AN ABSTRACT OF THE THESIS OF

Christina A. Mireles-DeWitt for the degree of Masters of Science in Food Science and Technology presented on February 28, 1994.

Title: Complex Mechanism of Chitosan and Naturally Occurring Polyanions

Redacted for privacy

Abstract approved: _____

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Reduction of biomass, COD, and BOD levels in processing waste through physico-chemical treatment is becoming an increasingly important environmental, as well as economic, concern for the food industry. Interest has therefore been generated in the use of chitosan as a flocculating agent for the recovery of protein and other bioproducts from food processing waste and for their subsequent use in animal feed. Chitosan, a polycation derivative of chitin, has been shown to be competitive and in some cases superior in flocculating ability to synthetic polymers. A hindrance to the use of chitosan has been cost-effectiveness and the current United States regulation allowing maximum amounts of only 0.1% chitosan in animal feed. Research has been conducted to investigate the mechanism of complexation of chitosan with three natural polyanions in order to better understand the influences affecting polymer interactions. Solutions were prepared at pH levels 3, 4.5, and 5.4, and ionic strength 0.15 and 0.30 in order to simulate varying waste environment conditions. Complex formation was measured by weighing an oven dried pellet recovered after centrifugation and subtracting salt determined from Cl^- measurements. Statistical analysis revealed that, depending on polyanion type, complex formation was affected by ionic strength. There was no main effect due to pH, however for alginate at initial pH=3 maximum complex formation occurred at a lower mixing ratio (MR=moles chitosan monomer/moles chitosan and polyanion monomers). The mole ratio of chitosan in the complex was determined through analysis of the supernatant using the MBTH reagent and Phenol-Sulfuric Acid methods. It was shown that binding did not always occur at a MR=0.5, but increased from 0 to 1 as

mixing ratio increased from 0 to 1. Supernatant pH measurements as well as infrared analysis indicated ionic interactions between COO^- or $SO3^-$ and NH_3^+ was the driving force behind complex formation.

Complex Mechanism of Chitosan and Naturally Occurring Polyanions

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Masters of Science

Completed February 28, 1994

Commencement June 1994

APPROVED:

Redacted for privacy

Associate Professor of Food Science and Technology in charge of major

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Date thesis is presented February 28, 1994

Typed by Christina A. Mireles-DeWitt

ACKNOWLEDGEMENTS

This paper is dedicated to:

Mom, Dad, and Tina
Tomorrow FINALLY came!

Grandpa and Grandma Arnold who welcomed us with open arms, a warm heart, and a full plate.

Grandpa and Grandma Mireles
Grandmother Lillian

All my gratitude and love goes to my husband,
Dr Bernard "Buddha" DeWitt
You got me through P-Chem,
You guided my research,
You shared my long nights with the thesis,
Thank you for putting up with it!

and Finally,
to Dr Torres who gave me the opportunity.

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COMPLEX MECHANISM OF CHITOSAN AND NATURALLY OCCURRING POLYANIONS

1. INTRODUCTION

Chitosan is a polymer composed of glucosamine residues which are linked by glycosidic bonds. It is derived from chitin, which is similar in structure to cellulose (a polymer of glucose units), particularly since the intermonomer bindings of both are of β-1,4 type. Polymer chain lengths of purified chitin have been shown to range in the thousands of monomer units (Carroad and Raymond, 1978). Chitin is the second most abundant polymer, the first being cellulose.

Global estimates of chitinous materials that is annually accessible range from 120 to 150 kilotons (Knorr, 1986; Knorr, 1991). According to Rawis (1984) the Japanese alone process 300 tons of chitin per year. Major sources of chitin are Dungeness crab (*Cancer magister*), King crab (*Paralithodis camschatica*), and Pacific shrimp (*Pandandalus borealis*) (Knorr, 1991). In addition, Sanford and Hutchings (1987) noted that there is always a supply of raw material since the principle sources of chitin (crab and shrimp) are harvested in different seasons.

Carroad and Raymond (1978) estimated shrimp and crab landings in the U.S. at 340 million kilotons in 1977. Solid waste made up 75% of the total weight from these shellfish, with chitin making up 20-58% of the dry weight of this waste depending on the processing method. Chitin comprises 12.3% of fresh water crayfish meal, 12.9% of crab meal, and 7.6% of shrimp meal (Patton and Chandler, 1975). Crawfish waste contains 23.5% chitin, on a dry basis. Shrimp waste contains 14-27% chitin, while crab contains 13-15% (No et al., 1989). Some interesting figures concerning the profitability of chitosan production were published by Bough and Landes (1977). They determined that from one ton of shrimp meal intended for animal feed and valued at $100/ton, 400 pounds of chitosan could be produced. The resulting chitosan could then be sold at $2/pound for approximately $800. Knorr

(1991) predicted sales of chitin/chitosan to range about $2 billion over the next ten years.

The overall scope of this project is to evaluate the effectiveness of chitosan-polyanion complexes for the removal of solids from typical processing wastewater. It is hypothesized that a multicharged polymer, acting as a "seed" to promote flocculation, would be much more effective in removing nutrients with poly-ionic natures than either a positively or negatively charged "seed" polymer by itself.

The research presented in this paper helps to achieve this by studying the interaction of chitosan at different mixing ratios (defined as the ratio at which two polymers are combined) with carrageenan, alginate and pectin. This is accomplished by quantitating complex formations and binding ratios. In addition, stability of complexes under conditions of industrial interest are examined by altering pH and ionic strength of polymer solutions. Investigation into the primary type of molecular interactions occurring during formation of polymer complexes is accomplished by measuring resulting pH's of complexed solutions and confirmed by FTIR. Final evaluation for the removal of solids from actual processing wastewater is left for subsequent investigation.

2. REVIEW OF LITERATURE.

2.1 Production and Purification.

Chitosan is produced from seafood exoskeletons through a series of treatments. First NaOH is added to remove proteins, followed by an addition of HCl to remove minerals, for example, $CaCO_3$ and $CaPO_4$. What remains is chitin. In order to form chitosan, the polymer is deacetylated with NaOH to hydrolyze the N-1 acetyl-linkage, rinsed, pH adjusted, and dried (Knorr, 1991; Bough et al., 1976). Water soluble chitosan is prepared by blending chitosan powder with an organic acid, such as adipic acid. Chitosan may be further purified by dissolving it in an organic acid (acetic acid) followed by filtration to remove extraneous material (Sanford and Hutchings, 1987).

No et al. (1989) determined optimum conditions for chitosan production from crawfish waste. Ideal deproteinization occurred when using 3.5% NaOH at 65°C for 2 hours with a solids to solvent ratio of 1:10 (w/v). Optimum demineralization was obtained with 1N HCl at room temperature for 30 minutes, with a solids to solvent ratio of 1:15 (w/v). Removal of carotenoid astaxanthin from the shell required extraction with acetone and then bleaching with 0.315% sodium hypochlorite solution for 5 minutes with a solids to solvent ratio of 1:10 (w/v). Bade and Wick (1988) proposed a method to improve chitosan production to make it a more "predictable" compound. The procedure preserved the original fibrous structure by using demineralization as the first step instead of mechanical degradation and deproteination.

2.2 Physical and Chemical Properties.

Much research has been conducted to investigate the various physical and chemical properties of chitosan. Primarily, researchers have been interested in chitosan's solubility. Chitosan, in its free amine form, is not soluble in water at pH's above 5.5, organic solvents, or alkali (Sanford and Hutchings, 1987; Filar and Wirick, 1978). Chitosan is insoluble in water at pH above 5.5 because of its compact α-, β-, and γ-conformations formed through hydrogen bindings (Hirano and Kitigawa, 1987). The pI for chitosan is 6.3. The majority of water soluble proteins have a pI < 5.0 (Senstad and Almas, 1986). However, in a low density ionic environment, as pH is reduced, chitosan becomes soluble. Filar and Wirick (1978) proposed that chitosan molecules uncoil and assume a rod-like or elongated shape, driving the equilibrium $NH_2 + H^+ \Leftrightarrow NH_3^+$ to the right, resulting in mutual repulsion of charged groups which provides the uncoiling force. Kienzle-Sterzer et al. (1982a) in a study of the dilute solution behavior of chitosan with respect to acetic acid concentration substantiated the proposal by noting an increase in specific viscosity as a result of the interactions between chitosan and acid.

Chitosan is soluble in nitric and hydrochloric acids at concentrations of 1% or less (Filar and Wirick, 1978). In this medium some degradation will occur at below pH 2 (Kent and Whitehouse, 1955). The best inert solvents for chitosan are N,N-dimethylacetamide-LiCl and N-methyl-2-pyrrolidone-LiCl. Their solvent power is derived from the addition of LiCl, as the two liquids alone are only swelling agents for chitin. Tertiary amide solvents with salts have been used for the dissolution of highly crystalline poly-amides. The LiCl works to reduce or break the crystalline forces, such as hydrogen bonding, by association with the polymer and solvent (Rutherford, 1978).

Various other properties of chitosan have been cited in the literature. It is a linear polyelectrolyte with a high charge density that adheres to negatively charged surfaces. Some examples of materials that chitosan interacts strongly with are

proteins, anionic polysaccharides, hair and skin (composed of negatively charged mucopolysaccharides and proteins), and nucleic acids (Sanford and Hutchings, 1987). The nitrogen content of purified chitin is 6.89% which is equivalent to 43.06% crude protein (Ortega and Church, 1979). Chitosan does not undergo thermal decomposition up to 150°C (Kent and Whitehouse, 1955). Depending on whether chitosan is a high, medium, or low viscosity grade, viscosity increases rapidly as concentration goes beyond 1%. Salt increases pH and minimizes viscosity variation (Filar and Wirick, 1978). Hydrophilic properties, solubility and water uptake of chitosan, are significantly higher than microcrystalline cellulose. Lipid bonding, an interphasic property of chitosan, can range up to 4-5 times its weight (Knorr, 1986).

Physicochemical characteristics of chitin from certain fungi and the crab, *Cancer magister*, have been compared. For the most part the chitin from crabs is similar to that of fungi, however differences were detected between the degree of crystallinity, the intensity of the absorption in the IR region, and the content of glucosamine, N-acetyls, and monosaccharides (Feofilova et al., 1980).

2.3 Uses of Chitosan.

The wide spread availability of chitosan and its unique properties has resulted in extensive research into a variety of areas for potential use.

2.3.1 Waste Recovery.

In the U.S., chitin is a substantial waste problem for the shrimp and crab-processing industries (Rawis, 1984). However, through use in waste recovery, chitosan can be bioconverted for the production of value added products and utilized for the removal and recovery of other waste materials or valuable by-products (Volesky, 1987). Since chitosan is a natural polymer, it is preferred over synthetic polymer flocculants, which may contain hazardous monomers (Sanford and Hutchings, 1987). A significant incentive for this type of research came when FDA approved chitosan as a feed additive (at levels no greater than 0.1%) and the US EPA approved it for potable water purification at levels up to 10 mg/L (Knorr, 1986; Sanford and Hutchings, 1987). Further incentive for this research comes from Section 204 of the Federal Water Pollution Control Act Amendments of 1972 (P.L. 92-500) which require proportionate sharing between municipal applicants and industrial users of costs for construction, operation, maintenance, and replacement of waste treatment facilities. Municipalities may also impose pretreatment standards on industrial users as a result of Section 307 of P.L. 92-500. The intent is to "prevent the discharge of any pollutant into such treatment works, which pollutant may interfere with such works" (Bough, 1975a). The result is food processors can expect to pay more for waste treatment. In addition there is an increased awareness of the ecological effects of toxic metals and their accumulation through the food chain (Knorr, 1991). Chitosan's ability to chelate metals has increased interest in its use to detoxify hazardous waste and clarify pools and spas (Sanford and Hutchings, 1987).

Bough and Landes have investigated the effectiveness of chitosan as a coagulant in various processing wastes. Bough (1975a) studied the treatment of egg breaking wastes with solutions of chitosan and Betz 1130, a negatively charged polymer. Suspended solids were reduced by 70-90% and the chemical oxygen demand (COD) by 55-75%. Estimated polymer cost for coagulation of the egg washer waste was $0.80/1000 gallons. Amino acid composition of egg washer waste was similar to the composition of whole eggs. Maximum coagulation using chitosan occurred at pH's 6.7-7.1 and at concentrations of 10, 60, and 100 mg/L. Tests showed that a combination of chitosan and Betz 1130 resulted in the largest floc formation and greatest reduction in turbidity and suspended solids.

The same year a study involving coagulation of chitosan with pimento and turnip greens was published (Bough, 1975b). Coagulation of chitosan with pimentos was optimal at pH 6, the pH of raw waste, and at increasing concentrations up to 30-40 mg/L chitosan. At levels over 50 mg/L turbidity increased. He states this as a common phenomenon in treatment with an excess polyelectrolyte concentration and is due to re-stabilization of solids from the coagulate to the suspended phase. At pH 4 effluent from turnip greens, when coagulated with chitosan and a carrageenan produced the lowest turbidity results when added at 10 mg/L and 15 mg/L respectively.

Bough and Landes (1976) found that chitosan coagulated suspended solids in cheese whey as effectively or better than ten commercial synthetic polymers. Concentrations of suspended solids were reduced by over 90% by coagulation at pH 6.0 with a ratio of chitosan to suspended solids of 2.15% (1:46.5). Complex composition after drying was 73% protein, 6% lactose, 10% ash, and 7% moisture. Rat feeding studies showed no difference in the protein efficiency ratio of casein, coagulated whey solids containing chitosan, and whey solids containing no polymer. In a separate report it was noted that research has shown chitosan to be particularly effective in reducing solids in protein containing waste (Bough et al, 1976).

Treatments with chitosan was found to reduce chemical oxygen demand by 60-80% in poultry, egg, meat, and shrimp wastes (Bough and Landes, 1977). Proximate composition of dry coagulated solids were found to contain significant amounts of protein (30-70%), and in poultry and egg wastes, 30-50% fat. No adverse effects were found when chitosan recovered waste products were fed to white rats at chitosan levels below 5% of the diet. Also, free chitosan mixed in the diet did not adversely effect growth rate, blood, or liver composition at levels of 5% or less.

Various other studies have helped to describe chitosan's effectiveness as a coagulating agent. No (1989b) demonstrated crawfish chitosan to be an effective ligand-exchange column material for recovery of amino acids from seafood processing wastewater. In comparison with commercial chitosan, crawfish chitosan loaded with copper or amino copper, showed higher recovery rates of amino acids. Recovery from amino copper-crawfish columns was pH dependent, with reduction at higher pH values. Eluate was completely free of copper ions when passed through a second crawfish chitosan column. Recovered amino acids could potentially be used as seafood flavors in terms of their sensory attributes. In another study, No (1989a) found crawfish chitosan to be as effective or better than two commercial chitosans and five synthetic commercial polymers at coagulating (pH 6) suspended solids in crawfish pigment extraction stickwater. At concentrations of 150 mg/L, suspended solids and turbidity were reduced 97% and 83% respectively and COD was reduced by 45%. In the control, turbidity was reduced by only 8%. Combinations of chitosan with $FeCl_3$ did not yield significant reductions in turbidity. The proximate composition of suspended solids, with a 5.9 g/L yield, was 27.1% crude protein, 51.7% fat, and 3.3% ash. The supernatant contained large concentrations of flavor-related free amino acids.

In addition to the use of chitosan as a coagulant in "food type" wastes, studies have been conducted to determine its effectiveness in removing more toxic compounds such as pesticides. Van Daele and Thome (1986) found the absorption

of PCB by chitosan powder to be efficient enough to retain up to 60% PCB under experimental conditions. Results suggested filtration of PCB contaminated water through chitosan should be sufficient to "protect" fish against intoxication. Thome and Van Daele also (1986) showed that one gram of chitosan was enough to eliminate >60% of PCB from 0.5 ppb polluted stream water and maintain the resultant contamination in fish at a non-toxic level of <1ppm. Larger amounts of chitosan (10g instead of 1g) did not increase PCB retention. The effectiveness of activated charcoal and chitosan together versus activated charcoal alone, using a closed loop system, was compared. By itself activated charcoal only removed 0.2 ppb PCB; however, in conjunction with chitosan it removed all of the PCB's. Activated charcoal absorbed a fraction of the PCB, but mainly filtered out particles in suspension that are able to saturate adsorption sites on chitosan.

There are many factors that affect the performance of a biosorbent. One of the more important is the interaction of the adsorbent with the solutes. Any solid surface in contact with a liquid is known to have a surface charge and this plays a dominant role in the adsorption of solutes from solution (Venkatroa et al., 1986). At low pH values, adsorbent surfaces that are negatively charged are neutralized and attract solute molecules much better. The nature of the solute is also important in determining the role of its adsorption on the sorbent surface. It has been suggested that there is a formation of a coordination complex between the metallic species and the chitin nitrogen or oxygen (Volesky, 1987). Chitosan can bind in vitro a variety of anions, such as bile acids of free fatty acids at low pH, by ionic bonds resulting from its amino groups (Sugano et al, 1988). The following are all studies into the effect of these and other factors on the complexation properties of chitosan or other dilute solution polymers.

Venkatroa et al. (1986) studied the kinetics of sorption by chitosan of two dyes C.I. Direct and Reactive Red 31 and 73. The sorption of dyes was markedly affected by temperature and chitosan particle size. Initial dye concentration uptake is influenced by boundary layer resistance and intra-particle diffusion. Temperature

effects indicate physical adsorption takes place. In a separate but similar study (McKay et al., 1986), isotherms at three temperatures were produced by plotting the amount of copper absorbed by a given mass particle size of chitosan versus final concentration of copper. In this study of chitosan, particle size was found to influence adsorption capacity only slightly and to decrease with temperature. The rate determining steps of adsorption were in the mass transfer of solute from the bulk solution to the particle surface and intraparticle diffusion. Adsorption at an interior site was found to be rapid and non-rate determining.

Senstad and Almas (1986) stated a high ratio of positively charged colloids will inhibit destabilization. Charge ratio depends on both pH and the molecular number ratio. They found that at lower pH values chitosan had a negative effect as a coagulating agent. Increasing the ratio of chitosan to protein caused the proteins to be less destabilized due to an excess of positive charged chitosan molecules. In the higher pH range (6.5-8.0) protein removal increased. The chitosan molecule had no net charge, but the amino groups were free to form chemical bridges with reactive sites on colloids. As pH increased, a larger portion of proteins assumed a net negative charge, and more chitosan was needed to form bridges. Sweep-floc (due to precipitation of chitosan) is an alternative mechanism for the removal of protein in the higher pH range. No significant differences in protein removal was observed for the ultrafiltered samples, implying that salt content had no effect on the destabilization of proteins. Hirano et al. (1978) reported that when a variable amount of the anionic solutions were added to N-acylated chitosan, changes in pH and ionic strength were found to be negligible. In contrast to Senstad and Almas (1986) however, their study showed no effect of pH on complexation.

Nauss et al. (1983) determined the stoichiometry of the precipitation obtained by mixing chitosan acetate with bile salts and their micelles under a variety of conditions. Like Senstad and Almas (1986) major variables affecting binding were shown to be pH and ionic strength. It was concluded the interactions were mainly ionic in nature. Chitosan was also shown as being capable of binding with 4-5 times

its weight with all lipid (fatty acid) aggregates tested. Researchers concluded that since there is more than one bile salt bound per glucosamine residue, there must be hydrophobic interactions operating at secondary binding sites. They further speculated that unprotonated glucosamine residues may be involved in the hydrogen bonding with some groups in the bile salts. The ability of chitosan to coacervate bile salts was also noted.

Kikuchi and Noda (1976) studied the reaction of chitosan with heparin and found the concentration of polymers to play an important role in determining the composition ratio of chitosan to heparin in the polyelectrolyte complex produced. IR studies revealed that the polyelectrolyte complexes had an adsorption band at 1520 cm^{-1} which neither appeared in chitosan, heparin, nor a mixture of both. A band around 1500 cm^{-1} was assigned to the N-H stretching frequency resulting from the -NH_3^+ group. Polyelectrolyte complexes failed to show this absorption. It was proposed that a band around 1520 cm^{-1} was due to an -NH_3^+ group participating in polyelectrolyte complex formation by binding heparin at its -SO_4 group. Polyelectrolyte complexes were insoluble and tests with toluidine blue showed an even dispersion of heparin in the complex.

Polyelectrolyte complexes formed by some acidic glycosaminoglycans with partially N-acylated chitosans were researched by Hirano et al. (1978). Complex formation of N-acetylated chitosans was generally stoichiometric. The interaction of chondroitin sulfate A, chondroitin sulfate C, and heparin with partially N-acetylated chitosans in aqueous acidic solutions (pH 2.8 and 4.5) was studied to examine the effect of the degree of substitution and the structure of N-acyl groups on complex formation. In the complex produced from the chondroitin sulfate A and N-myristoylchitosan at pH 2.8, the N-myristoylchitosan moiety was evident by the IR absorptions at 2950 (CH in fatty acyl) and at 1630 and 1550 cm^{-1} (C=O and NH in N-acyl). The chondroitin sulfate A moiety was evident by IR absorptions at 1240 and 820 cm^{-1} (S=O and equatorial C-O-S in σ-sulfate). For both chondroitin sulfate A & C the same mixing ratio maximum was found for complex formation with

chitosan. The mixing ratio maximum was higher at pH 4.5 than at 2.5 due to the higher degree of dissociation (α) for the dissociable groups. In chondroitin sulfate A & C, the COOH group has α=0.5 at pH 4.5 and α=0 at pH 2.8 while SO_3H has α=1.0 at pH 2.8 and 4.5. The NH_2 in chitosan has α=0.8 at pH 4.5 and 1.0 at pH 2.6. Almost the same mixing ratio maximum value was observed at the same degree of substitution value regardless of the N-acyl group structure.

2.3.2 Dietary Supplement.

Much research has been conducted on the toxicity of chitosan and its potential as an animal feed. Patton and Chandler (1975) tested the in vivo digestibility of chitin from various sources in fistulated Jersey steers using a variation of the suspended nylon bag technique. When comparing the loss of proximate composition (especially crude fiber) between solubility in vitro (water) and in vivo (rumen) they determined differences large enough to assume chitin digestion. The rumen digestive system solubilizes 5.0, 3.2, and 15 percent of the chitin in cockroaches, grasshoppers, and crab meal, respectively, over the solubilization occurring in water alone. Ortega and Church (1979) also evaluated the feeding value of crab meal and some other chitinous compounds by means of in vitro rumen digestion trials and concluded that nitrogen from crab meal was not used to any great degree by rumen microorganisms.

