CAPACITIES OF SOME CHITOSANS FOR ADSORPTION WITH IRON (II) IN 0.01 M HYDROCHLORIC SOLUTION

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KEY WORDS: CHITOSAN / IRON / ADSORPTION / CAPACITY / DEGREE OF DEACETYLATION

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Chitosan is the deacetylated derivative of chitin, a natural polymer derived from crab or shrimp shell. Chitosan has been used for adsorption of metal ions. Recently, chitosan capsules have been produced and sold as dietary supplements for reducing the absorption of fats and cholesterol.

In this work, three chitosans, designated as chitosan S, L and M, were studied and compared for their efficiencies in adsorption of Fe (II) in 0.01 M HCl to emulate the pH of gastric juce. These chitosans differ from one another in terms of their purity. Chitosan S in a pure sample containing no filler, and approximately 98% w/w. Chitosan L and chitosan M contained 5% (w/w) ascorbic acid and 32.6% (w/w) carbohydrate including starch, respectively. Adsorption capacities of chitosan was measured after two hours of contact with the acid solution. It was found that the order of capacities were chitosan L (167 ± 46 mg/g) > chitosan M (93 ± 9 mg/g) > chitosan S with 125-300 μm particle size (60 ± 6 mg/g) > chitosan S with >300 μm particle size (36 ± 4 mg/g). Chitosan S, having smaller particle size and larger surface area, exhibited a greater capacity of adsorption than chitosan S with larger size range. No correlation has been found between the capacity order and the degree of deacetylation, as measured by three techniques, namely first-derivative UV spectroscopy, IR technique and solid-state ^{13}C NMR. Fillers, such as starch and ascorbic acid added to chitosan M and chitosan L respectively, have shown to play a role on the Fe (II) adsorptivity. After washing off the fillers, the adsorptivities of these two chitosans were significantly decreased. A mechanism of adsorption of Fe (II) on chitosan flake is proposed.

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วรรณระวี จันทร์โท : ค่าความจุของการดูดซับอิออนของเหล็ก (II) ด้วยไคโตแซนบางชนิดในสารละลายกรดไฮโดรคลอริกเข้มข้น 0.01 โมลาร์ (CAPACITIES OF SOME CHITOSANS FOR ADSORPTION WITH IRON (II) IN 0.01 M HYDROCHLORIC SOLUTION). คณะกรรมการควบคุมวิทยานิพนธ์: ดวงใจ นาคะปรีชา, Ph.D., ประพิณ วิไลรัตน์, Ph.D., วเรศ วีระสัย, Dr. rer. nat. 130 หน้า. ISBN 974-664-466-1

ไคตินจัดเป็นพอลิเมอร์ในธรรมชาติที่พบในกระดองปูและเปลือกกุ้ง เมื่อไคตินถูกกำจัดหมู่อะซีติลออกจะเกิดเป็นอนุพันธ์ชนิดใหม่คือไคโตแซน จากงานวิจัยพบว่าไคโตแซนมีสมบัติดูดซับอิออนของโลหะได้ ในปัจจุบันนี้ไคโตแซนได้ถูกนำมาผลิตเป็นผลิตภัณฑ์อาหารเพื่อลดการดูดซึมไขมันและคลอเรสเตอรอลในร่างกาย

งานวิจัยนี้จะศึกษาและเปรียบเทียบสมบัติในการดูดซับไอออนของเหล็ก (II) ในสภาวะกรด (0.01 M HCl) เพื่อจำลองสภาพความเป็นกรดในกระเพาะอาหารด้วยไคโตแซน 3 ชนิดได้แก่ ไคโตแซน เอส แอล และ เอ็ม ซึ่งไคโตแซนทั้งสามชนิดนี้จะมีความบริสุทธิ์ต่างๆกัน กล่าวคือไคโตแซนเอสมีความบริสุทธิ์สูงสุดประมาณร้อยละ 98 โดยน้ำหนัก ในขณะที่ไคโตแซน แอล และเอ็ม มีกรดแอสคอร์บิคร้อยละ 5 โดยน้ำหนัก และคาร์โบไฮเดรต (รวมแป้ง) ร้อยละ 32.6 โดยน้ำหนักตามลำดับ ค่าความจุสำหรับการดูดซับของไคโตแซนจะถูกตรวจวัดหลังจากที่ไคโตแซนสัมผัสกับสารละลายนาน 2 ชั่วโมง ซึ่งพบว่าลำดับความสามารถในการดูดซับเป็นดังนี้คือไคโตแซน แอล (167 ± 46 mg/g) > ไคโตแซน เอ็ม (93 ± 9 mg/g) > ไคโตแซน เอส ขนาดอนุภาค 125-300 μm (60 ± 6 mg/g) > ไคโตแซน เอส ขนาดอนุภาค >300 μm (36 ± 4 mg/g) กล่าวคือไคโตแซน เอส ซึ่งมีขนาดอนุภาคเล็กกว่าจะมีพื้นที่ผิวภายนอกมากกว่า ทำให้มีความจุของการดูดซับมากกว่าไคโตแซนที่มีขนาดอนุภาคใหญ่กว่า อย่างไรก็ตาม พบว่าลำดับความสามารถในการดูดซับของผลิตภัณฑ์ไคโตแซนไม่มีความสัมพันธ์กับ degree of deacetylation ซึ่งตรวจวัดด้วยเทคนิค 3 เทคนิค ได้แก่ เทคนิค first-derivative UV spectroscopy เทคนิค IR และเทคนิค solid-state ^{13}C NMR นอกจากนี้ยังพบว่าตัวเติมเต็มได้แก่ แป้งซึ่งพบในไคโตแซนชนิดเอ็มและกรดแอสคอร์บิคซึ่งพบในไคโตแซนชนิดแอลนั้น มีบทบาทต่อความสามารถในการดูดซับไอออนของเหล็ก (II) ด้วย กล่าวคือเมื่อตัวเติมเต็มเหล่านี้ถูกกำจัดออกโดยการล้างพบว่าความสามารถในการดูดซับของไคโตแซนทั้งสองชนิดข้างต้นลดลงอย่างมีนัยสำคัญในงานวิจัยนี้ได้เสนอกลไกการดูดซับไอออนของเหล็ก (II) ด้วยไคโตแซน

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LIST OF ABBREVIATIONS

w/w	=	Weight by weight
M	=	Molar
ml	=	Milliliter
μm	=	Micrometer
°C	=	Degree celsius
Ci	=	Initial concentration of Fe (II) solution
Ce	=	Residual concentration of Fe (II) solution
FAAS	=	Flame Atomic Absorption Spectrometry
ZCP	=	Zero Crossing Point
DD	=	Degree of deacetylation
NMR	=	Nuclear Magnetic Resonance
FTIR	=	Fourier Transform Infrared
H_1	=	signal height of the pure N-acetyl glucosamine solution
H_2	=	signal height of the solutions of different percentages of N-acetyl glucosamine
Mp	=	molecular weight of Pullalan standards
W_0	=	Original weight of sample
W_1	=	Weight of pure chitosan after washing

LIST OF ABBREVIATIONS (CONTS.)

W_2	=	Weight of pure chitosan after solubilization during 2 hours shaken with 0.01 M HCl
W_{2a}	=	Residual chitosan after solubilization during 2 hours shaken with 0.01 M HCl
I_1	=	Impurity

CHAPTER I

INTRODUCTION

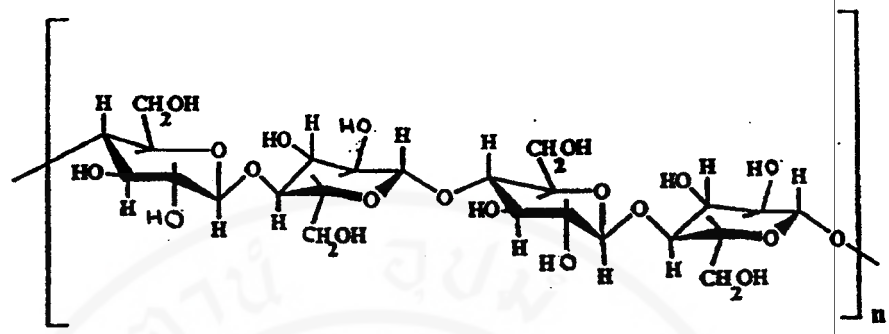
1.1 CHITOSAN

1.1.1 General introduction [1]

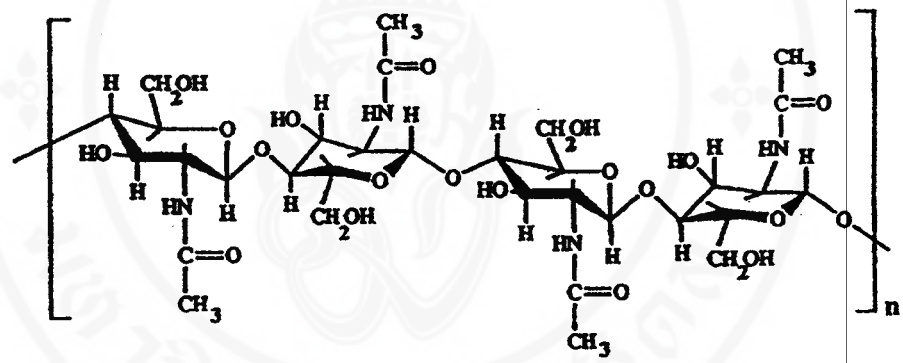
Chitosan was first discovered by Rouget in 1859 by boiling chitin with concentrated solution of potassium hydroxide. He reported that "the modified chitin" is soluble in dilute solutions of organic acids. This property of solubility distinguished it from chitin, which is insoluble in these solutions. "The modified chitin" was renamed "chitosan" by Hoppe-Seiler in 1894.

Chitosan is a random copolymer containing 1→4 linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glycopyranose residue. It is easily obtained by alkaline deacetylation of chitin, a cellulose-related polysaccharide widely distributed in nature (e.g. crustaceans, insects and certain fungi). It is recognized that most commercially prepared chitin is a linear copolymer consisting of approximately 70-90% N-acetyl-D-glucosamine and 10-30% D-glucosamine units, connected through (1→4)-linked β-glycosidic linkages. Most commercial grade of chitosan contains 75-95% glucosamine and 5-25% N-acetyl-D-glucosamine units. Molecular structure of cellulose, chitin and chitosan are shown in Figure 1.1

a) Cellulose



b) Chitin



c) Chitosan

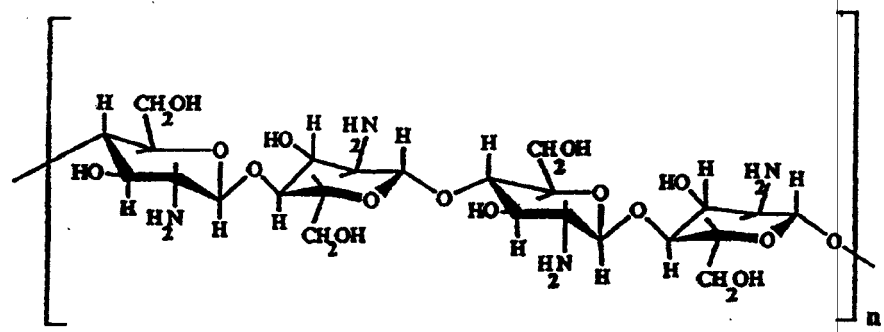


Figure 1.1 Molecular structure of cellulose, chitin and chitosan (100% deacetylated chitosan).

As shown Figure 1.1, the structures of chitin and chitosan are similar to cellulose. Cellulose consists of β-(1→ 4)-D-glucopyranose units. Chitin has the 2-hydroxyl replaced by an acetamide group on C-2 in each D-glucose unit resulting in mainly β-(1→ 4)-2-acetamido-2-deoxy-D-glucopyranose units. Chitosan contains an amino group at the C-2 of each D-glucose unit. Thus chitosan has three types of reactive functional groups, namely an amino group, a primary and a secondary hydroxyl group at the C-2, C-3 and C-6 positions, respectively. Chemical modifications of these groups have provided numerous useful materials for various applications.

1.1.2 Chemical and physical properties [1,2]

Chitosan has high concentration of primary amine groups, which makes it nucleophilic and basic. The properties presented in the following sections are limited to a few common organic reactions, chelation properties, enzymatic reactions and solubility.

1.1.2.1 Common organic reactions [1]

The structure of chitosan makes it useful to the synthetic chemist interested in site selective modification due to the distinctly different reactivities of the amino group at the C-2 position and secondary hydroxyl groups at C-6 and C-3 positions.

The nonbonding pair of electrons at the primary amine groups also makes chitosan a potent nucleophile. It should be noted that the primary amine sites on chitosan are more nucleophilic than the primary hydroxyl groups pendant at C6.

Weltrowski M. et al. [3] reported that chitosan reacts with 2-formylbenzene sodium sulfonate and 4-formyl-1,3-benzene sodium disulfonate in the presence of $NaCNBH_3$ to yield N-benzyl mono-and disulfonate derivatives of chitosan. The intramolecular interaction of the formylbenzene sulfonate group of chitosan is shown in Figure 1.2.

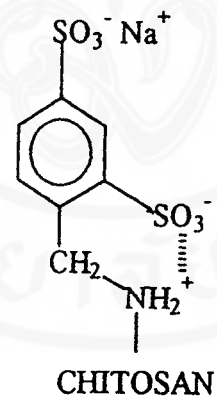


Figure 1.2 Intramolecular interaction between amino and sulfonate groups.

Although the majority of reactions involving chitosan involve the primary amine group, it is possible to modify selectively the hydroxyl groups. The hydroxyl group pendant at C6 is more reactive than that at C3 and is therefore preferentially derivatized.

Through these reactive groups, there are a number of organic reactions resulting in derivatized chitosan, such as sulfation, cyanoethylation, carboxylmethylation, hydroxyethylation (glycolation), xanthation, reaction with alkyl ethers and reaction with carbonyl compounds. Modification of chitosan has resulted in several forms of chitosan which are useful in various areas of applications, such as agricultural, medical, cosmetic etc.

1.1.2.2 Chelation [1, 2]

Chitosan are able to form complexes with many of the transition metals as well as some of the elements in the group 3-7 of the periodic table. The metal-chitosan complexes are formed, as a result of co-ordinate bonding. This involves the donation of the nonbonding pair of electrons from the nitrogen and/or the oxygen to a heavy metal ion.

One of the most useful chelation property of chitosan has been applied in the areas of food preparation, health care, water improvement and pharmaceuticals.

1.1.2.3 Enzymatic reactions [1]

Enzymatic hydrolysis of chitosan to glucosamine is carried out by two hydrolases; chitosanase and lysozyme. Chitosanase are enzymes synthesized by bacteria and fungi. Lysozyme (endo-β-N-acetylmuramidase) have been isolated from a great variety of sources; e.g. mammalian tissues, insects, plants, birds and microorganisms.

One of the most useful properties of chitosan is its biodegradability, as it is composed of glucosamine units found in most mammalian tissues. One of the pathways for breakdown of chitosan is enzymatic degradation, via chitosanase, which breakdown chitosan to dimers and monomers of glucosamine.

1.1.2.4 Solubility [2]

Chitosan is not soluble in water at neutral pH. At acidic pH, the amino groups ($-NH_2$) become protonated to form cationic amine groups ($-NH_3^+$), a soluble form. Chitosan is usually soluble at pH values less than 6 or 5.5. For practical purposes, chitosan is regarded as insoluble in sulfuric and phosphoric acid while certain solubility exists for other mineral acids like hydrochloric, nitric, and perchloric acid and for organic acids like acetic, formic, and propionic acid. Acetic acid is commonly used the reference solvent for solubility of chitosan.

Chitosan has been shown to precipitate a number of compounds such as hyaluronic acid, chondroitin sulfate, heparin, bovine submaxillary gland mucin and nucleic acids from solutions.

1.1.3 Applications of chitosan [4]

The number of published papers on chitosan has increased each decade since the 1930s. A significant number of these papers describe bioapplications of chitosan, such as agriculture, medical, cosmetic, and food-related uses.

This review focuses on various applications of chitosan, as well as current research on its physicochemical properties. Application areas include agriculture, water treatment, analytical chemistry, pharmaceuticals, medicine and cosmetics.

1.1.3.1 Agriculture and food-related use

Chitosan has many potential applications in agriculture because the polymer is essentially naturally occurring and biodegradable; therefore, it should not cause pollution problems. Chitosan is a good cationic polymer for membrane formation, and has been used as a coating of fruit.

Many researchers studied the potential of chitosan as an antibacterial preservative for prolonging the storage of fresh products. Joseph et al. investigated the effect of chitosan as a preservative coating on the postharvest life of tomato, bell pepper and strawberry fruit [5]. They found that chitosan coating reduced the respiration rate of tomato, bell peppers, cucumber and strawberry fruits and the production of ethylene in tomato fruits.

Chitosan has the potential for use in food in order to lower blood serum chloresterol and triglyceride levels [6]. One example is a chitosan-containing cookie, the chitosan being added to the food as part of a manufacturing process. Table 1.1 shows the use of chitosan as a food ingredient which are commercially available in Japan [1].

Table1.1 Foods containing chitosan [1].

Food	Manufacturer	Location
Dietary cookies	Nihon Kayaku, Inc.	Tokyo, Japan
Dietary noodles	Tanmi Foods, Inc.	Nagoya, Japan
Modified vinegar	Nakano, Inc.	Handa, Japan

1.1.3.2 Wastewater treatment

The largest application of chitosan is in the clarification of wastewater. The nitrogen in the amino group of chitosan molecule acts as an electron donor or centre of positive charge, which is presumably responsible for chelating with protein, dye and metal ions. Chitosan is employed for adsorption of dyes from wastewater. Ruey-Shin J. et al. [7] reported of the use of chitosan for removing vinyl sulfone and chlorotriazine reactive dyes from aqueous solution. Chelating toxic metal ions such as Pb (II) [8], Cu (II) [9], Hg (II) [10], Cr (VI) [11] and U (VI) [12] is one of the wastewater treatment application of chitosan.

1.1.3.3 Analytical chemistry

There are many applications of chitosan in analytical chemistry, such as preconcentrator for flow injection and electrochemical technique, or as a polymeric chromogenic reagent. The followings include some examples of applications in analytical chemistry.

Tang Y. et al. [13] studied the ability of chitosan to preconcentrate lead ions at the parts per billion level from 10-50 ml water samples, as

well as the conditions under which the preconcentration proceeds. The techniques have been used successfully to determine ultratrace lead in tap water.

Adriana G et al. [14] developed flow injection procedure comprising in-line concentration and spectrophotometric detection for lead determination in water samples. The preconcentration step is accomplished in a mini-column packed with chitosan. The eluted lead forms a complex with DPPH [2,2-dipyridyl-2-pyridylhydrazone] in an alkaline medium (pH = 11-12) in the presence of the cationic surfactant CTAB, with absorbance at 468 nm.

There are a few electrochemical studies on the analysis of precious metals. It can be used as a modifying reagent is the preparation of chemically modified electrode, because chitosan has a wide range of positive potential with complexing capacity group. It is ideal as the modifying reagent for chemical modified electrode to determine metals such as Au, Ag, Pt, Pd [15] and Pb [16]. Methods have been developed for adsorbing chitosan on to a glassy carbon electrode (GCE) by covalent bonding for use in anodic stripping voltammetry.

Ma HA. et al. [17] synthesized a new polymeric chromogenic reagent by condensing chitosan with chromogen, 7-(4-formylphynylphenylazo)-8-quinolinol-5-sulfonic acid (FPAQS) for spectrophotometric determination of iron (III) in acidic media.

1.1.3.4 Pharmaceutical

The pharmaceutical applications of chitosan have been reviewed, [18]. Recently, chitosan has been employed in pharmaceutical formulation and for drug delivery. Attention has been focused on its absorption-enhancing, controlled release [19, 20] and bioadhesive properties.

1.1.3.5 Medical use

The biomedical applications of chitosan have been summarized in the review article [21]. Chitosan was evaluated in a number of medical applications including use as a potential wound dressing where is was shown that it can enhance wound healing and/or blood clot formation [22, 23, 24].

There are many publications describing the lowering of cholesterol and lipid levels in test animals fed with chitosan [25]. There is a paper showing the lowering of cholesterol and lipid levels in test animals fed with chitosan particles that lead to increase in the cholesterol level of the feces although the mechanism for chitosan decreasing the cholesterol levels is not clearly understood.

1.1.3.6 Cosmetics/Personal care [26]

Chitosan is a non-toxic cationic polymer with excellent adhesive property. As a result, it is used for all kinds of purposes in the field of cosmetics.

The main application of chitosan in cosmetics has been in hair care, with commercial shampoos and conditioners already on the market in Japan, United States and Europe. Chitosan is also being used in both skin and hair applications such as shampoos, hair and skin fixatives, hair conditioners, and bath lotion.

