Chick data were analyzed as a completely randomized design using the GLM procedure
90 of a commercial statistical analysis software (SAS v9.4, SAS Corporation, Carry, N.C.). The data
91 was split into two experiments, 1) legumes and beans and 2) pea protein concentrate and pea
92 protein isolate and combinations of SDEG with either PPC or PPI. Treatment means were
93 separated by significant F with $\alpha = 0.05$. The relationship between protein efficiency ratio and

94 protein digestibility corrected amino acid score were analyzed using the Pearson correlation
95 coefficient (SAS v9.4, SAS Corporation, Carry, N.C.).

96 ## Results

97 ### Experiment 1

98 This experiment contained spray dried egg, ground chickpea, ground faba bean, ground
99 green pea, ground navy bean, ground pinto bean, and ground red lentil. Ground pinto bean had
100 the largest amount of moisture of all test proteins (Table 5-3). Spray dried egg had the highest
101 protein content (53.3%) of all protein sources. Ground faba bean had the highest protein content
102 of legumes tested (33.5%) and ground red lentil the second highest at 29.6%. Ground chickpea
103 contained the most fat (5.99%) of legumes tested, while spray dried egg had the highest fat
104 content of all samples (39.2%).

105 Lysine content was highest for ground green pea, followed by spray dried egg when
106 expressed as a percent of total protein (Table 5-9). The lysine level was lowest for ground faba
107 bean (6.86%) but was not substantially different than the other sources. Methionine content was
108 highest for spray dried egg (3.27%). Ground chickpea methionine at 1.52% was the highest
109 among the legume sources. The lowest methionine content was were ground faba beans at
110 0.76%. Cysteine content of the legumes were also lower compared to spray dried egg. Spray
111 dried egg contained 2.4% cysteine while ground green pea and ground pinto bean only contained
112 1.12%. Arginine content in legumes were 2-4% units higher than spray dried egg, except for
113 ground navy beans and ground pinto beans which were comparable to spray dried egg. Essential
114 to non-essential amino acid ratios for all test sources were near or above 1 (Table 5-13). Lysine
115 availability for all ingredients exceeded 90% (Table 5-13).

116 Feed intake, weight gain, and feed efficiency were greatest for chicks fed spray dried egg
117 ($P < 0.05$). Of the legume sources, ground chickpea fed birds had the highest ($P < 0.05$; 92.83 g)
118 weight gain and feed intake ($P < 0.05$). The lowest ($P < 0.05$) feed intake was observed for
119 ground red lentil, ground faba bean, ground green pea, and ground pinto bean. Lowest weight
120 gain was observed in birds that consumed ground pinto bean or the diet containing no protein.
121 Birds fed spray dried egg treatment had the highest PER ($P < 0.05$; 5.18) of all treatments.
122 Ground chickpea had a PER of 3.18 and was the highest ($P < 0.05$) among legume sources.
123 Ground green pea, ground navy bean, ground faba bean, and ground red lentil has similar PER
124 values. Ground pinto bean had the lowest PER (-0.35) and was similar to ground faba bean and
125 ground red lentil. Net protein ratio was highest for spray dried egg.

126 **Experiment 2**

127 Experiment two contained spray dried egg (SDEG), pea protein concentrate (PPC), pea
128 protein isolate (PPI), and combinations of spray dried egg with either PPC or PPI. Pea protein
129 isolate had the highest crude protein content at 79.8% (Table 5-4. Exp. 2 Ingredient composition
130 of evaluated test proteins.[1]Table 5-4; dry matter basis). The combinations of PPI and SDEG had
131 higher crude protein than the PPC (55.5%), SDEG (53.3%), and the combinations of PPC and
132 SDEG. Fat content was highest for SDEG (39.2%) and PPC had the highest moisture level
133 (10.06%).

