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# DEVELOPING VALUE-ADDED PEPTIDE ANTIOXIDANTS FROM RENDERING PRODUCTS

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# DEVELOPING VALUE-ADDED PEPTIDE ANTIOXIDANTS FROM RENDERING PRODUCTS

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# ABSTRACT

Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Vitamin E and ethoxyquin, have been commonly added to formulated animal feeds in order to delay discoloration and deterioration due to oxidation. However, these synthetic antioxidants have been limited in their applications as food additives because of potential health hazards. Specifically, synthetic antioxidants have been considered to be linked to various forms of cancer, most commonly kidney, liver and bladder cancer. In this work, value-added peptide antioxidants from rendering products were developed for use in potential aquaculture and pet food markets as natural antioxidant substitutes for synthetic antioxidants. The antioxidant peptides developed from the rendering protein were studied based on obtaining the hydrolysates fractions, together with evaluation antioxidant properties, ultrafiltration and analysis sequence of identified peptide.

In the initial studies, three typical rendering proteins (poultry meal, fish meal and premium pet meal) characteristic compositions of three rendering proteins were investigated, including moisture content, ash content, fat content, and protein content. Then, the antioxidant capacity of three alkaline rendering proteins hydrolysates, including poultry meal hydrolysates, fish meal hydrolysates and premium pet meal hydrolysates, were examined. The antioxidant activities of alkaline hydrolysates were evaluated using five methods including two based on the free radical scavenging capacity (DPPH, ABTS radical scavenging assay and one oxygen radical absorbance capacity (ORAC) assay). Hydrophobic antioxidant capacities of alkaline hydrolysates were determined by β-

Carotene Linoleic Acid System. Oxidation Stability Index (OSI) was used to evaluated the anti-lipid oxidation ability.

The antioxidant peptides developed from proteolytic hydrolysis of premium pet meal were the analyzed using Response surface optimization, Ultra-Filtration and LC-MS. The optimum hydrolysis condition on temperature, enzyme to substrate ratio, and the hydrolysis time were obtained by Response Surface Methodology (RSM). Abilities of scavenging ABT•+ free radicals and degree of the hydrolysis were employed as indicators of Response surface methodology. Under the optimum hydrolysis conditions, the proteolytic hydrolysates fractions were separated by the ultrafiltration to obtain different molecular weight proteolytic hydrolysates fractions. During this process, the antioxidant capacity was also evaluated by the ABTS•+ scavenging free radical assay. For the last step, peptides less than 1000Da peptide that were derived from premium pet meal were identified using LC-MS, indicating of the possible amino acid sequences of Leu-Thr-Cys or Iso-Thr-Cys and His-Cys.

# DEDICATION

This thesis is dedicated to my parents, Mr. Yulong Xing and Mrs. Liandi Zhang.

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# CHAPTER 1

# LITERATURE REVIEW

## 1.1 Introduction

Rendering is the recycling of raw animal tissue and used cooking fats and oils obtained from eating establishments into a variety of value-added products. During the rendering process, heat separation technology and filtering were applied to the material to destroy microbial populations, remove moisture, extract fat from the protein, and remove moisture and proteinaceous material from the fat (Bisplinghoff, 2006).

In the United States, the rendering industry is closely integrated with animal and meat production (**Figure 1.1**). These industries generate approximately 54 billion pounds per year of animal by-products and on-farm mortalities that were subsequently collected and processed by the rendering industry (Shurson, 2005). Rendering is the safest, most economical method of inactivating disease-causing microbes while recovering billions of dollars' worth of marketable commodities. It offers a safe and integrated system of animal raw material handling and processing that complies with all of the fundamental requirements of environmental quality and disease control (Meeker et al., 2006).

The rendering process converts raw animal tissue into various proteins, fats, and mineral products—rich granular-type meals and liquid fats with specific nutritional components. Annual volume in the United States was approximately 11.2 billion pounds of animal-derived proteins (Meeker & Hamilton, 2006). Eighty-five percent (85%) of this production is used as animal feed ingredients. Meanwhile, proteins in rendering are

constituents of all biological organisms and are found in all body tissues of animals. Proteins were found in higher concentration in organ and muscle tissue and range from very insoluble types in feather, hair, wool, and hoofs, to highly soluble proteins, such as those in serum or plasma. Animal-derived foods are primary sources of protein and other nutrients in the human diet; similarly, tissues from animal production and processing not utilized in human food are processed into an array of protein meals used in animal feed.

## 1.2 Rendering Industry

### 1.2.1 Rendering protein

Animal meat by-products were the main source of protein in pet diets and provide an important proportion of total fat in compound foods. Although raw animal by-product meals have very high protein quality (Cramer et al., 2007) because of their well-balanced amino acid profile and high availability, meat and bone meals (MBM) and poultry by-products currently found on the market show great variability in chemical composition and protein value. The high variability in the composition and protein quality of meat by-products was due to the heterogeneity of the raw material used (mainly the proportions of bone and connective tissue included) and the rendering conditions employed.

Raw material (Johnson & Parsons, 1997; Shirley & Parsons, 2001) and processing conditions (Wang & Parsons, 1998; Shirley & Parsons, 2001) have been shown to affect the protein quality of animal meals. Hence, simultaneous changes in raw material and technological processes were likely to have a large influence on the protein value of current MBM. Dawson and Savage (1983) found that the protein quality of MBM was

more affected by the type of offal rather than the rendering process used, whereas Skurray and Herbert (1974) showed that soft offal (cattle guts and rumens) had higher essential amino acids content and nutritive value than hard offal (sheep's heads and calves' heads and trotters(the feet of a goat, pig, lamb, or any cattle)) not only between, but also within processing plants.

The major animal protein ingredients, MBM (meat and bone meals) and PBM (poultry by-product meal), were important feed ingredients for livestock, poultry, aquaculture, and companion animal diets throughout the world. Annually, these products contribute over 3 million tons of ingredients to the U.S. feed industry. In addition to protein, these meals were excellent sources of essential amino acids, fat, essential fatty acids, minerals, and vitamins. The typical nutrient composition of the four most common animal proteins were shown in **Table 1.1**. As can be noted, all of these ingredients were higher in protein than soybean meal and other plant proteins. In addition, MBM was higher in phosphorus, energy, iron, and zinc than is soybean meal. The phosphorus level in MBM was seven-fold greater than that found in soybean meal and was in a form that was highly available to livestock and poultry. The phosphorus in both MBM and poultry meal was similar in bioavailability to feed-grade monodicalcium phosphate.

Collagen is more purified animal protein than MBM (meat and bone meals) and PBM (poultry by-product meal), which is one of the longest fibrous structural proteins. Collagen is an extracellular matrix protein, which plays an important role in many animal tissues within the skeletal (Fratzl, 2008) muscular, and cardiovascular network tissues

(Buehler, 2006). Native collagen is very hard to digest; its functions are quite different from those of globular proteins, such as enzymes. Tough bundles of collagen called collagen fibers are a major component of the extracellular matrix that supports most tissues and gives cells their structure from the outside, but collagen is also found inside certain cells.

Collagen has great tensile strength and is the main component of fascia, cartilage, ligaments, tendons, bone, and skin. As raw materials in the pharmaceutical and food industries, collagen peptides were used as important active components because of their excellent bioactivity, good biocompatibility, good penetrability, and lack of irritation to the body (Iwai et al., 2006). Modification of a protein is usually realized by physical, chemical, or enzymatic treatments, which change its structure and consequently its physicochemical and bioactive properties (Kamara et al., 2009). Hydrolyzed collagens from fish, porcine, and bovine origin were now used in such consumables as functional food, beverages, and dietary supplements.

Many reports indicated that collagen peptides may act as messengers and trigger the synthesis reorganization of new collagen fibers by stimulating fibroblast cells (Iwai et al., 2006). Recently, some studies showed that hydrolyzed collagen increases the fibroblast density and diameter of collagen fibrils in the dermis (Wu, Fujioka, & Sugimoto, 2004). Thus, hydrolyzed collagen may improve the mechanical strength of the skin by increasing decorin (a vital player in maintaining skin and tendon integrity at the molecular level) ratio (Lin, Yun et al., 2010). Another reason why collagen is widely

researched is that collagen is a cheap and resourceful meat by-product whose main product is gelatin, which is used extensively as a food additive to increase the texture, water-holding capacity, and stability of food products, or as an encapsulating material for making pharmaceutical soft gels (Kong, Chen, & Wang, 2006).

### 1.2.2 Rendered products in pet food

In 2005, pet food products were a $53 billion industry—with the market continuing to grow. In the United States, dog and cat food sales alone account for $14.5 billion with exports of nearly $1 billion. The global total for pet food and supplies for all pet animals approached $40 billion annually (Aldrich, 2006). These rising sales were driven, in part, by increasing ownership of pets, with more than 140 million dogs and cats and an estimated 200 million specialty pets, such as fish, pocket pets, and exotic animals. It is also moved by the trend that more people consider their pets to be members of the family, as demonstrated by everything from birthday and holiday celebrations, to family photos, to health insurance, to burial plots, to the preparation of special meals. Now, more than ever, pet foods were considered packaged goods that were co-mingled with other family food items. The top five pet food companies, representing over 65% of the market, were owned by household names like Mars, Nestle, Procter & Gamble, Colgate-Palmolive, and Del Monte. Traditional retail outlets, such as grocery and farm/feed stores, have lost some market share to big-box mass market stores, warehouse clubs, and pet specialty stores, but grocery stores still remain the largest outlet for the sale of pet foods.

Pet food choices have become almost limitless, with options for different price points, life-stage, shapes and sizes, package type, ingredient preferences, breed, size, and disease condition. Pet foods are also becoming more "humanized" and are development is parallel to human food trends. Nutrition research is showing that companion animals have some unique dietary requirements, e.g., arginine in dogs and cats and pre-formed vitamin A for the cat. Emerging nutritional benefits from omega-3 fatty acids, carotenoids, dietary fiber, mineral balance, and how meat proteins and fats are connected to optimal nutrition are actively under investigation. Rendered protein meals, such as meat and bone meal, poultry by-product meals, and fish meal, are almost universally used in pet foods.

Generally, they provide high-quality protein with a good balance of amino acids. Nutrient availability and dietary use can be hampered by excessive heat treatment, dilution of essential amino acids with connective tissue, high levels of ash, and oxidation. Rendered fats and oils like tallow, lard, poultry fat, and fish oil provide a supplementary source of energy, flavor, texture, and nutrients in pet foods. Balancing for essential and conditionally essential fatty acids has become a key driver for selection of specific fats in the diet. Application and oxidation issues are the most common challenges faced in their use. Much of the information for pet food ingredients has been gleaned from livestock and human nutrition research (Aldrich, 2006; Meeker, et al., 2006).

There is a fundamental need to expand these nutrient databases to address the unique nutritional idiosyncrasies of pets and to support this growing and continually

segmenting industry. Raw, fresh, human-edible, and alternative protein sources are competing to supply the protein and fat needs in pet foods. Opportunities for various rendered ingredients, especially those that are able to retain their species identity and maintain control over processing conditions while retaining nutrient quality, will be welcome (Aldrich, 2006; Meeker, et al., 2006).

### 1.2.3 The rendering industry's role in feed and food safety

A little over two decades ago, the industrialized societies of the world recognized the urgent necessity to address the broad realm of issues linked to safe food production. In the United States, this was exemplified by two major conferences in 1984. At the National Conference for Food Protection held in Washington, D.C., sponsored by the Food and Drug Administration (FDA), the keynote speaker extolled the country's "plentiful, wholesome, nutritious, and safe food supply" (Knauer, 1984), recognizing that a benevolent food supply took hard work, imagination, and cooperation among the food-producing industry, consumers, and the government. This initial conference was followed three months later by an international symposium held in New Orleans, Louisiana, that focused on *Salmonella*. The keynote speaker at the latter conference highlighted the challenging dimensions of *Salmonella* control internationally and addressed how this problem "confronts government, industry, and the scientific community as both a challenge and a reproach. It is a challenge because it taxes our ingenuity in dealing with its various dimensions. It is a reproach because it sometimes appears that with our science and technology we are better able to strive toward a certain well-defined

objective, like the moon, than to overcome a chronic food poisoning hazard" (Houston, 1984).

These two conferences clearly had an impact on the policy-making directions that government agencies took during that period, including the subsequent consideration of hazard analysis and critical control points (HACCP) as an interactive, scientifically-based protocol that could be used to eliminate food safety hazards, or at least reduce them to acceptable levels. It is interesting to note, although not necessarily surprising, that HACCP was operational as a concept in the private sector (the Pillsbury Company) as early as 1973 and was later embraced by the U.S. FDA as a regulatory mandate for canned acidified and low-acid foods packed in hermetically sealed containers (Corlett, 1998). Most importantly, these two early conferences had a definite impact on the United States' direction of food safety policy. Amplification followed in 1989 at an international symposium of the World Association of Veterinary Food Hygienists held in Stockholm, Sweden, co-sponsored by the European Association for Animal Production, the International Union of Food Science and Technology, and the World Health Organization.

Prevention of health risks due to food intake is central in food safety policy and demands an integrated approach, defining the role of all stakeholders and their individual responsibilities. Cooperation, collaboration, and communication among the affected parties are prerequisites to success. Food safety must be based on sound and verified science, and continued progress is dependent on the commitment of every level of

production to ensure the absence of hazards—from feed ingredient manufacturers that supply feed companies to processors responsible for the safe production of finished products for the table.

The quality of feed ingredients produced by the rendering industry plays an important role in this complex system because the practices of the industry are a reflection of the food cycle of production. Raw materials processed by the rendering industry are food residuals that did not enter human food channels but are recycled through innovative processing technology to produce proteins and fats of animal origin for livestock, poultry, aquaculture, and pets. In reality, we are describing the alpha and omega of the food chain. As a result, the rendering industry is conscious of its responsibility in this program of progressive integration. The industry concedes that feed ingredient safety is an important and attainable factor in total food safety objectives, hence the rationale for proactive testing for pathogens and toxins that could influence product integrity. It is also the reason for educational offerings to train the workforce to achieve safety, including the applicability of HACCP, the internationally accepted concept for safety assurance, and the promotion of the APPI Code of Practice, carefully constructed, with an adjunct program of third-party certification to demonstrate accountability and the industry's significant role in sustainable food safety. This assures that safe feed will produce healthy livestock that contributes to safe food and healthy people (Grossklaus, 1999; Franco, 1999 & 2006).

All in all, the role of the rendering industry in feed and food involves the formulation and administration of progressive, forward-looking programs under the auspices of the Animal Protein Producers Industry (APPI), the biosecurity arm of the rendering industry. While end-product testing for *Salmonella* has played a historic role in the industry's endeavors to assure the safety of feed ingredients of animal origin, the industry recognizes that the current and future challenges of feed/food safety necessitate innovation and new modeling. The industry has approved a robust Code of Practice that mandates long-term commitment and accountability while accepting that the success of such a program could only be realized through a comprehensive third-party certification audit.

The purpose of this chapter is to review the rendering industry from different perspectives and profile the contributions the industry makes in supplying safe feed ingredients and sources of energy to enhance the health of livestock in producing safe food. Clearly, inherent in safe food production are the acceptance and responsibility that feed ingredients meant for livestock, poultry, and aquaculture are part of the food chain. Manufacturers must conform to standards of sanitation and hygiene in production to preclude hazards that could impact the health of animals and humans, direct or indirect.

### 1.3 Bioactive Proteins and Peptides

Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts & Weiler, 2003). The activity of peptides is based on their inherent amino acid

composition and sequence. The size of active peptide sequences may vary from 2 to 20 amino acid residues, and many peptides may exert several bioactivities.

Food proteins are sources of peptides with various biological activities, such as anti-inflammatory, mineral carrying, antihypertensive, immune-stimulating, antithrombotic, and anti-gastric. These bioactive proteins and peptides are generated in vivo, in vitro, and during food processing. During the past decades, a growing body of scientific evidence has revealed that many food proteins and peptides exhibit specific biological activities in addition to their established nutritional value (Mine & Shahidi, 2006; Yalcin, 2006; Möller et al., 2008). Bioactive peptides have been found in enzymatic protein hydrolysates and fermented dairy products, but they can also be released during gastrointestinal digestion of proteins (Mine & Shahidi, 2010; Hartmann & Meisel, 2007). Bioactive peptides may help reduce the worldwide epidemic of chronic diseases that account for 58 million premature deaths annually. Functional proteins and peptides are an important category within the nutraceuticals food sector currently valued at $75 billion/year (Mine, 2009). Nevertheless, several challenges should be addressed to allow sustained growth within this sector. Some earlier health benefits claimed for protein nutraceuticals were based on in vitro models or limited clinical trials leading to equivocal findings. Technological and fundamental problems remain in relation to large-scale production, compatibility with different food matrices, gastrointestinal stability, bioavailability, and long-term safety (Murray & FitzGerald, 2007; Mine, 2007). Research into consumer perception and legislation is also necessary. Nutritionists, biomedical scientists, food scientists, and technologists are working together to develop improved

systems for discovery, testing, and validation of nutraceuticals proteins and peptides with increased potency and therapeutic benefits. Bioactive peptides and proteins are being developed that positively impact body function and human health by alleviating conditions such as coronary heart disease, stroke, hypertension, cancer, obesity, diabetes, and osteoporosis. Screening novel bioactive sources using high-throughput omics technologies, specific disease biomarkers, and comprehensive clinical trials will facilitate the development of nutraceuticals proteins and peptides for a further range of health conditions (Gilani et al., 2008; Mine et al., 2009).

### 1.3.1 Antioxidant proteins and peptides

Antioxidants in raw material play a key role in maintaining the quality of finished products, especially in the poultry industry. When antioxidants are added to raw material, oxidation is retarded, and good-quality pet food grade products can be recovered from a larger volume of the materials. Therefore, the control of lipid oxidation in food products is desirable. Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), may be added to food products to retard lipid oxidation. However, the demand for natural antioxidants has recently increased because of questions about the long-term safety and negative consumer perception of synthetic antioxidants (Yu et al., 2002). Many antioxidative substances have been and are being isolated from natural materials, including foods. Antioxidative action and the structure of those compounds have been reported by many researchers, and several antioxidants have already been developed (Bishov & Henick, 1975; Nagai, Inoue, Inoue, & Suzuki, 2003; Shahidi & Wanasundara, 1992). Water-soluble antioxidants, such as amino acids and

proteins, have been reported, because of their chelating effects on metal ions (Lu & Baker, 1986). Several studies have been performed to identify potential antioxidative peptides in fish skin gelatin (Kim et al., 2001; Mendis, Rajapakse, & Kim, 2005); there has yet to be any research on antioxidative peptide activity in porcine skin collagen. Furthermore, some protein hydrolysates from animal and plant sources have also been found to possess antioxidant activity (Amarowicz & Shahidi, 1997; Pena-Ramos & Xiong, 2002). These antioxidants have been investigated mainly in the prevention of lipid oxidation in foods.

During past decades, consumers have showed increased preference for the use of natural antioxidants and additives in food products (Lindberg Madsen & Bertelsen, 1995). Traditionally, natural food antioxidants were prepared from plants and plant by-products (Cao, Sofic, & Prior, 1996; Cho, 2004). However, due to interest in the utilization of animal by-products, many researchers have started evaluating animal peptide as a source of natural antioxidants (Lee, Kim, & Hwang, 2010). Some animal protein contains a large number of components that can be either pro-oxidative or antioxidative. For example, heme pigments present in the muscle tissue could promote lipid oxidation and oxidative rancidity (Richards & Hultin, 2002), whereas components from the press juice of fish muscle have been shown to inhibit hemoglobin-mediated lipid oxidation (Sannaveerappa, Carlsson, & Sandber, 2008). In addition, certain proteins and peptides found in animal protein have demonstrated high antioxidative activity (Kristinsson, 2007). Previous studies have shown that protein hydrolysates derived from rendering protein may have antioxidative effects (Lee, Kim, & Hwang, 2010).

Upon structural disruption, antioxidative amino acids, such as Cys, Met, Trp, Tyr, Phe, and His (**Table1.2**), (Wang & de Meija, 2005) can be exposed to function more effectively. However, studies on antioxidative peptides from rendering peptide are still limited. Two peptides, Trp, His (Davalos, Miguel, & Bartolome, 2004), showed the highest antioxidant activity among all amino acids, followed by Tyr and Met and then by Cys, His, and Phe, a purified antioxidative peptide as the sequence of Asp-Val-Cys-Gly-Arg-Asp-Val-Asn-Gly-Tyr (1096 Da) (Shen, Chahal, & Majumder, 2010). These peptides showed similar hydroxyl radical scavenging activity compared with that of vitamin C. Other amino acids (Arg, Asn, Gln, Asp, Pro, Ala, Val, Lys, Ile, Ser, Thr, Leu, Glu, and Gly) did not exhibit antioxidant activity (Pihlanto & Korhonen, 2003). Therefore, the presence of Trp in the structurally related peptides mentioned above is probably the driving force of their antioxidant activity. Similarly, the presence of Tyr and Phe can account for the high antioxidant activity observed with another group of structurally related peptides (Shen, Chahal & Majumder, 2010). Furthermore, one of the antioxidative peptides, Gln-Gly-Ala-Arg, was then synthesized and the antioxidant activities measured using the aforementioned methods. The results confirmed the antioxidant activity of this peptide added further support to its feasibility as a provider of natural antioxidants from porcine skin collagen protein.

### 1.3.2 Anti-inflammation proteins and peptides

Proteins and peptides from egg, milk, soy, and plant sources have been shown to have anti-inflammatory properties. In vitro and in vivo studies of these bioactive proteins and peptides have mostly been among egg proteins, milk proteins, and some special plant

proteins. A recent study by Lee et al. (2009) found that hen egg lysozyme (HEL) supplementation attenuated physiological markers of inflammation; reduced the colon expression of pro-inflammatory cytokines TNF - α, IL - 6, IFN - γ, IL - 8, and IL - 17; and increased the expression of anti-inflammatory IL - 4 and TGF - β in a porcine model of chemical colitis. The increase in TGF - β and Foxp3 mRNA expression points to the possibility that HEL may help in restoring gut homeostasis through the activation of T regulatory cells. Milk lysozyme was also postulated to possess a new anti-inflammatory activity through its inhibition of the hemolytic activity of serum complement when tested within the levels present in normal and inflamed breast milk samples (Ogundele, 1998). For milk peptides, bovine casein κ-casein is a phosphorylated glycoprotein composed of a C-terminal fragment, glycomacropeptide (GMP) (Otani & Hata, 1995).

Immunosuppressive results were also found with pancreatic and trypsin digests of alpha s1-casein and beta-casein, but not the native proteins. Similar suppressive effects on mitogen-stimulated lymphocyte proliferation were obtained by Sutas et al. (1996) using pepsin- and trypsin-digested alpha s1-casein and beta-casein. Further, *Lactobacillus* casein GG-derived enzymatic hydrolysis of these digests along with similar κ-casein digests resulted in reduced proliferation as well. *L. rhamnosus* GG-degraded bovine casein was also found to suppress T-cell activation (Mikkelsen et al., 2005). In chickens, it is often present in large amounts as an acute-phase protein during inflammation and infections. It has immunomodulation effects on chicken macrophages and heterophil-granulocytes (Xie et al., 2002) and has the ability to inhibit proliferation of mouse spleen lymphocytes.

Oxidative stress and inflammation are often related to chronic diseases involving the cardiovascular, neurological, and gastrointestinal systems. Ingestion of bioactive food proteins and peptides is a relatively safe way in which to prevent or treat these diseases. Proteins and peptides have been found not only to contribute to the body's energy supply and growth, but also to influence specific biological activities such as oxidative stress and inflammation. As our understanding of the efficacy and mechanism of action of antioxidative stress and anti-inflammatory proteins and peptides increases, so will the growing interest in their prophylactic, preventive, or therapeutic uses.