1.1.4 Chitosan in human [1]

When taken orally, chitosan is considered to be essentially indigestible by humans. It is recognized that human do not contain bacteria in the gut flora that possess chitosanases, whereas animals that have, such as fish, hens, and rabbits, are capable of digesting large percentages of chitosan. Low concentrations of lysozyme occur in the saliva and the stomach fluids of humans and the enzyme may degrade chitosan to a small extent. Hydrolytic degradation could also occur in the stomach, which has a pH between 1 and 2.

1.1.5 Chemical and physical characteristics

1.1.5.1 Molecular weight [27]

Molecular weight is one of the basic properties of materials. For small molecules, the molecular weight of a compound is obvious from its chemical formula. For polymer, the molecular weight of the individual macromolecule can vary widely, though the composition remains the same for all molecules, at least within a homopolymer sample. Therefore the average molecular weight should be specified used what average is being considered. In this work,

molecular weight, as determined by a gel permeation chromatography (GPC), is used to characterize the samples.

In GPC, as depicted in Figure 1.3, a dilute polymer solution is injected into the solvent stream, which then flows through a column or series of columns, packed with material of small particle size and controlled pore size (normally cross-linked sturene/divinyl benzene gels or silica), the pore size being comparable to the size of the molecules to be separated. The solvent molecules pass through and around the packing media, carrying the polymer molecules with them. The smaller molecules are able to pass through most of the pores and so have a relatively long flow path through the column. The larger polymer molecules are excluded from all but the largest of pores and hence have a shorter flow path, and elute first. The process relies on there being no interactions between the sample and the packing material. The concentration of the polymer in the eluting solution is continuously detected by measuring the refractive index difference or UV absorbance. The refractometer detects a difference in the refractive index of the eluent stream as the solute elutes from the column.

Calibration curve is the plot between the logarithm of the molecular weight (Mp) against elution volume. Standard polymers of known molecular weights are used for the calibration. Figure 1.4 shows an example of the plot. The linear range represents the working range of the GPC column.

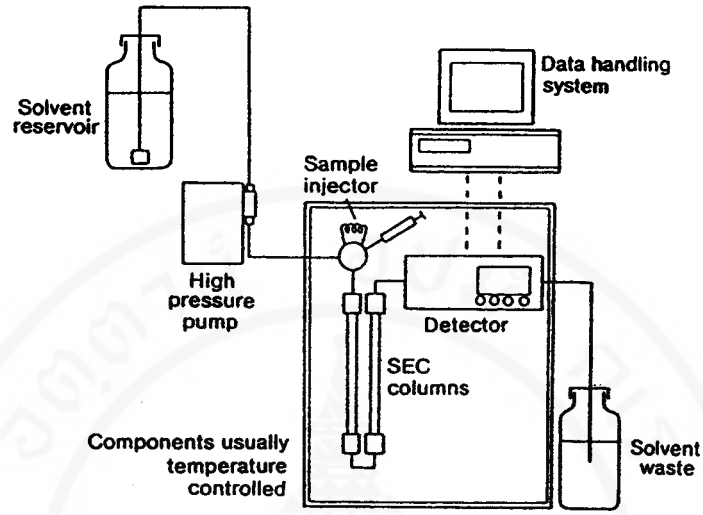


Figure 1.3 Schematic diagram of GPC apparatus.

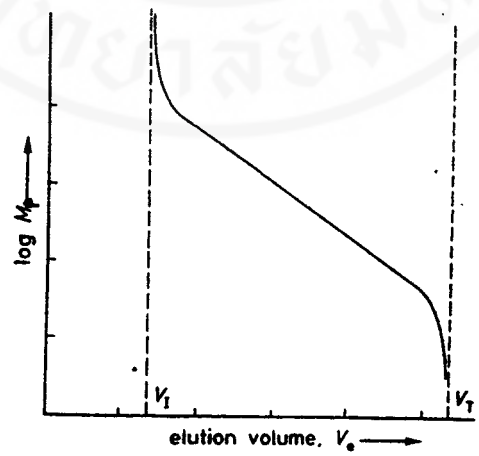


Figure 1.4 Gel permeation chromatography (GPC) calibration curve. V_I = Interstitial volume of the column; V_T = Total volume of solvent in the column.

1.1.5.2 Degree of deacetylation

The degree of deacetylation (DD) is an important chemical characteristics of chitosan that indicates the content of free amino groups i.e., the ratio of 2-acetamido-2-deoxy-D-glucopyranose to 2-amino-2-deoxy-D-glucopyranose structural units [28].

Methods such as infrared (IR) [29], ^{1}H-NMR spectrometry [30], solid-state ^{13}C NMR [31], first-derivative ultraviolet spectrometry [32], ninhydrin test [33], pyrolysis-gas chromatography [34], conductometric titration [35], thermal analysis [36], and enzymatic method [37] have been used for determination of the degree of deacetylation.

DD influences the physical, chemical and biological properties of chitosan such as the tensile strength of the film, ability to chelate metal ions, etc.

1.2 Adsorption on single binding site [38]

The adsorption of a substance from one phase to the surface of another in a specific system leads to a thermodynamically defined distribution of that substance between the phases when the system reaches equilibrium; that is when no further net adsorption occurs. The common manner in which to depict this distribution is to express the amount of substance adsorbed per unit weight of adsorbent, q_e, as a function of the residual equilibrium concentration, C_e, of substrance remaining in the solution phase. An expression of this type, termed and adsorption isotherm, defines

the functional equilibrium distribution of adsorption with concentration of adsorbate in solution at constant temperature. The common expression is the Langmuir equation given below:

$$q_e = \frac{Q^0 b C_e}{1 + b C_e} \qquad (1.1)$$

where q_e is the amount of solute adsorbed per unit weight of adsorbent.

Q^0 is the solid phase concentration corresponding to complete coverage of available sites, or the limiting adsoption capacity.

C_e is the residual liquid phase concentration at equilibrium.

b is constant.

The general shape of Langmuir equation corresponding to plot of q_e and C_e data in Figure 1.5.

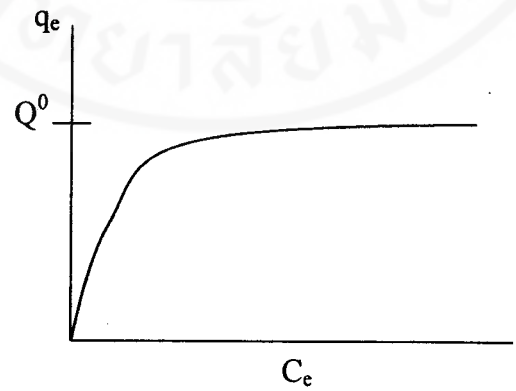


Figure 1.5 Graphical representation of isotherm Langmuir model.

This Langmuir model, originally developed for adsorption of gases onto solids, is predicated on the assumptions that adsorption energy is constant and

independent of surface coverage; that adsorption occurs on localized sites with no interaction between adsorbate molecules, and that maximum adsorption occurs when the surface is covered by a monolayer of adsorbate.

Experimental isotherms are useful for describing adsorption capacity to facilitate evaluation of the feasibility of this process for a given application, for selection of the most appropriate adsorbent, and for preliminary determination of adsorbent dosage requirements.

1.3 Importance of this work and Aims

Iron is one of the essential trace elements in human, and is present in two forms. One is essential body iron, present as hemoglobin in circulating red cells, myoglobin, tissue enzymes and plasma transferrin. The other is storage iron, present as ferritin and hemosiderin. These storage iron are found in the liver, spleen and bone narrow and are mobilized to cells when the body requires iron.

The total body iron is maintained at a constant level of about 4-5 gram by balancing the daily absorption (1-3 mg) with an average daily loss of an identical amount. Thus, if the body absorbs less or more iron than the normal requirement, it will result in iron deficiency or overload.

Iron deficiency is common in both the industrialized and developing populations of the world. A common definition of iron deficiency is anemia with either a circulating hemoglobin level of less than 12 gram per deciliter or packed red

cell volume (hematocrit) of less than 35%. In most cases this is relatively easily treated with iron supplements [39]. In contrast, serious toxic effects which are more difficult to treat can arise as a result of an excess of body iron known as iron overload [40].

Nowadays, chitosan capsules are produced and sold as dietary supplements for reducing the absorption of fats and cholesterol. In Thailand, various brands of chitosans are available. Some preparations are in powder form, some in capsule form. Because chitosan and its derivative are considered potentially safe material for human use and have reported chelation properties (see Section 1.1.2.2) of metals including iron. Chitosan dietary supplement could be used to control iron absorption. However adsorption studies of chitosan, especially of iron, at pH 1 and 2 are rare.

In this work, the capacity of some chitosans in acidic media for adsorption of Fe (II) will be estimated. Hydrochloric acid solution at pH 2 was used as the media.

The results from the capacity studies can be used to evaluate the inhibition of absorption of Fe (II) after administration of chitosan capsules. This will be used for evaluating whether further in vivo study should be carried out.

CHAPTER II

EXPERIMENTAL

2.1 Materials and chemicals

2.1.1 Chitosan sample

Three commercial chitosan samples were used in this work. One sample, designated as chitosan S, was obtained from shrimp shells. The sample is a pure chitosan in non-derivatized form. The sample was sieved through the standard sieves of <45, 125, 300 and 425 μm. Chitosan of each size range was kept in a desiccator before use.

The other two samples were commercially available as food supplements. The samples were designated as chitosan L and chitosan M. These samples were purchased from a local supermarket. Chemical compositions of chitosan L and chitosan M as shown the package label are summarized in Table 2.1.

Table 2.1 Chemical compositions of chitosan L and chitosan M.

Sample	Chemical compositions (% w/w)				
	Chitosan	Fillers			
		Ascorbic acid	Carbohydrate	Fiber	Unknown filler
Chitosan L	67.5	5.0	-	-	27.5
Chitosan M	49.0	-	32.6	18.4	-

According to the suppliers' labels, chitosan L were obtained from crab shells and chitosan M were obtained from crab and shrimp shells.

2.1.2 Chemicals

All chemicals used in this work were of the analytical grade. Table 2.2 contains list of chemicals and their suppliers.

Table 2.2 Lists of chemicals and their suppliers.

Chemicals	Formula	Supplier
Ferrous chloride tetrahydrate	$FeCl_2 \cdot 4H_2O$	Fluka (Buchs, Swizerland)
Stannous chloride dihydrate	$SnCl_2 \cdot 2H_2O$	E. Merck (Darmstadt, Germany)
D-glucosamine hydrochloride	$C_6H_{14}ClNO_5$	E. Merck (Darmstadt, Germany)
N-acetyl D-glucosamine	$C_8H_{15}NO_6$	Sigma-Aldrich (Steinheim, Germany)
Sodium acetate	CH_3COONa	E. Merck (Darmstadt, Germany)
Ninhydrin	$C_9H_6O_4$	E. Merck (Darmstadt, Germany)
Ethylene glycol mono-methyl ether (methyl Cellosolve)	$C_3H_8O_2$	E. Merck (Darmstadt, Germany)
Acetic acid	CH_3COOH	J.T Baker Chemical (Phillipsburg, USA)
Hydrochloric acid	HCl	E. Merck (Darmstadt, Germany)

2.2 Instruments and apparatus

2.2.1 Instruments

2.2.1.1 Flame Atomic Absorption Spectrometer (FAAS)

A Perkin Elmer atomic absorption spectrometer, model 3100 (USA) was used to determine Fe (II) concentration for the adsorption experiments (Section 2.4.2).

2.2.1.2 UV-Visible Spectrophotometer

The concentration of N-acetyl glucosamine in chitosan samples and all of reagents for first-derivative UV spectrometry studies were determined by using a JASCO UV-Vis spectrometer, UVIDEC-650 (Japan).

2.2.2 Apparatus

2.2.2.1 Sieve

Endoecotts test sieves (England) with the apertures of 45, 125, 300 and 425 μm were used for sieving chitosan samples on a Retsch 5657 sieve shaker.

2.2.2.2 Centrifuge

A Hettich Universal II centrifuge, model D7200 (Germany) was used to separate chitosan samples from the supernatants after equilibration.

2.2.2.4 Incubator shaker

A Gyrotory shaker (USA) was employed for kinetic and equilibration studies of Fe (II) binding. A fixed temperature of 37°C was used throughout the experiments.

2.2.2.5 pH measurement

A Fisher Scientific pH meter, Model 955, with a combination glass electrode (USA) was used for all pH measurements. Commercial standard buffers, pH 4.01 and 7.00, from E. Merck (Darmstadt, Germany) were employed for the instrument calibration.

2.3 Preparations of reagents and standard solutions

The followings sections describe the preparation procedures of standard solutions and other solutions employed in this work.

2.3.1 Hydrochloric acid solutions (0.01 and 0.1 M)

A stock solution of 5 M hydrochloric acid was prepared by diluting 310 ml of 37% HCl with deionized-distilled water and made up to 1000.00 ml in volumetric flask. The stock solution was then diluted to the desired concentrations of 0.01 and 0.1 M HCl by appropriate dilution with deionized water.

2.3.2 Iron (II) solution for adsorption studies.

Fe (II) solution was prepared using ferrous chloride tetrahydrate for adsorption studies. An accurately weighed ferrous chloride of 1.780 g was dissolved

with 0.01 M hydrochloric acid and made up to 250.00 ml with 0.01 M HCl in a volumetric flask to give 2000 mg l^{-1} of Fe (II). This stock solution was then diluted to different concentrations of Fe (II) (from 10 to 1,800 mg l^{-1}) which were used for studying adsorption of Fe (II) on chitosans.

Only freshly prepared Fe (II) solutions were used.

2.3.3 Iron (II) solutions for FAAS calibration.

Fe (II) solutions, used in determination of Fe (II) concentration by FAAS were diluted from the same solution of ferrous chloride solution (1,000 mg l^{-1}), described in the above Section. 10.0 ml of the 1,000 mg Fe (II) l^{-1} was diluted to 100.0 ml with 0.01 M HCl to give 100 mg l^{-1} of Fe (II). The determination of Fe (II) concentration in the adsorption studies (Section 2.4.2) was carried out using standard addition method. For the addition, the 100 mg Fe (II) l^{-1} solution was added to the sample solution to give the concentration of standard added of 0.5, 1 and 1.5 mg l^{-1}, respectively. Appropriate dilutions were made using 0.01 M HCl.

2.3.4 Ninhydrin reagent

The preparation of this reagent was carried out using the method of Leggett B. [41].

Approximately 2 g of ninhydrin was dissolved in 50 ml of ethylene glycol mono-methyl ether (Methyl Cellosolve) in a beaker. To the beaker, approximately 25 ml of 4.0 M sodium acetate buffer (pH 5.5) and 0.08 g of stannous chloride dihydrate ($SnCl_2 \cdot 2H_2O$) were added. The solution mixture was made up to 100.00 ml with deionized-distilled water in a volumetric flask.

Only freshly prepared ninhydrin reagent was used.

2.3.5 Sodium acetate buffer (4.0 M, pH 5.5)

Approximately 54.4 g of sodium acetate was added to 23 ml of 99.9% (w/v) acetic acid until the pH at 5.5 was reached. This solution was made up to 100.00 ml with deionized water in a volumetric flask.

This solution was used in the ninhydrin test.

2.3.6 N-acetyl-D-glucosamine (1,000 mg l^{-1})

N-acetyl-D-glucosamine solution of approximately 1,000 mg l^{-1} was prepared by dissolving 0.1000 g of N-acetyl-D-glucosamine in 100.00 ml with 0.01 M acetic acid. This solution was used as a reagent in determination percentage of degree deacetylation by first-derivative UV spectrometry.

Standard solutions of N-acetyl-D-glucosamine at the desired concentrations were prepared by appropriate dilution of this stock. The concentration range of the solution for calibration curve was from 5 to 40 mg l^{-1}.

2.3.7 D-glucosamine (1,000 mg l^{-1})

The stock D-glucosamine was prepared in 0.01 M acetic acid by dissolving 0.1256 g of D-glucosamine hydrochloride with 0.01 M acetic acid. This solution was made up to 100.00 ml in a volumetric flask to give 1,000 mg l^{-1} of D-glucosamine.

Standard solutions of D-glucosamine at the desired concentrations were prepared by appropriate dilution of this stock. These solutions were used in the

correction of the effect of D-glucosamine on signal height (H value) of N-acetyl-D-glucosamine in determination of percentage of degree deacetylation by first-derivative UV spectrometry (Section 2.4.1.5 b).

2.4 Procedures

The followings include the procedures for studying some characteristic properties of chitosans and the procedures for adsorption studies of Fe (II).

2.4.1 Measurement of some characteristic properties of chitosan.

Three samples of chitosan including, chitosan S, chitosan L and chitosan M, were studied for the following characteristic properties.

2.4.1.1 Particle size distribution

Particle size distribution was obtained by sieving the chitosan samples (60°C dried) through Endoecotts sieves (apertures 45, 125, 300 and 425 μm) using a Retsch 5657 sieve shaker. The chitosans falling into each size range was then accurately weighed. Percentages of size distribution were calculated based on the weight collected for each size range over the total weight.

2.4.1.2 Molecular weight

Molecular weights of three chitosan samples were determined by Gel Permeation Chromatography (GPC) using a PL-GPC 110 (England) with refractometer as detector. The column used was packed with ultralinear hydrogel.

An aqueous solution containing 0.5 M acetic acid and 0.5 M sodium acetate (1:1 by volume) was used as the eluent in the chromatography.

Three samples of chitosan were dissolved in the above eluent and filtered through 0.45 μm Nylon filter. The calibration was carried out using standard Pullulan which is a polysaccharide having molecular weight ranges of 5.80 x 10^3 to 1.66 x 10^6 Dalton. The operating conditions were set as following;

Injection volume 100 μl

Flow rate 0.6 ml/min

Temperature 30 °C

This analysis was carried out by scientist at the National Metal and Materials Technology Center (MTEC). The results of these measurements are discussed in Section 3.1.2 and summarized in Table 1A, Appendix I.

2.4.1.3 Solubility in water, 0.01 and 0.1 M HCl.

The solubilities of all chitosans used were determined in two concentrations of hydrochloric acid, 0.01 and 0.1 M HCl. In addition, solubilities in deionized-distilled water were also investigated.

The weight of three chitosan samples varied from 0.01 to 0.25 g. To an amount of sample in a centrifuged PTFE tube, 25.0 ml of, either 0.01 M or 0.1 M HCl was added. All samples were shaken for two hours at room temperature. The supernatant was then separated from the chitosan.

Rinsing solutions were tested for presence of chloride ion with a solution of 0.1 M $AgNO_3$. After rinsing, the residues of chitosans were dried in an oven at 60°C until constant weight. Determination of the solubility was carried out

using this constant weight and calculated as gram per 100 ml test solution. The general procedure for this solubility test is shown in the diagram in Figure 2.1.

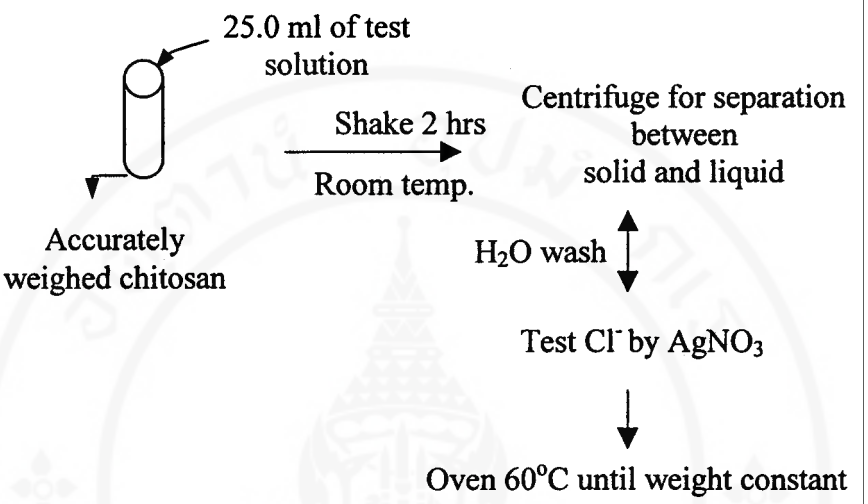


Figure 2.1 General procedure for measuring the solubility in some test solutions (0.01 M and 0.1 M HCl).

2.4.1.4 Characteristic spectra of chitosans by infrared and solid-state ^{13}C nuclear magnetic resonance technique

Characteristic spectra of the three chitosans for both infrared spectrometry and solid-state ^{13}C NMR were obtained as follow:

Infrared spectrometry

Approximately 2 mg chitosan samples were ground with 150 mg KBr in agate mortar. The mixture was compressed at 7 tons for 10 minutes to give

the KBr disc for IR measured. The IR spectra are depicted in Figure 3.3, Section 3.1.3.