134 Lysine content of all treatments were similar to that of the control (SDEG). Methionine
135 content was highest for SDEG (3.27%; Table 5-9Table 5-10). The combination of SDEG: PPC
136 (40:60) and SDEG:PPI (40:60) had the highest methionine levels but were still under 2%. Spray
137 dried egg contained the highest amount of cysteine (2.40%). The 40:60 combinations of SDEG
138 with PPC and PPI had the highest level of cysteine of test proteins but were not higher than

139 1.8%. Arginine content for PPC and PPI were approximately 2-3% higher than in SDEG and the
140 combinations were at least 1% unit greater. Lysine availability for all test proteins was above
141 95% (Table 5-14). The ratio of essential to non-essential amino acids was above 1.2 for all
142 sources.

143 Growth performance and PER results are presented in Table 5-16. Spray dried egg had
144 the highest feed intake (358.92 g) and was similar to the intake of SDEG:PPC (40:60; 350.46 g;
145 $P < 0.05$). Pea protein concentrate and PPI had the lowest feed intake (194.17 and 210.75,
146 respectively) while other combinations provided intermediate levels ($P < 0.05$). Weight gain was
147 greatest in birds fed SDEG and 40:60 SDEG:PPC (197.33 g and 171.36 g, respectively; $P <$
148 0.05). Spray dried egg and PPI (40:60) was similar to that of SDEG:PPC 40:60 but was lower
149 than spray dried egg (158.54 g; $P < 0.05$). Birds fed PPC and PPI had the lowest weight gain
150 through the study (33.25 g and 33.50 g, respectively; $P < 0.05$). Spray dried egg and SDEG:PPC
151 (40:60) had the greatest feed efficiency among treatments ($P < 0.05$). Protein efficiency ratio was
152 highest for SDEG and was similar to 40:60 SDEG:PPC ($P < 0.05$). Pea protein concentrate and
153 PPI had the lowest PER, 1.69 and 1.62 respectively ($P < 0.05$). Net protein ratio had similar
154 ranking of protein sources to that of PER.

155 Discussion

156 Spray dried egg, which served as the positive control, resulted in the highest PER value.
157 Spray dried egg is used as a control as it may be considered an ideal protein source due to its
158 balanced amino acid profile. The PER results were similar to previous research in which feeding
159 spray dried egg resulted in the highest PER (Donadelli et al., 2019; Smith, 2018; Johnson and
160 Parsons, 1997). Similar protein content and amino acid content of spray dried egg has also been
161 reported (Donadelli et al., 2019; Dust et al., 2005). Spray dried egg has been evaluated as a

162 protein source in weaned pigs with improvements in growth characteristics with 5% inclusion
163 Song et al., 2012). Andrade et al. (2019) fed beagles varying amounts of spray dried egg,
164 replacing poultry meal offal, and reported a linear improvement in crude protein digestibility.
165 In a series of experiments the amino acid profile and PER of various legumes were
166 examined (Nosworthy et al, 2018; Nosworthy et al., 2018; Nosworthy et al, 2017; Nosworthy et
167 al., 2017; Prandini et al., 2011). In these experiments, casein was used as the control and PER
168 values were adjusted to a casein PER of 2.5. One common finding in these reports were the
169 lower levels of methionine and cysteine in legume sources compared to the controls. Arginine
170 content was also reported to be 0.2-0.5% higher than the lysine content. In previous work, an
171 antagonistic effect between arginine and lysine has been reported (D'Mello and Lewis 1970;
172 O'Dell and Savage, 1966). These two amino acids are absorbed through the same transporter,
173 which leads to competition for nutrient uptake (Gropper and Smith, 2018).
174 Similar to the current study, Nosworthy et al., (2017) reported chickpea had the highest
175 PER among legumes that were tested. Ground navy beans also resulted in a similar PER value
176 compared to the current study (Nowworthy et al, 2017). Differences in the current work and
177 Nosworthy et al. (2017) occurred for red lentil, and green pea, and pinto bean. In their study red
178 lentil and pinto bean had higher PER values and green pea had a lower PER values. Pinto beans,
179 red lentils and green peas PER values have also been reported to be higher than what we
180 observed (Nosworthy et al, 2018; Nosworthy et al., 2018; Nosworthy et al, 2017).
181 The differences between the experiments may be a function of the legume samples being
182 fed in raw form; whereby, no heat treatment had been applied. The sources examined by
183 Nosworthy et al. (2017) were cooked in boiling water before evaluation. The effects of cooking
184 legumes have been reported to impact PER and animal growth positively (Nosworthy et al.,

