To the graduate council:

I am submitting herewith a thesis written by Gagan Rajpal entitled “Color and Mechanical Properties of Chitosan Films during Storage.” I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

Svetlana Zivanovic, Major Professor

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We have read this thesis
and recommend its acceptance

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Color and Mechanical Properties of Chitosan Films during Storage

A Thesis Presented for the Master of Science Degree

The University of Tennessee

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Gagan Rajpal

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Dedication

This thesis is dedicated to my parents Omparkash Baghi and Kailash Rajpal, my brother Dr.Girish Rajpal, sister-in-law Chinki Rajpal, nephew B Rajpal, rest of my family and my friends, for always believing in me, inspiring me, supporting me and encouraging me, to reach higher in order to achieve my goals.

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Abstract

Chitosan, a deacetylated product of chitin, is a copolymer of 2-amino-2-deoxy-D glucopyranose and 2-acetamido-2-deoxy-D glucopyranose. Chitosan has many functional properties and has various applications in food, agriculture, pharmaceutical, cosmetic industries. However, little is known about the fate of chitosan and its films during prolonged storage. Although it has been reported that chitosan films treated with dry heat or saturated steam became brown, data available on physical characterization of chitosan films is still conflicting.

This study was conducted to determine chemical stability and mechanical properties of chitosan films as affected by (1) type of acid used for film preparation (acetic, lactic, citric, and hydrochloric acid), (2) final pH of the films (regular vs. neutralized), (3) storage conditions (temperature of 4, 22, and 80°C, and relative humidity 20 and 70%), and (4) time (up to 5 months). Development of colored compounds was observed with a Hunter colorimeter and UV-Vis spectrometer, while polarized microscope was used to observe micro-structural changes in the film. Accumulation of hydroxymethyl-furfural (HMF) was monitored by high performance liquid chromatography. Neutralized films stored at high temperature and high humidity showed more darkening and higher HMF

levels than regular films stored at lower temperature under dry conditions. Polarized microscopy showed presence of birefringent crystals only in the films stored at high temperature.

Effects of type of acid and storage conditions on mechanical properties of chitosan films were evaluated on films stored for a period of 1 year. Dynamic mechanical analysis showed that citric acid films were brittle while lactic acid films were flexible even at room temperature. Thermal degradation of the films started at temperatures around 200 °C regardless on type of acid used for film preparation. Glass transition temperature (Tg') of chitosan films appeared to be between 40 ºC to 50 ºC.

Our study has shown accumulation of HMF and development of crystallinity at higher temperature and high humidity in chitosan films, whereas films stored at lower temperatures (4 ºC and 22 ºC) had low levels of HMF and no crystals were observed. Among all the films tested, acetic acid films were found to be the best for food application with least HMF accumulation and better overall mechanical properties compared with lactic, citric, and hydrochloric acid films.

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Abbreviations and Acronyms

DA	=	Degrees of N-deacetylation
DMA	=	Dynamic Mechanical Analysis
DSC	=	Differential Scanning Calorimetry
E’	=	Storage Modulus
E”	=	Loss Modulus
FD	=	Freeze Dried
FDA	=	Food and Drug Administration
GPa	=	Giga Pascal
HMF	=	Hydroxymethyl furfural
HPLC	=	High pressure liquid chromatography
LDPE	=	Low Density Polyethylene
NIOSH	=	National Institute of Occupational Safety and Health
NMAM	=	NIOSH Manual of Analytical Methods
PEG	=	Polyethylene Glycol
PEO	=	Polyethylene Oxide
PVDF	=	Polyvinylidene fluoride
PVOH	=	Polyvinyl alcohol
RH	=	Relative Humidity
Tg	=	Glass transition temperature of pure polymer
Tg’	=	Glass transition temperature of chitosan film
TGA	=	Thermo Gravimetric Analysis
TMA	=	Thermo Mechanical Analysis
UV-Vis	=	Ultra violet and Visible
WAXD	=	Wide Angle X-ray Diffraction

Part 1

Introduction

Food products undergo numerous physical, chemical, and microbial changes during storage. The changes occurring in the food components, such as proteins, lipids, carbohydrates, as well as pigments and vitamins, due to environmental and processing conditions (exposure to light, moisture, temperature, etc.) are the factors governing the stability of foods (1, 2). To preserve the food quality, physical and chemical processes such as sterilization, high pressure, radiation or addition of active agents have been developed. However, packaging is the important step in preservation process that not only retards deterioration of food, but may also enhance its quality. Traditionally, food packaging materials have been chosen to avoid unwanted interactions with food which may cause physical, chemical or biological damage (3, 4). Thus, polyethylene- or co-polymer based materials have been used in food industry for last 50 years. These materials are not only safe and inexpensive, but have versatile mechanical characteristics (5).

Food packaging is the largest growing sector within the plastic packaging market (6). An estimated 180 million tons is the global production of packaging films annually, and growth and demand are increasing every year (5). Of the estimated $100 billions in packaging market in the United States, 70% is attributed to beverages and food industry. However, one of the major limitations with the plastic packaging materials is that it has to be discarded, with very little being recycled. In fact, less than 10% of all the plastic packaging materials (not including bottles) was recycled by consumers during 1990s (6).

Production of plastic packaging materials requires the reliance on petroleum-based raw materials. As plastic materials used for food packaging are derived from petroleum, alternative packaging materials with the similar characteristics based on renewable resources need to be found (7). With the rising cost of petroleum, cost effective and environmental friendly ways to manufacture packaging materials have become essential.

In addition, today's consumers are becoming more aware and concerned about not only the quality of the food they consume but also about the environment they live in. Consumers are demanding natural, disposable, recyclable, and/or potentially biodegradable food packaging material (8). Examples of these packaging materials include bio-based polymers, bioplastic, made from materials originating from agricultural or marine sources (9).

The research presented here focused on stability and mechanical

properties of films prepared with natural, biodegradable, polysaccharide, chitosan, that have potential to be used as packaging material for food, pharmaceutical, and agricultural products.

Part 2

Literature Review

2.1. Biodegradable films

In recent years, a wide variety of packaging materials and numerous technical approaches have been employed to provide desirable effects to extend shelf-life of food and enhance product quality. For example, oxygen, moisture, or ethylene scavengers or antimicrobial agents may be incorporated into packaging material itself rather than to be added to food, or alternatively, these compounds can be placed onto inner surface of package to ensure direct contact but not mixing with the food. Such approaches designed to perform some desirable functions other than providing inert barrier, refer to concepts called ‘active packaging,’ ‘interactive packaging,’ and ‘intelligent packaging’ (10).

Packaging composed of materials originating from agricultural and marine

sources is defined as biopolymer-based packaging. The materials used for their preparation are mainly of polysaccharide, protein, or lipid nature (9). Due to their origin, some of these packaging materials may be edible but all of them have to fulfill requirements such as good sensory qualities, high barrier and mechanical efficiencies, sufficient biochemical, physico-chemical and microbial stability, free of toxic compounds and contaminants; and produced by environmentally friendly yet simple technology (11, 12). Preparation and application of biodegradable films from various proteins and polysaccharides have been investigated (13, 14). Pectin, being widely available from underutilized agricultural waste material and readily modified to give preparations that form excellent films, has been given considerable attention (14). Starch is a most commonly used raw material in biodegradable films. Films based on starch are easily biodegradable and cost effective but also very hydrophilic having moderate gas barrier properties (15). Amylose, the linear fraction of starch, is responsible for film forming capacity of starch. Amylose forms continuous and strong films, whereas, highly branched amylopectin forms brittle and noncontinuous films (16). Potassium sorbate incorporated into starch-based coating has been applied to extend the storage life of fruits (17).

Polysaccharides are generally nontoxic and widely available in the nature. Polysaccharide based films are selectively permeable to CO_2 and O_2, and may retard the respiration and ripening of many fruits and vegetables by limiting the

availability of O_2. Polysaccharides form hydrophilic films and thus are poor barrier to water vapor (18, 19, 20). The poor water vapor barrier property allows for the movement of water vapor across the film, and from one side of the package to another. Although this is disadvantageous for the dehydrated foods it can be used for packaging of horticultural commodities. High water vapor permeability of these films could prevent water condensation within the package that is considered as a potential cause of microbial spoilage in fresh produce (21).

Effect of ageing on the mechanical and barrier properties of whey protein films plasticized with glycerol and sorbitol was studied by Anker et al. (2001). It was found that increase in the concentration of glycerol and sorbitol led to an increase in water content, water vapor permeability, and elongation, while tensile strength, elastic modulus, and Tg of the films decreased (22). However, increased levels of xylitol did not have any effect on the permeability, moisture content, and Tg of the films, but decreased the elongation, tensile strength and elastic modulus (23).

Lipids are used in food coatings as they are hydrophobic in nature. They are commonly added to protein- and polysaccharide-based films due to their efficiency as water-vapor barriers in edible films. There are many factors influencing the functional properties of a film such as structure, degree of saturation and chain length of fatty acids, physical state, shape and dimension of lipid crystal, and distribution of lipids within the film (24). Waxes are non-polar

lipids and are insoluble in water. They do not spread to form monolayer on the surface as their hydrophobicity is high. It has been observed that a relationship exist between water vapor permeability of the film and weight loss in the fruits, but no correlation was found between CO_2 and O_2 permeability of coating and concentration of these gases in fruit (25).

2.2 Chitosan films

2.2.1 Chitosan

Chitosan is an edible and biodegradable polymer derived from chitin, the major organic skeletal substance in the exoskeleton of crustaceans, insects, and some fungi (26 - 29). Chitin is the second most abundant polysaccharide after cellulose on earth (30). It is commercially available from a stable renewable source, shell waste from fish industry (31). Chitin is available at various degrees of N-acetylation (DA), ranging from the fully N-acetylated to the totally deacetylated form. Chitin with high DA is soluble in only few solvents, such as $LiCl_2$, which limits its applications. Partial deacetylation that results in DA values below 50% makes the polymer soluble in aqueous acidic conditions. A deacetylated product of chitin, is a copolymer consisted of (1-4)-2-acetamido-2-deoxy-β-D-gluco-pyranose and (1-4)-2-amino-2-deoxy-β-D-glucopyranose, and is referred to as

chitosan (32).

Chitin, the source of chitosan, is insoluble in any of the common solvents due to extensive crystallinity. Chitosan is insoluble in pure water or organic solvents but is soluble in diluted organic and inorganic acids. Due to an increased viscosity upon hydration, chitosan or chitosan-based materials easily form biodegradable films or coatings (33).

Chitosan has a promising use in food applications because of its biocompatibility and nontoxicity. It can be used as a dietary fiber (34), antimicrobial agent, for formation of biodegradable films and coating (35, 36), production of membranes for reverse osmosis and pervaporation (34, 37), but also as a matrix in culturing plant cells, artificial skin, tablet binders, and surgical sutures (38, 39). Chitosan is one of the few naturally occurring cationic polysaccharides which not only form films without the addition of any additives but also exhibits good oxygen and carbon dioxide permeability (29). However, one disadvantage with chitosan is that it is highly sensitive to moisture.

2.2.2 Antimicrobial and binding properties of chitosan films

Chitosan possesses antimicrobial activity against bacteria, yeasts, and molds (40). Two hypotheses have been proposed for the mechanism of action of chitosan against microorganisms. According to one, disruption of cell membrane leads to leakage of cytoplasmic contents as a result of interaction between

positively charged chitosan and negatively charged proteins and phospholipids in microbial cell membrane (41). Second theory proposes permeation of chitosan oligosaccharides into the cell nucleus, interfering with the RNA transcription mechanism and thus with the synthesis of proteins (42). However, chitosan oligomers have been shown to possess weaker antimicrobial activity than high molecular weight chitosan (43).

Chitosan has shown antimicrobial activity against both gram-negative and gram-positive organisms, including food borne pathogens. Chitosan films made in dilute acetic acid solution were able to inhibit the growth of yeast and mold by direct application of the film on the colony-forming organisms (44). Antagonistic effect against *Escherichia coli*, *Staphylococcus aureus*, and *Saccharomyces cerevesiae* has been displayed by chitosan lactate and chitosan glutamate (45). On the other hand, chitosan films did not have any effect on lactic acid bacteria or had little effect when applied to inhibit surface spoilage bacteria in processed meat (45).

In comparison to other natural polymers obtained from seafood wastes and other naturally occurring substances, like peanut skins, bark from trees, activated sludge, and the synthetic polymer poly(4-aminostyrene) which is used in commercial chelating ion-exchange resins (46), chitosan has the highest chelating ability (47). Chitosan has been observed to remove mercury from the hard water at neutral pH (48) and also to lower the arsenic concentration in

ground water (49).The binding capacity of chitosan for heavy and toxic metals is, in most cases, more than 1 mM/g.

Chitin and chitosan molecules have both amino and hydroxyl groups that can bind with ligands under mild conditions, thus providing an excellent binding capacity for protein purification. However, their poor mechanical properties have prevented their wider commercialization (50). Chitosan-cellulose composite membranes had been produced, combining the advantages of both the polymers. Both of these polymers are natural, biodegradable and abundant. It has been observed that protein-chitosan-cellulose composite membranes possess good blood compatibility (51) and provide a relatively high dynamic binding capacity of human IgG (52).

2.2.3 Physical properties of chitosan films

The applicability of packaging films strongly depends on their physical and chemical properties. Many analytical techniques have been developed to determine the performances of the films, which can be used to predict the shelf life of the product.

Chitosan has two distinct crystal forms (53), both of them are orthorhombic having different unit cell dimensions. Form I has a unit cell of a=7.76 Å, b=10.91 Å, and c=10.30 Å, with the strongest reflection at 2theta=11.4º. The form II crystal has a unit cell of a=4.4 Å, b=10.0 Å, and c=10.3

Å, with the strongest reflection at 2theta=20.1º. According to Zong et al (2000), raw chitosan flakes show a strong reflection at 2theta=20º whereas, chitosan films prepared from chitosan aqueous acid solutions show a weak reflection at 2theta=20º and strong reflection at 2theta=10º (54). X-ray diffraction measurements have shown five crystalline polymorphs, four hydrated – "tendon" (most abundant) (55, 56, 57), "form II" (52), "L-2" (58), and "twisted" (59), which is unstable (60) and one anhydrous or "annealed", which is prepared by heating a hydrated crystal of chitosan in water at high temperature (56, 61). It has been observed that transformation from "tendon" to "annealed" occurred irreversibly (56) which involved change in the chain arrangement of chitosan but with no alteration in the molecular conformation (57, 59).

Aliphatic primary amino groups in chitosan molecule react with both organic and inorganic acids to form salts. Depending on the type of acid, various inorganic acid salts of chitosan can be classified into two types as revealed by X-ray diffraction (62) and 13C CP-NMR studies (63). One, called Type I salt, is anhydrous and the other, called Type II, is hydrated crystal. X-ray fiber diagram of many chitosan organic acid salts obtained so far has revealed that they also form two types of salts similar to those of inorganic acid salts. Chitosan salts of L-ascorbic acid (64, 65, 66) and D-isoascorbic acids (66) are Type I salts, along with L- and D-lactic acid salts prepared at high temperature (67). However, L- and D-lactic acid salts formed at low temperature (32) and those formed from

monocarboxylic acid falls under the category of Type II salts (68, 69). It has been seen that presence of anhydrous crystal of chitosan, having high crystallinity, makes it insoluble with any aqueous solution and it may not be biodegradable either (70). That is, in the anhydrous crystal, chitosan molecule looses an important property as biomaterial.

Glass transition is a second-order phase transition that occurs over the temperature range at which a glassy amorphous material enters the rubbery domain with a concomitant drop in the elastic moduli (Young's or shear modulus) (71). Changes in the mechanical properties of a polymer around Tg are of major practical interest. For example, glasses have high Tg (Tg silica > 1000 ºC) i.e. they are solid, hard and brittle, while polyethylene (Tg LDPE = -30 ºC) is highly flexible at room temperature (72). At temperature above Tg, in a polymer molecular mobility increases exponentially and decreases in viscosity affecting various physical properties (73, 74, 75). Extensive studies by many authors have shown the importance of glass transition temperature, Tg, in understanding the physical state and physicochemical properties of food materials (75 - 83). As determined by thermo-mechanical analysis, the *β*-relaxation corresponds to glass transition in an amorphous polymer. It reflects long range motions of long chain segments in an amorphous region of a polymer. The motion of chains stops at rubber-glass transition leading to an entangled mass of chains limiting their structural reorganization. Because diffusion-controlled physical and/or chemical

processes cease at glassy state, it is often described as a state of high stability. Mechanical, thermal and dielectric attributes are drastically changed as a result of small change in temperature in the vicinity of Tg (84).

The glass transition is an indispensable aid that can be applied in edible film research, as it affects their mechanical and barrier (gas, water vapor) properties (85, 86). There are few characteristic features of edible films which make them useful, such as, renewable nature of their ingredients, their ability to carry food additives (e.g. antioxidants, flavors, antimicrobials), and their potential use in the interior of heterogeneous food systems as selective barrier to the transport of vapors, gases, and solutes (85). Polysaccharides (chitosan, cellulose derivatives, pectin, starch, alginates, carragenan, pullulan) are well known for their film forming properties. These biopolymers and their blends form membranes and coatings that are generally considered effective gas barriers. Extensive intermolecular cross-linking may cause film to become brittle and addition of plasticizers (e.g. polyols) is often required to overcome this defect. Plasticizers reduce intermolecular forces and increase the mobility of polymer chains improving flexibility and extensibility of the film, avoiding cracking and breaking of the film during handling and storage (87). To understand and predict many aspects of stability and processability of food ingredients, it is important to know their glass transition temperature (84). Many researchers have tried to determine Tg of chitosan and chitosan films but conclusive data is still lacking.

Temperatures ranging from -23 to 67 ^{o}C (2), 110 to 140 ^{o}C (3), and 202 ^{o}C (4,5) have been reported as Tg of chitosan. Begin et al (1999), determined the tensile strength and elongation at break of chitosan films prepared with hydrochloric, formic, acetic, lactic and citric acid, after drying at 70 ºC for 4 hours without neutralization. They concluded that small molecular weight acids, such as hydrochloric, formic and acetic acid made thinner but hard and brittle films whereas, films made from lactic and citric acid were thicker and more elastic (88).

2.3 Chitosan co-polymer films

Generally, films composed of one primary component usually have certain disadvantages. They either act as good barriers or have good mechanical properties, but seldom both (18, 19). Hydrophilic nature of polysaccharides and proteins limits the resistance of water vapor transmission of their films, but in dry environments they act as efficient oxygen barriers (89). Films made of lipids, on the other hand, act as good moisture barriers but have other problems, such as waxy taste, cracking of film surface, heterogeneity, pinholes, and poor adherence. The desirable properties of different materials can be utilized to form composite films minimizing drawbacks of films made of only one component to form composite films or multilayer films, such as bilayer or emulsion films. Composite films made from combinations of fatty acids/oil and polysaccharides

(90, 91, 92) or proteins (93, 94, 95) have been evaluated by many researchers.

Chitosan has potential use in tissue engineering. There are few problems that are to be addressed before it is implemented, improving the fragile nature and membrane permeability. Modulus of elasticity is high in chitosan and it has low strain to break (96, 97). Copolymerizing or blending with other polymers can improve ductility and morphology. Various blends of chitosan with other synthetic polymers have been also studied. Polyethylene glycol (PEG) is of particular interest because of its hydrophilic, biocompatible, and biodegradable nature. Number of applications in therapeutic, research, and diagnostic has been found for PEG-based technologies (98). It has been found that chitosan/poly ethylene oxide (PEO) blends were miscible below 50% (w/w) of chitosan (99). It has also been shown that up to 20% PEO content, PEO/chitosan blends are amorphous. If added more than 20%, PEO tends to crystallize forming spherulites in the blend (100). Significant improvement in mechanical properties of chitosan upon addition of PEO has been suggested by many workers (101, 102).

Another synthetic water soluble polymer with excellent film forming, emulsifying, and adhesive properties is polyvinyl alcohol (PVOH). PVOH forms the hydrogels used in biomedical applications (103, 104, 105), offers resistance to grease, oil and solvents, has high tensile strength, flexibility, and serve as high oxygen and aroma barrier (106). However, PVOH alone cannot be used as food packaging as its films are difficult to extrude and is not approved by FDA.

However, blending PVOH and chitosan results in films with improved desirable traits for application. The blend films of chitosan/PVOH have been reported to have excellent mechanical properties for medical products and controlled drug delivery systems (107, 108).

Composite films prepared by mixing chitosan and keratin have been shown to possess superior properties compared to pure chitosan or keratin films (109). It was previously observed that keratin films were too fragile to handle when made without any additives. Glycerol was added to get transparent flexible films but it dissolved out in an aqueous environment (110). Blending chitosan with keratin gave films with superior mechanical properties. The composite films had strength and flexibility provided by chitosan and were softer than pure keratin films. In addition, keratin-chitosan blend film had improved waterproof characteristics. In acidic conditions, pure chitosan films easily dissolved and at neutral pH they swelled forming a fragile film after drying, while the composite films showed significantly less swelling with almost no change in mechanical properties before and after the swelling. It has also been observed that the composite films possessed similar antimicrobial properties as pure chitosan films and were equally biocompatible with mammalian cells (109).

Carrageenans, water soluble sulfonated polymers obtained from red algae, are commonly used in food and pharmaceutical industries as gelling and stabilizing agents, and for microencapsulation and immobilization of drugs and

enzymes. The preparations of κ- and ι-carragenans have excellent film forming properties (111). Composite films utilizing polyelectrolyte behavior of carrageenan and chitosan with opposite ionic charges have been prepared to further improve the mechanical and barrier properties. It was seen that mechanical strength, elongation, and water vapor permeability of carrageenan-chitosan composite film was dependent on organic acid used as a solvent and addition of ascorbic acid. The highest tensile strength and elongation with low water vapor permeability were shown when malic acid was applied, whereas, use of citric acid showed remarkably weaker mechanical properties regardless of the amount of ascorbic acid. Ascorbic acid alone generally increased tensile strength and elongation of the films, but had little effect on the water vapor permeability except in the presence of acetic acid (112).

To determine the possible use of chitosan films in food applications, the films need to be analyzed for their chemical stability during storage. It is also important to determine mechanical stability of chitosan films and determine their Tg which will tell not only the suitable temperature range of their application but also help in blending chitosan with other polymers to improve quality of films.

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Part 3

Mechanism of Color Development In Chitosan Films During Storage

3.1 Abstract

Chitosan, a deacetylated product of chitin, is a copolymer of 2-amino-2-deoxy-D glucopyranose and 2-acetamido-2-deoxy-D glucopyranose. Chitosan possesses many functional properties and has various applications in food, agriculture, pharmaceutical, and cosmetic industries. Its films have been proposed as an antimicrobial packaging material for various food products. However, little is known about the fate of chitosan films during prolonged storage. Since chitosan films have potential use in many diverse fields, our objective was to characterize the potential reactions in the chitosan films under different preparation and storage conditions.

Chitosan films, prepared with acetic, lactic, citric and hydrochloric acid, regular or neutralized, were stored at different temperature and humidity

conditions for 5 months. Color development was observed with Hunter colorimeter, crystallization with polarized microscope and accumulation of hydroxymethyl-furfural (HMF) and color compounds by UV-Vis spectrophotometer and liquid chromatography. Films, both regular and neutralized did not change color considerably when stored at 4 and 22 ºC. Darkening in films was promoted at high temperature and high humidity and regular films darkened more than neutralized films. Hydroxylmethyl-furfural levels were in a range of 100 to 300 ppm in the films stored for 5 months at 80 ºC. Polarized microscopy showed development of birefringent crystals over time in films stored at 80 ºC but no crystals were observed in films kept at lower temperatures. The results indicate that chitosan films have potential to be used for packaging of dehydrated food products to be stored at room temperature or refrigerated.

3.2 Introduction

It has been reported that degradation of chitosan by nitrous acid results in the formation of reactive 2,5-anhydro-D-mannose- (M-) unit at the reducing end. During degradation, two types of oligomers were found, one fully acetylated, which was stable, and the other deacetylated which quickly undergoes Schiff base reaction between the amino group and terminal M-unit, facilitating the cleavage of glycosidic bond next to the M-unit and the formation of HMF (1). It has also been observed that conversion of monosaccharides, such as D-fructose to HMF is affected by the amount of water in the system. Formation of HMF through fructose dehydration was promoted by reduction in water concentration (2). Studies on the influence of pH on the rates and yields in the dehydration of fructose to HMF at the temperature of 175 ºC, have shown that the conversion is minimum at pH 3.1 with no formation of HMF at pH higher than 3.9. Presence of weak-acid anion at pH 3 functioned as a base catalyst and lowered the yield of HMF (3). At pH above 4.5 there was isomerisation of D-fructose to D-glucose which further reduced rate of HMF formation (3).

During heating, depending on the amount of the water present, sugars first melt, than undergo dehydration, and polymerization of dehydrated products leads to browning as the final result of sugar degradation (4). The chemical reaction between reducing sugars and amino group that occurs during storage or heating of foods is called Maillard reaction or non-enzymatic browning which is different

from browning that occurs in freshly cut fruits and vegetables and is catalyzed by enzymes. The reversible reaction between reducing sugar and amine produces glycosylamine which undergoes Amadori rearrangement, especially at pH 5 or lower forms furan derivative and HMF. Under less acidic conditions (higher than pH 5), HMF and other reactive cyclic compounds polymerize into insoluble dark-colored nitrogen containing material. Another reaction which causes browning in foods due to heating of carbohydrates and reducing sugars is facilitated by acids and salts, is called caramelization. Dehydration is caused by thermolysis of sugar molecule, forming anhydrorings and introducing of double bonds, which leads to formation of furans. The reaction rate can be increased and directed by catalysts (5).

