USE OF CHITOSAN FOR THE REMOVAL OF METAL ION CONTAMINANTS AND PROTEINS FROM WATER

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Use of chitosan for the removal of metal ion contaminants and proteins from water

By

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A thesis submitted to the School of Graduate Studies

in partial fulfillment of the requirements

for the degree of Master of Science

Environmental Science Programme
Memorial University of Newfoundland

May 2003

St. John's Newfoundland & Labrador Canada

0-494-06602-4

Your file Votre référence
ISBN:
Our file Notre référence
ISBN:

ABSTRACT

Chitin is the second most abundant natural biopolymer after cellulose and is distributed in the shells of crustacea and cuticle of insects and also in the cell wall of some fungi. Chitosan is the deacetylated product of chitin. Chitosan has been used in a number of fields such as agriculture, the food industry and medicine. Both chitin and chitosan are recognized as excellent metal ligands, forming stable complexes with many metal ions. In particular, chitosan is considered one of the best natural chelators for transition metal ions and an effective protein coagulating agent.

The processing discards of shellfish pose major technological problems, mainly due to their insolubility in water and resistance to biodegradation. Direct use of crab processing discards is generally discouraged due to the obnoxious odour of putrefying shells, therefore, value-added utilization of their discards is of paramount importance.

In the treatment of crab processing discards to extend their usable shelf-life, five different chemical treatments were used. Fresh samples of crab processing discards were treated by dipping in 1% formaldehyde, formic acid, acetic acid, sodium bicarbonate or sodium hydroxide solution. Experiments were conducted at two temperatures ranges; 4-7°C and 20-25°C. The samples were evaluated for sensory characteristics by a seven - membered panel and the results were recorded. Chitin isolated from crab processing discards was deacetylated in order to produce three types of chitosan (Types 1, 2 and 3). Chitosans so prepared were evaluated for their capacity to chelate single metal ion solutions containing Co^{2+}, Fe^{2+}, Ni^{2+}, Cd^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+}. Then, the three chitosan types were tested for their capacity to chelate metal ions in samples of water obtained from a zinc minining site, Buchans, Newfoundland. Ethylenediaminetetraacetic acid (EDTA) was used as the reference chelating agent. The capacity to chelate single metal ions by chitosan and EDTA was determined by a colourimetric method and metal chelation capacity of wastewater was determined by inductively coupled plasma-mass spectrometry (ICP-MS) at three pH levels (5,6 and 7). Chelation and recovery of metals from aqueous solutions using a column containing chitosan was also determined by ICP-

MS. Ethylenediaminetetraacetic acid was used to recover metal ions that were chelated by chitosan in the column. Chitosan served as an effective coagulating agent in removing proteins from waste water.

Out of the tested treatments, 1% formaldehyde served best in controlling the foul odour development both at 4-7°C and 20-25°C followed by formic acid. Due to possible carcinogenicity of formaldehyde, formic acid could be best utilized in controlling foul odour of crab wastes. At all pH levels tested, EDTA exhibited the highest chelation capacity. At pH 5, chitosan served best for metal ion chelation and pH 7 was best for metal mixtures in waste water. Metal chelation by a column method for single metal ions showed over 98% chelation both at 50 and 100 ppm except for Co^{2+} while gave a metal recovery of 52-97%. For mixtures of metal ions, except Co^{2+}, exhibited 94% chelation and metal recovery was 24-90%. Chitosan Type 1 showed the best protein flocculation ability followed by Types 2 and 3 chitosan.

Encouraging results for metal chelation and recovery by the prepared chitosan and protein flocculation reveal that crustacean processing discards can be converted to useful environmentally friendly material for wastewater purification.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. F. Shahidi for providing invaluable guidance and financial support throughout my MSc programme. I also wish to extend my thanks to my supervisory committee members, Dr. N. Gogan, Chair Environmental Science Programme and Dr. A. Martin for their guidance and valuable suggestions. I wish to express my sincere thanks to Dr. Sue Ellen Maher, Environmental toxicologist and Mr. George Neary, Asarco Incorporated, Buchans, Newfoundland for providing me with wastewater samples. Thanks are extended to Mrs. Lakmali Hewa, Department of Earth Science for assisting me with ICP-MS analyses. I am also grateful to Dr. M-S. Heu for assisting me with column chromatography analysis and valuable suggestions. Special thanks are due to Dr. S.P.J.N. Senanayake, Mr. M.A Khan and Mr. J.A.L.P. Jayakody as well as other colleagues in Dr. Shahidi's research team. My Deepest gratitude is due to my husband Madhujith for his enormous support, encouragement and dedication and my little son Hasith from whom the time devoted to this work was withdrawn. I wish to express my deepest appreciation to my loving parents for their blessing and encouragement without which this work not have been successful.

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CHAPTER 1
INTRODUCTION

Heavy metals belong to the group of elements described geochemically as trace elements, because they collectively comprise <1% of the rock in the earth's crust (Alloway, 2001). Heavy metals are released into the aqueous environment through a variety of sources such as metal smelter effluents, textiles, microelectronics and usage in fertilizers and plastics. It is well known that most trace metals display strong bioactivities. The heavy metals play a dual role as essential nutrients and toxic chemicals in plant production and human health (Harada and Hatanka, 1988). The hazards associated with the pollution of water bodies caused by heavy metals have led to the development of various wastewater reclamation technologies (Bassi *et al.*, 2000). Heavy metals at low concentrations are difficult to remove. Chemical precipitation, reverse osmosis, and other available methods become inefficient when heavy metal contaminants are present in trace amounts in a large volume o f solution. Adsorption is one of few alternatives available in such a situation (Huang *et al.*, 1996). The use of adsorbents (eg. activated carbon, low cost industrial products and microbial materials) has received extensive attention (Huang *et al.*, 1996). All of these technologies have their inherent advantages and limitations. For instance, activated carbon still requires complexing agents to improve its performance in removing inorganic matters. On the other hand, ion-exchange resins are effective, but expensive.

The use of chelation ion exchange for wastewater remediation has been gaining attention in the recent past. Chelation ion exchange, in contrast to simple ion exchange, takes advantage of removing only toxic metal ions while the harmless ions move on into

the environment (Deans and Dixon, 1992). Some of the best chelation ion-exchange materials consist of different biopolymers and their derivatives because of the variety of functional groups, such as OH and NH_2 (Bassi *et al.*, 2000). Biopolymers are attracting increasing interest for their capacity as effective scavengers of heavy metals, thus making them environmentally safe (Huang *et al.*, 1996). Such materials include cellulose, alginates, proteins, chitin and chitin derivatives.

Chitin (ploy[β-(1-4)-2-acetoamido-2-deoxy-D-glucopyranose]), a polymer of *N*-acetyl-D-glucosamine is one of the most abundant organic materials produced annually by biosynthesis in animals, particularly in crustacea, mollusks and insects, and in certain fungi. Its derivative chitosan (ploy[β-(1-4)-2-amino-2-deoxy-D-glucopyranose]), a polymer of D-glucosamine, has reactive amino groups which are responsible for complex formation between metal ions and the polymer chain. Chitosan is a heteropolymer made of D-glucosamine and a small fraction of N-acetyl-D-glucosamine residues (Roberts, 1992). Therefore, the adsorption ability of chitosan is found to be much higher than that of chitin, which has relatively fewer amino groups. Chitosan also has many other useful features, including hydrophilicity, biocompatibility, biodegradability, antibacterial property, and a remarkable affinity for many proteins (Bassi *et al.*, 2000). Reclamation of proteins yields not only economically valuable products, but also the pretreatment of food, industrial wastewater which is becoming a common requirement prior to discharge to the municipal sewer systems (Selmer-Olsen *et al.*, 1996). Chitosan, with its partial positive charge, can be used for coagulation and recovery of proteinaceous materials present in such food processing operation (Knorr, 1991).

The processing of shellfish waste poses major technological problems as shells are largely water insoluble and very resistant to natural biodegradation (Healy *et al.*, 1994). Direct use of these discards for land manuring, or spreading, is generally discouraged by the uniquely obnoxious odours of putrefying shells. Therefore, value-added utilization of shell discards is of importance. Hence, the uses of chitin and chitosan have been explored for wastewater treatment such as removal of metals, recovery of precious metals and removal of proteinaceous materials from industrial wastewater.

Most previous studies have dealt with examining the capabilities of chitosan to adsorb metal ions and coagulation of proteinacous materials from industrial wastewater. However, little attempt has been made to understand the characteristics of different chitosans obtained by adapting different deacetylation times.

The objectives of the present study are to evaluate the efficacy of different chemicals for extending the shelflife of fresh crab processing discards prior to future processing. Preparation of chitosan from locally available crab discards was also attempted. In addition, the potential of chitosans produced under different conditions in chelating metal ions and flocculation of proteins was investigated. Finally, industrial wastewater containing a wide variety of metal ions was investigated.

CHAPTER 2
LITERATURE REVIEW

The environment, including the atmosphere, earth, water and space are affected by various activities of man; hence, the composition and the complex nature of the environment change. Over the past 200 years, a rapid increase in population worldwide has necessitated the need for even greater use of fuel and development of industrial chemicals, fertilizers, pesticides and pharmaceuticals to sustain and improve the quality of life (Chakrabarty *et al.*, 1988). Although many of these chemicals are utilized or destroyed, a high percentage of them is released into the atmosphere, water and soil; all of which represents a potential environmental hazard (Alexander, 1995). Unfortunately, it is not feasible to replace all industrial processes that generate polluting wastes with clean alternatives (Betts, 1991). Diverse environmental impacts are usually associated with high contaminant concentration formed in close proximity to input sources. Environmental pollution is classified into various groups, namely air pollution, water pollution (i.e., industrial effluent pollution and domestic effluent pollution), soil pollution and noise pollution.

2.1 Air Pollution

Amongst the various types of pollution, air pollution is of greater importance. A large variety and quantity of contaminants are released into the environment. Once released, these contaminants may become widely dispersed via the fluid dynamics of surface water and atmosphere. The atmosphere is responsible for the long-range dissemination of contaminants over regional, hemispherical and global scales due to its

dynamic nature and ability to move contaminants rapidly (Rahn and Lowenthal, 1984; Johnes *et al.*, 1989).

Through the process of atmospheric transport and deposition, toxic chemicals have found their way to remote environments far from emission sources (Eurtz, 1990). Many of these chemicals are toxic and persistent, thus, can bioaccumulate and remain biologically active for long periods of time. Airborn pollutants can be broadly defined as chemicals occurring in the atmosphere in concentration and exposure duration that may pose a threat to human health and the environment. This broad definition includes an array of chemicals ranging from criteria pollutants (e.g. ozone, sulphur dioxide), which have resulted in well-documented biological effects at local and regional scales, to the greenhouse gases (e.g. methane, chlorofluorocarbons) with their implications for global warming and stratospheric ozone depletion. Another group of chemicals, often referred to as air toxic or airborne contaminants, includes a large number and variety of chemical species broadly categorized as industrial organic, agricultural pesticide, trace metal and metalloid. These contaminants have strong anthropogenic emission sources and are known to be transported long distances (hundreds to thousands of kilometres) on the troposphere before being deposited into remote environments (Moser *et al.*, 1992).

Although humans have been responsible for emitting contaminants into the atmosphere for thousands of years, air pollution has increased exponentially, both in quantity and variety as a result of industrial revolution. Anthropogenic contaminants emanate from a host of different industrial, urban, and agricultural sources such as

chemical, metal, plastic, pulp and paper industries, fossil fuel processing plants, motor vehicles and aircrafts, municipal waste incinerators, agricultural practices such as pesticide usage and field burning, and small business practices, including dry cleaning among others (Freedman and Hutchinson, 1981). Emissions of toxic chemicals into the atmosphere may occur directly through deliberate or inadvertent release from industrial or urban sources or indirectly through volatilization following deliberate or accidental discharge of chemicals into water or soil. Considerable amounts of toxic chemicals enter the atmosphere from wind drift and volatilization during and following application of agricultural pesticides (Waddel and Bower, 1988).

Although numerous toxic chemicals are released from anthropogenic sources, organochlorine compounds and several trace metals are of particular concern. Organochlorines are significant environmental contaminants due to their chronic toxicity, their proven ability for bioaccumulation and biomagnification in the food chain, and the large quantities that have been manufactured, used, and released into the environment (Stickel, 1975; Harrison, 2001). Organochlorines represent a large class of chemicals that include synthetic organics and their by-products such as polychlorinated biphenyls (PCBs), hexachlorobenzene (HCB), polychlorinated dibenzodioxins (PCDD), and polychlorinated dibenzofurans (PCDF), and pesticides such as dichlorodiphenyl-trichloroethane (DDT), hexachlorocyclohexane (HCH) and toxapene.

All trace metals, even those with essential biochemical functions, have the potential to produce adverse biological effects at excessive levels of exposure. However,

trace metals such as mercury, cadmium, lead, and arsenic are of particular environmental concern because they are biologically nonessential and are toxic to most organisms at relatively low concentrations (Nriagu, 1988). The deposition of persistent synthetic organics and trace metals into terrestrial ecosystems not only results in increasing concentration of these contaminants in vegetation and soil, but also increases the risk to organisms at higher tropic levels such as herbivores, detrivores, and carnivores (Stickel, 1975; Martin and Caughtrey, 1981; Buckely, 1982).

2.2 Soil Pollution

Soil is an essential component of the terrestrial ecosystem because the growth of plants and biogeochemical cycling of nutrients depend upon it. Of the total area of the world's land (13.07×10^9 ha), only 11.3% is cultivated for crops; 34% account for forest and woodlands, 24.6% account for permanent grazing and 31% account for other land including urban industries and roads (World Resources Institutes, 1994-95). From a resource perspective, soil is vitally important for the production of food, fibre crops and timber, and it is therefore essential that the total productive capacity of the world's soils not be impaired. Pollution, along with other types of degradation, such as erosion and the continuing spread of urbanization pose a threat to the sustainability of soil resources. Soil pollution can also be a hazard to human health when potentially toxic substances are moved through the food chain or reach the ground water used for drinking purposes, among others (Alloway, 2001).

In comparison with air and water, soil is more variable and complex in composition and hence, it functions as a sink for pollutants, a filter which retards the passage of chemicals to the ground water, and a bioreactor in which many organic pollutants can be decomposed. As a consequence of its occurrence at the interface between the land and the atmosphere, soil is the recipient of a diverse range of polluting chemicals transported into the atmosphere. Further introduction of pollutants to the soil occurs as a result of agricultural and waste disposal practices, but in general, the most severe pollution usually arises from industrial and urban used lands (Alloway, 2001). Old industrial sites are generally characterized by being very heterogeneous, both with regard to the distribution of pollutants and also to the properties of the soil material that control the behaviour of these chemicals. In contrast, atmospherically deposited pollutants tend to be more evenly by distributed with gradual changes in concentration, which tend to decrease with distance for the source. The upper horizons of the soil are contaminated to the greatest extent by atmospheric deposition of pollutants (Alloway, 2001).

Soil pollutant sources include the following: heavy metals, hydrocarbon pollutants such as benzene, toluene, ethylbenzene and xylene; toxic organic micropollutants such as polycyclic aromatic hydrocarbons, polyheterocyclic hydrocarbons, polychlorinated biphenyls, polychlorinated dibenzodioxins, polychlorinated dibenzofurans and pesticide residues and metabolites; nutrient-rich wastes such as sewage sludge and livestock manures; radionuclides; as well as other industrial chemicals and pathogenic organisms. Pollutants reach the soil by four main pathways: these include atmospheric deposition of

particulates (washout or dry deposition), sorption of gases (e.g. volatile organic compounds) from the atmosphere fluvial transport and desorption/sorption from floodwaters, and placement (agricultural, amendments, dumping, injection, surface spreading etc). Placement of pollutants can occur in many ways. The most obvious being the spreading of wastes, such as sewage sludges or metal rich manures from cattle, pigs or poultry. The spraying of pesticides onto crops and soils is a good example of placement although they may subsequently be further dispersed into the environment. Industrially-contaminated land with its associated demolition of old buildings and manufacturing plants, the redevelopment of sites, leakages of stored chemicals, and accumulation of wastes provides other examples of the placement of contaminants into the soil (Alloway, 2001).

Soil pollution gives rise to toxicological problems in humans, livestock, crops and ecosystems and may also cause damage to structures and services. This can happen directly through contact with the soil itself or indirectly through soil pollution causing groundwater to become contaminated and thus giving toxicological and structural problems (Alloway, 2001). Large areas of soil and groundwater in the northern part of France are contaminated by trace metals as a result of former or contemporary processing of metal ions (Godin *et al.*, 1985).

2.3 Water pollution

Water is certainly the most important substance on earth, as it is the medium through which all life has evolved. The pollution of natural waters by both biological and

chemical contaminants is a worldwide problem. Only very few areas, whether in developed or developing countries, do not suffer from some form of water pollution. Pollution of water directly affects humans through drinking water while the aquatic life (plants and animals) is also affected. There are well in excess 1500 potential pollutants discharged to freshwaters (Mason, 2000). Water can become contaminated from both natural and anthropogenic sources (Fawell and Stanifield, 2001). All surface waters contain natural organic matter in the form of humic substances, which are large complex carbohydrates. The sources of contamination fall into two categories as point and diffuse sources; industrial or waste discharges being point sources while run-off from agricultural land and from hard surfaces being diffused sources (Fawell and Stanfield, 2001). Inorganic contaminants, examples being lead, arsenic, nitrate, aluminum and fluoride, and organic contaminants such as disinfectant by-products, pesticides, endocrine disrupters, polycyclic aromatic hydrocarbons and tri- and tetra-chloroethene, are among some of the most studied water pollutants.

Among the various means of environmental pollution, heavy metals released to the environment through various sources such as metal smelters, textile, microelectronic, fertilizer and pesticide manufacturing (Ross, 1994; Prasad and Hagemeyer, 1999) play an important role. The following sections discuss the different types of pollutants, role of heavy metals as an environmental pollutant, removal of heavy metal ions from wastewater as a means of reducing environmental pollution by heavy metals and the use of chitosan in removing heavy metals from wastewater.

2.4 Heavy metals

Heavy metals belong to the group of elements described geochemically as trace elements, because they collectively comprise < 1% of the rock in the earth's crust (Alloway, 2001). These are natural constituents of the earth's crust and are present in varying concentrations in all ecosystems. All trace elements are toxic to living organisms at excessive concentrations, but some are essential for the normal growth and reproduction by either plants or animals at low, but critical, concentrations. These elements are referred to as essential trace elements or micronutrients and their deficiency may lead to diseases and even death of the plant or animal. The essential trace elements include cobalt (Co; bacteria and animals), chromium (Cr; animals), copper (Cu; plants and animals), selenium (Se; animals) and zinc (Zn; plants and animals). In addition, boron (B; plants), chlorine (Cl; plants), iron (Fe; plants and animals), iodine (I; animals) and silicon (Si; plants and animals probably) are also essential trace elements but are not dense enough to be classified as heavy metals. Other elements including silver (Ag), arsenic (As), barium (Ba), cadmium (Cd), mercury (Hg), titanium (Ti), lead (Pb) and antimony (Sb) have not been known to exert any essential function, but similar to essential trace elements cause toxicity when present above a certain tolerance level. The most important heavy metals with regard to potential hazard and occurrence in contaminated soils include As, Cd, Cu, Cr, Hg, Pb and Zn (Alloway, 2001).

Heavy metals are stable and persistent environmental contaminants since many cannot be degraded or destroyed. Therefore, they tend to accumulate in soils, seawater, freshwater, and sediments. Excessive levels of metals in the marine environment can

affect marine biota and pose risk to human consumers of seafood. Heavy metals are also known to have adverse effects on the environment and human health. However for the sake of human health and that of the natural environment, particular attention must be paid to heavy metals that persist in the environment and in the tissue of plants and animals and which bioaccumulate in the food chain (Harrison, 2001).

2.4.1 Heavy metals in environmental pollution

Heavy metals occur naturally in rocks, soils, sediments and waters, but their anthropogenic component has increased greatly since the industrial revolution. This increase has caused serious environmental problems that have affected the food chain and consequently the organisms, including humans (Forstner, 1990).

2.4.1.1 Heavy metals in the atmosphere

Heavy metals are emitted from traffic and industrial sources into the atmosphere and spread over wide regions. Although heavy metal deposition has decreased considerably during the last 20 years in many parts of the world, there are many areas where the long-term accumulation of heavy metals, often associated with exposure to sulphur dioxide, has damaged the ecosystem. For instance, there are extensive damage areas surrounding the metallurgical industry complexes in the Kola Peninsula located in the NW of Russia. Coniferous trees efficiently filter pollutant particles from the air (Salemaa *et al.*, 2001). Stand throughfall and plant litter increases the load of heavy metals and sulphur on the forest floor (Heinrich and Mayer, 1980; Derome and Nieminess, 1988; Nieminen *et al.*, 1999).

Potentially toxic metals are emitted to the atmosphere as gases, aerosols and particles. They are ingested by organisms during respiration in high concentrations in proximate areas to a source and in lesser concentrations at more distant ones. Emitted metals are carried to the ecosystem at the earth's surface via atmospheric deposition. They enter the food web from the air, mix with soils and sediments and may create a health hazard. There are natural contributions of metals to the atmosphere from volcanic eruptions, hot springs and wildfires. These come in bursts and cause temporal distortion in atmospheric contents of potentially toxic elements that are then dispersed and diluted. Volatile elements such as mercury are emitted to the atmosphere naturally from soils, buried mineral deposits or subsurface rocks with high mercury contents (Siegel, 2002).

By far the major anthropogenic loading of metals in the atmosphere is from coal and oil-burning electrical power plants as well as from coal, oil and wood used in home heating and from sulphur smelting operations. For example, Pirrone *et al.* (1996) estimated that 40% of the mercury in the atmosphere over the former USSR originated from coal combustion. The masses of metals emitted to the atmosphere from these sources are enormous (Siegel, 2002).

Potentially toxic metals from atmospheric deposition on a land surface are integrated with soil. They can become part of the recharge to aquifers or runoff into surface water. Depending on an element's mobility in soils and vegetation, the capacity to accumulate one or more metals or discriminate against their uptake, potentially toxic

metals can access the food/water web. In addition, once in soils or associated waters the metals may be moved by erosion and in solution to fluvial systems (Siegal, 2002).

2.4.1.2 Heavy metals in soil

Soil provides the nutrient-bearing environment that sustains plant growth. Essential nutrient metals and other metals in food crops are translocated through soil into the food web. Natural contents of potentially toxic elements in soils are generally low unless the soil developed from rock with high contents of one or more elements to from ore-bearing rock metal in soils that may be greatly enhanced by human activities (Siegel, 2002).

Although metals such as copper, nickel and zinc are essential nutrients for living organisms (Alloway, 1995), they may be toxic at higher concentrations. Metal content in soil is determined by the nature of the parent material and by inputs of metals from sources such as sewage sludge, mining, smelting impurities in agricultural and horticultural materials, and fossil fuel combustion. Atmospheric deposition contamination arises where sewage sludge, metal-containing pesticides or fertilizers are used extensively. Owing to its relatively high concentration of metals, sewage sludge is a major potential source of metals in agricultural soils to which it is applied to improve fertility (Smith *et al.*, 1989) and physical conditions (Pagliai *et al.,* 1981). Coniferous forests are effective for trapping heavy metal aerosols because of the high adsorption surface area that their canopies present for interception (Lindberg and Turner, 1988). The humus layer of coniferous forest soils has often been found to be effective in

retaining heavy metals, particularly lead and copper (Tyler, 1978; Bergkvist, 1987; Derome and Nieminen, 1988; Bergkvist *et al.*, 1989). Zinc, being less strongly adsorbed by organic matter, and a plant nutrient subject to uptake and cycling, tends to show a weak depth-gradient in the soil and correlation with deposition (Anderson *et* al., 1991). Heavy metals are often associated with accessory minerals in acidic igneous rocks which dominate in the boreal zone (Koljonen, 1992).

High levels of metals are usually found in superficial soils and vegetation in areas affected by mining activities, metal factories and traffic emissions. Accurate measurements of the total metal contents in polluted soils are required to assess the potential risk of these areas; however, only soluble, exchangable and chelated metal species in the soils are the labile fractions available for plants (Kabata-Pendias, 1993). Heavy metal uptake by plants in contaminated soils has been extensively studied. However, in some cases the variability of soils and plants has prevented the obtaining of a direct relationship between the total metal concentration in the soil and plants (Voutsa *et al.*, 1996; Shallari *et al.*, 1998). Contaminated soils become a secondary environmental danger in two respects: (1) contamination of crops and vegetables which are particularly important when soils are used for agriculture or gardening; and (2) contamination of drained water by metal migration in gravitational waters (Denaix *et al.*, 2001).

Many countries have established guidelines limiting the loading of potentially toxic elements to soils (Sheppard *et al.*, 1992). Potentially toxic elements can be

classified into elements posing a threat mainly to human health (e.g. cadmium, lead and mercury) and those mainly of concern as phytotoxins (e.g. boron, copper, nickel and zinc) (Alloway, 1995). Intake of food is a potential human exposure pathway particularly where much food is locally sourced or home grown on contaminated soils (Webber,1992).

2.4.1.3 Heavy metals in water

Natural waters in streams and rivers, ponds and lakes, and aquifers and springs have a chemical element content that reflects the chemistry of rocks through or over which they flow. Thus, water chemistry can vary greatly in nature with rock compositions. In addition, during flow, factors such as temperature, pH, redox potential, adsorption capacity and bacterial activity may bring about changes to the water chemistry. High values of contaminant metals in water can be natural and may represent a rock type change or the existence of mineralization. The high values can also originate from industrial activities (e.g. acid mine drainage, effluent from chemical, plastics, metal plating, wood preserving), or leachate from waste disposal sites (Siegel, 2002).

Heavy metals are released into the aqueous environment through a variety of sources such as metal smelters, effluents from plastics, textiles, microelectronics, wood preservative-producing industries, as well as through the usage of fertilizers and pesticides (Ross, 1994; Prasad and Hagemeyer, 1999). Natural waters also contain toxic metals depending upon the bed rock (Gerger, 1999).

Heavy metal contamination in aquatic systems is one of the most critical environmental issues today (Nriagu *et al.*, 1998; Silva *et al.*, 1999). Aquatic organisms may bioaccumulate anthropogenic organic compounds and trace metals from water and sediments, and it is used as an aid in the monitoring of aquatic pollutants (Phillips, 1980).

Contamination of groundwater by metal ions can occur as a result of municipal or industrial chemicals. Increased usage of groundwater resources and increase of inputs of nonpoint surface pollutants into groundwater zones may cause contamination and general deterioration of groundwater quality (Yang *et al.*, 2000).

Contamination of soils and groundwater contamination by trace metals is becoming one of the major environmental concerns because these natural resources may impact human health through the food chain. Sources of trace metals are closely related to industrial and human activities, such as smeltering, wastewater from mining and manufacturing industries and solid wastes, among others. Trace metals such as Cd, Cr, Cu, Hg, Pb and Zn are mainly considered as being responsible for soil and groundwater pollution from the viewpoints of phytotoxicity of undesirable entrance into the food chain. Trace metal levels in arable soils are similar to their natural abundance, which is relatively safe for crop growth. Metals cause an adverse effect on nutrition availability properties of the soil by changing the ionic speciation and distribution in soil solution and decreasing the buffering capacity and urease activity. Where soils are subject to metal contamination, high metal content in crops may inhibit nutrient uptake and crop growth,

thus resulting in a yield decrease. Metal contents in major crops grown in unpolluted areas have been reported to be lower than pollution standards (Yang *et al.*, 2000).

Trace metal concentration in groundwater is in general similar to the natural abundance and lower than the pollution standards due to the geochemcial or chemical characteristics in groundwater; its impacts are known to be limited. A more detailed data base is necessary to describe the current status and impact of trace metals in groundwater (Yang *et al.*, 2000).

Drinking water is derived from three basic sources: lowland rivers and reservoirs, upland reservoirs, and groundwater. Lowland surface water sources contain a high level of natural and anthropogenic organic and inorganic matter and are much more prone to pollution. They receive the highest level of treatment in order to remove microorganisms, particulate matter and chemical contaminants. Upland sources usually suffer less from anthropogenic contamination but they contain high levels of naturally occurring organic matter form decaying vegetation. This in combination with iron is the cause of brown coloration seen in many upland streams. There can also, on occasion, be microbiological contamination from farm animals and wildlife. They are usually low in inorganic constituents and are therefore soft. Groundwaters are usually low in organic matter and are less vulnerable to both microbiological and chemical contamination. They frequently contain high levels of inorganic substances from the rocks through which the water is percolated (Fawell and Stanfield, 2001).

Maximum contaminant levels (MCLs) for potentially toxic metals and other inorganic and organic components in water and foods have been set by various organizations. These entities include the World Health Organization (WHO), the Pan American Health Organization (PAHO), and the Environmental Protection Agency (EPA) in the United States and the European Health Union.Table 2.1 shows a compilation of the MCLs established for potentially toxic metals in potable water. Data are also available from global and national organizations for allowable concentration of heavy metals in the atmosphere, in agricultural soils, in food and in the workplace (Seigel, 2002).

2.4.2 Heavy metal toxicity and human health

A toxic material is a substance that has an adverse effect on health. Many chemicals could be classified as toxic, but their toxicity level may be different. The level of toxicity of substances is related to the amount that causes an adverse effect and to some extent the type of effect (Nriagu, 1988). Many chemical elements may also be essential or at least beneficial to human health, but they also can become toxic when taken in excess. Some elements may have either antagonistic or synergistic effects on the biological properties of other elements; that is, a toxic element may either be helpful in reducing the toxic effects of another, or add to its toxic effect. For example, selenium is antagonistic to mercury and reduces its toxicity. Some toxic elements are used therapeutically for specific purposes, despite their toxic risks (Ferguson, 1990).

The toxic effects of an element are measured by its dose-response relationship, where the response is the sign of an adverse effect; doses are either acute or chronic. An acute dose is a large amount of a toxic material which produces a rapid onset of effects, often intense and can result in death. A chronic dose is usually a lesser amount but continued over a longer period of time. Therefore, toxic material has a chance to build up in a gradual manner for the onset of symptoms. At times symptoms and the effects may also differ (Ferguson, 1990).

Metals have a variety of effects on the human body, mostly at the cellular level. Some metals disrupt biochemical reactions while others block essential biological processes including the adsorption of nutrients. Some metals accumulate in the body and give rise to toxic concentrations after many years of exposure while others (including arsenic, beryllium, cadmium and chromium) are carcinogens.

Exposure to methyl mercury and high levels of lead can cause gross developmental deformities (Friberg *et al.*, 1986). Exposure to high levels of mercury, gold and lead has also been associated with the development of autoimmunity, in which the immune system starts to attack its own cells mistaking them for foreign invaders. Lead is particularly toxic to the brain, kidneys, reproductive and cardiovascular systems. Exposure can cause impairment in intellectual functioning, kidney damage, infertility, miscarriage and hypertension (Glover-Kerkvlient, 1995). Environmental lead exposure has been linked to a reduced IQ in children and to elevated blood pressure in adults, although the cause-effect relationship for the latter is not especially strong (Hare, 1986).

Table 2.1 Maximum contaminant levels (also, action levels or maximum allowable concentrations) for potentially toxic metals in drinking water[1].

Metal ion	Content in water (mg/L)	Content in air ($\mu g/m^3$)
As	0.01	0.01
Be	0.004	0.01
Cd	0.003	0.001-0.005*; 0.01-0.02**
Co	1.0	10
Cr	0.05	100
Cu	1.0	0.012*; 0.257**
Fe	0.2	6000
Hg	0.001	0.01-0.02
Mn	0.05	0.05*; 0.3**
Mo	0.04	0.1-3.2* ;10-30 **
Ni	0.02	0.002*; 0.15 **
Pb	0.0015	1-2
Sb	0.005	0.2-2
Se	0.01	0.02-0.07
Tl	0.002	0
Zn	5.0	NI

[1]Complied from several sources such as WHO, EPA, EC and others. Contents in air are given for some of the metals in concentrations as noted.
*Rural.
**Urban.
NI, not issued by health authorities

Adapted from Siegel (2002)

2.4.3 Analytical procedures for determination of heavy metals

The essence of speciation studies is to identify and quantify the many species that make up the heavy metal concentration. This may further lead to the understanding of the biological and agrochemical cycling of the elements of concern. Biological cycling involves the transport, adsorption, and precipitation of the element in the water system. There are two main techniques that have been applied in heavy metal monitoring, normally biological and chemical methods.

Biological monitoring involves surveillance by using the response of living organisms to the environment. This is carried out with the use of indicator species. Indicator species are those species or species assembly that have particular requirements for a known set of physical or chemical variables (Hellawell, 1986). Some of the desirable characteristics of indicator species used in biological monitoring include narrow and specific environmental tolerances, taxonomic soundness and composition distribution variability, well known ecological characteristics and suitability for experimental studies. Some forty years ago, colorimetry, spectrography and polarography were the dominating methods for quantitative determination of metals. Atomic absorption spectrophotometry was then in the introduction stages for metal analysis (Papp, 1994). The direct analytical techniques that have been successfully utilized in trace metal speciation include atomic absorption spectroscopy (AAS), inductively coupled plasma mass-spectrometry (ICP-MS) and electrochemical techniques such as anodic or cathodic stripping voltametry (ASV-CSV) (Florence, 1989; Papp, 1994).

2.4.3.1 Spectroscopic methods

Colorimetry and adsorption spectrophotometry colorimetry, the first modern method to be developed for metal analysis, is based on the formation of coloured complexes between metals and organic compounds, whose absorbance can be measured in a colorimeter or spectrophotometer, after their extraction into a suitable solvent. (Piscator and Vouk, 979). Colorimetric methods still have many applications as the more modern methods are not suitable, or in some cases have not yet been worked out. For some metals, the limitations of colorimetric assays are the labour intensive preparatory work required to isolate specific complexes (Taylor and Pollard, 1994). Such work is expensive, time-consuming and entails a high risk of contamination, as well as occupational hazard to the analysts. Accuracy and precision, especially when several manual sample preparation steps are involved, will depend on the analyst's skill (Taylor and Pollard, 1994).

2.4.3.2 Atomic emission spectroscopy

Atomic emission spectroscopy is based on the extraction of metals by flame or by electric discharge. The excited atoms then lose their acquired energy by emitting photons on returining to their ground state. This emission can be recorded on a spectrometer or spectrograph which has a monochromator that separates the emitted radiation according to wavelengths (i.e. the energy of photons). A spectrum is obtained and a very wide range of metals can be more or less quantitatively determined. Atomic emission spectroscopy is especially useful for multielement analysis. Atomic emission

spectroscopy is also used in the metal industry for quality control of products (Piscator and Vouk, 1979; Pollard, 1994).

2.4.3.3 Atomic absorption spectroscopy (AAS)

The basic principle behind AAS is that metals in the ground state will absorb radiation from a light beam with the same spectral composition as the light emitted by the element in consideration. The decrease in intensity as compared to a blank corresponds to the concentration of the metal. There are two main methods for atomization of samples: the flame method and the furnace method, the latter also known as electrothermal atomic absorption. Atomic absorption spectroscopy has found a number of applications and has been especially useful for the determination of Pb, Cd and Hg in biological materials and in exposure media as well as for the determination of many essential elements (Papp, 1994; Piscator and Vouk, 1979). Special modifications are required for highly volatile metals such as Hg and As (Pistor and Vouk, 1979). Atomic absorption spectroscopy has been used to demonstrate the ability to quantify chromium in a broad range of material at concentrations ranging from parts per hundred (i:e. percent) to parts per billion (Kaltz and Salem, 1994). One limitation of atomic absorption spectroscopy is that atomic absorption is not suitable for multielement analysis. Also, some non-specific absorption may occur due to the presence of other atoms and molecules in the flame salts such as sodium chloride and phosphate (Piscator and Vouk, 1979). Atomic absorption spectroscopy, however, is facing stiff competition from some newer techniques such as plasma emission and ICP-MS. As these techniques reach maturity, AAS, in particular flame AAS, may be relegated to a complementary role (Papp, 1994).

2.4.3.4 Inductively coupled plasma-mass spectrometry(ICP-MS)

This technique in heavy metal multielement analysis involves ionization of the analyte elements present in a sample in the ICP. In this method, freshwater samples are preferably filtered using disposable filters and acidified with high purity 0.2M HNO_3 and then placed in a clean sample container. The ions are then extracted into a MS, separated and detected. By scanning through the mass range within milliseconds, the ICP-MS acts as a highly sensitive multielemental technique allowing determination of about 30 elements per minute with detection limits of 0.1 to 10 µg/L (Kishi, 1997). The ICP has found applications as an ion source for mass spectrometery, and the ICP- MS technique has been employed for the determination of trace metals in biological and environmental materials (Kartz and Salem, 1994).

2.4.3.5 Voltammetry

This is an electroanalytical technique in which the current at an electrode is measured as a function of the potential, or voltage applied at the electrode. The potential is varied in some systematic manner and the resulting current-potential plot is called a voltammogram. This can be used to analyze any chemical species that is electroactive; hence, its applicability in heavy metal speciation of stripping voltammetry (Taylor, 1994). Stripping voltammetry can be carried out in two ways, namely cathode stripping voltammetry (CSV) and anode stripping voltammetry (ASV). Anodic stripping voltammetry is probably the most sensitive technique for Pb in very low levels and avoidance of contamination (Taylor, 1994). Advantages of ASV are the possibility of

simultaneous determination of four metals (Pb, Cd, Cu, Zn) in one sample (Harrison and Laxen, 1981).

2.5 Removal of metals from wastewater

The presence of toxic substances in wastewater has always been a matter of concern. This concern has become much more pressing with the intentional or unintentional release of an ever-larger variety of substances into the environment. The treatment of wastes containing toxic material may be technically difficult and/or expensive. Manufacturers or privatized water utilities may be reluctant to make large capital investments in wastewater treatment facilities (James, 2001).

Discharge of metals to the aquatic environment has been a major cause of concern and the treatment of these wastes has consequently attracted considerable attention. Wastes containing metals may arise from a variety of industrial and agricultural operations including tanneries, paint manufacture, battery manufacture, and pig wastes; however, but the main source being from metal processing. The sources of waste in metal processing are numerous and also extremely variable both in quantity and quality. Metals in the wastes may occur as large particles of pure metals in suspension or as metallic ions and complexes in solution. The technique most commonly employed in treating metal processing wastes is precipitation using pH adjustment (James, 2001).

Conventional methods for the removal of metals from industrial waste solution such as chemical precipitation or oxidation, filtration, electrochemical treatment, ion

exchange, application of membrane technology and evaporation recovery may be ineffective and expensive, especially when metals are present at low concentrations (Volseky, 1 987). A part from t heir e conomical and e cological d isadvantages, o nly i on exchange is able to remove trace levels of metal ions (Deans and Dixon, 1992). However, the effectiveness of ion exchange in removing metal ions from aqueous media is also suppressed by the presence of large quantities of metals ions from aqueous systems by various hydrophytic species (Moffat,1995). The vulnerability of these hyperaccumulators to high levels of toxic metal has ruled out the possibility of using them to clean wastewaters with high levels of heavy metals.

The reclamation of wastewaters through a process known as chelating ion exchange seems to be a better alternative to the existing remediation technologies. In contrast to simple ion exchange, chelating ion exchange permits the removal of the heavier toxic metals ions while permitting harmless ions to move into the environment (Deans and Dixon, 1992). Some of the best chelation exchange materials are biopolymers. Cellulose, alginates, proteins, chitin and chitin derivatives, which are particularly effective in adsorbing metals ions due to the presence of several functional groups such as -OH and -NH_2 that can reduce metal ion concentrations to the order of μg per gram from mg per gram levels (Kurita *et al.*, 1979; Deans and Dickson, 1992). Several investigators have reported the heavy metal binding capacities of chitin and chitosan (Muzzarelli *et al.*, 1989; Bassi *et al.*, 1999; Lasko and Hurst, 1999).

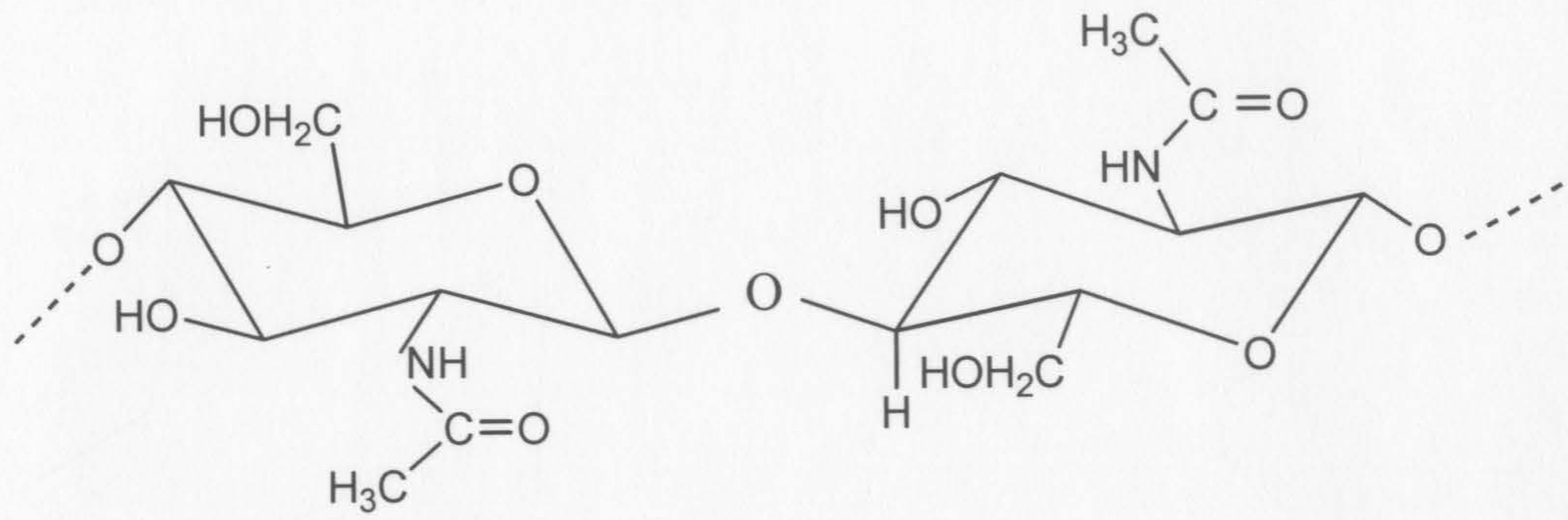
2.6 Chitin and chitosan

The name "chitin" is derived from the Greek word "Chiton" meaning a coat of nail (Lower, 1984). It was apparently first used in 1811 by French scientist Henri Braconnot (Muzzarelli, 1977; Brine, 1984), who isolated the substance from mushrooms. In 1823, Odier found this same compound in the cuticle of insects (Muzzarellli, 1977). Chitin is the major component of the exoskeleton of invertebrates, crustaceans, insects and the cell wall of fungi and yeasts (Knorr, 1984; Lower, 1984; Tan *et al.*, 1996). It 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, 1984). These units are linked together by β (1-4) glycosidic bonds (Ornum, 1992; Simpson *et al.*, 1994). Chitin is also known as *N*-acetyl-D-glucosamine polymer and is one of the most abundant polysaccharide that contains amino sugars (Bough, 1977; Austin *et al.*, 1981; Kumar and Jayachandran, 1993). Chitosan is the name used for the low acetyl substitute form of chitin. Chitosan polymers are composed of primarily of glucosamine, 2-amino-deoxy-β-D-glucose, known as (1→4)-2-amino-2-deoxy-β-D-glucose (Shahidi *et al.*, 1999) (Figure 2.1). Chitosan is derived from chitin by deacetylation in the presence of alkali which is a copolymer of glucosamine and some *N*-acetylglucosamine residues, of which the proportion of the former usually exceeds 80% (Arvanoyannis *et al.*, 1998). Chitosan was first discovered by Rouget in 1859 (Muzzarelli, 1977) when he boiled chitin in a concentrated solution of potassium hydroxide.

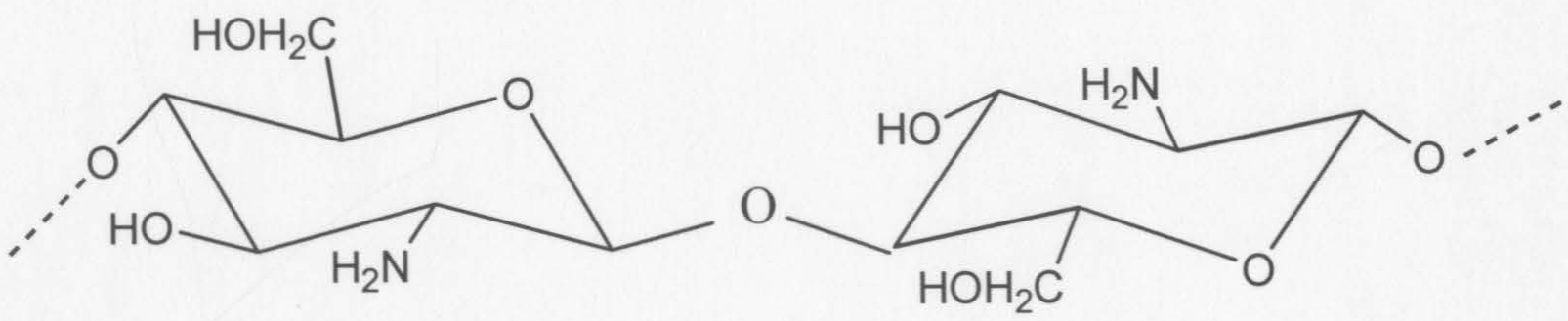
Chitosan is the simplest derivative that can be prepared from chitin and is the least expensive usable derivative of it (Ornum, 1992). Unlike most polysaccharides, the presence of positively charged amino groups regularly located along a polymer chain of chitosan allows the molecule to bind to negatively charged surfaces via ionic or hydrogen bonding (Muzzarelli, 1973; Rha, 1984; Shahidi, 1995).