Solid-state ^{13}C NMR

Solid-state ^{13}C NMR (Crossing Polarized Magic Angle Spin, CP-MAS) was used to examine the presence of six glucose carbons in the three chitosans. The spectra were obtained using a Bruker 300 MHz NMR spectrometer (Germany). The equipment was operated by scientist at the National Metal and Materials Technology Center (MTEC). In each run, approximately 50 mg of a solid sample was inserted into a 5 mm diameter probe. The solid-state ^{13}C NMR spectra are depicted in Figure 3.4, Section 3.1.3.

2.4.1.5 Degree of N-deacetylation

Three chitosan samples, chitosan S, chitosan L and chitosan M, were washed by deionized-distilled water until clean (see Appendix II for the procedure of cleaning) before determining degree of deacetylation. The followings include the procedures for determination of degree of N-deacetylation, by ninhydrin test, first-derivative UV spectrometry, infrared and solid-state ^{13}C NMR.

a) By ninhydrin test [33]

A stock solution of D-glucosamine (7×10^{-4} M) was prepared by dissolving 0.01 g of D-glucosamine in 100.00 ml 2% acetic acid. Standard solutions were prepared by pipetting 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the glucosamine solution into separate 10.0 ml volumetric flask. 1.5 ml of acetic/acetate buffer (pH

5.5, 4 M) was added to each flask. Finally, 6 ml of ninhydrin reagent (see Section 2.3.7) was added to each volumetric flask and the volume was made up to 10.00 ml with 2% acetic acid. All solutions were transferred to separate glass test tubes. All solutions were heated in boiling water for 10 minutes. The solutions were allowed to cool for 15 minutes before measuring the absorbance at 570 nm. The calibration curve was obtained by plotting the absorbance against the concentration of standard solutions.

Sample solutions consisted of 0.01 g of chitosan samples dissolved in 100.00 ml of 2% acetic acid. 2.0 ml of the chitosan sample solution was pipetted into separate volumetric flask to which was added the buffer, made up with 2% acetic acid and the ninhydrin reagents as above. The solution was heated as above and the absorbance value was recorded. The amount of chitosan in the sample was determined from the calibration curve of the standard glucosamine. Determination of the $-NH_2$ group on the glycoside repeat unit was performed in triplicate for each sample.

All procedure for the standard and sample were carried out at the atmospheric environment without using the N_2-gas.

Calculations of percentages of degree of deacetylation on chitosan samples are based on the equation 2A, Appendix II. The results determined on the three samples are summarized in Table 3A, Appendix II. Discussion of the use of the method for determination of degree of N-deacetylation are given in Appendix II (a).

b) First-derivative UV spectrophotometry [32]

Correction of the effect of D-glucosamine on signal height

A fixed concentration of 10 mg l^{-1} standard N-acetyl glucosamine in 0.01 M acetic acid was used. Various concentrations of D-glucosamine were added to twelve portions of this solution from 200, 150, 100, 90, 70, 50, 40, 30, 25, 20, 10 and 0 mg l^{-1} to give 4.8, 6.3, 9.1, 10, 12.5, 16.7, 20, 25, 28, 33, 50 and 100% (w/w) of N-acetyl glucosamine, respectively. These reagents were diluted from 1,000 mg l^{-1} standard solutions. Each solution mixture was made up 10.0 ml with 0.01 M acetic acid.

First-derivative spectra were obtained in the wavelength range of 190-300 nm. The reference curve was obtained by plotting H_1/H_2 against the corresponding N-acetyl glucosamine percentage where H_1 is signal height of the pure N-acetyl glucosamine solution and H_2 is signal height of the solutions of different percentages of N-acetyl glucosamine. The wavelength of 202 nm was for the measurement.

Calibration curve

N-acetyl glucosamine standards used for calibration, containing 5, 10, 15, 20, 25, 30 and 40 mg l^{-1} in 0.01 M acetic acid were prepared from 1000 mg l^{-1} N-acetyl glucosamine These aliquots were then diluted with 0.001 M acetic acid. Their first derivative spectra were carried out. A linear calibration curve was obtained by plotting the signal height (H value) at 202 nm against the corresponding N-acetyl glucosamine concentration.

Determination of the degree of deacetylation of chitosan samples

0.01 g of chitosan samples were dissolved in 10 ml of 0.1 M acetic acid solution and made up to 100.00 ml with distilled water. Three replicates of the first derivative spectra were performed on the three samples. The H values of the chitosan samples were measured at 202 nm and the contribution due to N-acetyl glucosamine was obtained from the calibration curve. The percentages of degree of deacetylation of chitosan samples were calculated by equation 3A in Appendix II (see formula in Note 2A, Appendix II). This method requires the use of reference curve as described in Figure 6A, Appendix II.

c) Infrared Spectrometry [29]

IR spectra of three chitosans were obtained using the Fourier Transform Infrared (FTIR) spectrometer as described in Section 2.4.1.4. The experiment was performed in duplicate for a sample.

Domzy J.G. and Roberts G.A.F. [29] proposed the determination of the degree of N-deacetylation of chitosan by infrared spectroscopic technique. The percentage of free amine groups was obtained by a linear relationship between the amide I band at 1655 cm^{-1} and hydroxyl band (internal standard) at 3450 cm^{-1} as shown in the equation 15A, Appendix II

The absorption band at 3450 cm^{-1} was determined from 3837 to 1902 cm^{-1} as an internal standard. The absorbance of the amide I band at 1655 cm^{-1} was calculated from 1902 to 1277 cm^{-1}. The ratio of 1.33, using the base lines indicated in Figure 2.2 of fully N-acetylated chitosan and A_{1655} maximum absorption values of hydroxyl band (A_{3450}) was obtained.

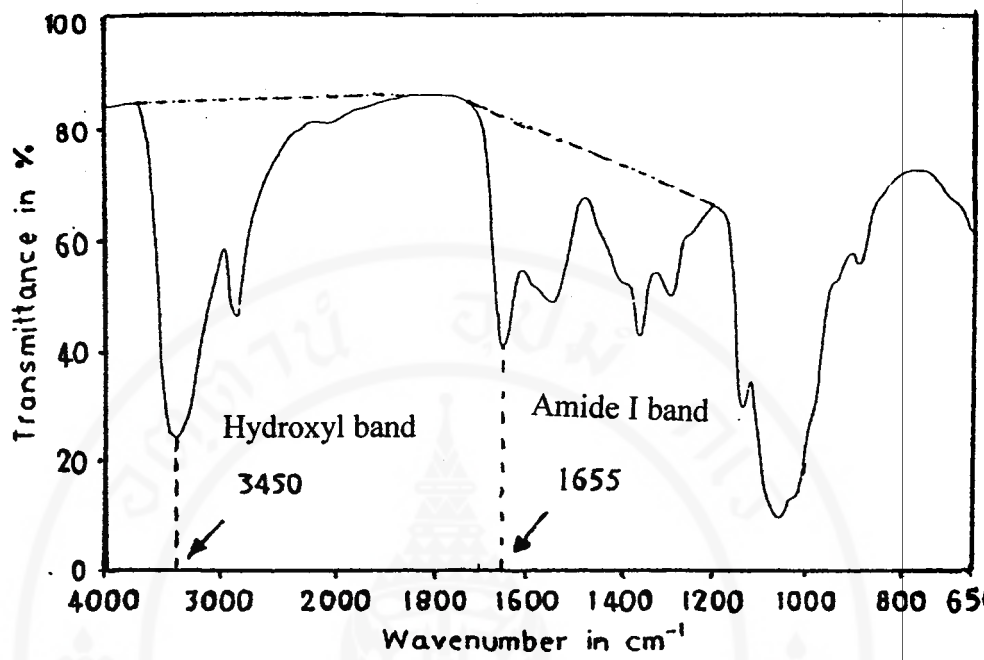


Figure 2.2 IR spectrum of partially N-acetylated chitosan showing the base lines used in determining the absorbance values A_{1655} and A_{3450} for the 1655 cm^{-1} and 3450 cm^{-1} bands [29].

The IR measurement was carried out by scientist at the Department of Chemistry, Faculty of Science, Mahidol University.

d) Solid-state ^{13}C Nuclear Magnetic Resonance [31]

Solid-state ^{13}C NMR (Crossing Polarized Magic Angle Spin, CP-MAS) was used in determination of the degree of deacetylation and performed using a Bruker 300 MHz NMR spectrometer (Germany) by scientist at the National Metal and Materials Technology Center (MTEC). Approximately 50 mg of chitosan samples was inserted into a 5 mm diameter probe.

Figure 2.3 depicts the ^{13}C NMR spectra obtained from a partially N-acetylated chitosan. The degree of N-deacetylation of chitosan was calculated using the area of the CH_3 resonance of the acetyl group (labeled as methyl) and the resonance of the glucose carbons (labeled C_1-C_6) as shown in equation 16A., Appendix II.

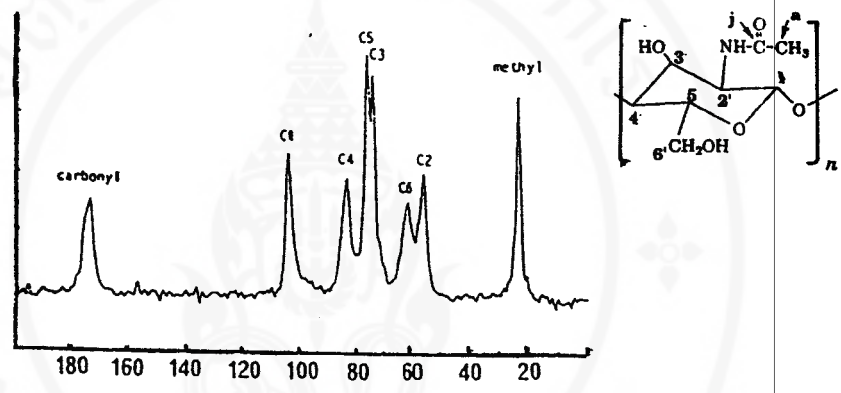


Figure 2.3 Solid-state ^{13}C NMR spectrum of partially N-acetylated chitosan (6% deacetylated) [42].

2.4.2 Adsorption studies of iron (II) on chitosans

2.4.2.1 Kinetic studies

The main purpose of the kinetic study is to determine the time required for adsorption to reach equilibrium.

For this experiment, the main fraction of chitosan S, that is > 300 μm, was used. Chitosan L and M were used as received after removing the capsules (45-300 μm). A fixed concentration of 600 mg l^{-1} Fe (II) in 0.01 M HCl was used for each kinetic run.

The kinetic experiments were carried out in 125 ml stoppered conical flasks, each containing 0.25 g of accurately weighed chitosan and 25.0 ml of Fe (II) solution. The mixture was shaken in an incubator shaker at 37 °C.

The contact time was varied from 0.25 to 4 hours. The mixtures were centrifuged in PTFE tubes. The supernatant was then separated from the chitosans. Determination of the initial and the residual concentrations of Fe (II) in solution were carried out using Flame Atomic Adsorption Spectrophotometry (FAAS) by standard addition. The adsorptivity of Fe (II) was calculated from these concentrations as mg Fe adsorbed per gram chitosan. The calculation of adsorptivity of Fe (II) on chitosan are shown by the formula below:

$$Adsorptivity = \left[\frac{Ci - Ce}{1000}\right] x \left[\frac{V}{W}\right] \qquad (2.1)$$

where Ci is initial concentration of Fe (II) solution (mg l^{-1}).

Ce is residual concentration of Fe (II) solution (mg l^{-1}).

V is volume of Fe (II) solution (ml).

W is weight of chitosan used (g)

pH of each supernatant was measured using a combined-glass electrode and a pH meter. The procedure of kinetic studies are shown as a diagram in Figure 2.4.

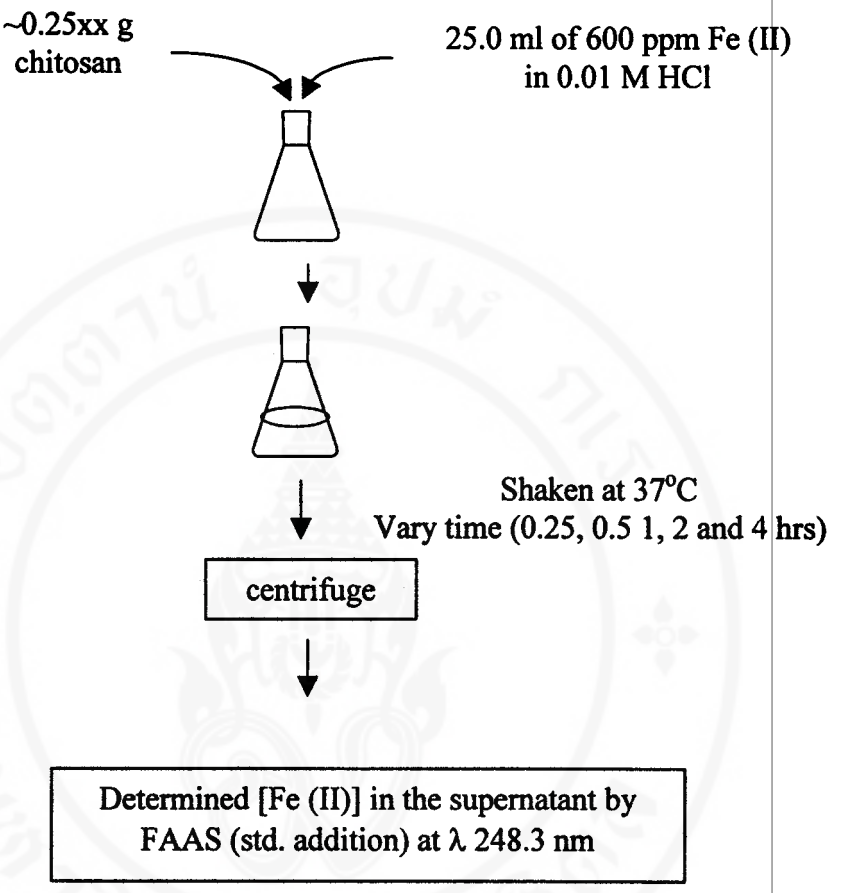


Figure 2.4 Diagram illustrating the procedure of kinetic studies of Fe (II) adsorption.

2.4.2.2 Isotherm studies

Adsorption capacities of Fe (II) were studied for chitosan S, chitosan L and chitosan M. A fixed weight of chitosan S sieved for 125-300 μm (0.1 g) and >300 μm (0.25 g), were added to 25.0 ml Fe (II) solutions in a 125 ml stopped conical flask. Chitosan L and chitosan M, after removing from the capsules, were homogeneously mixed and used at a fixed weight of 0.25 g. Initial concentrations of Fe (II) solution were between 10 to 1800 mg l^{-1} in 0.01 M HCl. All isotherm experiments were carried out in triplicate at 37°C after two hours contact time. The

initial concentrations (Ci) and the residual concentrations (Ce) of Fe (II) ion in solution were determined using FAAS by standard addition. Adsorption isotherms of the three samples were drawn by plotting the adsorptivity (calculated as mg Fe adsorbed per gram chitosan) against residual concentration (Ce). The calculations of adsorptivity of Fe (II) on chitosan are shown by the formula as describe in equation 2.1, Section 2.4.2.1. All data points derived from the triplicate experiments were plotted on the same graph for one isotherm. The procedure for isotherm studies are shown as the diagram in Figure 2.5.

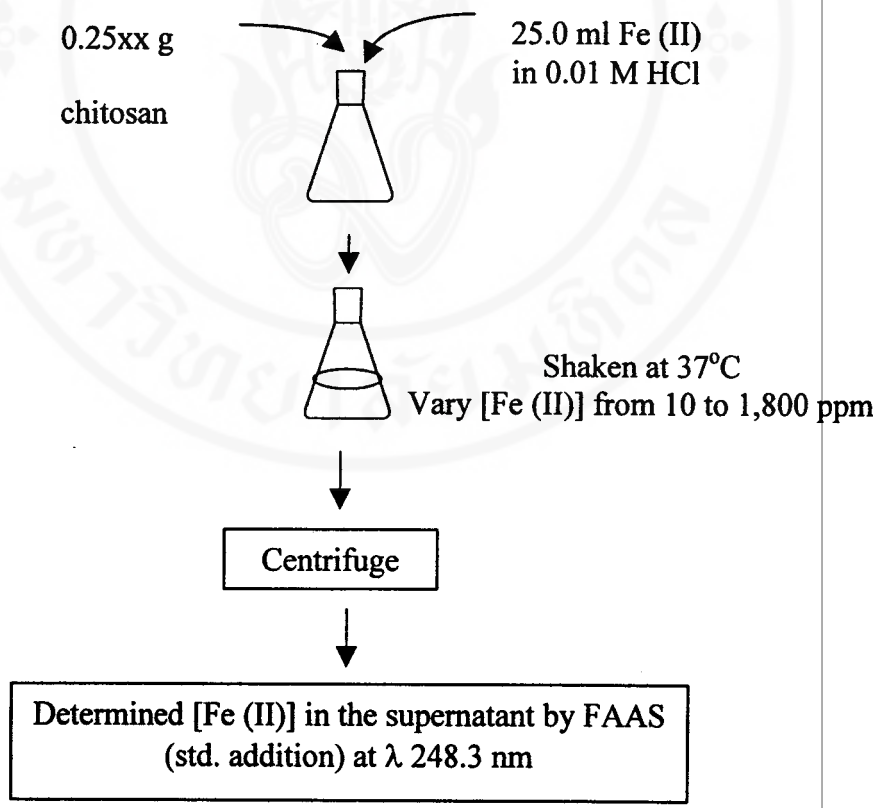


Figure 2.5 Diagram illustrating the procedure of adsorption isotherm studies of Fe (II) adsorption.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Characterization of chitosans

Three samples of chitosan (chitosan S, chitosan L and chitosan M) were studied for their characteristic properties. The following properties were measured as reported in Sections 3.1.1 to 3.1.4.

3.1.1 Particle size distribution

Particle size distribution was obtained by sieving the chitosans through Endicott sieves with apertures 45, 125, 300, and 425 μm, using a mechanical shaker. Percentages of size distribution were calculated based on the weight collected for each size range over the total weight. The results are shown in Table3.1 and Figure 3.1.

Table 3.1 Particle size distribution of chitosan S, L and M.

Particle size	%weight (w/w)		
(μm)	Chitosan S	Chitosan L	Chitosan M
>425	74.0	4.45	2.27
300-425	14.0	16.6	7.66
125-300	9.4	64.8	27.5
45-125	2.48	12.4	33.7
<45	-	1.9	28.9

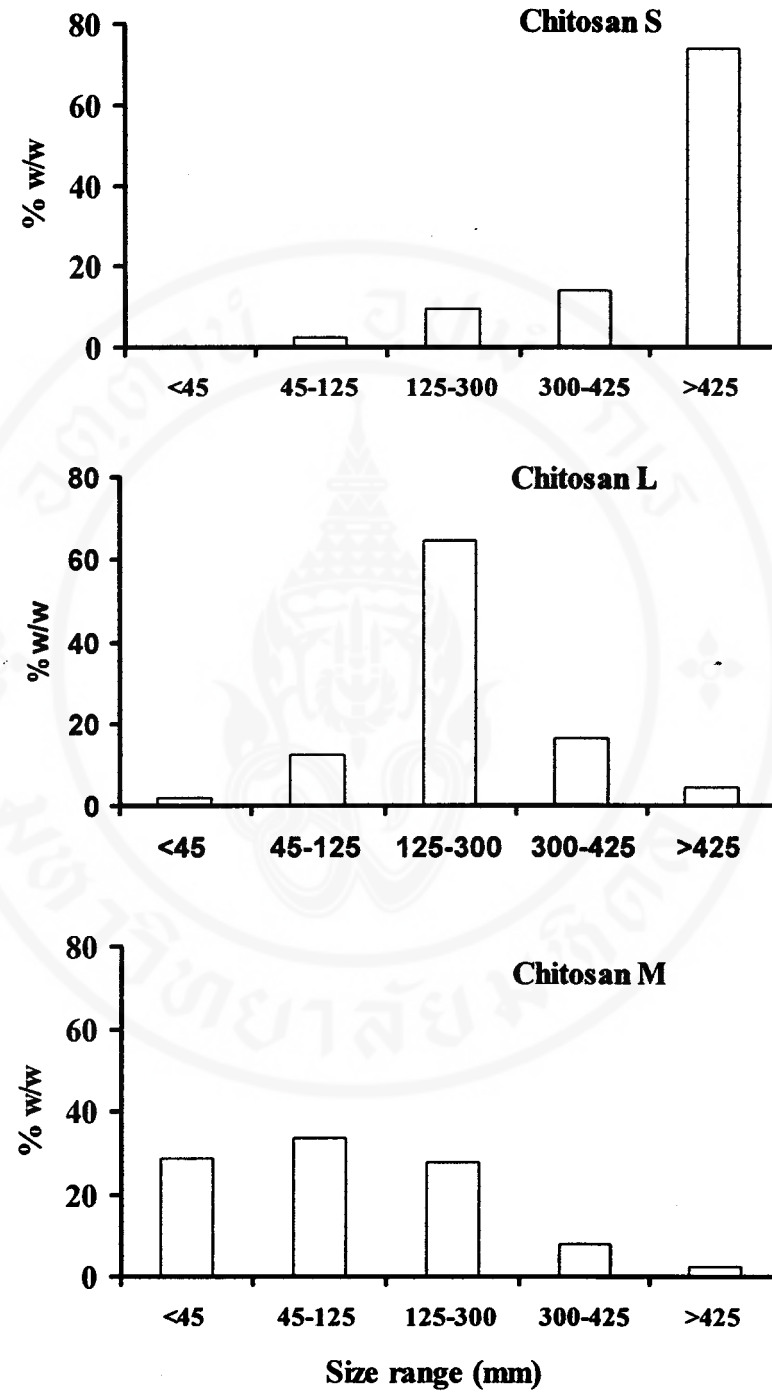


Figure 3.1 Particle size distribution of chitosan S, L and M.

