CHITOSAN AS AN EDIBLE FILM FOR PROTECTION OF SEAFOOD QUALITY

YATIYANA VIDANA ARACHCHILAGE JANAK KAMIL

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BY

©YATIYANA VIDANA ARACHCHILAGE JANAK KAMIL, B. Sc. (Agric.)

A thesis submitted to the School of Graduate

Studies in partial fulfilment of the

requirements for the degree of the

Master of Science

Department of Biochemistry

Memorial University of Newfoundland

August, 2000

St. John's Newfoundland Canada

THIS WORK IS DEDICATED TO

MY LOVING PARENTS

ABSTRACT

Preservation of seafood quality against oxidation of highly unsaturated lipids, autolysis reactions and microbial activities is important. Chitosan, a deacetylated form of chitin, has been identified as a versatile biopolymer for a broad range of food applications. However, very little information is available about the use of chitosan as an edible coating material for preservation of seafood quality and virtually nothing is known regarding the effect of viscosity of chitosan on its preservative efficacy for seafood. Effect of chitosan coating for extension of shelf-life of two different fish species, Atlantic cod (*Gadus morhua*) as a lean fish, and herring (*Clupea harengus*) as a fatty fish, was evaluated during a 12-days storage at refrigerated temperature (4 ± 1°C). Three chitosan preparations from snow crab (*Chinoecetes opilio*) processing wastes, differing in viscosities and molecular weights, were prepared and used in this study. The apparent viscosity of chitosans was dependent on the deacetylation time of their chitin precursors and were 14, 57 and 360 cP for samples prepared over a 20, 10 and 4 h period, respectively.

Prepared chitosan samples were used as edible coating material on the surface of the fish muscle and the quality of the coated samples were monitored. This was achieved by monitoring relative moisture loss (%) and peroxide value (PV), conjugated dienes (CD), 2-thiobarbituric acid-reactive substances (TBARS) and headspace (HS) volatiles, all which are related to lipid oxidation. Furthermore, total volatile basic nitrogen (TVB-N), trimethylamine (TMA) and hypoxanthine (Hx), which are related to chemical spoilage, as

well as total plate count (TPC) related to microbial spoilage were monitored. A significant ($p \leq 0.05$) reduction in relative moisture loss, by 37, 29, 29, 40, and 32%, over that of uncoated cod samples was observed for those coated with 360 cP chitosan after 4, 6, 8, 10, and 12 days of storage, respectively. Chitosan coating significantly ($p \leq 0.05$) reduced lipid oxidation, chemical spoilage and growth of microorganisms in both fish model systems compared to uncoated samples. The preservative efficacy and the viscosity of chitosan were inter-related; the efficacy of chitosans with a viscosity of 57 and 360 cP was superior to that with a 14 cP viscosity.

In another study, antioxidant efficacy of different viscosity chitosans in cooked comminuted herring and cod systems was investigated. Oxidative stability of treated fish species was determined and compared to those treated with conventional antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) at a level of 200 ppm. Progression of oxidation was monitored by employing peroxide value and TBARS tests. In general, all chitosans exhibited varying antioxidant activities in both types of fish model systems investigated. Formation of hydroperoxides and TBARS in herring samples containing 200 ppm 14 cP chitosan was reduced after day-8 of storage by 61 and 52%, respectively.

The mechanism of action of chitosan coating for extension of shelf-life of seafood appears to be due to chelation of metal ions contained in fish muscle proteins, controlling of gas exchange, particularly oxygen, between fish meat and the surrounding environment, and bactericidal effect of chitosan itself. Edible superficial chitosan coatings provide

supplementary and sometimes essential means to control physiological, microbiological and physicochemical changes in seafood products. The active edible chitosan coatings may thus be extended to new fully adapted superficial or internal applications for seafood products.

ACKNOWLEDGEMENTS

My sincere gratitude is conveyed to Dr. F. Shahidi for his support and the invaluable guidance throughout my M.Sc. degree programme. While shouldering voluminous responsibilities, his criticisms, detailed corrections and splendid suggestions for modifications enabled me to complete my course of studies in a very interesting and enlightened way. I would like to acknowledge the Canadian International Development Agency (CIDA), Marine Science Scholarship for providing me an opportunity and financial assistance to undertake this degree programme at Memorial University of Newfoundland. I would like to extend my appreciation to Dr. A. B. Dickinson and Mrs. Colleen Clarke for their invaluable assistance. I also wish to thank my supervisory committee members, Dr. A.M. Martin and Dr. J. Banoub, for their guidance throughout my programme. I would like to extend my appreciation to Drs. Y. J. Jeon, Drs. Udaya and Janitha Wansundara, and Dr. Mahinda Wettasinghe for their invaluable assistance and suggestions. Thanks to Dr. T. R. Patel for providing me access to his laboratory facilities. I would also express my sincere thanks to the Vice Chancellor, University of Peradeniya, Sri Lanka, for granting me study leave to pursue graduate studies at Memorial University of Newfoundland, St. John's, Canada.

I am very grateful to my friends, Ranjith Bandara, Anusha Rajapakse, Namal Senanayake, Samudra Siriwardhana, Metusalach, Ahmad Khan, Aman Bolar, Karthik

Kannan and Nishantha Wanasundara for helping me to succeed in all possible ways. Thanks to all my colleagues in Dr. Shahidi's research team for creating a pleasant environment to work. Ordinary words cannot convey the immense debt of gratitude I owe to my parents and family members for their consistent encouragement and help rendered throughout this study. Finally, I want tc thank all others at Memorial University of Newfoundland for their many help in different ways.

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LIST OF ABBREVIATIONS

AA	-Arachidonic acid
ANOVA	-Analysis of variance
AOAC	-Association of Official Analytical Chemists'
AOCS	-American Oil Chemists' Society
ADP	-Adenosine diphosphate
ATP	-Adenosine triphosphate
BHA	-Butylated hydroxyanisole
BHT	-Butylated hydroxytoluene
CD	-Conjugated dienes
cP	-Centipoise
CRD	-Completely randomized block design
DHA	-Docosahexaenoic acid
DIPEA	-Diisopropylethylamine
DMA	-Dimethylamine
DNA	-Deoxyribonucleic acid
EPA	-Eicosapentaenoic acid
FAME	-Fatty acid methyl esters
FAO	-Food and Agriculture Organization
FID	-Flame ionization detector
FDA	-Food and Drug Administration

GC	-Gas chromatography
GLM	-General linear model
HS	-Headspace
Hx	-Hypoxanthine
ICMSF	-International Commission on Microbiological Specifications for Foods
IMP	-Inosine monophosphate
MA	-Malonaldehyde
Mv	-Molecular weight
NADPH	-Nicotinamide adenine dinucleotide phosphate
nm	-Nanometer
PA	-Picric acid
ppm	-Parts per million
PUFA	-Polyunsaturated fatty acid
PV	-Peroxide value
r	-Correlation coefficient
RML	-Relative moisture level
SAS	-Statistical Analysis System
SD	-Standard deviation
TBA	-2-thiobarbituric acid
TBARS	-Thiobarbituric acid-reactive substances

TBHQ	-*tertiary*-Butyl hydroquinone
TCA	-Trichloroacetic acid
TMA	-Trimethylamine
TMAO	-Trimethylamine oxide
TPC	-Total plate count
TVA	-Total volatile aldehyde
TVB-N	-Total volatile-basic nitrogen
UHP	-Ultra high purity
v/v	-Volume by volume
XO	-Xanthine oxidase
WOF	-Warmed-over flavour
w/v	-Weight by volume
w/w	-Weight by weight

CHAPTER 1

INTRODUCTION

Consumer demand for high quality ready-to-eat foods with extended shelf-life has recently been on the rise. The potential health benefits associated with long-chain n-3 polyunsaturated fatty acids have stimulated consumer interest and increased consumption of seafoods (Kinsella, 1988).

On the other hand, seafood products are highly susceptible to quality deterioration caused by oxidation of their highly unsaturated lipids (Khayat and Schwall, 1983; Hasieh and Kinsella, 1989a). This is further accelerated by the presence of high concentrations of hematin compounds and metal ions in fish muscle (Tichivangana and Morrissey, 1982; Khayat and Schwall, 1983; Decker and Hultin, 1992). This problem may occur in both fatty and lean fish species. In some cases it has been observed that lipid oxidation may be even more severe in lean fish than fatty fish (Tappel, 1961; McGill *et al.*, 1974; Huang and Weng, 1998). A linkage between phospholipid hydrolysis and lipid peroxidation in low fat fish muscle during low temperature storage was reported by Han and Liston (1987). Thus, this is of great concern to the food industry because of development of undesirable off-flavours and potentially toxic reaction products and also loss of essential fatty acids and fat soluble vitamins in products (Pearson *et al.*, 1983; Shahidi and Wanasundara, 1992). Furthermore, seafood quality in terms of safety and keeping quality, is highly influenced by non-visible factors such as autolysis, contamination and growth of microorganisms and

loss of protein functionality (Sikorski *et al.*, 1990; Haard, 1992a; Huss, 1995). Autolysis of fish muscle proteins results in the formation of peptides and free amino acids, all of which act as suitable nutrients for microbial growth and production of biogenic amines which are known to affect the safety of fish meat (Gill, 1990; Fraser and Sumar, 1998). Cold storage and freezing do not always completely suppress deterioration of seafoods. Reactions leading to oxidative and enzymatic changes and protein degradation may still proceed at refrigerated storage (Haard, 1992a; Ashie *et al.*, 1996). Synthetic preservatives such as antioxidants, chelating agents and antimicrobial agents may be added to food products in order to improve their shelf life (Tichivangana and Morrissey, 1985; Huss *et al.*, 1997; Khalil and Mansour, 1998). However, the growing consumer demand for foods without synthetic antioxidants and preservatives has focused efforts in the discovery of new natural preservatives (Shahidi and Wanasundara, 1992; Madsen and Bertelsen, 1995). Several sources of natural presrvatives and antioxidants are known, and some are currently used in a variety of food products.

Chitosans which are low acetyl substituted forms of chitin have been identified as versatile biopolymers for a broad range of food applications (Shahidi *et al.*, 1999). Chitosan may be extracted from chitin which is present in exoskeleton of arthropods such as insects, crabs, shrimps, lobsters and certain fungal cell walls. The use of chitosan in food applications is particularly promising because of its "biocompatibility" and nontoxicity (Hirano *et al.*, 1990; Rao and Sharma, 1997). In 1992, Japan's Health Department approved chitin and its derivatives as a functional food (Subasinghe, 1999).

The characteristics of the chitosan, with respect to its non-digestibility, its biodegradability, and its bland taste, make it as an excellent choice as a food additive and this property is used in the preparation of low-calorie foods (Muzzarelli, 1996). Due to its ability to bind the lipids, chitosan may be considered as having a negative calorie value (Furda, 1980; Nauss *et al.*, 1983). Japan produces dietary cookies and noodles enriched with chitosan because of its hypolipidemic and hypocholesterolemic effects (Winterowd and Sandford, 1995; Hirano, 1996). Metal binding capacity of chitosan with metal ions such as those of copper, chromium, zinc, lead, vanadium and iron has been demonstrated (Winterowd and Sandford, 1995; Peng *et al.*, 1998). Recently, it has been indicated that chitosan and its derivatives exert antimicrobial effects against different groups of microorganisms such as bacteria, fungi and yeast (Papineau *et al.*, 1991; El Ghaouth *et al.*, 1992; Chen *et al.*, 1998).

Chitosan-based materials may serve as excellent edible films or coatings due to their unique property of increased viscosity upon hydration (Butler *et al.*, 1996). Furthermore, chitosan films are tough, long-lasting, flexible and very difficult to tear. Most mechanical properties of chitosan films are comparable to those of many medium strength commercial polymers (Butler *et al.*, 1996). Kittur *et al.* (1998) have shown that chitosan films have moderate water permeability values and could be used to increase the storage life of fresh produce and foodstuffs with a higher water activity. Studies on the extension of shelf-life of foodstuff by chitosan coating have been reported mainly for fruits and vegetables (El Ghaouth *et al.*, 1991; Zhang and Quantick, 1998). Despite the many

current and potential applications of chitosan, fundamental studies on chitosan coating of fish and seafood are lacking. On the other hand, even though chitosan has a wide range of viscosity when prepared, its efficacy as a coating material has not been investigated. Kaye (1985) reported that the viscosity of chitosan apperars to be a major factor determining its properties in many applications.

Based on the literature evidences for properties of chitin and its derivatives during the extraction process, an hypothesis was made that; (1) the deacetylation of chitin as affected by reaction time may produce chitosans with different viscosities. Also based on literature evidences for the use of carbohydrate polymers as edible coating materials for preservation of various food, a second hypothesis was made; (2) chitosan coating may extend the shelf-life of seafoods during refrigerated storage. Since the effectiveness of chitosan as an edible coating material depends mainly on its viscosity, this hypothesis was further extended that viscosity of chitosan plays an important role on the preservative efficacy of chitosan-coated seafoods. To examine these hypotheses, objectives considered were: (1) to prepare chitosans from snow crab (*Chinoecetes opilio*) processing waste using different deacetylation times, (2) to assess lipid oxidation, chemical spoilage and microbial spoilage of chitosan-coated fish species, Atlantic cod (*Gadus morhua*) as a lean fish and herring (*Clupea harengus*) as a fatty fish, during refrigerated storage condition, (3) to evaluate the preservative efficacy and viscosity of chitosans in the above model system (objective 2).

CHAPTER 2

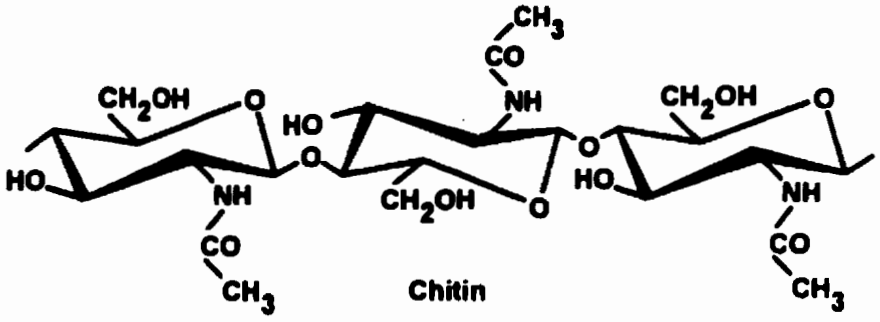
LITERATURE REVIEW

2.1 Chitin and Chitosan

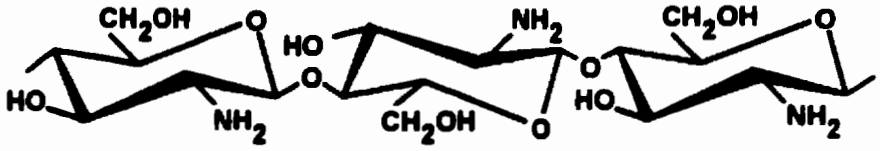
The name "chitin" is derived from the Greek word "chiton", meaning a coat of nail (Lower, 1984a), and was apparently first used by the French scientist Henri Bradconnot in 1811 (Muzzarelli, 1977; Brine, 1984), who isolated the substance from mushrooms. In 1823 Odier found the same compound in the cuticles of insects (Muzzarelli, 1977; Winterowd and Sandford, 1995). Chitin is the second most abundant natural polymer on earth after cellulose (Brzeski, 1987; Ornum, 1992) and is a linear homopolymer of 2-acetamido-2-deoxy-β-D-glucan, having 1000-3000 basic units (Austin *et al.*, 1981; Lower, 1984a). These units are linked together by β (1→4) glycosidic bonds (Ornum, 1992; Simpson *et al.*, 1994). Chitobiose, O-(2-amino-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-amino-2-deoxy-D-glucose, is the structural unit of native chitin (Muzzarelli, 1999). Chitin is also known as N-acetyl-D-glucosamine polymer and is one of the most abundant polysaccharides that contain amino sugars (Bough, 1977; Austin *et al.*, 1981; Kumar and Jayachandran, 1993).

Chitosan is the name used for low acetyl substituted form of chitin and chitosan polymers are composed primarily of glucosamine, 2-amino-2-deoxy-β-D-glucose, known as (1→4)-2-amino-2-deoxy-D-glucose (Shahidi *et al.*, 1999) (Figure 2.1). Chitosan was first isolated by C. Rouget in 1859 (Muzzarelli, 1977; Brine, 1984), and is a macromolecular material obtained by substantial or complete deacetylation of chitin

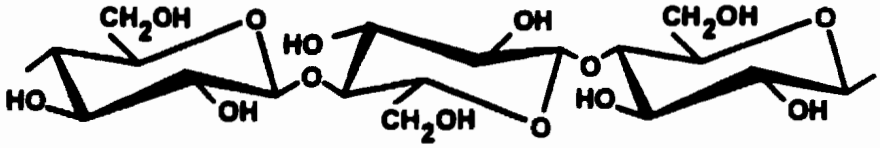
Figure 2.1 Chemical structures of chitin, chitosan and cellulose.



Chitin



Chitosan



Cellulose

without the distructioning its polymeric chain (Roberts, 1992; Lower, 1984b). Thus, acetamide groups in chitin are substituted by amino groups to afford chitosan. Chitosan is the simplest derivative that could be prepared from chitin and is the least expensive usable derivative of it (Ornum, 1992). Unlike most polysaccharides, presence of positively charged amino groups regularly located along a polymer chain of chitosan allows the molecule to bind to negatively charged surfaces via ionic or hydrogen bonding (Muzzarelli, 1973; Rha, 1984; Shahidi, 1995).

The term chitosan is preferred when the nitrogen content of the molecule is higher than 7% by weight (Muzzarelli, 1985) and the degree of deacetylation is more than 70% (Li *et al.*, 1992). Furthermore, Schoemaker (1991) reported that the ash content of chitosan should not exceed 1% and its insolubles should be $\leq 0.5\%$ by weight.

Interest in new applications of chitin and its derivatives grew in the 1930's and early 1940's leading to over 50 patents. However, commercialization of these products was hampered by lack of adequate manufacturing facilities and competition from synthetic polymers (Averbach, 1981; Subasinghe, 1999). However, after 1970's industrial exploitation of chitin and its derivatives has been emerging and expanding (Kaye, 1985). Furthermore, advancement in research and small-scale production of chitin and chitosan have expanded the number and variety of potential applications. In addition, environmental problems and cost for disposal of shellfish processing discard have increased and environmentally safe substitutes for many plastic or polymeric products are in demand (Ashford *et al.*, 1976; Berkeley, 1979; Shahidi and Synowiecki, 1991). Some food applications of chitin, chitosan and their derivatives are shown in Table 2.1.

Table 2.1. Food applications of chitin, chitosan and their derivatives

Area of application	Examples
Antimicrobial agent	Bactericidal
	Fungicidal
	Measure of mold contamination in agricultural commodities
Edible film	Controlled moisture transfer between food and the surrounding environment
	Controlled release of antimicrobial substances
	Controlled release of antioxidants
	Controlled release of nutrients, flavours and drugs
	Reduction of oxygen partial pressure
	Controlled rate of respiration
	Temperature control
	Controlled enzymatic browning in fruits
	Reverse osmosis membranes
Food additive	Clarification and deacidification of fruit juices
	Natural flavour extender
	Texture controlling agent
	Emulsifying agent
	Food mimetic
	Thickening and stabilizing agent
	Colour stabilization
Nutrition	Dietary fibre
	Hypocholesterolemic effect
	Livestock and fish feed additive
	Reduction of lipid absorption
	Production of single cell protein
	Antigastritis agent
	Infant food ingredient
Waste treatment	Affinity flocculation
	Fractionation of agar
Purification of water	Recovery of metal ions, pesticides, phenols and PCB's
	Removal of dyes
Other	Enzyme immobilization
	Encapsulation of nutraceuticals
	Chromatography
	Analytical reagents

Both chitin and chitosan have unusual multifunctional properties, including high tensile strength, bioactivity, and biodegradability which makes them an attractive specialty material (Berkeley, 1979; Ikejma and Inoue, 2000). Furthermore, these polymers have been identified as being biocompatible, non-antigenic, almost non-toxic, and biofunctional (Hirano *et al.*, 1990; Li *et al.*, 1992). Recently, Rao and Sharma (1997) reported that acute systemic toxicity tests in mice did not show any significant toxic effect of chitosan; all mice injected with the test material extract lived during the entire period (72 h) of observation. These authors further observed that eye irritation tests in rabbits and skin irritation tests in guinea pigs did not produce any undesirable toxic effect due to chitosan.

United States Food and Drug Administration (FDA) approved chitosan as a feed additive in 1983 and use of chitosan for portable water purification was approved by the US Environmental Protection Agency (EPA) up to a maximum recommended concentration of 10 mg/L (Knorr, 1986). In 1992, Japan's Health Department approved chitin and its derivatives as a functional food. A functional food should satisfy two of the following five functions: enhancement of immunity, prevention of illness, delaying of aging, recovery from illness, and control of biorhythm; chitin and chitosans have been identified as possessing most of the above attributes (Subasinghe, 1999).

2.1.1 Sources of chitin and chitosan

At least 10 gigatons (1.10^{13} kg) of chitin are synthesized and degraded each year in the biosphere (Muzzarelli, 1999). Nearly 10% of the global landings of aquatic products consist of species rich in chitinous material (Subasinghe, 1999). These include, species

such as shrimp, crab, lobster, squid, cuttlefish, oyster, clams, krill, crawfish and squilla, among others. The annual production of species containing chitin is approximately 13 million metric tons (FAO, 1999). Chitin usually occurs as mucopolysaccharide, that is conjugated with protein and inorganic material, mainly $CaCO_3$, pigments and lipids (Austin *et al.*, 1981; Knorr, 1984; Gopakumar, 1997) in the exoskeleton of crustaceans, insects and mollusks (Knorr, 1984; Lower, 1984a). The body parts and processing discards of these species contain approximately 10-55% chitin on a dry weight basis (Table 2.2). Since chitin and chitosan do not accumulate in the environment, chitinases and chitosanases must play a significant role in biochemical cycling of limiting elements including nitrogen (Bade and Wick, 1988). Certain fungi, algae, diatoms and protozoa also contain chitin and sometime chitosan as, cell wall and cellular constituents (Berkeley, 1979; Austin *et al.*, 1981).

The biosynthesis of chitin takes place in the membrane-bound protein complex chitin synthase. In arthropod exoskeleton and most of the fungi uridine diphosphate-N-acetyl-D-glucosamine is polymerized into chitin by chitin synthase (EC 2.4.1.16) (Cohen, 1993; Hirano, 1996; Merz *et al.*, 1999) (Figure 2.2). The natural pathway of chitin metabolism includes enzyme-catalyzed hydrolysis by chitinases. There are various forms of these enzymes which are usually divided into endo and exo-groups. The chitinases (EC 3.2.1.14) give *N,N'*-diaacetylchitobiose and *N,N',N''*-tricetylchitotriose as the final products (Peter, 1995). Chitin-N-deacetylase (EC 3.5.1.41) catalyzes the N-deacetylation reaction of chitin into chitosan (Muzzarelli, 1977; Cohen, 1993; Hirano, 1996). Diatoms which have protein-free chitin appendages are conceivable practical sources of very pure

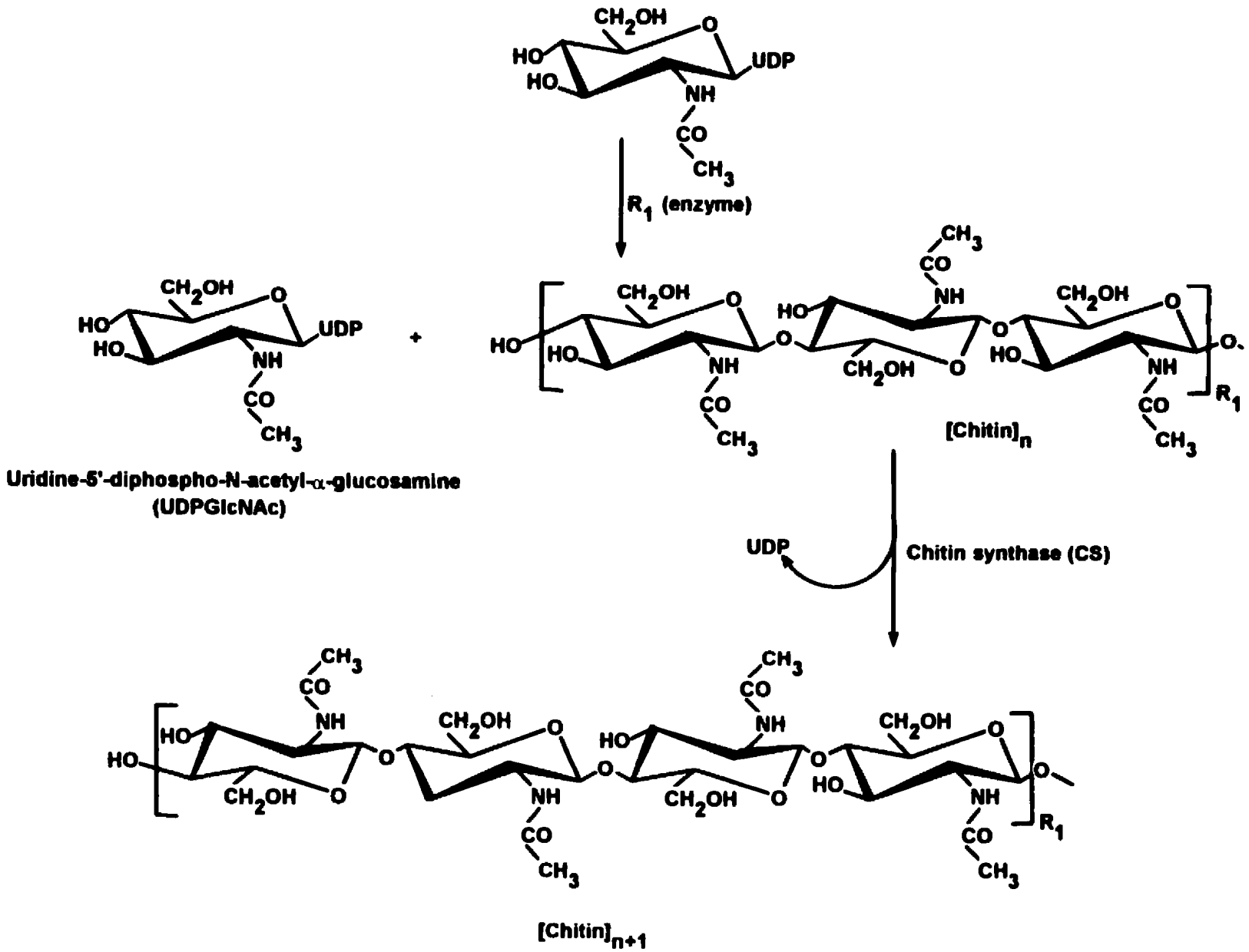
Table 2.2 Chitin content of selected crustaceans, insects and molluscan organs*

Organism	Chitin content (%)	Organsim	Chitin content (%)
Crustacea		**Insects**	
Cancer (crab)	72.1[c]	Periplaneta	2.0[c]
Carcinus (crab)	0.4-3.3[a]	(cockroach)	
	8.29[b]	Blatella	18.4[c]
	64.2[c]	(cockroach)	10.0[b]
Paralithodes	35.0[b]		35.0[c]
(King crab)		Coleoptera (beetle)	5.0-15.0[b]
Callinectes	14.0[a]		27.0-35.0[c]
(Blue crab)	14.9[d]	Tenebrio (beetle)	2.1[a]
Chinoecetes	29.0-40.0[b]		4.9[b]
(Snow crab)			31.3[c]
Pleuroncodes	1.3-1.8[b]	May beetle	16.0[b]
(red crab)	27.6[d]	Deptera (true fly)	54.8[c]
Pandadlus	40.0-41.0[b]	Pieris (sulfur	64.0[c]
(Pink shrimp)		butterfly)	
Crangon (shrimp)	5.8[b]	Grasshopper	2.0-4.0[a]
	69.1[c]		20.0[c]
Brine shrimp	27.2[d]	Bombyx	44.2[c]
Alaskan shrimp	28.0[d]	(silkworm)	
Nephrops (lobster)	69.8[c]	Calleria	33.7[c]
	6.7[b]	(Waxworm)	
Metanephrups	15.7[d]		
(lobster)		**Molluscan organs**	
Homarus (lobster)	60.8-77.0[c]	Clamshell	6.1
Lepas (barnacles)	58.3[c]	Oyster shell	3.6
Stone crab	18.1[d]	Squid(skeletal pen)	41.0
Horseshoe crab	26.4[d]	krill (deproteinized	40.2
Crawfish	23.5[d]	shell)	

[a]wet body weight
[b]dry body weight
[c]organic weight of cuticle
[d]total dry weight of cuticle
[e]dry weight of the cell wall

*Data adapted from Austin *et al.*(1981); Knorr (1984); Knorr *et al.* (1989); No *et al.* (1989); Shahidi (1994); Chung *et al.* (1996).

Figure 2.2 Sum equation for the reaction catalyzed by chitin synthase (CS; UDP-N-acetyl-D-glucosamine: chitin 4-β-N-acetylglucosaminyltransferase; EC 2.4.1.16. R_1 = UDPGlcNAc-tansferase: adapted from Merz *et al.*, 1999).


Uridine-5'-diphospho-N-acetyl-α-glucosamine
(UDPGlcNAc)
R1 (enzyme)
[Chitin]n
UDP
Chitin synthase (CS)
[Chitin]n+1

chitin. For special applications, requiring exceptionally high quality in relatively small quantities, diatoms might eventually become a practical source of pure chitin (Ashford *et al.*, 1976; Gopakumar, 1997). The vast majority of fungi including members of the Ascomycetes, Basidiomycetes, Deuteromycetes and Mastigomycetes have walls which contain chitin and glucans or mannans whereas those of Zygomycetes contain both chitin and chitosan (Bartnicki-Garcia, 1968; Gopakumar, 1997). In addition to that, large quantities of fungi, currently grown in fermentation systems for producing organic acids, antimicrobials and enzymes, constitute a potential source for chitin production (Ashford *et al.*, 1976; Rha, 1984; Anon, 1993). It is estimated that fungi could provide $3.2x10^4$ metric tons of chitin annually (Brine, 1984). Chitosan occurs naturally in fermented foods such as tempeh, sufu, and even in aged beef and these foods are already part of diet in people of many parts of the world (Bough, 1977; Berkeley, 1979).

Since the biodegradation of chitin is very slow in crustacean waste, accumulation of large quantities of shell discards from processing of crustaceans has become a major concern in the seafood industry (Shahidi and Synowiecki, 1991; Martin, 1998). Furthermore, pollution from wastes has become a serious problem because solid waste disposal and discharge of processing discards is tightened by regulation and in quite costly in most places (Schoemaker, 1991; Martin, 1998). Therefore, production of value-added products such as chitin, chitosan and their derivatives and subsequent application in different fields is of utmost interest (Brzeski, 1987; Shahidi *et al.*, 1999).

Approximately 25-60% by weight of shrimp raw material is discarded as waste when processed into headless shell-on products. Peeling process, which involves the

removal of the shell from the tail of prawn further increases the total waste production (Schoemaker, 1991). Thus, on a global basis shrimp processing industry produces over 700 000 metric tons of quality waste (Subasinghe, 1999). The annual landing of crab and crab-like species is estimated at about 1.35 million metric tons (FAO Fishery Satistics, 1999). Up to 80% of the crab raw material is discarded as waste during processing (Shahidi and Synowiecki, 1992). Production of frozen or canned crab meat gives rise to considerable amount of discard which total approximately 480 000 metric tons annually (Subasinghe, 1999). The annual amount of accessible chitin from seafood processing discards may account for up to 120 000-150 000 metric tons (Shahidi, 1994; Subasinghe, 1999). Shahidi and Synowiecki (1991) as well as No and Meyers (1995) have reported further extraction of pigments, proteins and carotenoproteins from processing discards of shrimp and crab. The total annual sale of chitin/chitosan, which was estimated at US$50 million in the early 80's is expected to surpass US$2 billion by the end of 2000 (Knorr, 1991; Schoemaker, 1991; Subasinghe, 1999).

2.1.2 Extraction of chitin and chitosan from shellfish processing discards

Over the last several years different procedures have been developed for preparation of chitin and chitosan from source materials (Roberts, 1992). These procedures are different mainly due to compositional differences in the original material and existing differences in conditions employed during the manufacturing process (No and Meyers, 1995). All of these lead to chitin and chitosan products which differ in their physicochemical properties.

2.1.3 Extraction of chitin from shellfish processing discards

Chitin in shellfish processing discards is tightly associated with proteins, lipids, pigments and mineral deposits (Roberts, 1992; Simpson *et al.*, 1994). Therefore, raw material has to be pretreated to remove non-chitinous components (Brzeski, 1987; Simpson *et al.*, 1994).

The entire manufacturing process of chitin may be divided into four different steps.

- Preparation of sample
- Removal of mineral fraction or demineralization
- Removal of protein fraction or deproteinization
- Removal of carotenoid pigments or decoloration

These four basic steps are continuously monitored so that removal of interfering components is best achieved. For example, careful control of time, concentration of chemicals and temperature is necessary in order to ensure the highest molecular weight chitin/chitosan (Ornum, 1992). Nevertheless, with careful control of above parameters, it might be possible to produce chitosan with molecular weights of 80, 000-1, 000, 000 Da with reproducible properties (Averbach, 1981).

The order of deproteinization and demineralization steps may be easily interchanged (Shahidi and Synowiecki, 1991). However, in commercial practice, generally proteins are first extracted using a base. The demineralization is achieved using a dilute hydrochloric acid solution (Shahidi, 1995). This acid treatment is important for the removal of minerals in order to ensure an ash content of less than 0.1% in the product (Kaye, 1985). Although the resultant calcium chloride may be used in the pulp and paper

manufacturing, the dehydration process required for its recovery is commercially unattractive. Another potential application of calcium chloride as a 30% solution as a dust control agent for spraying on mud roads has been reported (Shahidi, 1995). Recovery of chitin from wet shellfish processing discards averages 10% (Schoemaker, 1991).

2.1.3.1 Preparation of raw materials

Washing is important for the removal of soluble organics and adherent proteins where as grinding is necessary to increase the surface area and to obtain uniform size particles.

Carotenoproteins, by-products from shellfish processing, have potential for use as a feed supplement in aquaculture (Simpson and Haard, 1985; Ramaswamy *et al.*, 1991; Shahidi and Synowiecki, 1991; Shahidi, 1994) or as a colorant and flavour compound that may be used in food products (Simpson and Harrd, 1985). Extraction of carotenoid pigments with organic solvents or oil reduces both the ash and chitin levels and achieves a good recovery of pigments, but the product so obtained is devoid of protein and hence has decreased stability due to oxidation (Haard, 1992b). Since about one third of the dry matter in crustacean shellfish processing discards is protein, an enzymatic process has been developed to recover them along with the carotenoids in their native carotenoprotein state. Carotenoproteins may be efficiently extracted from shellfish processing discards such as shrimp, crab and lobster, if trypsin is added to the extraction buffer (Cano-Lopez *et al.*, 1987; Manu-Tawiah and Haard, 1987; Ramaswamy *et al.*, 1991). Cano-Lopez *et al.* (1987) reported that trypsin from Atlantic cod used for extraction of carotenoprotein from

shrimp processing discard was able to recover 64% of the astaxanthin and 81% of proteins, whereas bovine trypsin recovered only 49% of astaxanthin and 65% of proteins under similar experimental conditions.

Simpson *et al.* (1992) have shown the effect of trypsin from bovine pancreas and Atlantic cod offal (crude cod enzyme and semi purified extracts) on the recovery of carotenoproteins from lobster waste. However, commercial trypsin afforded a higher yield of pigment than cod enzyme preparations.

Shahidi and Synowiecki (1991) have shown the possibility of extracting carotenoid pigments prior to the deproteinization step. Fish oil was used for extracting carotenoids directly from shell wastes; the best recovery of carotenoids (72.23%) was achieved when the ratio of offal to oil was 1:2 (w/v) and the extraction temperature and time were adjusted to 60^0C and 30 min, respectively. Meanwhile, No and Meyers (1992) have reported oil extraction of astaxanthin from crawfish processing discards. In a similar study, Ramaswamy *et al.* (1991) reported the recovery of carotenoproteins from lobster waste using ethylenediaminetetraacetic acid (EDTA), as a chelating agent, and with the aid of trypsin which enhanced the yield of the process. These authors further recommended this method as a suitable procedure that prevented the oxidation of pigments due to the presence of proteins in the product.

2.1.3.2 Demineralization

Demineralization is carried out to remove calcium carbonate, which is the major mineral present in shellfish processing discards, and precipitate it as calcium chloride

(Simpson *et al.*, 1994). Demineralization is also important for removing of endotoxins which are present in the shell processing discards (Wachter *et al.*, 1999). Demineralization may be achieved using a variety of either inorganic or organic acids including HCl, HNO_3, H_2SO_3, CH_3COOH and HCOOH (Roberts, 1992) in different concentrations, time, temperature and solid to solvent ratios (No and Meyers, 1995). However, Chang and Tsai (1997) demonstrated that only HCl concentration and solution to solid ratio were important variables involved; demineralization temperature for shrimp processing discard was insignificant. Chang and Tsai (1997) also suggested that increasing HCl concentration or solution to solid ratio was significant whereas the effect of temperature for demineralization of shrimp shell waste was unimportant. Furthermore, these authors reported that increasing HCl concentration or solution to solid ratio decreased the residual calcium content and that use of 1.7N HCl and a solution to solid ratio of 9.00 mL/g at ambient temperature provided optimum conditions for the removal of calcium from shrimp processing discards. In a similar study, Shahidi and Synowiecki (1991) observed that ash content of deprotienized shell waste from shrimp and crab, on a dry basis, was 42% and 45%, respectively. After 30 min of demineralization at 20 ^{0}C [2.55 (w/v) HCl solution, at a ratio of shells to acid of 1:20 (w/v)], the ash content in chitin from shrimp and crab decreased to 0.10 and 0.25%, respectively. One of the disadvantage of using HCl for demineralization is that HCl at concentrations above 1.25N adversely affects the viscosity of the final product due to depolymerization of chitin chain (Muzzarelli, 1977). Extraction of minerals with EDTA under alkaline pH conditions has been suggested as a non-

degradative demineralization process in order to circumvent the above problems (Austin *et al.*, 1981; Roberts, 1992).

2.1.3.3 Deproteinization

Protein removal from shellfish processing discards is conventionally accomplished by alkali extraction (Bough *et al.*, 1978). Sodium hydroxide is often used for this purpose. Deproteinization is carried out by treating the demineralized intermediate products with aqueous alkali metal hydroxide solutions, preferably dilute 5 to 25% (w/w) solution of sodium hydroxide. This step is generally carried out at 50 to 110 ^{0}C, and preferably at 90 to 108 ^{0}C, and at a pH value of 12 -14 (Wachter *et al.*, 1999). However, deproteinization may be achieved using different alkali concentrations, time, temperature and solid to solvent ratios (No and Meyers, 1995). Nevertheless, a large variation exists for deproteinization conditions (Roberts, 1992). However, Chang and Tsai (1997) argued that influence of solution to solid ratio is insignificant and that optimal deproteinization condition used 2.5 N NaOH and 74 ^{0}C with a minimum solution to solid ratio of 5 mL/g. Two times alkali treatment, instead of once, was also reported by Anderson *et al.* (1978) for krill processing discards and by Chang and Tsai (1997) for pink shrimp waste who used 0.5 N NaOH for 6 h at ambient temperatures (initial alkali treatment). Austin *et al.* (1981) reported the use of mild alkaline conditions for isolation of proteins from the carapace of horseshoe crab (*Limulus polyphemus*) which contained a low amount of protein. Furthermore, these researchers observed that chitin manufactured via this process was probably typical type of native chitin and chemically leavorotatory in nature. Austin *et*

al. (1981) indicated that some other chitin which may require harsh acidic or temperature conditions for their isolation yield dextrorotatory products. Recovered protein from the deproteinization step may be utilized as a growth medium for microorganism or by the food and feed industries in selected applications (Bough *et al.*, 1978; Johnson and Peniston, 1982; No and Meyers, 1992). Limitations with alkali treatment include requirement of large amounts of alkali which results in deacetylation and a decrease in the molecular size of the product (Simpson *et al.*, 1994).