### 1.3.3 Antihypertensive proteins and peptides

Peptides, which are condensed amino acids, have been well documented as physiologically functional compounds in nature. In recent years, antihypertensive proteins and peptides have been reported widely, especially in relation to antihypertensive mechanisms as well as the development of antihypertensive food products.

Antihypertensive peptides are obtained from proteins of both animal and plant origin. Most antihypertensive peptides with proven effects on spontaneously hypertensive rats have angiotensin I-converting enzyme inhibitory activities. Clinical experiences for these antihypertensive peptides were also reviewed to discuss the potential of antihypertensive peptides for high blood pressure. Studies reporting an in vivo mechanism, based mainly on animal studies, were also included. Hypertension is a major risk factor in cardiovascular diseases such as heart disease and stroke. In order to prevent disease incidence, pharmacological substances can be used to decrease high blood

pressure to within the normal range. Angiotensin-I converting enzyme (ACE) plays an important role in blood pressure regulation. It is a dipeptidyl carboxypeptidase that catalyzes both the production of the vasoconstrictor angiotensin II and the inactivation of the vasodilator bradykinin. ACE is an unusual zinc metallopeptidase. Ferreira et al. (1970) isolated nine biologically active peptides from *Bothrops jararaca*, and the peptides contained 5–13 amino acid residues per molecule. Among them, a peptide with the sequence pyrrolidone carboxyl-Lys-Trp-Ala-Pro showed the highest ACEI activity. Ondetti et al. (1971) reported other strong ACEI peptides from the venom of *B. jararaca*. Thereafter, many other ACE inhibitors were discovered from enzymatic hydrolysates or the related synthetic peptides of bovine casein, human casein, zein, gelatin, soy sauce, soybean, corn, wheat, and other food proteins. Many studies have also been performed on fish products, such as sardine muscle, tuna muscle, and bonito. The most active ACEI peptides found in these studies are short peptides. These peptides contain 2–12 amino acid residues, several of them with proline in the C-terminus. These peptides show that it is activated by chloride and lacks narrow in vitro substrate specificity. ACE is predominantly expressed as a membrane-bound ectoenzyme in vascular endothelial cells and also in several other types of cells, including absorptive epithelial cells, neuroepithelial cells, and male germinal cells.

### 1.3.4 Anticancer proteins and peptides

Cancer is a major cause of mortality, with incidence rates increasing throughout the world. The World Health Organization predicts that, over the next decade, the number of cases of cancer will increase by 73% in the developing world and by 29% in the

developed world. Although the underlying causes of many cancers have not yet been fully defined, a wealth of evidence points to diet as one of the most important modifiable determinants of the risk of developing cancer, possibly due to the presence of potentially bioactive food components that are protective at different stages of cancer formation (Milner, 2004). There is tremendous interest in discovering novel peptides that may act as anticancer agents because of their multi-functionality, high sensitivity, and stability characteristics (Lee et al., 2005; Leng et al., 2005). Some natural and synthetic peptides have been reported to show antitumor or anticancer activities. A polysaccharide peptide extracted from *Coriolus versicolor* mushroom (Milner, 2004) and *Tricholoma mongolicum* mushroom (Lee et al. 2005) showed antitumor or immunoenhancing activities. Dolostatins are peptides isolated from marine sea hare *Dolabella auricularia*, and these peptides have been known to have antitumor activities on several cancer cell lines. Several antimicrobial peptides isolated from African *Streptomyces* frog and *Micromonospora* appeared to have not only antimicrobial, but also anticancer activities (Kim et al., 2000). Anticancer properties have been indicated in several egg proteins as well. In previous studies, lysozyme was shown to be an anticancer agent through the host-mediated immune response and probably through the inhibition of tumor formation and growth (Sava et al., 1989; Kovacs-Nolan et al., 2005). It has been established that avidin, an egg white protein, exhibits anticancer activity (Gasparri et al., 1999). The activity of avidin as an antitumor agent may involve changes in the host-tumor relationship or the host-mediated antitumor response. The rebirth of interest in cystatin research stems from its inhibition of cysteine proteinases, which has led to an attempt to

develop novel anticancer agents, especially after the unsuccessful exploration of serine- and metallo-protease inhibitors as anticancer agents in the last 30 years (Coussens et al., 2002). Cysteine proteases have been implicated in multiple steps of tumor progression, including early steps of immortalization and transformation, intermediate steps of tumor invasion and angiogenesis, and late steps of metastasis and drug resistance (Keppler, 2006). Cystatin inhibited tumor invasion in epithelial cells (Premzl et al., 2001) and was found to reduce the activity of the key proteolytic enzymes responsible for the growth of gastric cancer in vitro (Muehlenweg et al., 2000). In addition, egg cystatin was reported to affect the host's immune response through the cytokine network, which stimulates nitric oxide production, contributing to its anticancer properties (Verdot et al., 1999). Ovomucin was reported to have the potential to inhibit the growth of cancer cells by limiting angiogenesis (Oguro et al., 2001). Recently, a 70 kDa fragment from pronase-treated ovomucin has also demonstrated anticancer properties in a double-grafted tumor system (Watanabe et al., 1998).

### 1.3.5 Other bioactive proteins and peptides

Bioactive peptides have been recognized as natural processes within the body and are modulated almost exclusively by the interaction of specific amino acid sequences, either as peptides or as subsections of proteins. Including the bioactive activates antioxidant, anti-inflammatory, antihypertensive, and anticancer properties; many other properties have also been reported. With respect to human beings, proteins and peptides are involved in the modulation of cell proliferation, cell migration, angiogenesis, melanogenesis, and protein synthesis and regulation. The creation of therapeutic or

bioactive peptide analogs of specific interactive sequences has opened the door to a diverse new field of pharmaceutical and nutraceuticals in the food industry. In this project, we develop a new value-added peptide for the pet food and address its role in nature, its application to rendering, as well as the advantages and challenges posed by this new technology.

## 1.4 Analytical Methods

### 1.4.1 Methods for preparing peptide

Enzymatic Hydrolysis:

Hydrolysis of proteins by either acids or enzymes is the most widely used method to increase the exposure of antioxidant amino acids. Acid hydrolysis is less expensive and relatively simple, but it is more difficult to control, and amino acid damage may occur. In contrast, enzymatic hydrolysis is less harsh and more tractable and does not cause amino acid damage (Theodore & Kristinsson, 2008). Enzymatic hydrolysis is still the predominant method to prepare bioactive peptides from proteins; fermentation is another way to hydrolyze proteins but is considered to be less efficient. Protease hydrolysis is one of common ways of bone collagen pre-treatment. In Kristinsson study (2007), we estimated the degree of hydrolysis (DH) of collagen using different commercial proteases (neutrase, alcalase, papain, and acid protease). It was found that neutrase gave higher DH than the other enzymes. Therefore, the antioxidant activity was investigated on peptides obtained from neutrase hydrolysis. Protease was added to the mixture for hydrolysis process at a suitable temperature for the scheduled time. However, one drawback in the

preparation of antioxidants from rendering by-products is the presence of unstable lipids and heme proteins (Kristinsson, 2007), especially in fish products, which can be a rich source of polyunsaturated fatty acids and glycerides (Deckere, Korver, Verschuren, & Katan, 1998) and could potentially contaminate and decrease the antioxidant activity of protein hydrolysates.

Chemical Hydrolysis:

Alkaline hydrolysis technology was designed as an alternative disposal method for contaminated tissues and dead animals, and it has certainly proven to be effective. The economic justification of such a system without government intervention will be difficult. The most common procedure consists of washing, followed by saturated calcium bydroxide (liming period), which causes the non-collagen material to become more soluble and can be removed by later washing. Liming also causes hydrolytic reactions with limited solubilization. Next, the pH is lowered, and the lime is washed with cold water and removed from the stock. This is followed by washing with dilute acid and a final wash sulfate. The stock is then placed in extraction kettles, and extraction takes place in a series of cooks. The liquid extract is pressure filtered followed by evaporation. As indicated above, fish products can be a rich source of polyunsaturated fatty acids and glycerides (De Deckere, Korver, Verschuren, & Katan, 1998) and could potentially contaminate and decrease the antioxidant activity of protein hydrolysates. One way to overcome the problem is to prepare protein isolates using an alkaline solubilization technique; this technique involves solubilizing the myofibrillar and sarcoplasmic proteins

of fish muscle at alkaline pH (around pH 11.0) to separate contaminants such as lipids, connective tissues, and bones from soluble proteins. Protein isolates can then be prepared by precipitating the myofibrillar and sarcoplasmic proteins at their isoelectric pH (around pH 5.5). Undeland et al. (Undeland, Kelleher, & Hultin, 2002) had earlier shown that protein isolates prepared using an alkali solubilization technique has lower total lipid and phospholipid content. Hence, protein isolates prepared using the alkaline pH-shift method could yield a much purer protein substrate for enzyme hydrolysis. In our current study, we intended to evaluate the antioxidant properties of protein hydrolysates prepared from alkali-solubilized MBM (meat and bone meal) protein isolates.

### 1.4.2 Purification and separation proteins and peptides

#### 1.4.2.1 High-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) is now firmly established as the premier technique for the analysis and purification of a wide range of molecules. Peptides and proteins interact with the chromatographic surface in an orientation-specific manner, in which their retention time is determined by the molecular composition of specific contact regions. For larger polypeptides and proteins that adopt a significant degree of secondary and tertiary structure, the chromatographic contact region comprises a small proportion of the total molecular surface. Hence, the unique orientation of a peptide or protein at a particular stationary phase surface forms the basis of the exquisite selectivity that can be achieved with HPLC techniques. All biological processes depend on specific interactions between molecules, and affinity chromatography exploits these specific interactions to allow the purification of a biomolecule on the basis of its

biological function or individual chemical structure. In contrast, reversed-phase HPLC, ion-exchange, and hydrophobic-interaction chromatography separate peptides and proteins on the basis of differences in surface hydrophobicity or surface charge. These techniques therefore allow the separation of complex mixtures, whereas affinity chromatography normally results in the purification of one or a small number of closely related components of a mixture

#### 1.4.2.2 Cut-off peptide by ultrafiltration (UF) membranes

The hydrolysates of MBM by-products were fractionated through ultrafiltration (UF) membranes with a range of molecular weight cut-offs (MWCO) of 30, 10, and 5 kDa (Cho & Unklesbay, 2004), respectively, using a TFF system. The residue from the 10K membrane was then subjected to filtration with the 5K membrane, and the process was repeated until the maximal separation was obtained. In the same fashion, the 5K permeate was passed through the 3K membrane, and the 3K permeate was treated with the 1K membrane. Inclusions of the smaller MW fractions in the larger MW fractions were minimized while enough retentates and permeates were collected during each step. This was needed mainly because FP 900 is a highly hydrolyzed product with relatively low molecular weight peptides and cannot be fractionated well without diafiltration. The five peptide fractions were prepared and designated > 10K (10K retentate), 5-10K (10K permeate-5K retentate), 3-5K (5K permeate-3K retentate), 1-3K (3K permeate-1K retentate), and < 1K (1K permeate). The ultrafiltration process parameters obtained are shown in **Figure 1.2**.

### 1.4.3 Methods to evaluate antioxidant activity

There are a variety of methods to assess ‘‘total’’ antioxidant activity (Huang, Ou, & Prior, 2005; Prior & Cao, 1999; Prior, Wu, & Schaich, 2005). Due to technical variations and the fact that they are dependent on different chemistries, the sensitivity of the assays would be expected to vary both in total and to specific agents, thus making any “total” antioxidant assay differ from the next, and they are all but estimations of aggregate antioxidant activity. Most of these methods generally do not lend themselves to high-throughput studies.

Methods used to determine antioxidant capacities can roughly be classified into two types: assays based on hydrogen atom transfer (HAT) reactions and assays based on electron transfer (ET), depending upon the reactions involved. The majority of HAT-based assays apply a competitive reaction scheme, in which antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo compounds. These assays include inhibition of induced low-density lipoprotein autoxidation, oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP), and crocin bleaching assays. ET-based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes color when reduced. The degree of color change is correlated with the sample’s antioxidant concentrations. ET-based assays include the total phenols assay by Folin-Ciocalteu reagent (FCR), Trolox equivalence antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP), “total antioxidant potential” assay using a Cu (II) complex as an oxidant, ABTS and DPPH (Hu, Wu, & Cross, 2010). To study different aspects of

antioxidants comprehensively, validated and specific assays are needed in addition to these two commonly accepted assays. ET-based assays measure an antioxidant's reducing capacity, and HAT-based assays quantify hydrogen atom donating capacity. It is apparent that the hydrogen atom transfer reaction is a key step in the radical chain reaction. Therefore, the HAT-based method is more relevant to the radical chain-breaking antioxidant capacity.

Experimental evidence has directly or indirectly suggested that there are six major reactive oxygen species causing oxidative damage in the human body. These species are: (1) $O_2$•-Scavenging Capacity Assay. Classically, the SOD activity assay uses the competition kinetics of $O_2$•-reduction of cytochrome c (probe) and $O_2$•-scavenger (sample); (2) $H_2O_2$ Scavenging Capacity Assay; and (3) Hydroxyl Radical (HO) Scavenging Assay. Antioxidants in food (such as vitamin C) may act as pro-oxidants by reducing Fe(III) to Fe(II) and making HO• generation catalytic. Hydroxyl radical formation under the experimental conditions is indirectly confirmed by the hydroxylation of p-hydroxybenzoic acid. Fluorescein (FL) was used as the probe. The quantitation method is the same as that of the ORAC assay, except gallic acid is used as the standard. (4) Singlet Oxygen Scavenging Capacity Assay. Singlet oxygen is normally generated in the presence of light and photosensitizers. Beta-carotene is an excellent physical quencher of $O_2$. Singlet oxygen emits characteristic phosphorescence at 1270 nm. (5) Peroxynitrite (ONOO-) Scavenging Capacity Assay. Two methods are used for ONOO- scavenging measurements: inhibition of tyrosine nitration by ONOO- and inhibition of

dihydrorhodamine (DHR) oxidation (6) peroxyl radicals (ROO•) Scavenging Capacity Assay.

Rendering peptide is the essential in the United States, and hence, exploring new and creative methods to use protein would be a lucrative and environmentally responsible avenue. In the research work presented here, we have investigated the antioxidant activity of rendering protein hydrolysates using DPPH Radicals Scavenging Activity Assay, ABTS Radicals Scavenging Activity Assay, oxygen radical absorbance capacity (ORAC), and Beta-Carotene Linoleic Acid Assay in a washed muscle model system, in which oxidation was catalyzed using tilapia hemolysate.

#### 1.4.3.1 DPPH Free Radicals Scavenging Activity Assay

The scavenging effect of swine bone collagen on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical, was measured according to the method of Shimada et al. (1992) with little modification. The DPPH• radical (**Figure 1.3**) is one of stable organic nitrogen radicals, and they bears a deep purple color (Lin, Le, & Wang, 2010). It is commercially available. This assay is based on the measurement of the reducing ability of antioxidants toward DPPH•. The ability can be evaluated by electron spin resonance (EPR) or by measuring the decrease of its absorbance. The widely used decoloration assay was first reported by Brand-Williams and co-workers. Antioxidant assays are based on measurement of the loss of DPPH color at 515 nm after reaction with test compounds (Mendis, Rajapakse, & Byun, 2005), and the reaction is monitored by a spectrometer. The percentage of the DPPH remaining is calculated as when a DPPH stock solution is

mixed with a compound that can donate an electron, the DPPH radical will be reduced simultaneously with the loss of its violet color. However, it is expected that a residual pale yellow color from the picry1 group will remain present (Molyneux, 2004).

#### 1.4.3.2 ABTS Radicals Scavenging Activity Assay

The capacity of additives to diminish the formation of the 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) derived radical cation (ABTS•+) when the parent compound is incubated in the presence of a free radical source has been extensively studied to evaluate the free radical trapping capacity of pure compounds and complex mixtures (Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995). Interpretation of the data in these systems is not straightforward, as the decrease in radical cation concentration observed in the presence of the additives can be due to trapping of the primary radicals and (or) to the bleaching of the formed radical cations by the additives. In this regard, it is interesting to evaluate the capacity of compounds of biological interest to react with this stable free radical. Furthermore, methods have been proposed to evaluate the total antioxidant capacity of complex samples, from the bleaching extent of preformed radical cations (Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1996; Re, Pellegrini, Proteggente, Pannala, Tang, & Rice-Evans, 1999), so it would be interesting to identify which compounds are able to react with the ABTS radical cation, and at which rates (**Figure 1.4**). This radical cation can be easily formed by the one electron oxidation of the colourless parent compound. ABTS radical scavenging activities of MBM (meat bone meal) were determined by the method described by Re et al., with slight modification.

#### 1.4.3.3 Oxygen radical absorbance capacity (ORAC) assay

The assay measures the oxidative degradation of the fluorescent molecule (either beta-phycoerythrin or fluorescein) after being mixed with free radical generators, such as azoinitiator as compounds. Azo-initiators are considered to produce the peroxyl radical by heating, which damages the fluorescent molecule, resulting in loss of fluorescence. Antioxidant is able to protect the fluorescent molecule from oxidative degeneration. The degree of protection will be quantified using a fluorometer. Fluorescein (**Figure 1.5**) is currently used most as a fluorescent probe. Equipment that can automatically measure and calculate the capacity is commercially available (Biotek, Roche Diagnostics). The oxygen radical absorbance capacity (ORAC) (**Figure 1.6**) assay has found even broader application for measuring the antioxidant capacity of botanical samples (Cao, Alessio, & Cutler, 1995) and biological samples. A recent but widely accepted analysis, the ORAC (oxygen radical absorbent capacity) method (Cao, Alessio, & Cutler, 1993; Cao & Prior, 1999; Cao, Verdon, Wu, & Prio, 1995) with subsequent development (Huang, Ou, Hampsch-Woodill, Flanagan, & Deemer, 2002; Ou, Hampsch-Woodill, & Prior, 2001) was more sensitive than the iron-based assay (FRAP) (Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002) and may meet those needs. Originally, the ORAC assay developed by Cao et al. used 2, 20-azo-bis (2-amidinopropane) dihydrochloride (AAPH) as the radical source and phycoerythrin as the target molecule. Presently, fluorescein (FL) is the target molecule of choice in this type of experiment, as shown in the **Figure 1.5**.

#### 1.4.3.4 Beta-Carotene Linoleic Acid Assay

The cooxidation of β-carotene in the presence of linoleic acid was used to determine the antioxidant activity of the sample extracts against that of Trolox and a control containing no antioxidants. The relative decrease in absorbance of the treatments compared to that of the control was used as an indicator of antioxidant activity of treatments. In the oil-in-water emulsion system, lipophilic antioxidants are surface active and are oriented in the oil-water interface to provide better protection against oxidation. The β-carotene assay was conducted as described by Hammerschmidt (Hammerschmidt & Pratt, 1978). In brief, this method involved the addition of test samples at varying concentrations to a solution containing β-carotene, linoleic acid, and Tween 40 in chloroform, followed by evaporation of the chloroform under nitrogen flush. An aliquot of the emulsion containing each treatment was added to test tubes containing highly oxygenated distilled water. All treatments were incubated in aluminum-foil-covered test tubes at a certain temperature, and the absorbance was measured using a spectrophotometer.

#### 1.4.3.5 Rancimat assay

The term *oxidative stability* refers to the susceptibility of a food or edible oil to lipid oxidation, which causes rancid odors and flavors. Thus, the oil stability index (OSI; Basic Protocol) is an attempt to predict the length of time before a sample will go rancid. The test can be used to provide information regarding the efficacy of antioxidants. The induction period endpoint is determined by the time it takes for the sample to begin a rapid increase in conductivity. The time required for the sample to reach its induction period endpoint is termed the *Oil Stability Index* (OSI). Samples of the purified linoleic

acid or linolenic acid containing the antioxidants dissolved in methanol at a concentration, and induction periods (IP) at certain temperature and air flow automatically on a Rancimat device. IP is considered to be the time (h) over which the oil is resistant to oxidation with or without the presence of an antioxidant (Bountagkidou, Ordoudi, & Tsimidou, 2010).

### 1.4.4 Methods to identify peptide sequence

#### 1.4.4.1 Protein sequencing

Protein sequencing is a technique to determine the amino acid sequence of a protein, as well as which conformation the protein adopts and the extent to which it is complexed with any non-peptide molecules. Discovering the structures and functions of proteins in living organisms is an important tool for understanding cellular processes and allows drugs that target specific metabolic pathways to be invented more easily.

The two major direct methods of protein sequencing are Edman degradation reaction and the mass spectrometry. The Edman degradation is a very important reaction for protein sequencing because it allows the ordered amino acid composition of a protein to be discovered. Automated Edman sequencers are now in widespread use and are able to sequence peptides up to approximately 50 amino acids long. Digestion into peptide fragments longer than about 50-70 amino acids cannot be sequenced reliably by the Edman degradation. Because of this, long protein chains need to be broken up into small fragments that can then be sequenced individually. Digestion is done either by endopeptidases such as trypsin or pepsin or by chemical reagents such as cyanogen

bromide. Different enzymes give different cleavage patterns, and the overlap between fragments can be used to construct an overall sequence.

In this method, the peptide to be sequenced is adsorbed onto a solid surface—one common substrate is glass fibre coated with polybrene, a cationic polymer. The Edman reagent, phenylisothiocyanate (PITC), is added to the adsorbed peptide, together with a mildly basic buffer solution of 12% trimethylamine. This reacts with the amine group of the *N*-terminal amino acid. The terminal amino acid can then be selectively detached by the addition of anhydrous acid. The derivative then isomerises to give a substituted phenylthiohydantoin that can be washed off and identified by chromatography, and the cycle can be repeated. The efficiency of each step is about 98%, which allows about 50 amino acids to be reliably determined. The other major direct method by which the sequence of a protein can be determined is mass spectrometry. It will be discussed in the

**1.4.4.2.Mass spectrometry**

Liquid chromatography-mass spectrometry (LC-MS) is an effective method for separation and analysis peptide sequencing. Currently, the most sensitive identification of an unknown protein is mass spectrometric peptide mapping (peptide mass search, PMS). Using rapidly expanding protein databases based on nucleotide sequencing, it is possible to identify a protein based on the molecular masses of its constituent peptides if they are determined with sufficient precision. The beauty of the method is that it is not necessary to determine the mass of all peptides; usually six-seven peptides are sufficient for accurate protein identification. This method has been gaining popularity in recent years as new techniques and increasing computing power have facilitated it. Mass spectrometry

can, in principle, sequence any size of protein, but the problem becomes computationally more difficult as the size increases. Peptides are also easier to prepare for mass spectrometry than whole proteins because they are more soluble. One method of delivering the peptides to the spectrometer is electrospray ionization, for which John Bennett Fenn won the Nobel Prize in Chemistry in 2002. The protein is digested by an endoprotease, and the resulting solution is passed through a high-pressure liquid chromatography column. At the end of this column, the solution is sprayed out of a narrow nozzle charged to a high positive potential into the mass spectrometer. The charge on the droplets causes them to fragment until only single ions remain. The peptides are then fragmented, and the mass-to-charge ratios of the fragments are measured. (It is possible to detect which peaks correspond to multiply charged fragments, because these will have auxiliary peaks corresponding to other isotopes—the distance between these other peaks is inversely proportional to the charge on the fragment). The mass spectrum is analyzed by computer and often compared against a database of previously sequenced proteins in order to determine the sequences of the fragments. This process is then repeated with a different digestion enzyme, and the overlaps in the sequences are used to construct a sequence for the protein.