Watkins and Knorr (1983) evaluated the feeding value of commercially available chitin as a nonabsorbable carrier to azo food dyes. Food efficiency was slightly higher when chitin was incorporated into the diet with no significant difference in growth rate in rats fed chitin or cellulose. At levels up to 8.5% chitin no sign of toxic nutritional stress was evident and growth rates were slightly higher, implying chitin may be relatively inert nutritionally. However stress was noted at levels of 10% and higher.

In broilers fed up to 10% chitosan it was an effective dietary fiber promoting normal growth and vigor (Knorr, 1986). A combination of chitosan with whey in isonitrogenous, isocaloric diets enabled broilers to utilize whey more effectively. The growth of bifidobacteria in the gut of chickens increased when chitin was added to their diet. These bacteria block the growth of other types of microorganisms and generate the lactase required for digestion of milk lactose. This may be of significance for humans and animals with lactose intolerance. Moreover, 5% chitosan prevented rise of serum cholesterol in rats fed a 0.5% cholesterol enriched diet (Sugano et al.,1988). At 2%, both low and high molecular weight chitosan exerted a comparable cholesterol lowering action. Hypocholesterimic action of chitosans was independent of their molecular weight within tested viscosity range. In addition, chitosan appeared to interact with bile acid and/or cholesterol in the intestinal lumen and to stimulate fecal excretion of neutral steroids.

Finally, Hirano et al. (1990) reported chitosan to be safe, digestible, and hypolipidemic at appropriate oral dosages in rabbits, hens, and broilers. No abnormal symptom was observed in hens and rabbits for up to 239 days at 1.2-1.4 and 0.7-0.8 chitosan/kg bodyweight/day, respectively. Levels of chitosan in hens diets at 2% was not harmful. Chitosan over feeding, 3.6-4.2 and 14-18 chitosan/kg bodyweight/day for 140 and 56 days respectively, resulted in a decrease in egg-laying rate and appetite due to incomplete digestion. For rabbits chitosan was more digestible than chitin. Increasing from 41% on day 5 to 82% on day 15, indicating an adaption to chitosan feeding. Artificially raised cholesterol levels in rabbit serum, 850 and 320 mg/dL, was reduced to 300 and 210, respectively, by feeding 2% chitosan.

2.3.3 Bioconversion Scheme.

Cosio et al. (1978) studied the pretreatment of shrimp processing waste for a chitin bioconversion scheme to produce yeast single-cell protein. Chitin was pretreated by sieve reduction, deproteination and demineralization. Protein was recovered by precipitation. Some of the chitin was used as a substrate for microbial chitinase production (*Serratia marcescens QMB1466*). The remaining chitin was mixed with chitinase to hydrolyze the chitin to monomer N-acetyl glucosamine which served as the substrate for yeast (*Pichia kudriavzevii*) to grow and produce protein. The protein products were then to be sold as an animal feed.

2.3.4 Antimicrobial and Pesticide.

Popper and Knorr (1990) showed that on the surface of microorganisms, polycations such as chitosan, EDTA, TRIS, polylysine and some antibiotics, in contrast to divalent cations such as Mg^{2+} and Ca^{2+}, produce a destabilizing effect on the outer cell wall structure. Several concentrations of chitosan hydroglutamate were tested against suspensions of microorganisms with antimicrobial activity shown at 0.01 mg/mL. Initial counts were substantially decreased by increasing chitosan concentration. It was speculated that chitosan affects transportation mechanisms but not cell wall integrity and that a strong interaction of chitosan exists with the lipopolysaccharide layer of gram-negative bacteria as well as with the peptidoglycan of gram positive species.

Bade and Wick (1988) proposed production of predictable chitosan with predictable properties to formulate safe biodegradable pesticides. The contact of chitosan with plant cells has been shown to induce disease resistant responses biochemically identical to those induced by exposure to certain *Fusarium* species. Suppression of nematode damage to plants by chitin application has been observed

but not studied as much as fungi resistance. Suppression of nematodes by chitin may be due to a transient high spike of NH^{3+} formed in the soil which is harmful to parasitic roundworms. Alternatively, beneficial microorganisms could be favored when chitin is available as a substrate. Unfortunately, it seems that chitin in the quantities needed for nemocidal effect may be toxic to plants.

2.3.5 Beads and Coacervate Capsules.

Elution from chitosan gel beads containing the entrapped herbicide atrazine and the fertilizer urea showed an initial rapid release followed by a constant release rate (Teixeira et al., 1990). The chitosan gel beads were estimated to extend the release period of atrazine and urea by a factor of 50 to 180 times, respectively. Beads of 1% chitosan were found to be the most suitable for release of the herbicide.

Plant cells were immobilized in coacervate capsules using alginate or carrageenan crosslinked with water soluble chitosan in to investigate cell immobilization, capsule permeability, and subsequent cell viability (Beaumont et al., 1989). Cell outgrowth, respiration rate, and total protein recovery were equal to or greater than cells immobilized in the same polymers without the chitosan shell. Carrageenan beads allowed higher total protein recovery and respiration rate than those with alginate suggesting a greater diffusivity of carrageenan gels.

2.3.6 Fining Agent.

The use of acid-soluble and water-soluble chitosan as a fining agent for apple juice has been compared to conventional silica sol/gelatin/bentonite treatment (Soto-Peralta et al., 1989). Both proved equally effective in reducing juice turbidity. Imeri and Knorr (1988) reported that carrots treated with two different viscosity chitosans

at two different concentration solutions in 2% ascorbic acid produced no effect on juice yield, but lowered pH and soluble solids. Treatments also resulted in significant reduction of titratable acidity and color index. β-carotene was also reduced and correlated with a color index changes.

2.3.7 Films.

Kienzle-Sterzer et al. (1982b) studied the use of chitosan for the preparation of films and found swelling to show a salting-in effect up to ionic strength 0.4. Internal film stress decreased as ionic strength increased. In another study, chitosan was crosslinked with epichlorohydrin and a film was cast(Mayer et al., 1989). Underivitized chitosan had superior oxygen permeability resistance compared to synthetic packaging films such as mylar and polypropylene but considerable less tensile strength and flexibility. Tensile strength of cross-linked chitosan was 2-3 orders of magnitude greater than that of underivitized chitosan and approached tensile strength of mylar and polypropylene. Bade and Wick (1988) suggested that chitosan with predictable properties could result in the manufacture of high tensile strength biodegradable plastics. Chitosan-based plastics would be favorable because microorganisms degrading chitosan are abundant in all soil types. Chitosan films were reported to disappear completely within 3-4 weeks in North Atlantic coast waters. A unique application of chitosan films was proposed by Rawis (1984). Burn patients could be treated with a chitosan acetate solution which forms a tough, water-absorbent film over the wound. The film is permeable to oxygen, absorbs water strongly, and eventually could be degraded by the enzyme lysozyme present in wounds eliminating the need to remove the film.

2.3.8 Food Additive.

Finally, Knorr (1982) compared chitin, chitosan, and microcrystalline chitin with microcrystalline cellulose to examine their use as functional additives in food formulations. Water binding, fat binding, and emulsifying properties were studied. Water binding of the three forms of chitin ranged from 230-440% (w/w) and fat binding from 170-315% (w/w). Only microcrystalline chitin showed good emulsifying properties and was superior to cellulose. It also increased specific loaf volume of white and protein fortified breads.

3. COMPLEX FORMATION OF CHITOSAN AND NATURALLY OCCURRING POLYANIONS.

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3.1 Abstract.

Chitosan and polyanion mixtures were prepared in different mole ratios and at different pH values and ionic strengths. Complex formation was measured by weighing a dried pellet recovered by centrifugation. The mole ratio of chitosan in the complex was determined through analysis of the supernatant. Statistical analysis revealed that, depending on polyanion type, complex formation was affected by ionic strength. There was no main effect due to pH, however at initial pH=3 maximum complex formation occurred at a lower mixing ratio. Supernatant pH measurement showed that electrostatic interaction between COO^- or SO_3^- and NH_3^+ was the major force behind complex formation.

3.2 Introduction.

Today, reduction of biomass, COD, and BOD levels in processing waste through physico-chemical treatment is becoming an increasingly important environmental, as well as economic, concern for the food industry (Bough, 1976). Interest has therefore been generated in the use of chitosan as a flocculating agent for the recovery of protein and other bioproducts from food processing waste and for their subsequent use as animal feed. Examples reported in the literature of biomass recovery solely using chitosan have ranged from 70 to 97% (Knorr, 1984). In addition, chitosan has been shown to be competitive and in some cases superior in flocculating ability to synthetic polymers (Bough, 1976 and Bough et. al., 1976). A hindrance to the use of chitosan has been cost-effectiveness and the current U.S. regulation allowing maximum amounts of only 0.1% chitosan in animal feed (Publication PL1-002). Research in this laboratory has been conducted to investigate the complexation of chitosan with polyanions to form larger, more effective flocculating molecules possessing both positive and negative charge characteristics.

The purpose of this study was to investigate the effects of pH, ionic strength, and mixing ratio on chitosan-polyanion complex formation. Research to determine these effects have been conducted with a simple polyanion, polyacrylic acid, and also more complex, food grade polyanions, alginate (ALG), carrageenan (CAR), and pectin (PEC). A comparison of results from both studies should give insight into the complex formation process occurring with chitosan.

3.3 Materials and Methods.

3.3.1 Chitosan-Polyacrylic Acid Complexes.

Preparation. Chitosan (CHI, Lot 5112A) was purchased from Bioshell Inc., Albany, OR. To obtain a higher purity material, it was first dissolved in 0.1 N HCl, filtered through a medium-porosity fritted disk Buchner type filtration funnel, reprecipitated with NaOH, rinsed with deionized water, and finally freeze-dried. The molecular weight of CHI (220,000) was determined at 25°C with a Cannon-Fenske viscometer, following the procedures reviewed by Kienzle-Sterzer (1984). Purified CHI was dissolved in a solution of 27.5 g of NaCl in 1,000 mL of 1% acetic acid. The molecular weight of polyacrylic acid (PAA, Aldrich, Milwaukee, WI) was estimated to be 202,000 using dioxane as the solvent (Sutterlin, 1975).

3.3.2 Complex Formation.

CHI (0.1 g) and PAA (0.1 g) were dissolved in 100 mL of HCl and 100 mL of NaCl solutions, respectively. The ionic strength (IS), 0.025-0.300, was varied by adjusting the concentration of the HCl and NaCl solutions.

3.3.3 Complex Analysis.

The insoluble complex was separated by centrifugation at 34,800g for 40 min. The pellet was twice resuspended in distilled water and then recentrifuged. The washed complex was finally freeze-dried and weighed. The pH of the supernatant was recorded, and the CHI concentration was measured by using the Nessler reagent

method (Lang, 1958). A material balance was used to calculate the amount of PAA left in the supernatant.

3.3.4 Chitosan-Natural Polyanion Complexes.

Chitosan (Pro Floc HV) was obtained from Protan, Inc. (Commack, NY) and purified as above. The polyanions were carrageenan (CAR, Viscarin GP109, Marine Colloids Division, FMC Corp., Philadelphia, PA), alginate (ALG, Kelgin MV, Merck & Co., Inc., San Diego, CA) and pectin (PEC, from citrus fruit, Grade I, Sigma Chemical Co., St. Louis, MO).

3.3.5 Preparation.

Chitosan and polyanion solutions were prepared as follows. CHI (16.0g) was dissolved in 400 mL of a 1M HCl solution, transferred to a 2 liter volumetric flask, and brought to volume with distilled water. Six 250 mL portions were transferred to 2 liter beakers and diluted with 1,500 mL distilled water. Solutions were then adjusted to appropriate pH (3, 4.5, 5.4) and ionic strength (IS = 0.15 or 0.30), and brought to volume with pH adjusted water in a 2 liter volumetric flask. The amount of pH adjusted water added was recorded so as to make proper IS corrections.

The polyanion (POL), ALG, CAR, or PEC, (18.0g) was dissolved in 1,500 mL distilled water, transferred to a 2 liter volumetric flask, and then brought to volume. Six 250 mL portions were transferred to 1 liter beakers and diluted with 700 mL distilled water. Ionic strength levels were adjusted first using NaCl, followed by adjustment of pH with NaOH or HCl. The amounts used to adjust pH were recorded to calculate the solution IS. Finally, the polyanion solutions were

quantitatively transferred to 1 liter volumetric flasks and brought to volume with distilled water.

3.3.6 Complex formation.

Reactant solutions with equal pH values were mixed using a calibrated Manostat Automatic Dispenser (VWR). Volumetric ratios, in 2 mL increments, ranged from 4:16 to 16:4 when expressed as mL CHI:mL POL.

3.3.7 Complex Analysis.

The insoluble complex was separated by centrifugation at 12,500g for 30 min. The pellet was allowed to dry overnight in a convection oven at 40°C and weighed using an analytical balance. Salt analysis using a Cl^- specific electrode (Orion #94-17B & #96-17B) was performed on the resuspended dried complex. Complex weight was reported as the weight of the dried complex minus the amount of salt determined from Cl^- content measurements. The pH of the collected supernatant was recorded and the CHI concentration was measured using the 3-methyl-2-benzothiazone hydrazone hydrochloride (MBTH) reagent method (Tsuji *et al.*, 1969) and POL concentration was measured using the phenyl-sulfuronic acid method (Whistler & Wolfrom, 1962).

3.4 Results and Discussion.

The complex amount formed was reported as a function of a mixing ratio (MR) defined as:

$$MR = \frac{A}{A + B}$$

where A = moles CHI, and B = moles PAA or POL (CAR, ALG, or PEC). With PAA (Figure 3.1-3.4) and CAR (Figure 3.7) complex formation was not significantly influenced by solution ionic strength. ALG and PEC (Figures 3.5 and 3.6, respectively), however, did reveal a statistical, although not large, effect of ionic strength on overall complex formation. Measurements of supernatant pH were used to investigate the complex formation mechanism. For CHI:PAA complexes, changes in pH indicated that maximum increase (Figure 3.8) or decrease (Figure 3.9) in hydrogen ion concentration occurred at the MR of maximum complex formation. For CHI:POL complexes (Figures 3.10-3.12) no significant pH change occurred at initial pH = 3 (except with carrageenan, IS = 0.30). In a study of the formation of polyelectrolyte complexes of some acidic glycosaminoglycans with chitosan, Hirano *et al.* (1978) found also negligible pH changes after combining polymer solutions with initial pH 2.8. At pH 4.5, there was a slight decrease in hydrogen ion concentration as a result of CHI complexation with ALG and PEC, and a more significant decrease with CAR, mimicking the changes seen at initial pH = 5.4. At pH = 5.4 there was a decrease in hydrogen ion concentration analogous to that which occurs at pH = 6 for CHI:PAA. However, unlike PAA, maximum change was maintained from MR $\approx$ 0.2 to MR $\approx$ 0.4 and was far more significant. The pH changes after both CHI:PAA and CHI:POL complexation can, in part, be explained by rapid equilibrium adjustments due to complex formation involving carbonyl (COO^-) or sulfonyl (SO_3^-) and amino groups (NH_3^+).

$R\text{-}NH_3^+ \leftrightarrow R\text{-}NH_2 + -H^+$ $\qquad$ $Keq = [R\text{-}NH_2][H^+]/[R\text{-}NH_3^+]$

$R\text{-}COOH \leftrightarrow R\text{-}COO^- + -H^+$ $\qquad$ $Keq = [R\text{-}COO^-][H^+]/[R\text{-}COOH]$

Since theoretical calculations, using initial and final concentrations, which are based on the Henderson-Hasselbach equation did explain some of the observed changes in pH, it can be concluded that ionic interactions were the primary driving force behind complex formation. Studies of chitosan complexation with bile salts (Nauss *et al.*, 1983) and heparin (Kikuchi and Noda, 1976) have also noted the role of ionic interactions in the complex formation process. Infrared analysis of the CHI:PAA complex confirmed this conclusion (Chavasit and Torres, 1990). Discrepancies from predicted and actual pH changes were attributed to intramolecular hydrogen bonding, van der Waals forces, and stearic hindrances. The importance of these secondary interactions has been discussed by Domszy and Roberts (1986) in their study on ionic interactions between chitosan and oxidized cellulose.

Differences in polyanion conformation offer additional insight into the differences in pH behavior between CHI:PAA and CHI:POL complex formation. For example, it is possible for POL to react with CHI on a 1:1 basis due to structural similarity, this is not so with PAA. In addition, PAA can have bond rotation so as to optimize functional group distances. This makes PAA, in comparison to POL, a more flexible polymer with respect to bond rotation ability.

The determination of supernatant CHI indicated that the ratio at which the polyanions bind to CHI depends on MR. Kikuchi *et al.* (1976) also found MR to play an important role in determining the composition ratio of chitosan to heparin in the polyelectrolyte complex formed. The supernatant analysis of remaining CHI after formation of natural complexes, showed an increase in the binding ratio as mixing ratio was increased (e.g., MR $\approx 0.5 \rightarrow 1.0$, CHI:POL binding ratio $\approx 0.5 \rightarrow 1.0$). The capability of CHI to bind at different ratios with polyanions allows for future manipulation of the charge ratio (positive:negative ions) for specific processing waste applications (Figure 3.13).

The effect of pH on mixing ratio for maximum insoluble complex formation was also studied. Complexes formed by CHI with PAA showed the maximum insoluble complex formation to occur at a different mixing ratio for each pH (Figures

3.1-3.4). Supernatant analysis confirmed this by finding only trace CHI and PAA at these maximum complex formation mixing ratios. Complexes formed by CHI and CAR, ALG, or PEC, showed a maximum insoluble complex formation at the same mixing ratio for all levels of pH. The exception was ALG where maximum insoluble complex formation was slightly shifted to the left at pH 3 (MR $\approx$ 0.4). These observations were also consistent with supernatant analysis.

3.5 Conclusion.

Research on the mechanism of reaction of chitosan with polyacrylic acid demonstrated that maximum complex formation was affected by the initial pH of the solution. However, for more complex polymers, such as pectin, alginate and carrageenan, this was not so. This finding suggests that in the latter case, hydrogen bonding and other intramolecular interactions may be more involved in the complex formation process. It has also been shown that charge ratio can be altered by the chitosan-polyanion mixing ratio, therefore making possible the preparation of complexes with specific and controlled charged group ratios.

3.6 Acknowledgements.

This research was supported with funds from the Oregon State University Experiment Station and by Oregon Sea Grant with funds from the National Oceanic and Atmospheric Administration, Office of Sea Grant, Department of Commerce, under grant no. NA85AA-D-SG095 (project no. E/ISG-6) and from appropriations made by the Oregon State Legislature. This is Oregon Experiment Station publication no. 9716.

Figure 3.1 Effect of ionic strength (IS) and pH on complex formation of chitosan:polyacrylic acid at initial pH=3.

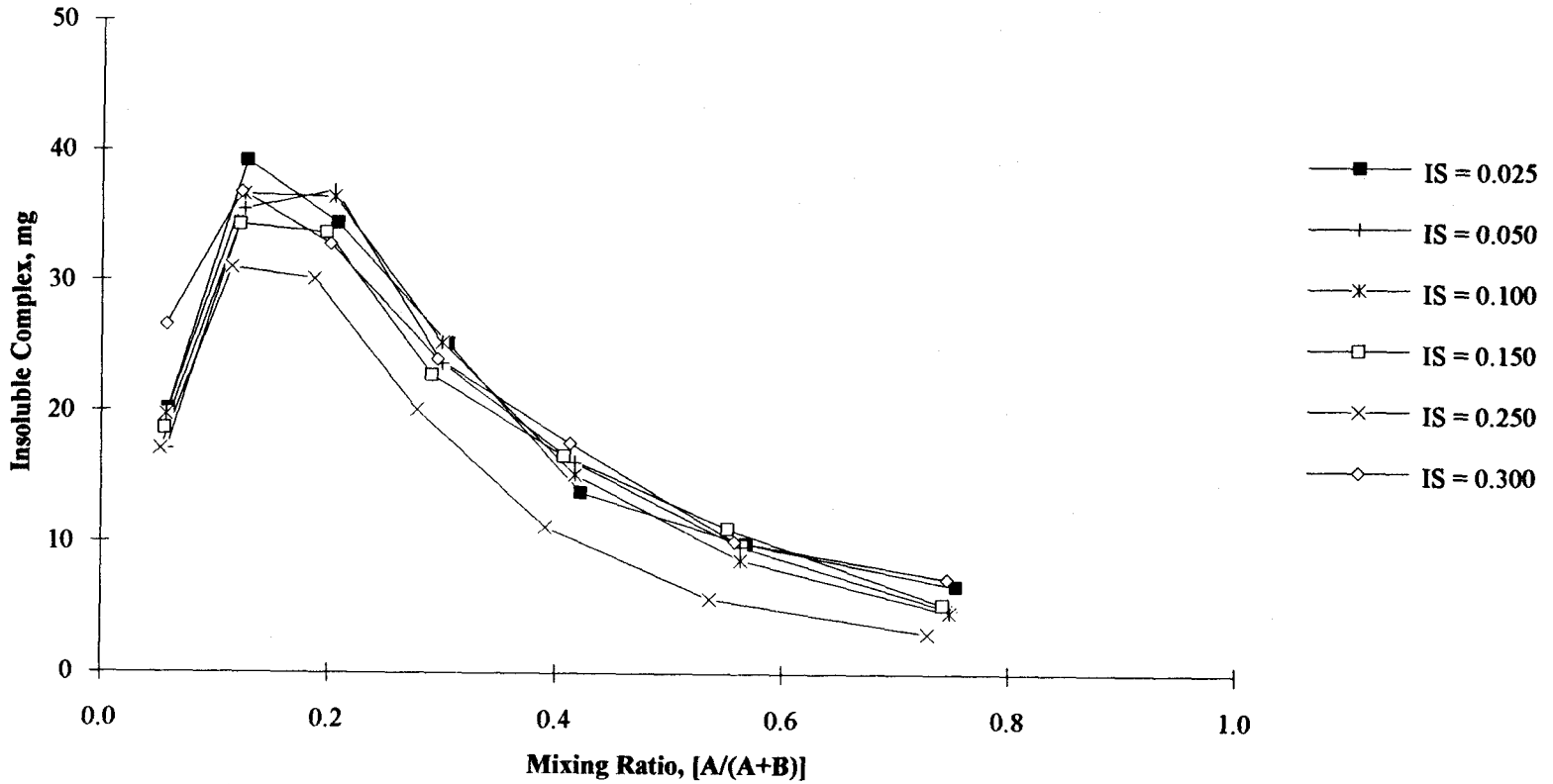


Figure 3.2 Effect of ionic strength (IS) and pH on complex formation of chitosan:polyacrylic acid at initial pH=4.