The use of enzymes for the removal of protein has been examined by a number of researchers. Possibility of using enzyme Rhozyme-62 concentrate at 60 ^{0}C for 6 h at pH 7.0 for deproteinization of crustacean shell wastes was described by Bough *et al.* (1978). Deproteinization of crustacean carapace was also achieved using proteolytic bacteria (*Pseudomonas maltophila* LC 102); this method of deproteinization is prefered because it avoids deacetylation (Shimahara *et al.*, 1982). Simpson *et al.* (1994) reported that out of various proteolytic enzymes, namely chymotrypsin, papain and bacterial protease used for deproteinization of crustacean shells, chymotrypsin was most effective, achieving a degree of deptoteinization comparable with that of the chemical deproteinization. Enzymatic deproteinization of the shells for production of protein hydrolysate during isolation of chitin from shrimp (*Crangon crangon*) processing discards was recently reported (Synowiecki and Al-Khateeb, 2000). This method was found suitable for isolation of chitin containing 4% of protein impurities and also for production of a protein hydrolysate with an adequate essential amino acid index and protein efficiency ratio. Enzyme treatment

doesd not affect the chitosan structure, however, complete removal of protein may not be achieved (No and Meyers, 1995).

Protein recovery reduces a considerable portion of the manufacturing cost of chitin and chitosan, and thus contributes significantly to the profitability of a chitin/chitosan enterprise (Johnson and Peniston, 1982; Shahidi and Synowiecki, 1991). The profile of amino acids recovered from shrimp and crab processing discards was better, except for lysine and tryptophan (Shahidi and Synowiecki, 1991).

2.1.3.4 Decoloration

Decoloration is an important unit operation for production of a white coloured chitin with added commercial value. The exoskeletons of crustaceans contain colouring matters, principally carotenoids, the main components being astaxanthine, astacene, canthaxanthine, lutein and β-carotene. These carotenoids do not appear to be complexed with either the inorganic material or the protein since treatments which remove them from shells do not remove carotenoids (Roberts, 1992). There are two steps involved in decoloration of shellfish processing discards; these are extraction of pigments using the necessary reagents and bleaching using appropriate chemicals. Decoloration of chitin may be achieved using organic solvents such as acetone, chloroform, ether or ethanol (Simpson *et al.*, 1994; No and Meyers, 1995). The second step is mainly important for commercial chitin preparation (Simpson *et al.*, 1994), for which acetone, sodium hypochlorite solution (No *et al.*, 1989) and hydrogen peroxide (Brine and Austin, 1981) have been used as the

bleaching agents. The colour of chitin recovered from shellfish processing discards may vary from white to pink (No and Meyers, 1995).

2.1.4 Production of chitosan from chitin

Chitosan is the simplest product that may be obtained from chitin (Ornum, 1992). In order to obtain chitosan from chitin, alkali treatment may be carried out to remove some or all of the acetyl groups from the chitin polymer; this process is known as deacetylation. Although amides may in principle be hydrolysed under either acidic or basic conditions, the use of acid hydrolysis is precluded because of the susceptibility of the glycosidic linkages in chitin to acid hydrolysis (Roberts, 1992). Furthermore, as pointed out by Horton and Lineback (1965), the trans arrangement of the C(2)-C(3) substituents in chitin increases the resistance of the C(2)-acetamido group to alkaline hydrolysis, therefore severe treatments are required to bring about deacetylation.

Deacetylation may be achieved using different reagents, concentrations, time, and temperature as well as weight of raw material to the volume of the extraction solution ratios in order to obtain chitosan from chitin (Table 2.3). Wachter *et al.* (1999) reported use of concentrated bases, such as 50 to 70% (w/w) sodium or potassium hydroxide at 70-110 ^{0}C, and more particularly, 90-108 ^{0}C, and at a pH of 12-14. The most frequently used alkali is NaOH, but KOH has been used in some instances and LiOH, $Ca(OH)_2$ and Na_3PO_4 have been claimed to be suitable (Roberts, 1992). Some limitations of this chemical method are high energy cost, use of high volumes of caustic concentrated NaOH and production of a liquid waste containing protein and non-protein nitrogenous

Table 2.3 Conditions used for deacetylation of shellfish waste for production of commercial chitosan*

Source	Type of alkali	Temperature (°C)	Material to solvent ratio (w/w or w/v)	Time (h)	Deacetylation (%)
Commercial chitosan	5% NaOH	100	-	3	100
Commericial chitosan	47% NaOH	110	-	1	94-95
Crab	47% NaOH	60	-	2x1-4[a]	57-90
		110	-	1x1-3[a]	78-96
		110	-	1x3[b]	99
	39% KOH[c]	reflux	1:17	20	-
	50% NaOH	100	-	1	93
Krill	50% NaOH	80-96	-	1/3	56-68
	39% KOH	reflux	1:17	20	-
Lobster	55% NaOH	100-140	1:100	0.5-15	65-81
Prawn	50% NaOH	30	1:56	24	87
	60% KOH	100	1:65	1	-
	50% NaOH	100	-	2	-
Shrimp	50% NaOH	100	-	0.5-5	68-78
	50% NaOH	145-150	1:10	1/12	-
	50% NaOH	60	1:4	2[d]	-
	50% NaOH	100	1:15	1/2x2	57
	30% NaOH	100	-	1	20
Squid	40% NaOH	80	1:20	3x1-3[a]	80-97
	30% NaOH	100	-	1	60
	40% NaOH	100	-	2x3	-

[a]times of separate alkali treatment
[b]3 times alkali treatment and additional alkali treatment after the transformation of the sample
[c]dissolved in 95% ethanol and ethylene glycol
[d]dry-heating

*Data Adapted from No and Meyers (1995); Aiba (1992); Benjakul and Sophanodora (1993); Dung *et al.* (1994); Kurita (1997); Rinaudo *et al.* (1997); Simpson *et al.* (1994); Shimojoh *et al.* (1998).

compounds (Simpson *et al.*, 1994; Hirano, 1996). Deacetylation may also be achieved by biological means using chitin deacetylase producing microorganisms such as *Mucor rouxii*, *M. meehei*, *Absidia butleri* and *Aspergillus niger* (Haard *et al.*, 1994; Simpson *et al.*, 1994). Some problems associated with enzymatic method are low yield and variation with age of fungal culture (Simpson *et al.*, 1994).

Complete deacetylation is rarely achieved nor is it normally necessary since solubility in dilute aqueous acids is obtained at ≥ 60% deacetylation (Roberts, 1992). However, Mima *et al.* (1983) established a method for preparation of chitosan product with a desired degree of deacetylation of up to 100%, without any serious depolymerization. Effective deacetylation was readily attained by intermittently washing the intermediate product in water two or more times during alkali treatment for less than 5h in 47% NaOH at 110 ^{0}C. Application of thermo-mechano-chemical treatment as an alternative method for chitin deacetylation was evaluated by Pelletier *et al.* (1990) who used a cascade reactor unit operation under reduced alkaline conditions of 10% (w/v) NaOH. Sudden decompression of the aqueous alkaline suspension of mercerized chitin resulted in near complete deacetylation of chitin. Domard and Rinaudo (1983) proposed a new method for preparation of fully deacetylated chitosan without any excessive decrease in molecular weight. Such an effective deacetylation was attained by use of thiophenol which traps oxygen, thus preventing degradation and exerting a catalytic effect during two or three successive alkali treatments for 1h at 100 ^{0}C.

Chitosan has been sold commercially in the form of a solution, flake and fine powder, and more recently in bead and fibre forms (Kumar and Jayachandran, 1993).

Some of the characteristics of the commercially produced chitosans are shown in Table 2.4.

2.1.5 Physicochemical properties of chitin and chitosan

2.1.5.1 Molecular conformation of chitin

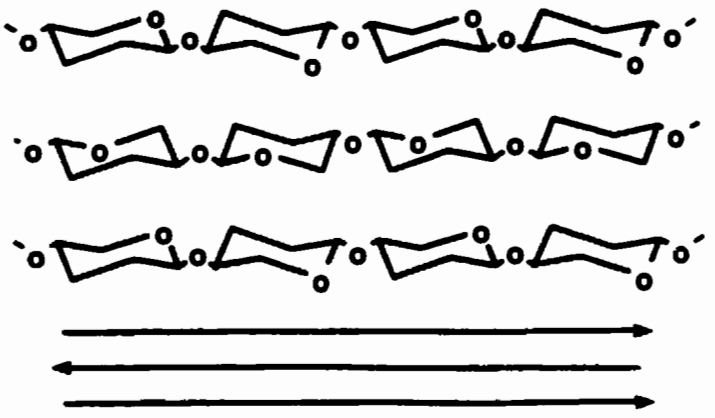
Chitin exists in three polymorphic forms with various degrees of crystalinity (Muzzarelli, 1985). These crystalline structures of chitin are evidenced by X-ray diffraction studies which showed three polymorphic forms of α-chitin, β-chitin, and γ-chitin (Roberts, 1992; Shimojoh *et al.*, 1998). The chitin from insect and crustacean cuticle occurs in the form of microfibrils, typically 10-25 nm in diameter and 2-3 μm in length (Peter, 1995). Generally, individual chains assume essentially a linear structure, which undergoes one full twist every 10.1-10.5 Å along the chain axis (Winterowed and Sandford, 1995). Chitin and chitosan are internally cross linked to a much greater degree than cellulose; each N-acetylglucosamine residue in chitin is linked by an estimated eight hydrogen bonds to residues in the surrounding chains which gives this material an exceptionally high tensile strength in three dimensions compared to cellulose or starch (Bade and Wick, 1988). Since each glycosidic unit in the chain is chiral and all units are connected by an oxygen atom that links C1 of one glycosidic unit to C4 of an adjacent unit, a distinct "left" and "right" direction may be assigned to each polymer chain (Muzzarelli, 1977; Roberts, 1992). In α-chitin, chains are anti-parallel, but in β-chitin chains are parallel and in γ-chitin, two chains are upwards direction to each downwards direction chain (Figure 2.3). Out of these different polymorphic forms, α-chitin is the most

Table 2.4 Specification for various grades of commercially available chitosan*

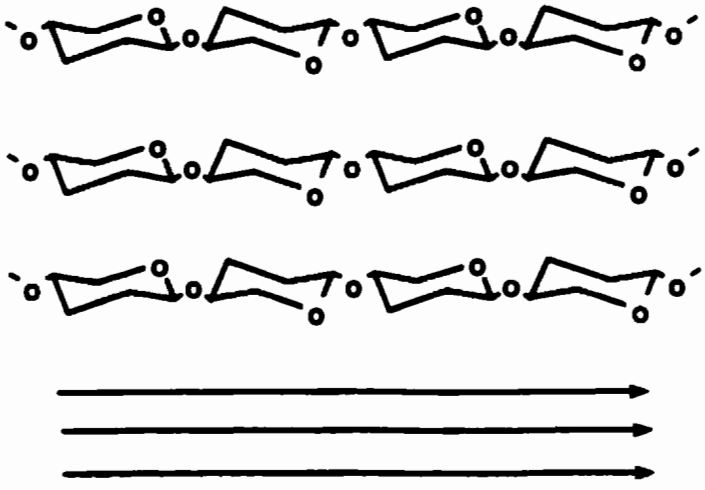
Specifications	Chitosan (Food grade)	Chitosan (Pharmaceutical grade)	Liquid Chitosan (Technical grade)
Appearance	white/yellow powder	white/yellow powder or flake	clear/yellow liquid
Moisture content	<10%	<10%	<10%
Residue on ignition	<0.5%	<0.2%	<0.5%
Protein content	<0.3%	<0.3%	<0.3%
Deacetylation	70-100%	70-100%	70-100%
Viscosity (0.5% solution)	50-100 cps	<5 cps	50 cps
Insolubles	<1.0%	<1.0%	<0.5%
Heavy metals			
Arsenic	<10 ppm	<10 ppm	<10 ppm
Lead	<10 ppm	<10 ppm	<10 ppm
pH	7-9	7-9	<5.5
Odour	No taste or smell	No taste or smell	No taste or smell

*Adapted from Subasinghe (1999).

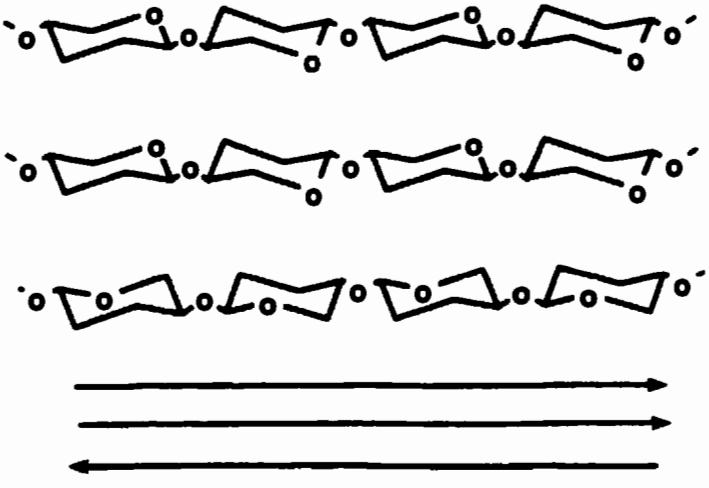
Figure 2.3 Arrangement of the polymer chains in the three forms of chitin.



α-Chitin



β-Chitin



γ-Chitin

abundant one and the other two might be converted to α-chitin under appropriate conditions (Roberts, 1992; Winterowd and Sandford, 1995). Since it is easy to access α-chitin compared to the other two polymorphic forms, most of the research has been carried out on α-chitin (Shimojoh *et al.*, 1998). However, chitin isolated from squid pens has a β-structure in contrast to the ordinary α-chitin. Deacetylation of squid chitin takes place much easier than that of α-chitin because of the loose arrangement of chitin molecules (Kurita, 1997). Differences between α-chitin and β-chitin are listed in Table 2.5.

Bade (1997) argued that, depending on these chain arrangements, there are four different orders of chitin present in nature, such as linear primary structure in which monomers linked to each other by covalent β-1-4-glycosidic bonds, three dimensional secondary structure which consists of primary chitin chains linked to form microfibrils, tertiary and quaternary structures with macrofibrils and sheets, respectively.

2.1.5.2 Molecular weight of chitin and chitosan

The molecular weight of natural chitin is normally greater than 1, 000, 000 Da and that of commercially available chitosan is around 100,000-1,200,000 Da (Lower, 1984b; Li *et al.*, 1992). Several external forces during manufacturing process may affect the molecular weight of chitosan. Factors such as high temperature (above 280 ^{0}C thermal degradation of chitosan takes place and the polymer chains rapidly break down), dissolved oxygen concentration and shear stress may cause these alterations (Muzzarelli, 1977; Li *et al.*, 1992). Brine and Austin (1981) observed chitin prepared from blue, red and stone crab

Table 2.5 Differences between α-chitin and β-chitin*

α-chitin	β-chitin
Quite resistant to modification reactions	Susceptible to modification reactions
Cannot undergo various reactions efficiently under mild conditions	Can undergo various reactions efficiently under mild conditions
Major sources: shrimp and crab shell	Major source: squid pen
Molecules are packed antiparallel	Molecules are packed parallel
Less affinity for organic solvents and water	High affinity for organic solvents and water
Deacetylation is relatively slow	Deacetylation is relatively fast

*Adapted from Kurita (1997).

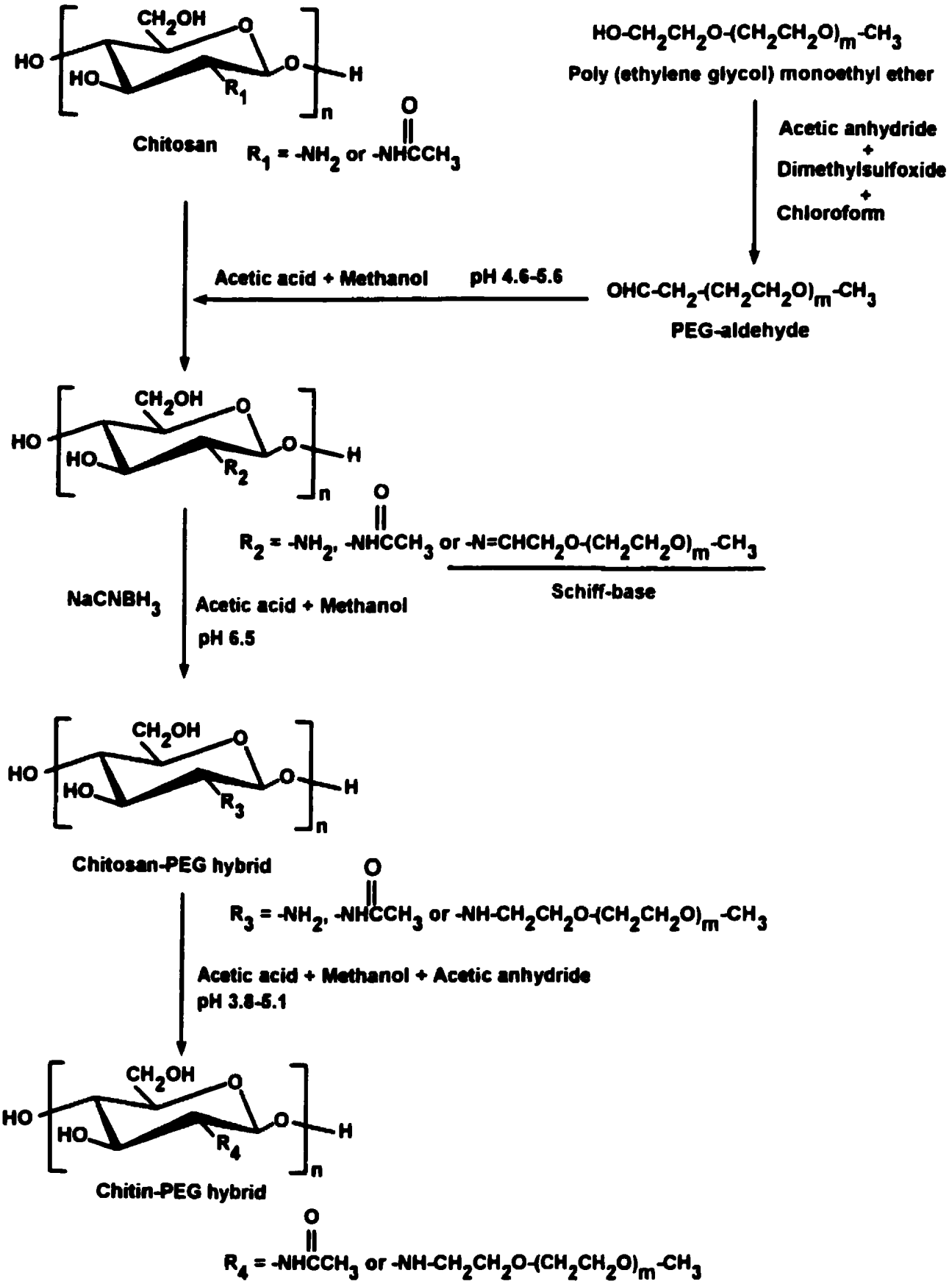
to have variations in their molecular weight depending on the method of demineralization employed. Furthermore, these authors reported that the greatest depolymerization occurred when HCl was used for demineralization, followed by acetic acid and sulphurous acid, with a minimum degradation occurring during EDTA demineralization process.

There are many methods to determine the molecular weight of chitin and chitosan, such as viscometry (Roberts and Domszy, 1982), laser light-scattering technique (Muzzarelli *et al.*, 1987), and chromatography (Wu and Bough, 1976). Out of these, viscometry is still widely used because of its speed and simplicity (Maghami and Roberts, 1988).

2.1.5.3 Solubility properties and common reactions of chitin and chitosan

Chitin is insoluble in aqueous solutions because of microcrystalline regions stabilized by hydrogen bonds (Claesson and Ninham, 1992). Unlike chitin, chitosan is readily soluble in various acidic solvents, forming viscous non-Newtonian solutions (Bough, 1977), when the pH of the solution is less than 6 (Li *et al.*, 1992). Furthermore, various studies have been conducted to produce water-soluble derivatives of chitin and chitosan using chemical modification techniques (Dung *et al.*, 1994; Sugimoto *et al.*, 1998). Production of a water soluble chitosan in the absence of acids is important if presence of acids may cause harmful effects, such as in cosmetics, medicine and certain foods (Li *et al.*, 1992). Sugimoto *et al.* (1998) used polyethylene glycol in order to improve solubility of chitin and chitosan in water (Figure 2.4).

Figure 2.4 Preparation of water soluble chitin/chitosan-PEG hybrid (adapted from Sugimoto *et al.*, 1998).

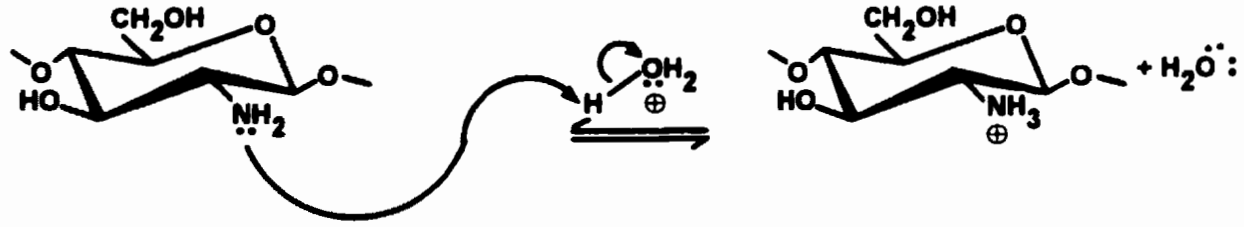

Chitosan
R_1 = -NH_2 or -$NHCCH_3$
$HO\text{-}CH_2CH_2O\text{-}(CH_2CH_2O)_m\text{-}CH_3$
Poly (ethylene glycol) monoethyl ether
Acetic anhydride
+
Dimethylsulfoxide
+
Chloroform
$OHC\text{-}CH_2\text{-}(CH_2CH_2O)_m\text{-}CH_3$
PEG-aldehyde
Acetic acid + Methanol
pH 4.6-5.6
R_2 = -NH_2, -$NHCCH_3$ or -$N{=}CHCH_2O\text{-}(CH_2CH_2O)_m\text{-}CH_3$
Schiff-base
$NaCNBH_3$
Acetic acid + Methanol
pH 6.5
Chitosan-PEG hybrid
R_3 = -NH_2, -$NHCCH_3$ or -$NH\text{-}CH_2CH_2O\text{-}(CH_2CH_2O)_m\text{-}CH_3$
Acetic acid + Methanol + Acetic anhydride
pH 3.8-5.1
Chitin-PEG hybrid
R_4 = -$NHCCH_3$ or -$NH\text{-}CH_2CH_2O\text{-}(CH_2CH_2O)_m\text{-}CH_3$

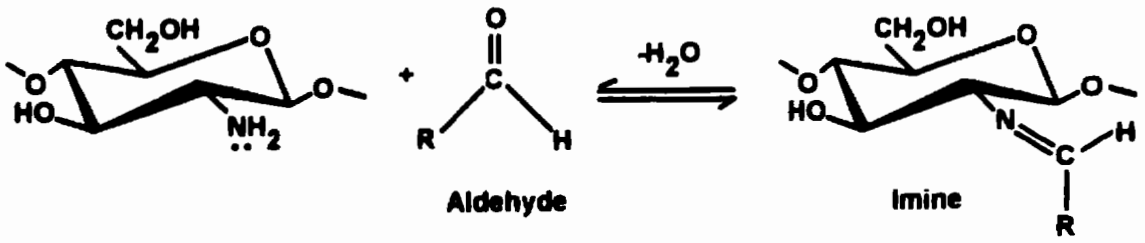
Solvents such as strong acids and fluoroalcohols may be used to solubilize chitin (Austin *et al.*, 1981). However, these solvents may cause degradation of chitin chain which in turn would limit its use (Muzzarellli., 1985). Austin *et al.* (1981) and Lower (1984a) have reported dimethylacetamide and methyl-2-pyrollidone containing 5% lithium chloride as effective solvent systems which are non-degradative to chitin. Upon heating, both chitin and chitosan decompose prior to melting. Thus, these polymers have no melting points (Winterowd and Sandford, 1995).

Both chitin and chitosan may be considered weak bases, and as such, they undergo the typical neutralization reactions of alkaline compounds. In these reactions the nonbonding lone pair electrons on the primary amine group of the glucosamine units perform the role of accepting protons (Winterowd and Sandford, 1995) (Figure 2.5). In addition, presence of the primary amino group in chitosan offers further possibilities for modification of the molecule such as *N*-acylation, *N*-alkylation, and *N*-alkylidenation (Figure 2.6).

The solution properties of chitosan are governed mainly by the degree of N-acetylation, the distribution of acetyl groups, the pH and the ionic strength (Anthonsen *et al.*, 1993). The amino group in chitosan has a pKa-value of 6.2-7.0, which makes chitosan a polyelectrolyte at low pH-values (Claesson and Ninham, 1992). The nonbonding pair of electrons on the primary amine groups also make chitosan a potent nucleophile, reacting readily with most aldehydes to form imines. Furthermore, acylchlorides react vigorously with chitosan to form the corresponding amide derivatives (Winterowd and Sandford,

Figure 2.5 Neutralization and nucleophilic reactions of chitosan (adapted from Winterowd and Sandford, 1995).


CH2OH
O
HO
NH2
OH2
H
NH3
+ H2O:


CH2OH
O
HO
NH2
R
H
-H2O
N
Aldehyde
Imine

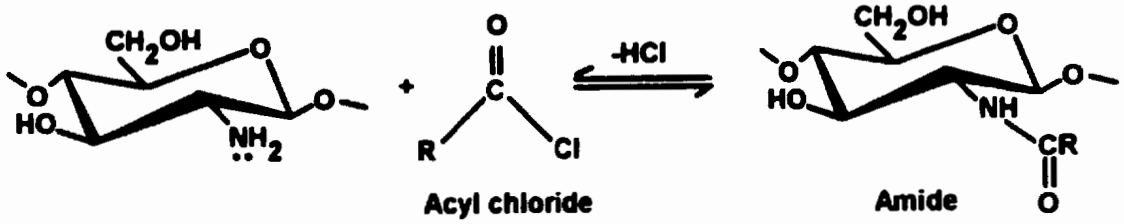
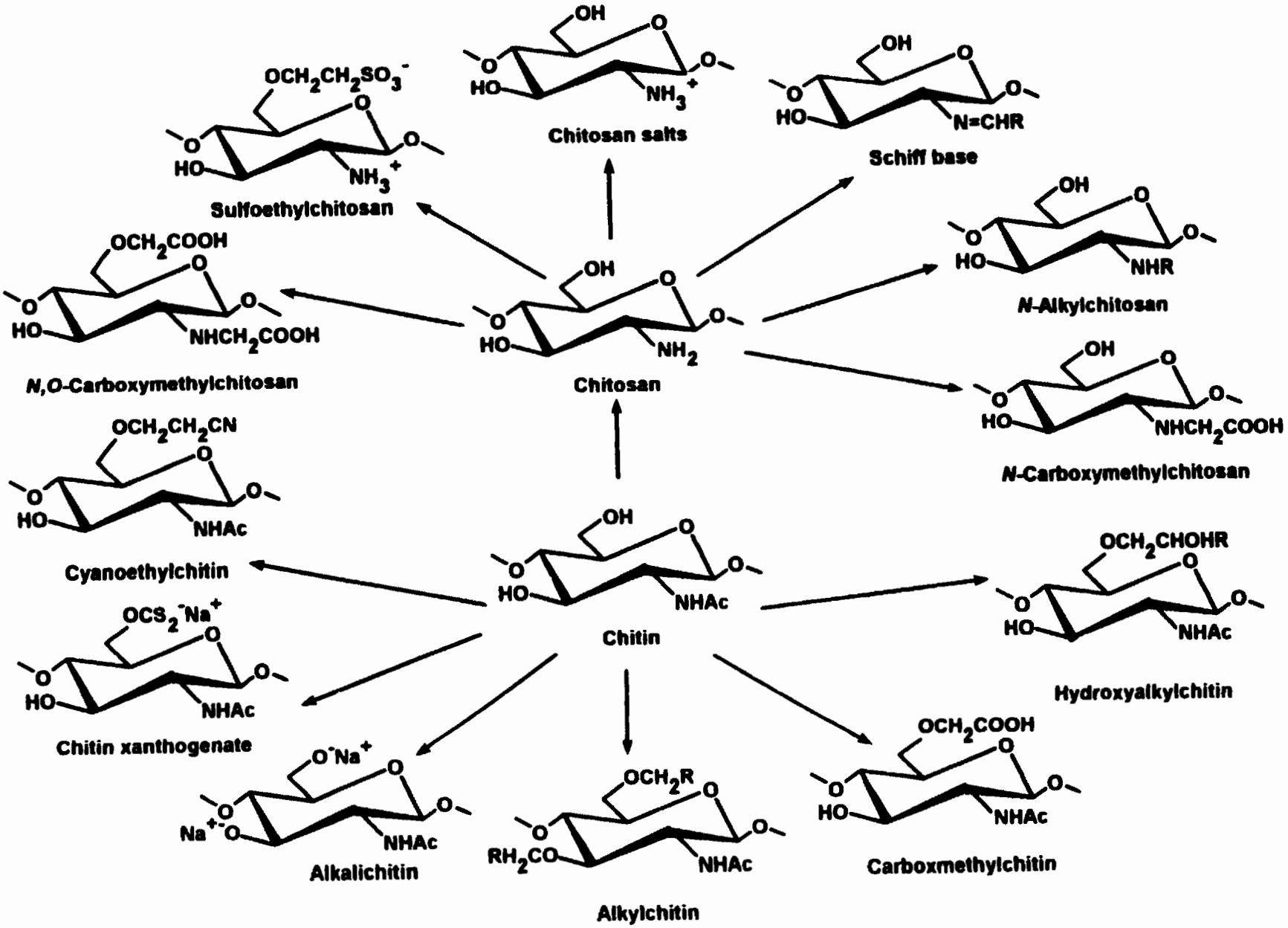

CH2OH
O
HO
NH2
R
Cl
-HCl
NH
CR
Acyl chloride
Amide

Figure 2.6 Chemical derivatization of chitin and chitosan (adapted from Peter, 1995).


Chitosan salts
Schiff base
Sulfoethylchitosan
N-Alkylchitosan
N,O-Carboxymethylchitosan
Chitosan
N-Carboxymethylchitosan
Cyanoethylchitin
Chitin
Hydroxyalkylchitin
Chitin xanthogenate
Alkalichitin
Alkylchitin
Carboxmethylchitin

1995) (Figure 2.5). Formaldehyde and glutaraldehyde are excellent cross-linking agents for chitosan (Uragami *et al.*, 1994) (Figure 2.7).

Both chitin and chitosan are able to form complexes with many of the transition metals as well as some of those from groups 3-7 of the periodic table (Muzzarelli, 1973). The heavy metal/polymer complexes are believed to form as a result of dative bonding. This involves the donation of nonbonding pair of electrons from the nitrogen, and/or the oxygen of the hydroxyl groups, to a heavy metal ion (Winterowd and Sandford, 1995). *N,O*-carboxymethyl chitosan has been found to form chemical bonding with ions of numerous heavy metals such as iron, copper, mercury and zinc, thus binding or squestering them from when they are present in even only dilute concentrations, e.g. 10 to 1000 ppm (Hayes, 1986). The cupric ion seems to form one of the strongest metal complexes with chitosan in the solid state (Chui *et al.*, 1996) (Figure 2.8). Complexing chitosan with polyanions results in the formation of larger, potentially more effective flocculating molecules. Chitosan reacts with polyanions such as alginate, carrageenan and pectin by electrostatic interactions between COO^- or SO_3^- and NH_3^+ groups (Mireles *et al.*, 1992).

It has been reported that the amino group of chitosan may be reacted with nitrite to form a relatively stable diazonium ($-N=N^+$) intermediate. Such diazonium intermediates could be used to couple a wide variety of aromatic groups to the chitosan polymer to produce novel derivatives with different properties (Ashford *et al.*, 1976).

Figure 2.7 Crosslinking of chitosan with glutaraldehyde (adpted from Uragami *et al.*, 1994)

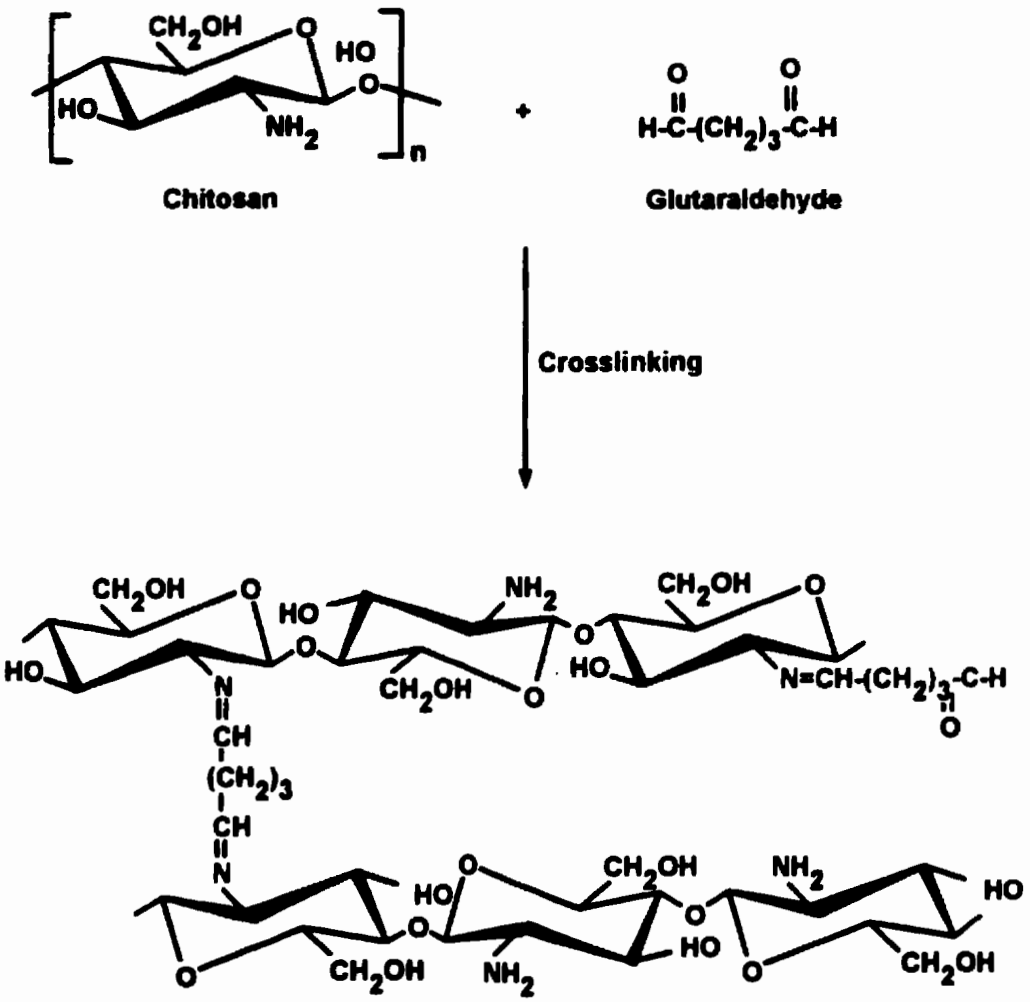
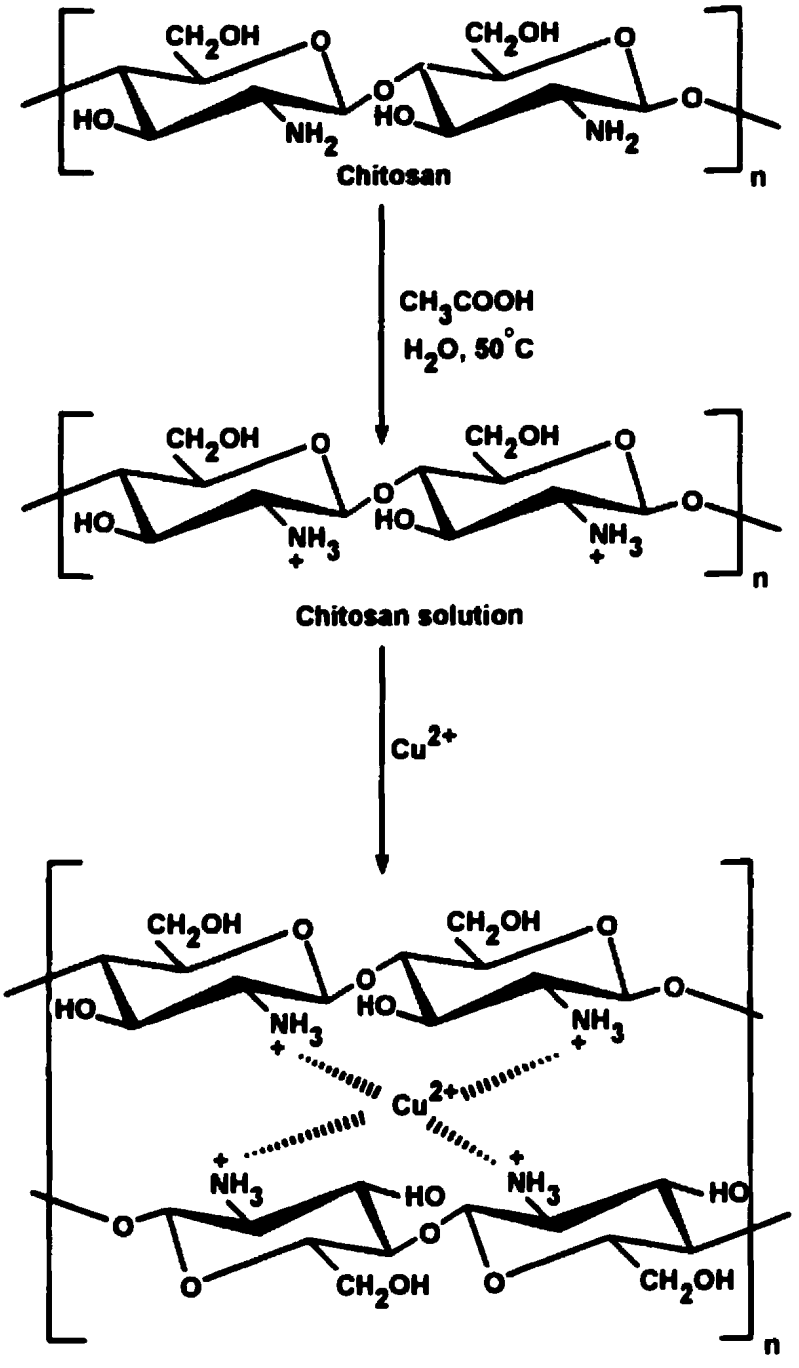

Chitosan
Glutaraldehyde
Crosslinking

Figure 2.8 Schematic representation of processes involved in the preparation of chitosan gel chelated by cupric ions.