### 1.4.5 Statistical methods: Response Surface Methodology (RSM)

Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes. The most extensive applications of RSM are in the particular situations where several input variables potentially influence some performance measure or quality characteristic

of the process. Thus, the performance measure or quality characteristic is called the *response*. The input variables are sometimes called independent variables, and they are subject to the control of the scientist or engineer. The field of response surface methodology consists of the experimental strategy for exploring the space of the process or independent variables, empirical statistical modeling to develop an appropriate approximating relationship between the yield and the process variables, and optimization methods for finding the values of the process variables that produce desirable values of the response.

A response surface analysis and analysis of variance (ANOVA) were employed to determine the regression coefficients, to determine the statistical significance of the model terms and to fit the mathematical models of the experimental data that aimed to optimize the overall region for both response variables. A second order polynomial model was applied to predict the response variables as given below:

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b^2_1X^2_1+b^2_2X^2_2+b^2_3X^2_3+b_1b_2X_1X_2+b_1b_3X_1X_3+b_2b_3X_2X_3 \qquad (1.4.4.2)$$

where Y is the predicted dependent variable; $b_0$ is a constant that fixes the response at the central point of the experiment; $b_1$, $b_2$, and $b_3$ are the regression coefficients for the linear effect terms; and $b_2$ and $b_3$ are the interaction effect terms, respectively. The adequacy of the model was predicted through regression analysis ($R^2$) and ANOVA analysis. The significance of the regression coefficients was analyzed through a *t*-test, and non-significant coefficients were removed to obtain a reduced model. The relationship

between the independent variables ($X_1$, $X_2$, and $X_3$) and the response variables ($Y_1$ and $Y_2$) was demonstrated by the response surface plots.

## 1.5 Figures and Table

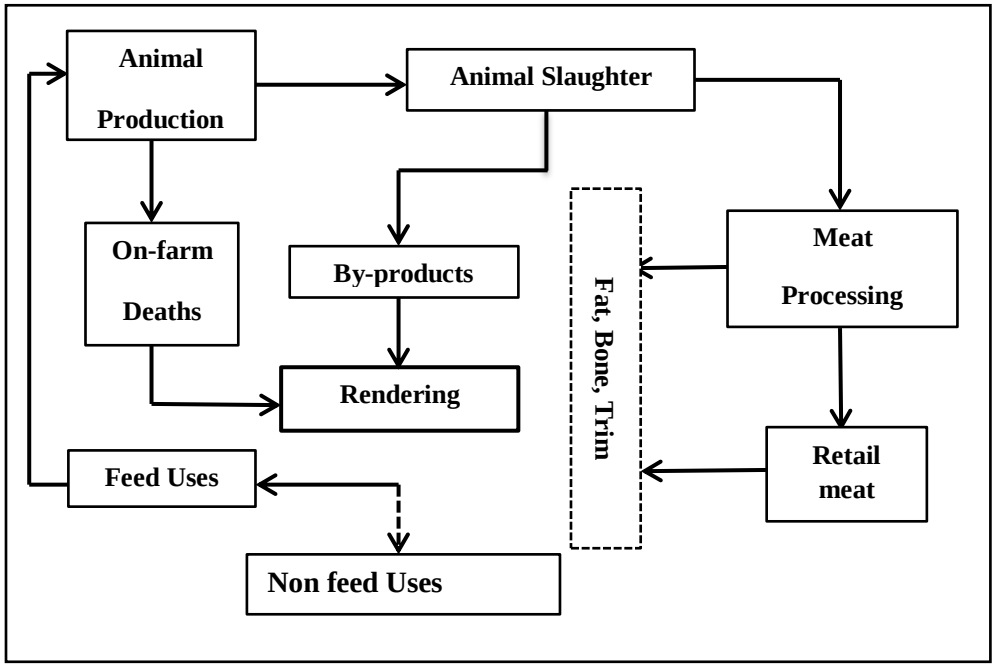



**Figure 1.1:** Interrelationships of Rendering with Animal Agriculture

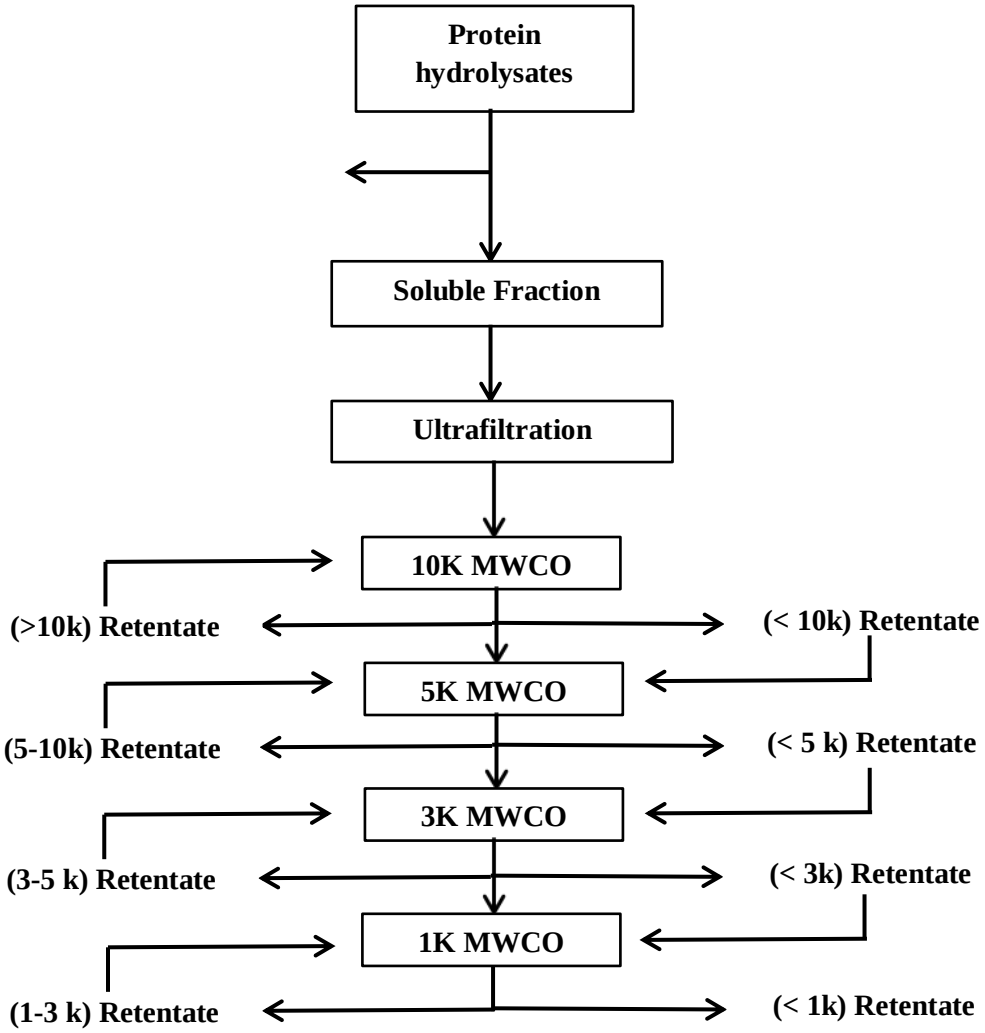



**Figure 1.2:** Process flow diagram of peptide sample preparation.

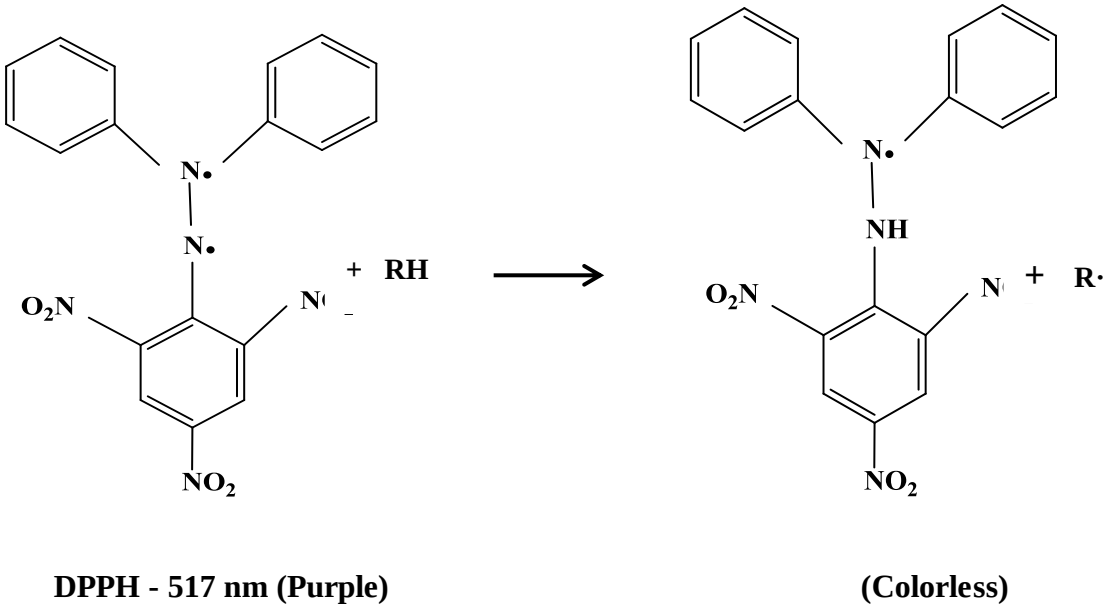



**Figure 1.3:** The scheme for scavenging the DPPH Radical by an antioxidant.

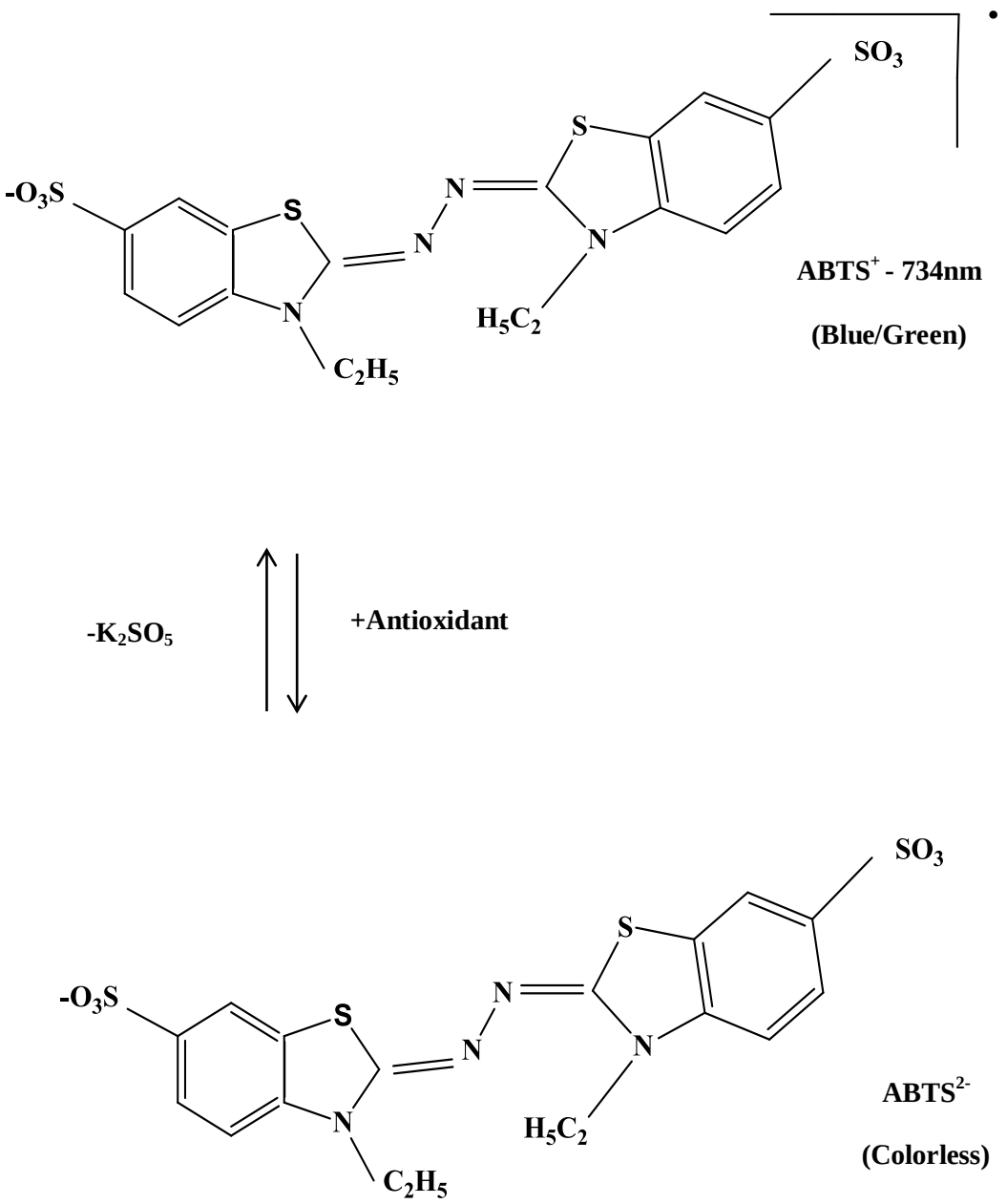
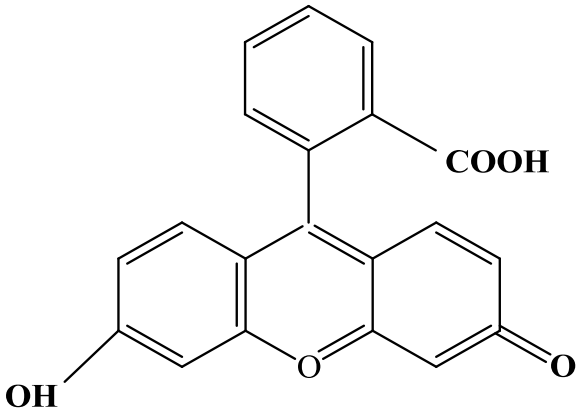
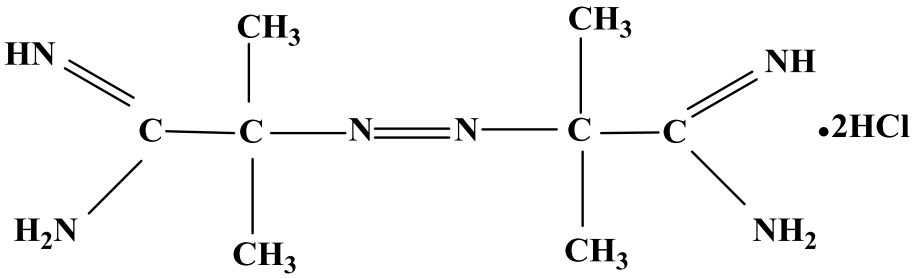


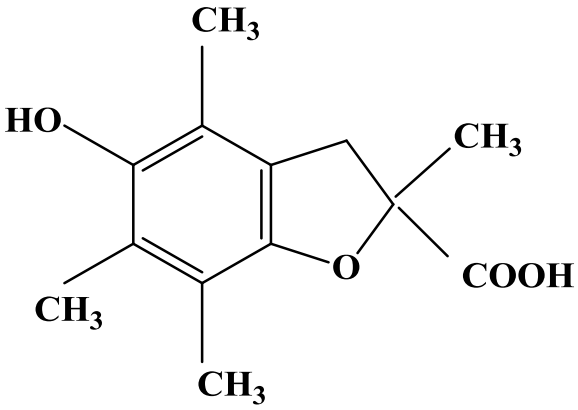

**Figure1. 4:** The Scheme for Scavenging the ABTS Radical by an Antioxidant.




**Fluorescein**




**AAPH**




**Trolox**

**Figure 1.5:** Structure of Fluorescein, AAPH, Trolox.

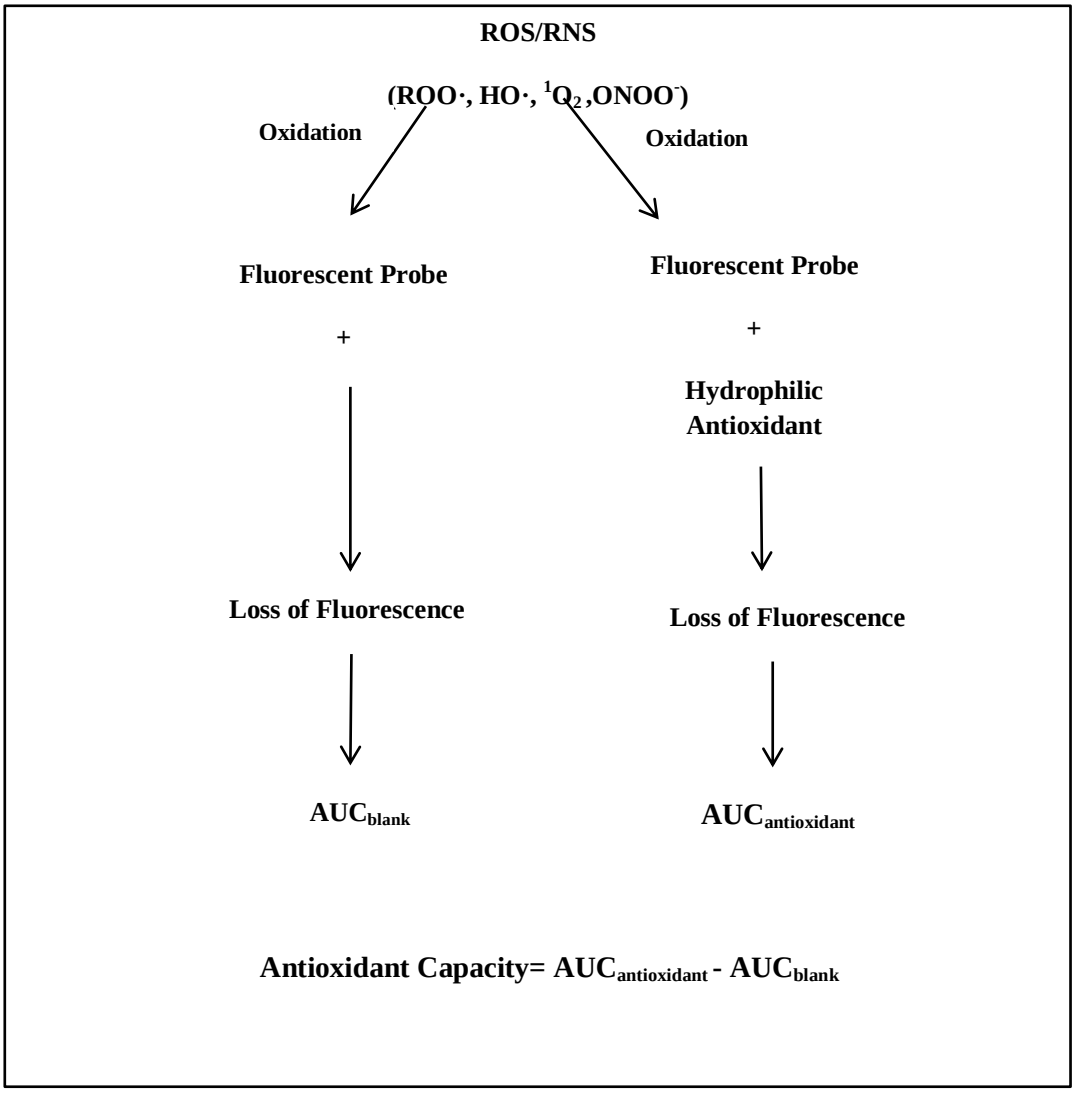



**Figure1.6:** Mechanism of ORAC

**Table 1. 1:** Nutrient composition of animal protein[1]

| Item | Meat and Bone Meal | Blood Meal[2] | Feather Meal | Poultry By-Product Meal |
|---|---|---|---|---|
| Crude Protein,% | 50.4 | 88.9 | 81.0 | 60.0 |
| Fat,% | 10.0 | 1.0 | 7.0 | 13.0 |
| Calcium,% | 10.3 | 0.4 | 0.3 | 3.0 |
| Phosphorus,% | 5.1 | 0.3 | 0.5 | 1.7 |
| $TME_{N,}$ kcal/kg | 2,666[3] | 3,625 | 3,276 | 3.120 |
| Amino Acids | | | | |
| Methionine,% | 0.7 | 0.6 | 0.6 | 1.0 |
| Cystine,% | 0.7 | 0.5 | 4.3 | 1.0 |
| Lysine,% | 2.6 | 7.1 | 2.3 | 3.1 |
| Threonine,% | 1.7 | 3.2 | 3.8 | 2.2 |
| Isoleucine,% | 1.5 | 1.0 | 3.9 | 2.2 |
| Valine,% | 2.4 | 7.3 | 5.9 | 2.9 |
| Tryptophan,% | 0.3 | 1.3 | 0.6 | 0.4 |
| Arginine,% | 3.3 | 3.6 | 5.6 | 3.9 |
| Histidine,% | 1.0 | 3.5 | 0.9 | 1.1 |
| Leucine,% | 3.3 | 10.5 | 6.9 | 4.0 |
| Phenylalanine,% | 1.8 | 5.7 | 3.9 | 2.3 |
| Tyrosine,% | 1.2 | 2.1 | 2.5 | 1.7 |
| Glycine,% | 6.7 | 4.6 | 6.1 | 6.2 |
| Serine,% | 2.2 | 4.3 | 8.5 | 2.7 |

[1]National Research Council,1994
[2]Ring or flash-dried.
[3]Dale,1997
$TME_N$=true metabolizable energy nitrogen corrected.

This table is modified from the National Research Council, 1994.

**Table 1. 2:** 20 amino acids and abbreviations

| Name | Abbreviation |
|---|---|
| Alanine | Ala |
| Arginine | Arg |
| Asparagine | Asn |
| Aspartic Acid | Asp |
| Cysteine | Cys |
| Glutamic Acid | Glu |
| Glutamine | Gln |
| Glycine | Gly |
| Histidine | His |
| Isoleucine | Ile |
| Leucine | Leu |
| Lysine | Lys |
| Methionine | Met |
| Phenylalanine | Phe |
| Proline | Pro |
| Serine | Ser |
| Threonine | Thr |
| Tryptophan | Trp |
| Tyrosine | Tyr |
| Valine | Val |

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# CHAPTER 2

# EVALUATION OF ANTIOXIDANT ACTIVITIES OF ALKALINE HYDROLYSATES OF RENDERING PROTEIN

## 2.1 Abstract

Alkaline hydrolysis is commonly used in the rendering industry, and it has certainly proven to be effective and economical. In this chapter, the antioxidant capacities of three alkaline rendering protein hydrolysates, poultry meal hydrolysates, fish meal hydrolysates, and premium pet meal hydrolysates, are studied. The antioxidant activities of alkaline hydrolysates were evaluated using four methods, including two based on the free radical scavenging capacity, that is, DPPH and ABTS radical scavenging assay. The hydrophobic antioxidant capacities of alkaline hydrolysates were determined by the β-Carotene Linoleic Acid System. The Oxidation Stability Index (OSI) was used to evaluate anti-lipid oxidation ability. The results showed that alkaline hydrolysates had concentration-dependent antioxidant activity and dissolved system-dependent antioxidant activity. The alkaline poultry meal hydrolysates and alkaline fish meal hydrolysates, hydrolysates dissolved in the methanol phase, were shown to have higher antioxidant capacity than in the water phase. Additionally, the alkaline rendering protein hydrolysates dissolved in methanol showed anti-lipid oxidation capacity. For alkaline poultry hydrolysates (300 ppm) protected, the protected lipid mixture can be extended to 9.35h, which was less than protected by 300 ppm BHT (14.4h) and more than protected by 100 ppm (8.4h). However, the β-Carotene Linoleic Acid System and Oxidation Stability

Index (OSI) were not effective analytical methods to evaluate the alkaline premium pet meal hydrolysates.

