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Chitosan-gallic acid films as multifunctional food packaging

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Chitosan-gallic acid films as multifunctional food packaging

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ABSTRACT

Chitosan is a good candidate for multifunctional food packaging because of its biocompatibility, biodegradability, antibacterial properties, secondary antioxidant activity, film forming ability, resistance to lipids and because of its structure which is very desirable for grafting various compounds to it. For this research, we took advantage of chitosan's amino group that has nuceleophilic character at a pH above its pKa, which is 6.3. Gallic acid, a phenolic compound with primary antioxidant properties was grafted to chitosan using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide. Grafting was evaluated using FTIR-ATR and ^{1}H and ^{13}CNMR. FTIR showed evidence of grafting on the amino group by differences of amide stretching in grafted chitosan (grafted) as compared to a mixture of chitosan and gallic acid, and non- modified chitosan. However, NMR results were inconclusive. Grafted chitosan showed significant primary antioxidant activity, containing 34.26 mg gallic acid/g, had DPPH scavenging ability of 90%, reducing power of ABS_{700}=0.5, and was soluble in aqueous acetic acid. The grafted chitosan readily formed films possessing excellent puncture strength and stability, evidenced by stable antioxidant activity over a 15 week storage period at 50°C. When evaluated as packaging material for peanuts, the films reduced malondialdehyde, peroxide and conjugated triene formation as compared to polyethylene bags. The grafted films also possessed antibacterial properties, showing a 2-log reduction of *Salmonella* Typhimurium. The grafted films were evaluated as packaging for potato chips and compared

with mixed chitosan and gallic acid films, on modified chitosan films and Ziploc® bags. The grafted chitosan pouches significantly reduced lipid oxidation throughout 8 weeks of storage at 50°C compared to Ziploc® pouches. Mixed chitosan and gallic acid pouches and films had similar properties to those exhibited by grafted films. Therefore, chitosan films prepared with free or grafted gallic acid have been demonstrated to be potentially valuable multifunctional food packaging. Chitosan films with the primary antioxidant properties of gallic acid show promise for multifunctional food packaging.

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CHAPTER I
INTRODUCTION AND LITERATURE REVIEW

Chitosan

Chitosan receives a lot of attention because of its numerous desirable qualities and that it is produced from chitin, which is the second most abundant biopolymer in the world (Friedman and Juneja, 2010). It is estimated that at least 10 gigatons of chitin are biosynthesized and degraded each year in the biosphere (Hermanson,1996). Chitosan is a partially deacetylated form of chitin, a linear polysaccharide consisting of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine units (Figure 1.1). Chitosan generally has a degree of acetylation lower than 40% and the removal of the acetyl group from chitin is usually performed with concentrated NaOH (Hermanson,1996). Chitosan is industrially processed from the shells of crustaceans such as crabs, shrimp and crayfish. Chitosan has potential application in the biomedical, food and chemical industries (Ocloo et al., 2011; Shepherd et al., 1997; Xia et al., 2011; Xing et al., 2005). The growing interest in chitosan is due to its biocompatibility, biodegradability, antibacterial properties, affinity for many proteins, and anti-oxidative properties (Friedman and Juneja, 2010; Gamage and Shahidi, 2007; Liu et al., 2012). It can be used as a flocculent, clarifier, chromatography column matrix, gas-selective membrane, plant disease resistant promoter, anti-cancer agent, wound healing promoting agent and antimicrobial agent (Friedman and Juneja, 2010; Goy et al. 2009; Ocloo et al., 2011; Shepherd et al., 1997)

There has been a lot of research, over 800 articles in the past ten years, relating to chitosan's antimicrobial properties. The mechanism, which seems to dictate the bacteriostatic and bactericidal effect of chitosan involves binding of its positively charged amino ($-NH_3^+$) group to negatively charged carboxylate ($-COO^-$) group on the surface of bacteria cell membrane or cell wall (Friedman and Juneja, 2010). Studies supported by electron micrographs demonstrate that the site of action is the outer membrane, where chitosan binds (Helander et al., 2001). The perturbation of the outer membrane is reflected by increased permeability of hydrophobic probes and increased sensitivity to the biocidal and inhibitory action of a range of inimical compounds including anionic detergents, dyes and bile acids (Helander et al., 2001). It was also shown by atomic force microscopy that the antibacterial action is probably via membrane disruption and leakage of cellular protein (Qi et al., 2004).

One of chitosan's properties, which can be very important to the food industry, is its ability to form films and possibly be used as packaging. Raw food, as well as countless processed food products need effective and efficient packaging for extending the shelf life and quality of the products. Film coatings prevent moisture loss, reduce drippings from meats and fishes as seen in plastic packaging, restrict flavor loss and foreign odor pick-up, can carry antioxidants and antimicrobials, and be used as direct treatment on the food's surface (Gennadios et al., 1997; Gomez-Estaca et al., 2009). Chitosan owes its film and fiber forming abilities to its linear structure which allows for strong inter and intra-

molecular hydrogen bonding (Demarger-Andre and Domard, 1994). Chitosan films are tough, flexible, transparent, and resistant to lipids, suggesting potential value as a food coating or packaging material (Tharanathan, 2003). It was shown *in vivo* that chitosan can limit the adsorption of lipids and is therefore used as a dietary supplement and for weight loss (Muzzarelli, 1996).

There are many contradictory studies related to chitosan's antioxidant activity. Numerous studies have shown that chitosan is a useful antioxidant when consumed as a dietary supplement, as well as having a lot of potential for the food industry (Anraku et al., 2008; Anraku et al., 2009; Feng et al., 2008; Sun et al., 2007; Tomida et al., 2009). Tomida and coworkers showed the low molecular weight chitosan may be absorbed into the gastrointestinal tract and inhibit nucleophilic activation and oxidation of serum albumin resulting in reduced oxidative stress (Tomida et al., 2009). Also when compared to vitamin C, chitosan was equally as effective in preventing carbonyl and hydroperoxide group formation for human serum albumin exposed to peroxyl radicals (Anraku et al., 2008)

There are studies showing that chitosan has primary antioxidant activity (Yen et al., 2007; Yen et al., 2008). Yen et al. (2007) found that chitosan from shiitake mushrooms had antioxidant activity via the conjugated diene method, reducing power, DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging, and hydroxyl radical scavenging ability of 62.4-81.9%, 0.42-0.57 Abs at 700nm, 7.44-22.4%, and 80.2-86.5% (1 mg/mL), respectively. A similar study carried out by Yen et al.

on crab chitosan found that crab chitosan had antioxidant activity, reducing power, DPPH scavenging, and hydroxyl radical scavenging ability of 58.3-70.2% (1 mg/mL), 0.32-0.44 Abs at 700 nm (10 mg/mL), 28.4-52.3% for 10 mg/mL, and 62.3-77.6% (0.1 mg/mL), respectively. A reference antioxidant that Yen et al (2008) compared their results with was butylated hydroxyanisole (BHA). They found that BHA had antioxidant activity, reducing power, DPPH scavenging, and hydroxyl radical scavenging ability of 89.2% (0.1 mg/mL), 0.96 Abs at 700 nm (0.1 mg/mL), 65.1% for 0.1 mg/mL and 22.8% for 20 mg/mL respectively. Antioxidant activity measured the ability of the tested antioxidant to reduce oxidation and reducing power, which expressed as the Abs at 700 nm, is the ability of an antioxidant to reduce the ferric form of iron to its ferrous form. DPPH scavenging is the ability of an antioxidant to reduce the radical, DPPH so it no longer exhibits an absorption at 517 nm. Hydroxyl scavenging was evaluated by the change in electron paramagnetic resonance signal. These results show that Yen and co-workers are claiming chitosan is an efficient primary antioxidant.

However, there are studies showing chitosan cannot be an efficient primary antioxidant because of its structure (Kanatt et al., 2008). Pasanphan and coworkers concluded that chitosan is chemically inert as an antioxidant based on strong intra- and intermolecular hydrogen bonds network and lack of on H-atom donor to serve as a good chain breaking antioxidant (Pasanphan et al., 2010). Similarly, Alexandrova et al. (1999) reported that chitosan has essentially zero antioxidant activity. Our preliminary research has shown that chitosan of varying

molecular weight and degree of deacetylation has no DPPH radical scavenging ability, no detectable levels of total phenolics (gallic acid equivalents), and no reducing power (Schreiber et al., 2011). It is probable that the cited studies that showed chitosan to have primary antioxidant activity were using chitosan that was not properly purified and contained residual carotenoids or other antioxidants.

Although data on chitosan's primary antioxidant activity is contradictory, the chelating activity of chitosan is well established. This makes it useful in removal of toxic metals from contaminated water supplies (Friedman and Juneja, 2010). Chitosan and its derivatives can selectively bind metal cations involving ion exchange, sorption, and chelating processes in near neutral pH conditions (Guibal, 2004; Synytsya et al., 2008). Nitrogen free electron doublet is responsible for the sorption of metal cations. Therefore, the degree of deacetylation, that represents the amount of free amino groups, affects binding capacity of the chitosan (Guibal, 2004). In the case of metal anions, the sorption proceeds by electrostatic attraction on protonated amine groups in acidic solutions (Guibal, 2004). Degree of deacetylation, crystallinity, and to a lesser degree molecular weight can influence chitosan's sorption properties (Guibal, 2004). One drawback that the application of chitosan can face is solubility in acidic media. However, cross-linking of chitosan overcomes this disadvantage while still keeping good adsorption properties (Qian et al., 2000). Although chitosan shows efficiency at adsorbing many ions, Bassi and coauthors found

that the adsorption of metal varied between ions. The authors found that the chitosan flakes were able to adsorb 0.99, 0.88, 0.50, and 0.45 mg of metal ion/g chitosan of Cu^{2+}, Pb^{2+}, Cd^{2+} and Zn^{2+}, respectively (Bassi et al., 2000).

Grafted Chitosans

Chitosan has a very desirable structure for grafting various compounds to it as it contains a high content of primary amino groups. At pH above the pK_a of chitosan's conjugate base, which is 6.3, the amino groups have nucleophilic character, and the unshared electron pair can undergo a variety of reactions (Wu, 2005). The reactive amine side groups offer enormous possibilities of modification, graft reactions, and ionic interactions (Peniche et al., 1998; Wu, 2005). There have been numerous studies grafting various compounds to chitosan (Hong et al., 2006; Janciauskaite et al., 2008; Ngo et al., 2011; Pasanphan and Chirachanchai, 2008; Synytsya et al. 2008).

Many studies, including the basis of this thesis, involve grafting a compound containing a carboxyl group. One method used to graft compounds containing carboxyl groups to chitosan is with enzymes (Chao et al., 2004; Vachoud et al., 2001; Vartianinen et al., 2008). Tyrosinase seemed to be the enzyme most often used to graft compounds to chitosan (Chao et al., 2004; Govar et al., 2003; Jayakumar et al., 2005; Kumar et al., 1999; Vartianinen et al., 2008;). For example, the enzyme tyrosinase can be used to catalyze the grafting of octyl and dodecyl gallate to the amino group of chitosan. (Chao et al., 2004;

Vartianinen et al., 2008). Tyrosinase converts a wide range of phenolic substrates into electrophilic o-quinones, the o-quinones can then undergo reactions with the nucleophilic amino group of chitosan (Figure 1.2) (Kumar et al., 1999)

Carbodimides are used to mediate formation of amide linkages between a carboxylate and an amine or phosphoramidite linkages between a phosphate and an amine (Hermanson, 1996). Use of the carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) with N-hyroxysuccinimide (NHS) is common in protein chemistry due to amino acids containing both amine and carboxylic groups. EDC and NHS can also be used to graft a compound containing a carboxylic group to chitosan. In one study Puronic®, a nonionic triblock copolymer, was first carboxylated with succinic anhydride and then grafted to chitosan using EDC and NHS (Chung et al., 2005). Another study focused on producing a drug delivery system by grafting carboxyl terminated poly(N-vinalcaprolactam) to chitosan using the same EDC/NHS system (Prabaharan et al., 2008). Caffeic acid was grafted to chitosan using EDC; however NHS was not included to stabilize this reaction (Aytekin et al., 2011).

Compounds such as polysaccharides and phenolics without carboxylic groups can also be grafted to chitosan. One study used peroxidase's ability to convert phenolic substrates into free radicals to graft dodecyl gallate to chitosan (Vachoud et al., 2001). The Maillard reaction was utilized to conjugate xylan from corncobs to chitosan by simply heating the mixture (Li et al., 2011). Eugenol was

grafted to chitosan using ceric ammonium nitrate, which is commonly used for oxidative addition reactions (Jung et al., 2006). Singh and Ray grafted 2-hydroxyethylmethacrylate to chitosan by using ^{60}Co gamma irradiation (Singh and Ray, 1994).

Common methods used to provide evidence of grafting are infrared (IR), nuclear magnetic resonance (NMR), and ultraviolet-visible spectroscopy (UV-VIS). IR was one of the most highly used techniques to show formation of bonding between chitosan and the compound that was being conjugated to it. Vachoud and colleagues used the increased absorbance in the C-H stretching region (2900 cm^{-1}) and carbonyl-stretching band (1716 cm^{-1}) to show an addition of the dodecyl moiety from the dodecyl gallate that was grafted to chitosan (Vachoud et al., 2001). A change in absorbance in the primary amine bending region (1590cm^{-1}) can indicate grafting on chitosan's primary amine group (Vachoud et al., 2001). Pasanphan and Chirachanchai grafted gallic acid to chitosan using EDC/NHS, and showed an increase in the IR ratio of the ester peak (1730 cm^{-1}) or the amide peak (1640cm^{-1}) and the internal C-O-C peak (895 cm^{-1}) of the pyranose ring. Ratios of FTIR absorbencies at specified wave numbers, A_{1640}/A_{895} and A_{1730}/A_{895} were used to show grafting of gallic acid to the amino group of chitosan (Pasanphan and Chirachanchai, 2008). Ngo and co-authors, who grafted gallic acid to chitooligosaccharides, referred to significant peaks at 1955 cm^{-1} and 1520 cm^{-1} to indicate ester and amide linkages, respectively (Ngo et al., 2011).

As seen in several papers, difficulty to obtain good scans is sometimes experienced with liquid-state NMR because of the high viscosity of the grafted chitosan solutions (DeAngelis et al., 1998; Vachoud et al., 2001; Yu et al., 1999). One way to enhance signals is by subjecting the sample to partial hydrolysis. Vachoud and co-authors were able to see signals that they attributed to the methylene groups and methyl protons from the dodecyl moiety of the dodceyl gallate they grafted to chitosan after partial hydrolysis by nitrous acid (Vachoud et al., 2001). Another way to overcome the possible issues of liquid NMR is by using either solid state ^{13}CP-MAS NMR, which can be a powerful tool for non-destructively studying grafting with proper spectral "editing" sequences, or ^{15}N NMR (DeAngelis et al., 1998; Yu et al., 1999). Pasanphan and Chirachanchai used ^{1}H NMR to show the presence of the phenyl protons in the gallic acid moiety as evidence of grafting (Pasanphan and Chirachanchai, 2008). However, showing presence of the compound grafted to chitosan just proves it is present in the final product and does not actually prove it is covalently bonded to chitosan.

UV absorption is another method used to provide evidence of grafting compounds to chitosan. Chen and coworkers, who grafted eugenol and carvacrol to chitosan, observed the presence of phenolic aldehydes through formation of a peak at around 310 nm which indicates n-π* transition of the aldehyde group and a peak around 275 nm, indicative of the π-π* transition of the phenolic portion (Chen et al., 2009). Another group, that used spectrophotometry to demonstrate grafting of dodecyl gallate to chitosan via peroxidase, observed a considerable

increase of UV-Vis absorbance, compared to the control (Vachoud et al., 2001). This appears to be a weak argument to justify the occurrence of grafting because it does not correlate a specific bond with an absorbance but just indicates increase in overall absorbance in the UV-Vis region. When a sample is turbid, it shows absorbance in broad UV-Vis range as opposed to completely colorless and transparent samples with no detectable absorbance in Vis range. Without specific peaks a reader may assume that their sample, which the author is claiming to be grafted, may have just been cloudy compared to the controls due to insolubility. When confirming grafting, it is important to use more than one analytical method. The best way to confirm grafting either by IR or NMR is to look for corresponding peaks that provide evidence of the covalent bonds being formed. Peaks corresponding to the compound being grafted just shows presence of the compound and not promotion of covalent bonds.

Lipid Oxidation and Analysis

Unsaturated fatty acyl-containing lipids are susceptible to oxygen attack, resulting in lipid oxidation. Lipid oxidation of foods products not only poses issues for food quality but it can also have health implications. There is evidence that products of lipid oxidation products, such as lipid peroxides, malonaldehyde and cholesterol oxidation products can be detrimental to consumer's health (Addis et al., 1983; Addis, 1986; Finocchiaro et al., 1984; Pearson et al., 1983). Lipid oxidation proceeds in three steps: initiation, propagation and termination.

Initiation takes place by either the abstraction of a hydrogen radical from an allylic methylene group or by addition of a radical to a double bond. Trace levels of metals, irradiation, light or heat can instigate the formation of the radical. Also, hydroperoxides already existing prior to oxidation can yield radicals by undergoing homolytic cleavage or bimolecular decomposition. Propagation is the second step in which free radicals cause the formation of additional radicals. In propagation the free radical reacts with molecular oxygen producing peroxy radicals. This continues as a chain reaction and the peroxy radicals form lipid hydroperoxides as well as lipid free radicals. Radicals are very reactive and when there is an insufficient amount of unsaturated fatty acids the radicals will react with each other forming a stable compound (Figure 1.3) (Jadhav et al., 1996).

Lipid oxidation in food is a serious problem that leads to the development of “off “ flavors and odors, and production of compounds destructive to proteins, enzymes, vitamins, and amino acids (Stangelo et al., 1975). There is evidence that lipid oxidation products can complex with protein affecting the latter’s solubility and possibly altering the texture of the food (Kanner and Rosenthal, 1992; Stangelo et al., 1975). There are many possible initiators of lipid oxidation including transition metals, oxidants, homolysis- prone substances, and enzymes (Kanner and Rosenthal, 1992). Due to the light absorbing abilities of dyes in food, they can serve as a source of singlet oxygen, which can readily react with unsaturated fatty acids (Kanner and Rosenthal, 1992).

The two food products that were used in the present research to demonstrate the ability of the chitosan-gallic acid films to reduce lipid oxidation were peanuts and potato chips. Because both foods are very popular, research to prevent their oxidative degradation and thereby increase their shelf life is valuable. Peanuts contain about 47.5% lipids. About 80% of the lipids in peanuts is composed of unsaturated fatty acids, mostly linoleic and oleic, which are prone to oxidation during storage (Agbo et al., 1992). One factor that can increase oxidation is presence of metal ions that may come in contact with peanuts during processing. Metals may catalyze lipid oxidation by enhancing the decomposition of hydroperoxides (Agbo et al., 1992). Many factors effect oxidation in peanuts and peanut products including moisture, temperature, oxygen concentration, and time (Evranuz, 1993). Generally, higher temperature, higher oxygen concentration, and lower relative humidity (RH) promote oxidation. In a study by Mate et al. (1996) it was found that in both dry-roasted and oil-roasted peanuts the peroxide value (PV) significantly increased throughout a 10-week incubation period at low RH (21%) and high oxygen concentration. The PV at the end of the 10-week storage was around 75 meq/Kg for both dry roasted and oil roasted peanuts kept at 21% RH and high oxygen concentration compared to around 20 meq/Kg or less for peanuts stored at 53% RH at both high and low oxygen concentration and 21% RH at low oxygen concentration (Mate et al., 1996). Evranuz's study showed the effect of temperature at the end of a 70-day

incubation period on peanuts. Peanuts at 15°C, 25°C, and 35°C had PV of around 50, 60 and 70 meq/Kg, respectively (Evranuz, 1993).

Potato chips are a very popular deep fried product. Because of the type of processing they are susceptible to oxidation and they frequently deteriorate due to oxidative rancidity (Quast and Karel, 1972). Deep frying is a cooking process of submerging a food in hot oil, around 180 °C and allowing heat to transfer from the oil to the food while water evaporates from the food and is partially replaced by oil (Yu et al., 2011). Because the frying oil is taken up by the food, the development of rancidity in the oil directly affects the final product (Yu et al., 2011). Volatile compounds such as pentane, 2-heptenal, isomers of 2,4 heptadienal and isomers of 2,4 decadienal were found in even fresh frying oil (Min and Schweizer, 1983). Min and Schweizer found high correlation, r=0.95 between the presence of these compounds in the potato chips and PV of the chip (Min and Schweizer, 1983). The environmental factors that can affect rancidity and stability of potato chips include the ability of light to penetrate the packaging and the moisture of the product (Quast and Karel, 1972).

Lipids are important macromolecules in food and contribute to the food's nutritional value as well as to its flavor, texture, general palatability, and storage stability (Pegg, 2001A). Therefore, it is very important to monitor and test for oxidation of lipids to determine shelf life of a food and to test for rancidity as a quality measure. Because food systems are dynamic and complex, it is crucial to use multiple analyses to more adequately quantify lipid oxidation. One of the

most well known methods for determining lipid oxidation is determination of peroxide value (PV). As previously mentioned, hydroperoxides are a primary product of lipid oxidation, which are produced during propagation. The PV is defined as the quantity of peroxide oxygen present in the sample (Pegg, 2001A). One method to quantify PV is by titration. The official method of the American Oil Chemists' Society(1998) is a titration method that uses acetic acid-isooctane or acetic acid-chloroform (solvent), potassium iodide, sodium thiosulfate and a starch indicator. In this reaction the iodine is liberated from potassium iodide by peroxides present in the oil being analyzed. However, determination of low PV levels can be difficult and inaccurate with this method because the disappearance of the pale-violet color produced from the reaction of starch with iodine is not always clear and definite (Oishi et al., 1992). Therefore, an electrochemical technique has been devised in which liberated iodine from the reaction is reduced at a carbon-felt electrode (Oishi et al., 1992).

Another more sensitive method to determine PV is the ferrous oxidation/ xylenol orange (FOX) method (Figure 1.4). The FOX method is able to quantify PV as low as 0.1 meq /kg sample (Pegg, 2001A). The FOX method is based on spectrophotometric determination of ferric ion concentration formed from oxidation of ferrous ions by peroxides in the presence of acid and xylenol orange (Shantha and Decker, 1994). Under acidic conditions, the ferric ion complexes with xylenol orange creating a purple color, which can then be measured as absorbance at 560 nm on a spectrophotometer (Jiang et al., 1992). In the AOAC

(Association of Official Analytical Chemists) method, equal amounts (50 µL) of iron(II)chloride solution and 10 mM xylenol orange are used (Shantha and Decker, 1994). However, we found that if twice the volume of xylenol orange is used (100 µL instead of 50 µL) for the oil samples as well as for the standard curve, the higher amounts of ferric ion can be read. This increases the linear region of the standard curve and allows for more accurate readings when the ferric ion is present in a high concentration. A negative aspect of the FOX method is the necessity to adhere to the 5 minute incubation period before the sample is read on the spectrophotometer. Because of this, it is only possible to prepare and read not more than two samples every five minutes, which is time-consuming when analyzing a large number of samples.

Conjugated dienes and trienes are primary lipid oxidation products, which can be measured as an indication of oxidation. Conjugated dienes or trienes possess two and three double bonds respectively, separated by single bonds. Conjugated bonds are not normally found in polyunsaturated fatty acids (PUFAs) contained in oils derived from plants or animals. PUFAs have a divinylmethane structure and the formation of conjugated dienes or trienes indicates oxidation of the PUFAs (Corongiu and Banni, 1994; Pegg, 2001A). The divenylmethane structure of PUFAs makes them susceptible to hydrogen abstraction by free radical attack that results in a PUFA-free radical intermediate that undergoes rearrangement forming a conjugated diene or polyene (Figure 1.5) (Pegg, 2001A). Conjugation can be measured by an increase in ultraviolet absorption.

This increase is seen via spectrophotometry around 233 nm for diene un-saturation and at 268 nm for triene un-saturation (Gray, 1978). This method is advantageous because it is a very simple and rapid procedure that involves diluting extracted oil with iso-octane and reading the absorbance on a spectrophotometer. There is a similar but more complex procedure to measure conjugable oxidation product. In this procedure, hydroperoxides of polyenoic fatty acids as well as hydroxyl and carbonyl compounds derived from them are converted to conjugated chromophores by reduction and then dehydration (Parr and Swoboda, 1976).

There are various analytical techniques to determine secondary lipid oxidation products. One common method is determining thiobarbituric acid (TBA) reactive substances (TBARS). This assay relies on a pigment being produced as a condensation product of one molecule of malonaldehyde with two molecules of TBA and the probable elimination of two molecules of water (Figure 1.6) (Sinnhuber and Yu, 1958). Aldehydes are produced when lipids break down in biological systems and foods. There is evidence that bicycloendoperoxides which are formed from polyunsaturated fatty acids are an important precursor of malondialdehyde (MDA) (Frankel, 1987). Malondialdehyde can be extracted for analysis by multiple methods, including direct extraction by an aqueous acid solution, distillation and direct determination in oil or lipid extract (Pegg, 2001B). We used the direct extraction method because it has a greater recovery of malonaldehyde and reduced the possibility of overestimation, as it does not

involve heating as the other extraction methods do (Pegg, 2001B). The results of this assay can be reported in multiple ways. For the research of this thesis a standard curve was used and the results were reported as mg malonaldehyde eq/kg of food sample. It is favorable to report it in malonaldehyde equivalents because it is known that other aldehydes are present in the extract, which are capable of producing the same pigment.

Antioxidants Activity and Analysis

The most common and effective method for reducing lipid oxidation in foods is by the addition of antioxidants (Halliwell et al., 1995). Most raw materials used in processed food contain natural antioxidants. However, during processing and storage, these antioxidants are degraded and/or removed creating a need for added antioxidants (Rajalakshmi and Narasimhan, 1996). Antioxidants are generally classified as either primary or synergistic/secondary, although there are antioxidants that exhibit more than one mechanism of function (Rajalakshmi and Narasimhan, 1996; Reische et al., 2008). Primary antioxidants terminate the free radical chain reaction by donating hydrogen or electrons to free radicals and converting them to stable products (Rajalakshmi and Narasimhan, 1996; Reische et al., 2008). Primary antioxidants can, therefore, delay or inhibit initiation. Examples of primary antioxidants include phenols, hindered phenols and compounds such as trolox-C, anoxomer and ethoxyquin. The second group of antioxidants, referred to as synergestic or secondary antioxidants, slow the rate of oxidation by several different actions such as chelating, replenishing hydrogen

to primary antioxidants, decomposing hydroperoxides, deactivating singlet oxygen, absorbing UV radiation, and acting as oxygen scavengers (Rajalakshmi and Narasimhan, 1996; Reische et al., 2008). Examples of secondary antioxidants include, ascorbic acid, ethylenediaminetetraacetic acid (EDTA), distearyl esters, and β–carotene (Rajalakshmi and Narasimhan, 1996, Reische et al., 2008). The role of antioxidants is not to enhance or improve quality but to maintain food quality and extend shelf life (Reische et al., 2008).