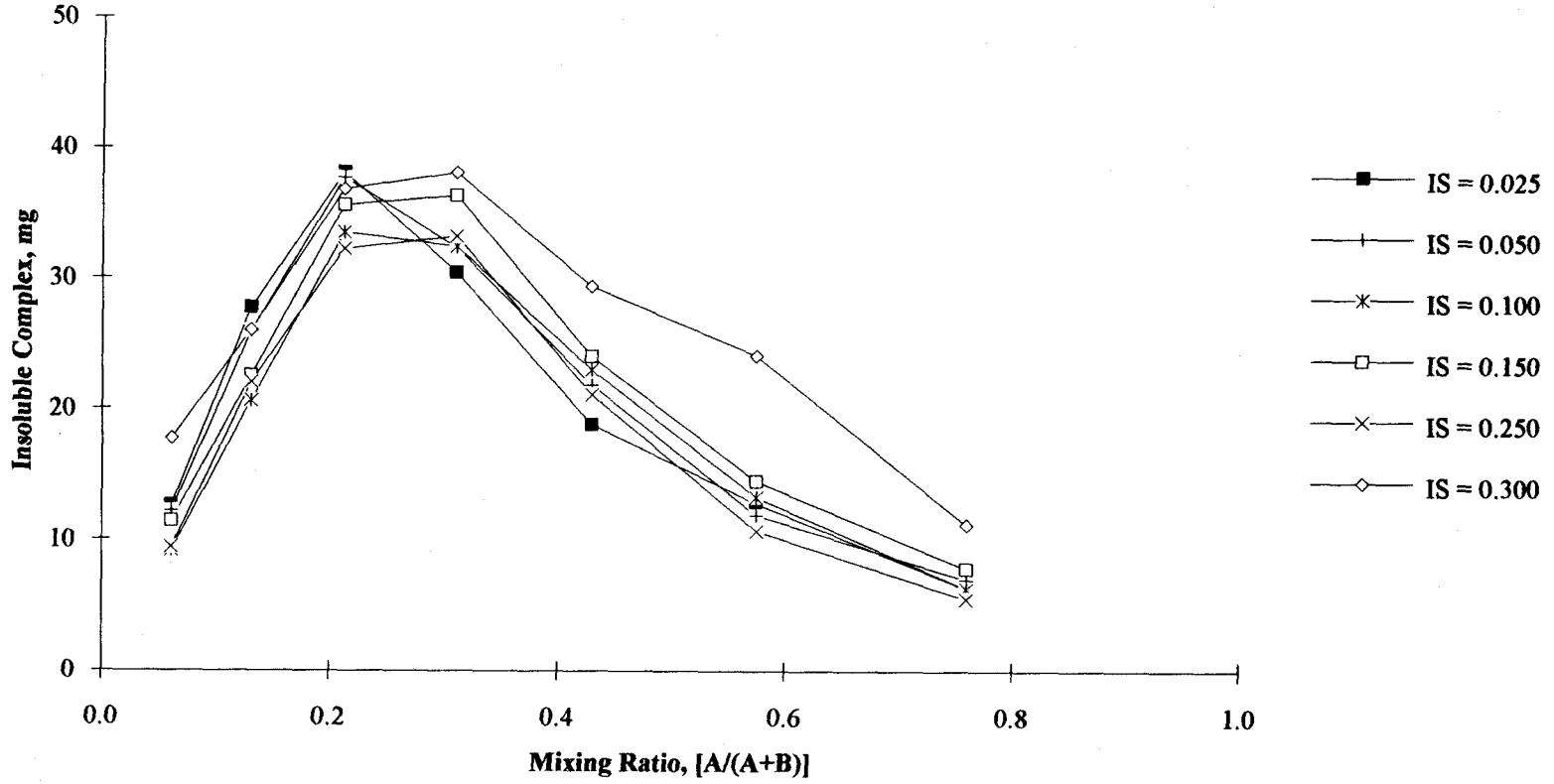


Figure 3.3 Effect of ionic strength (IS) and pH on complex formation of chitosan:polyacrylic acid at initial pH=5.

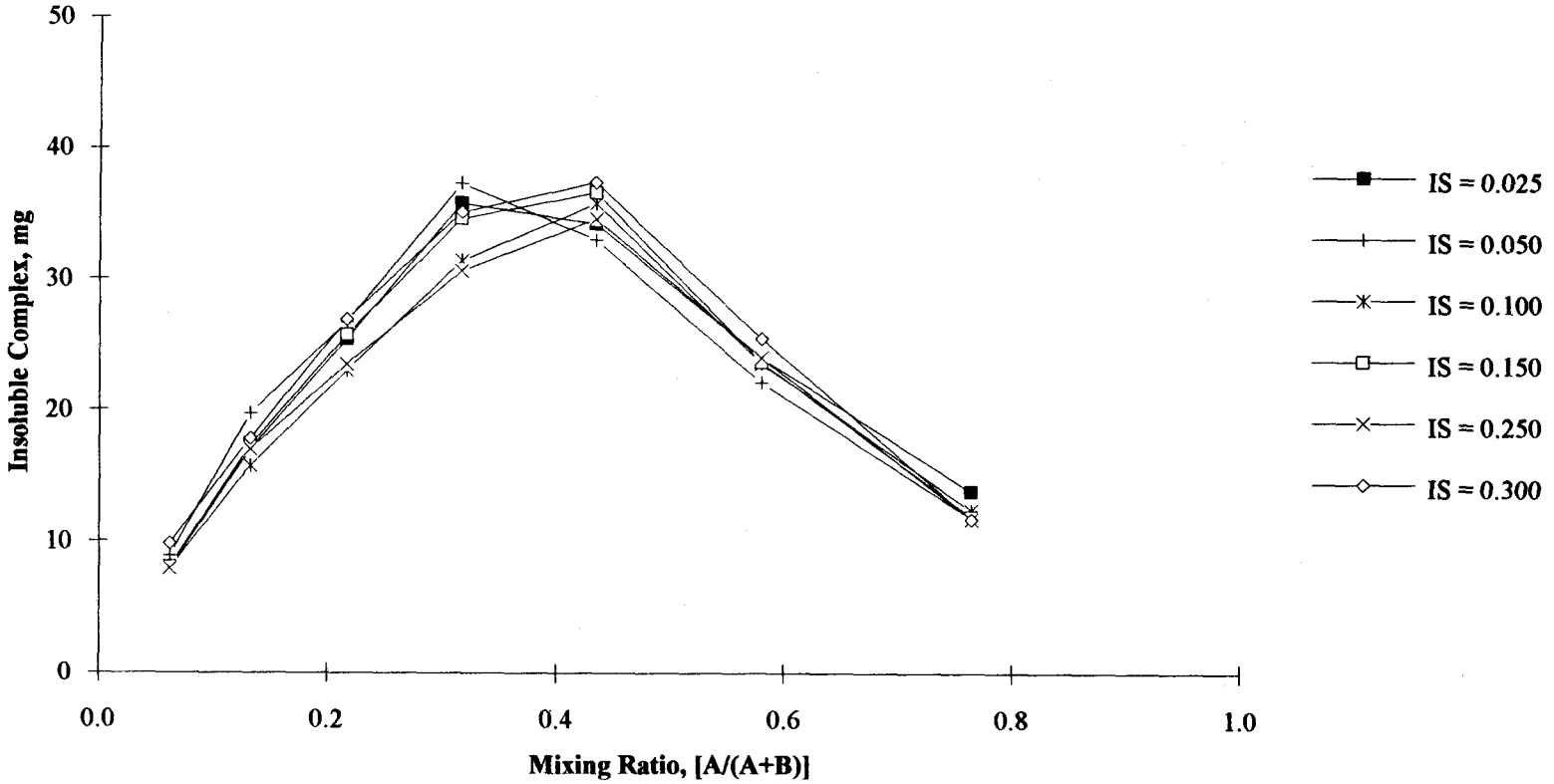


Figure 3.4 Effect of ionic strength (IS) and pH on complex formation of chitosan:polyacrylic acid at initial pH=6.

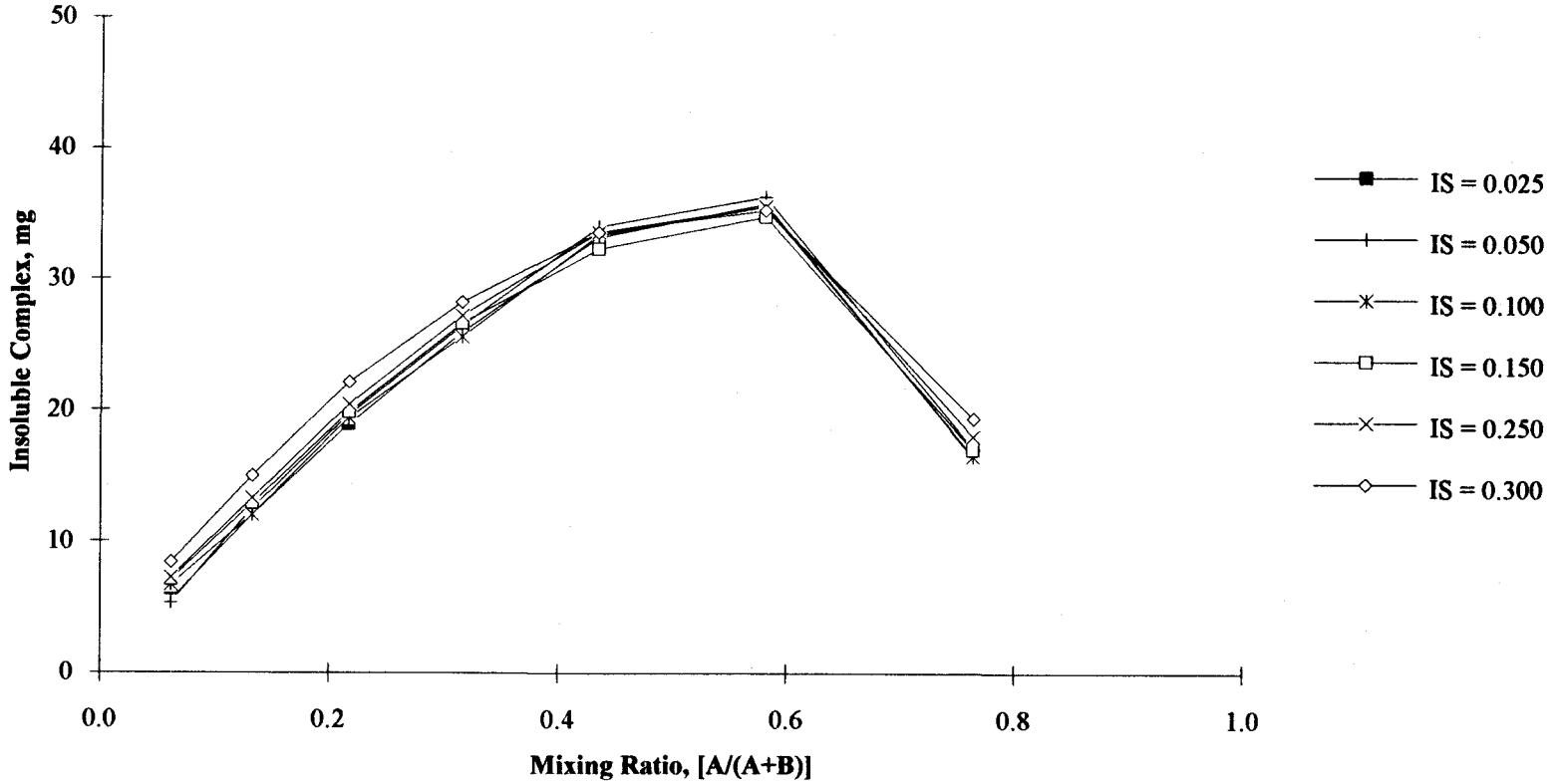


Figure 3.5 Effect of ionic strength (IS) and pH on complex formation of chitosan:alginate.

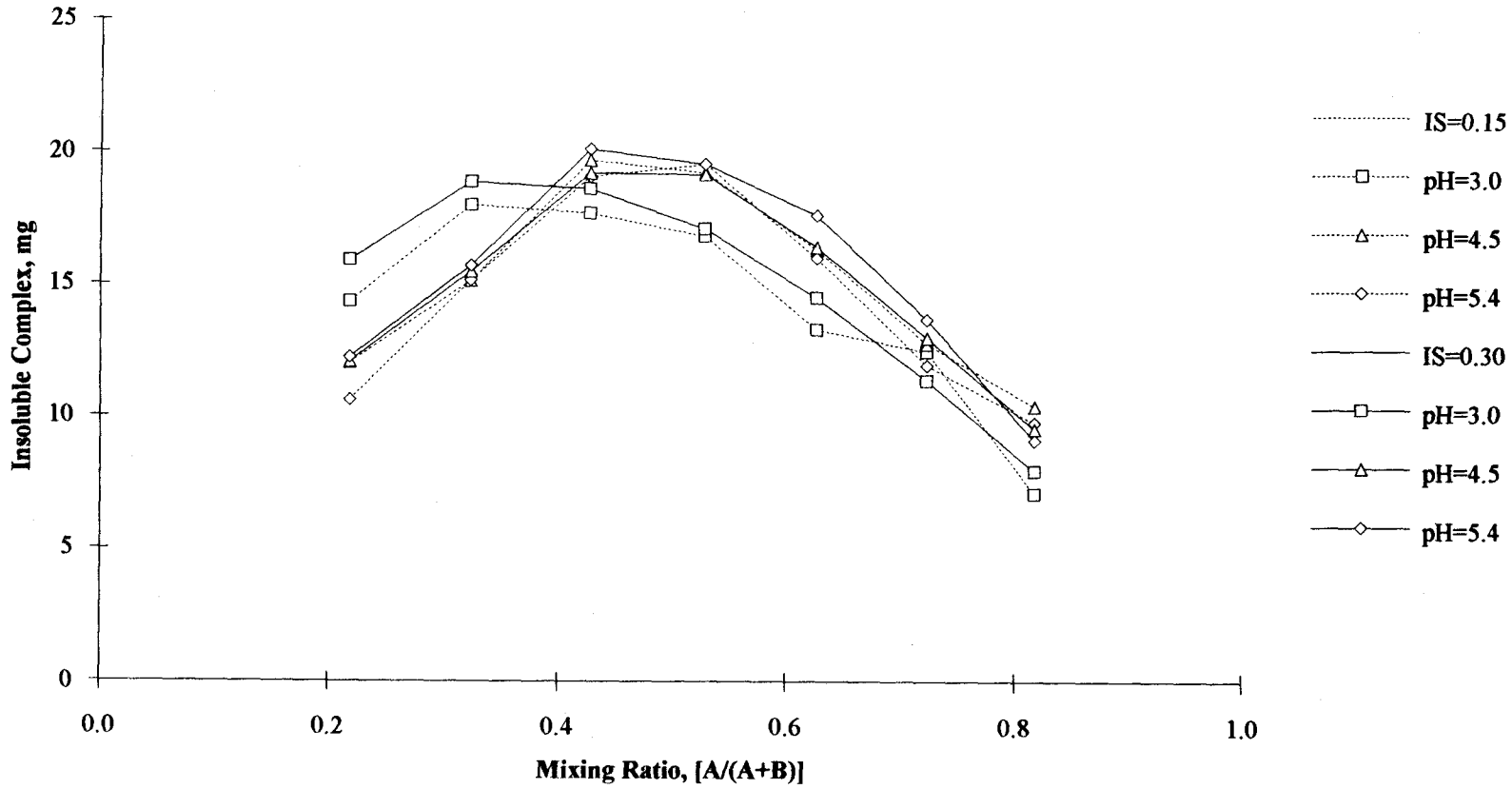


Figure 3.6 Effect of ionic strength (IS) and pH on complex formation of chitosan:pectin.

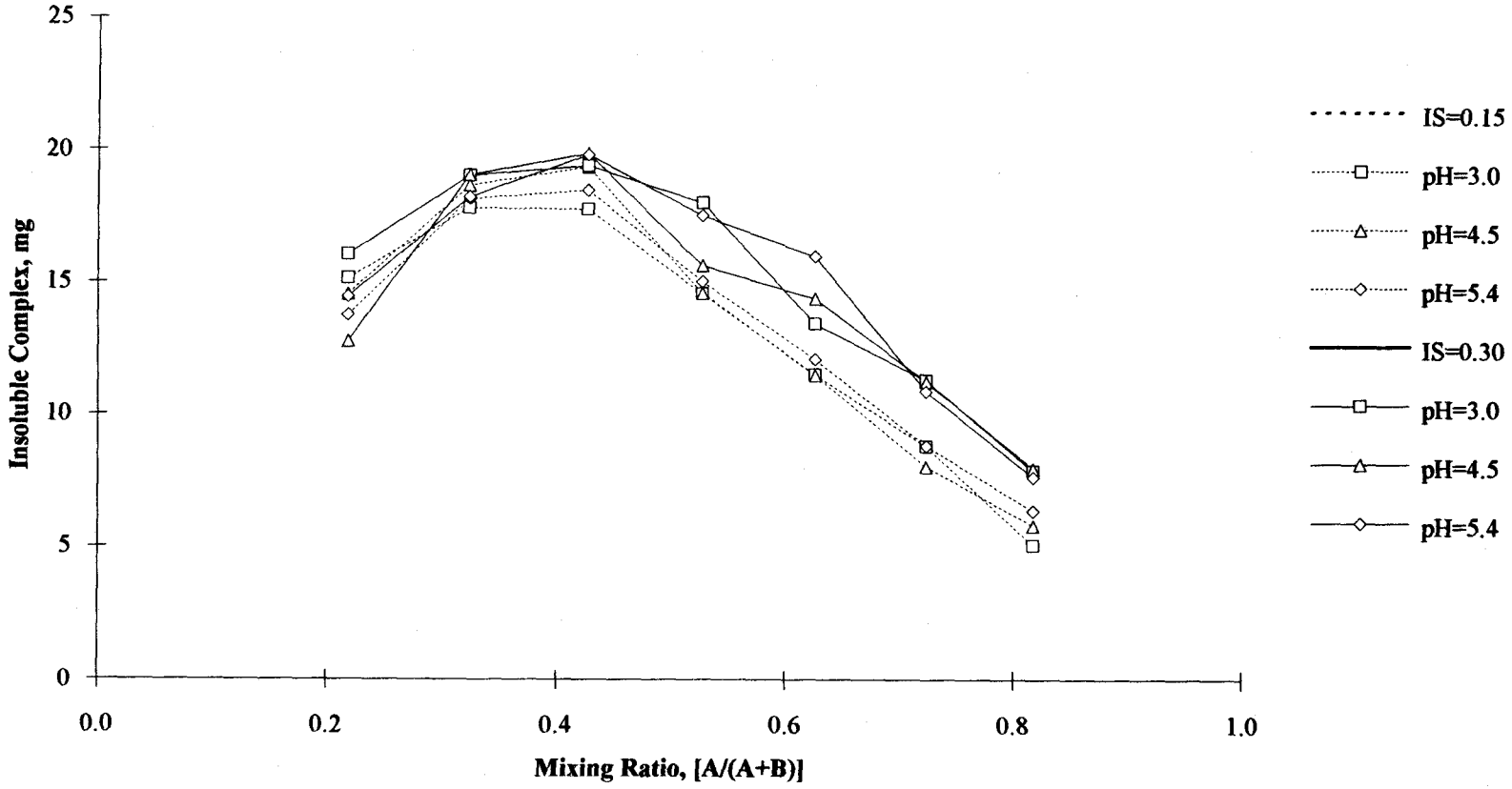


Figure 3.7 Effect of ionic strength (IS) and pH on complex formation of chitosan:carrageenan.

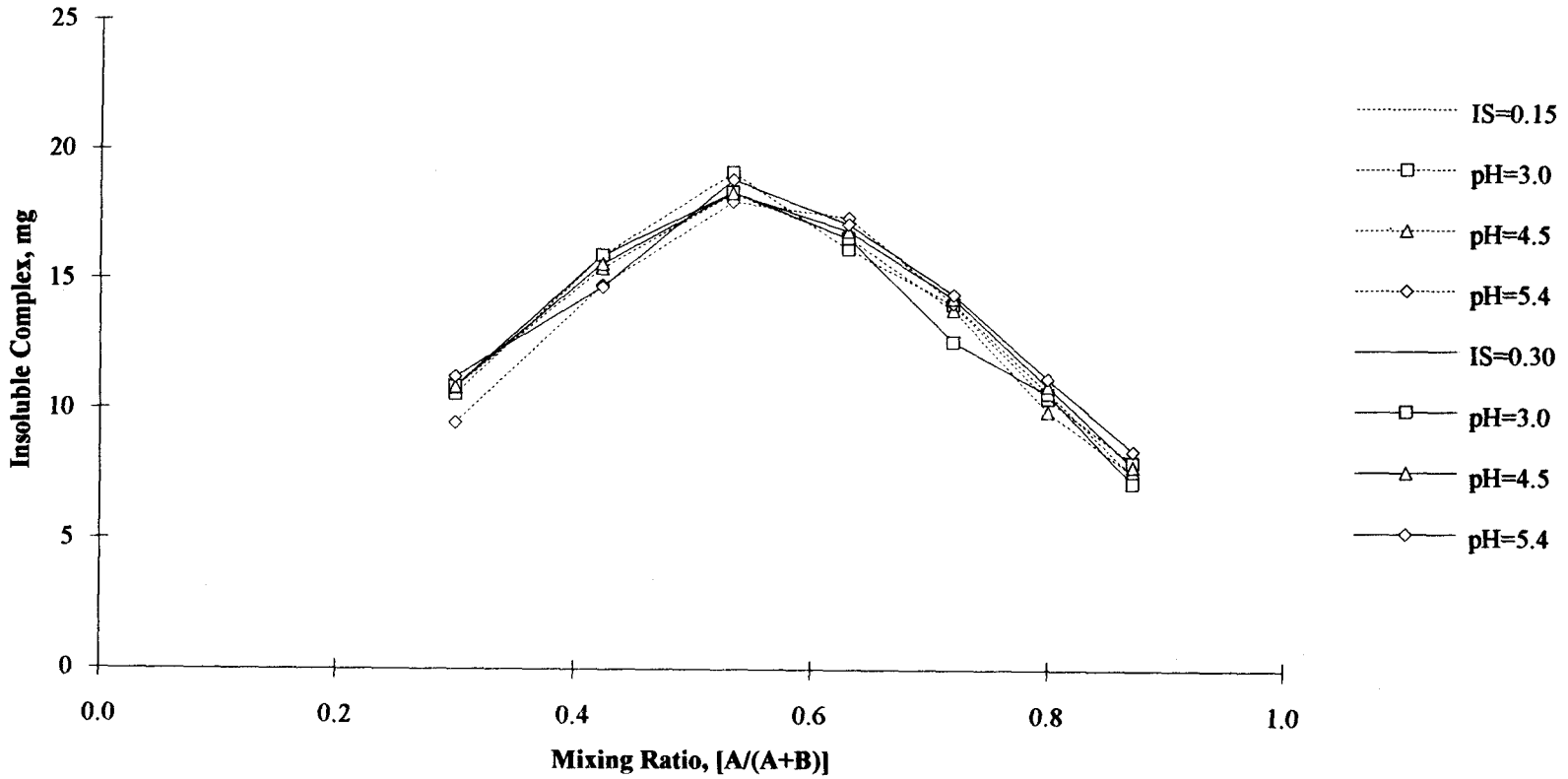


Figure 3.8 Effect of chitosan:polyacrylic acid complex formation at initial pH=4 on supernatant pH.