Research on chitin and chitosan accelerated in the early to mid 1900's and the first patent pertaining to chitin and chitosan was granted in the 1930s to G.W. Rigby, an employee of the Du Pont de Nemours and Company in the United States (Winterowd and Stanford, 1995). However, commercialization of chitin-based products was hampered by a lack of adequate manufacturing facilities and competition from synthetic polymers (Averbach, 1981; Subasinghe, 1999). However after the 1970s, industrial exploitation of chitin and its derivatives has been emerging and expanding (Kaye, 1985) (Figure 2.2). Furthermore, advancement in research and small-scale production of chitin and chitosan have expanded the number and variety of potential applications such as medical, cosmetic, agricultural and food-related uses (Winterword and Sandford, 1995). Both chitin and chitosan have been identified as being biocompatible, non-antigenic, almost non-toxic and biofunctional (Hirano *et al.*, 1990; Li *et al.*, 1992). The United States Food and Drug Administration (FDA) approved chitosan as a feed additive in 1983. Use of chitosan for potable water purification has been approved by the United States Environmental Protection Agency (EPA) up to a maximum level of 10 mg/L (Knorr, 1986). In addition, environmental problems and cost for disposal of shellfish processing discard have

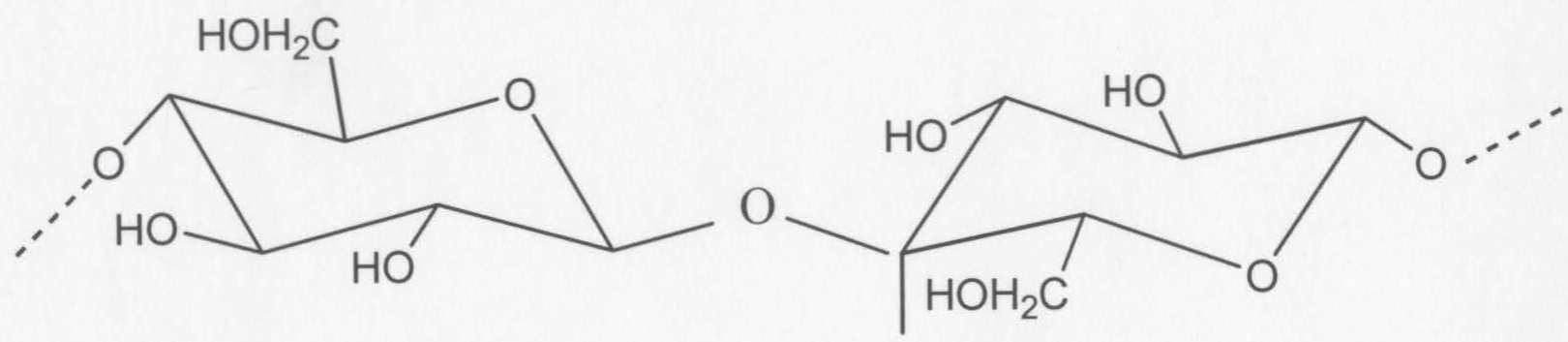
Figure 2.1 Chemical structures of chitin, chitosan and cellulose



Chitin

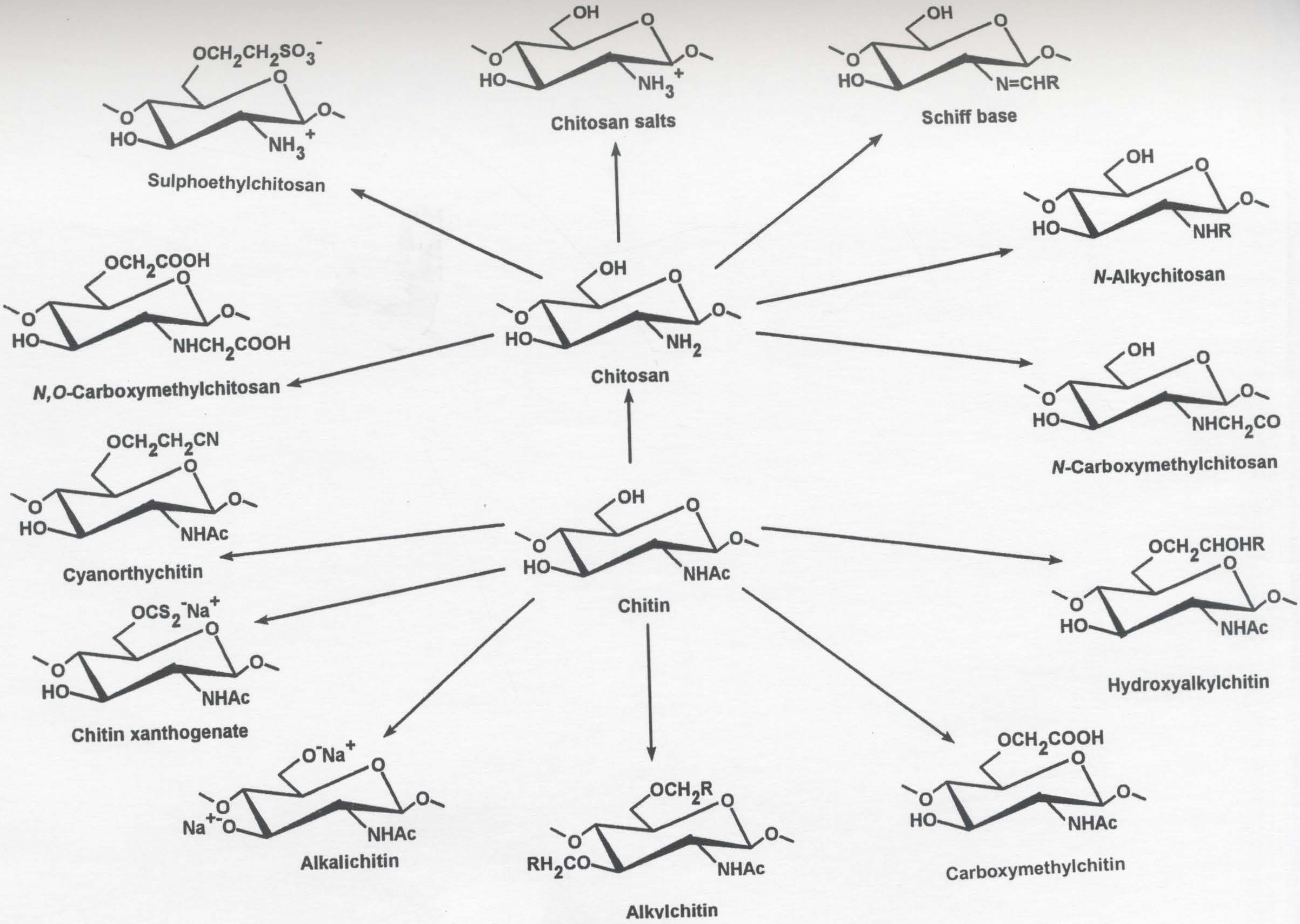


Chitosan



Cellulose

Figure 2.2 Chemical derivatives of chitin and chitosan adapted from Peter (1995)


Sulphoethylchitosan
Chitosan salts
Schiff base
N-Alkychitosan
N,O-Carboxymethylchitosan
Chitosan
N-Carboxymethylchitosan
Cyanorthychitin
Chitin
Hydroxyalkylchitin
Chitin xanthogenate
Alkalichitin
Alkylchitin
Carboxymethylchitin

become a pressing problem. On the other hand environmentally safe substitutes for many plastic or polymeric products are in demand (Ashford *et al.*, 1976; Berkeley, 1979; Shahidi and Synowiecki, 1991). Although not always accessible, the enormous abundance of chitin and its unusual chemical properties which can be readily modified, are factors underlying the current interest in developing its commercial potential (Winterowd and Sandford, 1995).

2.7 Sources of chitin and chitosan

Chitin is a waste product of the seafood processing industry with an estimated volume of 100,000 metric tonnes annually on a worldwide basis (Muzzarelli, 1999). Nearly 10% of the global landings of sea products consist of species rich in chitinous materials (Subasninghe, 1999). These include, species such as shrimp, crab, lobster, squid, krill, and crawfish, among others. The annual production of species containing chitin is approximately 13 million metric tonnes (FAO, 1999). Chitin occurs in association with protein and inorganic materials, mainly $CaCO_3$, pigments and lipids (Austin *et al.*, 1981; Knorr, 1984; Gopalkumar, 1997) in the exoskeleton of crustaceans, insects and mollusks (Knorr, 1984; Lower, 1984). Chitin constitutes more than half of the total organic matter in chitinous structures. Higher concentrations of up to 85% are found in arthropods which are able to synthesize chitin. There is no apparent relationship between the proportion of chitin and the degree of calcification, hardness or flexibility of the structure, as shells of gastropoda and lamellibranchia contain only small amounts of chitin (Muzzarelli, 1977). Certain fungi, algae, diatoms and protozoa also contain chitin and sometimes chitosan as cell wall and cellular constituents (Berkeley, 1979; Austin *et*

al., 1981). The body parts and processing discards of crustaceans, insects and mollusks 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 chitosanase must play a significant role in biochemical cycling of limiting elements including nitrogen (Bade and Wick, 1988). The biosynthesis of chitin takes place in the membrane-bound protein complex via the catalytic action of chitin synthase (EC 2.4.1.16) and by the addition of uridine diphosphate-*N*-acetyl-D-glucosamine to acceptors such as *N*-acetylglucosamine (G/CNAC) to afford *N*-acetylchitooligosaccharide. Chitin synthase has been found in crustaceans, insects and microorganisms (Koga, 1998).

The natural pathway of chitin metabolism includes enzyme-catalyzed hydrolysis by chitinases (Peter, 1995) to *N*-acetyl-chitooligosaccharide in an endo- or random-splitting fashion. This enzyme is found in many organisms, and plays a characteristic role in higher plants, seaweeds and mammals, insects and crustaceans, cell organisms, as well as spiders and snails (Koga, 1998). The chitinases (EC 3.2.1.14) give *N,N'*-diacetylchitobiose and *N,N',N''*-triacetylchitotriose as the final products (Peter, 1995). Chitin-*N*-deacetylase (EC 3.5.1.41) catalyses the *N*-deacetylation reaction of chitin into chitosan (Muzzarelli, 1977; Cohen, 1993; Hirano, 1996). Chitosanase (EC 3.2.1.132) hydrolyzes chitosan similarly to chitinase. This enzyme has been found mainly in microorganisms and is limited in certain plant such as orange (Koga, 1998).

Table 2.2 Chitin content of selected crustaceans and molluscan organs.

Organism	Chitin content, %
Crustaceans	
Crab	
Blue crab	14.9[a]
Stone crab	18.1[a]
Red crab	27.6[a]
Horseshoe crab	26.4[a]
Snow crab-claws	24.0[b]
Snow crab-legs	32.0[b]
Shrimp	
Brine shrimp	27.2[a]
Tiger shrimp-carapace	35[b]
Tiger shrimp-shell	37[b]
Molluscan organs	
Squid pen (β chitin)	30-35[b]
Krill-crude deproteinnized shell	34-49[b]
Krill-dried concentrated waste	25[b]
Clamshell	6.1[c]
Oyster shell	3.6[c]

All values are on a dry weight basis.
Data adapted from [a]Austin *et al*. (1981); [b]Knorr (1984); and [c]Subasinghe (1999).

Diatoms which have protein-free chitin appendages are conceivable practical sources of very pure chitin. For special applications, requiring exceptionally high quality and pure chitin in relatively small quantities, diatoms might eventually serve as a viable source (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 (Bartinicki-Garcia, 1968; Gopakumar, 1997). In addition, 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). It is estimated that fungi could provide 3.2×10^4 metric tonnes of chitin annually (Brine, 1984). Chitosan occurs naturally in fermented foods such as tempeh, tofu and even in aged beef, and is already part of the diet of 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 ad Synowiecki, 1991; Martin, 1998). The techniques which are available for disposal of processing by-products include ocean dumping, incineration or disposal to landfill sites (Revah-Moiseer and Carroas, 1981; Shahidi, 1994). Pollution from wastes has become a serious problem because solid waste disposal and discharge of processing discards are tightened by regulation and are quite costly in most places (Shoemaker, 1991; Martin, 1998). However, environmental restrictions and a better understanding of the potential value of applications have resulted

in efforts to find uses for these materials (Healy *et al.*, 1994). Direct use of crab wastes for land manuring or spreading is generally discouraged by the uniquely obnoxious odour of putrefying shells. Therefore, value-added utilization of chitin, chitosan and their derivatives in different fields is of utmost interest (Brzeski, 1987; Shahidi *et al.*, 1999). The constituents of crustacean shells include approximately 13-42% protein and 14-30% chitin (Shahidi and Synowiecki, 1991; Ferrer *et al.*, 1996).

2.8 Preparation of chitin and chitosan from shellfish processing discards

A variety of procedures have been developed over the years for the preparation of chitin and chitosan. Some of these form the basis of chemical processes for industrial production of chitosan from crab, shrimp, lobster or crawfish, which are the richest sources of chitin (in general 20-30% on dry basis) as well as being the only chitinous matter presently available in quantities sufficient to support a commercial chitin/chitosan industry (Johnson and Peniston, 1982; Ornum, 1992; Selmer-Olsen, 1996). Crustacean shell waste consists mainly of protein (30-40%), minerals (30-50%) and chitin (30-40%). These proportions may vary among species and with the season (Green and Kramer, 1984). Thus, the methods of chitin/chitosan preparation can vary with sources in order to address compositional differences. Further, physical and chemical characteristics of chitin and chitosan accordingly differ with species and preparation method (Brine and Austin, 1981).

2.8.1 Preparation of chitin from shellfish processing discards

Shellfish processing discards, chitin is associated with proteins, lipids, pigments and mineral deposits (Roberts, 1992; Simpson *et al.*, 1994). Therefore, the raw material has to be pretreated to remove non-chitionous components (Brzeski, 1987; Simpson *et al.*, 1994). The entire manufacturing process may be divided into four different steps: (1) preparation of sample, (2) removal of mineral fraction or demineralization, (3) removal protein fraction or deproteinization, (4) and 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 that the highest molecular weight chitin/chitosan is obtained (Ornum, 1992). Nevertheless with careful control of the 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 can be conducted in a reverse order, i.e. demineralization, followed by deproteinization. However, if protein recovery is an objective, its extraction before demineralization is preferred so as to maximize protein yield and quality (Johnson and Peniston, 1982). Demineralization is achieved using a dilute HCl solution (Shahidi, 1995). The 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 (Shahidi, 1995). The yield of chitin varies depending on the raw materials and species used. Yield of crawfish shell portion and whole meal were 25-28% and 17% respectively (No *et al.*, 1989). Crab waste contains 17-32% chitin whereas squid pen contains 30-35% chitin on a dry weight basis (Knorr, 1984; Shahidi and Synowiecki, 1991).

2.8.1.1 Preparation of raw materials

Washing of shellfish processing discards is important for the removal of soluble organics and adhered proteins whereas 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 potential colorant and possibly a flavourant in food products (Simpson and Haard, 1985). Extraction of carotenoid pigments with organic solvents or oil achieves a good recovery, but the product so obtained is devoid of protein and hence has decreased stability due to oxidation (Haard, 1992). 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 discards; the best recovery of carotenoids (74%) was achieved when the ratio of discards to oil was 1:2 (w/v) and the extraction temperature was adjusted to 60˚C.

2.8.1.2 Deproteinization

Crustacean shell waste is usually ground and treated with a dilute NaOH solution (1-10%) at elevated temperatures (65-100˚C) in order to dissolve the protein present. Deproteinzation may be achieved using different alkali concentrations, time, temperature and solid-to-solvent ratios (No and Mayers, 1995). Nevertheless, a large variation exists for deproteinzation conditions (Roberts, 1992).

Hackman (1954) extracted protein from lobster shells with 1M NaOH at 100˚C. Anderson *et al.* (1978) also treated shrimp shells with 3% NaOH at 100˚ C. No *et al.* (1989) extracted protein from crawfish shell waste with 3.5% NaOH at 65˚C. Optimal deprotienization also can be accomplished by treatment with 1% KOH for shrimp and 2% KOH for crab shell at 90˚C (Shahidi and Synowiecki, 1991). Exceptions to the above are seen in methods of Whistler and BeMiller (1962) and Brzeski (1982) involving deproteinization with 10% NaOH at room temperature, and with 3.5% NaOH at 25˚C, respectively. Cosio *et al.* (1982) carried out the deproteinization process at pH 11.5 and 30˚C. Reaction time usually ranges from 0.5 to 12 h depending on the preparation method, although Hackman (1954) and Whistler and BeMiller (1962) attempted to extract proteins for several days. Prolonged alkaline treatment under several conditions for isolation of proteins, from the carapace of horseshoe crab (*Limulus polyphemus*), which contained a low amount of protein was considered. Austin *et al.* (1981) indicated that some other chitin, which may require harsh acidic or temperature conditions for to 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 treatments include the requirement of large amounts of alkali which results in deacetylation and a decrease in the molecular size of the product explained above (Simpson *et al.*, 1994).

A number of researchers have attempted to remove proteins from shellfish by enzymatic digestion for production of chitin that has been minimally deacetylated. Commercially-available enzymes from plant and animal sources have also been used (Muzzarelli, 1973). Possibility of using the enzyme Rhozyme-62 at 60°C for 6 h at pH 7.0 for deproteinization of crustaceans shell waste was described by Bough *et al.* (1978). Simpson *et al.* (1994) reported that out of various proteolytic enzymes, chymotrypsin, papain and bacterial protease best served deproteinization of crustacean shells. Chymotrypsin was most effective, achieving a degree of deproteinization comparable to that of chemical deprotienzation. Enzymatic deproteinization of the shells for the production of protein hydrolysate during isolation of chitin from shrimp (*Crangon crangon*) processing discards was recently reported (Synoweicki and Al-Khateeb, 2000). This method was found suitable for isolation of chitin containing 4% protein impurities. The hydrolysate so obtained had an adequate essential amino acid index and protein efficiency ratio. Enzyme treatment did not affect the chitosan structure, however, complete removal of protein was not achieved (No and Meyers, 1995).

The recovered protein can be used as high-grade additive to livestock starter feed, thus resulting in a decreased manufacturing cost of chitin (Johnson and Peniston, 1982).

The profile of amino acids recovered from shrimp and crab processing discards were favourable, except for lysine and tryptophan (Shahidi and Synowiecki, 1991).

2.8.1.3 Demineralization

Demineralization is conventionally accomplished by extraction with dilute HCl (up to 10%) at room temperature in order to dissolve the calcium carbonate and provide calcium chloride (No and Meyers, 1995). Demineralization is also important for the removal of endotoxins which are present in the shell processing discards (Roberts, 1992). Demineralization can 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 ratio (No and Meyers, 1995). However, Chen and Tsai (1998) demonstrated that only the HCl concentration and solution-to-solid ratio were important whereas the effect of temperature for demineralization of shrimp shell waste was unimportant. Shahidi and Synowiecki (1991) observed that ash content of deproteinzed shell waste from shrimp and crab on a dry weight basis was 42 and 45%, respectively. After 30 min of demineralization at 20°C [2.55% HCl solution, of shell to acid ratio of 1:20 (w/v)], the ash content in chitin from shrimp and crab discards decreased to 0.10 and 0.25%, respectively. One of the disadvantages of using HCl for demineralization is that HCl at concentrations above 1.25 M adversely affects the viscosity of the final product due to depolymerization of the chitin chain (Muzzarelli, 1977). To avoid this, certain methods have been developed using mild acids to minimize degradation. Austin *et al.* (1981) used ethylenediaminetetraacetic acid (EDTA) for decalcification of crustacean shells.

Demineralization can usually be achieved in 2 to 3 h with proper agitation (Johnson and Peniston, 1982). A prolonged demineralization step, even 24 h, results in only a very slight drop in the ash content, but can bring about polymer degradation (Brzeski, 1982). For demineralization, it is also important that the amount of acid be stoichiometrically equal or greater than all minerals present in the shells to ensure complete reaction (Johnson and Peniston, 1982; Shahidi and Synowiecki, 1991).

2.8.1.4 Decoloration

Decoloration is an important unit operation for the production of a white coloured chitin with added commercial value. The exoskeleton of crustaceans contains coloured matters, principally carotenoids, the main components being astaxanthin, astacene, canthaxanthin, lutein and β-carotene (Roberts, 1992). They do not appear to be complexed with either the inorganic material or the protein since treatments which remove these components do not remove carotenoids (Roberts, 1992). There are two steps involved in decoloration of shellfish processing discards: they are extraction of pigments using the necessary reagents and bleaching using appropriate chemicals. Decoloration of chitin can 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) when 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).

2.8.2 Production of chitosan from chitin

Chitosan is a natural product derived from chitin. 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 can in principle be hydrolyzed 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 (Muzarelli, 1977; Roberts, 1992). Horton and Lineback (1965) reported that 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.

Conversion of chitin to chitosan is achieved by treatment with concentrated NaOH or KOH solution (40-50%) generally at 100˚C or higher. Deacetylation of chitin can be achieved with different reagents, concentrations, time and temperature as well as the ratio of the weight of raw material to the volume of the extraction solution (Table 2.3). Other factors affecting the extent of deacetylation include concentration of the alkali, temperature of deacetylation, time of reaction, previous treatment of the chitin, particle size and density of the chitin (No and Meyers, 1995).

Some limitations to this chemical method are high energy cost, use of high volumes of concentrated NaOH and production of a liquid waste containing protein and non-protein nitrogenous compounds (Simpson *et al.*, 1994; Hirano, 1996). Deaceytlation may also be achieved by biological means using chitin deacetylase-producing

microorganisms such as *Mucor rouxi*, *M. meehei*, *Absidia butleri* and *Aspergillus niger* (Haard *et al.*, 1994; Simpson *et al.*, 1994). Some problems associated with enzymatic methods are low yield and variation with age of fungal culture (Simpson *et al.*, 1994).

Mima *et al.* (1983) established a method for the preparation of chitosan products 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 and using alkali treatment for less than 5 h in 47% NaOH at 110˚C. Application of thermo-mechano-chemical technology to chitin deacetylation was evaluated by Pelletier *et al.* (1990) as an alternative method for chitosan production. This process consists of a cascade reactor unit opening under reduced alkaline conditions of 10% (w/v) NaOH. Sudden decompression of the aqueous alkaline suspension of mercerized chitin (at 4˚C for 24 h) resulted in a near complete deacytylation of chitin (Pelletier *et al.*, 1990). Chitosan is sold commercially in the form of a solution, flake and fine powder, and more recently in bead and fibre forms (Kumar and Jayachanndran, 1993).

2.9 Physiochemical properties of chitin and chitosan

2.9.1 Molecular structures

Chitin and chitosan are known to exhibit polymorphism (Muzzarelli, 1977). Chitin exists in three polymorphic forms with various degrees of crystallinity (Muzzarelli, 1985). These crystalline structures of chitin are evidenced by x-ray diffraction studies which

Table 2.3 Deacetylation conditions in chitosan production.

Source	Atmosphere	Alkali (%)	Temp (°C)	Time (h)	Ratio w/w or w/v)	Deacetylation %
Lobster	A/N[a]	KOH, 35	40-100	0.5-15	1:100	65-81.2
Crab	N	NaOH, 47	60	2x1-4*	-	57-90
	A		110	1x1-3*	-	78-96
				1x3**	-	99
		KOH, 39[b]	reflux	20	1:17	-
Shrimp	N	NaOH, 50	100	0.5-5	-	68-78
	N	NaOH, 50	145-150	1/12	1:10	-
	A	NaOH, 50	60	2***	1:4	-
Krill	A	NaOH, 50	80-96	1/3	-	56-68
	A	KOH, 39[b]	reflux	20	1:17	-
Squid	N	NaOH, 40	80	3x1-3*	1:20	80-97

[a]A = air, N = nitrogen.
[b]dissolved in 95% ethanol
*times separate alkali treatment and additional alkali treatment after the transformation of the sample form into thread-like pieces
***dry heating.
(Adapted from No and Meyers, 1995).

Table 2.4 Characteristics of chitin.

Specification	Description	Reference
Nitrogen (%)	7.01[a]	No *et al*. (1989)
	6.14-6.96	Brezski (1982)
	6.29, 6.42	Shahidi and Synowiecki (1991)
	6.27	Synowiecki *et al*. (1981)
Fat (%)	0.1-0.2	Brezski (1982)
Ash (%)	0.1	No *et al*. (1989)
	up to 3	Brezski (1982)
	<0.17	Mima *et al*. (1983)
	0.09, 0.10	Shahidi and Synowiecki (1991)
	0.06	Synowiecki *et al*. (1981)
Acetyl value (%)	19.6	No *et al*. (1989)
	15.9-19.7	Brine and Austin (1981)
Deacytylation (%)	7.5	No *et al*. (1989)
	42-58	Brezski (1982)
	0.08	Kurita *et al*. (1993)
Solubility (%)[b]	26.4	No *et al*. (1989)
	28-99	Brine and Austin (1981)
Optical rotation[b]	-38-+48	Brine and Austin (1981)
Colour	White	No *et al*. (1989)
		Mima *et al*. (1983)
		Kurita *et al*. (1993)
	Creamy white to light pink	Brezski (1989)
Residual amino acids (mg/g)	6.5	No *et al*. (1989)
	58.91-47.21	Shahidi and Synowiecki (1991)
Molecular weight (Da)	0.56-19.6 x 10^{6}[b]	Brine and Austin (1981)

[a] calculated in moisture free basis

[b] measured in *N*,*N*-dimethylacetamide containing 5% LiCl (DMAC-5% LiCl).

Table 2.5 Characteristics of chitosan

Specification	Amount	Reference
Nitrogen, (%)	7.06-7.63	Brezski (1982)
	7.80[a]-7.88[a]	Anderson *et al*. (1978)
	7.74-7.97	Bough *et al*. (1978)
Ash, (%)	<1	Brezski (1982); Bough *et al*. (1978)
	<0.01	Alimuniar and Zainuddin, (1992)
Colour	White	Anderson *et al*. (1978); Alimuniar and Zainuddin (1992)
	Pinky white	Brezski (1982)
	Tan	Anderson *et al*. (1978)
Molecular weight, (Da)	> 5.0×10^5	Mima *et al*. (1983)
	$0.7\text{-}1.5 \times 10^6$	Bough et al. (1978)

[a] corrected for ash content.

show three polymorphic forms of α-chitin, β-chitin, γ- chitin (Roberts, 1992; Shimojoh, 1998). The chitin of insect and crustacean cuticle occurs in the form of microfibrills typically 10-25 nm in diameter and 2-3 μm in length. These natural forms of chitin are known to differ from each other in orientation of the polysaccharide chains within the microfibrills, namely α- (antiparallel), β- (two parallel, one antiparallel) γ- (random orientation) chitin. The most abundant form is α-chitin (Peter, 1995). Generally, the individual chains assume an essentially linear structure which undergoes one full twist every 10.1 – 10.5 Å units along the chain axis (Winterowed and Sandford, 1995). Because each glycosidic unit in the chain is chiral and all units are connected by an oxygen atom that links C(1) of one glycosidic unit to C(4) of an adjacent unit, a distinct "left" and "right" direction can be assigned to each polymer chain (Muzzarelli, 1977; Roberts, 1992). Chitin and chitosan are internally cross-linked to a much greater degree than cellulose; each *N*-acetylglucosamine residue in chitins 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). Figure 2.3 shows an illustration of the parallel and antiparallel polymer arrangement of chitin.

As 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 pen 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). According to Bade (1997),

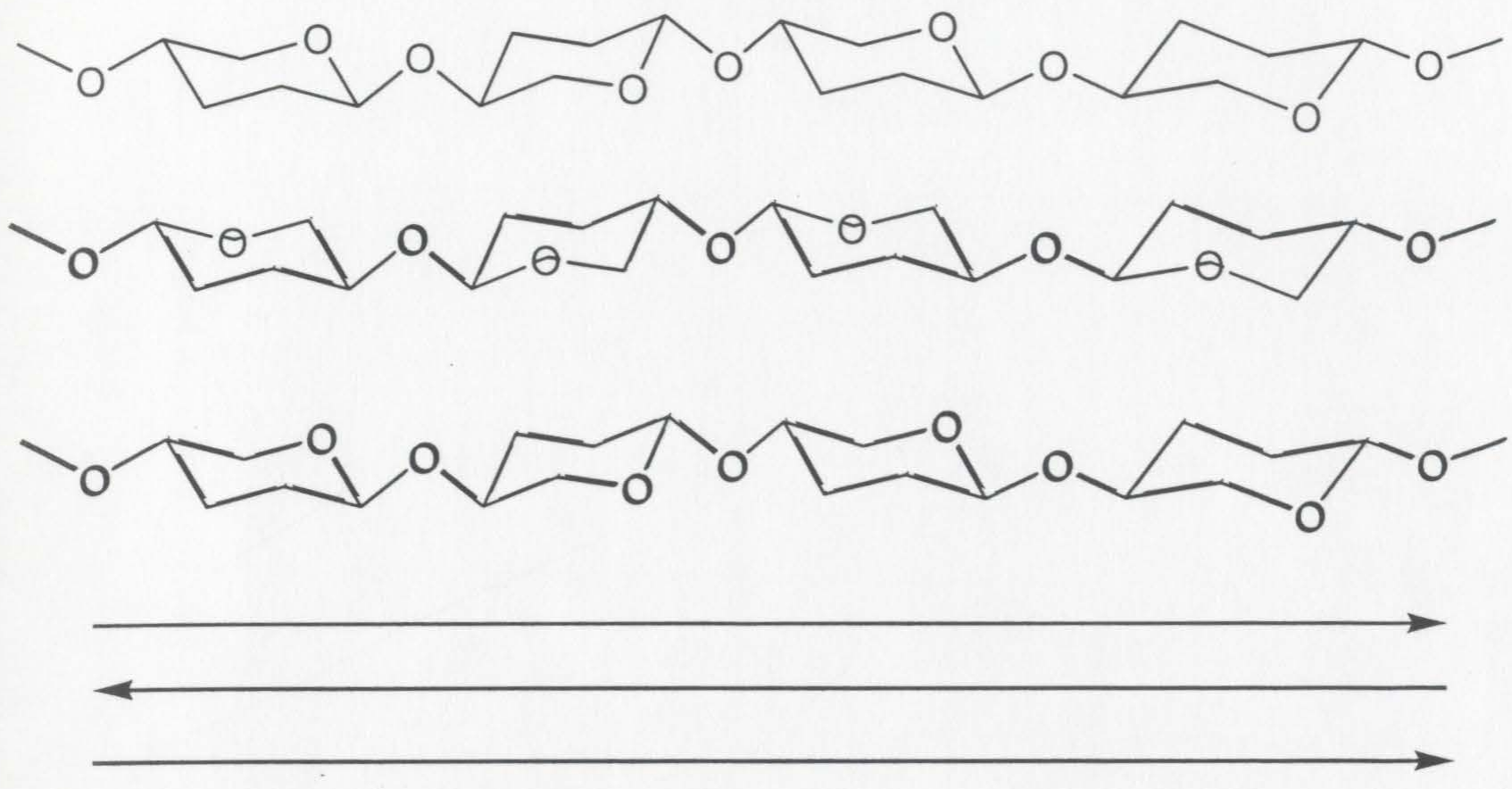
depending on these chain arrangements, there are four different orders of chitin present in nature, such as linear primary structure in which monomers are linked to each other by covalent β-(1-4)-glycosidic bonds, three dimensional secondary structure which consists of primary chitin chains linked to form microfibrills,tertiary and quarternary structures with macrofibrills and sheets, respectively. Differences between α-chitin and β-chitin are listed in Table 2.6.

2.9.2 Molecular weight of chitin and chitosan

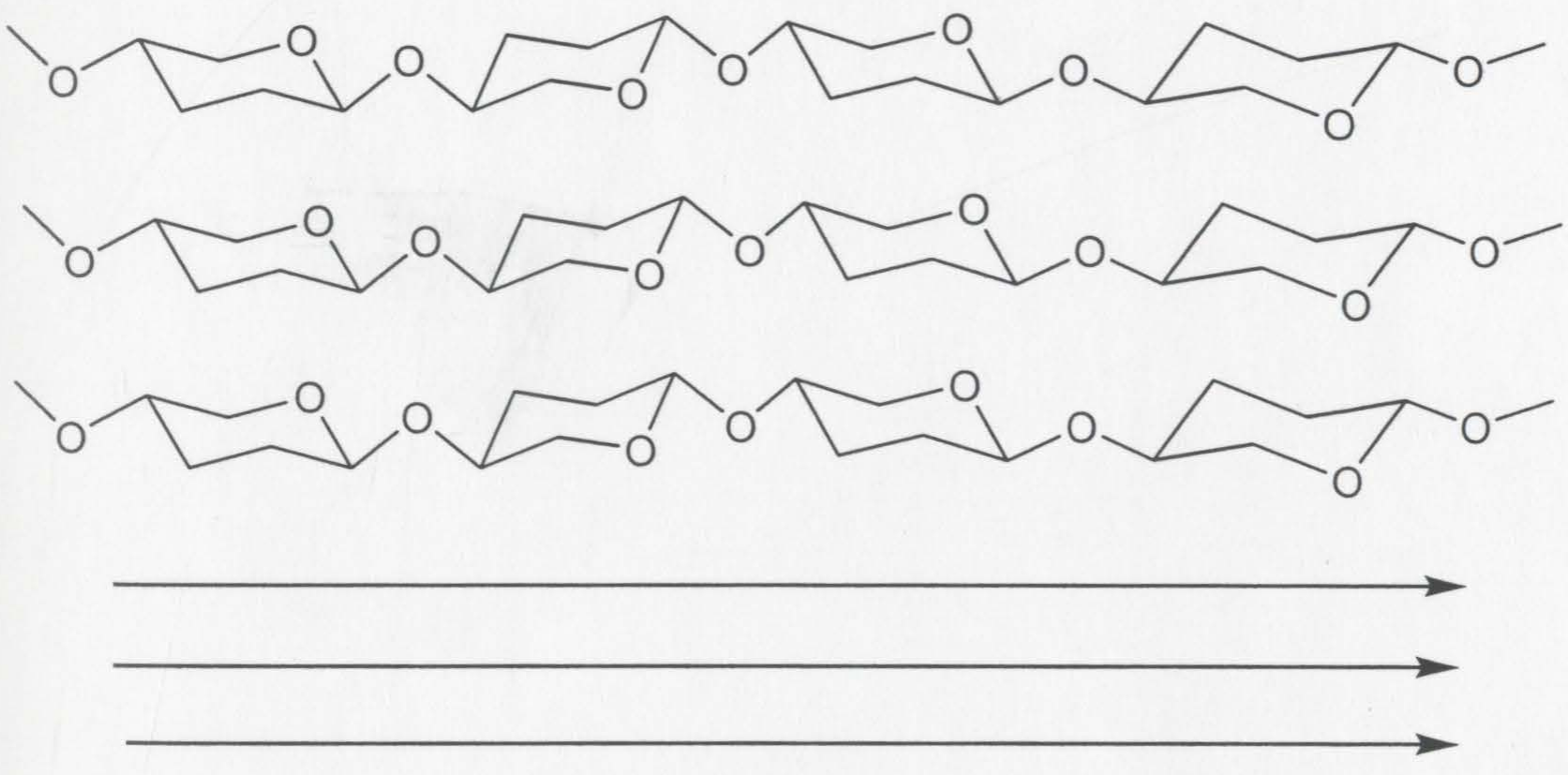
The molecular weight of natural chitin is normally greater than 1,000 kDa and that of commercially available chitosan varies from 100-over 1,000 kDa (Lower,1984; Li *et al.*, 1992). Several external forces during the manufacturing process can affect the molecular weight of chitosan. Factors such as high temperature (above 280°C) bring about degradation of chitosan and the polymer chains rapidly break down; dissolved oxygen concentration and shear stress may cause these alterations (Muzzarelli, 1977; Li *et al.*, 1992). Chitin prepared from blue, red and stone crab showed molecular weight variations that appeared to be closely related to the method of decalcification (Brine and Austin, 1981).

The maximum degree of depolymerization occurred when HCl was used for decalcification, followed by CH_3COOH and H_2SO_3 with minimal degradation occurring during EDTA decalcification process (Rutherford and Austin, 1978; Brine and Austin, 1981). There are many methods to determine the molecular weight of chitin and chitosan, such as viscometry (Roberts

Figure 2.3 Antiparallel (A) and parallel (B) chain arrangements of chitin.



(A)



(B)

Table 2.6 Differences between α-chitin and β-chitin.

α-chitin	β-chitin
Major sources-shrimp and crab shell	Major source-squid pen
Molecules are packed and 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
Quite resistant to modification reactions	Susceptible to modification reactions
Cannot undergo various reactions efficiently under mild condition	Can undergo reactions efficiently under mild conditions

Adapted from Kurita, (1997).

and Domszy, 1982), laser light scattering technique (Muzzarelli *et al.*, 1987), and gel permeation chromatography (Wu and Bough, 1976). Of these, viscometry is widely used because of its speed and simplicity (Maghami and Roberts, 1988).

2.9.3 Solubility

Chitin is insoluble in water, dilute aqueous salt solutions and all common organic solvents. It is depolymerized by strong mineral acids, but is partially soluble in mixtures of dimethylacetamide (DMAC) and LiCl (No and Meyers, 1995). Brine and Austin (1981) reported variations in the solubility (28-99%) of chitin from different species using a number of preparation methods. These workers noted that lower solubility resulted from incomplete removal of protein, and that molecular weight and acetyl content were not the controlling factors for solubility of chitin in 5% LiCl-DMAC solvent. Chitin is insoluble in aqueous solutions because its microcrystalline regions are stabilized by hydrogen bonds (Claesson and Ninham, 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 acid derivatives may cause harmful effects, such as in cosmetics, medicine and certain foods (Li *et al.*, 1992). Sugimoto *et al.* (1998) used polyethylene glycol (PEG) in order to improve solubility of chitin and chitosan in water (Figure 2.4).

The chitosan-PEG hybrid did not precipitate from aqueous solution by the addition of organic solvents such as methanol and acetone, because the chitosan-PEG

hybrid had a high hydrophilicity and some chitosan-PEG hybrids dissolved in water (Sugimoto, 1998). Table 2.7 lists some of the solvents for chitin and chitosan. It can be seen that while there are relatively few solvents for chitin, nearly all aqueous acids dissolve chitosan.

2.9.4 Chemical reactions of chitin and chitosan

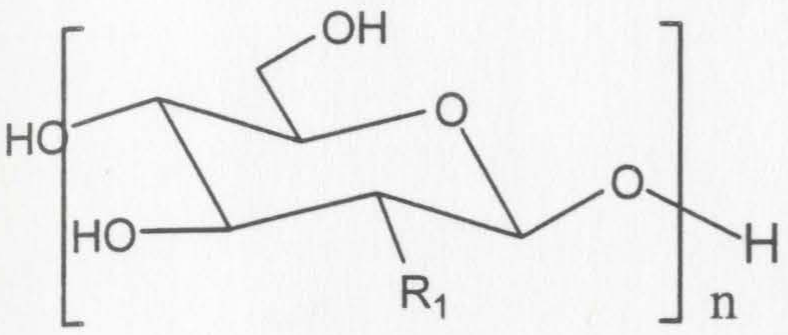
2.9.4.1 Neutralization

Chitin and chitosan can both be considered as weak bases; as such, they undergo the typical neutralization reactions of alkaline compounds. In these reactions the non-bonding pair of electrons on the primary amine group of glucosamine units performs the role of donating electrons. Thus, the primary amine groups become positively charged (Figure 2.5a). Chitin units may also include amino groups, but to a much lesser degree than chitosan.

2.9.4.2 Nucleophilic reactions

The non-bonding pair of electrons on the primary amine groups makes chitosan a potent nucleophile that can react readily with most aldehydes (Kurita *et al.*, 1988). Formaldehyde and glutaraldehyde are excellent cross-linking agents for chitosan. Acyl chlorides react vigorously with chitosan to form the corresponding amide derivative (Hirano *et al.*, 1976). The primary amine groups in chitin preparations also have the potential to participate in reactions with electrophiles, but in many cases are inaccessible due to the crystalline and intractable nature of chitin. It should be noted that the primary

Figure 2.4 Preparation of water-soluble chitin and chitosan derivatives
Adapted from Sugimoto *et al.* (1998).



$R_1 = NH_2$ or $NHCOCH_3$

$HO - CH_2CH_2O(CH_2CH_2O)_mCH_3$

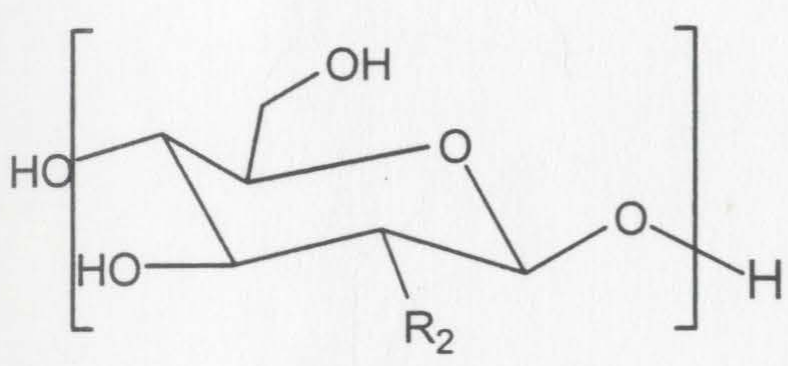
Poly(ethylene glycol) monoethyl ether

Ac_2O, DMSO, $CHCl_3$

AcOH, MeOH, pH 4.6-5.6

$OHC\ CH_2\ (CH_2\ CH_2O)_m\ CH_3$

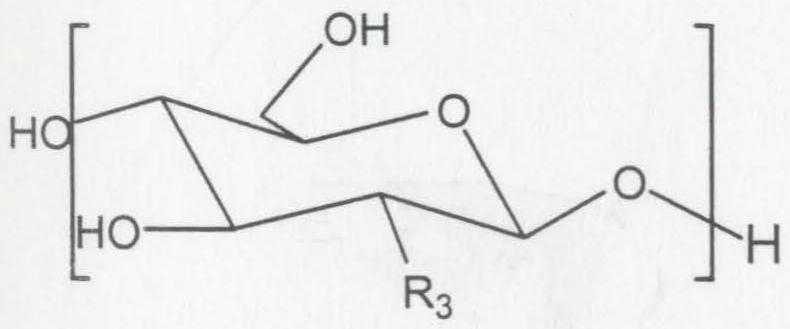
1 PEG -aldehyde



n

R_2=NH_2, $NHCOCH_3$ or
$N{=}CHCH_2O\ (CH_2\ CH_2\ O)_m\ CH_3$

$NaCN\text{-}BH_3$, AcOH, MeOH, pH 6.5

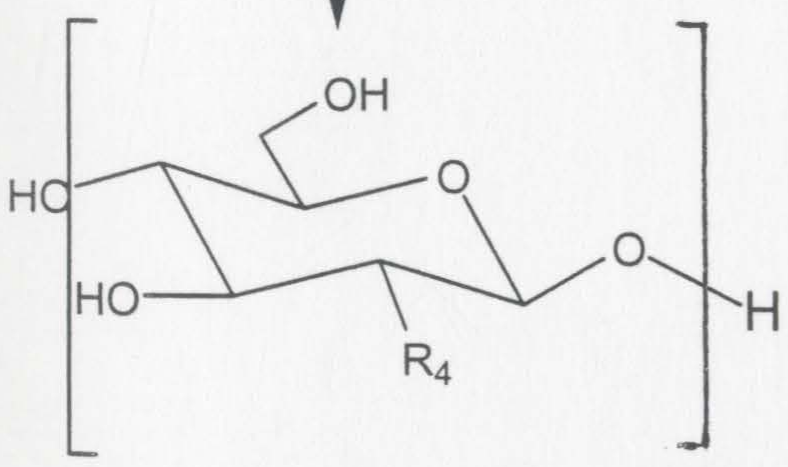


n

R_3=NH_2, $NHCOCH_3$ or
$NHCH_2CH_2O\ (CH_2\ CH_2O)_m\ CH_3$

2 Chitosan-PEG hybrid

Ac_2O | AcOH, MeOH, pH 3.8-5.1



n

R_4=$NHCOCH_3$ or
$NHCH_2CH_2O\ (CH_2\ CH_2\ O)_m\ CH_3$

3 Chitin-PEG hybrid

Table 2.7 Common solvents for chitin and chitosan.

Chitin solvents	Chitosan solvents
Dimethylformamide, lithium chloride	formic acid, water
Diethylformamide, lithium chloride	acetic acid, water
Hexafluoroisopropanol	lactic acid, water
Hexafluoroacetone, sequihydrate	glutamic acid, water
1,2-Chloroethanol, sulphuric acid	hydrochloric acid, water

Adapted from Winterowd and Sanford (1995).

amine sites on chitin and chitosan are more nucleophilic than the primary hydroxyl groups at the C(6) position (Figure 2.5b) (Winterowd and Sandford, 1995).

2.9.4.3 Acid- or base-assisted hydrolysis

Chitin and chitosan are susceptible to acid- or base-catalyzed hydrolysis. Under acidic or alkaline conditions, acetic acid can be liberated as the *N*-acetylamino groups at C(2) are converted to primary amines (Muzzarelli, 1977). Acidic conditions also result in some degree of depolymerization as hydrolysis of the β-glycosidic linkages takes place (Madhaven and Ramachandran, 1974). Depolymerization occurs to a lesser extent in alkaline environments. Chitosan can also be degraded by treatment with nitrous acid (Figure 2.6a) (Alan and Peyron, 1989).

2.9.4.4 Sulphation reactions

Although the majority of reactions of chitin and chitosan involves the primary amine group, it is possible to modify selectively their hydroxyl groups as well. This can be accomplished by protecting the amine group through the formation of polysaccharide formate or acetate and subsequent reaction of the salt with an electrophile (Muzzarelli, 1977). The hydroxyl group at C(6) is more reactive than that at C(3) and is therefore derivatized preferentially. The sulphation reactions as shown in Figure 2.6b, is a common example of this type of reaction. These reactions may also be carried out on chitin. The resultant products have blood anticoagulant properties similar to those of heparin (Wolform and Shen-Han, 1959).