Phenolic compounds in foods originate from one of the main classes of secondary metabolites in plants derived from phenylalanine and, to a lesser extent, tyrosine (Shahidi, 2000; Shahidi and Naczk, 2004; Van Sumere, 1989). They are compounds that have one or more hydroxyl groups attached directly to a benzene ring (Vermerris and Nicholson, 2008). Phenolic antioxidants use exothermic reactions to interfere with lipid oxidation by rapid donation of a hydrogen atom to lipid radicals (Shahidi and Naczk, 2004). 4Phenolics are excellent hydrogen or electron donors and their radical-intermediates are relatively stable due to resonance delocalization and lack of available positions for molecular oxygen attack (Nawar, 1996). Gallic acid, GA (3,4,5-trihydroxybenzoic acid), is a natural phenolic acid often obtained by alkaline or acid catalyzed hydrolysis of tannins or from hydrolysis of spent broths from *Penicillium glaucum* or *Aspergillus niger* (Aruoma et al., 1993). GA has high reducing potential, three hydroxyl groups which are positively correlated with antioxidant activity, and low OH- bond dissociation enthalpy of the three hydroxyl

groups on the benzene ring (Pasanphan and Chirachanchai, 2008; Sroka and Cisowski, 2003). The number of hydroxyl groups is found to correlate with antioxidant activity of the phenolic compound (Sroka and Cisowski, 2003). For example, Sroka and Cisowski found that % scavenging of hydrogen peroxide and of the DPPH radical by gallic acid, were 90 and 75%, respectively. This is compared to caffeic acid, having two hydroxyl groups (compared to three for GA) and 59 and 44% hydrogen peroxide and DPPH scavenging, respectively (Sroka and Cisowski, 2003).

There are many ways to analyze antioxidants in food. In fact some of the methods mentioned to evaluate lipid oxidation can also be used to evaluate antioxidants by the reduction of oxidation. Some antioxidants are often lost during processing so it is important that the finished product be analyzed for antioxidant content (Stuckey and Osborne, 1965).

A very well known assay used to determine content of total phenolics is the Folin Ciocalteu assay (Folin and Ciocalteu, 1927). The Folin Ciocalteu assay relies on the transfer of electrons in alkaline medium from phenolic compounds to the phosphomolybdic/phosphotungstic acid complex to form a blue complex with a UV absorbance at around 760 nm (Ainsworth and Gillespie, 2007; Singleton and Rossi, 1965; Singleton et al., 1999). There is controversy over whether only phenolic compounds are being quantified or chelators and reducing compounds as well. In addition, there are a number of other substances that interact with Folin Ciocalteu reagent such as sugars, aromatic amines, sulfur dioxide, and

ascorbic acid (Prior et al., 2005). However, this is a simple assay with high reproducibility and is shown to correlate well with ORAC as well as DPPH (Katsube et al., 2004; Prior et al., 2005).

Another method to evaluate antioxidant activity is by the scavenging of the free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Saha et al., 2008). This method is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors (Parkash et al., 2011) The DPPH radical is one of the few stable organic nitrogen radicals which bears deep purple color and is commercially available (Prior et al., 2005). In its radical form, DPPH absorbs at 515 nm because of its single electron, but upon reduction by an antioxidant or radical species the absorption disappears (Figure 1.7) (Parkash et al., 2011; Williams et al., 1994). There are generally two mechanisms for an antioxidant to scavenge DPPH; first, by a direct H abstraction process, and second, via a proton-concerted electron transfer process (Wang and Zhang, 2003). The original DPPH scavenging method is carried out on a spectrophotometer; however, variations of the method have been created (Saha et al., 2008; Yamaguchi et al., 1998). An HPLC method was evaluated so the DPPH method could be applied to colored foods whose pigments interfere with the spectrophotometric method. The HPLC DPPH method gave good correlation with the radical scavenging activity determined by the conventional colorimetric method (Yamaguchi et al., 1998). A disadvantage of the DPPH scavenging

method is that the stable DPPH radical greatly differs from the peroxyl radicals involved in lipid oxidation in a real food system (Prior et al., 2005).

Reducing power is another method used to evaluate antioxidant activity. One reducing power assay uses potassium ferricyanide, and ferric chloride to evaluate the ability of an antioxidant to reduce the Fe^{3+}/ ferricyanide complex to the ferrous Fe^{2+} form (Ferreira et al., 2007). This reaction of potassium ferricyanide with ferrous ions produces a blue pigment, which shows absorbance at 700 nm and can monitor Fe^{2+} concentration indicating successful reduction by the antioxidant being evaluated. An assay known as FRAP, Ferric Reducing/Antioxidant power assay, relies on a similar mechanisms. When a complex Fe^{3+}-tripyridyltriazine is reduced to the Fe^{2+} form by an antioxidant under acidic condition, an intense blue color develops with a maximum absorption at 593 nm (Moon and Shibamato, 2009). The results of the FRAP assay are generally presented in trolox equivalents (Madrona et al., 2011). Reducing power was evaluated throughout our research and the potassium ferricyanide method was used. The results were reported as absorbance at 700 nm and an increase in absorbance was positively correlated to an increased reducing power/antioxidant activity. We did not use a standard curve, however a gallic acid standard of 0.1 mg/mL was ran every time along with a distilled water control to make sure the test was accurate and had high reproducibility.

Overall Goals and Objectives

The overall goal of the research was to successfully develop multifunctional food packaging. The sub goals were to first graft the phenolic compound, gallic acid to chitosan using EDC and NHS to form amide bonds, and then to evaluate the grafted compound for covalent bonding. The second goal was to form films with the grafted chitosan-gallic acid and evaluate them for physical, antioxidant properties and antibacterial properties as well as for food packaging to reduce lipid oxidation. Shelf life studies were carried out to evaluate the grafted films as packaging using peanuts and potato chips as the packaged products. The storage studies were carried out in a forced air incubator at 50 °C and the peanuts and potato chips evaluated for PV, TBARS and conjugated dienes and trienes.

References

Addis, P. (1986). Occurrence of lipid oxidation-products in foods. *Food And Chemical Toxicology, 24*(10-11), 1021-1030.

Addis, P., Csallany, A., & Kindom, S. (1983). Some lipid oxidation-products as xenobiotics. *Acs Symposium Series, 234*, 85-98.

Agbo, O. F., Anderson, J. C., & Singh, B. (1992). Lipid Oxidation of Edible Peanut Pastes during Storage with Variation of Environmental and Processing Factors. *Peanut Science, 19*(2), 101-105.

Ainsworth, E. A., & Gillespie, K. M. (2007). Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. *Nature Protocols, 2*(4), 875-877.

Alexandrova, V. A., Obukhova, G. V., Domnina, N. S., & Topchiev, D. A. (1999). Modification of chitosan for construction of efficient antioxidant biodegradable macromolecular systems. *Macromol. Symp., 144*(1), 413-422.

Anraku, M., Fujii, T., Furutani, N., Kadowaki, D., Maruyama, T., Otagiri, M., Gebicki, J., & Tomida, H. (2009). Antioxidant effects of a dietary supplement: Reduction of indices of oxidative stress in normal subjects by water-soluble chitosan. *Food and Chemical Toxicology, 47*(1), 104-109.

Anraku, M., Kabashima, M., Namura, H., Maruyama, T., Otagiri, M., Gebicki, J., Furutani, N., & Tomida, H. (2008). Antioxidant protection of human serum albumin by chitosan. *International Journal of Biological Macromolecules, 43*(2), 159-164.

AOCS (American Oil Chemists' Society). (1998). AOCS. Official method Cd 8-53. Peroxide value. *In* Official Methods and Recommended Practices of the American Oil Chemists' Society, 5th ed. (D. Firestone, ed.). AOCS, Champaign, Ill.

Aruoma, O., Murcia, A., Butler, J., & Halliwell, B. (1993). Evaluation of the antioxidant and prooxidant actions of gallic acid and its derivatives. *Journal of Agricultural and Food Chemistry, 41*(11), 1880-1885.

Aytekin, A., Morimura, S., & Kida, K. (2011). Synthesis of chitosan-caffeic acid derivatives and evaluation of their antioxidant activities. *Journal of Bioscience and Bioengineering, 111*(2), 212-216.

Bassi, R., Prasher, S., & Simpson, B. (2000). Removal of selected metal ions from aqueous solutions using chitosan flakes. *Separation Science and Technology, 35*(4), 547-560.

Chao, A., Shyu, S., Lin, Y., & Mi, F. (2004). Enzymatic grafting of carboxyl groups on to chitosan - to confer on chitosan the property of a cationic dye adsorbent. *Bioresource Technology, 91*(2), 157-162.

Chen, F., Shi, Z., Neoh, K., & Kang, E. (2009). Antioxidant and Antibacterial Activities of Eugenol and Carvacrol-Grafted Chitosan Nanoparticles. *Biotechnology and Bioengineering, 104*(1), 30-39.

Chung, H., Go, D., Bae, J., Jung, I., Lee, J., & Park, K. (2005). Synthesis and

characterization of Pluronic((R)) grafted chitosan copolymer as a novel injectable biomaterial. *Current Applied Physics, 5*(5), 485-488.
Corongiu, F., & Banni, S. (1994). Detection of conjugated dienes by 2nd-derivative ultraviolet spectrophotometry. *Oxygen Radicals in Biological Systems, Pt C, 233*, 303-310.
De Angelis, A., Capitani, D., & Crescenzi, V. (1998). Synthesis and C-13 CP-MAS NMR characterization of a new chitosan-based polymeric network. *Macromolecules, 31*(5), 1595-1601.
Demarger-Andre, S., & Domard, A. (1994). Chitosan carboxylic-acid salts in solution and in the solid-state. *Carbohydrate Polymers, 23*(3), 211-219.
Evranuz, E. (1993). The effects of temperature and moisture-content on lipid-peroxidation during storage of unblanched salted roasted peanuts - shelf-life studies for unblanched salted roasted peanuts. *International Journal of Food Science and Technology, 28*(2), 193-199.
Feng, T., Du, Y., Li, J., Hu, Y., & Kennedy, J. (2008). Enhancement of antioxidant activity of chitosan by irradiation. *Carbohydrate Polymers, 73*(1), 126-132.
Ferreira, I., Baptista, P., Vilas-Boas, M., & Barros, L. (2007). Free-radical scavenging capacity and reducing power of wild edible mushrooms from northeast Portugal: Individual cap and stipe activity. *Food Chemistry, 100*(4), 1511-1516.
Finocchiaro, E., Lee, K., & Richardson, T. (1984). Identification and quantification of cholesterol oxides in grated cheese and bleached butteroil. *Journal of the American Oil Chemists Society, 61*(5), 877-883.
Folin, O., & Ciocalteu, V. (1927). On tyrosine and tryptophane determinations in proteins. *Journal of Biological Chemistry, 73*(2), 627-650.
Frankel, E. (1987). Secondary products of lipid oxidation. *Chemistry and Physics of Lipids, 44*(2-4), 73-85.
Friedman, M., & Juneja, V. K. (2010). Review of Antimicrobial and Antioxidative Activities of Chitosans in Food. *Journal of Food Protection, 73*(9), 1737-1761.
Gamage, A., & Shahidi, F. (2007). Use of chitosan for the removal of metal ion contaminants and proteins from water. *Food Chemistry, 104*(3), 989-996.
Gennadios, A., Hanna, M. A., & Kurth, L. B. (1997). Application of edible coatings on meats, poultry and seafoods: A review. *Food Science and Technology-Lebensmittel-Wissenschaft & Technologie, 30*(4), 337-350.
Gomez-Estaca, J., Bravo, L., Gomez-Guillen, M. C., Aleman, A., & Montero, P. (2009). Antioxidant properties of tuna-skin and bovine-hide gelatin films induced by the addition of oregano and rosemary extracts. *Food Chemistry, 112*(1), 18-25.
Govar, C., Chen, T., Liu, N., Harris, M., & Payne, G. (2003). Grafting renewable chemicals to functionalize chitosan. *Biocatalysis in Polymer Science, 840*, 231-242.
Goy, R., de Britto, D., & Assis, O. (2009). A review of the antimicrobial activity of chitosan. *Polimeros-Ciencia E Tecnologia, 19*(3), 241-247.

Gray, J. (1978). Measurement of lipid oxidation - review. *Journal of the American Oil Chemists Society, 55*(6), 539-546.
Guibal, E. (2004). Interactions of metal ions with chitosan-based sorbents: a review. *Separation and Purification Technology, 38*(1), 43-74.
Halliwell, B., Murcia, M. A., Chirico, S., & Aruoma, O. I. (1995). Free-radicals and antioxidants in food and in-vivo - what they do and how they work. *Critical Reviews in Food Science and Nutrition, 35*(1-2), 7-20.
Helander, I., Nurmiaho-Lassila, E., Ahvenainen, R., Rhoades, J., & Roller, S. (2001). Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. *International Journal of Food Microbiology, 71*(2-3), 235-244.
Hermanson, G. T. (1996). Zero-length cross linkers. In *Bioconjugate Techniques*, (pp. 169-176). San Diego, CA: Academic Press, Inc.
Hong, Y., Mao, Z., Wang, H., Gao, C., & Shen, J. (2006). Covalently crosslinked chitosan hydrogel formed at neutral pH and body temperature. *Journal of Biomedical Materials Research Part a, 79A*(4), 913-922.
Jadhav, S. J., Nimbalkar, S. S., Kulkarni, A. D., & Madhavi, D. L. (1996). Lipid Oxidation in Biological and Food Systems. In D. L. Madhavi, S. S. Deshpande & D. K. Salunkhe (Eds.), *Food Antioxidants: Technological, Toxicological and Health Perspective*, (pp. 5-60). New York, New York: Marcel Dekker.
Janciauskaite, U., Rakutyte, V., Miskinis, J., & Makuska, R. (2008). Synthesis and properties of chitosan-N-dextran graft copolymers. *Reactive & Functional Polymers, 68*(3), 787-796.
Jayakumar, R., Prabaharan, M., Reis, R., & Mano, J. (2005). Graft copolymerized chitosan - present status and applications. *Carbohydrate Polymers, 62*(2), 142-158.
Jiang, Z., Hunt, J., & Wolff, S. (1992). Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low-density-lipoprotein. *Analytical Biochemistry, 202*(2), 384-389.
Jung, B., Chung, S., & Lee, S. (2006). Preparation and characterization of eugenol-grafted chitosan hydrogels and their antioxidant activities. *Journal of Applied Polymer Science, 99*(6), 3500-3506.
Kanatt, S., Chander, R., & Sharma, A. (2008). Chitosan and mint mixture: A new preservative for meat and meat products. *Food Chemistry, 107*(2), 845-852.
Kanner, J., & Rosenthal, I. (1992). An assessment of lipid oxidation in foods - technical report. *Pure and Applied Chemistry, 64*(12), 1959-1964.
Katsube, T., Tabata, H., Ohta, Y., Yamasaki, Y., Anuurad, E., Shiwaku, K., & Yamane, Y. (2004). Screening for antioxidant activity in edible plant products: Comparison of low-density lipoprotein oxidation assay, DPPH radical scavenging assay, and Folin-Ciocalteu assay. *Journal of Agricultural and Food Chemistry, 52*(8), 2391-2396.
Kumar, G., Smith, P., & Payne, G. (1999). Enzymatic grafting of a natural product

onto chitosan to confer water solubility under basic conditions. *Biotechnology and Bioengineering, 63*(2), 154-165.
Li, X., Shi, X., Wang, M., & Du, Y. (2011). Xylan chitosan conjugate - A potential food preservative. *Food Chemistry, 126*(2), 520-525.
Liu, Z. Y., Ge, X. J., Lu, Y., Dong, S. Y., Zhao, Y. H., & Zeng, M. Y. (2012). Effects of chitosan molecular weight and degree of deacetylation on the properties of gelatine-based films. *Food Hydrocolloids, 26*(1), 311-317.
Madrona, A., Pereira-Caro, G., Bravo, L., Mateos, R., & Espartero, J. (2011). Preparation and antioxidant activity of tyrosyl and homovanillyl ethers. *Food Chemistry, 129*(3), 1169-1178.
Mate, J., Saltveit, M., & Krochta, J. (1996). Peanut and walnut rancidity: Effects of oxygen concentration and relative humidity. *Journal of Food Science, 61*(2), 465-+.
Min, D., & Schweizer, D. (1983). Lipid Oxidation In Potato-Chips. *Journal of the American Oil Chemists Society, 60*(9), 1662-1665.
Moon, J., & Shibamoto, T. (2009). Antioxidant assays for plant and food components. *Journal of Agricultural and Food Chemistry, 57*(5), 1655-1666.
Muzzarelli, R. (1996). Chitosan-based dietary foods. *Carbohydrate Polymers, 29*(4), 309-316.
Nawar, W. W. (1996). Lipids. In O. Fennema (Ed.), *Food Chemistry* Third ed., (pp. 225-320). New York, New York: Marcel Dekker, Inc.
Ngo, D., Qian, Z., Ngo, D., Vo, T., Wijesekara, I., & Kim, S. (2011). Gallyl chitooligosaccharides inhibit intracellular free radical-mediated oxidation. *Food Chemistry, 128*(4), 974-981.
Ocloo, F., Quayson, E., Adu-Gyamfi, A., Quarcoo, E., Asare, D., Serfor-Armah, Y., & Woode, B. (2011). Physicochemical and functional characteristics of radiation-processed shrimp chitosan. *Radiation Physics and Chemistry, 80*(7), 837-841.
Oishi, M., Onishi, K., Nishijima, M., Nakagomi, K., & Nakazawa, H. (1992). Rapid and simple coulometric measurements of peroxide value in edible oils and fats. *Journal of AOAC INTERNATIONAL, 75*, 507-510.
Parkash, A., Rigelhof, F., & Miller, E. (Internet) Medallion laboratories analytical progress: Antioxidant Activity, (Accessed 2011 October). Available from http://www.medlabs.com/Downloads/Antiox_acti_.pdf
Parr, L. J., & Swoboda, P. A. T. (1976). The assay of conjugable oxidation products applied to lipid deterioration in stored foods. *Journal of Food Technology*(11), 1-12.
Pasanphan, W., Buettner, G. R., & Chirachanchai, S. (2010). Chitosan gallate as a novel potential polysaccharide antioxidant: an EPR study. *Carbohydrate Research, 345*(1), 132-140.
Pasanphan, W., & Chirachanchai, S. (2008). Conjugation of gallic acid onto chitosan: An approach for green and water-based antioxidant. *Carbohydrate Polymers, 72*(1), 169-177.

Pearson, A., Gray, J., Wolzak, A., & Horenstein, N. (1983). Safety implications of oxidized lipids in muscle foods. *Food Technology, 37*(7), 121-129.
Pegg, R. (2001A). Measurement of primary lipid oxidation products. *Current protocols in food analytical chemistry*, D2.1.1-D2.1.15.
Pegg, R. (2001B). Spectrophotometric measurement of secondary lipid oxidation products. *Current protocols in food analytical chemistry*, D2.4.1-D2.4.18.
Peniche, C., Elvira, C., & Roman, J. (1998). Interpolymer complexes of chitosan and polymethacrylic derivatives of salicylic acid: preparation, characterization and modification by thermal treatment. *Polymer, 39*(25), 6549-6554.
Prabaharan, M., Grailer, J., Steeber, D., & Gong, S. (2008). Stimuli-responsive chitosan-graft-poly(N-vinylcaprolactam) as a promising material for controlled hydrophobic drug delivery. *Macromolecular Bioscience, 8*(9), 843-851.
Prior, R., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry, 53*(10), 4290-4302.
Qi, L. F., Xu, Z. R., Jiang, X., Hu, C. H., & Zou, X. F. (2004). Preparation and antibacterial activity of chitosan nanoparticles. *Carbohydrate Research, 339*(16), 2693-2700.
Qian, S., Huang, G., Jiang, J., He, F., & Wang, Y. (2000). Studies of adsorption behavior of crosslinked chitosan for Cr(VI), Se(VI). *Journal of Applied Polymer Science, 77*(14), 3216-3219.
Quast, D., & Karel, M. (1972). Effects of environmental factors on oxidation of potato chips. *Journal of Food Science, 37*(4), 584-&.
Rajalakshmi, D., & Narasimhan, S. (1996). Food Antioxidants: Sources and Methods of Evaluation. In D. L. Madhavi, S. S. Deshpande & D. K. Salunkhe (Eds.), *Food Antioxidants*, (pp. 65-75). New York, New York: Marcel Dekker.
Reische, D. W., Lillard, D. A., & Eitenmiller, R. R. (2008). Antioxidants. In C. C. Akoh & D. B. Min (Eds.), *Food lipids: chemistry, nutrition, and biotechnology* Third ed., (pp. 409-413). Boca Raton, FL: CRC Press.
Saha, M. R., Hasan, S. M. R., Akter, R., Hossain, M. M., Alam, M. S., Alam, M. A., & Mazumder, M. E. H. (2008). *In vitro* free radical scavenging activity of methanol extract of the leaves of *mimusops elengi linn*. *Bangladesh Journal of Veterinary Medicine, 6*(2), 197-202.
Schreiber, S., Bozell, J., Hayes, D., & Zivanovic, S. (Submitted). Chitosan-gallic acid films as multifunctional food packaging. *Journal of Agricultural and Food Chemistry*.
Shahidi, F. (2000). Antioxidants in food and food antioxidants. *Nahrung-Food, 44*(3), 158-163.
Shahidi, F., & Naczk, M. (2004). *Phenolics in Food and Nutraceuticals* Boca Raton, Florida: CRC Press.

Shantha, N., & Decker, E. (1994). Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. *Journal of Aoac International, 77*(2), 421-424.
Shepherd, R., Reader, S., & Falshaw, A. (1997). Chitosan functional properties. *Glycoconjugate Journal, 14*(4), 535-542.
Singh, D., & Ray, A. (1994). Graft-copolymerization of 2-hydroxyethylmethacrylate onto chitosan films and their blood compatibility. *Journal of Applied Polymer Science, 53*(8), 1115-1121.
Singleton, V., Orthofer, R., & Lamuela-Raventos, R. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Oxidants and Antioxidants, Pt a, 299*, 152-178.
Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic*(16), 144-158.
Sinnhuber, R., & Yu, T. (1958). Characterization of the red pigment formed in the 2-thiobarbituric acid determination of oxidative rancidity. *Food Technology, 12*(5), 59-59.
Sroka, Z., & Cisowski, W. (2003). Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids. *Food and Chemical Toxicology, 41*(6), 753-758.
Stangelo, A. J., Ory, R. L., & Brown, L. E. (1975). Comparison Of Methods For Determining Peroxidation In Processed Whole Peanut Products. *Journal of the American Oil Chemists Society, 52*(2), 34-35.
Stuckey, B., & Osborne, C. (1965). A review of antioxidant analysis in food products. *Journal of the American Oil Chemists Society, 42*(3), 228-&.
Sun, T., Zhou, D., Xie, J., & Mao, F. (2007). Preparation of chitosan oligomers and their antioxidant activity. *European Food Research and Technology, 225*(3-4), 451-456.
Synytsya, A., Blafkova, P., Synytsya, A., Copikova, J., Spevacek, J., & Uher, M. (2008). Conjugation of kojic acid with chitosan. *Carbohydrate Polymers, 72*(1), 21-31.
Tharanathan, R. N. (2003). Biodegradable films and composite coatings: past, present and future. *Trends in Food Science & Technology, 14*(3), 71-78.
Tomida, H., Fujii, T., Furutani, N., Michihara, A., Yasufuku, T., Akasaki, K., Maruyama, T., Otagiri, M., Gebicki, J., & Anraku, M. (2009). Antioxidant properties of some different molecular weight chitosans. *Carbohydrate Research, 344*(13), 1690-1696.
Vachoud, L., Chen, T., Payne, G., & Vazquez-Duhalt, R. (2001). Peroxidase catalyzed grafting of gallate esters onto the polysaccharide chitosan. *Enzyme and Microbial Technology, 29*(6-7), 380-385.
Van Sumere, C. F. (1989). Phenols and phenolic acids. *Methods in Plant Biochemistry: Plant Phenolics, 1*, 29-74.
Vartiainen, J., Ratto, M., Lantto, R., Nattinen, K., & Hurme, E. (2008). Tyrosinase-catalysed grafting of food-grade gallates to chitosan: Surface

properties of novel functional coatings. *Packaging Technology and Science, 21*(6), 317-328.
Vermerris, W., & Nicholson, R. (2008). *Phenolic compound biochemistry*: Springer Science.
Wang, L., & Zhang, H. (2003). A theoretical investigation on DPPH radical-scavenging mechanism of edaravone. *Bioorganic & Medicinal Chemistry Letters, 13*(21), 3789-3792.
Williams, M., Krootjes, B., Vansteveninck, J., & Vanderzee, J. (1994). The prooxidant and antioxidant properties of protoporphyrin-ix. *Biochimica Et Biophysica Acta-Lipids and Lipid Metabolism, 1211*(3), 310-316.
Wu, C. (2005). A comparison of the structure, thermal properties, and biodegradability of polycaprolactone/chitosan and acrylic acid grafted polycaprolactone/chitosan. *Polymer, 46*(1), 147-155.
Xia, W., Liu, P., Zhang, J., & Chen, J. (2011). Biological activities of chitosan and chitooligosaccharides. *Food Hydrocolloids, 25*(2), 170-179.
Xing, R., Liu, S., Guo, Z., Yu, H., Wang, P., Li, C., Li, Z., & Li, P. (2005). Relevance of molecular weight of chitosan and its derivatives and their antioxidant activities in vitro. *Bioorganic & Medicinal Chemistry, 13*(5), 1573-1577.
Yamaguchi, T., Takamura, H., Matoba, T., & Terao, J. (1998). HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1-diphenyl-2-picrylhydrazyl. *Bioscience Biotechnology and Biochemistry, 62*(6), 1201-1204.
Yen, M., Tseng, Y., Li, R., & Mau, J. (2007). Antioxidant properties of fungal chitosan from shiitake stipes. *Lwt-Food Science and Technology, 40*(2), 255-261.
Yen, M., Yang, J., & Mau, J. (2008). Antioxidant properties of chitosan from crab shells. *Carbohydrate Polymers, 74*(4), 840-844.
Yu, G., Morin, F., Nobes, G., & Marchessault, R. (1999). Degree of acetylation of chitin and extent of grafting PHB on chitosan determined by solid state N-15 MMR. *Macromolecules, 32*(2), 518-520.
Yu, X., Du, S., Zhang, J., & Song, L. (2011). Impact of Potato Chips Frying on the Quality Characteristics of Rapeseed Oil. *International Journal of Food Engineering, 7*(2), -.