Chitosan is a natural, non-digestible, biodegradable polymer with strong antimicrobial activity. It has been extensively investigated as a novel packaging material for the food and pharmaceutical industry (6, 7). The changes occurring in the food components, such as proteins, lipids, carbohydrates, and water, due to environmental and processing conditions (exposure to light, moisture, temperature, etc.) are the factors governing the stability of food (8, 9). However, packaging is the ultimate step in preservation process that not only retards detrimental deterioration of food, but may also enhance its quality. Suitable packaging often slows the deterioration rate and/or extends the shelf life of food. Traditionally, food-packaging materials have been chosen to avoid unwanted

interactions with food that may cause physical, chemical or biological damage (10, 11). Not much is known about the stability of chitosan films, especially about the fate of this polymer during prolonged storage. It has been reported that chitosan films treated with dry heat or saturated steam became brown and this darkening was attributed to the initial depolymerization and intermolecular cross-linking involving amino groups (12, 13). Furthermore, the considerable accumulation of 5-hydroxymethyl furfural (HMF) was detected in chitosan solutions under low pH (14).

Since chitosan films have potential use in many diverse fields, it is necessary to evaluate chemical stability of the films under different storage conditions. The objectives of this study were to determine potential development of crystallinity and quantitate the accumulation of HMF in chitosan films prepared with acetic, lactic, citric and hydrochloric acid, stored in different temperature (4, 22 and 80 ºC) and humidity conditions (20 and 70 % RH).

3.3 Materials and methods

3.3.1. Preparation of chitosan films

Chitosan films were prepared by casting method in polystyrene petri-dishes under ambient conditions. Chitosan solution was prepared by mixing 1 g of powdered high molecular weight (Brookefield viscosity 800.000 cps; Sigma

Aldrich, St. Louis, MO) chitosan in 90 g of deionized water. While stirring the mixture, 10 g of 10 % v/w acid solutions – acetic, lactic, citric acid or 1 % hydrochloric acid was added to dissolve the chitosan. The solution was stirred over night and filtered through miracloth filter (Calibiochem, Santa Barbara, CA). Aliquots of 20±1 g of chitosan solution were poured in 5cm-diameter petri-dishes and was allowed to dry under ambient conditions.

Dry films were either placed for conditioned storage (regular films) or were neutralized. Neutralization of the films was carried out by submerging them in 2 % NaOH solution for 1 hr and then rinsing thoroughly with deionized water till the pH of the water was approximately 7. The neutralized films were dried again at ambient temperature. All the films (regular and neutralized) were kept at 4º, 22º, and 80º C at dry or humid conditions. To ensure dry conditions at 4º, 22º C, films were kept in desiccators (20% RH) while relative humidity of ambient air was altered at 80º C storage. For all 3 temperatures at 70% RH, films were stored in an environmental chamber (IG420U, Yamato, Japan).

Sampling was done after 1, 2, 3, and 5 five months of incubation.

3.3.2. Colorimetery

The color of chitosan films was measured by a Hunter Colorimeter (Hunterlab, Reston, VA) and the L, a, and b values were recorded. The colorimeter was standardized with white reflectance standard tile and black tile.

The same white tile was used as a background during color determination. L value ranges from 0 (100% black) to 100 (100% white), measuring blackness/whiteness of the film. Values of 'a' indicate red (positive) or green (negative), while "b" values indicate yellow (positive) or blue (negative) coloration. All measurements were performed with three replications, using one film per replication.

3.3.3. Polarized microscopy

The morphological changes were examined with an Olympus BX 51 polarized microscope (Olympus American, Melville, NY) equipped with a DP 70 camera using normal and polarized light at 40 X optical magnification. The flat portion of the film was taken and placed onto a glass slide for observation. Minimum three films per treatment and sampling time were carefully examined for possible occurrence of birefringency.

3.3.4. UV-Vis spectrometry

To estimate formation of hydroxymethylfurfural (HMF) and colored compounds in chitosan films, films were first dissolved in phosphoric acid. Samples were prepared by dissolving 0.2 g films in 10 ml concentrated (85%) H_3PO_4 by constant stirring overnight at room temperature. Solutions were then filtered through 0.45μm pore size polyvinylidene fluoride (PVDF), Whatman

syringe filter (Sanford, ME) into a quartz cuvette with a 10 mm path (Fisher Scientific, Pittsburg, PA) and scans were taken by spectrophotometer (UV-2101PC, Shimadzu, Columbia, MD) from 220 to 800 nm. Pure concentrated phosphoric acid was used as a blank.

3.3.5. Hydroxymethylfurfural determination

Determination of HMF was carried out by HPLC-PDA, Dionex LC20 (Dionex, Sunnyvale, CA) at 210 nm on a C18 column (Waters Nova-pak, 4.6 X 250 mm) with a mobile phase of 95% KH_2PO_4 and 5% acetonitrile. HMF peak was identified by retention time combined with 3D spectra and quantitative analysis was performed at 210 nm. Sample preparation was same as in UV-Vis spectrometry.

3.3.6. Effect of temperature and aging on metal binding capacity of chitosan films

To test the effect of temperature and aging on metal binding capacity of chitosan films, the films prepared with 1 % acetic, lactic, and citric acid and stored at 22 °C and 80 °C in desiccators for 3 months were evaluated. Concentration of hexavalent chromium in solution was analyzed following NIOSH method (NIOSH Manual of Analytical Methods (NMAM), 8/15/94). In short, 1 mL sample solution was mixed with 6-7 mL 0.5 N H_2SO_4 in 25-mL volumetric flask,

0.5 mL sym-diphenylcarbazide in 50 % acetone was added, and the volume was adjusted with 0.5 N H_2SO_4 to 25 mL. Absorbance was read at 540 nm using spectrophotometer (UV-2101PC, Shimadzu, Columbia, MD). Approximately 0.2 g of film was submerged in 25 ml of solution. Readings were taken after films were submerged for 10-15 min and around 3 hrs. Chromium (VI) was measured in solution before and after the films were submerged in the solution. Measuring solution consisted of 40% Chromium solution, 20% diphenyl carbazide in acetone and 20% deionized water.

3.4 Results and discussion

3.4.1 Film color

Hunter colorimetry showed almost no change in L-value of chitosan films prepared with acetic acid when kept at 4 ºC and 22 ºC at high (70 % RH) or low (20 % RH) humidity, regardless if the films were regular (Figure 3.1, A) or neutralized (Figure 3.1, B). However, considerable decrease in L-value was observed in the films kept at 80 ºC at both levels of relative humidity, in both regular and neutralized films. The regular films kept at 80 ºC showed more browning (decrease in L-value) than neutralized films kept in the same environment. Both types of the films, regular and neutralized, kept at 80 ºC and 70 % RH showed the maximum browning, being the darkest after 5 months of

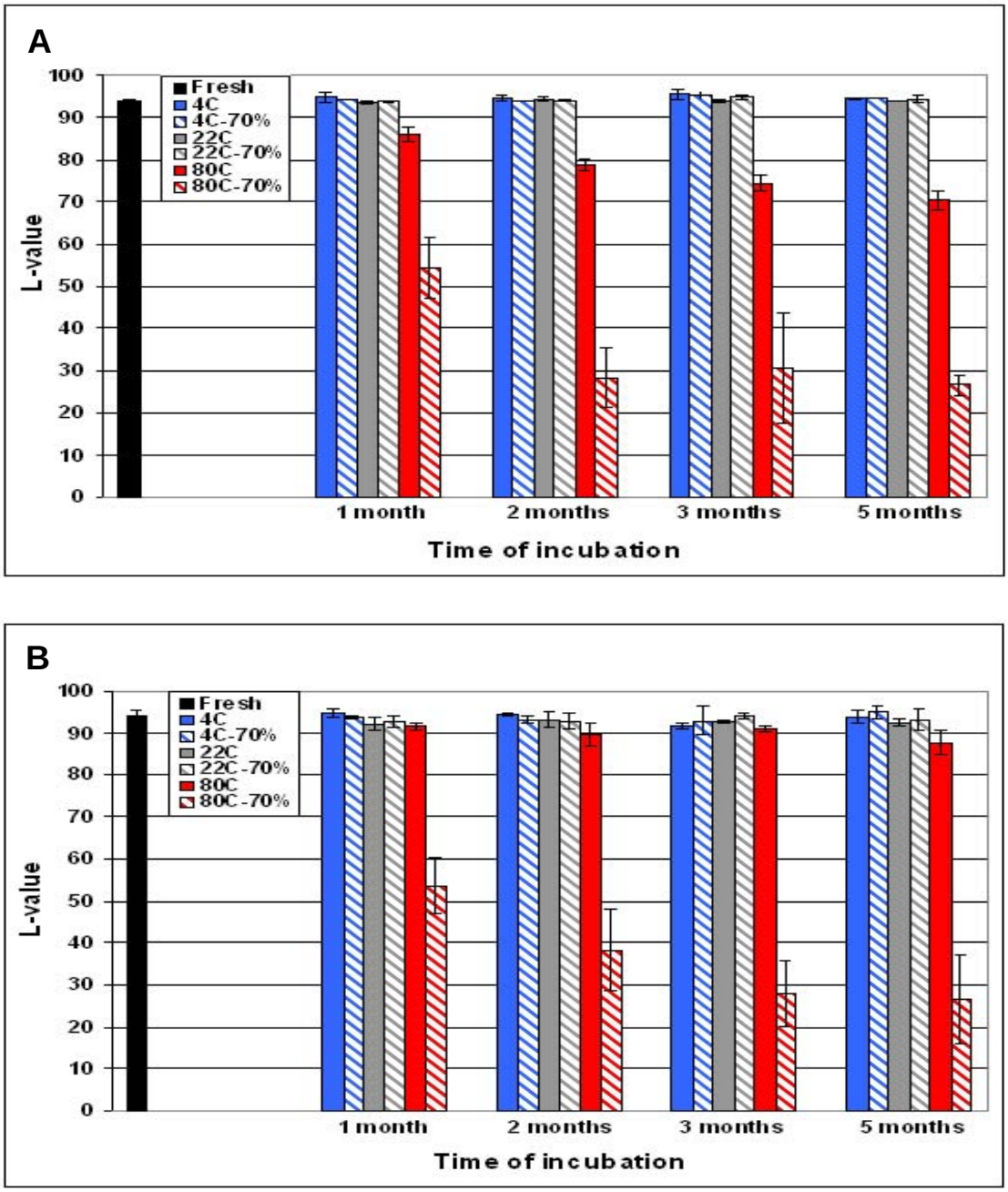


Figure 3.1. Change in L-value of regular (A) and neutralized (B) Chitosan films prepared in Acetic Acid, kept for 5 months in different environments. Error bars represent standard deviation of 3 replications.

storage.

As presented in Figure 3.2, films prepared with lactic acid, both regular and neutralized, when kept at 4 ºC and 22 ºC and 70 % RH or 20 % RH did not show any apparent change in color over the period of 5 months. As in films prepared with acetic acid, lactic acid films showed major changes in color when kept at 80 ºC regardless of the RH. Interestingly, in dry conditions and higher temperature, the neutralized films showed almost no browning as opposed to regular films which darkened continuously throughout the period of 5 months. The films kept at high temperature and higher humidity were the ones that showed maximum browning. The unexpected trend in L-values for regular lactic acid films kept at 80 ºC and 70 % RH, observed as first decrease (from L-value in fresh to L-value in films stored for 1 month) and later increase (L-value after 2, 3, 5 months, respectively) of L-values did not parallel true darkening of the films. Apparent 'whitening' i.e., higher L-value was possibly due to the thinning and cracking of the films which caused increase in light reflection from the background.

As seen with films prepared in acetic acid and lactic acid, the films prepared with citric acid when kept at lower temperatures (4 ºC and 22 ºC) with 20 % or 70 % RH, did not change in color over the period of 5 months (Figure 3.3), and the major changes in the L-value was observed when kept at 80 ºC. Similar to acetic and lactic acid films, the neutralized citric acid films at 80 ºC

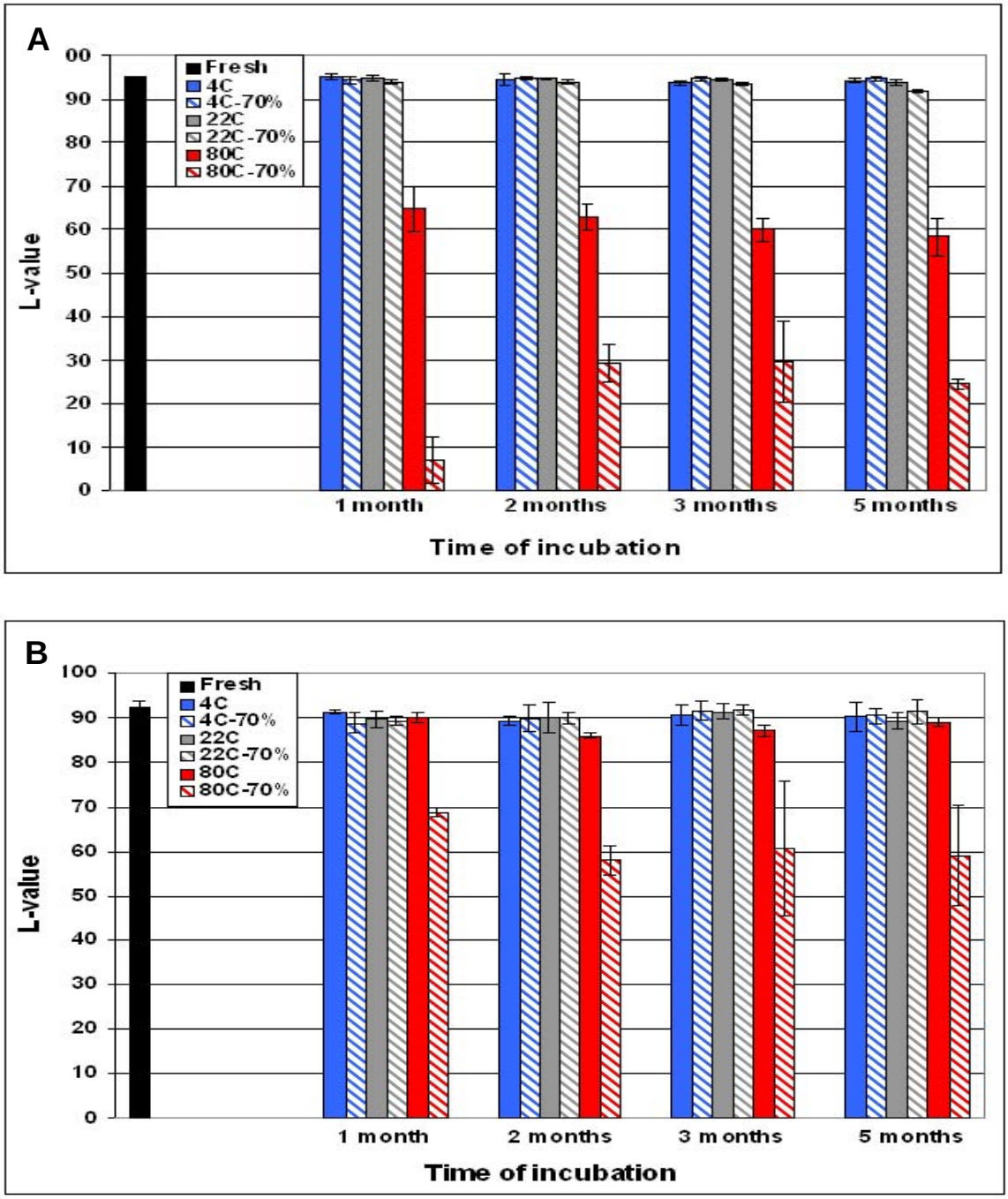


Figure 3.2. Change in L-value of regular (A) and neutralized (B) Chitosan films prepared in Lactic Acid, kept for 5 months in different environments. Error bars represent standard deviation of 3 replications.

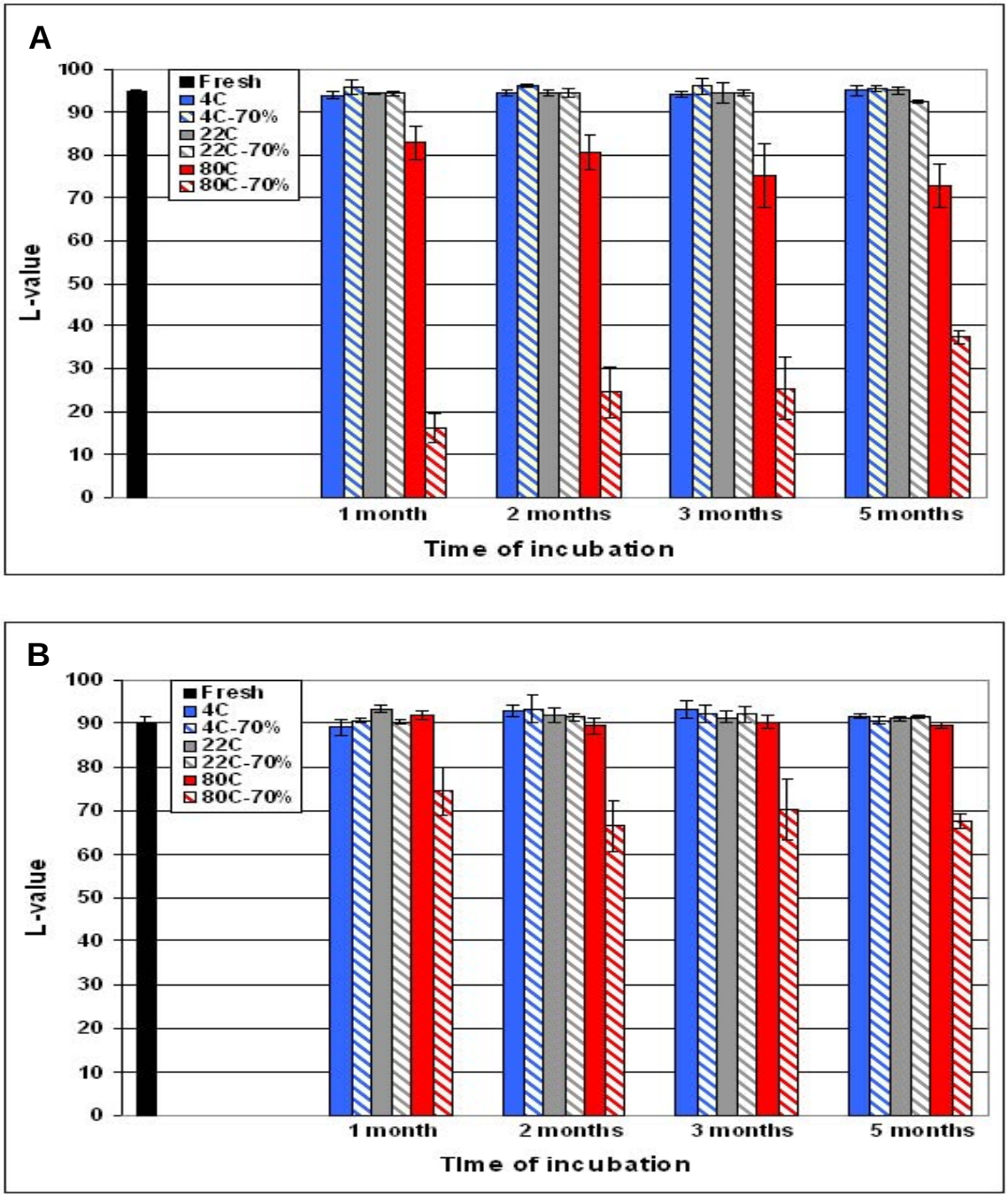


Figure 3.3. Change in L-value of regular (A) and neutralized (B) Chitosan films prepared in Citric Acid, kept for 5 months in different environments. Error bars represent standard deviation of 3 replications.

under dry conditions did not show any major change, whereas, regular films kept in same condition showed considerable browning. Both regular and neutralized films when kept at higher temperature and high humidity showed the maximum browning, regular films being more brown than neutralized. Increase in L-value of regular films at 80 ºC and 70 % RH, over the period of time also observed in lactic acid films was probably because of thinning and cracking of films causing light to reflect off the background.

The trend in case of films prepared with hydrochloric acid (HCl) was not much different from what was seen in other films (Figure 3.4). The film kept at 4 ºC and 22 ºC, did not show any change in color over the period of 5 months regardless of humidity. However, the films kept at higher temperature in both dry and humid conditions showed significant browning, maximum in humid conditions.

3.4.2 Polarized microscopy

Regular chitosan films kept at 80 ºC and 70 % relative humidity, when observed under polarized microscope showed the birefringent crystals or maltese cross (Figure 3.5). No birefringency was detected in fresh films and those kept at lower temperatures (4º and 22 ºC) during the length of experiment. The number of crystals apparently increased with time in the films kept at 80 ºC. However, the films incubated for 5 months at higher temperature were too dark to be

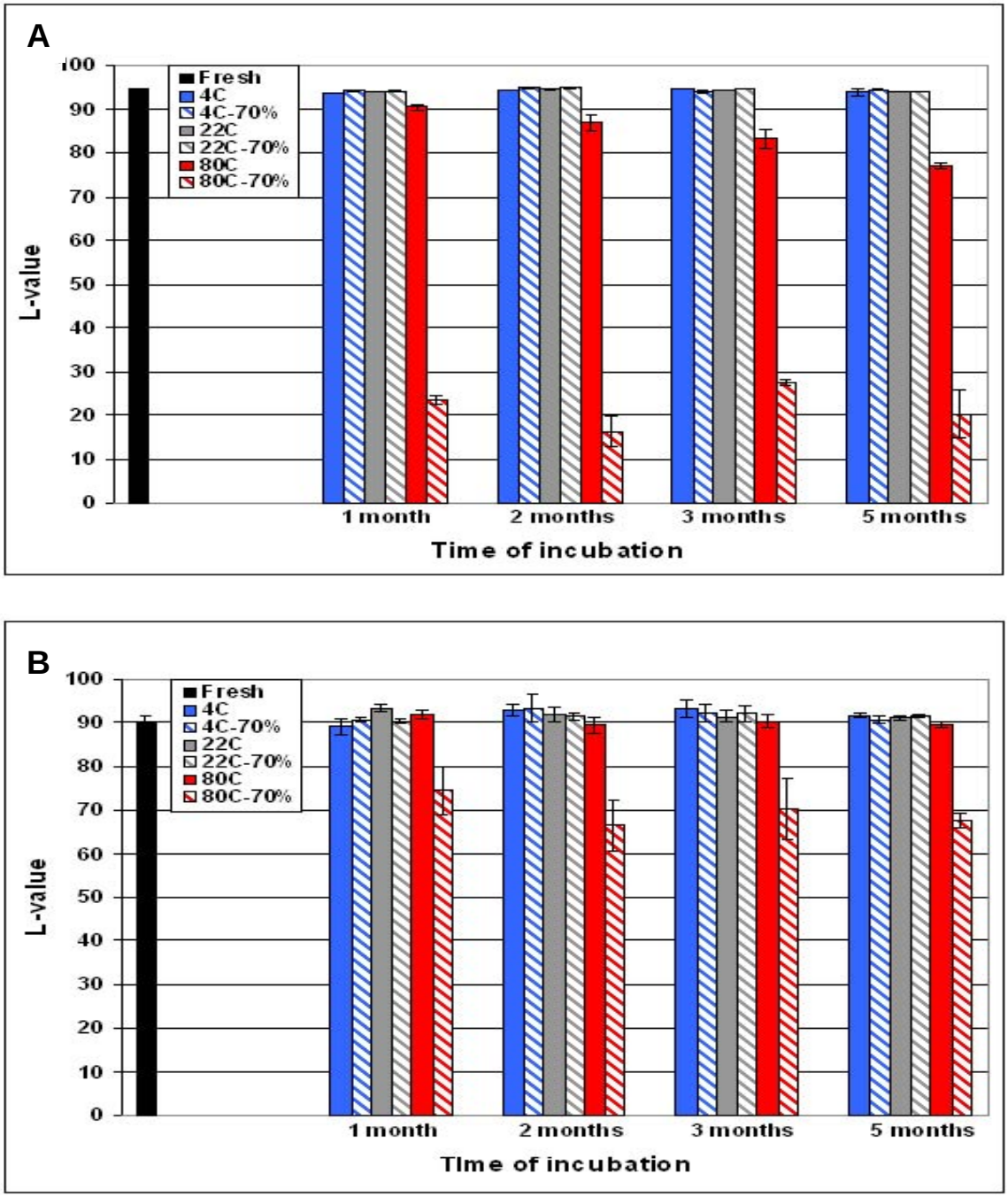
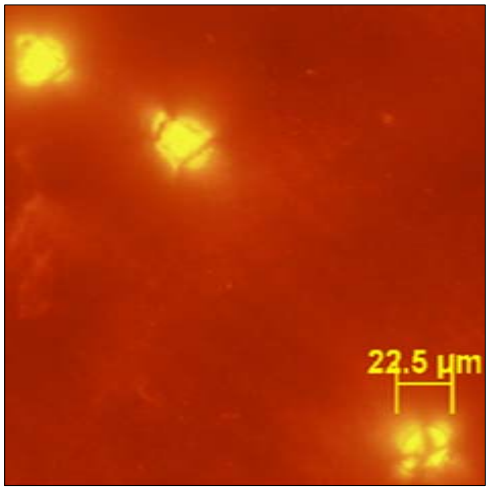
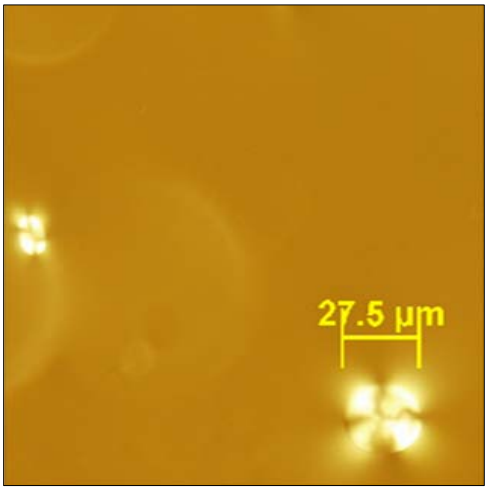


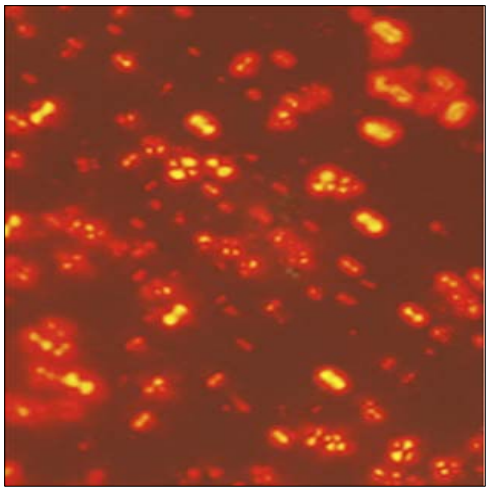
Figure 3.4. Change in L-value of regular (A) and neutralized (B) Chitosan films prepared in Hydrochloric Acid, kept for 5 months in different environments. Error bars represent standard deviation of 3 replications.