## 2.2 Materials and Methods.

### 2.2.1 Materials and sample preparation

Poultry meal and fish meal (**Figure 2.1a, b**) were obtained from Valley Proteins, Inc. (Winchester, VA) and premium pet meal (**Figure 2.1c**) was obtained from Carolina By-Products (Ward, SC). These three by-products were kept at a packed state in a deep freezer under -20℃. Poultry meal, labeled as "High antioxidant" and Fish meal labeled as "low antioxidant" in their original packages. , It was possible that poultry meal was already added high amount of synthetic antioxidant and fish meal was added low amount of synthetic antioxidant. In contrast, the "Premium pet meal" from the Carolina By-products was not labeled any words related to antioxidant. More detailed information about the added synthetic antioxidant was confidential to us.

Three samples (poultry meal, fish meal and premium pet meal) were randomly collected from one pack of each of the meals on Sep.3$^{rd}$, 2010, Sep.7$^{th}$, 2010 and Sep.15$^{th}$, 2010. Each time a 15g sample was collected respectively, using the Soxhlet method to remove the lipids independently on the same day. Then the lipid-free sample was placed in the plastic sample bag, sealed, labeled and held in a refrigerator -20 ℃.

### 2.2.2 Methods for characteristic composition of three rendering proteins

#### 2.2.2.1 Moisture determination

Mettler Toledo Halogen Moisture Analyzer (HB43-S) was used to determine moisture in three rendering meals. Each time 2 grams sample were placed in the clean

pan and spread evenly in the pan. Set the instrumental temperature at 105ºC. After 5-15 min, moisture contents were obtained from the LED display. Each sample was tested three times in order to get the average content.

**2.2.2.2 Ash determination**

Porcelain crucibles were dried for approximately 1 hour in a convection oven set at 100°C (AOAC International, 1995). The crucibles were removed from the oven with forceps and placed in desiccators to cool. The dried crucibles were weighed on an analytical balance and weight was recorded as "fired crucible weight." Next, Weigh 5 g samples were weighed. The crucibles were placed in a cold muffle furnace which was turned on set at 525 °C; Samples were left in the in muffle for at least overnight. The muffle furnace was turned off and using long tongs, remove crucibles were removed from muffle furnace and placed in desiccators to cool. Samples were weighed and ash content was calculated as follows:

$$\%\ ash\ (dry\ basis) = \frac{original\ wt\ after\ ashing - tare\ wt\ of\ crucible}{original\ sample\ wt \times dry\ matter\ coefficient} \times 100$$

Where: dry matter coefficient =% solids/100

The results were expressed as the mean ± standard deviations (n=3).

**2.2.2.3 Fat determination**

The Soxhlet method was used to determine fat content (Leefler et al., 2008). Briefly, 10 g sample was wrapped by thimble and placed in a soxhlet extractor. Fat was extracted for 3-4.5h using 250mL hexane as solvent. Hexane in the sample and thimble were first driven off by the hair dryer. Then, the sample and thimble were put into oven (set temperature at 60°C) for 20 minutes in order to dry totally. Weight sample and the

thimble, the loss of the weight were used to determine the fat content. Triplicate data were used to calculate the average and standard deviation. The fat content was calculated as the follows:

$$\%\, fat = \frac{original\ sample\ wt - original\ wt\ after\ soxhelt}{original\ sample\ wt} \times 100$$

**2.2.2.4 Protein (crude) determination**

The Kjeldahl method for total nitrogen determination remains the most universal and accurate method of determining total nitrogen (AOAC, 1995). The total N content of each rendering protein (0.500 g) was determined in duplicate by an automated Buchi Auto Kjeldahl Unit K-370 coupled with a Buchi Kjeldahl Sampler K-371 (Switzerland) according to the manufacturer's instructions. Protein content per 100 g of dry weight was calculated by multiplying the experimental N content by 6.25. (This experiment was conducted in the Agricultural Service Laboratory, Clemson University.)

**2.2.3 Methods for preparing protein hydrolysates**

Samples of poultry meal, fish meal, and premium pet meal (high antioxidant, low antioxidant, and pet grade protein) were extracted with 250 mL hexane using a Soxhlet apparatus for 4h. Then extracts were hydrolyzed by 0.5 N sodium hydroxide solutions, kept in 90 $^{\circ}$C water bathes for 2 hr. After the mixture was adjusted to pH to neutral condition (pH: 7.0-7.5) the sample solution was centrifuged at 4000 rpm/rcf speed for 10 min to separated layers. The supernatant was concentrated by an Efficient Rotary Evaporator (at 55-65 $^{\circ}$C). After that, the extracts were dried in a vacuum dryer at 65 $^{\circ}$C

for 2h. All extracts were stored at -20 °C until used. As evaluated the antioxidant activity capacity, distilled water or methanol was added into the extracts to prepare appropriate solutions with concentration 1.8%, 1.0%, 0.5%, and 0.2%. **Figure 2.3** was the flow chart of preparing alkaline protein hydrolysates.

### 2.2.4 Determination of antioxidant capacity by DPPH assay

The DPPH free radical scavenging activity of antioxidant extracts was measured by the Yamaguchi method with minor modifications. The DPPH radical was a stable free radical in purple in the presence of a hydrogen donor; its absorption strength decreases and is stoichiometric with respect to the number of electrons captured (Delgado-Andrade, Rufián-Henares, & Morales, 2005). In brief, a series concentration of samples (1.8%, 1.0%, and 0.5%) in methanol or distilled water was prepared, and then 0.5 mL sample solutions with concentrations 1.8%, 1.0%, and 0.5% were mixed with 0.5 mL of 0.18 mM DPPH radical solution freshly prepared in methanol, respectively. Methanol and the prepared samples were used as the positive control. After incubation for 1 h at room temperature in the dark, the absorbance of reactant in a 1-cm path length disposable plastic cuvette was measured at 517 nm on a UV-vis spectrometer. The decreasing absorbance of the DPPH solution indicates an increase in DPPH radical scavenging activity. The measurements of DPPH radical scavenging activity were carried out in triplicate. This activity was given as a percentage of DPPH radical scavenging, which was calculated and expressed as DPPH inhibition (%).

### 2.2.5 Determination of antioxidant capacity by ABTS assay

The method was based on the ability of antioxidant molecules to quench long-lived ABTS+, a blue-green chromophore with a characteristic absorption at 734 nm. The antioxidant activity was usually compared with that of Trolox, a water soluble antioxidant standard (Delgado-Andrade, Rufián-Henares, & Morales, 2005). A stable ABTS+ with 2.45 mM potassium persulfate (final concentration) and the mixture were allowed to stand in the darkness at room temperature for 12-16 hours before use. At the beginning of the analysis, the ABTS+ working solution was diluted by methanol from the stock solution. The final reaction mixture contained 2.80 mL ABTS+ solution and 200 μL 1.8%, 1.0%, and 0.5% sample solutions. Its absorbance was measured at 734 nm by a spectrophotometer. The scavenging capacity of each antioxidant was calculated and expressed as ABTS+ inhibition. The measurements of ABTS radical scavenging activity were carried out in triplicate.

### 2.2.6 Free radical scavenging capacity determined by the oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was used for this assay, as it determines the capacity of a sample to scavenge a variety of radicals initially triggered by the carbon-centered radicals generated upon decomposition of AAPH, thus giving a broader idea of the antioxidant capacity of the sample than the ABTS assay and DPPH assay, which measure the capacity to reduce a single type of radical. The ORAC assay was based on the procedure previously described by Delgado-Andrade, Rufián-Henares, & Morales (2004). Free radicals were produced by the radical generator AAPH, which oxidizes the fluorescent compound fluorescein, leading to a loss in fluorescence. All reagents were prepared in

phosphate buffer (pH 7.4 75 mM), and Trolox (40-200 μM) and used as a standard. Each well of a 96-well microplate contained, in a final volume of 250 μL of assay solution, 50 μL of fluorescein (0.75 μM) and 50μL of Trolox/sample (with concentration1.0%,0.5% and 0.2% ) was pre-incubated for 10 min at 37 °C, and then 150 μL of AAPH (200 mM) were added. After the addition of AAPH, the plate was shaken automatically for 3s, and the fluorescence was measured every 1 min for 80 min with emission and excitation wavelengths of 485 and 525 nm, respectively, using a microplate fluorescence reader (Synergy Multi-Detection Microplate Reader; Bio-Tek, Instruments, Inc., Winooski, VT) that was maintained at 37 °C. The ORAC values were calculated as area under the curve (AUC) and expressed as micromoles of Trolox equivalents per gram of sample (μmol of TE/g of sample).

### 2.2.7 Antioxidant assay using β-Carotene Linoleic Acid System (β-CLAMS)

The co-oxidation of β-carotene in the presence of linoleic acid was used to determine the antioxidant activity of the sample extracts against that of Trolox and a control containing no antioxidants (Taga, Miller, & Pratt, 1984). The relative decrease in absorbance of the treatments compared to that of the control was used as an indicator of the antioxidant activity of the treatments. In the oil-in-water emulsion system, lipophilic antioxidants were surface active and oriented in the oil-water interface to provide better protection against oxidation.

In this assay, antioxidant capacity was determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from

linoleic acid oxidation (Dapkevicius et al., 1998). A stock solution of β-carotene–linoleic acid mixture was prepared as follows: 20 mg β-carotene was dissolved in 2 mL of chloroform (HPLC grade). One mL linoleic acid and 6.4 mL Tween 80 were added. Chloroform was completely evaporated using a vacuum evaporator, followed by evaporation of the chloroform under nitrogen flush. Then distilled water was added with vigorous shaking until the volume of the mixture reached 75 mL; 2.8 ml of this reaction mixture were dispersed to test tubes, and 0.2 mL of various concentrations (1.0%, 0.5%, and 0.2%) of the extracts in methanol/water were added, and the emulsion system was incubated for up to 2 h at 60 °C. Then the absorbance was measured at 470 nm using a spectrophotometer. Absorbance values were taken immediately after preparation (i.e., time zero) and then tested 120 min later. The relative decrease in absorbance of the treatments compared to that of the control was used as an indicator of the antioxidant activity of the treatments.

### 2.2.8 Measurement of lipid oxidation induction period (IP)

A Metrohm Rancimat® apparatus (743 Rancimat, Brinkmann Instruments, Inc., Westbury, NY, U.S.) was used to determine induction point or time (IP) as described by Kim et al. (2010). The IP was defined as the time needed to produce volatile products such as aldehydes, acids, and alcohols, as a result of oil or fat oxidation. Alkaline hydrolysates were added at concentration 300 ppm and 100 ppm. For the water phase, emulsifier Tween 40 was used to make the solutions dissolved fully. Commercial antioxidant butylated hydroxytoluene (BHT) was added at 100 ppm and 300 ppm, separately. Air was circulated at 20 L/h flow through three gram samples at 110 + 0.2 °C.

This air was then passed through distilled water, and the conductivity of the water was measured. The induction period (IP) was measured as the intersection of the tangent lines (first derivative) using the software provided with the instrument (EN 14112, 2003; AOCS, 1996; Frankel, 1998).

### 2.2.9 Statistical analyses

Data were reported as the mean ± standard deviation. All statistical analysis was conducted on the SAS V9.1 software for Windows (SAS Institute Inc., Cary, NC, U.S.).

## 2.3 Results and Discussion

### 2.3.1 Hydrophobic antioxidant capacity of alkaline hydrolysates

The antioxidant activities of alkaline hydrolysates were evaluated using three methods, including two methods based on the free radical scavenging capacity--DPPH radical scavenging assay, ABTS radical scavenging assay, and oxygen radical absorbent assay. Radical scavenging capacities were determined using DPPH, ABTS, and ORAC assays. The results were shown in **Tables 2.2** and **2.3**. It was suggested that the alkaline hydrolysates have concentration-dependent antioxidant activity and dissolved system-dependent antioxidant activity.

As shown in **Table 2.2**, antioxidant capacities were expressed as the inhibition (%) of DPPH and ABTS free radicals. As the three rendering protein alkaline hydrolysates—poultry meal alkaline hydrolysates, fish meal alkaline hydrolysates, and pet premium protein hydrolysates—had different compositions, as shown in **Table 2.1** antioxidant

activities of their alkaline hydrolysates were different. At the concentration of 1.8%, the poultry meal alkaline hydrolysates dissolved in methanol had DPPH radical scavenging activity 94.14±0.03% and ABTS radical scavenging activity 91.35±1.26, which represented the highest antioxidant capacity in three different raw materials in the same dissolved system (**Table 2.2)**. In addition, in the other two level concentrations, the poultry meal hydrolysates had the highest antioxidant capacity, followed by premium pet meal and fish meal.

The alkaline hydrolysates had concentration-dependent antioxidant activity, and the higher the concentration of the alkaline hydrolysates was, the higher the antioxidant capacity. In the poultry meal hydrolysates, for both DPPH and ABTS, their antioxidant capacity had similar trends: HM (1.8%) > HM (1.0%) > HM (0.5%) or HW (1.8%) > HW (1.0%) > HW (0.5%) (Their inhibition percentages were 94.14±0.03 > 85.33±0.05 > 50.86±0.12 or 58.85±0.15 > 39.78±0.01 > 19.61±0.03, respectively.) (**Table 2.2**) This trend was not related to their dissolved system, raw materials, or evaluation methods.

For the alkaline poultry meal hydrolysates and alkaline fish meal hydrolysates, hydrolysates dissolved in the methanol phase were shown to have a higher antioxidant capacity than in the water phase. In **Table 2.2,** inhibition of DPPH and ABTS, HM (1.8%) was larger than HW (1.8%); HM (1.0%) was larger than HW (1.0%), HM (0.5%) was larger than HW (0.5%); LM (1.8%) was larger than LW (1.8%); LM (1.0%) was larger than LW (1.0%), and LM (0.5%) was larger than LW (0.5%), all of these differences were significant ($p<0.05$). This means that the antioxidant effective components of

poultry meal and fish meal were polar compounds. In contrast, the premium pet meal in the water phase had higher antioxidant capacity than its methanol solution. In **Table 2.3,** PM (1.8%) was larger than PW (1.8%), PM (1.0%) was larger than PW (1.0%), and PM (0.5%) was larger than PW (0.5%), all of these differences were significant ($p<0.05$).The antioxidant effective components of the premium pet meal were aqueous soluble compounds or nonpolar. As for the oxygen radical absorbent capacity (ORAC), it also had the trend whereby alkaline poultry meal hydrolysates and alkaline fish meal hydrolysates dissolved in the methanol phase were shown to have higher antioxidant capacity than in the water phase and the premium pet meal, reversely **(Table 2.4**).

### 2.3.2 Lipophilic antioxidant capacity of alkaline hydrolysates

The β-carotene linoleic acid method consists of the discoloration (oxidation) of β-carotene induced by oxidative degradation products from linoleic acid. This method estimates the ability of antioxidant compounds of alkaline-rendering protein hydrolysates to scavenge the radical peroxide (LOO•) from linoleic acid, which oxidizes the β-carotene present in an emulsion. The results in **Table 2.4 were shown** that the alkaline poultry meal hydrolysates and alkaline fish meal hydrolysates dissolved in the methanol phase were shown to have higher antioxidant capacity than in the water phase. For the lipophilic antioxidant capacity, HM (1.8%) (172.3±3.56 μ mol of TE/g of sample) was larger than HW (1.8%) (108.8±3.42 μmol of TE/g), which had a similar trend for the Hydrophobic antioxidant. However, the lipophilic antioxidant capacity of alkaline hydrolysates had certain concentration-dependent antioxidant activity. For the poultry hydrolysates, the antioxidant capacity of HW (1.0%) (73.28±3.52 μ mol of TE/g) was

poorer than HW (0.5%) (81.06±6.74 μ mol of TE/g). Therefore, alkaline hydrolysates showed lipophilic antioxidant capacity.

### 2.3.3 Oxidation Stability Index (OSI)

The oxidation stability of samples was evaluated with the commercial appliance Rancimat 743 applying the accelerated oxidation test (Rancimat test) (**Figure 2.4**) (Das et al., 2009). Seeking to compare the oxidation stability of the lipid mixture (the lipid mixture was removed from the rendering materials) with different alkaline hydrolysates added, the induction period (IP) was studied (**Figure 2.5, Figure 2.6**). Synthetic antioxidant BHT was used as a comparison control group. It was determined (**Figure 2.5**) that the basic lipid mixture induction period was 5.4h, which means that without the lipid, the mixture can be stored for 5.4h in the accelerated oxidation test without any antioxidant. After the addition of 300 ppm of different alkaline hydrolysates, the protected lipid mixture induction period was changed. For the alkaline poultry hydrolysates (300 ppm) protected, the protected lipid mixture can be extended to 9.35h, which was less than that protected by 300 ppm BHT (14.4h) and more than that protected by 100 ppm (8.4h) (**Figure 2.5**). Therefore, the alkaline rendering protein hydrolysates dissolved in methanol showed anti-lipid oxidation capacity.

## 2.4 Conclusion

The antioxidant capacities of three alkaline rendering protein hydrolysates were studied: poultry meal hydrolysates, fish meal hydrolysates, and premium pet meal hydrolysates. The antioxidant activities of alkaline hydrolysates were evaluated using five methods, including two based on free radical scavenging capacity, that is, DPPH and ABTS radical scavenging assay. The hydrophobic antioxidant capacities of alkaline hydrolysates were determined by the β-Carotene Linoleic Acid System. The Oxidation Stability Index (OSI) was used to evaluate anti lipid oxidation ability. The results showed that alkaline hydrolysates had concentration-dependent antioxidant activity and dissolved system-dependent antioxidant activity. The alkaline poultry meal hydrolysates and alkaline fish meal hydrolysates, hydrolysates dissolved in the methanol phase, were shown to have higher antioxidant capacity than in the water phase. Additionally, the alkaline rendering protein hydrolysates dissolved in methanol showed anti-lipid oxidation capacity. For alkaline poultry hydrolysates (300 ppm) protected, the protected lipid mixture can be extended to 9.35 h, which was less than that protected by 300 ppm BHT (14.4 h) and more than that protected by 100 ppm (8.4 h). However, the β-Carotene Linoleic Acid System and the Oxidation Stability Index (OSI) were not effective to evaluate the alkaline premium pet meal hydrolysates. Therefore, further investigation was suggested to study the antioxidant compounds in premium pet meal.

## 2.5 Figures and Tables

**A: Poultry Meal**

**B: Fish Meal**

**C: Premium Pet Meal**

**Figure 2. 1:** Three rendering proteins: Poultry meal, Fish meal and Premium pet meal

**Table 2. 1:** Crude proteins, moisture, ash, and fat content of three rendering proteins.

| Analysis (%) Content | Premium pet meal protein sample | Poultry meal sample (high antioxidant) | Fish meal sample (low antioxidant ) |
|---|---|---|---|
| Protein (%) | 65.68±0.75 | 52.43±0.75 | 50[1]minimum |
| Moisture (%) | 5.84±0.03 | 4.94±0.07 | 4.68±0.01 |
| Ash (%) | 14.44±0.02 | 16.5±0.02 | 16.61±0.19 |
| Fat (%) | 12.05±0.11 | 13.56±0.13 | 11.85±0.07 |
| **[1]This data was from Valley Protein Inc. Technical Data Sheet (Appendix B)<br>Data reported as analysis content (%) and expressed as means ± standard deviation (n=3).** | | | |

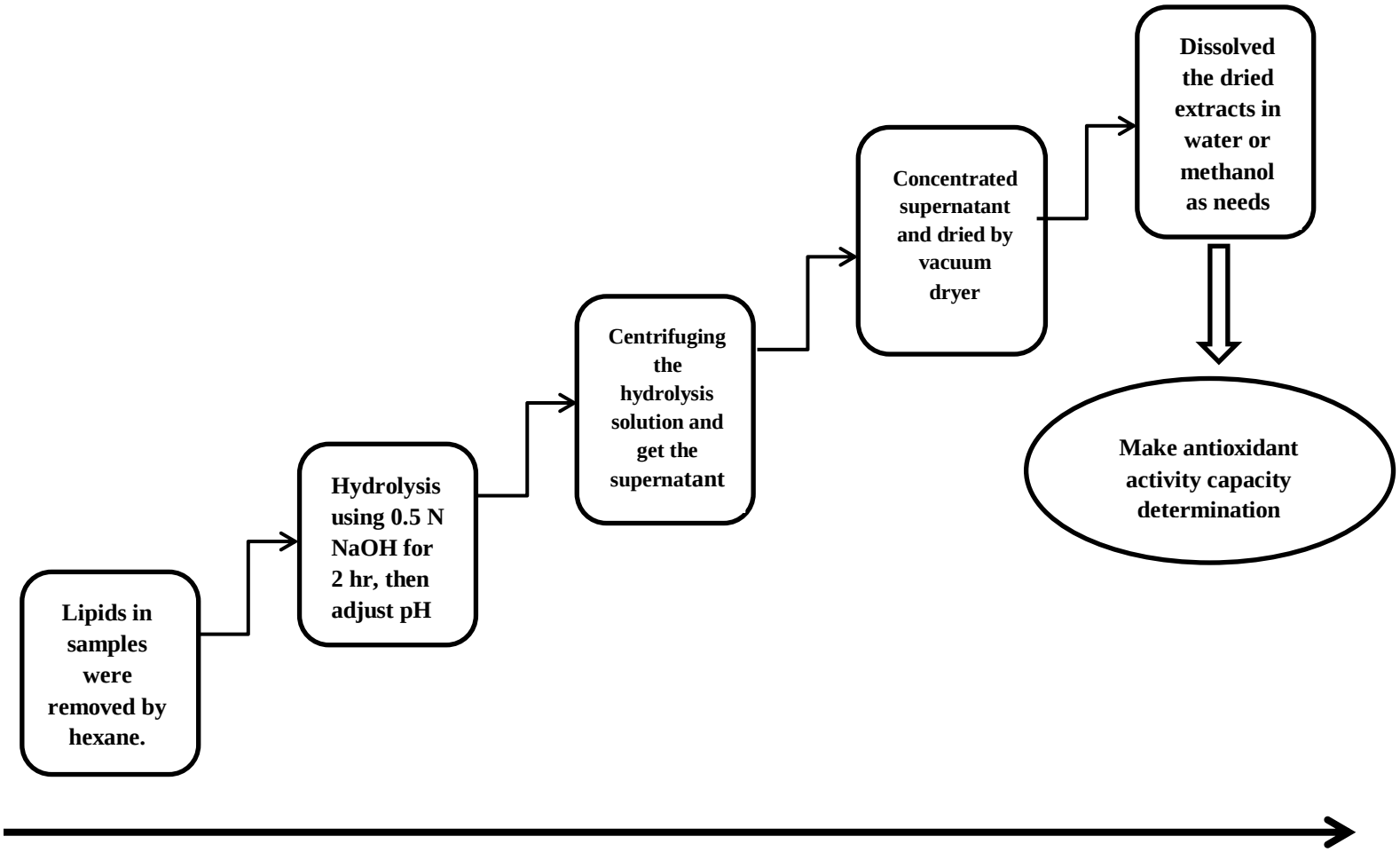
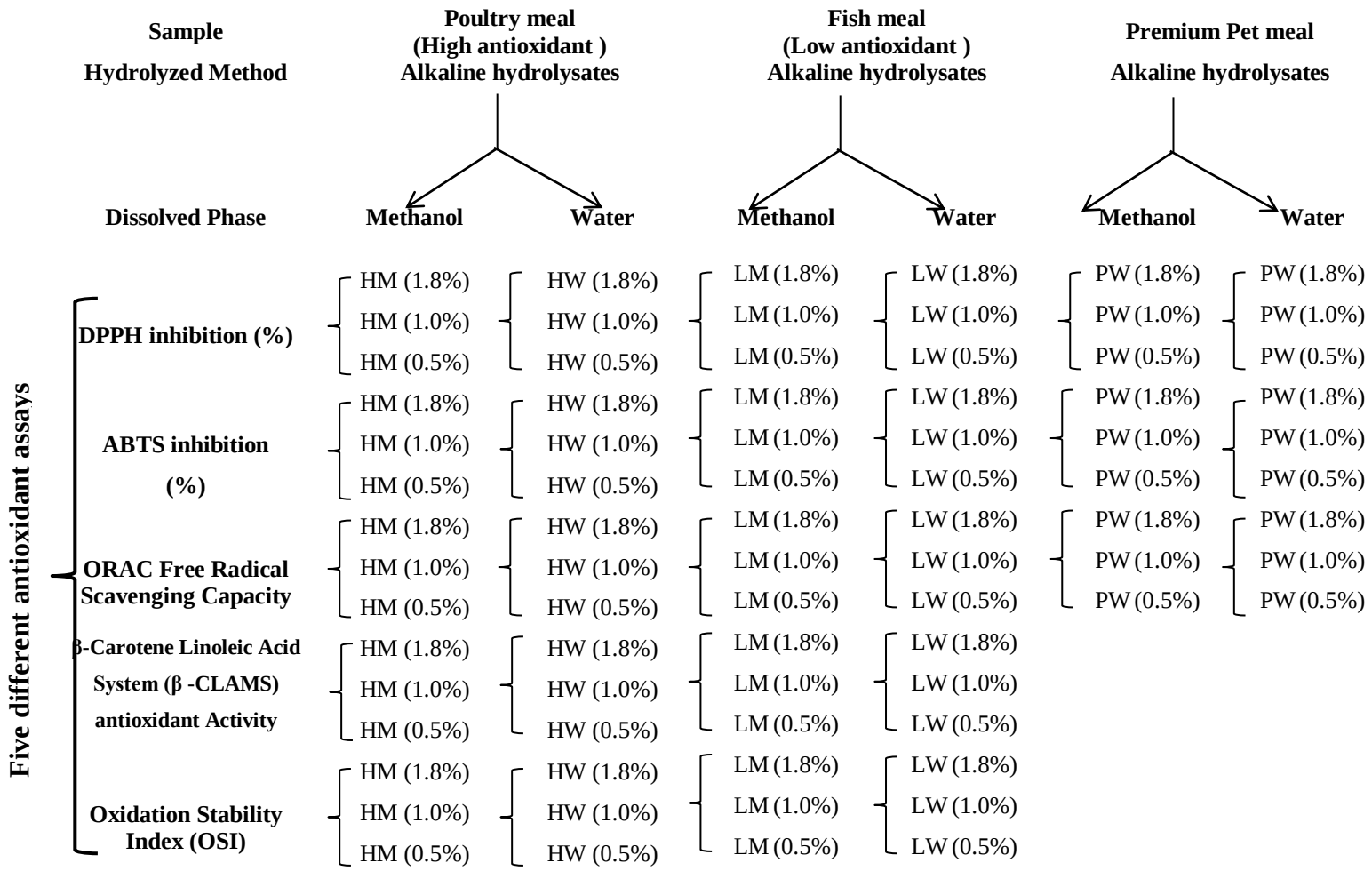



**Figure 2. 2:** Alkaline hydrolysates preparation flow chart.




(**HM**: High antioxidant Poultry meal sample dissolved in methanol； **HW**: High antioxidant Poultry meal sample dissolved in distilled water; **LM**: Low antioxidant Fish meal sample dissolved in methanol； **LW**: Low antioxidant Fish meal sample dissolved in distilled water; **PM**: Premium pet meal sample dissolved in methanol; **PW**: Premium pet meal sample dissolved in distilled water.)