CH2OH
O
HO
NH2
Chitosan
n
CH3COOH
H2O, 50°C
NH3+
Chitosan solution
Cu2+

2.1.5.4 Viscosity of chitosan

Viscosity is defined as resistance to flow, that is resistance to a applied force. It may also be defined as the shear stress divided by the shear rate. Shear stress is the applied force (pouring, mixing, pumping, chewing, swallowing, etc.) and shear rate is a value expressing how fast the liquid flows (Singh and Heldman, 1993). Viscosity of chitosan seems to be a major factor that determines its application in many fields (Kaye, 1985).

Chitosan in solution exhibits the polyelectrolyte effect: in the absence of salt, there is an abnormal increase in the viscosity of the more dilute solutions because of an enlarged effective volume due to charge repulsion and stretching out of the molecules. When sufficient salt is added to neutralize this charge effect, the viscosity behaviour is normal (Muzzarelli, 1977). For this reason, the solvent system of 0.5M acetic acid-0.5M sodium acetate buffer may be preferred for viscosity measurements of chitosan (Yomota *et al.*, 1993).

Factors such as concentration of alkali used for deacetylation, degree of deacetylation, molecular weight, pH of the solvent and temperature (Li *et al.*, 1992), exclusion of air and oxidation with bleaching agents (Bough *et al.*, 1978) may affect the viscosity of chitosan. Viscosity of chitosan solutions decreases as temperature is raised. However, the solutions regain their viscosities when cooled to initial temperatures (Muzzarelli, 1977).

The intrinsic viscosity [η], of a liner chain polymer like chitosan, depends on molecular weight (Mw) described in the Mark-Houwink equation where "k" and "a" are constants: $[\eta] = kMw^a$ (Roberts, 1992). The viscosities and rehelogical properties of

chitosans may be measured with rotational viscometers that measure torque (the resistance to a spindle or cylinder rotating at a given speed in a fluid). Shear rates (spindle speeds) may be changed, so one can obtain both reading at a given shear rate and plots of shear stress vs. shear rate.

2.2 Spoilage of seafoods

Freshness is essential for the quality of fish. Fresh fish and shellfish are highly perishable products due to their biological composition (Kinsella, 1988; Ashie *et al.*, 1996). The muscle tissue of fish spoils faster than mammalian muscle (Pedrosa-Menabrito and Regenstein, 1988). Fish tissues have high levels of non-protein nitrogenous compounds (free amino acids, trimethylamine oxide, and creatine), peptides, and proteins, but almost no carbohydrates; the content of connective tissues is low and the pH is generally above 6.0 (Pedrosa-Menabrito and Regenstein, 1988; Ray, 1996; Fraser and Sumar, 1998). The range of lipid content in edible parts of seafoods is approximately 0.5-25% (Ackman, 1990). The lipids of seafoods are unique in that they contain high proportions of long-chain polyunsaturated fatty acids, mostly of the ω-3 type. The content of poly-unsaturated fatty acids in fish is about 30% of the total fatty acids content (Pryor *et al.*, 1976). The breakdown of these compounds generally leads to unfavourable changes in the sensory characteristics of the fish meat which coincides in most cases with its physical deterioration (Fraser and Sumar, 1998). The higher ultimate pH and the colder water temperature in the temperate water fish facilitates the decreased lag time, and the

more rapid growth and reproduction of bacteria, even with refrigeration (Pedrosa-Menabrito and Regenstein, 1988).

"Spoilage refers to any change in the condition of food in which the latter becomes less palatable, or even toxic; these changes may be accompanied by alterations in taste, smell, appearance or texture" (Singleton and Sainsbury, 1978). Spoilage of muscle foods such as fish and shellfish results from changes brought about by biological reactions such as reactions due to activities of the fish's own enzymes (autolytic enzyme actions) and the metabolic activities of microorganisms (Haard, 1992a; Ray, 1996; Ashie *et al.*, 1996). The activity of the endogenous enzymes of fish muscle (proteases, cathepsins, peptidases, etc.) plays an important role in the degradation of peptides and proteins by providing an optimal medium for growth and reproduction and spoilage of microorganisms (Pedrosa-Menabrito and Regenstein, 1988). Autolysis is particularly important during, for instance, the first week or so of storage under the usual commercial conditions in melting ice (Burt, 1977; Huss, 1995). In addition to the enzymically induced reactions (microbial and autolytic), spontaneous chemical and physical changes may also occur. Of the former, lipid oxidation has by far the greatest importance while the latter includes processes such as protein denatutration, dehydration and migration of salts (Burt, 1977). Seafoods, containing a large proportion of C20:5 and C20:6 fatty acids (EPA, and DHA, respectively) in their lipids, are highly prone to autoxidation via a free radical chain mechanism (Shahidi, 1998). Furthermore, Shewfelt (1981) reported lipolysis of fish lipids due to phospholipase A and lipases from fish muscle which lead to increased oxidation. Because composition of muscle varies widely among fish species, there are obvious differences in modes of

spoilage. Fatty fish often undergo extensive rancidity prior to the onset of bacterial decomposition (Gill, 1990).

Very fresh fish aromas and flavours are characterized by mild, green, planty notes which are easily recognized and readily associated with fresh fish (Lindsay, 1990). The post-harvest biochemical and microbial changes in fish tissues depend very much upon factors which affect the concentration of substrates and metabolites in the tissues of live fish, the activity of endogenous enzymes, the microbial contamination, and the conditions after catching (Sikorski *et al.*, 1990). The freshness of fish is an important factor when determining whether the fish meat is edible or not, or in considering other possible uses (Fraser and Sumar, 1998).

The methods for assessment of fish quality may be conveniently divided into two broad categories: subjective (sensory evaluation), and objective (chemical and physical) (Gill, 1990). For centuries the freshness of fish was measured exclusively by subjective criteria. Subjective assessment is often prone to error and may be easily biased (Gill, 1990). Moreover, Gill (1990) reported that sensory evaluation must be performed scientifically under carefully controlled conditions so that effects of environment, personal bias, etc. may be reduced (Gill, 1990). Chemical methods of determining the degree of spoilage of refrigerated and preserved fish meat may give accurate assessment of fish quality (Wong *et al.*, 1967; Fraser and Sumar, 1998). Furthermore, Huss (1995) and Fraser and Sumar (1998) reported combination of chemical analyses that should be used in order to reduce any systematic errors. Certain specific requirements for fishery products

are included in the Food and Drug Regulations and Fish Inspection Act (1914 and as subsequently amended) in Canada (Blackwood, 1978).

2.2.1 Degradation of seafood lipids

The polyunsaturated fatty acids, particularly the trienoic, pentaenoic, and hexaenoic fatty acids are commonly found in seafoods are particularly sensitive to oxidative changes which limit their self life (Hsieh and Kinsella, 1989a; Shahidi, 1998). Tichivangana and Morrissey (1985) have shown that the oxidation of muscle foods occurs in the following order: fish>poultry>pork>lamb. Fish is subject to two principal oxidative deterioration: rancidity and rusting. Rusting is a light yellow to brown discoloration which occurs on exposed surfaces. The oxidation of lipids plays a very important role in the spoilage of both lean and fatty fish species (Tappel, 1961; Huang and Weng, 1998). Above the freezing point oxidation is mainly important in fish containing a high fat content (Yamaguchi and Nakamura, 1984) causing early off-flavour and off-odour development during the first few days on ice (Fujii *et al.*, 1989). Thus, oxidation of fatty fish is of great concern to the food industry because of the development of undesirable off-favours and potentially toxic reaction products as well as loss of essential fatty acids and fat soluble vitamins (Fujimoto *et al.*, 1990; Shahidi and Wanasundara, 1992). Furthermore, oxidized unsaturated lipid products bind to proteins and form insoluble lipid-protein complexes. This accounts for the toughened texture, poor flavour, and unappealing odour of poorly stored seafoods (Khayat and Schwall, 1983).

The factors which influence lipid oxidation in fish include lipid fatty acid composition, their disposition, presence or absence of activators and inhibitors (haeme, metal ions, pH, oxidative enzymes, tocopherol, carotenoids), and external factors such as storage temperature, time, light and oxygen pressure, water activity, and packaging conditions (Khayat and Schwall, 1983). Distribution of fat in the body; i.e., contact of fat in fish meat with an aqueous solution containing accelerators or inhibitors of rancidity, and the orientation of unsaturated fatty acids at an interface are also important factors lipid oxidation in fish (Flick *et al.*, 1992).

Although the process of lipid oxidation is thermodynamically favourable, the direct reaction between oxygen and highly unsaturated lipids is kinetically hindered (German and Kinsella, 1985; Hsieh and Kinsella, 1989a). Hence, an activating factor is necessary to initiate free radical chain reactions. Once the reaction has been initiated free radicals are formed which can react further thus making the mechanism self-propagating (German and Kinsella, 1985; Shahidi, 1998).

The ground state of unsaturated fatty acids corresponds to the singlet state which is diamagnetic, i.e., there are two paired electrons in the outer electronic shell. In contrast, ground state oxygen is in a triplet state (paramagentic) with two unpaired electrons that have the same spin but are in different orbitals. Hence the reactivity of triplet oxygen with unsaturated lipids is forbidden because of the spin restriction imposed by these spin states (Kanner *et al.*, 1987). It has been proposed that lipid oxidation in fish may be initiated and/or promoted by a number of mechanisms such as autoxidation, photosensitized oxidation, lipoxygenase, peroxidase, and microsomal enzymes (Slabyj and Hultin, 1982;

Frankel, 1985; Josephson *et al.*, 1987; Hsieh and Kinsella, 1989b). These include the production of enzymatic and non-enzymatic generated active oxygen species (i.e. hydrogen peroxide, hydroxyl radical); active oxygen iron complexes and thermally-mediated or iron-mediated homolytic cleavage of hydroperoxides (Frankel, 1980; Kubow, 1992).

2.2.1.1 Non-enzymatic initiation of lipid oxidation in seafoods

The production of active oxygen species such as superoxide anion and hydroxyl radicals are important in the initiation and promotion of rancidity in most biological tissues (Frankel, 1985; Hsieh and Kinsella, 1989a).

The superoxide anion ($O_2^{\cdot -}$) may be produced via a number of mechanisms including cytochrome oxidase which is a haem protein containing two iron and two copper residues and catalyses the transfer of electrons from cytochrome C to oxygen in the electron transfer chain (Hsieh and Kinsella, 1989a). Usually four electrons are transferred resulting in the production of two water molecules (Frankel, 1980). However, partial reduction of the oxygen generates a superoxide anion.

$$O_2 + e^{\cdot} \leftrightarrow O_2^{\cdot -}$$

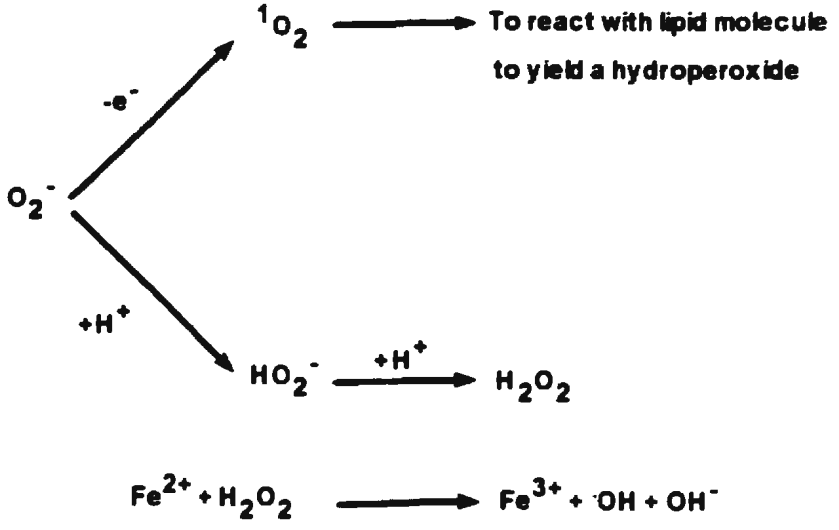
Decker and Hultin (1992) identified several sources of protein-bound iron that exist in biological tissues; these are myoglobin, haemoglobin, ferritin, transferrin, and haemosiderin. St. Angelo (1996) stated that iron bound to these proteins may be released during post-harvest storage, activating oxygen and initiating lipid oxidation. There is a range of concentrations of haematin compounds in muscles from different species of fish

than from different species of mammals. These componds are present in relatively large concentrations in the muscle of most fatty fish, especially in the lateral band dark muscle (Castell and Bishop, 1969). Autoxidation of oxymyoglobin and oxyhaemoglobin (both in the Fe^{2+} oxidation state) may also result in the formation of superoxide anion and metmyoglobin and methaemoglobin (both in the Fe^{3+} oxidation state), respectively. The formation of superoxide anions from oxymyoglobin/oxyhaemoglobin may be catalyzed by anions such as SCN^-, OCN^-, F^- and Cl^- (Satoh and Shikama, 1981). Flick *et al.* (1992) reported that increased oxidation of seafoods at lower humidities may be attributed to the concentration of prooxidants such as metal ions or haemoglobin. The main source of free iron or non-haem iron in cells is ferritin, which is a soluble iron storage protien found in liver, spleen and skeletal muscle, has a molecular mass of 450 kDa and contains 4500 iron atoms when fully loaded (Decker and Welch, 1990). Decker and Hultin (1990) observed that storage of unfrozen mackerel ordinary muscle at 4 ^{0}C for 7 days resulted in a 1.4-fold increase in low molecular weight iron from 0.16 to 0.23 μg Fe/g muscle. A small amount of iron is also found, bound to small molecules such as ATP, ADP, organic acids and DNA. These compounds are also capable of decomposing hydroperoxides (ROOH) to form free radicals (Kanner and Doll, 1991). Shahidi and Hong (1991) reported that metal ions such as copper and iron ions can enhance lipid autoxidation to a greater extent at their lower valance states.

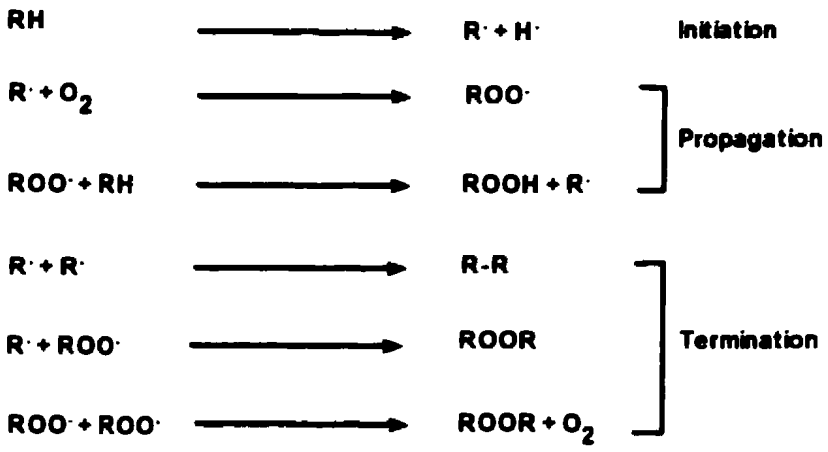
Fe^{2+} exposed to air can react with molecular oxygen to produce superoxide anion and can slowly oxidise to Fe^{3+} (Hsieh and Kinsella, 1989a).

$$Fe^{2+} + O_2 \leftrightarrow (Fe^{2+} - O_2 \leftrightarrow Fe^{3+} - O_2^{-}) \leftrightarrow Fe^{3+} + O_2^{-}$$

Tichivangana and Morrissey (1982 and 1985) reported that ferrous ion at 1-10 ppm levels acts as a strong pro-oxidant in cooked fish muscles. Castell *et al.* (1965) observed that the relative prooxidant activity of ions in fish muscle decreased in the following order: $Cu^{2+}>Fe^{2+}>Co^{3+}>Cd^{2+}>Li^{+}>Ni^{2+}>Mg^{2+}>Zn^{2+}>Ca^{2+}>Ba^{2+}$. Superoxide anion may be dismutated to form hydrogen peroxide, resulting in the formation of hydroxyl radicals via the reaction of H_2O_2 with Fe^{2+} (Frankel, 1980; Yen *et al.*, 1999).



Autoxidation of lipids involves initiation, propagation and termination steps, as given below (Shahidi, 1998).



Hydroperoxides (ROOH) are the primary products of lipid oxidation. The hydroxy radical initiates autoxidation by abstracting a hydrogen atom from another lipid radical thus propagating the chain reaction (Frankel, 1980). Each fatty acid may produce several hydroperoxides upon oxidation; however, hydroperoxides do not possess any flavour of their own (Shahidi, 1998). Due to the high reactivity of hydroperoxides, fading or colour deterioration of carotenoid pigments in the skin of red fish species may occur (Josephson *et al.*, 1987). Evidence suggests that hydroperoxides generated from oxygenase-mediated chemical processes in freshly harvested seafoods initiate lipid peroxidation (Josephson and Lindsay, 1986; Josephson *et al.*, 1987). Lipid peroxides are very unstable and break down to produce many types of secondary reaction products. Such products contribute to the oxidised flavour of seafood products (Khayat and Schwall, 1983; Shahidi, 1998).

Fresh seafoods contain very little, if any, of autoxidatively derived carbonyls. In the chromatographic fingerprints of most fresh fish, 2,4-heptadienal, 3,5-octadien-2-one,

and 2,4-decadienal are the primary carbonyls found in conjunction with fresh-fish five-, six-, eight- and nine-carbon volatiles (Josephson and Lindsay, 1986; Josephson, 1991). The mechanism for the formation of 2,4-heptadienal and 3,5-octadien-2-one from eicosapentaenoic acid (EPA) is shown in Figure 2.9 (Josephson, 1991). Malonaldehyde has been considered among the most important secondary oxidation products of lipids because of its well-documented cross-linking properties with amino group of proteins, enzymes and DNA (Pearson *et al.*, 1983; Frankel, 1985; Shahidi and Hong, 1991). Furthermore, malonaldehyde has been used as an indicator of secondary lipid oxidation products in muscle foods, under acidic conditions which would presumably release the bound malonaldehyde from its adducts. Figure 2.10 depicts the steps involved in the formation of the pink-coloured TBA-malonaldehyde adduct which has an absorption maximum at 532 nm (Kosugi *et al.*, 1989). Autoxidation of EPA produces unsaturated carbonyls, such as 2E, 4Z-heptadienal and 3E, 5Z-octadien-2-one (Josephson, 1991); finally, spontaneous retro-aldol condensation converts higher unsaturated aldehydes like 2E, 4Z-heptadienal to the lower saturated aldehydes such as propanal and acetaldehyde (Josephson, 1991) (Figure 2.11). Propanal has been identified as a dominant aldehyde formed upon oxidation of ω-3 PUFA such as eicosapentaenoic acd (EPA) and docosahexaenoic acid (DHA) in various fish species (He and Shahidi, 1997; Medina *et al.*, 1999; Girard and Durance, 2000).

Figure 2.9 Proposed degradation of eicosapentaenoic acid to 2,4-heptadienal and 3,5-octadien-2-one via lipid oxidation (adapted from Josephson, 1991)

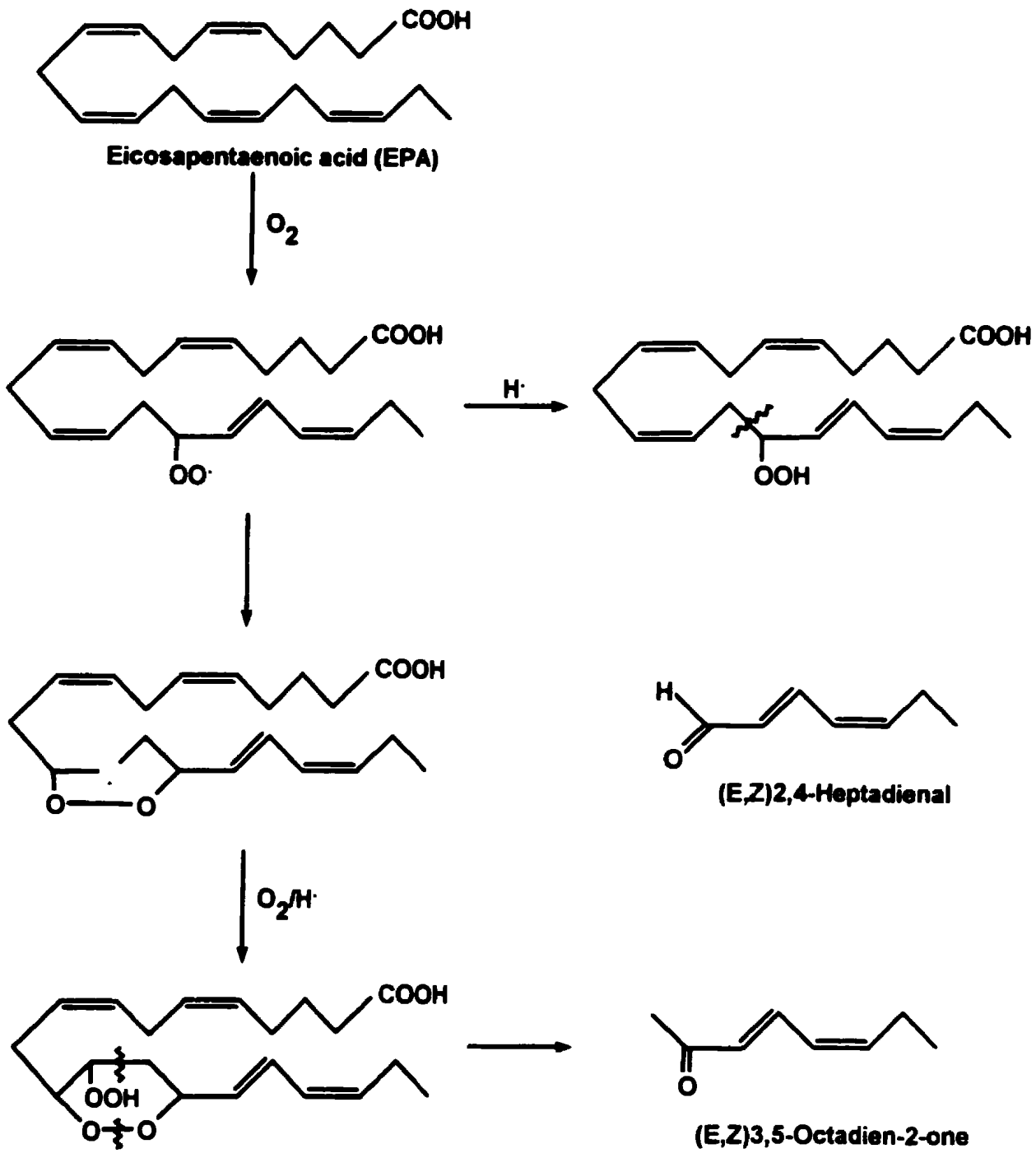

COOH
Eicosapentaenoic acid (EPA)
O_2
COOH
OO·
H·
COOH
OOH
COOH
O
O
H
O
(E,Z)2,4-Heptadienal
O_2/H·
COOH
OOH
O
O
O
(E,Z)3,5-Octadien-2-one

Figure 2.10 Possible mechanism between malonaldehyde and 2-thiobarbituric acid (TBA) in the classical TBA test for lipid oxidation (adapted from Pegg and Shahidi, 1993).

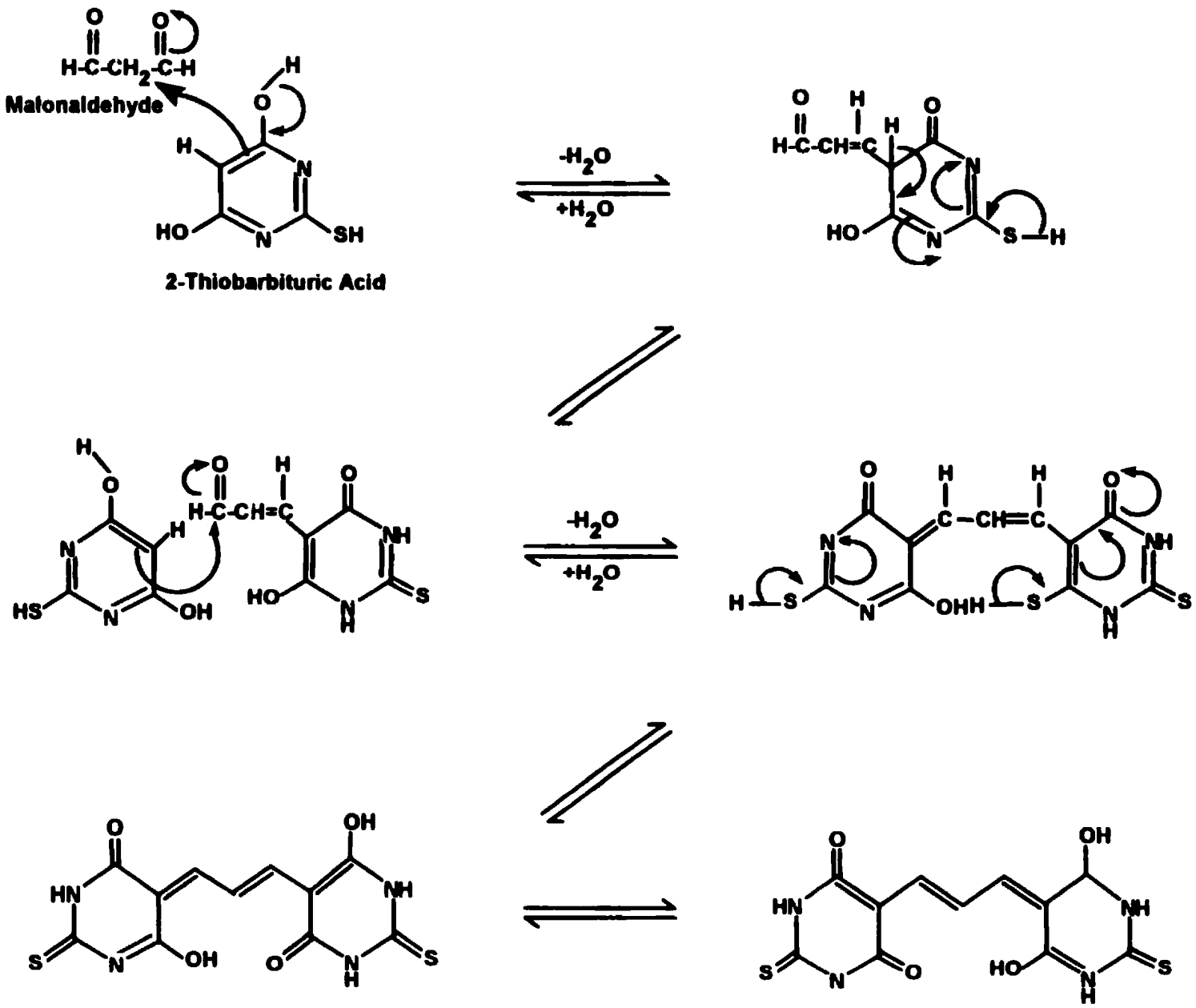
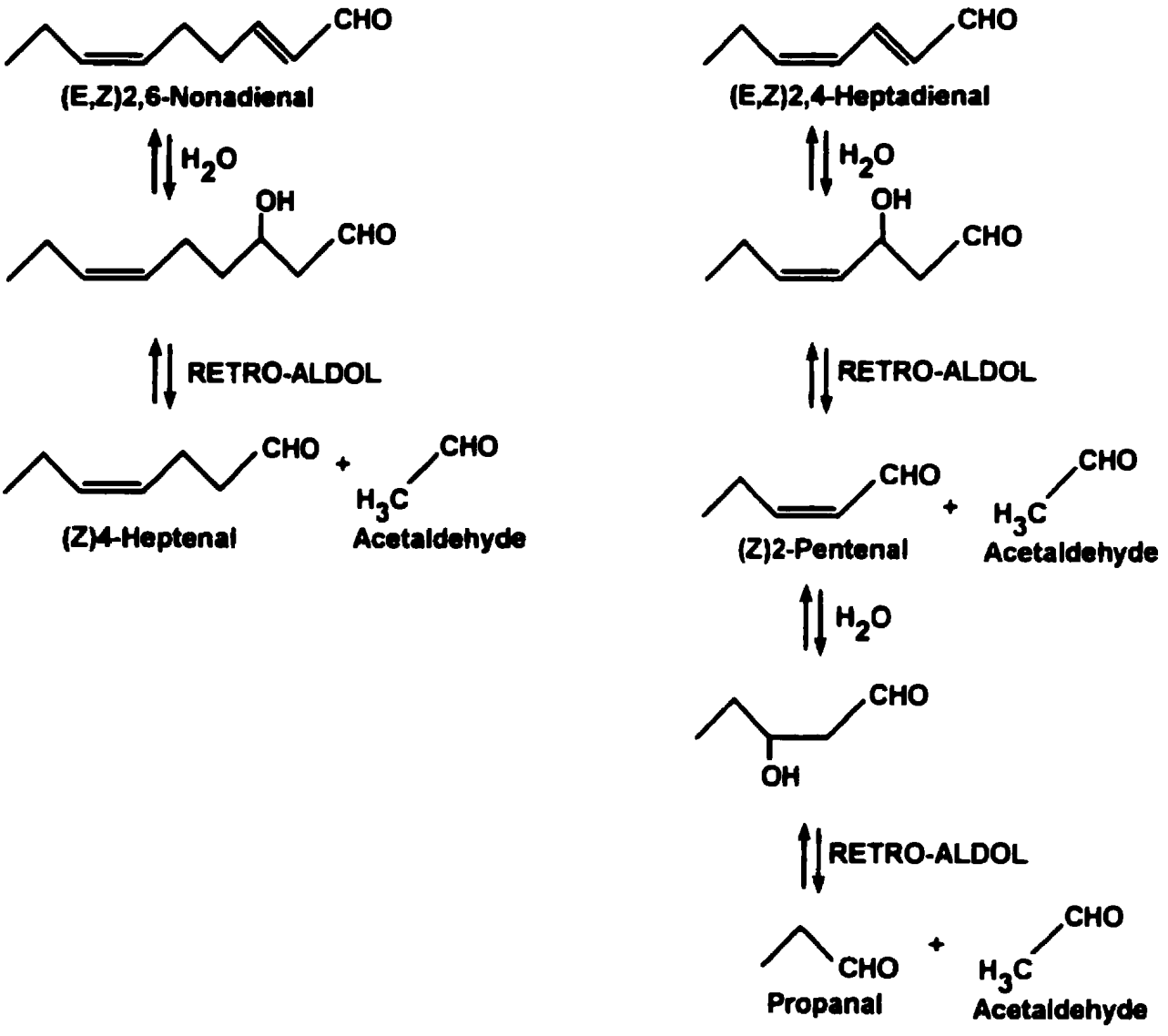

Malonaldehyde
2-Thiobarbituric Acid
-H2O
+H2O
-H2O
+H2O

Figure 2.11 Mechanism for the formation of 4Z-heptaenal from 2E, 6Z-nondienal and 2E-pentaenal and propanal from 2E, 4Z-heptadienal via α/β double-bond hydration and retro-aldol condensation (adapted from Josephson, 1991).


CHO
(E,Z)2,6-Nonadienal
H2O
OH
CHO
RETRO-ALDOL
CHO
+
CHO
H3C
(Z)4-Heptenal
Acetaldehyde
CHO
(E,Z)2,4-Heptadienal
H2O
OH
CHO
RETRO-ALDOL
CHO
+
CHO
H3C
(Z)2-Pentenal
Acetaldehyde
H2O
CHO
OH
RETRO-ALDOL
CHO
+
CHO
H3C
Propanal
Acetaldehyde

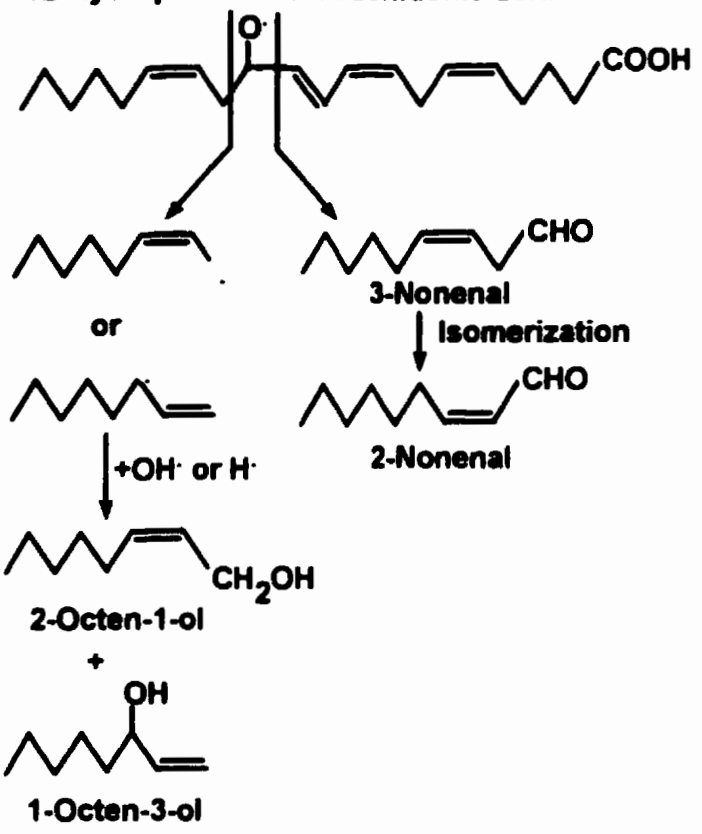
2.2.1.2 Enzymatic initiation of lipid oxidation in seafoods

There are two forms of enzyme-induced oxidation in seafoods. The true type involves the enzymes lipoxygenase or cyclo-oxygenase (Hsieh and Kinsella, 1989b; Kubow, 1992). These enzymes directly introduce an oxygen atom onto the crabon chain of the lipid molecule. Figure 2.12 depicts the proposed reaction scheme of lipoxygenase-catalyzed oxidation of arachidonic acid (AA) and eicosapentaenoic acid (EPA) and the subsequent generation of oxidative flavour componds (Hsieh and Kinsella, 1989b). Most of the work to date has been concerned with the presence of lipoxygenase in the gill and skin tissues of various fish species (German and Kinsella, 1985; Hsieh and Kinsella, 1989b). The gill lipoxygenase acts via a free radical mechanism and initiates the peroxidation of PUFA, such as arachidonic acid (AA), eicosapentaenoic acid (EPA), and dcosahexaenoic acid (DHA), to form specific 12- and 14-hydroperoxides (German and Kinsella, 1985). The breakdown of these unstable hydroperoxides and generation of secondary oxidation products may contribute to the onset of lipid autoxidation in fish tissues and produces reactive free radicals which subsequently initiate chain reactions causing further autoxidation (Hsieh and Kinsella, 1989a).

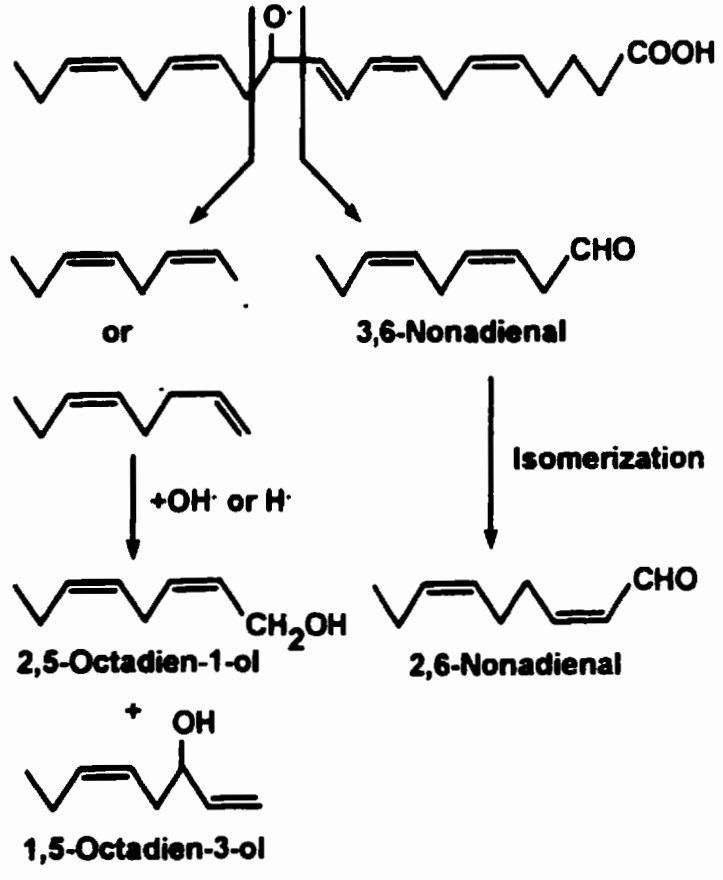
In microsomal lipid oxidation, the enzymes are involved in reducing iron complexes which are then capable of stimulating lipid oxidation (Kanner *et al.*, 1987). This microsomal enzyme system requires the presence of co-factor nicotinamide adenine dinucleotide/ nicotinamide adenine dinucleotide phospate (NADH/NADPH) as well as iron (Slabyj and Hultin, 1982). It is postulated that two electrons are transferred from NADPH/NADH via NADPH/NADH-cytochrome-P450-reductase and superoxide to iron

Figure 2.12 The proposed reaction scheme of lipoxygenase-catalyzed oxidation of arachidonic acid and eicosapentaenoic acid and the subsequent generation of oxidative flavour compounds (adapted from Hsieh and Kinsella, 1989b).

12-Hydroperoxide of Arachidonic acid



12-hydroperoxide of Eicosapentaenoic acid



chelators. Electrons are donated to the Fe(III)-complex and so generate Fe(II), which in turn stimulates lipid oxidation (Kanner *et al.*, 1987). Figure 2.13 shows the proposed reaction scheme of microsomal lipid oxidation in flounder muscle.

2.2.2 Changes in nitrogenous compounds in seafoods

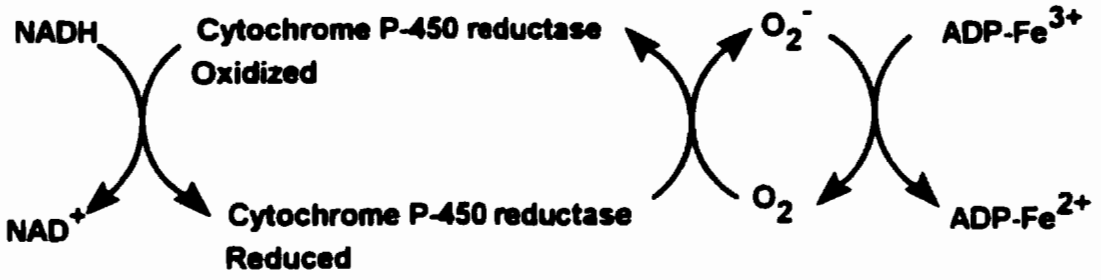
The post-mortem metabolism of nitrogenous compounds in fish flesh is mainly responsible for the gradual loss of the fresh appearance of the catch and for the development of signs of putrefaction (Sikorski *et al.*, 1990). This is due to decomposition of some non-protein components which contribute to the desirable flavour of seafoods, formation of volatile odorous compounds (Haard, 1992a; Huss, 1995), and partial degradation and changes of proteins causing undesirable rheological and colour affects in the muscles (Sikorski *et al.*, 1976).