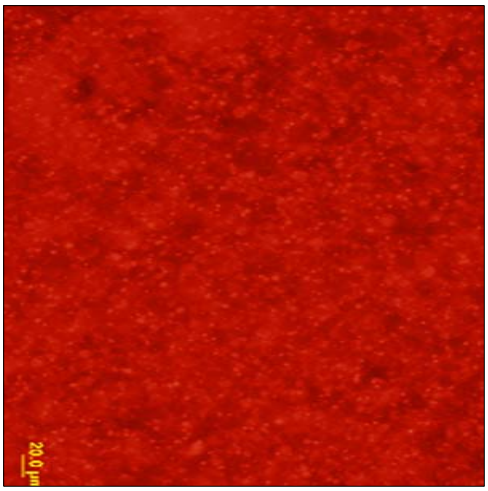
Acetic Acid



Lactic Acid



Citric Acid



Hydrochloric Acid

Figure 3.5. Microscopic changes in regular Chitosan films prepared with different acids and stored for 3 months at 80 ºC and 70% relative humidity.

successfully evaluated under a microscope. The crystals were the largest in films prepared with lactic acid and smallest in case of films prepared with citric acid. The films prepared with hydrochloric acid did not show any crystals even after 3 months, but pinhead size dots were present throughout the film making it hazy/foggy under the microscope. The number of crystals seemed to be the maximum in citric acid film and least in acetic acid film. Neutralized films were difficult to observe microscopically as they get deformed after neutralization.

3.4.3. UV-Vis spectroscopy

Figures 3.6, 3.7, 3.8 and 3.9 show UV-Vis spectra of chitosan films prepared with acetic, lactic, citric and hydrochloric acid respectively 80 ºC. The UV spectra of the films were taken to detect formation of hydroxy methyl furfural (MHF) while spectra in visible range should quantify overall development of coloration. Fresh solutions of pure HMF are colorless with the maximum absorption at 286 nm. As the HMF reacts and polymerizes producing dark compounds, the absorbance at 286 nm decreases and the products show absorbance in visible range.

Our fresh chitosan films had least amount of HMF and the absorbance at 286 nm increased over time in both regular and neutralized films. As the storage time period increased, the absorbance in visible region also increased, more in case of regular than in neutralized films. The reason for absorbance in the visible

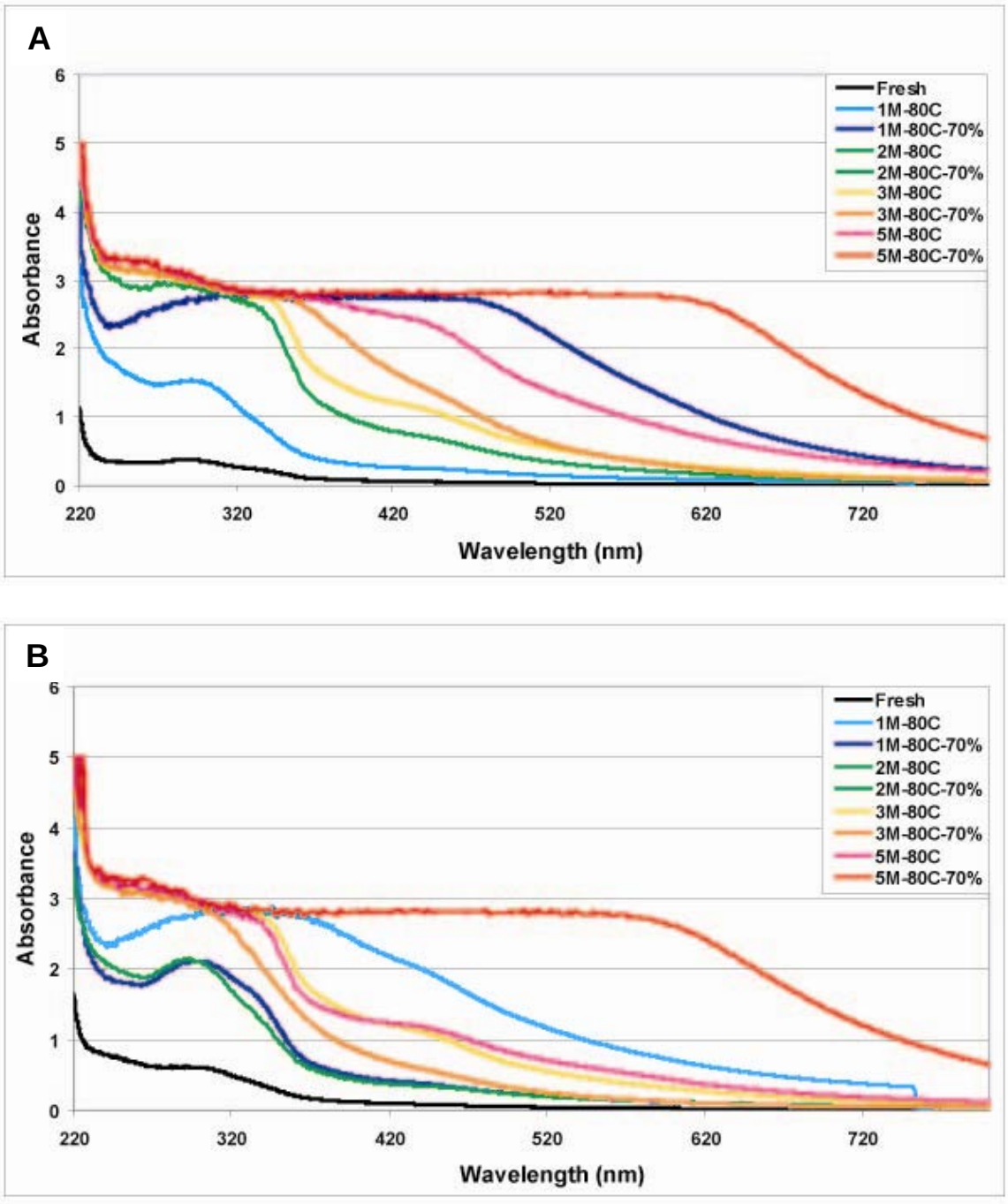


Figure 3.6. UV-Vis spectra of regular (A) and neutralized (B) Chitosan films prepared in Acetic Acid, kept for 5 months in different environments.

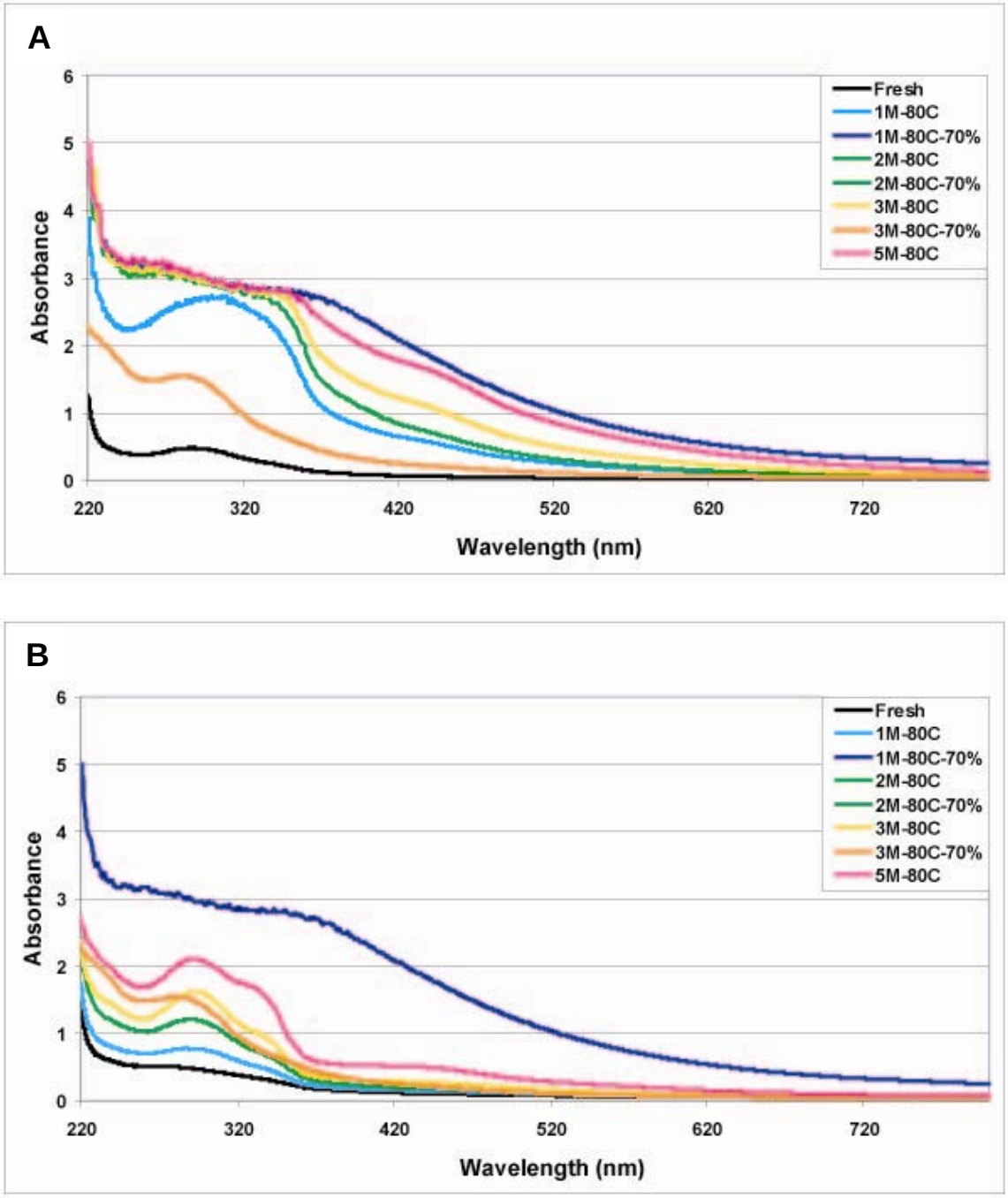


Figure 3.7. UV-Vis spectra of regular (A) and neutralized (B) Chitosan films prepared in Lactic Acid, kept for 5 months in different environments.

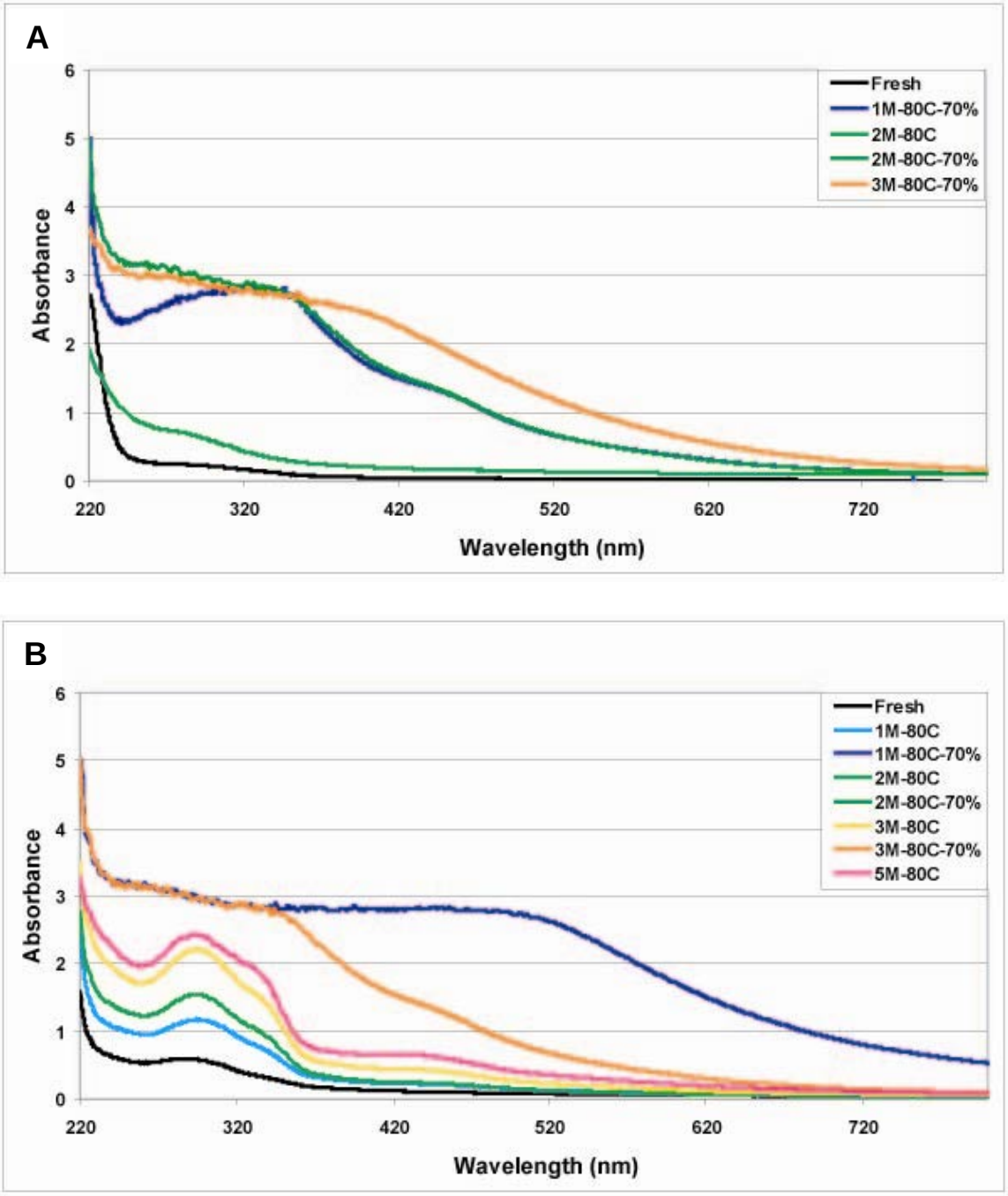


Figure 3.8. UV-Vis spectra of regular (A) and neutralized (B) Chitosan films prepared in Citric Acid, kept for 5 months in different environments.

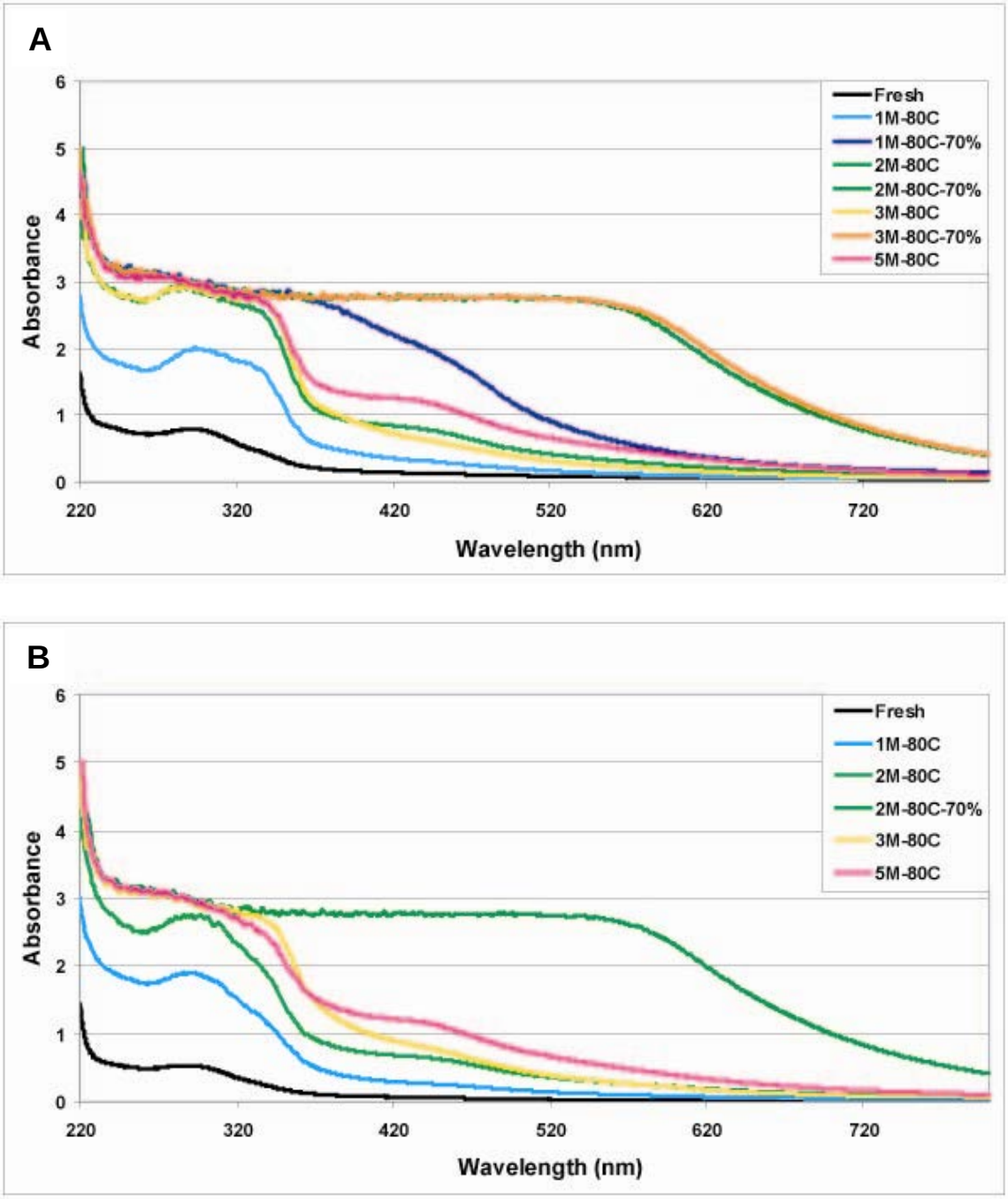


Figure 3.9. UV-Vis spectra of regular (A) and neutralized (B) Chitosan films prepared in Hydrocholoric Acid, kept for 5 months in different environments.

spectra could lie in the fact that HMF polymerizes and forms compounds visible to our naked eye. The increase in UV absorbance may be attributed to continuous formation of HMF from glucosamine.

As observed in chitosan films prepared with acetic acid, films made with lactic acid also showed increase in absorbance at 286 nm during storage (Figure 3.7). Spectra of the films (regular and neutralized) at 80 ºC and 70 % RH could not be obtained after 5 months, as the films did not dissolve. The trend (increase in absorbance) is seen better in the films kept at 80 ºC under dry conditions than in those kept under 70 % relative humidity. It was also observed that absorbance in visible spectra was higher in regular than in neutralized films.

The chitosan films prepared with citric acid were most difficult to dissolve. Most of the regular films kept at 80 ºC and 70 % RH did not dissolve and their spectra could not be taken (Figure 3.8, A). As seen in case of chitosan films made with acetic and lactic acid, the absorbance at 286 nm increased over the period of time, and regular films showed increase in absorbance in visible region more than by neutralized films.

Unlike other films, neutralized chitosan films prepared with hydrochloric acid kept at 80 ºC and 70 % relative humidity were more difficult to be dissolved and most of them did not dissolve and hence their spectra could not be obtained (Figure 3.9, B). But similar to other films, hydrochloric acid films showed increase in absorbance over the time with regular films showing higher absorbance in

visible region compared to neutralized films.

The increase in absorbance in visible spectra suggests that HMF polymerized causing darkening of the films. Formation of other compounds due to dehydration of carbohydrates and/or carboxylic acids as well as due to potential occurrence of Maillard reactions may further contribute to browning of the films.

3.4.4. Formation of Hydroxymethylfurfural in Chitosan Films

HPLC analysis of chitosan films showed presence of hydroxylmethylfurfural (HMF) in the samples, especially in the films stored at higher temperature. However, after prolonged storage even the films kept at lower temperatures showed the presence of HMF. Thus, in case of films formed with acetic acid, HMF was not detected in samples kept at lower temperatures (4 ºC and 22 ºC) for first 2 months, but there was significant amount of HMF in films kept at 80 ºC for only 1 month (Table 3.1). Samples kept at 80 ºC and 70 % relative humidity did not dissolve after 3 months of storage indicating extensive polymerization of colored compounds. Generally regular films had more HMF than neutralized films.

Table 3.2 shows the amount of HMF in lactic acid films. As seen in acetic acid films, lactic acid films kept at low temperatures (4 ºC and 22 ºC) till 2 months did not show presence of HMF but those stored at 80 ºC and 70 % RH did not

Table 3.1: Concentration of HMF (ppm) in regular and neutralized Chitosan films prepared in Acetic Acid, kept for 5 months in different environments.

		1 Month	2 Month	3 Month	5 Month
4°C	regular	-	-	103	109
	neutralized	-	-	-	-
4°C + 70% RH	regular	-	-	-	145
	neutralized	-	-	-	-
22°C	regular	-	-	-	-
	neutralized	-	-	-	74
22°C + 70% RH	regular	-	-	123	93
	neutralized	-	-	95	136
80°C	regular	437	-	112	302
	neutralized	-	-	292	104
80°C + 70% RH	regular	196	271	×	273
	neutralized	-	240	×	-

'-' HMF was not found in the sample
'×' Chitosan film did not dissolve

Table 3.2: Concentration of HMF (ppm) in regular and neutralized Chitosan films prepared in Lactic Acid, kept for 5 months in different environments.

		1 Month	2 Month	3 Month	5 Month
4°C	regular	-	-	-	76
	neutralized	-	-	-	-
4°C + 70% RH	regular	-	-	-	-
	neutralized	-	-	-	153
22°C	regular	-	-	-	-
	neutralized	-	-	-	208
22°C + 70% RH	regular	-	-	-	94
	neutralized	-	-	85	154
80°C	regular	88	128	-	177
	neutralized	71	202	-	77
80°C + 70% RH	regular	220	-	×	×
	neutralized	-	-	×	79

'-' HMF was not found in the sample
'×' Chitosan film did not dissolve

dissolve after being stored for 3 months or longer.

Citric acid films did not show accumulation of HMF in first 2 months regardless on storage conditions (Table 3.3). Films kept for 3 months or longer at 80 ºC did not dissolve, except for neutralized films that were kept at dry conditions.

Compared to acetic and lactic acid films where considerable accumulation of HMF started after 2 months of storage, accumulation in citric acid films started a month later indicating possible stabilization of chitosan by cross-linking of amino groups of glucosamine with carboxyl groups of citric acid. The cross-linking effect was evident with tricarboxylic acid (citric acid) but did not occur with monocarboxylic acids (acetic and lactic).

Table 3.4 shows results obtained from HPLC analysis of hydrochloric acid films. Although no trend was observed, amounts of HMF were higher in films that were kept for 5 months than in those kept for shorter period of time.

In general, accumulation of HMF was highest in acetic and lowest in citric acid films. Lactic acid and hydrochloric acid films produced intermediate amounts of HMF. Regular films kept at high temperature and high humidity accumulated more HMF than neutralized films or those kept at low temperatures. More humid conditions seemed to promote accumulation of HMF even in films kept at low temperatures. The possible reason for this could be in hydrolysis of chitosan by residual acid in the films. The oligomers and monomers produced by hydrolysis

Table 3.3: Concentration of HMF (ppm) in regular and neutralized Chitosan films prepared in Citric Acid kept for 5 months in different environments.

		1 Month	2 Month	3 Month	5 Month
4°C	regular	-	-	-	-
	neutralized	-	-	-	-
4°C + 70% RH	regular	-	-	-	80
	neutralized	-	-	-	99
22°C	regular	-	-	-	-
	neutralized	-	-	-	159
22°C + 70% RH	regular	-	-	-	72
	neutralized	-	-	-	213
80°C	regular	-	-	×	×
	neutralized	-	-	114	286
80°C + 70% RH	regular	-	-	×	×
	neutralized	-	-	×	×

'-' HMF was not found in the sample
'×' Chitosan film did not dissolve

Table 3.4: Concentration of HMF (ppm) in regular and neutralized Chitosan films prepared in Hydrochloric Acid kept for 5 months in different environments.