Appendix: Chapter 1

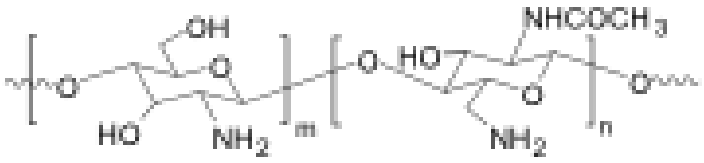


Figure 1. 1: Structure of chitosan (Pasanphan and Chirachanchai, 2008)

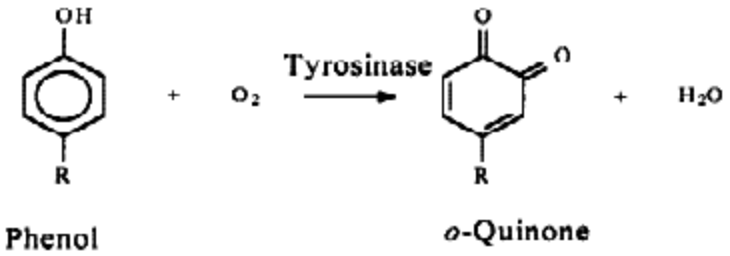


Figure 1. 2: Tyrosinase converts substrates compounds into electrophilic o-quinones (Kumar et al., 1999).

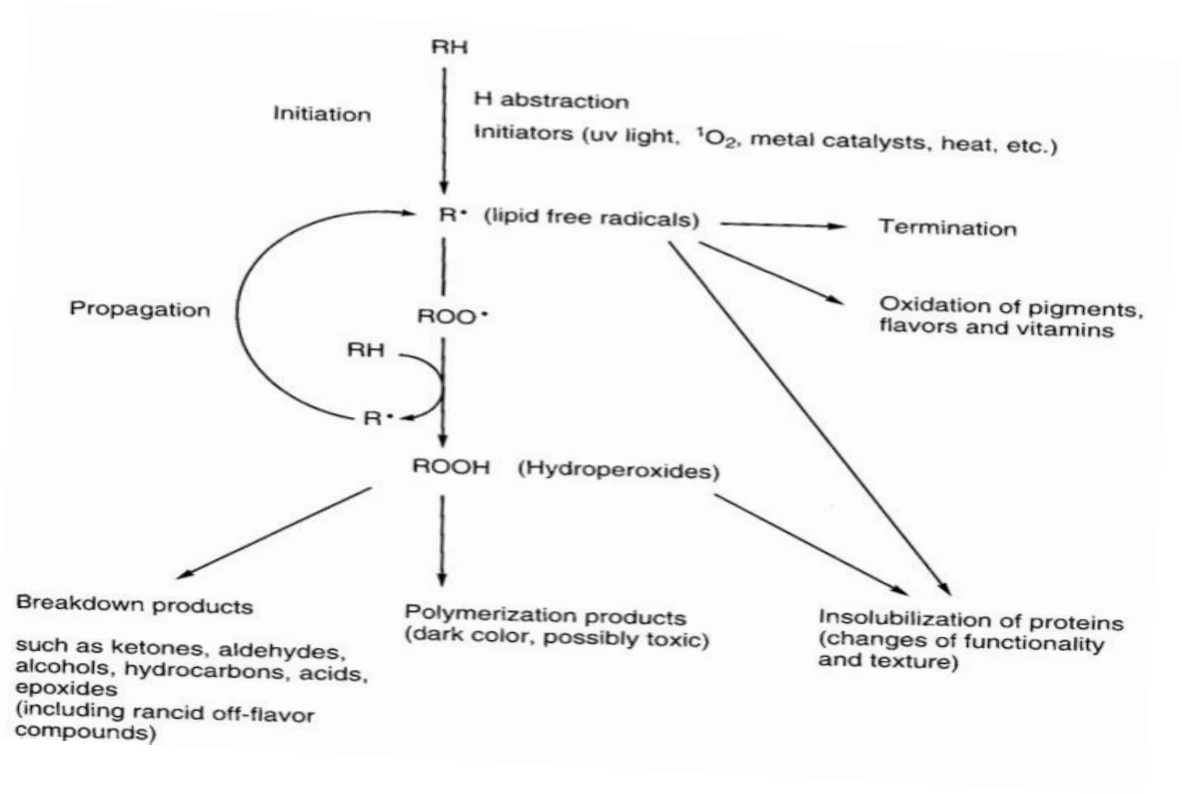


Figure 1. 3: Mechanism of lipid oxidation (Shahidi and Naczk, 2004).

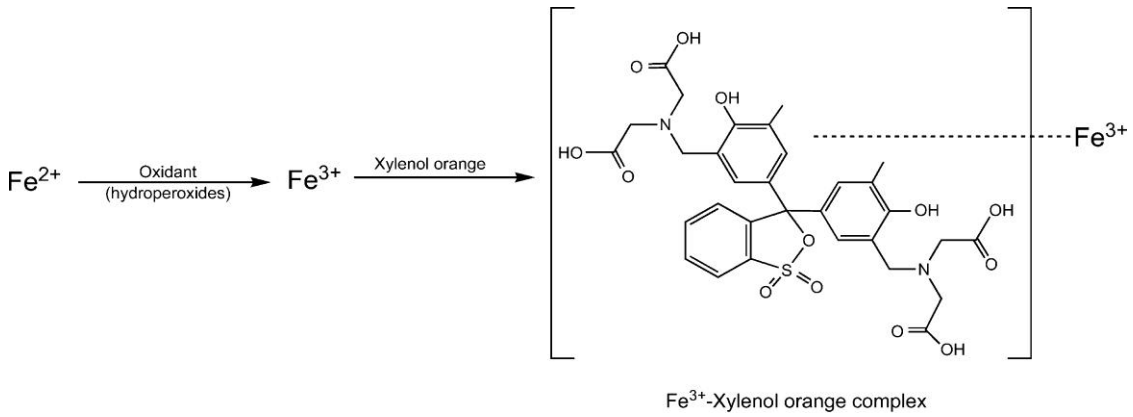


Figure 1. 4: Mechanism of the FOX method of PV (Moon and Shibamoto, 2009).

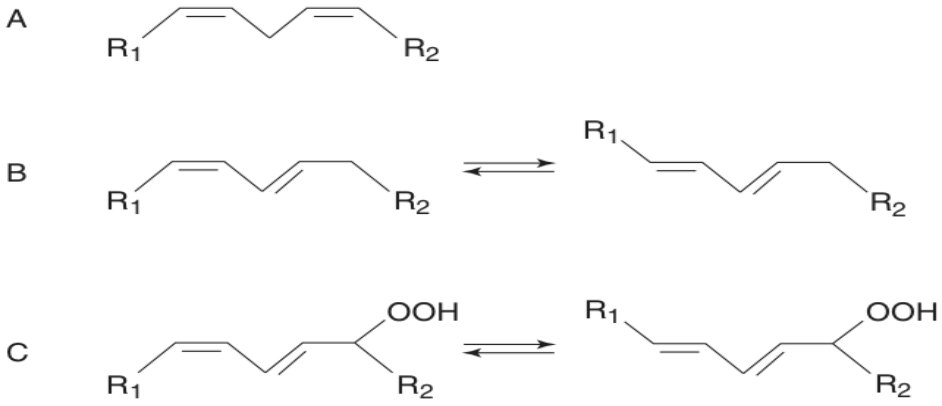


Figure 1. 5: Mechanism of PUFAs becoming conjugated (Corongiu and Banni 1994).

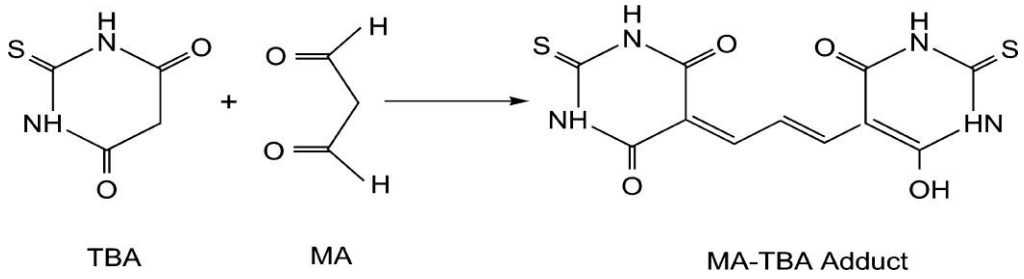


Figure 1. 6: Mechanism of TBARS (Moon and Shibamoto, 2009).

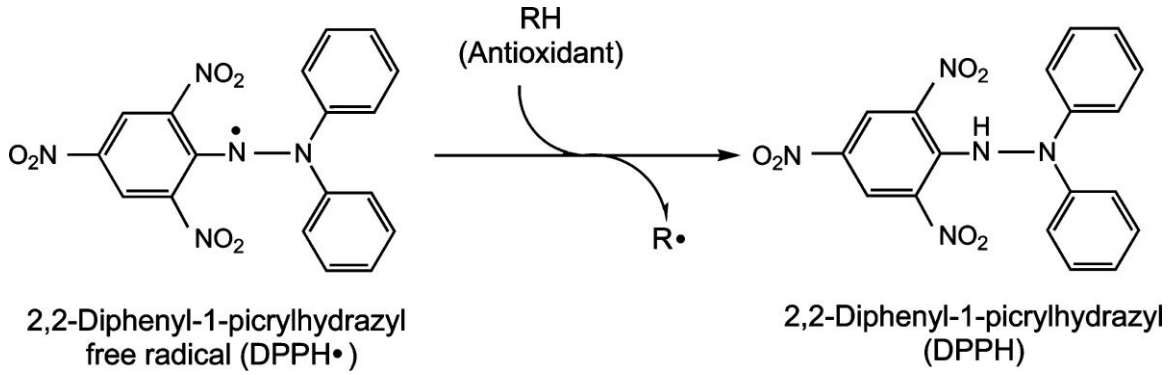


Figure 1. 7: Mechanism of DPPH scavanging (Moon and Shibamoto, 2009).

CHAPTER 2
INTRODUCTION OF PRIMARY ANTIOXIDANT ACITVITY TO CHITOSAN FOR APPLICATION AS MULTIFUNCTIONAL FOOD PACKAGING MATERIAL

This chapter is a lightly revised version of a paper by the same title submitted to the *Journal of Agricultural and Food Chemistry* by Stephanie Schreiber, Joseph Bozell, Douglas Hayes and Svetlana Zivanovic

Abstract

One of the strategies to enhance antioxidant properties of chitosan is to graft phenolics onto it. The objectives of this research were to evaluate 1) the effects of degree of deacetylation (DDA), ratio of reactants, and grafting time on the extent of grafting, and 2) efficiency of films made of grafted chitosan as packaging material for peanuts. The results indicate an inverse relationship between amount of conjugating reagents [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide] and grafting efficiency, with optimum molar ratio of $2 \cdot 10^{-4}$:1:0.05:0.05 for chitosan : gallic acid : carbodiimide : N-hydroxysuccinimide. The grafted product contained 34.26 mg gallic acid/g, had DPPH scavenging ability of 90%, reducing power of ABS_{700}=0.5, and was soluble in acetic acid. Chitin did not successfully undergo grafting and, an increase in DDA above 80% did not further improve the extent of grafting. When evaluated as packaging material for peanuts, the grafted chitosan reduced malondialdehyde, peroxide, and conjugated triene formation as compared to polyethylene bags, making it a good candidate for multifunctional food packaging material.

Introduction

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine units. Chitosan is a very desirable polymer for food packaging because it is one of the most abundant biopolymers in nature, biodegradable, antimicrobial, non-toxic, non-digestible, and easily forms films. It offers preventive antioxidant activity due its ability to selectively bind transition over non-transition metals, to establish ion-ion and ion-dipole interactions, and to act as an electron donor, through its nitrogen atoms. Most of chitosan's properties are due to the presence of the amine functionality, which confers both polyelectrolyte and chelate properties (Domard, 1987). However, chitosan lacks a hydrogen donor moiety in order to serve as a good chain breaking antioxidant. Adding the primary antioxidant capabilities to chitosan would be beneficial in creating a multifunctional packaging material that can inhibit lipid oxidation and reduce the development of off-flavors and rancidity in packaged food. Primary antioxidants donate a hydrogen or electron to free radicals converting them into a stable product inhibiting propagation. The primary antioxidants can also form complexes with lipid radicals and therefore inhibit or delay initiation. Phenolic compounds, categorized as primary antioxidants, form radical intermediates that are stable due to resonance delocalization so they do not initiate further free radical reactions and do not have suitable sites for molecular oxygen to attack (Shahidi and Naczk, 2005). When a phenolic compound reacts with a lipid radical, the resulting phenoxy radical is stabilized by

delocalization of unpaired electrons around the aromatic ring (Eskin and Przybylski, 2001). Therefore, grafting a phenolic compound to chitosan would be an efficient way of introducing primary antioxidant capabilities to the biopolymer.

There are multiple ways to graft phenolic compounds to chitosan. Chen and co-workers grafted eugenol and carvacrol to chitosan by synthesizing eugenol and carvacrol aldehydes and then grafting them on chitosan via Schiff base reactions (Chen et al., 2009). Vachoud and co-authors investigated grafting of dodecyl gallate to chitosan with horseradish peroxidase via a coupling reaction (Vachoud et al., 2001), while Aberg and his group used tyrosinase to catalyze a reaction between chitosan and the phenolic acid, arbutin (Aberg et al., 2002). A method for grafting gallic acid to chitosan using 1-ethyl-3-(3-dimethylaminopropyl) carbodimide (EDC) and N-hydroxysuccinimide (NHS) was developed by Pasanphan and Chirachanchai, (2008) which is illustrated in Figure 2.1. EDC is generally being used to mediate the formation of amide linkages between a carboxylate and an amine. EDC is a water-soluble compound that allows its direct addition to the reaction with no need for organic solvents (Hermanson, 1996). N-substituted carbodiimides, such as EDC, can react with carboxylic acids to form a highly reactive O-acylisourea intermediate, which can then react with a nucleophile, such as a primary amine from chitosan, to form an amide bond (Hermanson, 1996). However, hydrolysis is a major competing reaction and can cleave the activated ester intermediate forming an isourea and regenerating the carboxylate. NHS is added to form an active ester intermediate

from the reaction of the hydroxyl group of NHS with the EDC active ester (Hermanson, 1996). This is the method further investigated in this study.

The goal of this research was to graft a phenolic acid to chitosan to introduce significant primary antioxidant activity to the biopolymer, and to evaluate the grafted chitosan as food packaging material. Specifically, the objectives were 1) to evaluate the effects of degree of deacetylation (DDA), ratio of reactants, and grafting time on extent of grafting of a phenolic compound to chitosan; and 2) to determine efficiency of grafted chitosan films as packaging material for ground unroasted peanuts in comparison to non-modified chitosan films and polyethylene bags.

Materials and Methods

Reagents and Chemicals. Reagents used for grafting were 1-ethyl-3-(3-dimethylaminopropyl) carbodimide (EDC; 99.8% purity; CHEM-IMPEX, Wood Dale, IL) and N-hydroxysuccinimide (NHS; 98+% purity; Acros Organics, Gee, Belgium). If not otherwise noted, chitosan used in the experiments was 307 kDa chitosan with 80% DDA (as determined in our lab), kindly donated by Primex, Iceland. Other chitosans were water-soluble chitosan (MW 53 kDa; EZ Life Science Co. Ltd. Seoul, South Korea), 100% DDA chitosan (Carbomer, Inc, San Diego, CA), chitin (from crab shells, practical grade; Sigma-Aldrich, St. Louis, MO), and chitosan pentamer (Cape Cod Inc., East Falmouth, MA). Raw blanched peanuts were purchased from a local store, and plastic bags (low density polyethylene; 4 oz B01062) were from WHIRL-PAK (Fort Atkinson, WI).

Instruments and Equipment. All spectrophotometric assays were carried out on a UV-2101PC, Shimadzu (Columbia, MD), FT-IR analyses were carried out on a Nicolet NEXUS 670 FTIR (Thermo, Madison, WI), and NMR was carried out on a Varian 400-MR (Varian, Palo Alto, CA) instrument at 400 mHz for proton and 100.5 mHz for carbon and was referenced to the residual signal in the solvent. Thickness (mm) was measured using a hand-held microcaliper (Mitutoya Corp, Kawasaki, Kanagawa, Japan). Color was measured on a Hunter Lab Miniscan XE *Plus* (Hunter Associates Laboratory, Reston, VA), puncture strength was determined on a TA.XT *plus* Texture Analyzer using the TA-108S fixture and a 2 mm-diameter needle probe (TA-52; Texture Technologies Corp., Scarsdale, NY/Stable Micro Systems, Godalming, Surrey, UK), water permeability was evaluated with Fisher/Payne Permeability Cups (Thermo Fisher Scientific, Waltham, MA), and relative humidity was measured with a hydrometer (Thermo Fisher Scientific, Waltham, MA).

Grafting phenolics to chitosan. Grafting gallic acid to chitosan was conducted following a procedure modified from that of Pasanphan and Chirachanchai (2008) (Fig. 1). In short, 0.5 g gallic acid (GA) (3mmol), 0.03 g EDC (0.15 mmol), and 0 .017 g NHS (0.15 mmol) were dissolved in 20 mL 70% ethanol. The solution was stirred in an ice bath and after 1 hr was added to 0.16 g of purified chitosan dispersed in 30 mL 70% ethanol. The solution was further stirred in an ice bath for 30 min followed by stirring at room temperature for 24 hrs unless specified differently. The product was filtered through Whatman #4

filter paper, and washed 5 times by stirring for 45 min with 250 mL 70% ethanol. The final product was freeze-dried and analyzed the next day. The starting ratio of the grafting components was a $2 \cdot 10^{-4}$: 1 : 0.05 : 0.05 molar ratio of chitosan : GA : EDC : NHS. Reactants were varied to create the other ratios to test effects of varying different components of the grafting procedures. Any chitosan : GA : EDC : NHS ratio used in the experiments always refers to the molar ratio of the starting materials. Different ratios of the starting materials were evaluated for efficiency (Table 2.1). Grafting was carried out in at least duplicate and normally triplicate or more.

The antioxidant properties of the grafted chitosans were assessed by the DPPH scavenging method to determine their ability at scavenging radicals, total phenolics to determine the amount of gallic acid grafted to the chitosan, and reducing power to measure the electron donating ability. For all the antioxidant assays and solubility, 0.1% of each chitosan was prepared in 1% acetic acid, except for DPPH scavenging which was 0.01% chitosan in 0.1% acetic acid.

DPPH scavenging. The radical scavenging capacity of the grafted chitosan samples was determined using a DPPH method modified from literature (Saha et al., 2008). A solution of 4 mg 2,2-diphenyl-1- picrylhydrazyl (DPPH) was prepared in 100 mL methanol. Aliquots of of the DPPH solution (1 mL) were added to 1 mL of the sample or D.I. water (control). The samples were stirred for 30 min in the dark at room temperature. The absorbance (A) was read at 517 nm using a spectrophotometer. Samples were analyzed by calculating % scavenging

with the formula: % Scavenging= $[(A_{control} - A_{sample})/A_{control}]\cdot 100$. Measurements were performed in at least duplicate, and mostly triplicate.

Total Phenolics. The concentration of the phenolic acid grafted to chitosan was determined by the modified Folin-Ciocalteu method (Folin and Ciocalteu, 1927). Gallic acid standards were prepared in range of 0.01 to 0.1 mg/mL. For each sample, standard, or D.I. water (control), 1 mL-aliquots were added to a test tube followed by 7 mL D.I. water and 1 mL 3.35% Folin-Ciocalteau solution. After 3 min, 1 mL 12.4% sodium carbonate was added and samples were vortexed and put in a water bath for 30 min at 40°C. Absorbance of the samples was read at 725 nm with a spectrophotometer. Measurements were done in at least duplicate but normally triplicate.

Reducing Power. The reducing power was determined using the method reported by Yen and Chen (1995). Phosphate buffer pH=6.6, 0.2M (2.5 mL) was added to 1 mL of chitosan solution. Potassium ferricyanide (2.5 mL 1% solution) was added to the mixture and incubated at 50 °C for 20 min. Trichloroacetic acid (2.5 mL 10% solution) was added and centrifuged for 10 min at 3000 rpm. A, 2.5 mL aliquot of the upper layer was removed and added to a separate tube, followed by 2.5 mL D.I. water and 0.5 mL 0.1% iron chloride. Absorbance was immediately read at 700 nm using a spectrophotometer. Measurements were done in at least duplicate but normally triplicate.

Solubility. Transmittance (%) was measured at 600 nm using 0.1% grafted chitosan in 1% acetic acid. High transmittance reflects high solubility. Measurements were done in at least duplicate but normally triplicate.

FTIR. Grafted chitosan was ground up to a fine powder with a mortar and pestle and analyzed directly in an attenuated total reflectance (ATR) module. Transmittance (%) was read on FT-IR between 500 and 4000 cm^{-1}. The number of scans was 64, resolution 4 cm^{-1}. Measurements were done in at least duplicate but normally triplicate.

^{1}H and ^{13}C NMR was used to assess grafting sites. All compounds and grafted chitosan pentamer (not washed, dissolved in deuterated acetic acid /deuterated water) were run on a 400 MR NMR at room temperature. A chitosan pentamer was used due to the inability to dissolve the chitosan polymer in the NMR tube at high enough concentrations.

Film Preparation and Analysis. Chitosan film forming solution (FFS) was prepared with 0.7% grafted or non modified chitosan in 1% acetic acid. The films were cast in 5 and 10 cm-Petri dishes using 10 mL and 26 mL FFS, respectively. Petri dishes were left at room temperature until the films were dry (easy to peel off), usually 4 – 5 days.

Thickness, Color, Puncture strength and Water Vapor Permeability were measured in at least duplicate but normally triplicate. Thickness was determined with a hand-held microcaliper. Color was recorded using a colorimeter standardized with white and black tiles. Puncture strength was

calculated by dividing the force (N) by thickness. Water permeability was reported as mg of water that permeated a one-mm thickness of one square centimeter of the film over a 24 hour period at room temperature (Fisher Scientific, 1984).

Quality of Packaged Peanut Powder. Raw blanched peanuts were ground to a powder in a coffee grinder. The peanut powder was placed inside grafted chitosan pouches, non modified chitosan pouches, low density polyethylene bags, and open Petri dishes. Each pouch or bag contained 9 g of peanut powder. The pouches were all placed in a forced air incubator at 50 °C and the humidity was constantly monitored using a hydrometer. The relative humidity during the whole experiment was in the 20 – 30% range. Plain grafted-chitosan and non modified chitosan films (not used as pouches) were also placed in the incubator. Films, pouches, and peanut powder were analyzed after 0, 5, 9, 12 and 15 weeks. Two films and 3 pouches for each packaging type were analyzed at each sampling period**.**

Determination of **Thiobarbituric Acid Reactive Substances (TBARS), Peroxide Value (PV), Conjugated Dienes (CD) and Conjugated Trienes (CT)** in ground peanuts were conducted by methods modified from Current Protocols in Food Analytical Chemistry (Pegg, 2001A; Pegg, 2001B). The determination of TBA reactive substances was via a colorimetric assay using 2-thiobarbituric acid to determine malonaldehyde content and comparing it with a standard curve. PV was determined by a photometric method based on the ability of the lipid

peroxides to oxidize ferrous ions. The changes in conjugated dienes and trienes were assessed by measuring the change in maximum absorbance for a constant mass of sample around the peak associated with either conjugated dienes or conjugates trienes (Pegg, 2001A; Pegg, 2001B). Measurements were done in triplicate.

Statistical analysis. Tukey HSD comparison of means was performed using Jump (7.0.2).

Results and Discussion

Evidence of Grafting

Grafting of gallic acid to the chitosan was confirmed by FTIR. FTIR was run on non- modified chitosan, grafted chitosan with the starting molar ratio of $2 \cdot 10^{-4}$: 1 : 0.05 : 0.05 (chitosan : GA : EDC : NHS), and 35 mg gallic acid mixed with 1 g of chitosan. The mixture of gallic acid and chitosan represents the amount of gallic acid found in the $2 \cdot 10^{-4}$: 2 : 0.05 : 0.05 grafted chitosan. As seen in Figure 2.2, the peaks at 1450 to 1300 cm^{-1} show a difference between grafted chitosan and chitosan mixed with gallic acid. The FTIR scans of non-modified and grafted chitosan (Figure 2.2A and 2.2B) show the same -C-O stretching and –OH deformation vibrations at around 1050 cm^{-1} which indicates that the OH group remains unmodified and that glycosidic bonds are not formed. There is a significant difference between non-modified and grafted chitosan at peaks 1645 and 1548 cm^{-1} which are representative of –C=O stretch in chitosan

amide and N-H in plane deformation coupled with C-N stretching in chitosan respectively (Amaral et al., 2005). The height ratio of the peaks at 1645 and 1548 cm^{-1} is 0.95 for non-modified chitosan and 1.48 for modified chitosan indicating that there is significantly higher amide stretching in modified chitosan as compared to non-modified owing to the amide bond formed between gallic acid and chitosan in grafted chitosan.