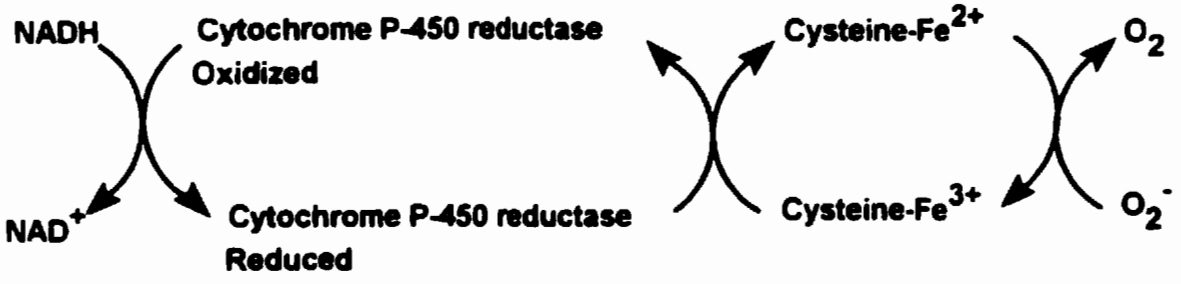
Some of the bacteria present on fish are able to carry out a respiration using certain molecules as electron acceptor. Many of the specific spoilage bacteria on fish use trimethylamine oxide (TMAO) as electron acceptor in an anaerobic respiration. The reduced component (trimethylamine) TMA, which is one of the dominant components of spoiling fish, has a typical fishy odour (Huss, 1995; Ashie *et al.*, 1996; Fraser and Sumar, 1998). TMAO apparently serves an osmoregulator in fish in saltwater environment (Lindasy, 1990) as well as being part of the body's buffer system.

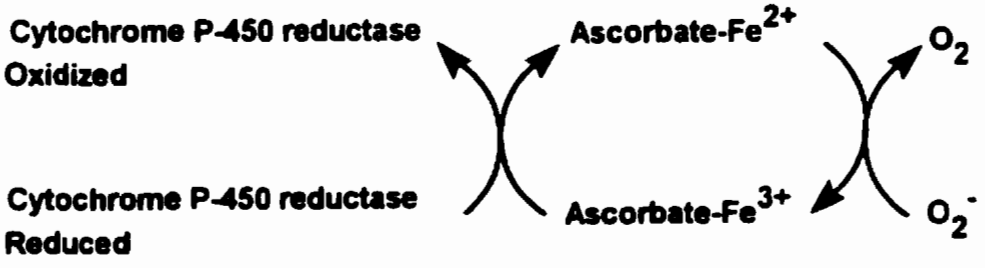
$$NADH + H^{+} + (CH_3)_3NO \rightarrow NAD+ + (CH_3)_3N + H_2O$$

TMAO may also be decomposed to dimethylamine (DMA) and formaldehyde by endogenous enzymes (Hebard *et al.*, 1982; Haard, 1992a; Sikorski *et al.*, 1990). The

Figure 2.13 The proposed reaction scheme of microsomal lipid oxidation in flounder muscle (adapted from Kanner *et al.*, 1987 and McDonald and Hultin, 1987).


NADH
Cytochrome P-450 reductase
Oxidized
NAD+
Cytochrome P-450 reductase
Reduced
O2-
O2
ADP-Fe3+
ADP-Fe2+


NADH
Cytochrome P-450 reductase
Oxidized
NAD+
Cytochrome P-450 reductase
Reduced
Cysteine-Fe2+
Cysteine-Fe3+
O2
O2-


Cytochrome P-450 reductase
Oxidized
Cytochrome P-450 reductase
Reduced
Ascorbate-Fe2+
Ascorbate-Fe3+
O2
O2-

responsible enzyme for this reaction has been identified as trimethylamine oxidase (TMAO-ase) or TMAO demethylase and is most commonly found in the gadoid fish (cod family) (Huss, 1995).

$$(CH_3)_3NO \rightarrow (CH_3)_2NH + HCHO$$

Formaldehyde induces cross-linking of the muscle proteins making the muscle tough and results in loss of water holding capacity in the muscle (Sikorski *et al.*, 1976; Hebard *et al.*, 1982) which is described as a "cottony" and "spongy" texture in seafoods (Pedrosa-Menabrito and Regenstein, 1988).

The suitability of using TMA content as a chemical idicator of evaluating freshness quality of seafood has been investigated extensively (Hebard *et al.*, 1982; Krzymien and Elias, 1990; Dalgaard *et al.*, 1993; Pacheco-Aguilar *et al.*, 2000). The picrate salt formation method has historically been one of the widely used procedures to determine TMA (AMC, 1979; Woyewoda *et al.*, 1986a). The level of TMA found in fresh fish rejected by sensory panels varies among fish species, but is typically around 10-15 mg TMA-N/100g in aerobically stored fish (Pedrosa-Menabrito and Regenstein, 1988; Connell, 1990) and at a level of 30 mg TMA-N/100g in packed cod (Dalgaard *et al.*, 1993).

The formation of other non-protein nitrogen compounds is also associated with spoilage in fish. In the muscles of cartilageous fish, ammonia may accumulate due to the activity of endogenous enzyme urease (Fraser and Sumar, 1998).

$$(NH_2)_2CO + H_2O \rightarrow 2NH_3 + CO_2$$

Large amounts of ammonia are also generated in fish muscle upon deamination of AMP (Sikorski *et al.*, 1990), deamination of free amino acids, and oxidation of amines (Gill, 1990). Ammonia itself has been found to be an excellent indicator of squid quality (LeBlanc and Gill, 1984).

Total volatile basic-nitrogen (TVB-N) is a general term which includes the measurement of TMA, DMA, ammonia and other volatile basic nitrogenous compounds associated with seafood spoilage (Gill, 1990; Botta, 1995; Huss; 1995). Both the speed and the low cost are major advantages of using TVB-N value, rather than other chemical indicators of evaluating freshness quality (Botta, 1995). Lang (1979) reported that fish is generally considered to be fresh if its TVB-N content is less than 20 mgN/100g. Moreover, the TVB-N content of 30 mgN/100g is considered by most authorities to indicate staled fish, whilst at a level of 40 mgN/100g the fish is regarded as unfit for consumption.

2.2.3 Degradation of organic phosphates in seafoods

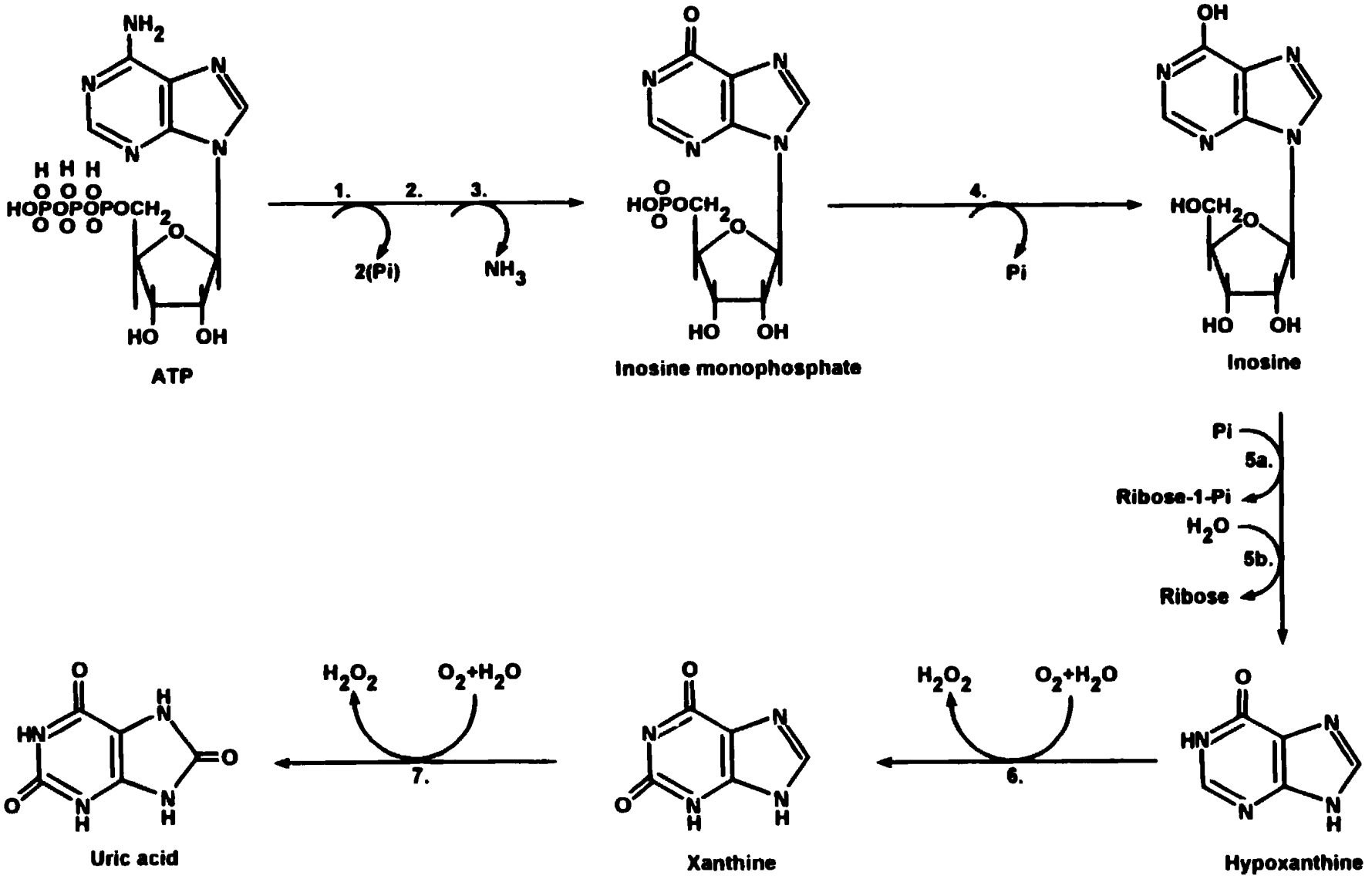
The post-mortem catabolism of ATP present in the muscle of fish plays an important role in its spoilage and may be used as an index of deterioration of fish quality (Harrd, 1992a; Fraser and Sumar, 1998). Several investigators have indicated a strong correlation between nucleotide catabolism and loss of freshness in muscle foods (Fatima *et al.*, 1981; Fletcher and Statham, 1988; Shahidi *et al.*, 1994). Furthermore, the depletion of ATP, which is normally associated with the transient accumulation of adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophospahte (IMP), is

the cause of rigor mortis (Haard, 1992a). Nucleotide degradation in seafoods is believed to be due to both autolytic as well as bacterial action (Sikorski *et al.*, 1990). The sequence of nucleotide catabolism has been determined to proceed via the reaction displayed in Figure 2.14.

Molecules of ATP are rapidly degraded after death to AMP and subsequently to IMP by partial dephosphorylation. The dephosphorylation of IMP is primarily autolytic and occurs during the early stages of chilled storage (Shahidi *et al.*, 1994; Ashie *et al.*, 1996). IMP is a known flavour enhancer contributing to sweetness (Gill, 1990; Haard, 1992a) and further dephosphorylation leads to the formation of inosine (INO) and hypoxanthine (Hx) both of which contribute to the bitter taste of spoiled fish (Gill, 1990; Sikorski *et al.*, 1990). The oxidation of Hx to xanthine and ultimately to uric acid is much slower (Gill, 1990; Haard, 1992a). The effect of Hx accumulation in fish tissues reflects the initial phase of autolytic deterioration and later includes contributions through bacterial spoilage (Shahidi *et al.*, 1994; Botta, 1995).

Contents of INO or Hx in fish muscles have been used as indicators of freshness (Burt, 1977; Sikorski *et al.*, 1990; Haard, 1992a). The content of Hx increases linearly with the time of storage to about 5 μmol/g wet weight and subsequently declines or remains at that level (Sikorski *et al.*, 1990) and correlates well with sensory assessment, particularly the flavour (Burt, 1977; Sikorski *et al.*, 1990). The following Hx values are proposed as a limit of acceptability of fish: 2 to 3 μmol/g for cod, 2 to 2.5 μmol/g for herring; 1 to 1.2 μmol/g for mackerel (Barile *et al.*, 1985a). For measurement of Hx the Analytical Methods Committee recommended the use of xanthine oxidase to convert Hx

Figure 2.14 Post-mortem ATP degradation in fish muscle: 1. ATP-ase; 2. myokinase; 3. adenosine monophosphate deaminase; 4. inosine monophosphate phosphohydrolase; 5a. nucleoside phosphorylase; 5b. inosine nucleosidase; 6,7. xanthine oxidase (adapted from Huss, 1995).


ATP
1.
2.
3.
2(Pi)
NH3
Inosine monophosphate
4.
Pi
Inosine
Pi
5a.
Ribose-1-Pi
H2O
5b.
Ribose
Hypoxanthine
H2O2
O2+H2O
6.
Xanthine
H2O2
O2+H2O
7.
Uric acid

into uric acid which is determined by reading the absorbance at 290 nm (AMC, 1979; Woyewoda *et al.*, 1986b).

While useful indices of freshness have been based on individual nucleotides or their breakdown products, indicators that incorporate the measurement of several of these nucleotide breakdown products are advantageous (Shahidi *et al.*, 1994). Thus, the concept of the *K* value was first introduced by Saito *et al.* (1959).

$$K\,(\%) = \frac{[INO] + [Hx]}{[ATP] + [ADP] + [AMP] + [IMP] + [INO] + [Hx]}$$

Very fresh fish have a *K*-value of less than 20% and reach the point of incipient spoilage at values above 60% (Ehira, 1976). Burns *et al.* (1985) have proposed another freshness indicator for fish, namely the *G* value, which is based on accumulation of Hx and disappearance of IMP, AMP, and INO in the muscle after slaughter. This index is useful over the entire iced shelf life of lean fish (Shahidi *et al.*, 1994).

$$G = \frac{[Hx] + [INO]}{[INO] + [IMP] + [AMP]}$$

2.2.4 Microbial spoilage of seafoods

Fish is generally spoiled faster than other muscle foods, is a phenomenon that was first realized in the early part of this century (Ashie *et al.*, 1996). The flesh of healthy live or newly-caught fish is sterile as the immune system of the fish prevents the bacteria from growing in the flesh. When the fish dies, the immune system collapses and bacteria are

allowed to proliferate freely (Reineccius, 1991; Huss, 1995). During storage, they invade the flesh by moving between the muscle fibres and hydrolyzing cells (Huss, 1995). The exposed muscle layers of fillets are especially vulnerable to bacterial penetration (Sikorski *et al.*, 1990). Another important factor related to spoilage of fish is the very high post-mortem pH (>6.0) in fish muscle. Most fish contain only very little carbohydrate (<0.5%) in their muscle tissues and only a very small amounts of lactic acid which is produced as a result of post-mortem glycolysis. The high pH and the presence of large amounts of non-protein-nitrogen which are readily available for bacterial metabolism, contribute to the generally favourable growth conditions for bacteria on or in fish flesh (Pedrosa-Menabrito and Regenstein, 1988; Huss *et al.*, 1997).

The skin microflora of fresh fish taken from cold waters is composed predominantly of Gram-negative bacteria, mainly *Pseudomonas*, *Accinetobacter*, *Moraxella*, *Shewanella*, *Flavobacterium*, *Alcaligenes* and *Vibrio* (Pedrosa-Menabrito and Regenstein, 1988; Ray, 1996). On the other hand, warm water species harbor predominantly mesophilic Gram-positive bacteria such as *Micrococcus*, *Coryneforms*, and *Bacilli* (Sikorski *et al.*, 1990). It has therefore been suggested that seafoods harvested from temperate waters spoil relatively more quickly than do their tropical or warm-water counterparts because they are "preinoculated" with psychrotrophic Gram-negative spoilage bacteria (Ashie *et al.*, 1996).

Microbial activity in fish may produce off-flavours in several ways: the production of undesirable primary metabolites, incidental chemical conversion of food constituents of otherwise little flavour significance, or through residual enzyme activity after cell death

(Sikorski *et al.*, 1990; Reineccius, 1991). Compounds such as TMA from TMAO, hydrogen sulphide, dimethyl sulphide, and methyl mercaptan from sulphur-containing amino acids; various amines and ammonia from amino acids; carbonyl compounds from lipids; and indole, skatole, putrescine, and cadaverine from proteins are produced as a result of microbial activity in seafoods (Ray, 1996).

Various microbial evaluation methods for fish have been developed during last decade and some of them are rapid and automated which may be of use when large numbers of samples are to be analyzed (Huss, 1995). Total plate count method is a commonly used method for evaluation of seafood quality and it represents the total number of bacteria that are capable of forming visible colonies (Ray, 1996). Blackwood (1978) has discussed monitoring of microbiological quality of fish at all stages from harvesting to domestic use.

Controlling surface microbial growth on food is important because it is the main source of contamination for many refrigerated food products, especially fresh muscle foods (Padgett *et al.*, 1998). Psychrotrophic spoilage bacteria are able to continue the spoilage process of seafoods under refrigerated conditions (Ashie *et al.*, 1996). Since refrigeration alone does not control growth of these microorganisms, it is essential that one or more additional safety factors be incorporated into food processing to inhibit or minimise the microbial growth during refrigerated storage of seafoods (Venugopal, 1995). It is therefore recommended that additional barriers or hurdles be used in combination with refrigeration to extend the shelf-life as well as to ensure consumer safety (Moberg, 1989). The "hurdle concept" involves incorporation of microbial barriers into products

during processing (Venugopal, 1995; Ashie *et al.*, 1996). The Showa Denko company (Tokyo, Japan) has developed a film which is in the form of a pillow with entrapped propylene glycol. When placed in contact for several hours with the surface of meat or fish, it absorbs water and causes injury to spoilage bacteria (Labuza, 1990). Furthermore, many chemical compounds, either present naturally or formed during processing legally added as ingredients, are capable of killing microorganisms or controlling their growth in foods (Huss *et al.*, 1997). However, many health-conscious consumers are interested in foods that do not contain any preservative, especially those that are not normally found in foods. This has resulted in the search for preservatives that are either naturally present in foods of plant and animal origin or produced by safe food-grade microorganisms used to produce fermented products and are also designated as "biopreservatives" (Ray, 1996).

2.3 Edible coatings in food preservation

In the last 20 years, petrochemical polymers, commonly called "plastics", have been the most widely used polymers for packaging of food because of their high performance and low cost (Callegarin *et al.*, 1997). However, accumulation of non-biodegradable wastes from the use of synthetic packaging materials, and the associated environmental pollution has raised concern and challenge for the industry (Bade and Wick, 1988; Gilbert *et al.*, 1996). Another problem in the use of plastic packaging is that polymeric materials generally are not completely inert. Transfer of substances from plastic packaging to the foods with which they come in contact may occur (Callegarin *et al.*, 1997). Furthermore, several methods may be used by the food processor to slow down or

inhibit deteriorative changes in foods, including chilled storage, freezing, heat processing, drying, and the use of chemical additives and preservatives (Labuza and Breene, 1989). However, increasing energy costs associated with freezing and drying, and growing consumer concerns about preservatives have forced the food industry to seek alternative methods of food preservation (Smith *et al.*, 1990). Although vacuum/gas packaging may be used to extend the shelf life and keeping quality of food, aerobic spoilage may still occur in such packaged products, depending on the level of residual oxygen in the package headspace (Kester and Fennema, 1986).

An edible coating or film is defined as a thin, continuos layer of edible material formed or placed, on or between, foods or food components to provide a barrier to mass transfer, to serve as a carrier of food ingredients and additives, or to provide mechanical protection (Krochta and Johnston, 1997). Additionally, edible coatings that are applied directly on the food surface are designed which create a modified atmosphere (Gilbert *et al.*, 1996). Various kinds of active substances may now be incorporated into the packaging material to improve its functionality and give it new or extra functions. Such active packaging technologies are designed to extend the shelf life of foods, while maintaining their nutritional quality and safety (Han, 2000).

Edible coatings and films have a variety of advantages such as biodegradability, biocompatibility (Han, 2000), and when consumed along with the food may provide additional nutrients and/or enhance sensory characteristics and possibly enhancing antioxidant and antimicrobial effects (Kester and Fennema, 1986; Gilbert *et al.*, 1996). Moreover, they are economical because of the low cost of raw material and are promising

in creating new markets for agricultural products (Callegarin *et al.*, 1997). In fact, activity of these compounds in coatings concentrates them at the product surface which is the place where protection is needed (Debeaufort *et al.*, 1998). Active packaging technologies involve interactions between the food, the packaging material, and the internal gaseous atmosphere (Labuza and Breene, 1989). Furthermore, use of biodegradable coating/film materials has the greatest potential in countries where landfill is the main waste management tool (Petersen *et al.*, 1999).

There are many mechanisms involved in extending the shelf life of food by coating films. These include controlled moisture transfer between food and the surrounding environment, controlled release of chemical agents like antimicrobial substances, antioxidants, reduction of oxygen partial pressure in the package that results in a decreased rate of metabolism, controlled rate of respiration, high impermeability to certain substances such as fats and oils, temperature control, structural reinforcement of food and coating of flavour compounds and leavening agents in the form of microcapsules (Labuza and Breene, 1989).

The formation of edible films or coatings based on hydrocolloids requires one of the following mechanisms (Kester and Fennema, 1986).

- Simple coacervation, where a hydrocolloid in an aqueous dispersion is precipitated or undergoes a change of phase by evaporation of the solvent, addition of a hydrosoluble nonelectrolyte in which the hydrocolloid is insoluble, addition of an electrolyte which causes "salting out" or bridging or even modification of the pH.

- Complex coacervation, where two hydrocolloid solutions of opposite charge are combined, causing the reaction and precipitation of polymer complexes.
- Gelification or heat coagulation, in which heating the macromolecule causes its denaturation followed by gelification or precipitation, or cooling of a dispersion of the hydrocolloid causing a sol-gel transition.

2.3.1 Edible coating/film materials

Edible films or coatings are generally composed of polysaccharides, proteins, lipids or their combinations (Baldwin, 1999). At least one component should be a high-molecular-weight, long chain-polymer, in order to yield film or coating matrices with appropriate cohesive strength when deposited from a suitable solvent (Kester and Fennema, 1986; Venugopal, 1998). Cohesive strength of a film is related to polymer structure and chemistry, nature of the solvent system, presence of additives such as crosslinking agents, and environmental conditions during film formation (Kester and Fennema, 1986). A uniform distribution of polar groups along the polymer chain increases cohesion by enhansing the likelihood of inter-chain hydrogen bonding and ionic interactions (Banker, 1966).

Several varieties of edible coatings and films have been prepared from polysaccharides such as alginate, pectin, carrageenan, starch, dextrins and cellulose. They comprise an abundant and renewable resource of hydrophilic film-forming agents with a wide range of viscosities, relatively low permeability to gases, but little resistance to water vapor transfer (Baldwin, 1999).

Films have also been developed from various plant proteins and some animal proteins as well. These include maize zein, wheat gluten, soya protein, peanut protein, collagen, gelatin, casein, milk whey proteins and egg albumin (Krochta and Johnston, 1997). Proteins are attractive to the polymer chemists as they possess a wide range of chemical functionalities and such molecules with a wide range of properties are available in nature (Petersen *et al.*, 1999). More recently protein materials, such as the milk protein whey and corn protein zein, have been used as edible coatings for preservation of roasted peanuts and cooked turkey meat, respectively (Mate and Krochta, 1996; Herald *et al.*, 1996). Chow and Lin (1986) described the possibility of using pork-skin collagen for manufacturing of edible collagen film for use as a casing material for sausages.

Fatty acids and alcohols lack saturated integrity and durability in their free form to be good film formers (Baldwin, 1999). Due to the fragile nature of these compounds, lipids are often incorporated into a structured matrix of some other compounds such as polysaccharides (Kester and Fennema, 1986).

Certain coating components, such as plasticizers, including polyhydric alcohols, waxes, and oils, are added to impart flexibility and elongation to polymeric substances (Baldwin *et al.*, 1995). Plasticizers function by weakening the intermolecular forces between adjacent polymer chains, resulting in decreased tensile strength and increased film flexibility (Banker, 1966). Edible coatings/films must meet a number of specific functional requirements (moisture barrier, solute and/or gas barrier, water or lipid solubility, colour and appearance, mechanical and rheological characteristics, non-toxicity, etc.) (Gilbert *et al.*, 1996). In any polymeric packaging film or coating, two sets of forces are involved:

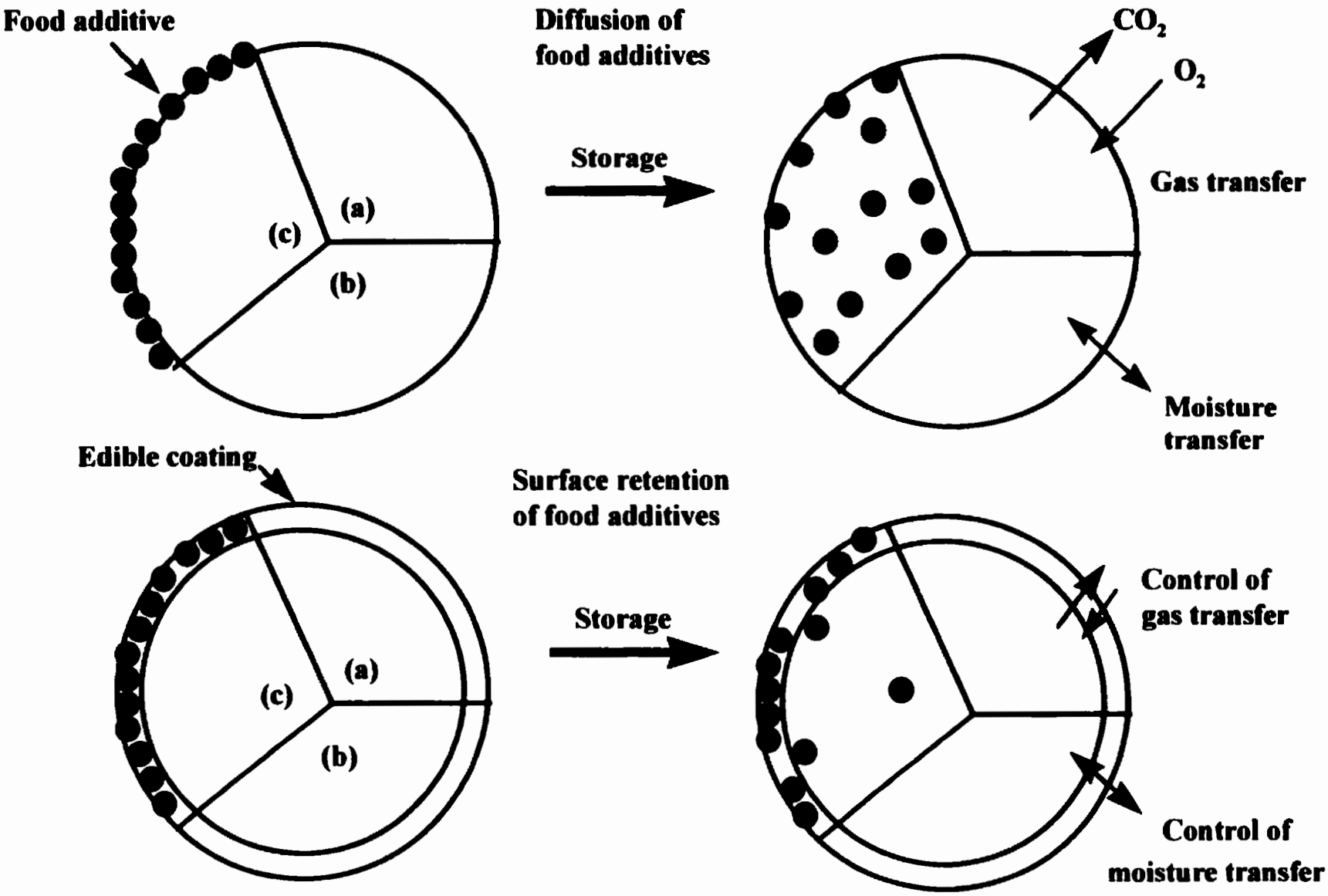
these are forces between the film-forming polymer molecules for all polymeric films or coatings (cohesion), and between the film and the substrate for coating only (adhesion) (Miller and Krochta, 1997).

2.3.2 Permeability characteristics of edible coating/film materials

Permeability of coatings and films to water vapour, gas, solutes, or lipids is an important property to consider when selecting film materials or for tailoring coating for specific commodities (Baldwin, 1999). Edible films may assist in retaining the characteristic food flavour via their aroma barrier properties and also limit quality deterioration due to oxidation via their oxygen barrier properties (Miller and Krochta, 1997). Because edible polymers can hydrogen bond effectively, they make good barrier films at low to intermediate relative humidity (RH) against oxygen, aroma, and lipids (Krochta and Johnston, 1997). Most edible films are quite moisture sensitive, but this inherent hydrophilicity makes them excellent barriers to nonpolar substances such as oxygen and some aroma compounds. As mentioned previously, an increase in crystallinity, density, orientation, molecular weight or crosslinking results in a decrease in polymer permeability (Miller and Krochta, 1997).

The structure of the film-forming polymer is important in terms of influencing permeability properties of a film (Kester and Fennema, 1986). Polymer chain packaging, whether it is tight or loose due to bulky side chains, results in increased or decreased permeability properties, respectively (McHugh and Krochta, 1994). Figure 2.15 gives a schematic representation of food preservation with edible coatings and films as active

Figure 2.15 Schematic representation of food preservation with or without edible coating as active layer (adapted from Rooney, 1995).


Food additive
Diffusion of
food additives
CO_2
O_2
Storage
Gas transfer
(a)
(c)
(b)
Moisture
transfer
Edible coating
Surface retention
of food additives
Storage
Control of
gas transfer
(a)
(c)
(b)
Control of
moisture transfer

layers when the first mode of deterioration results from respiration, from dehydration or moisture uptake, or from surface microbial development or oxidation.

2.3.2.1 Use of edible coatings/films to control water vapour transfer

Moisture level in food is critical for maintaining freshness, controlling microbial growth, and providing mouthfeel and texture (Cuq *et al.*, 1995; Krochta and Johnston, 1997). The loss of moisture causes the product to become hard and tough when water uptake involves spoilage and sometimes rancidity development (Debeauaufort *et al.*, 1998). Surface drying on some fresh and frozen foods or, inversely, moisture uptake in dry or semi-moist foods, may be hindered using edible coatings or films with a low water permeability (Cuq *et al.*, 1995; Krochta and Johnston, 1997; Baldwin, 1999). Moisture loss impairs the visual appearance of the product and leads to unacceptable product weight losses (Petersen *et al.*, 1999). Regulatory standards may also be violated because of water transmission during storage (Kester and Fennema, 1986). Moisture migration from the flesh of frozen, raw breaded shrimp to the surrounding breading during storage decreases the weight of the shrimp component (Gates *et al.*, 1985). The standard of identity for breaded shrimp marketed in the United States specifies that the product must contain at least 50% shrimp (by weight) (FDA, 1976).

The rate of moisture transfer between a food product and its surrounding atmosphere may be reduced by enrobing the entire product with an edible film or coating (Kester and Fennema, 1986). Polysaccharides and proteins establish polymer interactions and create a network responsible for the mechanical properties, but they are not efficient

water-vapour barriers because of their hydrophilic nature (Callegarin *et al.*, 1997; Krochta and Johnston, 1997). On the contrary, lipids provide for the film their water-vapour barrier property because of their hydrophobic character, but films made from lipids alone are usually too brittle (Callegarin *et al.*, 1997). Chitosan-lipid-based films display better efficiency against moisture transfer when the lipid is uniformly incorporated in the matrix such as films that contain lauric acid (Wong *et al.*, 1992). Moisture resistance of lipid films is inversely related to polarity of lipids (Baldwin, 1999). Hydrphobic alkanes and waxes, such as fatty acids, are less resistant to water vapour transmission since their polar groups attract migrating water molecules and thereby facilitate water transport (Cuq *et al.*, 1995).

2.3.2.2 Use of edible coating/films to control gas exchange

Oxygen is a major determinant of food shelf life because it contributes to the oxidation of lipids and is essential for the growth of aerobic food spoilage microorganisms and insect pests (Maloba *et al.*, 1996; Debeaufort *et al.*, 1998). The most obvious precaution against oxidative deterioration is preventing oxygen from coming into contact with products (Flick *et al.*, 1992). Oxygen sensitive foods should be stored in packages with initial headspace oxygen content of 2% or less (Rooney, 1981).

Enrobing of perishable produce in an edible film or coating offers the possibility of controlling respiratory gas exchange to suppress respiration and prolong storage life (Kester and Fennema, 1986). The chemical composition and structure of the film-forming polymer affects coating permeability in general (Miller and Krochta, 1997). Oxygen, carbon dioxide and water vapour permeability of some of the edible and synthetic

coatings/films are shown in Table 2.6. Highly polar materials with extensive degree of hydrogen bonding exhibit low gas permeability, especially under conditions of low humidity, but are poor barriers to moisture (Baldwin, 1999).

One major way to directly control oxygen levels in a package is through the use of an oxygen scavenger system which absorbs oxygen gas in the package and prevents rancidity of food (Rooney, 1981; Rooney *et al.*, 1981). Furthermore, Rooney *et al.* (1981) described polymers such as ethyl cellulose and cellulose acetate which contain a sensitizing dye and a singlet oxygen acceptor as scavenging medium for photochemical removal of headspace oxygen in oxygen-sensitive foods. A polymeric scavenger film, used for instance as an inner web of a package laminate, contains dissolved and immobilized dye which on illumination sensitizes ground-state oxygen which dissolves in the film to the singlet state (Rooney *et al.*, 1981; Maloba *et al.*, 1996). The singlet oxygen then reacts with suitable acceptor, also immobilized in the film, and is thereby consumed. As long as the system is illuminated essentially all the oxygen in the head space is removed (Rooney, 1995). The photochemical steps may be summarized as:

$$^1D_0 \rightarrow {}^1D_1$$

$$^1D_1 \rightarrow {}^3D_1$$

$$^3D_1 + {}^3O_2 \rightarrow {}^1D_0 + {}^1O_2$$

$$^1O_2 + A \rightarrow AO_2$$

$$^1O_2 \rightarrow {}^3O_2$$

Where, the superscripts refer to the singlet and triplet states of the dye (D) and oxygen, and the subscripts, "0" and "1" refer to the dye in its ground or first excited states. The

Table 2.6 Oxygen, carbon dioxide and water vapour permeabilities of commercial films and edible coatings*

Coating/film	Permeability		
	Oxygen[a]	Carbon dioxide[a]	Water vapour[b]
Chitosan	0.0014 ± 0.00	-	0.49 ± 0.00
Methyl cellulose	2.17 ± 0.45	69.0 ± 19.33	0.10 ± 0.00
Sucrose polyester	2.10 ± 0.00	-	-
Zein	0.36 ± 0.16	2.67 ± 1.09	0.12 ± 0.02
Wheat gluten	0.20 ± 0.09	2.13 ± 1.43	0.62 ± 0.01
Polyethylene	8.30 ± 0.00	26.1 ± 0.00	-
Polypropylene	0.55 ± 0.005	-	0.0007 ± 0.06
Hydroxypropyl cellulose	3.57 ± 0.03	143.9 ± 3.76	0.110 ± 0.004

[a]Unit of permeability is in fl.m/m^2.s.Pa; f is the abbreviation for femto (10^{-15})
[b]Unit of permeability is ng.m/m^2.s.Pa; n is the abbreviation for nano (10^{-9})

*Data adapted from Park (1999).

singlet oxygen acceptor or scavenger is denoted by "A" (Rooney *et al.*, 1981). "Longlife", is a commercially available oxygen-scavenging film (Aquantic Corp., USA) that may be placed in the package as a sachet, physically incorporated into closures, or blended into polymers that are used as packaging materials (Smith *et al.*, 1990). Furthermore, use of a resin composition formed by incorporating an oxygen scavenger against the permeation of oxygen in a plastic multi-layer vessel was patented by Koyama *et al.* (1992). Maloba *et al.* (1996) reported that the oxidative stability of sunflower oil stored in the presence of oxygen-scavenging film that contained polyfuryloxirane was more suitable than oil stored without the film, or than oil stored with 20 ppm BHT. Coatings based on whey protein were shown to reduce the oxygen uptake by dry-roasted peanuts, delaying oxidative rancidity as measured by the peroxide value and hexanal content of peanuts (Mate *et al.*, 1996). Wanstedt *et al.* (1981) observed that precooked, ground pork patties coated with calcium alginate significantly retard lipid oxidation as compared to that in uncoated controls. Earle and Snyder (1966) found that alginate coating improved the flavour and colour of frozen shrimps, probably because of a reduction in oxidative deterioration. For determining gas permeability of edible films, air porosimeters and specific permeability cells may be used, as it has been reported for a variety of products (Cuq *et al.*, 1995).

2.3.3 Chitin and chitosan as edible coatings/films for foods

The gelling properties of chitosan allows for a wide range of applications, the most attractive being coating of food and pharmaceuticals. Due to their film-forming properties, chitin (Knorr, 1986) and chitosan (Muzzarelli, 1986) have been successfully used as edible

coating material in fruits and vegetables. Recently, it has been found that carboxymethyl substituted chitosans (*N,O*-carboxymethyl chitosans) are water soluble and have distinctive and unique properties rendering them effective to selectively form permeable films or membranes (Hayes, 1986). A differentially permeable fruit coating called Nutri-Save (Nova Chem, Halifax, N.S) was developed from *N,O*-carboxymethyl chitosan. In such a coating, chitosan served as a both film former and a natural preservative and created a modified atmosphere for whole apples and pears in that it reduced the rate of respiration (50%) and desication for these commodities (Elson *et al.*, 1985).

Chitosan films are tough, longlasting, flexible and very difficult to tear. Most of these mechanical properties are comparable to many medium-strength commercial polymers (Butler *et al.*, 1996). Kittur *et al.* (1998) reported that chitosan films have moderate water permeability values and could be used to increase the storage life of fresh produce and foodstuffs with higher water activity values. Wong *et al.* (1992) and Butler *et al.* (1996) observed extremely good barrier to permeation of oxygen, while exhibiting relatively low vapour barrier characteristics for chitosan films.

Extension of the storage life and better control of decay of peaches, Japanese pears, kiwifruits (Du *et al.*, 1997), strawberries, raspberries (Zhang and Quantick, 1998) and apple (Savage and Savage, 1994) by application of chitosan coating/film has been documented. Similarly, cucumber, bellpeppers (El Ghaouth *et al.*, 1991), tomatoes (El Ghaouth *et al.*, 1992), and carrots (Cheah and Page, 1997) could be stored for long periods after coating with chitosan. These results may be attributed to decreased respiration rates, inhibition of fungal growth and delaying of ripening due to the reduction

of ethylene and carbon dioxide evolution (El Ghouth *et al.*, 1991; Du *et al.*, 1997; Zhang and Quantick, 1998).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Fresh samples of crab processing discards, comprised of intact cephalothorax, abdominal exoskeleton were collected from local source in Newfoundland and were thoroughly washed with distilled water and vacuum packed in Whirl plastic bags (Eastern Papaer, St. John's, NF) and stored at -60°C (Ultra Low, Revco, Inc., West Columbia, SC) until used. Fresh samples, namely cod and herring, were acquired from a local source in Newfoundland and immediately cleaned, gutted, filleted, and deskined. Processed fillets were vacuum packed in Whirl pack plastic bags (Eastern Paper, St. John's, NF) and frozen at -60°C until used. Reagents, namely 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane (TMP), trimethylamine (TMA), hypoxanthine (Hx), xanthine oxidase (XO), picric acid (PA), diisopropylethylamine (DIPEA), tert-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) were obtained from Sigma chemical Co. (St. Louis, MO). Aldehyde standards were purchased from Aldrich chemical Co. (St. Louis, MO) and trichloroacetic acid was acquired from Fisher Scientific (Nepean, ON). Plate Count Agar and peptone were obtained from Difco Laboratories (Detroit, MI). Fatty acid methyl esters were purchased from either Supelco (Oakville, ON) or Nu-Check (Elysian, MN) companies. High performance liquid chromatographic (HPLC)-grade chemicals were used for analysis and preparation as required. All other chemicals used were of American Chemical Society (ACS) grade or

better. Helium, hydrogen, nitrogen and compressed air were obtained from Canadian Liquid Air Ltd. (St. John's, NF).