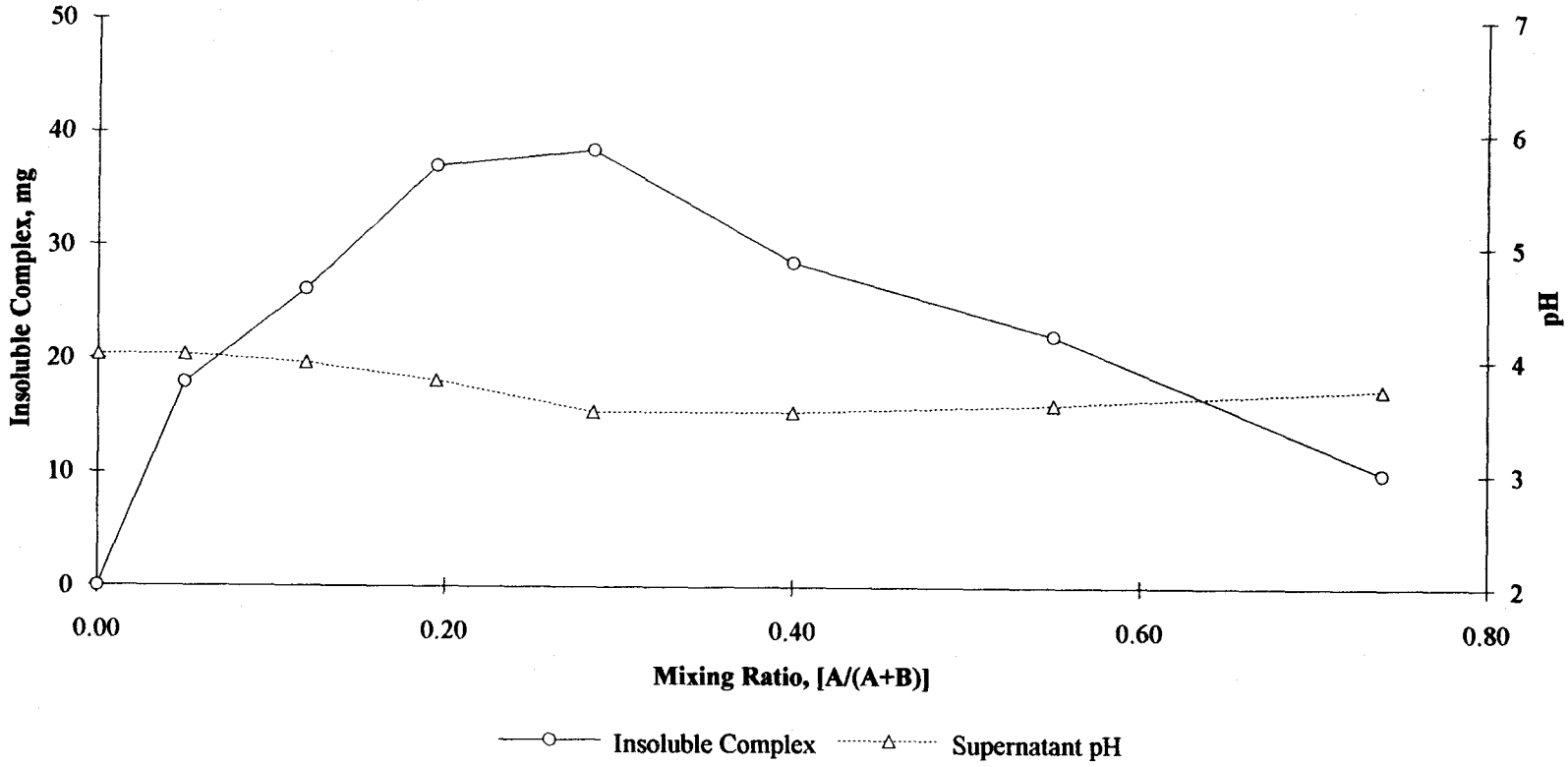


Figure 3.9 Effect of chitosan:polyacrylic acid complex formation at initial pH=6 on supernatant pH.

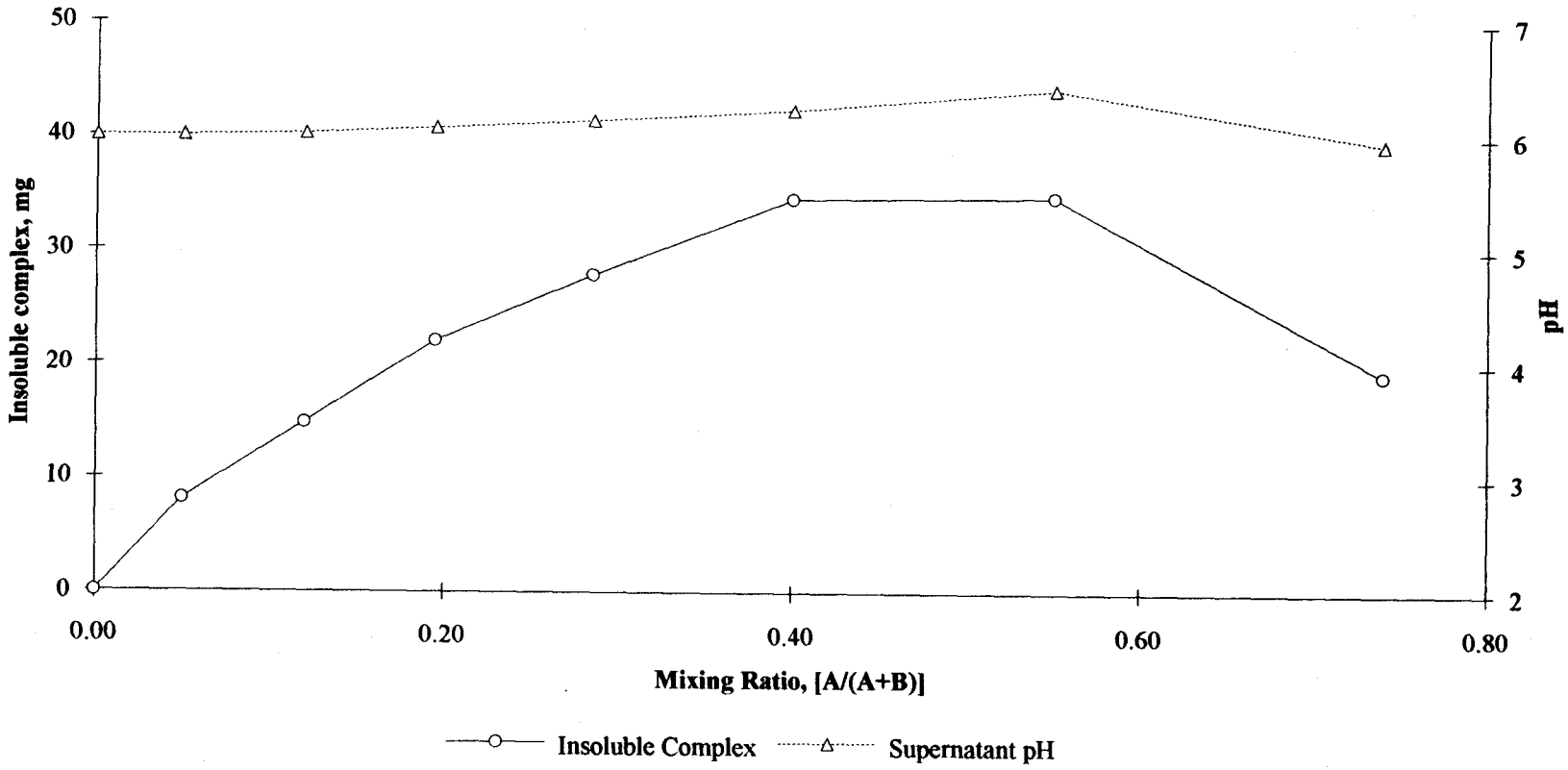


Figure 3.10 Effect of chitosan:alginate complex formation on supernatant pH.

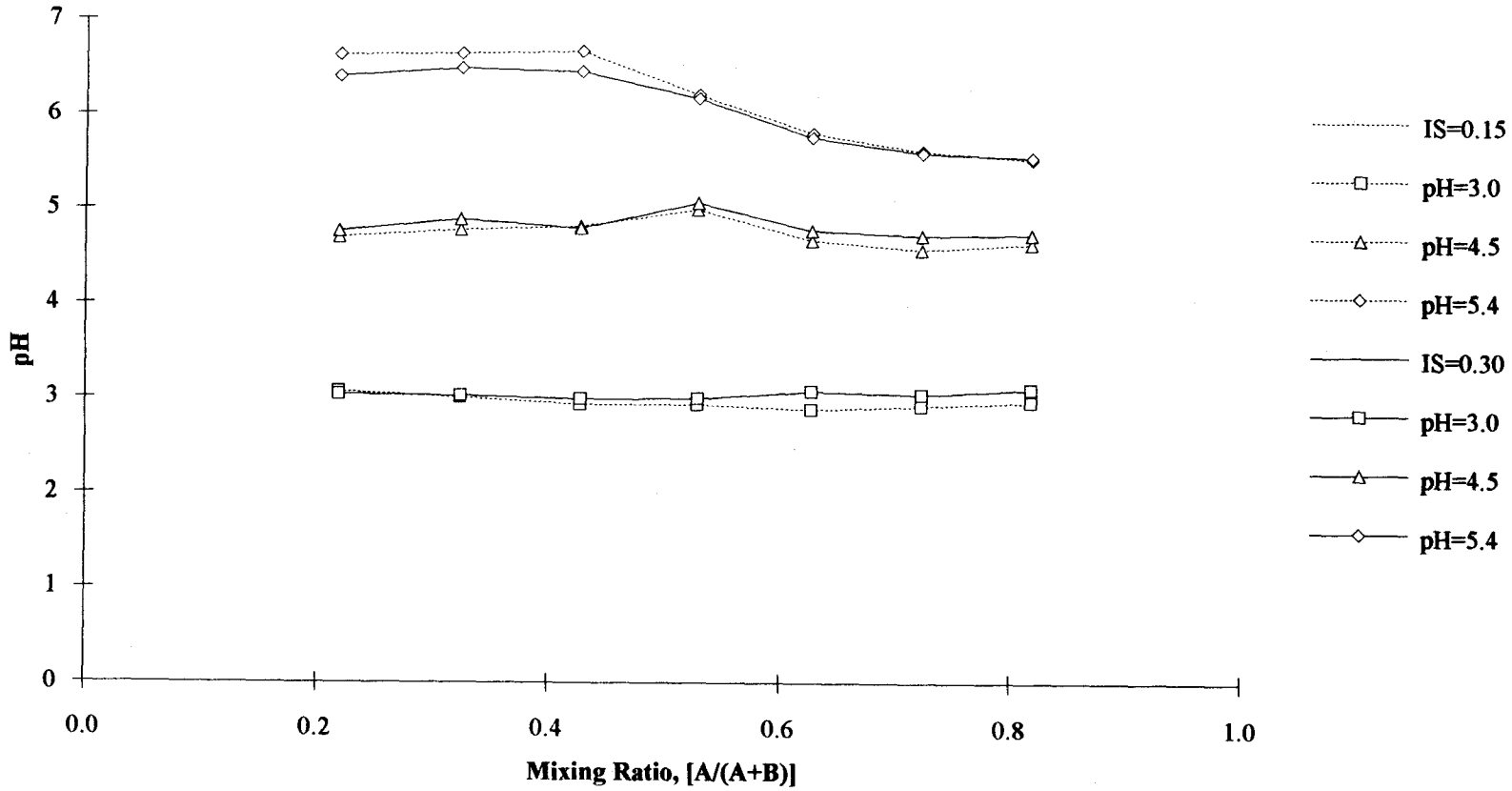


Figure 3.11 Effect of chitosan:pectin complex formation on supernatant pH.

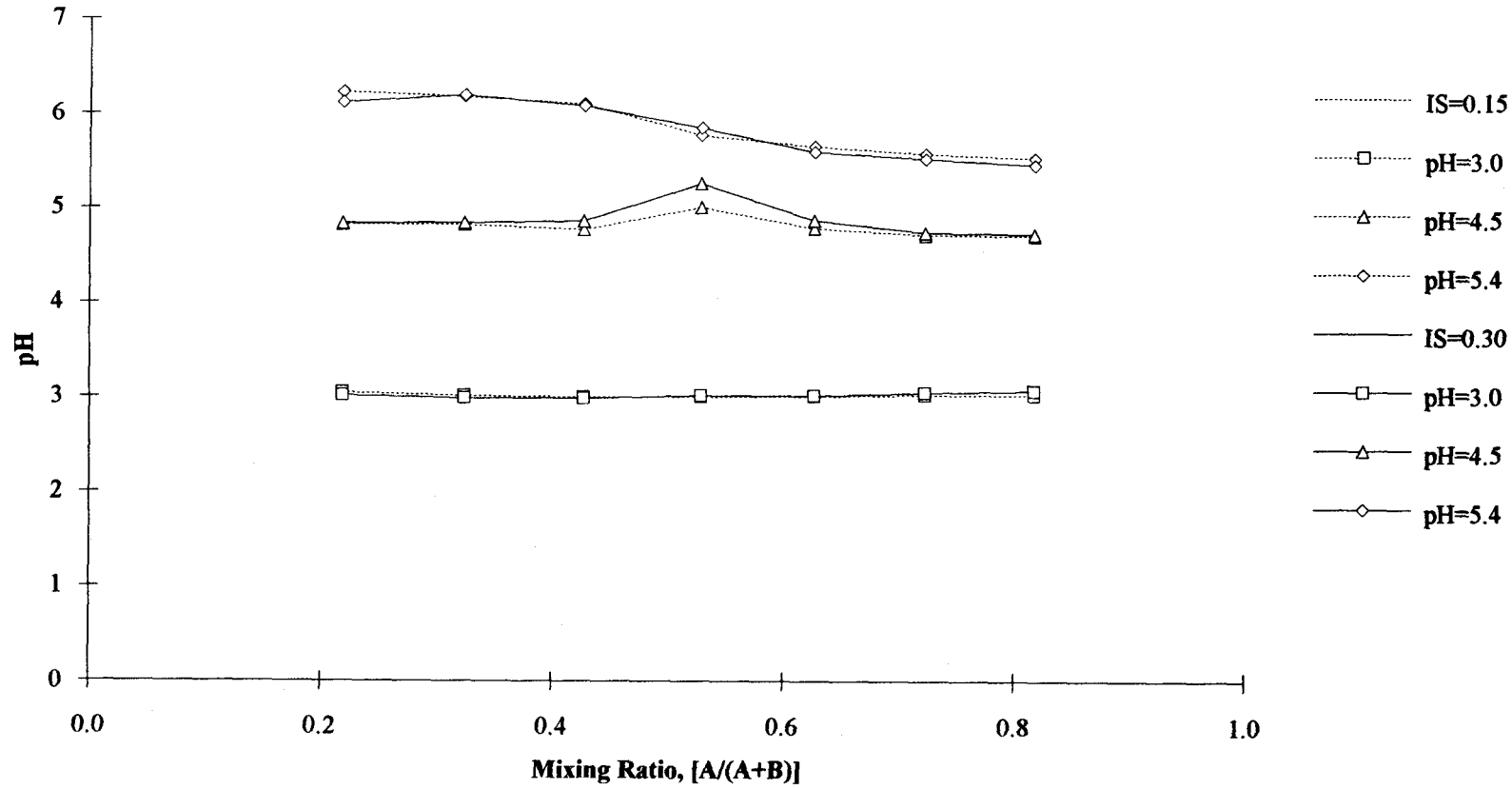


Figure 3.12 Effect of chitosan:carrageenan complex formation on supernatant pH.

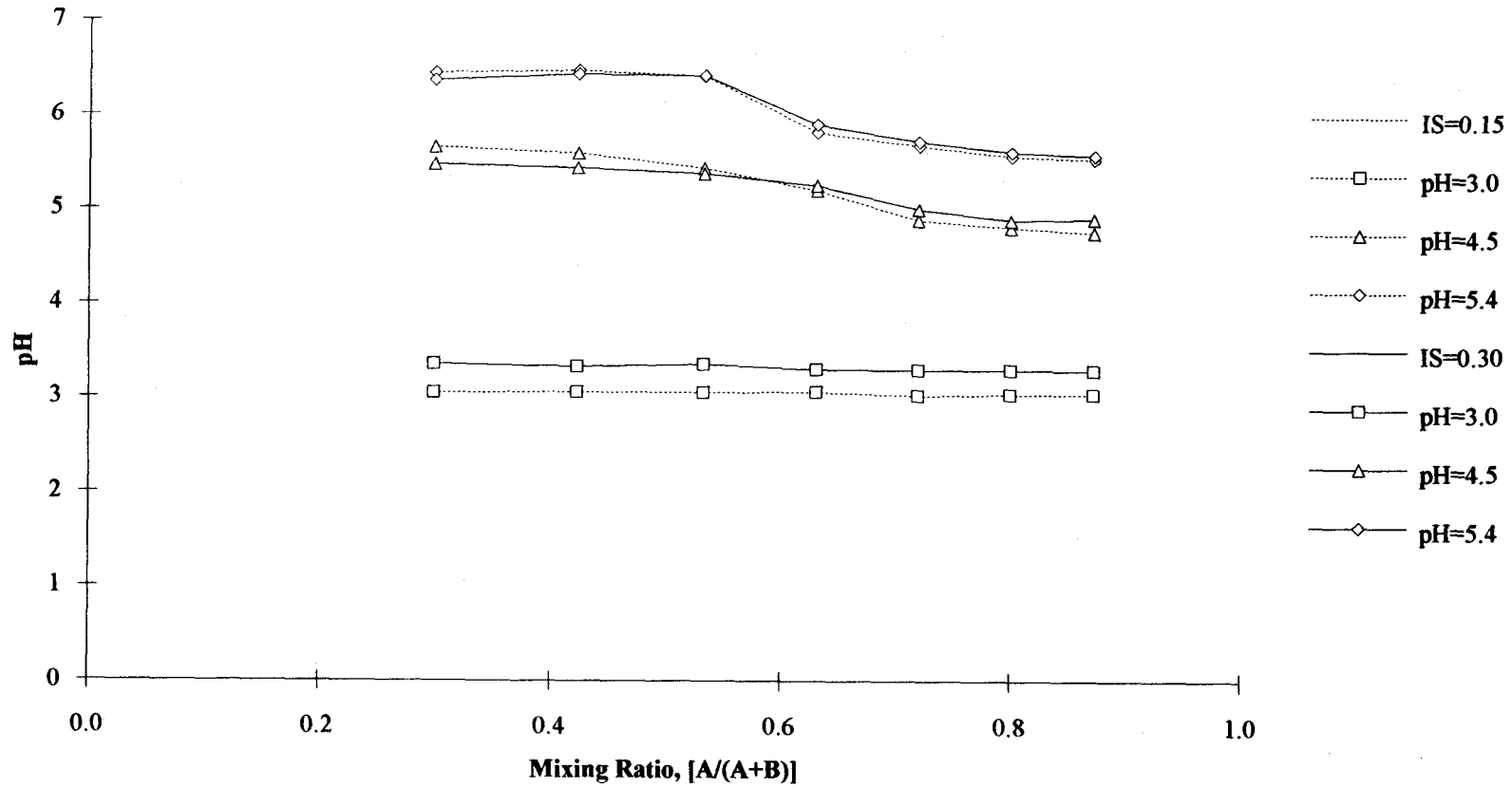
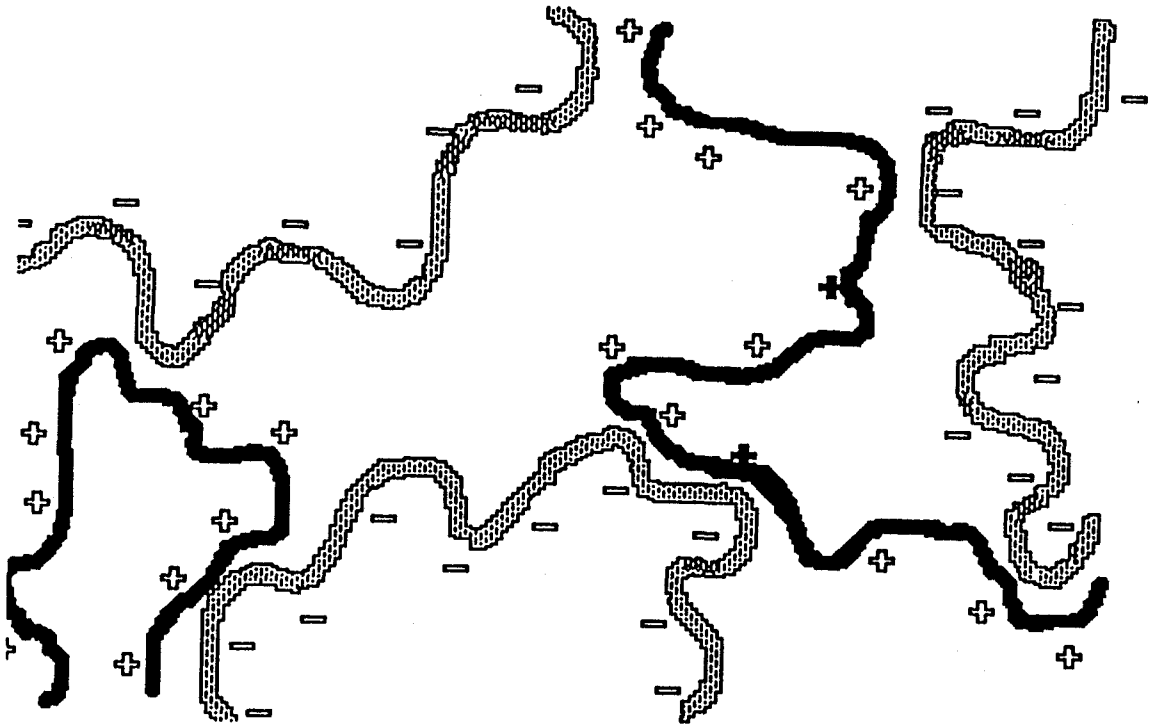


Figure 3.13 Schematic representation of a chitosan:polyanion complex.



