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Extraction of Antioxidants from Animal Blood and its Potential Application as a Pet Food Preservative

Chengyi Tu
Clemson University

EXTRACTION OF ANTIOXIDANTS FROM ANIMAL BLOOD AND ITS POTENTIAL APPLICATION AS A PET FOOD PRESERVATIVE

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Chengyi Tu
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Accepted by:
Dr. Alexey Vertegel, Committee Chair
Dr. Vladimir Reukov
Dr. Christopher Kitchens

ABSTRACT

Nowadays, more and more people are having pets as members of their family. To the year of 2012, there are 78.2 million dogs and 86.4 million cats owned in the U.S according to the report of the Humane Society of the U.S. The pet food industry as a result has been prosperous, with an estimated market size of $21 billion in the year of 2013. However, there is a common problem for the industry - fat rancidification. Pet foods usually contain relatively high levels of fat, which, if not well protected, are prone to oxidation and generate unfavorable products including acids, ketones and aldehydes. The resulting small volatile molecules will not only lead to unpleasant flavors and odors, but also could be unsafe if accumulated at high concentrations. In order to better preserve the quality of foods, it is a common and necessary practice to add antioxidant preservatives, which can scavenge free radicals and hence prevent or slow down the oxidation of fats. Currently available antioxidants can be generally divided into two categories: synthetic and natural antioxidants. Commonly used synthetic antioxidants include butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and *tert*-butylhydroquinone (TBHQ) and ethoxyquin (ETQ). Synthetic antioxidants are advantageous because of their high efficiency and low cost; however, they are criticized for having potential safety issues [3-7]. The natural options such as tocopherols and ascorbic acid are recognized as safer but less effective and are much more expensive compared to their synthetic counterparts. In spite of higher price and lower efficiency, there is a great customer demand for natural antioxidant, which is perceived to be beneficial for pet's health. Consequently, there is a need to develop an alternative natural antioxidant, which is effective, inexpensive and safe.

Animal blood is a good source for natural antioxidants. Erythrocytes as oxygen carrier are rich in antioxidants including superoxide dismutase (SOD), catalase (CAT) and glutathione

(GSH). Besides, animal blood is a by-product of rendering industry and it can be obtained easily at a low cost.

This work seeks an efficient and inexpensive way to extract antioxidants from animal erythrocytes as a novel pet food preservative. Two extraction methods were applied: a standard method and a novel simplified method. The standard method was originally designed to extract non-purified superoxide dismutase (SOD) from red blood cells and involves the use of several organic solvents. The product of this method is denoted as C-SOD. The second method is simplified to yield a crude protein mixture containing various antioxidants, as well as other proteins, and is denoted as CP. The characterization of the two products showed that C-SOD contained 1100 U/ml of SOD activity and 300 U/ml of catalase activity. In comparison, CP contained 224 U/ml of SOD activity. The following two assays were used to evaluate the antioxidant efficacy of the two products. Ferrous xylenol orange (FOX) assay and thiobarbituric (TBARS) assay showed that adding 5% (v/w) CP into ground chicken meat inhibited 100% of fat oxidation for 12 hours at 37 ℃, while C-SOD was less effective, reducing oxidation level by about 62%. In the chicken fat model, 5% (v/v) of C-SOD and CP inhibited 85% and 67% oxidation respectively for 12 hours at 50℃. Aging study showed that CP was quite stable, losing no more than 20% of its activity after accelerated aging for 10 days at 50℃. Overall, the results demonstrated that erythrocytes extracts CP and C-SOD were able to protect fats from oxidation in a number of food models. CP is more likely to be used by the industry because the extraction procedure is cheap and involves no organic solvents. In summary, CP is a very promising preservative for pet foods. In the future, with proper quality control and proof of safety, it may be potentially used in a number of animal and human food products.

DEDICATION

I would like to dedicate this work to my parents, Shixin Tu and Zhaoli Ji, for their unselfish and unwavering sacrifice and love.

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CHAPTER 1

INTRODUCTION

The pet food industry has been steadily growing for years, with a market size of $20 billion in the year of 2012 and it is estimated to be over $21 billion in 2013 according to the American Pet Products Association [9]. A good pet food is supposed to have the following characteristics. First, it should provide the necessary calories to support the animals' daily activities and nutrients such as proteins, fats, vitamins, fibers and minerals to maintain the health of the animals. Second, it needs to be tasty and palatable. Third, it should be inexpensive. Lastly and most importantly, it has to be safe for consumption.

However, a common problem for pet food is fat rancidification, which can compromise both its taste and nutrition value, and even more severely, cause safety problems. Pet foods normally contain relatively high levels of fats. If not well protected, fats are prone to oxidation and degrade into small volatile molecules like aldehydes and acids, releasing unpleasant flavors. Further, microbial growth can exacerbate the situation. Fats deterioration will not only lead to direct economic loss, but can also cause health problems [10, 11]. Therefore, it is a necessary and common practice to add preservatives into pet foods, which arrest or prevent the oxidation of fats and also inhibit the growth of microorganisms.

Antioxidants are the primary and indispensable preservatives for pet food because they prevent or slow down fat oxidation and degradation. Available antioxidants on market include: natural options such as tocopherols (vitamin E), ascorbic acid (vitamin C) and various kinds of plant oils; synthetic options such as butylated hydroxytoluene (BHT), butylated hydroxyanisole

(BHA) and *tert*-butylhydroquinone (TBHQ) and ethoxyquin (ETQ). Overall, synthetic antioxidants have stronger antioxidation activity and are also more cost effective in comparison with the natural alternatives. Unfortunately, there has always been a safety concern regarding the potential side effects of these synthetic materials [12-15]. In contrast, natural antioxidants are generally recognized as safe,[16], but they are more costly and less active and need to be added in larger quantity to achieve the same protection as their synthetic counterparts [17]. Hence, there is a need to develop a safe, inexpensive and effective alternative antioxidant.

Rendered materials, animal blood in particular, provide such an alternative. Erythrocytes function in the transport of oxygen in the blood and are constantly exposed to oxidative stress caused by radicals such as hydroxyl radical, superoxide anion and also hydrogen peroxide. As an adaptation to this oxidative stress, erythrocytes have successfully developed a comprehensive system of antioxidants to ensure those harmful molecules are eliminated effectively before they do any severe damages to the important cell organelles. Superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GHX) are the main antioxidant substances in erythrocytes. Therefore, we propose to find an inexpensive way to extract these antioxidants and apply them to pet food as a preservative to inhibit the oxidation of fat contents. To realize this goal, three challenges need to be addressed.

First of all, while the antioxidant systems in erythrocytes are effective at protecting the cells from the daily oxidative stress they are subjected to, their ability to protect food products need to be evaluated. Antioxidants in red blood cells are mostly enzymes, and as macromolecules, they are structurally complicated and sensitive to environmental changes. In other words, their activity depends on many factors including pH, temperature and ionic strength. Second, the cost of extracting them from the cells has to be low so it can be competitive with commercially available products, otherwise they may not be able to compete with other natural antioxidants

such as tocopherols and ascorbic acid. Lastly, most conventional protein extraction procedures involve the use of organic solvents, which may denature proteins and lead to additional safety issues if not removed completely. Therefore it is necessary to reduce or avoid the use of any hazardous or potentially hazardous chemicals during extraction.

To address these challenges, we conducted experiments to evaluate the proposed system using porcine blood obtained from a local slaughterhouse as an inexpensive source of erythrocytes. Two extraction methods were adopted. The first one was simplified method for extraction of superoxide dismutase enzyme, which we initially deemed to be the strongest antioxidant present in blood. This method involves several steps and a number of organic solvents. The idea of the second method is based on the elimination of hemoglobin, the most abundant pro-oxidant compound, from erythrocyte lysates. This procedure leaves a complex protein cocktail that contains a number of antioxidants naturally present in erythrocytes, in a mixture with many other proteins. The products of the two methods were tested with ground chicken breast meat and chicken fat as two important food models. Degree of oxidation was evaluated by two techniques, ferrous oxidation xylenol orange (FOX) assay and thiobarbituric reactive substances (TBARS) assay. PETOX, a commonly used synthetic antioxidant preservative containing 10% BHA and 10% BHT, was used for comparison and samples with no preservatives were used as negative controls. Finally, the potential application and future recommendations for future development of these products are discussed.

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CHAPTER 2

LITERATURE REVIEW

2.1 Pet food

Pet food typically refers to any plant or animal materials that are intended to be consumed by pets. Dog food and cat food constitute a large portion of the market. The pet food industry is tightly associated with the rendering industry since it utilizes a significant amount of rendered ingredients. Products produced by the rendering industry include bone meal, feather meal, and meat meal and these are the popular materials for making pet foods because they provide an inexpensive source of nutrients for pets. Also, rendered fats and oils are also added to pet foods to promote nutritive value and flavor.

Generally, there are two categories of pet foods: dry pet food and wet pet food. Dry food is made with a machine called an extruder. In brief, materials are blended according to the nutritive requirements and then subjected to high pressure in steam, being compressed and pushed through dies which will determine the final shapes of the food. As the mixture comes out of the extruder, they are cut into smaller pieces. At the same time, due to the decrease of pressure, they expand into their final shapes. The last step is to spray with fat (usually poultry fat which is a by-product of the rendering process) and compounds that make the food more palatable. Wet pet food production starts by using emulsified animal parts with additives. Then the mixture is cooked or canned directly and sterilized. Also, wet food is generally regarded as fresher than dry food. In terms of components, wet food generally contains higher protein content and less carbohydrates.

Compared to human food, pet food usually undergoes a longer storage time and contains relatively high levels of fat and oil content, leaving them very susceptible to lipid deterioration. Consequently, adding preservatives to inhibit fat deterioration and microbial growth is a common practice in the pet food industry. The most commonly used additives are synthetic ones such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and ethoxyquin, which are generally very effective but have raised a lot of controversies regarding safety issues. (Details will be discussed in **2.3).** Many efforts have been made to seek safe alternatives.

2.2 Food rancidification

Rancidity is the unpleasant and characteristic odor or flavor released from edible fats and oils as a result of oxidative and hydrolytic degradation [18]. Rancidity mostly occurs in foods that contain fats, including both raw food like fresh beef and processed food like pet foods or snacks. The unpleasant odor of rancidity not only turns customers away, but also decreases the nutritive value in the food (loss of unsaturated fatty acids) and even generates toxic chemicals (aldehydes, hydroperoxides).

Typically, rancidification is carried out in three ways: a) oxidation, b) hydrolysis and c) microbial degradation.

2.2.1 Oxidative rancidity

Oxidative rancidity is probably the most fundamental source of rancidity among the three pathways, and usually occurs through an auto-oxidation pathway[19]. First, lipid oxidation can be initiated by various factors, including heat, moisture, light, enzymatic activity, as well as pro-oxidants, and result into lipid free radicals, which will subsequently be attacked by oxygen and form peroxy radicals. Then, the newly formed peroxy radicals start to take hydrogen from other lipids, mostly unsaturated lipids, yielding another hydroperoxide and a new lipid free radical, indicating the onset of propagation. In the meantime, secondary oxidation begins and proceeds,

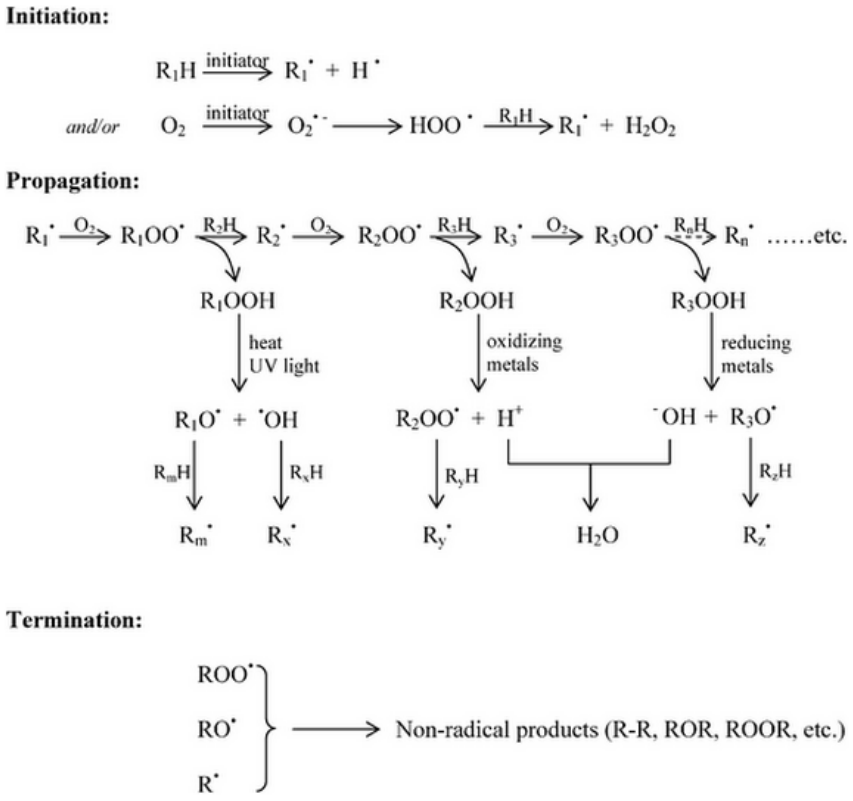

Figure 2-1 Lipids autoxidation pathway [2]

producing small volatile molecules such as aldehydes and ketones. Lastly, the chain reaction is terminated when those free radicals encounter antioxidants and new free radicals cannot be generated.

2.2.2 Hydrolytic rancidity

Hydrolytic rancidity, as indicated by its name, refers to the hydrolysis of triglycerides and the formation of free fatty acids (FFAs), monoglyceride, diglyceride or glycerol. It is a major concern to the dairy industry because both the indigenous and the microbial lipolytic enzymes in milk can facilitate the hydrolysis of fats and the release of small molecule of unpleasant flavor.