2.9.4.5 Heavy metal complexes

Both chitin and chitosan are able to form complexes with many of the transition metals (Muzzarelli, 1973). Complexes are not formed between chitin and chitosan and metals from groups 1 or 2. The heavy metal/polymer complexes are believed to form as a result of dative bonding. This involves the donation of the non-bonding pair of electrons from the nitrogen and/or the oxygen of the hydroxyl groups to a heavy metal ion. Cupric ion seems to form one of the strongest metal complexes with chitosan in the solid state. Ferrous ion is also capable of binding to chitosan (Winterowd and Sandford, 1995).

2.9.5 Viscosity of chitosan

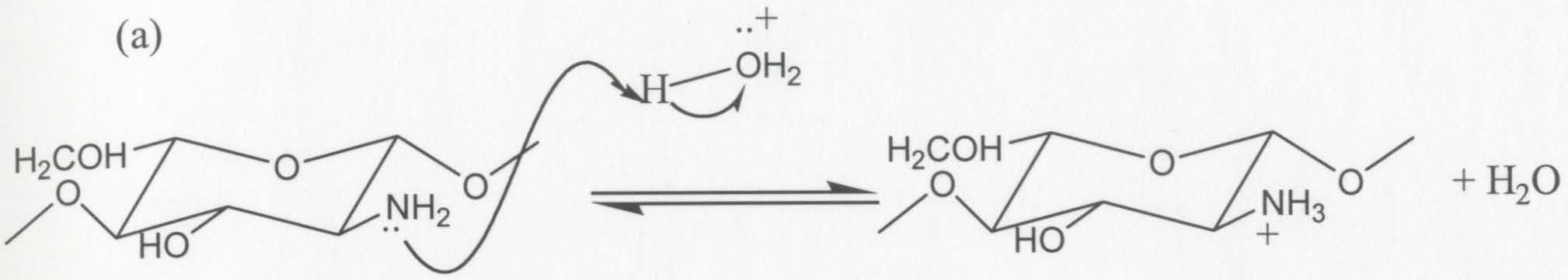
Viscosity is defined as resistance to flow or resistance to an applied force. It may also be defined as the shear stress divided by the shear rate. Shear stress is the applied force (pouring, mixing, pumping, chewing, swallowing) and shear rate is a value expressing how fast the liquid flows (Singh and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

Chitosan in solution exhibits a polyelectrolytic 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

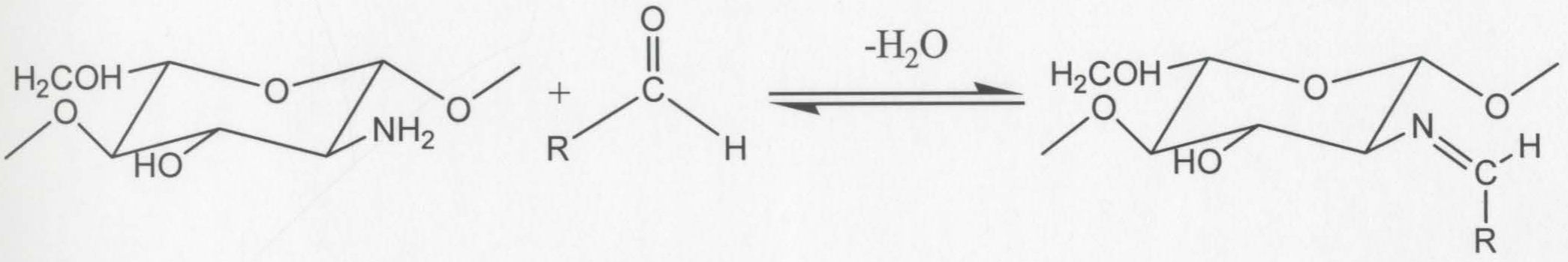
Figure 2.5a The neutralization reaction of chitosan.

Figure 2.5b Nucleophilic reactions of chitosan
(adapted from Winterowd and Sandford, 1995).

(a)

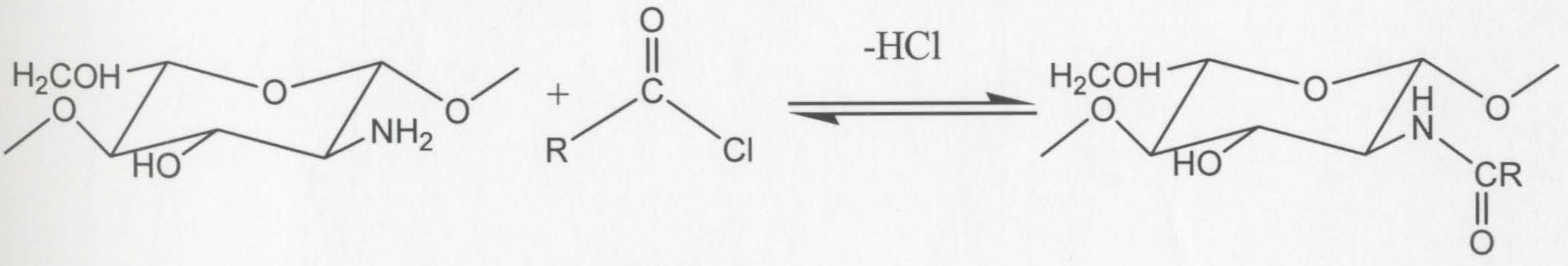


(b)



Aldehyde

imine



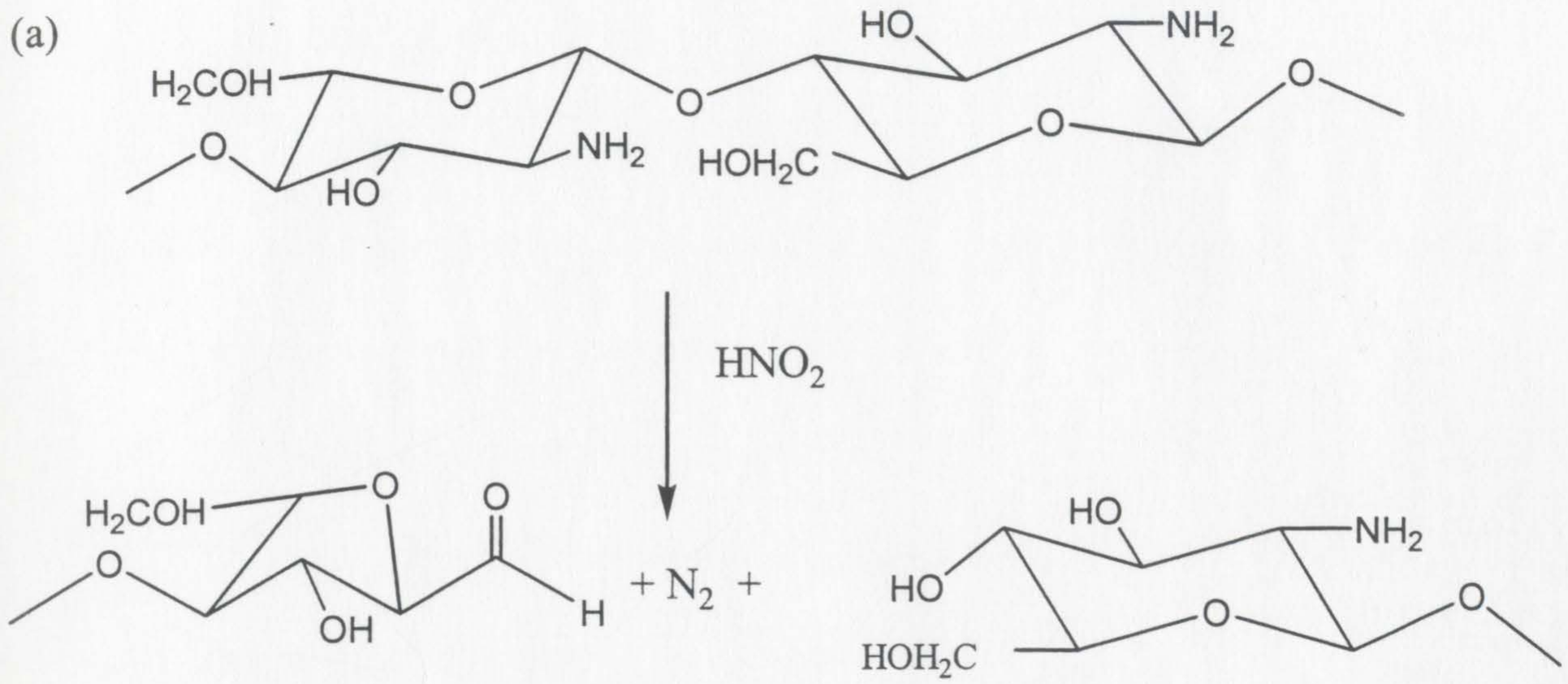
Acyl chloride

amide

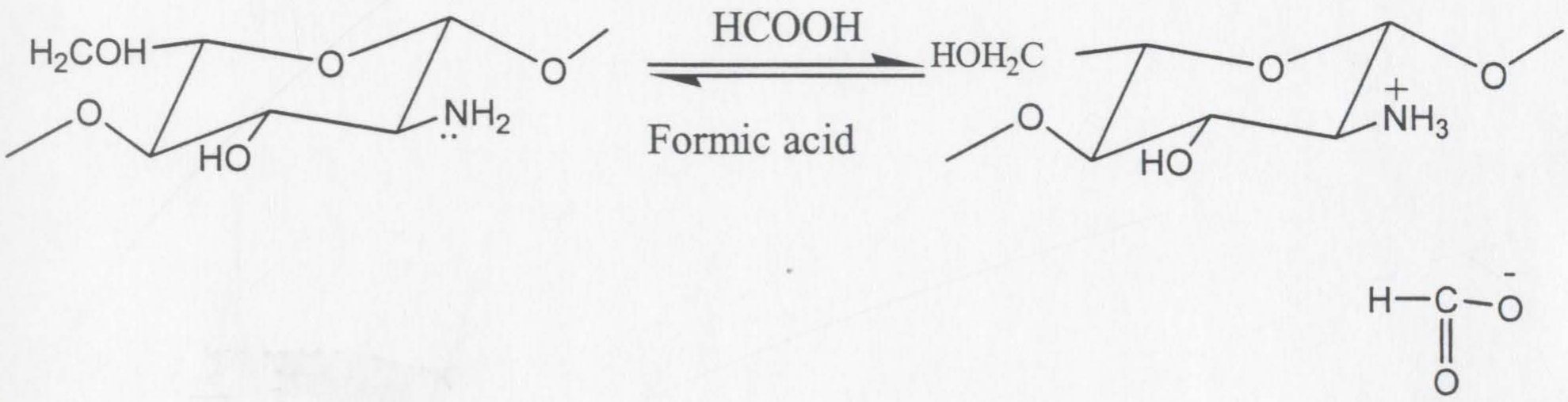
Figure 2.6a The degradation reaction of chitosan by use of nitrous acid

Figure 2.6b Sulphation of chitosan
(Adapted from Winterowd and Sandford, 1995).

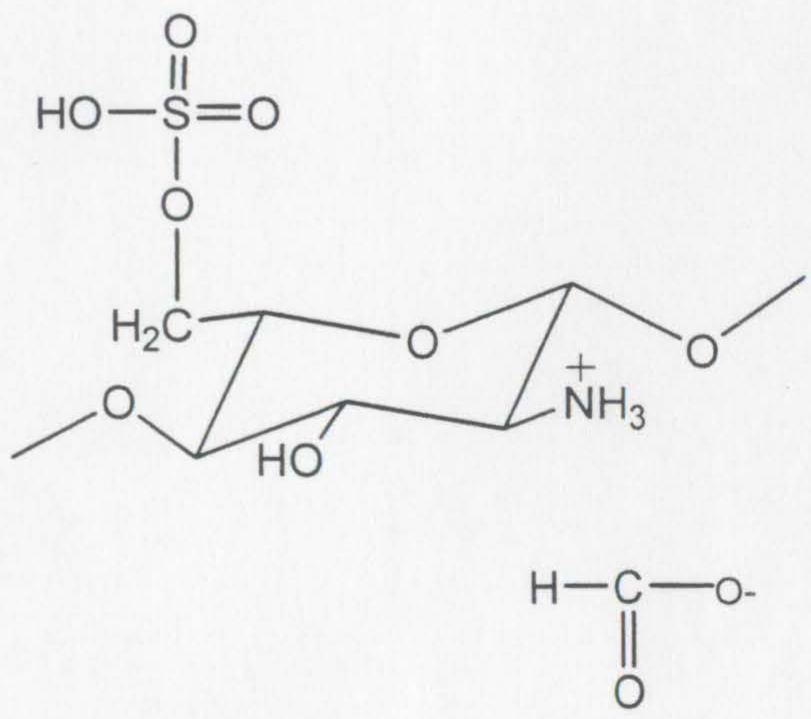
(a)



(b)



1 dissolve in formamide
2 chlorosulphonic acid



normal. For this reason, the solvent system of 0.2 M CH_3COOH and 0.1 M CH_3COONa is preferred for viscosity measurements (Muzzarelli, 1977).

The viscosity of the chitosan in solution is influenced by many factors, such as the degree of polymer deacetylation, molecular weight, concentration, ionic strength, pH and temperature. In general, as the temperature rises, the viscosity of the polymer solution decreases. However, a pH change in the polymer solution may give different results depending on the type of acid employed (Li *et al.*, 1992).

The intrinsic viscosity, [η], of a liner chain polymer such as chitosan depends on the molecular weight (M_w) described by the Mark-Houwink equation, where "K" and "a" are constants; $[\eta] = KM_w^a$ (Roberts, 1992). The viscosities and rheological properties of cations may be measured with a rotational viscometer that measures 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 have plots of shear stress versus shear rate (Roberts, 1992).

2.10 Applications of chitin and chitosan

Chitin and chitosan offer a wide range of applications, including clarification and purification of water and beverages, applications in pharmaceuticals and cosmetics, as well as agricultural, food and biotechnological uses (Knorr, 1991). Recent efforts for the use of chitin and chitosan have intensified since efficient utilization of marine biomass resource has become an environmental priority. Early applications of chitin and chitosan

include the treatment of wastewater and heavy metal adsorption agents in the industry, immobilization of enzymes and cells, resins for chromatography, functional membrane in biotechnology, seed coating and animal feed in agriculture, artificial skin, absorbable surgical suture, and wound healing accelerators in the medical field. Therefore, chitin and chitosan have been develop for new physiological materials lately as because they possess antitumour activity by immuno-enhancing antibacterial activity, hypocholeterolemic activity and antihypersensitive action (Jeon *et al.*, 2000).

2.10.1 Medical applications

The interest in chitin and chitosan for medical, ecological and pharmaceutical industries is evident in many publications and has been clearly indicated in several instances. Chitosan membranes have been proposed as artificial kidney membranes possessing high mechanical strength in addition to permeability to urea and creatine; they are impermeable to serum proteins and probably they are unique in offering the advantage of preventing emission of toxic metals into the blood stream, as currently happens with other membranes (Muzzarelli, 1977). Chitin and chitosan have been shown to have both material and bioactive properties that might be beneficial to enhance wound repair. Chitin is "physiologically" soluble because lysozyme, present in large amounts in recent and healing wounds, acts on it. Chitin derivatives suitable as healing accelerators are ethers and esters of salts. Examples are as follows: hydroxyethylchitin; carboxymethylchitin and its zinc salt; methylchitin and ethylchitin; chitin acetate, nitrate, citrate and phosphate; as well as ,*N*-formyl-, *N*-acetyl-, *N*-propionyl- and other acyl derivatives (Howling *et al.*, 2001; Muzzarelli, 1977). It was reported that the wound

recovering material composed of polyelectrolytic complexes of chitosan and sulphonated chitosan speed up wound healing and give a good-looking skin surface (Lahiji *et al.*, 2000). Chitosan has the ability to promote wound healing; this is due to the tendency to form polyelectrolytic complexes with polyanion heparin, which possesses anticoagulant and angiogenic properties. By forming a complex with heparin and actin to lengthen the half-life of growth factors, chitosan supports tissue growth and improves wound healing (Lahiji *et al.*, 2000). Chitosan is a very attractive candidate for burn treatment. This is true since chitosan can form tough, water-absorbent, biocompatible films. These films can be formed directly on the burn by application of an aqueous solution of chitosan acetate. The solution, although acidic, provides a cool and pleasant soothing effect when applied to the open wounds of burn patients. Deacetylated chitin, or chitosan, has been shown to aggressively bind to a variety of mammalian and microbial cells. This property of chitosan may lead to a variety of medical applications. These possible applications will use chitosan as a haemostatic, bacteriostatic and spermicidal agent.

In recent years chitosan has been introduced as a material in nutritional supplement markets, especially as a weight loss aid and a cholesterol-lowering agent (Shahidi *et al.*, 1999; Subasinghe, 1999). The mechanism behind this apparent effect of chitosan has been suggested to be its effect on the lipid transport mechanism in the gut, wherein free fatty acids (released from consumed fat), cholesterol, bile salts and other components form mixed micelles that comprise an essential step in the fat absorption process. Apparently the positively charged chitosan can bind to free fatty acids and bile salt components and disrupt lipid absorption (Sugano *et al.*, 1980).

2.10.2 Pharmaceutical and biotechnological applications

Chitosan has been extensively examined in the pharmaceutical industry for its potential in the development of controlled release drug delivery systems (Illum, 1998). This is due to its unique polycationic character and its gel- and film- forming properties. Such systems should allow the control of the rate of drug administration and prolong the duration of the therapeutic effect as well as perhaps the targeting of the drug to specific sites (Illum, 1998).

Chitin, 6-*O*-carboxymethylchitin, 6-*O*-(2-hydroxyethyl)chitin and 6-*O*-ethylchitin in the form of membranes have been used as enzymatically decomposable pharmaceutical carriers (Muzzarelli, 1977). Sawayanagi *et al.* (1982) reported that chitosan had excellent properties as disintegrant for direct compression of tablets where the addition of 50% chitosan resulted in rapid disintegration. The degree of deacetylation determined the extent of moisture absorption (Sawayanagi *et al.*, 1982). Chitosan, if added to tablets in a concentration higher than 5%, was superior to cornstarch and microcrystalline cellulose as a disintegrant. The efficiency was dependent on chitosan crystallinity, degree of deacetylation, molecular weight and particle size (Ritthidej *et al.*, 1994).

Akbuga (1993) studied the influence of physicochemical characteristics of drugs on their release characteristics from chitosan maleate matrix tablets and found that the drug solubility, degree of ionization and the molecular weight of the drug were the main factors of importance. A controlled release system has been produced by film coating of

theophylline granules with a polyelectrolyte complex of chitosan and sodium tripolyphosphate (Kawashima *et al.*, 1985). The rate of release of drug could be controlled by pH. At low pH values, reduced charge of the anionic tripolyphosphate reduced the electrostatic interaction in the complex and the network in the film loosened. Miyazaki *et al.* (1981) investigated the solubility of dried chitosan gels as vehicles for the sustained release of the poorly soluble drugs such as indomethacin and papaverine hydrochloride. Recently, chitosan, in the form of capsules, has been used for the specific delivery of insulin to the colon (Tozaki *et al.*, 1997).

Chitosan may also be used for biotechnological uses such as a carrier for enzymes and cells, porous beads for bioreactors, and a resin for chromatography and membrane materials. Immobilization technology has the potential for providing high cell densities and high product concentrations in a cell culture system. Enzymes immobilized as chitosan gels include α-chymotrypsin, α-galactosidase, invertase, β-galactosidase, α-amylase, lactose and cyclodextrin glucanotransferase (Li *et al.*, 1997).

2.10.3 Food and nutritional applications

Chitosan offers a wide range of food applications including bioconversion for the production of value-added food products, preservation of foods from microbiological deterioration, formation of biodegradable films, recovery of waste material from food processing industries, purification of water and clarification and deacidification of fruit juices (Shahidi *et al.*, 1999). The growing consumer demand for foods without chemical preservatives has focused efforts in the discovery of new natural antimicrobials (Wang,

1992). Antomicrobial activity of chitosan and its derivatives against different groups of microorganisms such as bacteria, yeast and fungi has received considerable attention in recent years (Yalpani *et al.*, 1992). Because of the positive charge on the C(2) of the glucosamine monomer at a pH below, chitosan is more soluble and has better antimicrobial activity than chitin (Chen *et al.*, 1998). Sudharshan *et al.* (1992) studied the antimicrobial effect of water-soluble chitosans such as chitosan lactate, chitosan glutamate and chitosan hydroglutamate and chitosan derived from *Absidia coerulea* fungi, on different bacterial cultures. They observed that chitosan glutamate and chitosan lactate were bactericidal against both Gram-positive and Gram-negative bacteria in the range of one to five log cycle reductions within one hour. Chitosan reduced the *in vitro* growth of numerous fungi with the exception of zygomycetes; that is, the fungi containing chitosan as a major component of its cell walls (Allan and Hadwiger, 1979).

The use of edible films and coatings to extend the shelf life and improve the quality of fresh, frozen and fabricated foods has been examined (Kester and Fennama, 1986). Chitin and chitosan possess film-forming properties and hence have been successfully used as food wraps (Shahidi *et al.*, 1999). The use of *N, O*-carboxymethylchitin films to preserve fruits over long periods has been approved in both Canada and the USA (Davides *et al.*, 1989). Due to its ability to form semi-permeable films, chitosan coatings modify the internal atmosphere as well as decrease transpiration loss and delay the ripening of fruits (El Ghaouth *et al.*, 1992). The feasibility of using a chitosan powder in a fluorescence sensor for monitoring lipid oxidation in muscle food was studied by Weist and Karel (1992). The primary amino groups of chitosan form a

stable fluorosphere with volatile aldehydes such as malondialdehyde, which is derived from the breakdown of fats (Weist and Karel, 1992).

The United Status Food and Drug Administration (USFDA) approved the use of chitosan as a feed additive in 1983 (Knorr, 1986). Chitosan is also used in the food industry as a food quality enhancer in certain countries. Japan produces dietary cookies, potato chips and noodles enriched with chitosan because of its hypocholesterolemic effect (Hirano, 1989). Furthermore, vinegar products containing chitosan are manufactured and sold in Japan, again because of their cholesterol lowering ability. The non-digestibility in the upper gastrointestinal (GI) tract, high viscosity, polymeric nature and high water binding properties, together with low water binding in the lower GI tract, are all responsible for the effective hypocholesterolemic potential of chitin and chitosan dietary fibres (Muzzarelli, 1996; Muzzarelli, 1997).

Hirano *et al.* (1990) demonstrated the nutritional significance of chitinous polymers in animals and indicated the effectiveness of chitin and chitosan as feed additives. Razdan and Pettersson (1994) observed increased high density lipoprotein (HDL) concentrations after feeding a chitosan-containing diet to broiler chickens. This could be attributed to enhanced reverse cholesterol transport in response to intestinal losses of dietary fats. The effect of chitin, chitosan and cellulose as dietary supplements on the growth of cultured red sea bream, Japanese eel and yellow tail has been investigated by Kono *et al.* (1987). Due to the ability of forming an ionic bond at low pH, it can bind *in vitro* to difficult types of anions such as bile acids or free fatty acids

(Muzzarelli, 1996). Large proportions of these bound lipids are thus excreted. Bound triacylglycerols would escape hydrolysis by the lipase, promoting the excretion of fatty materials including cholesterol, sterols and triacylglycerols (Zacour *et al.*, 1992; Muzzarelli, 1997). Inside the digestive tract, chitosan forms micelles with cholesterol, both endogenous and from dietary sources, in the alkaline fluids in the upper part of the intestine, resulting in the depression of absorption of dietary cholesterol and circulation of cholic acid to the liver. Because of the formation of cholic acid from blood cholesterol in the trends to decrease blood cholesterol concentration. Large intestinal microbials, which secrete chitinases, can digest these micelles; hence, the formed bile acids and sterols are excreted as free forms into faeces without absorption (Hirano and Akiyama, 1995).

2.10.4 Agricultural applications

Chitosan has many potential applications in agriculture because it is naturally occurring and biodegradable; therefore, it should not cause pollution problems. One application that is widely employed at present is seed coating (Li *et al.*, 1997). Hadwiger and coworkers (1985) found that chitosan treatment in coating seeds had many beneficial effects, such as inhibition of fungal pathogens in the vicinity of the seeds and enhancement of plant-resistant responses against diseases. Kokalis-Burelle (2001) reported that chitin contributes significantly to soil enrichment. He found that chitin can control plant pathogens, pathogenic nematodes and can provoke the development of host plant resistance against these pathogens. In addition to directly treating plants with chitosan, this polymer has been employed for improving soil properties and preparing

hydroponic fertilizes (Li *et al.*, 1997). Simther-Kopperl (2001) reported that when chitin decomposes it produces ammonia, which takes part in the nitrogen cycle. Furthermore, chitin is a main constituent in geochemical cycling of both carbon and nitrogen. Concerns about the environment have aroused great interest in the excessive use of agrochemicals such as fertilizers, herbicides and pesticides. Thus to reduce environmental damage caused by agrochemicals, the use of chitosan in controlled-released systems has gained momentum (Li *et al.*, 1997).

2.10.5 Waste treatment and purification of water

Both chitin and chitosan are recognized as excellent metal ligands, forming stable complexes with many metal ions (Muzzarelli, 1973; Kurita *et al.*, 1979). In particular, chitosan is considered as one of the best natural chelators for transition metals. Hence, chitin and chitosan have been utilized for waste water treatment, such as removal of toxic metals and radionuclides, recovery of precious metals, and recycling of metals from industrial wastewater for reuse and in order to reduce operational costs (Chui *et al.*, 1996). Chitosan can be utilized as a tool for the purification of water because of its high sorption capacity (Jeuniaux, 1986). The capacity of chitin and chitosan to form complexes with metal ions has been exploited in Japan for water purification (Simpson *et al.*, 1994). The NH_2 group of chitosan is of interest due to its ability to form coordinate covalent bonds with metal ions. Chitosan powder and dried films have more potential use in metal ion complexing because they will release most of their free amino groups above the pKa of the NH_2 group of chitosan (Tirmizi *et al.*, 1996).

The use of commercially available chitosan for potable water purification has been approved by the United States Environmental Protection Agency (USEPA) up to a maximum level of 10mg/L (Knorr, 1984). The effectiveness of cross-linked *N*-carboxymethylchitosan in removing lead and cadmium from drinking water has been demonstrated by Muzzarelli *et al.* (1989). A study of the metal binding capacity of chitosan has shown good binding with metals such as copper and vanadium (Micera *et al.*, 1986). Chitosan as a means of removing soluble silver from industrial waste streams has also been demonstrated (Lasko and Hurst, 1999). Commercially available chitosan's potential in the adsorption of heavy metals such as zinc, copper, cadmium and lead from aqueous solutions under various physicochemical conditions was investigated by Bassi *et al.* (2000). A new technique for preconcentration of trace elements and matrix elimination with a chitosan-based chelating resin was proposed as a useful pretreatment prior to measurements by ICP-MS. A small volume of the sample solution (80μL) was discretely introduced into the nebulizer of ICP-MS using a segment flow injection (SFI) system; a maximum of fifteen elements were simultaneously measured by this single injection (Lee *et al.*, 2000).

Chitosan is an excellent coagulating agent and flocculent due to the high density of amino groups on the polymer chain that can interact with negatively charged substances, such as proteins, solids and dyes, among others (Goosen, 1997). Wu *et al.* (1978) investigated the effectiveness of different chitosans for removing proteins from cheese whey. They found that the effectiveness in coagulating solids and proteins was inversely proportional to the molecular weight of the polymer. It is recognized that

chitosan with its partial positive charge can be used for coagulation and recovery of proteinaceous solids present in such food-processing wastewater (Knorr, 1991). Chitosan was also utilized as a coagulant for amino acids from crawfish processing wastewater (No and Meyers, 1989) and flocculation of microalgae at a chitosan concentration between 10 and 80 mg/L (Lubian, 1989).

Adsorption of dyestuff from processing effluents onto chitin has been reported by McKay *et al.* (1982) and the dye binding properties of chitin have been investigated by Knorr (1983). Dye binding was between 0.7 and 0.8 mg dye per gram chitin and chitosan. The kinetics of sorption of dyes on chitosan and the effects of temperature, particle size of chitosan, and pH of the dye solution have been examined by Venkatrao *et al.* (1986).

2.11 Seafood processing discards as environmental pollutants

The term shellfish, includes both molluscs and crustaceans and few other invertebrates such as sea urchins. Over the past two decades the shellfish industry has experienced a significant increase in processing volume with a corresponding increase in the available by-products, most of which are not presently utilized. The waste from shellfish processing plants is a serious concern for processors if strict environmental regulations are enforced (Brzeski,1987).

The most important commercially harvested crustaceans in Canada are crab, shrimp and lobster. In the crustacean industry, processing discards may account for up to 80% of the landed catch. These materials are mainly shells, viscera, heads and adhered

meat. The major components of shells are proteins, chitin and minerals, as well as small amounts of flavourant proteins and carotenoid pigments (Hansen and Illanes, 1994; Shahidi *et al.*, 1992; Ferrer *et al.*, 1996).

Spoilage of muscle foods, such as shellfish, results from changes brought about by the following: (1) biological reactions such as oxidation of lipids; (2) reactions due to activities of the fish's own enzymes; and (3) the metabolic activities of microorganisms. The predominance of these in any given food and under different storage conditions depends on several factors, including the chemical composition of the fish and shellfish. Chemically, shellfish has four major components: proteins, lipids, carbohydrates (chitin) and moisture (Ashie *et al.*, 1996). The relative proportions of all these components give fish and shellfish their characteristic structure, flavour, texture, colour and nutritional value. Besides these macrocontaminants, there are vitamins and other minor components, some of which become important in the spoilage process. One such group, generally referred to as extractives (i.e., extractable into water or aqueous solution), comprises primarily free amino acids, sugars and volatile nitrogenous bases such as ammonia, trimethylamine *N*-oxide (TMAO), creatine, taurine, the betaines, uric acid, anserine, carnosine and histamine (Jay, 1986).

In the pos-tmortem animal, enzymes from spoilage microorganisms primarily metabolize the extractive fraction of the fish muscle, producing a wide variety of volatile compounds resulting in off-flavours and off-odours. Trimethylamine *N*-oxide, which is found in a large number of marine fish and shellfish, is broken down to trimethylamine (TMA) by either endogenous enzymes or the bacterial enzyme, trimethylamine oxidase

(Phillippy, 1984). Trimethylamine *N*-oxide may also be decomposed to dimethylamine (DMA) and formaldehyde. The reactions involved are:

$$AH_2 + (CH_3)_3NO \longrightarrow (CH_3)_3N + H_2O + A$$

TMAO TMA

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

DMA formaldehyde

Where A is an oxidized substrate and AH_2 is the reduced form of the substrate. Trimethylamine reacts with lipids in the fish muscle to produce the characteristic fishy odour of low-quality fish. When the oxygen supply is depleted, many of the spoilage bacteria utilize TMAO as a terminal hydrogen acceptor, thus allowing them to grow under anaerobic conditions. This could be one reason why seafoods spoil more rapidly than other muscle foods. It has also been suggested that the formaldehyde produced in the above reaction cross-links with muscle proteins, contributing to the tough texture that develops during frozen storage (Sikorski *et al.*, 1976).

Other compounds produced as a result of microbial activity are hydrogen sulphide, dimethyl sulphide and methyl mercaptan from sulphur-containing amino acids; various amines and ammonia from amino acids; lower fatty acids from sugars such as glucose and ribose; carbonyl compounds from lipids; and indole, skatole, putrescine and

cadaverine from proteins. Indole production has also been correlated with the strong odour of decomposition which is characteristic of shrimp spoilage (Ashie *et al.*, 1996).

Despite the presence of valuable components in the discards, little developments of industrial scale have occurred in North America. Although some composting of processing discards has taken place, discards are generally dumped in-land or hauled into the ocean. Nonetheless, meal and silage production has also been used as a possible means of waste utilization (Shahidi, 1994). The processing of shellfish waste poses major technological problems, as shells are largely water insoluble and very resistant to natural biodegradation (Healy *et al.*, 1994). The constituents of such shells include protein and chitin, which are worthy of further processing.

Chitin is a biopolymer which could have many market applications. Chitosan is made either by chemical or biochemical deacetylation of chitin (Ornum, 1992). Chitin and chitosan can be used for agricultural, food, industrial and medical applications. Crab and shrimp fishery are of utmost importance to the economy of Newfoundland and Labrador. Therefore, value-added utilization of their discards is of importance (Shahidi and Synowiecki, 1991; Ferrer *et al.*, 1996).

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

Fresh samples of crab processing discards, comprised of intact cephalothoraxes and abdominal exoskeletons were collected from a local source in Newfoundland. Reagents, namely sodium hydroxide (NaOH), formaldehyde (HCHO), formic acid (HCOOH), acetic acid (CH_3COOH), hydrochloric acid (HCl), sulphuric acid (H_2SO_4), acetone, potassium chloride (KCl), hexamine, cadmium nitrate tetrahydrate ($Cd(NO_3)_2.4H_2O$), cupric sulphate ($CuSO_4$), ferrous sulphate ($FeSO_4$), zinc chloride ($ZnCl_2$), manganese chloride tetrahydrate ($MnCl_2.4H_2O$), nitric acid (HNO_3), chloroform, methanol, sodium hypochlorite (NaOCl), sodium carbonate, sodium tartarate and bovine serum albumin were obtained from Fisher Scientific Co. (Nepean, ON). Sodium bicarbonate ($NaHCO_3$), tetramethylmurexide (TMM), nickel(II) sulphate hexahydrate ($NiSO_4.6H_2O$), silver nitrate ($AgNO_3$), Folin & Ciocalteu's phenol reagent, ethylenediaminetetraacetic acid (EDTA), boric acid, picric acid (PA) and cobalt(II) chloride hexahydrate ($CoCl_2.6H_2O$) were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON). ICP-MS standards were obtained from SCP Science (St. Laurent, PQ). Wastewater samples were obtained from Asarco Inc (Buchans, NL).

3.2 Methods

3.2.1 Suitability of chemical treatments in extending shelf life of crab waste

Fresh samples of crab processing discards were treated by dipping them seperately in a 1% (w/v) solution of formaldehyde, formic acid, acetic acid, sodium

bicarbonate and sodium hydroxide. Experiments were conducted at cold room temperature (4-7°C) and at room temperature (20-25°C) separately. A control without any treatment was used for comparative purposes. The samples were evaluated for odour characteristics by a panel of 7 untrained panelists. Panelists were asked to judge the odour of the crab processing discards and to record the results on a 5-point scale [1-excellent (no foul odour), 2-good, 3-moderate, 4-bad, 5-very bad (foul odour)].

3.2.2 Proximate composition of crab processing discards

3.2.2.1 Moisture content

Two to 3 grams of ground crab processing samples were dried in a pre-weighed aluminium pan (Fisher) and placed in a forced-air convection oven (Fisher Isotemp 300). Samples were maintained at 105 ±1 °C until a constant mass was obtained. The moisture content was then calculated as percent ratio of the weight difference of the sample before and after drying to that of the original material (AOAC, 1990).

3.2.2.2 Ash content

Approximately 3 to 5 g of ground crab processing discards samples were accurately weighed into clean, dry, pre-weighed porcelain crucibles and charred over a Bunsen burner. The charred samples were heated in a muffle furnace (Blue M. Electro Co., Blue Island, IL) and maintained at 550 ±1°C until a gray ash was obtained. Crucibles were subsequently cooled in a desiccator and weighed. Ash content was calculated as percent ratio of the mass of the ash obtained after ignition to that of the original material (AOAC, 1990).

3.2.2.3 Crude protein content

Approximately 0.3-0.4 g of ground crab processing discards sample were weighed onto nitrogen-free weighing paper and transferred into a of Büchi digestion tube (Büchi 321, Büchi Laboratories, Fawil, Switzerland). The sample was digested with 20 mL of concentrated H_2SO_4 acid and two Kjeltab tablets (Profamo Analytical Service Inc., Dorval, PQ) for 45 min to obtain a clear solution. The digested samples were diluted with 50 mL of distilled water followed by the addition of 150 mL of a 25% (w/v) sodium hydroxide solution. The samples were steam distilled (Büchi 321, Büchi Laboratories, Fawil, Switzerland) to release the nitrogen in the form of ammonia, which was trapped in a 50 mL solution of 4% (w/v) boric acid containing N-Point indicator (EM Science, Gibbstown, NJ) in a receiving flask. Steam distillation was continued for 6 min and the contents in the receiving flask were titrated against a 0.1 N standard solution of sulphuric acid to determine the content of nitrogen (AOAC, 1990). The crude protein content of samples was calculated using a factor of 6.25.

$$\text{Percentage N} = \frac{(V_{sample} - V_{blank}) \times N \times 14.0067 \times 100}{W}$$

Where, V_{sample} = volume of titrate for sample (mL)
V_{blank} = volume of titrant for blank (mL)
N = normality of the H_2SO_4 solution used in the titration
W = weight of the sample (mg)

3.2.2.4 Lipid content

Total lipid content of samples was determined using the procedure described by Bligh and Dyer (1959). Briefly, Approximately 25 g of ground crab processing discards samples were accurately weighed and then extracted over a 3 min period with a mixture

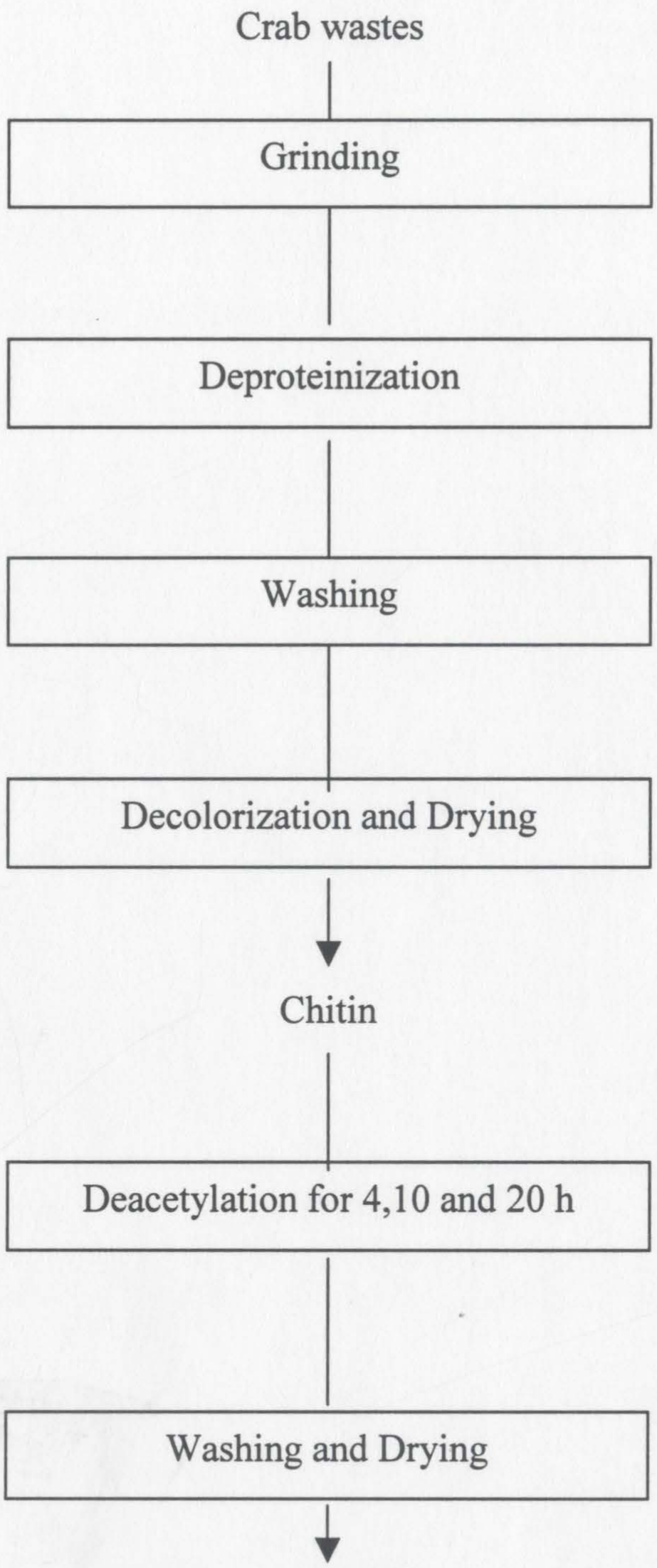
of 25 mL of chloroform and methanol followed by homogenization using a Polytron homogenizer (Model PT-3000, Brinkmann Instruments, Canada Ltd., Rexdale, ON). Approximately 25 mL of distilled water were added and the mixture was then filtered through a Büchner funnel under suction. The filtrate was allowed to separate overnight in a separatory funnel. Ten millilitres aliquots were transferred into a pre-weighed round bottom flask and the solvent was removed using a Büchi Rotorvapor (461,Büchi Laboratories, Fawil, Switzerland). The flask was then placed in the a forced-air convection oven at 80°C for 1 h. After cooling in a desiccator the round bottom flask containing the lipids was weighed and the total lipid content determined gravimetrically.

3.2.3 Preparation of chitin and chitosan and evaluation of their characteristics

Chitin was isolated from crab processing discards using a modified version of the methods of Mima *et al.* (1993) and Shahidi and Synoweicki (1991). Deproteinization and demineralization steps were carried out with 20 volumes of 4% (w/v) NaOH at 60°C for 3 h and 10 volumes of 10% (v/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 volumes of acetone and dried for 2 h at ambient temperature, followed by bleaching with 0.32% (v/v) sodium hypochlorite solution (containing 5.25% available chlorine) for 5 min with a solid-to-solvent ratio of 1:10 (w/v). Chitosan was prepared by alkali treatment of chitin using 10 volumes of 50% (w/v) NaOH in distilled water at 100 °C for 4, 10 and 20 h 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 X 10^{-3} mbar (Freezone 6, Model 77530, Labconco, Kansas City, MO). According to the deacytelation times three types of chitosan were obtained (Type 1, Type 2 and Type 3). A simplified flow diagram of chitin and chitosan preparation is given in Figure 3.1. Moisture, total nitrogen and ash contents were then determined. Particle sizes of chitosan types were determined by passing a sample of chitosan through a series of sieves with four sizes ranging from 250-1000 microns (250, 350 and 500 microns- Gilson Company Inc.,Worthinton, OH; 1000 microns- Tyler Co. of Canada Ltd., St. Catherines, ON). Apparent viscosity (at 2 N/m^2) of a 1% (w/v) chitosan level in a 1% (v/v) acetic acidsolution at 25°C was measured using a rotational viscometer (Cole-Parmer Co., Vernon Hills, IL). Measurments were made in triplicate using a No 5 spindle at 50 rpm on solutions at 25°C with values reported in centipose (cP) units. The degree of acetylation of chitosan was measured according to the picric acid method of Neugebauer *et al.* (1989). Dried and finely powdered chitosan (10-15 mg) 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 its introduction. In order to remove residual salts from the amino groups, a preliminary washing step was included; the polymer in the column was exposed for 15 min to 0.1 M diisopropylethylamine (DIPEA) in methanol, and then washed with methanol (10 mL). Binding of picric acid to the amino groups was performed by introducing 0.1 M picric acid in methanol (1.0 mL) and then allowing the reaction to proceed for 6 h. The column was subsequently washed with methanol (30 mL) at a rate of 0.5 mL/min in order to remove the unbound picric acid

Figure 3.1 A simplified flow diagram of chitin and chitosan preparation



Type 1, Type 2 and Type 3

completely. The bound picric acid was then quantitatively removed from the chitin-chitosan amino groups as subsequently described. Diisopropylethylamine in methanol (0.1 M, 1.0 mL) was introduced into the column and allowed to stand 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, Agilent Technologies, Wilmington, DE). A standard curve was prepared using different concentrations of DIPEA-picrate salt in methnolic solutions (Figure A1 in appendix). The fraction of amino groups acetylated (degree of *N*-acetylation, da) was calculated using the formula da = m-161n/m+42n where, m = mass of chitosan sample (mg), n = mmoles of picric acid eluted from sample, 161 Da = molecular weight of D-glucosamine unit, and 42 Da = molecular weight of *N*-acetyl-D-glucoamine less the molecular weight of D-glucosamine.

The molecular weight of chitosan was expressed as the viscosity molecular weight (Mv) using a ViscoTek model Y-500 relative viscometer (Viscotek Co., Houston, TX). Chitosan solution in 0.1 M 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 and η_s are the solution and the solvent viscosity, respectively. The values of intrinsic viscosity (η), of a liner chain polymer such as chitosan depends on the molecular weight (M_w) described by the Mark-Houwink equation, where "K" and "a" are constants; $[\eta]=Km_w^a$ (Roberts, 1992).

3.2.4 Metal chelation capacity of chitosan

Three different types of chitosan flakes (Type 1, Type 2 and Type 3) were used. Ethylenediaminetetraacetic acid was used as a reference chelating agent. For determination of metal chelation capacity, Fe^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+} were selected. Each experiment was replicated three times.

Chitosan (0.01 g) was weighed into seven beakers and 100 mL of the solution containing 400 μM metal ions were added separately. Metal solutions were prepared in hexamine-HCl buffer (pH 5) containing 10 mM KCl. The metal ion-chitosan mixtures were held for 24 h and the supernatants of metal ions were collected at 0, 1, 3, 6 and 24 h, respectively. At the same time EDTA and metal ion mixtures were kept for 24 h and supernatant of metal ion mixtures were collected at 0, 1, 2, 3, 6 and 24 h, respectively. Tetramethymurexide (TMM, 1mM) as an indicator was dissolved in hexamine-HCl buffer (pH 5) containing 10 mM KCl. The metal ions in the supernatant were estimated using a colorimetric method (Asakura *et al.*, 1990; Terasawa *et al.*, 1991). These experiments were carried out at pH 6 and 7.