185 2018; Erdaw et al., 2017; Goodband et al., 1987). Nosworthy et al. (2018) reported increased
186 PER as legume sources were extruded or cooked vs those that were baked. Erdaw et al (2017)
187 observed reduced CP and indispensable amino acid digestibility when raw soybean meal was
188 included in chick diets at 20%. Reduced feed efficiency has also been reported in pigs fed raw
189 soybeans (Goodband et al., 1987).

190 Raw legumes contain antinutritional factors, such as trypsin inhibitors, that can be
191 inactivated with heat (Vagadia et al., 2017; Krogdahl et al., 2010). Trypsin inhibitor activity
192 among legumes and can range from 94.1 U/mg in soybeans to 2.20 in peas U/mg (Yalcin and
193 Basman, 2015 ;Habiba, 2002). These inhibitors reduce enzyme activity in the gastrointestinal
194 tract and can reduce protein digestion (Choi, et al., 2019). Inactivation of trypsin inhibitors
195 allows trypsin to activate enzymes, such as chymotrypsin and carboxypeptidase that are
196 responsible for breakdown of proteins into smaller peptides and free amino acids (Gropper and
197 Smith, 2018). The use of cooking prior to feeding legumes may help explain why previous PER
198 values were higher than those in the current research. In addition to cooking, PER values were
199 compared to a casein PER value of 2.5 (Nosworthy et al, 2018; Nosworthy et al., 2017;
200 Nosworthy et al., 2018). If the current PER values were referenced to a SDEG PER value of 2.5,
201 then the raw legumes in the current work would be much lower than those reported in previous
202 work.

203 As the legumes were much lower in methionine and cysteine compared to the control, the
204 second experiment demonstrated that mixing a legume source with a source containing an
205 adequate amount of methionine and cysteine can help alleviate shortcomings in amino acid
206 profiles. The combination of pea protein concentrate and pea protein isolate improved the PER
207 value of chicks fed those combinations. However, the combination did not increase the values to

208 where they were similar to that of SDEG with the exception of the 40:60 SDEG:PPC treatment.
209 A classic example of improving growth by balancing amino acid profile, is the combination of
210 corn and soybean meal. This combination is heavily used in the swine and poultry industry as
211 corn has higher methionine levels than soybean meal. Corn has lower levels of lysine, but
212 addition of soybean meal provides a compensating level. Using legume sources in combination
213 with a protein containing higher amounts of methionine and cysteine may be necessary to
214 overcome deficiencies in legume amino acid profile.

215 Conclusion

216 Overall, the PER data was able to identify differences in growth that can be attributed to
217 the amino acid profile of the ingredients. As these sources are raw, evaluation of trypsin inhibitor
218 level may help further explain why chicks fed legumes had poor PER in addition to the reduced
219 level of sulfur amino acids. Cooking these sources in future work might provide a better
220 indication of their benefit in pet food which is a heat processed product. The use of *in-vivo* and
221 *in-vitro* digestibility studies of raw legumes might also highlight the importance of inactivating
222 trypsin inhibitors. *In-vitro* methods use preactivated enzymes, removing any impact the trypsin
223 inhibitors may have on nutrient digestion. This work helps demonstrate that these legume
224 sources will benefit from the addition of a complementary protein source to help balance the
225 amino acid profile. Future work with these legume sources in an *in-vitro* system may also
226 provide information on protein quality of legume sources. Methods that could be used are
227 pepsin-pancreatin protein digestibly, protein digestibility amino acid scores or protein
228 digestibility indispensable amino acid score (Schaafsma, 2012; De Godoy et al., 2009;
229 Nosworthy et al, 2018; Nosworthy et al., 2018; Nosworthy et al, 2017; Nosworthy et al., 2017).