		1 Month	2 Month	3 Month	5 Month
4°C	regular	-	-	-	-
	neutralized	-	-	-	-
4°C + 70% RH	regular	-	-	-	149
	neutralized	-	175	-	102
22°C	regular	-	173	-	87
	neutralized	-	-	-	107
22°C + 70% RH	regular	-	-	-	141
	neutralized	-	175	-	-
80°C	regular	-	-	-	224
	neutralized	58	-	-	188
80°C + 70% RH	regular	-	267	×	×
	neutralized	-	187	134	×

'-' HMF was not found in the sample
'×' Chitosan film did not dissolve

are more prone to dehydration and/or Maillard intra- and intermolecular reactions, and thus produce more HMF. It was observed by Kristoffer et al. (2001), that oligomers produced by degradation of chitosan by nitrous acid reacted intermolecularly by Schiff's base reaction what resulted in formation of HMF (10).

Our data showed more accumulation of HMF in films made from weak organic acids (acetic, lactic and citric) than in those made from hydrochloric acid, which is a strong acid. This is in agreement with results of Kuster and Temmink (1977), who found that yield of HMF decreased at pH < 3, possibly due to formation of formic esters from D-fructose or intermediate products under highly acidic conditions (12). Similarly, Wu and Zivanovic (2005) had shown that production of HMF from glucosamine is enhanced by acidic and lactic acid compared to hydrochloric acid (15).

3.4.5. Effect of temperature and aging on metal binding capacity of chitosan films

It was observed (Table 3.5) that acetic and lactic acid films bound 10 times more Cr (VI) than citric acid films. Films stored at 80 ºC although darkened, were stable in aqueous solution and effectively bound Cr (VI), whereas, lactic and citric acid films stored at room temperature (22 ºC) dissolved in Cr solution. The relative ineffectiveness of citric acid films (10 times less than acetic and

Table 3.5: Binding properties of Chitosan films prepared with Acetic, Lactic and Citric Acid.

Acid used in films and storage conditions		% Cr(VI) bound	mg Cr(VI) bound by 1g film
Acetic	22 C	94.7 ± 5.7	0.775 ± 0.10
	80 C	95.1 ± 2.5	0.997 ± 0.14
Lactic	22 C	*films dissolved*	
	80 C	92.3 ± 13.4	1.112 ± 0.20
Citric	22 C	*films dissolved*	
	80 C	9.0 ± 9.2	0.102 ± 0.13

Results are presented as mean values of three determinations ± standard deviation

lactic acid films) can be explained by possible cross-linking of chitosan molecules and by presence of large number of birefringent crystals at high temperature (as shown in result from Polarized microscopy).

3.5. Conclusion

Our results showed accumulation of HMF in chitosan films stored at high temperature and high humidity. UV-Vis and HPLC data along with Hunter colorimetry suggest the accumulation of not only HMF but other compounds, possibly polymerized products of HMF responsible for the darkening of the films. Polarized microscopy showed that high temperatures, especially when combined with high relative humidity, promote development of crytallinity in the films. To be efficiently applied as packaging material, chitosan films should be used for refrigerated products or those stored at room temperature at low humidity conditions.

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Part 4

Mechanical Properties of Chitosan Films During Storage

4.1 Abstract

Chitosan, a deacetylated product of chitin, is a copolymer of D-glucosamine and N-acetyl-D-glucosamine. It exhibits many functional properties and has various applications in food, agriculture, pharmaceutical, and cosmetic industry. However, it has been suggested that potential development of crystallinity in chitosan-based products may reduce biological activity of this biopolymer. Furthermore, the data available on physical characterization of chitosan films is still conflicting. Various attempts for determining the glass transition temperature (Tg') of chitosan films have failed to give conclusive results. Accurate determination of the glass transition temperature of chitosan and mechanical characterization of chitosan-based films under conditions common in the food industry would be beneficial in development of novel

biodegradable food packaging.

Study was conducted for mechanical characterization of chitosan films, prepared with acetic, lactic and citric acid, stored at room temperature in different humidity conditions over a period of one year. Dynamic mechanical analysis showed that citric acid films were stiff while lactic acid films were very flexible even at room temperature. Glass transition temperature of chitosan films (Tg') was found to be between 30 ºC to 40 ºC and the films begun to degrade around 200 ºC. X-ray diffraction analysis of chitosan films did not show considerable crystallinity even after one year of storage at room temperature and ambient laboratory conditions.

4.2 Introduction

Production of plastic packaging materials requires the reliance on petroleum products. As synthetic materials used for packaging are derived from petroleum, there is an urgent need to find alternative packaging materials based on renewable resources (1). With the rising costs of petroleum, cost effective and environmental friendly ways to manufacture packaging materials has become essential.

With the growing demand in the production and use of renewable material sources, natural polymers are replacing synthetic polymers in different applications, mainly because natural polymers are easily biodegradable (2, 3). Chitin, a structural polysaccharide in crustaceans, insects and fungi, is the second most abundant biopolymer on earth, just after cellulose, with estimated biosynthesis reaching around 100 billion tons annually (4). Deacytalated product of chitin, chitosan, gained a lot of attention from scientific community and industry due to its unusual functionality. Presence of degradable enzyme chitosanase that hydrolyses chitosan transforming it to readily available carbon and nitrogen source for various microorganisms, makes chitosan classified as biodegradable natural polymer. Chitosan is composed of β-1, 4-linked 2 acetamino-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose. Chitosan has many functional properties and is useful in making blends and alloys with other polymers (5). Great deal of research has been conducted in medical and

pharmaceutical field because of its nontoxic, nonantigenic, biocompatible, and biodegradable properties allowing its use in various applications, such as implantation (6), injection (7), or ocular implantation (8).

Food applications of chitin, chitosan, and their derivatives were reviewed by Shahidi et al. (9). Chitosan-based membranes have been proposed for use in food processing (10). Chitosan coating has been found to have a potential in extending the post-harvest life and maintaining the quality of longan fruit during storage at low temperature (11). Chitosan has shown antimicrobial activity against both gram-negative and gram-positive organisms, including food borne pathogens. Chitosan films made from dilute acetic acid solution are able to inhibit the growth of *Rhodotorula rubra* and *Penicillium notatum* by direct application of the film on colony-forming organisms. Antagonistic effect against *Escherichia coli*, *Staphylococcus aureus*, and *Saccharomyces cerevesiae* has been displayed by chitosan lactate and chitosan glutamate (12).

The suitable use of edible packaging films strongly depends upon their physical and chemical properties. Many tests have been developed to determine the performances of the film and predict the behavior of the film during shelf-life of a food product.

Glass transition is a second-order phase transition that occurs over the temperature range at which a glassy material enters the rubbery domain with a concomitant drop in the elastic moduli (Young's or shear moduli) (13). Changes

in the mechanical properties of a polymer around Tg are of major practical interest as glasses have high moduli, i.e. they are solid, hard and brittle (14). At temperature above Tg, their molecular mobility exponentially increase and viscosity decrease affecting various physical properties (15, 16, 17). Extensive research by many authors has shown the importance of precise determination of glass transition temperature, Tg, in understanding the physical state and physicochemical properties of food materials (17-25). However, the data available on physical characterization of chitosan films is still conflicting. Various attempts for determining the glass transition temperature of chitosan films have failed to give conclusive results. Thus, temperatures ranging from -23 to 67 ^{o}C (26), 110 to 140 ^{o}C (27), and 202 ^{o}C (28, 29) have been reported. Accurate determination of the glass transition temperature of chitosan and mechanical characterization of chitosan-based films under conditions common in the food industry would be beneficial in development of novel biodegradable food packaging.

Furthermore, since development of crystallinity in chitosan-based products has been proposed to reduce biological activity of this biopolymer (30), determination of optimum conditions for development of crystallinity in chitosan films would allow to target modifications of the films and prediction of potential changes in their functional properties during processing and storage.

4.3 Materials and methods

4.3.1. Preparation and aging of chitosan films

Chitosan films were prepared by casting method. The film forming solution was prepared by mixing 1 g flakes of high molecular weight chitosan, Brookfield viscosity of 800 cps (Sigma Aldrich, St. Louis, MO) in 90 g deionized water. The mixture was heated and boiled for 2 minutes with continuous stirring, cooled to approximately 50 ^{o}C, and 10 g of 10% acid solutions – acetic, lactic or citric acid was added, to achieve 1 % w/w chitosan concentration. Solutions were stirred over night and filtered through miracloth filter (Calbiochem, Santa Barbara, CA). Casting of films was done under ambient conditions by drying 20 ± 1 g of chitosan solution in petri-dishes (50 mm x 15 mm). For each of three acids, a set of 6 films was prepared. The films were either (a) analyzed as freshly prepared (within 2 days from being dried); (b) freeze dried immediately and analyzed the same day after being dried; (c) kept at room temperature (22 ºC) and 75 % relative humidity for 3 months; (d) kept in a desiccator (20 % RH) at room temperature for 3 months; (e) kept in ambient laboratory conditions for 3 months; or (f) stored for 1 year at room temperature and 20 % relative humidity.

4.3.2. Scanning electron microscopy

Hitachi S-4300SE/N scanning electron microscope was used to visually analyze the films. Films samples were cut with scissors and carbon-based tape with conductive glue was used to attach the samples to aluminum sample holder. Secondary electron (SE) signal with beam energy of 1.0 kV, working distance of 11.2 μm and magnification of 400 to 800 x magnification was used for film observation. Sample was tilted 30 degrees to the stage normal.

4.3.3. Thermo gravimetric analysis (TGA)

Mettler Toledo TGA SDTA 851e (Columbus, OH) was used to conduct thermo gravimetric analysis of chitosan films. Films samples were kept in an aluminum oxide crucible of 70 μl. The sample was first held at 40 ºC for 5 min and then heated from to 300 ºC at heating rate of 10 ºC/min. To prevent extensive degradation due to oxidation, the experiment was conducted in nitrogen environment.

4.3.4. Dynamic mechanical analysis (DMA)

Dynamic mechanical analyzer (DMA, Q800, TA Instruments, New Castle, DL), in the High Temperature Materials Laboratory (HTML) at the Oak Ridge National Laboratory (Oak Ridge, TN), was used to test the mechanical properties of the films. Rectangular film samples (5 x 50 mm) were mounted onto tension-

type clamp. Temperature ramp/frequency sweep mode at frequency of 10 Hz at the preload force of 0.5 N was applied for all samples. The temperature range was -100 ^{o}C to 200 ^{o}C with the heating rate of 3 ^{o}C/min. Isothermal segment at -100 ^{o}C for 5 min was set to equilibrate the samples before the measurement. The amplitude of 15 μm was applied for all acetic acid films, 10 μm for freeze dried lactic acid films, 7.5 μm for fresh lactic acid films, and 5 μm for 3-weeks old lactic acid films. Citric acid films were brittle and only fresh films and those kept at 70% relative humidity could be mounted onto clamps; the minimum amplitude (5 μm) was applied for these films. Three samples of each film type were analyzed and storage modulus, loss modulus, tan δ, and change in length were recorded.

4.3.5. Differential scanning calorimetry

Differential Scanning Calorimetry (DSC) analysis of polymer film samples was carried out using the Mettler Toledo DSC 822 (Columbus, OH). Tests were carried out in temperature range -100 ^{o}C to - 200 ^{o}C, at a heating rate of 3 ^{o}C/min in the nitrogen atmosphere. Sample films were placed in 40 μl Al-cups and sealed. A small hole was made on the top of the cup in order to allow release of water vapor. Weight of the samples was accurately determined before and after the analyses. An experiment consisted of four segments, starting with holding the sample isothermal at -100 ^{o}C for 5 min, segment 2 from -100 ^{o}C to +100 ^{o}C at a

heating rate of 3 ^{o}C/min, segment 3 cooling from +100 ^{o}C to -100 ^{o}C at the rate of 10 ^{o}C/min, and last segment was heating from -100 ^{o}C to +200 ^{o}C at the rate of 3 ^{o}C/min. Cooling was done with the help of liquid nitrogen and gaseous nitrogen was used to maintain inert environment in order to prevent oxidation of the sample. Three samples of each film type were analyzed.

4.3.6. Thermo mechanical analysis (TMA)

Thermomechanical analyzer (TMA SDTA 840, Mettler Toledo, Columbus, OH) was used to conduct thermo mechanical analysis of chitosan films. For each measurement, a sample was kept between 2 fused silica disks first isothermally at 30 ºC for 5 min and then heated to 250 ºC at heating rate of 10 ºC/min. A 3 mm ball point probe with a load of 0.1 N was used for all the samples. To maintain the inert environment and prevent film oxidation, gaseous nitrogen was used.

4.3.7. Wide angle X-ray crystallography

Wide angle X-ray diffraction was conducted using a Molecular Metrology SAXS/WAXD system (Northampton, MA) equipped with a monochromic CuKα (1.5418 Å) X-ray source, a three-pin-hole alignment, and two-dimensional detector operating at 45 kV and 0.66 mA with the 30 μm beam. The WAXD patterns were recorded on reusable Fuji image plates, with the sample-to-film

distance was 36.52 mm. The image plate was scanned in a FujiX BAS-1800II image analyzer and the resultant image was converted to intensity versus 2θ or q plot using Polar X-ray analysis software.

4.3.8. Karl-Fisher analysis

The moisture content in fresh films was analyzed using a coulometric Karl Fisher titrator (Denver Instruments, Aurora, CO). To analyze the moisture content, 0.2 g of film was dipped in 10 ml absolute alcohol overnight to extract residual water from the film into the alcohol. The known quantity of ethanol was injected with the help of syringe into the titrator. Absolute alcohol was used as a control. The value of moisture in control was deducted from value of moisture in alcohol with film.

4.4 Results and discussion

4.4.1 Scanning electron microscopy

Scanning electron microscopy was conducted on fresh chitosan films to visually evaluate homogeneity of the films and observe potential structural variations among the film types. There were no differences observed on the surface of the films. The transverse section of the films showed differences in the cut pattern. Cross-section of acetic acid films had vertical patterns (Figure 4.1,

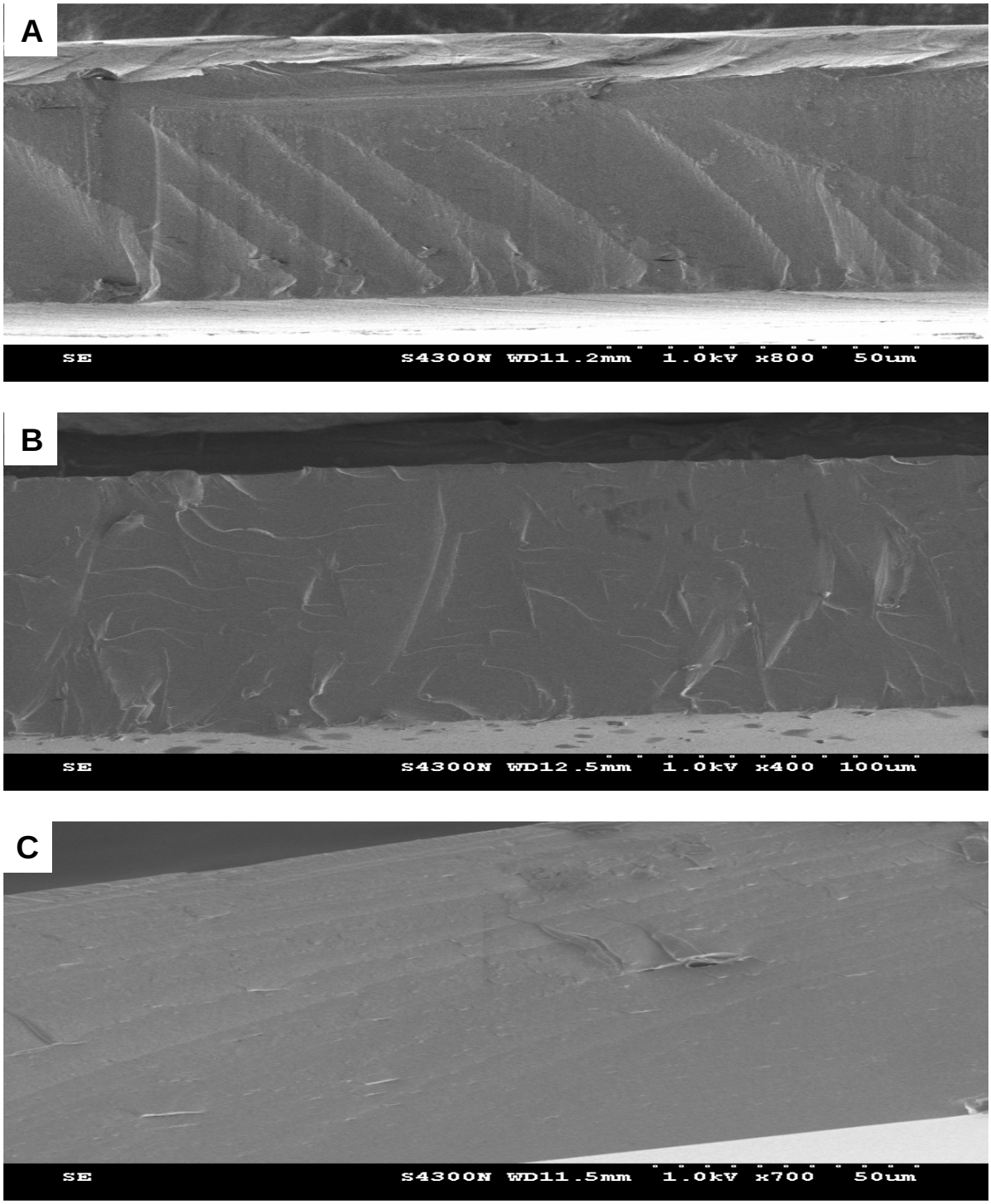

Figure 4.1. SEM micrographs of Transverse Section of Fresh dried Acetic Acid (A), Lactic Acid (B) and Citric Acid (C) films.

A), lactic acid films showed random patterns (Figure 4.1, B), whereas citric acid films had more horizontal patterns (Figure 4.1, C). Overall, films of all types appeared to be homogenous.

4.4.2 Thermo gravimetric analysis

Thermo-gravimetric analysis was conducted on fresh films to determine onset of thermal degradation of chitosan films (Figure 4.2). First derivative of the experimental data showed the endothermic peaks occurred with the increase of temperature, what corresponded to the weight loss either due to evaporation or degradation (Figure 4.2, B). The first endothermic peak in all films occurred between 75 and 100 ºC due to evaporation of water. However, in case of acetic acid films, the first peak occurred at 90 °C and may be the result of simultaneous evaporation of water and acetic acid (boiling point 112 ºC, 31). The second endothermic event in acetic acid films was detected at 165 °C and was probably due to thermal degradation of residual acid molecules. The onset of the second endothermic event in lactic acid films was at 99 °C and the peak was reached at 139 °C. This event most likely resulted from combined effects of evaporation (boiling point 122 ºC, 31) and thermal degradation of residual lactic acid. In case of citric acid films, the second peak occurred later as citric acid is non-volatile. The onset of this peak happened at 125 °C indicating melting of the acid (153 ºC,

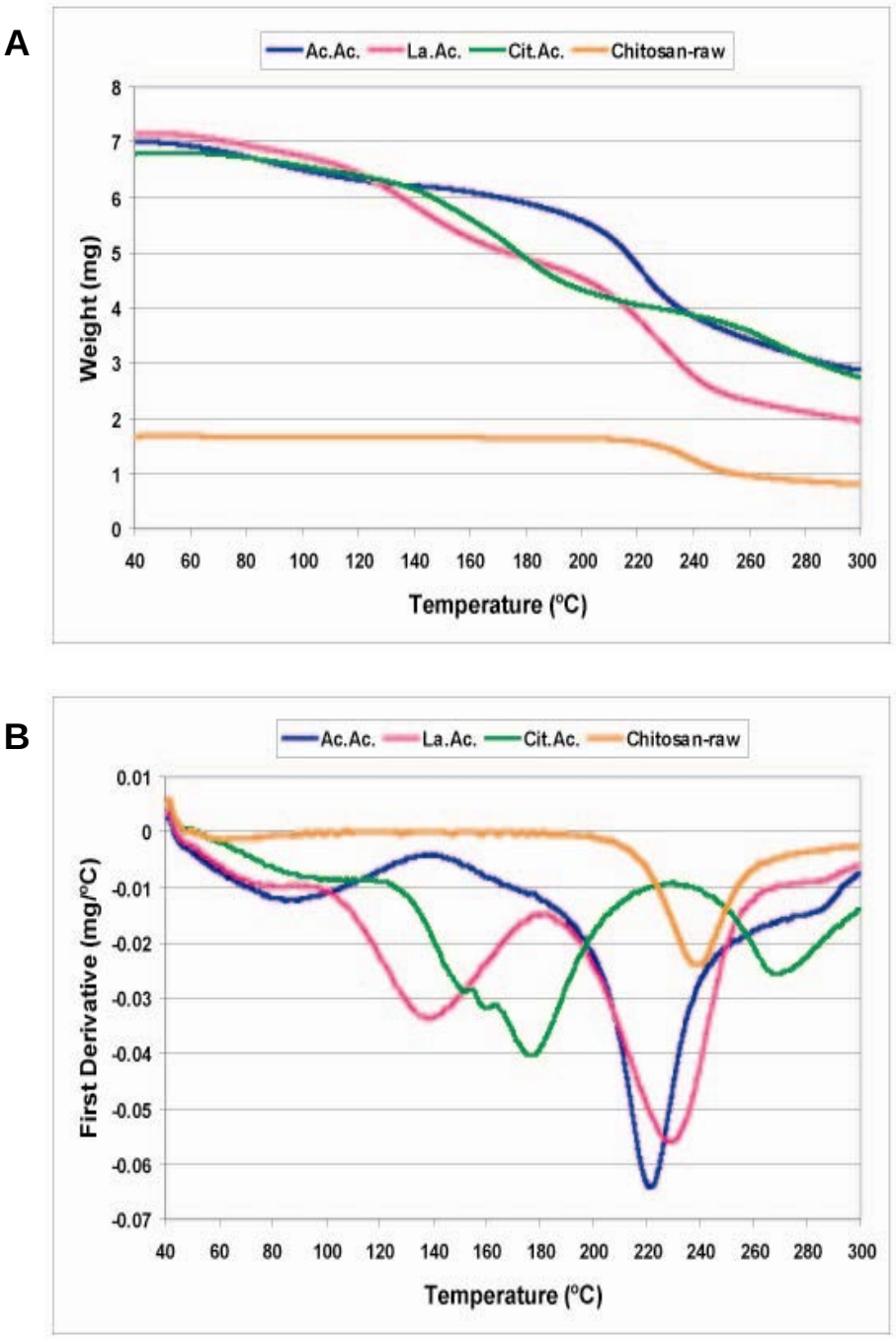

Figure 4.2. TGA thermogram of Fresh dried Chitosan films (A) and their 1st derivative (B).

31) while the minimum, reached at 176.5 °C, was result of degradation of this organic acid. There was no peak observed at any of these temperatures in case of pure chitosan powder confirming the effect of water and acid used in the preparation of films.

Pure chitosan was thermally degraded at 240 ºC (with the onset at 195 °C). However, the chitosan films prepared with acetic, lactic, and citric acid degraded at 223, 231, and 270 °C, respectively. Degradation of acetic and lactic acid films at lower temperatures was possibly because the molecules of these acids acted as spacers while delay in degradation of citric acid films seems to be due to cross-linking of polymer chains.

4.4.3 Dynamic mechanical analysis

As expected, dynamic mechanical analysis (DMA) of all the films showed decrease in storage modulus (E') as the temperature. The major drop in the storage modulus in all three types of the films occurred around 0 °C due to melting of water crystals. Comparing the E' values of fresh films at 4, 22 and 100 °C (Table 4.1) among the films, it can be noticed that although citric acid films were the most stiff at 4 °C (11 GPa) compared to acetic (5.6 GPa) and lactic (1.5 GPa) acid films, they behaved similar to acetic acid films at room temperature (4.2 and 4.7 GPa for acetic and citric acid films, respectively), but were

Table 4.1: Storage modulus (GPa) of Chitosan Fresh dried, Fresh Freeze dried (FD), stored for 3 months at 22 ºC and 20 and 70 % RH, and for 1 year at ambient conditions, films.

	Fresh			Fresh FD			22ºC + 20% RH			22ºC + 70% RH			1 year		
	4ºC	22ºC	100ºC	4ºC	22ºC	100ºC	4ºC	22ºC	100ºC	4ºC	22ºC	100ºC	4ºC	22ºC	100ºC
Acetic Acid	5.6	4.2	3.8	5.9	4.6	3.7	13	10	7.2	8.4	7.4	6.9	7.3	6.6	5.6
Lactic Acid	1.5	0.3	0.1	3.7	1.2	0.2	5.0	1.7	0.3	6.6	3.8	2.0	7.2	3.3	0.6
Citric Acid	11	4.7	0.03	-	-	-	-	-	-	13.5	8.3	0.09	-	-	-

considerably softer than other two film types at 100 °C (0.03 GPa compared to 3.8 and 0.1 GPa for citric, acetic and lactic acid films, respectively).

All films investigated in this study became considerably stiffer as they aged. However, there was no clear trend between values of E' and conditions of ageing. Thus, storage modulus of acetic acid films at room temperature changed from 4.2 GPa in fresh films to 10.0, 7.4, and 6.6 GPa in 3 months old films stored at 20 and 70 % RH and one year at 20 % RH, respectively. Citric acid films were generally very brittle and the dynamic mechanical analyses could not be performed on some samples as the films disintegrated during cutting and/or mounting on the DMA clamps.