3.2 Methods

3.2.1 Preparation of chitosans and evaluation of their characteristics

Chitin was isolated from crab processing discards using modified version of methods of Mima *et al.* (1983) and Shahidi and Synoweiki (1991). Deproteinization and demineralization steps were carried out with 20 vol. of 4% (w/v) NaOH at 60 ^{0}C for 3 h and 10 vol. of 10% (w/v) HCl at 25°C for 2 h, respectively. The alkali and acid treatments were repeated twice. Chitin residue, firmly complexed with the carotenoid pigments, was extracted with 20 vol. of acetone and dried for 2h at ambient temperature, followed by bleaching with 0.32% (v/v) sodium hypochlorite solution (containing 5.25% available chlorine) for 5 min with a solids to solvent ratio of 1:10 (w/v). Chitosan was prepared by alkali treatment of chitin using 10 vol. of 50% (w/v) NaOH in distilled water at 100°C for 4, 10 and 20 h in a nickel crucible under a nitrogen atmosphere (Mima *et al.*, 1983). The reactants were immediately filtered under vacuum after alkali treatment, washed with hot-deionized water to neutral pH and lyophilized for 72 h at -49°C and 62 * 10^{-3} mbar (Freezone 6, Model 77530, Labconco Co., Kansas City, MO).

Moisture, total nitrogen and ash contents were then determined (Shahidi and Synowiecki, 1991). Apparent viscosity (at 2 Nm^{-2}) of a 1% (w/v) chitosan in a 1% (v/v) acetic acid at 25°C was measured using a rotational viscometer (Cole-Parmer Co. Vernon Hills, IL). Measurements were made in triplicate using a No. 5 spindle at 50 rpm on

solutions at 25°C with values reported in centipoise (cP) units. The degree of acetylation of chitosans was measured according to the picric acid method of Neugebauer *et al.* (1989). Dried, Finely powdered chitosan (10-15mg) was placed in a small column for liquid chromatography, and the weight of the polymer was determined accurately by weighing the column before and after introduction of sample. In order to remove residual salts from the amino groups, a preliminary washing step was performed: the polymer in the column was exposed for 15 min to 0.1M diisopropylethylamine (DIPEA)-methanol, and then washed with methanol (10 mL). Binding of picric acid to the amino groups was performed by introducing 0.1M picric acid-methanol (1.0 mL) and the allowing the reaction to proceed for 6 h. The column was then washed with methanol (30 mL) at a rate of 0.5 mL/min in order to remove the unbound picric acid completely. The bound picric acid was then quantitatively removed from the chitin-chitosan amino groups by proceeding as follows. DIPEA in methanol (0.1M;1.0 mL) was introduced into the column and allowed to stay for 30 min and then eluted with methanol. The concentration of DIPEA-picrate in the eluate was measured at 358 nm using a Hewlett-Packard diode array spectrophotometer (Model 8452A, Hewlett Packard Co., Mississauga, ON). Standard line was prepared using different concentrations of DIPEA-picrate salt in methanolic solutions (Figure A1). The fraction of amino groups acetylated (degree of *N*-acetylation., d.a) was calculated using: $d.a = m - 161n/m + 42n$ where, m = weight of chitosan sample (mg), n = mmoles of picric acid eluted from sample, 161 = molecular weight of D-glucosamine unit, and 42 = molecular weight of N-acetyl-D-glucosamine - molecular weight of D-glucosamine.

Molecular weight of chitosan was expressed as the viscosity molecular weight (Mv) using ViscoTek model Y-500 relative viscometer (Viscotek Co. Houston, TX). Chitosan solution in 0.1M acetic acid-0.2 M sodium chloride was diluted to give four concentrations ranging from 0.05 to 2 g/L which were used for determination of specific viscosity (η_{sp}) at 25°C. Specific viscosity was determined as follows: $\eta_{sp} = (\eta-\eta_s)/\eta_s$, where η is the solution viscosity, η_s is the solvent viscosity. The values of intrinsic viscosity (η) obtained ($\eta = \lim_{c\to 0} \eta_{sp}/C$) by extrapolating ($\eta_{sp}$)/C versus C, where C is the concentration of the chitosan solutions, were fitted into the equation $Mv=[\eta/k]^{1/a}$, where constants of "k" and "a" are 1.81×10^{-5} and 0.93, respectively (Roberts and Domaszy, 1982; Anthonsen *et al.*, 1993; Kittur *et al.*, 1998).

3.2.2 Preparation of chitosan coating solution and treatment of fish

Each chitosan (10g) was added to deionized water (1 L) containing 10g of glacial acetic acid. The mixture was stirred at 40°C for 1h. The solutions in beakers were placed on a magnetic stirrer/hotplate and glycerol was added to them as a plasticizer at a level of 1.0 mL/g chitosan and stirred for 10 min. The resultant chitosan coating solution was filtered through a Whatman No.3 filter paper to remove any undissolved particles. For coating of fish (herring and cod) with chitosan solutions, each fish fillet (5x15cm) was immersed in the chitosan solution (5°C) for 30 s and, after a 2-min period, it was immersed again. Fish fillets of the control group were left untreated. Coated fish samples were dried at 40°C for 2 h in a forced air oven (Fisher Isotemp 300, Fair Lawn, NJ) in order to form edible coating and stored at 4 ± 1°C for quality assessment. All treatments of samples for

tests were done in a cold room (4 ± 1°C). Chemical and microbiological analyses were carried out at 2 days intervals to determine the overall quality of fish.

3.2.3 Proximate composition

Determination of moisture, crude protein, and ash contents of the two types of fish was carried out according to the AOAC (1990) method. Total lipid content was determined according to the method of Bligh and Dyer (1959).

3.2.3.1 Determination of moisture content of fish

Approximately 3-4 g of fish were accurately weighed into a preweighed aluminium dish and placed in a forced-air convection oven (Fisher Isotemp 300, Fair Lawn, NJ) which was preheated to 105 ± 1°C. Samples were held at this temperature overnight or until a constant mass was obtained. The moisture content was then calculated as percent ratio of the weight difference of samples before and after drying to that of the original material (AOAC, 1990). Relative moisture loss (RML) from chitosan coated fish fillets was calculated as: RML (%) = [(Initial moisture content - Final moisture content)/Initial moisture content] x 100.

3.2.3.2 Determination of crude protein content of fish

Approximately 0.3 - 0.4 g of fish were accurately weighed on a nitrogen-free paper and placed into a digestion tube of a Buchi 430 digester (Buchi Laboratories, Flawil, Switzerland). The nitrogen content in different samples was determined by digestion in 20

mL of concentrated sulphuric acid in the presence of two Kjeltab catalyst tablets (Profamo, Dorval, PQ) in the digester until a clear solution was obtained. Digested samples were diluted with 50 mL of distilled water followed by addition of 150 mL of a 25% (w/v) solution of sodium hydroxide. Nitrogen in the sample was converted to ammonium sulphate which was released as ammonia and steam-distilled (Buchi 321, Buchi laboratories, Flawil, Switzerland) into a 50 mL solution of 4% (w/v) boric acid containing a few drops of end point indicator (EM Science, Gibbstown, NJ). Approximately 200 mL of distillate were collected and the content of ammonia in the distillate determined by titrating it against a 0.1N standard solution of sulphuric acid (AOCS, 1990). The crude protein was calculated as N%*6.25.

3.2.3.3 Extraction of lipid and determination of total lipid content of fish

Total lipids were extracted into a mixture of chloroform and methanol as described by Bligh and Dyer (1959). Approximately 25 g of samples were accurately weighed and extracted with a mixture of 25 mL chloroform and 50 mL of methanol (1:2, v/v) by homogenizing for 3 min with a Polytron PT 3000 homogenizer (Kinematica, Littau, Switzerland) at a speed of 4. A further extraction was done with the addition of 25 mL of chloroform followed by homogenization. Approximately 25 mL of distilled water were then added and the mixture filtered through a Buchner funnel using a Whatman No.3 filter paper (Fisher scientific, Nepean, ON). The filtrate was allowed to separate in a separatory funnel. Dilution with chloroform and water resulted in separation of homogenate layers and inclusion of lipids in the chloroform. To minimize oxidation care was taken to exclude

oxygen at all stages by careful use of nitrogen blanketing techniques. The chloroform layer was separated and evaporated at 40°C under vacuum using a Buchi RE 111 rotovapor (Buchi Laboratories, Flawil, Switzerland). After nitrogen flushing, recovered lipids were stored at -60°C (Ultra Low, Revco, Inc., West Columbia, SC) for determination of peroxide value (PV), conjugated dienes (CD) and fatty acid composition.

A 10 mL aliquot of lipid extracts in chloroform, after drying over anhydrous sodium sulphate, were transferred into a tarred 50 mL round bottom flask and the solvent was removed under vacuum using a Buchi RE 111 rotovapor (Buchi Laboratories, Flawil, Switzerland). The flask was then placed in a forced-air convection oven (Fisher Isotemp 300, Fairlawn, NJ) at 80°C for 1h. After cooling in a desicator, lipid content was determined gravimetrically.

3.2.3.4 Determination of ash content of fish

Approximately 3 - 4 g of fish were weighed into a cleaned porcelain crucible and then charred over a Bunsen burner and subsequently placed in a temperature controlled muffle furnace (Blue M Electric Co., Blue Island, IL) which was preheated to 550°C. Samples were held at this temperature until a gray ash was produced. The crucible was then cooled in a desicator and weighed immediately. Ash content was calculated as percent ratio of the mass of the ash, obtained after ignition, to that of the original material (AOAC, 1990).

3.2.4 Quality assessment related to lipid oxidation

3.2.4.1 Analysis of fatty acid composition of lipids

Fatty acid composition of fish lipids was determined after their conversion to fatty acid methyl esters (FAMEs). About 10 mg of each oil were weighed into a 6 mL well-cleaned Teflon-lined, screw capped conical vials. The internal standard [250 mg methyl treicosanoate (C 23:0)/100 mL chloroform] was added to the vial and the solvent in the oil-internal standard mixture was evaporated under a stream of nitrogen. Transmethylation reagent (2 mL, freshly prepared 6 mL of concentrated sulphuric acid made up to 100 mL with spectral grade methanol and 15 mg of hydroquinone as an antioxidant) was added to the sample vial and mixed by vortexing. The mixture was incubated overnight at 60°C and subsequently cooled. Distilled water (1 mL) was added to the mixture and after thorough mixing, extracted three times with 1 - 5 mL of pesticide grade hexane. A few crystals of hydroquinone were added to each vial prior to extraction with hexane. Hexane layers were separated, combined and transferred to a clean tube and then washed two times with 1 - 5 mL of distilled water. In the first wash, the aqueous layer was removed and in the second wash the hexane layer was separated and evaporated under a stream of nitrogen. FAMEs were then dissolved in 1 mL of carbon disulphide and used for gas chromatographic analysis. A Hewlett-Packard 5890 Series II gas chromatograph (Hewlett Packard, Toronto, ON) equipped with a Supelcowax-10 column (0.25 mm diameter, 30 m length, Supelco Canada Ltd., Oakville, ON) was used for analyzing FAMEs. The oven temperature was initially 220°C for 10.25 min and then ramped to 240°C at 30°C/min and then held there for 9 min. The injector and flame ionization detector (FID) temperatures

were both at 270°C. Ultra high purity (UHP) helium was used as the carrier gas (15 mL/min). HP 3365 Series II ChemStation software (Hewlett-Packard, Toronto, ON) was used for data handling. The FAMEs were tentatively identified by comparison of their retention times with those of authentic standard mixtures (PUFA 1, Supelco Canada Ltd., Oakville, ON and GLC-416, Nu-Check, Elysian, MN). The area under each peak was calculated on a weight percentage basis using methyl tricosanoate (C23:0) as an internal standard.

3.2.4.2 Determination of peroxide value (PV)

Hydroperoxides (ROOH) are the primary products of lipid oxidation and the method for determining the content of peroxides in an oil is based on the reduction of the hydroperoxide groups (-OOH) with iodide ion (I^-) in an acidic medium. The method described by AOCS (1990) was used to determine PV of oil samples. Samples (2.0-4.0g) were weighed into 250 mL glass-stoppered Erlenmeyer flasks and 30 mL of acetic acid-chloroform (3:2, v/v) solution were then added. The contents were mixed until oil was dissolved and then 0.5 mL of saturated potassium iodide (KI) solution was added to it. The mixture was allowed to stand in stoppered flasks with occasional shaking for exactly 1 min and then 30 mL of distilled water were added to it. The liberated iodine was titrated with a standardized 0.01N solution of sodium thiosulphate ($Na_2S_2O_3$) with constant shaking until the yellow colour had almost disappeared. About 0.5 mL of starch indicator solution (1% w/v) was then added to the flask and titration was continued with vigorous shaking until the blue colour of the solution had disappeared. A blank titration was

conducted each time. PV was expressed as the uptake of milliequivalents of active oxygen (i.e. peroxide) per kilogram of oil.

$$PV = \{(V_{sample}-V_{blank}) \times N\ Na_2S_2O_3 \times 1000\}/\text{mass of sample (g)}$$

where V = volume of $Na_2S_2O_3$ solution (mL) and N = normality of $Na_2S_2O_3$

3.2.4.3 Determination of conjugated diene value (CD)

Marine lipids containing methylene-interrupted double bonds show a shift in their position during oxidation due to isomerization and conjugate formation. The resulting conjugated dienes exhibit intense absorption at 234 nm. Conjugated diene value of oil samples was measured according to the IUPAC (1987) method. Oil samples (0.02-0.04g) were weighed into a 25 mL volumetric flask, dissolved in isooctane (2,2,4-trimethylpentane) and made up to the mark with the same solvent. The solution was thoroughly mixed and its absorbance read at 234 nm using a Hewlett-Packard diode array spectrophotometer (Model 8452A, Hewlett Packard Co., Mississauga, ON). Pure isooctane was used as the reference. Conjugated diene value was calculated as CD = A/(C x d), where A = absorbance of the solution at 234 nm, C = concentration of the oil in g/100 mL and d = length of the cell (cm).

3.2.4.4 Determination of 2-thiobarbituric acid-reactive substances (TBARS)

A classical method to estimate secondary lipid oxidation products is the 2-thiobarbituric acid (TBA) test. This test measures the content of malonaldehyde and other TBA reactive substances (TBARS) in a sample. Fish samples were analyzed for their

TBARS values over a 12-day period according to the method of Siu and Draper (1978) as described by Shahidi and Hong (1991). Two grams of each sample were placed in a centrifuge tube to which 5 mL of a 10% (w/v) solution of trichloroacetic acid (TCA) were added and vortexed (Fisher Vortex Genie 2, Fisher Scientific, Nepean, ON) at high speed for 2 min. Five millilitres of a 0.02 M aqueous solution of 2-thiobarbituric acid was then added to each centrifuge tube which was further vortexed for 30 s. The samples were then centrifuged at 3000xg for 10 min and the supernatants filtered through a Whatman No.3 filter paper. Filtrates were heated in a boiling water bath for 45 min, cooled to room temperature in ice, and the absorbance of the resulting pigment read at 532 nm using a Hewlett-Packard diode array spectrophotometer (Model 8452A, Hewlett Packard Co., Mississauga, ON). TBARS values were calculated by multiplying the absorbance readings by a factor of 7.0, which was obtained from a standard line prepared using 1,1,3,3-tetramethoxypropane as a precursor of malonaldehyde (Figure A2). Inhibition of TBARS formation was determined using the following equation. % inhibition = 100-100 (TBARS value for the treated sample/TBARS value for the control sample).

3.2.4.5 Static headspace gas chromatographic analysis

A Perkin-Elmer 8500 gas chromatograph and an HS-6 headspace sampler (Perkin-Elmer Corp., Montreal, PQ) were used for volatile analysis of fish samples. A high polarity Supelcowax 10 fused silica capillary column (30 m x 0.32 m internal diameter, 0.10 mm film thickness, Supelco Canada Ltd., Oakville, ON) was used. Helium was the carrier gas employed at an inlet column pressure of 17.5 psig with a split ratio of 7:1. The oven

temperature was maintained at 40°C for 5 min and then ramped to 200°C at 20°C/min and held there for 5 min. The injector and flame ionization detector (FID) temperatures were adjusted to 280°C and held at this temperature throughout the analysis.

For headspace (HS) analysis, 2.0 g portions of homogenized fish samples were transferred to 5 mL glass vials. The vials were capped with teflon-lined septa, crimped and then frozen and kept at -60°C (Ultra Low, Revco, Inc., West Columbia, SC) until used. To avoid heat shock after removal from storage, frozen vials were tempered at room temperature for 30 min and then preheated in the HS-6 magazine assembly at 90°C for 45 min equilibration period. The heating time of the vial was 6 s and the volume of the vapor phase drawn was approximately 1.5 mL. Chromatographic peak areas were expressed as integrator count units. Individual volatile compounds were tentatively identified by comparing their relative retention times of GC peaks with those of commercially available standards. Quantitative determination of dominant aldehydes was accomplished using 2-heptanone as an internal standard.

3.2.5 Quality assessment related to chemical spoilage

3.2.5.1 Determination of trimethylamine (TMA)

The post-mortem metabolism of nitrogenous compounds in fish flesh is mainly responsible for gradual loss of fresh appearance of the catch and for the development of signs of putrefaction. Many of the spoilage bacteria on fish use trimethylamine oxide (TMAO) as an electron acceptor in an anaerobic respiration. The reduced component trimethylamine (TMA), which is one of the dominant components of stored as well as

spoiling fish, has a typical fishy odour. TMA determination was performed on trichloroacetic acid (TCA) extracts of fish using the modified picric acid methods described by Dyer (1959) and Woyewoda *et al.* (1986a). Ten grams of each sample were placed in a centrifuge tube (covered with ice) to which 20 mL of a 7% (w/v) TCA solution were added and homogenized for 3 min with a Polytron PT 3000 homogenizer (Kinematica, Littau, Switzerland) at a speed of 4, and then centrifuged at 4000xg in an automatic centrifuge (Sorvall RC-3, Ivan Sorvall Inc., CO) at 4°C for 15 min. Five millilitres of decanted supernatant were added into borosilicate-glass test tubes and, in progressive order 1 mL of 10% (v/v) formaldehyde, 10 mL of toluene and 3 mL of 25% (w/v) potassium hydroxide were added. Tubes were screw capped and thoroughly mixed in a Gyrotory water bath shaker (Model G76, New Brunswick Scientific Co. Inc., New Brunswick, NJ) for 15 min; 7 mL of upper toluene layer were passed through anhydrous sodium sulphate. Five millilitres of moisture-free toluene phase were mixed with 5 mL of picric acid reagent (2.0 g/100 mL toluene) and the absorbance was read at 410 nm using a Hewlett-Packard diode array spectrophotometer (Model 8452A, Hewlett Packard Co., Mississauga, ON). TMA content (μg) was calculated by multiplying the absorbance reading by a factor of 73.36, determined from a standard line prepared using standard TMA (Figure A3). Final TMA content was expressed as mg TMA-N per 100 g fish using following equation: $\{T \times [V_1 + (0.01 \times M \times W)]\}/ V_2 \times W \times 10$, Where T = equivalent TMA in μg determined from standard curve, M = Moisture of fish sample expressed in percent, V_1 = Volume (mL) of TCA added for 1:2 extraction, V_2 = Volume (mL) of extract added to test tube, W = Weight (g) of fish used in 1:2 extraction.

3.2.5.2 Determination of hypoxanthine (Hx)

The effect of hypoxanthine (Hx) accumulation in fish tissues reflects the initial phase of autolytic deterioration and later includes contributions through bacterial spoilage. Thus, measuring the concentration of Hx as an index of quality, capable of measuring changes in freshness quality of fish that occur during early stage as well as later stages of chilled storage. Monitoring of Hx levels in fish muscle during the storage was done according to the modified enzymatic method of Woyewoda *et al* (1986b). Fifty grams of each sample were placed in a glass beaker (covered with ice) to which 200 mL of 6% (w/v) perchloric acid were added and homogenized for 3 min with a Polytron PT 3000 homogenizer (Kinematica, Littau, Switzerland) at a speed of 4. Extracted solution was filtered through a fluted Whatman No. 3 filter paper and 10 mL of perchloric acid filtrate were neutralized to pH between 7.0 and 7.6 by carefully adding an equal volume (10 mL) of potassium hydroxide-phosphate buffer (pH 7.6) solution. Two millilitres of neutralized extract were added into borosilicate-glass test tubes and in progressive order 2 mL of 0.05 M phosphate buffer (pH 7.6), 0.5 mL of distilled water and 0.5 mL of xanthine oxidase enzyme were added. Tubes were screw capped and incubated in a thermostated water bath at 37 ± 1°C for 30 min and absorbance read at 290 nm using a Hewlett-Packard diode array spectrophotometer (Model 8452A, Hewlett Packard Co., Mississauga, ON). Hypoxanthine content (μg) was calculated by multiplying the absorbance reading by a factor of 63.69, determined from a standard line prepared using hypoxanthine as a standard (Figure A4). Final hypoxanthine content was expressed as micromole Hx per gram of fish using the following equation: $\{H \times [V_1 + (0.01 \times M \times W)] \times (V_2 + V_3)\}/ V_4 \times$

W x V_3 x G, Where H = µg Hx determined from standard curve, M = Moisture content of fish expressed in percent, V_1 = Volume (mL) of perchloric acid used in 1:4 extraction, V_2 = Volume (mL) of KOH/phosphate buffer used for neutralization, V_3 = Volume (mL) of extract neutralized by KOH / phosphate buffer, V_4 = Volume (mL) of sample extract added to test tube, W = Weight (g) of sample used in 1:4 extraction, G = gram molecular weight of Hx i.e. 136.1.

3.2.5.3 Determination of total volatile basic-nitrogen (TVB-N)

Total volatile basic-nitrogen (TVB-N) is one of the most widely used measurements of seafood quality. It is a general term which includes the measurement of trimethylamine, dimethylamine, ammonia and other volatile basic nitrogenous compounds associated with seafood spoilage. TVB-N was determined according to the modified micro-Kjeldahl distillation technique described by Cobb III *et al.* (1973). Ten grams of each sample were placed in a centrifuge tube (covered with ice) to which 50 mL of a 7% (w/v) solution of trichloroacetic acid were added and homogenized for 3 min with a Polytron PT 3000 homogenizer (Kinematica, Littau, Switzerland) at a speed of 4. Extracted solution was filtered through a fluted Whatman No. 3 filter paper and 5 mL of extract was filled into the micro-Kjeldahl distillation apparatus and mixed with a saturated sodium phosphate solution. Volatile nitrogen was trapped into a 5 mL solution of 2% (w/v) boric acid (with 1 mL N-point indicator). Distillation was continued until 10 mL of distillate had been collected. The content of the receiver flask was titrated with a standardized solution of 0.05 N H_2SO_4 until the original colour was obtained (pink reddish

colour). Total volatile basic-nitrogen was expressed as milligrams nitrogen per 100 g sample using the following equation: TVB-N = $\{(V_1 - V_2) \times N \times 100 \times 14 \times 50\}/ W \times 5$, Where V_1 = Volume (mL) H_2SO_4 used for sample, V_2 = Volume (mL) of H_2SO_4 used for blank, N = Normality of H_2SO_4, W = Weight of sample in grams.

3.2.6 Quality assessment related to microbial spoilage

A sample of 11 g fish with 99 mL of sterilized peptone water (10 g peptone and 5 g of NaCl in 1 L of distilled water, pH: 7.2-7.3) was put into sterilized plastic bags (Seward Medical Stomacher '400' bags) and homogenized in a laboratory stomacher (Type BA 7021, Seward Medical, London, UK) for 30 s. From this mixture 3, 4 and 5 dilutions were obtained by mixing with peptone water. A 1 mL aliquot from each diluent was applied on a sterilized standard plate count agar (23.5 g agar in 1 L of distilled water). The plates were triplicated and incubated at 20°C for 72 h. Afterwards, the colonies grown on the plates were counted using the colony counter and total aerobic psychrotrophic plate count values were indicated as colony forming units (CFU) per gram of fish.

3.2.7 Preparation of cooked comminuted fish model system

Fish model systems (cod and herring) were prepared according to the modified method described by Shahidi and Pegg (1990). Fish fillets (cod and herring) were homogenized using a Waring Blender (model 33BL73, Waring products, New Hartford, CT) and ground fish was mixed with 20% by weight of deionized water in Mason jars (height 10 cm, internal diameter 6 cm). Chitosans (50, 100, 150 and 200 ppm) and

commercial antioxidants butylated hydroxyanisole + butylated hydroxyanisole (BHA+BHT 200 ppm) and tert-butylhydroquinone (TBHQ 200 ppm) were added separately to fish and thoroughly homogenized. A control sample containing no chitosans/commercial antioxidants was also prepared. Samples were cooked in a thermostated water bath at 85 ± 2°C (internal temperature of 72 ± 3°C) for 40 min while stirring every 5 min with a glass rod. After cooling to room temperature, the fish systems were homogenized in a Waring Blender (model 33BL73, Waring products, New Hartford, CT) for 30 s, transferred into Whirl plastic bags (Eastern Papaer, St. John's, NF) and then stored under refrigerated conditions at 4 ± 1°C for further analysis.

3.3 Statistical analysis

All experiments used completely randomized block designs (CRD) and analyses were carried out in triplicate. Mean values with standard deviations (SD) were reported when and where necessary. SigmaStat was used to normalize the data, analysis of variance (ANOVA) performed, and differences in mean values determined using Tukey's procedures of statistical analysis system (SAS, 1990). Microbilogical results were compared using the $\log_{10}$ transformation of counts and data were analyzed using the General Linear Models Procedure (PROC GLM) of SAS (SAS Institute, Inc., 1990) and significant differences were determined using least square means ($p \leq 0.05$). Linear regressions were carried out to determine if there was a correlation between certain parameters.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Characteristics of chitosans extracted from crab processing discards

Characteristics of chitosans prepared with different deacetylation times are listed in Table 4.1. Preparation of chitosan samples (I, II, and III) shown in Table 4.1 involved deacetylation of chitin for 4, 10 and 20 h. The chitosans prepared from the snow crab processing discards showed variations in their viscosity which seem to be closely related to the duration of deacetylation time. The highest viscosity was observed when deacetylation was carried out for 4 h, followed by those prepared over a 10 and 20 h period. As shown in the Table 4.1, the nitrogen contents of chitosans were dependent on deacetylation time and were 7.55, 7.63, and 7.70%, respectively, for samples prepared over 4, 10 and 20 h, confirming a more effective deacetylation over longer periods. The corresponding apparent viscosity values were 360, 57, and 14 cP, respectively (Figure 4.1). The viscosity of product one (I) was 360 cP, compared to 57 cP for product two (II). This 6-fold difference in viscosity cannot be easily explained by the slight differences observed in nitrogen and ash contents. Similarly, the differences in nitrogen and ash contents between products two (II) and three (III) do not account for the different viscosity values of 57 and 14 cP, respectively.

The same trend was also observed with viscosity molecular-weight determinations. As shown in Table 4.1 there was a substantial difference in molecular weights measured by the viscometric method among products I, I, and III. The product deacetylated for 4 h had a molecular weight which was higher by a factor of 1.87 than that for the product which

Table 4.1 Characteristics of three different types of chitosans (I, II and III) prepared from crab shells[a]

Properties	Chitosan		
	I	II	III
Deacetylation time[b] (h)	4	10	20
Moisture (%)	4.50 ± 0.30	3.95 ± 0.34	3.75 ± 0.21
Nitrogen (%)	7.55 ± 0.10	7.63 ± 0.08	7.70 ± 0.19
Ash (%)	0.30 ± 0.03	0.25 ± 0.02	0.30 ± 0.00
Colour	cream white	cream white	cream white
Apparent viscosity (cP)	360 ± 0.53	57 ± 0.96	14 ± 0.34
Degree of deacetylation (%)	86.4 ± 2.1	89.3 ± 1.2	91.3 ± 1.3
Mv[c] (dalton)	1.8×10^6	9.6×10^5	6.6×10^5

[a]Results are expressed as mean value of three determinations ± standard deviation. [b]Deacetylation for preparation of chitosans I, II and III was achieved using a 50% NaOH solution at 100°C for 4, 10 and 20 h, respectively. [c]Viscosity molecular weight.

Figure 4.1 Different chitosans produced from crab processing discards due to changing of deacetylation time.


Chitosan 14 cP
Chitosan 57 cP
Chitosan 360 cP

was deacetylated for 10 h. Similarly, the product deacetylated for 20 h had a molecular-weight lower than that of product produced over a 10 h period, by a factor of 1.45. These molecular-weight differences were less than those observed for viscosity values. It was apparent that molecular weight was not linearly related to viscosity. Since both the viscosity and molecular weight were determined in solution, all observations made were based on solution behavior of chitosan products. Viscometry is one of the simplest and most rapid methods for determining the reactive molecular weights of polymers although it is not an absolute method and requires determination of constants through correlation of limiting viscosity number values with relative molecular weights determined by an absolute method (Maghami and Roberts, 1988). Furthermore, Maghami and Roberts (1988) observed that viscometric constants "K_m" and "a" in the Mark-Houwink equation received unchanged for a series of chitosans with *N*-acetyl contents of 0-40%.

Deproteinization is frequently referred to as the extraction of protein from crustacean shells. Non-chitinous protein is the main component which should be removed effectively to obtain a high quality chitin. It is evident that the protein and its binding characteristics affect the protein removal efficiency from shellfish processing discards (Austin *et al.*, 1981). Several possible covalent bonds might be involved in the chitin-protein link. Brine (1982) differentiated the types of bonding into four major groups. The schiff base type linkages, acetal (*O*-glycosidic), amide (*N*-glycosidic) and N-acylglucosaminyl type bonds contributed approximately 70-95% of the protein fractions of four different crab species. In this study 20 vol. of 4% (w/v) sodium hydroxide at 60°C for 3 h was used to remove protein from crab processing discards, similar to that used by No *et al.* (1989). These authors used a 3.5% (w/v) sodium hydroxide solution at 65°C for 2 h

with a solid to solvent ratio of 1:10 (w/v) for deproteinization of crawfish processing discards.

The efficacy of demineralization is generally followed by measuring the residual ash content of the isolated chitosan. The low ash content (0.25-0.30%) of all three types of chitosans indicates the effectiveness of the method used for demineralization. For demineralization, it is important that the amount of acid be stoichiometrically equal or greater than all minerals presents in the shells to ensure complete reaction with them (Shahidi and Synowiecki, 1991).

The degree of deacetylation was 86.4, 89.3, and 91.3% for chitosans extracted from chitin due to changing of deacetylation time 4, 10, and 20 h, respectively. The large positive charge density due to the high degree of deacetylation (86.4-91.3%) makes crab chitosan unique for industrial applications, because chitosan properties are highly dependent on charge density. Li *et al.* (1992) and Muzzarelli (1985) reported that the term chitosan should be used when the degree of deacetylation is above 70% and nitrogen content in product is higher than 7% by weight. Table 4.1 shows that the viscosity average molecular weight (M_v) of chitosan decreased with increasing deacetylation time. However, a very high degradation rate of chitin polymers was not observed in this study by increasing the deacetylation time from 10 h to 20 h, perhaps due to intermittently washing the intermediate product in distilled water two or more times during the alkali treatment. The procedure used for deacetylation in this investigation was similar to that of Mima *et al.* (1983) who produced 96% deacetylation with 3 alkali washing treatments involving a shorter time (1h) at a somewhat higher temperature (110°C).

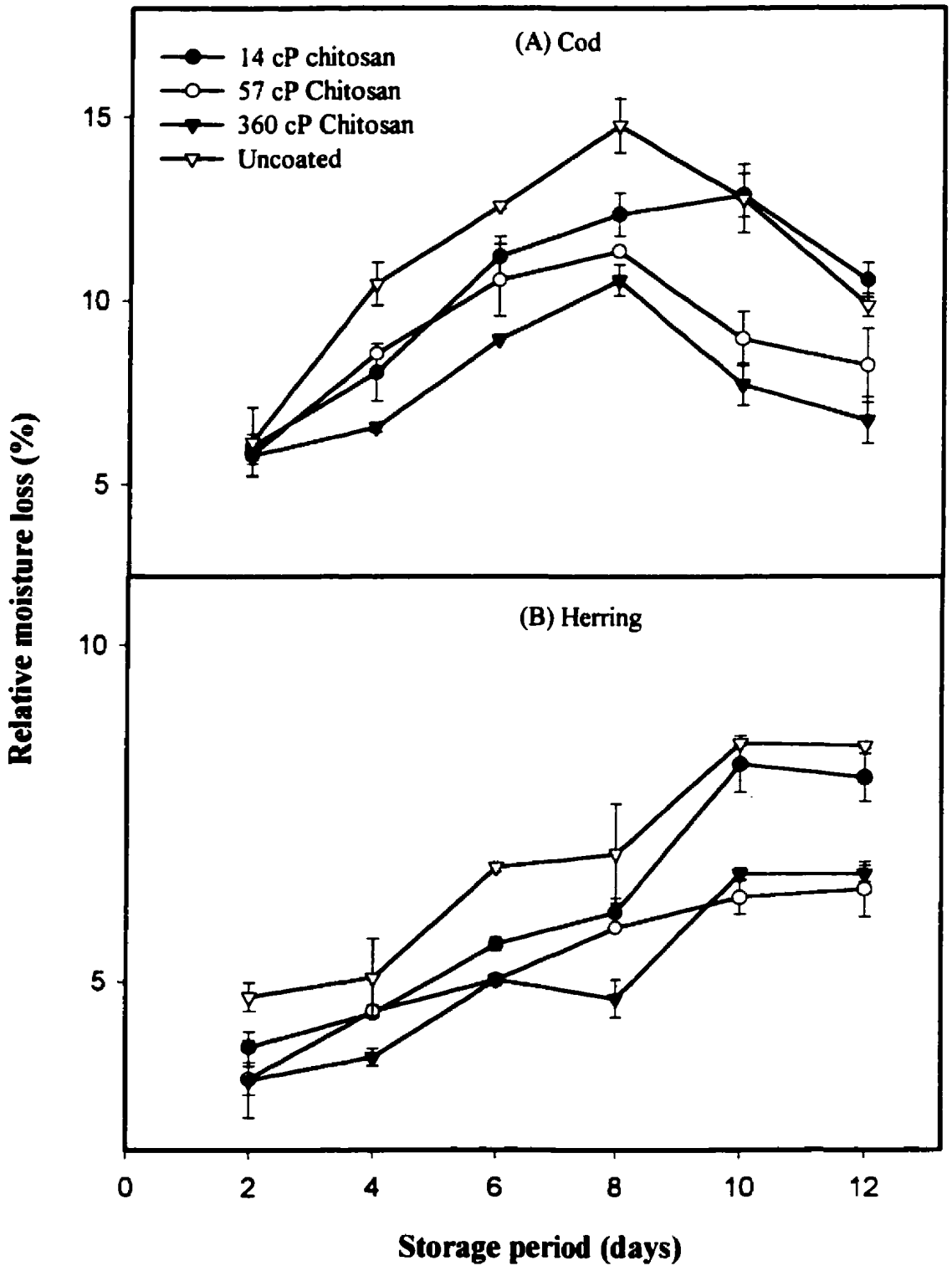
Different viscosity chitosans (14 cP, 57 cP and 360 cP chitosans) were used to investigate the effect of chitosan coating on fish quality during refrigerated storage. Edible films or coatings are generally composed of polysaccharides, proteins, lipids or their combinations. At least one component should be a high-molecular-weight, long-chain polymer, in order to yield film or coating matrices with appropriate cohesive strength when deposited from a suitable solvent. Chitosan has such a cohesive property due to its viscosity characteristics in aqueous solutions.

4.2 Effect of chitosan coating on relative moisture loss of fish

Moisture changes in a food may have adverse effects on its texture, wholesomeness and market value (Kester and Fennema, 1986). Furthermore, moisture loss impairs the visual appearance of products and leads to unacceptable product weight losses. Excessive water evaporation through the packaging material might result in desiccation of the packaged foodstuff or it may favour lipid oxidation (Vermeiren *et al.*, 1999).

The relative moisture loss of chitosan coated cod and herring fillets stored at 4 ± 1°C is summarized in Figure 4.2. The pattern of relative moisture loss in cod was different from that of herring stored under similar conditions (Figure 4.2). At 4 ± 1°C, the relative moisture loss was lower in herring fillets than in cod fillets regardless of the type of chitosan employed. Moisture in cod fillets was thought to be evaporated much faster during the first few days of storage; therefore, relative moisture loss was initially high. After day-6 of storage, relative moisture loss in cod samples decreased (except 14 cP chitosan coated sample). According to Pham and Willix (1984), the desiccated surface

Figure 4.2 Effect of chitosan coating on relative moisture loss (%) of fish stored at 4 ± 1°C. (A) Cod, (B) Herring.


(A) Cod
14 cP chitosan
57 cP Chitosan
360 cP Chitosan
Uncoated
15
10
5
(B) Herring
10
5
0
2
4
6
8
10
12
Relative moisture loss (%)
Storage period (days)

layer developed during cold storage produces a further resistance to mass transfer in the case of biological substances. Perhaps this could be the reason for observing a reduction in relative moisture loss in cod samples after a certain period of storage.

Cod samples coated with 57 and 360 cP chitosans exhibited a similar pattern of relative moisture loss as compared to that of the sample coated with 14 cP chitosan throughout the storage period. The relative moisture loss remained higher or similar in cod samples coated with 14 cP chitosan after day-8 of storage as compared to that of the uncoated samples. A significant ($p \leq 0.05$) reduction in relative moisture loss, by 37, 29, 29, 40, and 32%, over that of uncoated cod samples was observed for cod samples coated with 360 cP chitosan after 4, 6, 8, 10, and 12 days of storage, respectively. Corresponding values for 57 cP chitosan coated cod samples were 18, 16, 23, 30, and 17%. Water vapour permeability has been shown to be dependent on the relative polarity of the carbohydrate polymers. The more polar films tend to be more ordered and less porous, hence less water vapour permeable (Banker, 1966). Perhaps this could be the reason for comparatively lower relative moisture loss in 360 cP chitosan coated cod samples as compared to that of 14 cP chitosan and 57 cP chitosan coated cod samples. Water vapour transfers through hydrophilic coating/film by sorption and diffusion and is affected by many factors (Cuq *et al.*, 1995). Therefore, the mechanism involved in the water vapour transfer through the chitosan coatings may need to be studied further.

Unlike cod, a reduction in relative moisture loss due to chitosan coating was not clearly observed for herring samples. There was no statistically significant ($p > 0.05$) difference in relative moisture loss of herring fillets with respect to the different viscosity chitosan coatings, until day-6 of storage. After day-6 of storage at $4 \pm 1^{\circ}C$, there was a

significant ($p \leq 0.05$) treatment effect in relative moisture loss of 57 cP and 360 cP herring samples as compared to that of the uncoated herring. Both uncoated and 14 cP chitosan coated herring samples exhibited the highest relative moisture loss, and became dry on the surface (visual observation) after a day-10 storage period.