230 These methods would allow for determination of protein quality on a faster and more costly basis

231 while reducing the use of animals.

232

233

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313 # Tables

314 **Table 5-1. Composition of starter diet for experiments 1 and 2.**

Ingredient	Percentage (as fed)
Corn	55.250
Soybean meal	37.150
Limestone	1.450
Monocalcium phosphate	1.700
Salt	0.370
Methionine	0.325
Lysine	0.132
Threonine	0.044
Sodium bicarbonate	0.220
NB 3000	0.250
Soybean oil	3.100

315

316 **Table 5-2. Composition of N-Free diet for experiments 1 and 2.**

Ingredient	Percentage (as fed)
Corn Starch[1]	59.567
Dextrose[1]	29.727
Mineral Pre-mix[2]	5.365
Soybean Oil	5.365
Choline chloride	0.220
Vitamin Pre-mix[3]	0.203

317 [1] Protein sources added in experimental diets replaced a portion
318 of 2:1 cornstarch to dextrose mix.
319 [2]Percentage of the diet: Ca3(PO4)2, 2.8; CaCO3, 0.3; CoSO47H2O, 0.0001; CuSO45H2O, 0.002;
320 ferric citrate, 0.0415; H3BO4, 0.009; K2HPO4, 0.9; KI, 0.004; MgSO47H2O, 0.35; MnSO4H2O,
321 0.065; Na2-MoO42H2O, 0.0009; Na2SeO3, 0.00002; NaCl, 0.88; and ZnCO3, 0.01; total, 5.365.
322 [3]Supplied the following per kilogram of complete diet: vitamin A, 5,200 IU; vitamin D, 1,080 IU;
323 vitamin E, 30 mg; vitamin B12, 0.04 mg; riboflavin, 10.0 mg; niacin, 50.0 mg; pantothenic acid,
324 27.6 mg; vitamin K, 2.0 mg; folic acid, 4.0 mg; vitamin B6, 5.0 mg; thiamin, 17.8 mg; and biotin,
325 0.6 mg. [3]88.2 g/kg of Tylosin, Elanco Animal Health, Indianapolis, IN

326

327

328 **Table 5-3. Exp. 1 Ingredient composition of evaluated test proteins .[1]**

Ingredient	Moisture	Crude Protein	Ash	Fiber	Fat
Spray Dried Egg	5.20	53.30	4.58	0.83	39.2
Ground Chickpea	8.51	24.50	3.14	3.91	5.99
Ground Faba Bean	8.98	33.50	3.70	0.84	1.23
Ground Green Pea	8.45	25.20	2.37	1.36	1.14
Ground Navy Bean	6.98	23.30	3.20	5.01	2.38
Ground Pinto Bean	10.18	25.30	4.14	2.40	1.42
Ground Red Lentil	8.81	29.60	3.04	0.38	0.78

329 [1] Dry matter basis.
330 .
331
332 **Table 5-4. Exp. 2 Ingredient composition of evaluated test proteins.[1]**

Ingredient	Moisture	Crude Protein	Ash	Fiber	Fat
Spray dried egg	5.20	53.30	4.58	0.83	39.2
Pea protein concentrate (50% CP; PPC)	10.06	55.5	5.78	1.00	2.78
Pea protein isolate (72% CP; PPI)	6.74	79.8	4.8	1.78	3.41
20:80 SDEG:PPC[2]	9.088	55.06	5.54	0.966	10.064
40:60 SDEG:PPC[2]	8.116	54.62	5.3	0.932	17.348
20:80 SDEG:PPI[2]	6.432	74.5	4.756	1.59	10.568
40:60 SDEG:PPI[2]	6.124	69.2	4.712	1.4	17.726

333 [1] Dry matter basis.
334 [2]Calculated proximate components.