To further evaluate grafting sites, ^{1}H NMR and ^{13}C NMR were performed on $2 \cdot 10^{-4}$: 1 : 0.05 : 0.05 (chitosan : GA : EDC : NHS) grafted chitosan pentamer, non-modified chitosan pentamer, and the grafting components, gallic acid, EDC and NHS (Figure 2.3). Chitosan pentamer was used in these studies instead of the polymer due to the inability to dissolve chitosan in a sufficiently high concentration. The GA-grafted pentamer was not washed with ethanol due to its solubility in ethanol, as result of low molecular weight. The peaks not attributed to any of the starting materials were analyzed for evidence of amide bond formation. However, the unknown peaks were inconclusive in determining definite amide bond formation. According to Luo and coworkers, the ^{1}H NMR peak of the hydrogen in the –CONH bond is around 8.1 ppm (Luo et al., 2011). There is a small peak at around 8.1 ppm in the grafted ^{1}H NMR pentamer scan (Figure 2.3) however, the same peak is present in the non- grafted ^{1}H NMR pentamer scan. This is probably because there are remaining acetyl groups present in chitosan.

Effect of Degree of Deacetylation (% DDA)

Figure 2.4 shows the effect of variation in the degree of deacetylation on grafting efficiency estimated by three methods: DPPH % scavenging, total phenolics, and reducing power (Figure 2.4 A-C). Solubility of the grafted chitosan (Figure 2.4D) was included as an important factor for a material to be able to form films. After grafting, the completely deacetylated chitosan (100% DDA) was not significantly different from the 80% DDA chitosan in DPPH % scavenging, total phenolics, reducing power and solubility. On the other hand, water-soluble chitosan and chitin showed similar results for DPPH scavenging, total phenolics, and reducing power. Both showed insignificant antioxidant activity indicating lack of grafting. The fact that chitin did not graft supports the FTIR results that GA grafting occurs on the amine rather than on hydroxyl groups. Both chitin and chitosan have two hydroxyl groups per momomeric unit available for grafting; however, while the majority of amino groups in chitosan are deacetylated, most of amino groups in chitin are acetylated and thus unavailable for grafting. But, extensive deacetylation of chitosan (from 80 to 100%) did not result in further increase in grafting which may mean that the gallic acid is selectively binding to some of the amine groups or only binds to a certain extent.

Effect of Ratio of Reactants

The need to graft gallic acid to chitosan arises from the fact that chitosan exhibits no primary antioxidant activity. This is seen by non-grafted chitosan's DPPH scavenging ability, total phenolics and reducing power of only 9.40%, 1.84 mg/g GAE, and 0 Abs at 700 nm, respectively. These results all show negligible

primary antioxidant activity of chitosan. Our goal was to find a ratio of the reactants that results in grafted chitosan with optimized antioxidant activity and excellent solubility, while using minimal amounts of the conjugating agents.

Table 2.1 represents all ratios evaluated. Figure 2.5 shows the effect of the ratio the reactants on DPPH scavenging, total phenolics, reducing power, and solubility of grafted chitosan. Based on the method developed by Pasanphan and Chirachanchai (2008), the original ratio of the reactants was determined to be $2{\cdot}10^{-4}$: 1 : 3 : 3 for chitosan : GA : EDC : NHS, on a molar basis, and was used as a 'base line'.

The effect of the ratio of the reactants on DPPH scavenging is shown in Figure 2.5A. Samples with ratios $2{\cdot}10^{-4}$: 1 : 3 : 3 (B) and $1{\cdot}10^{-4}$: 1 : 3 : 3 (C) had the same ratio of GA : EDC : NHS; but, sample B had double the amount of chitosan ($2*10^{-4}$ vs. $1*10^{-4}$). Although sample C exhibited a higher % DPPH scavenging (44.9%) compared to sample B (31.5%) the values are not statistically different. Samples B, E, F, G, H and I had the same ratio of chitosan to gallic acid ($2{\cdot}10^{-4}$: 1); but the amounts of EDC and NHS varied from 0.05 : 0.05 to 3 : 3. Chitosan samples grafted with ratios E ($2{\cdot}10^{-4}$: 1 : 1 : 1) to I ($2{\cdot}10^{-4}$: 1 : 0.05 : 0.05) had significantly higher DPPH scavenging ability than sample B ($2{\cdot}10^{-4}$: 1 : 3 : 3); but, samples E to I were not significantly different between each other (Figure 2.5A). DPPH scavenging of samples E ($2{\cdot}10^{-4}$: 1 : 1 : 1) to I ($2{\cdot}10^{-4}$: 1 : 0.05 : 0.05) ranged from 65.3% to 89.5%, respectively.

The effect of the ratio of the reactants on the content of total phenolics in grafted chitosan, as the measure of the amount of gallic acid that was actually present in the grafted chitosan samples, is shown in Figure 2.5B. Ratios of the reactants (chitosan : GA : EDC : NHS) in all the samples (A to I) are expressed based on the amount of GA; in other words, amounts of all the starting materials were calculated having the GA as the reference ratio of 1. Samples A, B, C and D were designed to compare variation of chitosan amount ($1 \cdot 10^{-3}$ to $5 \cdot 10^{-5}$) keeping the GA : EDC : NHS ratio (1 : 3 : 3) constant. Chitosan grafted with ratio A had a significantly lower level of total phenolics than chitosan grafted with the ratio C, probably because sample A had 10 times lower amount of GA to chitosan in the reacting mixture than sample C ($1 \cdot 10^{-3}$: 1 vs. $1 \cdot 10^{-4}$: 1, respectively). Samples prepared with ratios E ($2 \cdot 10^{-4}$: 1 : 1 : 1) to I ($2 \cdot 10^{-4}$: 1 : 0.05 : 0.05) had significantly higher levels of total phenolics than sample B ($2 \cdot 10^{-4}$: 1 : 3 : 3); but samples E to I showed no significant differences (Figure 2.5B). Even though samples E to I were not statistically different, there does seem to be an inverse relationship between the amount of EDC and NHS, and total phenolics of the grafted chitosan samples. According to Hermanson (*8*), excess NHS can cause too much conjugation resulting in an insoluble and possibly inactive complex, which is mostly likely the reasoning behind inverse relationship. The values for total phenolics (GAE mg/g) of samples B, and E to I were 10.69, 25.03, 27.15, 31.15, 36.64 and 34.26 GA Eq (mg/g), respectively.

The effect of ratio of the reactants on reducing power of grafted chitosan is shown in Figure 2.5C. Samples A, B, C, and D prepared with excess of conjugating reagents (EDC and NHS) had similar reducing power, but had significantly lower reducing power than samples E to I, prepared with less EDC and NHS. The reducing power (as Abs_{700}) of samples B, and E to I were 0.22, 0.36, 0.40, 0.47, 0.55, and 0.51, respectively, indicating the same inverse relationship between the amount of EDC and NHS and efficiency of grafting, as seen with total phenolics (Figure 2.5B and 2.5C).

Solubility was determined as % transmittance in solutions of 0.1% grafted chitosan in 1% acetic acid and is shown in Figure 2.5D. With the same chitosan : GA ratio ($2 \cdot 10^{-4}$: 1), the decrease in amount of grafting reagents from 3 : 3 to 1 : 1 significantly improved solubility of the grafted chitosan. However, further reduction in amount of EDC and NHS did not have additional increase in solubility. As mentioned above, an excess of NHS can cause too much conjugation possibly resulting in an insoluble complex, which is mostly likely the reasoning behind the trend seen in Figure 2.5D. When we removed NHS from the grafting procedure, no antioxidant activity was seen (data not shown). Also, ratio $2 \cdot 10^{-4}$: 1 : 1 : 0.5 was analyzed and compared to ratio $2 \cdot 10^{-4}$: 1 : 0.5 : 0.5, to observe whether reduction of NHS without decreasing EDC have effect on grafting, since NHS has been shown to cause the reduced activity and solubility. Ratio $2 \cdot 10^{-4}$: 1 : 1 : 0.5 resulted in sample with significantly lower DPPH, total phenolics, and reducing power than $2 \cdot 10^{-4}$: 1 : 0.5 : 0.5 (data not shown).

Therefore, EDC and NHS probably need to be present in equal amounts for the reaction to be carried out optimally. This shows that with too much EDC as compared to NHS the unstable activated ester that is formed may become hydrolyzed and with too much NHS as compared to EDC the very stable activated ester results in too much conjugation and a less active and less soluble product.

The ratio $2 \cdot 10^{-4}$: 1 : 0.05 : 0.05 was chosen for continuing experiments because it resulted in high antioxidant activity and solubility with minimum amounts of the grafting agents, EDC and NHS needed. However, another ratio was tested with the goal of reducing the amount of gallic acid. Adding excess gallic acid to the grafting mixture increases cost and led to a large amount of free gallic acid being removed in the ethanol washes (data not shown). The molar ratio of chitosan to gallic acid in the starting ratio is 1 : 5000 and the ratio of final grafted product is 1 : 67. Only around 5% of NH_2 groups of chitosan are grafted.

The ratio of starting components with 1/3 of the original amount of gallic acid ($2 \cdot 10^{-4}$: 0.33 : 0.05 : 0.05) was prepared and analyzed to test whether chitosan can be efficiently grafted with less gallic acid present in the reactant mixture. However, this ratio showed a reduction in total phenolics and reducing power in grafted chitosan by 52% and 51%, respectively compared to the $2 \cdot 10^{-4}$: 1 : 0.05 : 0.05 ratio (data not shown). It appears gallic acid must be present in a molar ratio 20 times that of EDC and NHS to ensure maximum grafting activity.

This shows that not every amine group of chitosan is grafted and not all the gallic acid reacts with EDC and NHS.

Effect of Grafting Time

Figure 2.6 depicts the effect of grafting time on the antioxidant activity and solubility of the grafted chitosan. There was no significant difference between 6 hr and 24 hr; but grafting for 1.5 hr resulted in a sample with significantly lower DPPH scavenging ability, total phenolics, and reducing activity (Figure 2.6A-C). Varying the amount of grafting time from 1.5 to 24 hr showed that the majority of grafting seems to be complete after 6 hr, although limited grafting can be achieved within 1.5 hr. This is useful to note that in the future time can be saved by reducing grafting time from 24 to 6 hr.

Gallic Acid Grafted Chitosan Films as Multifunctional Packaging for Peanut Powder

The films cast from GA-grafted chitosan were significantly darker than the films prepared from non-modified chitosan (Figure 2.7); but, storage at 50 °C at low relative humidity (20-30%) for 15 weeks did not cause visible darkening of either of the films. Grafting did not change the physical properties of the films appreciably (Figure 2.8). Puncture strength of the chitosan films was statistically higher than that of the low density polyethylene bag and stayed unchanged during a 15-week incubation at 50 °C and low relative humidity (Figure 2.8A). Even when the films were used for packaging of peanuts, in form of pouches, the puncture strength stayed unchanged (Figure 2.8B). Although the puncture strength of the grafted and non-modified chitosan pouches appear to have

decreased at week 9 (Figure 2.8B), the results are not statistically different from the other weeks.

Grafting did not alter water vapor permeability (WVP) of chitosan films, although the WVP decreased in aged films (Figure 2.9A). WVP of the films in the grafted pouches was significantly higher at week 5 than the non-modified pouches (Figure 2.9B). However, the grafted and non-modified chitosan pouches did not significantly differ from week 9 through 15. Although the low density polyethylene bags were better at preventing water permeation, a sticky residue formed after week 5 on the polyethylene bags packed with peanut powder, which did not occur for any of the chitosan pouches.

Antioxidant properties (DPPH scavenging, total phenolics, reducing power) of the grafted films and pouches during 15-week storage at 50 °C are shown in Figure 2.10. The primary antioxidant activity of the films and pouches was not reduced during 15 weeks under the harsh conditions. In addition, the antioxidant activity of the films used in the pouches with peanut powder was similar to the activity of the films alone. This shows that the gallic acid providing the primary antioxidant activity to the films did not leach out of the pouches into the packaged product. If the antioxidant activity in the packaging was lower than in the films this could mean that the gallic acid was transferred to the food product and was probably not covalently bound.

The quality of peanut powder was evaluated based on malondialdehyde production, PV, and conjugated diene and triene formation (Figure 2.11). Non-

packaged peanut powder stored at the same condition had values of 1.65 mg (MDA) eq/kg food sample, 749 PV meq peroxide/ kg oil, 41 CD extinction value, and 8.5 CT extinction value at week 15 for malondialdehyde production, PV, and conjugated diene and triene formation respectively (data not shown). Overall, the peanuts packaged in the pouches made of chitosan films (both grafted and non-modified) had similar or better quality compared to those packaged in the significantly thicker low density polyethylene bags. The average thicknesses were 0.027mm, 0.028 mm and 0.058 mm for the grafted films, non-modified films, and polyethylene bags respectively. The peanut powder in the grafted chitosan pouches showed significantly lower level of malonaldialdehyde (a secondary lipid oxidation product) than peanut powder packaged in non-modified chitosan pouches and polyethylene bags during 12 weeks storage at 50 ^{o}C (Figure 2.11A). During the first 5 weeks of the stress storage, the peanut powder in the grafted chitosan pouches had a significantly lower PV than peanut powder packaged in non-modified chitosan pouches and polyethylene bag (Figure 2.11B). By the end of the storage, peanut powder in the polyethylene bags had a significantly higher PV than powder packaged in either type of chitosan pouches (Figure 2.11B). There is no significant difference for conjugated dienes regardless on the packaging material over the whole storage period (Figure 2.11C). However, the peanut powder in the polyethylene packaging showed a significantly higher conjugated triene extinction value than in chitosan pouches during 15 weeks of storage at 50 ^{o}C (Figure 2.11D). This shows that up to 12

weeks at 50 ^{o}C the production of the secondary lipid oxidation product malondialdehyde was significantly reduced in the peanut powder packed in the grafted chitosan pouches as compared to those packaged in non-modified chitosan pouches and the low density polyethylene bags. Chitosan films grafted with gallic acid efficiently reduce oxidation of peanut powder during 15 weeks at 50 ^{o}C compared to low density polyethylene films. None of the packaged peanut powders are considered rancid according to the minimal PV value found in literature for acceptability of peanuts which is 20 meg/kg of extracted oil (Evranuz, 1993; Mate et al., 1996; Min and Krochta, 2007). Also a 15 week incubation period at 50 ^{o}C is an acceptable shelf life study to measure lipid oxidation because Mate and co-researchers did 10 weeks at 37 ^{o}C which is a less stressed environment (Mate et al., 1996).

Conclusions

The optimum ratio of chitosan : GA : EDC : NHS was found to be $2{\cdot}10^{-4}$: 1 : 0.05 : 0.05 since it resulted in grafted chitosan with high antioxidant activity and solubility, while using minimal amounts of conjugating reactants. In order to obtain optimum antioxidant activity and solubility of the grafted chitosan, EDC and NHS must be present in significantly lower amounts than chitosan and gallic acid. Gallic acid, on the other hand, must be present in excess. Grafting of phenolic acid to chitosan apparently happens at the amino groups; but an increase in DDA over 80% does not further improve the efficiency of grafting.

Grafting time between 6 and 24 hr did not show a significant difference in conjugation, but shortening to 1.5 hr significantly reduced extend of reaction. Chitosan films grafted with gallic acid efficiently reduce oxidation of peanut powder during 15 weeks at 50 ^{o}C, significantly more than low density polyethylene films. Therefore, chitosan grafted with a phenolic compound has great potential ability to be used as food packaging material due to its primary and secondary antioxidant properties, film forming properties, and ability to reduce lipid oxidation.

References

Aberg, C. M., Chen, T. H., & Payne, G. F. (2002). Renewable resources and enzymatic processes to create functional polymers: Adapting materials and reactions from food processing. *Journal of Polymers and the Environment, 10*(3), 77-84.

Amaral, I., Granja, P., & Barbosa, M. (2005). Chemical modification of chitosan by phosphorylation: an XPS, FT-IR and SEM study. *Journal of Biomaterials Science-Polymer Edition, 16*(12), 1575-1593.

Chen, F., Shi, Z., Neoh, K., & Kang, E. (2009). Antioxidant and antibacterial activities of eugenol and carvacrol-grafted chitosan nanoparticles. *Biotechnology and Bioengineering, 104*(1), 30-39.

Domard, A. (1987). Ph and cd measurements on a fully deacetylated chitosan - application to cu-ii-polymer interactions. *International Journal of Biological Macromolecules, 9*(2), 98-104.

Eskin, N. A. M., & Robinson, D. S. (2000). Antioxidants and shelf life of foods. In N. A. M. Eskin & R. Przybylski (Eds.), *Food Shelf Life Stability: Chemical, Biochemical and Microbiological Changes*, (pp. 194). Boca Raton, FL: CRC Press LLC.

Evranuz, E. (1993). The effects of temperature and moisture-content on lipid-peroxidation during storage of unblanched salted roasted peanuts - shelf-life studies for unblanched salted roasted peanuts. *International Journal of Food Science and Technology, 28*(2), 193-199.

Fisher Scientific (1984). Fisher/Payne permeability cup instructions. In Fifth ed.

Folin, O., & Ciocalteu, V. (1927). On tyrosine and tryptophane determinations in proteins. *Journal of Biological Chemistry, 73*(2), 627-650.

Hermanson, G. T. (1996). Zero-length cross linkers. In *Bioconjugate Techniques*, (pp. 169-176). San Diego, CA: Academic Press, Inc.

Luo, Y., Peng, H., Wu, J., Sun, J., & Wang, Y. (2011). Novel amphoteric pH-sensitive hydrogels derived from ethylenediaminetetraacetic dianhydride, butanediamine and amino-terminated poly(ethylene glycol) Design, synthesis and swelling behavior. *European Polymer Journal, 47*(1), 40-47.

Mate, J., Saltveit, M., & Krochta, J. (1996). Peanut and walnut rancidity: Effects of oxygen concentration and relative humidity. *Journal of Food Science, 61*(2), 465-+.

Min, S., & Krochta, J. (2007). Ascorbic acid-containing whey protein film coatings for control of oxidation. *Journal of Agricultural and Food Chemistry, 55*(8), 2964-2969.

Pasanphan, W., Buettner, G. R., & Chirachanchai, S. (2010). Chitosan gallate as a novel potential polysaccharide antioxidant: an EPR study. *Carbohydrate Research, 345*(1), 132-140.

Pasanphan, W., & Chirachanchai, S. (2008). Conjugation of gallic acid onto

chitosan: An approach for green and water-based antioxidant. *Carbohydrate Polymers, 72*(1), 169-177.
Pegg, R. (2001). Measurement of primary lipid oxidation products. *Current protocols in food analytical chemistry*, D2.1.1-D2.1.15.
Pegg, R. (2001). Spectrophotometric measurement of secondary lipid oxidation products. *Current protocols in food analytical chemistry*, D2.4.1-D2.4.18.
Saha, M. R., Hasan, S. M. R., Akter, R., Hossain, M. M., Alam, M. S., Alam, M. A., & Mazumder, M. E. H. (2008). *In vitro* free radical scavenging activity of methanol extract of the leaves of *mimusops elengi linn*. *Bangladesh Journal of Veterinary Medicine, 6*(2), 197-202.
Shahidi, F., & Naczk, M. (2004). *Phenolics in Food and Nutraceuticals* Boca Raton, Florida: CRC Press.
Vachoud, L., Chen, T., Payne, G., & Vazquez-Duhalt, R. (2001). Peroxidase catalyzed grafting of gallate esters onto the polysaccharide chitosan. *Enzyme and Microbial Technology, 29*(6-7), 380-385.
Yen, G. C., & Chen, H. Y. (1995). Antioxidant activity of various tea extracts in relation to their antimutagenicity. *Journal of Agricultural and Food Chemistry, 43*(1), 27-32.

Appendix: Chapter 2

Table 2. 1: Molar ratio of starting compounds evaluated for efficiency of grafting.

	Chitosan	GA	EDC	NHS
A	$1*10^{-3}$	1	3	3
B	$2*10^{-4}$	1	3	3
C	$1*10^{-4}$	1	3	3
D	$5*10^{-5}$	1	3	3
E	$2*10^{-4}$	1	1	1
F	$2*10^{-4}$	1	0.5	0.5
G	$2*10^{-4}$	1	0.25	0.25
H	$2*10^{-4}$	1	0.1	0.1
I	$2*10^{-4}$	1	0.05	0.05

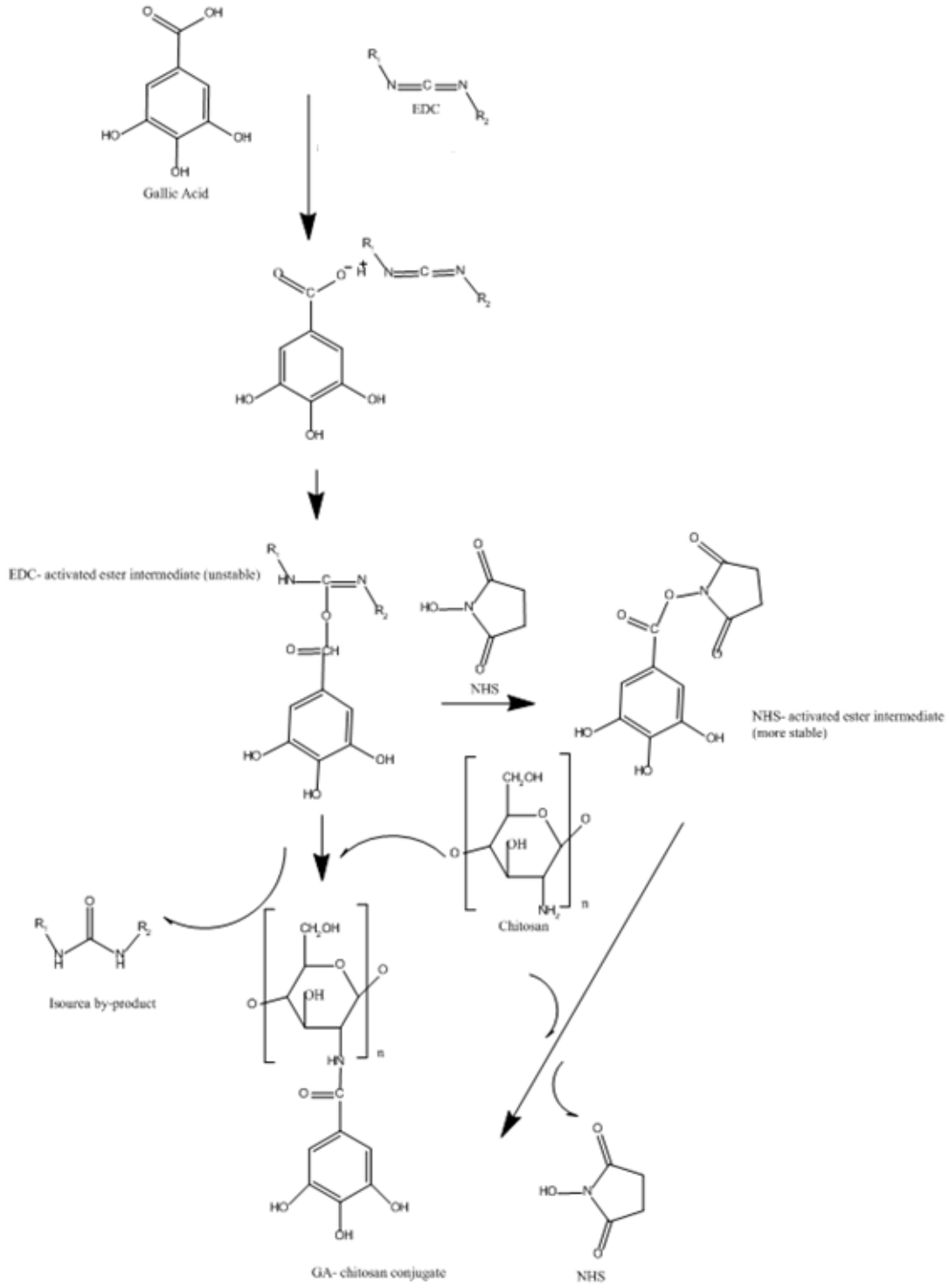


Figure 2. 1: Mechanism of grafting gallic acid to chitosan with EDC and NHS. After Pasanphan et al. (2010) and Hermanson et al. (1996)

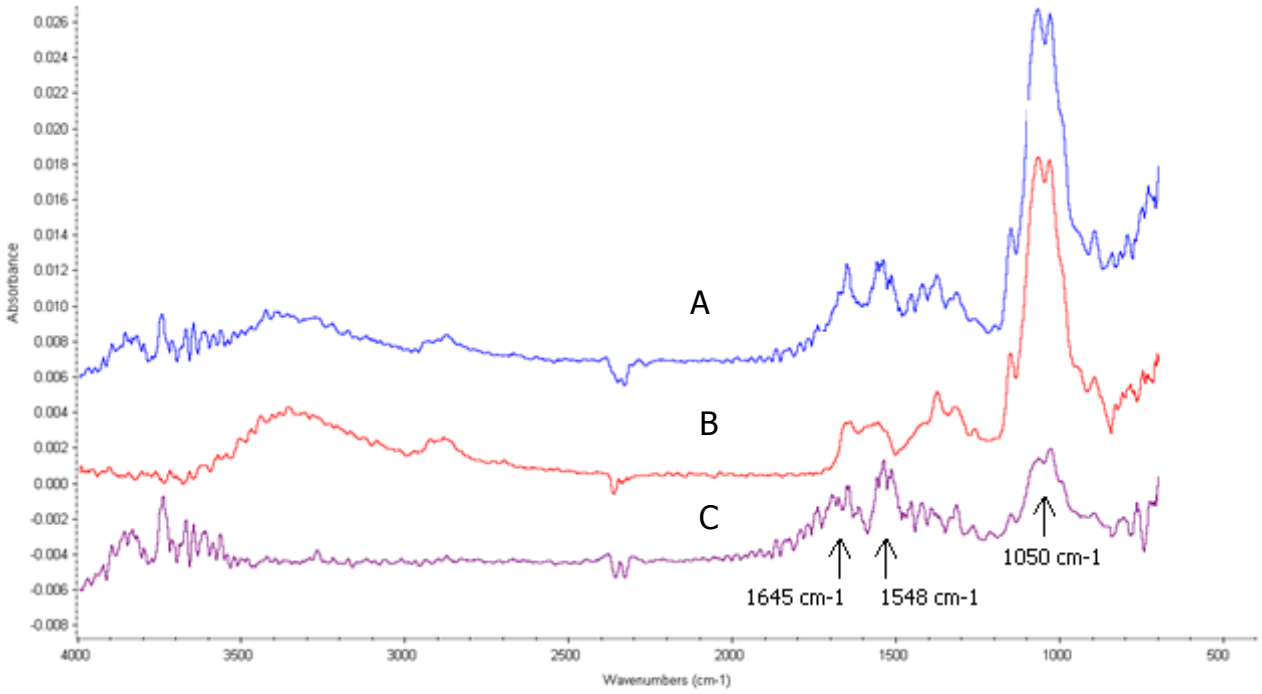


Figure 2. 2: ATR - FTIR scans of A) chitosan (non-modified), B) gallic acid grafted chitosan ($2 \cdot 10^{-4}$: 1 : 0.05 : 0.05 chitosan : GA : EDC : NHS), C) mixture of gallic acid and chitosan (34.26 mg gallic acid per 1 g chitosan). 1050 cm^{-1} shows the same -C-O stretching and –OH deformation vibrations indicating that the OH group remains unmodified and that glycosidic bonds are not formed. There is a significant difference between NM and grafted chitosan at peaks 1645 and 1548 cm^{-1} which are representative of –C=O stretch in chitosan amide and N-H in plane deformation coupled with C-N stretching in chitosan respectively (15).