4. CHARACTERIZATION OF CHITOSAN COMPLEXES.

4.1 Abstract.

Chitosan and polyanion mixtures were prepared in different mole ratios and at different pH values and ionic strengths. Binding ratio was calculated by quantitating excess polymer in the supernatant with either the Phenol-Sulfuric (polyanion specific) or MBTH (polycation specific) analytical methods. An effect of ionic strength on binding ratio was demonstrated only with PEC, while a pH effect was only seen in ALG. It was also noted that binding ratio did not always occur 1:1, but increased from 0 to 1 as mixing ratio increased from 0 to 1. Infrared analysis, used in conjunction with previous research on supernatant pH, indicated ionic interactions between COO^- or $SO3^-$ and NH_3^+ were the driving force behind complex formation.

4.2 Introduction.

Chitosan, a polymeric glucosamine, has been the subject of much research because of its unique role as a natural, non-synthetic polycation. Areas of investigation have included its use as a dietary supplement for animals, antimicrobial agent, pesticide, coacervate for the release of herbicides and fertilizers, film to cover burn wounds, fining agent in the production of juices, and food additive. Much research on chitosan has been focused on its use as a flocculent in the reduction of organics in food processing wastewater. The Water Pollution Control Act Amendments of 1972, FDA's approval of its use as a feed additive at levels of <0.1%, and EPA's approval for its use in potable water purification at levels up to 10 mg/L provide much incentive for using chitosan to reduce the Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) levels in wastewater.

Research into the treatment of egg breaking waste (Bough, 1975a), pimento and turnip greens (Bough, 1975b), cheese whey (Bough and Landes, 1976), and poultry, meat, and shrimp wastes (Bough and Landes, 1977) has shown chitosan to be as effective, if not more, than many synthetic flocculating agents.

Chavasit et al. (1988) studied the complexation of chitosan with a simple, negatively charged, synthetic polymer (polyacrylic acid, PAA) to better understand the properties effecting flocculation and suggested the use of multicharged complexes to enhance the flocculation of anionic and cationic nutrients present in wastewater. Chitosan and the "natural" polyanions carrageenan, pectin, and alginate were used in an earlier study to prepare such complexes (Mireles et al, 1992). The formation of these complexes under conditions of industrial interest were investigated by altering pH and ionic strength of polymer solutions combined at different mixing ratios. Upon combination of the polymers, complexation was noted to occur very rapidly and the resulting precipitate was reported as a function of mixing ratio (MR), defined as:

$$MR = A / (A+B)$$

where A = moles CHI monomer, and B = moles POL monomer (ALG or PEC). MR for CHI:CAR was reported as moles dimer, respectively. The amount of complex formed at a given initial pH was shown to be affected by ionic strength for all but CAR. Although statistically there was no main effect of pH on complex formation, altering initial solution conditions from pH 3 to pH 4.5 or 5.4 caused a shift in maximum complex formation in CHI:ALG complexes. Earlier study of chitosan complexation with polyacrylic acid (Chavasit and Torres, 1990) reported maximum complex formation to be affected by initial pH but not ionic strength. However, unlike that demonstrated with CHI:PAA complexation, no shifting of maximum complex formation occurred when CHI was combined with PEC or CAR.

Analysis of supernatant pH was conducted to confirm whether ionic interactions were the principle interactions involved in complex formation. At initial pH 3 and 4.5 there was little change of supernatant pH from initial levels. This was expected because the hydrogen ion concentration at these levels masked pH changes caused by polymer complexation. However at pH 5.4, when hydrogen ion concentration was comparable to polymer concentration, there was a measurable increase in pH from its initial values. The increase in pH was explained in part by Henderson-Hasselbach's equation, Le Chatelier's principle, and the general equation for the equilibrium constant, K_{eq}. Variations from the principles were attributed to stearic hindrances, Van der Waals forces, and hydrogen bonding (Mireles et al., 1992). In this study molecular binding ratios were measured in order to better understand the ionic nature of the insoluble complex. Infrared analysis was performed on the insoluble complex to characterize the primary molecular binding interactions involved in complex formation.

4.3 Materials and Methods.

4.3.1 Reagents.

Chitosan (CHI, Pro Floc HV) was obtained from Protan, Inc. (Commack, NY) and purified by dissolving in 0.1 N HCl and stirred over low heat for 24 hours. The solution was then filtered through four layers of cheese cloth and refiltered through highly porous Whatman #52 filter paper. After purification through filtration, dissolved chitosan was precipitated by adjusting the solution to pH 10 with 1N NaOH. Precipitate was then collected by centrifugation at 10,000g for 30 minutes using a Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge with SS34 rotor. The resulting supernatant was discarded and distilled water was added to wash the precipitate. Centrifugation was repeated and the chitosan washed again. Remaining moisture in the purified chitosan was removed by freeze drying.

Polyanions (POL) used were carrageenan (CAR, Viscarin GP109, Marine Colloids Division, FMC Corp., Philadelphia, PA), alginate (ALG, Kelgin MV, Merck & Co., Inc., San Diego, CA) and pectin (PEC, from citrus fruit, Grade I, Sigma Chemical Co., St. Louis, MO).

4.3.2 Complex Formation.

Each polymer was prepared at 0.1% concentrations and adjusted with HCl and NaCl to pH 3, 4.5, or 5.4 and ionic strength (IS) 0.15 or 0.30, respectively. Equivalent pH and ionic strength solutions of chitosan and polyanion were combined at seven ratios in the range 4:16 to 16:4 when expressed as mL CHI:mL POL (Mireles et al, 1992). Combined solutions were allowed to precipitate and held overnight at 4°C. Separation and collection of precipitate and supernatant was

achieved through centrifugation at 12,500g for 30 minutes. The precipitated pellet was dried overnight in a convection oven at 40°C and weighed using an analytical balance. Salt analysis using a Cl^- specific electrode (Orion #94-17B & #96-17B) was performed on the resuspended dried complex. Complex weight was reported as the weight of the dried complex minus the amount of salt determined from Cl^- content measurements. The pH of the collected supernatant was recorded, CHI concentration was calculated using the 3-methyl-2-benzothiazone hydrazone hydrochloride (MBTH) reagent method (Tsuji, et al., 1969), and POL concentration was measured using the phenyl-sulfuronic acid method (Whistler & Wolfrom, 1962).

4.3.3 Infrared Analysis.

Infrared analysis was used to study the molecular interactions in chitosan:polyanion complexes. Polymers were again prepared at 0.1% concentrations and adjusted to pH 3, 4.5, or 5.4. Ionic strength was adjusted at 0.15 or 0.30 for polymer solutions at pH 3 using NaCl. Solutions at pH 4.5 and 5.4 were only adjusted to ionic strength 0.30. Chitosan and polymer solutions with equivalent pH and ionic strengths were combined at 1:1 ratios. Precipitate was collected by centrifugation and freeze dried. The resulting freeze dried complex was mixed with KBr (1% complex in KBr) in order to form a KBr lens. Lens were also made of the following uncomplexed standards: CHI, CAR, PEC, ALG, and GLU (glucosamine-HCl). Lenses were placed in a Nicolet 510P FTIR set to scan 16 times with a resolution of 2.0. Scanning range was set from 4000-600 wavenumbers.

4.4 Results and Discussion.

Analysis using MBTH and Phenol-Sulfuric Acid allowed for the detection of CHI or POL in the supernatant and quantitation of the binding ratio (BR) defined as:

$$BR = A / (A+B)$$

where A = moles CHI monomer, and B = moles POL monomer (CAR, ALG, or PEC). Figures 4.1, 4.2, and 4.3 illustrate BR in the insoluble complex as compared to MR. Statistical analysis indicated BR to be affected by ionic strength for PEC (Figure 4.2), but not for ALG or CAR (Figure 4.1 and 4.3). There was also an affect of pH on BR for ALG, but not for PEC or CAR. The most significant effect on BR, regardless of polyanion used in the complex, was on MR. In all three figures the limited polymer in the MR was completely bound and not detected in the supernatant (Tables 4.1-4.3). For PEC and ALG complexes, the limited polymer (polyanion or polycation) in the MR also bound more effectively with the one in excess. For a reference, a dotted line was placed on the graphs to show where BR occurs 1:1 in the complex. As can be seen both MR and BR tend to coincide with one another. As MR approaches 1:1 so does BR and at about the same rate from either end of the scale. This is interesting because it suggests that changes in MR might change the ionic nature of the complex indicating that it may be possible to tailor a complex to have either a more positive or negative character simply by altering MR. For CHI:CAR complexes a similar effect is observed but MR and BR do not appear to coincide as much when CAR is in excess. At MR=0.2, where CHI is most limited, CHI binds in a 1:1 ratio with CAR, unlike PEC and ALG were the ratio is almost 1:3. Once the MR becomes greater than 0.5 for CHI, the BR begins to resume the same pattern shown with PEC and ALG. This suggests that CAR is the polymer affecting BR when it is excess in the MR. CAR differs from PEC and ALG in that instead of having linked monomeric units with one carboxyl group, it consists of dimer units with three (70%) or two (30%) sulfonate groups. Compared

to COO^-, the negative charge in the SO_3^{2-} is more widely distributed over three oxygens perhaps making it less active in binding with CHI.

Finally infrared analysis was performed on the individual polymers and their complexes to confirm whether ionic interactions were the primary reaction forces. Comparison of complexed and uncomplexed spectra showed that in each case, regardless of pH or ionic strength, a peak between λ1500 and λ1600 (as indicated by the arrow) occurred in the complexes that was not in the individual polymers (Figures 4.5-4.20). Overlapping of standard chitosan and polyanion spectra with that of complexed spectra helped to confirm the new peak. Review of literature (Rappoport, 1967) showed NH_3^+RCOOH (or NH_3^+) bonds create a strong peak in the same region (λ1500-λ1600). Infrared analysis of GLU (Figure 4.4), the free amine (NH_3^+) form of chitosan, revealed through overlapping of complex spectra that it also displayed the new peak found in the complexes. Further investigation revealed a similar study was performed on the characterization of chitosan-collagen interactions by comparing i.r. spectra from free amine chitosan, collagen, and chitosan-collagen complexes revealed no new bonds, such as the covalent bonds created in certain polymerization reactions, in the complex (Domard and Taravel-Brun, 1992). It was therefore concluded that the only bands corresponding to the amino function of chitosan in the complex was the NH_3^+ form, demonstrated by a peak at λ1510 in the spectrum, further substantiating the theory that the interaction between the two polymers was electrostatic (ionic).

4.5 Conclusion.

Analysis of chitosan complexation with naturally occurring anionic polymers shows that although the process was influenced by several properties, it was driven primarily by electrostatic forces. The effect of ionic strength and pH, with two exceptions, did not significantly influence complex binding ratios. In addition, it was demonstrated that by adjusting MR binding ratios could be altered to adjust the overall ionic property of the complex. However, complex recovery efficiencies shown in earlier research (Mireles et al, 1992) due to different mixing ratios should be taken into account since maximum BR efficiency for the respective polymer complexes did not coincide with maximum complex formation.

Table 4.1 Absorbances of supernatant pectin at different mixing ratios (A/A+B) as measured by phenol-sulfuric acid.

mixing ratio	IS=0.15 pH=3.0	IS=0.15 pH=4.5	IS=0.15 pH=5.4	IS=0.30 pH=3.0	IS=0.30 pH=4.5	IS=0.30 pH=5.4
0.22	0.48	0.55	0.42	0.48	0.32	*
0.22	0.53	0.55	0.48	0.40	0.35	*
0.32	0.61	0.61	0.56	0.57	0.49	0.62
0.32	0.61	0.64	0.56	0.57	0.41	0.55
0.43	0.66	0.37	0.63	0.62	0.48	0.35
0.43	0.64	0.71	0.60	0.62	0.48	0.33
0.53	0.63	0.59	0.51	0.59	0.47	0.38
0.53	0.48	0.27	0.51	0.59	0.45	0.36
0.63	tr	tr	tr	tr	tr	tr
0.63	tr	tr	tr	tr	tr	tr
0.72	tr	tr	tr	tr	tr	tr
0.72	tr	tr	tr	tr	tr	tr
0.82	tr	tr	tr	tr	tr	tr
0.82	tr	tr	tr	tr	tr	tr

* data lost
tr = trace amounts

Table 4.2 Absorbances of supernatant alginate at different mixing ratios (A/A+B) as measured by phenol-sulfuric acid.

mixing ratio	IS=0.15 pH=3.0	IS=0.15 pH=4.5	IS=0.15 pH=5.4	IS=0.30 pH=3.0	IS=0.30 pH=4.5	IS=0.30 pH=5.4
0.22	0.22	0.33	0.37	0.40	0.32	0.24
0.22	0.36	0.34	0.47	0.37	0.25	0.25
0.32	0.26	0.37	0.38	0.35	0.24	0.19
0.32	0.33	0.36	0.42	0.38	0.21	0.21
0.43	0.25	0.29	0.03	0.26	0.14	0.12
0.43	0.29	0.28	0.25	0.28	0.13	0.11
0.53	0.22	0.06	0.04	0.15	0.04	0.00
0.53	0.22	0.07	0.05	0.17	0.04	0.01
0.63	tr	tr	tr	tr	tr	tr
0.63	tr	tr	tr	tr	tr	tr
0.72	tr	tr	tr	tr	tr	tr
0.72	tr	tr	tr	tr	tr	tr
0.82	tr	tr	tr	tr	tr	tr
0.82	tr	tr	tr	tr	tr	tr

tr = trace amounts

Table 4.3 Absorbances of supernatant carrageenan at different mixing ratios (A/A+B) as measured by phenol-sulfuric acid.

mixing ratio	IS=0.15 pH=3.0	IS=0.15 pH=4.5	IS=0.15 pH=5.4	IS=0.30 pH=3.0	IS=0.30 pH=4.5	IS=0.30 pH=5.4
0.20	0.36	0.29	0.23	0.29	0.26	0.20
0.20	0.40	0.30	0.29	0.37	0.29	0.27
0.32	0.37	0.37	0.24	0.39	0.32	0.20
0.32	0.39	0.33	0.27	0.39	0.28	0.23
0.43	0.29	0.33	0.19	0.32	0.23	0.17
0.43	0.38	0.29	0.24	0.36	0.24	0.19
0.53	0.24	0.24	0.19	0.26	0.14	0.11
0.53	0.29	0.28	0.17	0.25	0.13	0.14
0.62	tr	tr	tr	tr	tr	tr
0.62	tr	tr	tr	tr	tr	tr
0.70	tr	tr	tr	tr	tr	tr
0.70	tr	tr	tr	tr	tr	tr
0.87	tr	tr	tr	tr	tr	tr
0.87	tr	tr	tr	tr	tr	tr

tr = trace amounts

Figure 4.1 Effect of ionic strength (IS) and pH on binding ratio of chitosan:alginate.

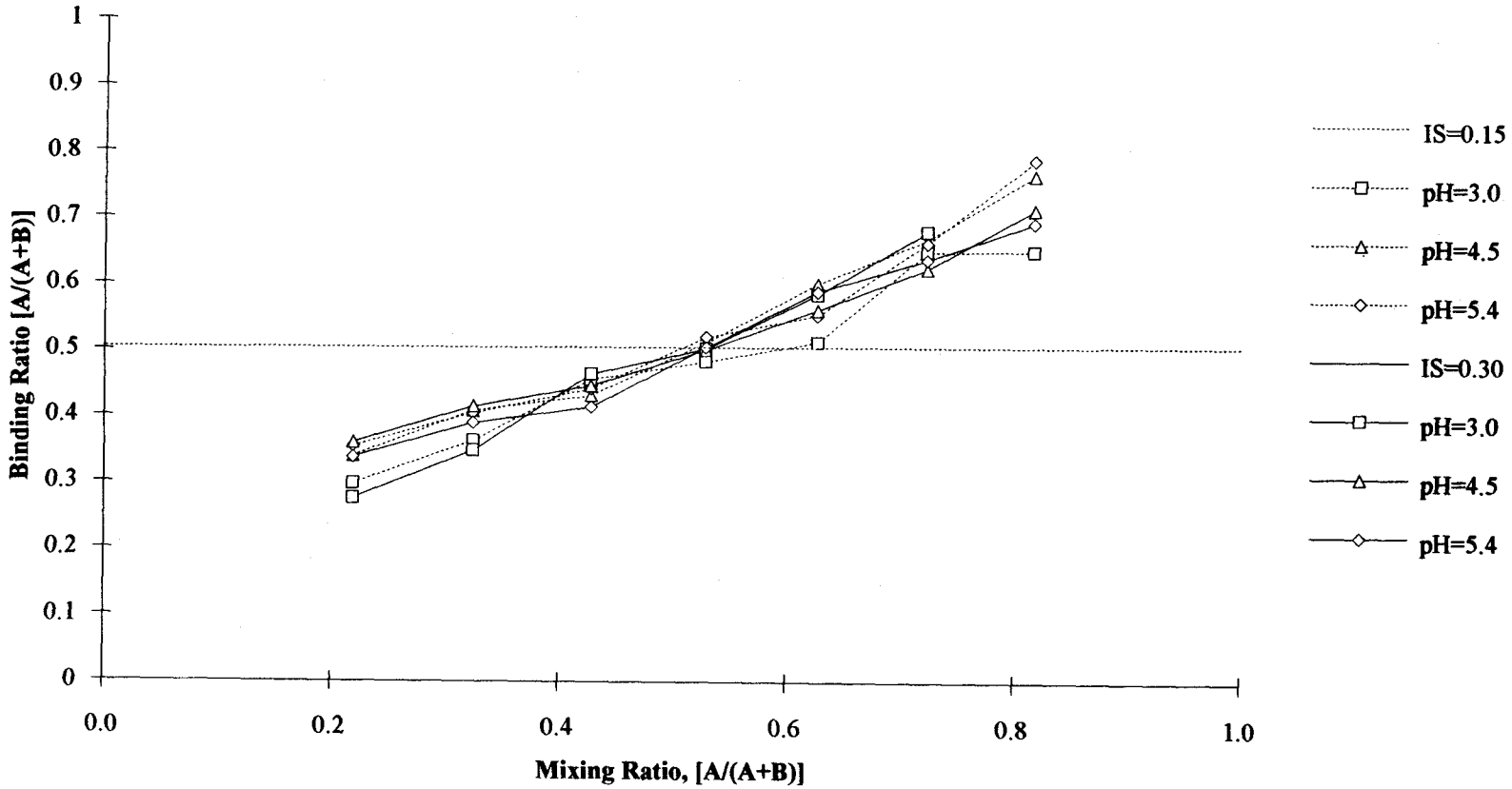


Figure 4.2 Effect of ionic strength (IS) and pH on binding ratio of chitosan:pectin.