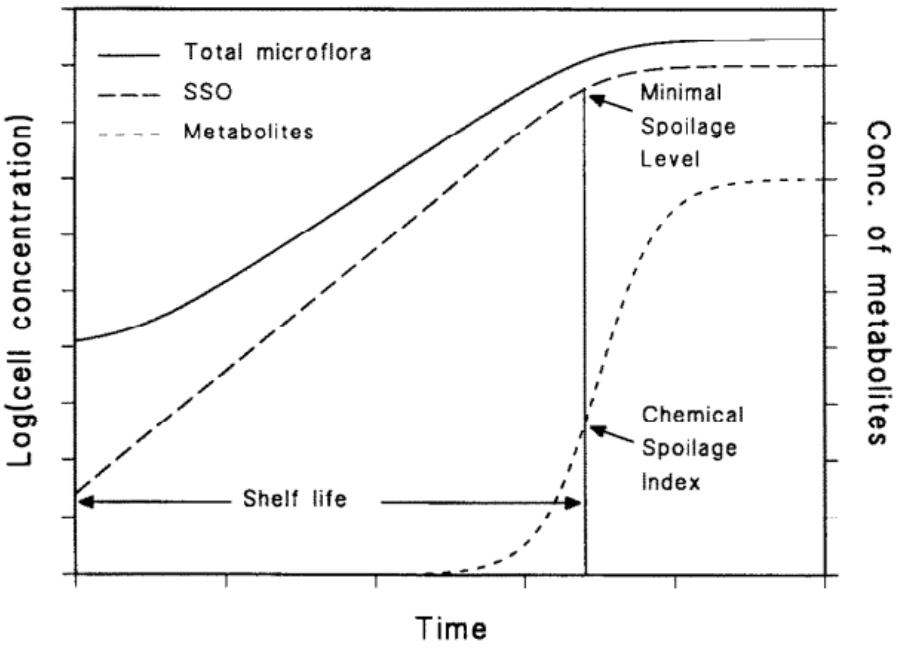

Figure 2-2 Generalized scheme of food spoilage due to the over growth of specific spoilage organisms (SSO) [8].

2.2.3 Microbial rancidity

Microbial growth causing food spoilage is very familiar to everyone in our daily life. Notably, it is not independent of the other two pathways (oxidation and hydrolysis). In fact, bacteria in many cases accelerate food hydrolysis and decomposition, releasing metabolites that may be toxic and change the pH of food, and result into the off-odors and off-flavors. Bacterial spoilage is most evident and rapid in proteinaceous foods such as meat, fish and milk since they are highly nutritious, pH neutral or slightly acidic and possess high moisture contents [8]. Specific spoilage organisms (SSO) usually comprise only a small proportion of the initial microflora on fresh food, but they are well-adapted to the storage conditions and generally good at utilizing the nutrients in foods, and consequently, they are able to overgrow and prevail in the

end (**Fig 2-2**). For example, *pseudomonas* species are the most common spoilage organisms. They consume non-protein nitrogen fraction (NPN) in animal foods and produce lipases or proteases which hydrolyze fats as described above. Lastly, the over-growth of the bacteria and accumulation of extracellular substances is directly visible by eyes[20].

The three pathways, independently or in combinations, are responsible for the rancidification of foodstuffs and have caused economic loss over the world which can hardly be calculated exactly.

2.3 Antioxidants as food additives

A food additive is a substance or a mixture of substances that are not normally consumed as food by itself, whether or not it has nutritive value, but is added to food to preserve flavor or enhance the taste and appearance. For example, artificial sweeteners are used to make diet Cokes sweet but free from calories; emulsifiers are added to make a better mixture of oil and water as in ice cream and homogenized milk; food coloring agents are processed into drinks or foods to make it look tasty as in green ketchup or red wine. The most frequently used additives are antimicrobial agents and antioxidants, which are responsible for tackling the rancidity problems caused by bacterial growth and oxidation respectively.

Antioxidants (AOs), in general, refer to any molecule that inhibits the oxidation of other molecules. Basically, food antioxidants, according to theirs sources, can be divided into two categories: synthetic ones which are synthesized from smaller molecules and natural ones which are extracted mostly from natural plants. Alternatively AO can be categorized according to their mechanisms into primary ones, which contribute hydrogen to free radicals and thus break the chain reaction, and secondary ones which trap radicals or chelate metals.

2.3.1 Synthetic antioxidants

In food industry, overall, synthetic antioxidants are more popular than natural ones, primarily because of their better antioxidant activity [21] and lower cost of production. Most of them have phenolic chemical structures and serve as primary antioxidants (meaning they directly eliminate free radicals instead of inhibiting pro-oxidants). Common examples include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), tert-butylhydroquinone (TBHQ) and ethoxyquin (ETQ).

2.3.1.1 Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT)

Butylated hydroxyanisole and butylated hydroxytoluene share similar chemical structures (**Fig 2-3**). Both of them are derivatives of phenol and are fat-soluble thus they have been used to protect fats from oxidation. BHT is prepared by the reaction between 4-methylphenol and isobutylene catalyzed by sulfuric acid [22].The FDA regulates the purity of BHT to be at least 99% as food additive. BHA is prepared from 4-methoxyphenol and isobutylene and its required minimum purity is 98.5%. They both act as free radicals scavengers by forming non-radical products with free radicals to break the chain reaction. The bulk price of BHT can be as low as 10 dollars per kilogram.

Nowadays BHT and BHA are used in enriched rice, in margarine, as an emulsion stabilizer for dehydrated potato, in dry breakfast cereals, in potato granules and in essential oils. However, although almost the entire population has been exposed to BHT and BHA for years, the safety concern on their use has not been resolved yet. Actually, it is as controversial as ever before. For years, many animal studies have indicated that BHT not only caused short-term damages to lungs, but also induced the development of pulmonary tumor and skin tumor in the animal models [3, 23-25]. However, in those animal studies, the dosages (100mg/ kg, or g/kg) used were generally much higher than the approved level by the FDA, many of those negative effects became less

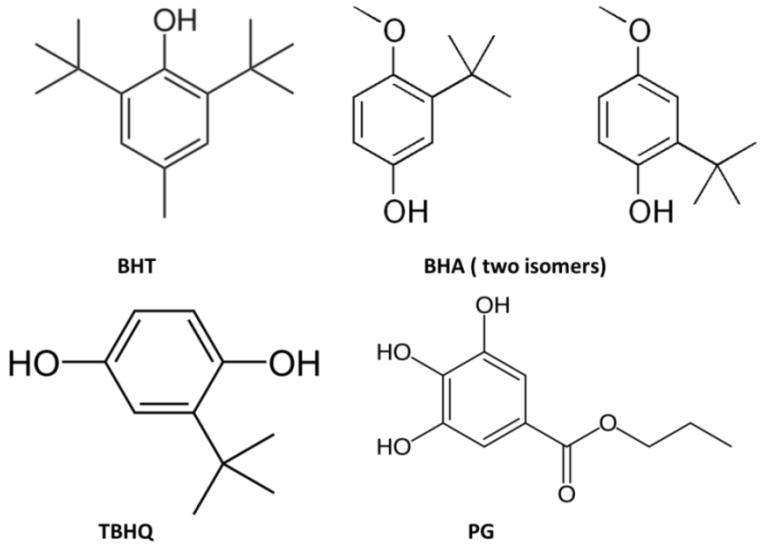

Figure 2-3 The chemical structures of 4 common synthetic antioxidants with phenolic structures

evident as smaller doses were used. Besides, a retrospective study on human showed there was no connection between BHT or BHA intake and stomach cancer risk [26]. Currently, FDA allows the use of BHT or BHA, in combination or alone, up to 200 ppm in fats or oil contents, 50 ppm in dry breakfast cereals or sweet potato flakes, and 33 ppm in rice [27]. The regulation, to some extent, has limited their performance.

2.3.1.2 *Tert*-butylhydroquinone (TBHQ)

Another antioxidant, tert-butylhydroquinone (TBHQ) is also a phenol-based compound and it is also a highly effective antioxidant with similar mechanism. Like the debate on BHT and BHA, conclusion regarding the safety of TBHQ has not been drawn yet. On one hand, the two major authorities, the United States Food and Drug Administration (FDA) and the European Food safety Authority have evaluated TBHQ and claimed that it is safe to consume under the approved levels [28]. On the other hand, at a higher doses level, it can be problematic. Animal studies have

shown TBHQ was responsible for the development of stomach cancer or proliferative lesion in forestomach [29, 30] . Over all, the adverse effects of TBHQ observed in animals studies have been confirmed, but only at very high dose levels, hundreds or thousands times higher than that permitted by FDA for human consumption.

2.3.1.3 Propyl gallate (PG)

Propyl gallate (PG) is also a phenolic compound, which is an ester formed by the condensation of gallic acid and propanol. PG is usually used in conjugation with BHT and BHA and added to fats, oil contents, margarine, and chewing gums. The permitted dose level of PG is close to that of BHT and BHA. Like all the other phenolic antioxidants, PG has also been criticized for its potential carcinogenic effects [29, 31]. In 2009, PG was found to be an estrogen antagonist [32], rendering its use in food even more controversial.

2.3.1.4 Ethoxyquin (ETQ)

Ethoxyquin (1, 2 dihydro-6-ethoxy-2, 2, 4-trimethylquinoline) is a quinolone based antioxidant and it is sacrificed in its antioxidant action, which means it scavenges free radicals and change its own structures. Oxidation products of ethoxyquin include 2, 4-dimethy-6-ethoxyquinoline, quinone-imine N-oxide, ethoxyquin 1, 8 dimer.

ETQ has been used in some animal feeds since the 1950s and over the decades, its application has been extended to the whole pet food industry. ETQ has been proved to be effective at retarding the oxidation of vitamin E, vitamin A, carotene in animal feeds and preventing oxidation in pet food. Currently, According to FDA regulation, it is approved to be added into animal feeds at the quantity of no more than 150 parts per million (ppm). However, FDA has received complaints with regard to the adverse effects of this compound since 1988 [33]. Numerous studies have pointed the potential safety issues of ETQ. For instance, Blaszczyk found that ETQ could induce DNA damages in human lymphocytes in a dose-dependent manner [34];

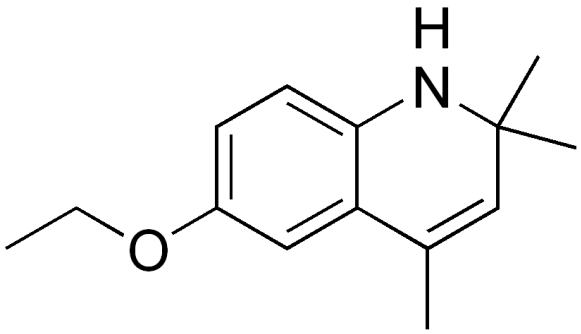

Figure 2-4 The chemical structure of ethoxyquin

Ornsrud showed that a major metabolite of ethoxyquin, ethoxyquin dimer, could lead to adverse effects in liver and kidney in rats[35].

2.3.2 Natural antioxidants

In comparison to synthetic antioxidants, which are not consumed normally as food, natural antioxidants are mostly necessary nutrients themselves and have been used as supplements in many ways in addition to their part in a regular diet. Also, due to the heated debate and concern regarding the safety of synthetic antioxidants, it is not surprising that natural alternatives are receiving more and more attention from researchers and also better acceptance from consumers. The most commonly used natural antioxidants are tocopherols (vitamin E), tocotrienols (vitamin E) and ascorbic acid (vitamin C).

2.3.2.1 Vitamin E

Tocopherols and tocotrienols, or vitamin E, is a group of eight fat-soluble compounds, termed as alpha, beta, gamma, delta tocopherols and tocotrienols. Tocopherols were first identified from a dietary factor in rats back in 1936, thus given the name “tocopherol” meaning “birth”, and the discovery of tocotrienols occurred much later, in 1964 by Pennock and Whittle, who isolated this chemical from rubber. Both tocopherols and tocotrienols have a relatively hydrophilic chromanol head and a hydrophobic tail. Their slight structural difference lies in the

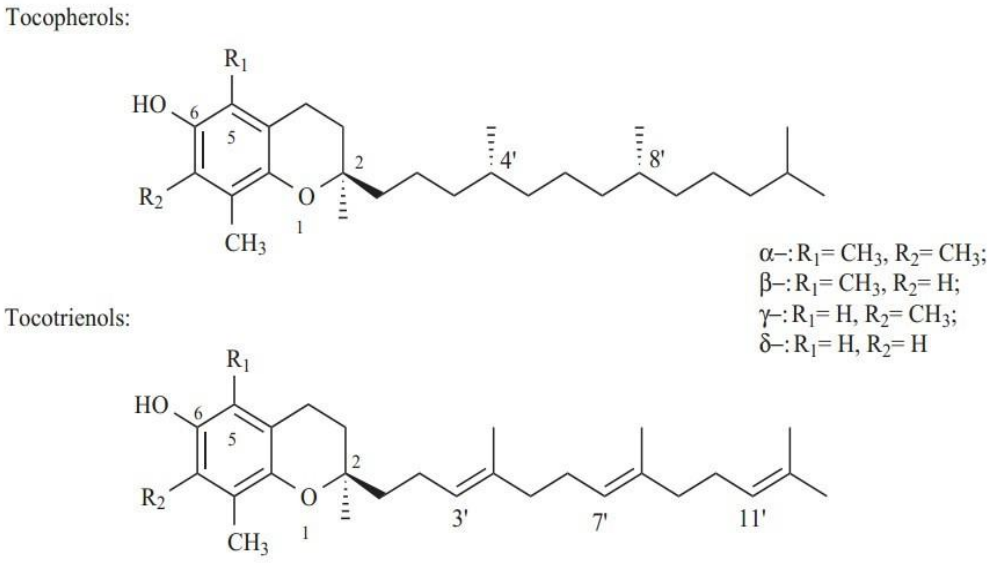

Figure 2-5 Chemical structures of tocopherols and tocotrienols [1].

presence of 3 double bonds in the lipid tail of tocotrienols. All members of vitamin E family are all fat-soluble.