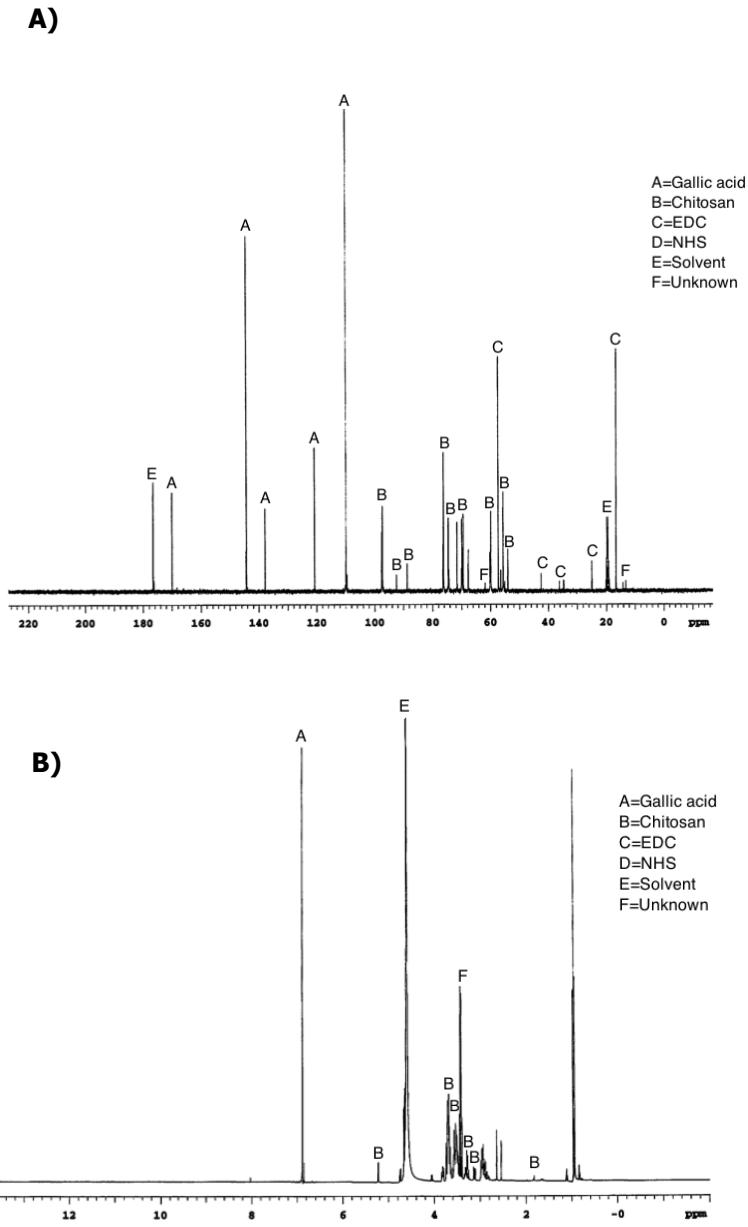


Figure 2. 3: NMR scans of gallic acid grafted chitosan pentamer; A) ^{13}C NMR, B) ^{1}H NMR. The peaks were identified by separately running the pure compounds.

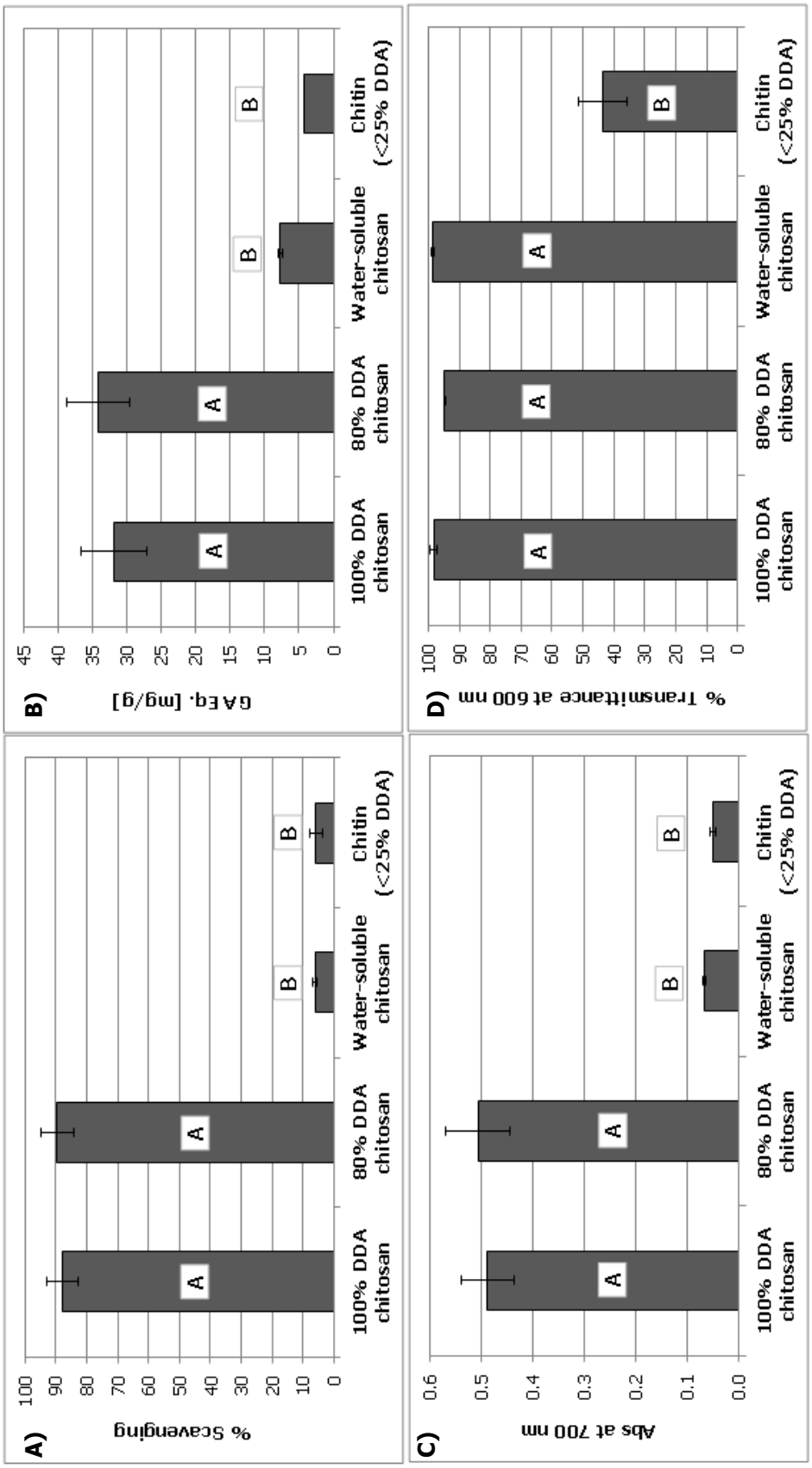


Figure 2. 4: Effect of degree of deacetylation of chitosan (DDA) on efficiency of grafting; A) DPPH scavenging (% Scavenging), B) Total Phenolics (GA Eq. [mg/g]), C) Reducing Power (Abs at 700 nm), D) Solubility (% Transmittance at 600 nm). All chitosans and chitin were grafted at $2{\cdot}10^{-4}$: 1 : 0.05 : 0.05 (chitosan/chitin : GA : EDC : NHS) ratio. Values are presented as means with standard deviation. Bars with different letters are significantly ($p<0.05$) different.

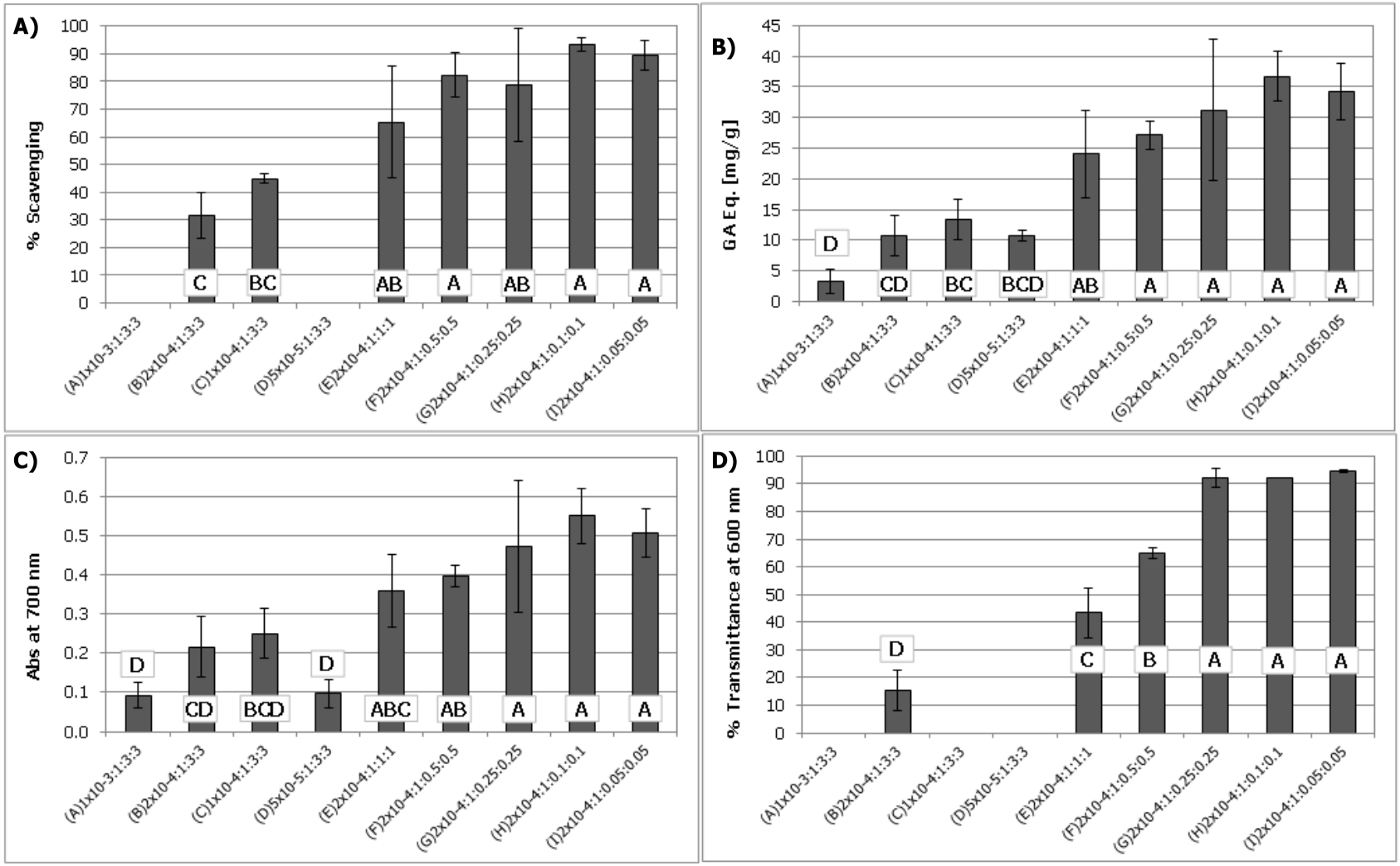


Figure 2. 5: Effect of ratio of the compounds used for grafting on the grafting efficiency; A) DPPH scavenging (% Scavenging), B) Total Phenolics (GA Eq. [mg/g]), C) Reducing Power (Abs at 700 nm), D) Solubility (% Transmittance at 600 nm). Key refers to molar ratios of chitosan : GA : EDC : NHS. Values are presented as means with standard deviation. Bars with different letters are significantly ($p<0.05$) different.

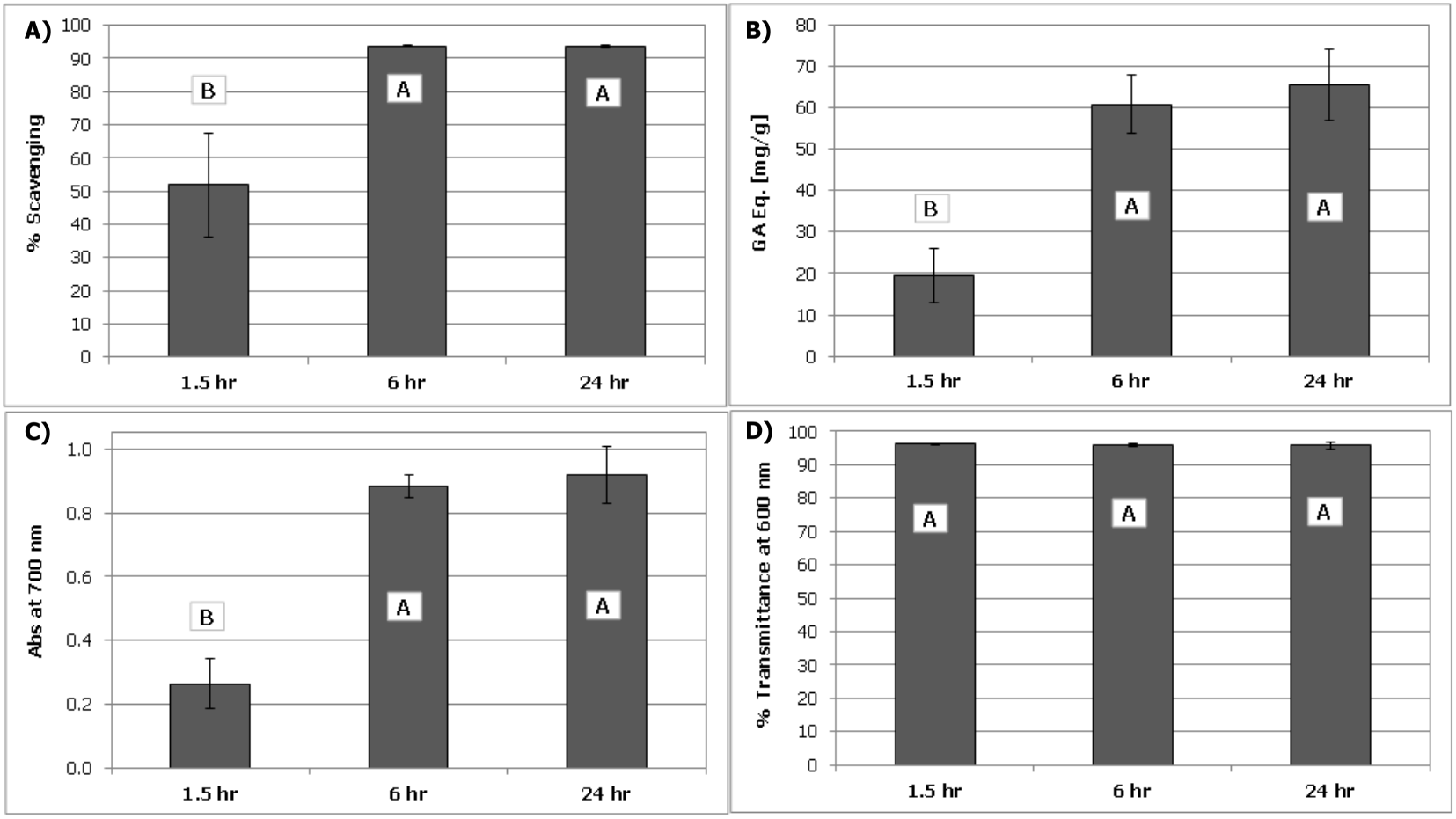


Figure 2. 6: Effect of reaction time on the grafting efficiency; A) DPPH scavenging (% Scavenging), B) Total Phenolics (GA Eq. [mg/g]), C) Reducing Power (Abs at 700 nm), D) Solubility (% Transmittance at 600 nm). Ratio of the reactant was $2 \cdot 10^{-4}$: 1 : 0.05 : 0.05 for chitosan : GA : EDC : NHS. Values are presented as means with standard deviation. Bars with different letters are significantly ($p<0.05$) different.

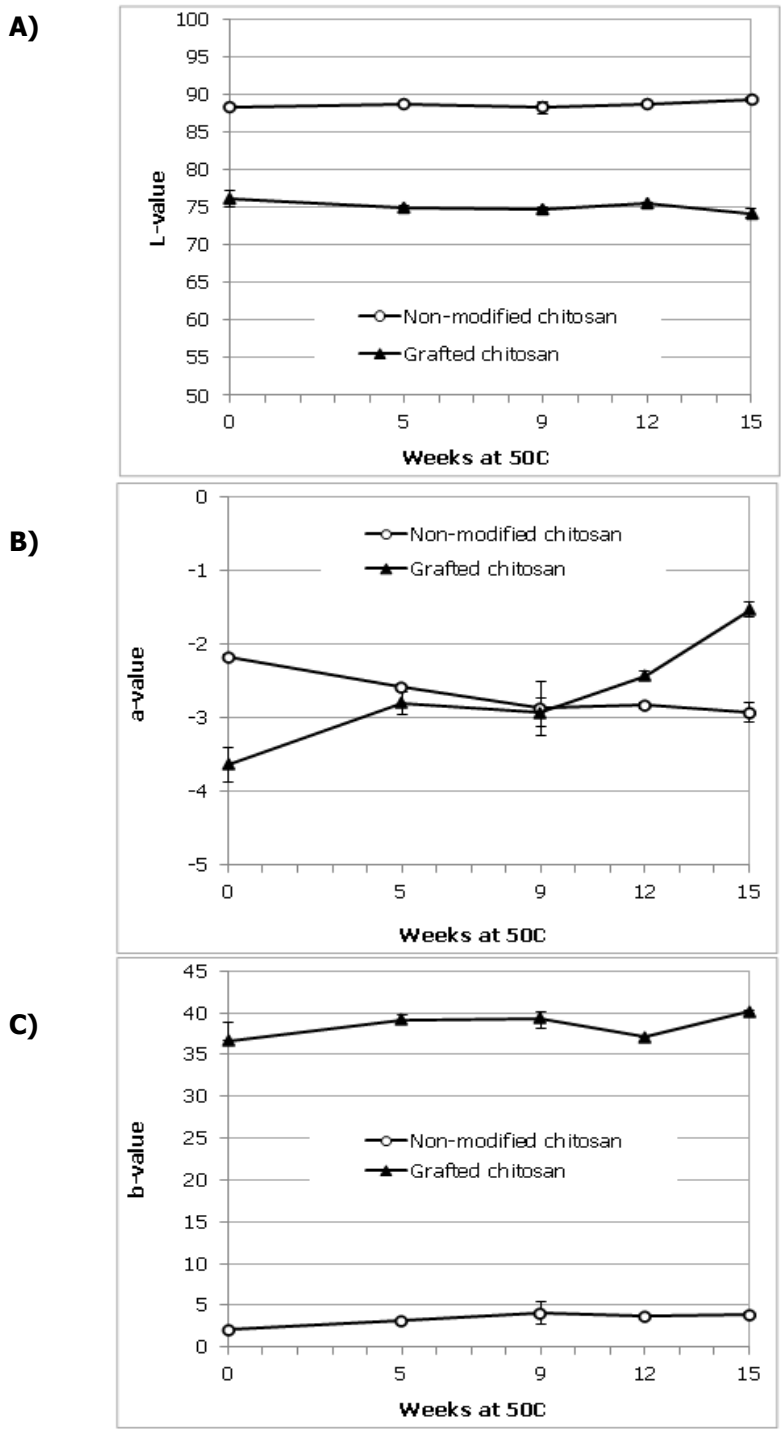
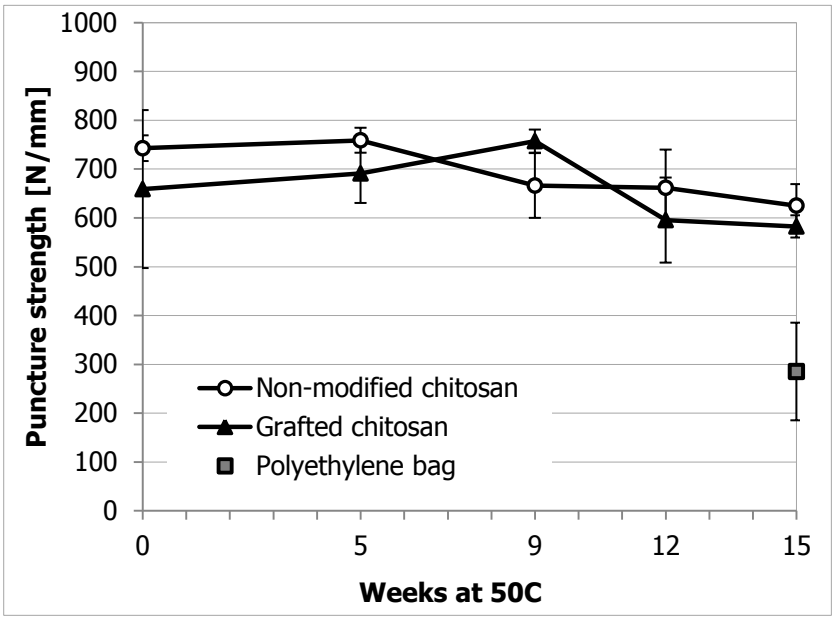
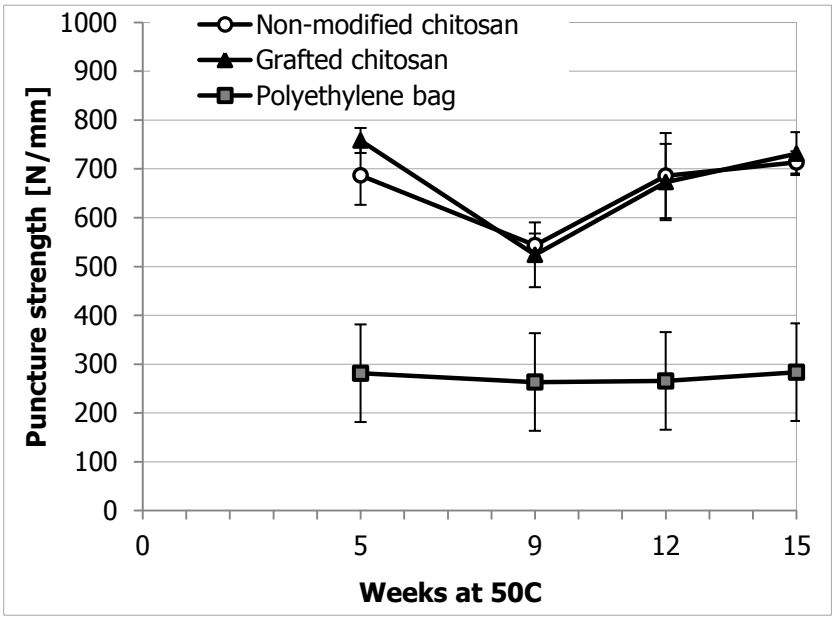


Figure 2. 7: Effect of 50°C incubation on color of the films made with GA-grafted chitosan and non-modified chitosan. A) L-value (lightness), B) a-value (redness), C) b-value (yellowness). Values are presented as means with standard deviation.



A)



B)

Figure 2. 8: Effect of 50 °C incubation on puncture strength of A) films (GA-grafted chitosan film, non-modified chitosan film, polyethylene bag), and B) pouches (package material tested after removal of ground peanuts stored in the pouches). Values are presented as means with standard deviation.