The determination of chelation capacity of metal ions was carried out by the method described by Askura *et al.* (1990). To two millilitres of supernatant of metal ion solutions, 0.2 ml of TMM were added. Absorbance was read at 460 and 530 nm using a the HP diode array spectrophotometer and absorbance ratios A_{460nm}/A_{530nm} were calculated. The absorbance ratios were then converted to the corresponding free metal

ion concentrations using a standard curve (Figure A2 in appendix to Figure A13). Metal ion chelating capacities of chitosans were calculated using the following equation.

$$\text{Metal chelating capacity, \%} = \frac{C_{Initial} - C_{Final}}{C_{Initial}} \times 100$$

Where $C_{Initial}$ and C_{Final} refer to the initial and final millimolar concentration of metal ion, respectively.

3.2.5 Determination of heavy metals in waste water samples by inductively coupled plasma-mass spectrometry (ICP-MS)

Samples of chitosan (0.01 g of Type 1, 2 and 3) were mixed with 50 mL industrial waste water (obtained from a zinc mining site in Buchans, NL) in centrifuge tubes. Industrial wastewater samples were adjusted at different pH levels (pH 5, 6 and 7). The original wastewater sample was used as a control. Industrial wastewater sample-chitosan mixtures were held for 3 h at room temperature (22°C). The contents were then centrifuged for 5 min at 4000xg (ICE Centra M5, International Equipment Co., Needham Heights, MA) and supernatants were collected. Supernatants were filtered through a 0.45μm filter (Millipore Corporation, Bedford, MA). The metal ions, namely Co^{2+}, Ni^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Cr^{2+}, Pb^{2+}, Ag^{+}, Hg^{2+} and Mo^{2+} were analyzed for residual metal concentrations using ICP-MS (HP 4500, Agilent). The concentrations of the aforementioned metal ions in the original industrial wastewater were separately analyzed using ICP-MS as a control.

Before the samples were introduced to the ICP-MS, supernatants were acidified with HNO_3 and (one litre water sample should have 25 mL of 8 M HNO_3 acid added to it) rinsed with ultra pure water.

The HP 4500 ICP-MS was used to acquire raw data from unknown water samples, known reference materials and calibration solutions. Water samples were diluted ten fold with approximately 0.2 M HNO_3. Samples were introduced to the ICP-MS via 10 mL test tubes arranged in a CETEC autosampler (Agilent, Palo Alto,CA). This enables automated runs of up to 30 h in length. Data was acquired for 39 elements, namely Li, Be, Mg, Al, Si, P, S, Cl, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Br, Rb, Sr, Mo, Ag, Cd, Sn, Sb, I, Cs, Ba, La, Ce, Hg, Ti, Pb, Bi and U. The data for elements used as internal standards were also acquired. Five calibration standards were analyzed and one blank per cycle was used for background correction. The blank was followed by 7 unknown and one water reference material ran as an unknown for quality control purposes. Raw data in counts per seconds (cps) format was exported from the ICP-MS data acquisition package for concentration calculation using inhouse created spreadsheet-based software. Matrix and drift correction was accomplished with the use of internal standards, which were continuously added to the sample introduction system via online dilution. All standards were prepared using ICP standard solutions (SCP Science, St. Laurent, PQ for elements of interested. High purity distilled acids were used to dilute these standards to required concentrations. All limits of detection (LOD) for elements of interested were quoted at the ppb (μg/kg) levels. Table 3.1 shows the LOD of elements for ICP-MS.

For ICP-MS, Ar was used as the plasma carrier and auxiliary gas with sample uptake and a rate of 1 mL/min was employed. A sample acquisition time of 579.79 s with rinse time of 180 s (0.5 M HNO_3), 10 s integration time, and one repetition were employed.

3.2.6 Metal chelation capacity and recovery of metals from aqueous solutions using small-sized columns containing chitosan by ICP-MS

Removal and recovery of metal ions from aqueous solutions was done according to a modified version of the method explained by Chui *et al.* (1996).

3.2.6.1 Column preparation

Ten grams of chitosan (Type 1) were taken into a 500 mL beaker containing 200 mL of deionized water (pH 7). The mixture was stirred for 24 h at ambient temperature. The pre-soaked chitosans were packed into five glass columns (15 cm x 0.9 cm).

3.2.6.2 Metal chelation and recovery of single metals from aqueous solutions

Samples of 50ppm and 100ppm of Ni^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+} and Ag^{+} prepared by dissolution in pH 7 deionized water from the corresponding salt Ten millilitres of metal ion solutions were eluted from each separate column at neutral pH at a rate of 2 mL/min, and at room temperature (22˚C). Twenty millilitres of deionized water (pH 7) were passed through each column to wash the remaining metal ions, and the eluate was collected. The

Table 3.1 Limit of detection of elements for ICP-MS.

Element	Limit of detection, ppb
Ag	0.09
As	2.00
Cd	0.04
Co	0.03
Cr	0.08
Cu	0.02
Fe	0.03
Hg	1.00
Mn	0.02
Mo	0.03
Ni	0.10
Pb	0.30
Zn	1.00

combined eluates were used for ICP-MS analysis. Ten millilitres of a 0.1 M solution of EDTA were used to desorb each metal ion from the column and the eluate was collected again. Twenty millilitres of deionized water were passed through the column to wash the remaining EDTA, and the eluate was collected. The amount of metal ions recovery in the eluate was similarly determined by ICP-MS using the procedure given in section 3.2.5. Metal chelation capacity and recovery values were then calculated.

3.2.6.3 Metal chelation and recovery of multiple metals from aqueous solution

Samples of 50 and 100 ppm of each of Ni, Co, Cd, Cu and Ag metal ion mixture was dissolved in pH 7 deionized water. Metal chelation capacity and recovery values were calculated using the method described in section 3.2.5.

3.2.7 Determination of protein flocculation by chitosans

Three types of chitosan (Type 1, Type 2 and Type 3) were selected in this experiment.

3.2.7.1 Preparation of columns

The column preparation was done as described in section 3.2.6.2. Three glass columns were used for protein flocculation and the dimensions of each column was 4 cm x 0.9 cm. The experiment was carried out in triplicate.

3.2.7.2 Determination of protein flocculation of chitosan

Bovine serum albumin (BSA) was dissolved in deionized water to obtain different concentrations (2-8 mg/mL). Solutions of BSA were passed through the three chitosan columns at a flow rate of 12m L/min and 5mL of eluted solutions were collected in three glass tubes. The content of protein in each tube was measured using the method described by Lowry *et al.* (1951).

3.2.7.3 Determination of protein by Lowry's method

Reagents A, B and C were prepared as follows: for reagent A, $Na_2CO_3$was dissolved in 0.1M NaOH to obtain a 2% (w/v) solution; for reagent B, $CuSO_4$ was dissolved in 1% (w/v) sodium tartarate to obtain a 0.5%(w/v) solution; and reagent C was prepared by using 50 mL of solution A with 1 mL of solution B.

Three millilitres of reagent C were transferred to a glass tube. The sample (0.3mL) was added to the glass tube and shaken vigorously. Lowry's solution (0.3 mL; half diluted Folin & Ciocalteu's phenol reagent) was added and it was shaken vigorously. The mixtures were kept for 20 min at ambient temperature. Absorbance of the mixture was read at 660 nm. Analytical linearity range of the Lowry's method was 10 μg to 1.00 mg protein (Lowry *et al.*, 1951). Working standards (Figure A14) were prepared using BSA at concentration ranging between 50 and 700 μg/mL.

3.2.8 Statistical analysis

All experiments in this study were replicated at least three times. Data were reported as mean ± standard deviation (SD). Analysis of variance (ANOVA) was performed and differences in mean values determined using Tukey's studentized range test at $p<0.001$ or 0.05 and employing ANOVA and least significance difference procedures of statiatical analysis system (Snedecor and Cochran, 1980; SAS, 1990). A simple linear regression was also performed using the same software in the general linear model.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Suitability of chemical treatments for extending the shelf life of crab processing discards

The effectiveness of methods and storage conditions to control fish and shellfish spoilage depends largely on the mechanisms involved. Thus, an understanding of postmortem biochemical changes in fish and shellfish and their relationship with various factors and spoilage mechanisms involved is essential in order to formulate and rationalize effective strategies for controlling deterioration processes (Ashie *et al.*, 1996).

Shellfish processing discards pose major technological problems, as shells are largely water insoluble and very resistant to natural biodegradation (Healy *et al.*, 1994). The scrapped exoskeletons and other parts of processed shellfish such as American lobster (*Homarus americanus*), snow crab (*Chinocetes opilio*) and shrimp (*Pandalus borealis*) constitute 30-80% of their total weights. The processing discards from the crustacean industry are mainly shells, viscera, heads and adhered meat. The major components of shells are proteins, chitin and minerals, as well as small amounts of flavourant proteins and c arotenoid pigments (Shahidi *et al.*, 1992; Hansen and Illanes, 1994; Ferrer *et al.*, 1996). Direct use of the crab waste for land manuaring or spreading is generally discouraged by the uniquely obnoxious odours of putrefying shells.

When the wastes are buried or land filled decomposition of the fresh wastes occurs under the anaerobic conditions that generate particularly malodorous compounds. Anaerobic composting of fish wastes therefore poses problems of smell, transport,

application and aesthetic utilization without adverse environmental impacts. The impacts may be mainly due to most of the nitrogen in the product being in forms that can be easily volatilized or washed away, not in the form of the stable humus that is generated by aerobic composting (Hayes *et al.*, 1994). Low technology land burial and open surface dumping of seafood wastes is likely to be prohibited and regulations enforced in more and more jurisdictions, due to the nuisances of malodours and scavengers, and the eventual contamination of surface and ground water (Hayes *et al.*, 1994). In order to prevent development of putrefying odours and extend shelf-life, five chemical spraying treatments of crab processing discards were employed. The chemical treatments were 1% (v/v) solutions of formaldehyde, formic acid, acetic acid, sodium hydroxide and sodium bicarbonate.

Crab processing discards, which had been treated with all five treatments and the control, remained acceptable up to 5 days of storage under the cold room temperatures (4-7°C) without developing any putrefying odour. Acceptability percentage was calculated based on the scores given by the panelists for the first three categories; that is, excellent, good and moderate, in terms of odour (Table A1 in appendix). Acceptability percentage was calculated by adding these three catagories (Acceptability%= Excellent% +Good% +Moderate%). Formaldehyde- and formic acid-treated samples could be kept for 11 days and 7 days, respectively, without any foul smell developing (Table 4.1 and Table A2 in appendix). After 14 days all treatments were unable to prevent the development of putrefying odours. The efficiency of treatments was, however, in following order.

formaldehyde> formic acid> $NaHCO_3$> acetic acid> NaOH> Control

According to Table 4.2 and Table A2 in the appendix, crab waste treated with all five treatments as well as the control could be kept for only 2-3 days at room temperature (20-25°C) without developing any putrefying odour. Formaldehyde- and formic acid-treated samples, however, could be kept for 3 and 2 days, respectively. The order of efficiency of other treatments was as follows:

formaldehyde> formic acid> acetic acid> $NaHCO_3$> NaOH> Control

Foul odour is produced mainly due to microbial activities and enzymatic degradation of crab meat residues attached to the shells. The volatile compounds produced are mainly alcohols, aldehydes, ketones and hydrocarbons (Pan and Kau, 1994). Microbial activities in crab wastes produce a number of odorous gases leading to an objectionable smell. Formaldehyde and formic acid can act as antimicrobial compounds and reduce microbial activity (Merck Index, 1996).

Low-temperature storage prevents spoilage or contaminating microorganisms from metabolizing, growing, and causing spoilage without necessarily killing them. The refrigeration process usually results in an initial reduction in the microbial population followed by a gradual decline during storage, with the survival of spoilage microorganisms (Ashie *et al.*, 1996). Crab processing discards can be held longer at 4-7°C than 20-25°C.

Table 4.1 Sensory evaluation[1] of crab processing discards treated with different reagents and stored at 4-7°C.

	Treaments[2]					
Storage time, days	Formaldehyde	Formic acid	Acetic acid	Sodium bicarbonate	Sodium hydroxide	Control
0	100	100	100	100	100	100
1	100	100	100	100	100	100
2	100	100	100	100	100	100
3	100	100	100	100	100	100
4	100	100	100	100	100	100
5	100	100	100	100	100	100
6	100	100	100	43	100	43
7	100	100	57	57	57	29
8	100	71	29	43	0	0
9	100	29	0	0	0	0
10	100	0	0	0	0	0
11	100	0	0	0	0	0
12	71	0	0	0	0	0
13	29	0	0	0	0	0
14	0	0	0	0	0	0

[1]Number of untrained panelists = 7
(Acceptability % = Excellent% + Good% + Moderate%)
[2]Treatments were applied to crab processing discards at a 1% (v/v) level.

Table 4.2 Sensory evaluation[1] of crab processing discards treated with different reagents and stored at 20-25˚C.

	[2]Treatments					
Storage Time, days	Formaldehyde	Formic acid	Acetic acid	Sodium bicarbonate	Sodium hydroxide	Control
0	100	100	100	100	100	100
1	100	71	71	58	58	29
2	86	43	0	0	0	0
3	14	0	0	0	0	0
4	0	0	0	0	0	0

[1]Number of untrained panelists = 7
(Acceptability % = Excellent% + Good% + Moderate%)
[2]Treatments were applied to crab processing discards at a 1% (v/v) level.

Although samples treated with formaldehyde were best in terms of off-odour prevention at both 4-7˚ and 20-25˚C, formaldehyde may act as a carcinogen (Merck Index, 1996). Most adults react to formaldehyde at a concentration of 0.5 to 1.5 ppm in the air. Infants, the elderly, those with specific allergic reactions, and individuals with respiratory problems may react to even lower levels. Symptoms of formaldehyde exposure include nausea, vomiting, abdominal pain, or diarrhea (Merck Index, 1996). When the reaction is allergic, symptoms may include minor respiratory irritation and watery eyes. Extreme cases of exposure or chronic exposure has been linked to respiratory cancer (Sittig, 1991).

According to the current Occupational Safety and Health Administration (OSHA, 2001), the permissible exposure limit (PEL) for formic acid is 5 ppm in the air. Inhalation of formic acid results in coughing and bronchitis. Extensive exposure can produce depression of the central nervous system, severe metabolic acidosis and nephropathy (Seiler *et al*., 1988).

4.2 Proximate analysis of crab wastes and isolation of chitin

The chemical composition of crab (*C. opilio)* processing discards is crude protein, 21.5±0.53 %; ash, 43.7±0.1 %; lipid, 0.35±0.1 % and chitin 35.3±1.9 % (all measurements are in dry weight basis). Crab processing discards comprise a composite of dried waste of shell, cephalathrorax, legs and claws. Non-chitinous protein was the main component which should be removed effectively to obtain a high quality chitin (Austin *et al.,* 1981). For isolation of chitin, a deproteinization step was included

followed by demineralization to remove inorganic matters. Crab processing discards were ground and treated with 4% (w/v) NaOH at a solid-to-solvent ratio of 1:20 (w/v) at elevated temperatures (65-100°C) for 3 h in order to dissolve the protein present, similar to that used by No and Mayers (1995). Prolonged alkaline treatment under severe conditions causes depolymerization and deacetylation of chitin (No and Mayers, 1995). Demineralization was accomplished by extraction with 10% (v/v) HCl solution at a solid-to-solvent ratio of 1:10 (w/v) at room temperature for 2 h in order to dissolve calcium salts, similar to that used by No *et al.* (1989) and No and Mayers (1995). A prolonged demineralization time of 24 h resulted in only a very slight drop in the ash content, but this can cause polymer degradation (Brzeski, 1982). For demineralization, it is important that the amount of acid be stoichiometrically equal or greater than all minerals present in the shells to ensure complete reaction with inorganic salts (Shahidi and Synowiecki, 1991). Acid and alkali treatments may produce a coloured chitin product because of the carotenoids remaining in the preparations. When a bleached chitinous product is desired, pigments can be removed using appropriate solvents (No and Mayers, 1995). Chitin was treated with acetone at a 1:20 (w/v) solid-to-solvent ratio and dried at ambient temperature, followed by bleaching with 0.32% sodium hypochlorite. No *et al.* (1989) prepared a white coloured crawfish chitin using an acetone wash, followed by bleaching with 0.315% (v/v) sodium hypochlorite. The yield of chitin from crab processing discards was 27.8%. Austin *et al.* (1981) and Shahidi and Synowiecki, (1991) reported isolation of 14-30% chitin from crab processing discards.

4.3 Production and characteristics of chitosan extracted from crab processing discards

Chitosan was prepared by alkali treatment of chitin using a 1:10 (w/v) solid-to-solvent ratio of 50% (w/v) sodium hydroxide in distilled water at 100°C for 4, 10 and 20 h under a nitrogen atmosphere (Mima *et al.*, 1983). According to the length of deacetylation time, three types of chitosan were obtained (Types 1, 2 and 3). The degree of deacetylation was 86.4, 89.3, and 91.3% for chitosans prepared from chitin under deacetylation times of 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 could be used only when the degree of deacetylation is above 70% and nitrogen content in the product is higher than 7% by weight. Table 4.3 shows that the viscosity average molecular weight (M_v) of chitosan decreased with increasing deacetylation time. However, a very high deacetylation rate of chitin polymers was not observed in this study by increasing the deacetylation time from 10 to 20 h. The procedure used for deacetylation in this investigation was similar to that of Mima *et al.* (1983) who produced 96% deacetylation with alkali treatment at 110°C.

Characteristics of chitosans prepared with different deacetylation times are presented in Table 4.3. Preparation of chitosan samples (Types 1, 2 and 3) shown in Table 4.3 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 10-20 h period. As shown in Table 4.3, the nitrogen contents of chitosans were dependent on deacetylation time and were 7.52, 7.64 and 7.73% respectively, for samples prepared over 4, 10, and 20h, confirming a more effective deacetylation over longer periods. The corresponding apparent viscosity values were 360, 57, and 14 cP.

As shown in Table 4.3 there was a substantial difference in molecular weights measured by the viscometric method for Types 1, 2 and 3 chitosan. The Type 1 chitosan prepared upon deacetylation for 20 h had a molecular weight 1.45 times lower than that of the Type 2 product prepared over a 10 h period. These molecular weight differences were less than those observed for viscosity values. The 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 behaviour of chitosan types. 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 (Maghami and Roberts, 1988). Furthermore, Maghami and Roberts (1988) observed that viscometric constants "K_m" and "a" in the Mark-Houwink equation remained uncharged for a series of chitosans with *N*-acetyl contents of 0 - 40%.

Table 4.3 Characteristics of three different types of chitosans (Types 1,2 and 3) prepared from crab shells[a].

Properties	Type 1	Type 2	Type 3
Deacetylation time[b] (h)	20	10	4
Moisture (%)	3.75 ± 0.21	3.95 ± 0.34	4.50 ± 0.30
Nitrogen (%)	7.70 ± 0.19	7.63 ± 0.08	7.55 ± 0.10
Ash (%)	0.30 ± 0.00	0.25 ± 0.02	0.30 ± 0.03
Colour	cream white	cream white	cream white
Apparent viscosity (cP)	14.0 ± 0.34	57.0 ± 0.96	360 ± 0.53
Degree of deacetylation (%)	91.3± 1.3	89.3± 1.2	86.4± 2.1
Mv[c] (Dalton)	6.60×10^5	9.60×10^5	1.80×10^6

[a]Results are expressed as mean value of three determinations ± standard deviation.
[b]Deacetylation for preparation of chitosans Types 1, 2 and 3 was achieved using a 50% (w/v) NaOH solution at 100°C for 4, 10 and 20 h, respectively.
[c]Viscosity molecular weight

4.4 Metal chelation capacity of chitosans determined by a colourimetric method

The chemical definition of metal chelation can be defined as firm binding of metal ions with an organic molecule (ligand), which gives a ring structure (Muzzarelli, 1977). The resulting ring structure protects the mineral from entering into unwanted chemical reactions. Capacity of adsorption of metal ions expressed as a percentage is referred to as chelation capacity.

Tetramethylmurexide (TMM) is a chelating agent with an absorption maximum at 530 nm. However, shifting of the absorption maximum to 460 nm occurs when metal ions are chelated by tetramethylmurexide (Figure 4.1). The ratio of absorbance at 460 nm to that at 530nm is linearly correlated with the metal ion concentration (Askura *et al.*,1990). When a known concentration of metal ion is added to a buffered solution of chitosan, some of the metal ions chelates with chitosan while leaving any untreated or free metal ion in the solution. When TMM is added to the solution, it chelates the remaining metal ions, and the characteristic absorbance maximum shifts from 530 nm to 460 nm. The untreated metal ions can then be determined from the calibration line and the concentration of metal ions chelated by chitosan is calculated by subtracting the free ion concentration from that initially present (Askura *et al.,* 1990) (Table A3-A5 in appendix). The slope of the standard curve became sharp as the pH of the system increased, because high pH conditions favour TMM-metal complex formation. The reason for using the hexamine buffer system in this study was due to the weak interaction of hexamine with metallic ions, which would be advantageous to the stability of the metal ligand complex (Kohn and Furda, 1967). The metal chelation capacity depends on the

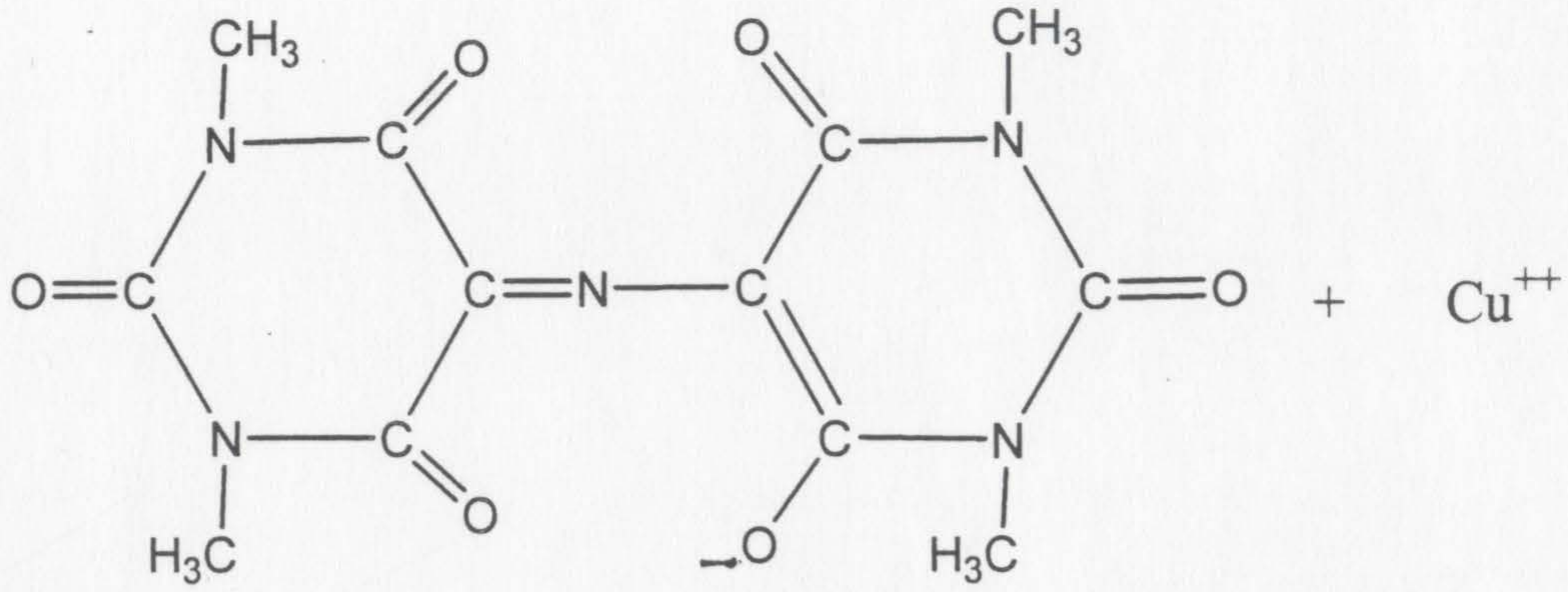
type of chitosan (e.g. Type 1, 2 or 3) pH, reaction time and the metal type at its initial concentration.

4.4.1 pH of the solution

According to the metal chelation capacity values at pH 5, all three types of chitosan tested showed the same chelation pattern (Type 1 > Type 2 > Type 3) for Zn^{2+}, Cu^{2+}, Fe^{2+} and Cd^{2+} while Ni^{2+}, Mn^{2+} and Co^{2+} showed different patterns. Metal chelation capacities at pH 6 for Zn^{2+}, Ni^{2+}, Fe^{2+} and Mn^{2+} showed the same pattern (Type1 > Type 2 > Type 3) at 6h, while Cu^{2+}, Co^{2+} and Cd^{2+} showed different patterns. However, there was no significant difference ($p < 0.05$) between the three types of chitosan at 3h except for Fe^{+2}. Considering pH 7, Co^{+2} and Zn^{+2} showed the highest chelation capacity at 3 h (Type 1 chelation capacity was higher than those of types 2 and 3). Chelation capacity of the three types of chitosan chelators were significantly different ($p \leq 0.05$) from that of EDTA.

Generally, the adsorption of metal ions by chitosans is expected to be pH sensitive (Roberts, 1992). Adsorption of Zn^{2+}, Cu^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+} and Mn^{2+} on chitosan flakes in solutions was studied at three pH levels (Figure 4.2 - Figure 4.8). The uptake of metal ions by chitosan showed a decrease as the pH increased from 5 to 7. This could be due to the pH dependance of complex formed between chitosan and metals and the pH complex formation of the remaining metal ions and TMM, which was also pH dependent. Some researchers have observed that pH 5 is best for metal chelation (Cu^{2+}

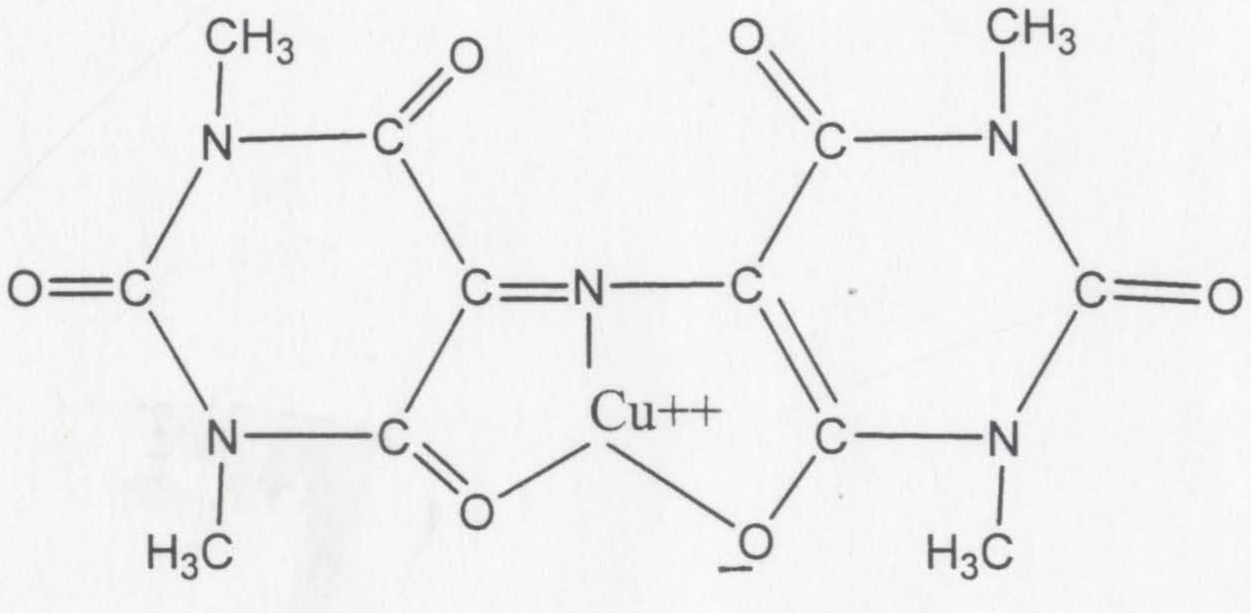
Figure 4.1 The binders of Cu^{2+} by tetramethylmurexide. Adapted from Terasawa *et al*. (1991)



+ Cu^{++}

Tetramethylmurexide

530 nm (purple)



Tetramethylmurexide - Cu^{++}

480 nm (orange)

Zn^{2+} and Fe^{2+}) of plant phenolic compounds using the TMM method (Asakura *et al.*, 1990; Terasawa *et al.*, 1991; Wettasinghe and Shahidi, 2002). Bassi *et al.* (2000) observed that the uptake of metal ions by chitosan increased as the pH rose from 4 to7 using atomic absorption spectrophotometry. This could be due to the better availability of amino groups at higher pH values. The reduced adsorption of metal ions under acidic pH conditions could be attributed to the fact that at a lower pH, the metal ions that would coordinate with the lone pair electrons of nitrogen would have to compete with H_3O^+ for an active site (Jha *et al.*, 1988). Muzzarelli and Tubertini (1969) observed a decrease in the uptake capacity for many metal ions by chitin and chitosan when solution pH decreased from 7 to 2.5. However, hexavalent chromium uptake by chitosan decreased as the pH increased from 3 to 5 (Udaybhaskar *et al.*, 1990). The correspondence between rising pH and adsorption of metal ions was not found above pH 7. The experimentally observed decrease in metal removal at higher pH values may be explained by rapid changes in protonated and unprotonated forms of chitosan (Udaybhaskar *et al.*, 1990). According to Bassi *et al.* (2000), the maximum decrease in the uptake of metal ions was found at pH 6 and 7, but when using a colourimetric method, the maximum decrease of metal ions was found at pH 5.

4.4.2 Reaction time

The chitosan used reduced the concentration of metal ions regardless of the contact time. However, changing the contact time between chitosan and metal ions had a significant ($p \leq 0.05$) effect on the chelation capacity of chitosans. The maximum chelation capacity was achieved at pH 5 by all three types of chitosan upon 6 h standing

Figure 4.2 Metal chelation capacity of Cd^{2+} by chitosans and EDTA under three different pH conditions over time.

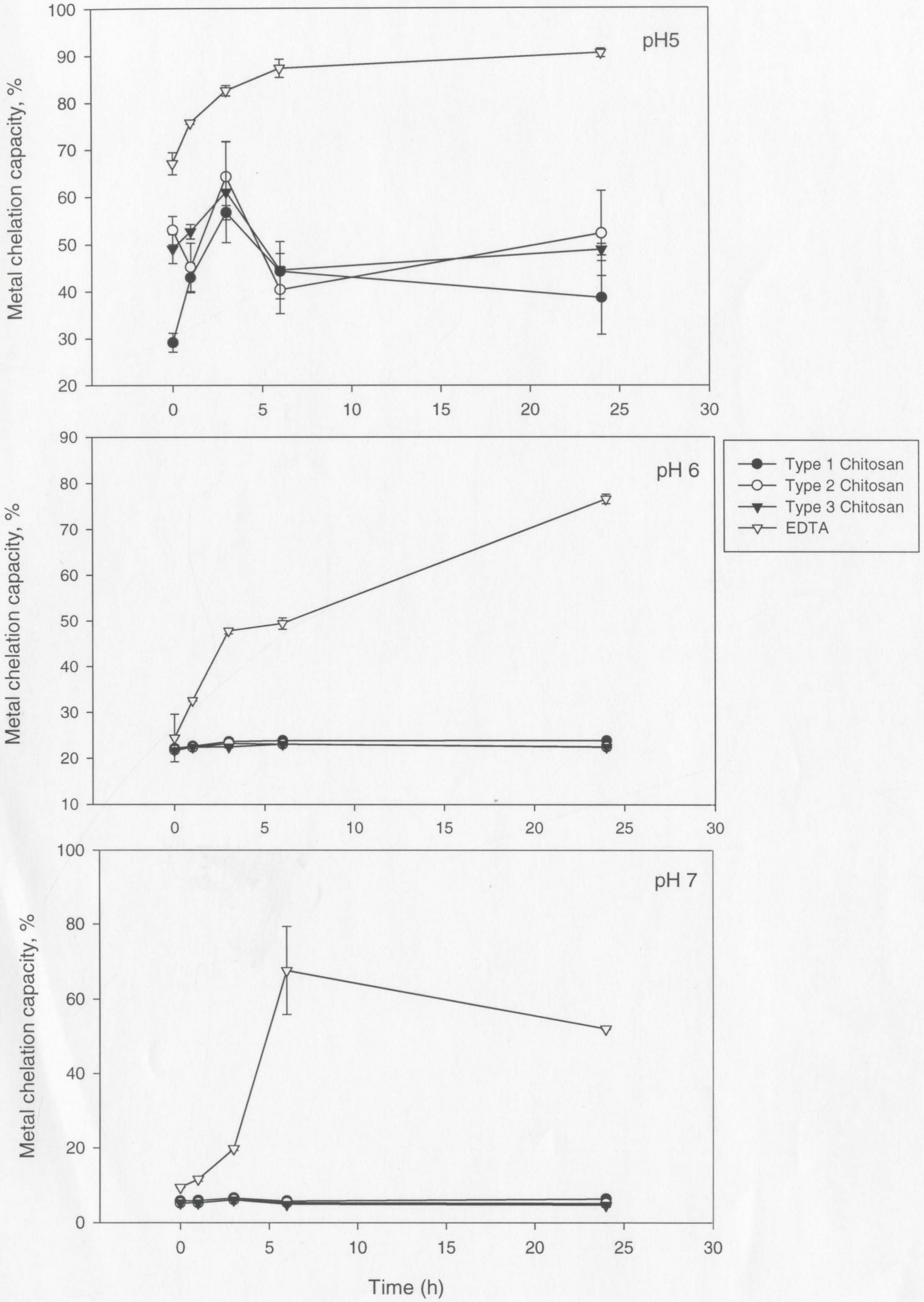

pH5
pH 6
pH 7
Metal chelation capacity, %
Time (h)
Type 1 Chitosan
Type 2 Chitosan
Type 3 Chitosan
EDTA

Figure 4.3 Metal chelation capacity of Co^{2+} by chitosans and EDTA under three different pH conditions over time.

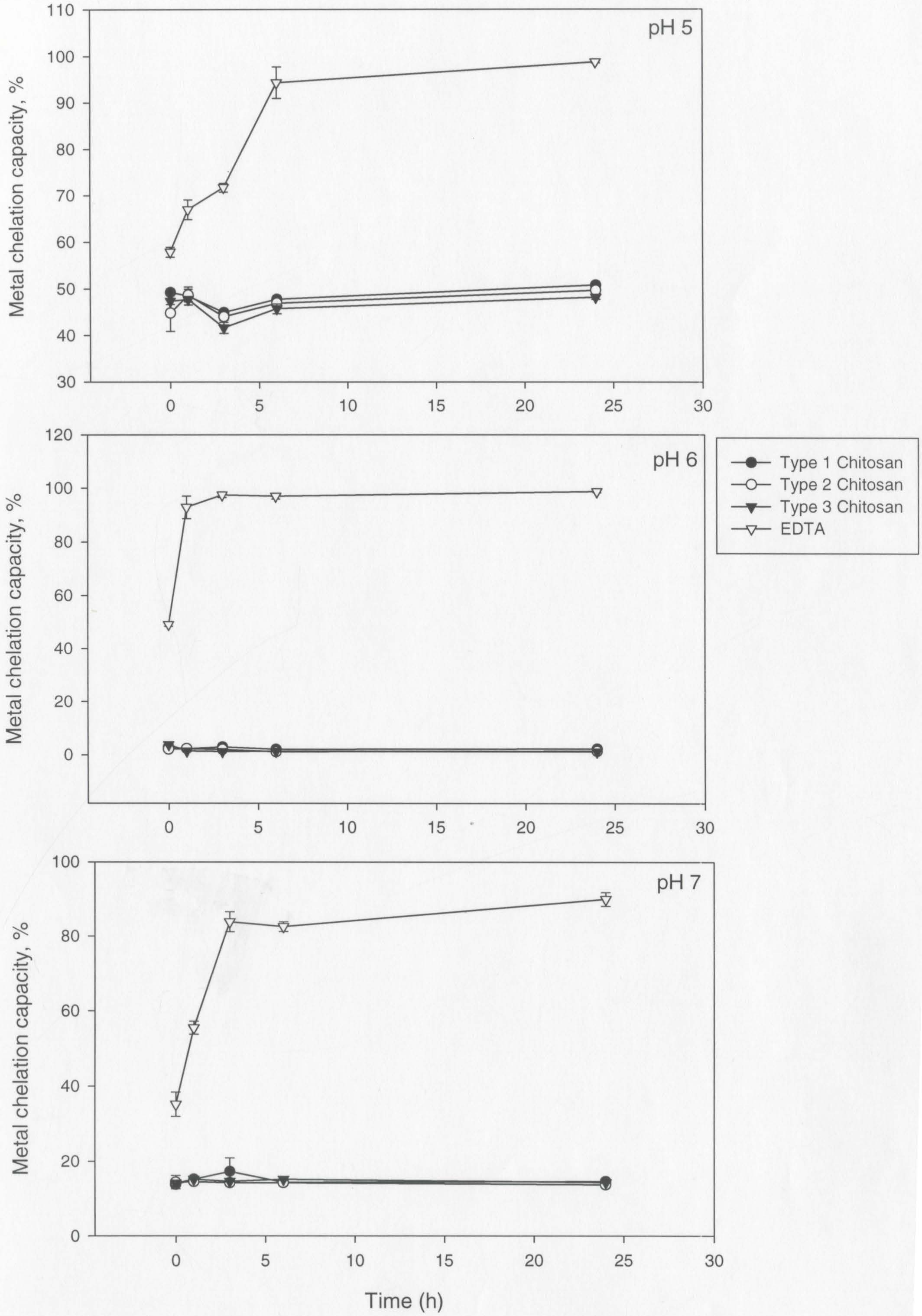

pH 5
pH 6
pH 7
Metal chelation capacity, %
Time (h)
Type 1 Chitosan
Type 2 Chitosan
Type 3 Chitosan
EDTA
110
100
90
80
70
60
50
40
30
120
20
0
5
10
15
25

Figure 4.4 Metal chelation capacity of Cu^{2+} by chitosans and EDTA under three different pH conditions over time.

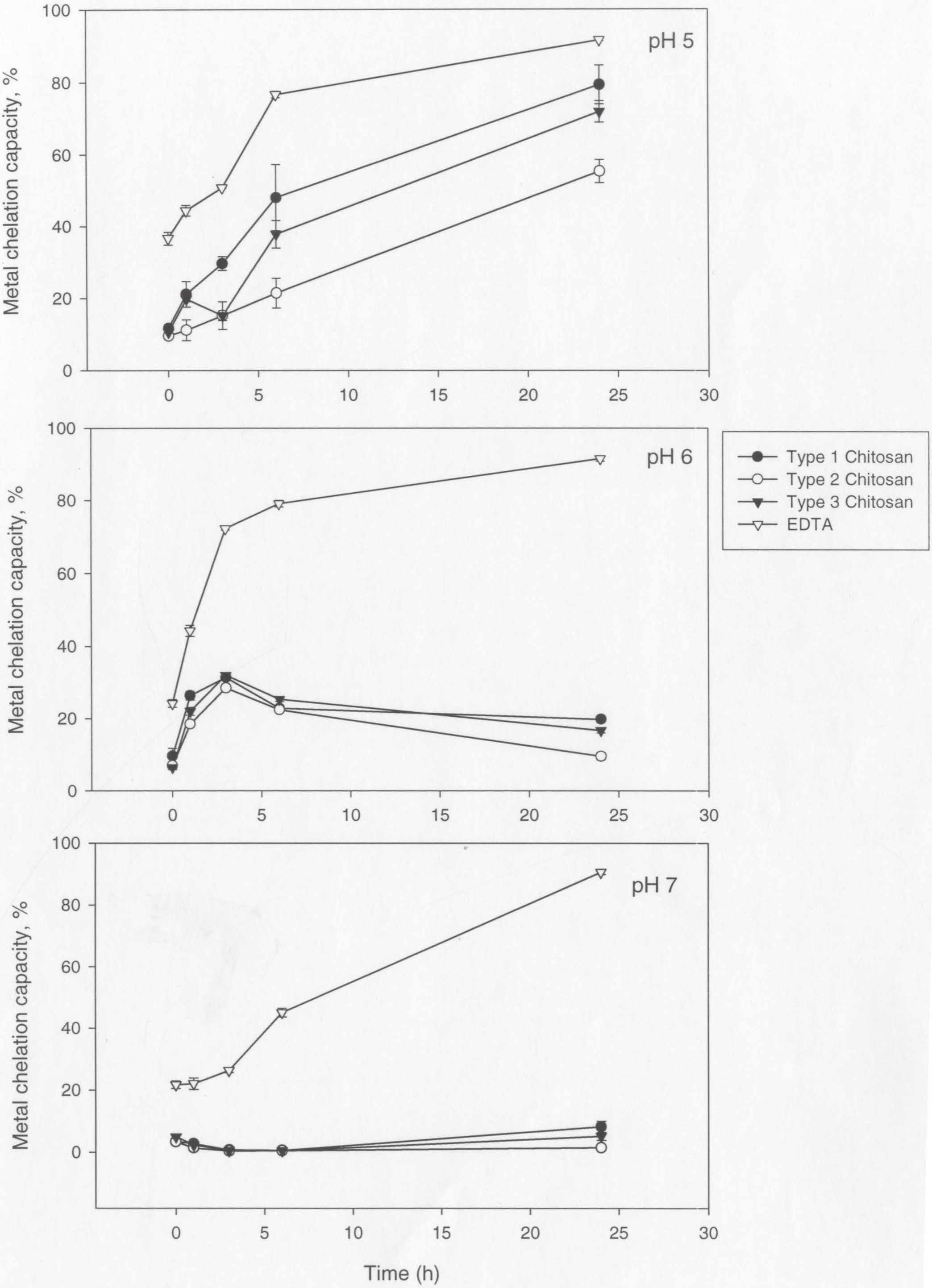

pH 5
pH 6
pH 7
Metal chelation capacity, %
Time (h)
0
20
40
60
80
100
0
5
10
15
20
25
30
Type 1 Chitosan
Type 2 Chitosan
Type 3 Chitosan
EDTA

Figure 4.5 Metal chelation capacity of Fe^{2+} by chitosans and EDTA under three different pH conditions over time.

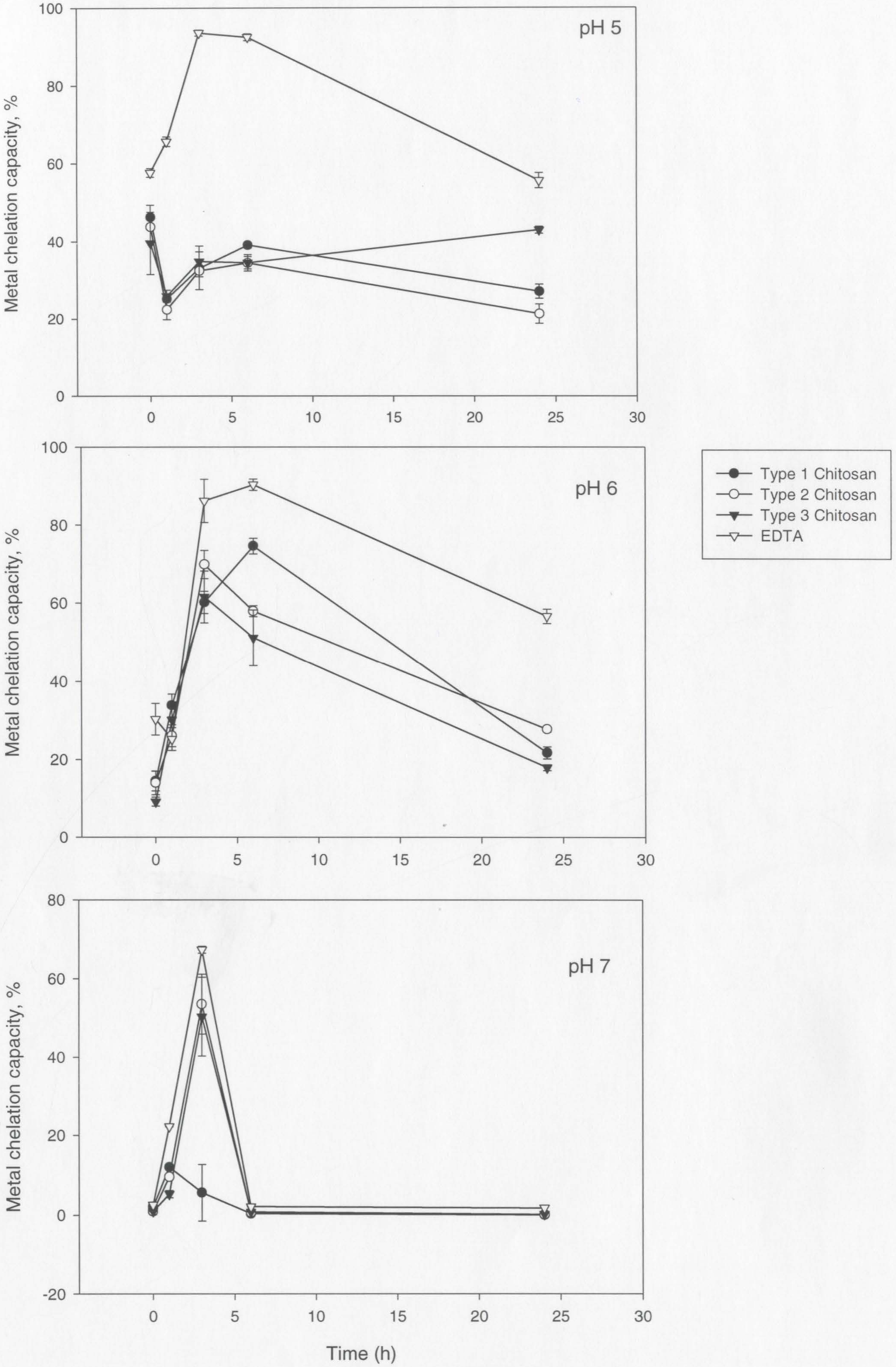

pH 5
pH 6
pH 7
Metal chelation capacity, %
Time (h)
Type 1 Chitosan
Type 2 Chitosan
Type 3 Chitosan
EDTA
100
80
60
40
20
0
-20
0
5
10
15
20
25
30

Figure 4.6 Metal chelation capacity of Mn^{2+} by chitosans and EDTA under three different pH conditions over time.