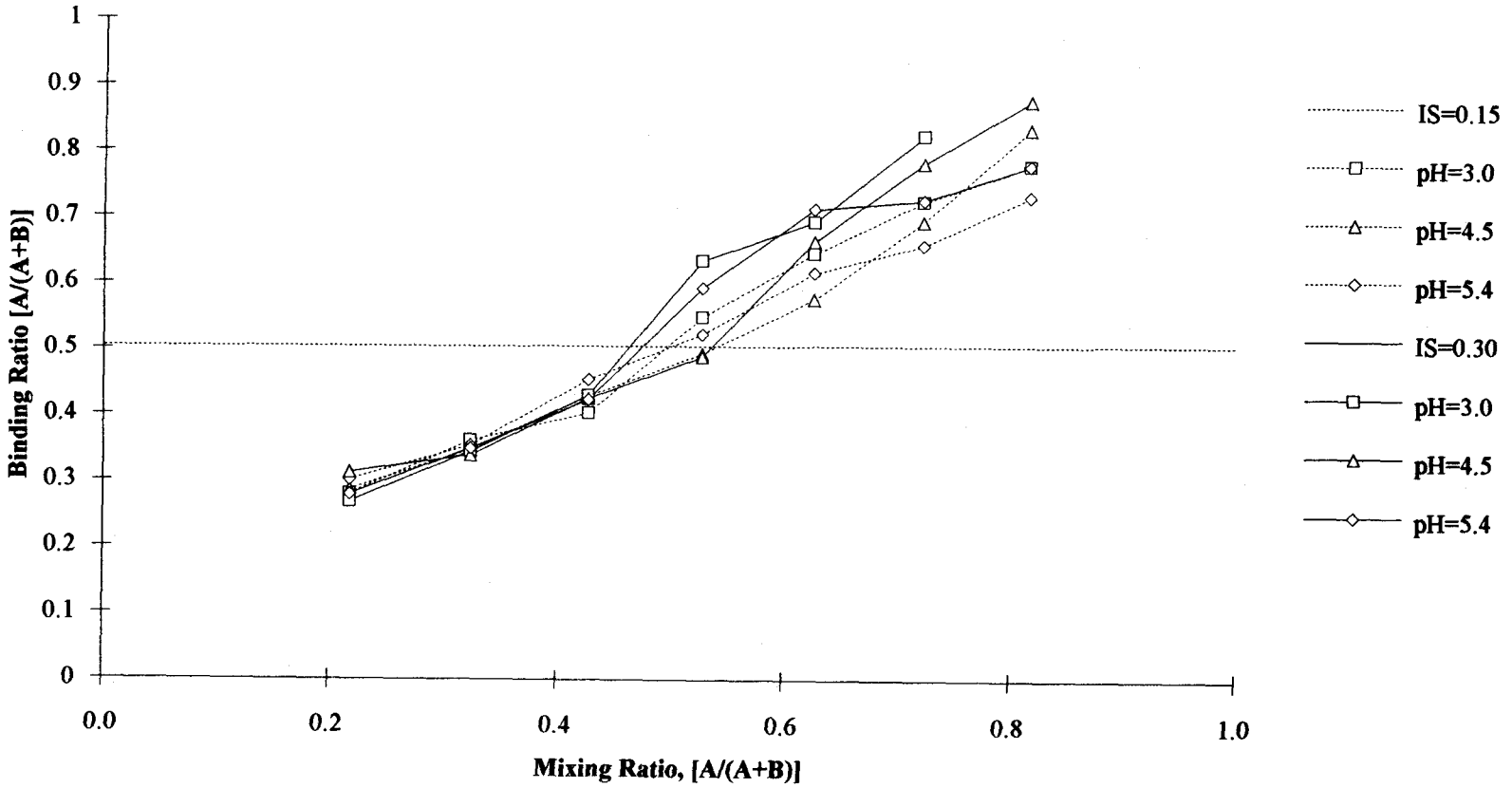
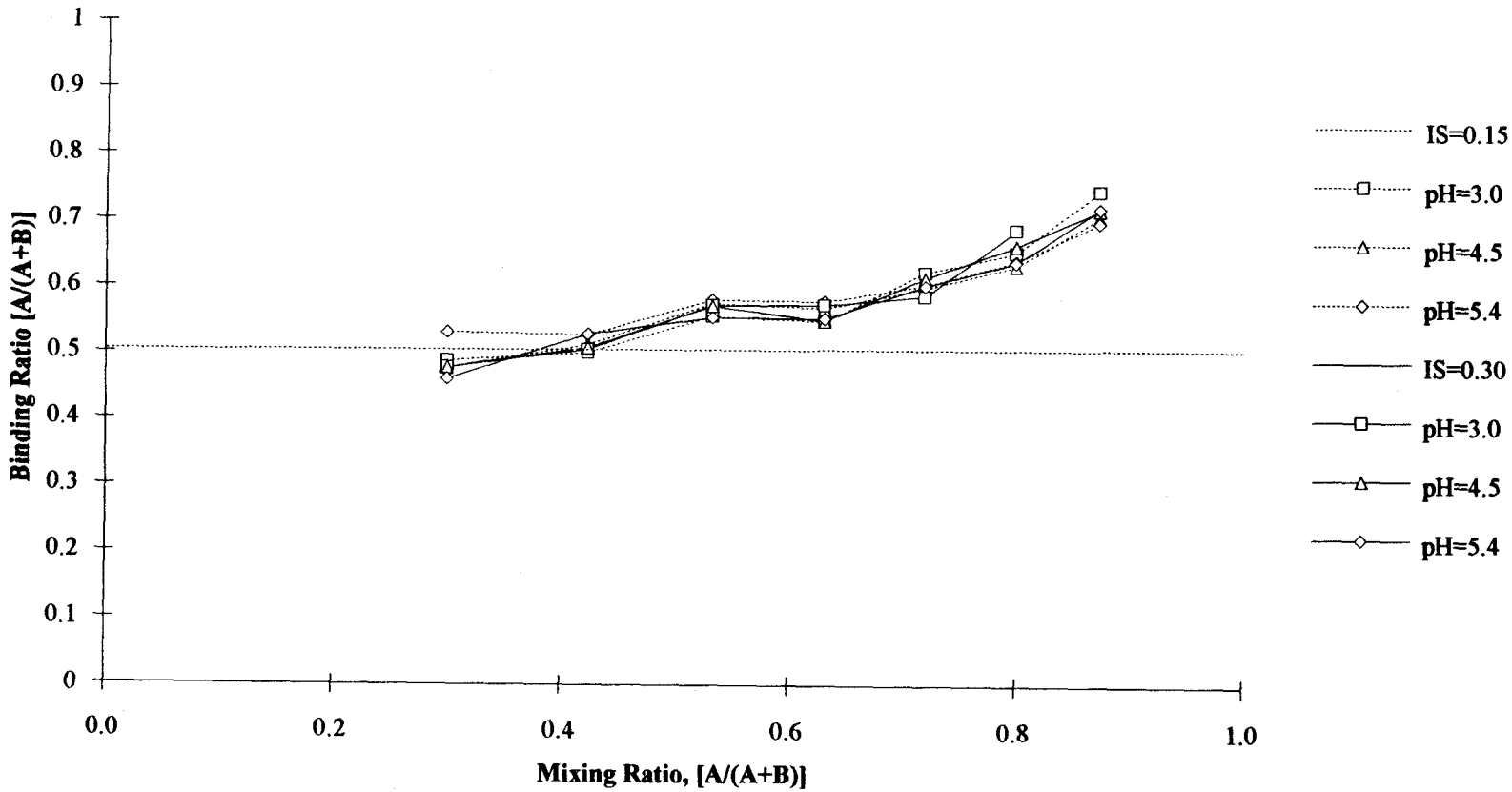
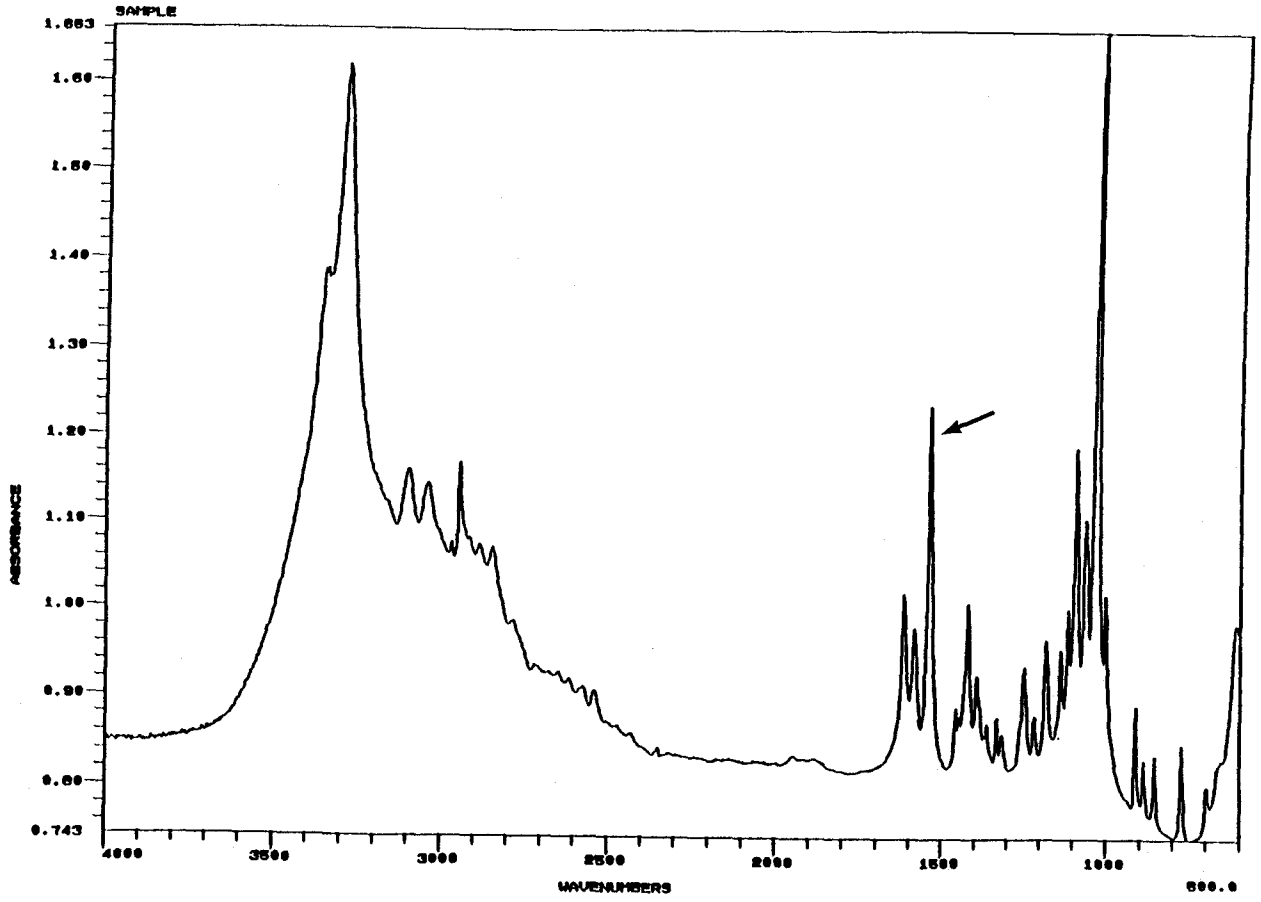


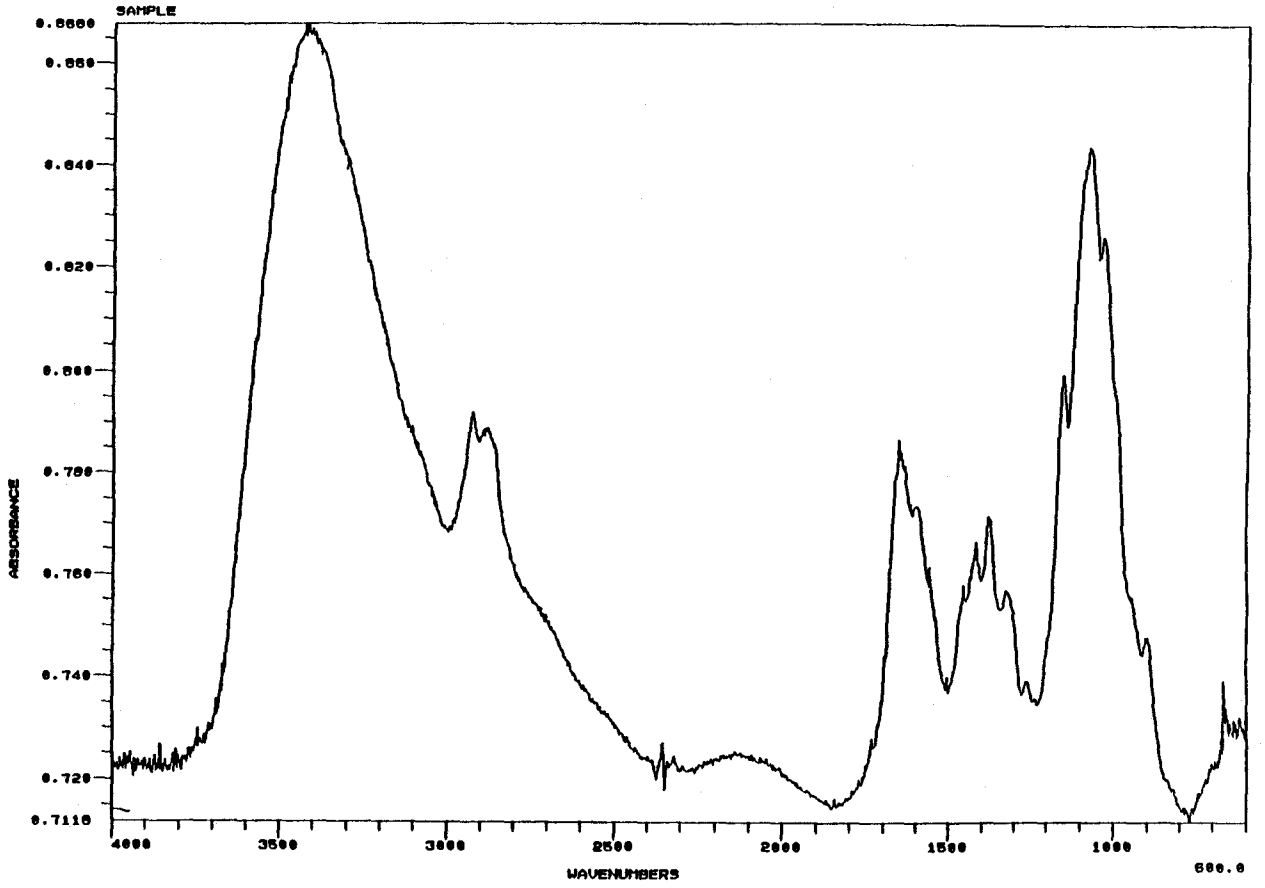
Figure 4.3 Effect of ionic strength (IS) and pH on binding ratio of chitosan:carrageenan.



4.4 Infrared analysis of glucosamine



4.5 Infrared analysis of chitosan

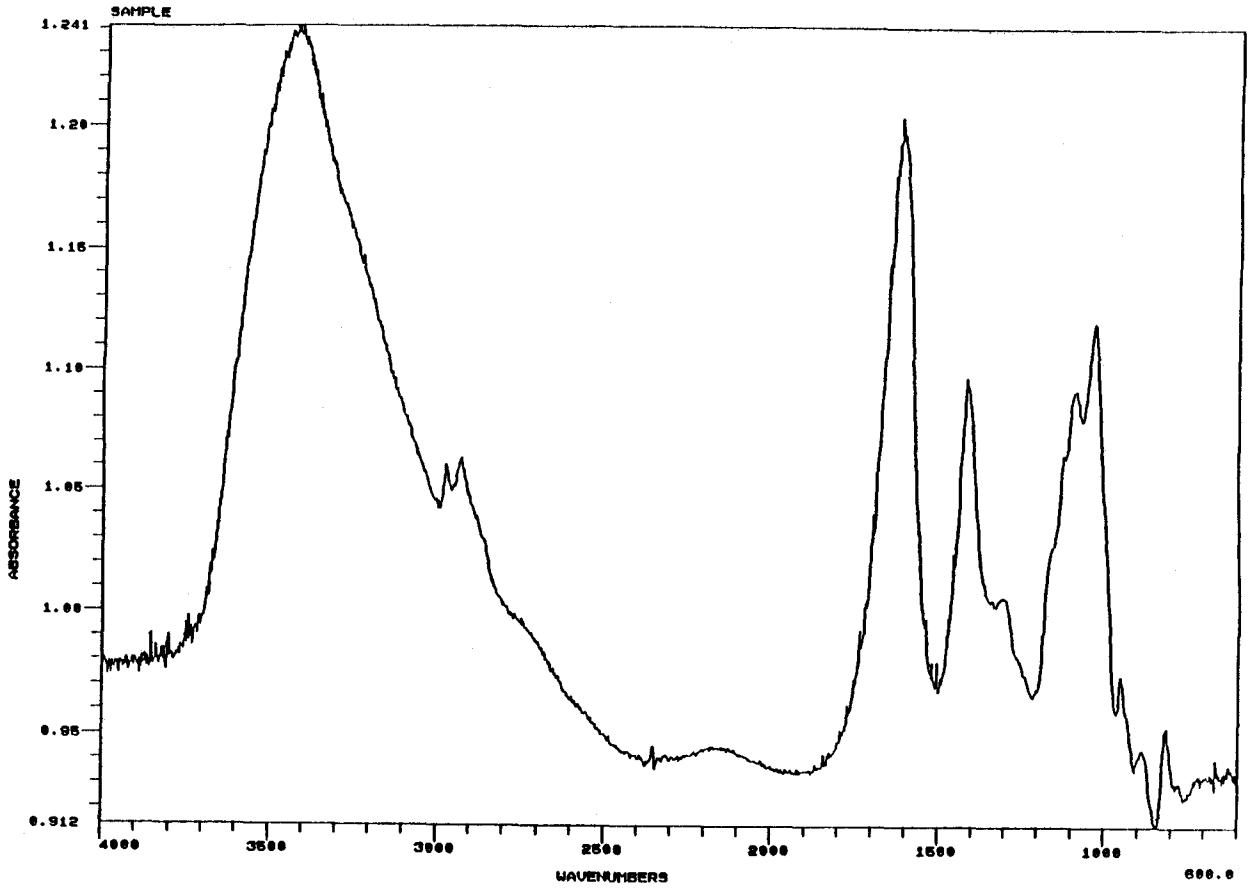


Nicolet_510P_FTIR___
OSU_Chemistry_Dept._
Gilbert_Addition_416

CHITOSAN STANDARD

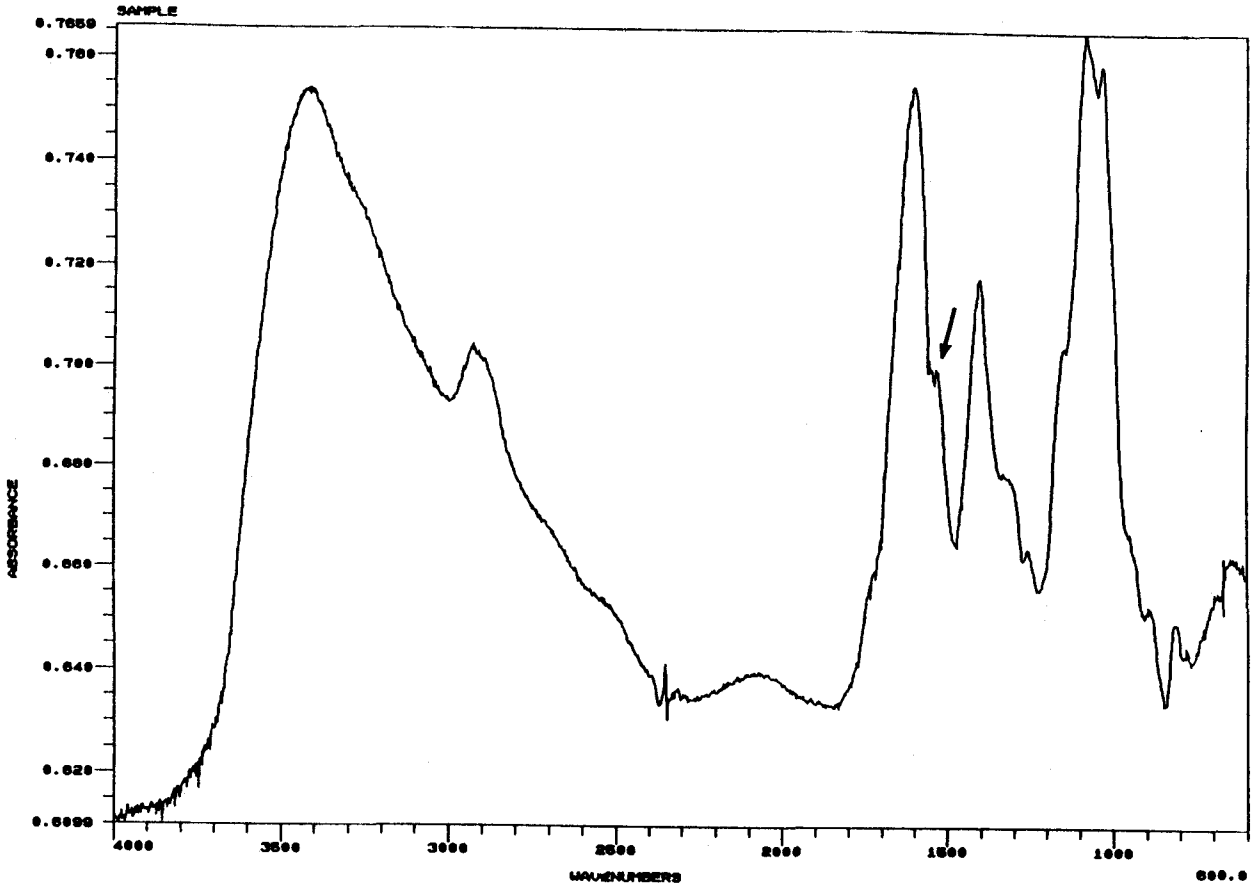
SCANS: 16 RES: 2.0 TIME: 12/04/ 12:26:10

4.6 Infrared analysis of alginate

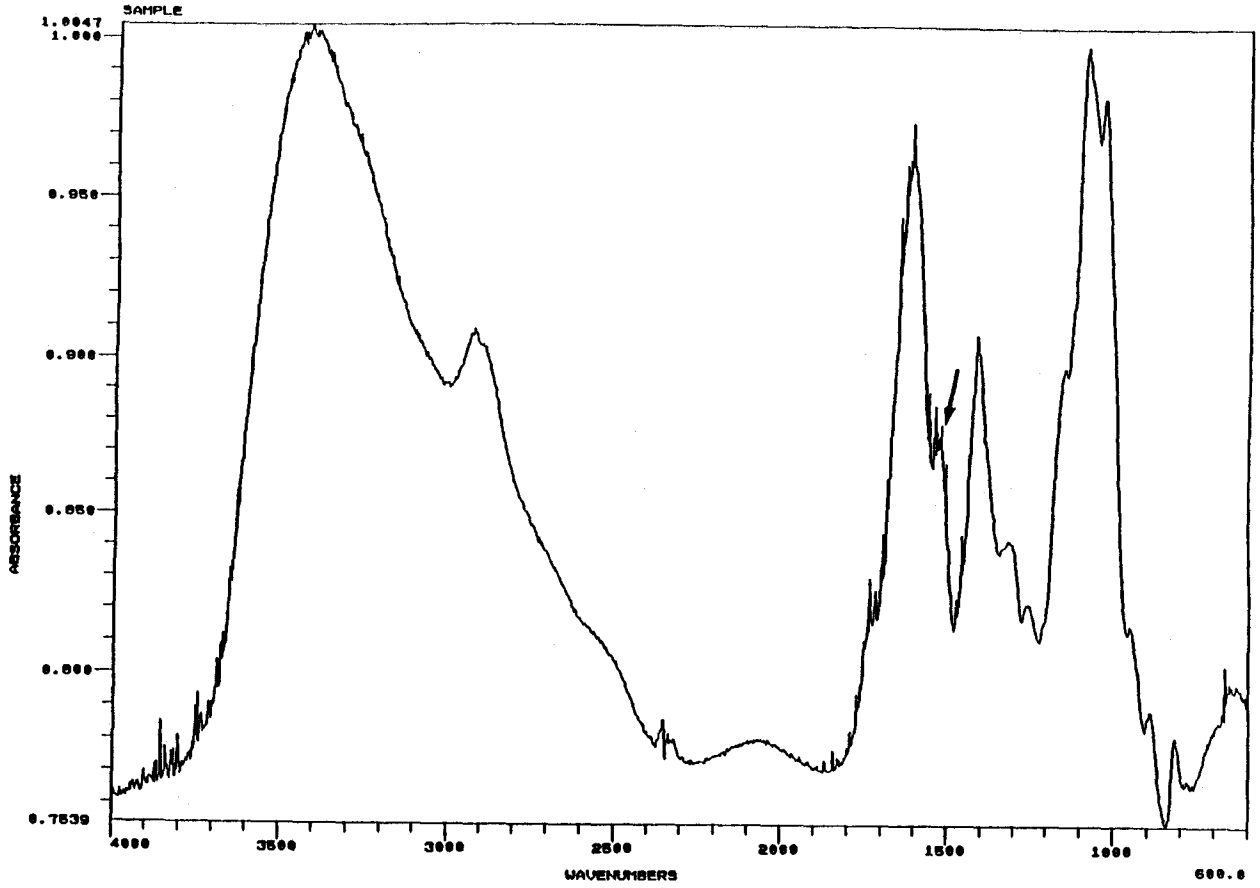


Nicolet_510P_FTIR___ ALGINATE STANDARD
OSU_Chemistry_Dept._
Gilbert_Addition_416 SCANS: 16 RES: 2.0 TIME: 12/06/ 11:13:27