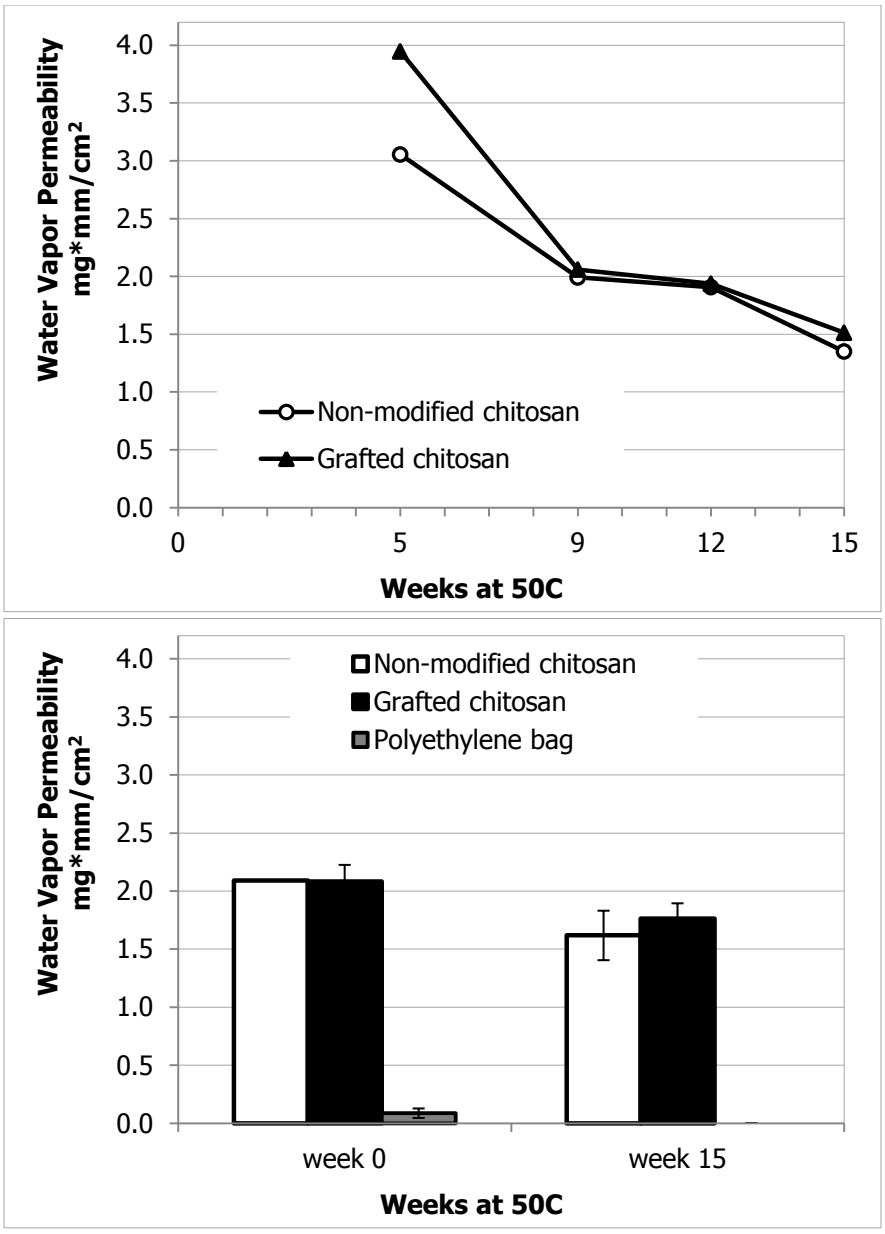
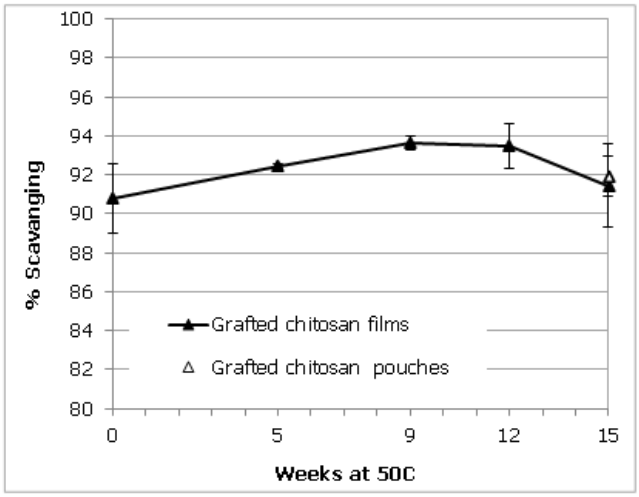
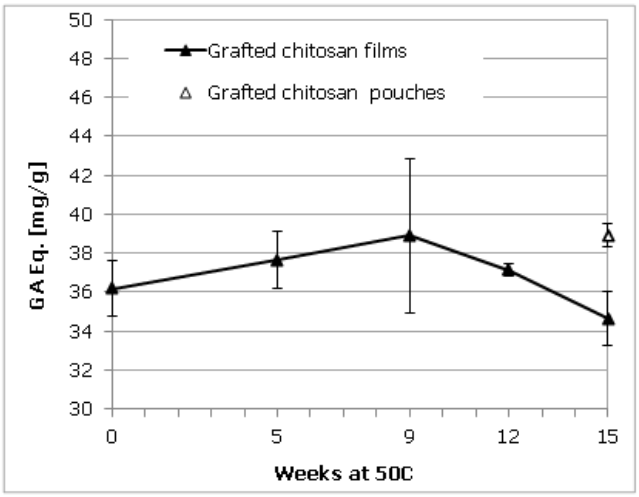


Figure 2. 9: Effect of 50 °C incubation on water vapor permeability of A) films (GA-grafted chitosan film, non-modified chitosan film, polyethylene bag), and B) pouches (package material tested after removal of ground peanuts stored in the pouches). Values are presented as means with standard deviation. Permeability was measured over a 24 hr period.

A)



B)



C)

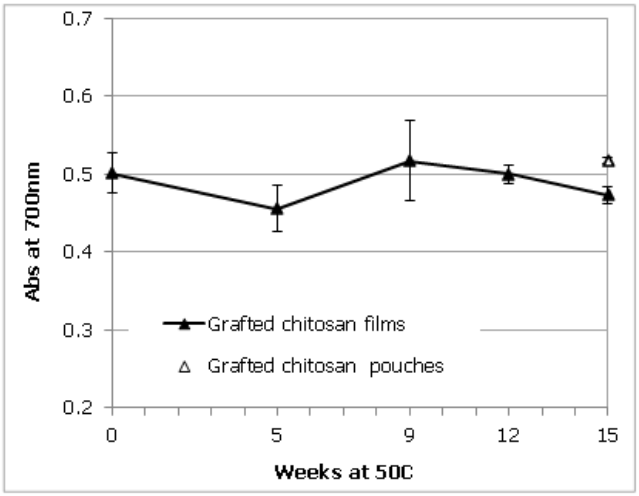


Figure 2. 10: Effect of 50 °C incubation on A) DPPH scavenging (% Scavenging), B) Total Phenolics (GA Eq. [mg/g]), and C) Reducing Power (Abs at 700 nm) of grafted films and pouches. Values are presented as means with standard deviation.

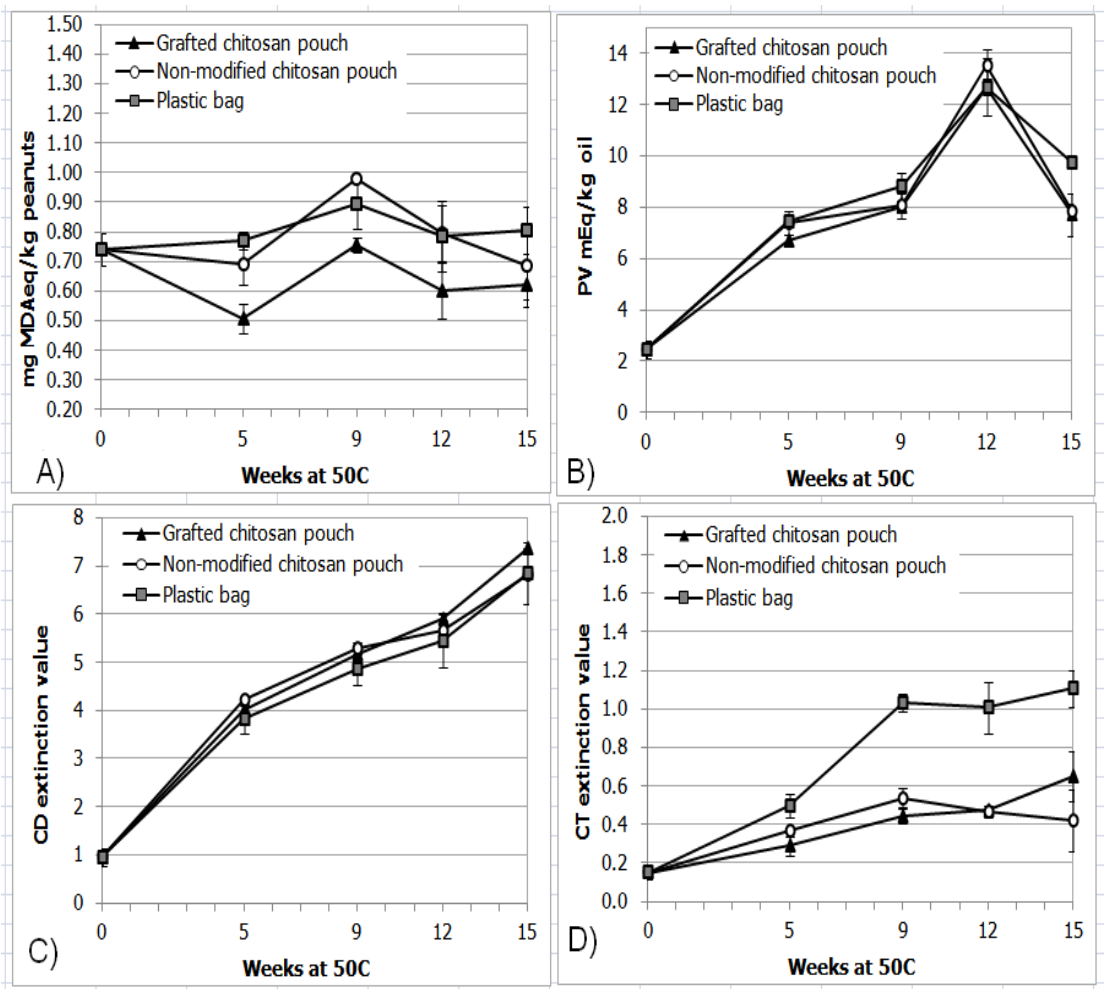


Figure 2. 11: Effect of packaging on quality of ground peanuts during 15 weeks storage at 50°C. A) TBA Reactive Substances, B) PV, C) Conjugated Diene formation, and D) Conjugated Triene formation. Values are presented as means with standard deviation.

CHAPTER 3
CHITOSAN-GALLIC ACID FILMS AS MULTIFUNCTIONAL POTATO CHIP PACKAGING

This chapter is a lightly revised version of a paper by the same title to be submitted to the *Journal of Food Science* by Stephanie Schreiber and Svetlana Zivanovic

Abstract

Potato chips are very susceptible to lipid oxidation because their preparation via deep-frying process. It is necessary for packaging to reduce lipid oxidation because direct addition of antioxidants to the oil seems to be inefficient. The objective of this study was to evaluate grafted chitosan-gallic acid pouches as packing material to reduce oxidation in potato chips. The grafted films/pouches were also evaluated for physical characteristics, antioxidant and antibacterial properties. The grafted films/pouches were compared to material prepared from mixed chitosan and gallic acid, non-modified chitosan and Ziploc® bags. Grafted chitosan pouches significantly reduced peroxide value compared to the Ziploc® pouches throughout the entire 8 week storage period at 50°C. Both grafted and mixed chitosan films had lower water permeability than non-modified films. The grafted and mixed chitosan films both yielded a 2-log reduction of *Salmonella* Typhimurium. However compared to non-modified chitosan films both had significantly lower antibacterial property most likely due to less available amine groups. The chitosan films/pouches show promise as potato chip packaging.

Introduction

Potato chips are a very common deep fried snack product and are relished all over the world (Vijayalakshmi et al., 2009). They undergo the cooking method of deep-frying which is a process involving heat and mass transfer whereby water is evaporated from the food and partially replaced by oil (Man et al., 1999). Because of the cooking process, potato chips contain a high percentage of oil and consequently oxidation is a major concern which results in both off-flavors and odors (Quast and Karel, 1972). Potato chips are very susceptible to lipid oxidation instigators such as sun light and fluorescent light which chips are frequently displayed under (Kubiak et al., 1982). Because potato chips are such a popular item significant effort has been focused on research to extend their shelf-life. Currently, a common way to reduced oxidation of the product is by adding antioxidants to the frying oil (Gorden and Kourismska, 1995; Lalas and Dourtoglou, 2003; Lolos et al., 1999; Man and Tan, 1999).

Having effective potato chip packaging to inhibit or reduce oxidation once chips are produced and packaged is necessary even if antioxidants have been added to the oil because antioxidants are easily lost and degraded during the frying process (Augustin and Berry, 1983; Man and Tan, 1999). In Man and Tan's research, a significant decrease of malonaldehyde development in potato chips was detected when fried in antioxidant enriched oil compared to those fried in oil without added antioxidants (Man and Tan, 1999). However, the chips still had a significant increase in malonaldehyde production at every 2 week testing period

during a 14 week incubation at ambient temperature with the chips packaged in aluminum laminate bags (Man and Tan, 1999). Also, Augustin and Berry evaluated the ability of antioxidants BHT and BHA to reduce lipid oxidation in palm olein that was maintained at 108°C 4 hr/day for two consecutive days while frying potato chips hourly (Augustin and Berry, 1983). They evaluated the oil for antioxidant loss and found that the concentration of BHT and BHA in the frying oil decreased by 86% and >90% respectively at the end of the two day experiment. Augustin and Berry also found that BHT and BHA were relatively ineffective at retarding oxidation in the final product (Augustin and Berry, 1983). Therefore optimizing the packaging is critical because it is impossible to completely prevent deterioration of the oil or antioxidants added to the oil.

Potato chips are currently packed are packed under nitrogen in multilayered polymeric films characterized by low permeability to oxygen and water (Del Nobile, 2001). However, once the potato chip bag is open the nitrogen gas is dispersed and oxygen enters. Having a packaging with primary antioxidant activity would increase shelf life of a product even after the bag has been opened. Chitosan grafted with gallic acid films could be advantageous as potato chip packaging. Chitosan is a biodegradable, non-toxic, bio-active, film-forming polysaccharide with ion adsorbing and antimicrobial properties. Because of chitosan's primary amino group, phenolic compounds can be grafted to it. In our previous study, we grafted gallic acid to chitosan via amide bonding using 1-ethyl-3-(3-dimethylaminopropyl) carbodimmide (EDC) and N-hydroxysuccinimide

(NHS) and used the films made from the grafted chitosan as packaging to reduce lipid oxidation in peanut powder (Schreiber et al., 2011). We found that the chitosan-gallic acid films significantly reduced malondialdehyde production in ground peanuts compared to non-modified chitosan pouches and polyethylene bags during 12-week storage at 50°C and RH of 20-30%. The chitosan- gallic acid films also significantly reduced peroxide value of ground peanuts compared to non- modified chitosan films and polyethylene bags after 5 and 15 weeks of storage, respectively (Schreiber et al., 2011). Additionally, the peanut powder in the polyethylene bags showed a significantly higher formation of conjugated trienes after 15 weeks compared to the chitosan-gallic acid films. Therefore, we are proposing that these films can have a similar effect as potato chip packaging. Objectives of the work presented here were to 1) evaluate chitosan-gallic acid as packaging for reducing lipid oxidation in potato chips and 2) to evaluate chitosan-gallic acid packaging for physical, antioxidant and antibacterial properties.

Materials and Methods

Reagents and Chemicals. Reagents used for grafting were 1-ethyl-3-(3-dimethylaminopropyl) carbodimide (EDC; 99.8% purity; CHEM-IMPEX, Wood Dale, IL) and N-hydroxysuccinimide (NHS; 98+% purity; Acros Organics, Gee, Belgium). Chitosan used in the experiments was 307 kDa chitosan with 80% DDA (as determined in our lab), kindly donated by Primex, Iceland. Russet potatoes, peanut oil and Ziploc® bags were purchased from a local grocery store.

All spectrophotometric **assays** were carried out on a UV-2101PC, Shimadzu (Columbia, MD). Relative humidity was measured with a hydrometer (Thermo Fisher Scientific, Waltham, MA). **Color, Puncture strength and Water Vapor Permeability** were measured in at least duplicate but normally triplicate. Color was recorded using a using a Hunter Lab Miniscan XE *Plus* colorimeter (Hunter Associates Laboratory, Reston, VA) standardized with white and black tiles. Puncture strength was measured with a TA.XT *plus* Texture Analyzer using the TA-108S fixture and a 2 mm-diameter needle probe moving with a test speed of 1 mm/s (TA-52; Texture Technologies Corp., Scarsdale, NY/Stable Micro Systems, Godalming, Surrey, UK). Water vapor permeability (WVP) was measured using Fisher/Payne Permeability Cups (Thermo Fisher Scientific, Waltham, MA) and reported as mg of water that permeated a 1 mm thickness of 1 cm^2 of the film over a 24-hour period at room temperature (Fisher Scientific, 1984).

Grafting phenolics to chitosan. Grafting gallic acid to chitosan was conducted following procedure modified from Pasanphan and Chirachanchai (2008) procedure (Fig. 1) as described in Schreiber et al., (2011). In short, 0.5 g gallic acid (GA) (3 mmol), 0.03 g EDC (0.15 mmol), and 0.017 g NHS (0.15 mmol) were dissolved in 20 mL 70% ethanol. The solution was stirred in an ice bath and after 1 hr was added to 0.16 g of purified chitosan dispersed in 30 mL 70% ethanol. The solution was further stirred in an ice bath for 30 min followed

by stirring at room temperature for 24 hrs. The product was filtered through Whatman #4 filter paper, and washed 5 times by stirring for 45 min with 250 mL 70% ethanol. The final product was freeze-dried (VitTiz advantage Plus, Stone Ridge, New York) and analyzed the next day. The starting ratio of the grafting components was a $2 \cdot 10^{-4}$: 1 : 0.05 : 0.05 molar ratio of chitosan : GA : EDC : NHS.

The antioxidant properties of the grafted chitosan were assessed by the DPPH scavenging method to determine their ability at scavenging radicals, total phenolics to determine the amount of gallic acid grafted to the chitosan, and reducing power to measure the electron donating ability. All analyses were done in at least duplicate. For all the antioxidant assays and solubility, 0.1% of chitosan was prepared in 1% acetic acid, except for DPPH scavenging which was 0.01% chitosan in 0.1% acetic acid.

DPPH scavenging. The radical scavenging capacity of the grafted chitosan samples was determined using a DPPH method modified from literature (Saha et al., 2008). A solution of 4 mg 2,2-diphenyl-1- picrylhydrazyl (DPPH) was prepared in 100 mL methanol. Aliquots of of the DPPH solution (1 mL) were added to 1 mL of the sample or D.I. water (control). The samples were stirred for 30 min in the dark at room temperature. The absorbance (A) was read at 517 nm using a spectrophotometer. Samples were analyzed by calculating % scavenging with the formula: % Scavenging= $[(A_{control} - A_{sample})/A_{control}] \cdot 100$.

Total Phenolics. The concentration of the phenolic acid grafted to chitosan was determined by the modified Folin-Ciocalteu method (Folin and Ciocalteu, 1927). Gallic acid standards were prepared in range of 0.01 to 0.1 mg/mL. For each sample, standard, or D.I. water (control), 1 mL-aliquots were added to a test tube followed by 7 mL D.I. water and 1 mL 3.35% Folin-Ciocalteau solution. After 3 min, 1 mL 12.4% sodium carbonate was added and samples were vortexed and put in a water bath for 30 min at 40°C. Absorbance of the samples was read at 725 nm with a spectrophotometer.

Reducing Power. The reducing power was determined using the method reported by Yen and Chen (1995). Phosphate buffer pH=6.6, 0.2M (2.5 mL) was added to 1 mL of chitosan solution. Potassium ferricyanide (2.5 mL 1% solution) was added to the mixture and incubated at 50 °C for 20 min. Trichloroacetic acid (2.5 mL 10% solution) was added and centrifuged for 10 min at 3000 rpm. A, 2.5 mL aliquot of the upper layer was removed and added to a separate tube, followed by 2.5 mL D.I. water and 0.5 mL 0.1% iron chloride. Absorbance was immediately read at 700 nm using a spectrophotometer.

Film Preparation and Analysis. Chitosan film forming solution (FFS) was prepared with 0.7% grafted or non-modified chitosan in 1% acetic acid. Films made of mixed chitosan and gallic acid were prepared by blending 32 mg of gallic acid per gram of chitosan and using this to form a 0.7% solution in 1% acetic acid. The films were cast in 5 and 10 cm-Petri dishes using 10 mL and 26 mL FFS, respectively. Petri dishes were left at room temperature until the films

were dry (easy to peel off), usually 4 – 5 days. The 5 cm- diameter films were used for analysis of physical, antioxidant and antibacterial properties. The larger 10 cm- diameter films were evaluated as packaging for the potato chips.

Preparation of potato chips. Fifteen pounds of Russet potatoes were purchased from the local grocery story. They were washed, peeled, and sliced with a potato slicer to 1/15 inch thickness. The sliced potatoes were immediately placed in cold water to avoid any enzymatic browning. Before frying, the potato slices were drained on a stainless steel screen and dried with a fan. They were fried in a deep fryer in peanut oil until virtually no bubbles were seen, roughly 3 minutes and 45 seconds. After frying, the potato chips were placed on a stainless steel screen covered in absorbent paper towel and lightly shaken to remove excess oil. Once potato chips were cooled, they were ground in a food processer for 30 seconds to increase surface area. The ground potato chips were packaged in grafted chitosan pouches, pouches made from a mixture of gallic acid and chitosan with the ratio found in grafted chitosan, non-modified chitosan pouches, pouches made from Ziploc® bags, and placed in open Petri dishes. The pouches were made by attaching two 10 cm- diameter films or two 10 cm- diameter circular cutouts from Ziploc® bags with adhesive. Each pouch contained 9 g of ground potato chips. The pouches were all placed in a forced air incubator at 50 °C and the humidity was constantly monitored using a hydrometer. The relative humidity during the whole experiment was in the 20 – 30% range. Plain grafted-chitosan, mixed (gallic acid and chitosan), and non-modified chitosan films, (not

used as pouches) were also placed in the incubator. Pouches were analyzed after 1, 3, 6, and 8 weeks. Films were analyzed at the beginning and end of storage (week 0 and week 8). Two pouches (made of two films each) and three films for each packaging type were analyzed at each sampling period**.**

Antibacterial activity of films. Antibacterial activity was carried out using a procedure modified from Zivanovic et al., (2007). Test films were removed from the 50°C forced air over after 8 weeks and peeled off the petri dish they were originally cast on. They were cut into strips and placed into sterile tubes. Sterile phosphate buffer (0.05 M, pH 7.08, 4.5 mL) and 0.5 mL of a 10^{-4} dilution of *Salmonella* Typhimurium DT104, strain 2486, were added to the tube and mixed by vortexing, and incubated with constant shaking on a Vari Mix platform rocker (Thermo Scientific, Barrington, IL) for 6 h at room temperature. The survival of *S.* Typhimurium was determined using the spread-plate method on trypticase soy agar (TSA) medium. All measurements were performed with three replications.

Leaching of gallic acid from grafted and mixed chitosan films. Grafted chitosan films, and films made from a mixture of gallic acid and chitosan representative of the ratio found in grafted chitosan were submerged in 95% ethanol and were agitated on a Labquake, (Barnstead/Thermolyne, Dubuque, IA). for 94 to 96 hours a room temperature. Samples of the ethanol were regularly taken and analyzed for total phenolics.

Determination of **Thiobarbituric Acid Reactive Substances (TBARS), Peroxide Value (PV), and, Conjugated Dienes (CD) in potato chips** were conducted by methods modified from Current Protocols in Food Analytical Chemistry (Pegg, 2001A; Pegg, 2001B). The determination of TBARS was carried out with a color assay using 2-thiobarbituric acid to determine malonaldehyde content and comparing it with a standard curve. PV was determined by a photometric method based on the ability of the lipid peroxides to oxidize ferrous ions. The change in conjugated dienes was assessed by measuring the change in maximum absorbance for a constant mass of sample at the peak (233 nm) associated with conjugated dienes. All measurements were done in duplicate.

Statistical analysis. Tukey HSD comparison of means and Student's t-test were performed using Jump (7.0.2).

Results and Discussion

Effect of Storage on Physical Properties of grafted Packaging

For weeks 3, 6 and 8 of storage the L-values differed for grafted (g), mixed (m), non-modified (nm) and Ziploc® (z) pouches z>nm>m>g indicating grafted pouches were the darkest and Ziploc® were the lightest in color although, at week one, non-modified pouches did not significantly differ in color from Ziploc® (Figure 3.1A). The a-value was not significantly different during weeks 1, 3, 6, and 8 for grafted, mixed or non-modified chitosan pouches however for all 4

testing points the Ziploc® pouches had an a-value significantly higher than all the chitosan pouches (Figure 3.1B). For weeks 1, 3, and 6 the b- value differed for grafted, mixed, non-modified and Ziploc® pouches g>m>nm>z indicating grafted pouches were the most yellow and Ziploc® pouches were the least yellow. However, at week 8, grafted and mixed pouches were similar (Figure 3.1C). Color of each of the four pouches was evaluated over times. Grafted chitosan films did not darken or become more yellow at the end of the storage period compared to the beginning. Mixed pouches did become darker, and more yellow at the end of the 8 week storage compared to the first week. The non- modified pouches became more yellow over time but not darker. The color of Ziploc® pouches stayed unchanged during storage. Overall, grafting gallic acid to chitosan make the films significantly darker, which can possibly be advantageous in reducing the amount of light permeating the packaging. The chitosan films with gallic acid were significantly darker than those without gallic acid probably because of gallic acids sensitivity to light however this did not alter the antioxidant property. The color of chitosan films from literature varied. Fernandez-Saiz and co-authors found that the L, a, and, b values for chitosan film ranged from 93 to 95, -2 to 0, and 0.9 to 18 respectively depending on storage conditions (Fernandez-Saiz et al., 2009). Fernandez-Saiz and co-workers also found that an increase in temperature and an increase in relative humidity resulted in darker and more yellow films. We saw similar results with an increase of time at an already high temperature. Another study found chitosan

films to be much darker with L, a, and, b values being 48, -2.4, and 4.9 respectively (Rao et al., 2010).

The puncture strength and elasticity did not significantly differ between grafted, mixed or non-modified chitosan pouches during the entire 8-week storage at 50°C (Figure 3.2A and 3.2B). However, the puncture strength of the Ziploc® pouches was significantly lower and the elasticity was significantly greater compared to all the chitosan pouches for every testing period. The puncture strength of the grafted pouches did not change over time but the elasticity at week 1 was significantly higher than at all proceeding measurements. The mixed pouches had significantly higher puncture strength at week 8 than week 1, and the elasticity at week 1 was higher than at all following weeks. The puncture strength of the non- modified pouches did not significantly change over time but the elasticity at week 8 was lower than at week 1. The puncture strength and elasticity of the Ziploc® pouches did not significantly change over the entire storage period. The puncture strength of all the chitosan films were high and ranged from ≈600 to ≈1000 N/mm. Zivanovic and co-authors found the puncture strength of 1% chitosan (150 kDa) films with to be 416± 24.7 N/mm (Zivanovic et al., 2007). Butler and co-authors found that % elongation decreases with storage time which, is similar to our results of elasticity decreasing with storage time (Butler et al., 1996).

Water vapor permeability (WVP) was not significantly different for grafted or mixed chitosan films however, both had significantly less water permeability

than the non- modified chitosan films (Figure 3.3). The values were 0.77, 0.70 and 0.99 mg*mm/cm^2 for grafted, mixed and non-modified films respectively. Therefore grafting or mixing gallic acid with chitosan may enhance the films ability to reduce WVP. The lower WVP of the grafted films is probably due to the covalent interaction between chitosan and the gallic acid. Siripatrawan and Harte stated that hydrogen and covalent interactions between the chitosan network and other compounds can decrease WVP due to the limiting availability of free hydrogen containing groups to form hydrophilic bonding with water, resulting in chitosan's decreased affinity towards water (Siripatrawan and Harte, 2010). This is consistent with Zivanovic et al., (2007) indicating that the main factor in water vapor permeability is diffusivity of water molecules through the film matrix. Intermolecular interaction can also be the reason that mixed chitosan and gallic acid films showed a decrease in WVP because Siripatrawan and co-authors saw this effect when they incorporated green tea extracts with chitosan without grafting (Siripatrawan and Harte, 2010).