Note: Chitosan L and M were sieved without separation between chitosan and fillers.

Results in Figure 3.1 show that chitosan S and chitosan L namely had particle sizes, of >425 and 125-300 μm, respectively. Particle size range of chitosan M varied from 45 to 300 μm. However, effect of particle size on adsorption was carried out only on two particle size ranges;using >300 and 125-300 μm for chitosan S. Adsorption studies were carried out on chitosan L and chitosan M as homogeneous mixture as obtained from the capsules.

3.1.2 Molecular weight

Molecular weights of three chitosans were determined by Gel Permeation Chromatography (GPC) as described in Section 2.4.1.2. The experiments were performed in duplicate for each sample. GPC chromatogram, calibrations curve and the results (retention time and GPC molecular weight of samples, Mp) are presented in Appendix I. The results are shown as bar chart in Figure 3.2.

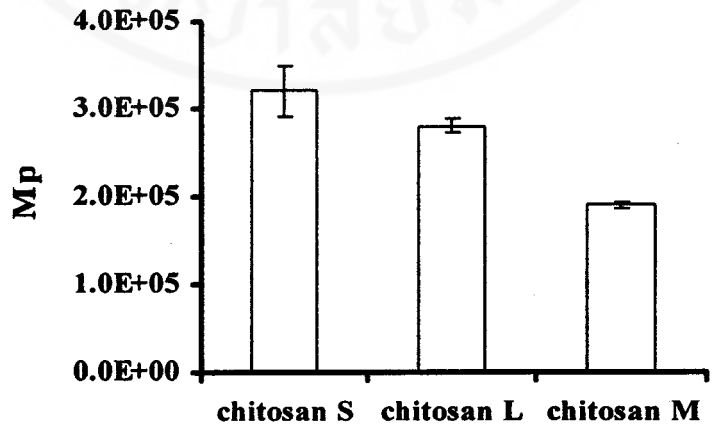


Figure 3.2 Bar graph of average molecular weight of chitosan S, L and M obtained from GPC method.

Note: Chitosan L and M were studied without separation of chitosan and fillers.

The results of GPC molecular weight in Figure 3.2 indicated that all chitosan samples have the molecular weight of the order of 10^5. The order of molecular weight is chitosan S > chitosan L > chitosan M.

3.1.3 Characteristic spectra of chitosans by infrared and solid-state ^{13}C nuclear magnetic resonance techniques

IR spectra and solid-state ^{13}C NMR spectra of three chitosans were obtained by using a Fourier Transform Infrared (FTIR) spectrometer and a Bruker 300 MHz NMR spectrometer respectively (Section 2.4.1.4). The experiments were performed in duplicate for each sample. The IR spectra and the solid-state ^{13}C NMR spectra of all samples are shown in Figure 3.3 and Figure 3.4 respectively. The major absorption bands (Figure 3.3) such as hydroxyl and amide I (C=O) are presented for all samples.

Solid-state ^{13}C NMR spectra (Figure 3.4) show characteristic chemical shifts (ppm) of the six glucose carbons (C_1 to C_6), the methyl carbon of the acetyl group and the carbonyl carbon (C=O) of the acetyl group. It was observed that these two latter signals were very weak for all chitosans, in comparison to the sample shown in Figure 2.3 due to the high degree of deacetylation (DD) of the samples (see discussion of the DD in Section 3.1.4).

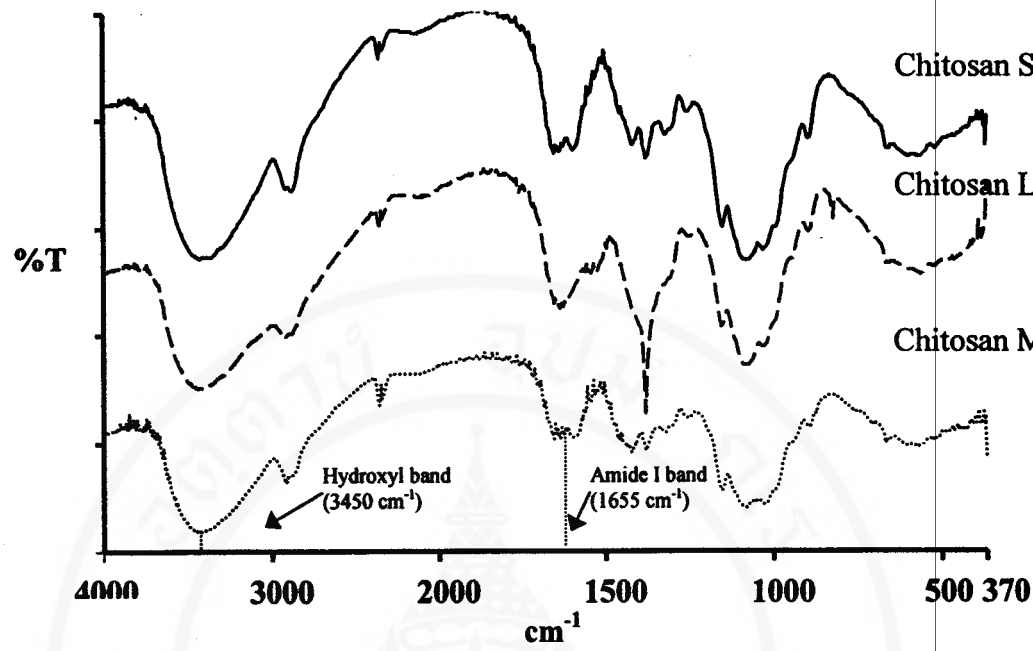


Figure 3.3 IR spectra of chitosan S, L and M.

Note: Chitosan S, L and M were employed after washing (separation between chitosan and fillers).

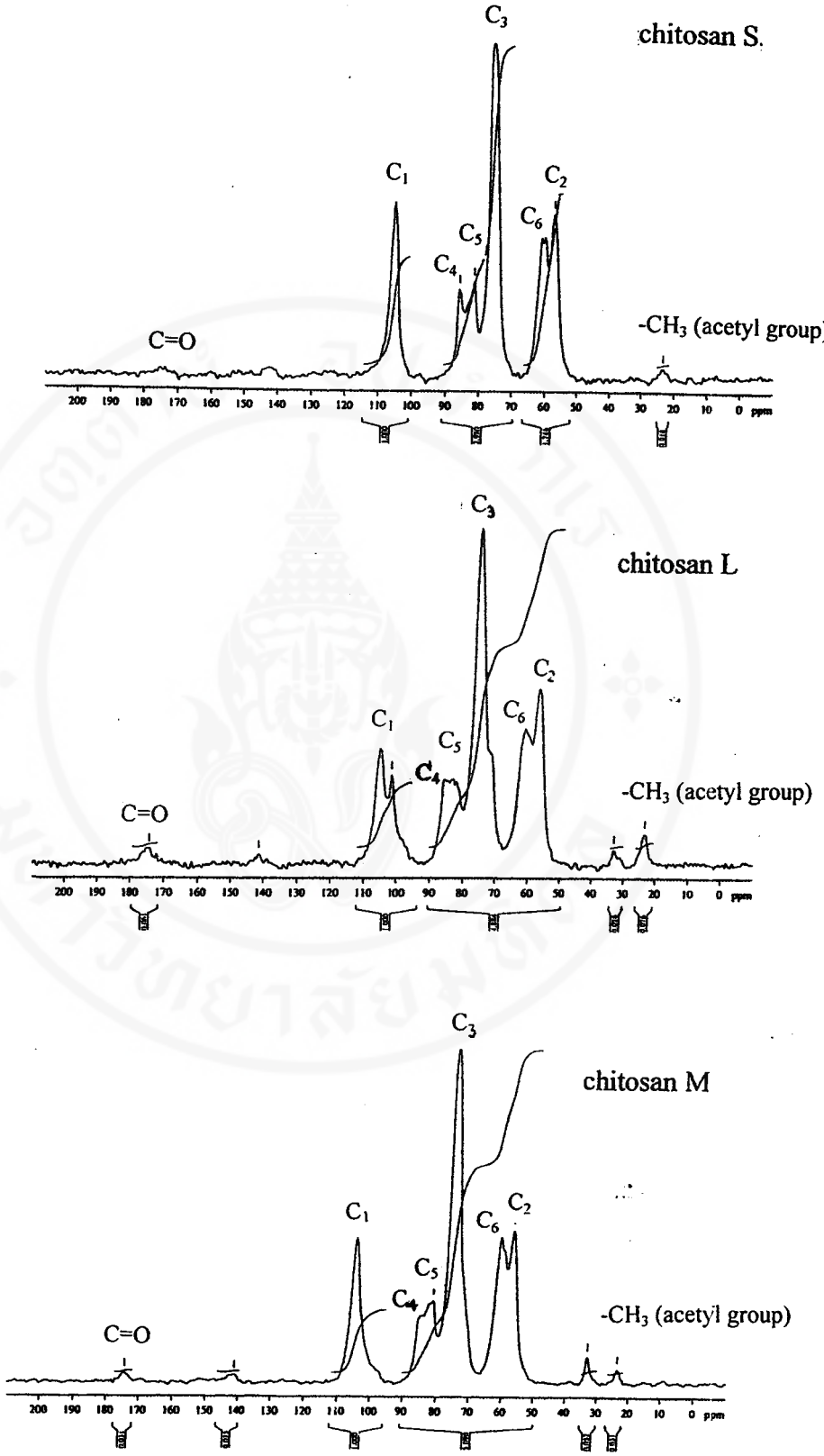


Figure 3.4 Solid-state ^{13}C NMR spectra of chitosan S, L and M.

Note: Chitosan S, L and M were employed after washing (separation between chitosan and filers).

3.1.4 Degree of N-Deacetylation

The degree of deacetylation of chitosan has some effect on their physical and chemical properties. There are several methods available for determination of degree of N-deacetylation of chitosan. In this work, three samples of chitosan (chitosan S, L and M) were subjected to determination of the %deacetylation after elimination of fillers (see Appendix II for this procedure).

Determination of the percent deacetylation was carried out using four methods, using ninhydrin test, first-derivative UV spectrometry, infrared spectrometry and solid-state ^{13}C nuclear magnetic resonance. Besides solid-state ^{13}C NMR and IR techniques, the percent deacetylation for all methods were calculated based on a per mol basis. Descriptions of the calculation of percent deacetylation for ninhydrin and first-derivative UV spectrometric methods and raw data of each method are in Appendix II. The details of procedure were described in Section 2.4.1.5. Comparisons of degree deacetylation of all chitosan samples using those four techniques are shown in Table 3.2.

Table 3.2 Percentage of degree of N-deacetylation of chitosans determined by four methods.

Sample	degree of N-deacetylation (%)			
	Ninhydrin test[a] (n=3)	First derivative UV-spectrometry[a] (n=3)	Solid-state ^{13}C NMR (n=2)	Infrared spectrometry (n=2)
Chitosan S	27.9 ± 4.4	93.4 ± 0.1	98.3 ± 0.1	91.8 ± 0.1
Chitosan L	23.7 ± 2.8	91.1 ± 0.5	91.7 ± 0.7	85.1 ± 0.9
Chitosan M	10.4 ± 2.9	93.7 ± 0.2	97.2 ± 0.3	92.1 ± 0.2

[a] Calculation was based on per mol basis.

In the cases where degree of deacetylation were calculated on the per mol basis, using ninhydrin test and first-derivative UV spectrometry, it was found that the results from the latter method are much greater than from the ninhydrin test. This finding agree with the work reported by Khor E. et al. [43]. It was found also that the intensity of color faded with time after boiling step. This could cause error of measurement. The results of this method will be discarded for further discussion.

The results of IR and solid-state ^{13}C NMR were calculated in similar way based on the ratio of one indicator signal against the internal standard. The results of both agree well with one another.

Results in Table 3.2 indicate that the degree of deacetylation as obtained from first- derivative UV spectrometry, infrared spectrometry and solid-state ^{13}C NMR correlate well with each other. The order of degree of deacetylation of chitosan samples is: chitosan S $\approx$ chitosan M > chitosan L. The values of all three techniques are mostly above 90% which means that all samples contain very small number of acetyl groups on the amine sites.

3.2 Adsorption studies of iron (II) on chitosans

This study of Fe (II) adsorption deals with two aspects of adsorption, using kinetics and adsorption capacity. Adsorption capacity on chitosan was measured at two hours.

This section reports the adsorption kinetics and isotherms of Fe (II) on the three samples of chitosan, namely, chitosan S, chitosan L and chitosan M.

3.2.1 Kinetic studies

In this experiment, the main fraction of chitosan S (>300 μm) was used. Chitosan L and M were used as received after opening the capsules (45-400 μm).

Approximately 0.25 g of chitosans were accurately weighed and stirred at 37°C with 25.0 ml of 600 mg l^{-1} Fe (II) solution in 0.01 M HCl. The contact time varied from 0.25 to 4 hours. Determination of the initial and the residual concentrations of Fe (II) in solution were carried out using Flame Atomic Absorption Spectrometry (FAAS) by standard addition. The adsorptivity of Fe (II) was calculated from these concentrations as mg Fe adsorbed per gram chitosan. The results of Fe (II) adsorption for the three samples of chitosan measured at different contact times are shown in Figure 3.5.

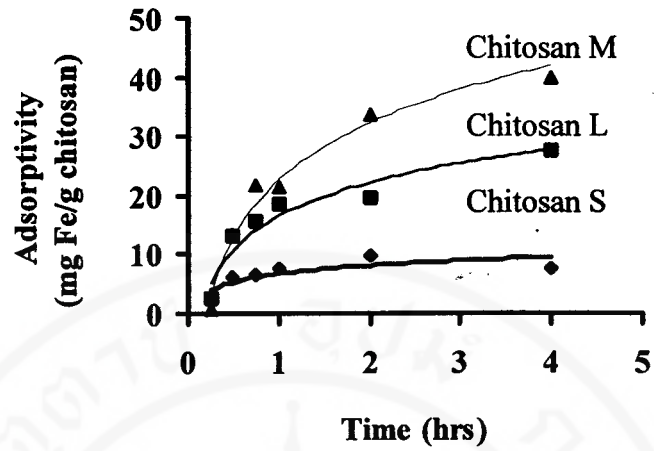


Figure 3.5 Adsorption kinetics of Fe (II) on chitosan S, L and M. The experiments were carried out using the initial Fe (II) concentration of 600 mg l^{-1} in 0.01 M HCl; 37°C.

Note: Chitosan S, L and M were obtained without separation of chitosan from fillers.

The kinetic studies of chitosan S showed that the adsorption approached equilibrium after two hours contact time. Both chitosan L and chitosan M required longer contact time than four hours to reach the adsorption equilibrium.

Thus, within two hours of gastric digestion, the capacity of adsorption of Fe (II) on some chitosans may not be the maximum value. However, for the adsorption experiment, the capacities measured at two hours contact time to imitate the time of gastric digestion.

3.2.2 Adsorption of iron (II) on chitosans

Adsorption capacities of Fe (II) were studied on chitosan S, chitosan L and chitosan M. A fixed weight of chitosan S, sieved to, 125-300 μm (0.1 g) and > 300 μm (0.25 g), were added to Fe (II) solutions. Chitosan L and chitosan M, after taken out from capsules, were homogeneously mixed and used at a fixed weight of 0.25 g. Initial concentrations of Fe (II) solution were between 10 to 1800 mg l^{-1} all of which were prepared in 0.01 M HCl. All isotherm experiments were carried out in triplicate at 37°C for two hours. The initial concentrations (Ci) and the residual concentrations (Ce) of Fe (II) ion in solution were determined using FAAS by standard addition. Adsorption isotherms of the three samples were drawn by plotting the adsorptivity, calculated as mg Fe adsorbed per gram chitosan, against residual concentration (Ce). All data points derived from the triplicate experiments were plotted on the same graph for one isotherm. The isotherms are depicted in Figure 3.7 (chitosan S), Figure 3.8 (chitosan L and chitosan M). The program ENZFITTER was used for non-linear least square fitting and the results summarized in Table 3.3 and Figure 3.6.

It is observed from all of the isotherms, shown in Figure 3.7 and 3.8 that most data points of a chitosan did not lie on the fitted Langmuir curve. This could possibly be due to difference in conditions during each course of experiment.

Table 3.3 Adsorption capacities for Fe (II) adsorption on chitosan S (125-300 μm and >300 μm), chitosan L and chitosan M The capacities were measured under constant temperature of 37°C where the media is 0.01 M HCl.

Adsorption capacity[a] ± error[b] (mg Fe/g chitosan)	Chitosan S		Chitosan L	Chitosan M
	> 300 μm	125-300 μm		
	36.2 ± 4.1	60.1 ± 6.2	166.8 ± 46.0	93.0 ± 8.5

[a] The capacities were calculated based on weight loss during the two hours of contact (see Appendix VIII).

[b] standard error given by the ENZFITTER program.

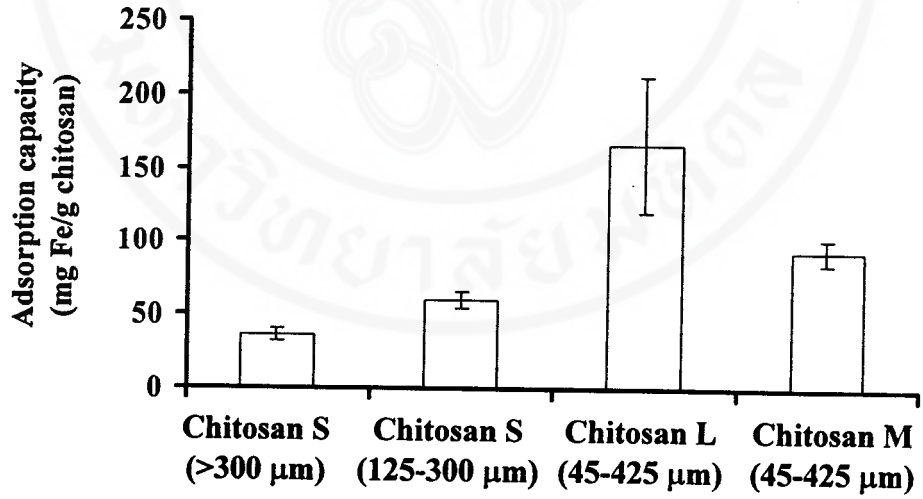
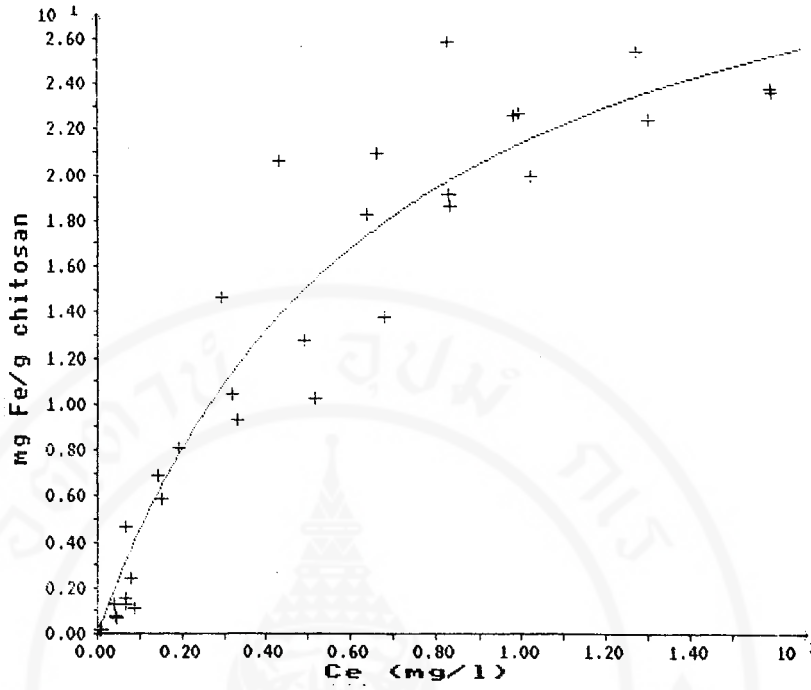


Figure 3.6 Bar charts showing the adsorption capacities for Fe (II) measured on three samples of chitosan, chitosan S (125-300 μm and >300 μm), chitosan L and chitosan M. The error bars indicate the variation as given by ENZFITTER program of the capacity value. Experimental condition: 0.01 M HCl at 37°C.