For decades, vitamin E has been extensively studied and numerous functions and applications of it have been discovered. One primary function of vitamin E is antioxidation. All 8 forms of vitamin E have antioxidant properties because the hydroxyl group on the chromanol ring can donate one hydrogen atom to eliminate a free radical. This antioxidation activity is of crucial biological significance. For example, *in vivo* vitamin E can be incorporate into cell membranes and protect them from oxidative stresses. Besides, independent of its free radical scavenging ability, vitamin E regulates enzymatic activity. For instance, alpha-tocopherol inhibits protein kinase C (PKC) and consequently leads to the stop of smooth muscle growth [36]. As a gene expression regulator, vitamin E may decrease the expression of CD36 in human monocyte-derived macrophages [37]. Vitamin E also plays a role in the inhibition of platelets aggregation

[38, 39], and researchers have found that supplementation of vitamin E at a dose level of 400-800IU/day for 2 years in coronary atherosclerosis patients has significantly reduced cardiovascular death incidence by 77% [40]. Vitamin E is also thought to be essential in maintaining neurological functions [41].

Most early studies focused on tocopherols, especially alpha-tocopherol, partially because it is preferentially absorbed and accumulated in humans, while studies on the other half of the family - tocotrienols, were much fewer [42]. However, later, researchers started to recognize the significance of tocotrienols and pay more attention to them. Since the 1990s, the anti-cancer properties of tocotrienols [43], along with its neurological protection effects [44], started to be unveiled. In ehrlich sarcoma, tocotrienols were reported to suppress tumor cell proliferation[45], and similar result was discovered with liver and lung cancer [46]. In a breast cancer model constructed in mice, tocotrienols modulate the immune responses and might explain the reason why tocotrienols inhibited the development of breast cancer [47]. Besides, amazingly, in vitro studies showed that tocotrienols possess high antiangiogeneic properties, and its antiangiogenesis effect is even higher than tocopherols [48] [49]. In 2009, a group of scientists in Hong Kong University found that when treated with gamma-tocotrienol with chemotherapy drugs, skin cancer cells were reduced [50].

Given the multiple important biological activities that vitamin E is involved in, it is expected that the deficiency of it would result in many problems of different severities. So far, it has been reported and confirmed that vitamin E deficiency would cause spinocerebellar ataxia [51], myopathies [52], peripheral neuropathy [53], retinopathy [53], impairment of immune response, red blood cell destruction. The recommended daily intake of vitamin E for adults is 15 mg. However, even though it is true that vitamin E is so necessary and beneficial, supplementation of vitamin E did not exhibit expected health benefits on humans [54].

Consequently, it seems very tempting to use vitamin E as a food additive given its high nutritive value, less safety concern and decent protective effects on lipids. However, there are also several disadvantages of it. First, vitamin E, like many other redox-active compounds, has both anti- and pro-oxidant effects in presence of certain molecules. The pro-oxidative activity of alpha-tocopherol has been observed in LDL isolated from volunteers [55]. Second, the bulk price of vitamin E is generally higher than synthetic antioxidants. Third, vitamin E is not as effective as synthetic antioxidants. One study compared the free radicals scavenging capability of synthetic and natural antioxidants, finding alpha-tocopherols were not as effective as TBHQ and BHT [56].

2.3.2.2 Ascorbic acid

Ascorbic acid, as one form of vitamin C, is familiar to most people because of its essential role in maintaining our health. The first scientific story of vitamin C dates back to the middle of 18^{th} century, when people found that drinking lemon juice can prevent sailors from getting scurvy, though they had no knowledge about what was the magical thing in the juice that was responsible for the therapeutic effects. The chemical structure of ascorbic acid was determined by Norman Haworth, who as a result received the Noble prize in 1937.

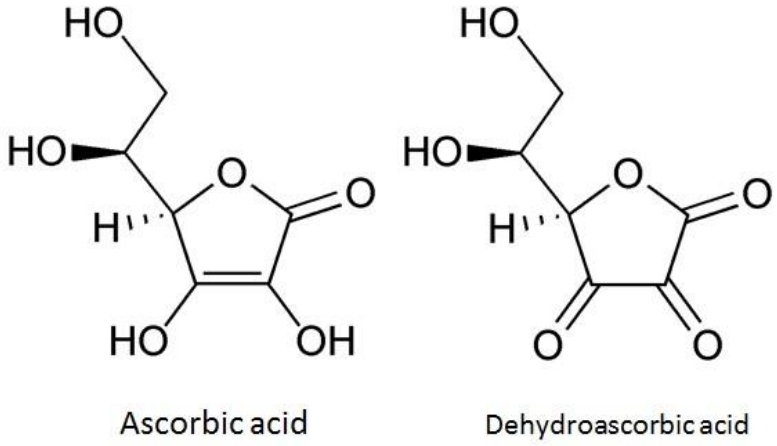

Figure 2-6 Reduced and oxidized forms of ascorbic acid.

Distinct from all the antioxidants discussed above, ascorbic acid is water-soluble because of the multiple hydroxyl groups and the relatively small size. One primary function of vitamin C is to serve as an enzyme cofactor in mammals used for synthesis of collagen, and its antioxidant activity is equally, if not more, important in biological system. Ascorbate protects lipids from oxidation by donating electrons to become dehydroascorbic acid. This is of crucial biological significance since oxidative stress, if not controlled properly, can lead to severe diseases including cardiovascular disease, hypertension and even diabetes [57]. Interestingly, despite its essential biological role, vitamin C cannot be synthesized in the human body. As a result, it is necessary to take in this substance through diet. According to UK Food Standards Agency, it is recommended to take 40 mg of vitamin C per day or 280 mg per week at once.

Therefore, adding vitamin C into food is safe and promotes nutritive value. However, it suffers from the same drawbacks as vitamin E in that its antioxidant activity is not competitive compared to the synthetic options, and, as it is water-soluble, its use in fat is limited.

2.4 Measurement of lipids oxidation

As discussed previously, lipid oxidation in food causes the loss of nutritive value and the release of unpleasant flavor. However, there is no uniform method that can be used to evaluate the oxidation for all the foods, because different fats are present in different substrates, under different oxidative stresses, and can be oxidized via various pathways and generate different products. One method usually determines the level of one oxidation by-product, and therefore, may not give a comprehensive picture.

As a result, numerous analytical methods have been developed to evaluate lipids oxidation. Some of the tests are physical and some are chemical. Some measure primary oxidation products such as lipid peroxide and some measure secondary products like malonaldehyde (MA). Therefore, it is important to carefully select an appropriate measurement method for a particular application.

2.4.1 Measurement of primary oxidation products

In the initial stage of fat oxidation, lipid hydroperoxides and some dienes will form, and they are identified as the primary oxidation products. Following methods are commonly used to determine the levels of the primary oxidation products as an indicator of oxidation.

2.4.1.1 Measurement of Peroxide Value (PV).

PV stands for the total concentration of hydroperoxide and is one of the most commonly used parameters for determination of fat quality. The autoxidation of lipids continuously generates hydroperoxides, which are not stable and can break down into secondary volatile and nonvolatile products. Initially, peroxide is generated at higher rate than it breaks down, so PV increases as the oxidation proceeds. Therefore, PV is a good parameter for oxidation at initial stage [58]. However, when the oxidation enters later stage, the situation is totally reversed.

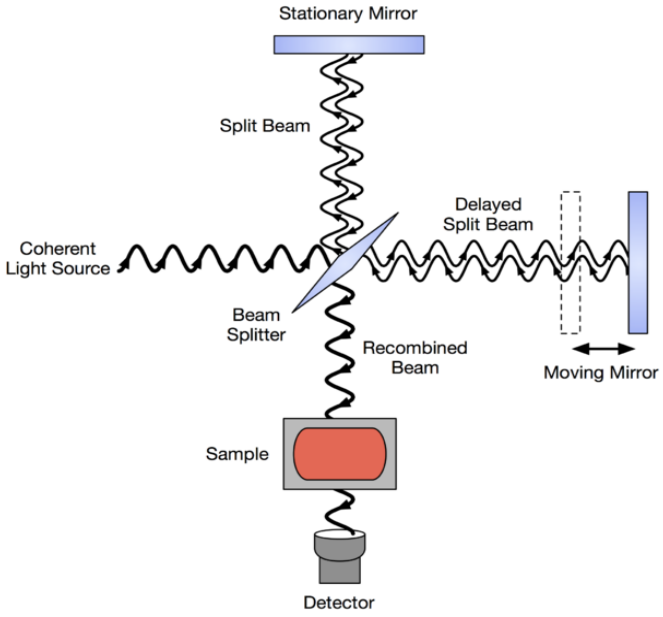

Figure 2-7 Schematic diagram of interferometer for FTIR use (from Wikipedia)

Multiples methods have been proposed to determine PV, mainly including ferric ion complex method, iodometric titration method and Fourier transformation infrared spectroscopy (FTIR).

Iodometric titration method is based on the oxidation of iodide ion (I^-) into iodine (I_2) by lipid hydroperoxide. Then, the iodine is titrated with sodium thiosulfate solution. This iodometric method has been commonly used to determine PV and has become a standard reference method for other methods. However, it has several drawbacks. First, the procedure includes too many steps and is therefore time-consuming, which would be especially problematic when it comes to a large amount of samples to measure at different time points. Besides, the sensitivity of this test is not very satisfying as well.

The ferric ion complexes method is based on the oxidation of ferrous ions (Fe^{2+}) into ferric ions (Fe^{3+}) under acidic condition. Ferrous ions can be oxidized by lipid hydroperoxide, and the

resulting ferric ions form a complex with a dye (thiocyanate or xylenol orange) and show strong absorption at specific wavelengths (505nm for ferric-thiocyanate and 550nm-600nm for ferric-xylenol orange). Thus the PV is determined by reading absorbance at those specific wavelengths. Thiocyanate method is sensitive, easy, and reproducible [59]. Ferrous oxidation of xylenol orange (FOX) assay is also sensitive, rapid and inexpensive. It has been used to evaluate the PV of various substrates including snacks, plant tissues, plasma and chicken meat [60-62]. Also, importantly, it has shown good agreement with the standard iodometric method.

Unlike chemical colorimetric methods, Fourier transformation infrared spectroscopy (FTIR) relies on more sophisticated principles. FTIR detects the absorption, emission and photoconductivity in infrared spectrum and it is capable of collecting a wide range of spectral data simultaneously, which is the major advantage of FTIR over traditional dispersive spectroscopy technique. It directly provides information on different functional groups present in the samples. Also, this technique requires no sample preparation before analysis, thus it is a fast and convenient technique which can be used to monitor the oxidation process in a kinetic fashion. However, though free from sample preparation, researchers using this technique have to interpret the spectral data carefully because it contains information for more than one compound. Similarly, Raman spectroscopy has also been applied to characterize edible oil, mainly for determination of total unsaturation, *cis/trans* isomers and free fatty acids contents [63].

2.4.1.2 Measurement of conjugated dienes.

Oxidation of polyunsaturated fatty acids generates conjugated dienes and lead to a rise in the absorption in the ultraviolet region (234 nm). Lipids containing dienes and polyenes show a shift in double-bond position during oxidation and conjugation. Similarly, conjugated trienes strongly adsorbs at wavelength of around 264 nm. Based on this principle, the oxidation change is detected by direct reading at those two wavelengths, which makes it a convenient and simple

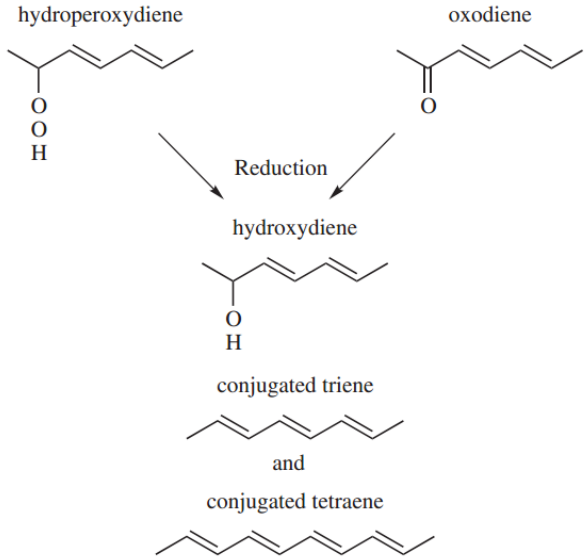

Figure 2-8 Formations of conjugated dienes and trienes in oil

method. Further, a good agreement between PV and the levels of conjugated dienes has been found [64].

However, this technique suffers from some drawbacks as well. First of all, many biological materials share very close absorption peaks in the same UV region and may interfere with each other. Carotenoids are such compounds which may strongly interfere with reading in this region. In terms of specificity and sensitivity, this technique is not very satisfying in comparison with PV methods [65].

2.4.2 Measurement of secondary products

Primary oxidation products are very unstable themselves and they accumulate only at the initial stage of oxidation. At the later stage, primary oxidation products like lipid hydroperoxides start to degrade into relatively stable secondary products including aldehydes, ketones, alcohols, hydrocarbons and so on. Many of them are volatile and directly contribute to the unpleasant flavor in food. Numerous techniques have been developed to analyze these compounds.