The water vapour barrier properties of edible films and coatings have served as important factors in their selection for use in several food systems. Stuchell and Krochta (1995) reported efficient protection of salmon against water loss using a coating composed of a mixture of whey protein and acetylated monoglycerols. El Ghaouth *et al.* (1991) used edible chitosan coatings to reduce water loss from cucumber and bell pepper fruits. However, Ishikawa and Nara (1991) observed that the chitosan film coated on apple surfaces efficiently inhibited the permeation of sucrose into the apple while it practically did not affect the dehydration rate. Furthermore, lamb carcasses coated with alginates gelled with calcium chloride were not preserved from water loss, but exhibited a reduction in microbial growth (Lazarus *et al.*, 1976).

4.3 Quality assessment related to lipid oxidation

Assessment of lipid oxidation during refrigerated storage of fish fillets (moisture, protein, lipid and ash contents were 73.53 ± 0.38, 13.21 ± 0.02, 12.43 ± 0.13, and 0.35 ± 0.01% for herring and 80.92 ± 0.93, 15.44 ± 0.07, 1.25 ± 0.04, and 0.52 ± 0.00% for cod, respectively) coated with chitosans was achieved by measuring peroxide value (PV) and conjugated diene value (CD) for primary lipid oxidation products and thiobarbituric acid reactive substances (TBARS) and static headspace gas chromatographic analysis (HS) for secondary lipid oxidation products. Due to the limitations of each of these assays, it is

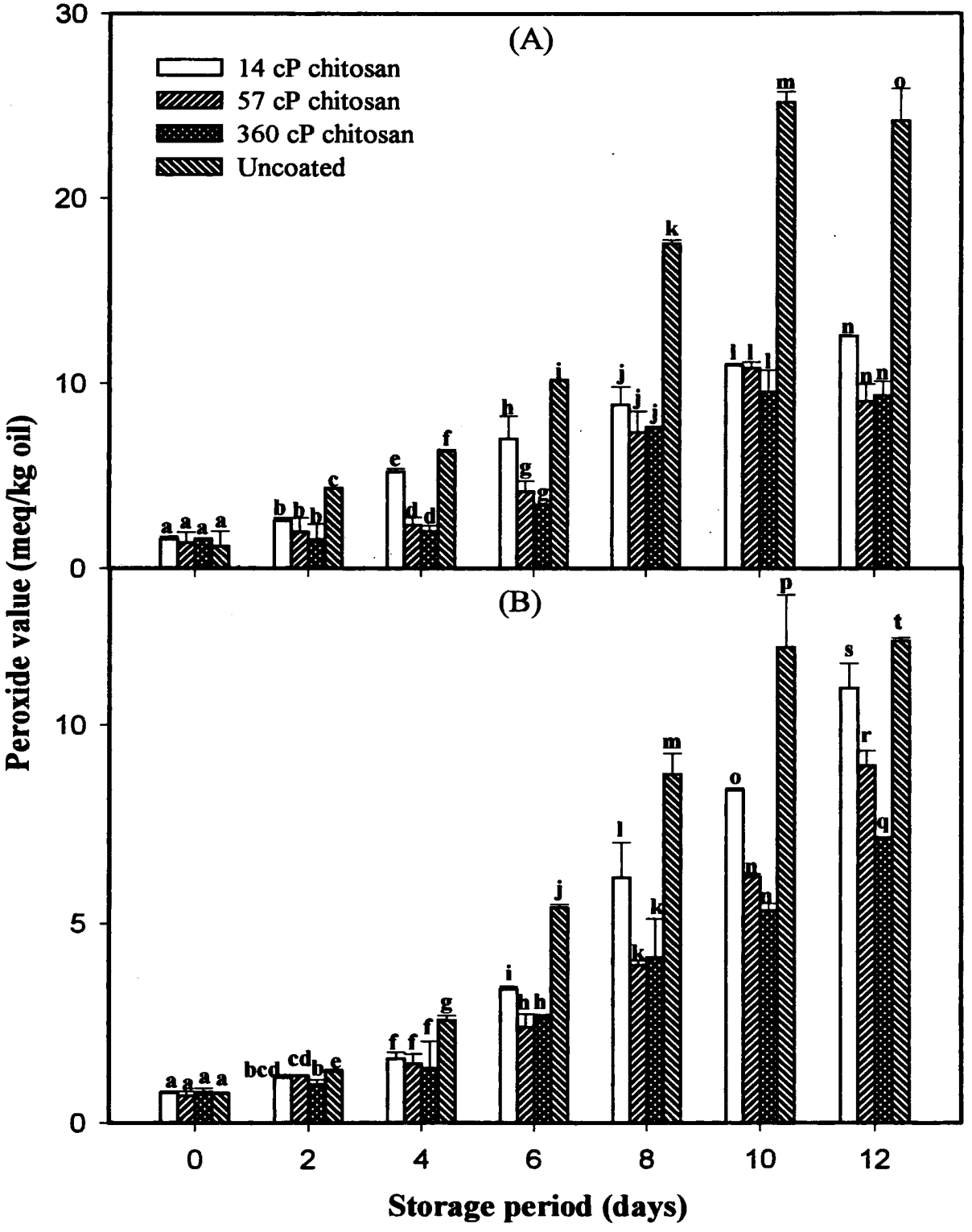
often recommended that more than one method of measuring lipid oxidation be used (Dillard and Tappel, 1989; Shahidi, 1998).

4.3.1 Effect of chitosan coating on primary lipid oxidation products of fish

The use of peroxide value (PV) test for measurement of early to intermediate stages of oxidation in lipids extracted from marine products has been found to be appropriate (Woyewoda *et al.*, 1986c). The hydroperoxide content, generally expressed as PV, is determined by an iodometric method. This is based on the reduction of the hydroperoxide group (ROOH) with iodide ion (I^-). The amount of iodine (I_2) liberated is proportional to the concentration of peroxide present. Released iodine (I_2) is assessed by titration against a standardized solution of sodium thiosulphate ($Na_2S_2O_3$) using a starch indicator (Shahidi and Wanasundara, 1998). Lipids containing methylene-interrupted dienes or polyenes show a shift in their double bond position during oxidation due to isomerization and conjugate formation (Logani and Davies, 1980). The resulting conjugated dienes exhibit an intense absorption at 234 nm; similarly conjugated trienes absorb at 268 nm (Shahidi and Wanasundara, 1998).

The effect of chitosan coating on changes of PV of fish lipids was examined after their extraction from samples. As depicted in Figure 4.3A, peroxide value of the uncoated herring sample increased progressively up to day 10 of storage. On day-12 of storage, peroxide values of samples coated with chitosans were about 48-63% lower than that of the control (Figure 4.3A). Meanwhile, the peroxide value of all chitosan-treated herring samples were less than 10 meq/kg oil up to day 8 of storage, but values for uncoated samples exceeded this level even after day 4 of storage. However, variable results were

Figure 4.3 Effect of chitosan coating on peroxide value of fish stored at 4 ± 1°C. (A) Herring, (B) Cod. Each bar represents mean ± standard deviation of triplicate analyses. In each panel, bars sharing the same letter in a group of bars are not significantly different ($p > 0.05$) from one another.


(A)
14 cP chitosan
57 cP chitosan
360 cP chitosan
Uncoated
(B)
Peroxide value (meq/kg oil)
Storage period (days)
0
2
4
6
8
10
12
0
5
10
20
30

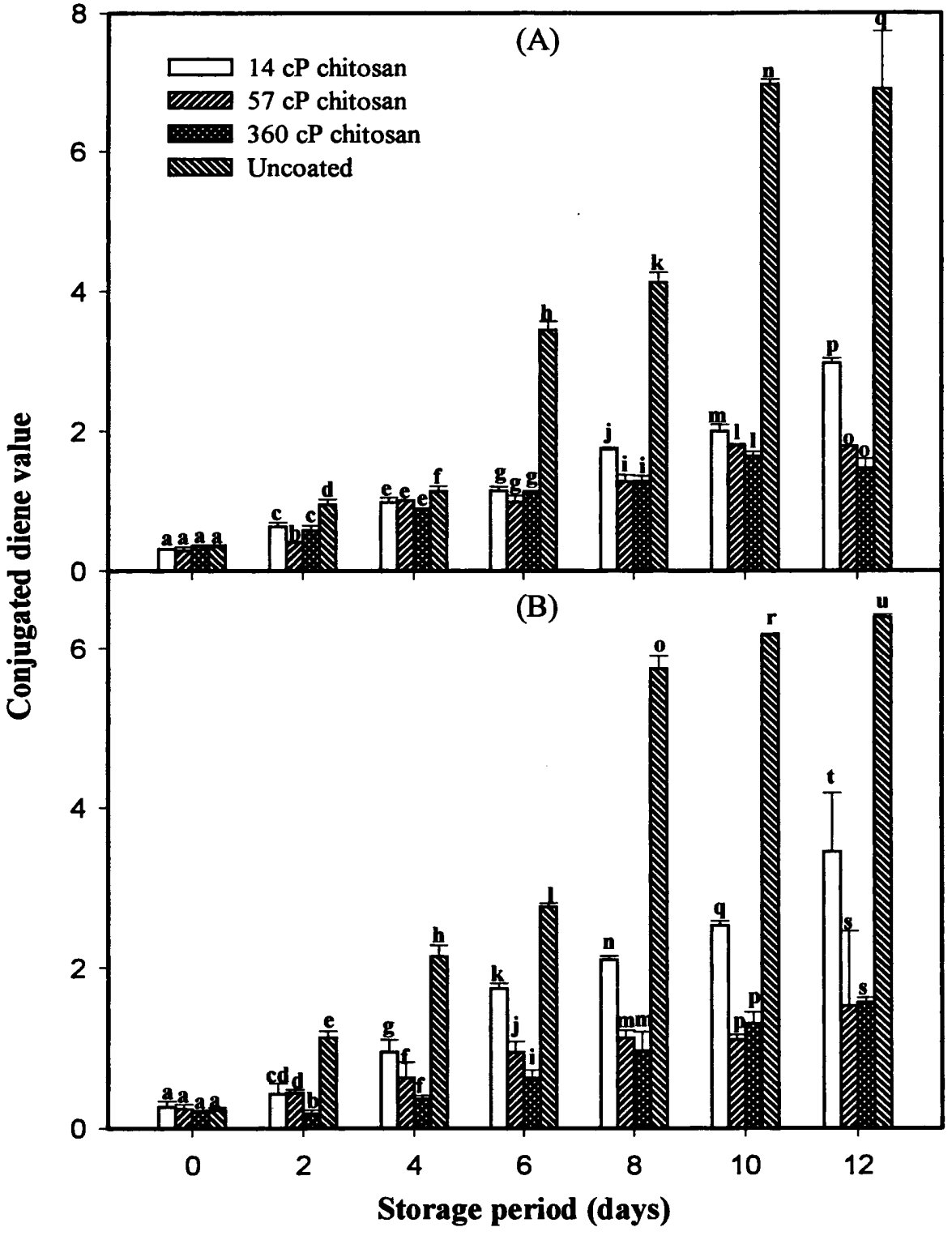
observed with 14 cP chitosan-treated herring samples, where significantly ($p \leq 0.05$) different peroxide values were obtained compared to the other two types (57 cP and 360 cP) of chitosan coated herring samples after 4, 6 and 12 days. Under conditions of actual food use, oxidation proceeds, but usually peroxide values remain below 10 meq/kg (Frankel, 1985). The treatment of herring samples with 57 and 360 cP chitosans effectively decreased the production of peroxides over the entire storage period. However, after 6 days of storage, PV among three types of chitosan coated herring samples were not significantly ($p > 0.05$) different from one another. Regardless of chitosan treatment, all herring samples showed lower peroxide values (< 6.31 meq/kg oil) up to 4 days of storage.

As shown in Figure 4.3B, similar peroxide value patterns were observed for chitosan coated cod samples. Chitosan coated cod samples, regardless of the viscosities of chitosan used, exhibited lower peroxide values throughout the entire storage period when compared with those of the control. However, the inhibitory effect of chitosans against oxidation was viscosity-dependent as evidenced by lower PV for samples coated with 57 and 360 cP chitosans compared to that coated with 14 cP chitosan. Furthermore, cod coated with 57 and 360 cP viscosity chitosans exhibited a PV which held the same trend as those of herring samples, but with lower levels; the effects were also more pronounced than those exerted by 14 cP chitosan. After day-4 of storage, PV of coated cod samples with all three types of chitosans were not significantly ($p > 0.05$) different. However, after day-6 of storage, significantly ($p \leq 0.05$) different peroxide values were observed between the 14 cP chitosan coated cod sample and those coated with 57 and 360 cP chitosans. This trend was observed during the entire storage period.

As depicted in Figure 4.3B peroxide values of cod samples coated with different viscosity chitosans increased over time, but at a slower rate as compared to that of the control. The differences in peroxide values of samples coated with 57 cP and 360 cP chitosan were either marginal or insignificant ($p > 0.05$) over the entire storage period, except after 12 days of storage. The 360 cP chitosan coated cod sample showed a PV about 40% lower than that of the uncoated samples after 12 days of storage. The peroxide value (11.95 meq peroxides/kg oil) of uncoated cod samples exceeded 10 meq peroxides/kg oil, the maximum acceptable level, after 10 days of the storage period (Frankel, 1985).

In concurrent studies conjugated diene (CD) values of chitosan coated herring and cod samples were also monitored as shown in Figure 4.4A and 4.4B, respectively. The pattern of CD values for herring and cod samples was similar to that of peroxide values of the corresponding samples. All chitosan-coated herring and cod samples had significantly ($p \leq 0.05$) lower CD values throughout the entire storage period. However, this effect was better demonstrated after day 6 of storage of herring samples where differences among all coated and uncoated samples were more than double. A similar trend was observed in chitosan coated cod samples after day-8 of storage. Furthermore, herring and cod samples treated with 14 cP chitosan showed higher CD values than those treated with 57 and 360 cP chitosans under similar experimental conditions. Sklan *et al.* (1983) determined the content of conjugated dienes, trienes and tetraenes, referred to as total conjugated products of oxidation, in total lipids extracts of turkey meat during a 60-day storage at 4°C. Similar studies were carried out by Ahmad and Augustin (1985) for fried fish during a 40-day storage at 60°C. These authors indicated that the level of both dienes and trienes

Figure 4.4 Effect of chitosan coating on conjugated diene values of fish stored at 4 ±1 °C. (A) Herring, (B) Cod. Each bar represents mean ± standard deviation of triplicate analyses. In each panel, bars sharing the same letter in a group of bars are not significantly different ($p > 0.05$) from one another.


(A)
14 cP chitosan
57 cP chitosan
360 cP chitosan
Uncoated
(B)
Conjugated diene value
Storage period (days)
0
2
4
6
8
10
12

increased with increasing storage time. Medina *et al.* (1999) reported that conjugated diene values in cooked tuna stored at both 40°C and 60°C was increased continuously during 4-days of storage.

Conjugated diene values of chitosan coated herring samples increased by about 4-9-fold at the end of a 12-day storage period, whereas the increase for the uncoated herring sample was 19-fold. In cod samples corresponding values were 6-12-fold and 27-fold, respectively. Storage of herring fillets beyond 10 days resulted in a reduction in both PV and CD of uncoated, 57 cP and 360 cP chitosan-treated samples, perhaps due to the breakdown of hydroperoxides or their interaction with muscle proteins.

As hydroperoxides are the primary products of lipid oxidation, PV provides a clear indication about the initial oxidation potential of different lipids, similar to that of CD values which exhibited a trend in the same direction.

The fatty acid composition of total lipids of herring and cod fillets is shown in Table 4.2. Results in this table indicate the highly unsaturated nature, and hence oxidative susceptibility, of lipids from herring and cod fillets. The high PV and CD of lipids from herring and cod fillets during initial stages of storage of fillets may be attributed to their nigh content of polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Moreover, characteristic features of lipid oxidation in fish flesh are influenced by factors such as lipid content (Ke *et al.*, 1982), the level of microsomal associated lipid oxidation system present (Slabyj and Hultin, 1982), the level of haem compounds (Castell and Bishop, 1969) and the presence of metal ions (Tichivangana and Morrissey, 1985).

Table 4.2 Fatty acid composition (weight %) of herring and cod fillets lipid[a]

Fatty acid (weight %)	Cod	Herring
12:0	0.10 ± 0.00	-
14:0	2.65 ± 0.00	1.03 ± 0.10
15:0	0.29 ± 0.01	0.36 ± 0.00
16:0	19.3 ± 0.17	18.2 ± 0.03
16:1ω7	2.40 ± 0.13	3.81 ± 0.05
17:0	0.65 ± 0.00	0.29 ± 0.00
17:1	0.49 ± 0.05	0.85 ± 0.01
18:0	2.93 ± 0.00	1.38 ± 0.08
18:1ω9	16.9 ± 0.73	13.5 ± 0.03
18:3ω6	0.47 ± 0.11	1.20 ± 0.03
20:0	0.09 ± 0.00	0.75 ± 0.04
20:1ω9	4.31 ± 0.14	4.17 ± 0.03
20:5ω3	9.93 ± 0.01	11.3 ± 0.16
22:1ω11	1.13 ± 0.06	0.06 ± 0.00
22:4ω6	0.93 ± 0.05	1.76 ± 0.02
22:5ω3	1.60 ± 0.13	3.14 ± 0.03
22:6ω3	27.3 ± 0.18	31.3 ± 0.07

[a]All values are mean of three determinations ± standard deviation

As expected, both PV and CD values were higher in herring samples as compared to those of cod throughout the storage period. The herring fillet has a large proportion of dark muscles which are generally more prone to lipid oxidation than cod muscle which are generally light. This is probably not only because dark herring muscle has a higher content of total lipids, including phospholipids per unit weight of tissue, but the fact that it also contains higher amounts of pro-oxidant metals (Mai and Kinsella, 1979; Decker and Hultin, 1992). Herring and cod samples treated with the 14 cP chitosan, showed higher PV and CD values as compared to those treated with 57 and 360 cP chitosans. Furda (1990) reported that the degree of polymerization of glucosamine units is determines the viscosity of chitosan solutions. The above results may thus be interpreted as arising from differing molecular weights of chitosans. Furthermore, this might have effect on gas permeability of different viscosity chitosans.

Many foods are extremely sensitive to autoxidation due to their high level of iron and unsaturated fats which occur in close proximity. Oxygen levels above 1% in the headspace of food containers may cause undesirable texture changes, discoloration, flavour changes, nutritional loss and microbial safety hazards (Graf and Eaton, 1990). Therefore, effort has been made to reduce these effects by eliminating oxygen from the package and/or adding primary antioxidants and/or metal sequestrants. Hwang and Regenstein (1989) tested several antioxidants as well as vacuum packaging and studied their effects on peroxide values of frozen minces from Gulf menhaden (*Brevoortia patronus*) and Atlantic menhaden (*Brevoortia tyrannus*). Vacuum packaging was more effective in retarding oxidative rancidity than the antioxidants. Furthermore, these authors suggested that the removal of oxygen served as the most critical factor related to lipid

oxidation in these species. The effect of packaging material on the oxidation of fish sausage lipid was reported by Satomi *et al.* (1988). During a 5-week storage at 37°C, both degradation of nitrosopigments and development of off-flavour proceeded in proportion to oxygen permeability of the packaging material. When the sausages were packaged in zero oxygen permeability material, oxidative deterioration did not occur.

Chitosan films show extremely good barrier to oxygen permeation (Butler *et al.*, 1996) and chitosan coating applied directly on the surface of fish meat might act as a barrier between fish meat and its surrounding, thus retarding diffusion of oxygen to the fish meat surface. Chen and Hwa (1996) observed that tensile strength, tensile elongation, and enthalpy of the membrane prepared from high molecular weight chitosans were higher than those of low molecular weight chitosans. Furthermore, these authors reported that the permeability characteristics of high molecular weight chitosans (high viscosity) were lower than those of their low molecular weight counterparts (low viscosity). The results of the present study indicate that chitosan coating is effective in retarding the production of primary lipid oxidation products in herring fillets stored at 4 ± 1°C. These results are in agreement with those of Stuchell and Krochta (1995) who reported the effectiveness of whey protein isolate as an edible coating material for reduction of primary lipid oxidation products in salmon. The results of the present study indicate that chitosan coating is effective in retarding the production of primary lipid oxidation products in herring fillets stored at 4 ± 1°C. These results are in agreement with those of Stuchell and Krochta (1995) who reported the effectiveness of whey protein isolate as an edible coating material for controlling the formation of primary lipid oxidation products in salmon.

Figure 4.5 shows excellent correlation between PV and CD ($r = 0.9230$). Jackson (1981) indicated that formation of hydroperoxides normally coincides with CD formation upon oxidation of lipids. The CD assay is faster than PV determination and does not depend on chemical reactions such as colour development for its determination and requires a smaller sample size.

4.3.2 Effect of chitosan coating on secondary lipid oxidation products of fish

The 2-thiobarbituric acid (TBA) test measures the content of variety of aldehydes, including malonaldehyde, which are formed during oxidation of fish lipids. Malonaldehyde is a minor decomposition product of lipid peroxides formed in muscle foods. As depicted in Figures 4.6A and 4.6B, all chitosan coated herring and cod samples showed significantly ($p \leq 0.05$) lower TBA reactive substances (TBARS) values compared to the uncoated sample throughout the storage period. However, TBARS values of herring samples were always higher than those of cod samples under similar storage conditions. This is not surprising considering the higher content of total lipids and polyunsaturated fatty acids (PUFA) (12.4% and 48.77%, respectively) in herring as compared to that of cod (1.2% and 40.24%, respectively) (Table 4.2). Coated herring and cod fillets with different viscosity chitosans displayed different TBARS values. Among the three different types of chitosan tested, 360 cP chitosan was most effective in retarding TBARS formation, irrespective of the type of fish being coated. This is probably due to the fact that presence of a large number of ionic functional groups creates strong polymer interactions which restrict the chain motion in high viscosity chitosans which results in

Figure 4.5 Relationship between conjugated diene values and peroxide values of chitosan-coated herring samples stored at 4 ± 1°C.

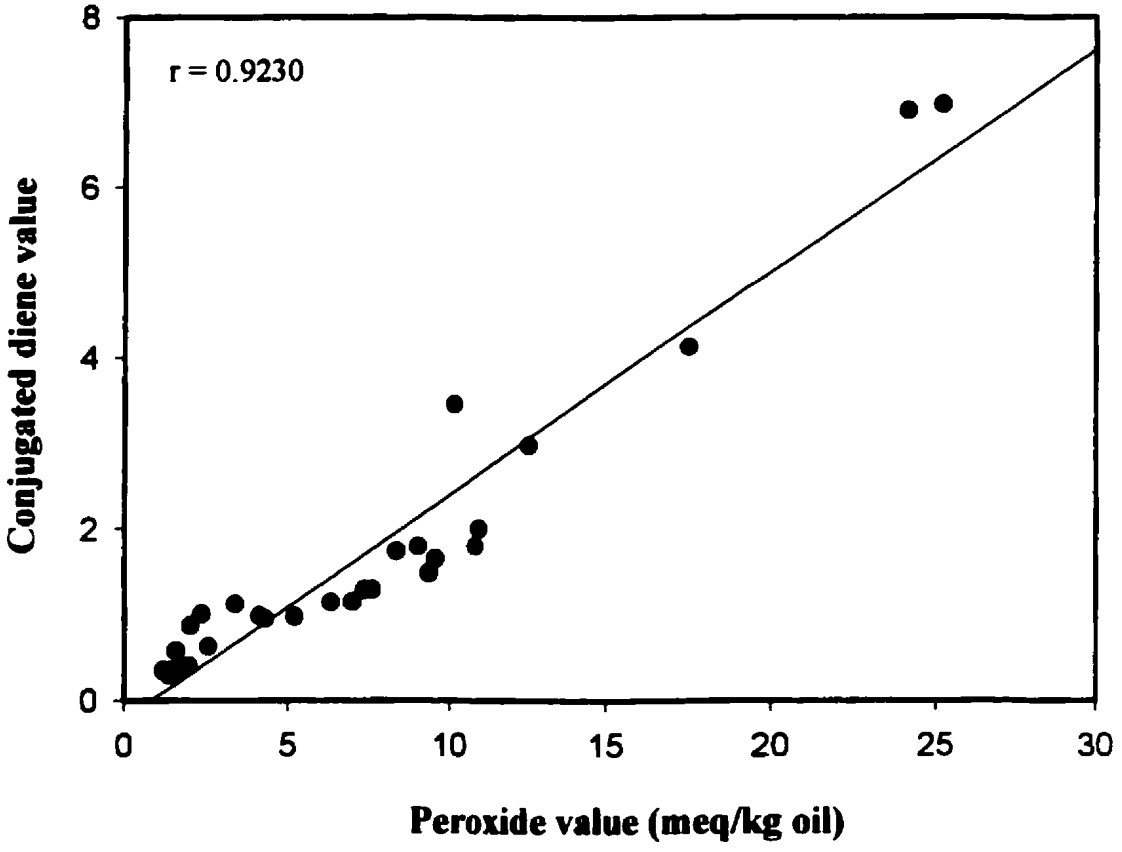
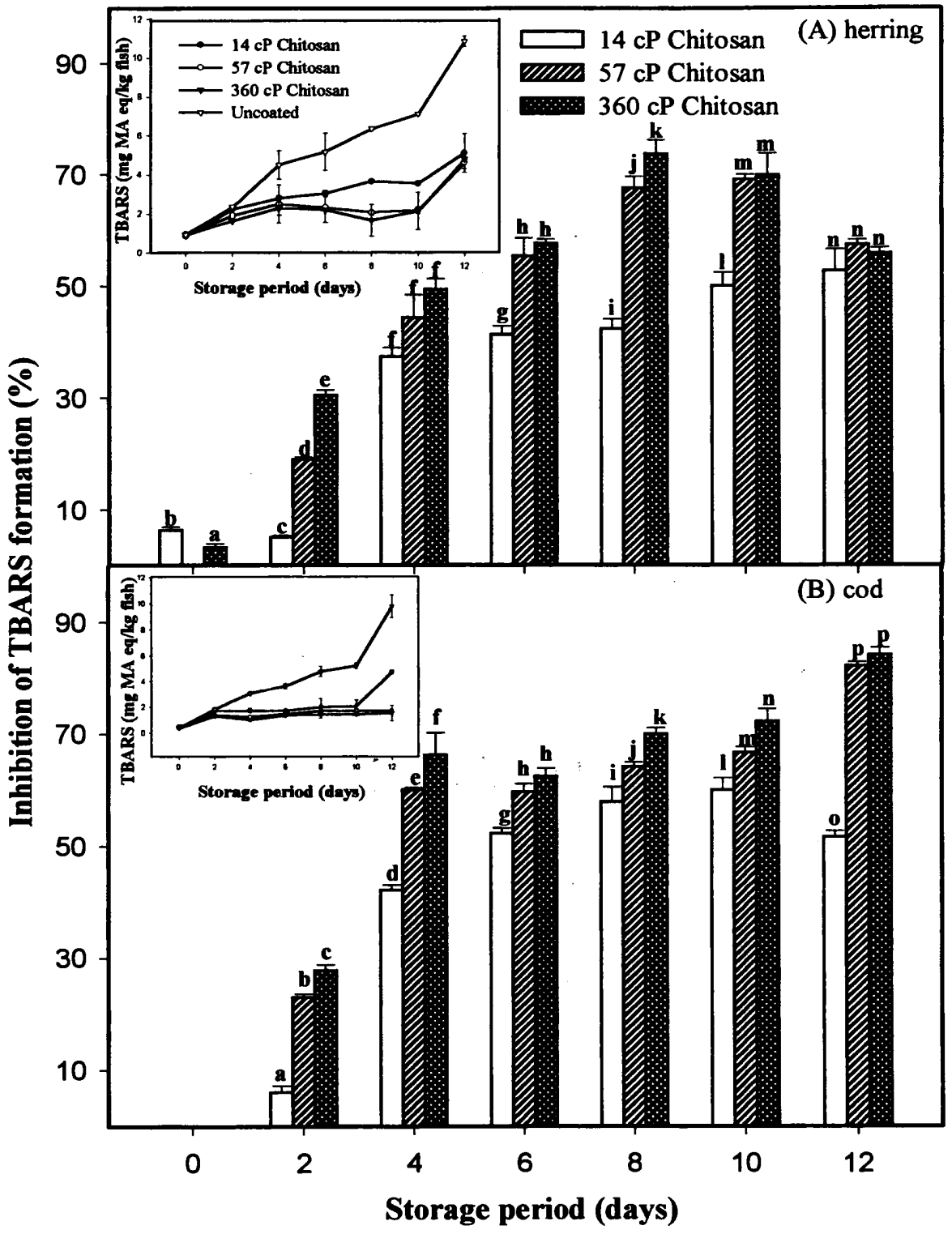

r = 0.9230
Conjugated diene value
Peroxide value (meq/kg oil)
0
2
4
6
8
0
5
10
15
20
25
30

Figure 4.6 Inhibition of TBARS formation of chitosan-coated fish samples stored at 4 ± 1°C. (A) Herring, (B) Cod. Each bar represents mean ± standard deviation of triplicate analyses. In each panel, bars sharing the same letter in a group of bars are not significantly different ($p > 0.05$) from one another.


(A) herring
14 cP Chitosan
57 cP Chitosan
360 cP Chitosan
Uncoated
TBARS (mg MA eq/kg fish)
Storage period (days)
(B) cod
Inhibition of TBARS formation (%)
Storage period (days)

good oxygen barrier properties. The inhibitory effect of chitosan against herring oxidation increased with storage time, especially after day 4, especially for higher viscosity (57 and 360 cP) chitosans as compared that of 14 cP chitosan. These differences could be due to the existing differences in oxygen permeability of chitosans with different viscosity characteristics. However, after day 10, percentage inhibition of TBARS formation in both 57 and 360 cP samples were reduced from 69 and 70 to 57 and 56, respectively. After 12-day of storage percentage inhibitions of TBARS formation among the three chitosans used for coating of herring samples were not significantly different ($p > 0.05$).

Unlike herring, percentage inhibition of TBARS formation for cod samples increased throughout the entire storage period. The TBARS values represent the content of secondary lipid oxidation products with low threshold values which contribute to off-flavour perception in oxidized seafoods and marine lipids. Results of this study indicate that chitosan coating has a marked effect on the inhibition of TBARS in both herring and cod. Use of other edible coating materials for protection of muscle foods against oxidation has been reported in the literature. Wanstedt *et al.* (1981) observed that calcium alginate coating served as an effective means for controlling lipid oxidation in ground pork patties as reflected in TBARS values. Corn zein coatings were also found to lower TBARS formation of pre-cooked pork chops during a 9-day refrigerated storage (Hargens-Madsen *et al.*, 1995). Carrageenan films have also been used successfully to inhibit the oxidation of fresh mackerel mince patties as measured by peroxide and TBA values (Hwang *et al.*, 1997).

The static headspace gas chromatographic analysis measures volatile carbonyls which are formed in detectable quantities during the oxidation process (Frankel, 1985).

Furthermore, gas chromatographic measurement of headspace volatiles resulting from lipid oxidation has often been used as an objective tool to compare chemical measurements of oxidation with sensory panel evaluation of off-aroma and off-flavour. Changes of propanal and total volatile aldehyde (TVA) are reliable indicators for assessment of the oxidative status during storage of chitosan-coated herring as shown in Tables 4.3 and 4.4, respectively. Figure 4.7 shows the chromatograms of headspace volatiles of uncoated and chitosan-coated herring samples after 12 days of storage at 4 ± 1°C. Of the identified volatiles (formaldehyde, acetaldehyde, propanal, isobutanal, butanal, pentanal, hexanal and heptanal), formaldehyde and propanal were most prominent. However, in fish muscle formaldehyde may also accumulate as a result of decomposition of trimethylamine oxide by endogenous and microbial enzymes. Therefore, formaldehyde was not considered as a volatile aldehyde that could form only from autoxidation of fish muscle lipids. Propanal is an oxidation product of ω-3 fatty acids and was present, in abundance, in both herring and cod (45.81% and 38.84%, respectively). Frankel *et al.* (1994) observed that propanal formation in fish oil highly and significantly correlated with the content of ω-3 PUFA ($r = 0.950$, $p < 0.001$). Ota (1958) showed that acetaldehyde and butanal were present in mackerel, sardine, and flat fish and their content increased during room temperature storage of samples examined. Diemair and Schams (1962), in a comprehensive investigation of carbonyl compounds, identified alkanals, alkenals, alkadienals, and methyl ketones in fatty fish, but only alkanals were present in stored lean fish. The content of propanal in the control herring sample on day 0 was 1.2 mg/kg and increased to about 8.0 mg/kg on day 6 and to 29 mg/kg on day 12 (Table 4.3). Propanal formation was detected, to different extents, due to coating with chitosans tested. Both 57

Table 4.3 Content of propanal (mg/kg fish) in headspace volatiles of chitosan-coated herring samples stored at 4 ± 1°C[a]

Chitosan	Storage period (days)						
	0	2	4	6	8	10	12
Uncoated	1.15±0.85[a]	5.93±1.05[b]	7.48±1.05[b]	8.58±0.48[b]	14.03±1.00[c]	17.58±0.60[c]	29.32±0.78[c]
14 cP	1.45±0.53[a]	4.58±0.30[ab]	6.15±0.30[ab]	7.73±0.73[b]	8.25±0.20[b]	9.93±0.23[b]	11.15±1.13[b]
57 cP	1.15±0.75[a]	3.88±0.53[a]	4.93±0.65[a]	6.23±0.40[a]	5.70±0.48[a]	6.05±0.48[a]	7.09±0.09[a]
360 cP	1.55±0.60[a]	3.93±0.65[a]	4.40±0.55[a]	5.05±0.35[a]	4.58±0.60[a]	5.68±0.33[a]	7.15±0.13[a]

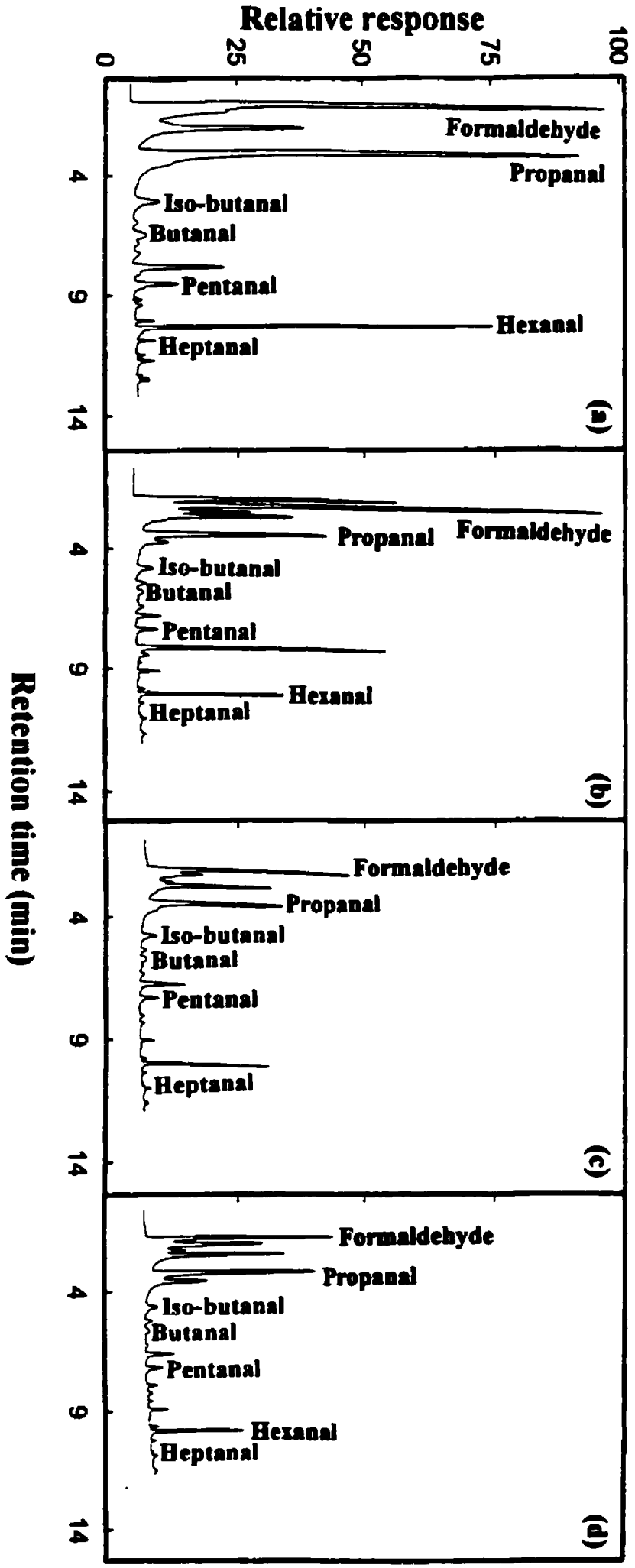
[a]Results are expressed as mean ± standard deviation of three determinations. Values with the same superscripts within each column are not significantly different (P > 0.05)

Table 4.4 Content of total volatile aldehydes (mg/kg fish) in headspace volatiles of chitosan-coated herring samples stored at 4 ± 1°C[a]

Chitosan	Storage period (days)						
	0	2	4	6	8	10	12
Uncoated	14.38±3.50[a]	28.18±3.35[a]	40.88±4.83[b]	44.05±5.03[b]	58.93±5.88[b]	69.38±1.73[c]	87.56±3.11[c]
14 cP	15.53±1.52[a]	30.33±6.85[a]	33.35±6.03[ab]	36.75±3.98[ab]	39.75±3.58[a]	42.05±3.83[b]	58.17±2.30[b]
57 cP	14.23±2.47[a]	22.78±3.15[a]	31.00±2.93[ab]	32.08±5.33[a]	34.40±5.33[a]	35.98±1.60[a]	44.03±0.13[a]
360 cP	14.05±2.65[a]	24.43±1.03[a]	28.75±2.50[a]	32.55±1.58[a]	34.85±1.85[a]	36.08±0.88[a]	46.11±1.33[a]

[a]Results are expressed as mean ± standard deviation of three determinations. Values with the same superscripts within each column are not significantly different (P > 0.05)

Figure 4.7 Day-12 gas chromatograms of chitosan-coated herring samples stored at 4 ± 1°C. (a) Uncoated, (b) 14 cP chitosan coated, (c) 57 cP chitosan coated, (d) 360 cP chitosan coated.


Relative response
0
25
50
75
100
Retention time (min)
4
9
14
Formaldehyde
Propanal
Iso-butanal
Butanal
Pentanal
Hexanal
Heptanal
(a)
(b)
(c)
(d)

and 360 cP chitosans exerted an inhibitory effect on the formation of propanal throughout the storage period. The 360 cP chitosan had the strongest inhibitory effect and reduced the formation of propanal in herring samples by 34, 70 and 76%, on days 2, 6 and 12, respectively. After 2 days of storage at 4 ± 1°C, there was no significant ($p > 0.05$) difference in the content of total volatile aldehydes in coated and uncoated herring samples. However, after 4 days of storage, herring coated with 360 cP chitosan had significantly ($p \leq 0.05$) lower total volatile aldehydes than its uncoated counterpart. After 8 days of storage approximately 40 % reduction in total volatile aldehydes in all treated herring samples was observed as compared to that of the uncoated control sample. This reduction was 48% after 12 days in both 57 and 360 cP chitosan-coated herring samples.