Tan δ curves (Figure 4.3 B, 4.4 B, and 4.5 B) were expected to have only one peak corresponding to glass transition temperature but for all tested films the curves had more than one major peak. Thus, acetic acid films had peaks at 0,

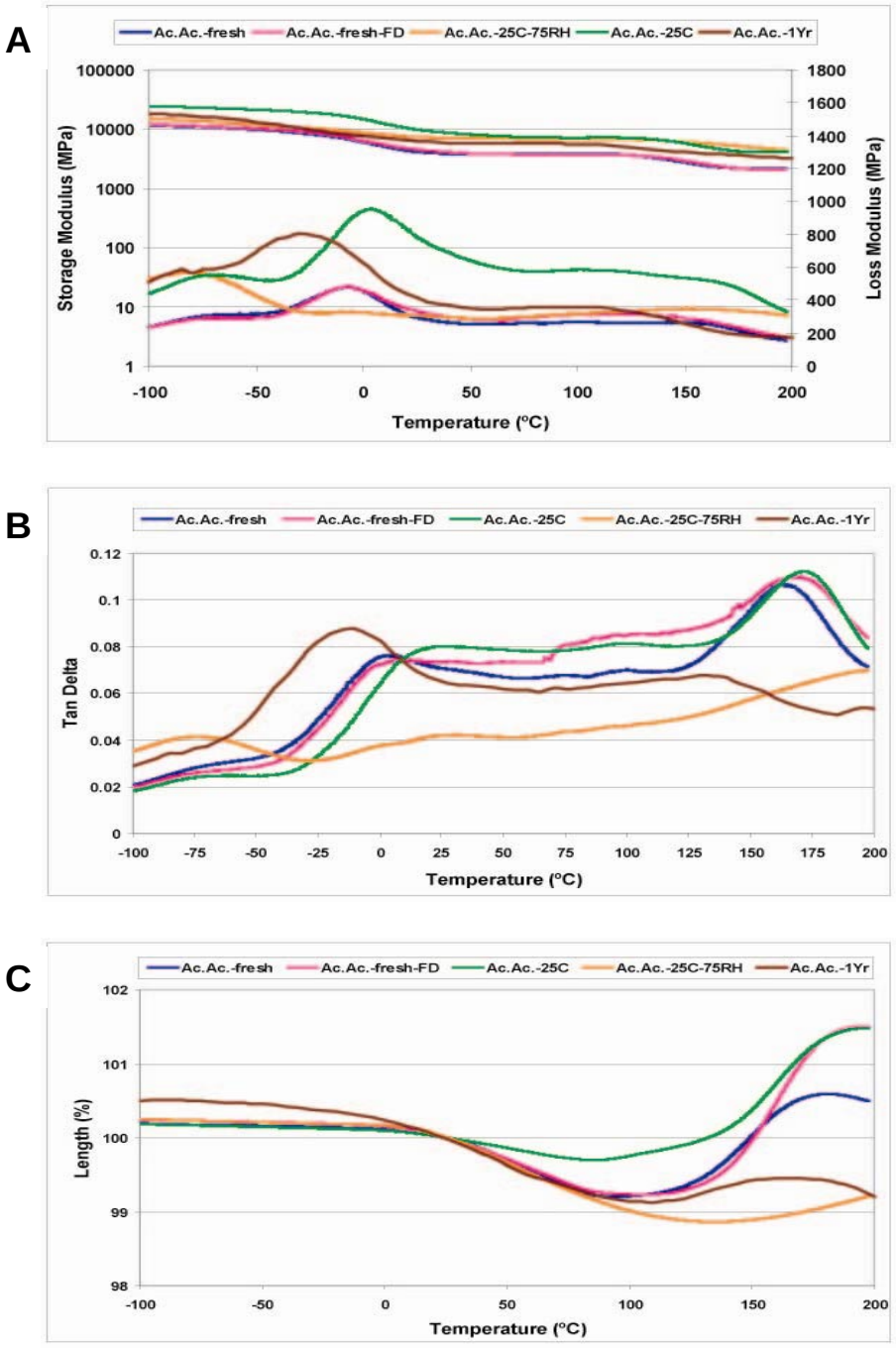

Figure 4.3. DMA thermogram (A), tan δ (B) and percent length (C) of Acetic Acid Fresh dried, Fresh Freeze dried (FD), stored for 3 months at 22 ºC and 20 and 70 % RH, and for 1 year at ambient conditions, films.

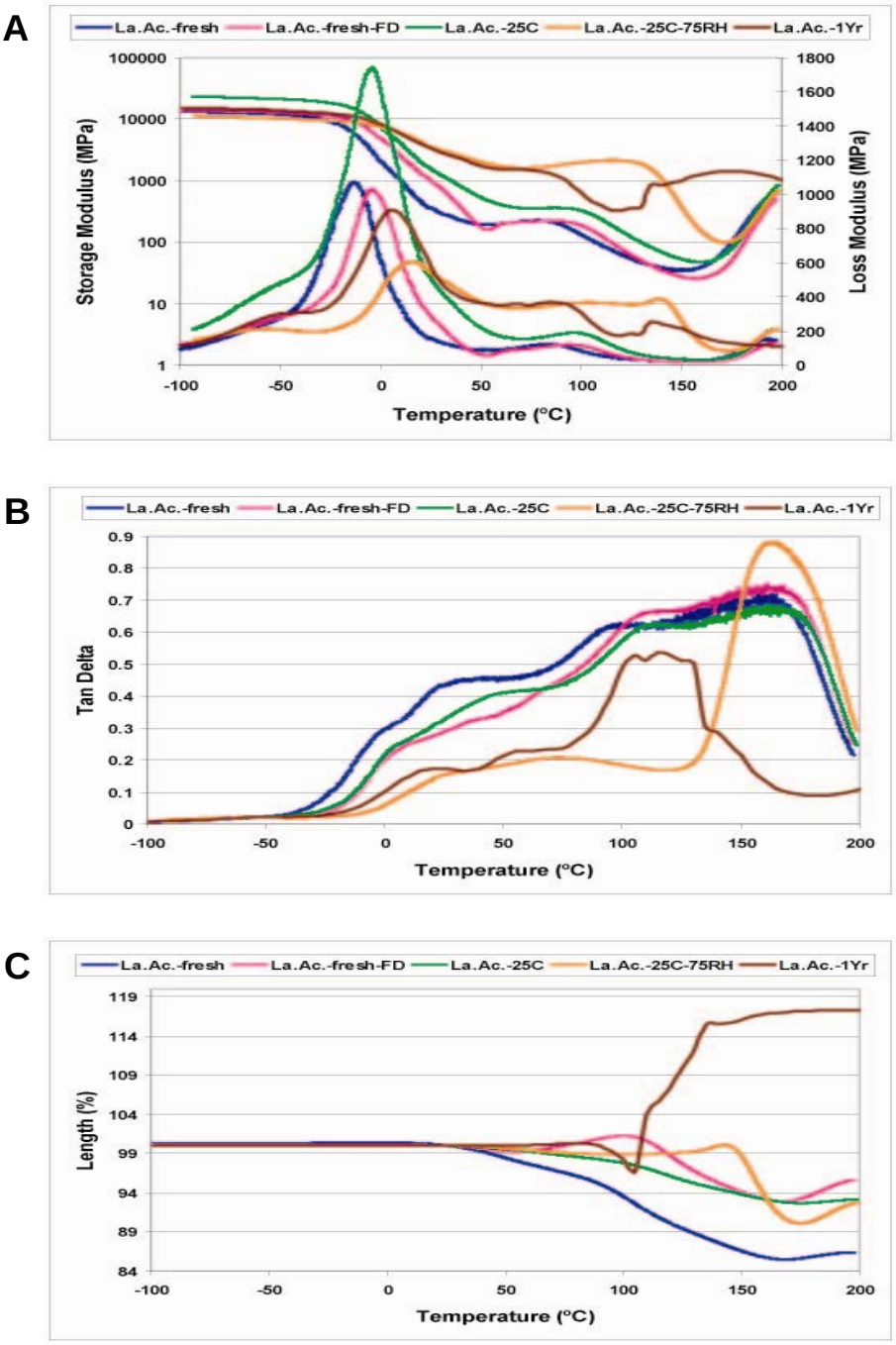

Figure 4.4. DMA thermogram (A), tan δ (B) and percent length (C) of Lactic Acid Fresh dried, Fresh Freeze dried (FD), stored for 3 months at 22 ºC and 20 and and 70 % RH, and for 1 year at ambient conditions, films.

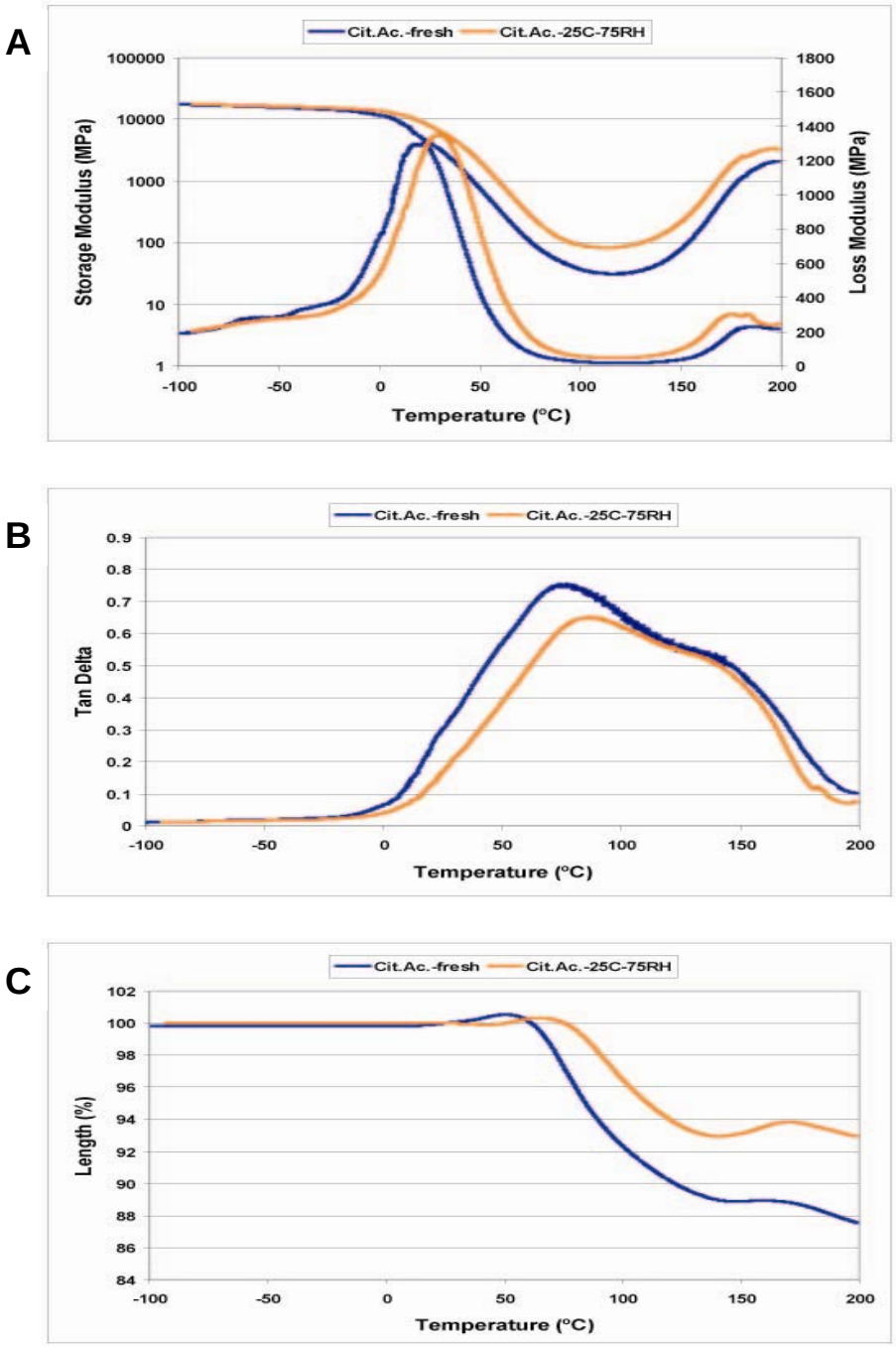

Figure 4.5. DMA thermogram (A), tan δ (B) and percent length (C) of Citric Acid Fresh dried and stored for 3 months at 22 ºC and 70 % RH, films.

40, 100 and 170 °C. The peaks at 0 and 100 °C corresponded to melting and evaporation of ice and water, respectively, at 170 ºC to thermal degradation of chitosan, and the peak at around 40 ºC probably to β relaxation in chitosan film, or Tg'.

The length of the films during the DMA analysis decreased at first due to evaporation of water and consequent shrinking of the films, but than increased at temperatures above 160 °C possibly due to chitosan degradation and breaking of intra and intermolecular bonds (Figure 4.3 C, 4.4 C, and 4.5 C).

4.4.4 Differential scanning calorimetery

Fresh chitosan films had relatively large amounts of residual water (Table 4.2) what affected observation of true thermal changes and detection of Tg'. To reduce the amount water in the films, DSC was run with two heating segments, first from -100 ºC to 100 ºC to evaporate residual water and second from -100 ºC to 200 ºC.

Table 4.2: Moisture percent in Fresh Chitosan films analyzed by Karl Fisher Method

	Acetic Acid	Lactic Acid	Citric Acid
Fresh	14.52% (0.00)	10.91% (0.00)	1.72% (0.00)

During the first heating segment of fresh films (Figure 4.6 A), endothermic peaks were observed at 65, 73, and 75 ºC in lactic, acetic, and citric acid films, respectively. These peaks were most likely result of water evaporation (32) and were not seen in the second run. When the same films were run again (the second segment), acetic acid films had endothermic peaks at 102 and 169 ºC. The first peak was probably due to evaporation of free residual acid molecules (boiling point of acetic acid 112 °C) and the second was due to thermal degradation of chitosan. Thermal degradation of the films was evident by the burnt appearance of the samples after the DSC runs as well as by the weight losses beyond the weight of residual water in the films (Table 4.3). Lactic and citric acid films had peaks only at 160 and 166 °C, respectively showing degradation of carbohydrate polymer. However, step transition at 41 and 42 °C was noticed in thermographs of acetic and lactic acid films, respectively, indicating glass transition temperature in these films.

Similar to fresh acetic acid films, all the films prepared with this acid and stored under different conditions had a dominant endothermic peak between 61 and 73 °C in the first run due to evaporation of water (Figure 4.7 A). In the second run, however, all the samples exhibited three events, first as a step transition at 47 °C, second as endothermic peak at 105 - 109 °C, and third at 170 - 179 °C (Figure 4.7, B). Interestingly, there was no trend between time and conditions

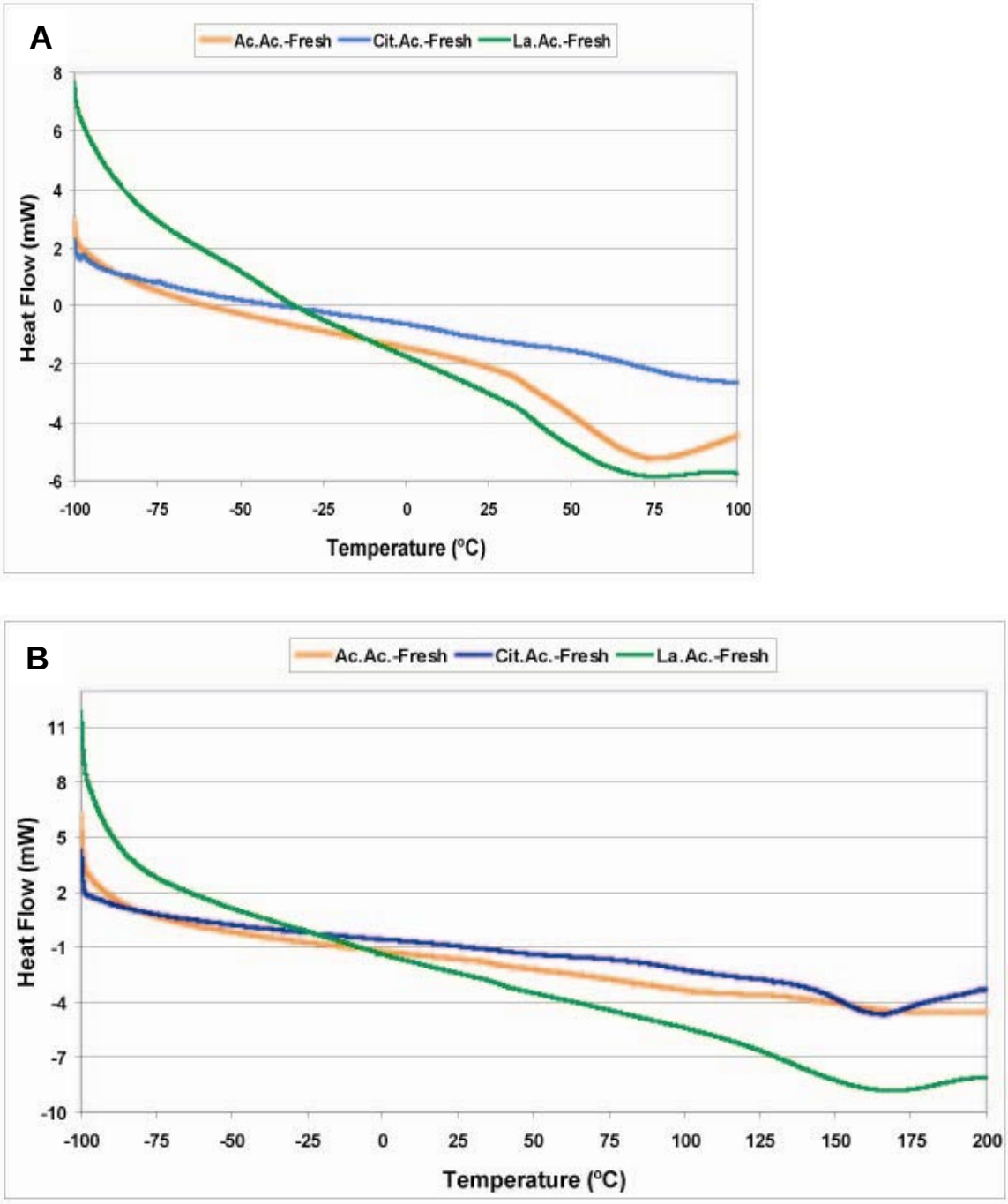

Figure 4.6. DSC thermogram, segment 1 (A) and segment 2 (B) of Fresh dried films.

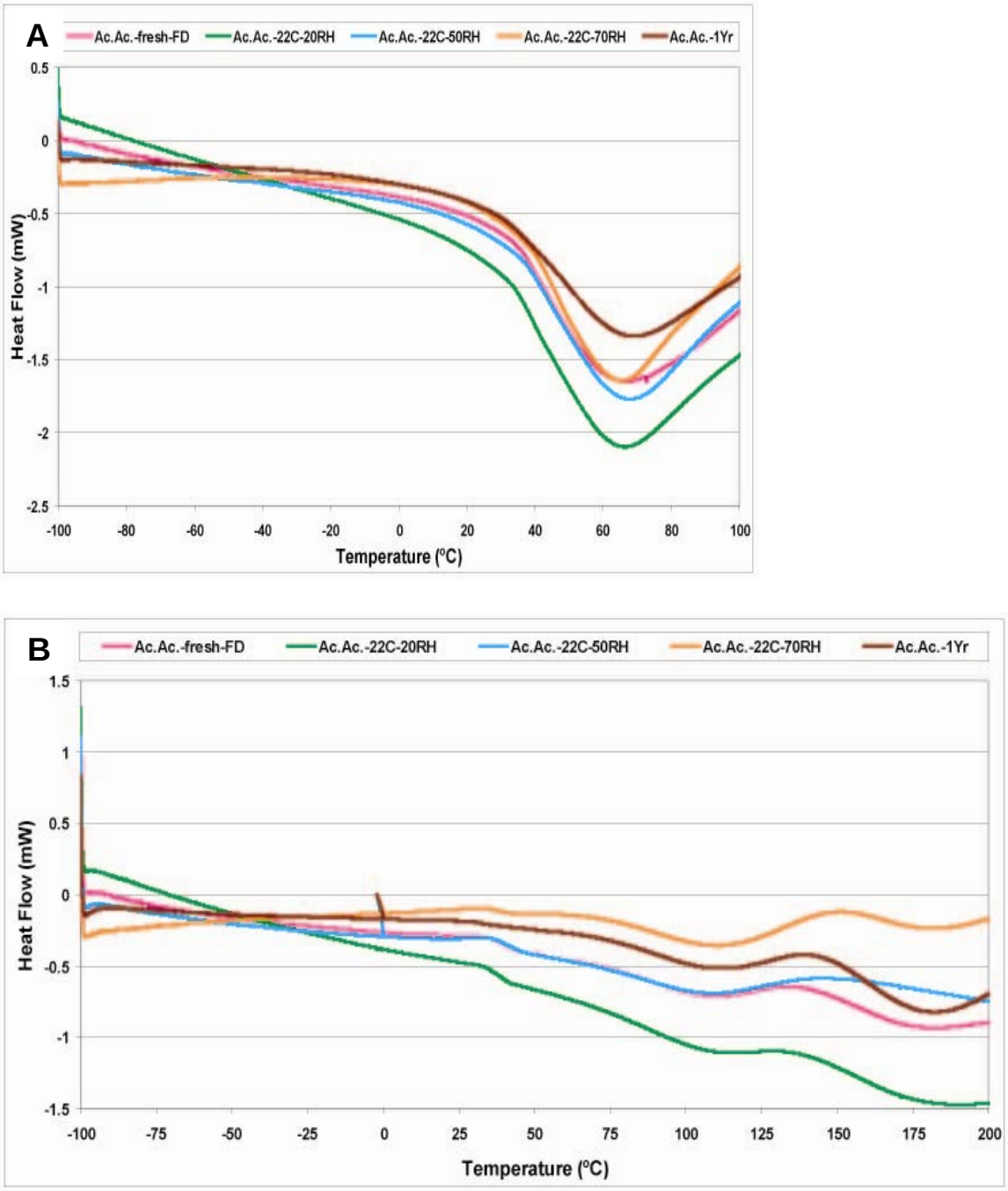

Figure 4.7. DSC thermogram, segment 1 (A) and segment 2 (B) of Acetic Acid Fresh Freeze dried (FD), stored for 3 months at 22 ºC and 20, 50 (ambient) and 70 % RH, and for 1 year at ambient conditions, films.

Table 4.3: Weight loss in Chitosan Fresh dried, Fresh Freeze dried (FD), stored for 3 months at 22 ºC and 20, 50 (ambient) and 70 % RH, and for 1 year at ambient conditions, films at the end of DSC.

	Acetic Acid	Lactic Acid	Citric Acid
Fresh	21.5% (0.94)	30.6% (2.29)	24.9% (0.44)
Fresh-FD	21.7% (2.81)	39.2% (13.26)	28.2% (0.34)
20% RH	19.6% (1.47)	28.5% (0.97)	24.6% (3.69)
50% RH	15.5% (1.13)	18.4% (2.9)	24.8% (1.06)
70% RH	24.8% (6.9)	27.7% (1.96)	26.6% (2.98)
1 year	20.0% (1.94)	21.5% (4.98)	23.3% (0.76)

of storage and the temperature at which the peaks occurred. In other words, the range of peak temperatures was quite narrow (e.g., 105 to 109 °C) and the differences were not significant indicating that no detectable changes in thermal properties of these films happened within one year storage at room temperature regardless on the relative humidity of ambient air.

Figure 4.8 shows the DSC thermographs of lactic acid films that were stored for three months and one year at different conditions. As with the acetic acid films, these thermographs had one endothermic peak at 60 - 68 °C in the first run (Figure 4.8, A), and three notable regions, at 39 - 51 °C, 107 - 108 °C, and 159 - 175 °C in the second segment (Figure 4.8, B).

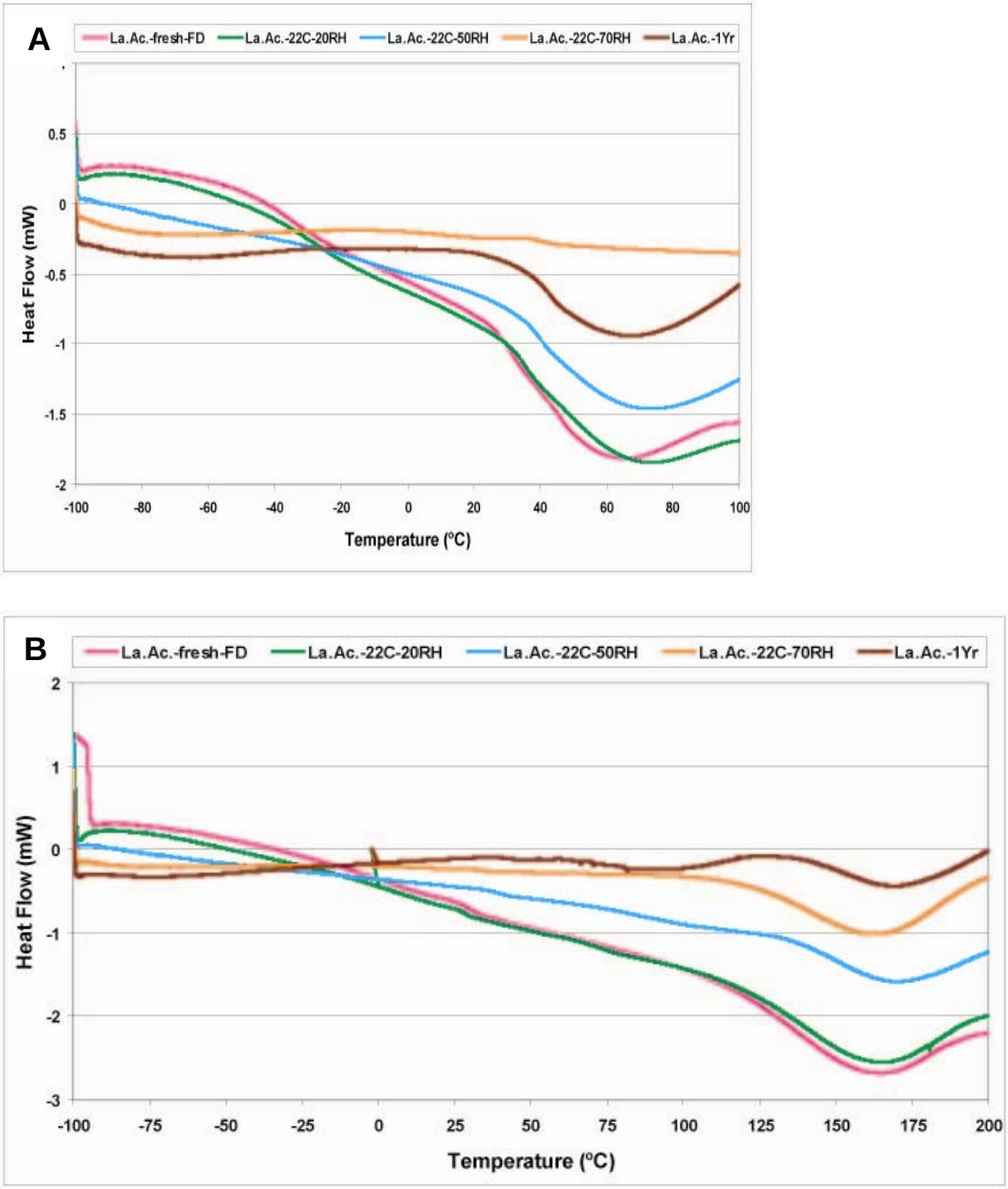

Figure 4.8. DSC thermogram, segment 1 (A) and segment 2 (B) of Lactic Acid Fresh Freeze dried (FD), stored for 3 months at 22 ºC and 20, 50 (ambient) and 70 % RH, and for 1 year at ambient conditions, films.