2.4.2.1 Thiobarbituric acid (TBA) assay.

Currently, TBA assay is one of the most commonly used techniques for oil quality analysis [66]. Polyunsaturated lipids are prone to oxidize and break down into many volatile small molecules such as aldehydes and acids. Malondialdehyde (MDA) is a common one and has been used as an indicator of lipids oxidation. TBA is able to react with MDA in acid medium and results into a compound called TBA-MDA adduct which strongly absorbs at 532 nm (or it can be excited at 520nm and emit at 560nm). The result is usually expressed as milligrams of MDA equivalents per kilogram sample or as micromoles of MDA per gram of sample.

However, TBA assay is limited by its cross-reactivity. In other words, not just MDA form adducts with 2-thiobarbituric acid. Substances such as alkenals and other aldehydes also complex with TBA and therefore interfere with the results. For instance, TBA forms yellow color compounds with many other aldehydes and result in a strong absorption at 455nm [67], which may overlap with the absorption of TBA-MDA adduct. Therefore, another term, called thiobarbituric acid reactive substances (TBARS) has been used instead.

In order to improve the sensitivity and accuracy of the assay, many different procedures and modifications have been proposed. Basically, TBA assay has been performed (1) directly on the food samples followed by the extraction of colored adducts [68]; (2) on steam distillated from samples because secondary oxidation products are mainly volatile; (3) on liquid extractions of the samples by acid; (4) on lipids extraction. It should be noted that different procedures may result in quite different TBA numbers for the same sample. For example, distillation method usually yields about twice larger TBA numbers than the lipid extraction method, probably because the heat applied to the sample during distillation leads to additional oxidation. On the other hand, TBA assay is usually a good option for comparative study.

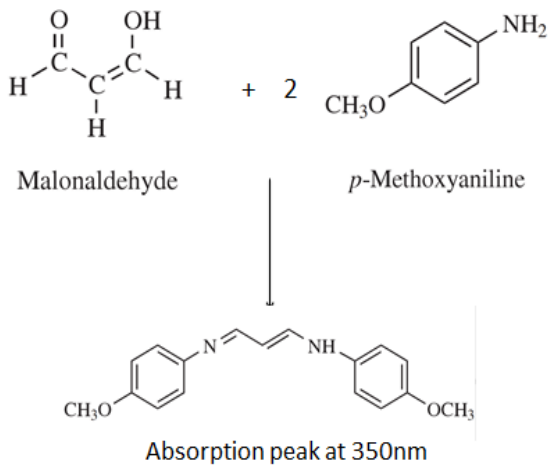

Figure 2-9 The formation of chromogen from p-anisidine and aldehydes

FOX assay provides oxidation information regarding the primary oxidation products (hydroperoxide lipids) and TBARS assay measures the level of secondary oxidation products. Therefore, in our work, both assays were used to provide comprehensive information regarding the oxidation degree of the samples.

2.4.2.2 P-anisidine value (p-AnV).

P-anisidine value measures the amounts of alpha and beta unsaturated aldehydes, which are mainly 2-alkenals and 2, 4-dienals). Under acidic conditions, p-AnV reacts with aldehydes and form yellowish compounds, which have maximum absorption at 350 nm. This method has been reported to show excellent correlation with the total amount of volatile substances [69]. Therefore, it is suitable for estimating the off-odor of samples.

Figure 2-10 Rancimat 743 OSI machine model.

In industry, p-anisidine test is usually combined with PV, termed as total oxidation (TOTOX):

$$TOTOX = 2\,PV + p\text{-}AnV$$

As discussed previously, at early stage of oxidation, PV rises because of the accumulation of lipids hydroperoxide, while these primary products are unstable and degrade to secondary products, mainly various aldehydes. However, in reality, for different substrates and under different conditions, it is hard to determine when the oxidation is in early stage and when it is in the later stage. Therefore, to comprehensively evaluate the oxidation change of foods, a combination measurement is preferred.

2.4.2.3 Oil stability index.

OSI also measures the secondary products of oxidation, but its principle is quite different. During lipids oxidation, big molecules are degraded into small and volatile molecules including aldehydes, alcohols and acids. In the OSI machine, these volatile molecules will be carried away

by an air flow and re-dissolved in water, and the electrical conductivity of the water will increase as a result. The OSI value is defined as point of the maximum rate of oxidation, or, the maximum rate of rise in conductivity.

OSI test is convenient and simple since it requires no additional chemical reagents, and the procedure has been automated. Besides, it is able to monitor the oxidation process in a kinetic way. However, a drawback of this method is that it can only work at relatively high levels of oxidation; in other words, it lacks sensitivity. Also, it is more suitable for measuring bulk oil sample instead of fat in foods.

2.5 Antioxidants in erythrocytes

Antioxidant enzymes are present in almost all the organisms, ranging from as simple as bacteria to as intelligent as us human beings. The main purpose of antioxidant enzymes is to cope with the oxidative stress brought by reactive oxygen species (ROS), including hydroxyl radicals, superoxide anions and hydrogen peroxide. ROS is involved in multiple cell behaviors including growth, differentiation and progression. And it serves as an important defense mechanism when pathogens are present [70]. However, when cells dysfunction and lose the control of it, ROS may be over-produced and accumulated in cells, causing damages to the cells, or on a larger scale, leading to chronic inflammation.

Red blood cells, as oxygen transporter, are constantly exposed to the threat of oxidative stress, but they rarely show any sign of severe damage. The key lies in the fact that they are especially rich in antioxidants such as superoxide dismutase, catalase, and glutathione, which work close together to maintain the normal operation of the “oxygen transporter”, as is shown in **Fig 2-11** [71].

2.5.1 Superoxide dismutase (SOD)

SOD is one of the most extensively studied antioxidant enzymes, due to its crucial importance in dealing with oxidative stress, and it is universally present in organisms that need to cope with oxidative challenge. In human red blood cells, the SOD activity is around 496 units/g Hb [72]. SOD catalyzes the dismutation of highly reactive superoxide anion (O_2^-) into less reactive hydrogen peroxide (H_2O_2) and oxygen (O_2). According to the metal cofactors they bind to, SOD can be categorized into three groups: copper and zinc SOD, iron or manganese SOD and nickel SOD. Cu/Zn-SOD is a homodimer with a molecular weight of around 32,500, and it is usually present in eukaryotes. Currently, most commercially available SOD is extracted from bovine erythrocytes. Mn-SOD is often found in prokaryotes, protists and mitochondria. Ni-SOD is used in prokaryotes.

Superoxide anion (O_2^-) is very reactive and can damage critical cellular organelles even in very short exposure time. Besides, the half-life of superoxide is very short at high concentrations but much longer at low concentration. Fortunately, SOD eliminates this potentially harmful substance effectively. SOD has the largest catalytic efficiency among any known enzymes (about $7\times10^9 M^{-1}S^{-1}$) [73], in other words, this dismutation reaction is almost only diffusion limited. Given the excellent antioxidant activity, it is not surprising that in mammal cells SOD serves as the primary anti-inflammatory enzyme. The disruption of SOD activity is reponsible for many human diseases. For example, depressed SOD expression in the kidney is partially responsible for chronic renal failures [74], amyotrophic lateral sclerosis has been found to be associated with mutations in the Cu-Zn-SOD gene, and chemical modification of SOD in the brain is linked to degenerative disorders such as Alzeimer disease (AD) and Parkinson disease (PD) [75]. Futher, many efforts have been made to apply SOD to treat various ROS-related

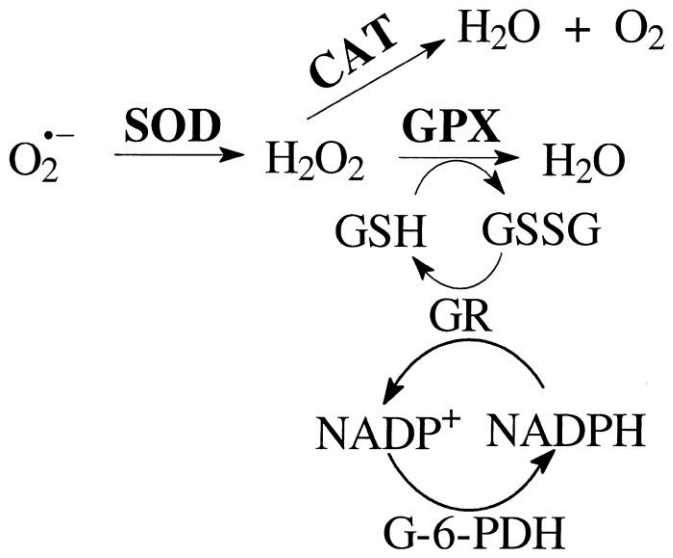

Figure 2-11 Illustration of antioxidant enzymes cooperating to work in cells

problems. For instance, recombinant human Cu/Zn-SOD has been given to premature infants to improve pulmanory outcome [76]. For microbial organisms, SOD is also of crucial importance because it counteracts the deleterious effects from oxygen metabolites [77].

With regard to the determination of SOD activity, many methods have been proposed to analyze different types of sample. Nebot proposed to make use of the autoxidation of tetracyclic catechol and kinetically measure the spectral absorbance at 525 nm [78]. Another method is on the inhibition of NADH oxidation by SOD and it has been applied to determine SOD activity in tissue extracts [79]. Similarly, the principle of xanthine oxidase method is the inhibition of oxidative enzyme.

2.5.2 Catalase (CAT)

Catalase is another important antioxidant enzyme, which works together with SOD to protect cells or tissues against oxidative damages and it is found in almost all organisms exposed to oxygen. Catalase converts two molecules of hydrogen peroxide into two molecules of water

and one molecule of oxygen. Like SOD, catalase is also a very powerful enzyme and it has one of the highest turnover numbers among all known enzymes. Catalase is primarily an intracellular enzyme. It consists of four polypeptide chains and contains four porphyrin heme groups, which allow the enzyme to react with hydrogen peroxide. One unit of catalase will remove 1.0 μmole of hydrogen peroxide per minute at pH 7.0 and 25℃.

Catalase gene mutation has been associated with many diseases, including diabetes, hypertension and vitiligo [80]. Animal study shows that catalase deficiency also increased the chance of tissue injury and renal fibrosis in kidney [81]. In contrast, overexpression of catalase targeted to mitochondria increased the life span of mouse [82]. Therefore, its activity in patients has been an important physiological parameter.

Notably, the endogenous catalase activity in beef *longissimus dorsi* (LD) is around 500 units per gram of the meat, and the number for pork LD and for chicken breast is 750 U/g and 148 U/g respectively. This remarkable catalase activity was shown to play an important role in meat stability during storage, and inhibition of the endogenous catalase activity would render the meat to be more vulnerable to oxidation [83].

2.5.3 Glutathione (GSH) and Glutathione peroxidase (GPX)

Glutathione is a tripeptide, which can be synthesized from L-cysteine, L-glutamic acid and glycine. The thiol group (SH) serves as a proton donor and this is the key for its antioxidant activity. Glutathione exists in two states: the reduced glutathione form (GSH) and the oxidized glutathione disulfide (GSSG). In human blood, the normal concentration of GSH is around 1.7 μmol/ml [84].

Glutathione peroxidase is a general name for a family of enzymes, which catalyzes the reduction of lipid hydroperoxide and hydrogen peroxide to corresponding alcohols and water respectively using glutathione as substrate. In this process, glutathione is converted into

glutathione disulfide, which will later be reduced back by glutathione reductase using NADPH as electron donor. This cycle is quite important for protecting cells from oxidation damages. In human red blood cells, the GPX activity is about 17 U/g Hb [85].

Similar to SOD and catalase, GPX activity has been taken as an important pathological parameter for diagnosis of multiple diseases. For instance, a gradual decrease of GPX activity was observed in patients with chronic kidney disease mainly because this organ is responsible for the synthesis of this enzyme [85]. In patients with chronic obstructive pulmonary disease (COPD), a significant decrease in GPX activity was found, along with the increase in lipid oxidation products-MDA [86]

CHPATER 3

EXTRACTION AND CHARACTERIZATION OF ANTIOXIDANTS FROM ANIMAL ERYTHROCYTES

3.1 Introduction

As is known, extraction of proteins from cells is a ubiquitous procedure in both industry and in scientific research. For example, in many biological science studies, proteomics provide important clues on the state of the cells. Commercially, many valuable proteins have been extracted from animal cells for sale, such as superoxide dismutase (SOD) from bovine erythrocytes and catalase from bovine liver cells. Generally, protein extraction from cells can be divided into three steps a) disruption of cell membrane; b) crude separation c) further purification.

a) Extraction of protein from cells typically starts with the disruption of cell membranes, which encapsulate the proteins. There are many factors to be considered for this procedure. Proper methods should be selected to effectively disrupt various types of cells with different toughness but protein denaturation should be avoided. For instance, osmotic pressure is a relatively gentle method that can usually break mammalian cell membranes quite successfully, but does not work very well with bacterial membranes whose structure is able to resist high pressure. Therefore, addition of enzymes or sonication may be required to lyse these tiny but tough microorganisms. To break red blood cells, osmotic pressure can do the work.

b) Following cell disruption is the crude separation step. Most undesired proteins and other substances like DNA and RNA must be removed in this step. Nucleic acids should be removed at early stage because they may interfere with protein isolation later. Selective precipitation of

protein can be carried out to remove/keep the undesired/desired proteins. One of the most commonly used techniques for selective precipitation is salting out. When salts concentration increases, water molecules are attracted by the salt ions and hence pulled away from proteins, leaving fewer available water molecules to interact with the charged or hydrophilic parts of protein. As a result, proteins are no longer able to maintain the soluble conformation and precipitate out. In a protein cocktail, different proteins precipitate out at different salts concentrations. Typically, SOD precipitates at 40%-80% saturation point of ammonium sulfate [87], while catalase precipitates at 10%-50% saturation point of ammonium sulfate [88]. Salting out procedure can help remove most of the undesired proteins before the final purification.

c) Lastly, after crude separation, further purification may be or may not be required, mainly depending on the future application of the product. For biological analysis, the isolated protein would certainly need to be relatively pure, while for industrial application, such as production of a nutritive supplemental protein, cost may be the primary concern. A popular technique to purify proteins is high performance liquid chromatography (HPLC). It utilizes the unique physicochemical properties (hydrophilic or hydrophobic), binding affinity as well as biological activity of the target protein to selectively isolate it. However, the price of this procedure is high and may be impractical for a large-scale production. Hence, it is not used in our extraction methods.