Boyd *et al.* (1992) have shown that propanal is present in high amounts when samples of EPA and DHA are heated at 80°C and Girard and Durance (2000) confirmed that propanal was a dominant aldehyde arising from oxidation of canned pink and sockeye salmon sample. Medina *et al.* (1999) observed no induction or lag period for propanal formation during oxidation of canned tuna which was stored at 40°C for four days.

A linear relationship existed between TBARS and propanal content ($r = 0.9127$; Figure 4.8A) and between TBARS and content of TVA ($r = 0.9214$; Figure 4.8B). The correlation coefficient ($r = 0.9127$) between propanal and TBARS indicates that propanal may serve as an alternative to TBARS and as a useful indicator for evaluation of oxidation state of seafoods. Appearance of chitosan coated herring and cod filets on day-6 of storage are shown in Figure 4.9 and Figure 4.10, respectively.

Figure 4.8 Relationship between propanal contents and TBARS values (A) and total volatile aldehyde contents and TBARS values (B) of chitosan coated herring samples stored at 4 ± 1°C.

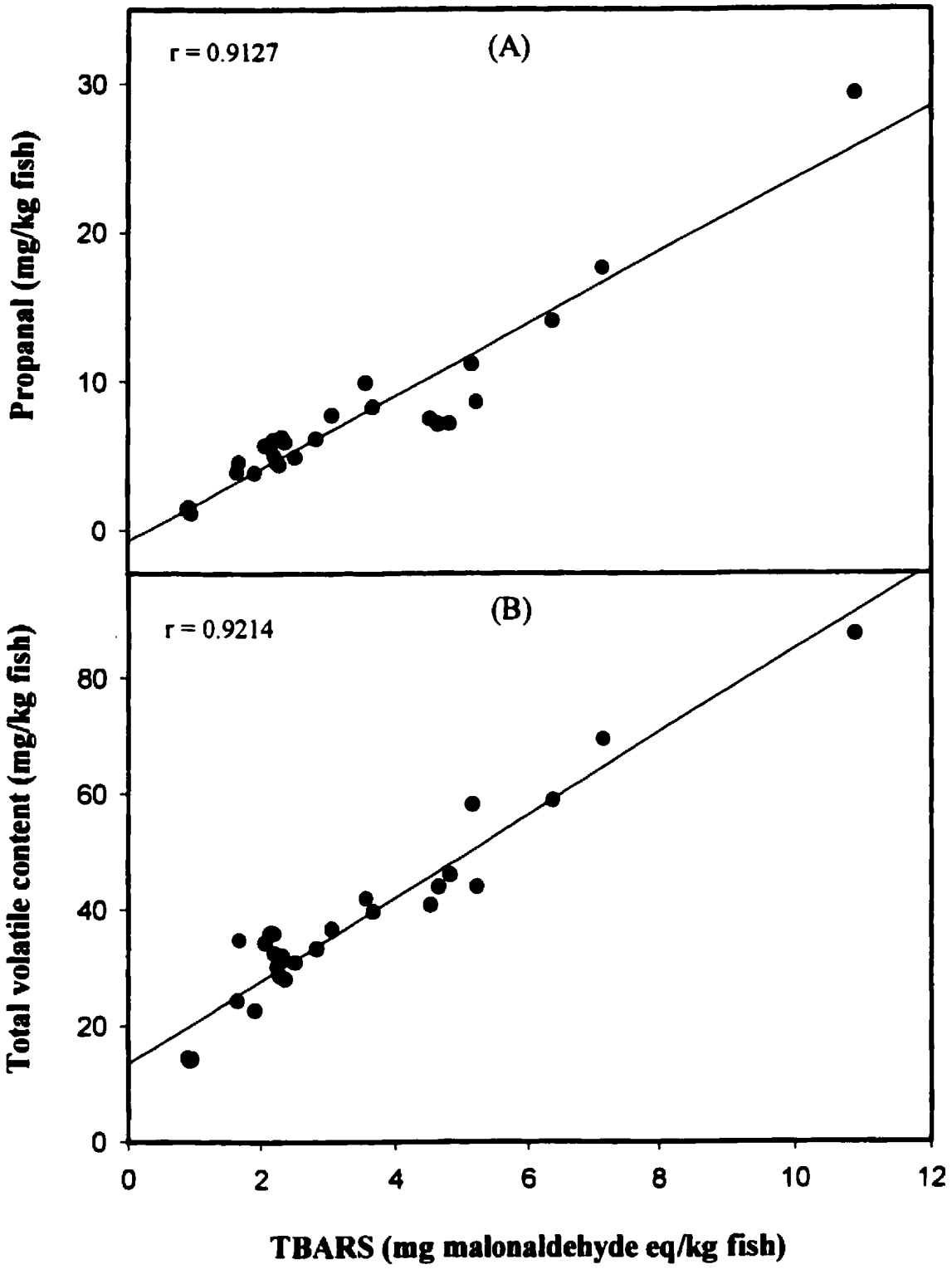

r = 0.9127
(A)
Propanal (mg/kg fish)
30
20
10
0
r = 0.9214
(B)
Total volatile content (mg/kg fish)
80
60
40
20
0
0
2
4
6
8
10
12
TBARS (mg malonaldehyde eq/kg fish)

Figure 4.9 Appearance of chitosan coated herring fillets on day-6 of storage.

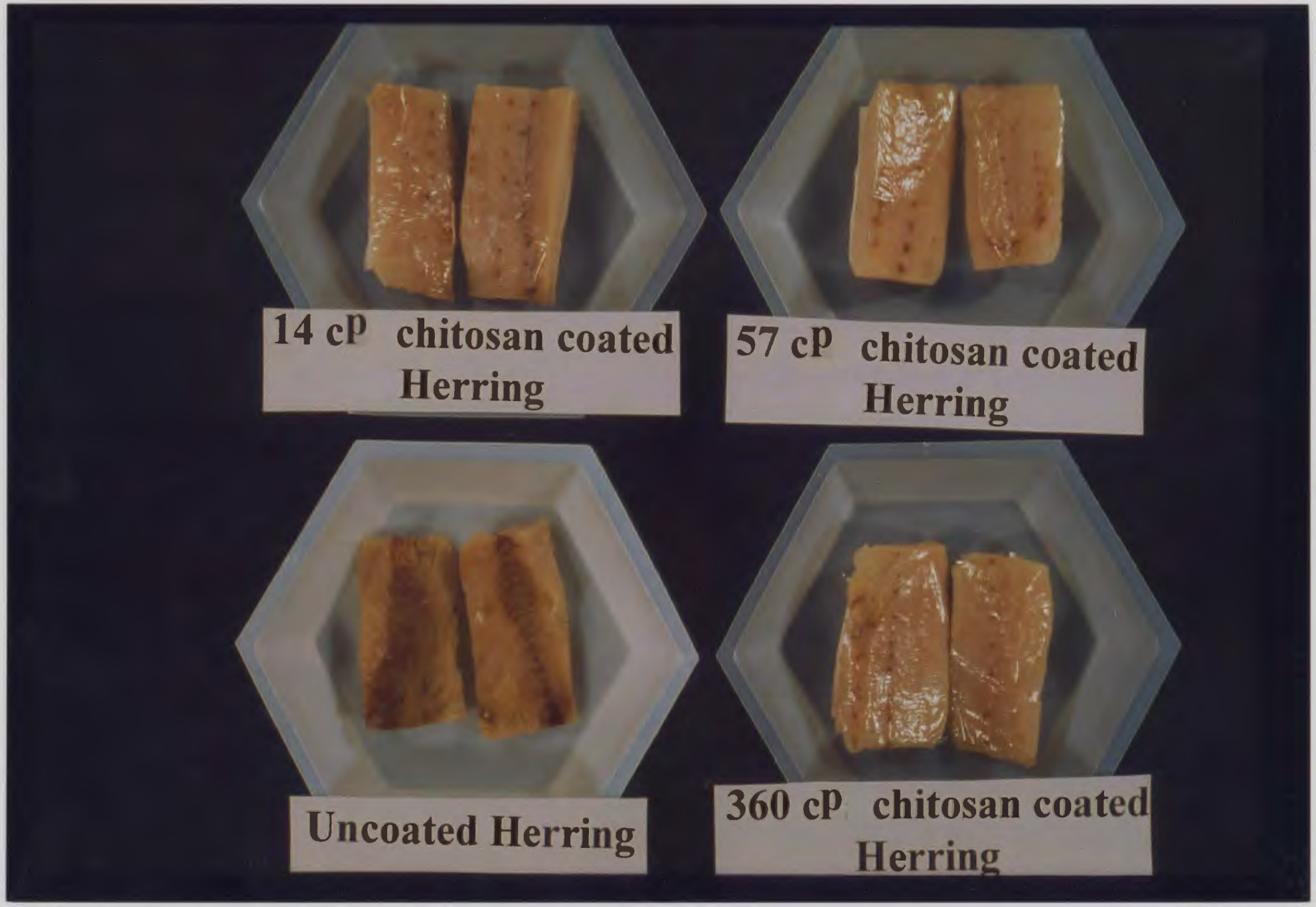
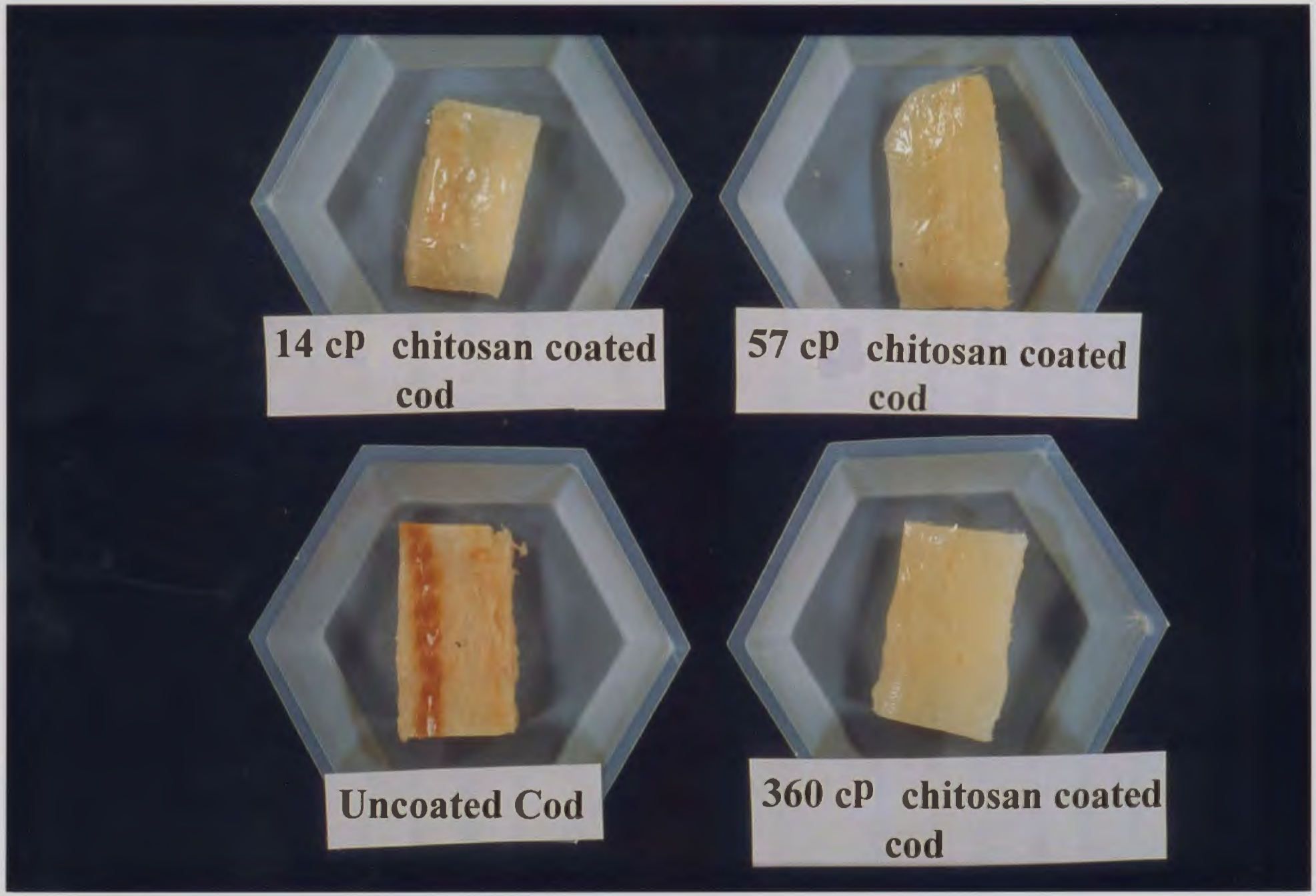

14 cP chitosan coated Herring
57 cP chitosan coated Herring
Uncoated Herring
360 cP chitosan coated Herring

Figure 4.10 Appearance of chitosan coated cod fillets on day-6 of storage.


14 cP chitosan coated cod
57 cP chitosan coated cod
Uncoated Cod
360 cP chitosan coated cod

4.3.3 Antioxidant efficacy of different viscosity chitosans in a cooked comminuted fish model systems

Different types of fish species have been used as models for evaluation of antioxidants (Tichivangana and Morrissey, 1984; He and Shahidi, 1997). Incorporation of an antioxidant into cooked fish meats or even glazing of fish fillets could slow down their deterioration, thus extending shelf-life of products (Khali and Mansour, 1998). This fact provides the basis for use of fish model systems for evaluation of antioxidant activity of various substances. In addition, use of the model system may shed further light into the mechanism by which control of oxidation in coated samples is achieved.

Cooked comminuted fish model systems were prepared according to the method described in the section 3.2.7 and oxidative stability of cod and herring fish model systems stored at $4 \pm 1^{\circ}C$ was monitored using the peroxide value and TBARS analyses.

Peroxide values of cooked comminuted herring and cod samples treated with different viscosity chitosans at 50, 100 and 200 ppm are presented in Figures 4.11A-C and 4.12A-C, respectively. Control herring and cod samples devoid of chitosan or commercial antioxidants had significantly ($p \leq 0.05$) higher peroxide values throughout the storage period and values in all samples increased up to the day-8 of storage, but then gradually decreased. In general, both herring and cod samples treated with the 14 cP chitosan, regardless of the concentration used, had lower peroxide values as compared to those of 57 and 360 cP chitosans. After day-8, peroxide values in herring and cod samples treated with the 14 cP chitosan at 200 ppm level were reduced by 61% and 54% as compared to that of their control counterparts, respectively. Corresponding values for 57 cP chitosans and 360 cP chitosans at same concentration in herring samples were reduced

Figure 4.11 Effect of different viscosity chitosans and commercial antioxidants on peroxide value in a cooked comminuted herring model system. (A) 14 cP chitosan, (B) 57 cP chitosan, (C) 360 cP chitosan. Each bar represents mean ± standard deviation of triplicate analyses. In each panel, bars sharing the same letter in a group of bars are not significantly different ($p > 0.05$) from one another.

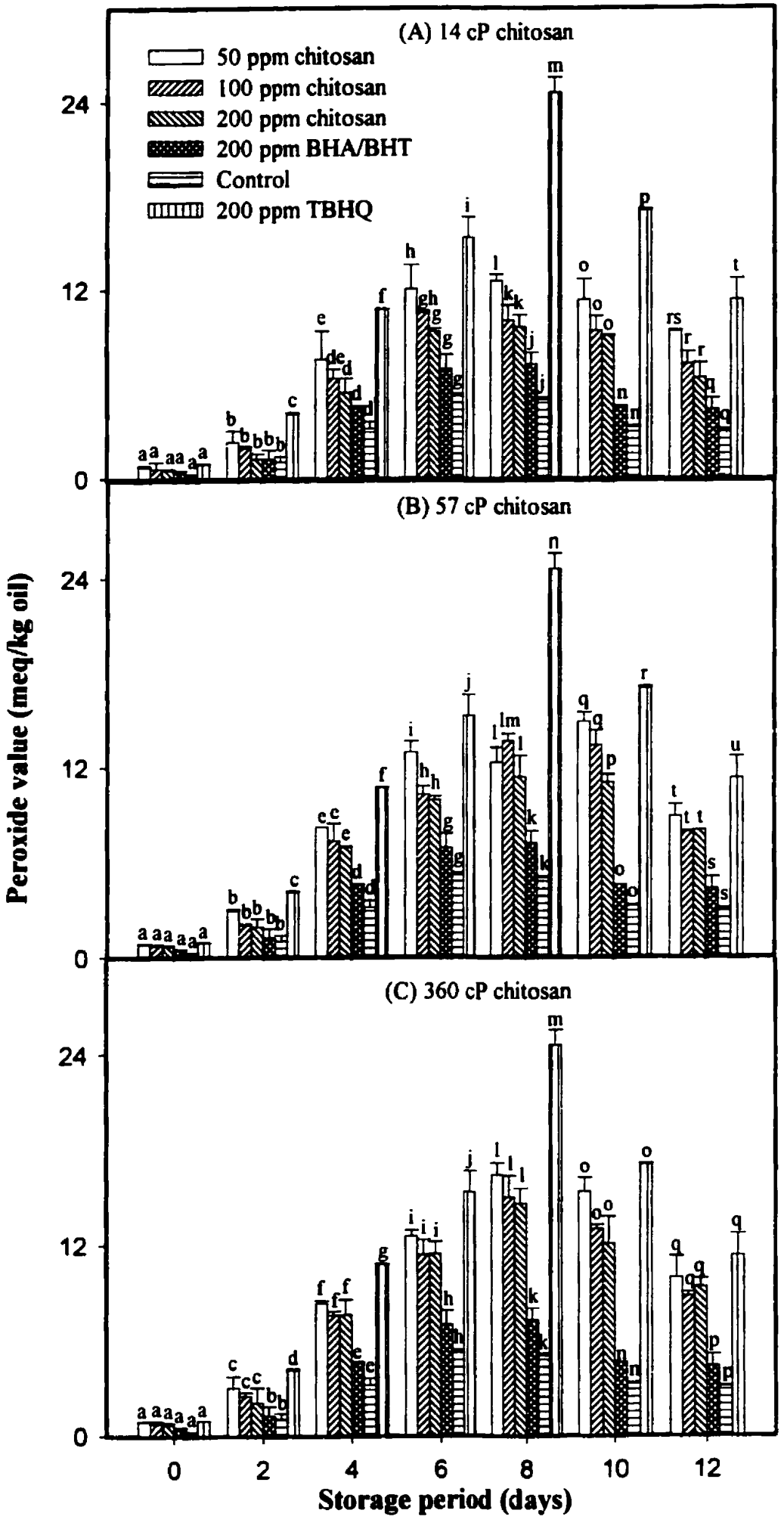
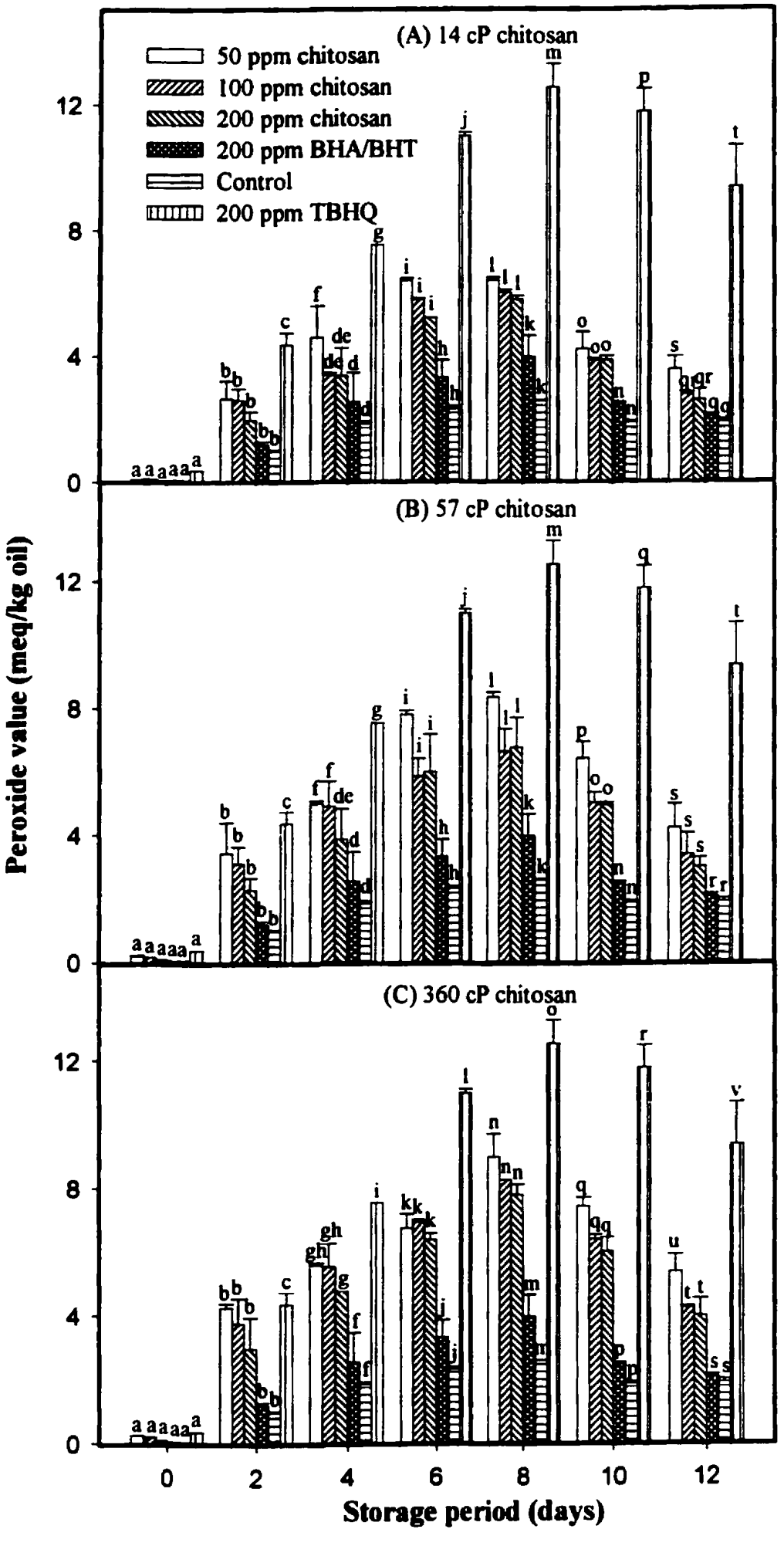

(A) 14 cP chitosan
50 ppm chitosan
100 ppm chitosan
200 ppm chitosan
200 ppm BHA/BHT
Control
200 ppm TBHQ
(B) 57 cP chitosan
(C) 360 cP chitosan
Peroxide value (meq/kg oil)
Storage period (days)
0
12
24
0
2
4
6
8
10
12

Figure 4.12 Effect of different viscosity chitosans and commercial antioxidants on peroxide value in a cooked comminuted cod model system. (A) 14 cP chitosan, (B) 57 cP chitosan, (C) 360 cP chitosan. Each bar represents mean ± standard deviation of triplicate analyses. In each panel, bars sharing the same letter in a group of bars are not significantly different ($p > 0.05$) from one another.


(A) 14 cP chitosan
(B) 57 cP chitosan
(C) 360 cP chitosan
50 ppm chitosan
100 ppm chitosan
200 ppm chitosan
200 ppm BHA/BHT
Control
200 ppm TBHQ
Peroxide value (meq/kg oil)
Storage period (days)
0
2
4
6
8
10
12

by 53% and 40%, respectively. Among reference antioxidants, TBHQ exhibited the greatest antioxidant effect on the formation of peroxides. Reduction in peroxide contents of herring samples treated with 200 ppm BHA/BHT and TBHQ was in the range of 71 to 80% on day-8 of storage. Lower peroxide values were evident for herring and cod samples containing 200 ppm chitosan (in all viscosities) possibly due to a concentration effect. Peroxide values of cooked comminuted cod samples treated with 14 cP chitosans at 50 ppm were not significantly different ($p > 0.05$) from those for the 100 ppm treated samples.

As shown in Figure 4.13A-C and Figure 4.14A-C, TBARS values of cooked comminuted herring and cod samples containing different viscosity chitosans and commercial antioxidants (BHA, BHT and TBHQ) increased over time, but at slower rates compared to those of the control samples. The differences in TBARS values of herring samples containing different viscosity chitosans (14 , 57 and 360 cP) were significant ($p \leq 0.05$) over the entire storage period. The 14 and 57 cP chitosans at 50, 100 and 200 ppm levels resulted in a significantly ($p \leq 0.05$) lower TBARS values with marginal differences between lower and higher concentrations in both fish model systems. After day-6 of storage, TBARS values of herring samples containing 14 cP chitosan were approximately 49-56% lower than that of the control (Figure 4.14A). The 360 cP chitosan, at 50 ppm, reduced TBARS by 14-34% in treated herring samples whereas the effects for 14 and 50 cP chitosans at the same concentration were significantly ($p \leq 0.05$) higher than that of the control.

As expected, TBHQ exerted the strongest antioxidant effect at all concentrations in both fish model systems. However, difference of TBARS values between BHT/BHA

Figure 4.13 Effect of different viscosity chitosans and commercial antioxidants on TBARS formation in a cooked comminuted herring model system. (A) 14 cP chitosan, (B) 57 cP chitosan, (C) 360 cP chitosan.

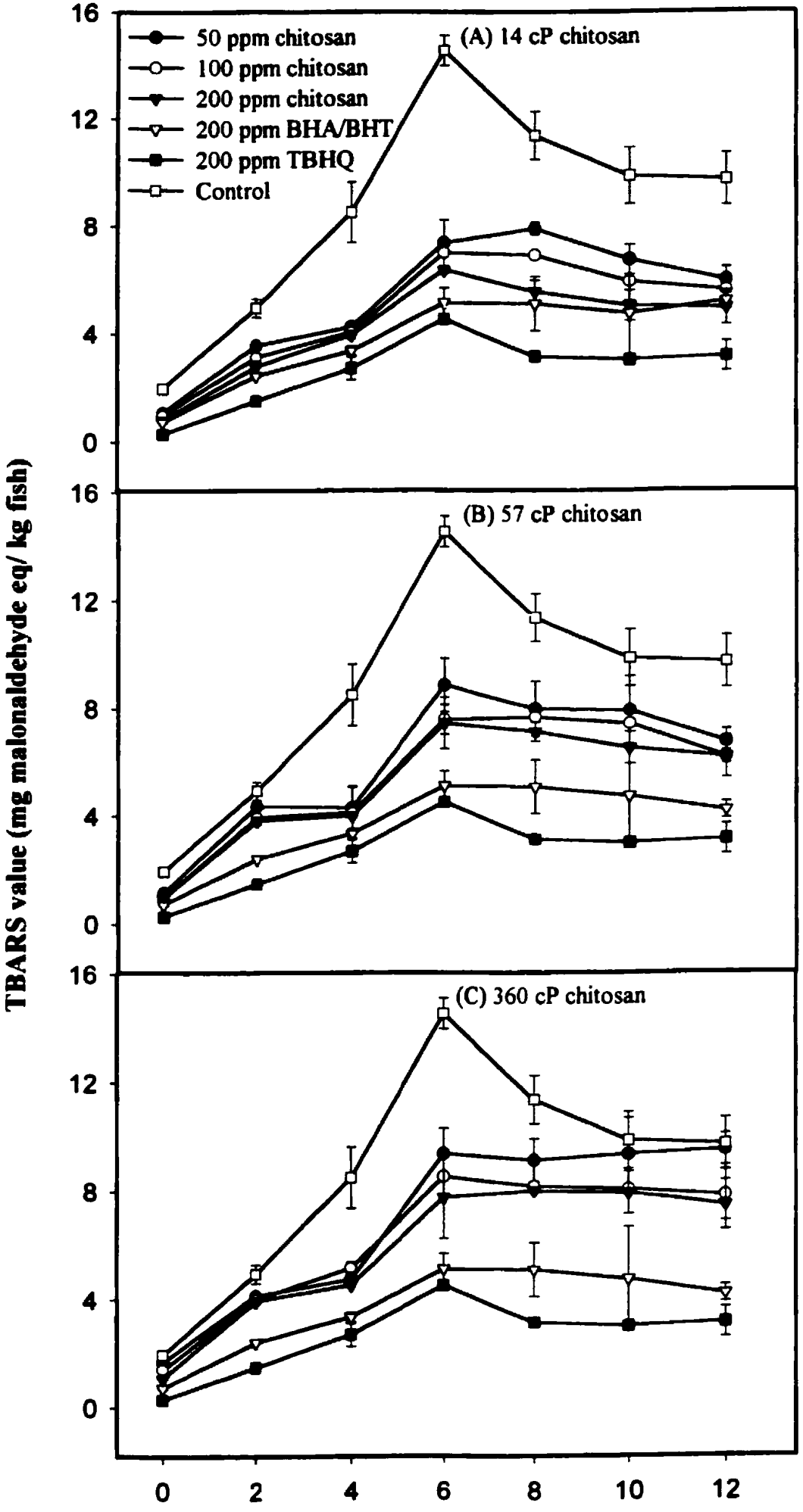
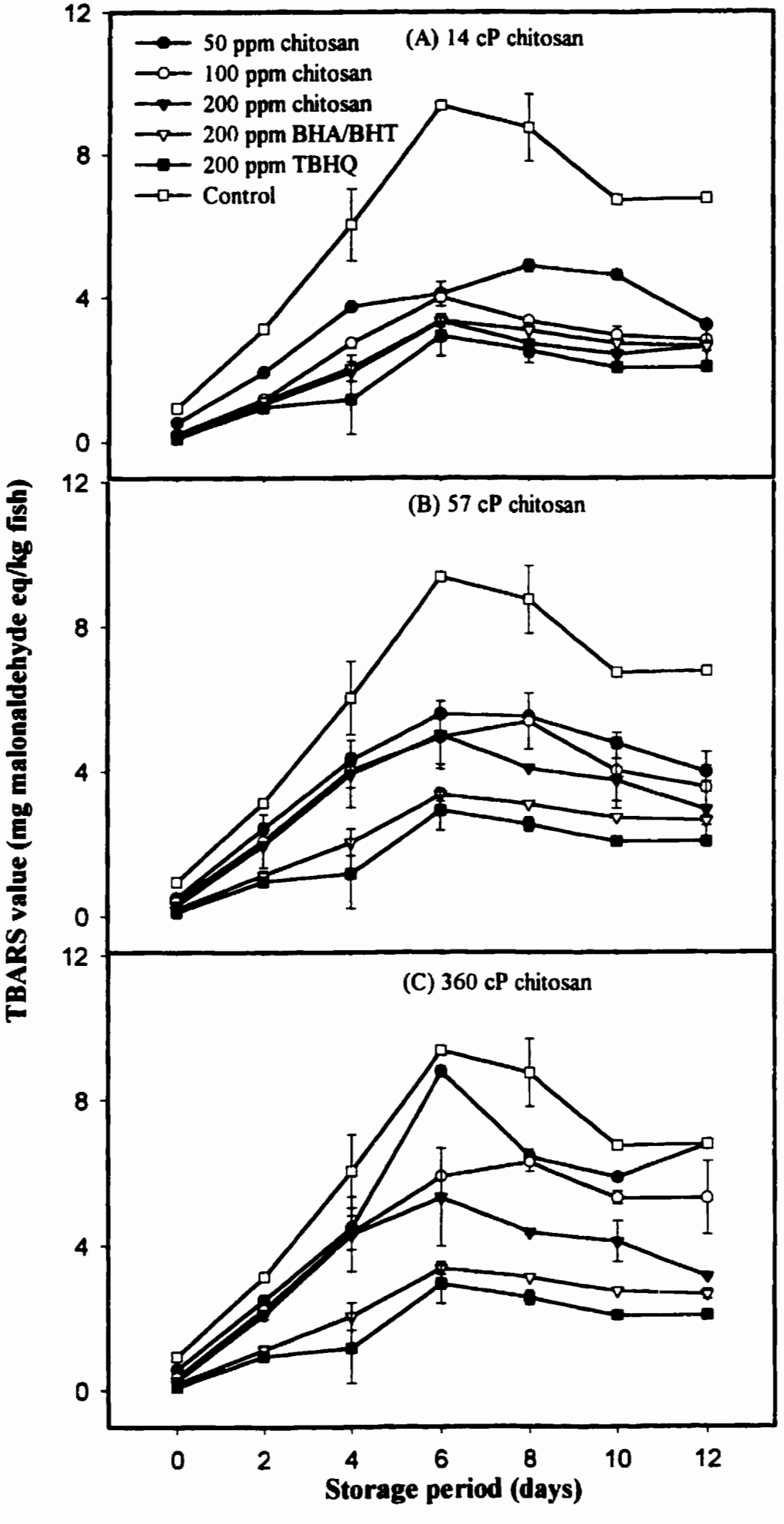

50 ppm chitosan
100 ppm chitosan
200 ppm chitosan
200 ppm BHA/BHT
200 ppm TBHQ
Control
(A) 14 cP chitosan
(B) 57 cP chitosan
(C) 360 cP chitosan
TBARS value (mg malonaldehyde eq/ kg fish)
0
4
8
12
16
0
2
4
6
8
10
12

Figure 4.14 Effect of different viscosity chitosans and commercial antioxidants on TBARS formation in a cooked comminuted cod model system. (A) 14 cP chitosan, (B) 57 cP chitosan, (C) 360 cP chitosan.


50 ppm chitosan
100 ppm chitosan
200 ppm chitosan
200 ppm BHA/BHT
200 ppm TBHQ
Control
(A) 14 cP chitosan
(B) 57 cP chitosan
(C) 360 cP chitosan
TBARS value (mg malonaldehyde eq/kg fish)
Storage period (days)
0
2
4
6
8
10
12

and TBHQ treated cod samples was not significant ($p > 0.05$) during the entire storage period. At 200 ppm, 14 cP chitosan exerted an effect similar to that of the commercial antioxidants (BHA/BHT and TBHQ) at the same level in both fish model systems. Among different viscosity chitosans, 14 cP chitosan was more effective than higher viscosity chitosans in prevention of lipid oxidation in both fish model systems.

Antioxidant activity of different viscosity chitosans in cooked comminuted herring and cod may be attributed to their metal-binding capacity. Several sources of protein-bound iron exist in fish tissues such as myoglobin, haemoglobin, ferritin and transferrin. The iron bound to these proteins may be released during storage and, particularly cooking, activating oxygen and initiating lipid oxidation (St. Angelo, 1996). Oxidative rancidity usually occurs more rapidly in cooked ground fish than in raw fish (Ramanathan and Das, 1992). Lee and Toledo (1977) also found that the cooking significantly increased the TBA values of minced mullet (*Mugil* spp.) during refrigerated storage. The cooking process disrupts the muscle membrane system, thereby exposing the lipid components to oxygen and/or other reaction catalysts such as iron (Love and Pearson, 1976).

Chitosans may retard lipid oxidation by chelating any ferrous ion present in the system, thus eliminating the prooxidant activity of ferrous ion or preventing its oxidation to ferric ion. Furthermore, Chitosans possess many amino groups in their chemical structure which are known to chelate metal ions (Winterowd and Sandford, 1995; Peng *et al.*, 1998). The varying antioxidant effect of different viscosity chitosans in cooked comminuted fish model systems may be attributed to molecular weight differences in chitosans which determine their chelation of metal ions. In their charged state, the cationic amino groups of chitosans impart intramolecular electric repulsive forces, which increase

the hydrodynamic volume by the extended chain conformation (Anthonsen *et al.*, 1993). Perhaps this phenomenon may be responsible for poor chelation by high viscosity (high molecular weight) chitosans. Furda (1983) has reported that the degree of polymerization of the glucosamine unit is a major factor determining the viscosity of chitosan. Since the extent of deacetylation is similar in all three types of chitosans examined in this study, the above results may thus be interpreted as the effect of differing molecular weight of chitosans.

Xue *et al.* (1998) reported that the liposoluble marine polysaccharides, hexanoyl chitin and *N*-benzoylhexanoyl chitosan solutions, retard the accumulation of hydroperoxide of methyl linoleate by effectively trapping peroxy radicals in organic solvents when the radical chain reaction had been initiated by 2,2'-azobis (2,4-dimethylvaleronitrile). Moreover, Xue *et al.* (1998) reported that water-soluble chitosans may chelate metals or combine with lipids to display a significant antioxidative effect. Effectiveness of chitosan treatment on inhibition of oxidation of beef was studied by Darmadji and Izumimoto (1994) who observed that addition of 1% chitosan resulted in a 70% reduction in TBARS values of meat after 3 days of storage at 4°C. The effect of N-carboxymethylchitosan (NCMC) in prevention of warmed-over flavour (WOF) in uncured meat was studied by St. Angelo and Vercellotti (1989) who reported in 93 and 99% inhibition of TBARS and hexanal content in ground beef. Similarly, Shahidi (1995) reported that NCMC and its derivatives were effective in controlling the oxidation and flavour deterioration of pork over a nine day storage at refrigerated temperatures.

4.4 Quality assessment related to chemical spoilage

The post-mortem metabolism of nitrogenous compounds in fish flesh is mainly responsible for its gradual loss of fresh quality (Sikorski *et al.*, 1990). Chemical spoilage of fish samples coated with chitosans during storage was evaluated by measuring changes in the contents of TVB-N, TMA and Hx.

4.4.1 Effect of chitosan coating on total volatile basic nitrogen (TVB-N) production in fish

Table 4.5 shows the effect of chitosan coating on TVB-N production in cod and herring samples stored at 4 ± 1°C. Total volatile basic nitrogen content of treated cod samples increased by about 3-4 fold as compared to a 6-fold increase for the control sample at the end of a 12-day storage period. Reduction of TVB-N contents of herring samples treated with chitosans was in the range of 26 to 51% after 12 days of storage. Treatment with 57 cP and 360 cP chitosans of both cod and herring samples reduced ($p \leq 0.05$) TVB-N formation throughout the entire storage period. After day 6, the TVB-N levels in uncoated cod sample exceeded the acceptable level of 30 mg N/100g of flesh suggested for fish and shellfish (Cobb III *et al.*, 1973; Connell, 1990). For herring samples, this level was reached after 8 days for the uncoated sample. Meanwhile, 14 cP chitosan-treated samples exceeded this acceptable level after 8 and 10 days for cod and herring, respectively. In general, the TVB-N levels were higher in the uncoated cod samples than the uncoated herring samples in contrast to what would have been expected from the observed trends in total microbial counts (Figure 4.16A and Figure 4.16B) because TVB-N production is mainly due to microbial activity. However, existing

Table 4.5 Content of total volatile basic-nitrogen (mg-N/100 g fish) of chitosan-coated fish samples stored at 4 ± 1°C[a]

Chitosan	Storage period (days)						
	0	2	4	6	8	10	12
[Cod]							
Uncoated	9.83±0.95[a]	11.98±0.15[a]	18.18±0.14[c]	29.83±1.15[c]	37.10±0.99[c]	41.25±0.08[c]	53.39±1.15[c]
14 cP	10.05±0.7[a]	15.88±0.95[b]	15.93±1.13[b]	22.13±1.37[b]	31.93±1.18[b]	36.90±1.95[b]	38.10±0.95[b]
57 cP	8.13±1.01[a]	11.55±1.31[a]	14.45±0.97[b]	16.75±0.95[a]	20.88±2.31[a]	23.18±0.78[a]	25.33±2.13[a]
360 cP	8.65±0.95[a]	9.80±0.94[a]	11.33±0.86[a]	15.70±0.19[a]	20.23±0.09[a]	20.98±0.93[a]	21.94±0.68[a]
[Herring]							
Uncoated	8.65±1.03[a]	10.70±0.13[a]	17.00±0.09[c]	21.88±0.39[c]	27.50±0.38[d]	37.33±0.12[d]	48.91±0.07[c]
14 cP	8.09±1.74[a]	14.85±0.66[b]	14.40±0.18[b]	19.35±0.18[b]	23.18±1.16[c]	30.75±0.09[c]	36.35±0.18[b]
57 cP	9.60±0.93[a]	9.83±0.71[a]	13.23±0.14[a]	16.50±0.11[a]	20.75±0.57[b]	22.15±1.74[b]	24.19±1.32[a]
360 cP	7.50±0.99[a]	10.63±0.11[a]	14.10±0.54[b]	16.18±0.14[a]	17.53±0.12[a]	19.55±0.54[a]	24.33±0.89[a]

[a]Results are expressed as mean ± standard deviation of three determinations. Values with the same superscripts within each column are not significantly different (P > 0.05)

differences in the level of endogenous enzymes and their activity in cod and herring muscles might be responsible for this observation. Furthermore, factors such as age, locality and culture method may influence the content of non-protein nitrogenous compounds in fish muscle (Kyrana *et al.*, 1997) and this could in turn affect TNB-N levels. Increase in TVB-N levels in fish during storage has been attributed to several enzymatic processes, namely deamination of free amino acids, degradation of nucleotides and oxidation of amines (Gill, 1990).