**Figure 2. 3:** General View of all the assays evaluated in chapter three.

**Table 2. 2:** Power (% inhibition) of Scavenging DPPH and ABTS+ Radicals by Three Rendering Protein Hydrolysates

| Materials | | Dissolved system | DPPH inhibition (%) | ABTS inhibition (%) |
|---|---|---|---|---|
| **Poultry meal sample** | HM (1.8%) | Methanol | 94.14±0.03 | 91.35±1.26 |
| | HM (1.0%) | Methanol | 85.33±0.05 | 88.73±0.13 |
| | HM (0.5%) | Methanol | 50.86±0.12 | 32.37±1.13 |
| | HW (1.8%) | Water | 58.85±0.15 | 52.56±0.05 |
| | HW (1.0%) | Water | 39.78±0.01 | 41.38±1.12 |
| | HW (0.5%) | Water | 19.61±0.03 | 24.60±0.06 |
| **Fish Meal** | LM (1.8%) | Methanol | 68.45±0.05 | 71.25±0.12 |
| | LM (1.0%) | Methanol | 51.26±0.04 | 60.22±1.25 |
| | LM (0.5%) | Methanol | 31.26±0.06 | 34.51±0.03 |
| | LW (1.8%) | Water | 43.00±0.13 | 46.72±0.42 |
| | LW (1.0%) | Water | 21.32±1.02 | 28.15±0.13 |
| | LW (0.5%) | Water | 15.06±0.08 | 18.34±0.12 |
| **Premium pet Meal** | PM (1.8%) | Methanol | 51.28±0.13 | 45.32±0.08 |
| | PM (1.0%) | Methanol | 39.40±0.15 | 31.52±0.24 |
| | PM (0.5%) | Methanol | 21.61±0.26 | 18.92±0.56 |
| | PW (1.8%) | Water | 80.56±2.32 | 86.60±1.24 |
| | PW (1.0%) | Water | 64.28±1.20 | 69.45±1.05 |
| | PW (0.5%) | Water | 41.27±0.03 | 46.00±0.07 |

**HM**: High antioxidant Poultry meal sample dissolved in methanol; **HW**: High antioxidant Poultry meal sample dissolved in distilled water; **LM**: Low antioxidant Fish meal sample dissolved in methanol; **LW**: Low antioxidant Fish meal sample dissolved in distilled water; **PM**: Premium pet meal sample dissolved in methanol; **PW**: Premium pet meal sample dissolved in distilled water.

Data reported as inhibition of total free radical scavenging and expressed as means ± standard deviations (n=3)

**Table 2. 3:** ORAC Free Radical Scavenging Capacity of Three Rendering Protein Hydrolysates.

| Materials | | Dissolved system | ORAC( µmol of TE/g of sample) |
|---|---|---|---|
| **Poultry meal sample** | HM (1.0%) | Methanol | 402.12±3.52 |
| | HM (0.5%) | Methanol | 582.46±3.64 |
| | HM (0.2%) | Methanol | 820.75±3.02 |
| | HW (1.0%) | Water | 363.65±1.23 |
| | HW (0.5%) | Water | 371.96±2.36 |
| | HW (0.2%) | Water | 476.45±7.82 |
| **Fish Meal sample** | LM (1.0%) | Methanol | 220.92±3.56 |
| | LM (0.5%) | Methanol | 297.90±13.35 |
| | LM (0.2%) | Methanol | 363.68±23.64 |
| | LW (1.0%) | Water | 201.28±12.37 |
| | LW (0.5%) | Water | 256.21±15.35 |
| | LW (0.2%) | Water | 290.85±3.31 |
| **Premium pet Meal sample** | PM (1.0%) | Methanol | 132.78±2.64 |
| | PM (0.5%) | Methanol | 193.45±15.67 |
| | PM (0.2%) | Methanol | 210.56±23.32 |
| | PW (1.0%) | Water | 190.14±12.43 |
| | PW (0.5%) | Water | 245.62±18.56 |
| | PW (0.2%) | Water | 353.52±13.27 |

**HM**: High antioxidant Poultry meal sample dissolved in methanol; **HW**: High antioxidant Poultry meal sample dissolved in distilled water; **LM**: Low antioxidant Fish meal sample dissolved in methanol; **LW**: Low antioxidant Fish meal sample dissolved in distilled water; **PM**: Premium pet meal sample dissolved in methanol; **PW**: Premium pet meal sample dissolved in distilled water.

The ORAC values were calculated as area under the curve (AUC) and expressed as micromoles of Trolox equivalents per gram of sample (µmol of TE/g of sample). (Means ± standard deviations (n=3)

**Table 2. 4** β -Carotene Linoleic Acid System (β -CLASMS) Antioxidant Activity of Three Protein Hydrolysates

| **Materials** | | **Dissolved system** | **(β-CLAMS µmol of TE/g of sample)** |
|---|---|---|---|
| **Poultry meal sample** | HM (1.0%) | Methanol | 172.3±3.56 |
| | HM (0.5%) | Methanol | 108.8±3.42 |
| | HM (0.2%) | Methanol | 101.3±5.63 |
| | HW (1.0%) | Water | 73.28±3.52 |
| | HW (0.5%) | Water | 81.06±6.74 |
| | HW (0.2%) | Water | 109.2±5.32 |
| **Fish Meal sample** | LM (1.0%) | Methanol | 44.92±3.14 |
| | LM (0.5%) | Methanol | 51.00±1.53 |
| | LM (0.2%) | Methanol | 66.20±3.42 |
| | LW (1.0%) | Water | 82.72±5.67 |
| | LW (0.5%) | Water | 75.16±7.20 |
| | LW (0.2%) | Water | 99.07±11.32 |

**HM**: High antioxidant Poultry meal sample dissolved in methanol; **HW**: High antioxidant Poultry meal sample dissolved in distilled water; **LM**: Low antioxidant Fish meal sample dissolved in methanol; **LW**: Low antioxidant Fish meal sample dissolved in distilled water.

The β-CLAMS values were calculated by inhibition of the linoleic acid oxidized and expressed as micromoles of Trolox equivalents per gram of sample (µmol of TE/g of sample), (Means ± standard deviations (n=3)

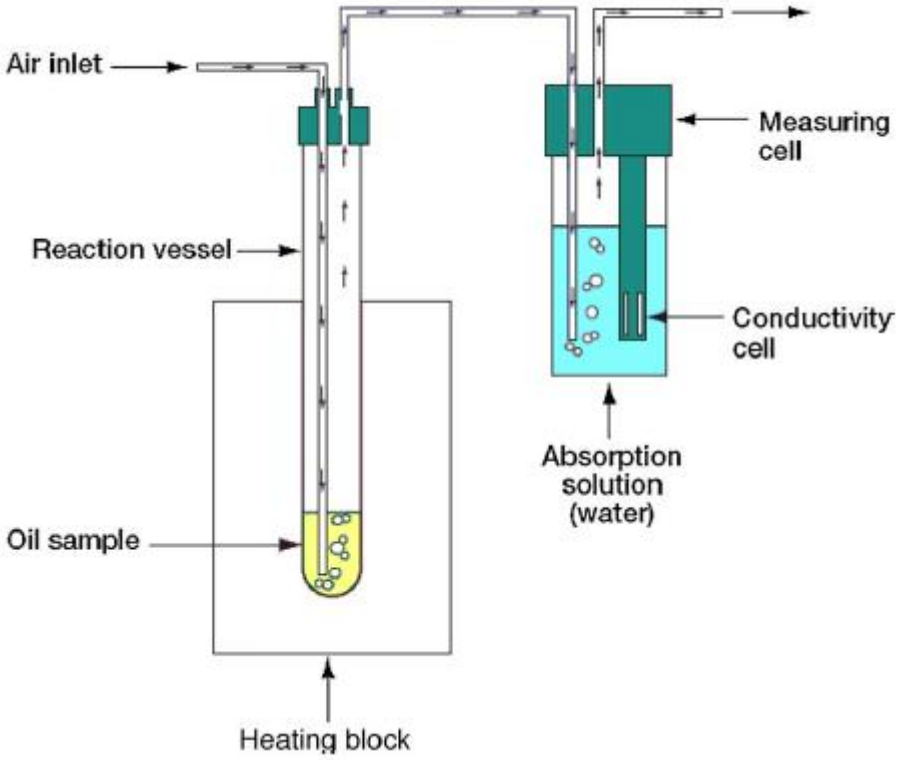
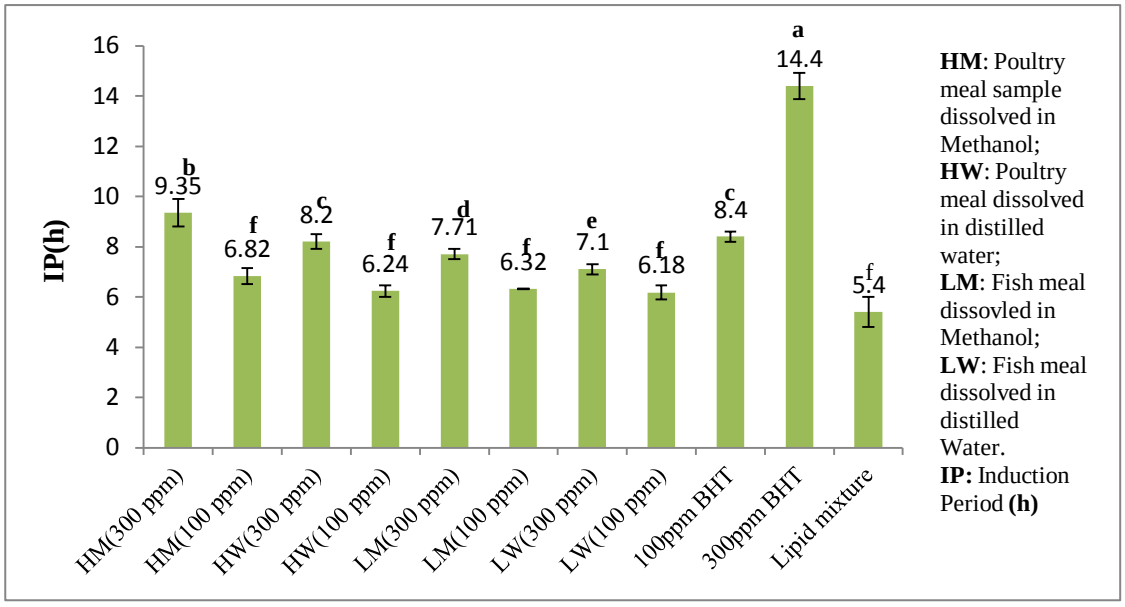



**Figure 2. 4:** Schematic of Rancimat test (modified from Das LM, et al, 2009)



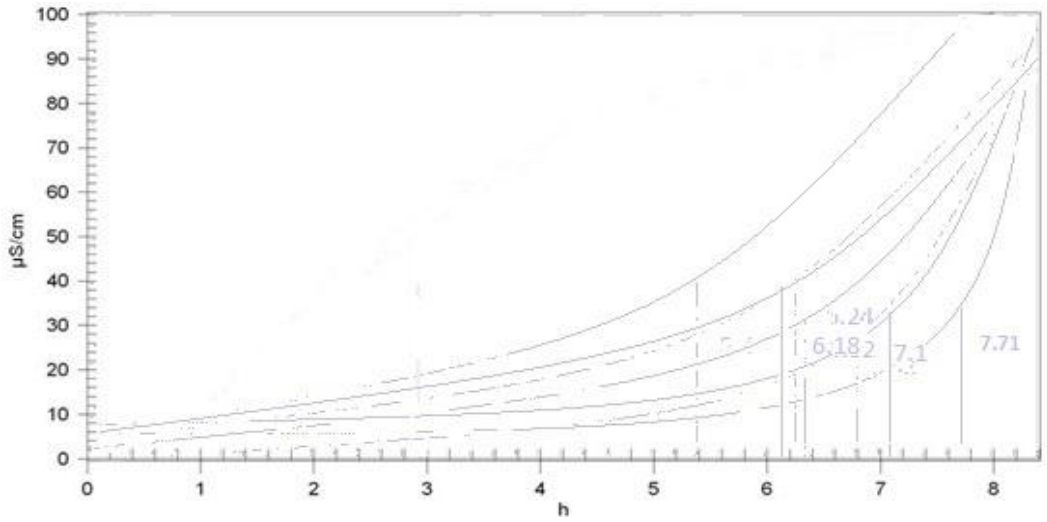




**Figure 2. 5:** Result of Rancimat 743.**Figure 2.5a**: lipid mixture (5.4h), HM (100 ppm)(6.82h), HW ( 300 ppm )(8.2h), HW ( 100 ppm )(6.24h), LM ( 300 ppm )(7.71h), LM( 100 ppm ) (6.32h), LW ( 300 ppm ) (7.1h), LW ( 100 ppm ) (6.18h) 300ppm BHT (14.4h), 100ppm BHT(8.4h) induction time (t) $^{a\text{-}f}$ Different letters indicate significant differences between groups ($p < 0.05$).; **Figure.2.5: b**: Graphical determination of different hydrolysates induction time (t) by the tangent method (automatically evaluation) from equipment Rancimat 743.

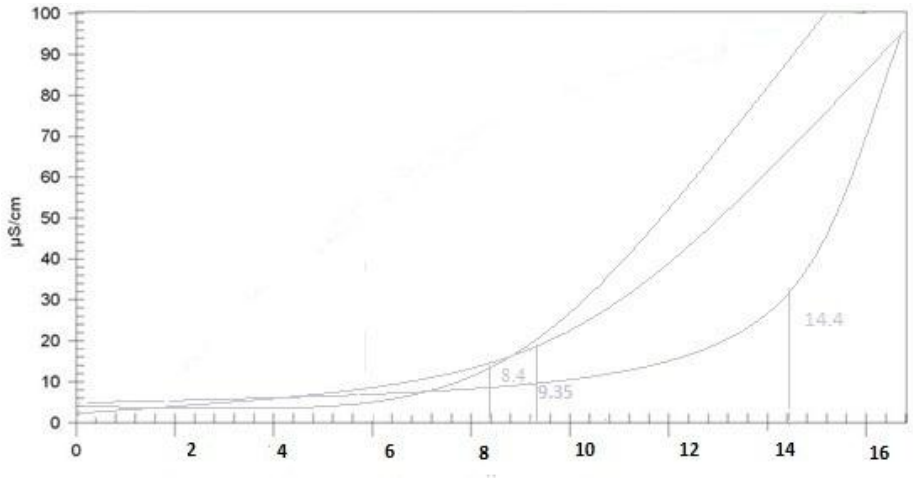



**Figure2. 6:** Graphical determination of Rancimat 743.Graphical determination of 300ppm BHT (14.4h), 100ppm BHT(8.4h), 300ppm HW( 300 ppm ) (9.35h) induction time (h) from equipment Rancimat 743.

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# CHAPTER 3

# EVALUATION OF ANTIOXIDANT ACTIVITY OF PROTEOLYTIC HYDROLYSATE OF PET MEAL AND IDENTIFICATION OF ANTIOXIDANT PEPTIDE SEQUENCE

## 3.1 Abstract

The aim of this study was to investigate antioxidant peptides from proteolytic hydrolysis of premium pet meal using response surface optimization, ultrafiltration, and LC-MS. Abilities of scavenging ABT·+ free radicals and degree of hydrolysis were employed as the indicators of response surface methodology. A second order polynomial model produced a satisfactory fitting of the experimental data with regard to total antioxidant capacity ($R^2$ = 0.9863, $p$ < 0.0001) and degree of hydrolysis ($R^2$ = 0.9450, $p$ < 0.0001). The optimum hydrolysis conditions for TAC were temperature 50.2 ℃, E/S (w/w) 2.59, time 5.64h. Under this condition, the DH was 37.73%. The 1K-3k fractions (10mg/mL) showed the highest total antioxidant capacity (TAC) yield with 1.96 ± 0.12 mM Trolox, followed by the < 1K fraction with 1.82±0.08 mM Trolox. Two antioxidant peptides derived from premium pet meal were identified using LC-MS.

## 3.2 Materials and Methods

### 3.2.1 Materials and chemicals

In premium pet meal (Carolina By-Products (CBP)), the by-product was kept in a packed state in a deep freezer under -20 ℃. Premium pet meals applied in this part of the study were used same collected sample method as it shown in the chapter three. **Table 3.1** lists the typical chemical composition of the sample. Protease from the bovine pancreas (≥ 5 units/mg solid) (From Sigma-Aldrich Co. St. Louis, MO); ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid); Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; trichloroacetic acid; and Folin & Ciocalteu's phenol reagent were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). All the other chemicals used were of an analytical grade.

### 3.2.2 Methods of sample preparation

Before hydrolysis, lipids of the commercial premium pet meal were removed by hexane with the traditional Soxhelt apparatus. To digest the premium pet meal protein, the protease was used according to the hydrolysis guidelines from the manufacturer with slight modification. The delipidated protein was homogenized in a 0.75 mM PBS (phosphate buffer solution) at pH = 7.5 and hydrolyzed by the individual proteases at different concentrations (w/w, enzyme/substrate) and temperatures. During the process of hydrolysis, the reaction mixture was adjusted to pH = 7.5 using 0.5 M NaOH or 0.5 M HCI during hydrolysis. The enzymatic reactions were terminated by heating the mixtures for 15 min at 80 ℃. After the mixture was adjusted to pH = 7.5 the mixture sample solution was centrifuged at 4000 rpm/rcf speed to establish separated layers, and the

soluble fraction was then filtered through Whatman 4 filtration paper for further fractionation. The flow chart of this chapter was shown in **Figure 3.1**.

### 3.2.3 Total antioxidant activity determination

For the ABTS assay, the method outlined by Re et al. (1999) was used with several modifications. The ABTS radical cation (ABTS•+) was generated by reaction of aqueous ABTS solution (7 mM) and 2.45 mM concentration of a potassium persulfate solution. The mixture was held in the dark at room temperature for 12-14 h before being used. Then it was diluted with distilled water in order to obtain an absorbance of 1.5 ± 0.03 units at 734 nm using a UV–vis spectrophotometer. Diluted hydrolysis solution (20 $\mu$L) or reference substances (Trolox) were allowed to react with 280 µL of the resulting blue-green ABTS radical solution in a dark condition upon a 96-well plate. The mixture was incubated for 60 min at room temperature, and the absorbance at 734 nm was measured with a microplate reader. Then the decrease of absorbance was calculated. The standard curve was linear between 0–0.15mM Trolox (final concentration). The results were expressed as TAC (total antioxidant capacity in mM Trolox equiv.) value. Absorbance response (y) of Trolox ($y = 372.49x + 8.3188$, $R^2 = 0.9927$) was in linearity within the concentration range. The activity of extracts was estimated at least by three different concentrations. All the tests were performed in triplicate.

### 3.2.4 Measurement of degree of hydrolysis of protein hydrolysates

Degree of hydrolysis was measured by a slightly modified method from Haslaniza et al. (8). One mL of enzymatic hydrolysate solution was put into a test tube, and 2 mL of 0.3 M trichloroacetic acid was added. This was mixed with a Voltex mixer, and the

mixed solution was placed for 20 min at an ambient temperature. The solution was filtered with a filter paper (Whatmans No. 4). Then 75 µL of the filtered solution, 1 mL of 0.5 N NaOH, and 0.125 mL of 2.0 N Folin & Ciocalteu's phenol reagent were added and mixed. The mixture reacted for 15 min at room temperature. The insoluble materials of the reacted solution were removed by using a filter paper. The absorbance of the filtered solution was measured at 750 nm, and degree of hydrolysis was calculated by the following formula.

$$\text{Degree of hydrolysis (\%)} = \frac{Dt-D0}{Dmax-D0} \times 100$$

Where $D_0$ was the absorbance of unhydrolyzed sample; $D_{t\ was}$ the absorbance of the sample hydrolyzed for t hours; and $D_{max}$ was the absorbance of the sample hydrolyzed with 4 mL 6 N HCl into 0.1 g sample for 12 hours at 110 ℃.