Table 5-5. Exp 1. Essential amino acid profile of test proteins fed to 7-day old chicks.

	Percentage[1]											
Ingredient	Arg	Cys	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Tyr	Val
Spray Dried Egg	2.99	1.18	1.20	2.75	4.25	3.81	1.61	2.91	2.29	0.77	1.96	3.41
Ground Chickpea	1.84	0.33	0.54	0.97	1.58	1.52	0.31	1.25	0.77	0.20	0.58	1.02
Ground Faba Bean	3.02	0.42	0.79	1.35	2.28	1.98	0.22	1.34	1.05	0.27	1.00	1.42
Ground Green Pea	1.88	0.30	0.54	1.02	1.66	1.73	0.20	1.15	0.81	0.20	0.69	1.10
Ground Navy Bean	1.19	0.26	0.63	1.07	1.80	1.55	0.27	1.30	1.00	0.26	0.69	1.30
Ground Pinto Bean	1.31	0.24	0.64	1.10	1.83	1.60	0.27	1.34	0.94	0.19	0.73	1.31
Ground Red Lentil	2.05	0.29	0.65	1.20	1.95	1.87	0.21	1.37	0.93	0.22	0.80	1.34

[1] Dry matter basis.

Table 5-6. Exp 2. Essential amino acid profile of test proteins fed to 7-day old chicks.

	Percentage[1]											
Ingredient	Arg	Cys	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Tyr	Val
Spray Dried Egg	2.99	1.18	1.20	2.75	4.25	3.81	1.61	2.91	2.29	0.77	1.96	3.41
Pea Protein Isolate (72%; PPI)	5.57	0.76	1.66	3.65	6.03	5.34	0.76	4.11	2.66	0.59	1.95	3.86
Pea Protein Concentrate (50%; PPC)	4.31	0.66	1.27	2.32	3.78	4.00	0.49	2.59	1.95	0.47	1.75	2.50
20:80 SDEG:PPC[2]	5.05	0.84	1.57	3.47	5.67	5.03	0.93	3.87	2.59	0.63	1.95	3.77
40:60 SDEG:PPC[2]	4.54	0.93	1.48	3.29	5.32	4.73	1.10	3.63	2.51	0.66	1.95	3.68
20:80 SDEG:PPI[2]	4.05	0.76	1.26	2.41	3.87	3.96	0.71	2.65	2.02	0.53	1.79	2.68
40:60 SDEG:PPI[2]	3.78	0.87	1.24	2.49	3.97	3.92	0.94	2.72	2.09	0.59	1.83	2.86

[1]Dry matter basis

[2]Calculated amino acid concentration.

Table 5-7. Exp. 1 Non-essential amino acid profile of test proteins fed to 7-day old chicks.

	Percentage[1]										
Ingredient	Ala	Asp	Glu	Gly	HyLys	HyPro	Lan	Orn	Pro	Ser	Tau
Spray Dried Egg	2.82	4.86	5.73	1.67	0.03	0.00	0.00	0.06	1.81	3.08	0.02
Ground Chickpea	0.91	2.39	3.26	0.87	0.03	0.00	0.00	0.02	0.87	0.83	0.27
Ground Faba Bean	1.25	3.32	5.01	1.24	0.04	0.00	0.00	0.03	1.33	1.22	0.29
Ground Green Pea	0.96	2.54	3.79	0.96	0.03	1.00	0.00	0.07	0.92	0.87	0.30
Ground Navy Bean	0.96	2.63	3.08	0.90	0.04	0.00	0.00	0.03	0.84	1.10	0.28
Ground Pinto Bean	0.94	2.63	3.38	0.89	0.03	0.01	0.00	0.02	0.81	1.00	0.26
Ground Red Lentil	1.11	2.97	4.19	1.06	0.04	0.00	0.00	0.10	1.04	0.98	0.29

[1] Dry matter basis.