Olfsdottir *et al.* (1997) observed that the level of TVB-N in capelin stored at 0°C reached approximately 50 mg N/100 g in 5 days, while that in capelin stored at 5°C reached the same level in 4 days. According to Al-Kahtani *et al.* (1996), TVB-N values for irradiated tilapia and Spanish mackerel remained within the permissible range (30 mg TVB-N/100g) up to 20 days of storage at 2°C.

4.4.2 Effect of chitosan coating on trimethylamine (TMA) production in fish

During chilled or iced storage of marine fish, trimethylamine oxide (TMAO) is reduced to the odoriferous compound TMA by facultative bacteria (Woyewoda *et al.*, 1986a). In cold water fish stored in ice, the main spoilage organism is *Shewanella putrefaciens* which has the ability to produce TMA (Kyrana *et al.*, 1997). Trimethylamine has been used as a freshness quality indicator in fish and shellfish during early stages of storage. As depicted in Table 4.6, chitosan coating resulted in a significant ($p \leq 0.05$) reduction in TMA production in both cod and herring samples. The low initial TMA-N contents (0.08-0.30 mg TMA-N/100g) indicated that the fillets (cod and herring) were procured in very good condition. Furthermore, slight increase ($p > 0.05$) in TMA over the

Table 4.6 Content of trimethylamine (mg/100 g fish) of chitosan-coated cod samples stored at 4 ± 1°C[a]

Chitosan	Storage period (days)						
	0	2	4	6	8	10	12
[Cod]							
Uncoated	0.30±0.02[a]	0.80±0.09[a]	1.45±0.24[b]	5.05±0.28[c]	5.10±0.36[c]	6.39±0.09[c]	7.13±0.05[c]
14 cP	0.30±0.04[a]	0.97±0.17[a]	1.00±0.05[a]	2.01±0.05[b]	2.65±0.17[b]	2.73±0.11[b]	3.98±0.60[b]
57 cP	0.34±0.04[a]	0.76±0.07[a]	0.73±0.08[a]	1.32±0.10[a]	1.83±0.07[a]	2.09±0.10[a]	3.01±0.01[ab]
360 cP	0.33±0.03[a]	0.75±0.14[a]	0.84±0.06[a]	1.00±0.12[a]	1.72±0.20[a]	1.88±0.13[a]	2.75±0.63[a]
[Herring]							
Uncoated	0.09±0.00[a]	0.73±0.07[b]	1.20±0.00[c]	2.86±0.95[b]	3.07±0.02[c]	3.58±0.17[d]	3.98±0.11[c]
14 cP	0.09±0.01[a]	0.49±0.03[a]	0.67±0.13[b]	1.58±0.04[a]	2.33±0.00[b]	2.79±0.06[c]	2.89±0.04[b]
57 cP	0.09±0.01[a]	0.34±0.13[a]	0.21±0.07[a]	1.01±0.13[a]	1.18±0.31[a]	1.38±0.09[b]	2.05±0.41[a]
360 cP	0.08±0.00[a]	0.36±0.05[a]	0.26±0.07[a]	0.90±0.03[a]	0.95±0.04[a]	1.02±0.10[a]	1.93±0.13[a]

[a]Results are expressed as mean ± standard deviation of three determinations. Values with the same superscripts within each column are not significantly different ($P > 0.05$)

initial storage period (4-days) reflects the low starting level of TMAO in the flesh of cod and herring fillets and precludes the usefulness of this compound as a freshness indicator.

On day-12 of storage, trimethylamine nitrogen content of cod samples coated with chitosans was 44-61% lower than that of the control (Table 4.6). The level of 5-10 mg TMA/100g flesh, which is thought to indicate rejection limit for cod (Sikorski *et al.*, 1990), was reached after 6 days in uncoated samples, whereas in all treated samples, the levels were well below the rejection limit throughout the storage period. However, significantly ($p \leq 0.05$) lower TMA levels were observed for 57 cP and 360 cP chitosan-coated cod samples compared to that of the sample treated with the 14 cP chitosan after 6 days. Meanwhile, herring samples did not reach the rejection limit of 5 mg/100g, in agreement with values reported by Gill (1990) and Sikorski *et al.* (1990) for fatty fish species such as herring. Furthermore, comparatively higher amounts of TMA were produced in cod as compared to the herring samples. This could be due to the high content of TMAO in cod (gadoid fish) tissues (Huss, 1995), which serves as an osmoregulator. However, significantly ($p \leq 0.05$) higher levels of TMA were in uncoated herring samples as compared to those for their uncoated counterparts after 2 days.

4.4.3 Effect of chitosan coating on hypoxanthine (Hx) production in fish

Considerable enzymatic dephosphorylation of inosine monophospahte (IMP) via inosine (Ino) to hypoxanthine (Hx) occurs within the edible shelflife period of iced-stored fish and these changes are implicated in the loss of fresh flavours and development of undesirable aromas in several fish species (Huss, 1995). The Hx test is applicable to all species of fish during the early stages of refrigerated storage (Woyewoda *et al.*, 1986b).

However, a wide variation in the rate of nucleotide catabolism has been observed in different species of fish. Therefore, it is important to establish the rate of accumulation of Hx in a particular species before applying the Hx as an index of freshness or quality (Ehira, 1976). Table 4.7 shows that regardless of the viscosity of chitosan, significantly ($p \leq 0.05$) different Hx levels were reached for the treated and untreated samples of cod and herring after 4 days. After day-6, hypoxanthine formation in cod and herring samples treated with chitosan was reduced by 41-52% and 23-29% as compared to that of their uncoated counterparts, respectively. The critical level of Hx proposed for cod rejection is 2-3 μmol/g fish (Barile *et al.*, 1985a) and this level was reached after 2 days, 4 days, and 6 days in uncoated, 14 and 57 cP chitosan coated samples, respectively. However, 360 cP chitosan-coated sample did not reach this level (2.5 μmol/g fish) throughout the entire storage period. The content of Hx was first increased, but later decreased with progression of spoilage (Field *et al.*, 1986; Woyewoda *et al.*, 1986b) in seafoods. A similar pattern was observed in this study, perhaps due to the breakdown of Hx to xanthine and subsequently to uric acid.

The pattern of Hx accumulation in the chitosan coated herring samples was highly variable (Table 4.7). While this assay may provide for a chemical index in assessing the quality of the chitosan coated-cod fillets, it does not seem to be a test that is applicable to herring samples. Hypoxanthine values of treated herring increased by about 2-4-fold after day-6 of storage, whereas the increase for the control was about 3-fold. However, herring samples also exhibited the expected increase and decrease pattern similar to cod. This was in contrast to the findings of Kyrana *et al.* (1997) who observed a linear increment of the Hx content in ice stored gilthead sea bream over a 24-day storage period. The steady

Table 4.7 Content of hypoxanthine (μmole/ g fish) of chitosan-coated fish samples stored at 4 ± 1°C[a]

Chitosan	Storage period (days)						
	0	2	4	6	8	10	12
[Cod]							
Uncoated	1.32±0.10[a]	2.17±0.36[b]	2.33±0.50[ab]	4.93±0.13[b]	5.42±0.90[b]	5.40±0.61[b]	4.13±0.13[c]
14 cP	1.64±0.29[a]	1.64±0.29[ab]	3.13±0.43[b]	2.87±0.10[a]	2.89±0.37[a]	3.13±0.97[a]	2.65±0.34[b]
57 cP	1.34±0.42[a]	1.39±0.42[ab]	1.93±0.08[a]	2.74±0.76[a]	2.05±0.06[a]	2.26±0.33[a]	1.45±0.06[a]
360 cP	1.26±0.09[a]	1.27±0.09[a]	2.07±0.45[a]	2.39±0.66[a]	2.10±0.78[a]	1.96±0.58[a]	1.72±0.09[a]
[Herring]							
Uncoated	0.73±0.11[a]	1.09±0.66[a]	2.15±0.90[b]	2.44±0.42[b]	2.73±0.76[b]	2.01±0.39[c]	1.98±0.09[c]
14 cP	0.94±0.06[a]	1.45±0.60[a]	1.69±0.63[a]	1.77±0.08[a]	1.79±0.87[ab]	1.82±0.09[bc]	1.80±0.14[bc]
57 cP	0.44±0.08[a]	0.96±0.91[a]	1.74±0.47[a]	1.73±0.04[a]	1.13±0.19[a]	1.10±0.00[a]	0.95±0.09[a]
360 cP	0.63±0.39[a]	1.41±0.01[a]	1.69±0.19[a]	1.89±0.17[a]	1.07±0.09[a]	0.86±0.08[a]	0.72±0.10[a]

[a]Results are expressed as mean ± standard deviation of three determinations. Values with the same superscripts within each column are not significantly different ($P > 0.05$)

increase in the concentration of Hx was also noted up to 19 days in seal meat stored at 0-4°C (Shahidi *et al.*, 1994). In general, samples coated with chitosans, regardless of their viscosity, had a lower hypoxanthine content throughout the entire storage period.

The development of Hx in many species parallels the production of TMA (Huss, 1995). Dalgaard *et al.* (1993) showed a linear correlation (r = 0.9000) between the contents of TMA and Hx during storage of vacuum-packed cod fillets stored at 0°C. However, in this study the observed correlation (r = 0.6862) between TMA and Hx was not strong (Figure 4.15), perhaps due to acceleration of breakdown of Hx during storage temperature at 4 ± 1°C. Moreover, this could be due to a reduction in microbial and autolytic activities under vacuum-pack conditions encountered in their study.

4.5 Quality assessment related to microbial spoilage

The metabolic processes of the microflora contribute, in part to the gradual loss of taste substances in iced fish and ultimately lead to spoilage due to partial proteolysis and accumulation of unpleasant metabolites (Barile *et al.*, 1985b; Sikorski *et al.*, 1990). The surface bacteria of cold-water fish are mainly psychrotropic (Huss, 1995).

Total aerobic psychrotropic bacterial count in both fish species coated with chitosans was compared with uncoated samples and those treated with 1% acetic acid used for dissolving chitosans (Figure 4.16A & B). Fresh cod and herring fillets had initial total aerobic psychrotropic count around 3-3.7 $\log_{10}$ cfu/g. Samples of cod and coated with chitosan contained less than 10^6 c.f.u/g fish (psychrotropic bacterial count) during the entire storage period while uncoated and 1% acetic acid-treated cod samples exceeded this

Figure 4. 15 Relationship between hypoxanthine (Hx) and trimethylamine (TMA) contents of chitosan-coated cod samples stored at 4 ± 1°C.

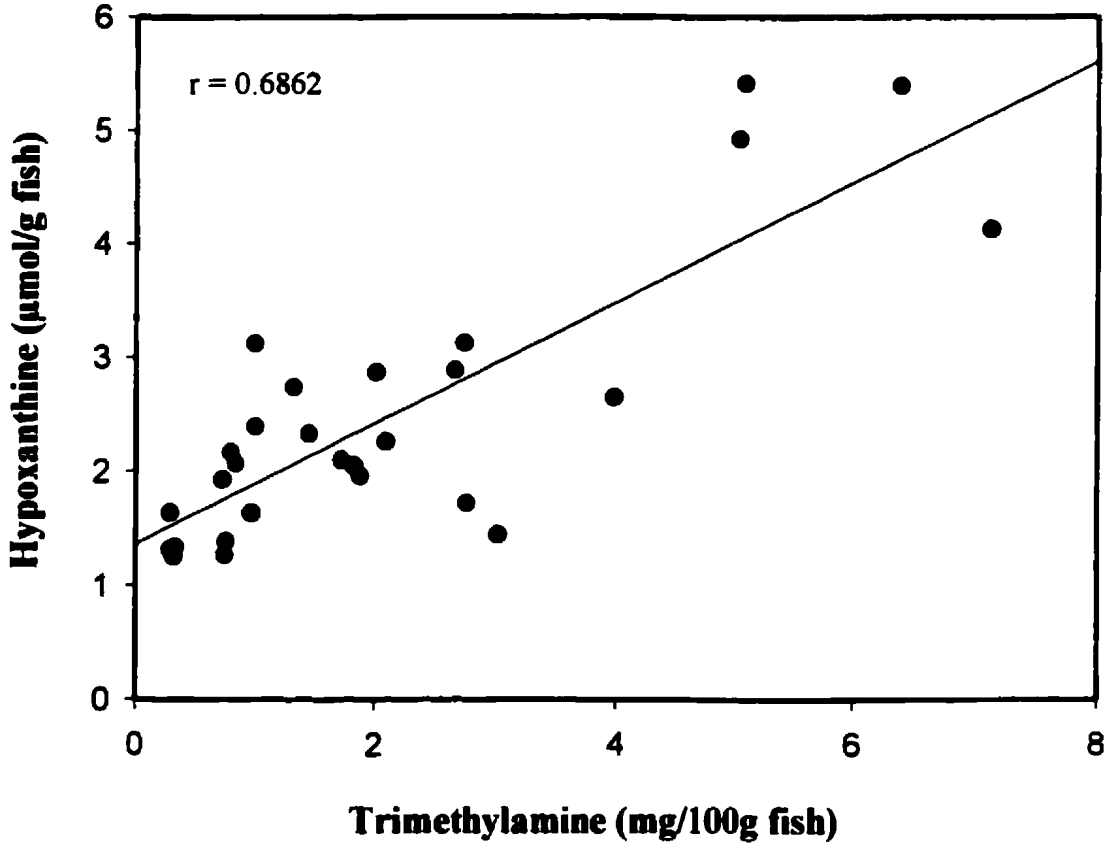
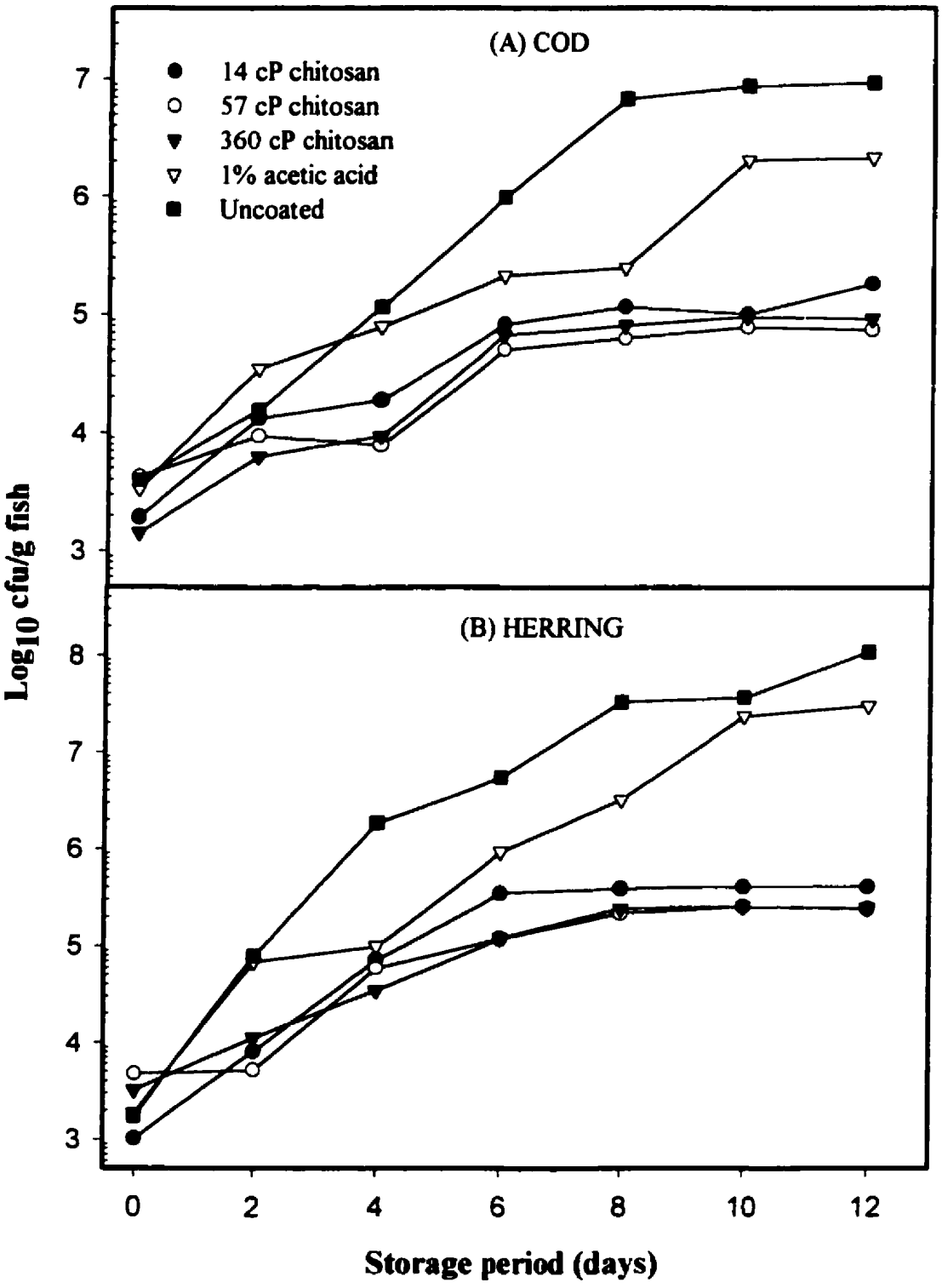

r = 0.6862
Hypoxanthine (µmol/g fish)
Trimethylamine (mg/100g fish)
0
1
2
3
4
5
6
0
2
4
6
8

Figure 4.16 Effect of chitosan coating on total plate count of cod (A) and herring (B) samples stored at 4 ± 1°C.


(A) COD
14 cP chitosan
57 cP chitosan
360 cP chitosan
1% acetic acid
Uncoated
(B) HERRING
Log10 cfu/g fish
Storage period (days)
0
2
4
6
8
10
12
3
4
5
6
7
8

level after 6 days and 10 days, respectively. This acceptability limit of 10^6 c.f.u/g has been proposed for fresh fish (ICMSF, 1986). Stenstrom (1985) observed that cod fillets stored at 2°C exceeded the maximum acceptable level of total plate count value of 10^6 cfu/g after 6 days of storage. A similar pattern ($<10^6$ c.f.u/g) was observed with chitosan-coated herring samples. However, this acceptable limit was reached much faster in herring samples and exceeded it after 4 and 6 days for uncoated and 1% acetic acid-treated samples, respectively. Herring fillets treated with 57 and 360 cP chitosans had a lower ($p \leq 0.05$) aerobic psychrotropic count than 14 cP chitosan treated fillets after day-6 of storage. Furthermore, a logarithmic increase of bacterial population was clearly observed only with uncoated and 1% acetic acid-treated samples. After 6 days, there were no further significant ($p \leq 0.05$) increases in total plate count (TPC) showing that stationary phase in bacterial growth had been attained in all chitosan-coated cod and herring samples. Moreover, data indicated that treatments with chitosan resulted in reductions of 10^3 and 10^2 TPC of herring and cod samples, respectively, after 12 days of refrigerated storage.

Parkin *et al.* (1981) reported the effectiveness of a modified atmosphere (80% CO_2 and 20% air) in extending the shelf-life of fresh rockfish fillets stored in the dark at 2-4°C. The modified atmosphere storage of fish resulted in a significantly lower aerobic plate counts than that held in the air. Gray *et al.* (1983) studied refrigerated perch, seatrout, croaker, and bluefish packed with carbon dioxide. They found 45-55% increase in stability, mainly due to an extension in the lag phase of psychrotropic organisms and their reduced growth rate in the logarithmic phase.

Treweek and Morgan (1977) described the destabilization of negatively charged bacterial cells by a cationic polymer (polyethyleneimine, PEI). It was proposed that such a

flocculation may be caused by one of three mechanisms, namely double layer coagulation, adsorption coagulation or polymer bridging. It was further concluded that high molecular weight PEI produced rapid flocculation at low doses due to adsorption coagulation. Chitosan is believed to chelate certain ions from the lipopolysaccharide (LPS) layer of the outer membrane of bacteria. Thus, it has been suggested that alterations in LPS layer may cause the outer cell surface to become more permeable, thereby releasing cellular components of bacteria (Papineau *et al.*, 1991; Chen *et al.*, 1998). Furthermore, chitosan coating acts as a barrier to protect oxygen transfer and leads to inhibition of growth of the aerobic bacteria. Tsai and Su (1999) noted that chitosan caused leakage of glucose and lactate dehydrogenase from *E.coli* cells. Furthermore, these authors reported that the antibacterial mechanism of chitosan involves a cross-linkage between the polycations of chitosan and the anions on the bacterial surface that changes the membrane permeability. Cuero (1999) reported that the antimicrobial action of chitosan is influenced by intrinsic and extrinsic factors such as the type of chitosan (e.g. plain or derivative), degree of polymerization, host natural nutrient constituency, substrate chemical and/or nutrient composition, and environmental conditions.

SUMMARY AND CONCLUSIONS

This study has demonstrated that chitosans prepared from snow crab processing discards possess varying viscosities which are closely related to the duration of the deacetylation period. Different viscosity chitosans (14, 57, and 360 cP chitosans) were used to investigate the effect of chitosan coating on fish quality during refrigerated storage. Several analytical techniques were used to evaluate the preservative efficacies of chitosan edible coating used in cod and herring fillets. This study demonstrated the potential of chitosan as a preservative coating for herring and cod in reducing or preventing moisture loss, lipid oxidation, and microbial growth. Cod samples coated with 57 and 360 cP chitosans exhibited a significantly ($p \leq 0.05$) lower relative moisture loss as compared to those of the uncoated samples and fish coated with 14 cP chitosan throughout the storage period. However, a reduction in relative moisture loss due to chitosan coating was not clearly observed for the herring samples. Furthermore, chitosan produced from crab processing discards exhibited a moderate to strong viscosity-dependent preservative effect in both fish model systems. In general, 360 cP chitosan exerted a better preservative effect (comparable or better than 57 and 14 cP chitosans) in both fish model systems stored at $4 \pm 1^{\circ}C$.

In another experiment, the effects of different viscosity chitosans on lipid oxidation of cooked comminuted fish were examined. The chitosans exhibited antioxidant activity in cooked comminuted fish model systems, as reflected in their peroxide values and their

content of 2-thiobarbituric acid-reactive substances, in a concentration-dependent manner. However, the antioxidant efficacy of relatively high viscosity chitosan (360 cP) in both fish model systems was lower than that of the low viscosity chitosan (14 cP) at the same concentration.

The mechanism of action of chitosan coating for extension of shelf-life of seafoods appears to be due to chelation of metal ions contained in fish muscle proteins, control of gas exchange, particularly oxygen, between fish meat and the surrounding environment, and bactericidal effect of chitosan itself. However, generation of further data regarding permeability characteristics of different viscosity chitosans would help in the selection of a suitable chitosan for packaging of seafoods. Sensory evaluation of chitosan coated samples should eventually be conducted to determine the relationship with the analytical methods used in this study. Additional research is also needed to optimize chitosan coatings/film formulations for seafood applications and assessment of the cost-benefit of chitosan edible coatings with respect to seafood preservation. Finally, more extensive investigations are needed for better understanding of the relationships reported in the present research, especially in view of the current worldwide interest in commercial utilization of chitosan as it relates to its health benefits.

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APPENDIX

Figure A.1 Dependence of the absorbance of diisopropylethylamine (DIPEA)-picrate concentration at 358 nm on the concentration of DIPEA-picrate.

Correlation coefficient (r) = 0.9945
Equation of the line was Y = aX + b where,
Y = absorbance at 358 nm ($A_{358\ nm}$)
X = concentration of DIPEA-picrate in μmol (C)
a = 0.0126
b = 0.0
Therefore, $C = 79.37 * A_{358\ nm}$

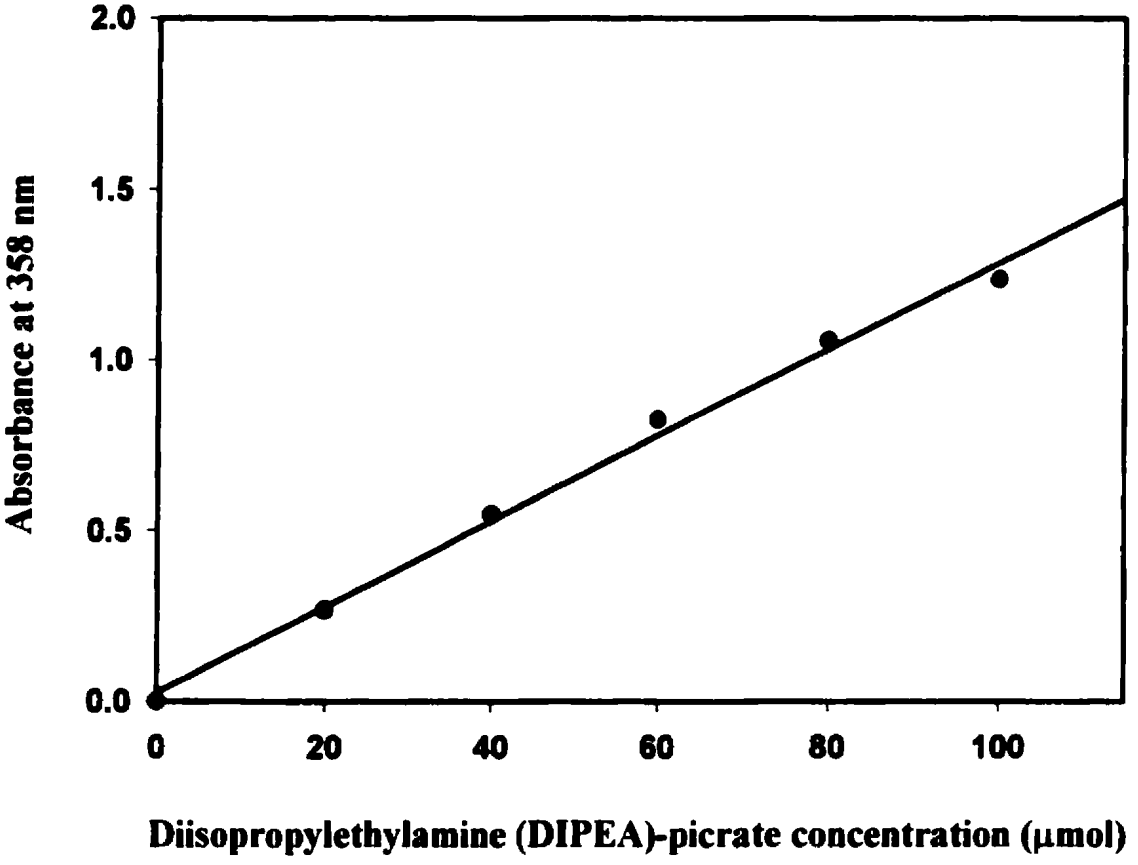

2.0
1.5
1.0
0.5
0.0
Absorbance at 358 nm
0
20
40
60
80
100
Diisopropylethylamine (DIPEA)-picrate concentration (μmol)

Figure A.2 Dependence of the absorbance of malonaldehyde (MA)-TBA complex at 532 nm on the concentration of MA.

Correlation coefficient (r) = 0.9976
Equation of the line was Y = aX + b where,
Y = absorbance at 532 nm (A_{532nm})
X = concentration of MA in mg MA eqivalents/kg fish (C)
a = 0.143
b = 0.0
Therefore, C = 7.00*A_{532nm}

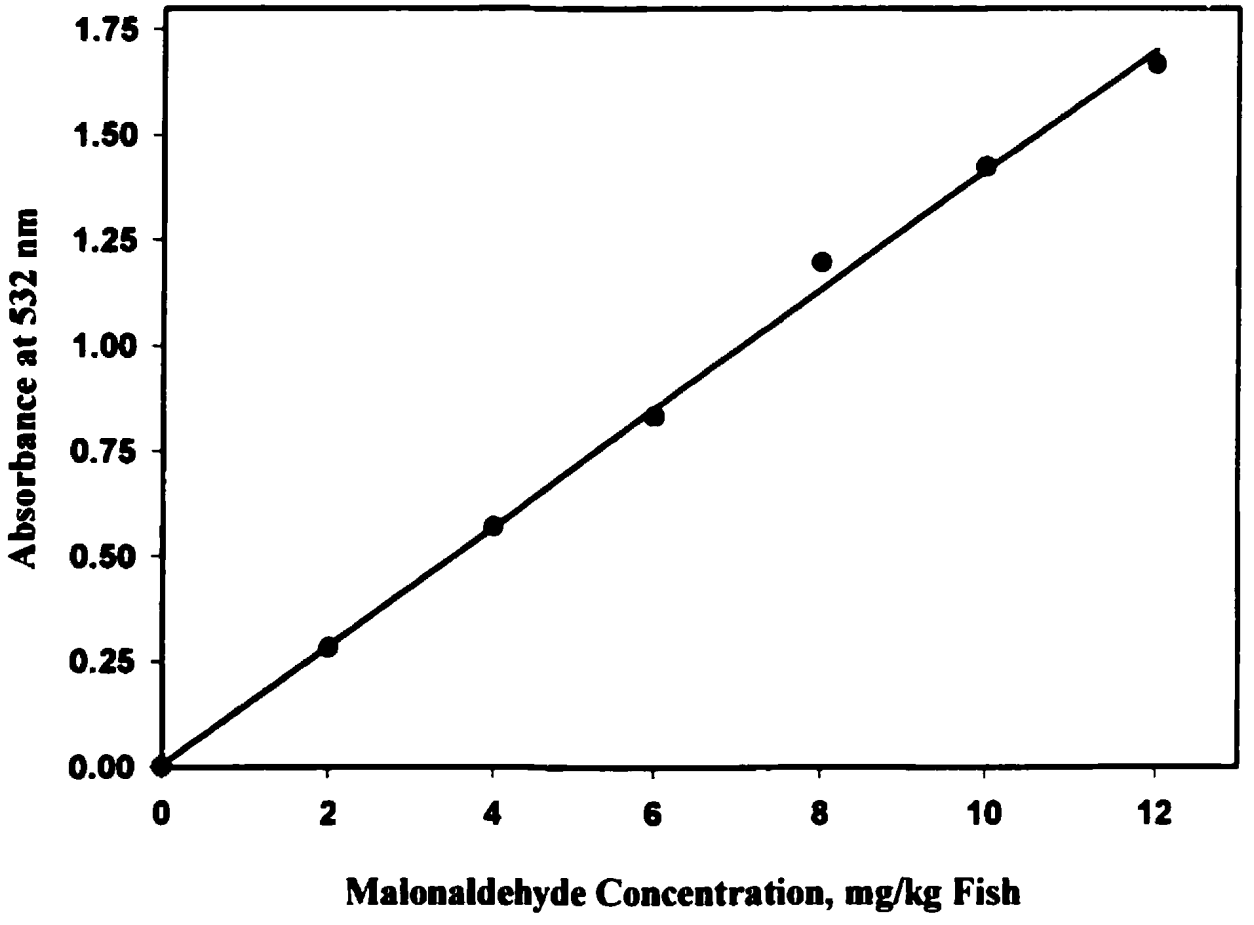

1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Absorbance at 532 nm
0
2
4
6
8
10
12
Malonaldehyde Concentration, mg/kg Fish

Figure A.3 Dependence of the absorbance of trimethylamine at 410 nm on the concentration of trimetylamine.

Correlation coefficient (r) = 0.9848
Equation of the line was Y = aX + b where,
Y = absorbance at 410 nm ($A_{410\ nm}$)
X = concentration of trimethylamine in μg (C)
a = 0.0126
b = 0.0
Therefore, C = 73.36*$A_{410\ nm}$

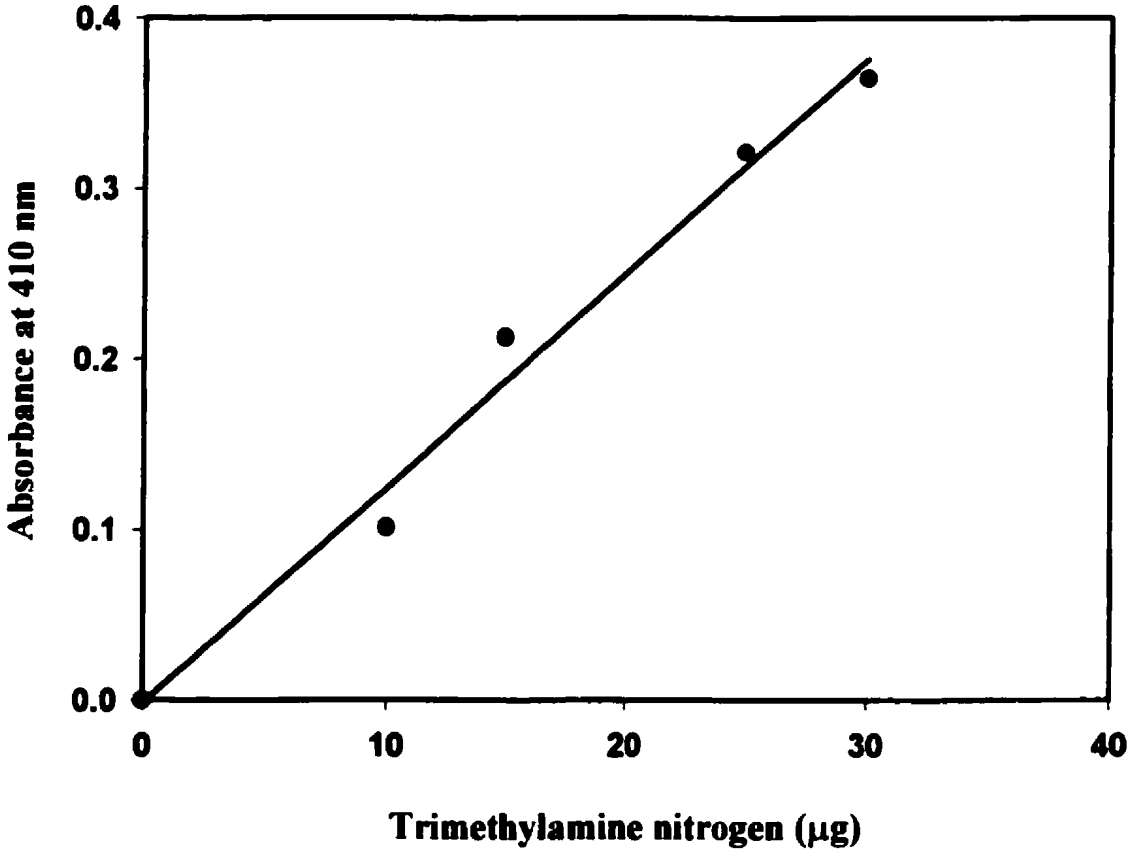
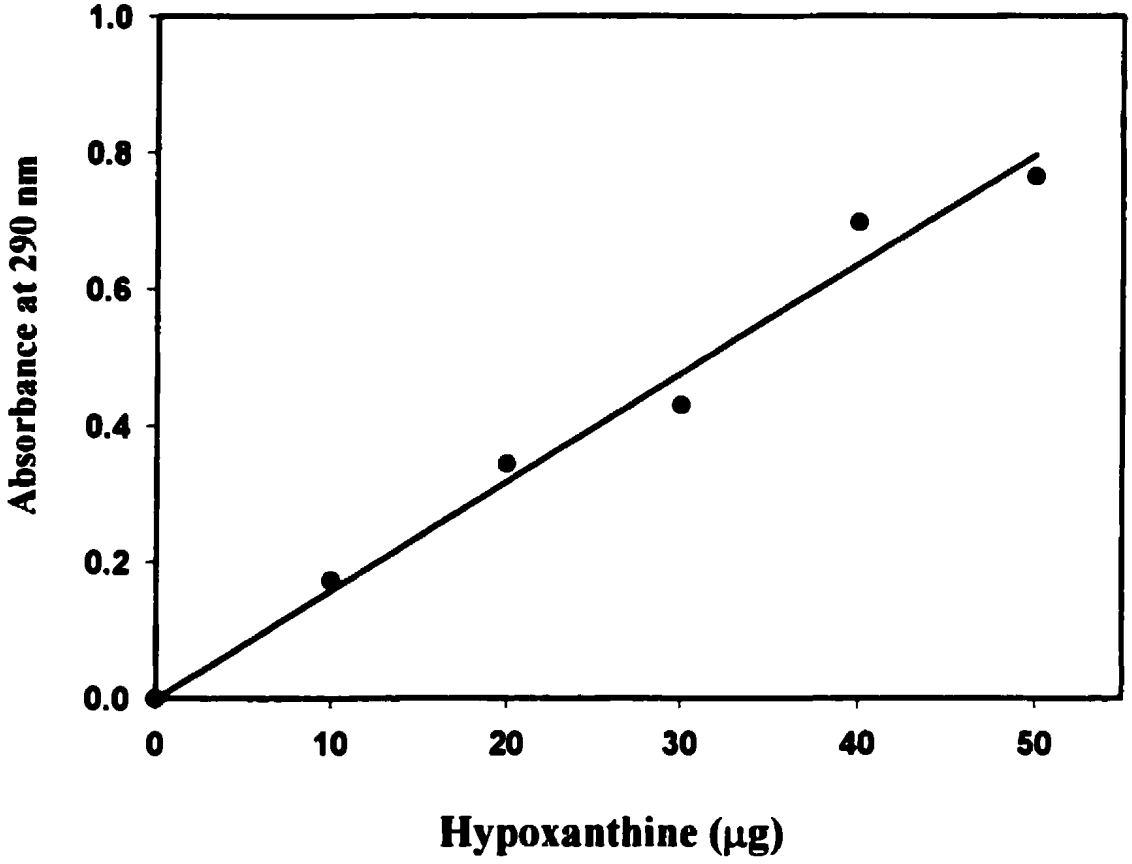

0.4
0.3
0.2
0.1
0.0
Absorbance at 410 nm
0
10
20
30
40
Trimethylamine nitrogen (μg)

Figure 4.4 Dependence of the absorbance of hypoxanthine at 290 nm on the concentration of hypoxanthine.

Correlation coefficient (r) = 0.9823
Equation of the line was Y = aX + b where,
Y = absorbance at 290 nm ($A_{290\ nm}$)
X = concentration of hypoxanthine in μg (C)
a = 0.0157
b = 0.0
Therefore, C = 63.69*A_{290nm}


1.0
0.8
0.6
0.4
0.2
0.0
Absorbance at 290 nm
0
10
20
30
40
50
Hypoxanthine (μg)