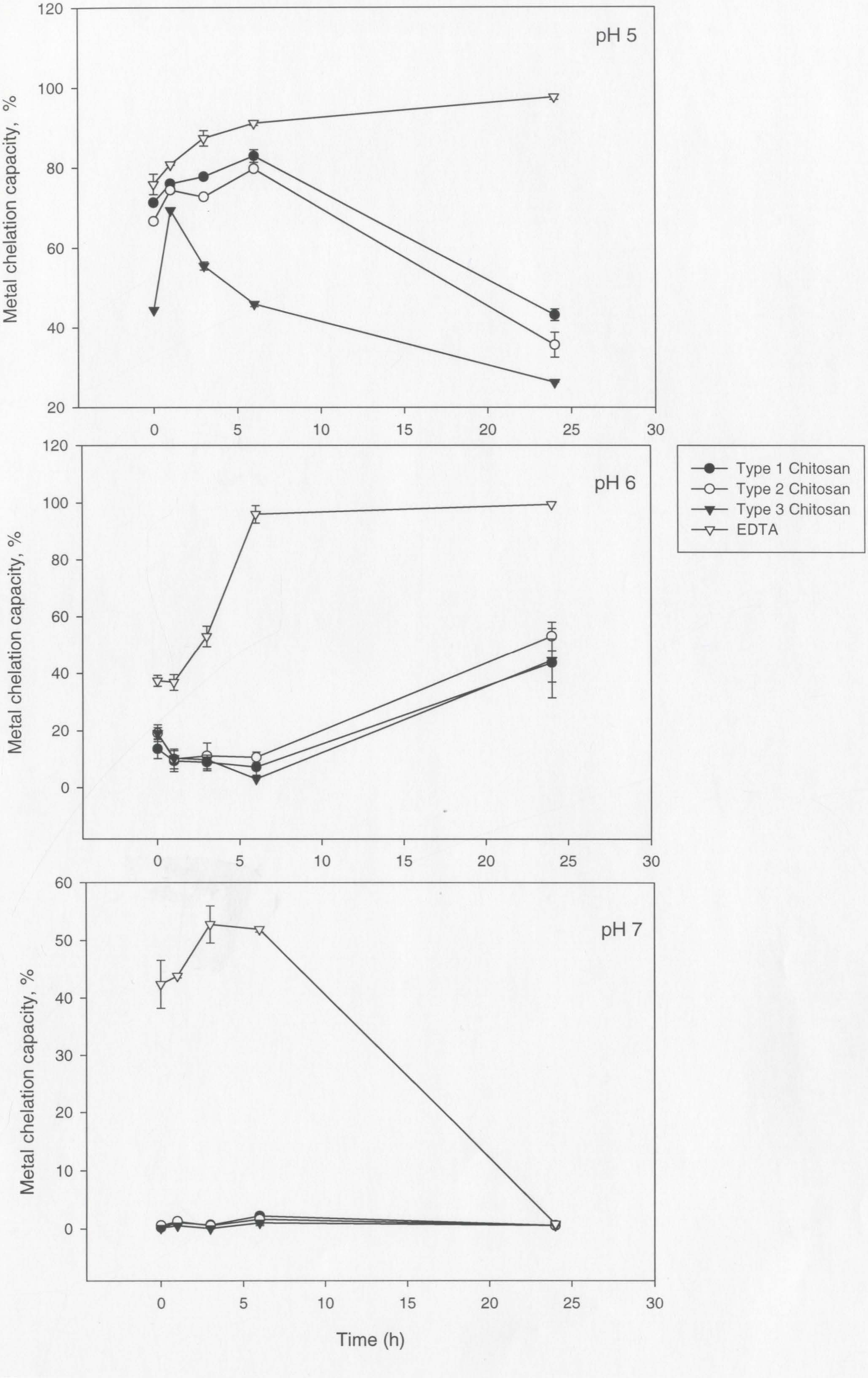

pH 5
pH 6
pH 7
Metal chelation capacity, %
Time (h)
Type 1 Chitosan
Type 2 Chitosan
Type 3 Chitosan
EDTA

Figure 4.7 Metal chelation capacity of Ni^{2+} by chitosans and EDTA under three different pH conditions over time.

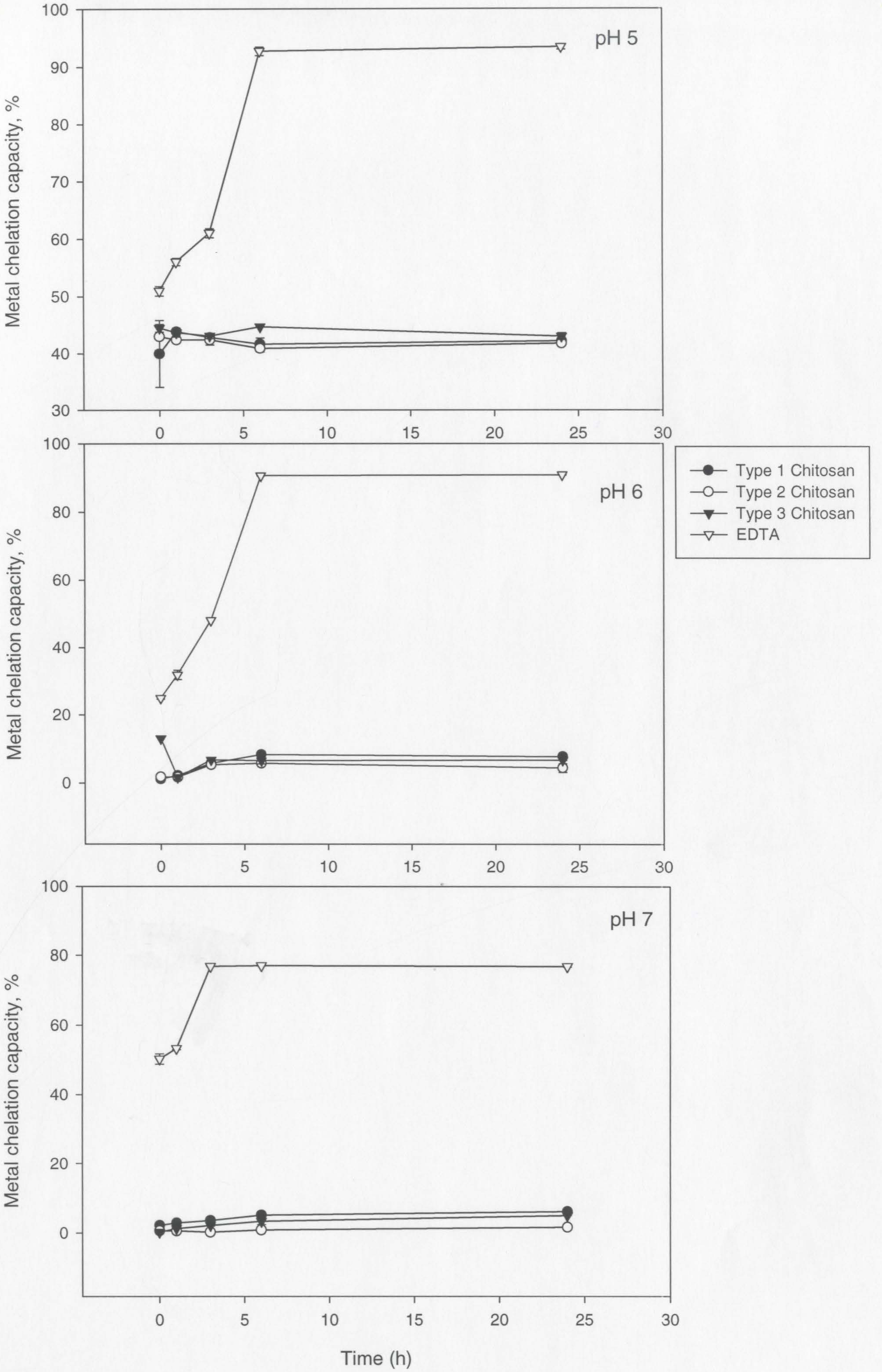
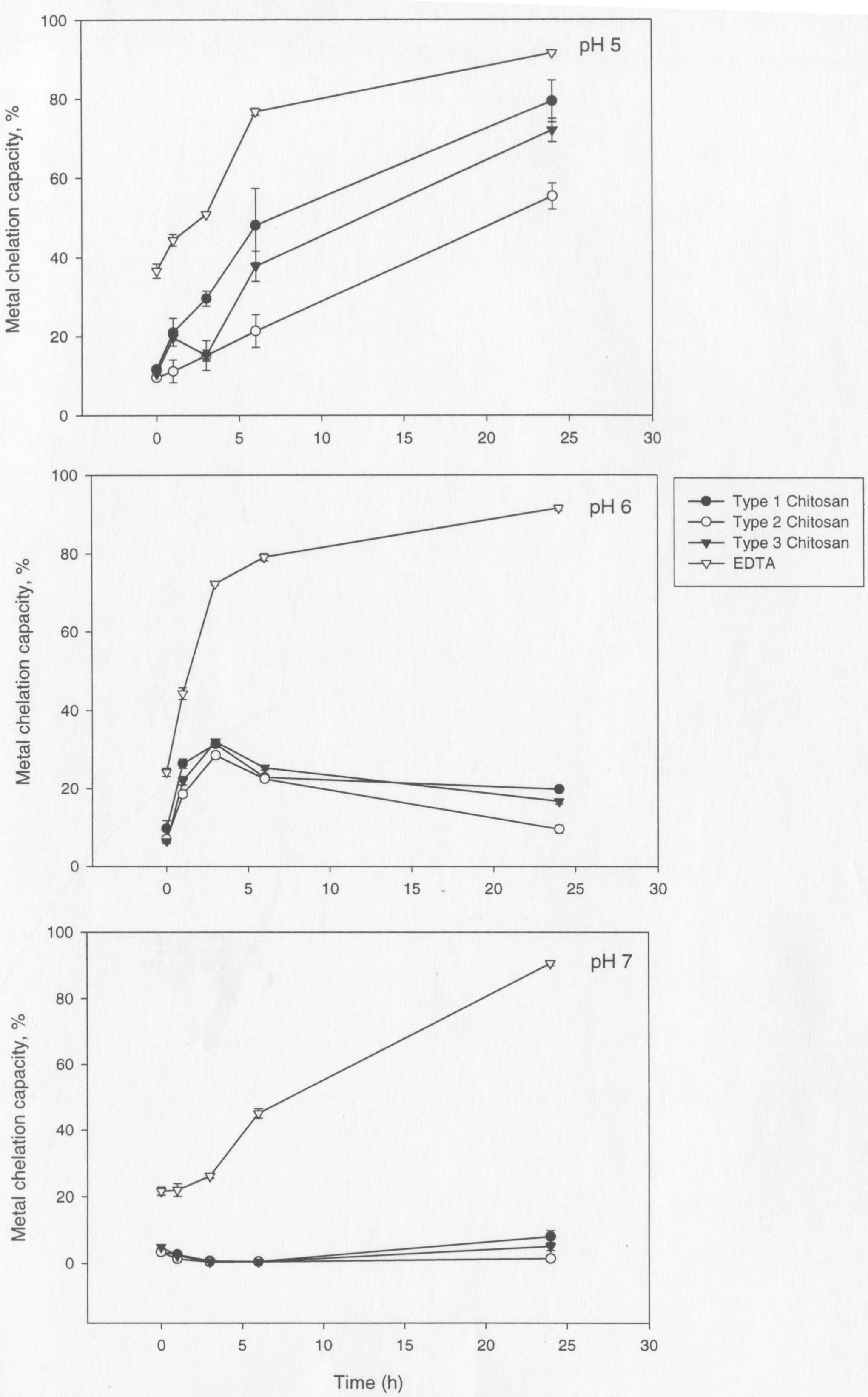

pH 5
pH 6
pH 7
Metal chelation capacity, %
Time (h)
Type 1 Chitosan
Type 2 Chitosan
Type 3 Chitosan
EDTA
0
5
10
15
20
25
30
20
40
60
80
100
30
90
70
50

Figure 4.8 Metal chelation capacity of Zn^{2+} by chitosans and EDTA under three different pH conditions over time.


pH 5
pH 6
pH 7
Metal chelation capacity, %
Time (h)
0
20
40
60
80
100
0
5
10
15
20
25
30
Type 1 Chitosan
Type 2 Chitosan
Type 3 Chitosan
EDTA

with Zn^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+} and Fe^{2+}. The maximum chelation capacity was achieved after 3 h of standing at pH 6 by all three types of chitosan with Zn^{2+}, Cd^{2+}, Cu^{2+}, Co^{2+} and Fe^{2+}. The maximum chelation capacity was achieved after 3h of standing at pH 7 by all three types of chitosan with Fe^{2+}, Co^{2+} Cu^{2+} and Cd^{2+} (Figures 4.2-4.7). Figures 4.2-4.8 show that the amount of chitosan used under different reaction times exhibited a considerable uptake of metal ions. The increase in the adsorbed metal ion contents leveled off after 6h of standing at pH 5 (Zn^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+} and Fe^{2+}) and 3h of standing at pH 6 (Zn^{2+}, Cd^{2+}, Cu^{2+}, Co^{2+} and Fe^{2+}), indicating the attainment of adsorption equilibrium. Longer reaction times (> 3 h) between chitosan and metal solutions were reported and indicated no result in an increase in metal ion uptake (Dean and Dixon, 1992; Peniche-Cocas *et al.*, 1992; Bassi *et al.*, 2000). Bassi *et al.* (2000) observed that shaking of the mixture of chitosan flakes and metal ions did not to affect the adsorption capacity of chitosan for metal ions. Ethylenediaminetetraacetic acid in comparison with different chitosan types showed the highest chelation capacity, as it is known to be a strong chelating agent (Juang *et al.*, 1999). Interaction between EDTA and metal ions in natural aquatic systems has been investigated by several researchers. For example, Barica *et al.* (1973) examined the effects of EDTA on increased metal concentrations in lake water as a result of solubilization from associated metal-contaminated sediments. In the present study, out of three chitosans examined Type 1 showed the best chelation capacity.

4.4.3 Partical size of chitosan

Experimental results show that the chelation capacity increased with decreasing particle size (Type 1 > Type 2 > Type 3). Particle size of the Type 1 product was 350-500 microns, Type 2, 350-1000 microns and Type 3, 500-1000 microns. According to Huang *et al.* (1996), the number of adsorption sites per unit mass increased with decreasing particle size. Chitosan can be considered as a microporous material. After chitosan was crushed into small particles, adsorption sites on the chitosan chain increased thus allowing better binding of metal ions. According to Roberts (1992) the adsorpion capacity was found to be independent of particle size but to decrease with increasing temperature.

4.4.4 Metal identity

Upon chelation, the polymers assume typical colours: yellow for Fe^{2+}, pink for Co^{2+}, green for Ni^{2+} and blue for Cu^{2+}. A number of metal ions including Zn^{2+}, Mn^{2+} and Cd^{2+} while being effectively chelated did not develop any colour as expected. The order of metal ions chelation of chitosan at pH 5 was $Cu^{2+} > Fe^{2+} > Ni^{2+} > Co^{2+} > Zn^{2+} > Cd^{+2} > Mn^{+2}$. The order of metal chelation at pH 6 was $Fe^{2+} > Co^{2+} > Cd^{2+} > Ni^{2+} > Cu^{2+} > Zn^{2+} > Mn^{2+}$ and pH 7 was $Fe^{2+} > Co^{2+} > Cd^{2+} > Ni^{2+} > Cu^{2+} > Zn^{2+} > Mn^{2+}$. Thus, different patterns were obtained at different pH values. However, the order found for the adsorption of metal ions by chitosan appears to vary in different studies. According to Peter (1995), the chelation order was $Zn^{2+} > Ni^{2+} > Cu^{2+} > Cd^{2+} > Co^{2+} > Mn^{2+} > Fe^{+2}$.

Figure 4.9 Metal chelation capacities of three types of chitosan after 3 h.

Type 1 Chitosan

Type 2 Chitosan

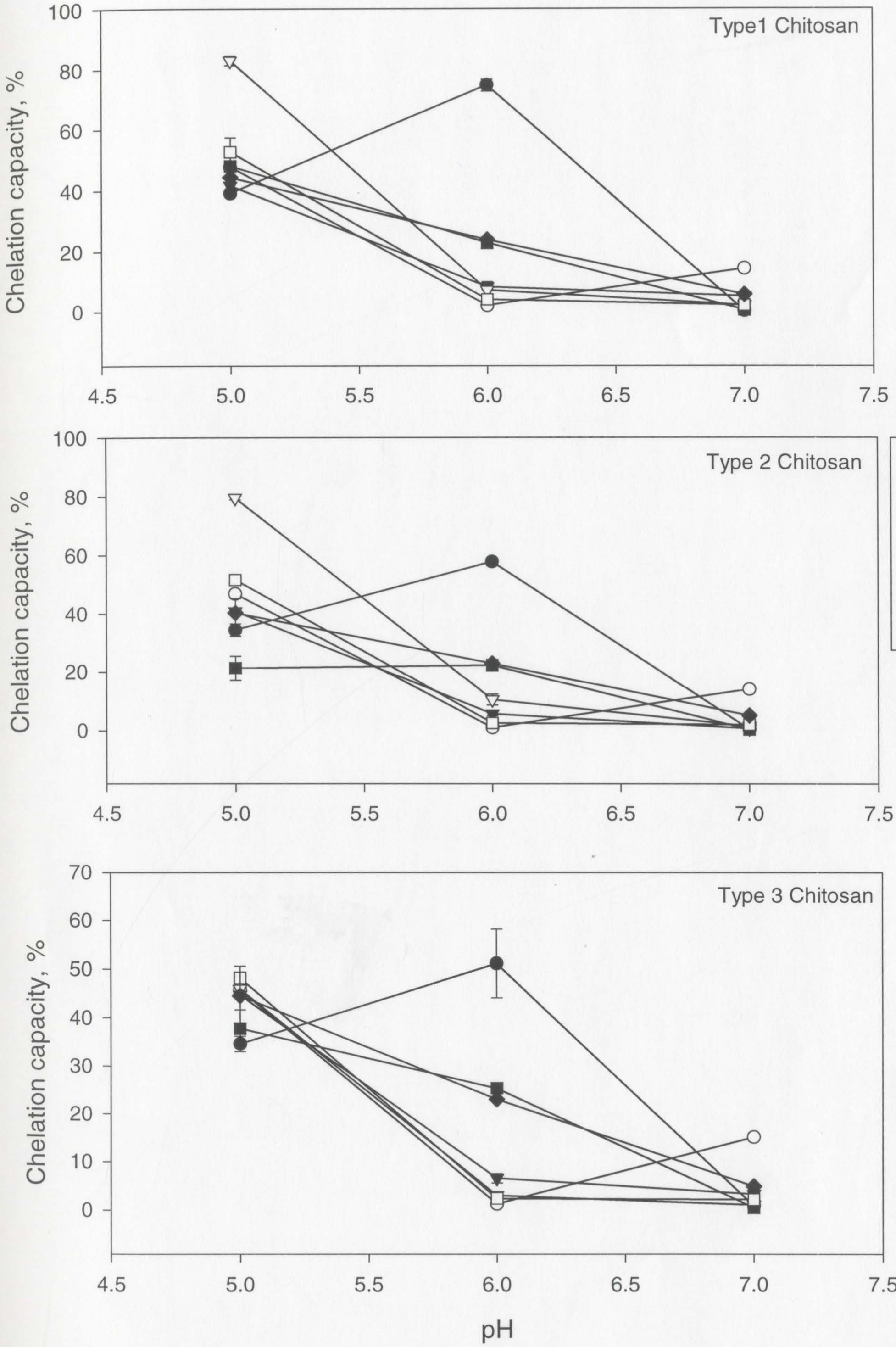
Type 3 Chitosan

Chelation capacity, %

pH

Fe2+
Co 2+
Ni 2+
Mn 2+
Cu 2+
Zn 2+
Cd 2+

Figure 4.10 Metal chelation capacities of three types of chitosan after 6 h.


Type1 Chitosan
Type 2 Chitosan
Type 3 Chitosan
Chelation capacity, %
pH
Fe2+
Co 2+
Ni2+
Mn2+
Cu2+
Zn2+
Cd2+
100
80
60
40
20
0
70
50
30
10
4.5
5.0
5.5
6.0
6.5
7.0
7.5

A colorimetric method is was for measuring metal chelation capacity using the absorption ratio of $460_{nm}/530_{nm}$ for all metal ions. Several authors have employed the same $460_{nm}/530_{nm}$ absorption ratio for Zn^{2+}, Cu^{2+} and Fe^{2+} metal ions (Asakura *et al.*, 1990; Terasawa *et al.*, 1991; Wettasinghe and Shahidi, 2002).

4.5 Determination of heavy metals in waste water samples by inductively coupled plasma mass spectrometry (ICP-MS)

Mining activities result in pollution of aquatic systems. Water samples from a zinc mining site were analyzed using ICP-MS, which is a powerful detection system for elemental analysis at very low concentrations (trace and ultratrace level) (Lee *et al.*, 2000). Since ICP-MS has a very low detection limit and a very wide analytical concentration range, it is often used for elemental analysis (Harada and Hatanaka, 1998) and studies on environmental materials (Yamasaki and Tsumura, 1992; Lee *et al.*, 2000). Elements present in the water samples collected from a zinc mining site (Buchans, NL) are included in Table 4.4. The average pH of the waste water samples collected was 6.2-6.5.

Wastewater samples obtained from the zinc mining site, Buchans, NL, contained excessive amounts of Mn^{2+}, Co^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+}, Cu^{2+} and Zn^{2+} based on the Canadian water quality guidelines (1999) for protection of fresh water. Figures 4.11 to 4.13 show the effectiveness of chitosan in metal ion chelation from wastewater at different pH levels. Out of the three pH conditions used, pH 7 was the best for chelation of multiple metal ions. Considering the three types of chitosan, Type 1 was significantly

Table 4.4 Elements present in the wastewater samples from the zinc mining site (Buchans, NL) as determined by ICP-MS and their concentrations.

Elements	Concentration, ppb or µg/L
Mg	1191.1
Al	19.40
S	110.35
Ca	14700.00
Cl	1669.00
Cr	0.70
Fe	161.50
Mn	158.50
Co	0.47
Ni	1.11
Cu	15.39
Zn	1330.00
As	0.43
Mo	1.40
Ag	0.03
Cd	4.99
Hg	0.59
Pb	32.93
U	0.02

Figure 4.11 Effectiveness of chitosan in metal ion chelation from wastewater at pH 5.

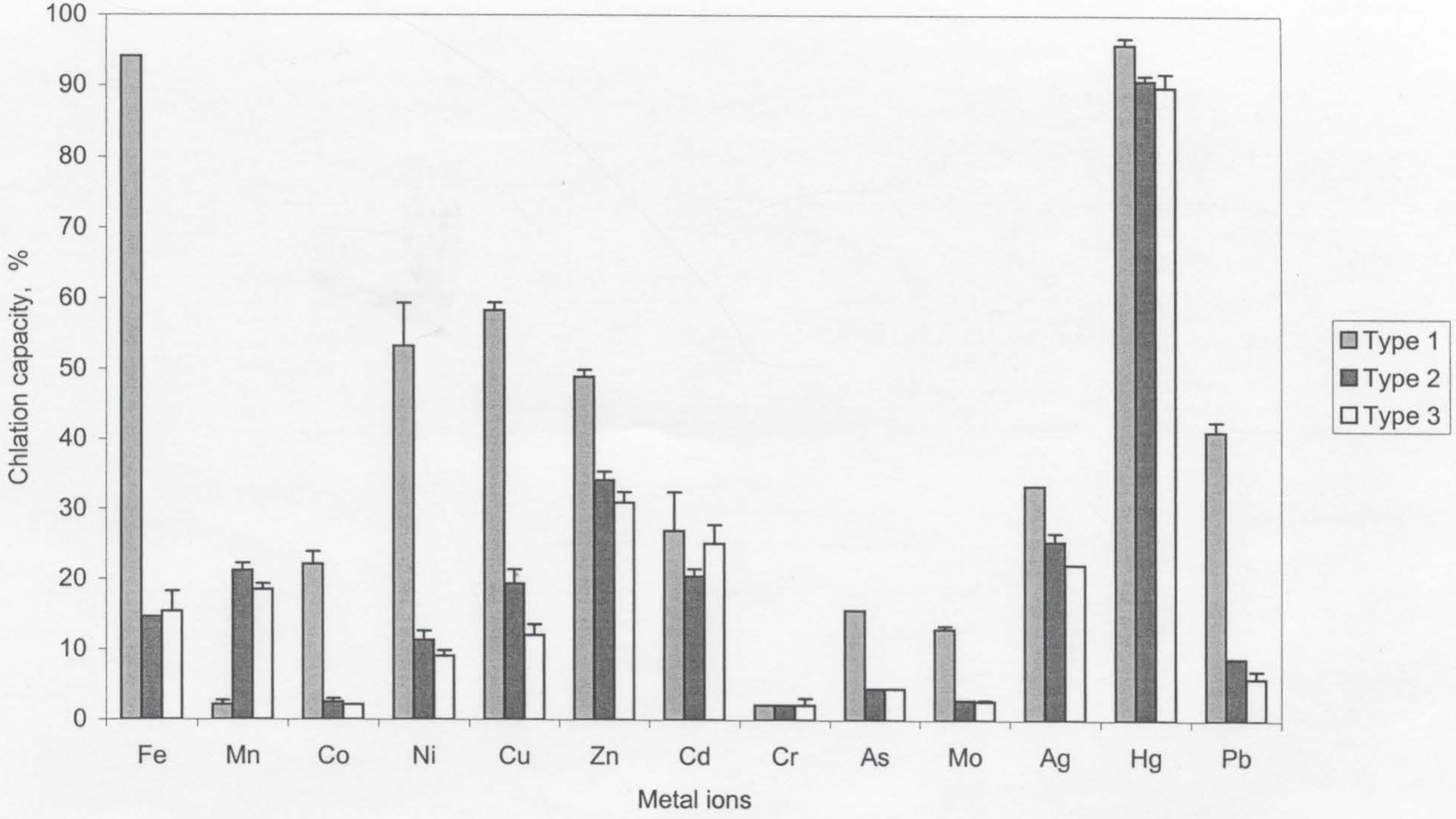

100
90
80
70
60
50
40
30
20
10
0
Chlation capacity, %
Fe
Mn
Co
Ni
Cu
Zn
Cd
Cr
As
Mo
Ag
Hg
Pb
Metal ions
Type 1
Type 2
Type 3

Figure 4.12 Effectiveness of chitosan in metal ion chelation from wastewater at pH 6.

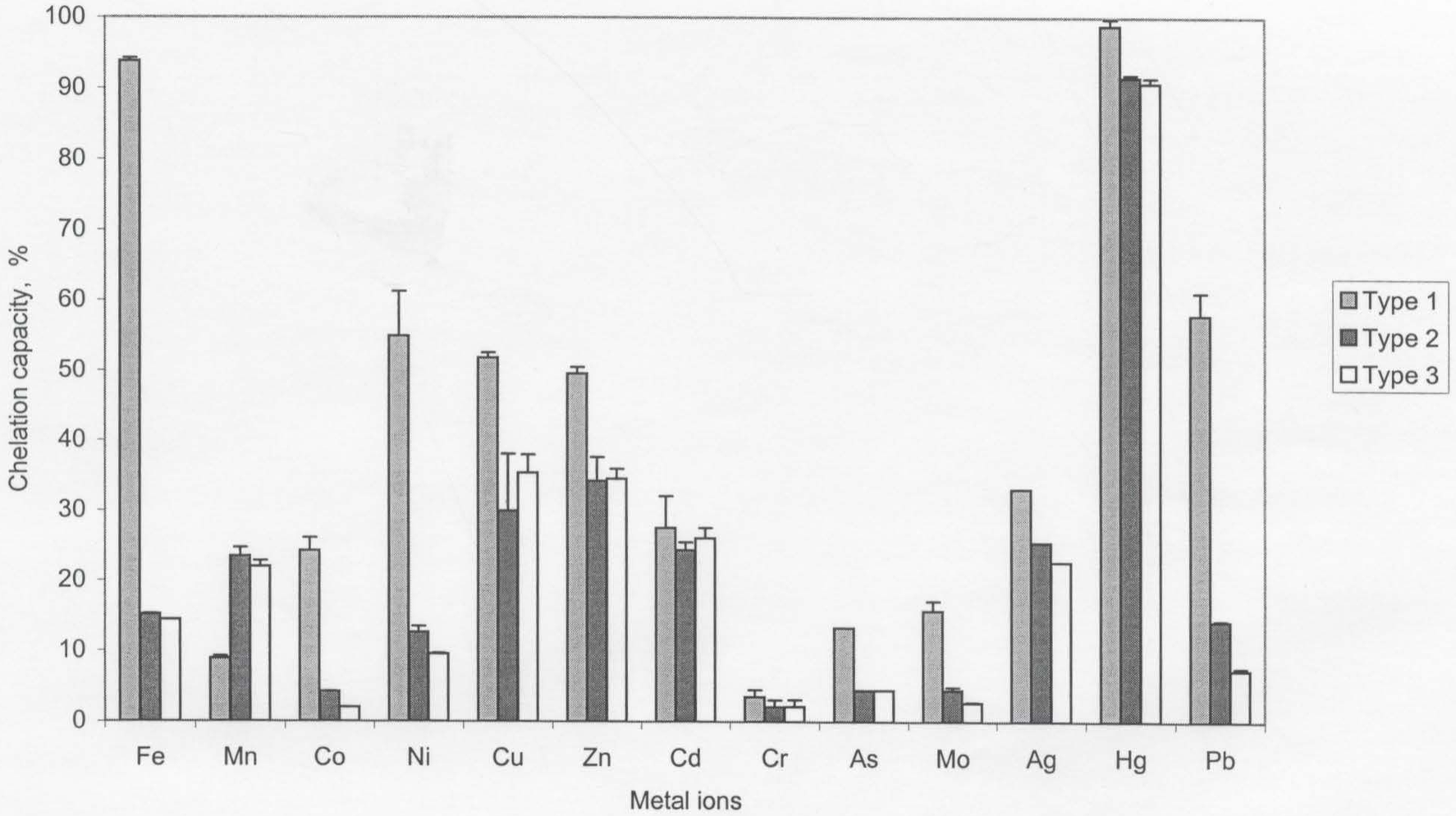
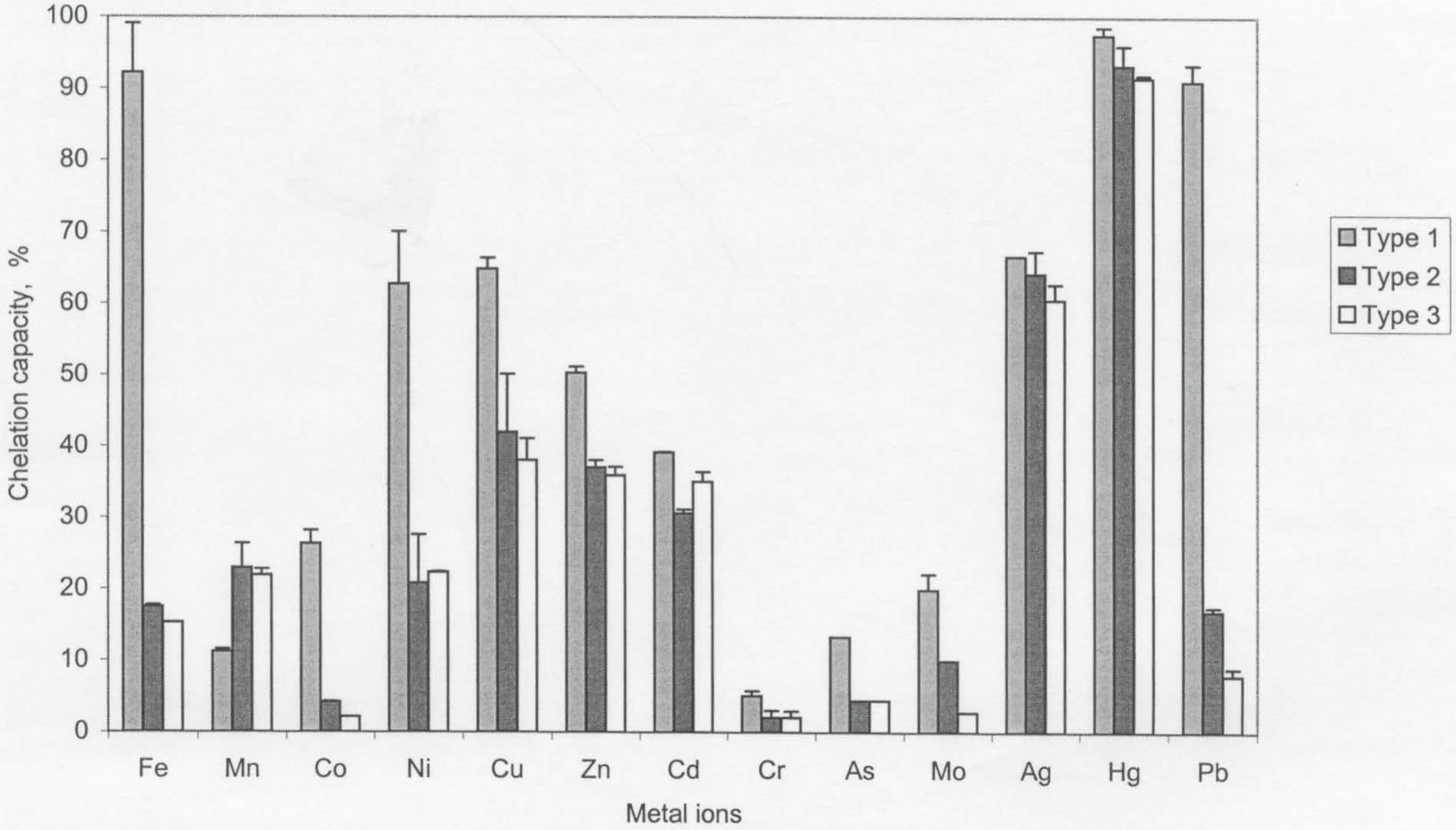

100
90
80
70
60
50
40
30
20
10
0
Chelation capacity, %
Fe
Mn
Co
Ni
Cu
Zn
Cd
Cr
As
Mo
Ag
Hg
Pb
Metal ions
Type 1
Type 2
Type 3

Figure 4.13 Effectiveness of chitosan in metal ion chelation from wastewater at pH 7.


100
90
80
70
60
50
40
30
20
10
0
Chelation capacity, %
Fe
Mn
Co
Ni
Cu
Zn
Cd
Cr
As
Mo
Ag
Hg
Pb
Metal ions
Type 1
Type 2
Type 3

($p < 0.05$) different from Types 2 and 3 (Table A6 in appendix). Chitosan Type 1 was best for metal chelation followed by Type 2 and 3. Chitosans Type 2 and 3 showed the same chelation pattern. Of the metal ions tested, Hg^{2+} was best chelated under all pH conditions by all three types of chitosan. Ferrous ions were best chelated at pH 5. The order of chelation capacity percentage of metal ions was in the decreasing order of: $Hg^{2+} > Pb^{2+} > Fe^{2+} > Cu^{2+} > Ni^{2+} > Zn^{2+} > Cd^{2+} > Co^{2+} > Mn^{+2}$.

According to the Canadian water quality guidelines (1999), the wastewater sample tested contained high amounts of Zn^{2+} (Table 4.5). Using chitosan (Type 1, Type 2 and 3) the concentration of zinc ions could be reduced upto 50%. However, it is still higher than the level suggested in the Canadian water quality guidelines, (1999) for protection of aquatic life. Therefore, multiple passage through chitosan might be necessary. Zinc is an essential element mediating a variety of metal enzymes and the biosynthesis of nucleic acids and polypeptides. The non-stoichiometric accumulation of heavy metals by the carrier protein metal thionein could lead to zinc deficiencies in humans. Symptoms of zinc deficiency include delayed healing, suppression of enzymatic activity, and immune response (Moore and Ramaoorthy, 1984). Toxicity symptoms of zinc include nausea, vomiting and diarrhea, in several cases with blood, and abdominal cramps (Elinder and Piscator, 1979).

The toxicity of zinc ions to aquatic plants is highly variable, with LC_{50}'s (lethal concentration) ranging from 0.0075 to 50 mg/L. LC values usually refer to the concentration of a chemical in air, but in environmental studies it can also mean the

concentration of a chemical in water. The concentration of a chemical in water that kills 50% of the test animals in a given time is referred to as the LC_{50} value. Part of the extreme inconsistency is due to the ability of many species to adapt to high zinc levels in water. In addition, physicochemical factors may significantly influence the bioavailability of zinc. Under most circumstances, mercury and copper are more toxic to aquatic plants than chromium, cadmium, nickel, lead and arsenic which may be more or less toxic, depending on the conditions considered (Moore and Ramamoorthy, 1984). Acute toxicity of zinc to freshwater invertebrates is relatively low. Although the 48-96 h LC_{50}'s generally range from 0.5 to 5 mg/L, some species are particularly sensitive to zinc. Considering fish, the 48 – 96 h LC_{50} for zinc usually falls within 0.5 to 5.0 mg/L (500-5000 μg/L), physicochemical and biological factors may extend the range from 0.09 to >100 mg/L. Susceptibility to zinc poisoning is largely species dependent (Moore and Ramamoorthy, 1984). Zinc toxicity of soil decreased considerably after ageing but there was a parallel increase in pH of more than one unit (Lock and Janssen, 2002).

Research has been done on the use of chitosan for the removal of some heavy metal ions from actual industrial waste water. Masri and Randall (1978) investigated chitosan binding of lead, copper, nickel, cadmium and zinc ions from a number of different industrial waste solutions. The use of commercially available chitosan for potable water purification has been approved by the USEPA up to a maximum level of 10 mg/L (Knorr, 1984). It not only removes toxic metal ions such as those of Cd and Pb when present in the environment, but it also prevents accidental contamination by radioactive isotopes such as cobalt-60. Chitosan also eliminates undesirable though harmless metal ions such

as Fe^{2+}, which are known to impart unpleasant organoleptic properties (Muzzarelli *et al.*, 1989).

Apart from the advantage of reusing the industrial waste, chitosan has the capability of adsorbing either metal ions or complexing metal ions at the same time. Under alkaline conditions, chitosan exhibits a strong cation exchange behaviour to remove metal ions from industrial wastewater. In acidic condition, chitosan causes anion exchange sites to be made available to bind the nickel- cyanide anion. Adjustment of pH may be on developed to replace the conventional process for cyanide wastewater treatment. The density of adsorption sites on chitosan increased with decreasing particle size (Huang *et al.*, 1996).

4.6 Metal chelation capacity and recovery of metals from aqueous solutions using a chitosan column.

4.6.1 Individual metals.

Out of the metal ions investigated there was no significant ($p < 0.05$) difference in metal chelation ability of chitosan between 50 and 100 ppm metal concentration, except for Co^{2+}. According to Deans and Dixon (1992), a significantly reduced adsorption efficiency of chitosan was noticed upon raising the initial concentrations of copper and lead ions from 10 to 100 ppm. Increasing metal ion concentration in the solutions seems to reduce the external diffusion of the adsorbate and enhance intraparticular diffusion (Jasson-Charrier *et al.*, 1996). Chitosan removed over 99% of metal ions from 50 ppm metal solutions, except for Co^{2+} and over 98% from 100 ppm (Table 4.6).

Table 4.5 Summary of guidelines for protection of aquatic life and dinking water.

Metal ions	Canadian water quality guideline for protection of aquatic life*	Drinking water MCL**
Cr^{3+}	1	50
Fe^{2+}	300	200
Mn^{2+}	-	50
Co^{2+}	-	1000
Ni^{2+}	25-150	20
Cu^{2+}	-	1000
Zn^{2+}	30	5000
As^{2+}	5	50
Mo^{2+}	73	40
Ag^{+}	0.1	-
Cd^{2+}	0.017	3
Hg^{2+}	0.1	1
Pb^{2+}	1-7	1.5

*Adapted from Canadian water quality guideline (1999) ; values are given in ppb or μg/L .

**MCL-Maximum contamination levels for potentially toxic metals in dinking water; Adapted from Siegel (2002).

The lone pair electrons present on the amino nitrogen can establish dative bonds with transition metal ions. Some hydroxyl groups in chitosan may function as second donors; hence, deprotonated hydroxyl groups can be involved in coordination with metal ions (Micera *et al.*, 1989). Acidic pH would favour protonation of the amino sites, resulting in reversal of charges, and may greatly diminish the metal chelating ability of chitin and chitosan (Chui *et al.*, 1996). Hence, neutral pH was maintained in the chitosan columns for metal adsorption. When comparing the uptake capacity for the metal ions, the performance of chitosan, as expressed by the order of its affinity, was in the order of $Ag^{+} > Cu^{2+} > Ni^{2+} > Cd^{2+} > Co^{2+}$ for 50ppm and $Ag^{+} > Co^{2+} > Cu^{2+} > Cd^{2+} > Ni^{2+}$ for 100 ppm. The amount of metal adsorbed by chitosan was directly proportional to the increase in metal ion concentration, demonstrating a linear relationship (Chui *et al.*, 1996). In the present study there was no significant difference between solutions containing 50 and 100 ppm of single metal ions.

EDTA could satisfactorily recover 52-97% and 56-97% of Ni^{2+}, Co^{2+}, Cd^{2+} and Cu^{2+} from solutions containing 50 and 100 ppm of metal ions, respectively. Chui *et al.* (1996) found that metal removing efficiency of chitosan was over 93% for Cu^{2+} and Cr^{3+} from aqueous metal solutions ranging in concentration from 20 to 100 ppm. In this study, the removal efficiencies of chitosan ranged from 82 to 99 % depending on the concentration of Ni^{2+} in solution. The recovery efficiencies of Ni^{2+} and Cu^{2+} were 66-100 % for Type 1 chitosan.

Chelation formulations based on EDTA are used in a wide variety of applications in agriculture, food processing and water treatment industries to sequester metal ions (Gonsior *et al.*, 1997). Concern has arisen that EDTA, because of its high binding constants with metals, has the potential to solubilize metals from solid phases (i.e. sediments and colloidal particulates). This process could occur by several possible interrelated mechanisms affecting overall metal ion speciation. The chelatant may complex free metal ions in solution, thus shifting precipitation and sorption equilibria toward increased dissolution of metals. Alternatively, EDTA could interact directly with solid phases by complexing metal ions present in, and/or adsorbed to precipitated solids and minerals (Gonisor *et al.*, 1997). Because of wide spread usage, high water solubility and slow biodegradation rate, EDTA has been detected in various water sources, including waste water treatment plant effluents and river waters in both the USA and Europe (Alder *et al.* 1990). Concentrations of EDTA measured in river water typically range from 0.01 to 0.1 μM, similar to the concentration of several metal ions (Xue *et al.*, 1995).

4.6.2 Metal ion mixtures

Considering metal ion mixtures, chitosan can remove 89% of Ni^{2+}, Cd^{2+}, Cu^{2+} and Ag^{+} from aqueous metal ion solutions (Table 4.7). For the metal mixture, there is a significant difference ($p < 0.05$) between 50 and 100 ppm for all metal ions. Order of metal chelation for 50 ppm was as follows: $Ag^{+} > Cd^{2+} > Ni^{2+} > Cu^{2+} > Co^{2+}$. The metal chelating ability of chitosan for Ag^{+} was higher than that for other metal ions.

Table 4.6 Comparison of metal chelation and recovery of chitosan Type 1.

Metal ion	Chelation, %		Recovery, %	
	50 ppm	100 ppm	50 ppm	100 ppm
Ni^{2+}	99.6±0.01^{c}	98.9±0.00^{a}	87.0±10.9^{cd}	97.9±1.69^{d}
Co^{2+}	76.6±0.17^{a}	99.7±0.00^{d}	52.2±0.00^{b}	84.7±3.18^{c}
Cd^{2+}	99.1±0.01^{b}	99.1±0.00^{b}	79.8±0.21^{c}	74.3±1.56^{b}
Cu^{2+}	99.9±0.06^{c}	99.5±0.00^{c}	97.5±2.26^{d}	56.2±0.51^{a}
Ag^{+}	99.9±0.00^{c}	99.9±0.00^{d}	0.08±0.00^{a}	0.00±0.00

Results reported are mean vales of three determinations ± standard deviation
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.

Ethyelenediaminetetraacetic acid could recover 61-89 % of Ni^{2+}, Cd^{2+} and Cu^{2+} at 50ppm and 77-90% for Cd^{2+}, Cu^{2+}, Co^{2+} and Ni^{2+} at 100 ppm. Considering metal recovery, there was a significant ($p<0.05$) difference between 50 and 100 ppm.

Chitin and chitosan have been explored for wastewater treatment, such as removal of toxic metals and radionuclides, recovery of precious metals, and recycling of metals from industrial wastewater for reuse and to reduce operational costs (Rorrer and Hsien, 1993; Coughlin, 1990).

4.7 Determination of protein flocculation by chitosan

Chitosan, with its partial positive charge, can effectively function as a polycationic coagulating agent in wastewater treatment (Peniston and Johnson, 1970). Chitosan as a coagulating agent for wastewater streams is particularly effective in removing protein from wastes; the coagulated by-products could serve as a source of protein in animal feed (Bough, 1976). In the present study three types of chitosan were used for determination of protein flocculation. In order to determine protein flocculation ability of chitosan, BSA solutions were passed through the three glass columns at a rate of 12 mL/min.