Note: Chitosan S, L and M were obtained without separation of chitosan and fillers.

a)



b)

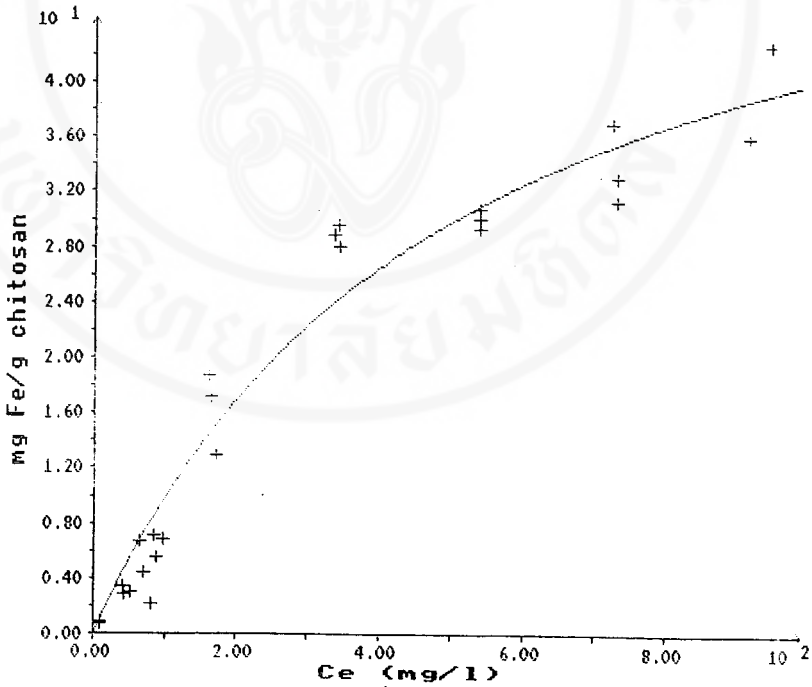
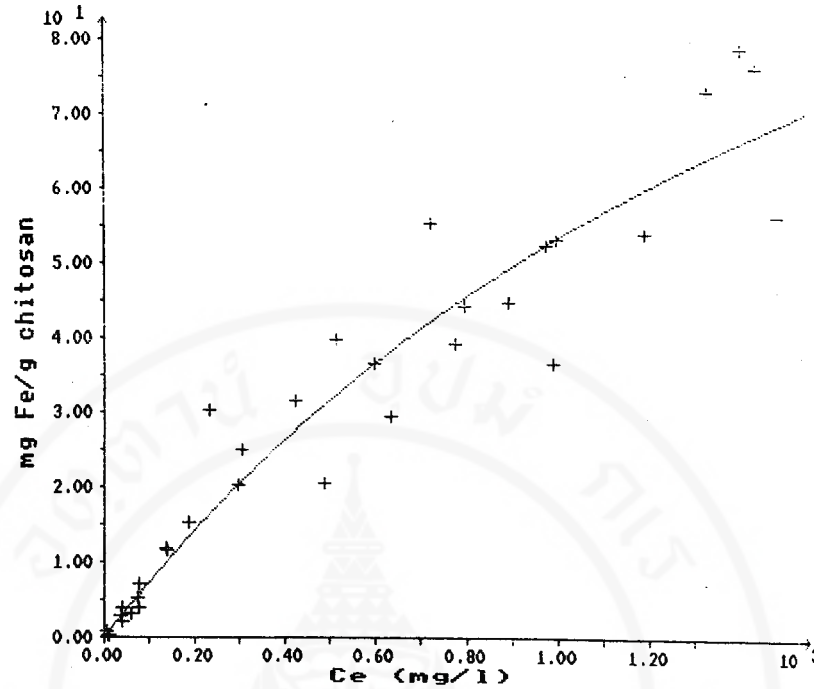


Figure 3.7 Adsorption capacities for Fe (II) adsorption on chitosan S: a) >300 μm b) 125-300 μm which derived from the triplicate experiments (set I to III in Appendix IV) were plotted on the same graph for fitting one isotherm by the program ENZFITTER. Experimental conditions; 0.01 M HCl, shaking at 37°C for 2 hours.

Note: Chitosan S, L and M were obtained without separation of chitosan and fillers.

c)



d)

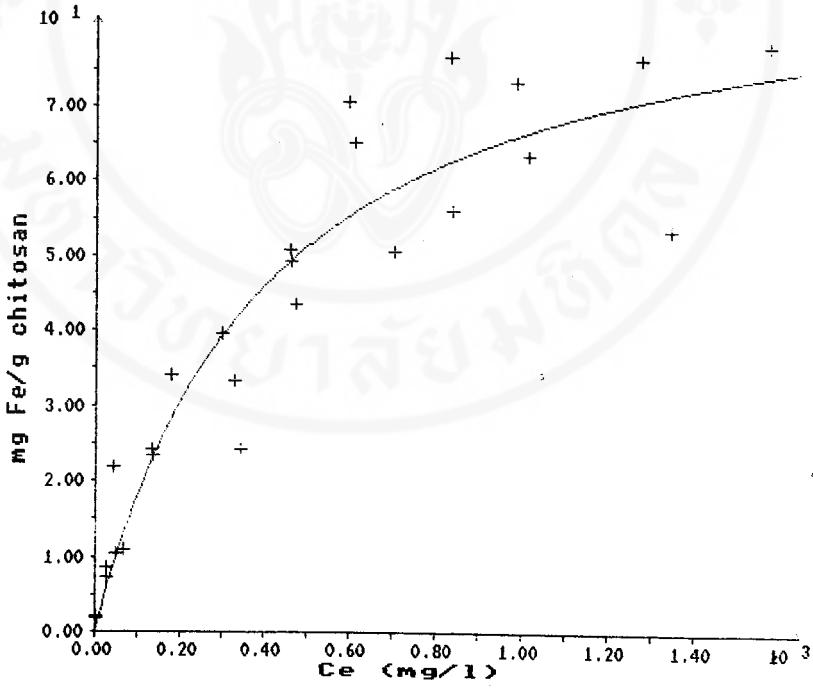


Figure 3.9 Adsorption capacities for Fe (II) adsorption on chitosan: c) chitosan L d) chitosan M which derived from the triplicate experiments (set I to III in Appendix IV) were plotted on the same graph for fitting one isotherm by the program ENZFITTER. Experimental conditions; 0.01 M HCl, shaking at 37°C for 2 hours.

Note: Chitosan S, L and M were obtained without separation of chitosan and fillers.

Results in Figure 3.6 indicates that the order of capacity for Fe (II) adsorbed on solid chitosans is: chitosan L > chitosan M > chitosan S (125-300 μm) > chitosan S (>300 μm). However, these capacities were measured under the assumption that chitosans do not solubilize in 0.01 M HCl during the 2 hrs contact. However, this is not exactly true since it was observed that all three chitosans are slightly solubilize in 0.01 M HCl (Appendix VII). Therefore the capacity data reported for the adsorption of Fe (II) on the chitosans are only estimated values. These estimated capacities will be less than the true value because the solubilized chitosan can bind with metal ions [44].

3.2.3 Possible mechanism for iron (II) adsorption

It is known that the adsorption of transition metals on chitosan mainly takes place via coordination with the amine groups ($-NH_2$) on chitosan. However, under acidic condition, one favourable reaction is protonation on the amino sites of chitosan, resulting in positive charges according to equation (3.1).

$$RNH_2 + H^+ \rightleftharpoons R\text{-}\overset{+}{N}H_3 \qquad (3.1)$$

R is a glucose group.

Figure 3.9 shows results which support protonation at chitosan amino sites. It was observed that the pH of the mixture of chitosan and 0.01 M HCl increased form 2 to 5.8, 5.5 and 5.8 for chitosan S, L and M respectively. This means

that there was less amount of H^+ in solutions after protonation on the amine sites had taken place (equation 3.1).

Figure 3.9 also shows that after two hours contact with chitosans the final pH values were always less than the initial pH for every concentrations of Fe (II). It was observed that the pH decreased, as the concentration of Fe (II) increased, to a maximum value. The drop of the solution pH indicated that the amine group on chitosan undergoes chelation with Fe (II) by through release of the hydrogen ion. Hence, the adsorption of Fe (II) probably proceeds by the following process.

$$R\text{-}\overset{+}{N}H_3 + Fe^{2+} \rightleftharpoons R\text{-}\underset{\displaystyle Fe^{2+}}{\overset{|}{N}}H_2 + H^+ \qquad (3.2)$$

When the adsorption approaches the maximum capacity, the pH remained unchanged, indicating saturation of active amine sites.

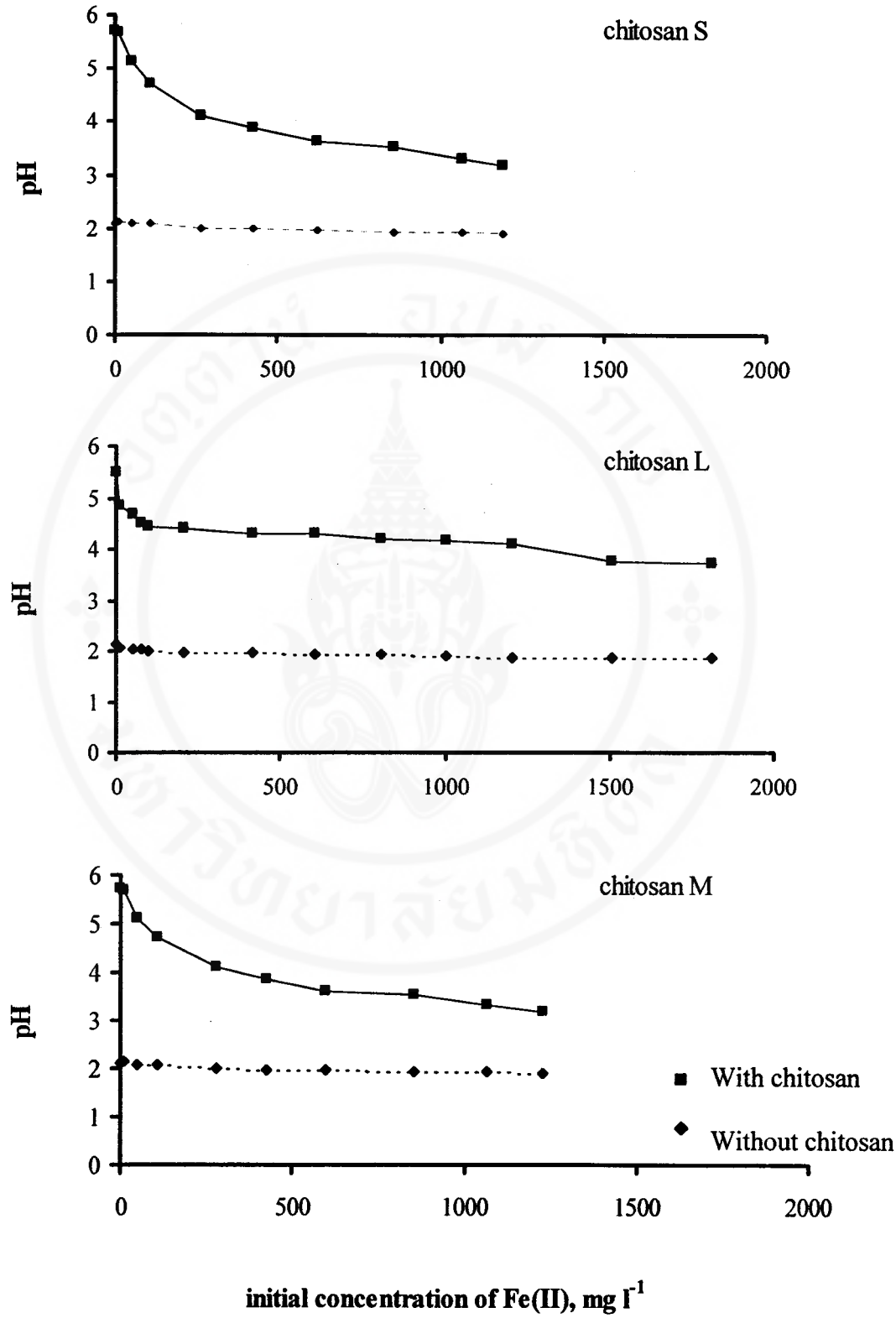


Figure 3.9 Plot between the final pH of solution, after 2 hours of contact of three chitosan samples, and initial concentration of Fe (II) in 0.01 M HCl. The results were obtained from the isotherm experiment.

3.2.4 Adsorption capacity and particle size

Effect of particle size of chitosan S on adsorption of Fe (II) is shown in Figure 3.6.

It was observed that chitosan of smaller size (125-300 μm) gave higher capacity than the larger size (>300 μm). The increase in adsorption capacity for smaller particles is perhaps due to the increase in external surface area for non-porous material such as chitosan. Smaller particles of chitosan S contain larger area of surface having amine sites than the larger particle. Guibal E. et al. [45], has found similar results for the effect of particle size on adsorption capacity of vanadium (IV) on chitosan flakes.

3.2.5 Effect of ascorbic acid on Fe (II) adsorption chitosans

It is observed from the capacity results (Figure 3.6) that chitosan L, which is a commercial supplement containing, vitamin C gave rather high capacity in comparison with the other two samples which contained, no vitamin C. Moreover chitosan M, with the same size range as chitosan L, gave approximately the same capacity as chitosan S.

This seems not to be the effect of particle size because both chitosan L and chitosan M are much smaller in their average size ranges than chitosan S which had the lowest capacity for Fe (II). Vitamin C content is therefore suspected to play a role for the adsorption of Fe (II) on solid chitosan. Therefore, an experiment, in which the ascorbic acid was washed off the surface of chitosan L, was carried out.

Chemical composition of chitosan L, as reported in Table 2.1, Section 2.1.1 shows that the sample is a mixture with chitosan (as deacetylated glucosamine) of 67.5% (w/w). The label on the package of this sample indicates that the sample also contains 15 mg ascorbic acid per 250 mg capsule (5.0% w/w). This ascorbic acid is one of the solid mixture present in the 32.5 % (w/w) filler. In this experiment, the sample was washed with deionized-distilled water several times (approximately 30 ml water per 4 gram sample) until the sample became ascorbic acid free. To confirm this, the rinsing solution was tested by adding 1.0 ml of 0.001 M $KMnO_4$ to 5.0 ml of this solution. The test employs the redox reaction:

$$2MnO_4^- + C_6H_8O_6 + 14H^+ \longrightarrow C_6H_6O_6 + 2Mn^{2+} + 8H_2O \qquad (3.3)$$

Ascorbic acid Dehydroascorbic acid

The 4 gram portion of chitosan L was washed until the color of permanganate ion (MnO_4^-) persisted in the rinsing solution, or until the concentration of MnO_4^- is constant. A spectrometer was used to measure the absorbance of permanganate ion (purple in color) at 525 nm. The results of this test are shown in Table 17A and Figure 12A (Appendix V). The absorbance of the rinsing solution of chitosan L increased as the number of washing step increased. The absorbance finally became constant which confirmed that the elution of ascorbic acid from chitosan L was complete.

Similar experiments were carried out on the other two chitosans (S and M). It was observed, in Figure 12A and Table 17A (Appendix V), that the absorbance

of MnO_4^- never changed through out the steps of washing. This means that there were no other reducing agents (such as ascorbic acid) being washed away from chitosan S or chitosan M. Thus, it can be concluded that these two samples do not contain ascorbic acid like chitosan L.

To study the effect of ascorbic acid on Fe (II) adsorption on chitosan L, the experiment similar to the usual adsorption experiments was performed but using the washed sample (ascorbic free). However for this particular experiment adsorptivity for Fe (II) was used instead of capacity. The adsorptivity was measured using three initial concentrations of Fe (II) which are 200, 600 and 1,200 mg l^{-1}. The measured adsorptivities of chitosan L, with and without the presence of ascorbic acid, are shown in Table 19A, Appendix VI Figure 3.6 shows the results as bar charts. In the same Figure, the results obtained from chitosan S which is a pure chitosan used as control were also plotted. Only two initial concentration of Fe (II) were used for the control chitosan.

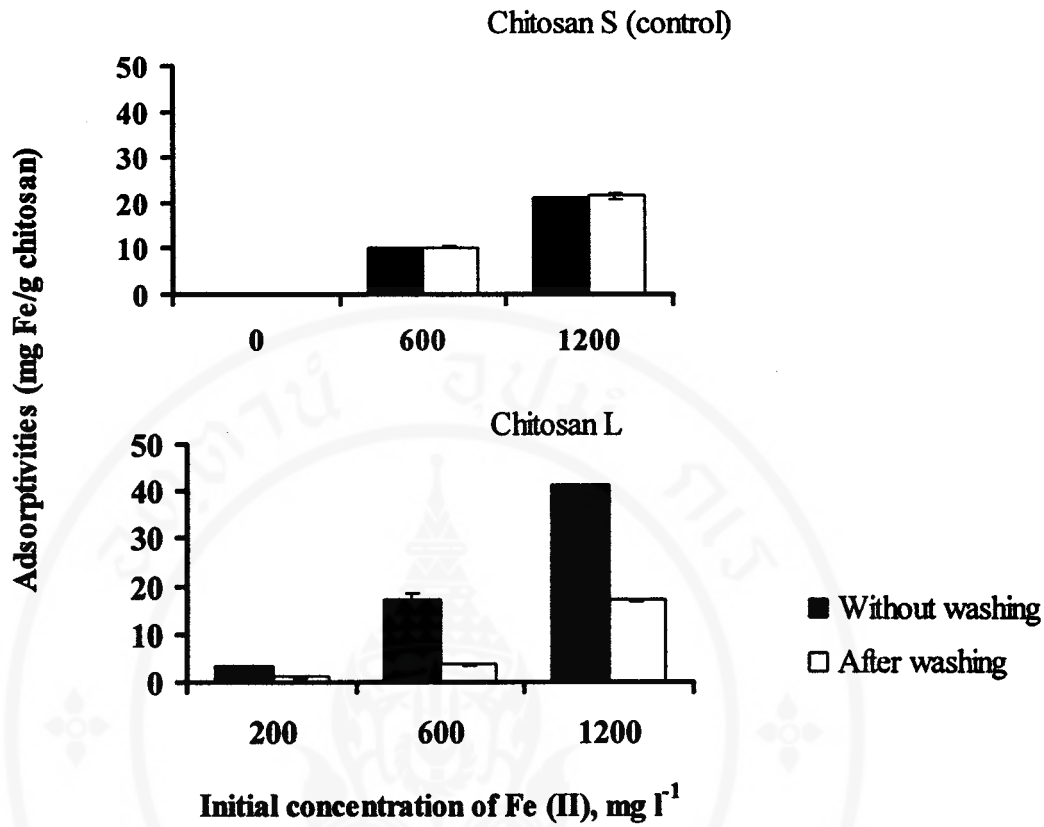


Figure 3.10 Bar charts showing the effect of ascorbic acid on the average adsorptivities for Fe (II) adsorption of chitosans S and L. The error bars represent the variation of the results performed in duplicate. Experimental condition: 0.01 M HCl, 37°C, 2 hours contact time.

Results in Figure 3.10 shows that the adsorptivity of Fe (II) on pure chitosan, chitosan S, did not change after washing. In contrast, the adsorptivities of chitosan L decreased after the ascorbic acid was washed away from the sample. These results have shown that ascorbic acid may enhance the adsorptivity of chitosan L for Fe (II). A clear explanation for this is not yet known. However it is well known that ascorbic acid is after used to reduce Fe (III) ions to Fe (II) ions. The iron species which is adsorbed on amine should be Fe (II). For chitosan S and chitosan M where

there is no ascorbic acid as filler, some Fe (II) ions could be oxidized to Fe (III) ions by air. This can be seen as a slight orange color of the solution mixture after 2 hours contact. For chitosan L, the intensity of this color was less then the other two chitosans. The oxidation of Fe (II) to Fe (III) would lead to a lower concentration of Fe (II) in solution and hence an elevated calculated absorbance.