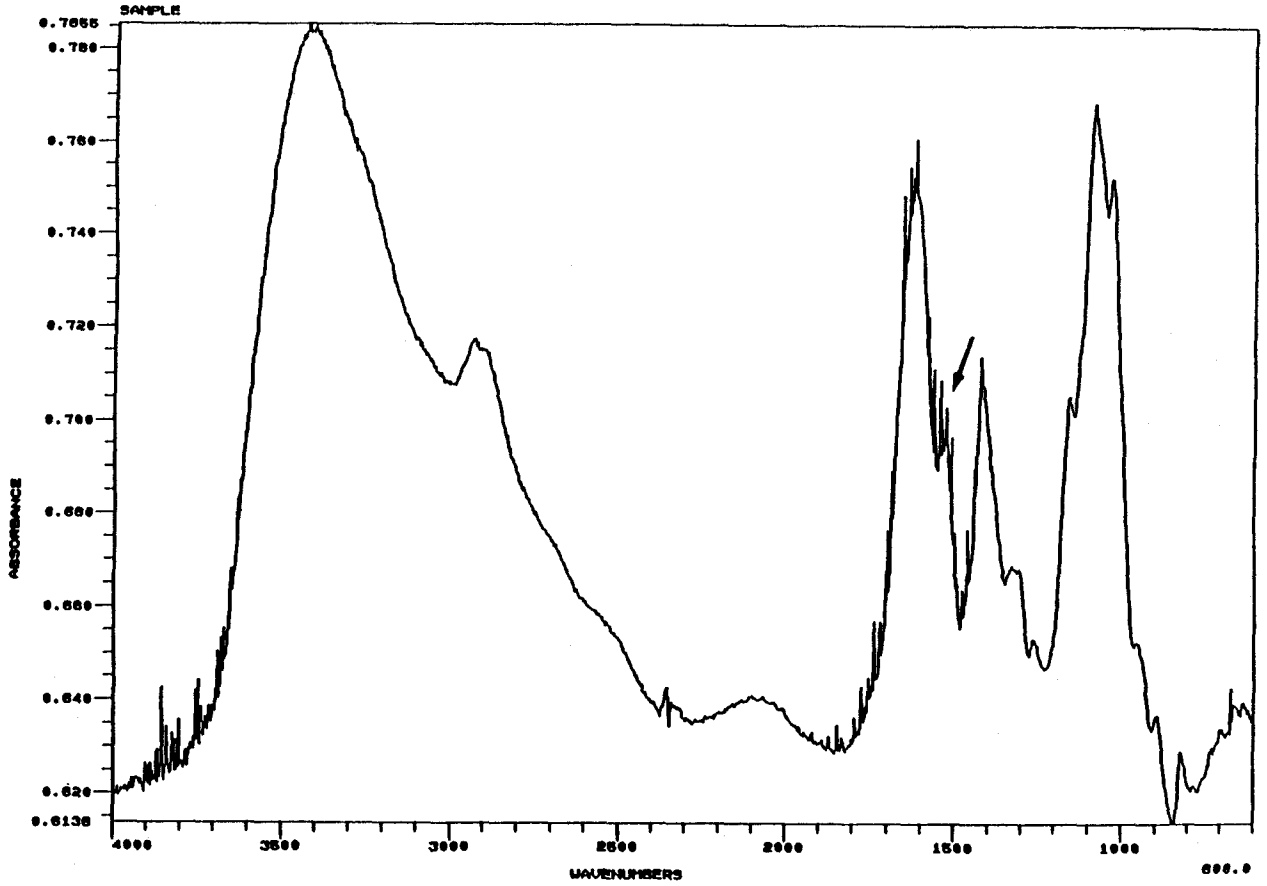
4.7 Infrared analysis of chitosan:alginate at pH=3.0 and ionic strength 0.15



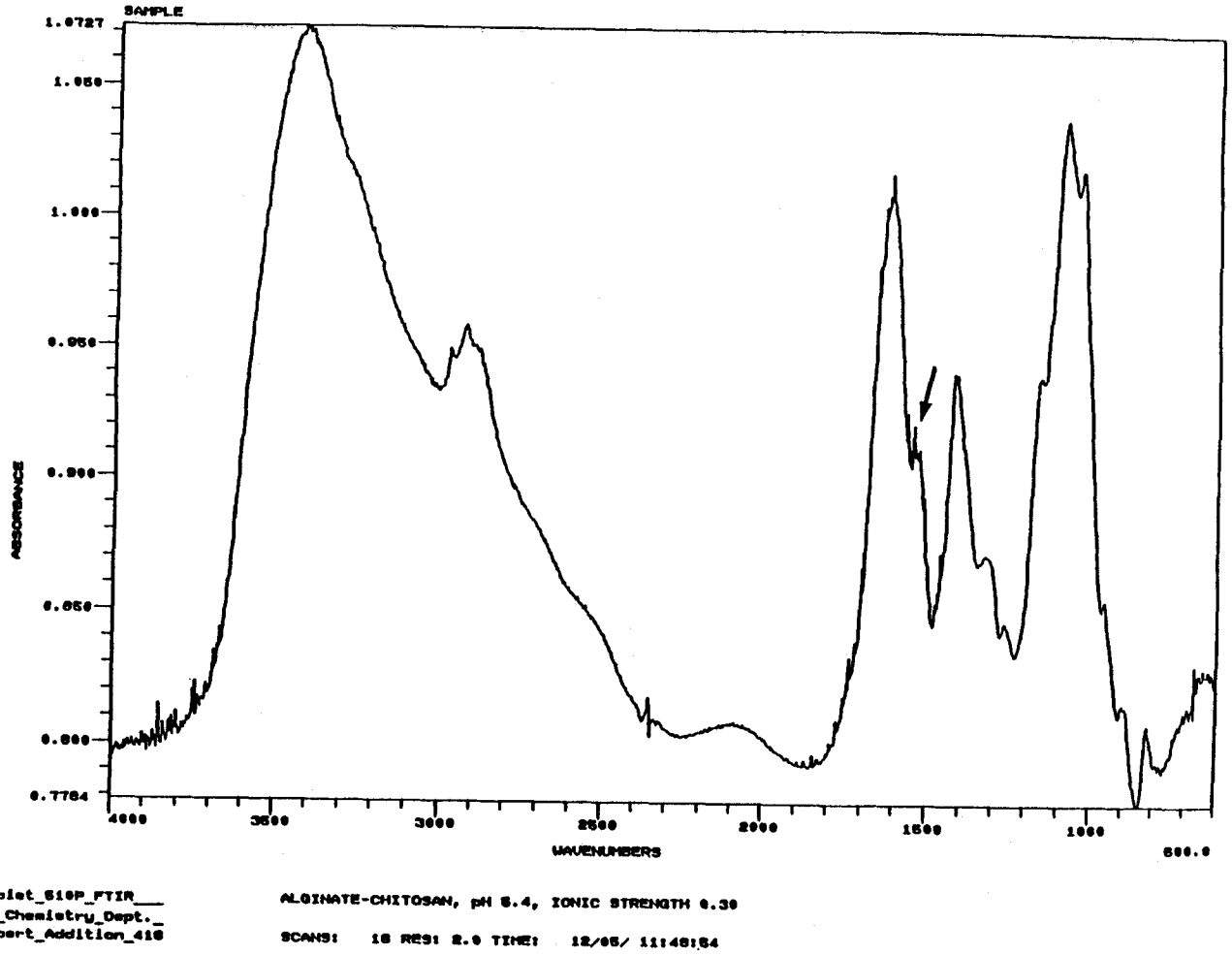
4.8 Infrared analysis of chitosan:alginate at pH=3.0 and ionic strength 0.30



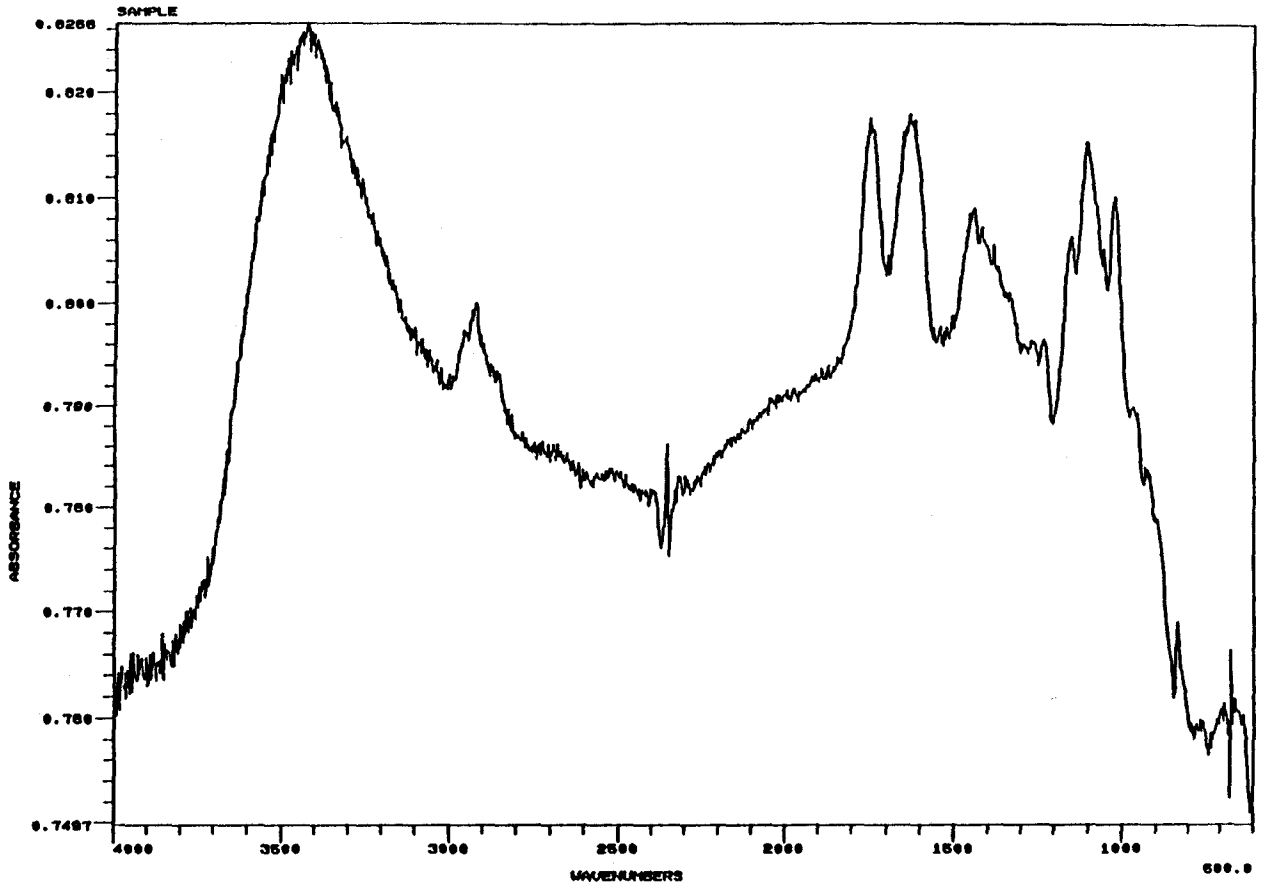
4.9 Infrared analysis of chitosan:alginate at pH=4.5 and ionic strength 0.30



4.10 Infrared analysis of chitosan:alginate at pH=5.4 and ionic strength 0.30

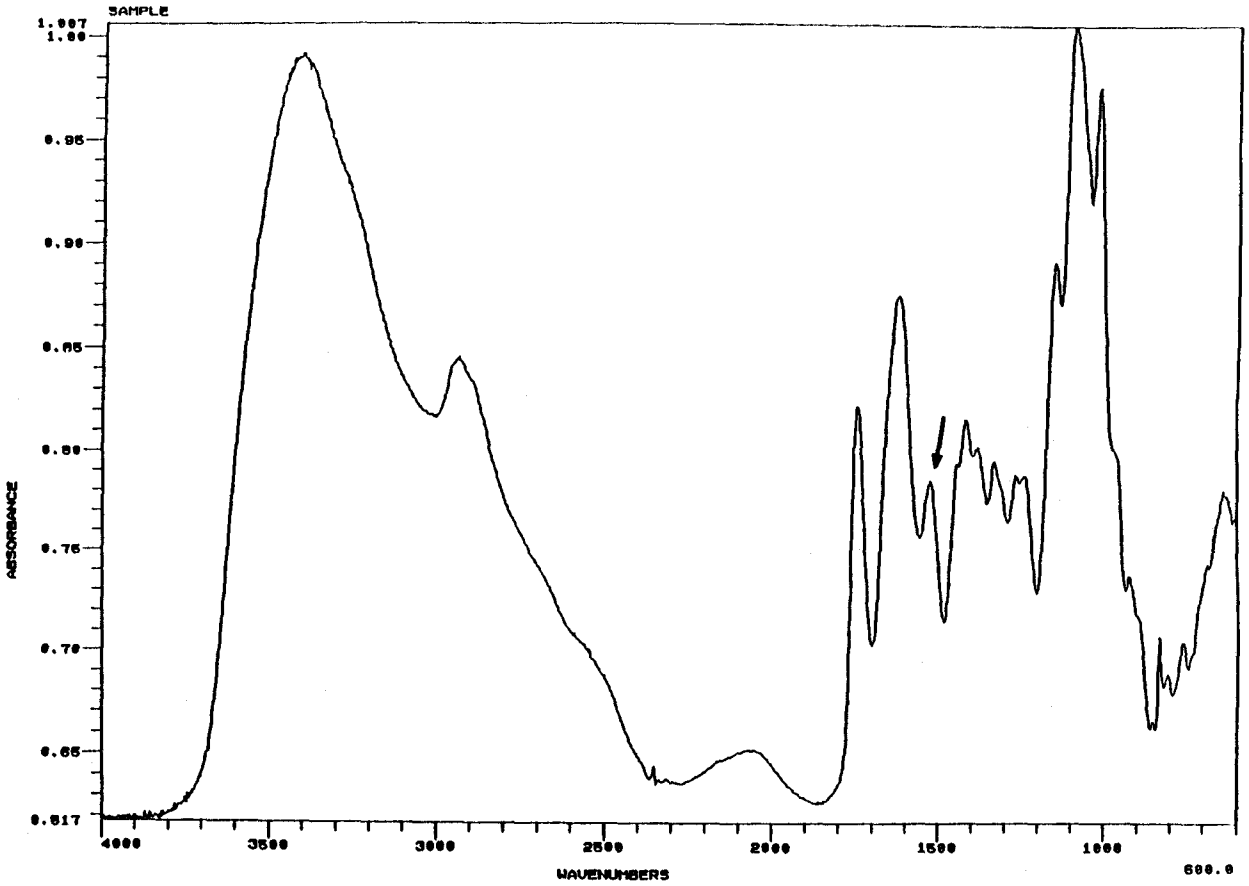


4.11 Infrared analysis of pectin



Nicolet_510P_FTIR___ PECTIN STANDARD
OSU_Chemistry_Dept._
Gilbert_Addition_416 SCANS: 16 RES: 2.0 TIME: 12/04/ 15:04:44

4.12 Infrared analysis of chitosan:pectin at pH=3.0 and ionic strength 0.15

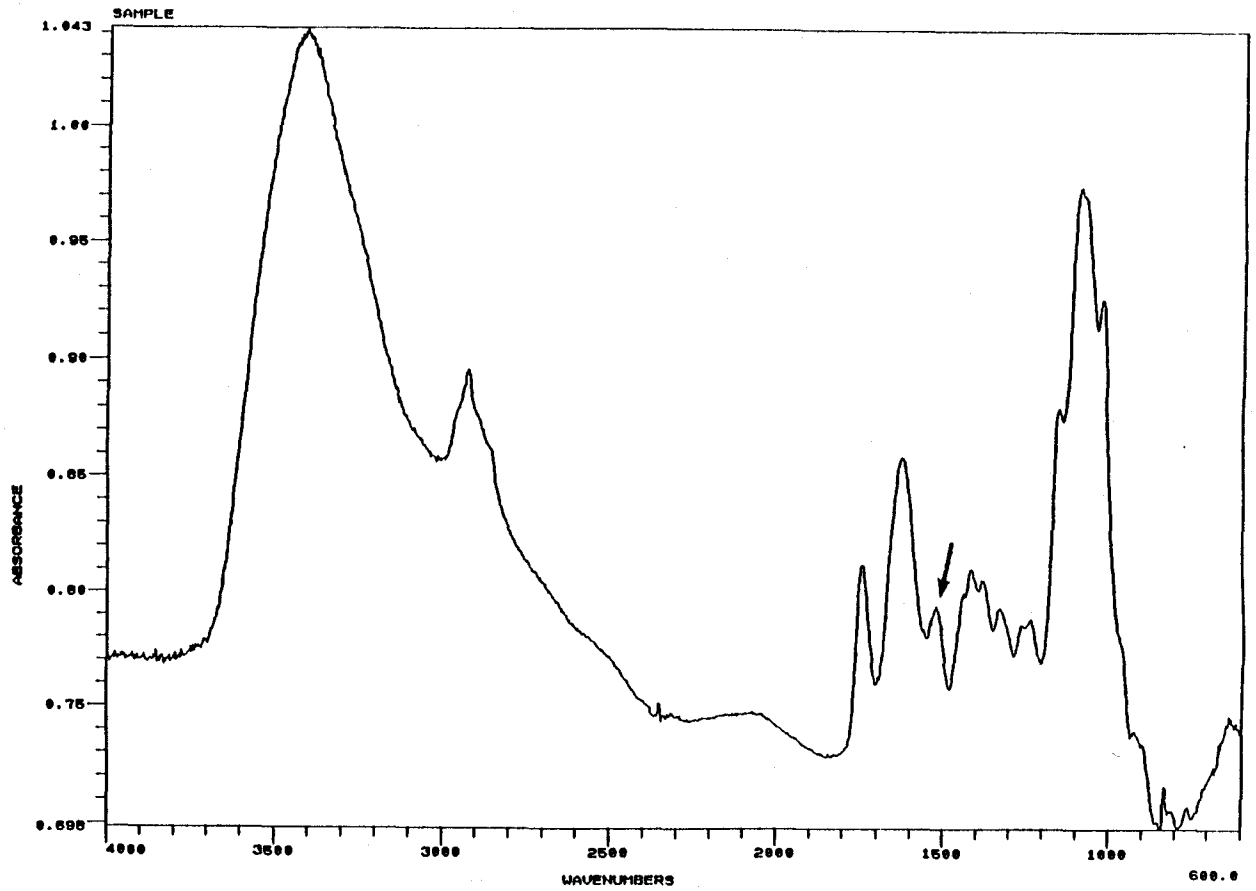


Nicolet_510P_FTIR___
OSU_Chemistry_Dept._
Gilbert_Addition_416

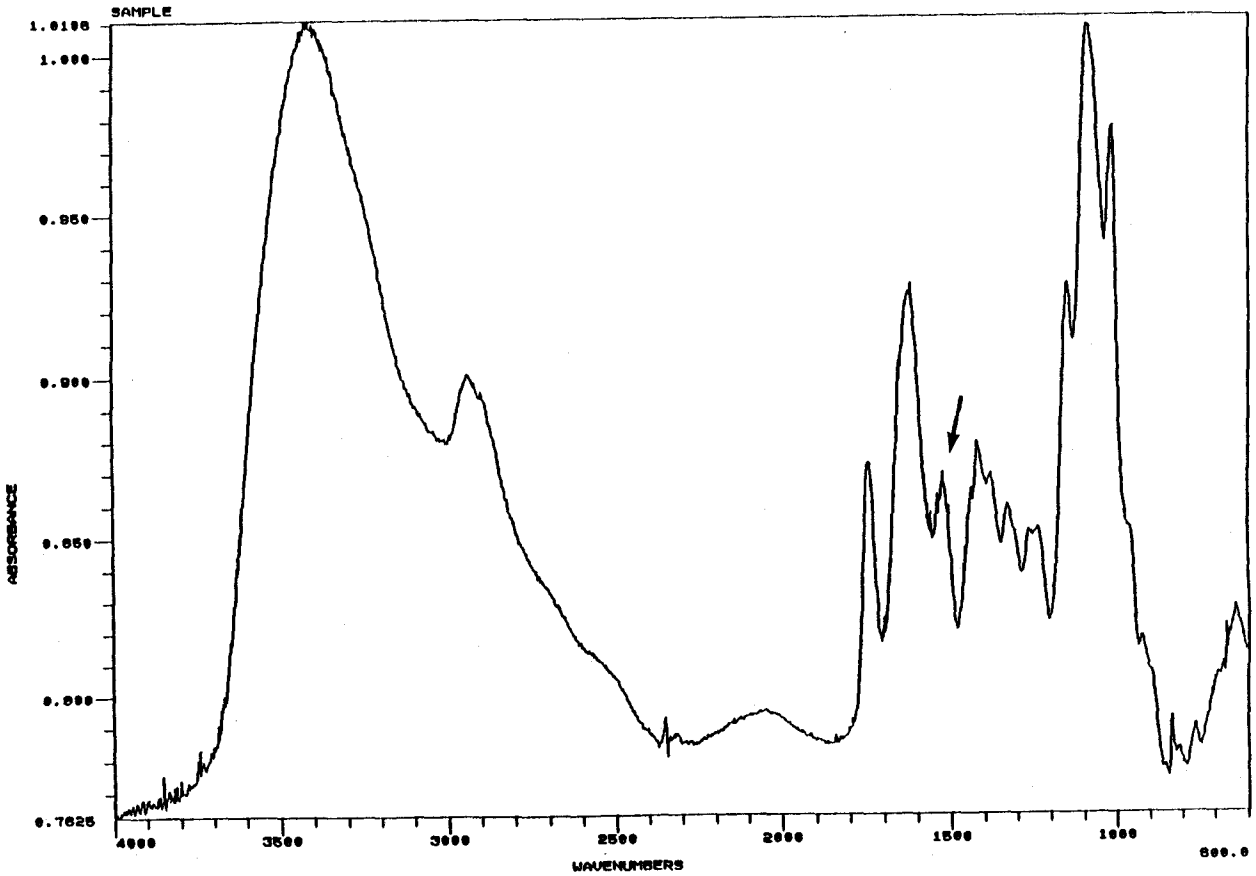
PECTIN-CHITOSAN, pH 3.0, IONIC STRENGTH 0.15