**3.2.5 Cutoff peptide by ultrafiltration (UF) membranes**

Under the optimum hydrolysis condition (antioxidant activity capacity was selected as the indicator), the temperature 50℃, E/S (w/w) 2.53, and the hydrolysis time 5.28 h, triplicate premium pet meal proteolytic hydrolysates (10mg/mL)were rehydrated then fractionated consecutively through UF membranes having MWCO of 10k, 5k ,3k and 1 kDa (Millipore, Inc., Marlborough, MA). The ultrafiltration unit Cells 8010 and MWCO membranes were assembled, installed, and prepared according to manufacturer manuals. Prior to operation, make sure the holder were flushed and cleaned according to the procedure described in the user guide. Before we conducted experiment make sure the holder, cartridges, and tubes were drained completely. Then the feed tubes were placed in prepared feed solution (10mg/mL proteolytic hydrolysates solutions) at the room

temperature. Turned on the nitrogen tank valve counterclockwise to the open position and make sure the pressure was not greater than 296 kPa (40psi). Under this nitrogen flush pressure, 5-10 mL targeted retentate (1st time was the molecular weight less than 10kDa) was obtained. After this, feeding solution and distilled water were used to wash the cell unit and tubes. Then, changed the membrane and repeat the above steps until all the desired retentates (proteolytic hydrolysates fractions less than 5kDa, 3kDa and 1kDa) were obtained. Different retentates were collected and labeled each time. This allowed optimization of the separation efficiency of peptides and estimation of yields for preparing sufficient samples for evaluation.

### 3.2.6 Identification of peptides sequence by LC-MS

Identification of peptides in the two most active fractions from the UFF separation (MW < 1000) were carried out by an Agilent 6100 quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA) connected with electrospray ionization modes. The operating parameters of the ESI source, including nebulizing gas pressure, drying gas pressure, drying gas temperature, needle voltage, and shield voltage, were all optimized with regard to maximum signal intensity of pseudo-molecular ion by flow injection of 10 μL samples. The samples were separated on a 150mm" 75mm" 3.5μm" columnRaw spectra were processed using Agilent MS solution software. A precursor ion scan of the purified antioxidant fraction was used to identify unique peptide mass, which was then fragmented using low energy collision induced dissociation to reveal the peptide fragment for de novo sequencing. The molecular mass and proteolytic peptide sequencing of the peptide from protease digestions was done on negative ion mode on the

electrospray ionization–mass spectrometry (ESI–MS). Each peptide sample was directly injected into the electrospray ion source and sampled for 10 min using a mobile phase of 98% water and 2% acetonitrile containing 0.1% formic acid at a flow rate of 4 mL/min. The mass scanning range was from 100 to 1000 msu at 1 second with a 0.1-s inter-scan delay in continuum mode.

### 3.2.7 Response Surface Methodology ---Central Composite Design

Central composite design is an experimental design, useful in response surface methodology, for building a second order (quadratic) model for the response variable without needing to use a complete three-level factorial experiment. In our design, three variables - temperature ( ℃), enzyme to substrate ratio (E/S), hydrolysis time (h) were coded in **Table 3.2**.

**Figure 3.2** shows a CCD for 3 design variables; the design involves $2^N$ factorial points, 2*N* axial points and n (n≥3) central point. In our experiment, there were three factors, temperature (℃), enzyme to substrate ratio (E/S), hydrolysis time (h). Therefore, the group numbers we used (**Table 3.3**) involved first-order ($2^3$) factorial points, 2×3 axial points, and n≥3 (6 replicates central point's replicates were used in this process). Then we converted these coded 20 groups into real hydrolysis conditions and applied them in the real hydrolysis experiments. Two hydrolysis indicators (Degree of hydrolysis and Total antioxidant capacity) were evaluated on these 20 groups. Matlab V7.0 (Mathworks, Portola Valley, CA) software was used to conduct the optimization analyses.

### 3.2.8 Statistical analysis

MATLAB V7.0 (Mathworks, Portola Valley, CA) software was used to conduct the statistical analyses. Results for antioxidant capacity and degree of hydrolysis were expressed as means ± standard deviations (n=3). A response surface analysis and analysis of variance (ANOVA) was employed to determine the regression coefficients, to ascertain the statistical significance of the model terms and to fit the mathematical models of the experimental data that aimed to optimize the overall region for both response variables. A second order polynomial model was applied to predict the response variables as given below:

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b^2_1X^2_1+b^2_2X^2_2+b^2_3X^2_3+b_1b_2X_1X_2+b_1b_3X_1X_3+b_2b_3X_2X_3$$

where Y was the predicted dependent variable; $b_0$ was a constant that fixes the response at the central point of the experiment; $b_1$, $b_2$, and $b_3$ were the regression coefficients for the linear effect terms; and $b_2$ and $b_3$ were the interaction effect terms, respectively. The adequacy of the model was predicted through the regression analysis ($R^2$) and the ANOVA analysis ($p < 0.05$). The significance of the regression coefficients was analyzed through a *t*-test, and non-significant coefficients were removed to obtain a reduced model. The relationship between the independent variables ($X_1$, $X_2$, and $X_3$) and the response variables ($Y_1$ and $Y_2$) was demonstrated by the response surface plots.

## 3.3 Results and Discussion

### 3.3.1 Fitting the response surface models

The experimental values for total antioxidant capacity (TAC, $Y_1$) and hydrolysis degree ($Y_2$) were employed in a multiple regression analysis performed using response surface analysis to fit the second-order polynomial equations. In this study, the values

obtained experimentally for both response variables were near the predicted values (**Table 3.3**), indicating a satisfactory model (**Table 3.4**). Coefficients of determination ($R^2$), probability values (*p*), and lack-of-fit values for both dependent variables were given in **Table 3**. The quality of fit to the second-order polynomial models was established based on the coefficients of determination ($R^2$), which were 0.9863 and 0.9450 for TAC and degree of hydrolysis, respectively. The ‘‘fitness’’ of the model was studied through the *p*-value of less than 0.05, which indicated the suitability of the model to predict variation accurately.

### 3.3.2 Influence of the hydrolysis parameters on total antioxidant capacity and hydrolysis degree

For antioxidant activity capacity, temperature and enzyme-to-substrate (E/S) ratio were significant ($p < 0.05$) in three linear effects ($X_1$, $X_2$, and $X_3$), two quadratic effects ($X_{12}$ and $X_{22}$), and three interactive effects ($X_1X_2$, $X_1X_3$, and $X_2X_3$). The predicted model obtained for $Y_1$ was given in **Table 3.3**. **Figure 3.3** shows profiles of the response surface plots showing the combined effect of temperature and E/S (a), time and temperature (b), and E/S and time (c) on the total antioxidant capacity of the hydrolysis solution. Through MATLAB software, we can obtain the best optimized condition, which consists of a temperature of 49.7 ℃, E/S (w/w) of 2.53, and time of 5.28 h. Under this condition, the TCA was 1.549 mM, equal to Trolox.

For hydrolysis degree, the trend was very similar to those for total antioxidant capacity, temperature, and enzyme-to-substrate ratio, which were significant ($p < 0.05$) in three linear effects ($X_1$, $X_2$, and $X_3$), two quadratic effects ($X_{12}$ and $X_{22}$), and three

interactive effects ($X_1X_2$, $X_1X_3$, and $X_2X_3$). The predicted model obtained for $Y_2$, given in **Table 3.3** and **Figure 3.4**, shows the response surface plots with the combined effect of temperature and E/S (a), time and temperature (b), E/S and time (c) on the degree of hydrolysis solution. Through MATLAB software, we were able to get the best optimized condition that included temperature 50.2 ℃, E/S (w/w) 2.59, and time 5.64h. Under this condition, the DH was 37.73%.

### 3.3.3 The fractionation of antioxidant peptides by ultrafiltration

The five antioxidant peptide fractions from preotolytic hydrolysates were prepared and designated >10K (10K retentate), 5-10K (10K permeate-5K retentate), 3-5K (5K permeate-3K retentate), 1-3K (3K permeate-1K retentate), and < 1K (1K permeate). Their antioxidant capacities were evaluated in **Table 3.5**. The 1K-3k fraction showed the highest total antioxidant capacity (TAC) yield with 1.96 ± 0.12 mM Trolox, followed by the < 1K fraction with 1.82±0.08 mM Trolox, and the >10K showed the lowest TCA by 1.55 mM Trolox. Guo et al. (2009) reported that the low molecular weight fraction (< 1k) from the protease N-hydrolysate of royal jelly proteins had the greatest antioxidant activity. Park et al. (2001) also noted that < 5k hydrolysate from egg yolk protein had the highest antioxidant activity. Soluble peptides fractionated from proteolytic protein hydrolysates into various molecular mass ranges between < 1000 and 10000 Da have different antioxidant capacity and amino acid compositions from their parent proteins and from each other. Because the total antioxidant capacity (TAC) of fraction 1-3KDa (1.96 ± 0.12 mM Trolox) was not much higher than fraction < 1KDa with 1.82±0.28 mM Trolox,

and the fraction <1KDa was easier for identification of the peptide sequence, the fraction < 1KDa was selected for further investigation.

### 3.3.4 Identification of antioxidant peptide sequence by LC-MS

#### 3.3.4 1. De novo peptide sequencing analysis

De novo peptide sequencing was an indispensable tool in drug discovery work, for example, from bioactive compounds, the need to identify peptides to be used as therapeutic peptide drug. Peptide sequencing was initially achieved by means of the Edman degradation method (Edman, 1950; Heinrikson, 1984) which was time consuming, low throughput and poor sensitivity when compare to mass spectrometric approach. Tandem mass spectrometry is now a widely used approach for peptide sequencing. Briefly, a precursor peptide ion, usually protonated with one or multiple charges generated from soft electrospray ionization method is selected and collided with non-reactive molecule in gas phase at low kinetic energy (10eV to 50 eV). The collision facilitated defragmentation produced daughter ions can be used to identify amino acid sequence. A typical LC-MS or LC-MS/MS spectrum with fragmentation chemistry, however, doesn't illustrate peptide sequence directly; it is the relationship between successive ions containing the sequence information. Peptide sequencing is achieved by software using iterative calculations. These software either looking for continuous sequence at N- terminal and/or C-terminal fragments differing by just one amino acid. In this manner, the sequence can be obtained from the complete series of fragments, such as one reported by Hernández-Ledesma (2005). However, this is often unlikely to occur. Indeed, the peptide usually does not break at every conjunction of amino acids.

Especially for small peptide (within six amino acid residues), it is difficult to break into several fragments with good intensities. If the collision energy is increased, the peptide may break at locations which were not a conjunction of amino acids and this make peptide sequencing very difficult. According to Bruni (2005), each peak in the spectrum can be due to the presence of one of the various classical types of fragment (a-ion, b-ion, c-ion, x-ion, y-ion, z-ion, **Figure 3.5**). Each ion has a weight depending on its components, according to the following criteria: a-ions, molecular mass of amino acid residue plus -27; b-ions, plus 1; c-ions, plus 18; x-ions, plus 45; y-ions, plus 19; z-ions, plus 3, y-$NH_3$-ions, plus 37; y-$H_2O$-ions, plus 37; etc.

In **Figure 3.7** and **Figure3.8**, the Biolynx peptide sequencer (a kind of software used to identified the peptide sequence) failed to obtain the amino acid sequences of each peptide, but every mass signals and their corresponding fragmentation spectra obtained by LC-MS could be matched with single peptide fragment by manual calculation. In fact, the analysis gave two sequences, Leu-Thr-Cys or Iso-Thr-Cys, and the molecular weight was 335 Da (**Figure 3.7**). Because the de novo approach cannot distinguish between isoleucine and leucine (Altelaara, 2012), there were two sequences for one molecular weight.

Notice that a non-unique or a partial peptide sequence may still be considered as satisfactory if the emphasis was on the identification of the protein from database blasting. In our case, Leu-Thr-Cys was part of chicken muscle protein (165kDa), which was a structural constituent of the myofibrillar band in chicken striated muscle (Noguchi et al., 1992). It was regret that we cannot find the database of rendering meal, but the

sequence of peptide from poultry should be similar.

### 3.3.4 2. Identification of four peaks LC-MS fragmentation.

For peptide fraction< 1KDa identification, four major peaks were separated by liquid chromatography into four peaks (Peak 1, Peak 2, Peak 3, and Peak 4). In **Figure 3.6**, the UV at absorbance 251 nm was shown for four major peaks at retention time, $R_1$ = 1.328 min, $R_2$ = 1.661 min, $R_3$ = 1.898 min, and $R_4$ = 1.898 min, respectively. The Peak 1 precursor ion scan mass spectrum was shown in **Figure 3.7** In the MS spectrum, major charged ions with m/z were 113.1(Da), 229.0(Da), 317(Da), 186.9(Da), 165.0(Da), and 204.9(Da), according to fragment intensity from high to low. Similarly, in the peak 2 MS spectrum, major charged ions were 113.1(Da), 131.2(Da), 198.0(Da), and 103(Da). Consequently, the Peak 1 fraction was analyzed for amino acid sequencing, and the result was shown in **Figure 3.8**.

Protonation at the amine was followed by cleavage at any link of the peptide. In our study, in Peak 1, the spectrum indicates that 317(Da) ion was the peptide fragment minus one $H_2O$ molecule weight, $b_1$-ion (113.1Da, both in the Peak 1 spectrum and the Peak spectrum): from N-terminus to the broken marked bond; $x_1$-ion (131.2 Da, in the Peak 2 spectrum): from C-terminus to the broken marked bond; $y_1$-ion (103Da, in the Peak 2 spectrum): from C-terminus to the broken marked bond; 186.9 Da ion: from N-terminus to the broken marked bond and fragment of the $x_1$-ion; 204.9 Da ion: 186.9Da ion plus one $H_2O$; 214.0 Da ions: from N-terminus to the broken marked bond and fragment of the $y_1$-ion; other ions were collision residues (**Figure 3.8**). Therefore, this antioxidative peptide had an amino acid sequence of Leu-Thr-Cys or Iso-Thr-Cys, and

the molecular weight was 335 Da (**Figure 3.8**). Because the de novo approach cannot distinguish between isoleucine and leucine (Altelaara, 2012), there were two sequences for one molecular weight.

In the same way, in Peak 4, the spectrum indicates that 239.9.4(Da) ion was the peptide fragment minus one $H_2O$ molecule weight, and 257.9(Da) was the whole peptide molecule weight. $b_2$ -ion (137 Da in the Peak 4 spectrum, **Figure 3.9**): from N-terminus to the broken marked bond; $a_2$-ion (109 Da, in the Peak 4 spectrum, **Figure 3.9**): from C-terminus to the broken marked bond. Therefore, this antioxidative peptide had an amino acid sequence of His-Cys, and the molecular weight was 257.9Da (**Figure 3.10**).

Peptide Leu-Thr-Cys was part of chicken muscle protein (165kDa), which was a structural constituent of the myofibrillar band in chicken striated muscle (Noguchi et al., 1992). Elvin A. Kabat (1992) noted that peptide Leu-Thr-Cys was part of the chicken (shark) heavy chain FR1, which has anti-DNP (antidinitrophenyl) bioactive benefits. Chen Zhinan et al. (2006) reported that peptide Leu-Thr-Cys-Ser-Leu-Asn-Asp-Ser-Ala-Thr-Glu-Val can be used as target for medicaments for treating tumor recurrence, metastasis, rheumatoid arthritis, and osteoarthritis. However, the high radical scavenging activity has never been reported. In the free radical-mediated lipid peroxidation system, antioxidant activity of peptide or protein was dependent on molecular size and chemical properties such as hydrophobicity and electron transferring ability of amino acid residues in the sequence. Guo et al. (2009) also reported 29 antioxidative peptides that were isolated from royal jelly proteins hydrolysate and found that some small peptides with 2-4 amino acid residues (Phe-Lys, Ala-Leu, Arg-Tyr, Tyr-Tyr, and Lys-Asn-Tyr-Pro) had

strong antioxidant activity. Additionally, the lower the molecular weight, the higher their chance to cross the intestinal barrier and exert biological effects (Kumar, Nazeer, & Jaiganesh, 2011). Among 20 nature amino acids, Trp showed the highest antioxidant activity among all amino acids, followed by Tyr and Met and then Cys, His, and Phe (Shen, 2010). Other amino acids (Arg, Asn, Gln, Asp, Pro, Ala, Val, Lys, Ile, Ser, Thr, Leu, Glu, and Gly) did not exhibit antioxidant activity (Hernandez-Ledesma, 2007 and 2005). Our result indicated that the antioxidant activity of the peptides isolated from proteolytic hydrolysates depends on their special amino acid sequences. In the present work, the size of the peptide was very small (335 Da and 257.9 Da), and the presence of hydrophobic amino acid residue Leu has a significant role in the positive inhibition of free radicals, which was different from what Shengwen Shen (2010) reported. Accordingly, further detailed studies on purified peptide in regard to the characterization of meat-derived peptide in structural properties and antioxidative activity in cells and animal will be needed.

## 3.4 Conclusion

In conclusion, our study reports, for the first time, the antioxidant properties of peptides derived from premium pet meal protein enzymatic hydrolysis. Response surface optimization was reliable for obtaining the most desirable bioactive peptides with potent bioactive activities. The sequences of the antioxidant peptides identified in the present study were novel. Developing value-added peptide antioxidants demonstrated that some commercial hydrolysates of rendering products possessed antioxidant activities, which may enable them to be used as natural antioxidants as substitutes of some commercial antioxidants such as BHT, vitamin E, and ethoxyquin, which are widely used in formulated animal feeds.

## 3.5 Figures and Tables

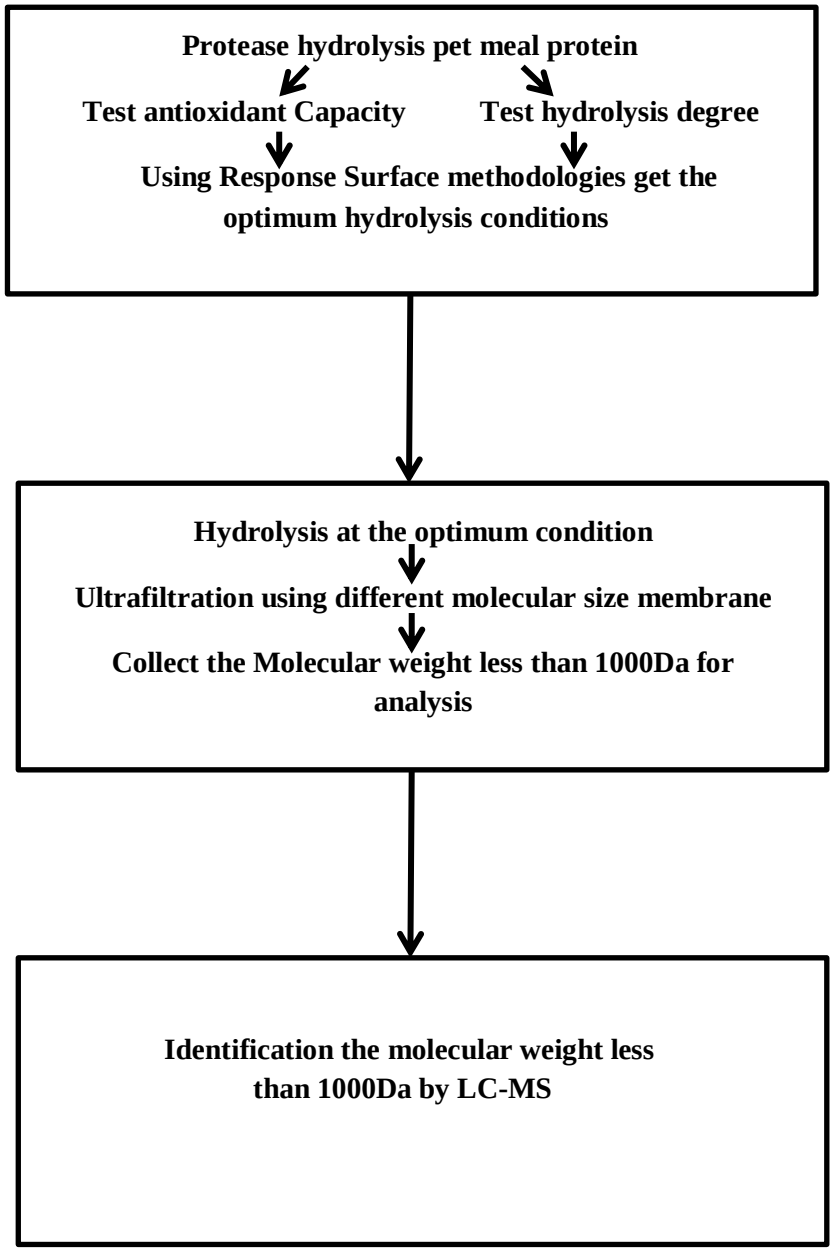



**Figure 3. 1:** Flow chart of the chapter three.

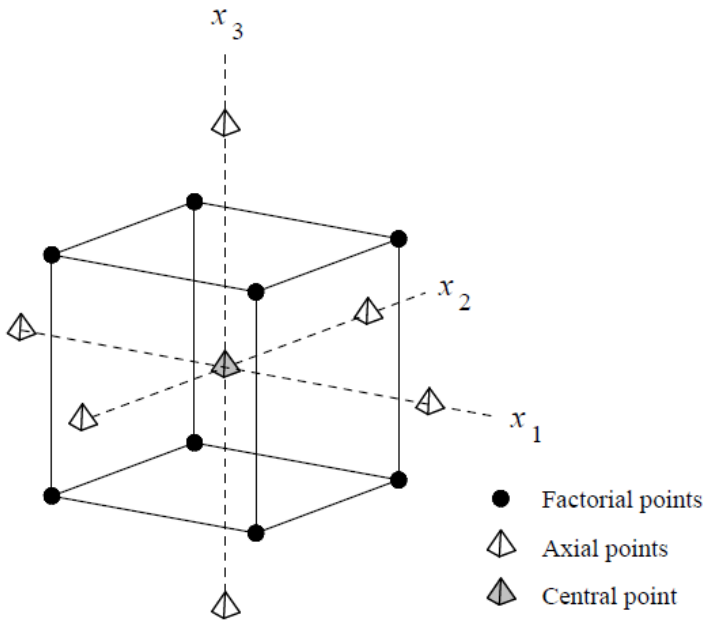



**Figure 3. 2:** Central composition design for 3 variables and 2 levels

**Table 3. 1:** Chemical composition of premium pet meal.

| Analysis (%, wet basis) Content | Premium pet meal |
|---|---|
| Moisture (%) | 5.84±0.03 |
| Ash (%) | 14.44±0.02 |
| Fat (%) | 12.05±0.11 |
| Protein (%) | 65.68±0.75 |

Values represent means of 3 determinations ± standard deviations

**Table 3. 2:** Independent variables and their coded and actual values used for optimization.

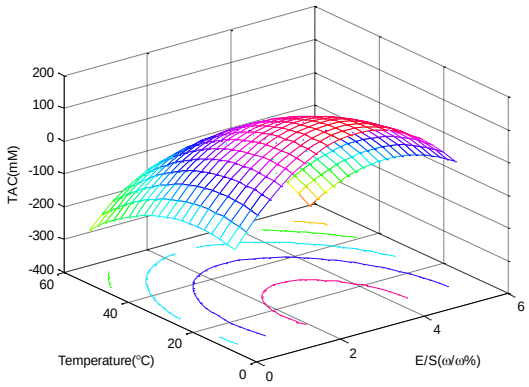
| Independent variables | Units | Symbol | Coded levels | | | | |
|---|---|---|---|---|---|---|---|
| | | | -1 | 0 | 1 | Axial (-α) | Axial (α) |
| Temperature | ◦C | $X_1$ | 25 | 35 | 45 | 10 | 60 |
| E/S-Enzyme /Substrate ratio(ω/ ω) | % | $X_2$ | 0.5 | 1.5 | 2.5 | 0.25 | 6 |
| Hydrolysis time | h | $X_3$ | 4 | 5 | 6 | 3 | 7 |

**Table 3. 3:** Three-factor central composite design used for RSM with experimental and predicted values for the independent variables.