In this work, which is aimed to the extraction of antioxidants from animal erythrocytes as a food preservative, there are three guidelines:

1) It has not yet been determined, which particular antioxidant (or perhaps their synergistic action) is the most effective as preservative in food and there is no requirement for protein purity, therefore, the extraction procedure should aim at obtaining a cocktail with as high antioxidant activity as possible;

2) Currently, treatment by commercially available food antioxidants such as PETOX (containing 10% BHA and 10% BHT) costs about $5-7 per ton of food. Therefore, producing antioxidants from erythrocytes will only be practical when the cost is competitive with those antioxidants on market.

3) Safety. Substances from red blood cells themselves should be safe as additives in animal feeds. However, during the procedure of extraction, some hazardous chemicals may be added/not completely removed from the system. Therefore, the use of dangerous chemicals should be avoided or reduced.

Based on these three guidelines, two extraction protocols were adopted. The first method is a standard procedure used in the literature for the extraction of SOD, which skips the most expensive chromatographic purification step. It utilizes ethanol and chloroform to specifically remove hemoglobin, which is a potential pro-oxidant and precipitate the desired proteins with acetone and potassium phosphate. The product of this method is denoted as crude SOD (C-SOD). The second method is yet more simplified. In this method, zinc chloride was employed to specifically remove hemoglobin from cell lysates and no further purification was performed. The resulting product is denoted as crude protein (CP).

To characterize the two products (C-SOD and CP), SOD activity and catalase activity of them were measured because these two enzymes are the most common and effective antioxidant enzymes in cells. Total protein content was analyzed by bicinchoninic acid assay (BCA). Since a large amount of zinc was used in the second method, it might lead to additional safety/regulatory issues. Thus, zinc content was measured by inductively coupled plasma mass spectrometry (ICP-MS) to ensure safe zinc levels according to the regulation.

3.2 Materials and methods

Fresh porcine blood was generously donated by Wilson Processing, Inc. (Seneca, SC). Chloroform was purchased from VWR Inc. (West Chester, PA). HPLC Grade Acetone, anhydrous dipotasium hydrogen phosphate (K_2HPO_4), phosphate buffered saline tablets (PBS), sodium chloride ($NaCl_2$) and anhydrous zinc chloride ($ZnCl_2$) were obtained from Fisher Scientific Inc. (Fair Lawn, NJ). Pierce BCA protein assay kit was bought from Thermo Scientific Inc. (Rockford, IL). WST-1 was from Dojindo Molecular Technologies Inc. (USA). Xanthin/Xanthine and Catalase (from bovine liver) was bought from Sigma-Aldrich, LLC. (St. Louis, MO). Superoxide dismutase (bovine erythrocytes) was from Calbiochem Inc. (Darmstadt, Germany). Xanthine Oxidase (from cow milk) was purchased from Roche Diagnostics Inc. (Mannheim, Germany). Hydrogen peroxide (H_2O_2 , 3%) was purchased from Rite Aid (Camp Hill, PA).

3.2.1 Extraction of crude SOD (C-SOD) by the standard method

The standard method to extract SOD from blood was adopted with minor modifications [89](as is detailed in **Fig 3-1**). In brief, fresh porcine blood was centrifuged at 5000g for 15min to obtain packed red blood cells (RBCs), followed by washing using 0.9% sodium chloride twice. The clean and packed cells were lysed by equal volume of 1% tween-20 in DI water. Hemoglobin was then removed by Tsuchihashi treatment (ethanol/chloroform 62.5/37.5 v/v). Then, salting out was carried out with K_2HPO_4 (1/3 w/w) to remove extraneous proteins. Lastly, acetone (75% v/v) was applied to precipitate and obtain crude SOD. The resulting product was washed briefly by 1X PBS and re-dissolved in the same buffer with minimum volume.

3.2.2 Extraction of crude protein mixture (CP) by a simplified method

The procedure is detailed in **Fig 3-2**. Briefly, fresh porcine blood was centrifuged at 5000g for 15min to obtain a pellet of packed RBCs. Subsequent cell lysis was conducted with 3-4 times cell volume of DI water, followed by vigorous shaking for 30 min to ensure complete lysis. $ZnCl_2$ (1.2% w/v) was then added, followed by incubation and occasional shaking for about 1hour or longer. The hemoglobin was precipitated and removed by centrifugation at 5000g for 15min. Resulting transparent solution was stored refrigerated (4 ℃).

3.2.3 Total protein content analysis

The total protein contents of C-SOD and CP were determined by bicinchoninic acid (BCA) assay using the assay kit purchased from Pierce Biotechnology Inc. Its basic principal is as follow. In an alkaline solution Cu^{2+} is reduced to Cu^{1+} by proteins, and bicinchoninic acid specifically complexes with cuprous cation (Cu^{1+}) and the resulting compound strongly absorbs at 562 nm. In brief, 50 parts of solution "A" is mixed with 1 part of solution "B" to obtain working solution. Then 0.1 ml of sample was mixed with 2.0 ml of working solution, followed by incubation at 37 ℃ for 30 min. The absorbance at 562 nm of the resulting solution was measured using a microplate reader. Total protein contents were calculated by comparing with the standard curve.

3.2.4 SOD activity assay

SOD activity was determined by the xanthine-xanthine oxidase method. This method is based on a water soluble tetrazolium salt, WST-1 which is converted into formazan dye upon interaction with superoxide anion generated by the xanthine/xanthine oxidase system. Therefore, SOD activity is reversely proportional to the production of the yellow formazan dye. A 20 μl aliquot of each sample (n=3) was mixed with reaction reagents containing xanthine, xanthine oxidase and WST-1 in 96-well plate (Costar). The reaction was carried out at 37 ℃ for 1hour. The absorbance at 450 nm was read with a microplate reader. SOD activity was calculated based

on the standard curve (0.1 U/ml - 100 U/ml).For CP, since zinc will interfere with xanthine oxidase activity, 10 kilo-Dalton membrane was used to remove over 99% of the zinc in CP.

3.2.5 Catalase activity

Catalase activity was determined using the method described by Pardha [83]. In brief, each sample (0.1 ml) was reacted at room temperature with 2.9 ml of 30 mM hydrogen peroxide in HEPES buffer (pH=7.0). The loss of H_2O_2 was continuously monitored by measuring the absorbance at 240 nm. A unit of catalase would decompose 1 μmole of H_2O_2 per minute at room temperature at pH 7.0.

3.2.6 Elemental analysis

Zinc elements analysis was conducted by Public Agriculture Service Center at Clemson University using inductively coupled plasma mass spectra (ICP-MS).

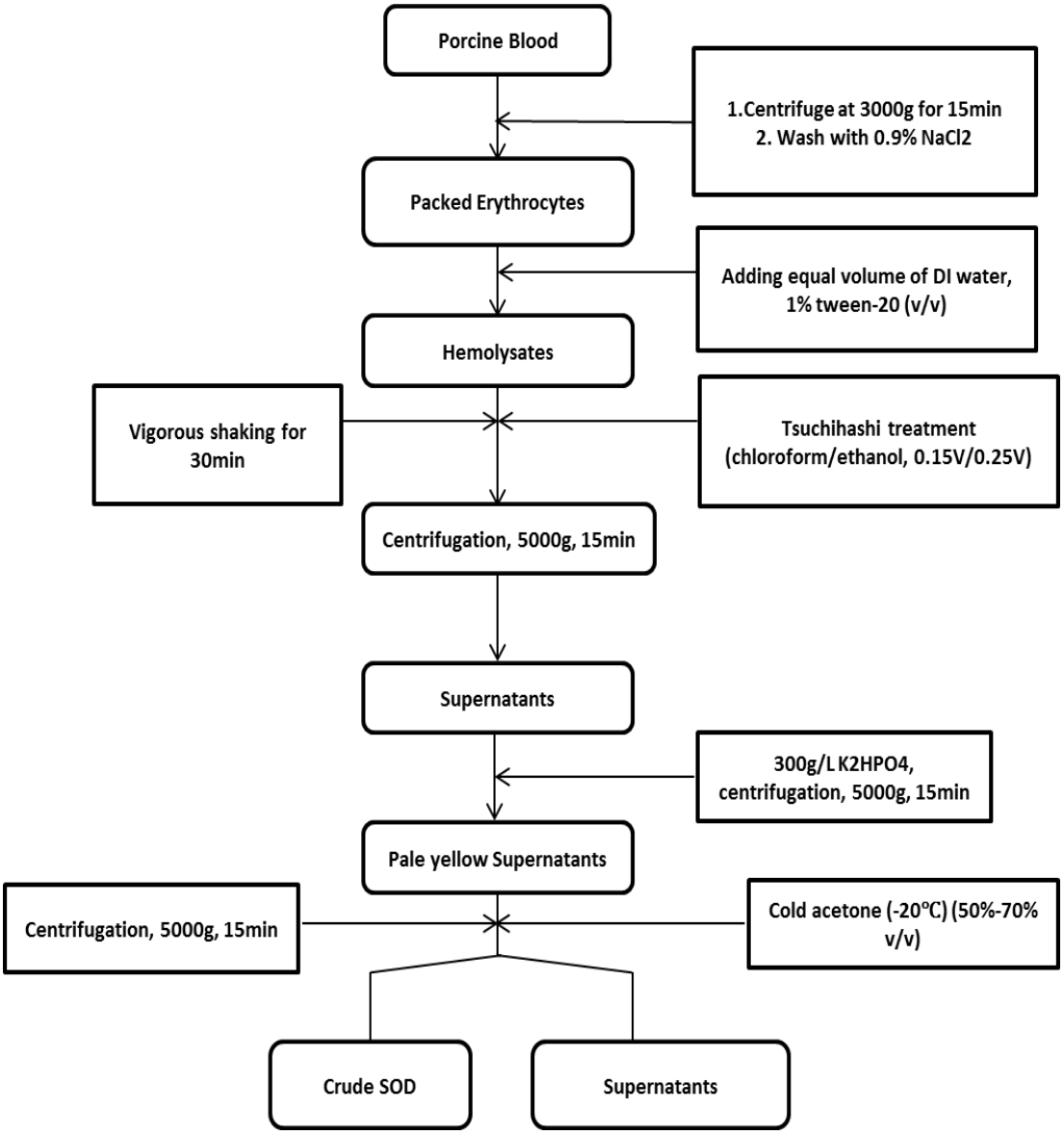

Figure 3-1 Extraction of crude SOD by the standard method (the HPLC step was removed to decrease the cost)

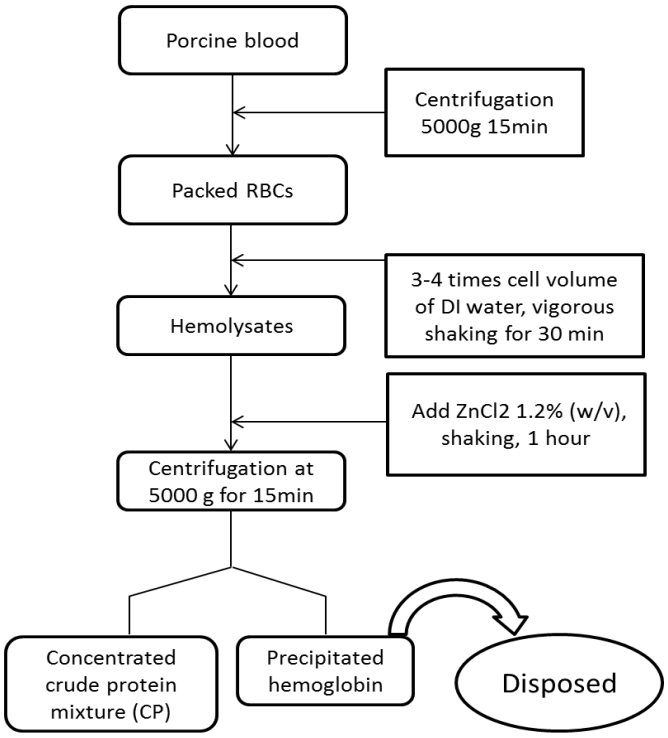

Figure 3-2 Extraction of crude protein mixture (CP) from red blood cells by a simplified method

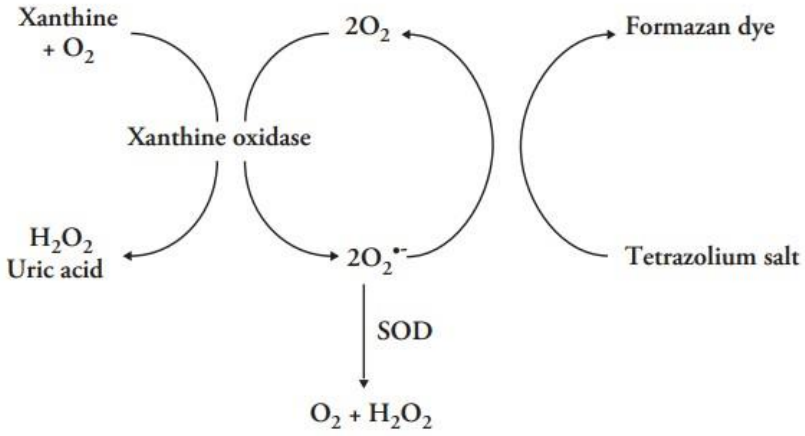

Figure 3-3 Illustration of xanthine/ xanthine oxidase method of SOD activity assay.