SCANS: 16 RES: 2.0 TIME: 12/05/ 19:51:26

4.13 Infrared analysis of chitosan:pectin at pH=3.0 and ionic strength 0.30



4.14 Infrared analysis of chitosan:pectin at pH=4.5 and ionic strength 0.30

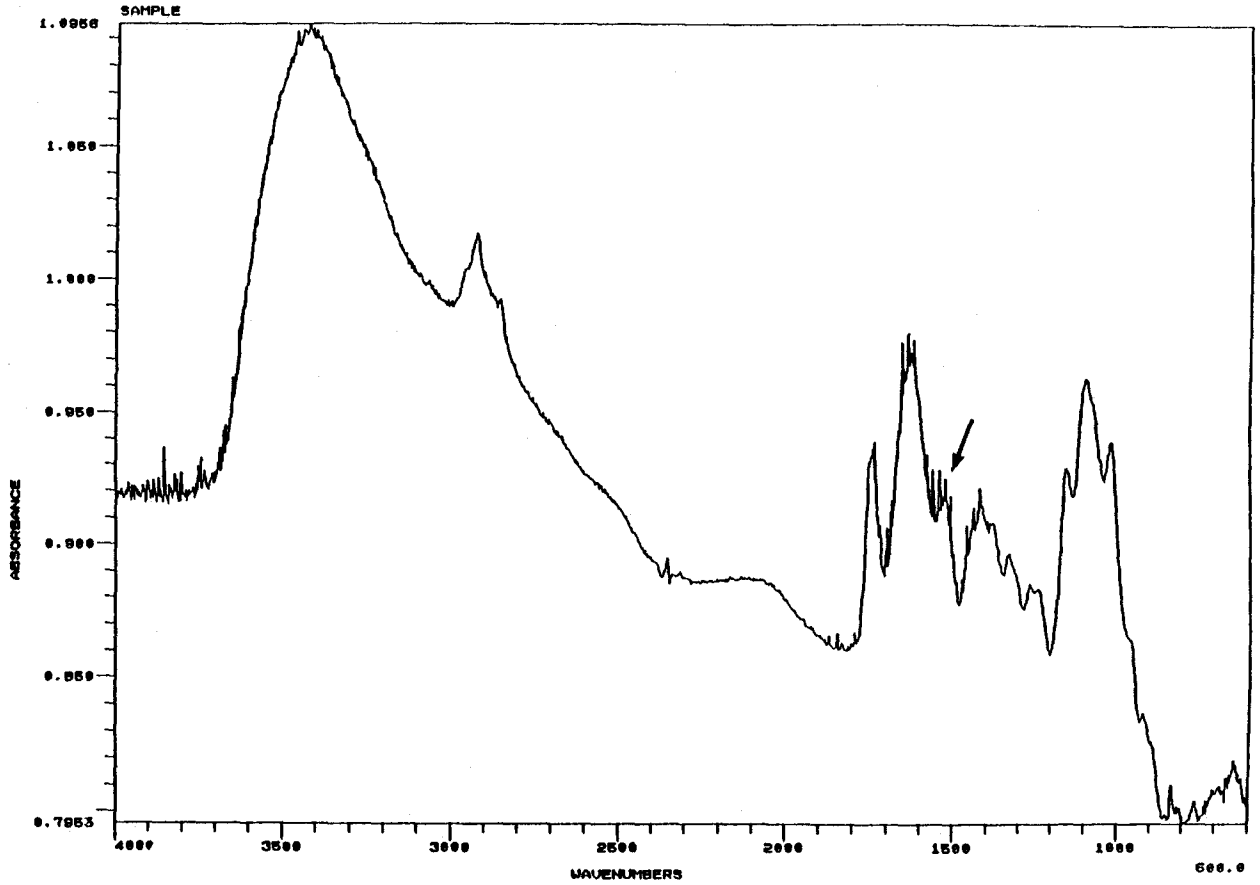


Nicolet_510P_FTIR___
OSU_Chemistry_Dept._
Gilbert_Addition_416

PECTIN-CHITOSAN, pH 4.5, IONIC STRENGTH 0.30

SCANS: 16 RES: 2.0 TIME: 12/06/ 10:43:51

4.15 Infrared analysis of chitosan:pectin at pH=5.4 and ionic strength 0.30

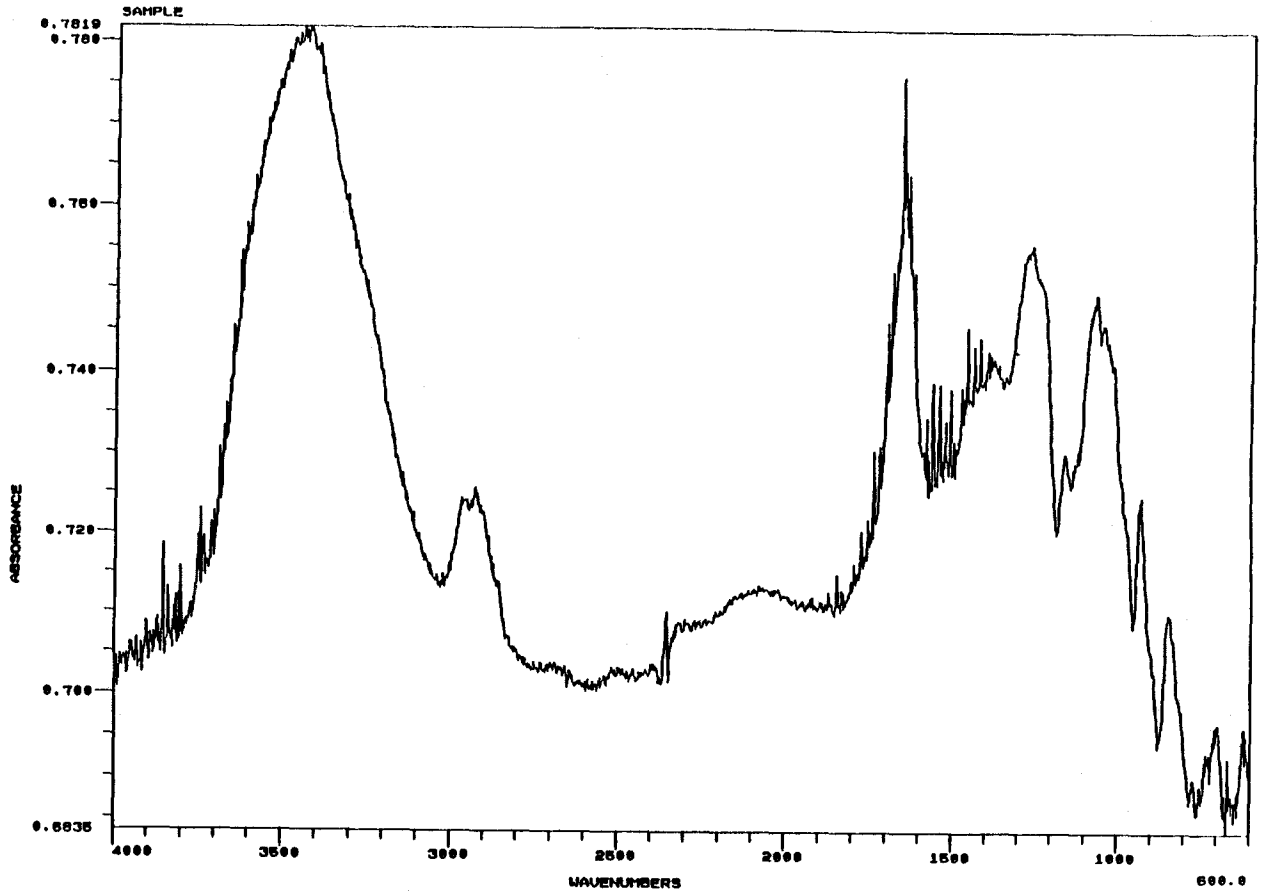


Nicolet_510P_FTIR___
OSU_Chemistry_Dept._
Gilbert_Addition_418

PECTIN-CHITOSAN, pH 5.4, IONIC STRENGTH 0.30

SCANS: 16 RES: 2.0 TIME: 12/04/ 16:10:55

4.16 Infrared analysis of carrageenan

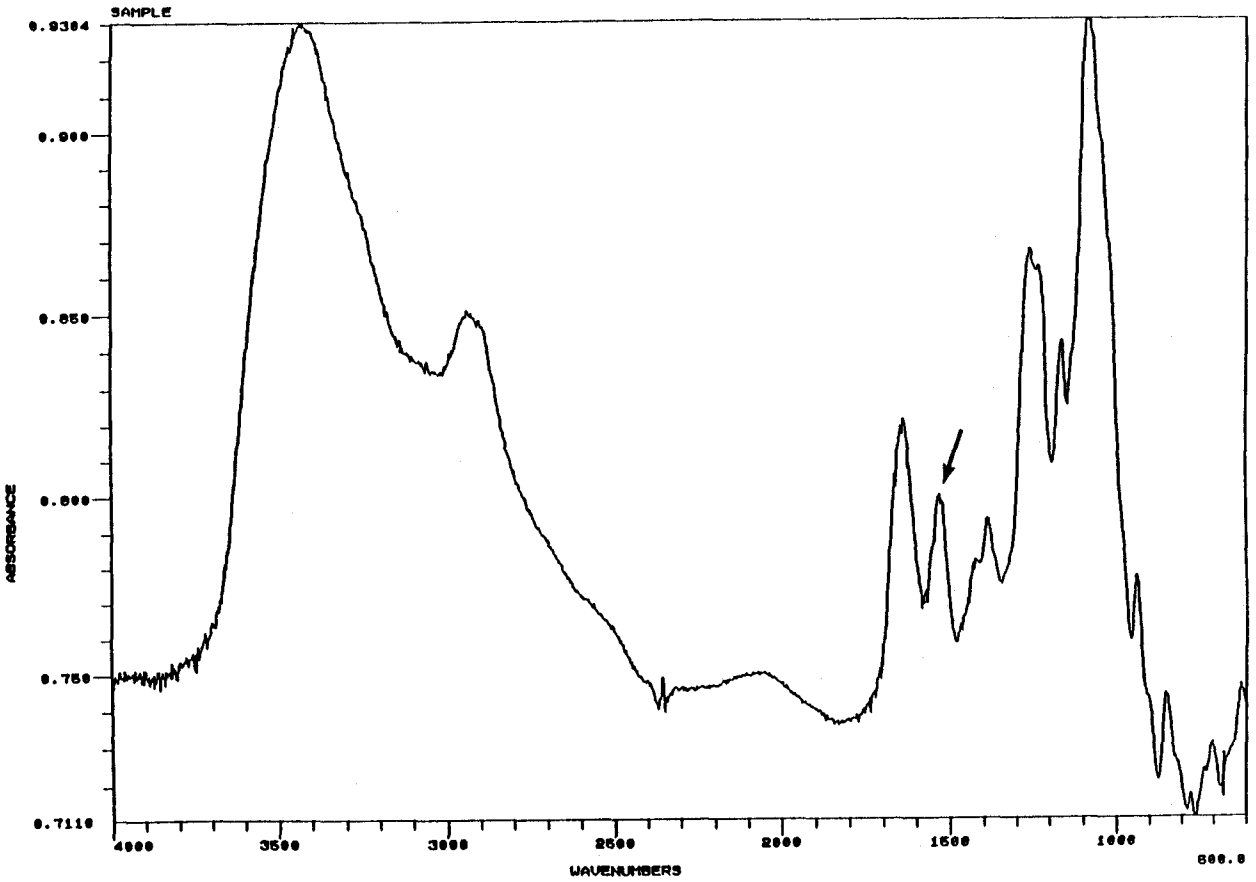


Nicolet_510P_FTIR___
OSU_Chemistry_Dept._
Gilbert_Addition_416

CARRAGEENAN STANDARD

SCANS: 16 RES: 2.0 TIME: 12/06/ 12:12:46

4.17 Infrared analysis of chitosan:carrageenan at pH=3.0 and ionic strength 0.15

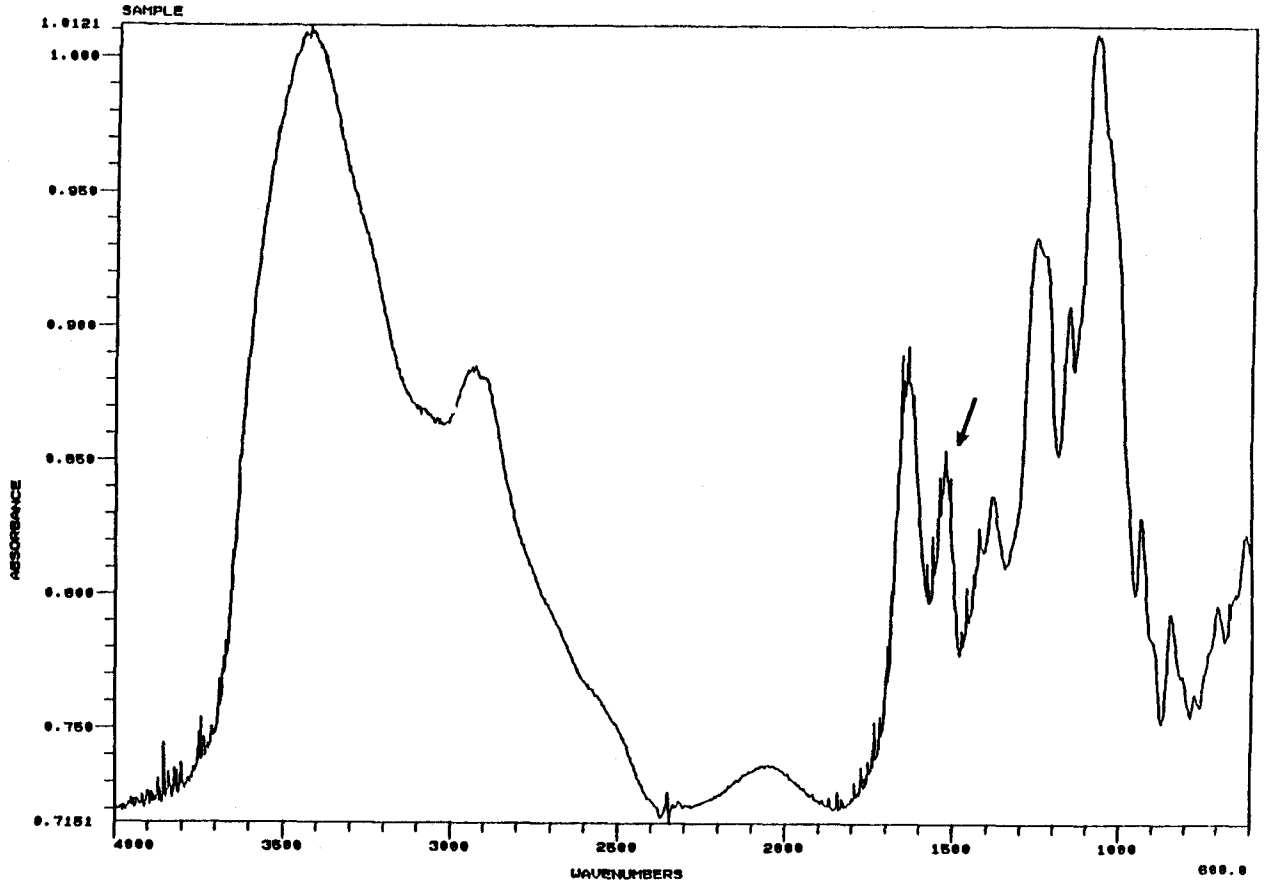


Nicolet_510P_FTIR___
OSU_Chemistry_Dept._
Gilbert_Addition_416

CARRAGEENAN-CHITOSAN, pH 3.0, IONIC STRENGTH 0.15

SCANS: 16 RES: 2.0 TIME: 12/06/ 19:36:20

4.18 Infrared analysis of chitosan:carrageenan at pH=3.0 and ionic strength 0.30

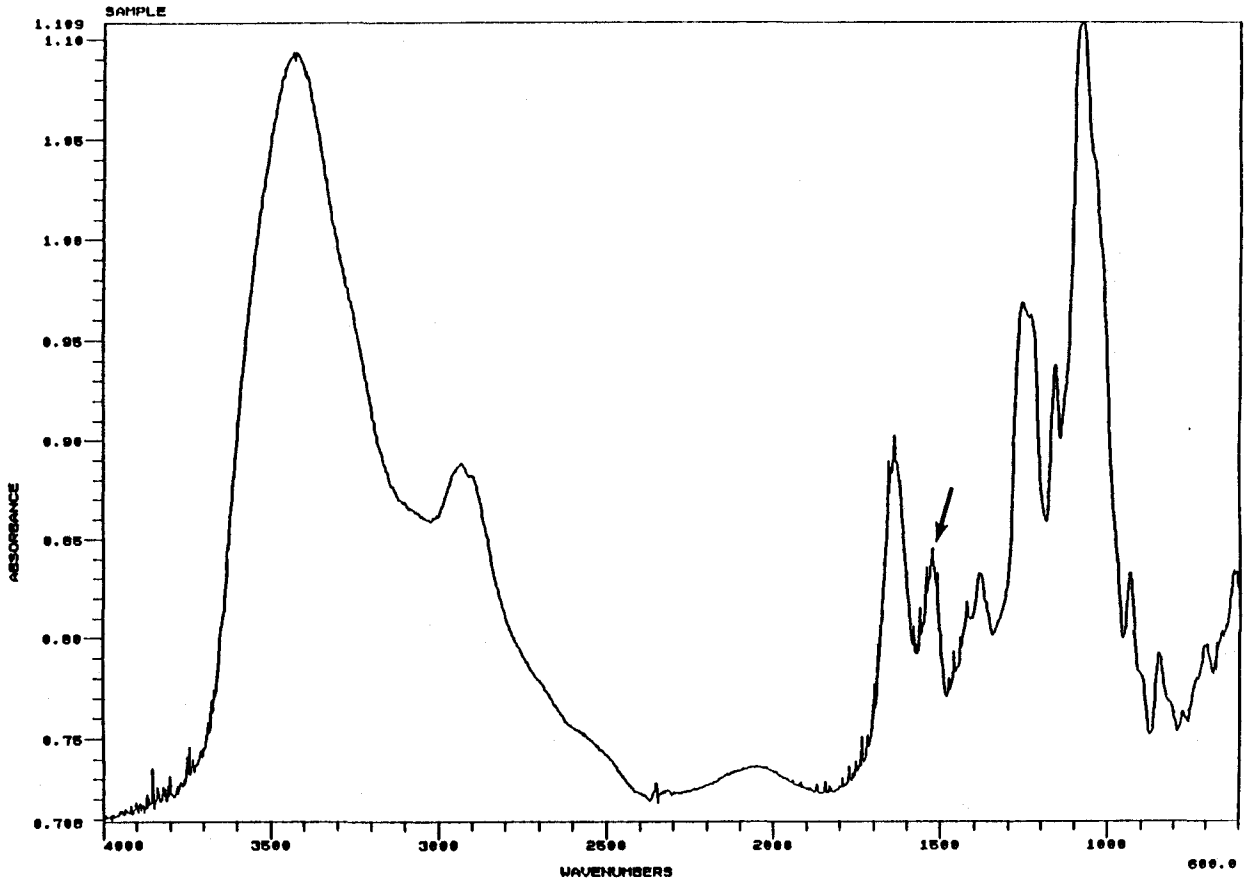


Nicolet_510P_FTIR___
OSU_Chemistry_Dept._
Gilbert_Addition_416

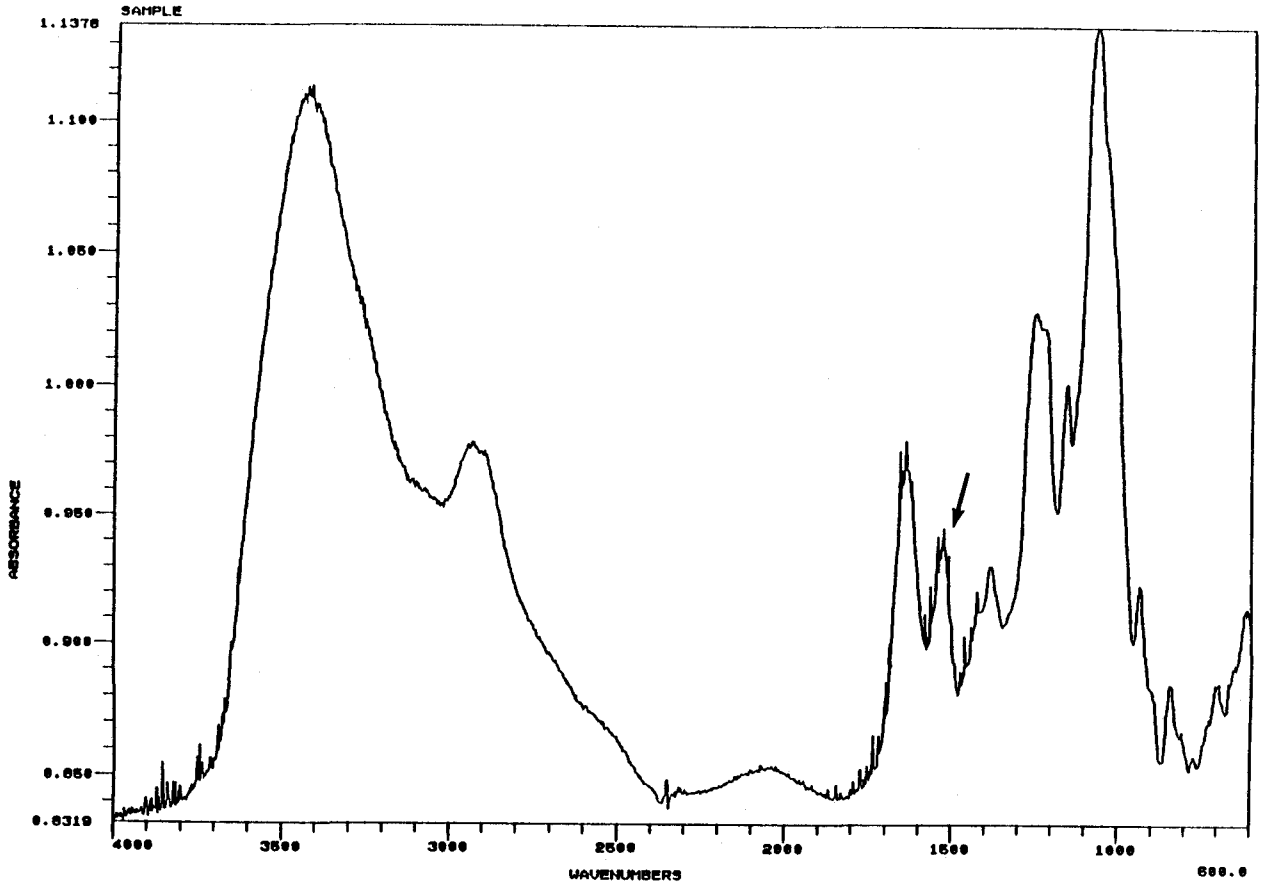
CARRAGEENAN-CHITOSAN, pH 3.0, IONIC STRENGTH 0.30

SCANS: 16 RES: 2.0 TIME: 12/05/ 12:33:14

4.19 Infrared analysis of chitosan:carrageenan at pH=4.5 and ionic strength 0.30



4.20 Infrared analysis of chitosan:carrageenan at pH=5.4 and ionic strength 0.30



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