In case of aged citric acid films, endothermic peak in first run was detected at 75 - 84 °C (Figure 4.9, A). This shift to higher temperature range compared to films prepared with other acids may be result of cross-linking of chitosan molecules by carboxyl groups of citric acid what slower the water mobility. In the second run, step transition happened at 39 - 52 °C, first endothermic peak at 106 - 111 °C and the second peak at 161 - 164 °C.

Based on our results, glass transition temperature in chitosan films appears to be between 39 and 52 °C, depending on the acid used for film preparation. These values are different than those found in literature. However, the Dong et al. (2004) and Sakurai et al. (2000) used chitosan films dehydrated in diethyl ether and lyophilized prior to mechanical analyses (33, 29). Since all the residual water was eliminated from the films, the Tg values determined by these authors were 140 - 150 °C and 203 °C, respectively. Thus, it appears that heating in our first DSC run eliminated only free water and the residual bound water served as a plasticizer decreasing the Tg to 40 - 50 °C. Nevertheless, our results are of great practical importance since the biodegradable films that can be used in food industry, such as chitosan films, if extensively dried during preparation, would absorb water from the ambient air regardless how low relative humidity was. This absorbed water would, in turn, shift the Tg to lower temperatures making the films more susceptible to physical and chemical changes.

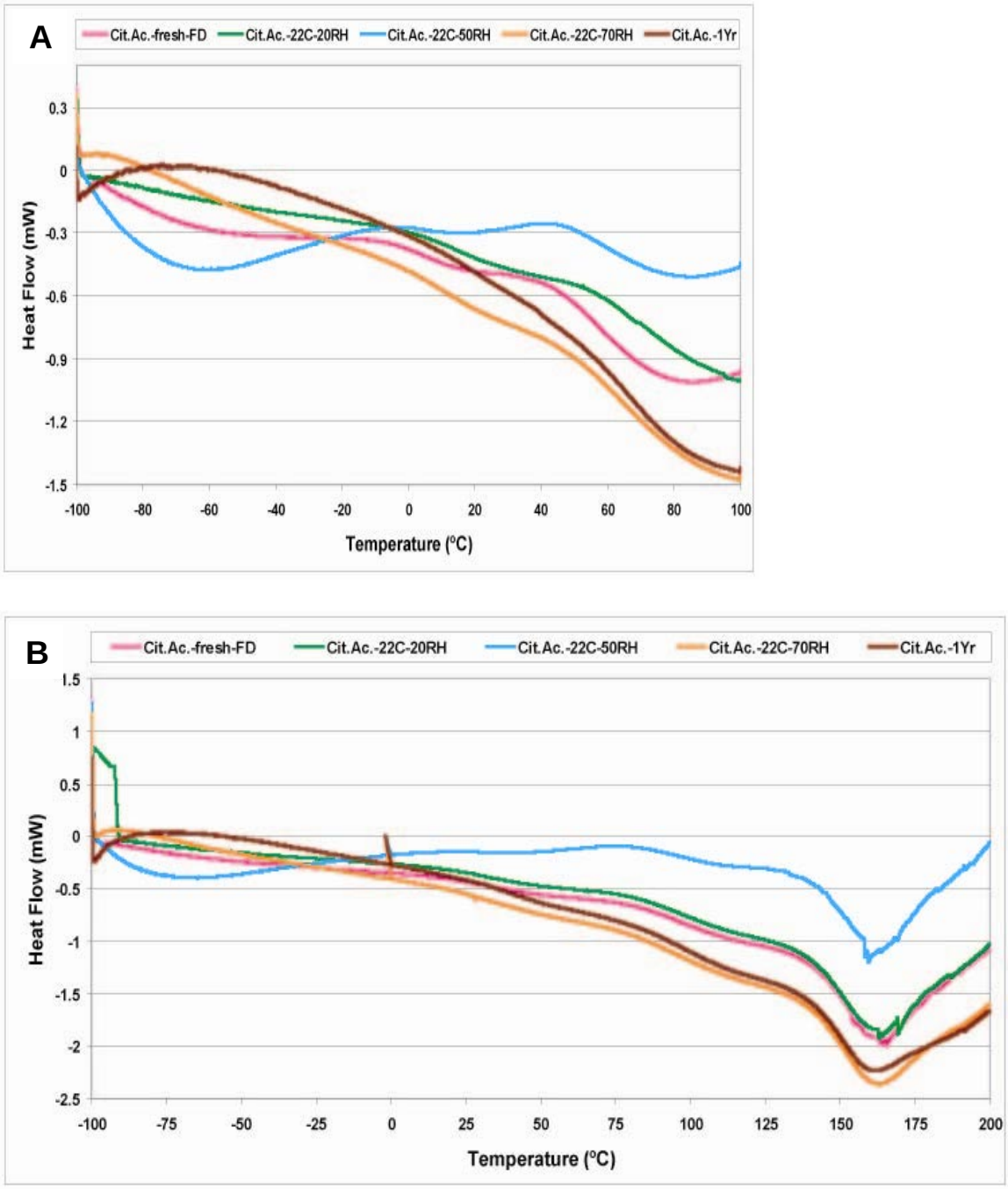

Figure 4.9. DSC thermogram, segment 1 (A) and segment 2 (B) of Citric Acid Fresh Freeze dried (FD), stored for 3 months at 22 ºC and 20, 50 (ambient) and 70 % RH, and for 1 year at ambient conditions, films.

4.4.5 Thermo mechanical analysis

Thermo mechanical analysis of fresh films (Figure 4.10) showed that the lactic acid films were the thickest (180 μm) and acetic acid films were the thinnest (73.3 μm) of the three. It was also observed that there was least deformation in acetic acid films and maximum deformation in citric acid films especially around the temperature of 190 ºC. There was continuous decrease in the thickness of the films during heating, initially due to evaporation of water and later, above 160 °C, due to degradation of chitosan molecules.

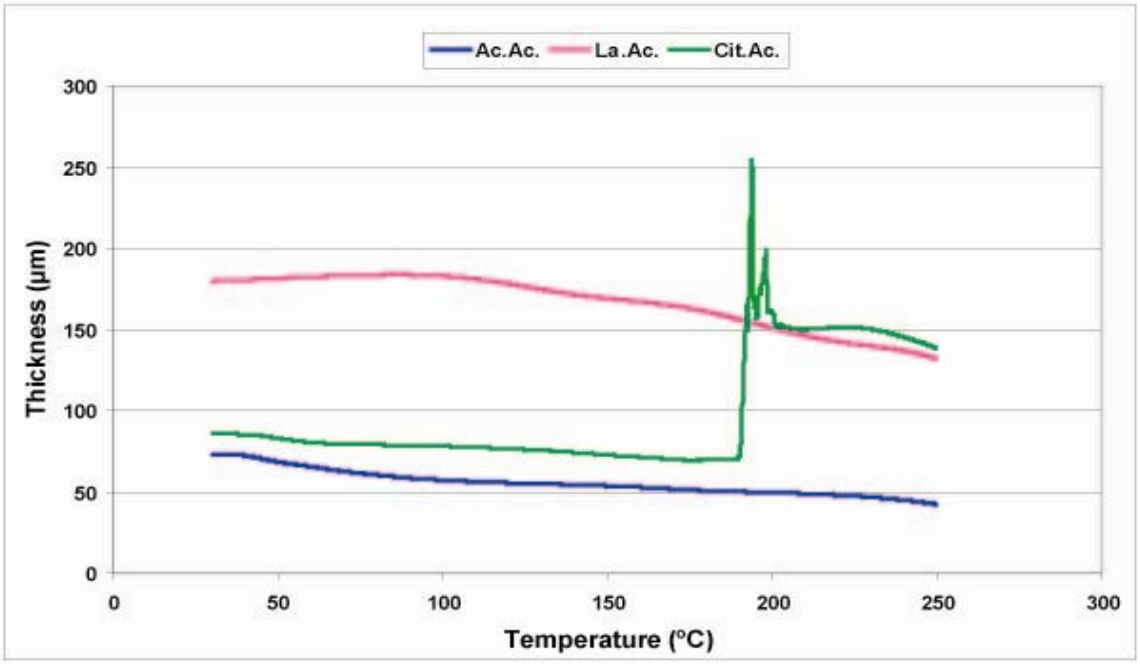

Figure 4.10. TMA thermogram of Fresh dried Chitosan Films.

4.4.6 X-ray diffraction

X-ray crystallography of acetic (Figure 4.11, A), lactic (Figure 4.11, B) and citric acid films (Figure 4.11, C) that were stored for one year at different environmental conditions, showed the absence of crystallinity development in the films. Although the chitosan flakes usually give crystalline peak at diffraction angle of 20° due to relatively regular crystal lattice that remains from original chitin arrangement (34, 35), the broad peak at 2θ = 20.2° detected in our films was too broad and of minor intensity that could not be identified as occurrence of crystallinity.

4.5 Conclusion

Our results show that there was no development of crystallinity in chitosan films during 3 months storage at 22 °C at different relative humidity or even after one year at ambient conditions. Glass transition temperature (Tg') of chitosan films seems to lie between 40 ºC to 50 ºC. Acetic acid films seem to be best suitable as packaging films since they were most mechanically and thermally stable in a temperature range of 4 ºC to 100 ºC, where as lactic acid films are too soft and citric acid films are too stiff at low temperatures and significantly soften as the temperature increased.

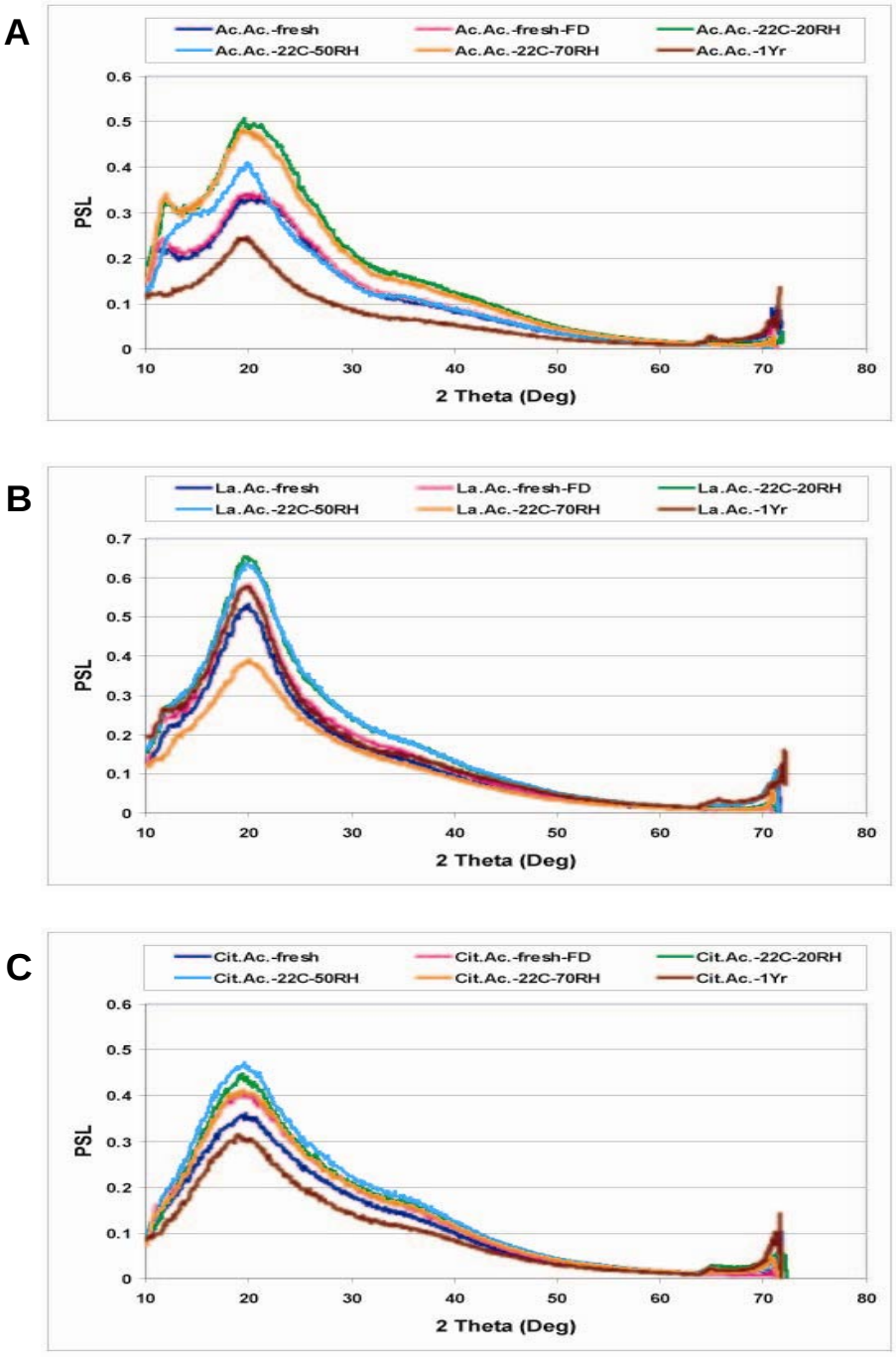

Figure 4.11. X-ray diffractogram of Acetic Acid (A), Lactic Acid (B) and Citric Acid (C) Fresh dried, Fresh Freeze dried (FD), stored for 3 months at 22 ºC and 20, 50 (ambient) and 70 % RH, and for 1 year at ambient conditions, films.

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Part 5

Overall Conclusions

The studies we conducted to evaluate color and mechanical properties of chitosan films stored in different environments have shown that chitosan films prepared with different acids stay chemically stable for a minimum of one year if stored at refrigeration or room temperatures. However, darkening, accumulation of hydroxymethylfurfural (HMF), and development of crystallinity take place if the films are kept at high temperature (80 °C). Neutralization of the films immediately after casting slows down darkening at high temperatures.

The glass transition temperature (Tg') of the chitosan films seems to lie between 40 °C to 50 °C depending on the acid used for film preparation. However, the Tg' could be detected only after the residual water was evaporated from the films confirming significant plasticizing effect of water and acid. Storage modulus (E') of the films was affected by the acid and length of storage. Among the fresh films, citric acid films appeared to be the most stiff (E' 4.7 GPa) while

lactic acid films were the softest (E' 0.3 GPa). However, fresh citric acid films extensively softened as temperature increased but become brittle during storage. The mechanical properties of acetic acid films changed the least over the temperature range from 4 °C to 100 °C regardless on the age of the films.

Our studies have shown that chitosan films prepared with acetic acid were more chemically and mechanically stable than films prepared with lactic or citric acid. Citric acid films seem to be inadequate for packaging material as their physical properties considerably change with temperature and age. Although lactic acid films may have application due to their flexibility, the films darken rapidly and neutralization of the films only reduces the process.

Chitosan films prepared with acetic acid by casting have potential to be used as packaging material for food products to be stored at cold and dry conditions.

APPENDIXES

Appendix A – Mechanism of color development in chitosan films during storage

Table A-1: Change in a-value of regular (A) and neutralized (B) Chitosan films prepared in Acetic Acid, kept for 5 months in different environments.

A	Fresh	1 Month	2 Month	3 Month	5 Month
4ºC	-0.99 ± 0.05	-1.25 ± 0.05	-1.20 ± 0.04	-1.28 ± 0.08	-1.41 ± 0.05
4ºC + 70% RH	-0.99 ± 0.05	-1.31 ± 0.06	-1.25 ± 0.07	-1.16 ± 0.04	-1.45 ± 0.09
22ºC	-0.99 ± 0.05	-1.33 ± 0.04	-1.44 ± 0.05	-1.38 ± 0.06	-1.53 ± 0.04
22ºC + 70% RH	-0.99 ± 0.05	-1.41 ± 0.07	-1.32 ± 0.11	-1.41 ± 0.07	-1.72 ± 0.08
80ºC	-0.99 ± 0.05	-0.54 ± 0.34	6.27 ± 1.34	12.01 ± 1.70	16.68 ± 1.83
80ºC + 70% RH	-0.99 ± 0.05	34.19 ± 5.56	20.00 ± 9.81	19.35 ± 1.33	3.14 ± 2.23

B	Fresh	1 Month	2 Month	3 Month	5 Month
4ºC	-1.35 ± 0.12	-1.21 ± 0.10	-1.10 ± 0.06	-1.08 ± 0.06	-1.20 ± 0.04
4ºC + 70% RH	-1.35 ± 0.12	-1.37 ± 0.04	-1.33 ± 0.03	-1.16 ± 0.09	-1.30 ± 0.02
22ºC	-1.35 ± 0.12	-1.16 ± 0.06	-1.20 ± 0.02	-1.22 ± 0.04	-1.58 ± 0.55
22ºC + 70% RH	-1.35 ± 0.12	-1.29 ± 0.11	-1.24 ± 0.06	-1.40 ± 0.10	-1.78 ± 0.10
80ºC	-1.35 ± 0.12	-1.89 ± 0.05	-2.61 ± 0.25	-2.59 ± 0.44	-2.97 ± 0.31
80ºC + 70% RH	-1.35 ± 0.12	30.69 ± 7.37	19.08 ±3.95	16.69 ±3.60	8.81 ± 4.34

Table A-2: Change in b-value of regular (A) and neutralized (B) Chitosan films prepared in Acetic Acid, kept for 5 months in different environments.

A	Fresh	1 Month	2 Month	3 Month	5 Month
4°C	9.35 ± 0.65	8.74 ± 0.83	8.66 ± 0.30	9.32 ± 0.63	9.07 ± 0.72
4°C + 70% RH	9.35 ± 0.65	8.86 ± 0.42	8.86 ± 0.41	8.61 ± 0.42	8.64 ± 0.54
22°C	9.35 ± 0.65	9.27 ± 0.30	9.23 ± 0.25	9.67 ± 0.39	9.31 ± 0.36
22°C + 70% RH	9.35 ± 0.65	9.51 ± 0.33	9.04 ± 0.76	9.25 ± 0.52	9.32 ± 0.61
80°C	9.35 ± 0.65	40.69 ± 3.39	59.66 ± 2.27	67.95 ± 3.56	75.23 ± 2.39
80°C + 70% RH	9.35 ± 0.65	50.10 ± 4.02	11.05 ± 8.73	9.96 ± 8.34	0.40 ± 0.92

B	Fresh	1 Month	2 Month	3 Month	5 Month
4°C	9.02 ± 1.42	8.66 ± 0.63	8.12 ± 0.52	9.37 ± 0.55	7.93 ± 0.59
4°C + 70% RH	9.02 ± 1.42	9.90 ± 0.57	9.99 ± 0.69	9.22 ± 0.18	7.95 ± 0.32
22°C	9.02 ± 1.42	8.99 ± 0.33	8.17 ± 0.20	9.66 ± 1.02	8.55 ± 0.32
22°C + 70% RH	9.02 ± 1.42	9.64 ± 1.63	9.11 ± 0.71	9.48 ± 0.88	10.57 ± 0.69
80°C	9.02 ± 1.42	14.23 ± 2.93	20.57 ± 1.29	23.97 ± 2.72	34.19 ± 1.71
80°C + 70% RH	9.02 ± 1.42	44.82 ± 1.53	14.63 ± 1.95	8.49 ± 9.31	2.86 ± 1.85

Table A-3: Change in a-value of regular (A) and neutralized (B) Chitosan films prepared in Lactic Acid, kept for 5 months in different environments.

A	Fresh	1 Month	2 Month	3 Month	5 Month
4ºC	-1.48 ± 0.02	-1.04 ± 0.04	-1.69 ± 0.02	-1.76 ± 0.02	-1.84 ± 0.09
4ºC + 70% RH	-1.48 ± 0.02	-1.89 ± 0.06	-1.86 ± 0.10	-1.85 ± 0.07	-2.11 ± 0.04
22ºC	-1.48 ± 0.02	-1.84 ± 0.08	-2.06 ± 0.13	-2.06 ± 0.14	-2.27 ± 0.12
22ºC + 70% RH	-1.48 ± 0.02	-1.86 ± 0.06	-1.66 ± 0.04	-1.76 ± 0.02	-1.64 ± 0.08
80ºC	-1.48 ± 0.02	23.35 ± 4.06	26.62 ± 2.13	30.00 ± 2.52	31.78 ± 3.16
80ºC + 70% RH	-1.48 ± 0.02	13.19 ± 7.95	4.33 ± 2.78	4.59 ± 6.64	1.96 ± 2.57

B	Fresh	1 Month	2 Month	3 Month	5 Month
4ºC	-0.60 ± 0.04	-0.67 ± 0.04	-0.42 ± 0.02	-0.49 ± 0.10	-0.58 ± 0.17
4ºC + 70% RH	-0.60 ± 0.04	-0.28 ± 0.29	-0.34 ± 0.33	-0.36 ± 0.09	-0.64 ± 0.11
22ºC	-0.60 ± 0.04	-0.62 ± 0.20	-0.47 ± 0.29	-0.59 ± 0.20	-0.66 ± 0.08
22ºC + 70% RH	-0.60 ± 0.04	-0.70 ± 0.21	-0.53 ± 0.28	-0.70 ± 0.26	-0.93 ± 0.22
80ºC	-0.60 ± 0.04	-0.37 ± 0.45	0.15 ± 0.14	0.13 ± 0.34	-0.33 ± 0.09
80ºC + 70% RH	-0.60 ± 0.04	13.43 ± 1.72	4.54 ± 0.78	1.25 ± 0.73	-0.10 ± 0.38

Table A-4: Change in b-value of regular (A) and neutralized (B) Chitosan films prepared in Lactic Acid, kept for 5 months in different environments.

A	Fresh	Month	2 Month	3 Month	5 Month
4ºC	10.32 ± 0.30	6.72 ± 0.28	11.77 ± 0.35	12.86 ± 0.35	11.52 ± 0.16
4ºC + 70% RH	10.32 ± 0.30	13.85 ± 1.74	13.41 ± 0.97	14.34 ± 0.74	13.67 ± 0.67
22ºC	10.32 ± 0.30	11.75 ± 0.85	12.68 ± 0.95	13.18 ± 0.98	13.47 ± 0.62
22ºC + 70% RH	10.32 ± 0.30	14.26 ± 0.96	15.21 ± 1.05	17.90 ± 0.59	20.46 ± 1.23
80ºC	10.32 ± 0.30	68.75 ± 2.94	76.97 ± 9.85	73.98 ± 0.62	60.08 ± 3.52
80ºC + 70% RH	10.32 ± 0.30	5.69 ± 4.66	1.36 ± 2.42	2.28 ± 2.81	-0.19 ± 1.12

B	Fresh	1 Month	2 Month	3 Month	5 Month
4ºC	9.39 ± 0.88	9.60 ± 1.14	13.17 ± 3.08	13.15 ± 2.08	14.00 ± 2.92
4ºC + 70% RH	9.39 ± 0.88	13.90 ± 3.47	13.82 ± 3.36	11.23 ± 2.96	11.92 ± 3.56
22ºC	9.39 ± 0.88	14.05 ± 4.22	12.93 ± 4.06	11.72 ± 3.07	13.60 ± 1.73
22ºC + 70% RH	9.39 ± 0.88	13.75 ± 0.85	12.94 ± 2.07	11.07 ± 2.31	10.46 ± 3.94
80ºC	9.39 ± 0.88	15.71 ± 1.06	21.44 ± 2.27	21.02 ± 3.06	16.97 ± 2.57
80ºC + 70% RH	9.39 ± 0.88	18.06 ± 2.59	4.11 ± 0.33	3.39 ± 0.39	2.52 ± 0.22

Table A-5: Change in a-value of regular (A) and neutralized (B) Chitosan films prepared in Citric Acid, kept for 5 months in different environments.