Of three types of chitosan used, Type 1 showed the best flocculation ability followed by Types 2 and 3 (Table A7 and Figure 4.14). Considering Type 1 chitosan; protein flocculation was high between 2 and 4 mg/mL protein concentration. The flocculation percentage was reduced with increasing protein concentrations thereafter.

Table 4.7 Comparison of metal chelation and recovery of chitosan Type 1 for mixtures of metal ions.

Metal ion	Chelation, %		Recovery, %	
	50 ppm	100 ppm	50 ppm	100 ppm
Ni^{2+}	94.3±0.66[b]	97.2±0.07[b]	62.3±0.18[b]	90.9±9.16[a]
Co^{2+}	71.6±2.61[a]	99.4±0.39[c]	24.1±0.09[a]	89.4±14.5[a]
Cd^{2+}	94.8±0.55[b]	99.1±0.02[c]	61.2±0.07[b]	77.9±15.5[a]
Cu^{2+}	89.8±6.52[b]	95.8±0.12[a]	70.4±1.31[c]	84.3±10.0[a]
Ag^{+}	99.4±0.00[b]	99.6±0.03[c]	85.0±0.02[d]	0.86±0.17[a]

Results reported are mean vales of three determinations ± standard deviation
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.

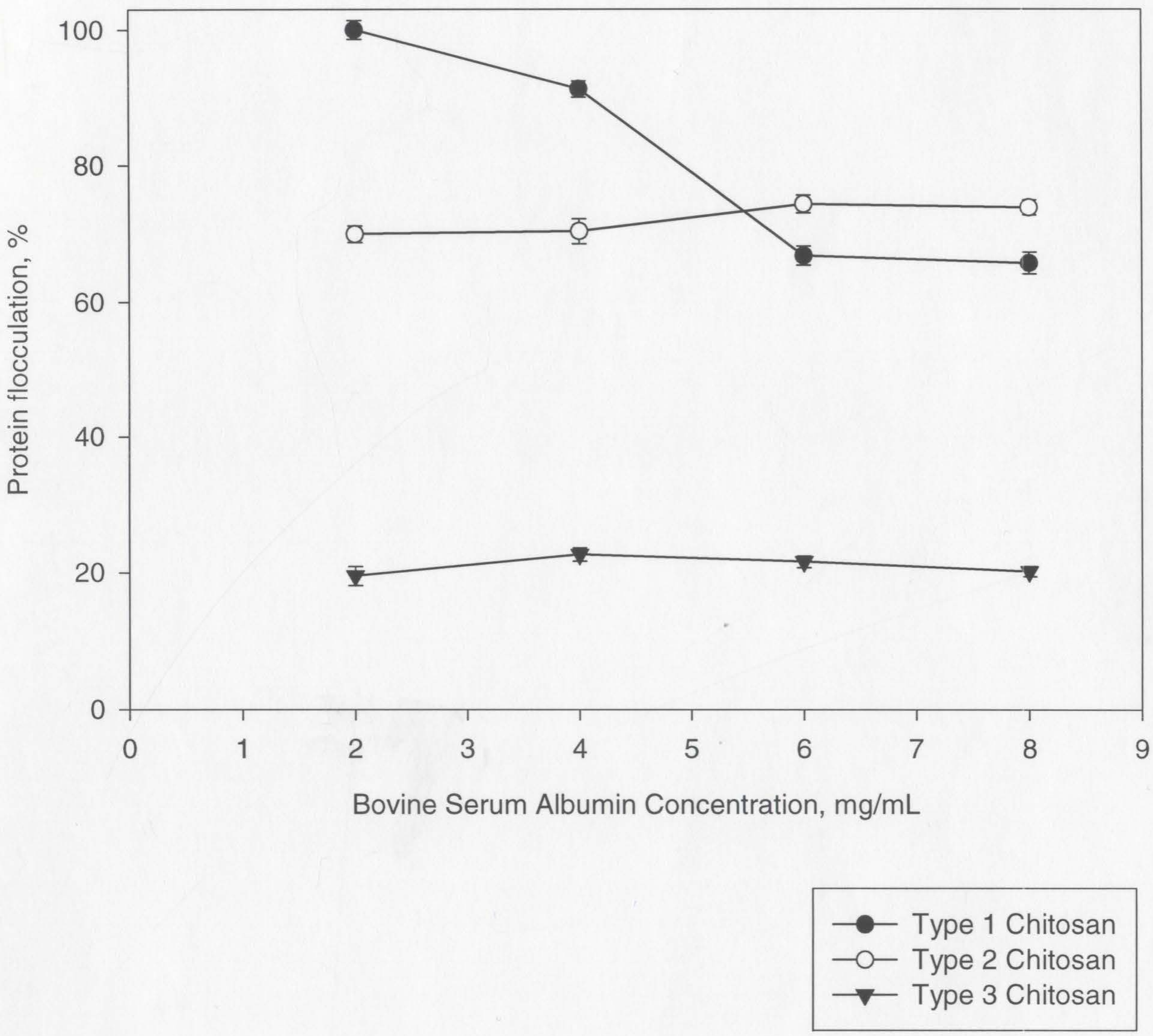
Type 2 showed the same flocculation efficiency for all concentrations of protein employed. In the present study pH 7 was used for determination of protein flocculation. The effects of pH and concentration of chitosan on reduction of turbidity in wastewater have been reported by several research groups (No and Mayers, 1989). The effect of pH on reduction of turbidity in crawfish waste water showed that lowest turbidity of 597 NTU (Nephelometric Turbidity Unit) at pH 6, and at pH 7 the turbidity was reduced to 610 NTU (No and Mayers, 1989). According to the Jun *et al.* (1994), the lowest turbidity can be achieved by treatment of water containing proteins with an optimum concentration of chitosan at pH 5.8. The mechanism of flocculating lipids and proteins from food processing wastes is due to the pKa of the amino group of the glucosamine residues which is about 6.3 (Muzzarelli, 1985); hence, chitosan is polycationic at acidic pH values (Hwang and Damodaran, 1995; Fernandez and Fox, 1997). In contrast to chitin, chitosan is soluble at pH below 6.3 and higher pH values (Senstand and Mattiasson, 1989).

Chitosan is an excellent coagulating agent and flocculant due to the high density of amino groups on the polymer chain that can interact with negatively charged substances such as proteins, solids and dyes (Li *et al.*, 1997). Wu *et al.* (1978) investigated the effectiveness of different chitosans for removing proteins from cheese whey. They found that the effectiveness of chitosan in coagulating solids and proteins was inversely proportional to its molecular weight. In the present study, Type 1 chitosan had a lower molecular weight ($6.6x10^5$ Da) than Type 2 ($9.6x10^5$ Da) and Type 3 ($1.8x10^6$ Da), as determined by. The present results support those of previous workers (Wu, 1978; Li *et*

al., 1997), in that chitosan Type 1 had the highest protein flocculation ability when compared to Type 2 and Type 3 chitosans.

Proteins and fats can be reclaimed from wastewaters by a multitude of physical/chemical and biological techniques. Reclamation of proteins yields not only economically valuable products, but also the pretreatment of food industry wastewater which is becoming a common requirement prior to discharge to the municiple sewer systems (Selmer-Olsen *et al.*, 1996). The most widely researched application for chitosan is perhaps its use as a coagulant for suspended matter in food processing wastes. Chitosan has been used to treat the waste effluents of a wide number of food process operations including egg breaking, vegetable, shrimp, cheese, meat, beer and apple juice processing. In these operations, chitosan was demonstrated to be a very good coagulating agent. The minimum reduction of suspended solids reported was 70% (Enriquez and Flick, 1989).

Figure 4.14 Protein flocculation capacity of chitosan using different concentrations of bovine serum albumin.


Protein flocculation, %
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
Bovine Serum Albumin Concentration, mg/mL
Type 1 Chitosan
Type 2 Chitosan
Type 3 Chitosan

5.0 CONCLUSIONS AND RECCOMMENDATIONS

Formaldehyde served best in controlling the foul odour development both at 4-7°C and 20-25°C. Formic acid could also be utilized in controlling foul odour development at a 1% (v/v) level considering its safety aspects.

Chitosan was effective in the removal of metal ions including Mn^{2+}, Cd^{2+}, Zn^{2+}, Fe^{2+}, Ni^{2+}, Co^{2+} and Cu^{2+} from wastewater samples obtained from a zinc mining site, Buchans, NL. Based on the colorimetric estimations, chitosan Type 1 performed better than Type 2 and Type 3 in its capacity to remove metal ions from single metal ion solutions. Ethylenediaminetetraacetic acid displayed the highest chelation capacity compared to all three chitosan types with single metal ions at all three pH values (pH 5, 6 and 7). The highest chelation capacity was observed at pH 5 followed by pH 6 and pH 7 with single metal ions. Type 1 chitosan served best for the removal of individual metal ions and metal ion mixtures compared to Types 2 and 3 chitosan. Removal of metal ions (Hg^{2+}, Fe^{2+}, Ag^{+}, Ni^{2+}, Pb^{2+}, Cu^{2+} and Zn^{2+}) from industrial wastewater was most effective at pH 7 as measured by ICP-MS. Mercury was most chelated the best under all pH conditions with all three types of chitosan.

Chitosan packed in columns removed more than 98% of Ni^{2+}, Cd^{2+}, Cu^{2+} and Ag^{+} from aqueous solutions at both 50 and 100 ppm levels of metal ion concentrations. Out of the metal ions tested, there was no significant difference among metal chelation both at 50 and 100 ppm metal concentration for single metal ions and metal mixtures, except for Co^{2+}. More than 89% of metal ion mixtures were removed by

chitosan from aqueous solutions of Ni^{2+}, Cd^{2+}, Cu^{2+}, Ag^{+} at both 50 ppm and 100 ppm levels. EDTA satisfactorily removed 52-97% and 56-97% of Ni^{2+}, Co^{2+}, Cd^{2+} and Cu^{2+} when used at 50 and 100 ppm, respectively. Ethylenediaminetettracetic acid could recover 61-89 % of Ni^{2+}, Cd^{2+} and Cu^{2+} at 50 ppm metal concentrations and 77-90 % for Cd^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+} for 100 ppm concentrations of metal ion mixtures.

In the protein flocculation study, Type 1 chitosan exhibited the best flocculation ability followed by Types 2 and 3 chitosans. Considering Type 1, protein flocculation was highest between 2 and 4 mg/mL protein. The flocculation percentages were reduced with increasing protein concentration after 4mg/mL. Type 2 showed the same flocculation percentages for 2 to 8 mg/mL protein.

Chitosans were effective in the removal of metal ions from wastewater samples. Chitosan Type 1 performed better than Types 2 and 3 in removing single metal ions, as measured a by a colorimetric method. A three hour reaction time and pH 5 yielded satisfactory results in removing metal ions from single metal ion solutions. However, pH 7 may be recommended for removal of metal ions from mixtures. The present study established that chitosan can effectively remove metal ions and proteins from waste and contaminated waters. Chitosan can be used at a rate of 0.2g /L wastewater. High efficiency of metal removal for metal ion mixtures can be achieved using Type 1 chitosan, over a 3h period at pH 7. In general, wastewaters rich in protienaceous material increase biological oxygen demand (BOD). The present study

reveals that chitosan is not only a metal remover but also good as a protein flocculating agent. High efficiency of protein flocculation can be achieved by using the Type 1 chitosan at neutral pH at less than 4 mg/mL protein in the wastewater.

Based on the above, it is suggested that crustacean shellwaste serves best for production of chitosan which may in turn be used as an environmentally friendly material for wastewater purification.

FUTURE WORK

The major commercial application of chitosan is its use in industrial wastewater treatment and recovery of a feed grade protein from food-processing plant discharges. Chitosan prepared from crab chitin has been extensively studied for its metal chelation potential, but not widely employed in wastewater treatment. Acid solubility of chitosan is a disadvantage from a practical stand point of view for metal collection as regular chitosan may dissolve in acidic wastewater. Cross-linking can change the solubility characteristics of chitosan for use in acidic media. Therefore, it is important to conduct further studies with cross-linked and non cross-linked chitosan for metal chelation and metal recovery from wastewater. Since chitosan carries a partial positive charge, it effectively functions as a polycationic coagulating agent in wastewater treatment. Further research on the evaluation of coagulating and recovery of protenaceous material in wastewater discharge from food processing plants is necessary. It is also important to study the metal chelation characteristics and protein flocculation ability of chitosan prepared from other crustacean sources, especially squid which has a different chitin and hence chitosan crystallization pattern.

Increasing demand for chitin and chitosan may, however, lead to exploitation of natural resources such as crab, shrimp, lobster and squid. Thus, planning of large scale utilization of chitin must also include their production via biotechnological means in order to address a possible shift in the ecological balance in the marine environment.

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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 mol/L
a = 0.0126
b = 0.0
Therefore, X=79.37* $A_{358\ nm}$

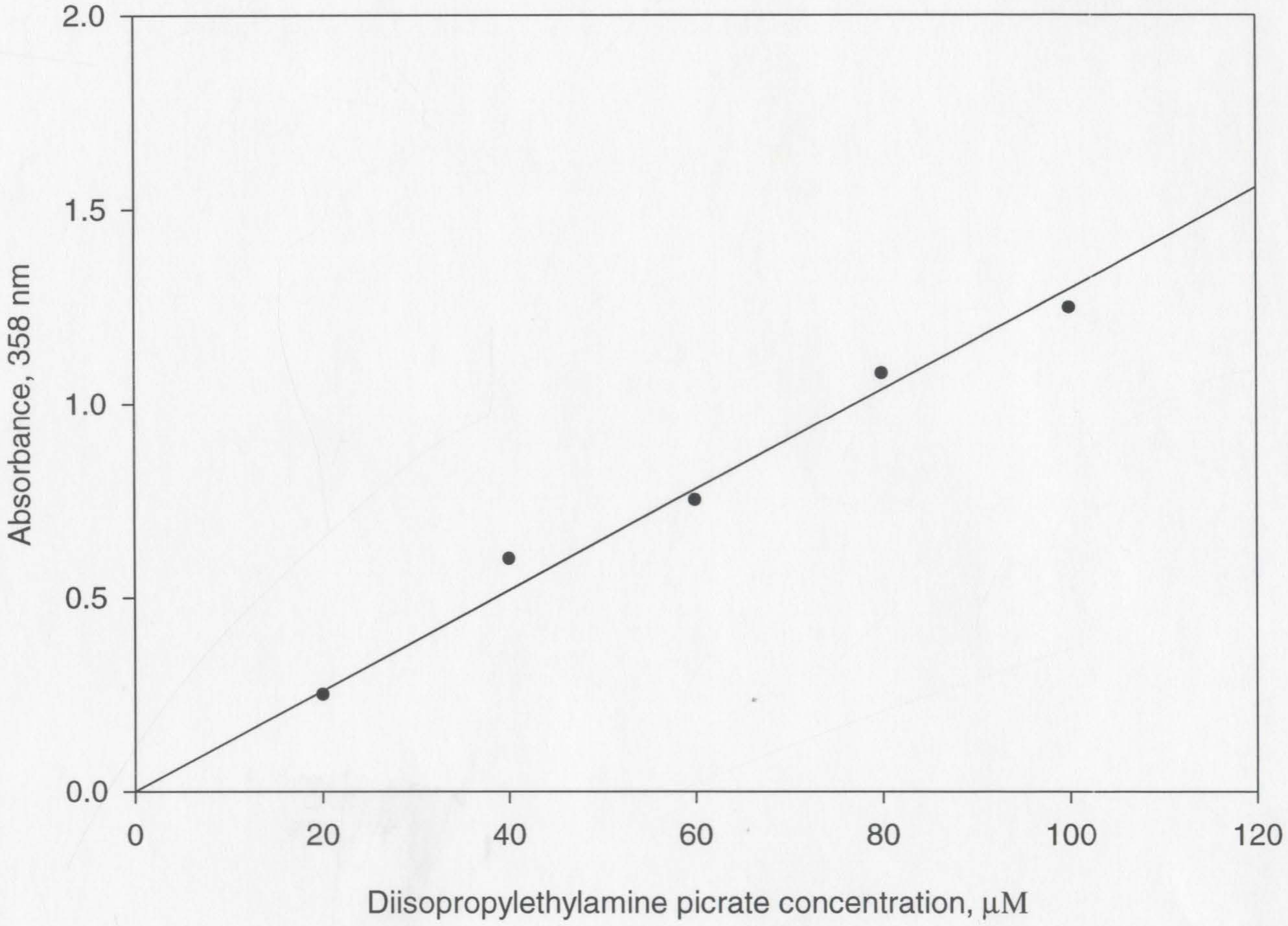

2.0
1.5
1.0
0.5
0.0
Absorbance, 358 nm
0
20
40
60
80
100
120
Diisopropylethylamine picrate concentration, μM

Table A.1 Sensory evaluation of crab waste at cold room temperature (4-7˚C).

Days	HCHO	HCOOH	CH_3COOH	$NaHCO_3$	NaOH	Control
0	1 - 100	1 - 100	1 - 100	1 - 100	1 - 100	1 - 100
	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00
	3 - 00	3 - 00	3 - 00	3 - 00	3 - 00	3 - 00
	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00
	5 - 00	5 - 00	5 – 00	5 – 00	5 – 00	5 - 00
1	1 - 85.71	1 - 57.14	1 - 57.14	1 - 28.57	1 - 28.57	1 - 00
	2 - 00	2 - 14.28	2 - 14.28	2 - 42.85	2 - 42.85	2 - 71.42
	3 - 14.28	3 - 28.57	3 - 28.57	3 - 28.57	3 - 28.57	3 - 28.57
	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00
	5 – 00	5 - 00	5 – 00	5 – 00	5 – 00	5 - 00
2	1 - 57.14	1 - 57.14	1 - 57.14	1 - 57.14	1- 71.42	1 - 57.14
	2 - 42.85	2 - 42.85	2 - 42.85	2 - 42.85	2- 28.57	2 - 28.71
	3 - 00	3 - 00	3 - 00	3 - 00	3 - 00	3 - 14.28
	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00
	5 - 00	5 - 00	5 – 00	5 – 00	5 – 00	5 - 00
3	1 - 57.14	1- 42.85	1 - 57.14	1 - 57.14	1 - 14.28	1 - 14.28
	2 - 42.85	2- 57.14	2 - 28.57	2 - 14.28	2 - 42.85	2 - 57.14
	3 - 00	3 - 00	3 - 14.28	3 - 28.57	3 - 42.85	3 - 28.57
	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00
	5 – 00	5 - 00	5 – 00	5 – 00	5 – 00	5 - 00
4	1 - 00	1 - 00	1 - 00	1 - 00	1 - 00	1 - 00
	2 - 100	2 - 100	2 - 57.14	2 - 57.14	2 - 57.85	2 - 57.14
	3 - 00	3 - 00	3 - 42.85	3 - 42.85	3 - 42.85	3 - 42.85
	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00
	5 – 00	5 - 00	5 - 00	5 – 00	5 – 00	5 - 00
5	1 - 00	1 - 00	1 - 00	1 - 00	1 - 00	1 - 00
	2 -100	2 - 100	2 - 57.14	2 - 57.14	2 - 28.57	2 - 00
	3 - 00	3 - 00	3 - 42.85	3 - 42.85	3 - 71.42	3 - 100
	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00
	5 – 00	5 - 00	5 - 00	5 – 00	5 – 00	5 - 00
6	1 - 00	1 - 00	1 - 00	1 - 00	1 - 00	1 -00
	2 - 100	2 - 100	2 - 42.85	2 - 00	2 - 57.14	2 - 00
	3 - 00	3 - 00	3 - 57.14	3 - 42.85	3 - 42.85	3 - 42.85
	4 - 00	4 - 00	4 - 00	4 - 57.14	4 - 00	4 - 57.14
	5 – 00	5 - 00	5 - 00	5 – 00	5 – 00	5 - 00
7	1 - 00	1 - 00	1 - 00	1 - 00	1 - 00	1 -00
	2 - 100	2 - 28.57	2 - 57.14	2 - 00	2 - 57.14	2 - 00
	3 - 00	3 - 71.42	3 - 42.85	3 - 57.14	3 - 00	3 - 28.57
	4 - 00	4 - 00	4 - 00	4 - 42.85	4 - 42.85	4 - 71.42
	5 – 00	5 - 00	5 - 00	5 – 00	5 – 00	5 - 00
8	1 - 00	1 - 00	1 - 00	1 - 00	1 - 00	1 - 00

	2 - 57.14	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00
	3 - 42.85	3 - 71.42	3 - 28.57	3 - 42.85	3 - 00	3 - 00
	4 - 00	4 - 28.57	4 - 71.42	4 - 57.14	4 - 100	4 -100
	5 - 00	5 - 00	5 - 00	5 – 00	5 – 00	5 - 00
9	1 -00	1 - 00	1 - 00	1 - 00	1 - 00	1 - 00
	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00
	3 - 100	3 - 00	3 - 00	3 - 00	3 - 00	3 - 00
	4 - 00	4 - 100	4 - 100	4 - 100	4 - 42.85	4 - 00
	5 - 00	5 - 00	5 - 00	5 – 00	5 - 57.14	5 - 100
10	1 - 00	1 -00	1 - 00	1 - 00	1 - 00	1 - 00
	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00
	3 - 100	3 - 00	3 - 00	3 - 00	3 - 00	3 - 00
	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00
	5 - 00	5 - 100	5 - 100	5 - 100	5 – 100	5 - 100
11	1 - 00	1 -00	1 - 00	1 - 00	1 - 00	1 - 00
	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00
	3 - 100	3 - 00	3 - 00	3 - 00	3 - 00	3 - 00
	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00
	5 - 00	5 - 100	5 - 100	5 - 100	5 – 100	5 - 100
12	1 - 00	1 -00	1 - 00	1 - 00	1 - 00	1 - 00
	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00
	3 -71.42	3 - 00	3 - 00	3 - 00	3 - 00	3 - 00
	4 - 28.57	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00
	5 - 00	5 - 100	5 - 100	5 - 100	5 – 100	5 - 100
13	1 - 00	1 -00	1 - 00	1 - 00	1 - 00	1 - 00
	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00
	3 - 28.57	3 - 00	3 - 00	3 - 00	3 - 00	3 - 00
	4 - 71.42	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00
	5 - 00	5 - 100	5 - 100	5 - 100	5 – 100	5 - 100
14	1 -00	1 -00	1 - 00	1 - 00	1 - 00	1 - 00
	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00
	3 - 00	3 - 00	3 - 00	3 - 00	3 - 00	3 - 00
	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00
	5 - 100	5 - 100	5 - 100	5 - 100	5 – 100	5 - 100

1- Excellent
2- Good
3- Moderate
4-Bad
5- Very bad

Table A.2 Sensory evaluation of crab wastes at room temperature (20-25˚C).

Days	HCHO	HCOOH	CH_3COOH	$NaHCO_3$	NaOH	Control
0	1 - 100	1 - 100	1 - 100	1 - 100	1 - 100	1 - 100
	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00
	3 - 00	3 - 00	3 - 00	3 - 00	3 - 00	3 - 00
	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00
	5 - 00	5 - 00	5 - 00	5 - 00	5 – 00	5 - 00
1	1 - 00	1 - 00	1 - 00	1 - 00	1 - 00	1 - 00
	2 - 57.14	2 - 28.57	2 - 28.57	2 - 00	2 - 00	2 - 00
	3 - 42.85	3 - 71.42	3 - 42.85	3 - 57.85	3 - 57.14	3 - 28.57
	4 - 00	4 - 00	4 - 00	4 - 42.85	4 - 14.28	4 - 71.42
	5 - 00	5 - 00	5 - 28.57	5 - 00	5 - 28.57	5 - 00
2	1 - 00	1 - 00	1 - 57.14	1 - 00	1- 00	1 - 00
	2 - 00	2 - 00	2 - 42.85	2 - 00	2- 00	2 - 00
	3 - 85.71	3 - 42.85	3 - 00	3 - 00	3 - 00	3 - 00
	4 - 00	4 - 57.85	4 - 00	4 - 00	4 - 00	4 - 00
	5 - 14.28	5 - 00	5 - 00	5 - 100	5 – 100	5 - 100
3	1 - 00	1 - 00	1 - 00	1 - 00	1 - 00	1 - 00
	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00
	3 - 14.28	3 - 00	3 - 00	3 - 00	3 - 00	3 - 00
	4 - 85.71	4 - 100	4 - 100	4 - 100	4 - 100	4 - 100
	5 - 00	5 - 00	5 - 00	5 - 00	5 – 00	5 - 00
4	1 - 00	1 - 00	1 - 00	1 - 00	1 - 00	1 - 00
	2 - 00	2 - 100	2 - 00	2 - 00	2 - 00	2 - 00
	3 - 00	3 - 00	3 - 00	3 - 00	3 - 00	3 - 00
	4 - 100	4 -100	4 - 00	4 - 00	4 - 00	4 - 00
	5 - 00	5 - 00	5 - 100	5 - 100	5 – 100	5 - 100
5	1 - 00	1 - 00	1 - 00	1 - 00	1 - 00	1 - 00
	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00
	3 - 00	3 - 00	3 - 00	3 - 00	3 - 00	3 - 00
	4 - 100	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00
	5 - 00	5 - 100	5 - 100	5 - 100	5 – 100	5 - 100
6	1 - 00	1 - 00	1 - 00	1 - 00	1 - 00	1 -00
	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00	2 - 00
	3 - 00	3 - 00	3 - 00	3 - 00	3 - 00	3 - 00
	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00	4 - 00
	5 - 100	5 - 100	5 - 100	5 - 100	5 – 100	5 - 100

1-Excellent
2-Good
3-Moderate
4-Bad
5-Very bad

Table A3 Chelation capacity (%) of chitosan and EDTA over 24 hour at pH 5.

Metal ion	Chelator	0h	1h	3h	6h	24h
Fe^{2+}	Type 1	46.40 ± 3.03^{Db}	25.26 ± 2.28^{Aa}	33.12 ± 0.69^{Ba}	39.13 ± 0.79^{Cb}	27.14 ± 1.87^{Ab}
	Type 2	43.85 ± 0.45^{AbC}	22.46 ± 2.62^{aA}	32.53 ± 4.90^{aB}	34.56 ± 2.11^{aB}	21.33 ± 2.52^{aA}
	Type 3	39.64 ± 8.03^{aC}	25.96 ± 1.59^{aA}	34.92 ± 3.96^{aB}	34.62 ± 1.59^{aB}	43.04 ± 0.95^{cD}
	EDTA	57.69 ± 1.11^{cA}	65.59 ± 1.23^{bB}	93.63 ± 0.79^{bC}	92.52 ± 0.89^{cC}	55.66 ± 1.93^{dA}
Co^{2+}	Type 1	49.23 ± 0.20^{BCb}	48.50 ± 1.92^{ABCa}	44.98 ± 0.58^{Aa}	47.77 ± 0.28^{ABCa}	50.85 ± 0.16^{Ca}
	Type 2	44.79 ± 3.91^{ABa}	48.61 ± 1.36^{BCa}	44.04 ± 0.83^{Aa}	46.91 ± 0.95^{ABCa}	49.68 ± 0.62^{Ca}
	Type 3	47.36 ± 0.69^{Bab}	47.79 ± 0.37^{Ba}	41.65 ± 1.18^{Aa}	45.75 ± 1.06^{Ba}	48.20 ± 0.42^{Ba}
	EDTA	$57.89\pm1.08Ac$	66.96 ± 2.09^{Ba}	71.71 ± 0.97^{Cb}	94.30 ± 3.38^{Db}	98.73 ± 0.19^{Db}
Ni^{2+}	Type 1	39.97 ± 5.90^{Aa}	43.85 ± 0.69^{Aa}	42.83 ± 0.36^{Aa}	41.64 ± 1.12^{Aa}	42.20 ± 0.16^{Aa}
	Type 2	42.98 ± 0.25^{Aa}	42.44 ± 0.41^{Aa}	42.44 ± 0.91^{Aa}	40.91 ± 0.62^{Aa}	41.77 ± 0.32^{Aa}
	Type 3	44.49 ± 0.69^{Aa}	43.68 ± 0.74^{Aa}	43.10 ± 0.47^{Aa}	44.70 ± 0.02^{Aa}	43.03 ± 0.61^{Aa}
	EDTA	50.96 ± 0.83^{Ab}	56.08 ± 0.63^{Bb}	61.01 ± 0.79^{Cb}	92.70 ± 0.75^{Db}	93.42 ± 0.45^{Db}
Mn^{2+}	Type 1	71.28 ± 0.26^{Bc}	75.94 ± 0.72^{BCb}	77.70 ± 0.00^{Cb}	82.89 ± 1.59^{Db}	43.19 ± 1.45^{Ac}
	Type 2	66.72 ± 0.00^{Bc}	74.40 ± 0.58^{Cb}	72.70 ± 0.00^{Cb}	79.64 ± 0.58^{Db}	35.68 ± 3.14^{Ab}
	Type 3	44.46 ± 0.00^{Ba}	69.43 ± 0.00^{Da}	55.50 ± 1.00^{Ca}	45.94 ± 0.64^{Ba}	20.26 ± 0.01^{Aa}
	EDTA	$75.89\pm2.56Ac$	$80.76\pm0.19Ac$	87.29 ± 1.93^{Bc}	91.00 ± 0.49^{Bc}	97.54 ± 0.66^{Cd}
Cu^{2+}	Type 1	11.76 ± 0.19^{Aa}	21.12 ± 3.50^{Bb}	29.63 ± 1.91^{Cb}	48.07 ± 9.25^{Dc}	79.31 ± 5.32^{Ec}
	Type 2	9.59 ± 0.27^{Aa}	11.19 ± 2.89^{ABa}	15.19 ± 1.39^{Ba}	21.40 ± 4.14^{Ca}	55.30 ± 3.24^{Da}
	Type 3	10.56 ± 0.22^{Aa}	19.76 ± 0.72^{Bb}	15.22 ± 3.82^{Ba}	37.79 ± 3.83^{Cb}	71.97 ± 2.94^{Db}
	EDTA	36.62 ± 1.80^{Ab}	44.40 ± 1.48^{Bc}	50.83 ± 0.15^{Cc}	76.68 ± 0.93^{Dd}	91.61 ± 0.25^{Ed}
Zn^{2+}	Type 1	49.66 ± 2.01^{Aa}	51.28 ± 0.27^{Ab}	52.04 ± 0.00^{Ab}	52.65 ± 0.04^{Ab}	51.48 ± 0.79^{Ab}
	Type 2	46.06 ± 5.39^{Aa}	49.00 ± 0.87^{Aba}	50.32 ± 0.15^{Bab}	51.64 ± 0.81^{Bab}	50.75 ± 0.86^{Bab}
	Type 3	48.24 ± 0.81^{Aa}	45.98 ± 0.70^{Aa}	46.25 ± 0.81^{Aa}	48.20 ± 0.54^{Aa}	46.80 ± 0.36^{Aa}
	EDTA	66.12 ± 0.59^{Aa}	71.11 ± 0.70^{Bc}	86.15 ± 1.55^{Cc}	95.23 ± 0.77^{Db}	97.00 ± 0.30^{Ec}
Cd^{2+}	Type 1	29.19 ± 2.02^{Aa}	42.95 ± 3.13^{Ca}	56.69 ± 1.49^{Da}	44.29 ± 3.79^{Cb}	38.67 ± 7.78^{Ba}
	Type 2	52.94 ± 2.93^{Cc}	45.18 ± 5.08^{Bb}	64.22 ± 7.51^{Dc}	40.36 ± 5.03^{Aa}	52.25 ± 8.90^{Cc}
	Type 3	49.05 ± 3.04^{BCb}	52.69 ± 1.53^{Cc}	61.00 ± 10.64^{Db}	44.54 ± 6.08^{Ab}	48.85 ± 1.21^{Bb}
	EDTA	67.03 ± 2.35^{Ad}	75.69 ± 0.27^{Bd}	82.55 ± 1.12^{Cd}	87.30 ± 1.94^{Dc}	90.49 ± 0.95^{Dd}

Types. 1,2 and 3 -Types of chitosan.
Results reported are mean vales of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.

Table A4 Chelation capacity (%) of chitosan and EDTA over 24 hour at pH 6.

Metal ion	Chelator	0h	1h	3h	6h	24h
Fe^{2+}	Type 1	14.45 ± 2.53^{Ab}	33.87 ± 2.88^{Cb}	60.14 ± 2.82^{Da}	74.63 ± 2.01^{Ec}	21.73 ± 1.57^{Bb}
	Type 2	13.99 ± 3.04^{Ab}	26.01 ± 2.79^{Db}	69.86 ± 3.62^{Db}	57.90 ± 1.39^{Cb}	27.74 ± 0.71^{Bc}
	Type 3	9.14 ± 1.18^{Aa}	30.15 ± 4.63^{Ea}	61.57 ± 6.64^{Ea}	51.13 ± 7.07^{Da}	17.94 ± 0.76^{Ba}
	EDTA	30.32 ± 4.07^{Ac}	55.19 ± 2.93^{Cc}	86.20 ± 5.56^{Cc}	90.38 ± 1.48^{Cd}	56.67 ± 1.84^{Bd}
Co^{2+}	Type 1	2.75 ± 0.18^{Aa}	2.26 ± 0.22^{Aa}	2.94 ± 0.00^{Aa}	2.08 ± 0.00^{Aa}	2.23 ± 0.42^{Aa}
	Type 2	$2.01\pm0.71A^{Ba}$	2.21 ± 0.16^{Ba}	1.91 ± 0.06^{Aa}	1.09 ± 0.00^{Aa}	1.32 ± 0.00^{Aa}
	Type 3	3.68 ± 0.10^{Ba}	1.40 ± 0.24^{Aa}	1.19 ± 0.10^{Aa}	1.30 ± 0.20^{Aa}	1.19 ± 0.00^{Aa}
	EDTA	49.07 ± 0.62^{Ab}	92.90 ± 4.19^{Bb}	97.49 ± 0.99^{BCb}	96.97 ± 1.04^{BCb}	98.69 ± 0.34^{Cb}
Ni^{2+}	Type 1	1.12 ± 0.00^{Aa}	2.17 ± 1.07^{Aa}	5.75 ± 0.00^{Ba}	8.31 ± 1.00^{Bb}	7.83 ± 0.67^{Bc}
	Type 2	1.74 ± 0.00^{Aa}	1.72 ± 0.00^{Aa}	5.35 ± 0.00^{Ba}	5.80 ± 0.63^{ba}	4.48 ± 1.27^{Ba}
	Type 3	1.33 ± 0.00^{Aa}	1.69 ± 0.68^{Aa}	6.80 ± 0.00^{Ba}	6.58 ± 0.96^{Bab}	6.80 ± 0.10^{Ba}
	EDTA	25.02 ± 0.31^{Ab}	31.92 ± 1.38^{Bb}	48.05 ± 0.00^{Cb}	90.63 ± 0.10^{Dc}	90.87 ± 0.08^{Dd}
Mn^{2+}	Type 1	13.56 ± 3.34^{Ba}	9.32 ± 3.64^{Aa}	8.87 ± 2.90^{Aa}	7.19 ± 2.74^{Ab}	43.69 ± 12.16^{Ca}
	Type 2	19.15 ± 1.98^{Bb}	10.08 ± 3.48^{Aa}	11.16 ± 4.52^{Aa}	10.65 ± 1.86^{Ac}	53.03 ± 5.04^{Cb}
	Type 3	19.13 ± 2.93^{Cb}	10.28 ± 0.14^{Ba}	9.82 ± 1.62^{Ba}	3.01 ± 0.00^{Aa}	44.56 ± 7.57^{Da}
	EDTA	37.43 ± 1.95^{Ac}	36.85 ± 2.79^{Ab}	53.04 ± 3.61^{Bb}	95.92 ± 3.08^{Cd}	99.40 ± 0.00^{Cc}
Cu^{2+}	Type 1	9.68 ± 2.08^{Ab}	26.38 ± 1.19^{Cc}	31.35 ± 0.80^{Da}	22.84 ± 0.08^{Ca}	18.83 ± 0.59^{Bb}
	Type 2	6.95 ± 0.73^{Aa}	18.50 ± 0.69^{ba}	28.52 ± 0.41^{Da}	22.44 ± 0.57^{Ca}	9.59 ± 1.04^{Aa}
	Type 3	6.46 ± 0.60^{Aa}	22.05 ± 1.10^{Cb}	32.00 ± 0.07^{Da}	25.34 ± 0.59^{Cb}	16.73 ± 0.66^{Bb}
	EDTA	24.20 ± 1.04^{Ac}	44.30 ± 1.51^{Bd}	72.32 ± 0.44^{Cb}	79.14 ± 0.94^{Dc}	91.60 ± 0.76^{Ec}
Zn^{2+}	Type 1	2.64 ± 0.34^{Aa}	3.75 ± 0.49^{Aba}	4.39 ± 0.36^{Ba}	4.08 ± 0.67^{Bb}	5.88 ± 0.82^{Bb}
	Type 2	1.93 ± 0.31^{Aa}	2.81 ± 0.44^{Aa}	3.31 ± 0.45^{Ba}	2.63 ± 0.26^{Aa}	3.59 ± 0.36^{Ba}
	Type 3	1.96 ± 0.49^{Aa}	2.67 ± 0.09^{Aa}	3.66 ± 0.42^{Ba}	2.43 ± 0.37^{Aa}	3.07 ± 0.25^{Aa}
	EDTA	20.56 ± 1.31^{Ab}	58.14 ± 0.04^{Bb}	72.37 ± 0.00^{Cb}	92.94 ± 0.33^{Dc}	94.02 ± 0.24^{Dc}
Cd^{2+}	Type 1	22.18 ± 0.21^{Aa}	22.60 ± 0.11^{Aa}	23.62 ± 0.28^{Aa}	23.82 ± 0.08^{Aa}	23.90 ± 0.69^{Aa}
	Type 2	21.78 ± 0.25^{Aa}	22.36 ± 0.11^{Aa}	23.19 ± 0.35^{Aa}	23.02 ± 0.08^{Aa}	22.50 ± 0.15^{Aa}
	Type 3	21.70 ± 0.19^{Aa}	22.39 ± 0.26^{Aa}	22.85 ± 0.22^{Aa}	23.05 ± 0.28^{Aa}	22.48 ± 0.11^{Aa}
	EDTA	24.41 ± 5.11^{Ab}	32.54 ± 0.16^{Bb}	47.79 ± 0.65^{Cb}	49.43 ± 1.25^{Cb}	76.60 ± 1.05^{Db}

Types 1, 2 and 3 -Types of chitosan.
Results reported are mean vales of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.

Table A5 Chelation capacity (%) of chitosan and EDTA over 24 hour at pH 7.

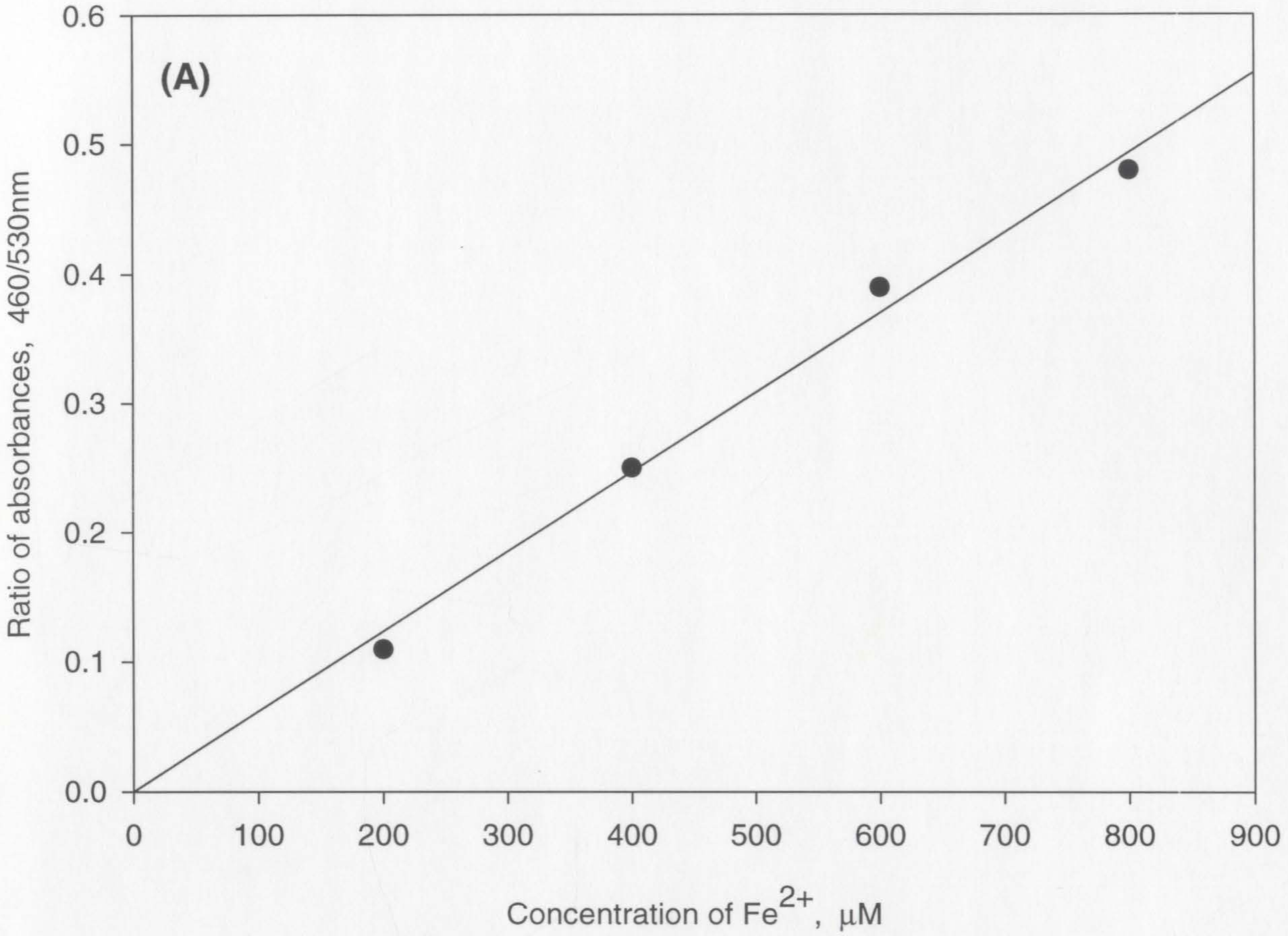
Metal ion	Chelator	0h	1h	3h	6h	24h
Fe^{2+}	Type 1	2.09 ± 0.15^{Bb}	11.99 ± 0.29^{Cb}	55.62 ± 7.08^{Da}	0.38 ± 0.16^{Aa}	0.09 ± 0.03^{Aa}
	Type 2	0.94 ± 0.79^{Aa}	6.57 ± 0.00^{Ba}	53.53 ± 7.64^{Ca}	0.60 ± 0.02^{Aa}	0.14 ± 0.06^{Aa}
	Type 3	0.97 ± 0.08^{Aa}	5.29 ± 0.93^{Ba}	50.32 ± 10.01^{Ca}	0.85 ± 0.05^{Ab}	0.25 ± 0.00^{Ab}
	EDTA	2.55 ± 0.29^{Bb}	22.23 ± 0.12^{Cc}	67.36 ± 0.89^{Db}	2.15 ± 0.08^{Bc}	1.82 ± 0.00^{Ab}
Co^{2+}	Type 1	13.74 ± 0.37^{Aa}	15.15 ± 0.71^{Ba}	17.31 ± 3.59^{Cb}	14.38 ± 0.07^{Aa}	14.69 ± 0.28^{Aa}
	Type 2	14.41 ± 1.77^{Aa}	14.52 ± 0.14^{Aa}	14.22 ± 0.26^{Aa}	14.27 ± 0.25^{Aa}	13.75 ± 0.27^{Aa}
	Type 3	14.12 ± 0.16^{Aa}	15.16 ± 0.10^{Aa}	14.63 ± 0.19^{Aa}	15.17 ± 0.29^{Aa}	14.34 ± 0.14^{Aa}
	EDTA	35.18 ± 3.24^{Ab}	55.51 ± 1.71^{Bb}	83.81 ± 2.71^{Cc}	82.51 ± 1.31^{Cb}	89.96 ± 1.83^{Db}
Ni^{2+}	Type 1	2.19 ± 0.22^{Ab}	2.86 ± 0.08^{Ab}	3.57 ± 0.09^{Ab}	5.15 ± 0.53^{Bc}	6.17 ± 0.72^{Bb}
	Type 2	0.62 ± 0.35^{Ba}	0.59 ± 0.03^{Ba}	0.24 ± 0.06^{Aa}	0.89 ± 0.52^{Ba}	1.76 ± 0.23^{Ca}
	Type 3	0.23 ± 0.12^{Aa}	$1.23\pm0.06A^{Ba}$	2.17 ± 0.21^{Bb}	3.32 ± 0.89^{Cb}	5.04 ± 0.04^{Db}
	EDTA	50.27 ± 1.49^{Ac}	53.26 ± 0.34^{Ac}	76.81 ± 0.02^{Bc}	77.04 ± 0.12^{Bd}	76.80 ± 0.21^{Bc}
Mn^{2+}	Type 1	0.27 ± 0.03^{Aa}	1.09 ± 0.46^{Cb}	0.67 ± 0.08^{Bb}	2.25 ± 0.07^{Db}	0.62 ± 0.09^{ABa}
	Type 2	0.62 ± 0.49^{Ab}	1.29 ± 0.04^{Bb}	0.58 ± 0.00^{Ab}	1.69 ± 0.89^{Ba}	0.58 ± 0.07^{Aa}
	Type 3	0.17 ± 0.00^{Ba}	0.56 ± 0.02^{Ba}	0.06 ± 0.01^{Aa}	1.07 ± 0.49^{Ca}	0.63 ± 0.08^{Ba}
	EDTA	42.38 ± 4.17^{Bc}	43.82 ± 0.32^{Bc}	52.74 ± 3.19^{Cc}	51.91 ± 0.51^{Cc}	0.84 ± 0.09^{Ab}
Cu^{2+}	Type 1	3.47 ± 0.83^{Ba}	2.74 ± 0.04^{Ba}	0.76 ± 0.52^{Aa}	0.62 ± 0.25^{Aa}	8.14 ± 1.84^{Cb}
	Type 2	3.37 ± 0.21^{Ca}	1.38 ± 0.82^{Ba}	0.43 ± 0.26^{Aa}	0.53 ± 0.02^{Aa}	1.53 ± 0.85^{Ba}
	Type 3	4.97 ± 0.53^{Cd}	2.33 ± 0.19^{Ba}	0.57 ± 0.22^{Aa}	0.51 ± 0.01^{Aa}	5.17 ± 1.26^{Cb}
	EDTA	21.66 ± 1.23^{Ab}	22.01 ± 1.88^{Ab}	26.19 ± 0.65^{Bb}	45.05 ± 1.48^{Cb}	90.63 ± 0.72^{Dc}
Zn^{2+}	Type 1	0.68 ± 0.09^{Bb}	1.44 ± 0.32^{Cb}	1.33 ± 0.05^{Cb}	2.20 ± 0.00^{Da}	0.18 ± 0.13^{Aa}
	Type 2	1.38 ± 0.13^{Bb}	1.28 ± 0.08^{Bba}	0.40 ± 0.01^{Aa}	1.93 ± 0.03^{Ca}	0.85 ± 0.09^{Ab}
	Type 3	0.14 ± 0.00^{Aa}	0.74 ± 0.39^{Ba}	0.17 ± 0.01^{Aa}	2.18 ± 0.03^{Da}	1.94 ± 0.15^{Cc}
	EDTA	21.48 ± 1.08^{Ac}	42.11 ± 0.03^{Bc}	58.63 ± 4.46^{Cc}	99.00 ± 0.05^{Eb}	93.36 ± 0.19^{Dd}
Cd^{2+}	Type 1	5.76 ± 0.26^{Aa}	5.87±0.15Aa	6.55±0.05Aa	5.78±0.05Aa	6.29 ± 0.16^{A}
	Type 2	5.20 ± 0.12^{Aa}	5.29±0.05Aa	6.08±0.06Aa	5.28±0.12Aa	5.04 ± 0.02^{A}
	Type 3	5.20 ± 0.00^{Aa}	5.26±0.19aa	6.05±0.33Ba	4.87±0.33Aa	4.68 ± 0.55^{A}
	EDTA	9.40 ± 0.09^{Ab}	11.60 ± 0.58^{Ab}	19.76 ± 0.43^{Bb}	67.62 ± 11.73^{D}	52.05 ± 0.13^{C}

Types 1, 2 and 3 -Types of chitosan.
Results reported are mean vales of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.