3.3 Results

The results have been summarized in **Table 3-1**. The two extraction methods started with same volume (100 ml) of blood so they would be directly comparable. The standard method yielded 20 ml of crude SOD solution (C-SOD) with protein concentration of 2.2 mg/ml. In comparison, the simplified method obtained 91 ml of final solution (CP) with protein concentration of only 0.6 mg/ml. It can be calculated from these numbers that the total protein yields for C-SOD and CP are 44.0 mg and 54.6 mg respectively.

In terms of SOD activity, C-SOD was much more concentrated than CP, with SOD activity of as high as 1100 units/ml compared to 224 units/ml of CP. Further, considering that the standard method was specifically designed to extract SOD, it was not surprising C-SOD also showed a higher SOD specific activity (500 units/mg) over CP (373 units/mg). However, it should be noted that the total SOD activity yields for the two methods are close (22000 units for C-SOD and 20384 units for CP from 100 ml of blood).

Interestingly, C-SOD was shown to have a decent amount of catalase activity (300 units/ml) whereas CP contained no discernible catalase activity, indicating that high concentration of zinc might have inhibited catalase or precipitated it along with hemoglobin.

Table 3-1 Characterization of C-SOD and CP

	Initial blood volume (ml)	Ending volume (ml)	Protein concentration (mg/ml)	SOD activity (U/ml)	SOD specific activity (U/mg)	Catalase activity (U/ml)	Zinc Concentration (ppm)
C-SOD	100	20	2.2	1100	500	300	NA
CP	100	91	0.6	224	373	<10	4800

3.4 Discussion

Due to the excellent antioxidant ability and crucial biological significance of SOD, many researchers have extracted and characterized SOD from different sources for various purposes. Blood is a favorable and inexpensive source of this enzyme. Early work has shown that a significant amount of SOD can be extracted from porcine blood (13300 units SOD from 100ml of blood) [90]. Also, SOD and catalase were also extracted from human blood for medical application [88]. Besides blood, many plants are also rich in SOD. Hadji's group purified Cu-Zn-SOD from garlic to investigate its antioxidant effects on tumor cells [91].

However, most of these researchers utilized chromatography or dialysis to achieve a desirable purity, which greatly increases the cost and renders it impractical for large scale industrial applications such as food preservatives. Consequently, in this work, efforts were made to achieve a balance between purity and cost-crude products were extracted without any further purification in order to lower the cost to a level that is competitive to current food preservatives on market.

The comparison between the two methods used here is summarized in **Table 3-2**. By the standard method, SOD was successfully extracted with the specific activity of 500 units/mg and total activity of 22000 units. The simplified method was just slightly less effective, yielding SOD with the specific activity of 373 units/mg and 20384 units in total. With respect to cost and safety, the simplified method does not employ any organic solvents and use much less reagents hence it is preferred over the standard method. A major drawback of the simplified method is that it yielded very little catalase activity (<10 units/ml), which may compromise its antioxidant activity.

The residual zinc (4800 ppm) in CP complicates the story. On one hand, in terms of safety, zinc is classified as a GRAS (Generally recognized as safe) material by FDA while it is regulated to be no more than 250 ppm in animal feeds in Europe. On the other hand, there are numerous

benefits of zinc that have been discovered. First, zinc is an essential trace element required for both humans and animals due to its indispensable role in over 100 enzymes [92, 93], including several antioxidant enzymes. Zinc supplementation has been reported to improve clinical outcomes of several diseases including spinal cord injury [94],aid in the recovery of burn patients [95] and in the case of acute diarrhea [96]. Second, zinc may stabilize and improve the performance of SOD.

To sum up, the simplified method is more likely to be amplified to an industrial scale because it is less expensive, simpler and also safer. But one crucial question remains: is the erythrocytes extract, either C-SOD or CP, capable of arresting or slowing down fats oxidation in foods, which will be addressed in next chapter.

Table 3-2 Comparison between the standard method and the simplified method

	Standard Method	**Simplified Method**
Denotation of Products	C-SOD	CP
Concentration	High (1100 U/ml)	Low, 224 U/ml (if this concentration does not work, it may need to be concentrated, which may lead to additional cost for the procedure)
Total antioxidant enzyme yield	High SOD activity and some catalase activity	High SOD activity, no discernible catalase activity
Purity	Relatively high	Relatively low
Cost	High	Low
Safety	Organic solvents residue need to be removed	Not much safety concern as long as Zinc concentration is well controlled
Time of procedure	Around 4 hours	Around 3 hours

CHAPTER 4

EVALUATION OF ERYTHROCYTES EXTRACTS TO INHIBIT FAT OXIDATION BY FOX ASSAY AND TBARS ASSAY

4.1 Introduction

Fat rancidification, as discussed in chapter **2.1**, is mainly caused by the oxidation of fats and lipids. The oxidation products are mainly lipid hydroperoxides and they are prone to degradation into small molecules including acids, ketones and aldehydes, which are responsible for the off-flavor of foodstuffs. Auto-oxidation is the primary pathway of fats deterioration. Free radicals such as superoxide anion, hydroxyl radicals and hydroxyl peroxide are the main players in the auto-oxidation process. As a result, in pet food industry, it is a common practice to add antioxidant preservatives scavenge those free radicals and prevent oxidation. At present, commercially available antioxidant preservatives include: synthetic options such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and *tert*-butylhydroquinone (TBHQ) and ethoxyquin (ETQ) and natural options such as tocopherols (vitamin E) and ascorbic acid (vitamin C). Synthetic antioxidants are generally effective and relatively inexpensive but they suffer from a notorious reputation of being potentially carcinogenic [5, 14, 97, 98]. The natural antioxidants are safe, but not as effective as the synthetic ones, and they are generally more expensive. Consequently, there is a need to seek for an inexpensive, safe and effective alternative.

Erythrocytes are rich in antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH), which are responsible for eliminating free radicals in the cells. Therefore, these antioxidants can be extracted from red blood cells as a novel alternative preservative. In our work, two methods were used to extract the antioxidants: a standard method which was specifically designed for SOD and the product is denoted as C-SOD; a novel and simplified method which simply removed hemoglobin (a potential pro-oxidant) and its product is denoted as CP. We hypothesize that addition of C-SOD and CP into foodstuffs can effectively prevent or slow down fat oxidation.

This chapter focuses on validating the hypothesis by evaluating the protective effects of C-SOD and CP. To fulfill this goal, it is necessary to choose: a) proper food or fat samples relevant to pet foods; b) appropriate treatments to oxidize the samples in a way that resembles the oxidation process occurring to foodstuffs in the real conditions; c) appropriate methods to measure lipids oxidation.

a) In the pet food industry, rendered poultry fat is added into pet foods because it promotes flavor and nutritive value and chicken fat is a commonly used one. Besides, chicken fat is rich in linoleic acid (17.9%-22.8%) and other omega-6 and omega-9 acids which are susceptible to oxidation. Therefore, chicken fat can serve as an appropriate oxidation model for our research. Chicken meat was also used because it appropriately models canned wet pet foods, which are made from emulsified animal organs and tissues.

b) Many factors can accelerate lipids oxidation, such as UV irradiation, moisture and heat. UV irradiation catalyzes the formation of free radicals in unsaturated acids [99]; High temperature can increase the rate of auto-oxidation and hence speed up oxidation of fats exponentially [100]. The effect of moisture is a little more complicated. It was proposed that a food sample is most stable at its monolayer water content [101], but as water content is above this

"monolayer level", oxidation can be increased by moisture. With respect to the oxidation of pet foods, the exposure to high temperature and induction of unintended moisture are the common causes for oxidation while UV irradiation is less likely to happen in the process of manufacturing and storage. Consequently, heating treatment was applied to the meat/fat samples and moisture was purposely induced to the fat samples to simulate the naturally occurring oxidation process in an accelerated way.

c) To evaluate the oxidation of fats, various methods are available and must be chosen carefully depending on the particular application. Ferrous oxidation xylenol-orange (FOX) assay is a sensitive, inexpensive and accurate method which has been extensively used to measure the level of lipids peroxides in various types of samples including meats and fats [59] [102, 103]. Thiobarbituric reactive substances (TBARS) assay is also a popular technique used in food industry to evaluate lipids oxidation. This method specifically detects secondary oxidation products representative by malonaldehyde. Besides, TBARS assay is also sensitive, cheap, simple and suitable for different types of samples. Therefore, FOX assay and TBARS assay were employed to measure the lipids oxidation in the food samples.

In order to compare the performance of the erythrocytes extracts (CP and C-SOD) with commercially available antioxidants, PETOX, which contains 10% BHT and 10% BHA, was used as an internal standard. Further, in order to identify the antioxidant activity of individual antioxidant enzymes, we also incorporated commercial purified SOD and catalase in the experiments. Lastly, accelerated aging experiment was conducted to estimate the shelf-life of extracts.

4.2 Materials and methods

Ground chicken breast meat (10% fat content) was purchased from local supermarket (Clemson, SC). Chicken fat was homemade by Dr Vladimir Reukov. PETOX was obtained from

Kemin, Inc. (Des Moines, Iowa). Methanol, Methanolic Sulfuric acid and Catalase (from bovine liver) were bought from Sigma-Aldrich, LLC. (St. Louis, MO). Commercially available superoxide dismutase from bovine erythrocytes was purchased from Calbiochem Inc. (Darmstadt, Germany). Xylenol orange was bought from MP Biomedical, LLC, (Solon, OH). Ferrous ammonium sulfate was obtained from Avantor Performance Materials, Inc. (Center Valley, PA). Dichloromethane was bought from Thermo Fisher Scientific, Inc. (Fair Lawn, NJ). Trichloroacetic acid was bought from VWR, Inc. (West Chester, PA). Thiobarbituric acid was purchased from TCI, Inc. (Tokyo, Japan).

4.2.1 Antioxidants treatment and oxidation

a) Oxidation of chicken meat. For each sample, 1.5 gram of ground chicken breast meat containing about 10% fat was weighed into a 15-ml tube and 75 μl of following antioxidants samples (or controls) are added and mixed with the meat:

1) CP (the final concentration of zinc element in the meat would be around 250 ppm);

2) C-SOD;

3) Commercial purified SOD, dissolved in deionized water, 1000 U/ml;

4) Commercial purified catalase, dissolved in deionized water, 1000 U/ml;

5) 2000 ppm PETOX in water, emulsified by an ultra-sonicator (the final concentration would be 1000 ppm based on fat, which is the current the dose level used in industry)

6) Deionized water (negative control).

7) Deionized water (this sample was to be refrigerated as a positive control).

After antioxidants treatments, all samples except the positive control were incubated at 37 ℃ for 12 hours. The resulting meat samples were ready for analysis. All the samples were prepared in duplicates (n=2).

b) Oxidation of chicken fat. Similar to treatments for chicken meat, 500 µl of chicken fat was mixed with 25 µl of the same antioxidants samples used in the meat experiment in a 1-ml centrifuge tube. The mixture was ultra-sonicated for 15 seconds to form emulsion. All samples, except the positive control, were incubated at 50 ℃ for 12 hours. All the samples were prepared in duplicates (n=2).

4.2.2 FOX assay

An improved FOX assay method [104] was applied with some modifications.

a) For meat samples, 4 ml of cold (-20 ℃) dichloromethane/ethanol (v/v 3/2) was added into each meat sample, followed by ultra-sonication for 1 min to liberate the hydroperoxide lipids in meat. Centrifugation at 8000g for 5 min was applied so that the organic phase containing lipids would separate out (the bottom phase) and was ready for assay. In the meantime, the FOX reaction medium was prepared. It consists of 10 µl 5mM aqueous ferrous ammonium sulfate, 20 µl 0.25 M methanolic sulfuric acid, 20 µl 1mM methanolic XO, and 130 µl dichloromethane/ethanol (v/v 3/2). Lastly, 20 µl of the lipids solution was taken and mixed with the reaction medium in a quartz 96-well plate and incubated at room temperature for about 15 min. Absorbance at 560 nm was read.

b) For fats samples, 100 µl of the fat was taken and dissolved in 4 ml of cold (-20 ℃) dichloromethane/ethanol (v/v 3/2). Then, the lipids solution was ready to be analyzed using the same FOX reaction medium as described above.

The oxidation inhibition rate of sample was calculated by the following equation:

Oxidation inhibition (%) $= (\frac{OD_{NC} - OD_S}{OD_{NC} - OD_{PC}}) \times 100\%$. (OD: optical density; NC: negative control; PC: positive control; S: sample).

4.2.3 TBARS assay

TBARS assay was only applied to the meat samples. First, 50 μl of 7.2% BHT (in ethanol) was added to the meat sample. Then, 4 ml of deionized water was added into each meat sample, followed by ultra-sonication for 1 min to homogenize the tissue and hence fully dissolve the volatile oxidation products such as aldehydes and acids. 250 μl of this homogenate was taken and mixed with 250 μl of TBA reaction medium containing 15 mM TBA and 15% TCA (w/v). The mixture was incubated at 90 ℃ for 15 min to allow the formation TBA-MDA adducts. The resulting compounds can be excited at 520 nm and strongly absorb at 560 nm.

4.2.4 Dosage effects

Three concentrations of CP, the original concentration, 2-fold dilution and 3-fold dilution, were tested on both ground chicken meat and chicken fat using the method described above.