Table 5-8. Exp. 2 Non-essential amino acid profile of test proteins fed to 7-day old chicks.

	Percentage[1]										
Ingredient	Ala	Asp	Glu	Gly	HyLys	HyPro	Lan	Orn	Pro	Ser	Tau
Spray Dried Egg	2.82	4.86	5.73	1.67	0.03	0.00	0.00	0.06	1.81	3.08	0.02
Pea Protein Isolate (72%; PPI)	3.04	8.08	10.72	2.87	0.03	0.03	0.00	0.09	3.12	3.00	0.03
Pea Protein Concentrate (50%; PPC)	2.22	5.63	8.36	2.19	0.02	0.09	0.01	0.02	0.09	2.29	0.13
20:80 SDEG:PPC[2]	2.34	5.47	7.83	2.08	0.022	0.07	0.01	0.028	0.43	2.44	0.10
40:60 SDEG:PPC[2]	2.46	5.32	7.30	1.98	0.02	0.05	0.01	0.036	0.77	2.60	0.08
20:80 SDEG:PPI[2]	2.99	7.43	9.72	2.63	0.03	0.02	0.00	0.084	2.85	3.01	0.02
40:60 SDEG:PPI[2]	2.95	6.79	8.72	2.39	0.03	0.01	0.00	0.078	2.59	3.03	0.02

[1]Dry matter basis

[2]Calculated amino acid concentration.

Table 5-9. Exp. 1 Essential amino acid profile of test proteins as a percent of total amino acids.

	Percentage[1]											
Ingredient	Arg	Cys	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Tyr	Val
Spray Dried Egg	6.08	2.40	2.44	5.59	8.64	7.74	3.27	5.91	4.65	1.56	3.98	6.93
Ground Chickpea	9.04	1.62	2.65	4.76	7.76	7.47	1.52	6.14	3.78	0.98	2.85	5.01
Ground Faba Bean	10.46	1.45	2.74	4.68	7.90	6.86	0.76	4.64	3.64	0.94	3.46	4.92
Ground Green Pea	8.68	1.12	2.49	4.71	7.66	7.98	0.92	5.31	3.74	0.92	3.18	5.08
Ground Navy Bean	5.62	1.23	2.97	5.05	8.50	7.32	1.27	6.14	4.72	1.23	3.26	6.14
Ground Pinto Bean	6.10	1.12	2.98	5.12	8.52	7.45	1.26	6.24	4.38	0.88	3.40	6.10
Ground Red Lentil	8.31	1.18	2.64	4.87	7.91	7.58	0.85	5.56	3.77	0.89	3.24	5.43

[1] Dry matter basis.

Table 5-10. Exp. 2 Essential amino acid profile of test proteins as a percent of total amino acids.

	Percentage[1]											
Ingredient	Arg	Cys	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Tyr	Val
Spray Dried Egg	6.08	2.40	2.44	5.59	8.64	7.74	3.27	5.91	4.65	1.56	3.98	6.93
Pea Protein Isolate (72%; PPI)	8.2	1.38	2.44	5.37	8.87	7.86	1.12	6.05	3.91	0.87	2.87	5.68
Pea Protein Concentrate (50%: PPC)	9.14	1.4	2.69	4.92	8.02	8.49	1.04	5.49	4.14	1	3.71	5.3
20:80 SDEG:PPC[2]	8.53	1.60	2.64	5.05	8.14	8.34	1.49	5.57	4.24	1.11	3.76	5.63
40:60 SDEG:PPC[2]	7.92	1.80	2.59	5.19	8.27	8.19	1.93	5.66	4.34	1.22	3.82	5.95
20:80 SDEG:PPI[2]	7.78	1.58	2.44	5.41	8.82	7.84	1.55	6.02	4.06	1.01	3.09	5.93
40:60 SDEG:PPI[2]	7.35	1.79	2.44	5.46	8.78	7.81	1.98	5.99	4.21	1.15	3.31	6.18

[1] Dry matter basis.
[2] Calculated values.