Figure A.2 Dependence of the absorbance ration of (A) Fe^{2+} and (B) Co^{2+} concentration at 460 nm/530 nm on the concentration of metal solutions – pH 5

(A) Regression coefficient (r^2) = 0.9901
Equation of the line was Y = aX + b where,
Y= absorbance at $A_{460\ nm/530\ nm}$
X= concentration of Fe^{2+} in μM
$a = 6.156x\ 10^{-4}$
b = 0
Therefore, $X = 1624.43^*\ A_{460\ nm/\ 530\ nm}$

(B) Regression coefficient (r^2) = 0.9994
Equation of the line was Y = aX + b where,
Y= absorbance at $A_{460\ nm/530\ nm}$
X= concentration of Co^{2+} in μM
$a = 1.3347x\ 10^{-3}$
b = 0
Therefore, $X= 749.23^*A_{460\ nm/530\ nm}$


(A)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Ratio of absorbances, 460/530nm
0
100
200
300
400
500
600
700
800
900
Concentration of Fe^{2+}, μM

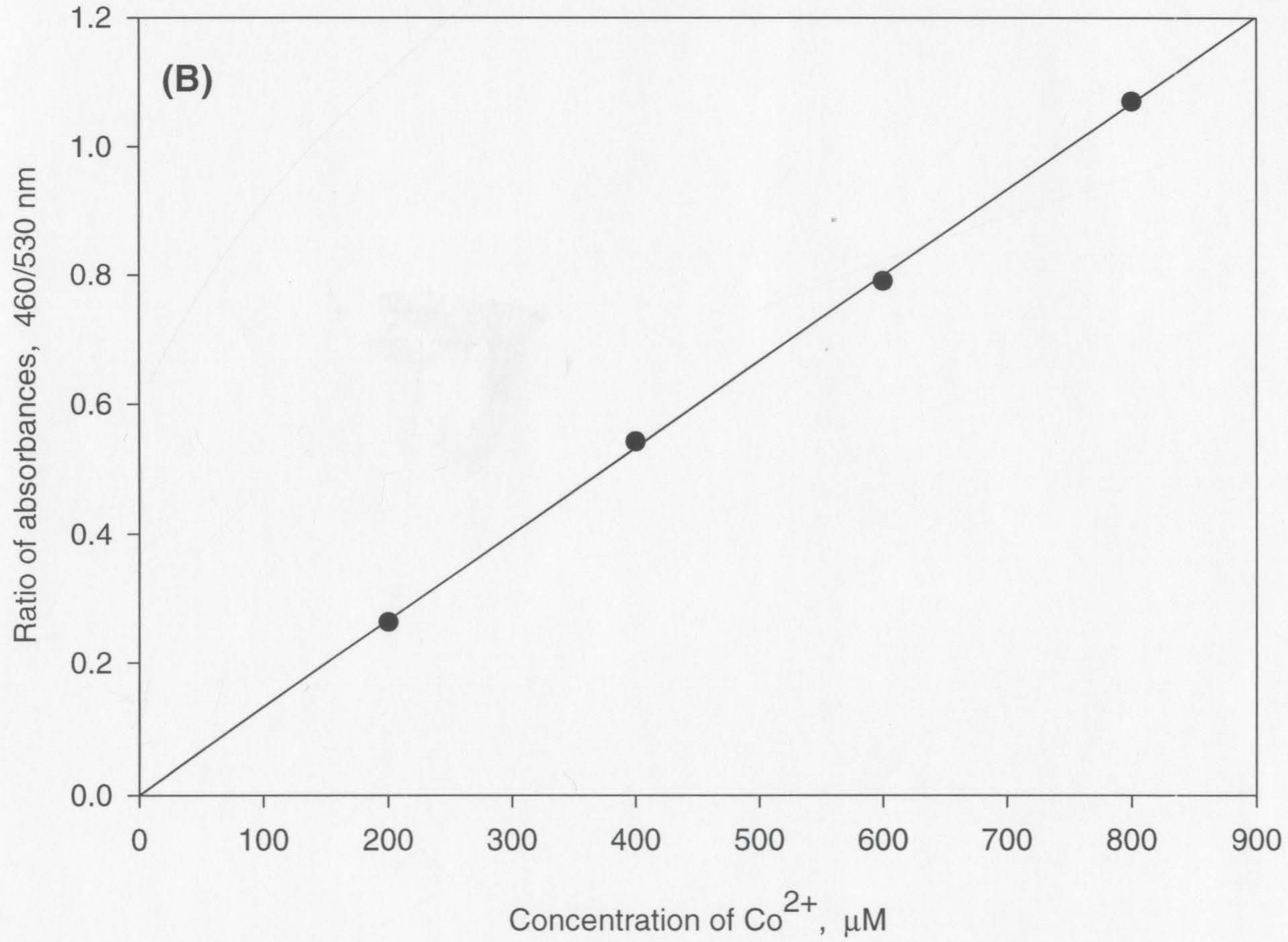

(B)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ratio of absorbances, 460/530 nm
0
100
200
300
400
500
600
700
800
900
Concentration of Co^{2+}, μM

Figure A.3 Dependence of the absorbance ratio of (A) Ni^{2+} and (B) Mn^{2+} concentration at 460 nm/530 nm on the concentration of metal solutions- pH 5

(A) Regression coefficient (r^2) = 0.9956
Equation of the line was $Y = aX + b$ where,
Y= absorbance at $A_{460\ nm/530\ nm}$
X= concentration of Ni^{2+} in μM
$a = 9.93 \times 10^{-3}$
b = 0
Therefore, $X = 100.70^* A_{460\ nm/530\ nm}$

(B) Regression coefficient (r^2)=0.9989
Equation of the line was Y=aX + b where,
Y= absorbance at $A_{460\ nm/530\ nm}$
X= concentration of in Mn^{2+} μmol (C)
$a = 2.221X\ 10^{-4}$
b = 0
Therefore, $C = 4524.88^* A_{460\ nm/530\ nm}$

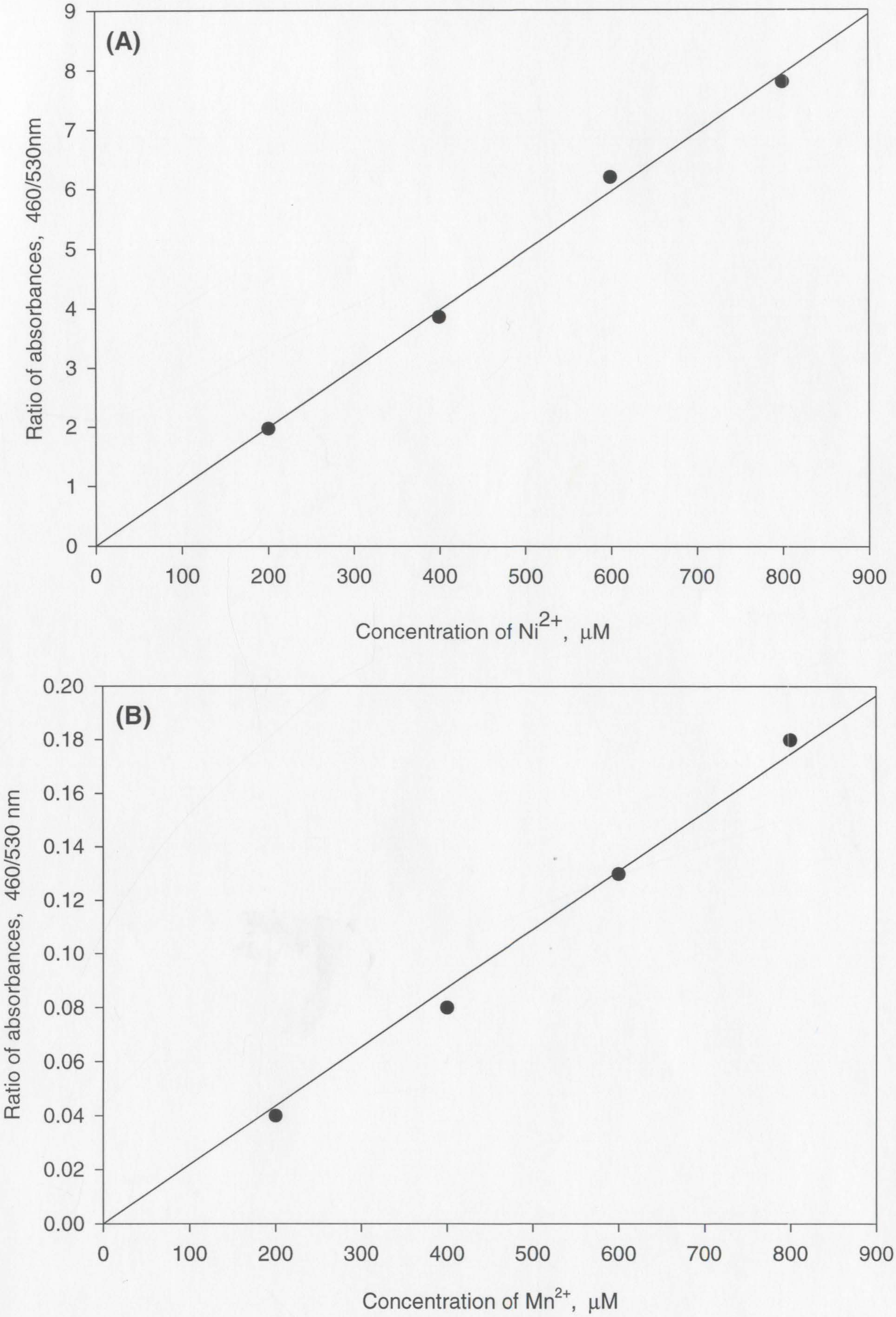
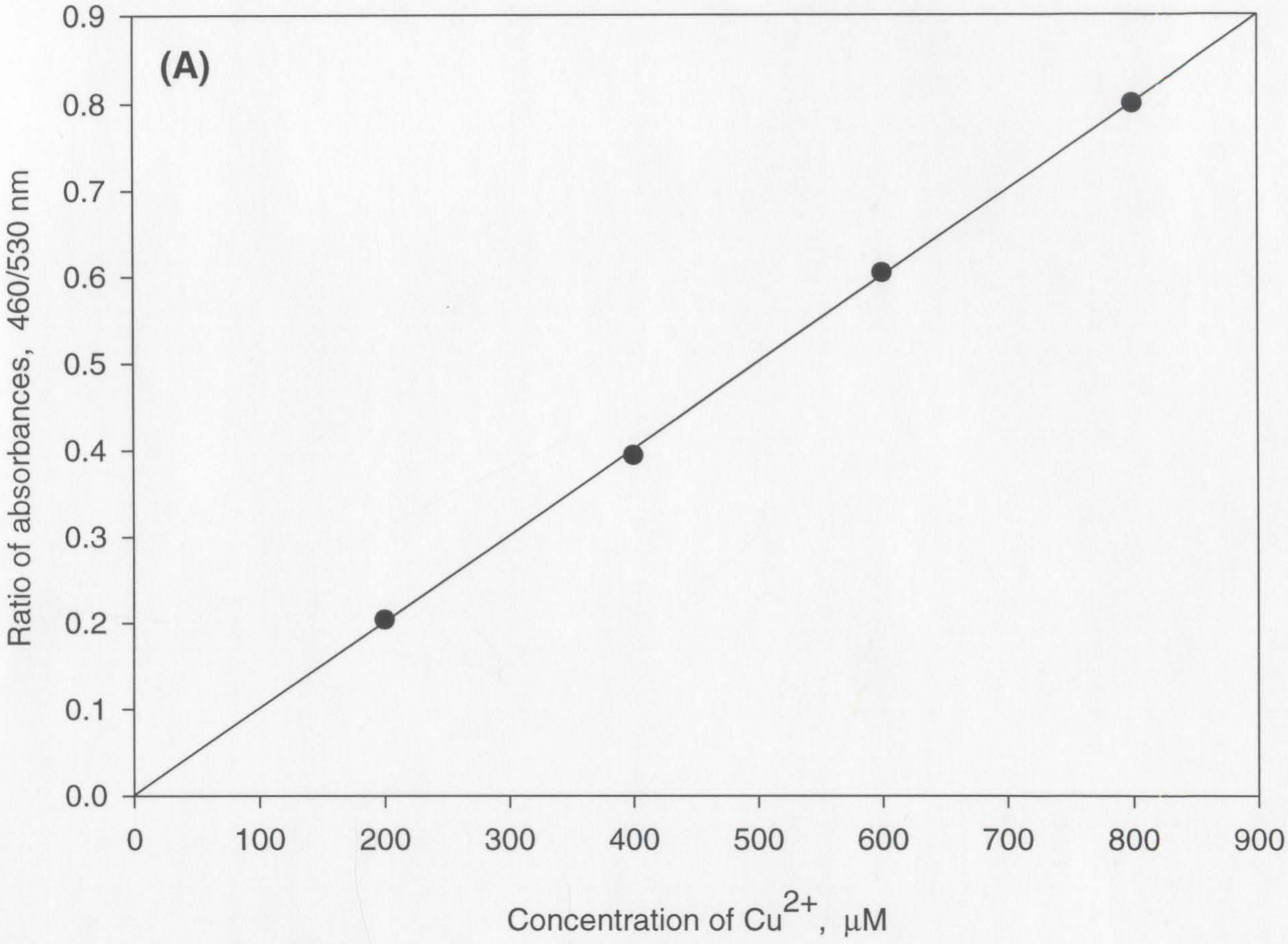

(A)
Ratio of absorbances, 460/530nm
0
1
2
3
4
5
6
7
8
9
0
100
200
300
400
500
600
700
800
900
Concentration of Ni^{2+}, μM
(B)
Ratio of absorbances, 460/530 nm
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0.20
0
100
200
300
400
500
600
700
800
900
Concentration of Mn^{2+}, μM

Figure A.4 Dependence of the absorbance ratio of (A) Cu^{2+} and (B) Zn^{2+} concentration at 460 nm/530 nm on the concentration of metal solutions – pH 5

(A) Regression coefficient (r^2) = 0.99966
Equation of the line was Y = aX + b where,
Y= absorbance at $A_{460\ nm/530\ nm}$
X= concentration of Cu^{2+} in μM
$a = 9.99 \times 10^{-4}$
b = 0
Therefore, $X = 1010.10 * A_{460\ nm/530\ nm}$

(B) Regression coefficient (r^2) = 0.9969
Equation of the line was Y = aX + b where,
Y= absorbance at $A_{460\ nm/530\ nm}$
X= concentration of in Zn^{2+} μM
$a = 4.21 \times 10^{-3}$
b = 0
Therefore, $X = 237.52 * A_{460\ nm/530\ nm}$


(A)
Ratio of absorbances, 460/530 nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
100
200
300
400
500
600
700
800
900
Concentration of Cu^{2+}, μM

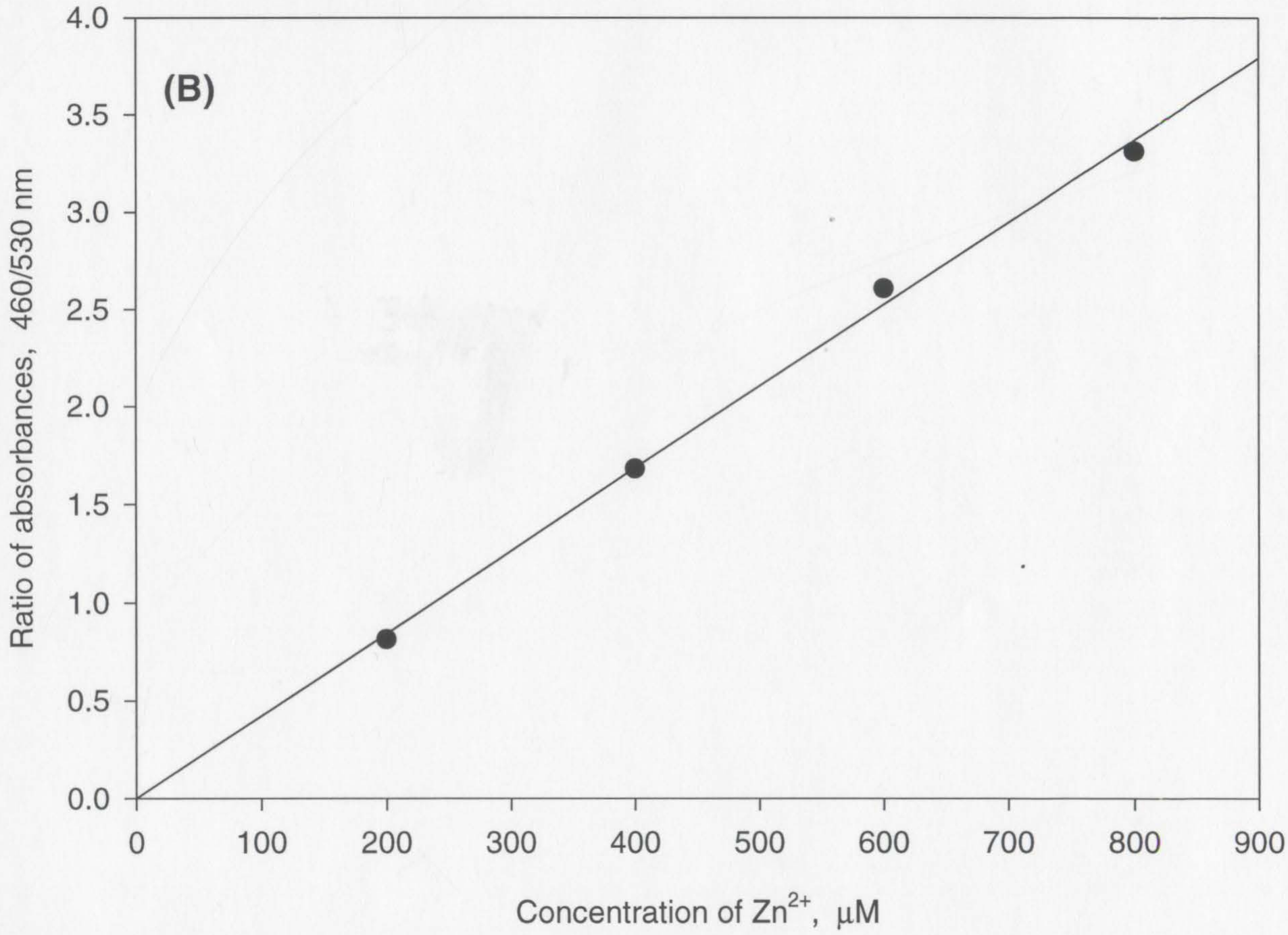

(B)
Ratio of absorbances, 460/530 nm
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0
100
200
300
400
500
600
700
800
900
Concentration of Zn^{2+}, μM

Figure A.5 Dependence of the absorbance ratio of Cd^{2+} concentration at 460 nm/530 nm on the concentration of metal solutions- pH 5

Regression coefficient (r^2) = 0.9962
Equation of the line was $Y = aX + b$ where,
Y= absorbance at $A_{460\ nm/530\ nm}$
X= concentration of Ca^{2+} in μM
$a = 5.016 \times 10^{-4}$
b = 0
Therefore, $C = 1993.62 * A_{460\ nm/530\ nm}$

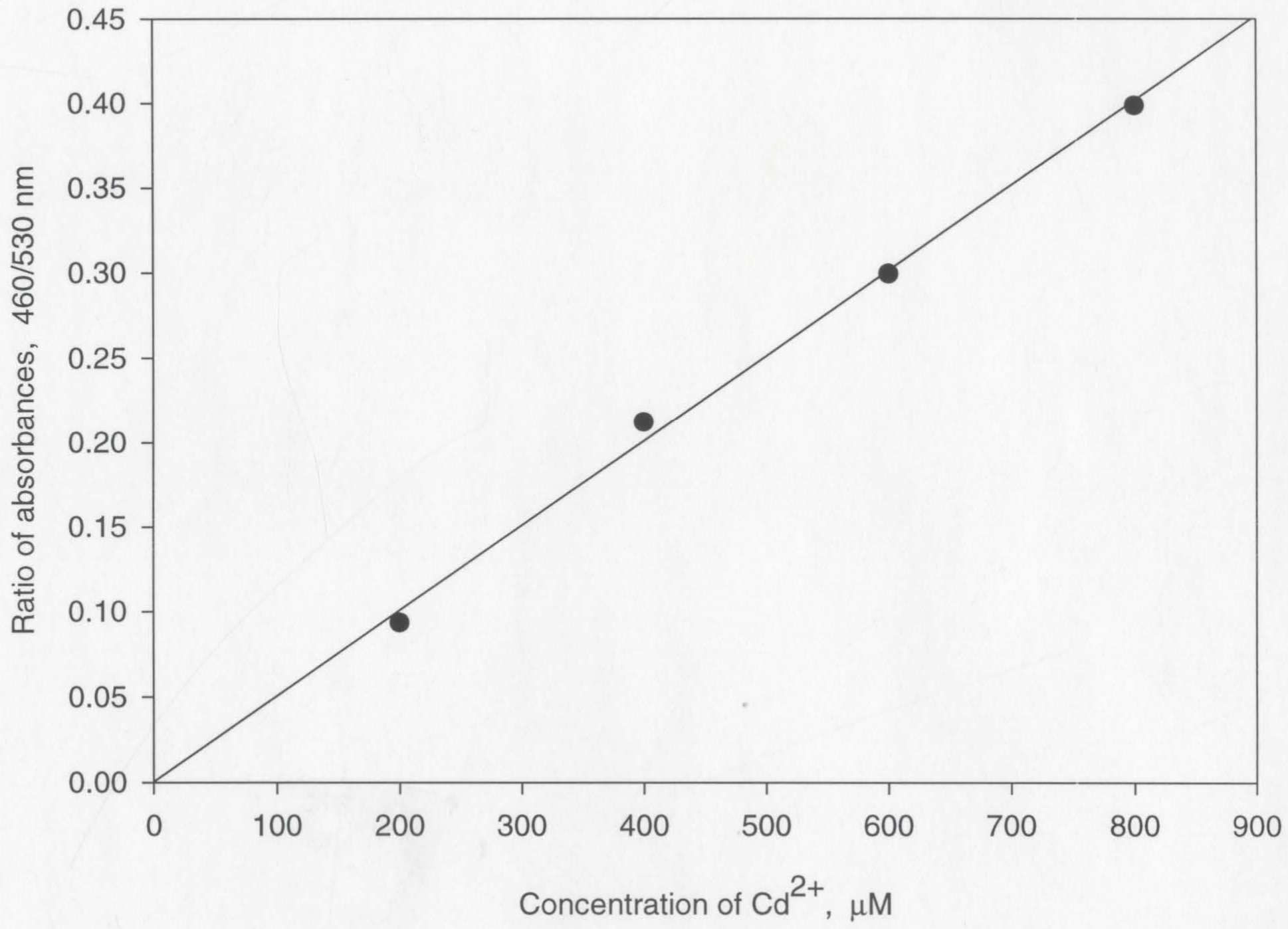

0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Ratio of absorbances, 460/530 nm
0
100
200
300
400
500
600
700
800
900
Concentration of Cd^{2+}, μM

Figure A.6 Dependence of the absorbance ratio of (A) Ni^{2+} and (B) Zn^{2+} concentration at 460 nm/530 nm on the concentration of metal solutions – pH 6

(A) Regression coefficient (r^2) = 0.99779
Equation of the line was Y = aX + b where,
Y= absorbance at $A_{460\ nm/530\ nm}$
X= concentration of Ni^{2+} in μM
$a = 9.144 \times 10^{-3}$
b = 0
Therefore, $X = 109.35 * A_{460\ nm/530\ nm}$

(B) Regression coefficient (r^2) = 0.99915
Equation of the line was Y = aX + b where,
Y= absorbance at $A_{460\ nm/530\ nm}$
X= concentration of in Zn^{2+} μM
$a = 4.180 \times 10^{-3}$
b = 0
Therefore, X = 239.21

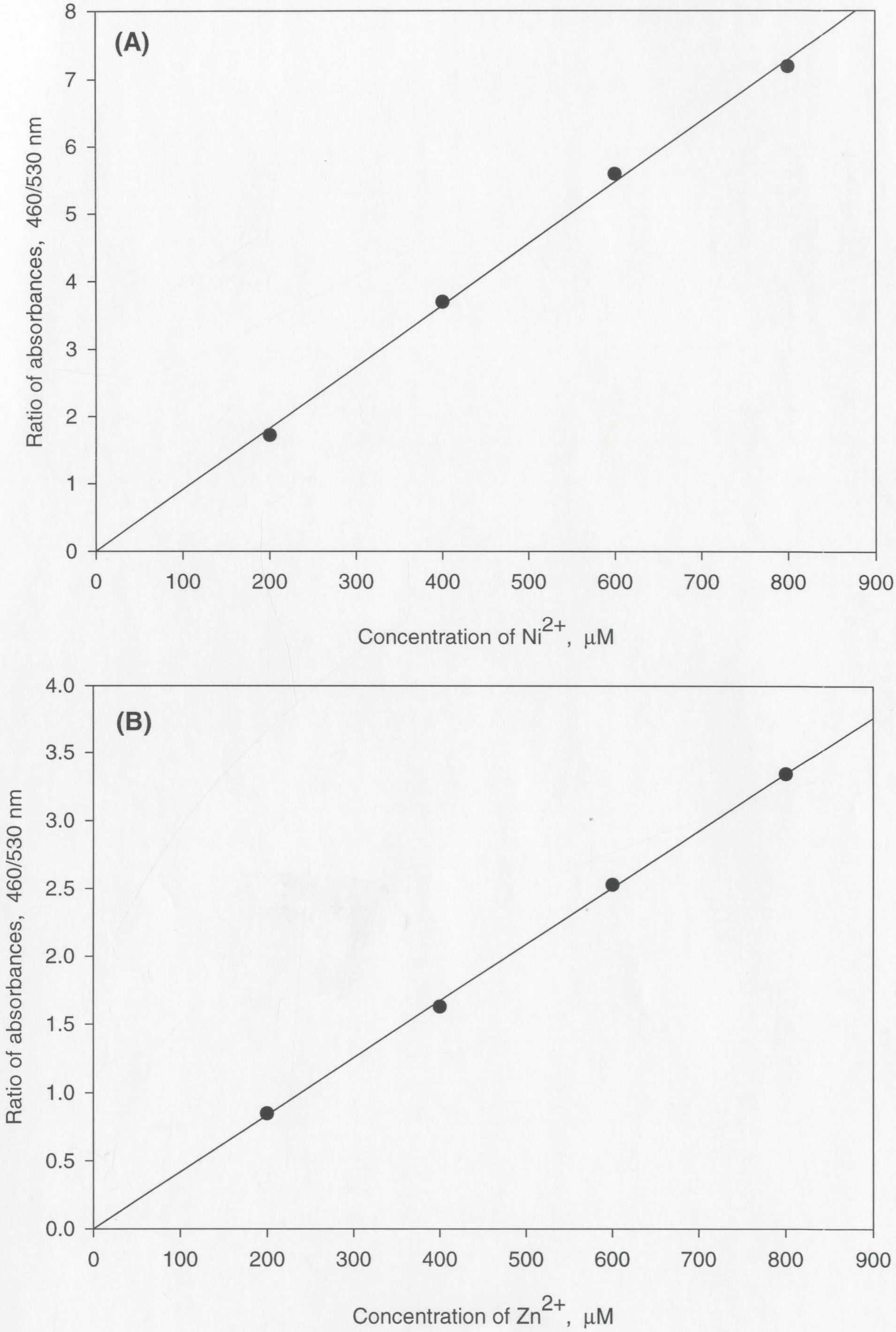
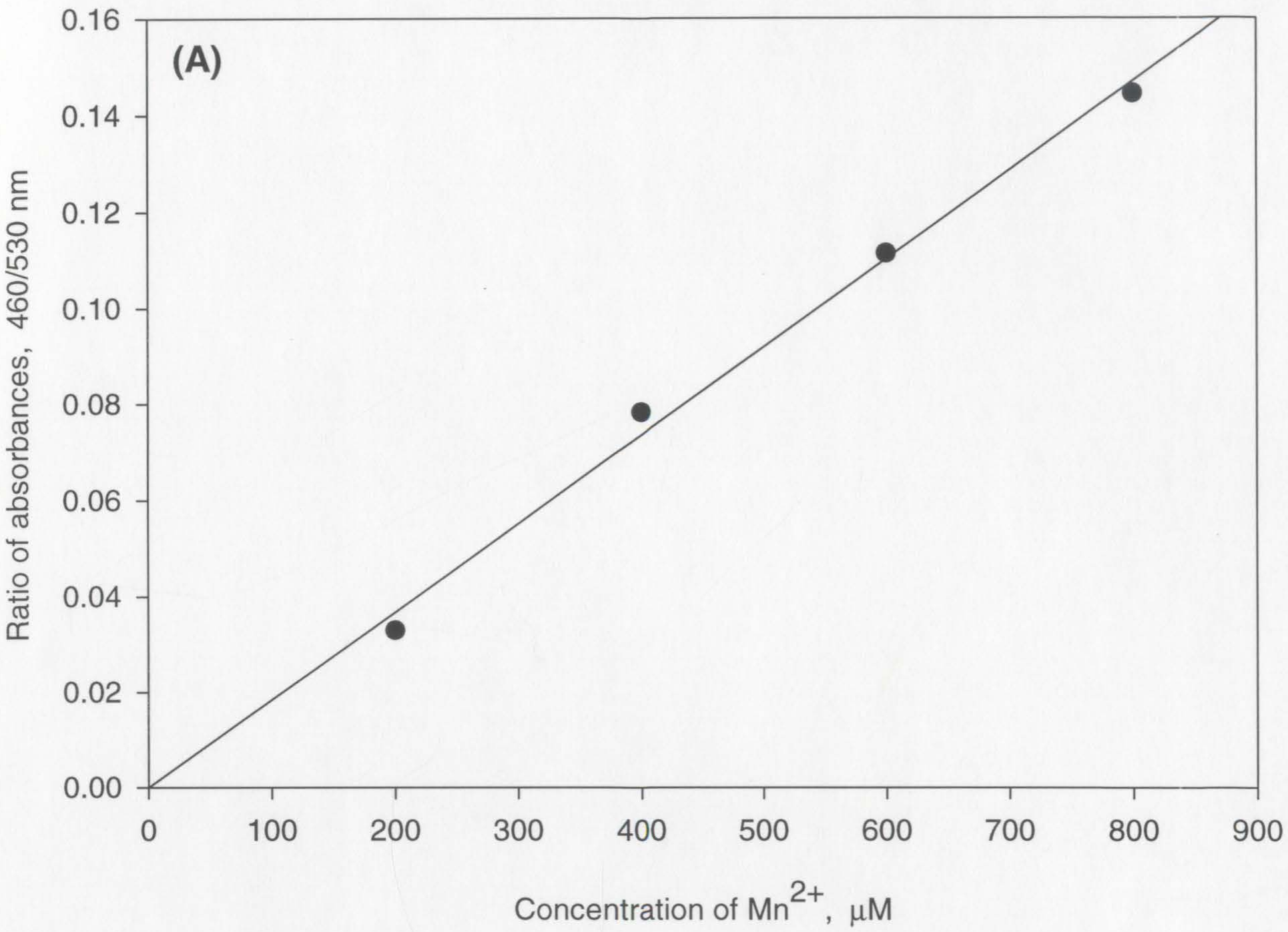

(A)
Ratio of absorbances, 460/530 nm
0
1
2
3
4
5
6
7
8
0
100
200
300
400
500
600
700
800
900
Concentration of Ni2+, μM
(B)
Ratio of absorbances, 460/530 nm
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0
100
200
300
400
500
600
700
800
900
Concentration of Zn2+, μM

Figure A.7 Dependence of the absorbance ratio of (A) Mn^{2+} and (B) Cd^{2+} concentration at 460 nm/530 nm on the concentration of metal solutions – pH 6

(A) Regression coefficient (r^2) = 0.9933
Equation of the line was $Y = aX + b$ where,
Y = absorbance at $A_{460\ nm/530\ nm}$
X = concentration of Mn^{2+} in μM
a = 1.836×10^{-4}
b = 0
Therefore, $X = 5447.51 * A_{460\ nm/530\ nm}$

(B) Regression coefficient (r^2) = 0.9915
Equation of the line was $Y = aX + b$ where,
Y= absorbance at $A_{460\ nm/530\ nm}$
X= concentration of in Cd^{2+} μM
a = 2.065×10^{-3}
b = 0
Therefore, $X = 485.43 * A_{460\ nm/530\ nm}$


(A)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Ratio of absorbances, 460/530 nm
0
100
200
300
400
500
600
700
800
900
Concentration of Mn^{2+}, μM

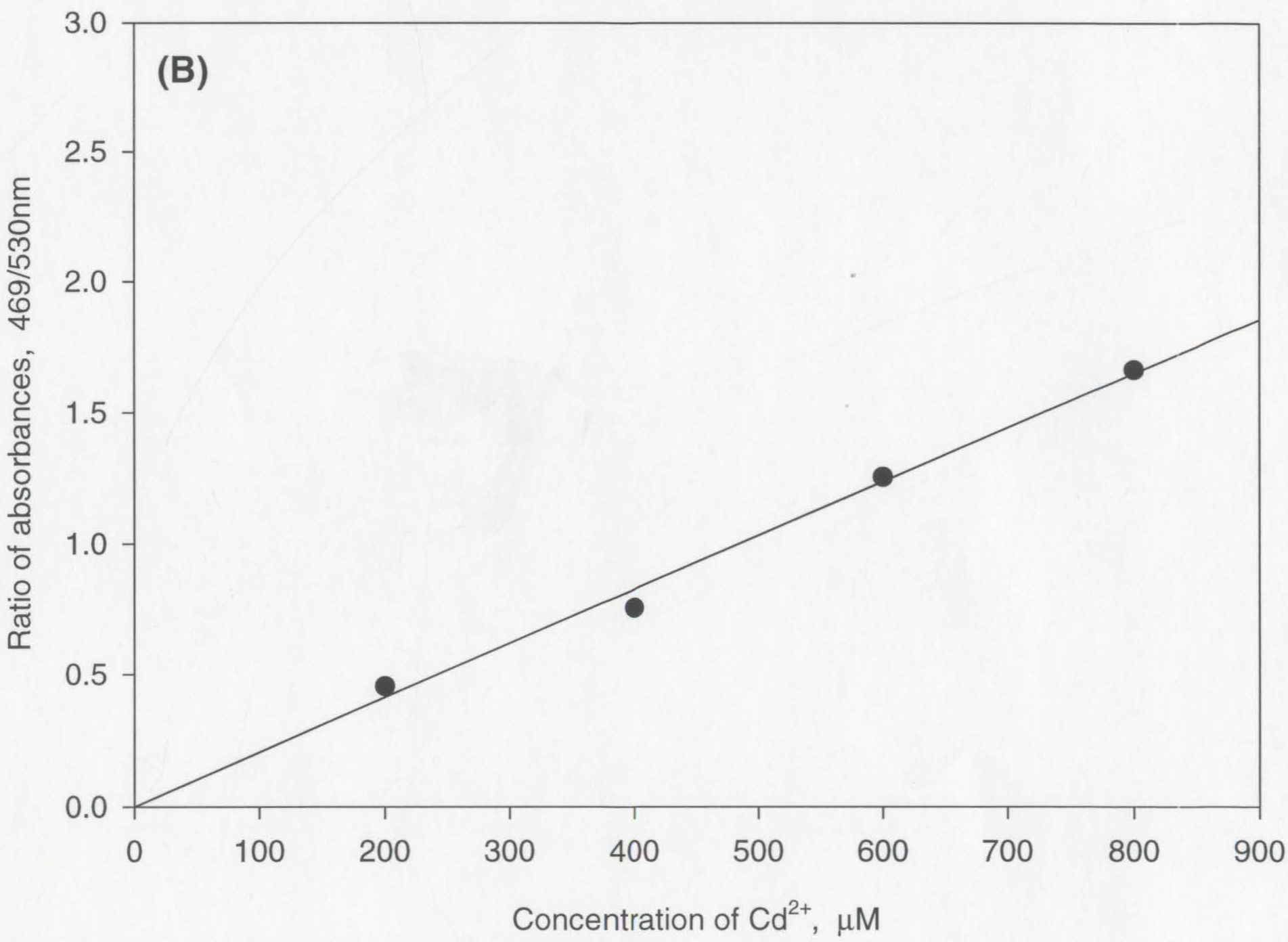
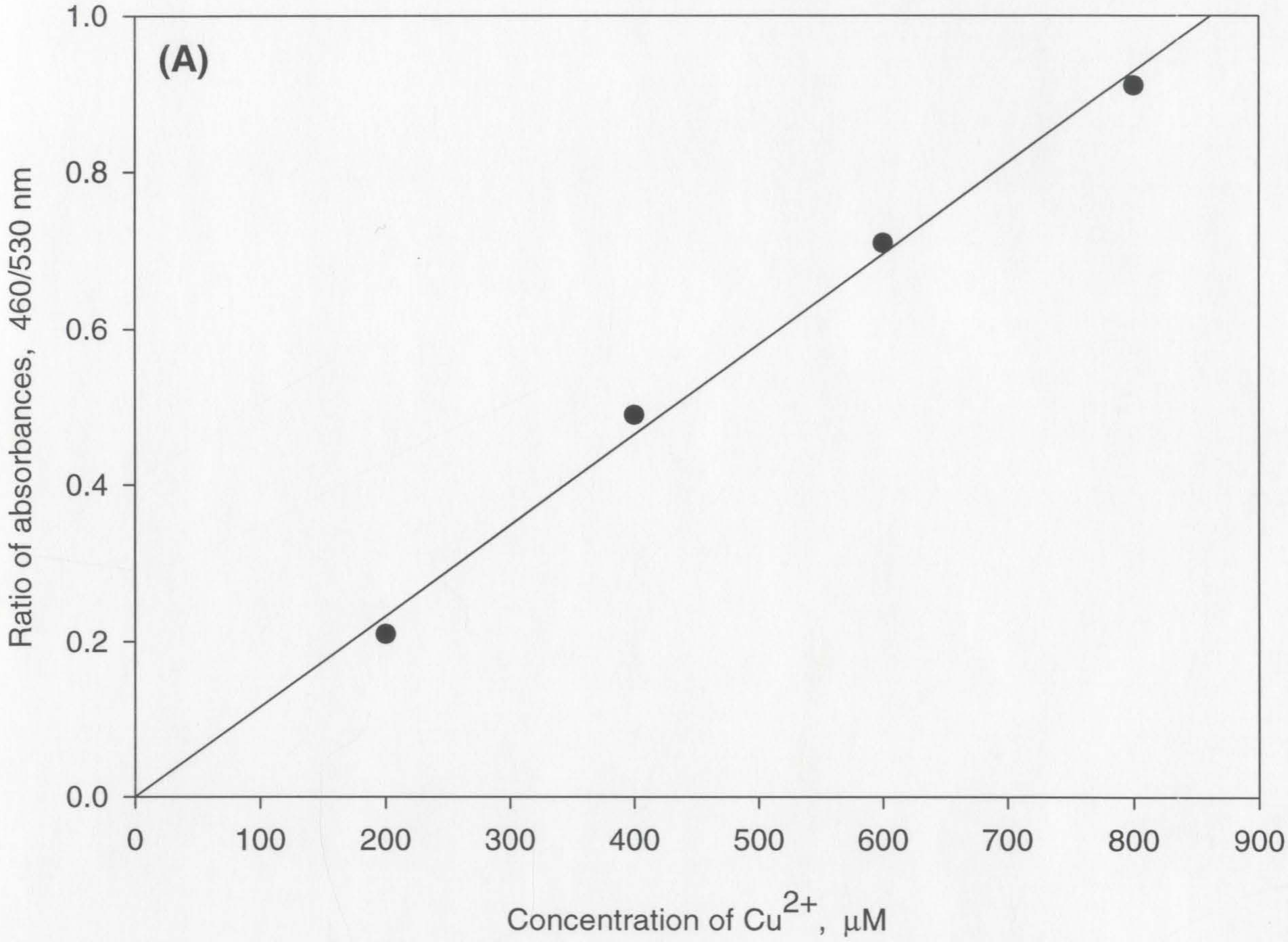

(B)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Ratio of absorbances, 469/530nm
0
100
200
300
400
500
600
700
800
900
Concentration of Cd^{2+}, μM

Figure A.8 Dependence of the absorbance ratio of (A) Cu^{2+} and (B) Co^{2+} concentration at 460 nm/530 nm on the concentration of metal solutions – pH 6

(A) Regression coefficient (r^2) = 0.9937
Equation of the line was Y = aX + b where,
Y= absorbance at $A_{460\ nm/530\ nm}$
X= concentration of Cu^{2+} in μM
$a = 1.16 \times 10^{-3}$
b = 0
Therefore, $X = 862.06 * A_{460\ nm/530\ nm}$

(B) Regression coefficient (r^2) = 0.9939
Equation of the line was Y = aX + b where,
Y= absorbance at $A_{460\ nm/530\ nm}$
X= concentration of in Co^{2+} μM
$a = 7.7 \times 10^{-4}$
b = 0
Therefore, $X = 1298.70 * A_{460\ nm/530\ nm}$


(A)
1.0
0.8
0.6
0.4
0.2
0.0
Ratio of absorbances, 460/530 nm
0
100
200
300
400
500
600
700
800
900
Concentration of Cu^{2+}, μM

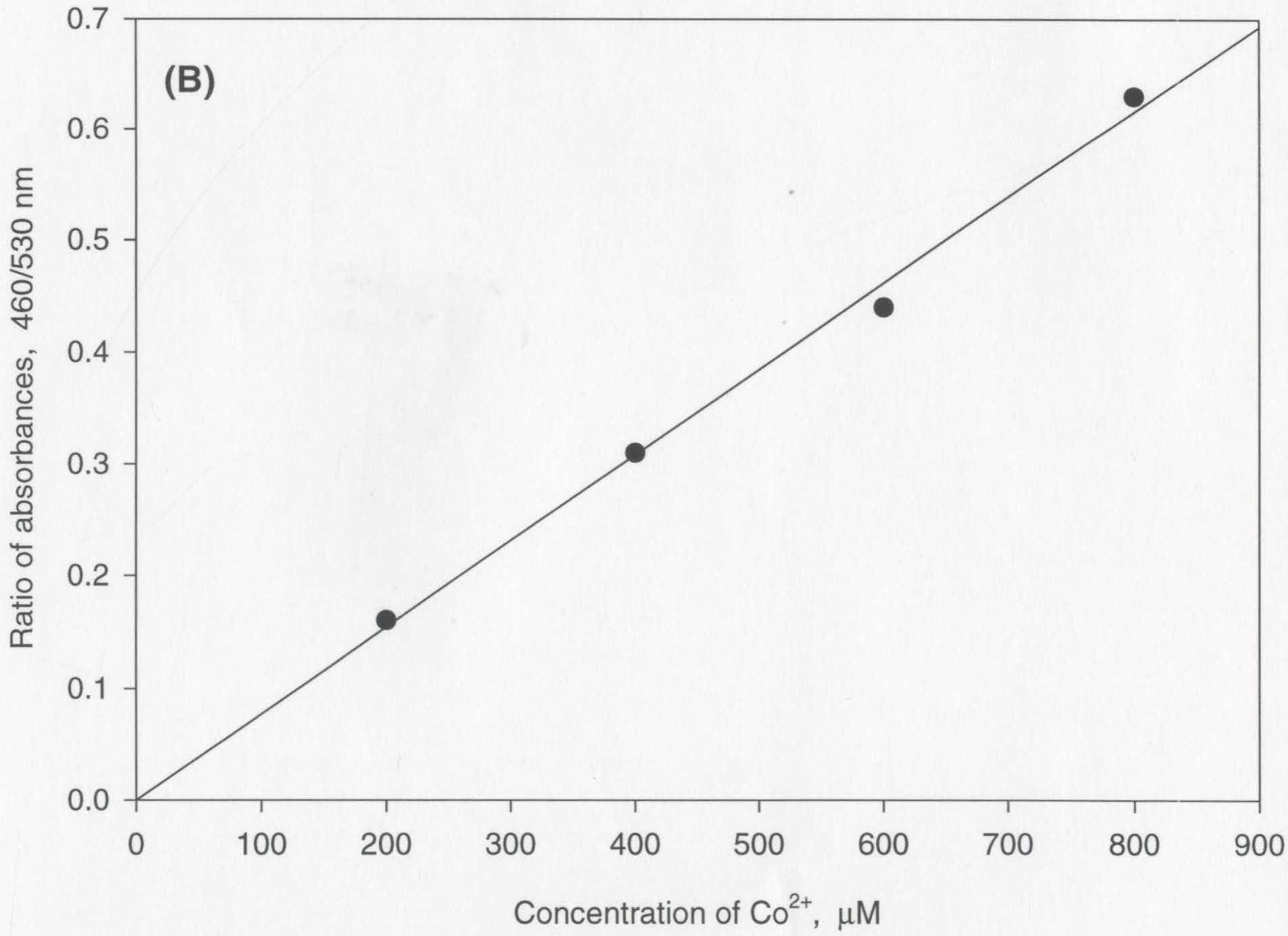

(B)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Ratio of absorbances, 460/530 nm
0
100
200
300
400
500
600
700
800
900
Concentration of Co^{2+}, μM