A	Fresh	1 Month	2 Month	3 Month	5 Month
4ºC	-1.07 ± 0.03	-1.80 ± 0.05	-1.01 ± 0.06	-1.06 ± 0.02	-1.11 ± 0.03
4ºC + 70% RH	-1.07 ± 0.03	-1.17 ± 0.04	-1.18 ± 0.06	-1.24 ± 0.03	-1.43 ± 0.03
22ºC	-1.07 ± 0.03	-1.02 ± 0.07	-1.12 ± 0.01	-1.09 ± 0.18	-1.13 ± 0.08
22ºC + 70% RH	-1.07 ± 0.03	-1.21 ± 0.01	-1.11 ± 0.01	-1.14 ± 0.02	-1.06 ± 0.01
80ºC	-1.07 ± 0.03	3.66 ± 3.76	5.92 ± 3.74	12.64 ± 8.19	14.67 ± 5.89
80ºC + 70% RH	-1.07 ± 0.03	-0.42 ± 0.16	-0.17 ± 0.02	-0.39 ± 0.10	-0.10 ± 0.04

B	Fresh	1 Month	2 Month	3 Month	5 Month
4ºC	-0.90 ± 0.06	-0.39 ± 0.20	-0.26 ± 0.88	-0.74 ± 0.06	-0.99 ± 0.03
4ºC + 70% RH	-0.90 ± 0.06	-0.81 ± 0.08	-0.83 ± 0.12	-0.84 ± 0.04	-1.02 ± 0.12
22ºC	-0.90 ± 0.06	-0.76 ± 0.23	-0.84 ± 0.16	-0.76 ± 0.14	-0.92 ± 0.16
22ºC + 70% RH	-0.90 ± 0.06	-0.80 ± 0.04	-0.89 ± 0.11	-0.95 ± 0.11	-1.10 ± 0.08
80ºC	-0.90 ± 0.06	-0.91 ± 0.10	-0.76 ± 0.12	-0.49 ± 0.12	-0.23 ± 0.25
80ºC + 70% RH	-0.90 ± 0.06	10.98 ± 4.56	3.96 ± 0.75	1.03 ± 0.37	0.45 ± 0.42

Table A-6: Change in b-value of regular (A) and neutralized (B) Chitosan films prepared in Citric Acid, kept for 5 months in different environments.

A	Fresh	1 Month	2 Month	3 Month	5 Month
4°C	6.99 ± 0.09	12.19 ± 0.75	6.75 ± 0.29	7.48 ± 0.27	6.40 ± 0.10
4°C + 70% RH	6.99 ± 0.09	7.44 ± 0.41	7.90 ± 0.27	9.42 ± 0.06	8.85 ± 0.17
22°C	6.99 ± 0.09	6.77 ± 0.47	7.13 ± 0.18	7.39 ± 0.87	6.68 ± 0.65
22°C + 70% RH	6.99 ± 0.09	10.05 ± 0.21	11.56 ± 0.40	13.68 ± 0.34	14.83 ± 0.77
80°C	6.99 ± 0.09	43.94 ± 1.21	50.50 ± 8.25	62.27 ± 1.44	68.51 ± 7.44
80°C + 70% RH	6.99 ± 0.09	-0.17 ± 0.14	-0.83 ± 0.08	0.45 ± 0.47	2.07 ± 0.75

B	Fresh	1 Month	2 Month	3 Month	5 Month
4°C	10.71 ± 1.81	13.28 ± 1.21	8.17 ± 1.01	7.98 ± 1.61	10.44 ± 1.37
4°C + 70% RH	10.71 ± 1.81	10.24 ± 1.38	7.97 ± 3.80	9.77 ± 2.18	10.08 ± 1.45
22°C	10.71 ± 1.81	7.99 ± 1.00	9.38 ± 1.41	9.35 ± 0.89	10.06 ± 0.50
22°C + 70% RH	10.71 ± 1.81	9.98 ± 0.56	9.32 ± 1.68	10.25 ± 2.68	9.65 ± 0.36
80°C	10.71 ± 1.81	11.08 ± 1.58	18.25 ± 3.47	17.46 ± 4.61	18.27 ± 3.94
80°C + 70% RH	10.71 ± 1.81	20.94 ± 8.18	4.22 ± 0.32	3.76 ± 0.35	3.22 ± 0.29

Table A-7: Change in a-value of regular (A) and neutralized (B) Chitosan films prepared in Hydrochloric Acid, kept for 5 months in different environments.

A	Fresh	1 Month	2 Month	3 Month	5 Month
4°C	-1.13 ± 0.01	-1.02 ± 0.00	-0.77 ± 0.01	-1.03 ± 0.01	-1.06 ± 0.03
4°C + 70% RH	-1.13 ± 0.01	-1.03 ± 0.01	-0.84 ± 0.02	-0.94 ± 0.01	-1.15 ± 0.03
22°C	-1.13 ± 0.01	-1.06 ± 0.01	-0.91 ± 0.01	-1.04 ± 0.01	-1.24 ± 0.02
22°C + 70% RH	-1.13 ± 0.01	-1.24 ± 0.03	-1.10 ± 0.04	-1.37 ± 0.02	-1.87 ± 0.28
80°C	-1.13 ± 0.01	-2.20 ± 0.22	0.58 ± 1.57	3.59 ± 2.52	10.56 ± 0.98
80°C + 70% RH	-1.13 ± 0.01	4.33 ± 1.63	-0.02 ± 0.30	0.14 ± 0.16	-0.18 ± 0.39

B	Fresh	1 Month	2 Month	3 Month	5 Month
4°C	-1.13 ± 0.05	-0.85 ± 0.02	-0.62 ± 0.05	-0.95 ± 0.21	-0.84 ± 0.09
4°C + 70% RH	-1.13 ± 0.05	-0.93 ± 0.03	-0.67 ± 0.03	-0.73 ± 0.05	-0.99 ± 0.04
22°C	-1.13 ± 0.05	-0.88 ± 0.02	-0.72 ± 0.05	-0.79 ± 0.07	-0.97 ± 0.03
22°C + 70% RH	-1.13 ± 0.05	-0.99 ± 0.03	-0.85 ± 0.08	-1.12 ± 0.07	-1.36 ± 0.02
80°C	-1.13 ± 0.05	-2.53 ± 0.04	-1.85 ± 0.72	-0.35 ± 1.67	-0.09 ± 1.13
80°C + 70% RH	-1.13 ± 0.05	25.36 ± 1.06	12.96 ± 4.17	5.48 ± 1.61	13.19 ± 7.47

Table A-8: Change in b-value of regular (A) and neutralized (B) Chitosan films prepared in Hydrochloric Acid, kept for 5 months in different environments.

A	Fresh	1 Month	2 Month	3 Month	5 Month
4°C	8.08 ± 0.13	8.30 ± 0.04	9.08 ± 0.05	8.48 ± 0.04	7.80 ± 0.09
4°C + 70% RH	8.08 ± 0.13	7.69 ± 0.29	8.61 ± 0.15	8.58 ± 0.18	7.57 ± 0.16
22°C	8.08 ± 0.13	7.91 ± 0.10	8.81 ± 0.07	8.57 ± 0.12	8.16 ± 0.10
22°C + 70% RH	8.08 ± 0.13	8.45 ± 0.29	9.15 ± 0.26	9.67 ± 0.15	10.81 ± 1.54
80°C	8.08 ± 0.13	27.31 ± 4.55	45.32 ± 6.15	56.19 ± 6.26	71.42 ± 1.91
80°C + 70% RH	8.08 ± 0.13	1.28 ± 0.58	-0.34 ± 0.05	-0.31 ± 0.18	-0.11 ± 0.71

B	Fresh	1 Month	2 Month	3 Month	5 Month
4°C	9.28 ± 0.20	8.39 ± 0.41	9.12 ± 0.74	8.86 ± 1.17	7.70 ± 1.04
4°C + 70% RH	9.28 ± 0.20	8.38 ± 0.23	8.44 ± 0.33	8.49 ± 0.73	7.81 ± 0.09
22°C	9.28 ± 0.20	8.02 ± 0.46	8.75 ± 0.35	8.59 ± 0.25	7.99 ± 0.25
22°C + 70% RH	9.28 ± 0.20	8.30 ± 0.33	8.76 ± 0.25	8.96 ± 0.29	8.64 ± 0.22
80°C	9.28 ± 0.20	25.38 ± 2.99	35.06 ± 6.29	44.98 ± 7.64	46.82 ± 4.00
80°C + 70% RH	9.28 ± 0.20	16.95 ± 2.43	5.23 ± 2.40	1.43 ± 0.57	5.88 ± 4.80

Table A-9: UV-Vis absorbance of regular (A) and neutralized (B) Chitosan films prepared with Acetic Acid kept for 5 months in different environments.

A	Fresh		1 Month		2 Month		3 Month		5 Month	
	286	420	286	420	286	420	286	420	286	420
4ºC	0.375	0.065	0.801	0.229	0.723	0.122	0.916	0.138	0.860	0.179
4ºC + 70% RH	0.375	0.065	0.567	0.124	1.009	0.142	0.627	0.109	1.192	0.208
22ºC	0.375	0.065	0.637	0.119	0.773	0.126	1.099	0.174	1.297	0.241
22ºC + 70% RH	0.375	0.065	0.773	0.149	1.086	0.147	1.196	0.178	1.475	0.275
80ºC	0.375	0.065	1.524	0.268	2.896	0.795	3.024	1.216	3.093	2.492
80ºC + 70% RH	0.375	0.065	2.670	2.748	3.028	1.671	3.028	1.671	3.068	2.810

B	Fresh		1 Month		2 Month		3 Month		5 Month	
	286	420	286	420	286	420	286	420	286	420
4ºC	0.613	0.102	0.590	0.133	0.769	0.121	0.926	0.141	1.471	0.242
4ºC + 70% RH	0.613	0.102	0.754	0.148	0.732	0.115	0.648	0.109	1.317	0.238
22ºC	0.613	0.102	0.643	0.119	0.792	0.128	0.882	0.141	0.895	0.215
22ºC + 70% RH	0.613	0.102	0.590	0.114	0.812	0.128	1.073	0.154	1.459	0.267
80ºC	0.613	0.102	2.708	2.185	2.116	0.376	3.024	1.216	3.089	1.242
80ºC + 70% RH	0.613	0.102	2.058	0.425	2.966	0.697	2.966	0.697	3.118	2.810

Table A-10: UV-Vis absorbance of regular (A) and neutralized (B) Chitosan films prepared with Lactic Acid kept for 5 months in different environments.

A	Fresh		1 Month		2 Month		3 Month		5 Month	
	286	420	286	420	286	420	286	420	286	420
4ºC	0.480	0.070	0.335	0.093	0.421	0.088	0.526	0.088	0.546	0.122
4ºC + 70% RH	0.480	0.070	0.327	0.101	0.412	0.085	0.430	0.098	0.480	0.115
22ºC	0.480	0.070	0.338	0.090	0.523	0.101	0.668	0.112	0.964	0.160
22ºC + 70% RH	0.480	0.070	0.371	0.108	0.555	0.118	0.792	0.128	1.226	0.196
80ºC	0.480	0.070	2.636	0.652	2.982	0.839	3.005	1.225	3.089	1.780
80ºC + 70% RH	0.480	0.070	2.817	0.543	1.532	0.250	1.532	0.250	×	×

'×' Chitosan film did not dissolve

B	Fresh		1 Month		2 Month		3 Month		5 Month	
	286	420	286	420	286	420	286	420	286	420
4ºC	0.494	0.121	0.604	0.144	0.620	0.111	0.526	0.088	1.292	0.197
4ºC + 70% RH	0.494	0.121	0.519	0.128	0.619	0.108	0.484	0.099	1.233	0.185
22ºC	0.494	0.121	0.547	0.120	0.523	0.101	0.942	0.143	1.341	0.203
22ºC + 70% RH	0.494	0.121	0.520	0.124	0.659	0.115	1.073	0.146	1.376	0.207
80ºC	0.494	0.121	0.779	0.164	1.197	0.201	1.572	0.291	2.075	0.519
80ºC + 70% RH	0.494	0.121	2.720	1.966	2.133	0.438	2.133	0.438	3.068	2.810

Table A-11: UV-Vis absorbance of regular (A) and neutralized (B) Chitosan films prepared with Citric Acid kept for 5 months in different environments.

A	Fresh		1 Month		2 Month		3 Month		5 Month	
	286	**420**	**286**	**420**	**286**	**420**	**286**	**420**	**286**	**420**
4ºC	0.236	0.043	0.255	0.076	0.347	0.069	0.419	0.078	0.469	0.130
4ºC + 70% RH	0.236	0.043	0.379	0.094	0.407	0.077	0.342	0.081	0.582	0.140
22ºC	0.236	0.043	0.281	0.084	0.403	0.071	0.500	0.093	0.654	0.116
22ºC + 70% RH	0.236	0.043	0.281	0.105	0.413	0.083	0.627	0.105	0.795	0.133
80ºC	0.236	0.043	×	×	0.676	0.184	×	×	×	×
80ºC + 70% RH	0.236	0.043	2.684	1.494	3.011	1.551	2.931	2.252	×	×

'×' Chitosan film did not dissolve

B	Fresh		1 Month		2 Month		3 Month		5 Month	
	286	**420**	**286**	**420**	**286**	**420**	**286**	**420**	**286**	**420**
4ºC	0.596	0.121	0.514	0.129	0.549	0.100	0.740	0.114	1.423	0.255
4ºC + 70% RH	0.596	0.121	0.415	0.117	0.762	0.120	0.454	0.087	1.175	0.188
22ºC	0.596	0.121	0.444	0.126	0.647	0.110	0.905	0.137	1.409	0.256
22ºC + 70% RH	0.596	0.121	0.482	0.114	0.618	0.104	0.951	0.134	0.896	0.199
80ºC	0.596	0.121	1.138	0.231	1.501	0.251	2.138	0.449	2.379	0.662
80ºC + 70% RH	0.596	0.121	3.004	2.804	3.011	1.551	3.011	1.551	×	×

'×' Chitosan film did not dissolve

Table A-12: UV-Vis absorbance of regular (A) and neutralized (B) Chitosan films prepared with Hydrochloric Acid kept for 5 months in different environments.

A	Fresh		1 Month		2 Month		3 Month		5 Month	
	286	420	286	420	286	420	286	420	286	420
4ºC	0.788	0.145	0.654	0.107	1.066	0.145	0.527	0.089	0.737	0.155
4ºC + 70% RH	0.788	0.145	0.648	0.108	1.028	0.139	0.493	0.092	0.904	0.138
22ºC	0.788	0.145	0.551	0.100	1.069	0.154	0.533	0.103	1.131	0.180
22ºC + 70% RH	0.788	0.145	0.575	0.105	1.040	0.146	0.589	0.109	0.656	0.120
80ºC	0.788	0.145	1.948	0.354	2.926	0.845	2.918	0.726	3.043	1.260
80ºC + 70% RH	0.788	0.145	3.028	2.203	3.027	2.760	3.005	2.758	×	×

'×' Chitosan film did not dissolve

B	Fresh		1 Month		2 Month		3 Month		5 Month	
	286	420	286	420	286	420	286	420	286	420
4ºC	0.537	0.072	0.670	0.097	1.127	0.152	0.562	0.093	0.854	0.175
4ºC + 70% RH	0.537	0.072	0.571	0.090	1.138	0.150	0.555	0.097	0.919	0.183
22ºC	0.537	0.072	0.561	0.085	1.072	0.147	0.580	0.099	1.009	0.187
22ºC + 70% RH	0.537	0.072	0.622	0.093	1.201	0.172	0.585	0.102	1.298	0.191
80ºC	0.537	0.072	1.894	0.297	2.737	0.687	2.971	0.902	3.018	1.222
80ºC + 70% RH	0.537	0.072	×	×	3.005	2.758	×	×	×	×

'×' Chitosan film did not dissolve

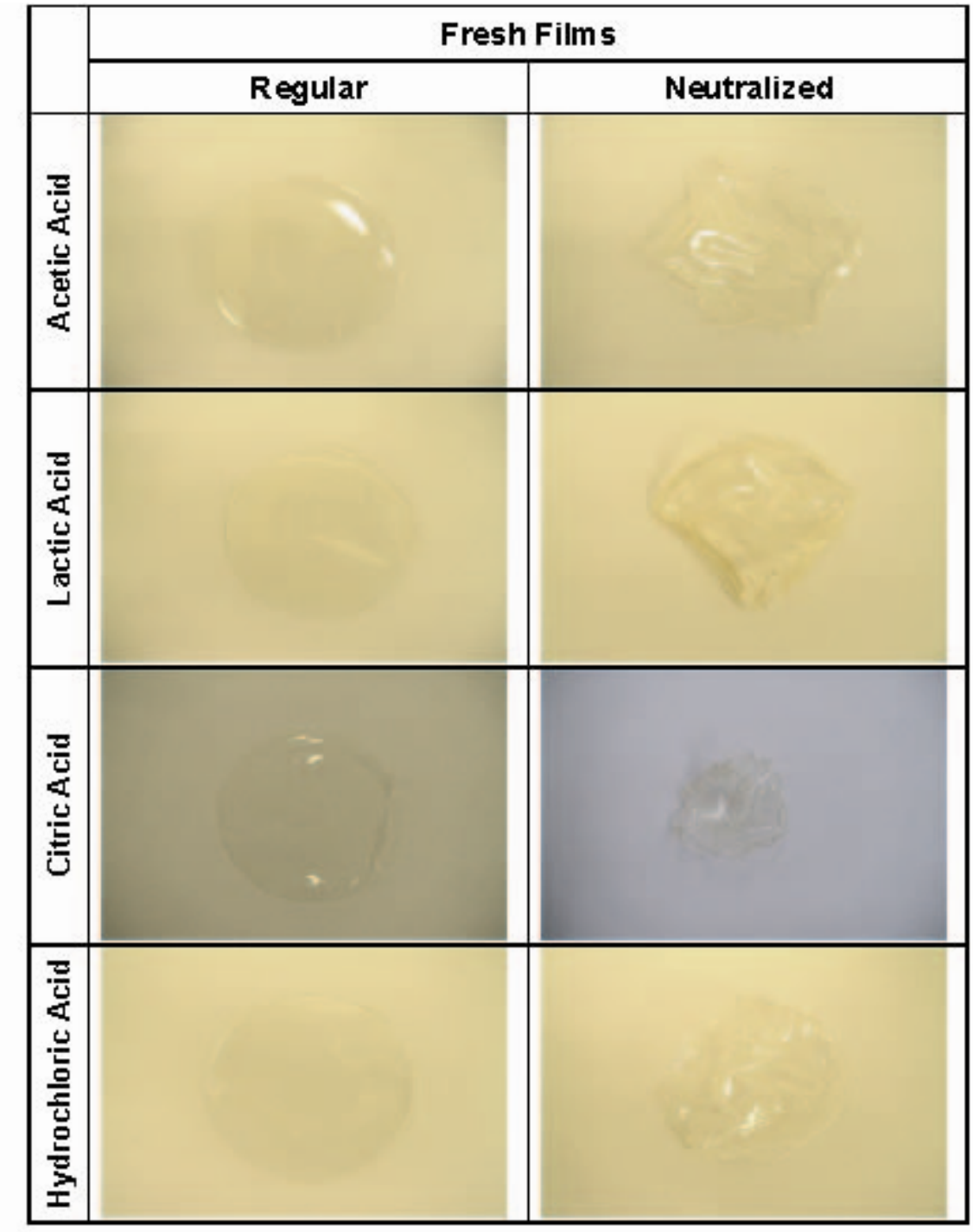

Figure A-1. Pictures of Fresh dried regular and neutralized Chitosan films.

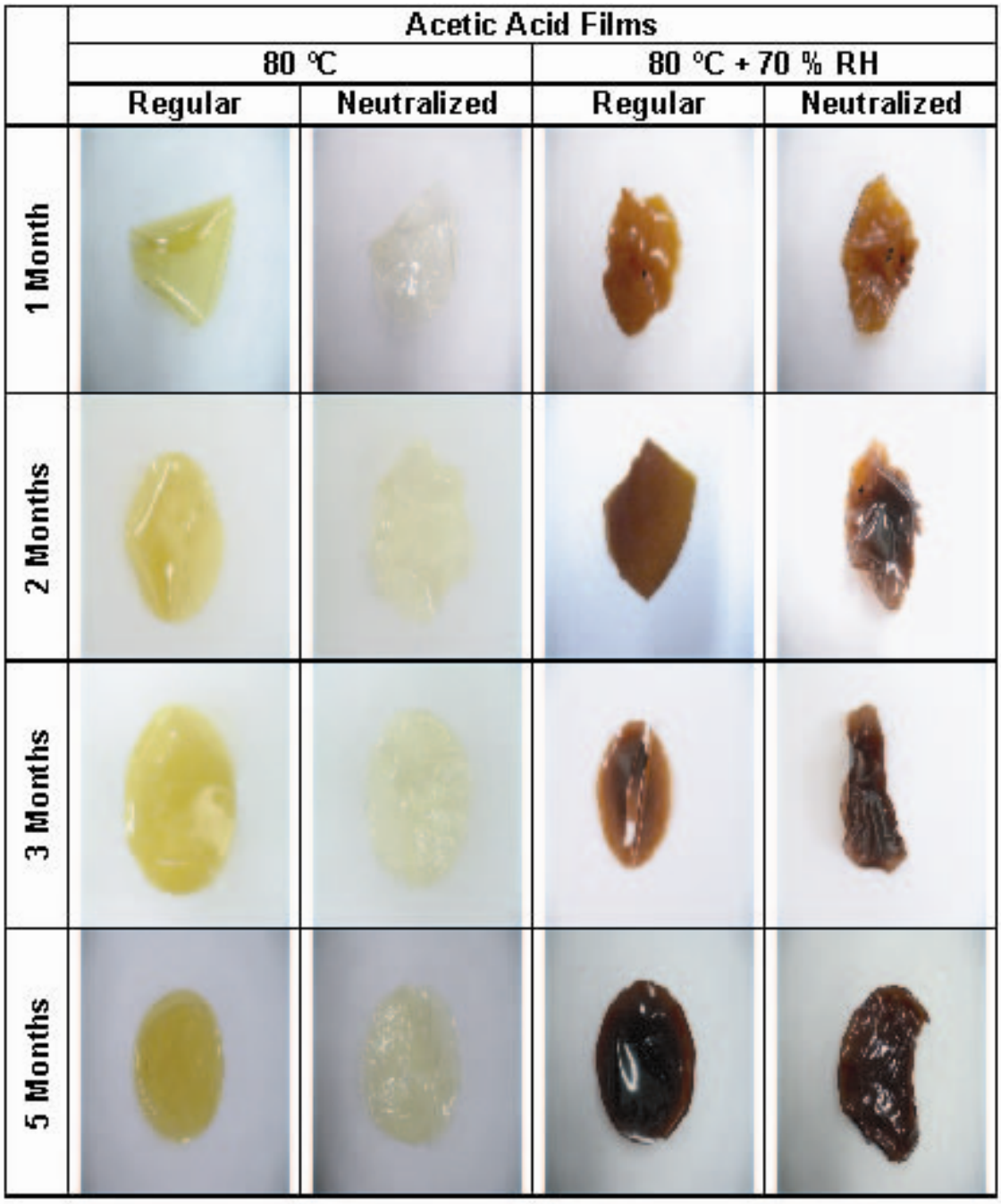

Figure A-2. Pictures of Acetic Acid (regular and neutralized) films kept for 5 months at 80 ºC and 80 ºC and 70% RH.

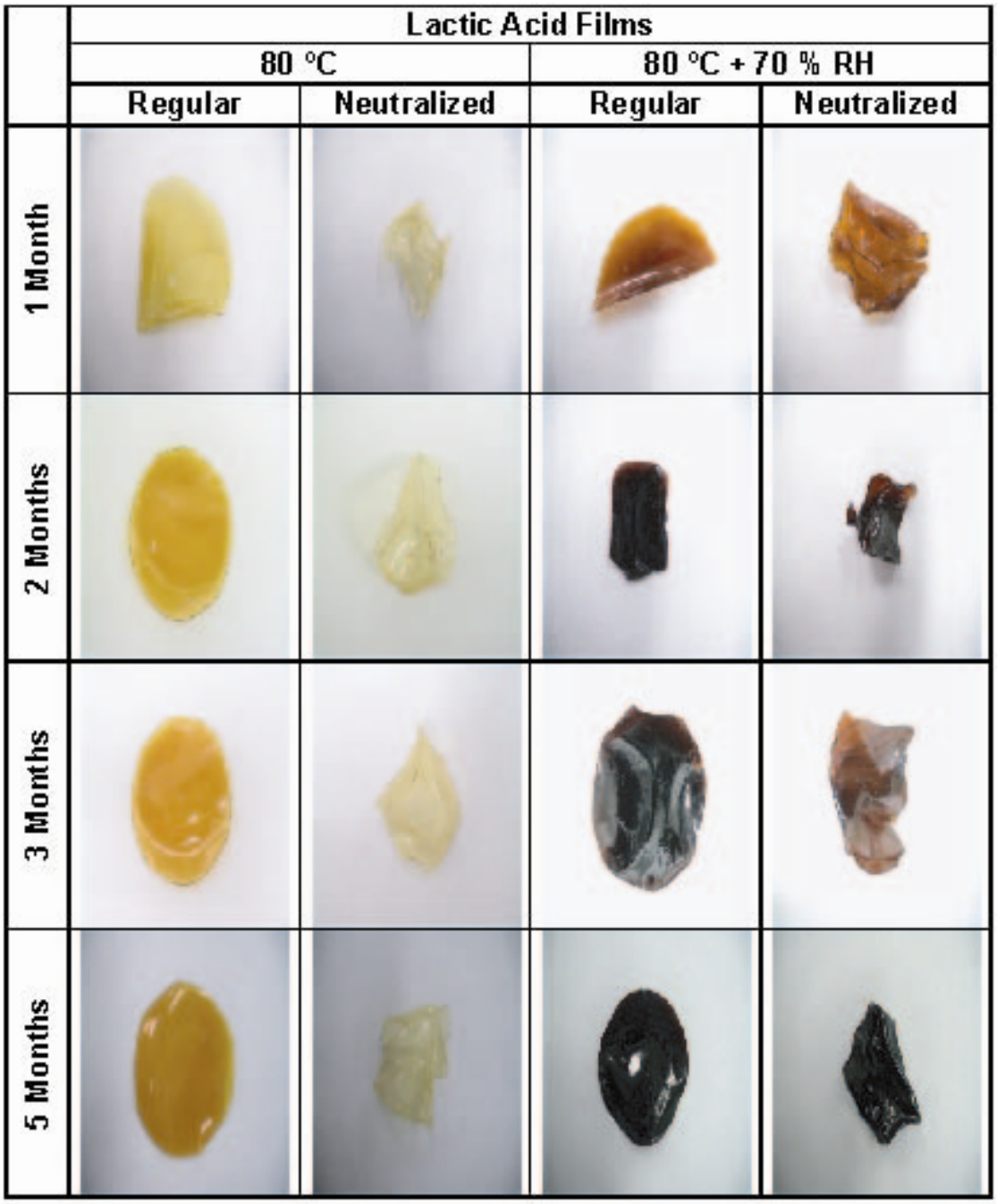

Figure A-3. Pictures of Lactic Acid (regular and neutralized) films kept for 5 months at 80 ºC and 80 ºC and 70% RH.