4.2.5 Accelerated aging experiment

The production of C-SOD involves the use of organic solvents including ethanol, chloroform and acetone which may lead to safety issues, and the cost of C-SOD is also relatively high. Therefore it is less likely to be commercialized. Hence, the aging experiment was conducted with only CP. CP was aliquoted in 0.5 ml centrifuge tubes (300 μl each tube), followed by incubation at 50 ℃ for12 hours, 1 day, 2 days, 4 days and 8 days, and 10 days. Antioxidation efficacy of the samples from different time points was tested on both ground chicken meat and fat as described above. Oxidation change was determined by FOX assay. The oxidation inhibition rate was plotted versus the time of aging.

4.3 Results and discussion

4.3.1 Antioxidation efficacy in meat

FOX assay (**Fig 4-1A**) shows that CP, PETOX and the positive control have the lowest levels of peroxide value and they are not significantly different from each other. The meat sample mixed with water was most seriously oxidized as expected. Both CP and PETOX successfully inhibited 100% oxidation within 12 hour-incubation at 37 ℃. C-SOD, however, though containing more SOD activity and catalase activity than CP, turned out to be less effective, reducing oxidation by 62%. Commercially purified SOD and catalase decreased the oxidation level by only approximately 23% and 32% respectively.

Overall, the TBARS assay results (**Fig 4-1B**) agreed with the FOX assay very well with only minor differences. CP and PETOX were equally effective, successfully preventing oxidation within the time frame of 12 hours. C-SOD, commercial purified SOD and catalase suppressed the oxidation by 66%, 37% and 18% respectively.

It can be seen that adding individual natural antioxidant enzymes, SOD or catalase (75 U/gram of the meat), was able to improve the stability of fats and lipids in chicken meat slightly, but far away from practical requirements.

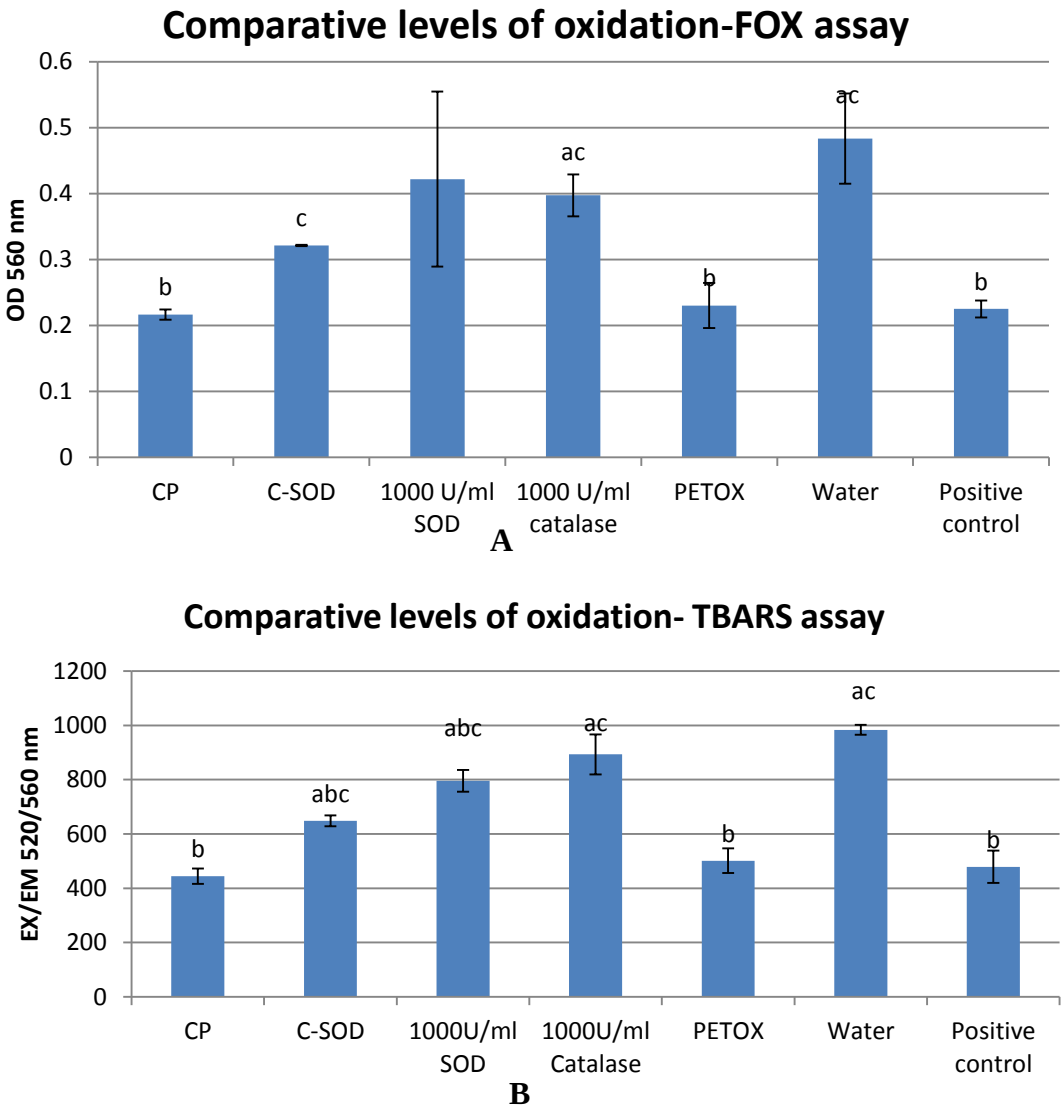

Figure 4-1 The comparative levels of oxidation of in meat model with different antioxidants treatments. A: the oxidation was evaluated by FOX assay. B: the oxidation was evaluated by TBARS assay. PETOX as a commercial food antioxidant was used as an internal control. Water was a negative control Samples stored at 4 ℃ was the positive control. P^a <0.05: comparison with PETOX was significant. P^b <0.05: comparison with water was significant P^c <0.05: comparison with positive control was significant.

It is important to note that the endogenous enzyme activity of meat is crucial for its stability. Chicken meat itself contains a significant amount of antioxidant enzymes. The endogenous SOD activity in chicken breast is around 58-70 U/g and the catalase activity is about 40 U/g depending on the species of chicken and ways of raising [105]. One study found endogenous antioxidants enzymes were important for the stability of lipids in meat[106] and it has been reported that endogenous catalase effectively protected lipids from oxidation in meat whereas addition of high concentration of exogenous purified catalase did not improve the lipids stability much [107]. The reason could be that antioxidant enzymes like SOD and catalase tend to be more effective when working together. Besides, enzymes are water-soluble and hence can barely eliminate free radicals inside of fat tissues. Further, high moisture (40%-50%) content in meat dilutes the antioxidant enzymes while fat-soluble antioxidants such as BHT and BHA can easily dissolve and concentrate in fat to scavenge free radicals.

Though C-SOD was more concentrated than CP, containing more SOD and catalase, its performance was less satisfying than CP. One reason may be that CP could contain some other antioxidants which may actually be equally or even more important than SOD and catalase. Glutathione and glutathione peroxidase, for example, are responsible for reducing oxidized lipids in cells and therefore they may play a vital role in protecting fats, however they are not studied in this work. Besides, the presence of zinc in CP can be extremely helpful because zinc may stabilize both the endogenous and exogenous SOD and improve their activity [108, 109], which is important for the prevention of the fats oxidation in the meat.

Further, it is also easy to realize the add-in value of zinc. Nowadays, people are quite familiar with zinc because of its essential role in maintaining human health. Dietary supplementation of zinc has become common. Numerous studies have demonstrated the health benefits of taking dietary zinc. For instance, zinc rich diet was believed to restore impaired

immune responses in elderly people, especially for those with specific IL-6 polymorphism [110]; Zinc supplementation was proved to improve pregnancy and infant outcome [111]; Children with protein energy malnutrition (PEM) supplemented with 10 mg of zinc in the form of zinc sulfate made significant gains in terms of albumin levels, probably reflecting the rehabilitation of the PEM [112]. Also, zinc has also been reported to be a potential preservative due to its capability in the inhibition of microbial growth in food. For instance, a study tested zinc chloride as a preservative in cracked table olive and found 0.05% and 0.1% (w/v) $ZnCl_2$ reduced *E.coli* growth significantly and even promoted sensory profile [113].

Regarding the safety of zinc (in forms of zinc chloride or zinc sulfate), FDA classifies it as a GRAS (generally recognized as safe) material and puts no clear dose restriction on the use of it as a food additive; in Europe it is regulated to be less than 250 mg/kg (or ppm) in animal feeds. The dose level applied in our experiment did not exceed this maximum limit (the background zinc in ground chicken meat can be neglected). Consequently, using zinc at this concentration to protect meat can be regarded as safe.

In summary, being effective, inexpensive, and natural, CP is a promising product as a future alternative antioxidant preservative for meat and meat based products.

4.3.2 Antioxidation efficacy in fat

The fat model turned out to be much more stable since it contained much fewer pro-oxidants and lower moisture level. Therefore, the difference between the positive control (the sample refrigerated at 4 ℃) and the negative control was not large, only 0.075 in terms of optical absorbance. Consequently, the variation was relatively big. The result was summarized in **Fig 4-2.**

Both C-SOD and PETOX inhibited 85% of the oxidation. CP was slightly less effective, inhibiting 67% of the oxidation. Purified commercial SOD and catalase reduced the oxidation by 34% and 13% respectively, similar to the results of the meat model.

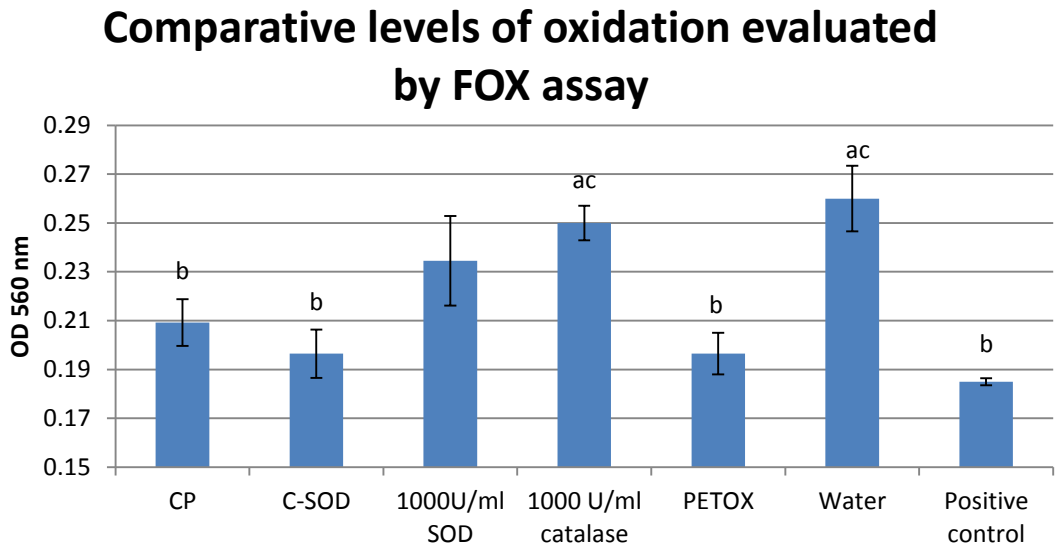

Figure 4-2 The comparative levels of oxidation in fat model. PETOX as a commercial food antioxidant was used as an internal standard. Water was used as the negative control Samples stored at 4 ℃ was the positive control. P^a <0.05: comparison with PETOX was significant. P^b <0.05: comparison with water was significant P^c <0.05: comparison with positive control was significant.

In the fat model, the moisture content was 5%, much lower than that of the meat model. Therefore, the water-soluble antioxidants in C-SOD could easily concentrate in the water phase and effectively scavenge free radicals generated near the water-fat interface, where much of the oxidation may occur. In the meat model, however, high moisture content greatly diluted C SOD and compromised its effectiveness.

In the meat model, CP was shown to be better than C-SOD since zinc may stabilize and enhance both endogenous and exogenous SOD as discussed previously. However, in the chicken fat model, there is no native SOD to speak of; therefore the impact of zinc weighed less. As a result, C-SOD, which has much higher SOD activity and catalase activity did a better job in the fat model.

Overall, both CP and C-SOD work in the fat model, but whether they can be applied to bulk fat as a preservative needs to be further investigated. Adding water-soluble antioxidants into bulk fat is a paradoxical practice because it inevitably increases the moisture content greatly (the normal moisture level in bulk poultry fat is less than 0.1%), which will actually accelerate fat oxidation. Besides, mixing water-soluble antioxidants with fat requires emulsifiers or strong physical energy like ultra-sonication, which increase the cost of the manufacturing. But for foods which containing some levels of moisture, such as canned pet food, CP and C-SOD can be used as alternatives for synthetic antioxidants.

4.3.3 Dosage effects

Generally, the result shows that the antioxidant ability of CP decreased quickly along with the decrease of dosage in both meat model and fat model (**Fig 4-3**).

With respect to the protection of meat (as is shown in **Fig 4-3A**), 5% (v/w) of the original CP (1xCP) inhibited 91% of the oxidation. However, the 2-fold diluted CP and the 3-fold diluted CP only inhibited 50% and 25% of the oxidation, not comparable to the performance of PETOX which inhibited as much as 85% of the oxidation. The result for fat is similar. As is shown in **Fig 4-3B**, 5% (v/v) of the original CP inhibited around 86% of the oxidation. The 2-fold dilution also showed decent antioxidant ability, reducing peroxide value by 70% while the 3-fold dilution only inhibited 35% of the oxidation. It should be noted that in this experiment, the performance of CP was better than the result of **Fig 4-2B.** The reason is that the fat model was not a very sensitive one. The margin between the positive control and negative control is too small (<0.05 in terms of optical absorbance). Therefore, the variation is relatively large.

Overall, it can be seen that in order to achieve the antioxidant effects of commercially available antioxidant (PETOX), it is necessary to use at least 5% of the CP of original or even higher concentration (or it may also be viable to use very concentrated CP in a small volume).