Figure A.9 Dependence of the absorbance ratio of Fe^{2+} concentration at 460 nm/530 nm on the concentration of metal solutions – pH 6

Regression coefficient (r^2) = 0.9977
Equation of the line was Y = aX + b where,
Y= absorbance at $A_{460\ nm/530\ nm}$
X= concentration of Fe^{2+} in μM
$a = 7.9 \times 10^{-4}$
b = 0
Therefore, $X = 1265.82 * A_{460\ nm/530\ nm}$

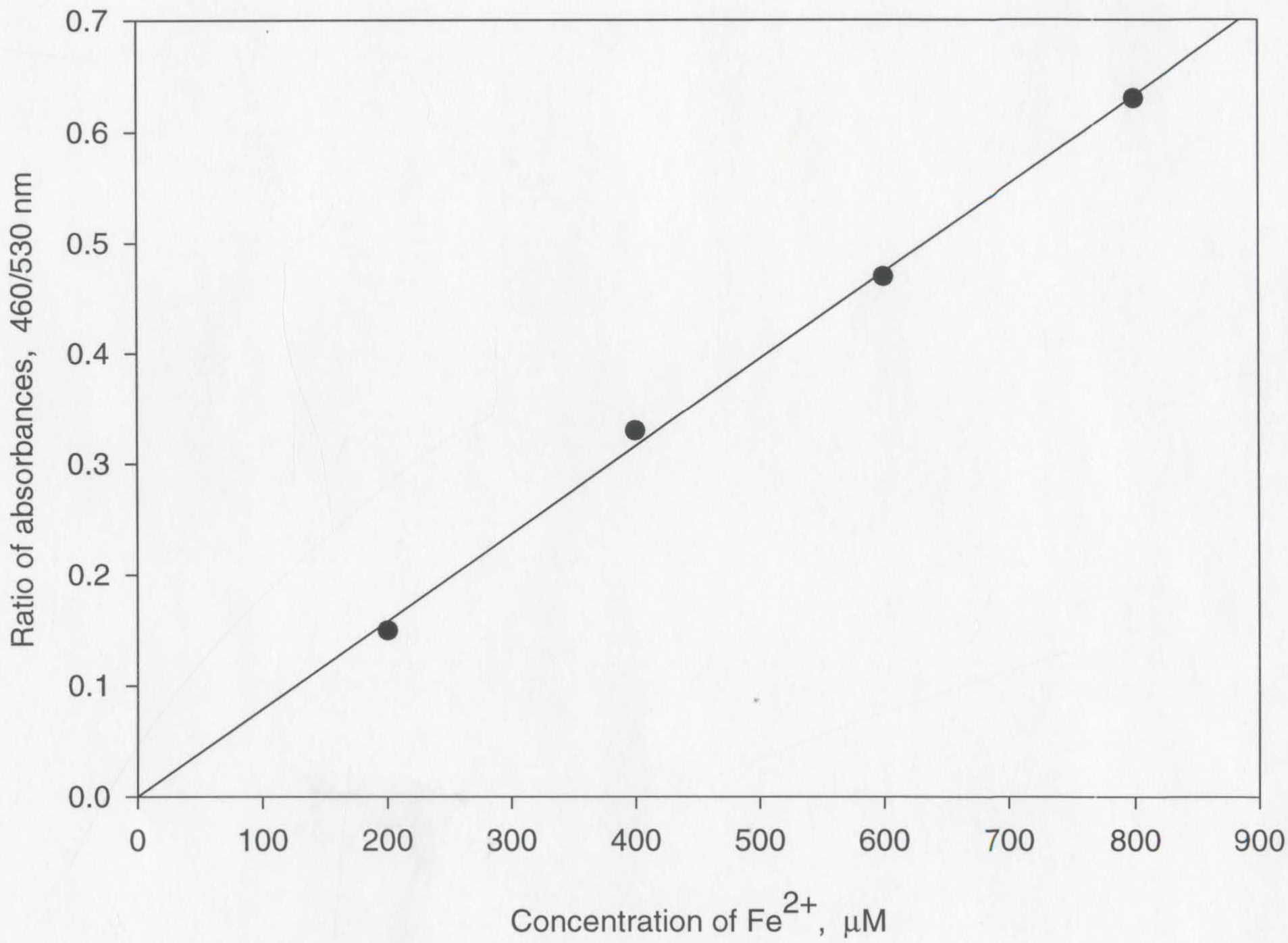

Ratio of absorbances, 460/530 nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
100
200
300
400
500
600
700
800
900
Concentration of Fe^{2+}, μM

Figure A.10 Dependence of the absorbance ratio of (A) Ni^{2+} and (B) Mn^{2+} concentration at 460 nm/530 nm on the concentration of metal solutions – pH 7

(A)Regression coefficient (r^2) = 0.9921

Equation of the line was Y = aX + b where,

Y= absorbance at $A_{460\ nm/530\ nm}$

X= concentration of Ni^{2+} in μM

$a = 8.95 \times 10^{-3}$

b = 0

Therefore, $X = 111.73 * A_{460\ nm/530\ nm}$

(B)Regression coefficient (r^2) = 0.9939

Equation of the line was Y = aX + b where,

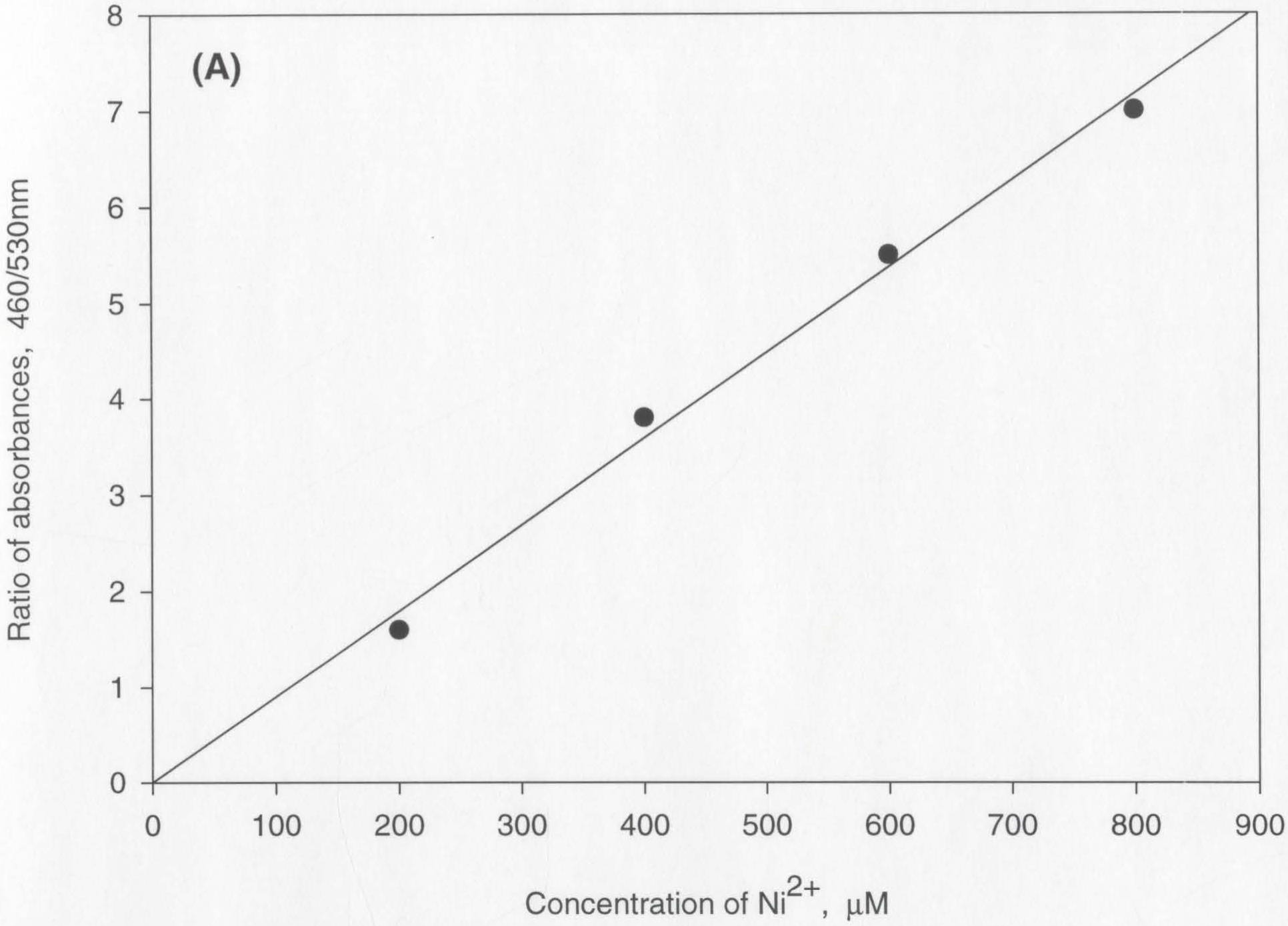
Y= absorbance at $A_{460\ nm/530\ nm}$

X= concentration of in Mn^{2+} μM

$a = 2.8676 \times 10^{-4}$

b = 0

Therefore, $X = 3487.23 * A_{460\ nm/530\ nm}$


(A)
Ratio of absorbances, 460/530nm
0
1
2
3
4
5
6
7
8
0
100
200
300
400
500
600
700
800
900
Concentration of Ni^{2+}, μM

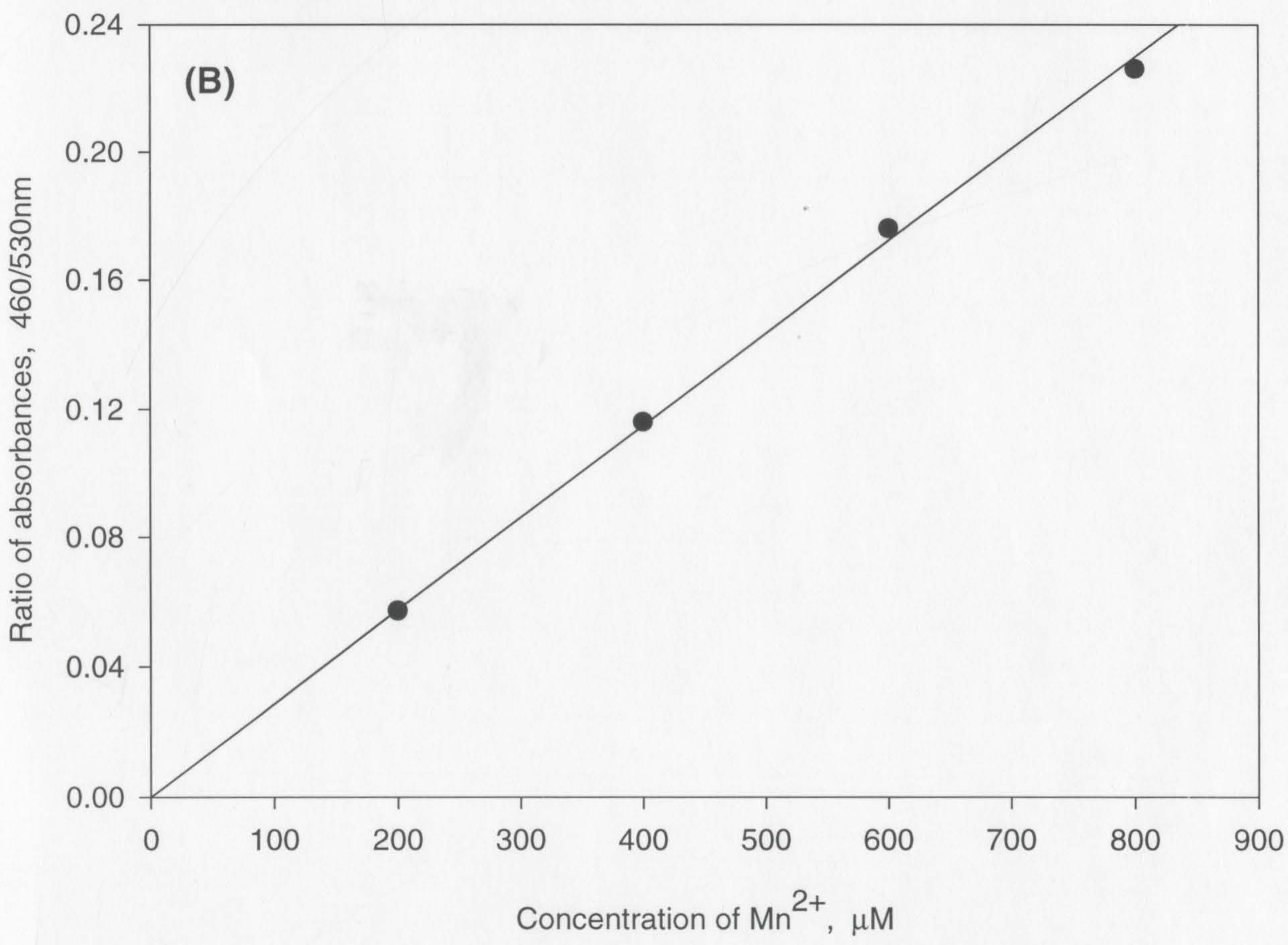
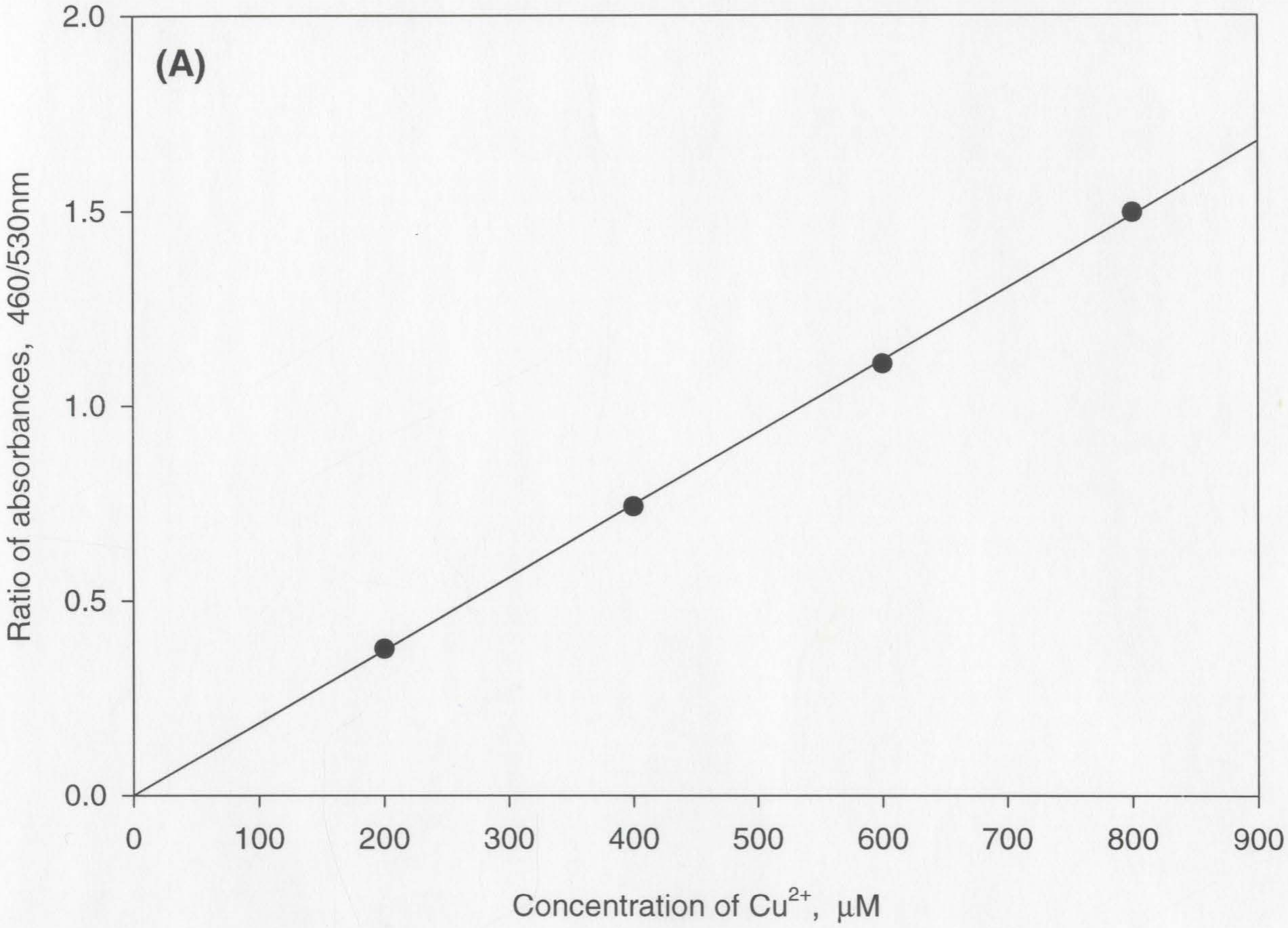

(B)
Ratio of absorbances, 460/530nm
0.00
0.04
0.08
0.12
0.16
0.20
0.24
0
100
200
300
400
500
600
700
800
900
Concentration of Mn^{2+}, μM

Figure A.11 Dependence of the absorbance ratio of (A) Cu^{2+} and (B) Zn^{2+} concentration at 460 nm/530 nm on the concentration of metal solutions – pH 7

(A)Regression coefficient (r^2) = 0.9997
Equation of the line was $Y = aX + b$ where,
Y= absorbance at $A_{460\ nm/530\ nm}$
X= concentration of Cu^{2+} in μM
$a = 1.8635 \times 10^{-3}$
b = 0
Therefore, $X = 536.60 * A_{460\ nm/530\ nm}$

(B)Regression coefficient (r^2) = 0.9962
Equation of the line was $Y = aX + b$ where,
Y= absorbance at $A_{460\ nm/530\ nm}$
X= concentration of in Zn^{2+} μM
$a = 4.42 \times 10^{-3}$
b = 0
Therefore, $X = 226.24 * A_{460\ nm/530\ nm}$


(A)
Ratio of absorbances, 460/530nm
2.0
1.5
1.0
0.5
0.0
0
100
200
300
400
500
600
700
800
900
Concentration of Cu^{2+}, μM

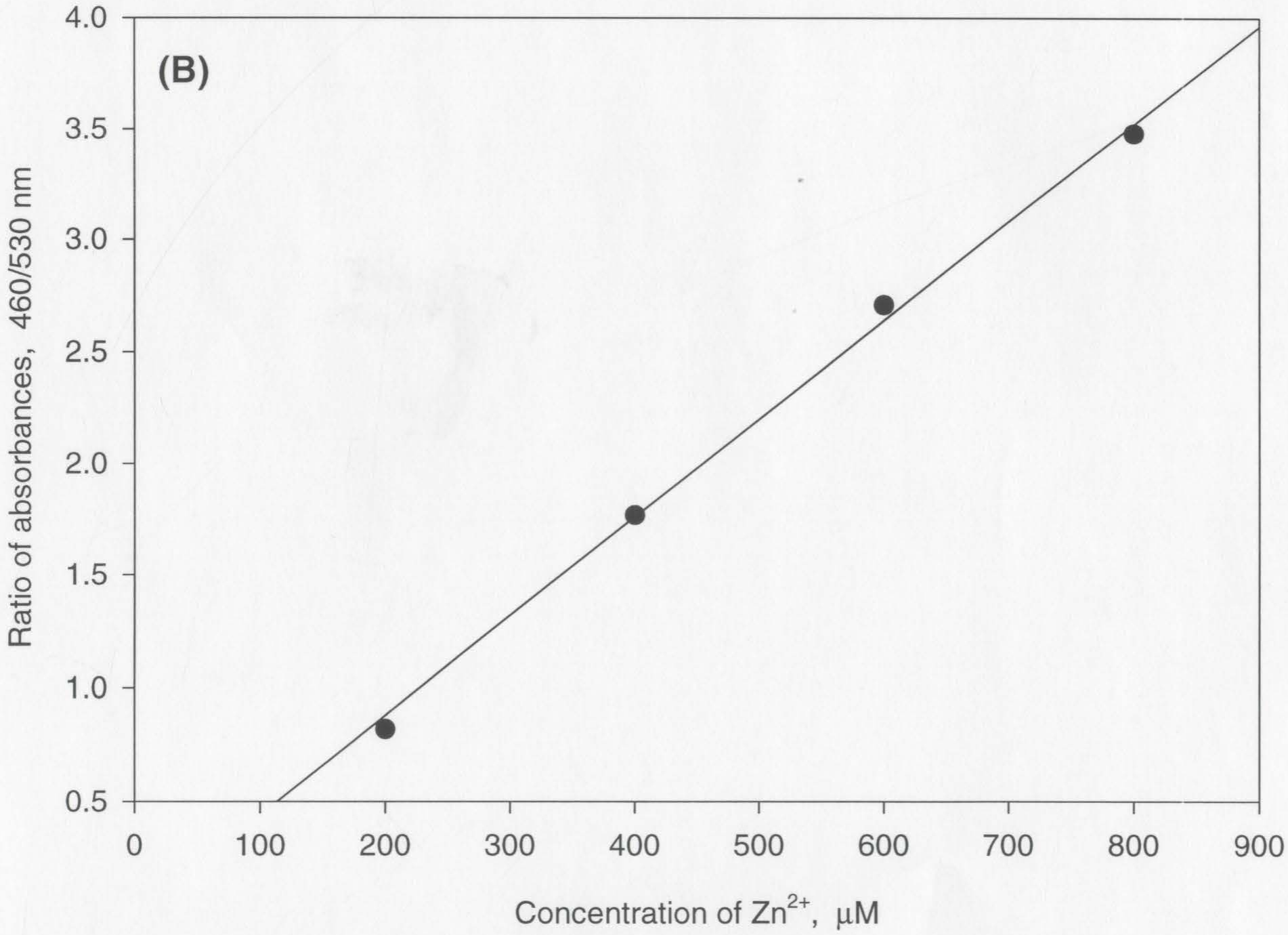

(B)
Ratio of absorbances, 460/530 nm
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
100
200
300
400
500
600
700
800
900
Concentration of Zn^{2+}, μM

Figure A12 Dependence of the absorbance ratio of (A) Fe^{2+} and (B) Co^{2+} concentration at 460 nm/530 nm on the concentration of metal solutions – pH 7

(A) Regression coefficient (r^2) = 0.9959

Equation of the line was $Y = aX + b$ where,

Y= absorbance at $A_{460\ nm/530\ nm}$

X= concentration of Fe^{2+} in μM

$a = 1.18 \times 10^{-3}$

$b = 0$

Therefore, $X = 847.45 * A_{460\ nm/530\ nm}$

(B)Regression coefficient (r^2) = 0.9975

Equation of the line was $Y = aX + b$ where,

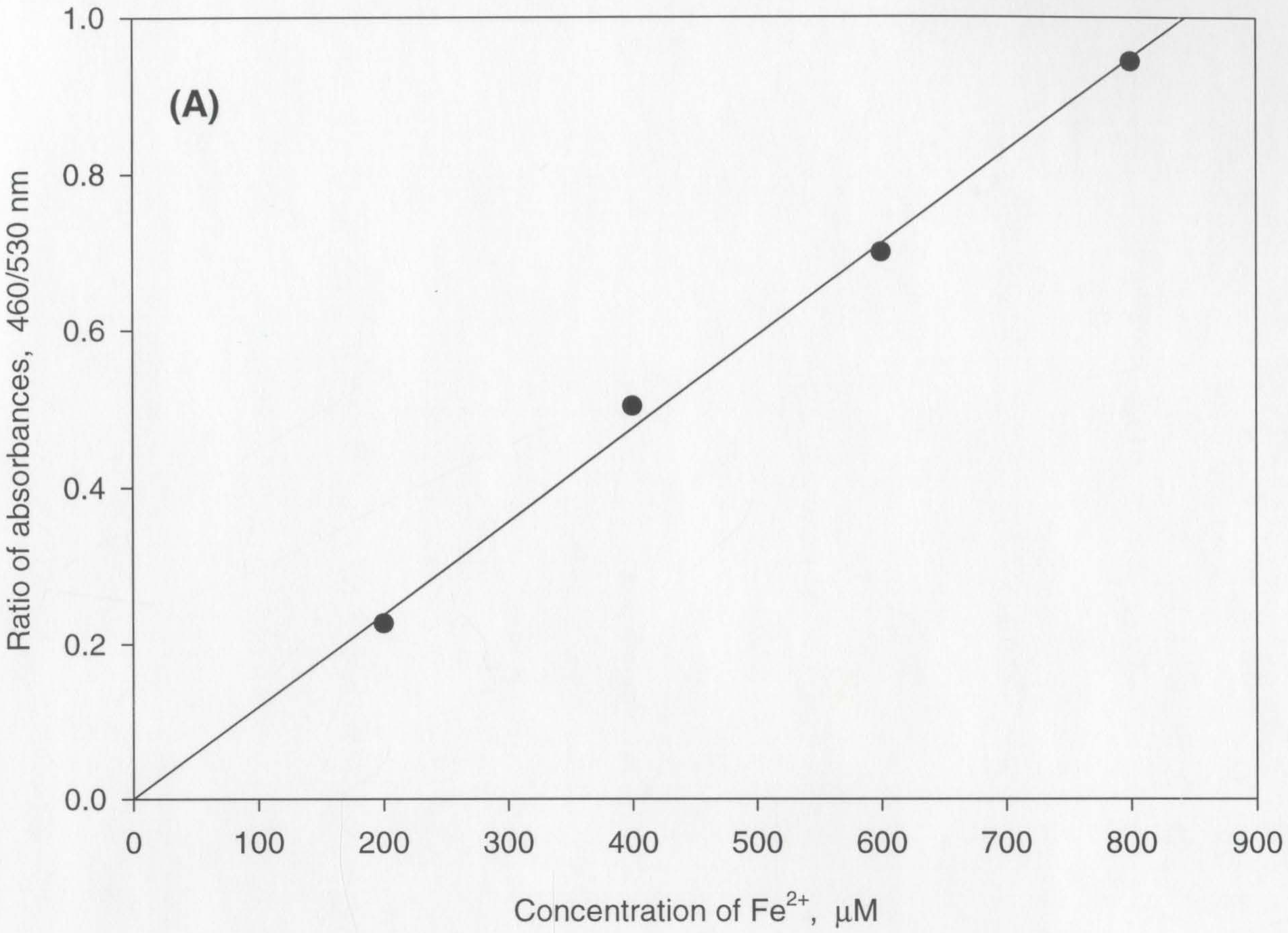
Y= absorbance at $A_{460\ nm/530\ nm}$

X= concentration of Co^{2+} in μM

$a = 1.26 \times 10^{-3}$

$b = 0$

Therefore, $X = 793.65 * A_{460\ nm/530\ nm}$


(A)
1.0
0.8
0.6
0.4
0.2
0.0
Ratio of absorbances, 460/530 nm
0
100
200
300
400
500
600
700
800
900
Concentration of Fe^{2+}, μM

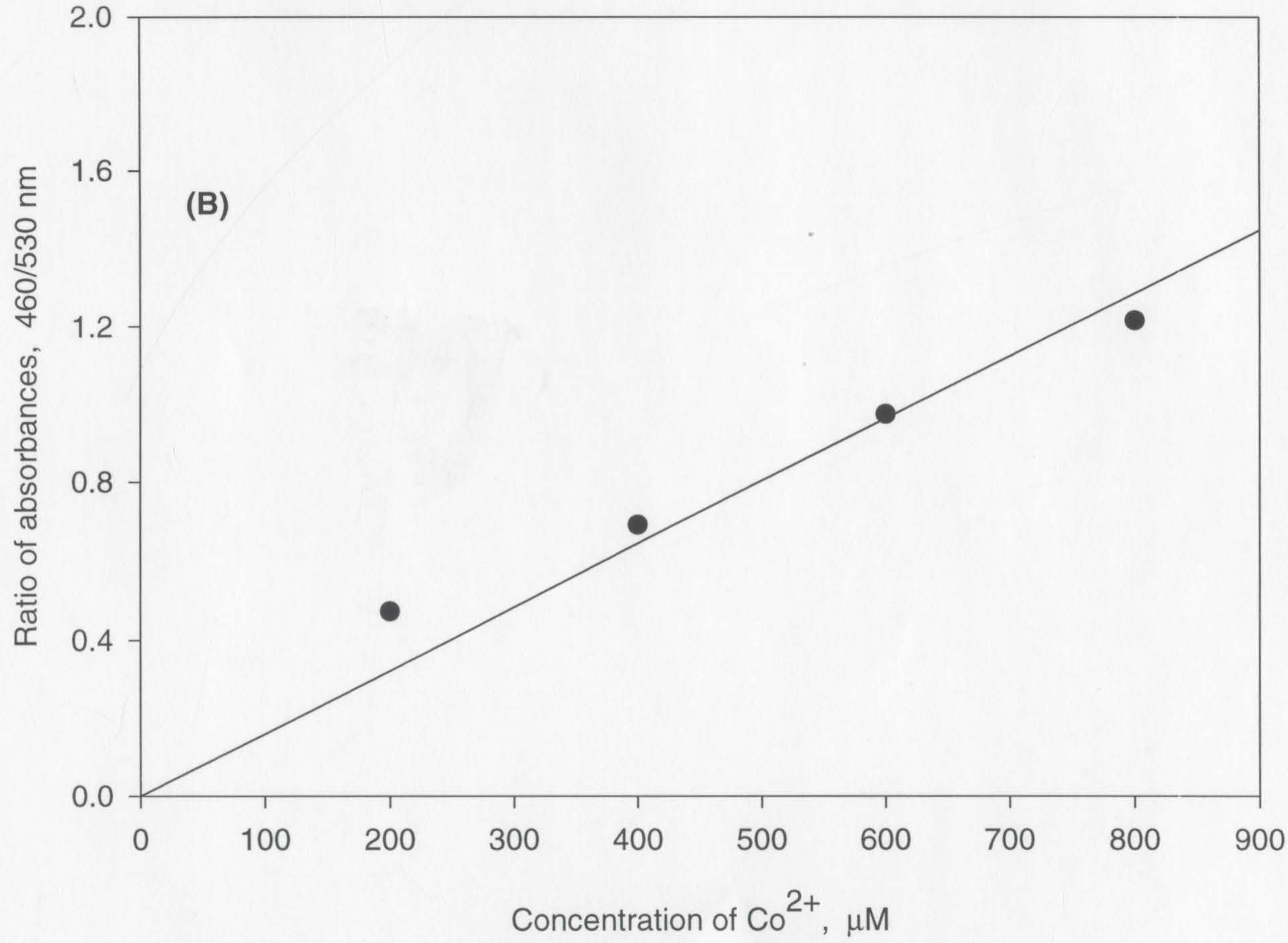

(B)
2.0
1.6
1.2
0.8
0.4
0.0
Ratio of absorbances, 460/530 nm
0
100
200
300
400
500
600
700
800
900
Concentration of Co^{2+}, μM

Figure A.13 Dependence of the absorbance ratio of Cd^{2+} concentration at 460 nm/530 nm on the concentration of metal solutions – pH 7

(A)Regression coefficient (r^2) = 0.9992

Equation of the line was $Y = aX + b$ where,

Y= absorbance at $A_{460\ nm/530\ nm}$

X= concentration of Cd^{2+} in μM

$a = 2.035 \times 10^{-3}$

$b = 0$

Therefore, $X = 491.40 * A_{460\ nm/530\ nm}$

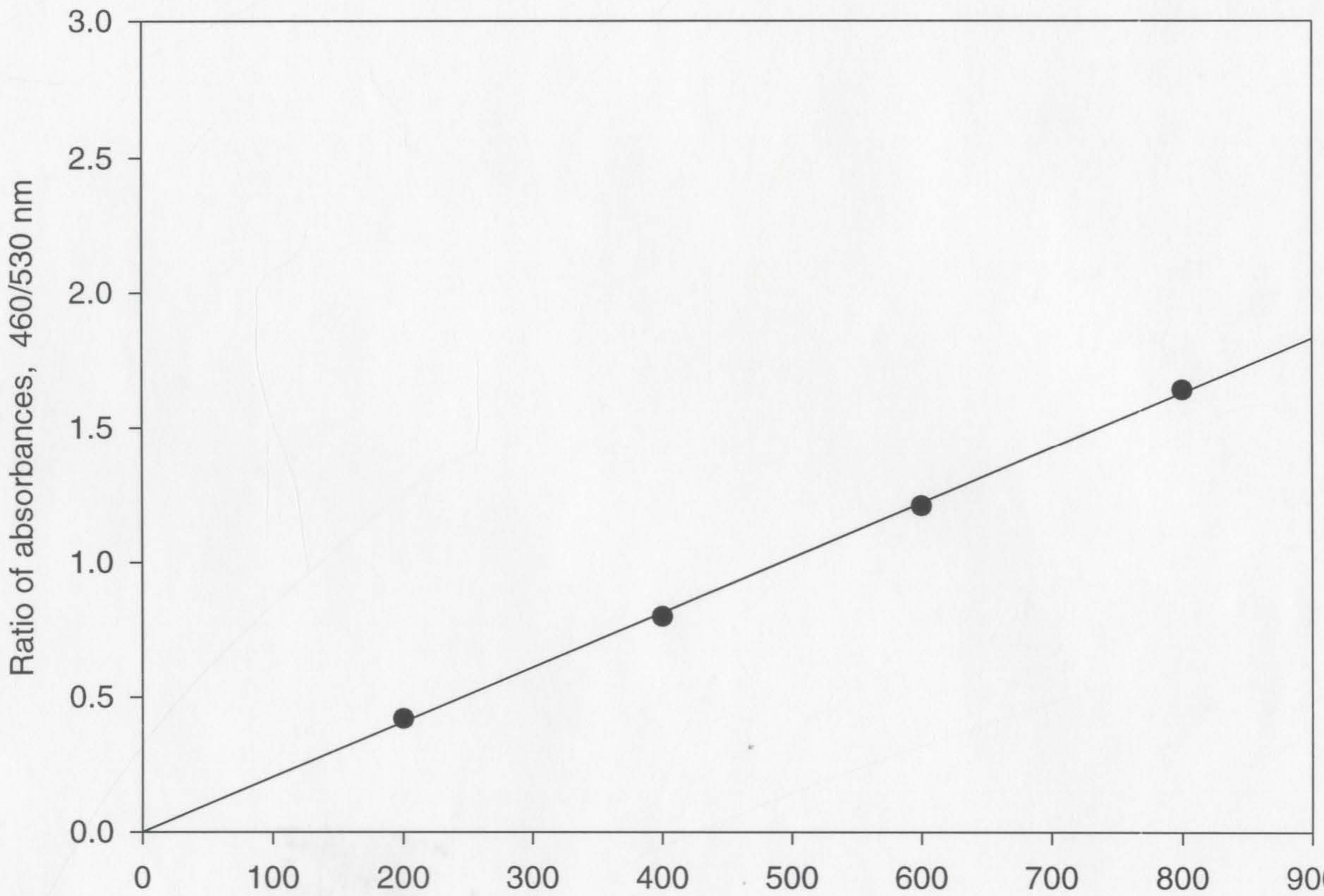

Ratio of absorbances, 460/530 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
100
200
300
400
500
600
700
800
900
Concentration of Cd^{2+}, μM

Table A.6 Effectiveness of chitosan in metal ion chelation from industrial wastewater

Heavy metals	Original Concentration (ppb)	Types of chitosan	Chelation capacity %		
			pH 5	pH 6	pH 7
Cr^{3+}	0.71±0.01	Type 1 Type 2 Type 3	2.08±0.98^{a} 2.08±0.98^{a} 0	3.52±0.98^{b} 2.08±0.98^{a} 0	5.06±0.70^{b} 2.08±0.98^{a} 0
Fe^{2+}	161.5±12.0	Type 1 Type 2 Type 3	94.1±0.00^{b} 14.6±0.00^{a} 15.4±2.89^{a}	93.8±0.45^{b} 15.2±0.07^{a} 14.4±0.00^{a}	92.2±6.85^{b} 17.5±0.24^{a} 15.3±0.00^{a}
Mn^{2+}	158.5±0.91	Type 1 Type 2 Type 3	2.11±0.59^{a} 21.2±1.03^{c} 18.5±0.82^{b}	8.92±0.29^{a} 23.4±1.27^{b} 21.9±0.85^{b}	11.2±0.37^{a} 22.9±3.42^{b} 21.9±0.83^{b}
Co^{2+}	0.47±0.00	Type 1 Type 2 Type 3	22.1±1.81^{c} 2.47±0.50^{b} 2.10±0.00^{a}	24.2±1.85^{b} 4.20±0.06^{a} 2.10±0.00^{a}	26.3±1.83^{b} 4.20±0.06^{a} 2.10±0.00^{a}
Ni^{2+}	1.15±0.13	Type 1 Type 2 Type 3	53.2±6.15^{b} 11.3±1.31^{a} 9.04±0.78^{a}	54.9±6.36^{b} 12.6±0.84^{a} 9.51±0.12^{a}	62.8±7.29^{b} 20.8±6.76^{a} 22.3±0.09^{a}
Cu^{2+}	15.4±0.47	Type 1 Type 2 Type 3	58.3±1.14^{c} 19.3±2.07^{b} 12.0±1.51^{a}	51.8±0.74^{b} 29.9±8.08^{a} 35.3±2.60^{a}	64.9±1.56^{b} 41.9±8.21^{a} 38.0±3.05^{a}
Zn^{2+}	1331±27.8	Type 1 Type 2 Type 3	48.9±1.01^{b} 34.1±1.28^{a} 30.9±1.55^{a}	49.5±0.96^{b} 34.2±3.34^{a} 34.5±1.43^{a}	50.3±0.88^{b} 37.0±0.98^{a} 35.9±1.17^{a}
As^{2+}	0.43±0.02	Type 1 Type 2 Type 3	15.6±0.00^{b} 4.44±0.00^{a} 4.44±0.00^{a}	13.3±0.00^{b} 4.44±0.00^{a} 4.44±0.00^{a}	13.3±0.00^{b} 4.44±0.00^{a} 4.44±0.00^{a}

Mo^{2+}	1.40±0.05	Type 1 Type 2 Type 3	12.9±0.51[b] 2.77±0.01[a] 2.64±0.19[a]	15.7±1.38[b] 4.47±0.43[a] 2.70±0.05[a]	19.9±2.22[c] 9.96±0.03[b] 2.78±0.00[a]
Ag^{+}	0.03±0.00	Type 1 Type 2 Type 3	33.3±0.00 0 0	33.3±0.00 0 0	66.7±0.00 0 0
Cd^{2+}	4.99±0.08	Type 1 Type 2 Type 3	26.9±5.56a 20.4±1.07a 25.1±2.69a	27.5±4.50a 24.3±1.14a 26.0±1.53a	39.1±0.09c 30.6±0.52a 35.0±1.38b
Hg^{2+}	0.59±0.02	Type 1 Type 2 Type 3	95.8±0.99b 90.7±0.75a 89.8±1.91a	95.8±0.99b 91.5±0.41a 90.7±0.75a	97.5±1.07b 93.2±2.80a 91.5±0.41a
Pb^{2+}	32.9±1.15	Type 1 Type 2 Type 3	41.1±1.43c 8.75±0.03b 5.94±1.07a	57.9±3.24b 14.2±0.15a 7.26±0.34a	91.0±2.33c 16.9±0.57b 7.91±0.97a

Figure A.14 Dependence of the absorbance of bovine serum albumin protein at 660 nm on the concentration of protein in the solutions

Regression coefficient (r^2) = 0.9944
Equation of the line was Y = aX + b where,
Y= absorbance at $A_{660\ nm}$
X= concentration of protein in μg/mL
a = 0.0011
b = 0.0486
Therefore, X = 909.04 * A_{660nm}

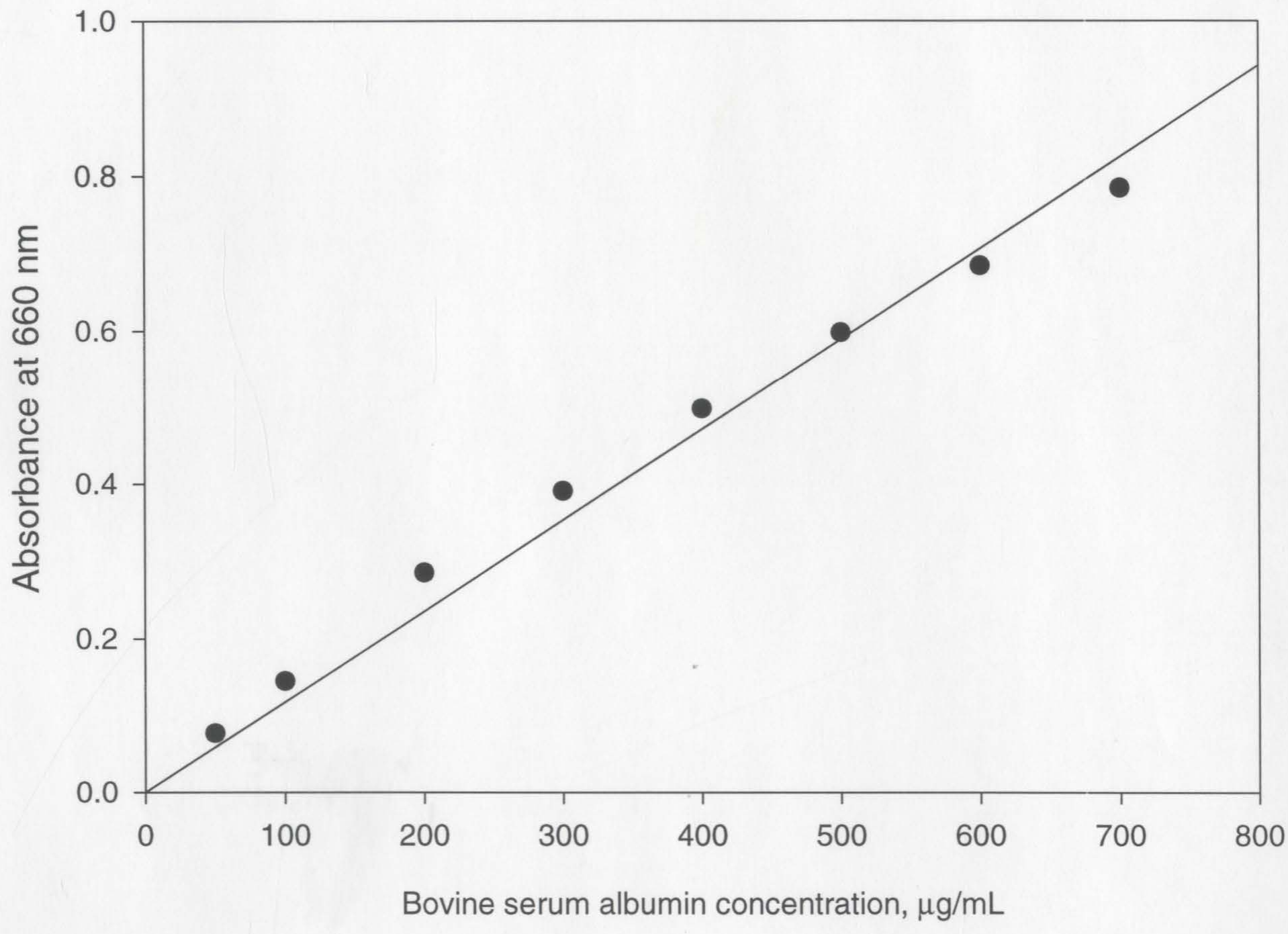

Absorbance at 660 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
600
700
800
Bovine serum albumin concentration, µg/mL

Table A.7 Protein flocculation capacity of chitosan using different concentrations of bovine serum albumin.

Protein concentration (mg/mL)	Type 1	Type 2	Type 3
2	100.00±1.4^{c}	70.00±1.2^{a}	19.55±1.40^{a}
4	91.25±1.2^{b}	70.00±1.8^{a}	22.70±0.98^{b}
6	66.83±1.4^{a}	74.35±1.3^{b}	21.66±0.88^{a}
8	65.76±1.6^{a}	73.86±1.1^{b}	20.26±0.76^{a}

Results reported are mean vales of three determinations ± standard deviation.
Means in each column sharing the same superscript are not significantly ($p>0.05$) different from one another.