Effect of Storage on Antioxidant Activity of Chitosan- Gallic Acid Packaging

Antioxidant properties (DPPH scavenging, phenolics and reducing power) of grafted and mixed chitosan films during the 8-week storage at 50 °C are shown in Figure 3.4. There was no significant reduction in % DPPH scavenging, GAEq (mg/g) or reducing power (Abs700 nm) over the storage period for grafted and mixed pouches and films. There were some significant differences according to statistics between mixed and grafted pouches/films as well as each pouch/film

over time however, these differences were very small and of no consequence. In addition, the antioxidant activity of the films used in the pouches with the potato chips was similar to the activity of the films alone. This shows that the gallic acid providing the primary antioxidant activity to the films did not leach out of the pouches into the packaged product.

Effect of Storage on Antimicrobial Properties of grafted Packaging

The antimicrobial effect of the packaging would be very beneficial for a variety of food products. The antibacterial properties of the grafted films were compared to mixed (gallic acid and chitosan) and non-modified chitosan films. The log reduction of the *S.* Typhimurium by the grafted, mixed and non-modified films were 1.87, 2.12 and 2.88 respectively (Figure 3.5). The log reduction by the grafted and mixed films were not statistically different from each other but were both statistically lower than the non-modified films. The possible explanation for the reduction in the grafted film's antibacterial activity is that some of its amino groups have formed an amide bond with gallic acid making them unable to act as antimicrobials.

The speculated reasoning for the reduced antimicrobial activity of the films made of a mixture of chitosan and gallic acid is that when the films were formed in an aqueous acetic acid solution the chitosan was protonated and underwent ionic interaction with the de-protonated carboxylic end of gallic acid. The pK_a for the gallic acid's carboxyl group is about 4.4 and the pK_a of chitosan is 6.3. The pH of the chitosan film forming solution was 3.81 (0.7% chitosan in 1% acetic

acid), at which some gallic acid carboxyl groups are partially de-protonated. The gallic acid may be partially inhibiting the chitosan's amino group from acting as an antimicrobial. It has been shown that the anionic carboxyl group from collagen may be capable of forming ionic interactions with the $–NH_3^+$ of chitosan (Sionkowka et al., 2004; Taravel and Domard, 1993). We can assume that the –COOH group of gallic acid have a similar interaction with the $–NH_3^+$ of chitosan. In this proposed situation, the mixed chitosan and gallic acid are acting similar to the grafted grafted. This was also seen when we washed grafted films and mixed chitosan and gallic acid films were washed in 95% ethanol over a 94-96 hour period (Figure 3.6). The amount of gallic acid removed from grafted and mixed films were 0.5 and 0.44 mg of gallic acid per film respectively. This shows that only a small portion, around 13%, of the mixed gallic acid is actually leaching out and the majority is somehow incorporated with the chitosan film.

Phenolic compounds are known antimicrobials and their hydroxyl groups are crucial to antibacterial activity (Puupponen-Pimia et al., 2001; Dorman and Deans, 2000; Ultee et al., 2002). The hydroxyl groups of gallic acid are not hindered by either grafted grafted or mixed chitosan and gallic acid films. However phenolic compounds need to be able to permeate the bacterial cell membrane to cause destruction and in this experiment gallic acid was either grafted or bound to chitosan by ionic interaction (Di Pasqua et al., 2007; Fogg and Lodge, 1945). Also, the antimicrobial activity of simple phenolics such as gallic acid has been debated. For example, Chanwitheesuk and co- authors

showed gallic acid exhibited activity against the bacteria *S. typhi* and *S. aureus* with the minimum inhibitory concentration (MIC) values of 2500 and 1250 µg/ml, respectively (Chanwitheesuk et al., 2007). However, study by Rauha and co-authors provided evidence that simple phenolic compounds such as caffeic, gallic and procathecuic acids have antibacterial activity that is restricted to only a few bacterial strains (Rauha et al., 2000).

Effect of grafted Packaging on Lipid Oxidation of Potato Chips

The quality of the potato chips was evaluated based on TBARS, PV, and conjugated diene formation (Figure 3.7). After week 1 of storage there were no significant differences in malondialdehyde production between the potato chips stored in grafted, mixed, non-modified and, Ziploc® pouches or potato chips kept in an open petri dish. After three weeks of incubation at 50°C the Tukey test showed no significant differences for malondialdehyde production but Student's t-test showed that the p-values of the chips kept in grafted, mixed, non-modified and Ziploc® pouches compared to chips stored in open petri dishes were 0.058, 0.054, 0.083 and 0.305, respectively. At weeks 6 and 8 the potato chips in the open petri dishes were significantly higher in malondialdehyde production than the potato chips in any of the pouches. Also, potato chips in the open petri dishes did not significantly increase in malondialdehyde production until week 6 as compared to the early weeks. The lack of sensitivity of the TBARS test was also noted by Kuibak et al., (1982) who stated that the TBA numbers for potato chips were not indicative of the development of oxidized flavors.

PV is a more sensitive test than TBARS and therefore changes in oxidation of the potato chips are more easily seen. After 1 week of storage there was significant difference between all packaged potato chips compared to open except for the chips in Ziploc® pouches. The PV for potato chips in grafted and mixed pouches were significantly lower than the chips packaged in Ziploc® pouches but, chips in non- modified chitosan pouches were not significantly different from Ziploc® bags. At week 3, through the remainder of the storage, the PV value of the potato chips stored in an open petri dish was so much higher than all packaged chips that they were excluded from statistical analysis. At week 3, the Tukey test showed that only the chips packaged in grafted pouches were significantly lower in PV value from those packaged in Ziploc® pouches however, Student's t-test showed that all other packaged chips had a significantly lower PV value than chips in Ziploc® pouches. At week 6, the Tukey test showed no significant differences; however, Student's t-test showed the p-values of chips packed in grafted, mixed and non-modified pouches compared to Ziploc® pouches were 0.047, 0.033 and 0.034, respectively. At week 8, the final week of analysis, the Tukey test showed the PV of the chips packaged in Ziploc® bags were significantly higher than all other chips except for those in mixed pouches; however Student's t test showed the p-value was 0.017 for the relationship between chips in mixed pouches to those in Ziploc® pouches. When the potato chips packed in pouches were compared overtime, the PV value was significantly higher at the end of the storage period compared to weeks 0, 1, and 3 for grafted

pouches. The PV value was significantly higher after the 8 week storage period than at any other week for mixed pouches. For non-modfied pouches the PV value over time increased 8 weeks>6 weeks>3 weeks >1 week. For Ziploc® pouches the PV value at week 8 was significantly higher than at all other weeks except for at week 6. Min and Schweizer, (1983) conducted a study in similar conditions in which they also used ground potato chips, a forced air oven and a similar temperature (55°C). The potato chips from this study were packed in an airtight serum bottle and after 27 days at 55°C of storage they had a PV of 34.9 (Min and Schweizer, 1983). From our study we found that the PV value of the potato chips packaged in grafted chitosan films was 35.8, after a much longer incubation period of 56 days.

Measurement of conjugated dienes (CD) was another assay used to evaluate lipid oxidation. At week 1, the potato chips in the open petri dish were already significantly higher in CDs than the packaged chips. After week one the potato chips in the open petri dishes were not included in statistical analysis because their conjugated diene content was so much higher that it would negate any other significant differences from being seen. At weeks 3 and 6, there were no significant differences among the packaged potato chips. At week 8, the potato chips packaged in the Ziploc® pouches had a significantly higher conjugated diene extinction value than all other packaged chips except for mixed pouches. According to the Tukey test, the CD extinction value of potato chips packaged in both grafted and mixed pouches had no significant changes over

time. The CD extinction value of the potato chips packaged in Ziploc® pouches were significantly higher at week 8 than at weeks 0, 1 and 3. The potato chips in the open petri dish significantly increased at every testing point except for at week 1, which was not significantly different from week 0.

Conclusions

Grafted pouches were significantly darker than mixed chitosan and gallic acid pouches, non-modified chitosan pouches and Ziploc® bags. Grafted chitosan films were significantly more yellow than all other packaging for 6 weeks of storage at 50°C, but at week 8 mixed pouches were not significantly different from grafted pouches. The puncture strength of the grafted, mixed and non-modified chitosan films did not differ from each other and were all significantly stronger than Ziploc® pouches. All chitosan films had a higher elasticity at week 1 than at the end of storage (week 8). Grafted and mixed chitosan films had significantly lower water vapor permeability than non- modified chitosan films. The antioxidant activity did not decrease in grafted and mixed pouches/films throughout the 8 week 50°C storage period. Grafted and mixed chitosan films showed a 1.87 and 2.12 log reduction, respectively of *Salmonella* Typhimurium which was significantly lower than non-modified chitosan which reduced *S.* Typhimurium 2.88 logs. Grafted chitosan films significantly reduced PV and conjugated diene formation compared to Ziploc® bags.

References

Augustin, M. A., & Berry, S. K. (1983). Efficacy of the anti-oxidant bha and bht in palm olein during heating and frying. *Journal of the American Oil Chemists Society, 60*(8), 1520-1523.

Butler, B. L., Vergano, P. J., Testin, R. F., Bunn, J. M., & Wiles, J. L. (1996). Mechanical and barrier properties of edible chitosan films as affected by composition and storage. *Journal of Food Science, 61*(5), 953-&.

Chanwitheesuk, A., Teerawutgulrag, A., Kilburn, J. D., & Rakariyatham, N. (2007). Antimicrobial gallic acid from Caesalpinia mimosoides Lamk. *Food Chemistry, 100*(3), 1044-1048.

Del Nobile, M. (2001). Packaging design for potato chips. *Journal of Food Engineering, 47*(3), 211-215.

Di Pasqua, R., Betts, G., Hoskins, N., Edwards, M., Ercolini, D., & Mauriello, G. (2007). Membrane toxicity of antimicrobial compounds from essential oils. *Journal of Agricultural and Food Chemistry, 55*(12), 4863-4870.

Dorman, H., & Deans, S. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology, 88*(2), 308-316.

Fernandez-Saiz, P., Lagaron, J., & Ocio, M. (2009). Optimization of the Film-Forming and Storage Conditions of Chitosan as an Antimicrobial Agent. *Journal of Agricultural and Food Chemistry, 57*(8), 3298-3307.

Fogg, A. H., & Lodge, R. M. (1945). The mode of antibacterial action of phenols in relation to drug-fastness. *Transactions of the Faraday Society, 41*(7), 359-365.

Folin, O., & Ciocalteu, V. (1927). On tyrosine and tryptophane determinations in proteins. *Journal of Biological Chemistry, 73*(2), 627-650.

Gordon, M. H., & Kourimska, L. (1995). The effects of antioxidants on changes in oils during heating and deep frying. *Journal of the Science of Food and Agriculture, 68*(3), 347-353.

Kubiak, C. L., Austin, J. A., & Lindsay, R. C. (1982). Influence of package construction on stability of potato-chips exposed to fluorescent lighting. *Journal of Food Protection, 45*(9), 801-805.

Lalas, S., & Dourtoglou, V. (2003). Use of rosemary extract in preventing oxidation during deep-fat frying of potato chips. *Journal of the American Oil Chemists Society, 80*(6), 579-583.

Lolos, M., Oreopoulou, V., & Tzia, C. (1999). Oxidative stability of potato chips: effect of frying oil type, temperature and antioxidants. *Journal of the Science of Food and Agriculture, 79*(11), 1524-1528.

Man, Y. B. C., Liu, J. L., Rahman, R. A., & Jamilah, B. (1999). Shelf-life of fried potato chips using RBD palm olein, soybean oil and their blends. *Journal of Food Lipids, 6*(4), 287-298

Man, Y., & Tan, C. (1999). Effects of natural and synthetic antioxidants on

changes in refined, bleached, and deodorized palm olein during deep-fat frying of potato chips. *Journal of the American Oil Chemists Society, 76*(3), 331-339.
Min, D., & Schweizer, D. (1983). Lipid oxidation in potato-chips. *Journal of the American Oil Chemists Society, 60*(9), 1662-1665.
Pasanphan, W., & Chirachanchai, S. (2008). Conjugation of gallic acid onto chitosan: An approach for green and water-based antioxidant. *Carbohydrate Polymers, 72*(1), 169-177.
Pegg, R. (2001). Measurement of primary lipid oxidation products. *Current protocols in food analytical chemistry*, D2.1.1-D2.1.15.
Pegg, R. (2001). Spectrophotometric measurement of secondary lipid oxidation products. *Current protocols in food analytical chemistry*, D2.4.1-D2.4.18.
Puupponen-Pimia, R., Nohynek, L., Meier, C., Kahkonen, M., Heinonen, M., Hopia, A., & Oksman-Caldentey, K. (2001). Antimicrobial properties of phenolic compounds from berries. *Journal of Applied Microbiology, 90*(4), 494-507.
Quast, D. G., & Karel, M. (1972). Effects of environmental factors on oxidation of potato chips. *Journal of Food Science, 37*(4), 584-&.
Rao, M., Kanatt, S., Chawla, S., & Sharma, A. (2010). Chitosan and guar gum composite films: Preparation, physical, mechanical and antimicrobial properties. *Carbohydrate Polymers, 82*(4), 1243-1247.
Rauha, J., Remes, S., Heinonen, M., Hopia, A., Kahkonen, M., Kujala, T., Pihlaja, K., Vuorela, H., & Vuorela, P. (2000). Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *International Journal of Food Microbiology, 56*(1), 3-12.
Saha, M. R., Hasan, S. M. R., Akter, R., Hossain, M. M., Alam, M. S., Alam, M. A., & Mazumder, M. E. H. (2008). *In vitro* free radical scavenging activity of methanol extract of the leaves of *mimusops elengi linn*. *Bangladesh Journal of Veterinary Medicine, 6*(2), 197-202.
Schreiber, S., Bozell, J., Hayes, D., & Zivanovic, S. (Submitted). Chitosan-gallic acid films as multifunctional food packaging. *Journal of Agricultural and Food Chemistry*.
Scientific., F. (1984). Fisher/Payne permeability cup instructions. In Fifth ed.).
Sionkowka, A., Wisniewski, M., Skopinska, J., Kennedy, C. J., & Wess, T. J. (2004). Molecular interactions in collagen and chitosan blends. *Biomaterials, 25*(5), 795-801.
Siripatrawan, U., & Harte, B. (2010). Physical properties and antioxidant activity of an active film from chitosan incorporated with green tea extract. *Food Hydrocolloids, 24*(8), 770-775.
Taravel, M., & Domard, A. (1993). Relation between the physicochemical characteristics of collagen and its interactions with chitosan .1. *Biomaterials, 14*(12), 930-938.
Ultee, A., Bennik, M. H. J., & Moezelaar, R. (2002). The phenolic hydroxyl group

of carvacrol is essential for action against the food-borne pathogen Bacillus cereus. *Applied and Environmental Microbiology, 68*(4), 1561-1568.

Vijayalakshmi, N. S., Indiramma, A. R., Rangarao, G. C. P., & Ramesh, B. S. (2009). Package design for chips based on their deteriorative characteristics, size and permeability of packages. *Journal of Food Science and Technology-Mysore, 46*(5), 450-454.

Yen, G. C., & Chen, H. Y. (1995). ANTIOXIDANT ACTIVITY OF VARIOUS TEA EXTRACTS IN RELATION TO THEIR ANTIMUTAGENICITY. *Journal of Agricultural and Food Chemistry, 43*(1), 27-32.

Zivanovic, S., Li, J., Davidson, P., & Kit, K. (2007). Physical, mechanical, and antibacterial properties of chitosan/PEO blend films. *Biomacromolecules, 8*(5), 1505-1510.

Appendix: Chapter 3

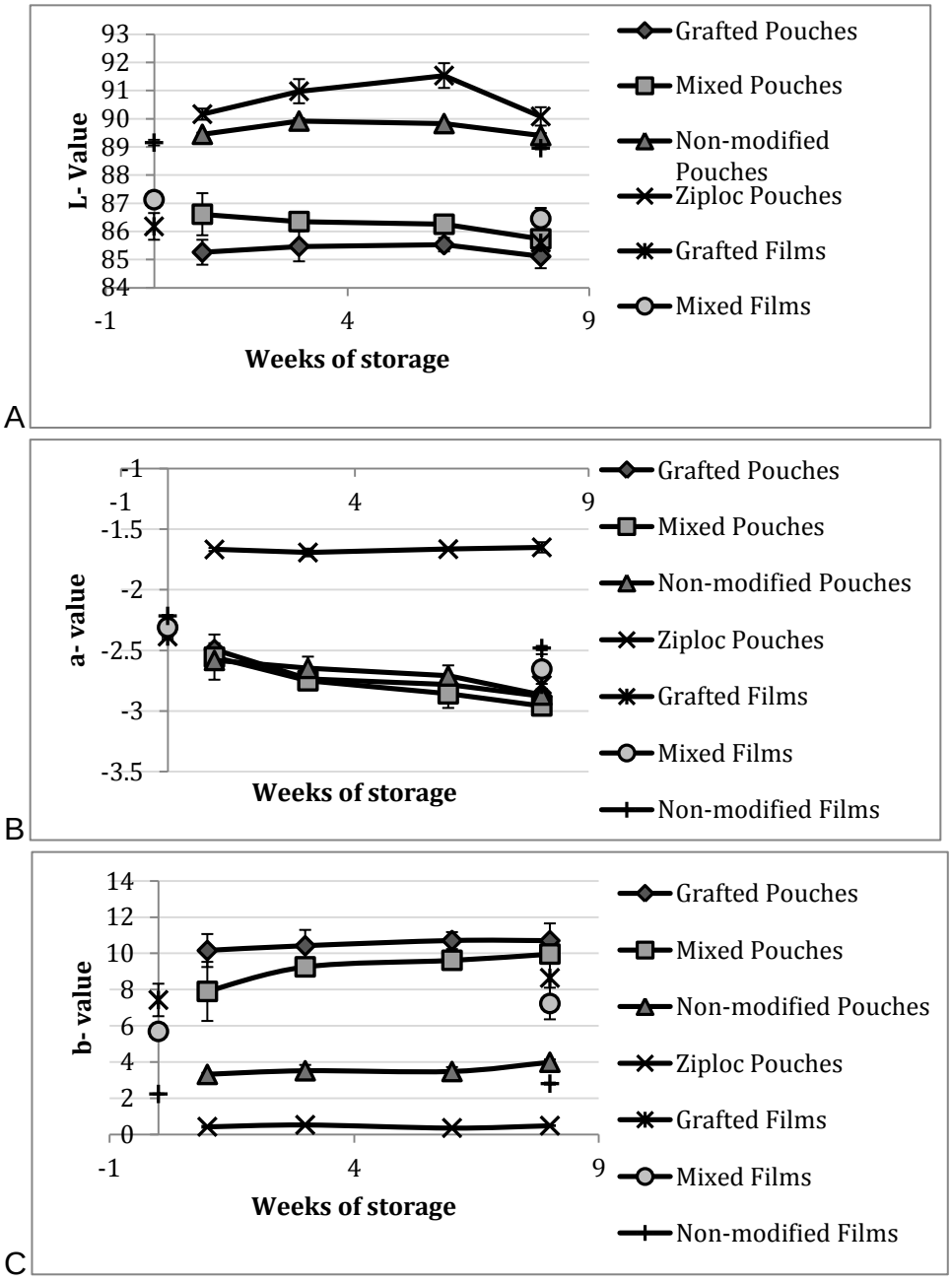


Figure 3. 1: Effect of 50°C storage on color of the pouches and films made with GA-grafted chitosan, mixed gallic acid and chitosan, non-modified chitosan and Ziploc® bag. A) L-value (lightness), B) a-value (redness), C) b-value (yellowness). Values are presented as means with standard deviation.

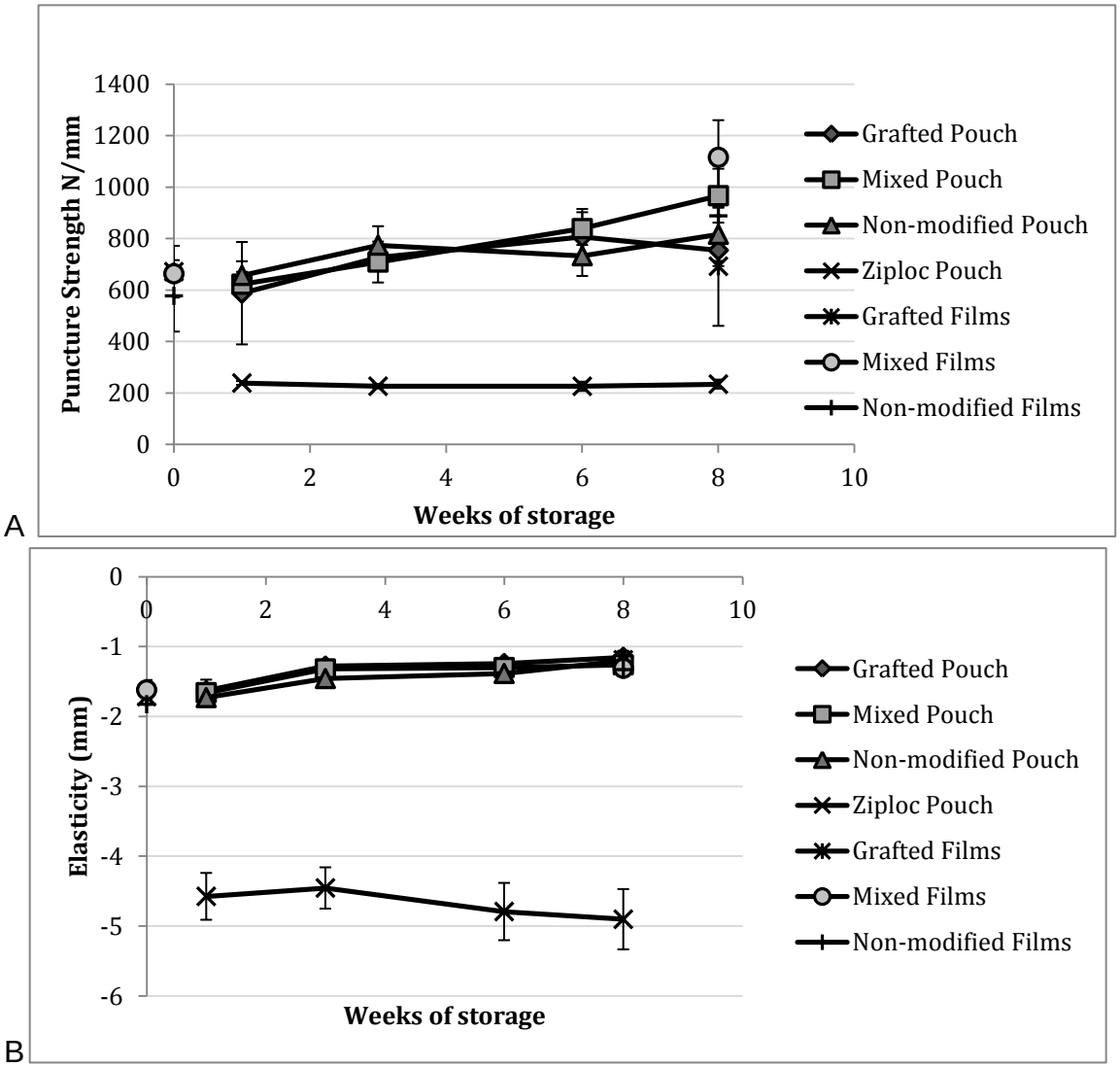


Figure 3. 2: Effect of 50°C storage on A) puncture and B) elasticity of the pouches and films made with GA-grafted chitosan, mixed gallic acid and chitosan, non-modified chitosan and Ziploc® bag. Values are presented as means with standard deviation.

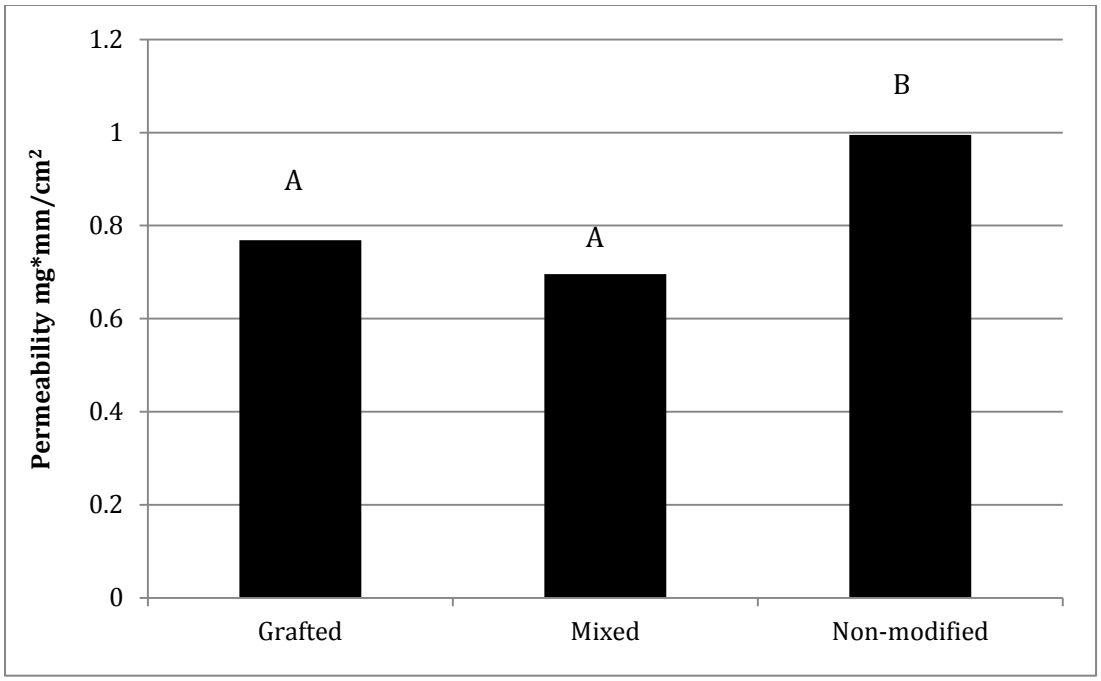


Figure 3. 3: Effect of 50°C storage on water vapor permeability of the pouches and films made with GA-grafted chitosan, mixed gallic acid and chitosan, and, non-modified chitosan. Values are presented as means with standard deviation. Bars with different letters are significantly ($p<0.05$) different over time.