It was observed that the color of residual chitosan L solid changed from pale white to slight orange. Some particles turned black in color. This better observation was found only for chitosan L.

However this sample of chitosan L also contains unknown fillers which contributed to 27.5% (w/w) of sample. The enhancement of the adsorptivity may have been caused these unknown filler.

3.2.6 Effect of ascorbic acid on chitosan S

It was observed that ascorbic acid which contributed to 5% (w/w) of chitosan L enhanced, the adsorptivity for Fe (II) in Section 3.2.5. In this experiment, the adsorptivities for Fe (II) were measured, using chitosan S (which is a pure chitosan) and two initial concentrations of Fe (II), using 600 and 1,200 mg l^{-1}. The measured adsorptivities of chitosan S, with and without the addition of 5% (w/w) ascorbic acid are shown in Table 21A, Appendix VI. Figure 3.11 show the results as bar charts.

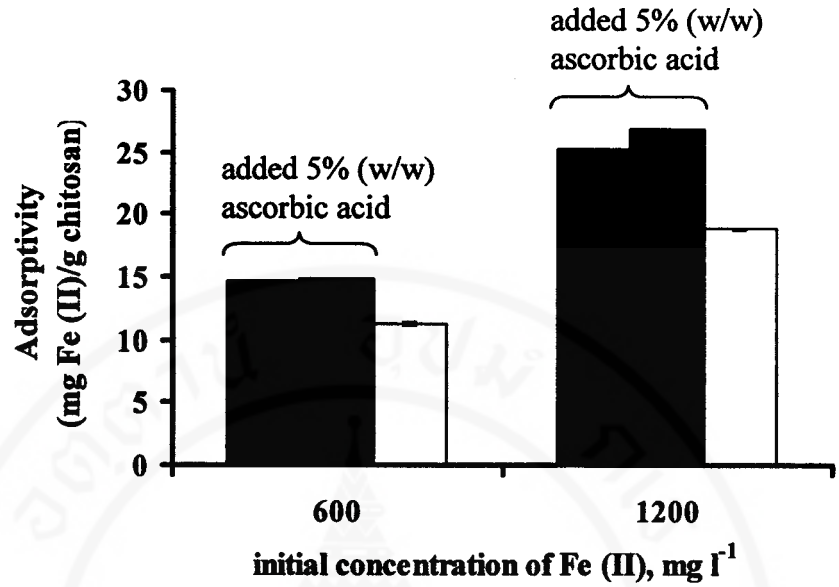


Figure 3.11 Bar charts showing the effect of 5% (w/w) ascorbic acid on the adsorptivities for Fe (II) adsorption of chitosans S. The error bars represent the variation of the results performed in duplicate of control chitosan S. Experimental condition: 0.01 M HCl as media, 37°C, 2 hours contact time.

The results in Figure 3.11, show that Fe (II) adsorption on chitosan S with 5% (w/w) ascorbic acid are higher than the control chitosan (pure chitosan). Therefore, these results confirm that ascorbic acid can enhance adsorption of Fe (II) on solid chitosan.

To study the effect of percentage of ascorbic acid on Fe (II) adsorption on chitosan S, the experiment similar to the usual adsorption experiments was carried out at different concentration of ascorbic acid ranging from 3 to 15% (w/w). A fixed concentration of 600 mg l^{-1} Fe (II) in 0.01 M HCl was used. The adsorptivities of

chitosan S measured at different percentages of ascorbic acid are shown in Table 22A, Appendix VI and in Figure 3.12.

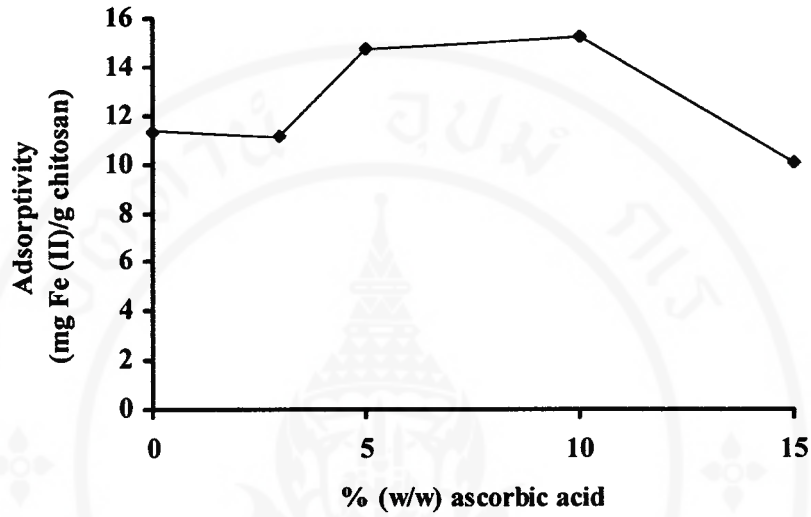


Figure 3.12 The plot of adsorptivities of Fe (II) on chitosan S and % (w/w) ascorbic acid. The experiments were carried out using the initial Fe(II) concentration of 600 mg l^{-1} in 0.01 M HCl at 37°C.

In Figure 3.12, it can be seen that adsorptivities of chitosan S for Fe (II) adsorption tend to increase from 5 to 10% (w/w) ascorbic acid, but there was no increase at 15% (w/w) ascorbic acid.

3.2.7 Effect of starch and fiber on chitosans

It is observed (Figure 3.6) that chitosan M (45-425 μm), which is a commercial supplement with some added fillers, gave the second highest capacity out of the four samples. This may not be the effect of particle size although its capacity is greater than the capacity obtained for a larger size range, such as chitosan S (125-300

μm). Filler such as starch and fiber powder added to this sample by the manufacturer may play, a role in the enhancement of adsorption of Fe (II). Therefore, a washing step was carried out to study this effect.

Chemical composition of chitosan M reported in Table 2.1, Section 2.1.1 shows that the sample contains chitosan (as deacetylated chitosan, glucosamine) at 49.0% (w/w). The label on the package indicates that it also contains 5.8 mg carbohydrate per 500 mg capsule (32.6 % carbohydrate w/w) and 250 mg fiber per 500 mg per capsule (18.4 %w/w). Washing of chitosan M was carried out to remove these fillers. Since the test for residual starch is more feasible than a test for residual fiber, the starch test was used.

In the experiment, the starch was removed from chitosan M by washing the sample with warm deionized-distilled water (60°C) several times until, there was no starch in the rinsing solution. Approximately 5 grams sample was used and 30 ml volume of water used in each rinsing step. To confirm that the sample became starch-free, the rinsing solution was tested by adding 100 μl of iodine solution (approximately 0.01 M I_2) and 500 μl of 0.008 M I^- to 5.0 ml of this solution. The test employs the well known I_3^- -starch reaction, where tri-iodide (I_3^-) reacts with starch to give I_3^- -starch complex. Without the presence of starch, there should only be the brown or yellow color of I_3^-.

The 5 grams portion of chitosan M was washed until the yellow color of I_3^- persisted in the rinsing solution or until there was no blue color of starch-I_3

formed. A spectrometer was used to measure the absorbance of the I_3^--starch complex at 582 nm and the absorbance of I_3^- (yellow in color) at 289 and 352 nm, which are the maximum absorption bands of tri-iodide. The results of this test are shown in Table 17A, Appendix V. It was found that the absorbance of I_3^-starch complex measured at 582 nm of the rinsing solution of chitosan M, decreased as the number of washing step increased. When, the absorbance of this almost became constant and near to the absorbance of the reagent blank, the absorbance of I_3^- measured at 352 nm were used. The absorbance of I_3^- should not changed if there is no starch present in the rinsing solution. The results confirmed that the remove of starch from chitosan M was more or less complete.

Similar washing experiments were carried out on the other two chitosan (S and L). It was observed, in Table 17A (Appendix V), that there were no I_3^--starch formed in all rinsing steps for chitosan S and L (absorbance readings at 582 nm were equal to the blank reading). This means that there were no starch being washed away from the chitosans. Thus, it can be concluded that these two samples do not contain starch like chitosan M. During this washing, other types of filler such as that reported as fiber may have been also washed away from chitosan M.

To study the effect of starch on Fe (II) adsorption on chitosan M, the experiments similar to the usual adsorption experiments were performed on this sample before and after washing. However for this particular experiment, the adsorptivity for Fe (II) was carried out using two initial concentrations of Fe (II) using 600 and 1,200 mg l^{-1}. The measured adsorptivities of chitosan M, with and without

the presence of starch, are shown in Table 20A, Appendix V. Figure 3.13 contains the results as bar charts. Similar experiment was carried out on chitosan S which is a pure chitosan.

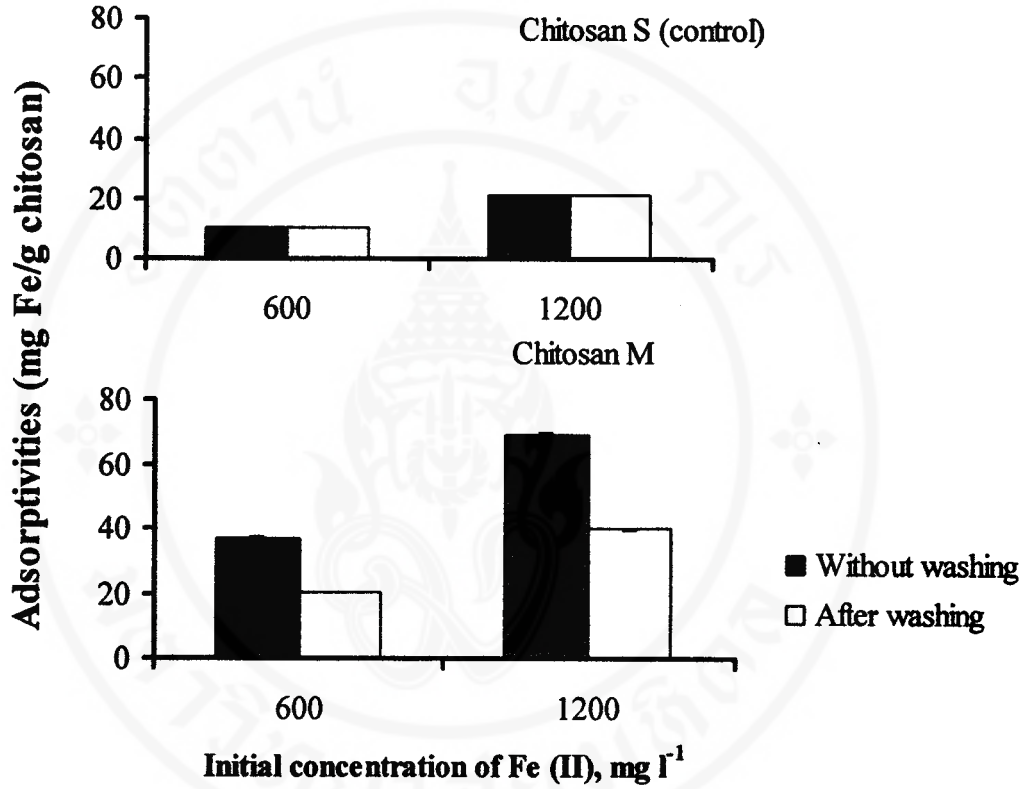


Figure 3.13 Bar charts showing the effect of starch on the average adsorptivities for Fe (II) adsorption of chitosans S and M. The error bars represent the variation of the results performed in duplicate. Experimental condition: 0.01 M HCl as media, 37°C, 2 hours contact time.

The results in Figure 3.13, indicated that Fe (II) adsorption on chitosan M with starch resulted in higher value of adsorptivity than the washed sample. Therefore, these results confirm that starch or fiber can enhance adsorption of Fe (II)

on solid chitosan. The adsorptivity results for the control (chitosan S) show the adsorptivity stayed unchanged after washing.

There is a report of the enhancement of binding of Fe (II) by fiber Fulgencio SC. et al (1995) who [46] have found that natural food materials containing fibre exhibited a higher capacity to bind Fe (II) than Ca (II) and Fe (III).

CHAPTER IV

CONCLUSION

In this work, adsorption of Fe (II) ions was studied for three types of chitosan, chitosan S, chitosan L and chitosan M in 0.01 M hydrochloric acid. This acid solution of pH 2 was selected as the medium to imitate the pH of human stomach. However the experiments were not carried out under the exact condition of the stomach where there are other types of compounds such as tripsin. The three samples differ from one another in terms of purity. Chitosan S is a pure chitosan containing no filler. Chitosan L contains 5% (w/w) ascorbic acid with unknown filler of 27.5% (w/w), whereas chitosan M contains 32.6% (w/w) carbohydrate and 18.4% (w/w) fiber.

The adsorption experiments were carried out for 2 hours. A non-linear least square program ENZFITTER was used to calculate the capacities of Fe (II) adsorption. It was found that the order of capacities were chitosan L (167 ± 46 mg/g) > chitosan M (93 ± 9 mg/g) > chitosan S of 125-300 μm (60 ± 6 mg/g) > chitosan S of >300 μm (36 ± 4 mg/g). This capacity order has no relationship with the degree of deacetylation. However for pure chitosan, such as chitosan S, smaller size range exhibited a better efficiency in adsorption of Fe (II). This is due to the larger area of the outer surface when the particles are smaller.

Impurities or fillers added on chitosan have shown to play role on the capacity for Fe (II) adsorption. Chitosan L which contains 5% (w/w) of vitamin C and 27.5% (w/w) of unknown exhibited the highest capacity. Chitosan M which contains approximately 50% (w/w) of fillers, that are 32.6% (w/w) of carbohydrate and 18.4 % (w/w) of fiber, was the second best absorbent for Fe (II). After washing off the fillers it was found that adsorptivities of these two chitosans decreased.

Adsorption of Fe (II) at the amino functional group of chitosan can be shown from the pH of the solution. The final pH of the chitosan mixture, containing no Fe (II), always increase from the initial pH value after two hours contact. The difference between these pH values was more than three units. In the presence of Fe (II), the final pH was found to decrease with increasing initial Fe (II) to a constant value. This indicated the adsorption of Fe (II) on the quarternary ammonium sites, thus releasing H^+ into solution.

Chitchumroonchokchai C. [47] has studied the daily intake of iron in Thai Population and found that the average intake to be 33.8 mg/day. The value of the daily intake may be divided by three to estimate the intake per meal of 11.3 mg Fe/meal. Therefore, the capacities of the chitosan samples have shown that administration of chitosan capsules (approximately 0.3, 0.06 and 0.1 g chitosan S, L and M, respectively per capsules). In this work, capacity of three chitosans obtained may inhibit gastric absorption of iron.

APPENDIX I

Average molecular weight of chitosan

In this work, the average molecular weight of three chitosan samples, chitosan S, chitosan L and chitosan M were determined by Gel Permeation Chromatography (GPC) using a PL-GPC 110 with refractometer as detector. The procedure and conditions are described in Section 2.4.1.2. The molecular weights determined are GPC molecular weight (Mp), employing calibration against polysaccharide standards of known molecular weights (Pullalan). The calibration graph of the Pullulan standards was obtained by plotting log Mp against retention time (Figure 1A). The results of average molecular weights (Mp) of the chitosans are shown in Table 1A.

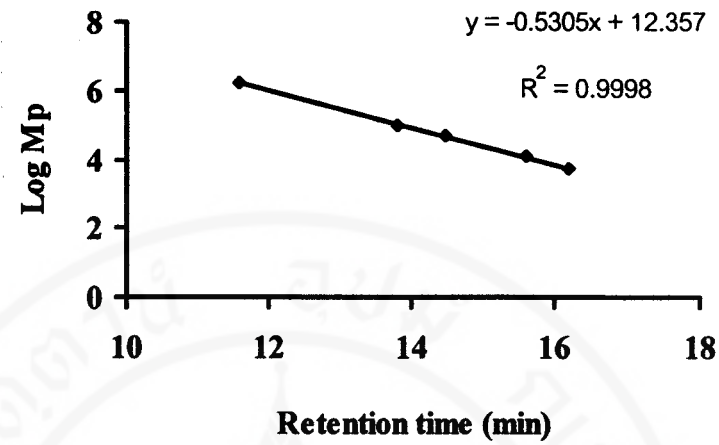


Figure 1A Calibration graph log Mp with retention time.

Mp is the molecular weight of Pullalan standards.

Table 1A Average molecular weight (Mp) of chitosan samples measured by GPC.

Sample	Retention time (min) (n = 2)	Mp ± variation (n = 2)	Polydispersity
Chitosan S	12.9	$(3.20 \pm 0.3) \times 10^5$	7.32
Chitosan L	13.0	$(2.80 \pm 0.086) \times 10^5$	4.89
Chitosan M	13.3	$(1.90 \pm 0.038) \times 10^5$	5.05

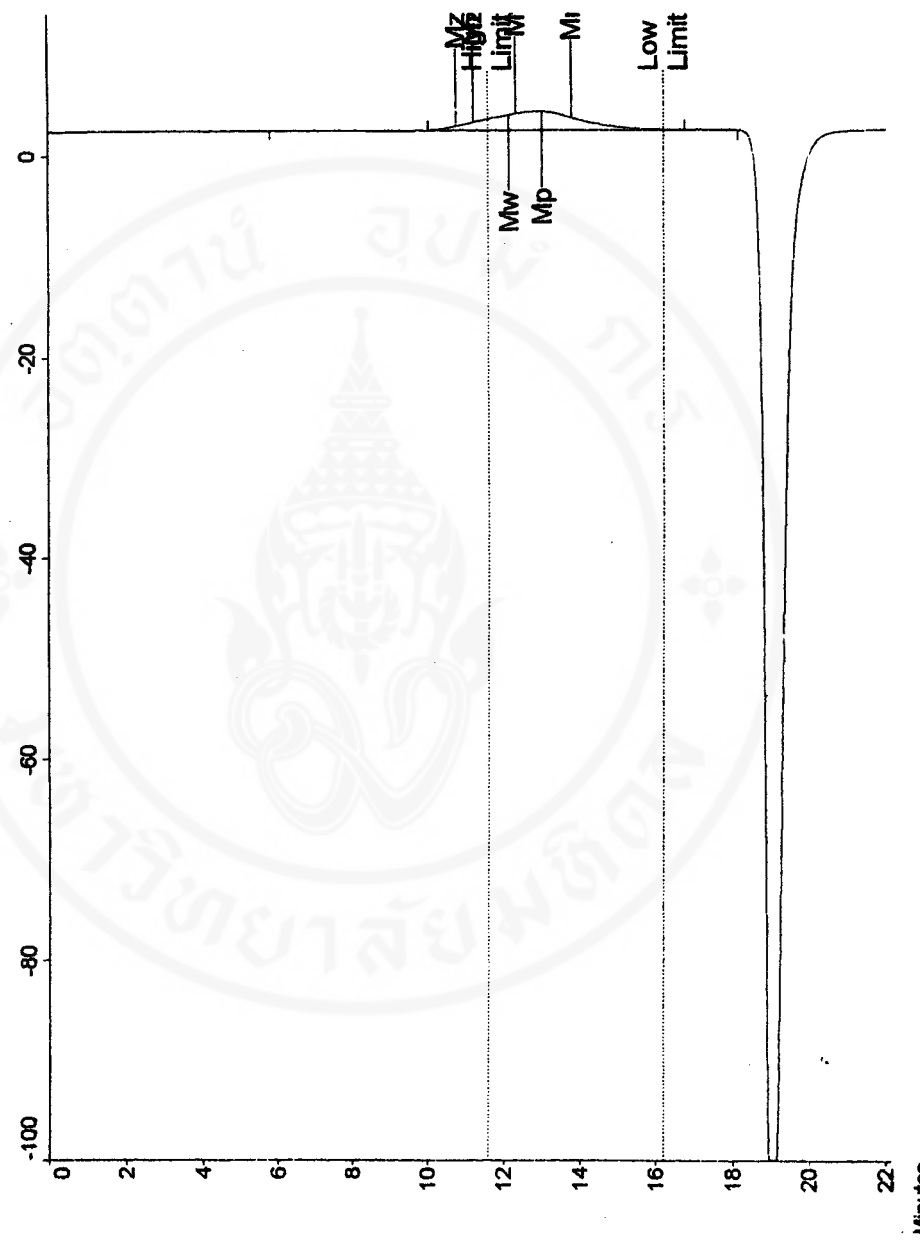


Figure 2A Chromatogram of molecular weight for chitosan S by GPC method.