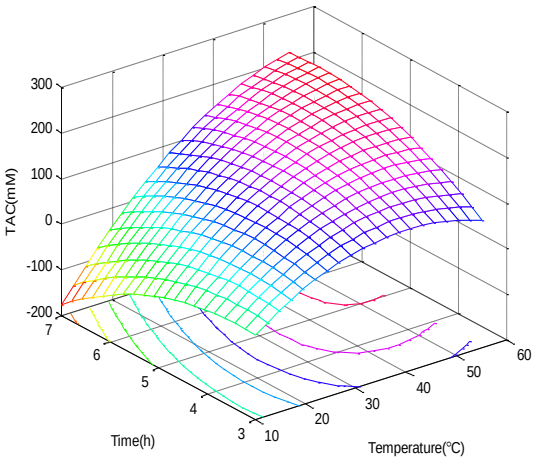
| Standard order | Factor 1 ($X_1$) Temperature (°C) | Factor2 ($X_2$) E/S ratio (ω/ω %) | Factor 3 ($X_3$) Hydrolysis time (h) | Response 1 (Y1) TAC(Total antioxidant capacity )( mM ) | | Response2 (Y2) DE (Degree hydrolysis %) | |
|---|---|---|---|---|---|---|---|
| | | | | Experimental | Predicted | Experimental | Predicted |
| Corner Points | | | | | | | |
| 1 | -1 | -1 | -1 | 0.73 | 0.73 | 13.23 | 12.75 |
| 2 | 1 | -1 | -1 | 0.93 | 0.87 | 18.77 | 20.76 |
| 3 | -1 | 1 | -1 | 1.32 | 1.30 | 15.69 | 20.32 |
| 4 | 1 | 1 | -1 | 1.43 | 1.46 | 32.15 | 32.05 |
| 5 | -1 | -1 | 1 | 0.66 | 0.64 | 9.230 | 10.89 |
| 6 | 1 | -1 | 1 | 1.00 | 1.04 | 22.31 | 19.23 |
| 7 | -1 | 1 | 1 | 1.02 | 1.09 | 25.23 | 24.80 |
| 8 | 1 | 1 | 1 | 1.49 | 1.50 | 34.82 | 36.85 |
| Axial Points | | | | | | | |
| 9 | -1.68 | 0 | 0 | 0.93 | 0.92 | 18.62 | 16.16 |
| 10 | 1.68 | 0 | 0 | 1.39 | 1.38 | 32.77 | 33.02 |
| 11 | 0 | -1.68 | 0 | 0.52 | 0.55 | 7.69 | 8.39 |
| 12 | 0 | 1.68 | 0 | 1.46 | 1.41 | 32.46 | 29.56 |
| 13 | 0 | 0 | -1.68 | 1.15 | 1.17 | 26.07 | 23.24 |
| 14 | 0 | 0 | 1.68 | 1.18 | 1.13 | 25.08 | 25.71 |
| Center Point Replicates | | | | | | | |
| 15 | 0 | 0 | 0 | 1.32 | 1.33 | 29.85 | 30.22 |
| 16 | 0 | 0 | 0 | 1.32 | 1.33 | 29.23 | 30.22 |
| 17 | 0 | 0 | 0 | 1.36 | 1.33 | 31.08 | 30.22 |
| 18 | 0 | 0 | 0 | 1.36 | 1.33 | 32.00 | 30.22 |
| 19 | 0 | 0 | 0 | 1.32 | 1.33 | 29.08 | 30.22 |
| 20 | 0 | 0 | 0 | 1.29 | 1.33 | 29.69 | 30.22 |

**Table 3. 4:** Statistical parameters calculated for central composite experimental design.

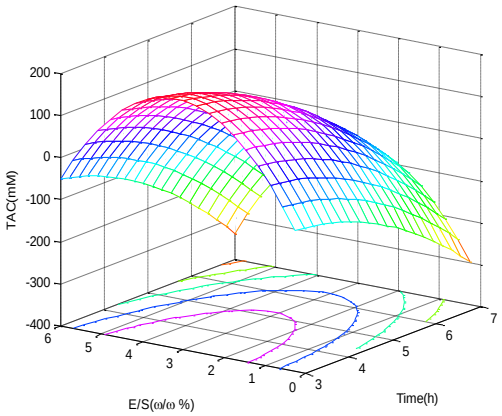
| Regression coefficient | 2nd order polynomial equation | $R^2$ | Regression (*p* -value) |
|---|---|---|---|
| TAC-total antioxidant capacity($Y_1$) | $Y=-204.7491+6.1167X_1+199.5356X_2+69.2262X_3-0.1397X^2_1-30.3392X^2_2-12.6642X^2_3-0.8450X_1X_2-2.4454X_1X_3+1.6737X_2X_3$ | 0.9863 | 0.0001 |
| Hydrolysis Degree($Y_2$) | $Y= -66.0473+1.6880X_1+7.0946X_2+ 18.4491X_3-0.0196X^2_1 -3.9862X^2_2-2.0375X^2_3+ 0.0929X_1X_2+1.5827X_1X_3+ 0.0083X_2X_3$ | 0.9450 | 0.0001 |




**a**

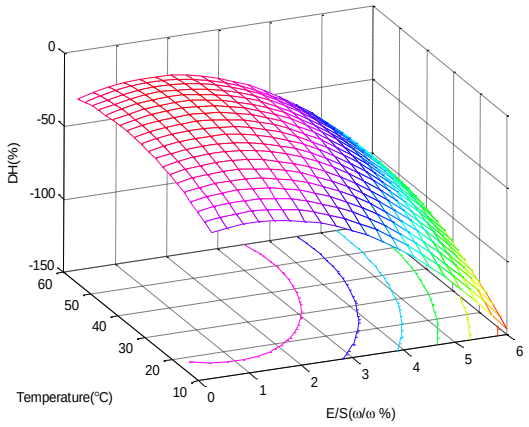



**b**

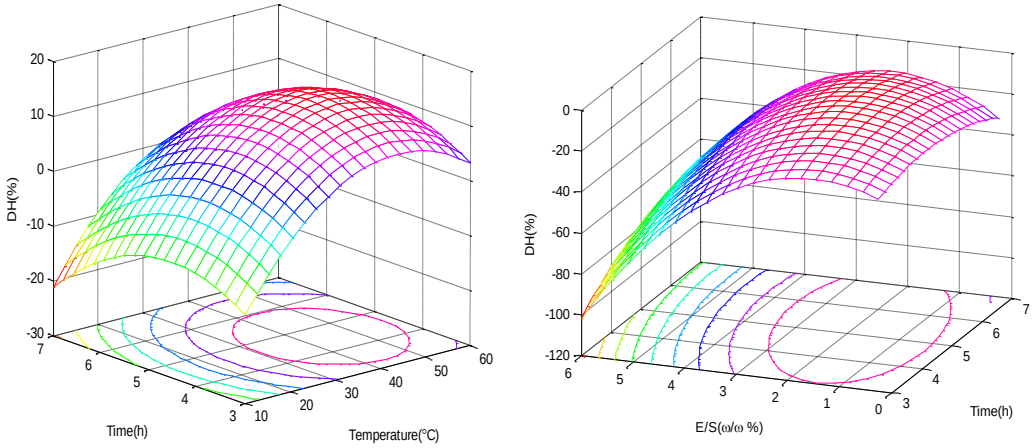



**c**

**Figure 3. 3:** Response surface plot showing the combined effect of temperature and E/S (a), time and temperature (b), E/S and time (c) on the total antioxidant capacity of hydrolysis solution.




**a**




**b** **c**

**Figure 3. 4:** Response surface plot showing the combined effect of temperature and E/S (a), time and temperature (b), E/S and time (c) on the hydrolysis degree.

**Figure 3. 5:** Antioxidant activity of peptide fractions (10mg/mL) of hydrolysates (DH of 37.12%) with different molecule weight.

| Fraction(k) | Total antioxidant capacity(TAC)(mM) |
|---|---|
| >10k | $1.55\pm0.13^{e}$ |
| 5k-10k | $1.69\pm0.04^{d}$ |
| 3k-5K | $1.73\pm0.06^{c}$ |
| 1k-3k | $1.96\pm0.12^{a}$ |
| <1k | $1.82\pm0.08^{b}$ |

[a-e] Different letters in each row denote significant differences between each fraction ($p < 0.05$) Total antioxidant capacity was expressed as the means ±deviations(n=3)

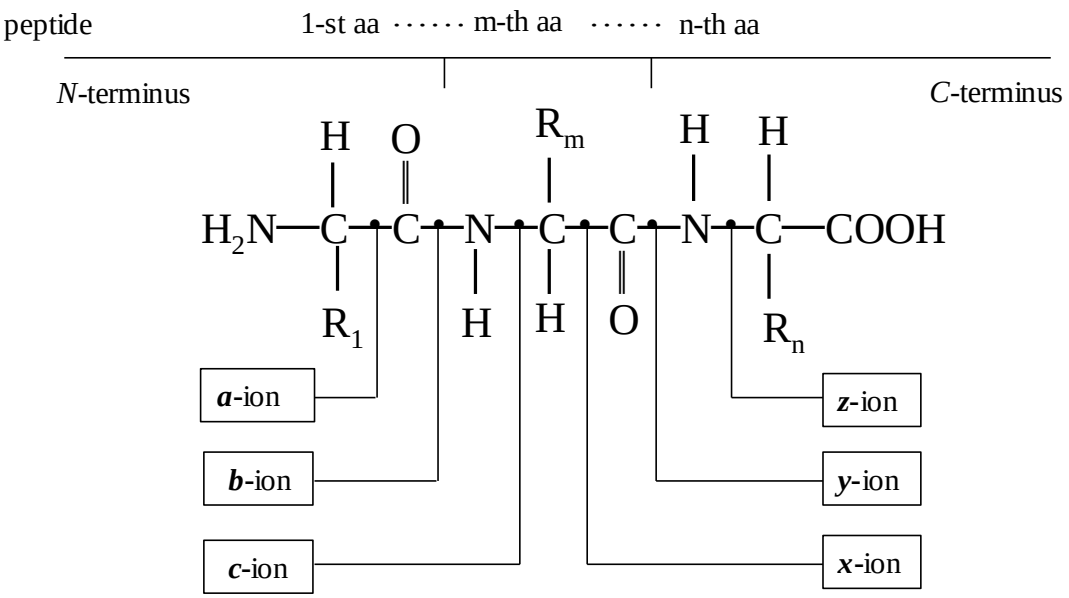



**Figure 3. 6:** Classical fragmentation of a peptide chain which modified from Bruni et al, 2005.

Protonation at the amine was followed by cleavage at any link of peptide. ***a***-ion: from *N*-terminus until any link like the marked one; ***b***-ion: from *N*-terminus until any link like the marked one; ***c***-ion: from *N*-terminus until any link like the marked one; *z*-ion: from *C*-terminus until any link like the marked one; *y*-ion: from *C*-terminus until any link like the marked one; *x*-ion: from *C*-terminus until any link like the marked one.

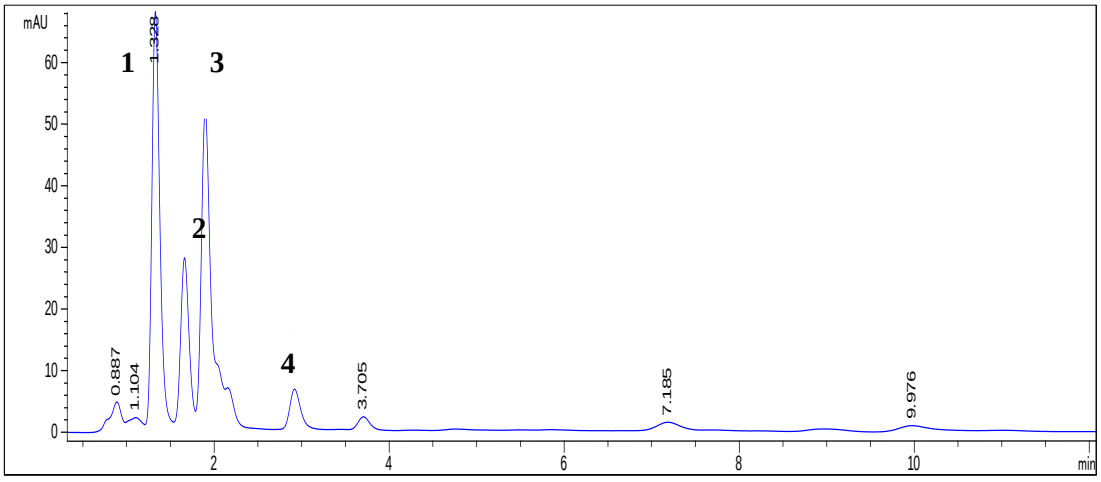



**Figure 3. 7:** The molecular weight less than 1kDa proteolytic peptide on LC-ESI-MS.The molecular mass and proteolytic peptide sequencing of the peptide from protease digestions was done on the electrospray ionization– mass spectrometry (ESI–MS) with UV detector (Agilent Technologies, Santa Clara, CA). Molecular weight less 1Da peptide sample was directly injected into the electrospray ion source and sampled for 10 min using a mobile phase of 98% water and 2% acetonitrile containing 0.1% formic acid at a flow rate of 4mL/min. The mass scanning range was from 100 to 1000 msu at 1 second with a 0.1-s inter-scan delay in continuum mode. The UV at absorbance 251 nm was shown four major peaks (Peak 1, Peak2 , Peak3 and Peak4)at retention time, R1=1.328 min, R2=1.661min, R3=1.898 min and R4=1.898 min, respectively.

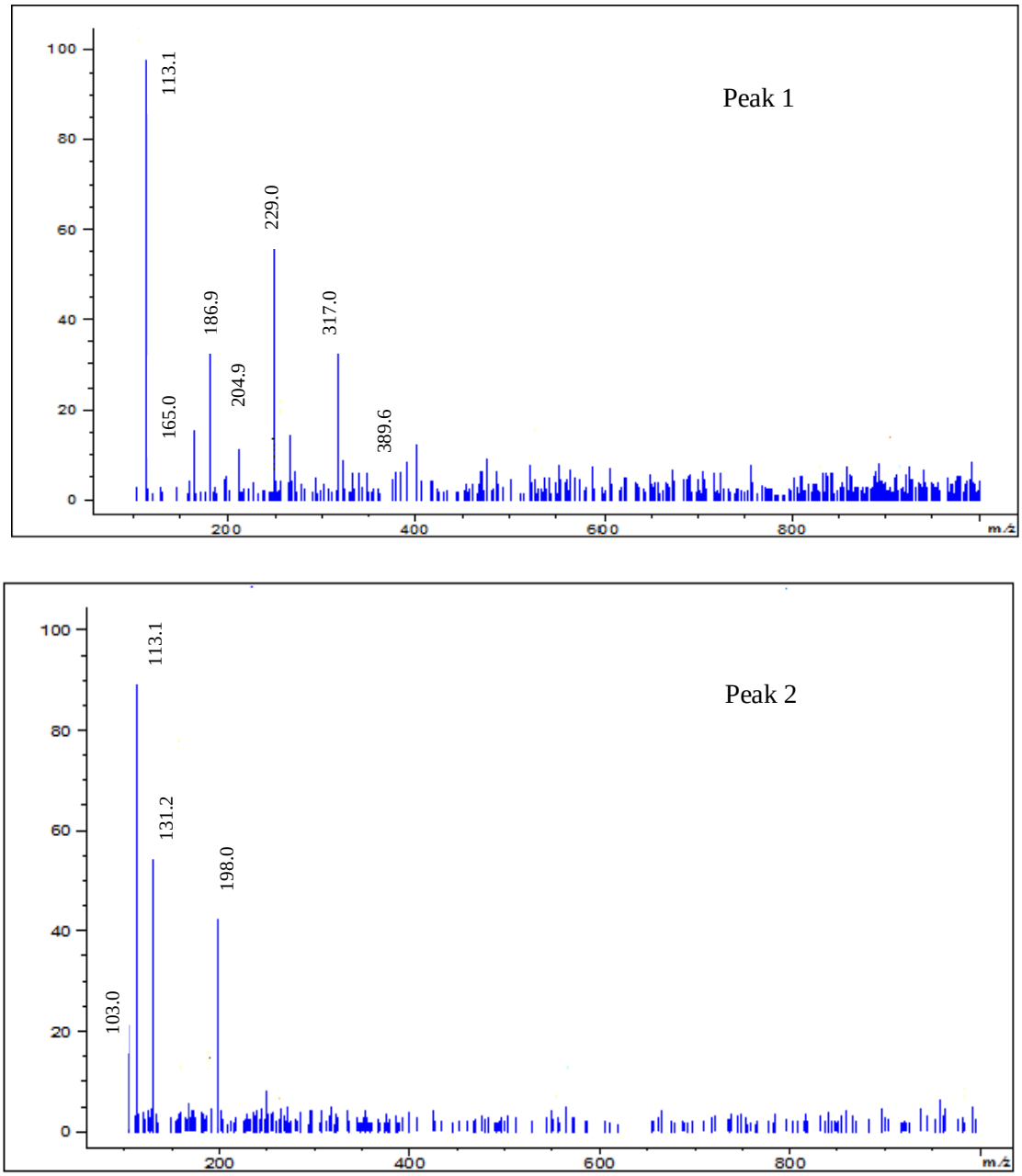



**Figure 3. 8:** Mass spectrums of Peak 1 and Peak 2.

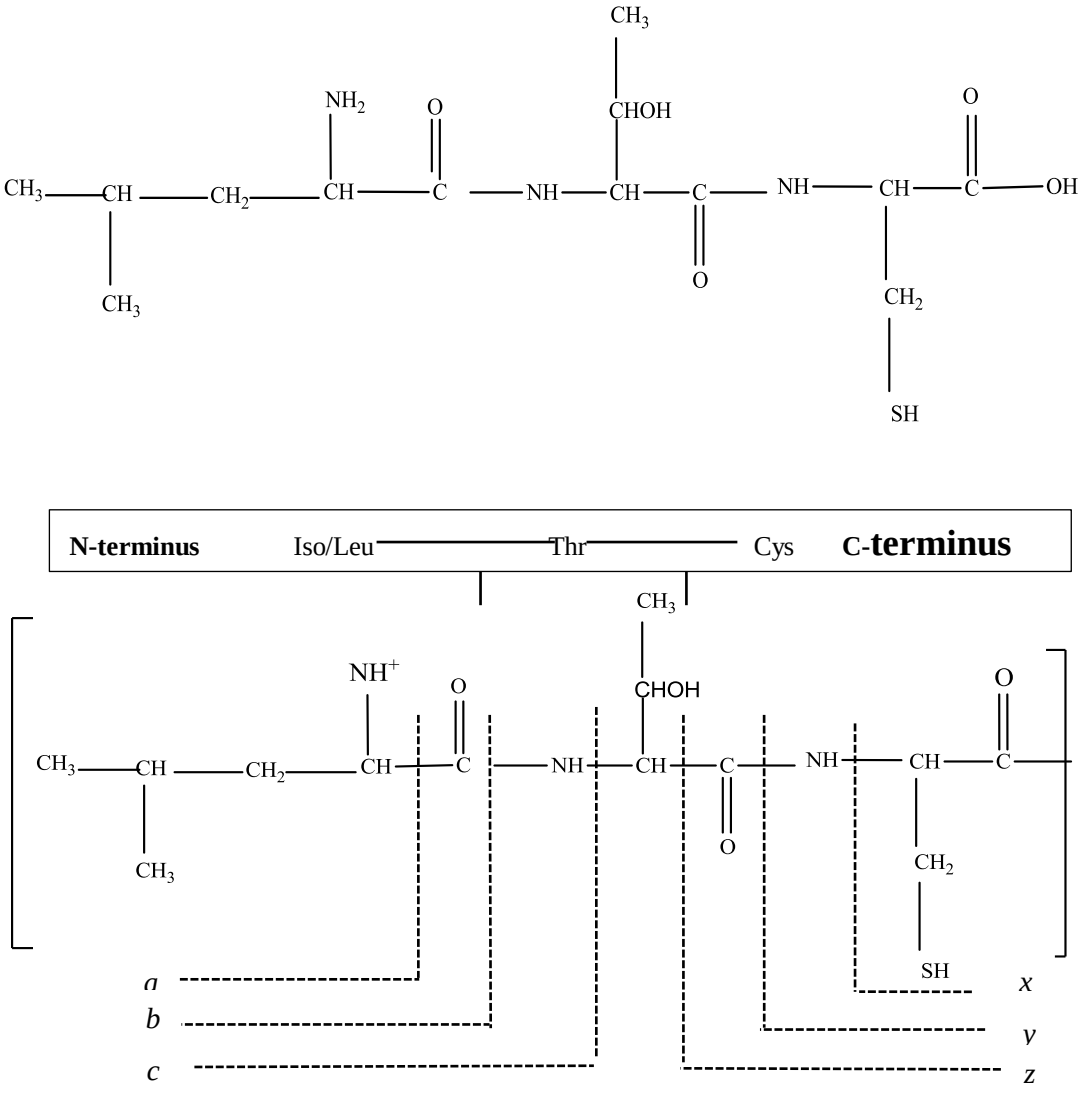
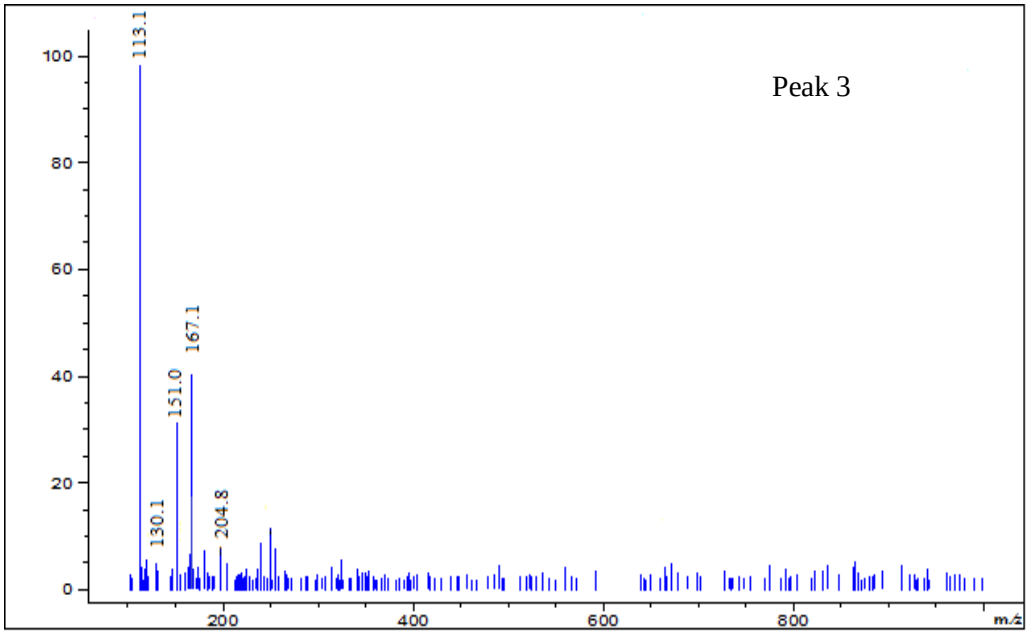



**Figure 3. 9:** Fragmentation of peptide Leu-Thr-Cys peptide chain.Protonation at the amine was followed by cleavage at any link of peptide. $a_1$-ion: from N-terminus until any link like the marked one; $b_1$-*ion*: from N-terminus until any link like the marked one; $c_1$-*ion*: from N-terminus until any link like the marked one; $z_1$-*ion*: from C-terminus until any link like the marked one; $y_1$-*ion*: from C-terminus until any link like the marked one; $x_1$-*ion*: from C-terminus until any link like the marked one.