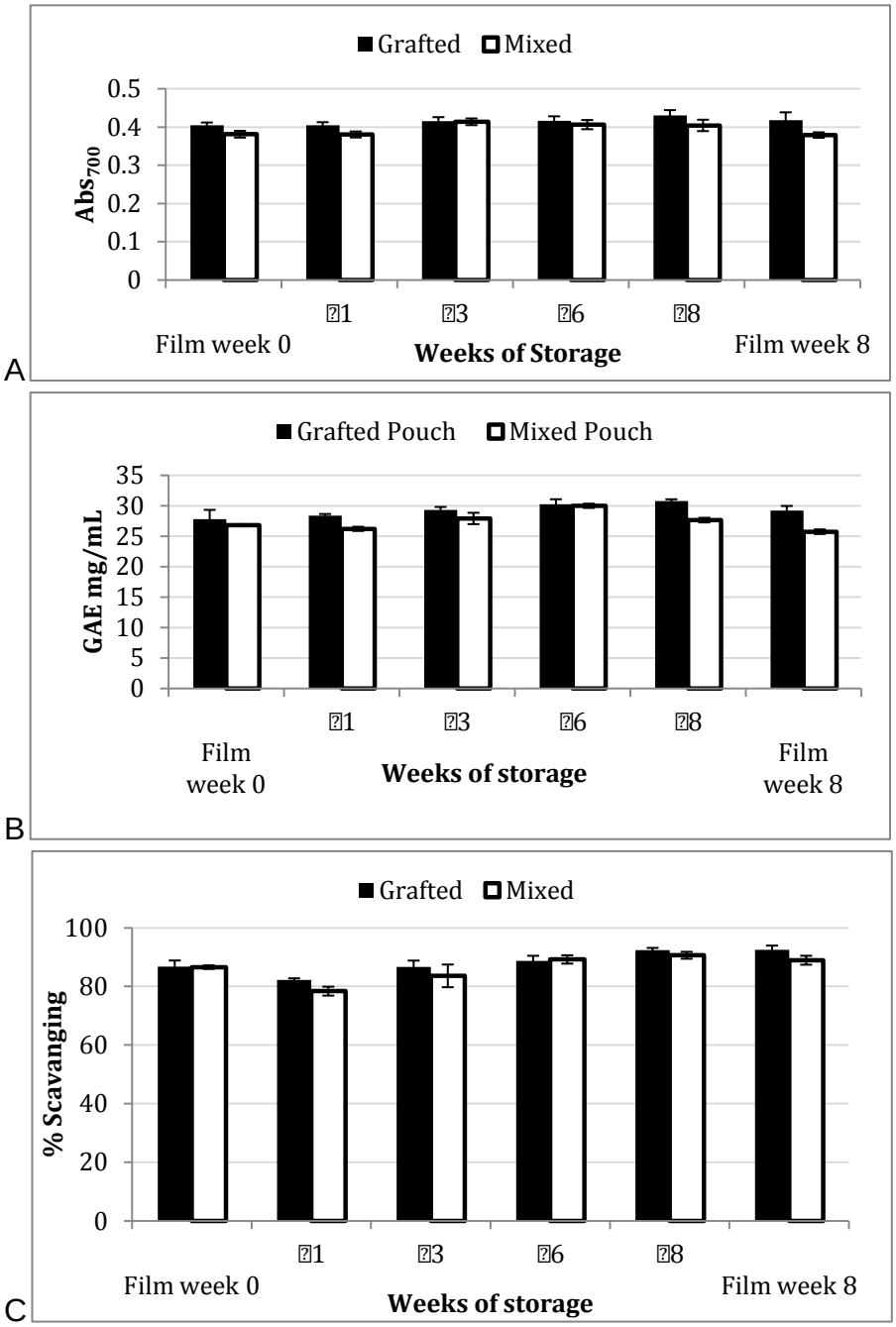


Figure 3. 4: Effect of 50°C incubation on A) DPPH scavenging (% Scavenging), B) Total Phenolics (GA Eq. [mg/g]), and C) Reducing Power (Abs at 700 nm) of grafted films and pouches and mixed films and pouches. Values are presented as means with standard deviations.

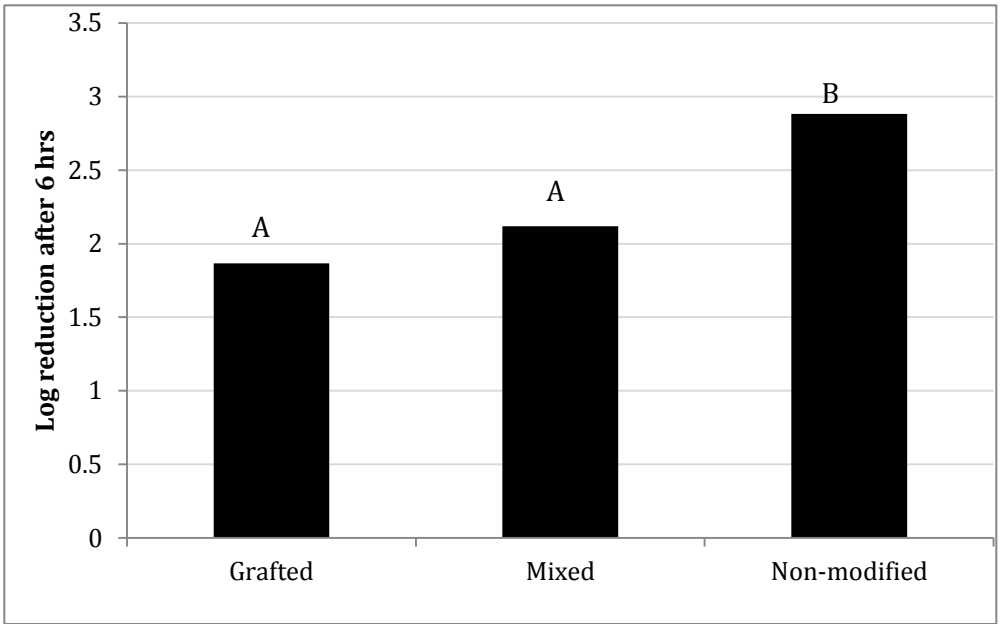


Figure 3. 5: Effect of grafted, mixed, and non-modified chitosan films on log reduction of *S*. Typhimurium. Values are presented as means. Bars with different letters are significantly ($p<0.05$) different.

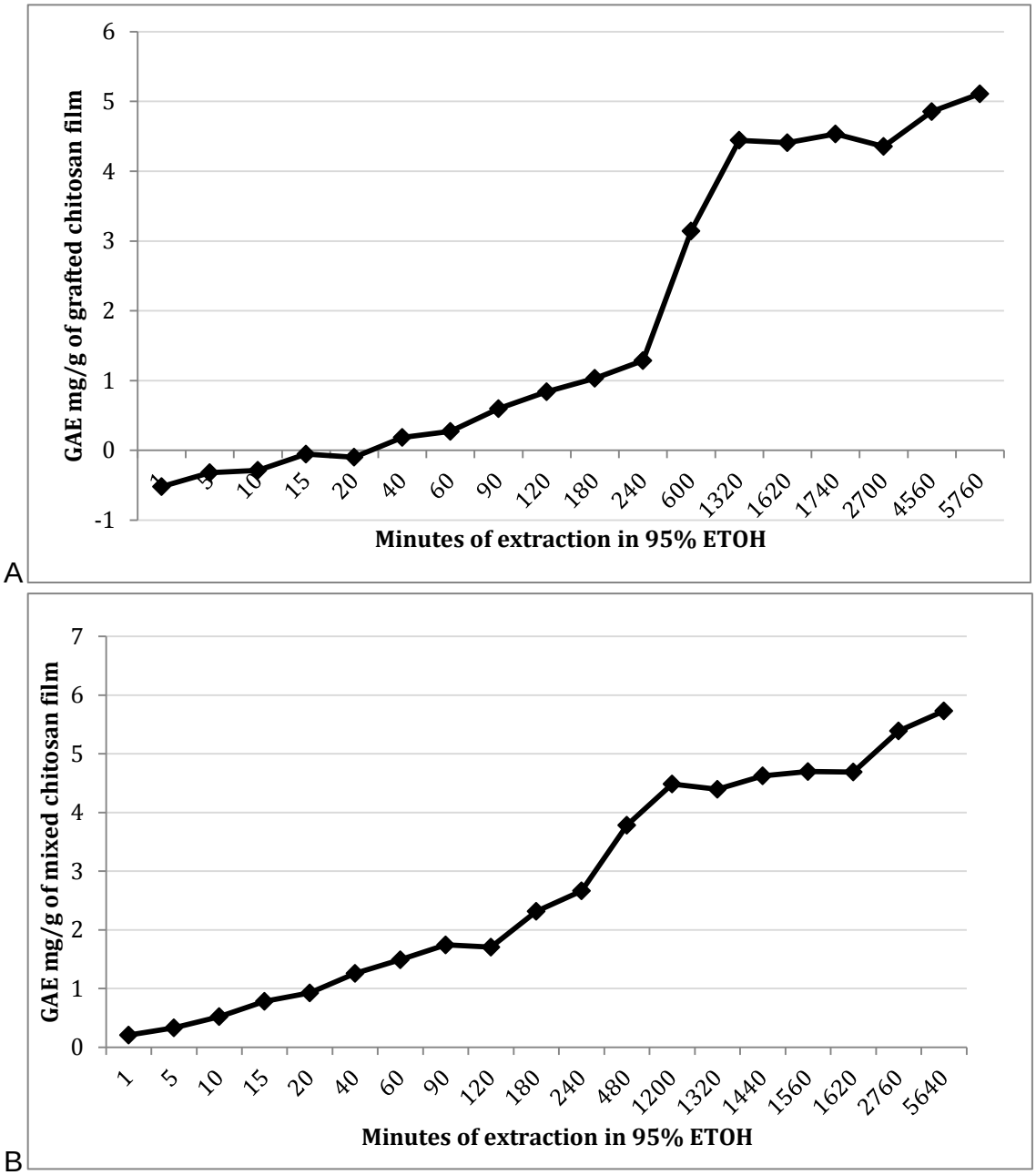
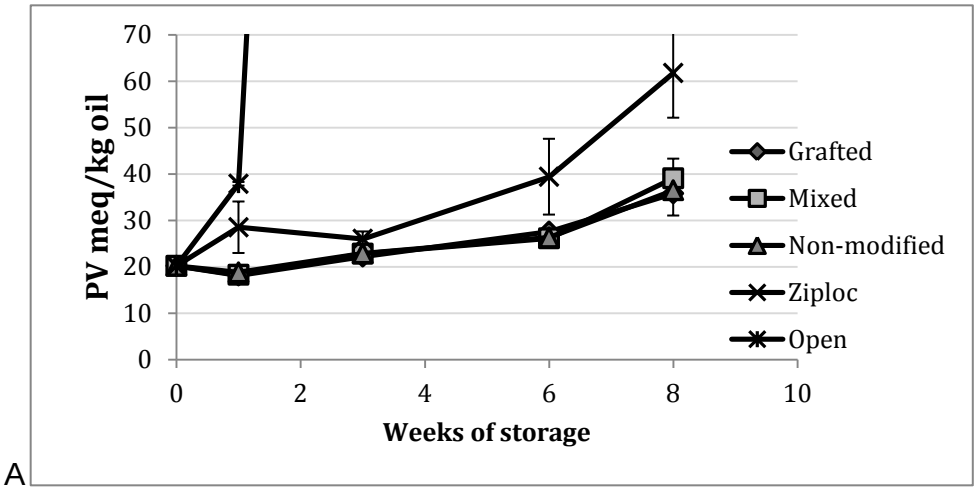
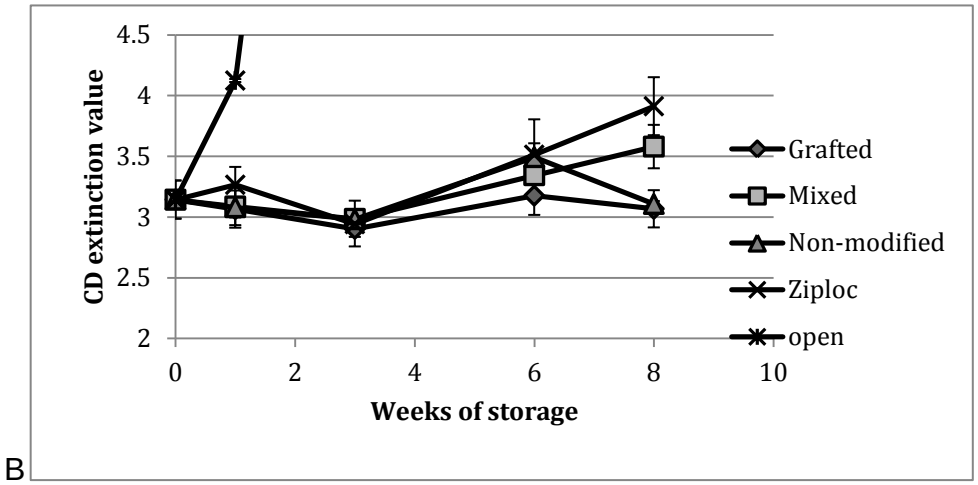


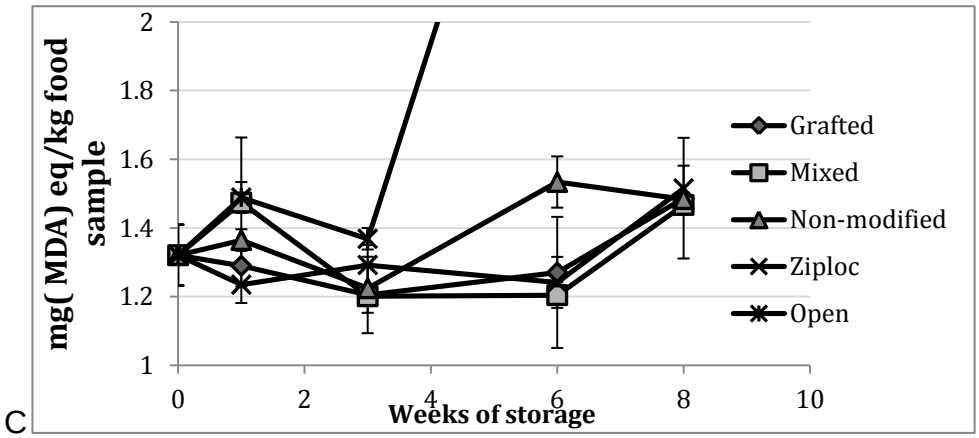
Figure 3. 6: Leaching of gallic acid from A) grafted and B) mixed chitosan films.



A



B



C

Figure 3. 7: Effect of packaging on quality of potato chips during 8 weeks storage at 50°C. A) TBA Reactive Substances, B) PV, C) Conjugated Diene formation. Values are presented as means with standard deviation.

CHAPTER 4
OVERALL CONCLUSIONS AND RECOMMENDATIONS

The optimum ratio of starting materials (chitosan : GA : EDC : NHS) for grafting was found to be $2 \cdot 10^{-4}$: 1 : 0.05 : 0.05 since it resulted in grafted chitosan with high antioxidant activity and solubility, while using minimal amounts of conjugating reactants. The formation of covalent bonds between chitosan and Gallic acid was confirmed by FTIR but NMR showed inconclusive results. Grafted chitosan films were significantly darker than films made from a mixture of chitosan and gallic acid and significantly darker than non-modified films. All chitosan films evaluated had a high puncture strength compared to packaging made of polyethylene material. Grafted chitosan films and mixed gallic acid and chitosan films had lower water vapor permeability than non-modified chitosan. Grafted chitosan films retained their antioxidant properties when used as packaging for both peanuts and potato chips under a stressed environment (50°C and forced air flow and RH 20-80%). The grafted chitosan films had around a 2-log reduction of *Salmonella* Typhimurium*, which* was significantly lower than non-modified chitosan films. Chitosan films grafted with gallic acid efficiently reduced oxidation of peanut powder during 15 weeks at 50°C, significantly more than low-density polyethylene films. The grafted films also reduced oxidation in potato chips compared to Ziploc® bags during 8 weeks at 50°C. Packaging made of a chitosan and gallic acid mixture showed similar characteristics as grafted chitosan.

The first recommendation for future research is evaluate formation of covalent bonding between chitosan and gallic acid by other methods such as

chromatography or MALDI Mass Spec. Another recommendation is to blend the grafted chitosan with plastic material such at polyethylene in order to have a more practical application for industry. The last recommendation for future research is to determine whether grafting gallic acid to chitosan has any benefit over simply mixing chitosan and gallic acid together. Overall, chitosan films have practical application as packaging for the food industry and introduction of primary antioxidant activity can possibly be advantageous.

Appendix: Overall

Antioxidant and Lipid Oxidation Assays

DPPH: (Saha et al., 2008)

Preparing solutions:

- DPPH in methanol= 0.004g in 100mL of methanol
- Gallic acid standard curve: $1*10^{-4}$, $5*10^{-4}$, $1*10^{-3}$, $5*10^{-3}$
- 0.01% chitosan in 0.1% acetic acid

1. 1 mL DPPH solution + 1 mL sample
2. 1 mL DPPH solution + 1 mL distilled H_2O= blank
3. Shake and incubate for 30 min at RT
4. Read absorbance at 517 nm
5. % scavenging= ((abs of blank-abs of sample)/(abs of blank)

Reducing Power: (Yen and Chen, 1995)

Preparing solutions:

- Make phosphate buffer pH 6.6. Add 3.484 grams K_2HPO_4 and 12.907 mL HCl (1M) bring to 100 mL with distilled H_2O.
- Make 1% $K_3[Fe(CN)_6]$ = 1 gram in 100 mL water
- Make 0.1% $FeCl_3$= 0.1 g in 100 mL water
- 0.1% chitosan in 1% acetic acid

1. 1 mL chitosan solution and 2.5 mL phosphate buffer
2. Add 2.5 mL 1% $K_3[Fe(CN)_6]$
3. Incubate at 50C for 20 min
4. Add 2.5 mL 10% trichloroacetic acid
5. Centrifuge for 10 min at 3000 rpm
6. Remove 2.5 mL of upper layer and put in separate tube
7. Add 2.5 mL distilled H_2O to this tube
8. Then to the separate tube add 0.5mL 0.1% FeCl3
9. Read at 700 nm

Total Phenolics: (Folin and Ciocalteu, 1927)

Preparing solutions

- Folin Ciocalteau: 12.5 mL of 2N Folin Ciocalteu in 100 mL distilled H_2O
- Sodium carbonate: Dissolve 12.4 g Na_2CO_3 in 100 mL distilled H_2O
- GA stock solution: 0.05 g gallic acid and 3 mL ethanol bring to 50 mL with distilled H_2O
- Standard curve: In 10 mL volumetric flasks take 100 μL, 250 μL, 500 μL, 1000 μL of gallic acid stock solution, fill to volume with distilled H_2O

1. Add 1 mL sample or standard to test tube
2. Add 7 mL distilled H_2O
3. Add 1 mL Folin solution
4. After 3 min add 1 mL sodium carbonate and vortex
5. Incubate in water bath for 30 min at 40°C
6. Read absorbance at 725 nm

TBARS: (Pegg, 2001)

Preparing solutions:

- Antioxidant solution
  Weigh 0.5 g propyl gallate and 0.5 g ethylenediaminetetraacetic acid, disodium salt dihydrate (EDTA) into a 100-ml volumetric flask, dissolve crystals in a small volume of ethanol/water and dilute to mark with ethanol/water solution. Prepare fresh daily.
- 1,1,3,3-Tetramethoxypropane (TMP), 0.2 mM
  Prepare a 20 mM TMP stock solution by accurately weighing 328 mg TMP into a 100-ml volumetric flask and diluting to mark with water. Store up to 3 months at 4°C. Prepare a 0.2 mM working solution by diluting 100-fold in a volumetric flask. Store up to 1 month at 4°C.
- Trichloroacetic acid (TCA) reagent
  Weigh 200 g TCA (20% final) in a beaker and dissolve crystals in a small volume of water. To a 1-liter volumetric flask, add some water and then add 16 ml 85% (w/w) o-phosphoric acid (1.6% final). Swirl the solution and then quantitatively transfer the TCA mixture from the beaker to the volumetric flask using water. Fill to mark with water and mix contents thoroughly. Store up to 4 to 6 weeks at 4°C.
- Standard curve: Add 0.5, 1, 3, 5 mL standards 0.2 mM TMP in 100 mL volumetric flask. Fill to volume with 1:1 (TCA:Water)

1. 2 grams sample
2. Add 1 mL antioxidant solution and 20 mL ice cold TCA reagent and mix 2 min
3. Add 20 mL ice cold distilled water and mix for 1 minute
4. Vacuum funnel with # 1 Whatman filter paper and then filter with 0.45 μm filters
5. Pipette 5 mL extract or standard into 50 mL centrifuge tube. Add 5 mL 0.02 M Thiobarbituric acid (TBA).
6. Blank: 5 mL 1:1 (TCA:Water) and 5 mL 0.02 M TBA

7. Vortex then heat in boiling water for 35 min
8. Cool for 5 min in ice bath
9. Read at 532. Zero with the blank.
10. Calculate mg(MDA) eq/kg food sample

Oil Extraction: (Agbo et al., 1992)

1. Add 4 g of food sample in 50 mL tube
2. Add 24 mL of hexane and vortex
3. Rotate constantly for 1 hr
4. Centrifuge twice
5. Decant and remove solvent with nitrogen

Peroxide Value: (Pegg, 2001)

Preparing solutions:

- Iron(II) chloride solution
  Dissolve 0.5 g $FeSO_4 \oplus 7H_2O$ in 50 ml water. In a separate flask, dissolve 0.4 g $BaCl_2 \oplus 2H2O$ in 50 ml water. Slowly add the barium chloride solution to the iron(II) sulfate solution with constant stirring, followed by 2 ml of 10 M HCl. Filter off barium sulfate precipitate by gravity using Whatman no. 1 filter paper. Store <1 month in a brown bottle in the dark at room temperature.
- Iron(III) chloride standard solution
  Dissolve 0.5g $FeCl_3$ in 50 ml of 10 M HCl and add 1 to 2 ml of 30% (v/v) H_2O_2. Boil solution <5 min to remove excess H_2O_2 and allow to cool to room temperature. Dilute to 500 ml with water and transfer a 1-ml aliquot to a 100-ml volumetric flask. Dilute to mark with 7:3 (v/v) chloroform/methanol solution (final 10 µg/ml $FeCl_3$). Store up to 2 weeks at room temperature.

1. Accurately weigh (and record the weight of) 0.01 to 0.50 g oil or lipid extract sample. And then dilute.
2. Add 7:3 chloroform/methanol solution until 10 mL volume. Vortex sample 2 to 4 sec.
3. Turn on a spectrophotometer and set wavelength to 560 nm.
4. Zero spectrophotometer with solvent (chloroform/methanol solution) blank using a glass cuvette.
5. Add 100 µl of 10 mM xylenol orange solution to sample, vortex 2 to 4 sec, and then add 50 µl iron(II) chloride solution and vortex again.
6. Allow solution to stand **exactly** 5 min at room temperature and then determine its absorbance at 560 nm.

7. Construct a standard curve by repeating steps without using oil or lipid extract sample. Instead of the sample, varying aliquots of an iron(III)–chloride standard solution (10 µg/ml), 100 µl of 10 mM xylenol orange solution, and enough 7:3 (v/v) chloroform/methanol solution to a final volume of 10 ml.
8. Appropriate volumes of the iron(III) chloride standard solution should range from 1 to 10 ml.
9. Calculate PV, expressed as meq active oxygen/kg sample, using the following equation:
10. PV=[(AS -AB)×mi]/(W×55.84×2)
11. Where *A*S is the absorbance of the sample, *A*B is the absorbance of the blank, *m*i is the inverse of the standard curve slope (1/slope), *W* is the weight of the sample (g), and 55.84 is the atomic weight

Conjugated Diene and Triene: (Pegg, 2001)

1. Accurately weigh (and record the weight of) 0.01 to 0.05 g oil or lipid extract sample into a 25-ml volumetric flask.
2. Each sample should be assayed at least in duplicate.
3. Dissolve sample in 2,2,4-trimethylpentane (iso-octane) and bring to volume. Mix solution thoroughly.
4. Turn on as spectrophotometer and set the wavelength to 233 nm for CD measurements and 268 for CT.
5. Zero spectrophotometer with a solvent blank (i.e., 2,2,4-trimethylpentane) using a quartz cuvette.
6. Measure absorbance of the dissolved oil sample using a quartz cuvette.
7. E = Aλ / (cL ×l)
8. Where E is the extinction value, Aλ is the absorbance measured at either 233 nm (for CDs) or 268 nm (for CTs), cL represents the concentration of the lipid solution in g/100 ml, and l is the path length of the cuvette in cm.

Micro Procedure: (Zivanovic et al., 2007)

1. Make 0.7% chitosan films in 1% acetic acid, cast on 5 cm petri dishes
2. Cut films into strips and place 0.04 g in sterilized tubes
3. Loop Salmonella (Typhimurium DT 104) strain 2486 in BHA broth for 24 hrs at 35°C. Loop in new BHA broth for another 24 hrs at 35°C.
4. Plate salmonella dilutions on TSA plates -5 to -8 dilutions and count colonies after 24 hrs at 35°C
5. In test tubes with film strips add 4.5 mL phosphate buffer and 0.5 mL of -4 dilution of bacteria
6. Incubate for 6 hours at room temperature, constantly shaking
7. Streak plates with 0, -1 and -2 dilutions of inoculated phosphate buffer
8. Incubate for 24 hours at 35°C
9. Run positive and negative controls. Positive= no film, Negative= no bacteria

VITA

Stephanie Beth Schreiber was born in Pittsburgh, Pennsylvania on September 12, 1987. She grew up in Wilmington, Delaware and graduated from Concord High School. She continued her education at the University of Maryland where she earned a B.S. degree majoring in Food Science. She later earned an M.S degree in Food Science and Technology with a concentration in Food Chemistry from the University of Tennessee, Knoxville. Stephanie will pursue a career in the food industry concentrating in product development.