APPENDIX II

Determination of degree of deacetylation

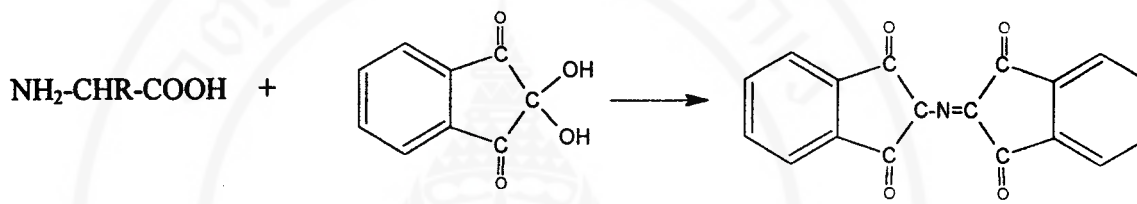
The determination of degree of deacetylation (DD) was carried out in co-operation with co-worker in the same laboratory using four methods. The four methods are ninhydrin test, first-derivative UV spectrometry, infrared spectrometry and solid-state ^{13}C nuclear magnetic resonance. Infrared spectrometry measurements were carried out by a scientist at the Faculty of Science, Mahidol University. The method of solid-state ^{13}C NMR was carried out by an operator at the National Metal and Materials Technology Center (MTEC).

The ninhydrin test involves determination of the free amine group content and other techniques involve determination of the acetyl group content. Besides solid-state ^{13}C NMR and IR technique, the percent deacetylation (DD) were calculated based on a per mol basis. Descriptions of the calculation for ninhydrin and first-derivative UV spectrometry are described in Note 1A in this Appendix.

In this work, three samples of chitosan (chitosan S, chitosan L and chitosan M) were subjected to determination of the percent deacetylation after remove of fillers. Procedures are described in Sections 2.4.1.5 (a) to 2.4.1.5 (d). Degree of deacetylations of chitosan, as determined by each methods, are discussed as followings.

a.) By ninhydrin test [33]

Ninhydrin test is a method which estimates the amount of chitosan by direct detection of the $-NH_2$ group on the glycoside repeat unit of chitosan. This method is based on the reaction of ninhydrin reagent with amine group on chitosan. The ninhydrin test employs the reaction:



Amino acid Ninhydrin Blue complex

Usually the test is used in the determination of amino acid. Ninhydrin reacts with α amino group in amino acid and in protein to give the blue complex. The absorbance of the complex is directly proportional to the number of amino group.

A method for determining the degree of N-deacetylation of chitosan using ninhydrin reaction was proposed by Curotto E. and Atos F. [33]. They reported that the percentage of degree deacetylation of the chitosan samples was determined using the ratio of slopes of calibration between chitosan sample and the glucosamine standard. Calculations of percentage of degree of deacetylation were based on equation 1A,

$$\% \text{ degree of deacetylation} = \frac{\text{slope of sample}}{\text{slope of standard}} \times 100 \qquad (1A)$$

where the glucosamine was used as a representative of one hundred percent pure $-NH_2$ containing substance. However this method was not used in this work.

In this work, determination of percent deacetylation was carried out using ninhydrin reaction by following the method of Khor E. et al. [43]. The amount of glucosamine of a chitosan sample (mg l^{-1}) was determined by direct calibration against standard glucosamine. The percentage of degree of deacetylation was calculated based on per mol using equation 2A (for derivation of formula see Note 1A).

$$\text{Degree of deacetylation by ninhydrin} = \left[\frac{B}{B + [(W - 179B)/221]} \right] \times 100 \qquad (2A)$$

where B is mole of D-glucosamine in chitosan, which is the weight of D-glucosamine in grams /179.
(molecular weights of N-acetyl-D-glucosamine and D-glucosamine are 221 and 179 respectively). W is the sample weight (in grams).

The method of ninhydrin test was employed in this work for determination of the degree of deacetylation of the three samples (Section 2.4.1.5 (a)).

The calibration curve (Figure 3A) was obtained by plotting the absorbance against the concentration of glucosamine standard solutions. The calibration data are shown in Table 2A. Concentrations of D-glucosamine of samples and degrees of deacetylation, as calculated using equation 2A, are summarized in Table 3A.

Table 2A Absorbance data of glucosamine standard in concentration ranges 1.18 to 11.80 mg l^{-1} by ninhydrin test.

Volume of D-glucosamine (ml)	Concentration of D-glucosamine (mg l^{-1})	Absorbance*
0.1	1.18	0.082
0.2	2.36	0.165
0.4	4.72	0.322
0.6	7.08	0.526
1.0	11.80	0.798

* Absorbance value of glucosamine standard whith corrected blank reagent at λ 570 nm (absorbance of blank reagent is 0.197).

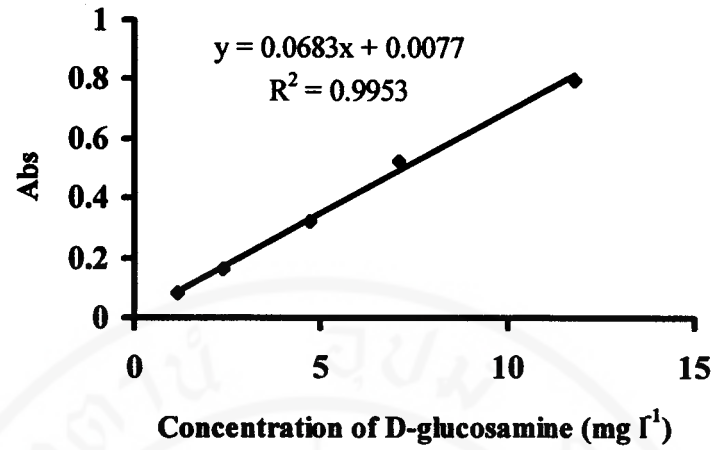


Figure 3A Calibration curve of D-glucosamine for determination of degree of deacetylation by ninhydrin test.

Table 3A Concentration of D-glucosamine on chitosans and the degree of deacetylation (DD) of the samples as determined by the ninhydrin test.

Sample	D-glucosamine (mg l^{-1}), (DD)*			DD ± SD (n = 3)
	Exp. 1	Exp. 2	Exp. 3	
Chitosan S	(5.71), **(32.16)**	(4.95), **(28.10)**	(4.35), **(23.40)**	27.88 ± 4.38
Chitosan L	(4.51), **(23.96)**	(3.90), **(20.83)**	(5.03), **(26.32)**	23.70 ± 1.76
Chitosan M	(1.20), **(7.06)**	(2.35), **(11.90)**	(2.17), **(12.35)**	10.43 ± 2.93

* in percentage.

b.) By first-derivative UV spectrometry

The first-derivative UV spectrometry has been used as a method for determination of the DD as first proposed by Muzzarelli A. and Rocchetti R. in 1985 [32]. This method is based on detection of the N-acetyl glucosamine which is left on repeat unit of chitosan after the process of deacetylation of chitin. The determination was carried out using the amount (in grams) of N-acetyl glucosamine which can be obtained from direct calibration against the standard curve constructed using standard solutions of N-acetyl glucosamine (mg l^{-1}). This amount was then converted into moles. The percentage of degree of deacetylation was calculated based on a per mol basis using equation 3A (see derivation of formula in Note 2A).

$$\text{Degree of deacetylation by first-derivative UV spectrometry} = 100 - \left\{ \left[\frac{A}{A + [(W - 221A)/179]} \right] \times 100 \right\} \quad (3A)$$

where A is mole of D-glucosamine in chitosan, which is the weight of D-glucosamine in grams /179.

(molecular weights of N-acetyl-D-glucosamine and D-glucosamine are 221 and 179 respectively). W is the sample weight (in grams).

Muzzarelli et al [32] reported using the first derivative absorption spectra of glucosamine and N-acetyl glucosamine in the presence of acetic acid, that the maximum absorbance of these two compounds were found at 197 and at 193 nm, respectively. They found that the presence of acetic acid can greatly disturb the determination of both compounds, especially N-acetyl glucosamine if the wavelength used to measure the absorbance signal is not carefully selected. In this work, the

absorption spectra and first-derivative spectra for N-acetyl glucosamine and glucosamine and the effect of acetic acid concentration was re-examined. The zero order and first derivative spectra (against water) for N-acetyl glucosamine and glucosamine are shown in Figure 5A. Results have shown that both compounds contribute to the absorption spectrum in the narrow UV wavelength. The results of the first derivative spectra (against water) for various concentrations of acetic acid (0.01, 0.02 and 0.03 M) and for 40 mg l^{-1} of N-acetyl glucosamine are shown in Figure 4A. The raw data of absorbance of each concentrations of acetic acid at various wavelengths by first-derivative UV mode are given in Table 4A.

Table 4A The absorbance values of 0.01, 0.02 and 0.03 M acetic acid at various wavelengths.

Wavelength (nm)	**[acetic acid], M**		
	0.01	**0.02**	**0.03**
200.0	-0.011	-0.008	-0.002
200.4	-0.010	-0.009	-0.005
200.8	-0.011	-0.010	-0.003
201.2	-0.012	-0.010	-0.005
201.6	-0.012	-0.011	-0.007
202.0	**-0.011**	**-0.010**	**-0.010**
202.4	-0.013	-0.013	-0.012
202.8	-0.013	-0.014	-0.013
203.0	-0.013	-0.015	-0.017
203.4	-0.013	-0.017	-0.020
203.8	-0.013	-0.019	-0.024

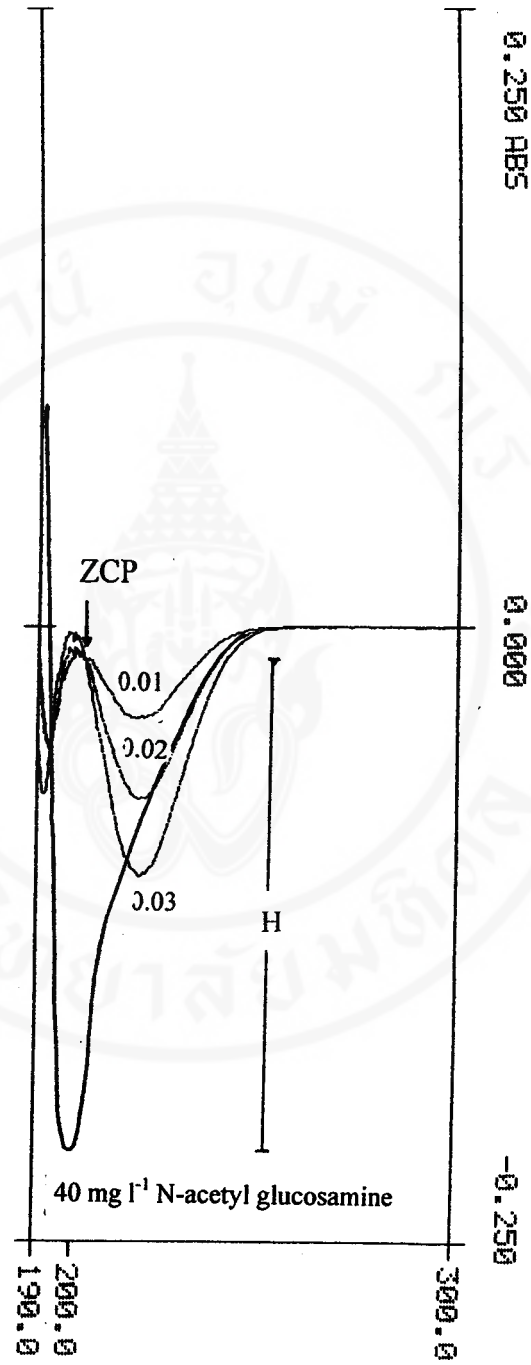
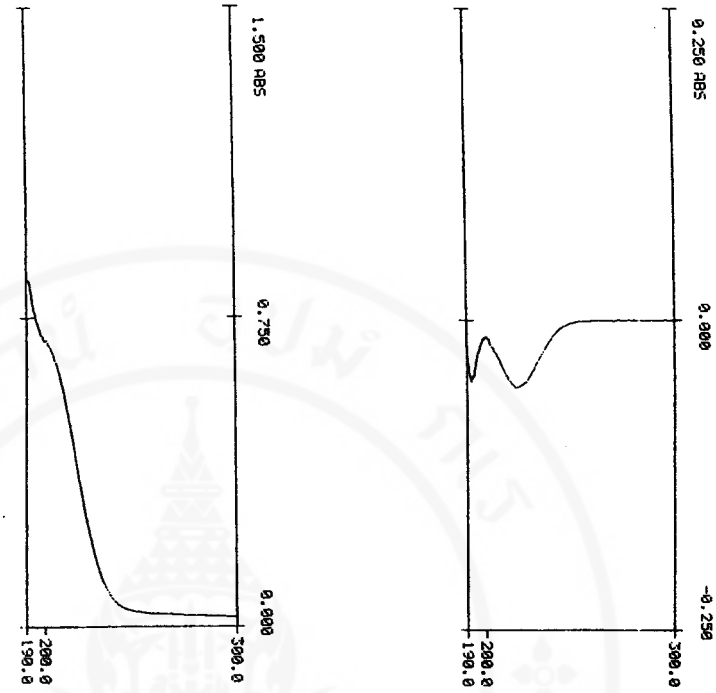


Figure 4A First-derivative spectra of 0.01, 0.02 and 0.03 M acetic acid against water and of 40 mg l^{-1} of N-acetyl glucosamine in 0.01 M acetic acid against water.

a) Glucosamine



b) N-acetyl glucosamine

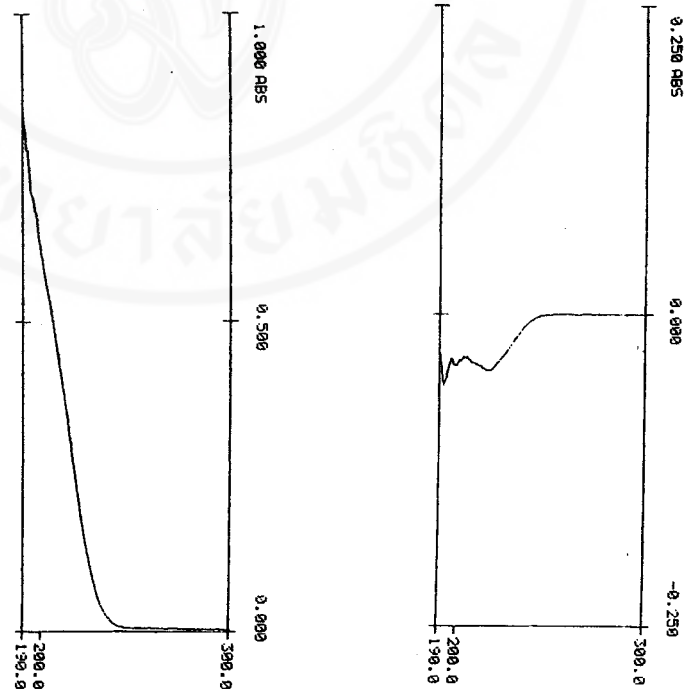


Figure 5A The spectra of 5 mg l^{-1} of N-acetyl glucosamine and 10 mg l^{-1} of glucosamine in 0.01 M acetic acid against water.

Data in Figure 4A and in Table 4A have shown that all acetic acid spectra share a common point at 202 nm, denoted as the zero crossing point (ZCP). Muzzarelli et al. have suggested that measuring the signal of N-acetyl glucosamine at the ZCP wavelength can avoid the matrix interferent caused by variation in concentration of acetic acid, especially within the concentration range studied (0.01–0.03 M). In this work, this method of Muzzarelli was employed in determination of N-acetyl glucosamine on chitosans when the solvent is 0.01 M acetic acid.

Since both N-acetyl glucosamine and D-glucosamine unit on chitosan absorb light are the same wavelength ranges, the signal measured from the first derivative spectra of a sample is not resulted only from pure N-acetyl glucosamine. Therefore some kind of correction is necessary. In this work, the effect of glucosamine concentration was examined to give correction factor.

Effect of percent (w/w) glucosamine

The procedure followed the experiments of Muzzarelli et al. and of Khor E. et al. The first derivative absorption spectra of N-acetyl glucosamine in the presence of various concentration of glucosamine were measured. A fixed concentration of 10 mg l^{-1} standard N-acetyl glucosamine in 0.01 M acetic acid was used. Concentrations of standard D-glucosamine were varied from 0 to 200 mg l^{-1} to give 100 to 4.8 % (w/w) of N-acetyl glucosamine, respectively. Each solution was mixed and made up to 10.0 ml with 0.01 M acetic acid.

First-derivative spectra were obtained in the wavelength range of 190-300 nm. The reference curve was obtained by plotting H_1/H_2 against the corresponding N-acetyl glucosamine percentage where H_1 is signal height of the pure N-acetyl glucosamine solution and H_2 is signal height of the solution which contained different percentages of N-acetyl glucosamine. These signal heights were measured at the ZCP wavelength of 202 nm as depicted in Figure 5A. The data are given in Table 4A. The reference curve is shown in Figure 6A.

Table 5A The experiment data for correction factor of signal height at 202 nm; fixed 10 mg l^{-1} of N-acetyl-D-glucosamine and varied concentration of N-glucosamine in ranges 10 to 200 mg l^{-1} to give 50 to 4.8 % (w/w) N-acetyl glucosamine.

% N-acetyl glucosamine	Signal height at 202 nm* (H_2)	Ratio of (H_1/H_2)
4.8	19.0	0.63
6.3	15.0	0.80
9.1	14.0	0.86
10.0	14.0	0.86
12.5	13.5	0.89
16.7	13.0	0.92

Table 5A (continued)

% N-acetyl glucosamine	Signal height at 202 nm* (H_2)	Ratio of (H_1/H_2)
20.0	13.0	0.92
25.0	13.5	0.89
28.6	13.0	0.92
33.3	13.5	0.89
50.0	12.0	1.00
100.0 (H_1)	12.0	1.00

H_1 is signal height of the pure N-acetyl glucosamine solution.

H_2 is signal height of the solutions of different percentages of N-acetyl glucosamine

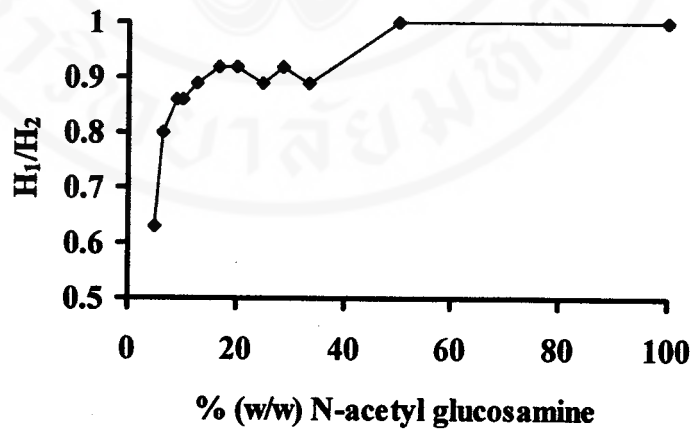


Figure 6A Reference curve for the determination of N-acetyl glucosamine in the presence of defined quantities of glucosamine, percent by weight. The experiments were carried out using the fixed concentration N-acetyl glucosamine of 10 mg l^{-1} in 0.01 M acetic acid and varied concentration of glucosamine from 0 to 200 mg l^{-1} to give 100 to 4.8 % (w/w) of N-acetyl glucosamine, respectively.

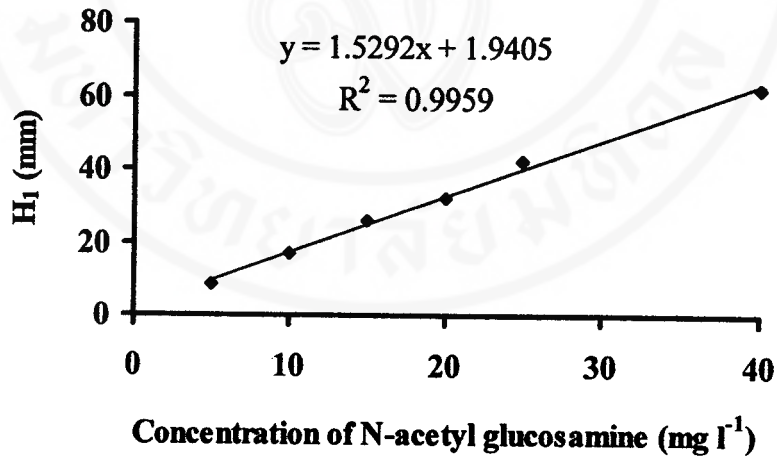
In Figure 6A, it was found that concentration of glucosamine influence the signal height of N-acetyl glucosamine when N-acetyl glucosamine is less than 15 % (w/w). Correction factor for determination of percentage of degree acetylation are necessary. This agrees with the original work of Muzzarelli et al. and Khor B. et al.

In determination of degree of deacetylation in the three chitosan samples, the first-derivative spectra of standard N-acetyl glucosamine solutions in 0.01 M acetic acid were first collected using eight concentrations in the range of 5 to 40 mg l^{-1} (Section 2.4.1.5 (b)). The percentages of degree of deacetylation of chitosan samples were calculated by correction of signal height using Figure 6A as described in Note 3A.