Table 5-11. Non-essential amino acid profile of test proteins as a percent of total amino acids fed to 7-day old chicks.

	Percentage[1]										
Ingredient	Ala	Asp	Glu	Gly	HyLys	HyPro	Lan	Orn	Pro	Ser	Tau
Spray Dried Egg	5.73	9.88	11.64	3.39	0.06	0.00	0.00	0.12	3.68	6.26	0.04
Ground Chickpea	4.47	11.74	16.01	4.27	0.15	0.00	0.00	0.10	4.27	4.08	1.33
Ground Faba Bean	4.33	11.50	17.35	4.30	0.14	0.00	0.00	0.10	4.61	4.23	1.00
Ground Green Pea	4.43	11.72	17.49	4.43	0.14	0.00	0.00	0.09	4.25	4.01	1.38
Ground Navy Bean	4.53	12.42	14.54	4.25	0.19	0.00	0.00	0.14	3.97	5.19	1.32
Ground Pinto Bean	4.38	12.25	15.74	4.15	0.14	0.05	0.00	0.09	3.77	4.66	1.21
Ground Red Lentil	4.50	12.04	16.99	4.30	0.16	0.00	0.00	0.41	4.22	3.97	1.18

[1] Dry matter basis.

Table 5-12. Exp. 2 Non-essential amino acid profile of test proteins as a percent of total amino acids fed to 7-day old chicks.

	Percentage[1]										
Ingredient	Ala	Asp	Glu	Gly	HyLys	HyPro	Lan	Orn	Pro	Ser	Tau
Spray Dried Egg	5.73	9.88	11.64	3.39	0.06	0.00	0.00	0.12	3.68	6.26	0.04
Pea Protein Isolate (72%; PPI)	4.47	11.89	15.78	4.22	0.04	0.04	0.00	0.13	4.59	4.42	0.04
Pea Protein Concentrate (50%; PPC)	4.71	11.94	17.73	4.65	0.04	0.19	0.02	0.04	0.19	4.86	0.28
20:80 SDEG:PPC[2]	4.91	11.52	16.51	4.39	0.04	0.15	0.01	0.05	0.88	5.14	0.23
40:60 SDEG:PPC[2]	5.11	11.11	15.29	4.14	0.04	0.11	0.01	0.07	1.58	5.42	0.18
20:80 SDEG:PPI[2]	4.72	11.48	14.95	4.05	0.04	0.03	0.00	0.12	4.40	4.78	0.04
40:60 SDEG:PPI[2]	4.97	11.08	14.12	3.88	0.04	0.02	0.00	0.12	4.22	5.15	0.04

[1]Dry matter basis.
[2]Calculated values.

Table 5-13. Summary of amino acid composition of experimental ingredients.

Ingredient	Available Lys	Total Lys	Lys Availability	Total AA	EAA[1]	NEAA[2]	EAA:NEAA
Spray Dried Egg	3.67	3.81	96.33	49.21	29.13	20.08	1.45
Ground Chickpea	1.50	1.52	98.68	20.36	10.91	9.45	1.15
Ground Faba Bean	1.94	1.98	97.97	28.87	15.14	13.73	1.10
Ground Green Pea	1.70	1.73	98.27	21.67	11.28	11.44	0.99
Ground Navy Bean	1.49	1.55	96.13	21.18	11.32	9.86	1.15
Ground Pinto Bean	1.58	1.60	98.75	21.47	11.50	9.97	1.15
Ground Red Lentil	1.84	1.87	98.40	24.66	12.88	11.78	1.09

[1]Essential amino acids (EAA; Arg, Cys, His, Ile, Leu, Lys, Met, Phe, Thr, Tyr, and Val)
[2]Nonessential amino acids (NEAA; Ala, Asp, Glu, Gly, HyLys, HyPro, Lan, Orn, Pro, Ser, Tau)

Table 5-14. Summary of amino acid composition of experimental ingredients.