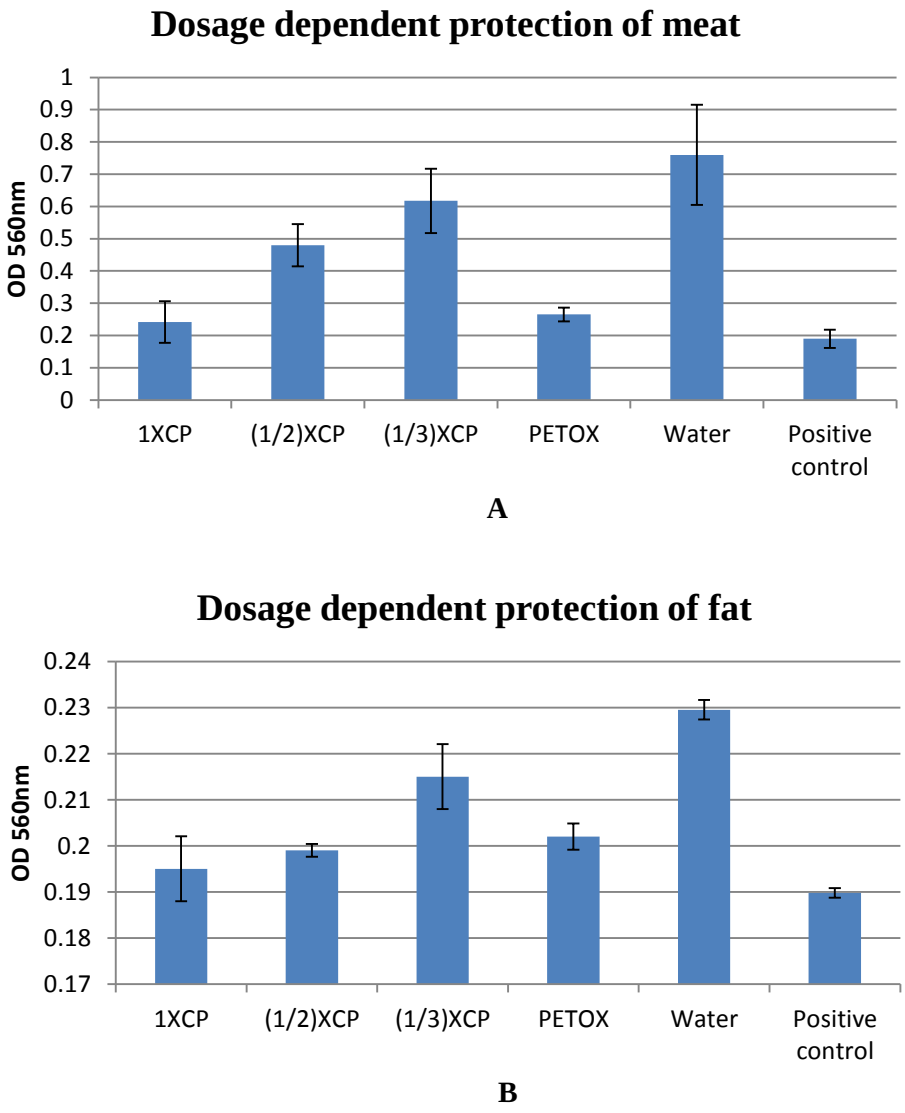

Figure 4-3 Dosage dependent protection of meat (A) and fat (B). 5% (v/w for meat, v/v for fat) of three concentrations of CP (original, 2-fold dilution, 3-fold dilution) were added into meat or fat. The comparative oxidation level was determined by FOX assay.

4.3.4 Accelerated aging experiment

During the accelerated aging at 50 ℃， some proteins became unstable and started to precipitate out of the solution at day 1. The precipitates were removed before experiments on meat and fat. The result with meat model was shown in **Fig 4-3A**. The oxidation inhibition ability of CP with meat did not decrease much. The fast decrease occurred between day 1and day 2, from 100% to around 85%. In the following days, its antioxidation ability remained impressively stable, staying at around 85%-90%.

The result with fat was shown in **Fig 4-3B.** The oxidation inhibition rate was around 70% initially, and dropped to 59% after one day. Later, CP's activity did not go down much. After 10-day incubation at 50℃, CP could still inhibit around 50% of the oxidation, indicating the effective ingredient responsible for the protection of fat was also quite stable.

The excellent stability of CP can be partially explained by the presence of zinc. First, with respect to the protection of meat, zinc can be very important since it can stabilize the native SOD in meat and improve its activity [108, 114], and as a metal element, zinc itself does not lose this ability during the accelerated aging. In contrast, macromolecules such as proteins tend to denature over time. Even small molecules like BHT and BHA, they tend to lose activity inevitably gradually because they are sacrificial antioxidants in essence and always ready to get oxidized. Second, SOD is also an effective ingredient in CP, which can also be stabilized by zinc.

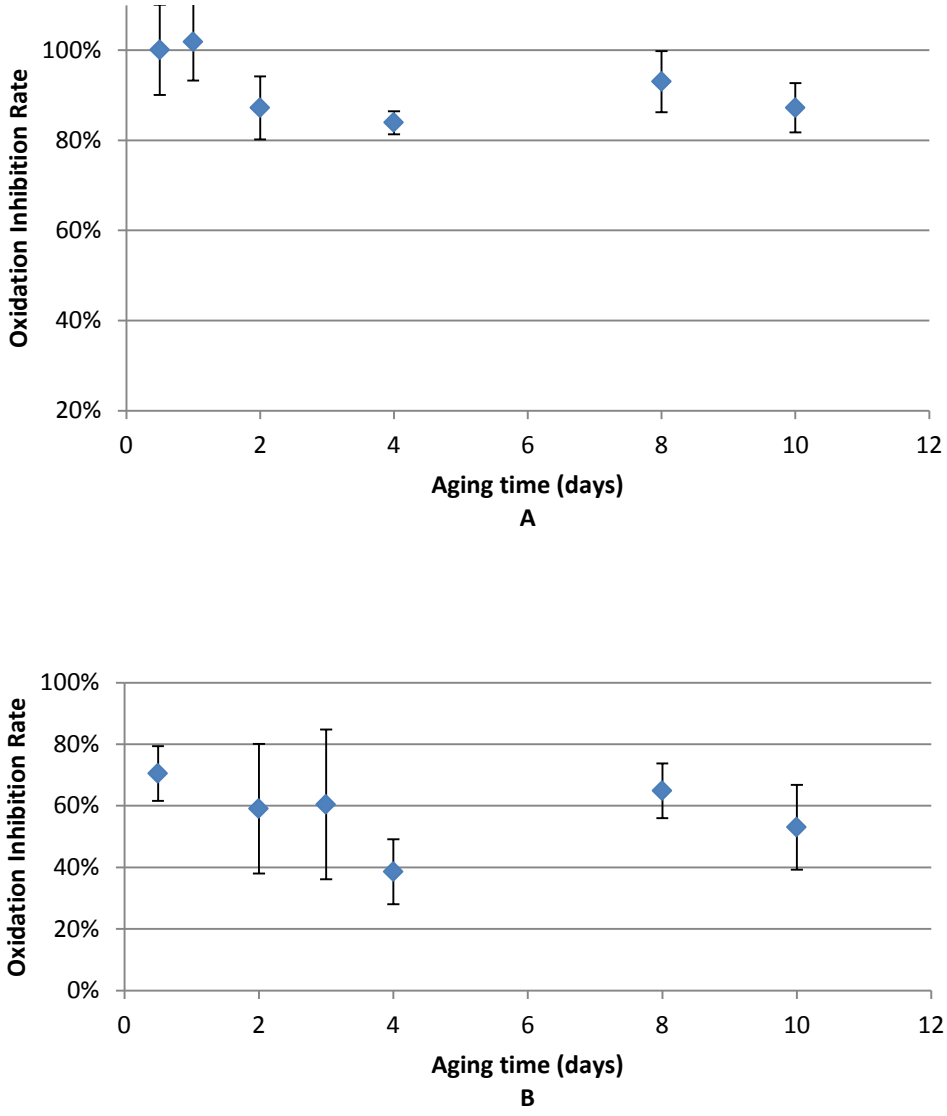

Figure 4-4 The accelerated aging of CP at 50 ℃. CP was stored at 50 ℃. and taken out at different time points to test its antioxidant efficacy in both the meat model (A) and the fat model (B). Oxidation inhibition rate was calculated and plotted versus the aging time.

From an industry perspective, the storage of CP is inconvenient since it is not concentrated. Specifically, to protect a ton of food, 50 liters of CP will be needed, while in comparison, only 0.1-1liter of PETOX will be sufficient for the same work. Storing large volume of liquid in a manufacturing facility will increase the cost and also make it difficult to operate. Therefore, in the future, it may be necessary to concentrate CP using appropriate centrifuge membranes. Besides, though zinc has been shown to be antibacterial previously [115], CP as a cocktail of various biological substances including proteins and nucleic acids, may still be susceptible to bacterial contamination and pose additional safety issues which needs to be studied in the with caution.

CHPATER 5

CONCLUSIONS AND FUTURE RECOMMENDATIONS

5.1 Conclusions

Nowadays more and more people are paying attention to the safety of the petfood they feed to their pets. The safety of synthetic antioxidants such as BHT, BHA and ethoxyquin is controversial. Though being approved by FDA to use in animal feeds for a long time, they have been shown to be causing tumors and many other problems in animal experiments. In response to customer's need, there is a trend of looking for natural alternatives in recent years. At present, the most popular natural antioxidants on market are vitamin E and vitamin C as well as herbs such as thyme, dittany, marjoram, lavender and rosemary. High cost and relatively low efficacy are the main drawbacks of these natural antioxidants as food preservatives. Besides, herbs extracts have strong flavors which may change the original taste of food.

Erythrocytes are a good source of antioxidants. Erythrocytes function to transport oxygen for blood and are constantly exposure to the oxidative stress. As a result, they are rich in antioxidants including superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) to eliminate the oxidative free radicals before they cause any severe damages to the cells. This work looks to find a way to extract these natural antioxidants from erythrocytes and add them into pet foods to retard the oxidation of the fats contents.

To fulfill this goal, two extraction methods were implemented. One is a standard procedure which was designed to obtain SOD from red blood cells. This standard method uses several organic solvents including acetone, ethanol and chloroform which can be problematic when it

comes to safety issues. The product of this procedure is denoted as C-SOD. The other method was a novel and simplified one using no organic solvents during the procedure and the product of this method is denoted as CP. The two products were partially characterized. The effectiveness of CP and C-SOD was tested using ground chicken breast meat and chicken fat, evaluated by ferrous xylenol orange (FOX) assay and thiobarbituric (TBARS) assay. CP and C-SOD were also compared to a commonly used antioxidant in the industry-PETOX. Lastly, accelerated aging experiment was conducted with CP.

The work on extraction and characterization demonstrated that:

a) Both the standard method and the novel simplified method successfully obtained a decent amount of total SOD activity. But, the former also yielded some catalase activity while the latter did not.
b) Since the standard method involves more extraction and purification procedures than the simplified one, it is not surprising that its product (C-SOD) has higher specific activity than the product of the simplified method (CP).
c) In terms of safety and price, the simplified method would be preferred since it involves no organic solvents which can lead to health concerns and raise the cost

The work on antioxidant efficacy of CP and C-SOD test demonstrated that:

a) CP was very effective at inhibiting oxidation in meat probably because 1) CP contains some other strong antioxidants that were not identified; 2) zinc may stabilize both the exogenous and endogenous SOD activity.
b) Both CP and C-SOD were effective in the fat model which contained 5% moisture. C-SOD was better. The reason may be that it is more concentrated with SOD activity of 1100U/ml in comparison of 224 U/ml of CP.

c) Different types of food undergo oxidation via different mechanisms. For meat products, the presence of pro-oxidants (especially iron) greatly accelerates fat oxidation, however in pure fat, pro-oxidants are much fewer and the moisture may be a critical factor instead. As a result, an appropriate antioxidant should be selected for a specific type of food.

The work on accelerated aging experiment demonstrated that:

a) In the meat model, CP's antioxidant activity did not decrease much over the course of 10-day incubation at 50℃, from 100% inhibition to around 85% inhibition.
b) In the fat model, CP's performance also remained relatively constant because the antioxidant SOD could have been stabilized and improved by the presence of zinc.

5.2 Future recommendations

For future study, several questions need to be addressed.

a) Identify the proper type of petfood to which the erythrocytes extracts, C-SOD and CP, can be applied.

   As mentioned previously, both CP and C-SOD are antioxidants in water solution, so it would be difficult to apply them to pure bulk fat with extremely low moisture because that will actually induce oxidation. Wet canned pet food contains a high level of moisture and it essentially resembles the meat model in this work, therefore, it may be tested in the future.

b) Test the samples in petfood in a real industrial manufacturing, packaging and storage conditions.

   Though CP and C-SOD were demonstrated to be effective in the two models in the experiments, the real industrial conditions can be more complicated. Moisture, UV irradiation and presence of pro-oxidants are all reasons for fast oxidation of fats while

it is unknown whether the erythrocytes extracts can successfully work against all of them. They need to be tested on the real pet food in the manufacturing facility to validate their efficacy.

c) Identify other effective ingredients in the erythrocytes

This work mainly looks at only two antioxidants in erythrocytes, SOD and catalase, however, there are some other antioxidants which may be equally or even more effective for preventing lipids oxidation. Glutathione and glutathione peroxidase can be a good research object to look into in the future work.

d) Verify the safety of CP.

Though no hazardous chemical is involved in the production of CP, the safety of it still needs to be carefully studied. Further, with proper quality control, it may be added into human foods as well.

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