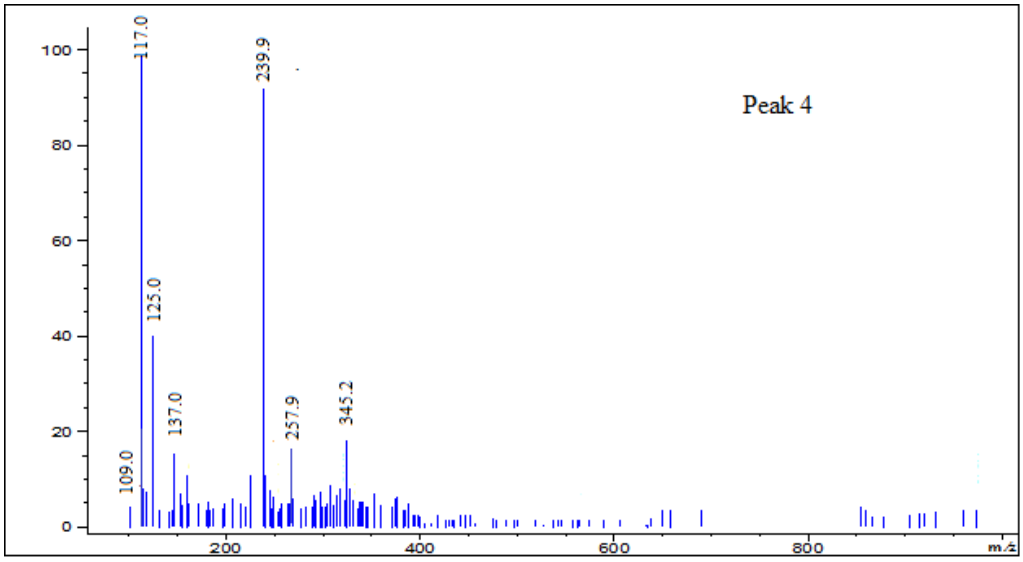




**Figure 3. 10:** Mass spectrums of Peak 3 and Peak 4.

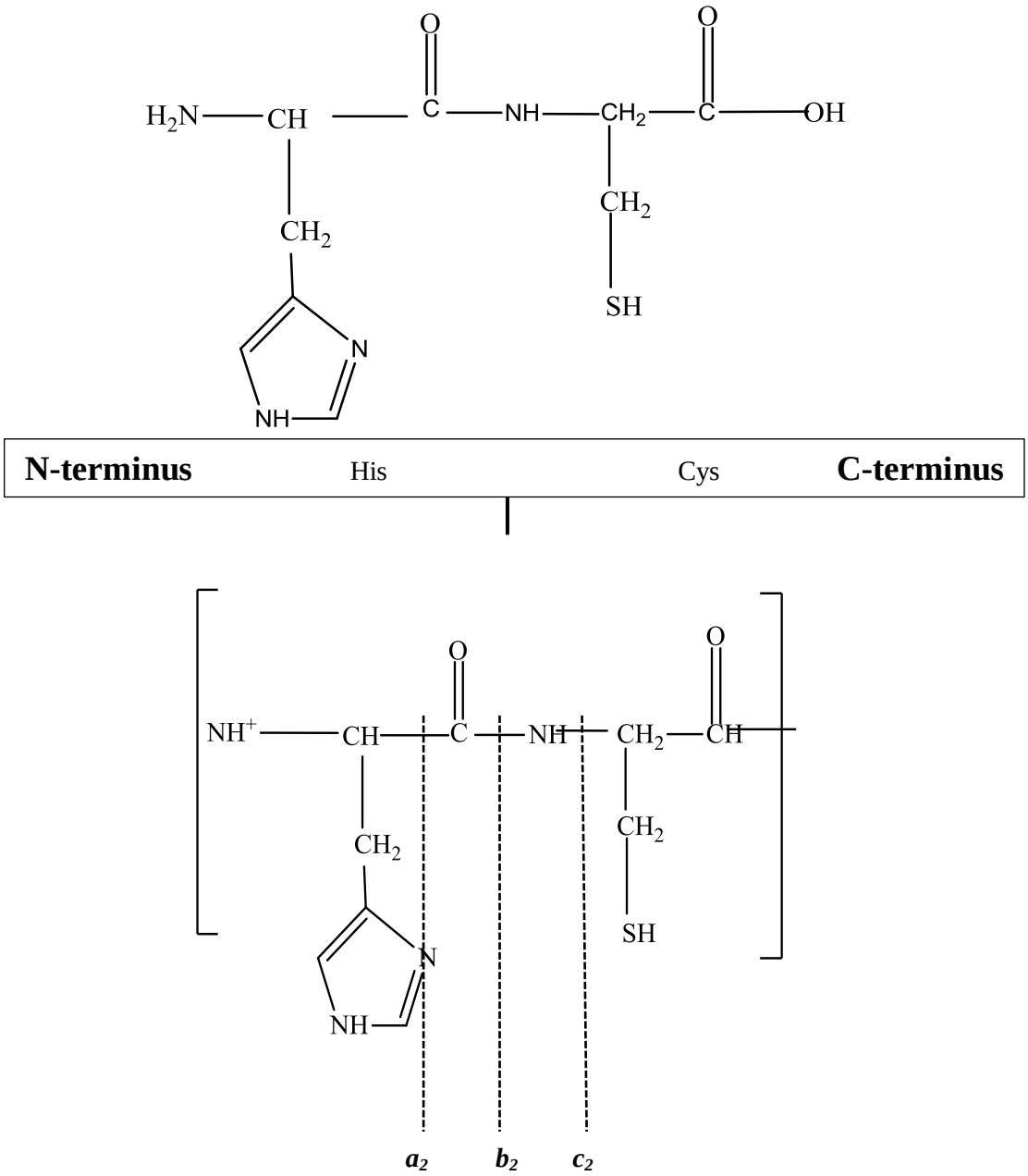



**Figure 3. 11:** Fragmentation of peptide His-Cys peptide chain.Protonation at the amine was followed by cleavage at any link of peptide. $a_2$ -ion: from N-terminus until any link like the marked one; $b_2$-ion: from N-terminus until any link like the marked one; $c_2$-ion: from N-terminus until any link like the marked one.

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## Significance of the Project

In the United States, approximately 54 billion pounds of inedible animal tissue were generated annually, which represents approximately 37% to 49% of the live weight of each slaughtered food animal (Shurson, 2005). Rendering was the safest, most economical method of inactivating disease-causing microbes while recovering billions of dollars' worth of marketable commodities.

Our project "Developing value-added peptide antioxidants from rendering products for potential aquaculture and pet food market" demonstrated that some hydrolysates of rendering products possessed antioxidant activities, which enable them to be used as natural antioxidants as substitutes for some commercial antioxidants such as BHT, vitamin E, and ethoxyquin, which are widely used in formulated animal feeds. Rendered animal co-products have been demonstrated to be an excellent source for producing strong antioxidant hydrolysates that are suitable and feasible to be developed into safe and possible substitutes for current commercial antioxidants used in rendering industries.

For human being, the peptides have the potential to be a good dietary supplement for the prevention of oxidative stress. Dietary antioxidants may protect against various diseases such as cancer, atherosclerosis, hypertensive, aging, and diabetes. Food-derived peptides are potential natural antioxidants without marked adverse effects. Therefore, food-derived antioxidative peptides play important roles in preventing these diseases and are commonly used in food and pharmaceutical applications.

## Limitation

When analyzing the results of our research, certain limitations need to take into account. No matter how long a research takes place, there always will be other aspects of the context that were not examined. Usually, we are unable to observe the entire population and therefore we must be content with gathering data from a subset of the population. There was one limitation in our research; the sample should be collected three times from the rendering industry to form at least three lots for the sample collection. Since there was only one lot sample from rendering industry in our research, the result of the experiment was not perfect as it was designed. Although three different dates sample triplicate collection were conducted from this lot, it was not perfect to analyzing the results of our research.

## Future work

In the present work, the antioxidant peptides derived from rendering protein were studied. Further research should be done to synthesize the purified peptides as the sequence analyzed in our research and evaluate its antioxidant activity. In this way, the purified peptides were employed to verify the results of theoretical analysis peptide sequence.

More studies should be investigated regarding to the characterization of antioxidant peptide in structural properties and antioxidative activity in cells and animal are needed. In our experiment, all the antioxidant capacity was tested out of the animal or human body. It is better to interpret those data and relate it to human health, sometimes, a compound in the test tube may act differently than it does in physiological conditions, in cells, or in the human body.

It is interesting to know whether the peptides developed from rendering proteins were nutraceuticals peptides with other beneficial effects on increased potency and therapeutic benefits. Bioactive peptides and proteins are being developed that positively impact body function and human health by alleviating conditions such as coronary heart disease, stroke, hypertension, cancer, obesity, diabetes, and osteoporosis. In this way, rendering bioactive proteins and peptides will facilitate the development of nutraceuticals proteins and peptides for a further range of health conditions.

# APPENDIX A

## Analysis two commercial antioxidants

### 1. Materials and methods

Two commercial antioxidants, Petox and santoquin (**Figure 1**), were supplied by Carolina By-Products, which are currently used to inhibit the oxidation of formulated products in the rendering industry. Petox was indicated as having the components BHA and BHT, and Santoquin was indicated as having the component ethoxyquin. Standard chemicals butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, U.S.). All HPLC analytical grade solvents were purchased from Fisher Scientific (Suwanee, GA, U.S.).

### 1.1 Qualitative analysis of commercial antioxidant Petox using LC-MS

An Agilent 6100 quadrupole mass spectrometer was used with electrospray ionization in negative modes. Full scan spectra were taken in the mass range of m/z 100–800. The operating parameters of the ESI source, including nebulizing gas pressure, drying gas pressure, drying gas temperature, needle voltage, and shield voltage, were all optimized with regard to the maximum signal intensity of pseudo-molecular ion by flow injection of 10 µL samples.

HPLC analysis was carried out on an Agilent pump (Colorado Springs, CO, U.S.). Chromatographic separation was performed on a SHIMADZU column (premier C18 column, 3µm, 4.6 X 150 mm). Solvent A was 100% water, and solvent B was 100%

methanol. The mobile phase was prepared daily, degassed using an in-line degasser (Waters), and delivered at a flow rate of 0.8 at room temperature. The gradient program conditions are given as follows. From 0 to 12 min, flow rate was kept at 0.8 ml/min (t = 0 min B 10%, t = 5 min B 10%, t = 50 min B 90%, t = 55 min, B 10%). The negative model was used to inject the sample to the mass spectrometer.

**1.2 Qualitative analysis of commercial antioxidant Santoquin using GC-MS**

A Shimadzu gas chromatograph—GC-17 (Columbia, MD U.S.) was equipped with a GCMS-QP5050 Detector. The inlet was set at 250 °C, and the detector was kept at 280°C. A DB5-MS (60m x 0.25 mm x 0.25 um film) column from J. & W. Scientific (Folsom, CA) was used. Carrier grade helium was purchased from Air Products and Chemicals, Inc. (Allentown, PA) to be used as the carrier gas. A split ratio of 20:1 was used. Temperature programming was used with the initial temperature of 50 °C for 5 minutes with ramping of 10 °C/minute to a final temperature of 260 °C. Total run time for each injection was 44 minutes. The mass selective detector scanned from 33 to 500 mass units in each scan. A three-minute solvent delay was used for all injections. 0.5 µL of each sample was injected manually with sample wash with hexane.

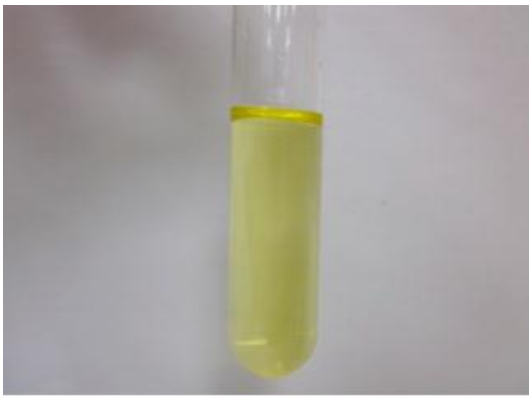
**2. Results and Discussion**

**Figure 2** was the result of identification of Petox by LC-MS. A UV-chromatogram of Petox in standard solution was shown, and this demonstrates the good LC-MS separation possible in a single run. The retention times of BHA (peak A) and BHT (peak B) were 41.708 min and 53.174 min, respectively. The mass spectra of BHA

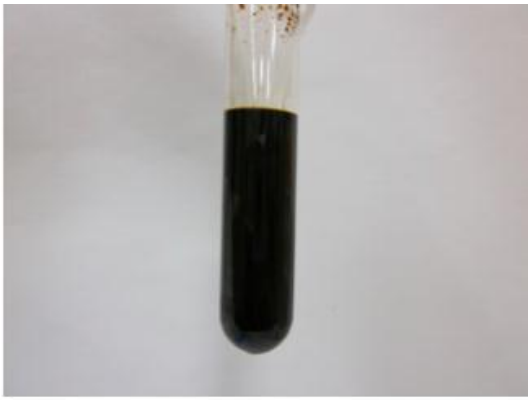
and BHT are shown in **Figure 2.** In negative ion mode, the chromatogram with the retention time of 41.708 min was BHA (m/z = 179.2), and the chromatogram with the retention time of 53.174 min was BHT (m/z = 219). For each antioxidant, the ion with the highest abundance was chosen for qualification. Therefore, BHA and BHT are included in Petox.

Ethoxyquin is an ingredient that is used as a fat stabilizer and preservative and is found primarily in premium pet foods that have high fat levels, although there are other pet foods that contain it as well. **Figure 3** lists the major antioxidant peaks of the commercial antioxidant Santoquin (retention time was 24.051 min). In the GS-MS library, it was marked by name 2, 4-dimethyl-6-ethoxyquinoline. The electron impact (EI) mass spectrum (**Figure 3**) shows the following peaks: 217 ($M^+$), 202 ($M^+$ - $CH_3$), 188 ($M^+$ - $C_2H_5$), 174 (202–$C_2H_4$), 145 (174–CHO).

## 3. Figures

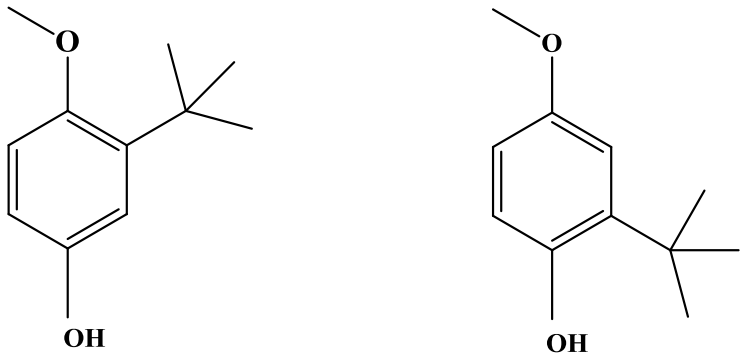


a: Petox

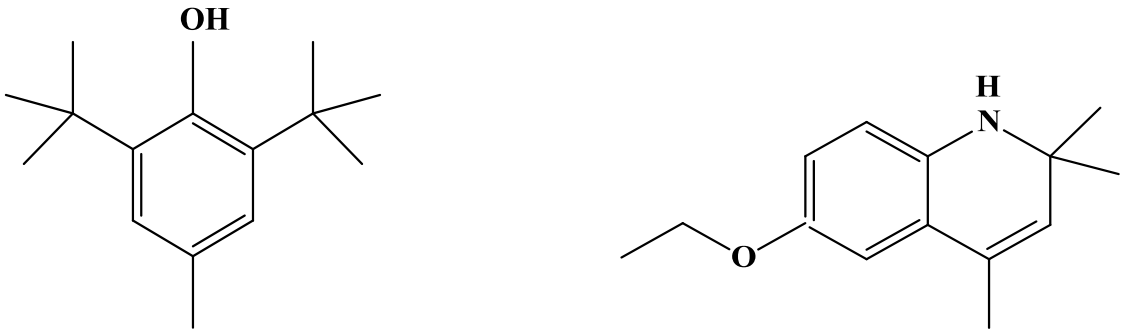


b: Santoquin

**Figure 1:** Two commercial antioxidants Petox(a) and Santoquin (b)




**a :Butylated Hydroxyanisole (BHA)**




**b: Butylated Hydroxytoluene (BHT)**

**c: Ethoxyquin**

**Figure 2:** Structures of butylated hydroxyanisole (BHA) (**a**), butylated hydroxytoluene (BHT) (**b**) and ethoxyquin(**c**).

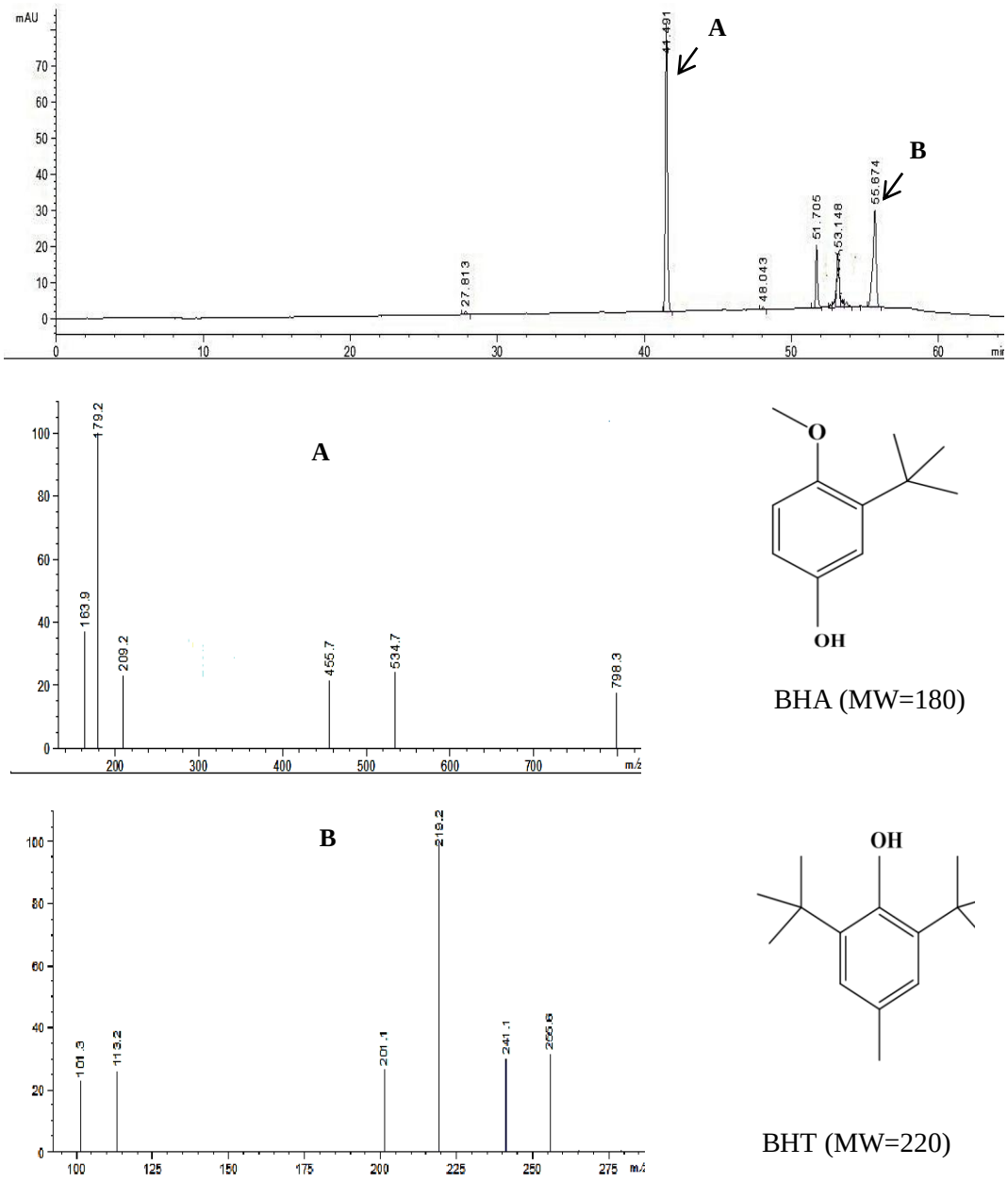



**Figure 3:** Liquid chromatographic profiles of the petox and its components peaks (A: BHA; B: BHT).The chemicals listed were two major antioxidant constituents that were identified by LC-MS.

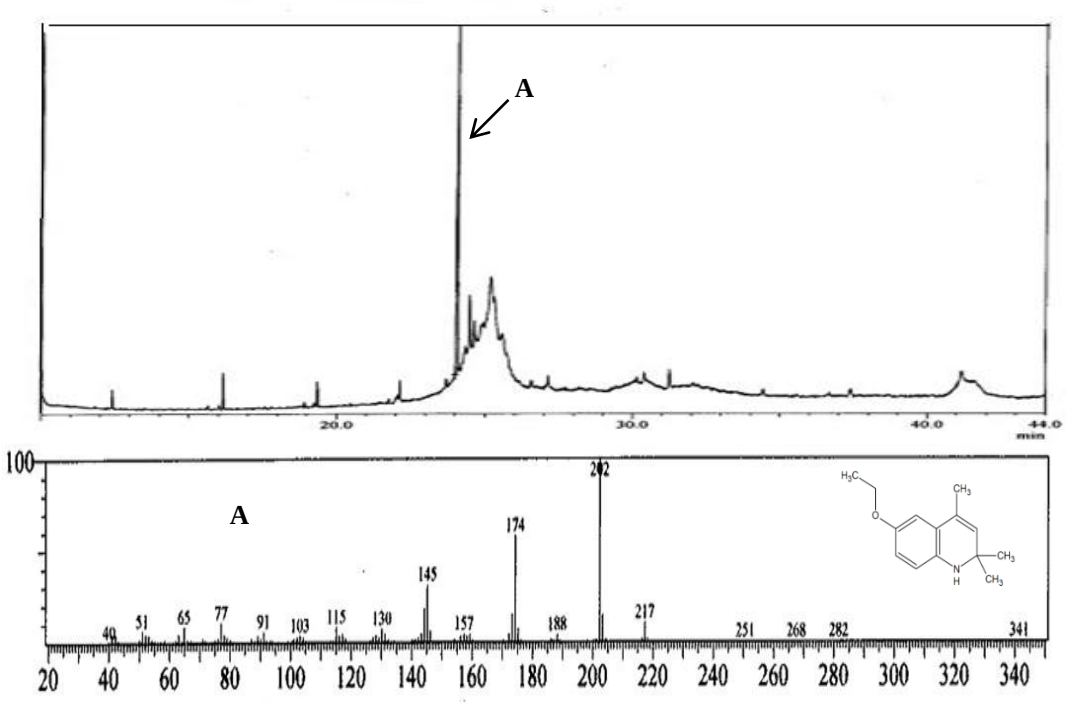



**Figure 4:** Gas chromatographic profiles of the santoquin and its major components peak A (Peak A: ethoxyquin).The chemicals listed was major antioxidant constituents that was identified by GC-MS.

# APPENDIX B

VALLEY PROTEINS, INC.

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www.valleyproteins.com

TECHNICAL DATA SHEET

## FISH MEAL

| Typical Analysis | |
|---|---|
| Protein | 50 % Minimum |
| Fat | 8 % Minimum |
| Fiber | 3 % Maximum |
| Phosphorus | 3 % Minimum |
| Calcium | 6 % Minimum – 7.2% Maximum |

Valley Proteins, Inc. has the ability to make special animal protein blends to your specification. Please contact us at sales@valleyproteins.com for additional information.

*Recover. Recycle. Replenish. Renew.*