Ingredient	Available Lys	Total Lys	Lys Availability	Total AA	EAA[1]	NEAA[2]	EAA:NEAA
Spray Dried Egg	3.67	3.81	96.33	49.21	29.13	20.08	1.45
Pea Protein Isolate (72%; PPI)	5.18	5.34	97.00	67.95	36.94	31.01	1.19
Pea Protein Concentrate (50%; PPC)	3.95	4.00	98.75	47.14	26.09	21.05	1.24
20:80 SDEG:PPC[1]	3.89	3.96	20.06	47.55	26.70	20.86	1.28
40:60 SDEG:PPC[1]	3.84	3.92	39.12	47.97	27.31	20.66	1.32
20:80 SDEG:PPI[1]	4.88	5.03	98.27	64.20	35.38	28.82	1.24
40:60 SDEG:PPI[1]	4.58	4.73	97.78	60.45	33.82	26.64	1.29

[1]Caclulate values.

Table 5-15. Exp. 1 Growth performance, protein efficiency ratio, and net protein ratio of 7-day old chicks fed experimental protein sources for 10 days.

Treatment	Feed Intake (g)	Weight Gain (g)	Feed Efficiency	PER	%PER	NPR
Spray dried egg	340.12^{a}	177.33^{a}	0.51^{a}	5.18^{a}	---	5.18^{a}
Ground chickpea	292.31^{b}	92.83^{c}	0.31^{b}	3.18^{b}	61.24^{b}	3.06^{b}
Ground faba bean	147.25^{de}	8.88^{de}	0.05^{cde}	0.59^{cd}	11.41^{cd}	2.21^{cd}
Ground green pea	161.42^{d}	17.04^{de}	0.10^{cd}	1.05c	20.29^{c}	2.53^{c}
Ground navy bean	206.13^{c}	32.5^{d}	0.15^{c}	1.56^{c}	30.12^{c}	2.72^{c}
Ground pinto bean	178.42^{cd}	1.25^{ef}	0.03^{e}	-0.35^{d}	-6.74^{d}	1.04^{d}
Ground red lentil	150.46^{de}	8.04^{de}	0.04^{de}	0.40^{cd}	7.72^{cd}	2.29^{cd}
Negative control	128.17^{e}	-23.75^{f}	-0.17^{f}	---	---	---

$^{a\text{-}f}$ Means within a column with unlike superscripts differ ($p < 0.05$).

Table 5-16. Exp. 2 Growth performance, protein efficiency ratio, and net protein ratio of 7-day old chicks fed experimental protein sources for 10 days.

Treatment	Feed Intake (g)	Weight Gain (g)	Feed Efficiency	PER	%PER	NPR
Spray dried egg (SDEG)	358.92^{a}	197.33^{a}	0.5348^{a}	5.35^{a}	---	5.94^{a}
Pea protein concentrate (50%; PPC)	194.17^{c}	33.25^{e}	0.1690^{d}	1.69^{d}	31.57^{c}	2.82^{d}
Pea protein isolate (72%; PPI)	210.75^{c}	33.50^{e}	0.1620^{d}	1.62^{d}	30.29^{c}	2.68^{d}
20:80 SDEG:PPC	242.29^{bc}	75.71^{d}	0.3108^{c}	3.11^{c}	58.10^{b}	4.02^{c}
40:60 SDEG:PPC	351.09^{a}	171.36^{ab}	0.4848^{ab}	4.85^{ab}	90.57^{a}	5.47^{ab}
20:80 SDEG:PCI	276.46^{b}	93.75^{cd}	0.3358^{c}	3.36^{c}	62.73^{b}	4.01^{c}
40:60 SDEG:PPI	350.46^{b}	158.54^{b}	0.4503^{b}	4.50^{b}	84.17^{a}	5.13^{b}
Negative control	129.17^{d}	-21.79^{f}	-0.1740^{e}	---	---	---

$^{a\text{-}e}$ Means within a column with unlike superscripts differ ($p < 0.05$).