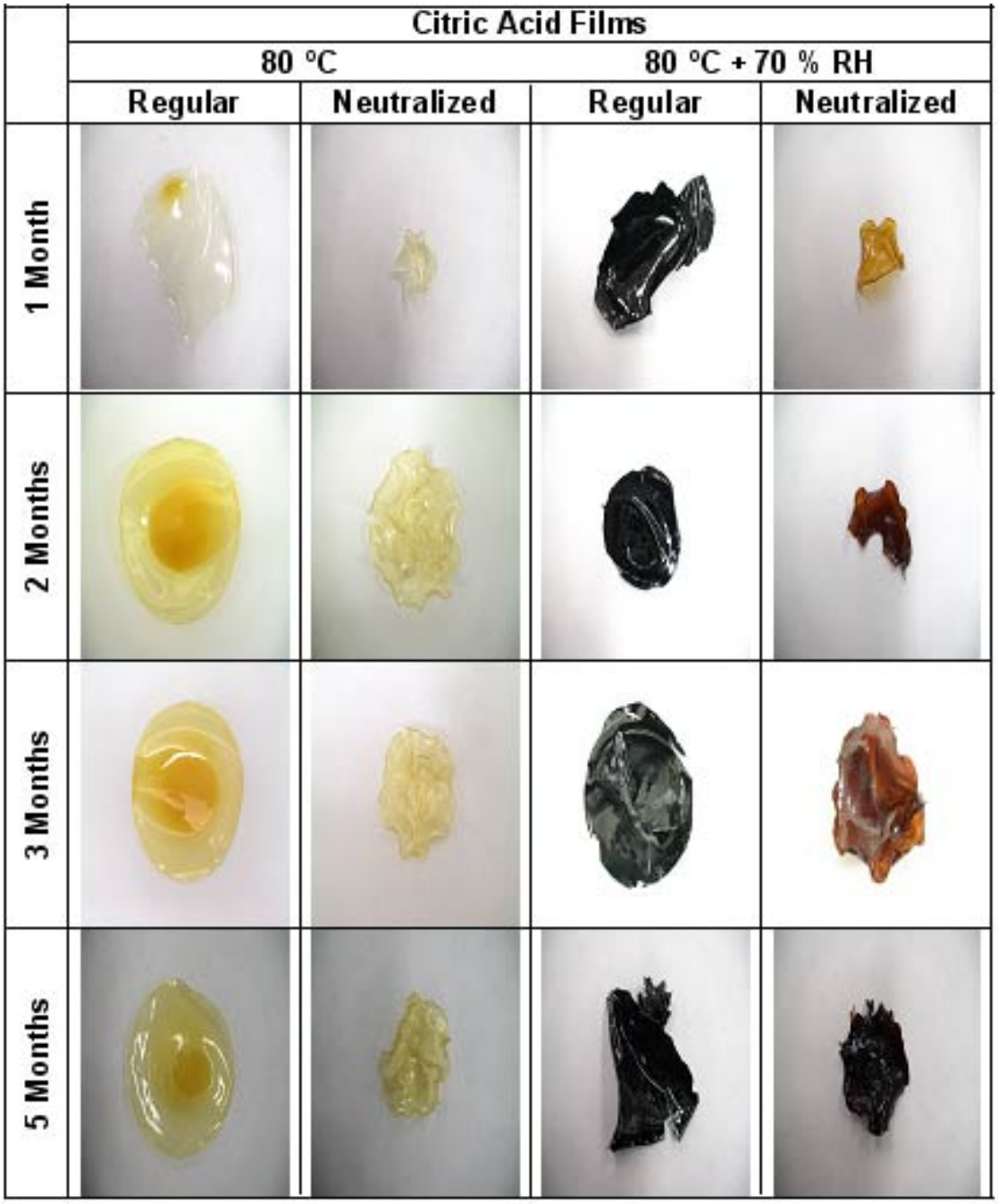

Figure A-4. Pictures of Citric Acid (regular and neutralized) films kept for 5 months at 80 ºC and 80 ºC and 70% RH.

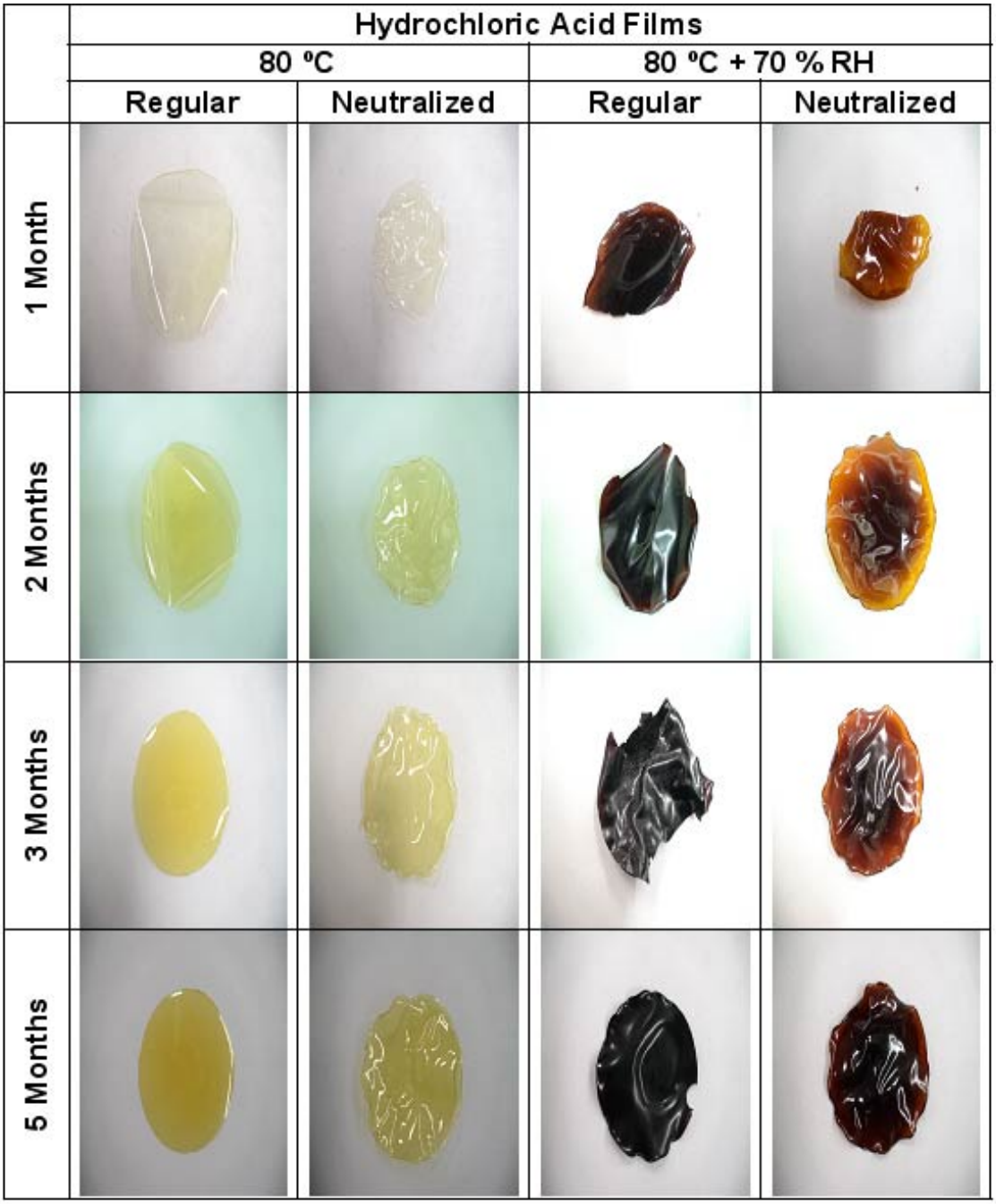

Figure A-5. Pictures of Hydrochloric Acid (regular and neutralized) films kept for 5 months at 80 ºC and 80 ºC and 70% RH.

Appendix B – Mechanical properties of chitosan films during storage

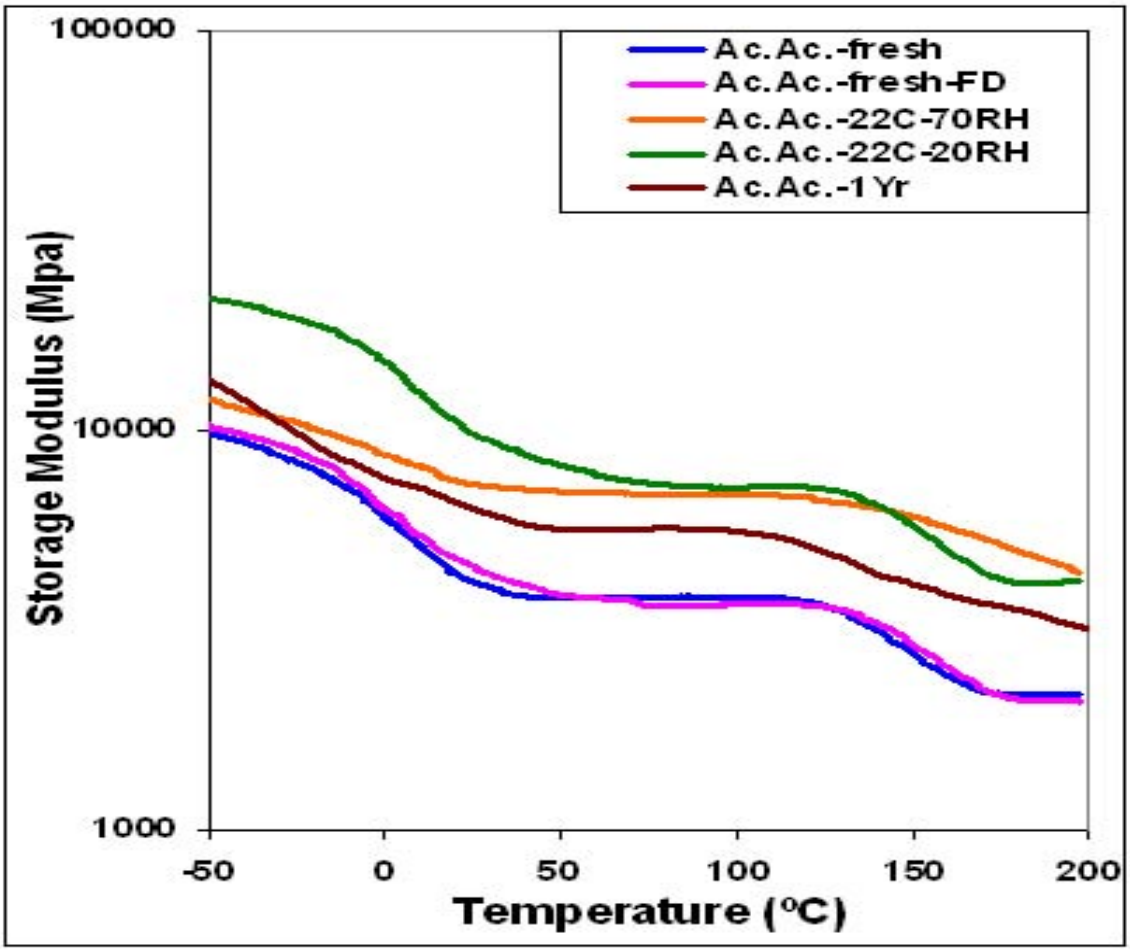

Figure B-1. DMA thermogram (A of Acetic Acid Fresh dried, Fresh Freeze dried (FD), stored for 3 months at 22 ºC and 20 and 70 % RH, and for 1 year at ambient conditions, films.

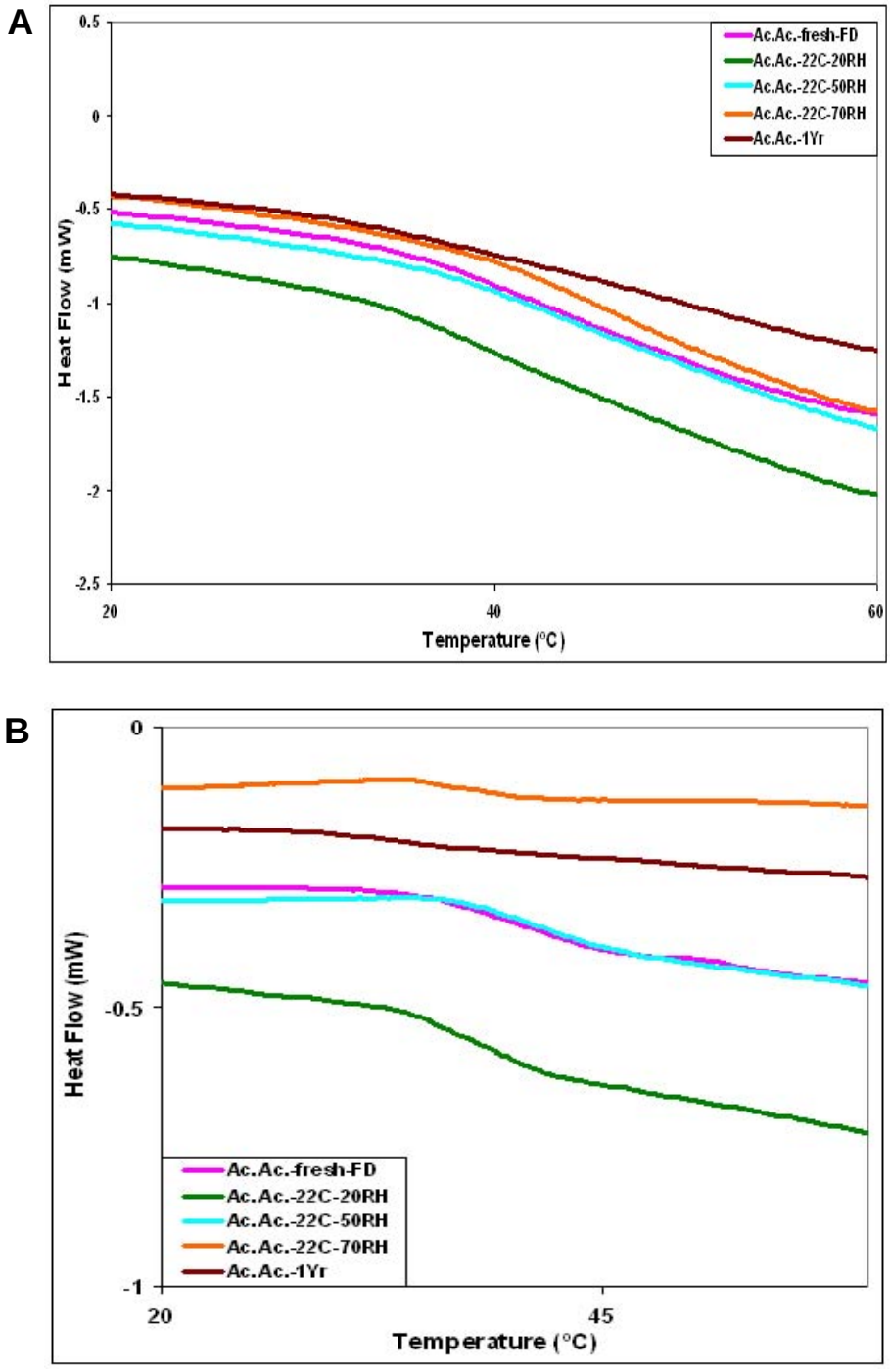

Figure B-2. DSC thermogram, segment 1 (A) and segment 2 (B) of Acetic Acid Fresh Freeze dried (FD), stored for 3 months at 22 ºC and 20, 50 (ambient) and 70 % RH, and for 1 year at ambient conditions, films.

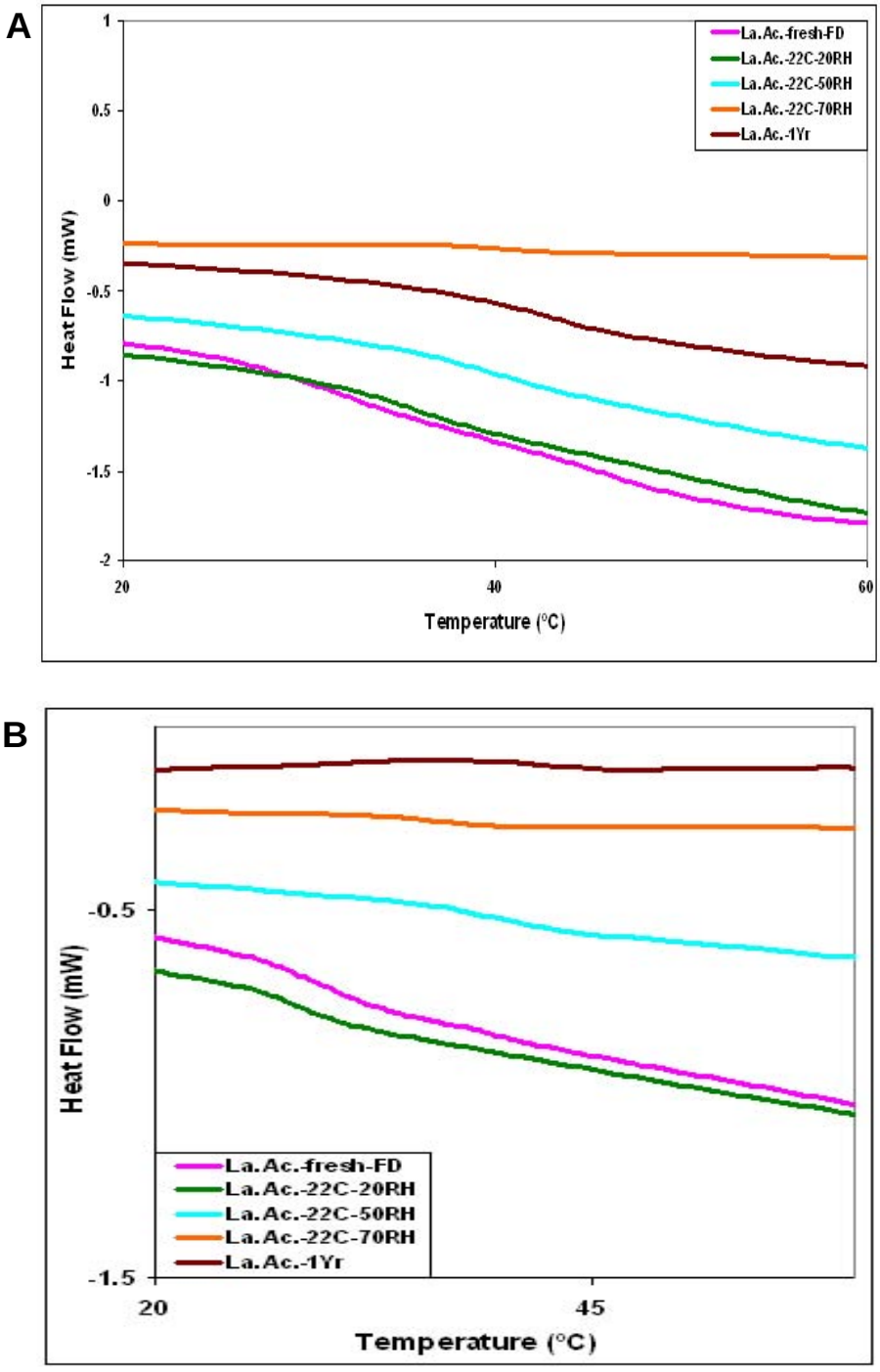

Figure B-3. DSC thermogram, segment 1 (A) and segment 2 (B) of Lactic Acid Fresh Freeze dried (FD), stored for 3 months at 22 ºC and 20, 50 (ambient) and 70 % RH, and for 1 year at ambient conditions, films.

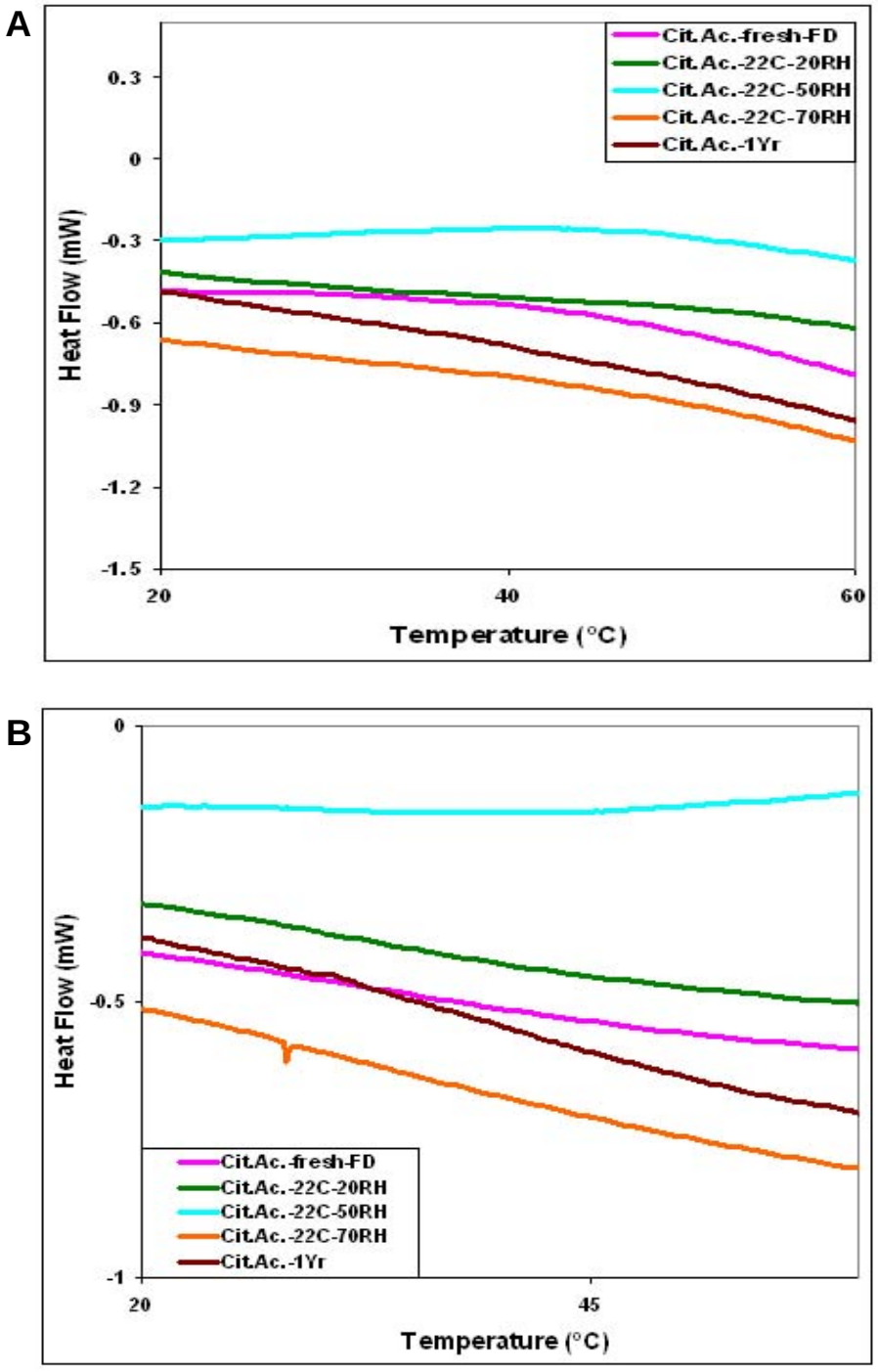

Figure B-4. DSC thermogram, segment 1 (A) and segment 2 (B) of Citric Acid Fresh Freeze dried (FD), stored for 3 months at 22 ºC and 20, 50 (ambient) and 70 % RH, and for 1 year at ambient conditions, films.

Vita

Gagan Rajpal was born in Sirhind, Punjab, India on the 1st of November 1976. His interest in interacting with different people and love for animals always motivated him to become a Veterinarian. There is an adage “God helps those who help themselves, but we (veterinarians) help those who cannot help themselves”. He pursued Bachelors of Veterinary Sciences and Animal Husbandry, from CCS H.A.U., Hisar, India. However, lust for knowledge and influence of his elder brother brought him to United States of America to pursue his post-graduate studies in fall 2003. He completed his Masters in Food Science and Technology. His aim is to work in the area of research and development and serve his community.