The experimental data and first derivative spectra of acetyl glucosamine standard calibration are shown in Figure 7A and degree of deacetylation of chitosan samples are given in Table 6A.

Table 6A The experiment data of glucosamine standard in concentration range 5 to 40 mg l^{-1}.

Concentration of N-acetyl glucosamine (mg l^{-1})	Signal height (mm) at 202 nm
5	8.5
10	17
15	26
20	32
25	42
40	62



H_1 is signal height of the pure N-acetyl glucosamine solution.

Figure 7A Calibration curve of N-acetyl glucosamine for determination of degree of deacetylation by first-derivative UV spectrometry.

Table 7A Concentration of N-acetyl glucosamine and degree of deacetylation of chitosan S, L and M, as determined by the first-derivative UV spectrometry.

Sample	N-acetyl glucosamine (mg l^{-1}), *(DD)*[a] , **(DD)**[b]			DD* ± SD
	No 1	No 2	No 3	
Chitosan S	9.9, *92.1*, **93.4**	10.5, *92.3*, **93.5**	9.2, *92.1*, **93.4**	93.4 ± 0.1
Chitosan L	13.1, 89.*2*, **90.5**	11.2, *89.9*, **91.2**	12.5, *90.3*, **91.6**	91.1 ± 0.5
Chitosan M	9.9, *92.4*, **93.6**	10.5, *92.3*, **93.5**	9.2, *92.7*, **93.8**	93.7 ± 0.2

DD is calculated as percentage.

[a] is percentage of deacetylation was obtained by not corrected signal height (H_1/H_2).

[b] is percentage of deacetylation was obtained by corrected signal height (H_1/H_2).

* Average percentage of deacetylation was obtained from correction factor (H_1/H_2).

In Figure 7A, the calibration has shown to have a satisfactorily good correlation coefficient at 0.9959.

Results in Table 7A indicates that the order of degree of deacetylation of chitosan samples is: chitosan S ≈ chitosan M > chitosan L. The values of degree of deacetylation of chitosan M was higher than chitosan L.

Note 1A

Ninhydrin Test

Ninhydrin test is a test which estimates the amount of chitosan by direct detection of the amino group on the glycoside repeat unit of chitosan.

Consider the total weight of chitosan (W) taken for this measurement as in gram unit. This weight is from

$$W = (W - Y) + Y \quad (4A)$$

where Y is the weight of D-glucosamine in gram unit, and the weight of N-acetyl-D-glucosamine within the sample is W-Y. In order to determine percentage of degree of deacetylation in mole, it is convenient to define the total mole of N-acetyl glucosamine and glucosamine (N).

$$N = [(W - Y)/221] + (Y/179) \quad (5A)$$

or

$$N = [(W - 179B)/221] + B \quad (6A)$$

where B is the number of moles of D-glucosamine and molecular weight of N-acetyl-D-glucosamine and D-glucosamine are 221 and 179, respectively.

Since the percentage of degree of deacetylation is defined as the ratio of mole of D-glucosamine per total mole of N-acetyl glucosamine and glucosamine, the relationship in equation can be written.

$$\text{Mole fraction of N-deacetylglucosamine} = B/N \quad (7A)$$

Hence

$$\%\text{mole of N-deacetylglucosamine} = [B/N] \times 100 \quad (8A)$$

or degree of deacetylation by ninhydrin test $= \left[\frac{B}{B + [(W - 179B)/221]} \right] \times 100$

Note 2A.

First-derivative UV spectrometry

Determination of degree deacetylation of all chitosans can be calculated based on the per mol basis through the following steps.

A general equation which represents the total weight of chitosan could be written as

$$W = X + (W - X) \qquad (9A)$$

where X is the weight of N-acetyl-D-glucosamine in gram unit and the weight of glucosamine (W-X) was obtained. In order to determine percentage of degree of deacetylation in mole, it is convenient to define the total mole of N-acetyl glucosamine and glucosamine (N).

$$N = (X/221) + [(W - X)/179] \qquad (10A)$$

or

$$N = A + [(W - 221A)/179] \qquad (11A)$$

where A is the number of mole of N-acetyl-D-glucosamine. The molecular weights of N-acetyl-D-glucosamine and D-glucosamine are 221 and 179, respectively.

Since the percentage of degree of deacetylation is defined as the ratio of mole of N-acetyl-D-glucosamine per total mole of N-acetyl glucosamine and glucosamine, the relationship in equation 11A can be written.

Mole fraction of N-acetylglucosamine = A/N (12A)

Hence %mole of N-acetylglucosamine = [A/N] x 100 (13A)

or Degree of acetylation = $\left[\frac{A}{A + [(W - 221A)/179]}\right] \times 100$ (14A)

Thus, degree of deacetylation = $100 - \left\{\left[\frac{A}{A + [(W - 221A)/179]}\right] \times 100\right\}$

by first-derivative UV spectrometry

Note 3A.

Method of calculation of percent deacetylation of the first-derivative UV spectrometry is given as following.

An example of calculation method for DD is described for chitosan S.

Consider H_2 of chitosan S = 17 mm

From calibration curve (linear equation; $H_1 = 1.5292\ X + 1.9405$)

Therefore, estimation for concentration of N-acetyl glucosamine (X) = 9.85 mg l^{-1}

Mole of N-acetyl glucosamine in 100.0 ml = $\frac{9.85 \times 100 \times 10^{-3}}{1000 \times 221}$

= 4.46×10^{-6}

According to equation 3A,

% (mol/mol) acetylation = $\left[\frac{A}{A + [(W - 221A)/179]}\right] \times 100$

Therefore, for chitosan S

$$\% \text{ (mol/mol) acetylation} = \left[\frac{4.46 \times 10^{-6}}{(4.46 \times 10^{-6}) + [(0.0103 - (221 \times 4.46 \times 10^{-6})/179]} \right] \times 100$$

$$= 7.89$$

$$\%\text{(w/w) acetylation} = \left[\frac{(7.89 \times 221)}{(7.89 \times 221) + [(100 - 7.89) \times 179]} \right] \times 100$$

$$= 9.56$$

From the reference curve where % (w/w) acetyl glucosamine is 9.56, the correction factor is 0.86, thus

$$H_1 / H_2 = C = 0.86\ (9.56)$$

$$H_1 = 0.86 \times 17 = 14.62 \text{ mm}$$

From calibration curve (linear equation; $H_1 = 1.5292\ X + 1.9405$)

$$14.62 = 1.5292\ X + 1.9405$$

$$X = 8.29 \text{ mg } l^{-1}$$

$$\text{Mole of N-acetyl glucosamine in 100.0 ml} = \frac{8.29 \times 100 \times 10^{-3}}{1000 \times 221}$$

$$= 4 \times 10^{-6}$$

$$\% \text{ acetylation} = \left[\frac{A}{A + [(W - 221A)/179]} \right] \times 100$$

$$\% \text{ acetylation} = \left[\frac{4 \times 10^{-6}}{(4 \times 10^{-6}) + [(0.0103 - (221 \times 4 \times 10^{-6})/179]} \right] \times 100$$

$$= 6.62 \% \text{ mole}$$

Therefore degree of deacetylation = 100 - 6.62 = 93.38

DD of the other two chitosans were calculated in similar range.

c.) Infrared Technique

An infrared spectroscopic technique for determining the degree of N-acetylation of chitosan was proposed by Domzy J.G. and Roberts G.A.F. [29]. The method involves use of the amide I band of the sample at 1655 cm^{-1} as a measure of the N-acetyl group content and the hydroxyl band at 3450 cm^{-1} as an internal standard. Figure 2.2 illustrate these two bands. The IR spectra obtained for the three samples in this work are given in Figure 8A.

The percentage of the degree of N-deacetylation is calculated by following formula below:

$$\% \text{ N-deacetylation} = 100 - [(A_{1655}/A_{3450}) \times (100/1.33)] \quad (15A)$$

where A_{1655} is the area of the amide I band (maximum absorption about 1655 cm^{-1}) from 1277 cm^{-1} to 1902 cm^{-1}. The base line is the straight shown transmittance of 1277 cm^{-1} to transmittance of 1902 cm^{-1}).

A_{3450} is the area of the hydroxyl group (maximum absorption about 3450 cm^{-1}) from 1902 cm^{-1} to 3837 cm^{-1}. The base line is the straight shown transmittance of 1902 cm^{-1} to transmittance of 3837 cm^{-1}).

The results of percent deacetylation of the three samples are shown in Table 8A.

Table 8A Peak area values and percent deacetylation.

Wavelength (cm^{-1})	Peak area of chitosan (arbitary unit)					
	Chitosan S		Chitosan L		Chitosan M	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
1277 – 1902	37.2403	27.5219	59.3344	31.5648	29.6282	16.9716
1902 -3837	336.3784	267.6010	281.8244	176.0926	176.0926	246.0284
% deacetylation	91.68	92.27	84.17	86.52	87.35	94.81
Average %deacetylation (n=2)	91.94 ± 0.26		85.07 ± 0.90		91.70 ± 4.35	

Results in Table 8A indicates that the order of degree of deacetylation of chitosan samples is: chitosan S ≈ chitosan M > chitosan L.

One possible error of using the 3450 cm^{-1} absorption band as an internal standard is that errors may arise due to the effect of adsorbed water on the intensity of this band.

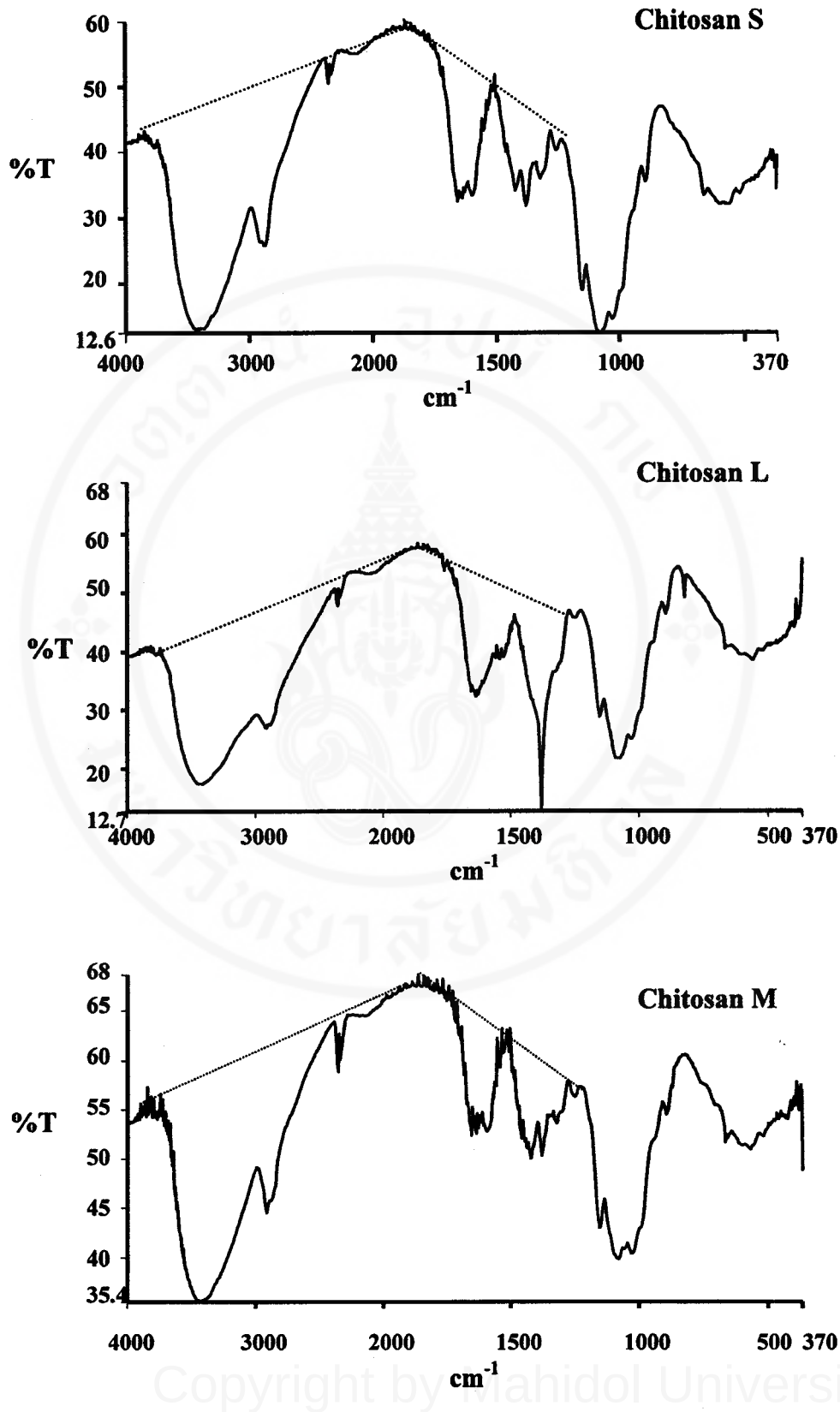


Figure 8A IR spectra of chitosan S, L and M.

d.) Solid-state ^{13}C NMR [31]

The percentage of deacetylation of a chitosan was calculated from solid-state ^{13}C NMR data by comparing the area of the CH_3 resonance of the acetyl group to the resonance of the glucose carbons. Degree of deacetylation was calculated from formula below:

$$\% \text{ N-deacetylation} = 100 - \left\{[(I_{CH3})/(I_{C1\text{-}C6}/6)] \times 100\right\} \qquad (16A)$$

where I_{CH3} is the area of the CH_3 resonance of acetyl group

$I_{C1\text{-}C6}$ is the sum of area of the glucose carbons

The results of the integral intensities and % deacetylation of chitosan samples are shown in Table 9A and NMR spectra of three samples are presented in Figure 9A.

Table 9A The experimental data from solid-state ^{13}C NMR spectra of chitosan S, L and M.

Sample	Integral intensity (arbitary unit)				%deacetylation (n = 2)
	$-CH_3$ (in acetyl group)		Summation of C_1-C_6 (in glucose)		
	Exp.1	Exp.2	Exp.1	Exp.2	
Chitosan S	0.017	0.018	5.915	5.726	98.25 ± 0.05
Chitosan L	0.076	0.090	5.884	6.263	91.70 ± 0.70
Chitosan M	0.031	0.025	6.090	6.009	97.20 ± 0.30

Results in Table 9A indicate that the order of degree of deacetylation of chitosan samples is: chitosan S ≈ chitosan M > chitosan L.

Discussions

Degree of deacetylation determined on the three samples (chitosan S, chitosan L and chitosan M) are summarized in Table 3.3, Section 3.1.4. From the results, ninhydrin test gave the lowest %deacetylation among all methods. The method, in comparing with the first derivative method, gave much lower values values of DD although the calculations were similar (based on a per mol basis). For this work, DD of samples given by the ninhydrin test, were therefore not taken in consideration. The method itself is quite complicated in terms of handling of reagents. It requires also a skillful technician. This rather low values of DD was formerly reported by Khor E et al. [43] and was not recommended by this group of researcher.

First-derivative UV spectrometry is very sensitive for N-acetyl glucosamine detection. The procedure is simple and convenient among all four methods. Khor E. et al. [48] advocated the use of first derivative UV-spectrometry as the standard method for determining the degree of deacetylation of chitosan. However the method seems to be suitable if the sample contains relatively low content of glucosamine, for example partially deacetylated chitin. For these types of sample the correction factor is near to 1. When the sample is above 90% deacetylated, the method could not give the true value of DD. The error begins in the first step of determination where the signal height of sample (with unknown contribution to signal from

glucosamine) was used directly in calibration equation to find the concentration of N-acetyl glucosamine

Solid-state ^{13}C NMR is recommended as a standard for determinations at high acetyl content. However the method require high cost instrument.

IR technique is difficult the in mixing step between chitosan and KBr to make a homogeneous form of disk. Chitosan flake is stiff and difficult to grind. The method, in our opinion should not be used as standard method. One possible of error in the using IR technique is that the 3450 cm^{-1} absorption band might be sensitive to adsorbed water.

APPENDIX III

Experimental data obtained for kinetic studies of Fe (II) and pH changes

Table 10A Adsorption kinetics of Fe (II) on **chitosan S.** The experiments were carried out using the initial Fe (II) concentration of 600 mg l^{-1} in 0.01 M HCl; 37°C.

Time (hrs)	Residual conc of Fe (II) (mg l^{-1})	Weight of chitosan (g)	Adsorptivity (mg Fe/g chitosan)	pH	
				start	final
0.25	592.33	0.2233	2.17	2.27	3.98
0.5	555.27	0.2227	6.33	2.27	3.70
0.75	553.82	0.2228	6.49	2.27	3.72
1	543.86	0.2226	7.62	2.27	3.73
2	525.42	0.2238	9.64	2.27	3.75
4	544.43	0.2231	7.54	2.27	3.59

Table 11A Adsorption kinetics of Fe (II) on **chitosan L**. The experiments were carried out using the initial Fe (II) concentration of 600 mg l^{-1} in 0.01 M HCl; 37°C.

Time (hrs)	Residual conc of Fe (II) (mg l^{-1})	Weight of chitosan (g)	Adsorptivity (mg Fe/g chitosan)	pH	
				start	final
0.25	597.90	0.1462	2.36	2.27	3.65
0.5	535.84	0.1462	12.97	2.27	3.56
0.75	520.98	0.1457	15.57	2.27	3.69
1	502.58	0.1465	18.62	2.27	3.76
2	498.17	0.1460	19.44	2.27	3.94
4	450.00	0.1460	27.68	2.27	4.00

Table 12A Adsorption kinetics of Fe (II) on **chitosan M**. The experiments were carried out using the initial Fe (II) concentration of 600 mg l^{-1} in 0.01 M HCl; 37°C.

Time (hrs)	Residual conc of Fe (II) (mg l^{-1})	Weight of chitosan (g)	Adsorptivity (mg Fe/g chitosan)	pH	
				start	final
0.25	609.09	0.0752	0.87	2.27	3.73
0.50	571.17	0.0756	13.41	2.27	3.69
0.75	545.70	0.0755	21.86	2.27	3.77
1.00	546.93	0.0755	21.44	2.27	3.75
2.00	510.45	0.0752	33.64	2.27	3.75
4.00	491.13	0.0755	39.90	2.27	3.58

APPENDIX IV

Experimental data obtained for isotherm studies of Fe (II) and pH changes

Table 13A Adsorption capacities for Fe (II) adsorption on **chitosan S (>300 μm).** The capacities were measured under constant temperature of 37°C where the media is 0.01 M HCl.

Set I

Initial conc. of Fe (II) ($mg\ l^{-1}$)	Residual conc of Fe (II) ($mg\ l^{-1}$)	Weight of chitosan (g)	Adsorption capacity (mg Fe/g chitosan)	pH	
				start	final
9.33	7.58	0.2239	0.19	2.13	5.69
50.23	38.47	0.2245	1.31	2.08	5.12
108.70	66.53	0.2262	4.66	2.07	4.71
263.29	191.02	0.2225	8.12	2.00	4.10
426.28	294.68	0.2253	14.60	1.98	3.87
621.10	436.85	0.2244	20.53	1.96	3.62
854.82	668.29	0.2228	20.93	1.94	3.53
1063.64	833.54	0.2229	25.81	1.92	3.32
1189.04	987.92	0.2234	22.50	1.90	3.17
Blank (0.01 M HCl)				2.09	5.72

Table 13A (continued)

Set II

Initial conc. of Fe (II) ($mg\ l^{-1}$)	Residual conc of Fe (II) ($mg\ l^{-1}$)	Weight of chitosan (g)	Adsorption capacity (mg Fe/g chitosan)	pH	
				start	final
9.53	8.33	0.2238	0.13	2.06	4.70
52.12	45.28	0.2246	0.76	2.04	4.75
79.37	65.77	0.2241	1.52	2.02	4.67
98.98	88.89	0.2230	1.13	2.00	4.60
205.70	144.14	0.2225	6.92	1.98	4.23
414.54	321.09	0.2237	10.44	1.97	4.17
607.79	516.75	0.2222	10.24	1.93	4.00
805.56	642.86	0.2234	18.20	1.92	3.68
1003.32	833.33	0.2225	19.10	1.90	3.64
1202.38	1000.00	0.2239	22.60	1.88	3.55
1504.52	1305.29	0.2225	22.39	1.87	3.40
1807.27	1597.99	0.2223	23.54	1.86	3.35
Blank (0.01 M HCl)				2.12	4.74

Table 13A (continued).

Set III

Initial conc. of Fe (II) ($mg\ l^{-1}$)	Residual conc of Fe (II) ($mg\ l^{-1}$)	Weight of chitosan (g)	Adsorption capacity (mg Fe/g chitosan)	pH	
				start	final
9.53	8.19	0.2232	0.15	2.06	4.68
52.12	45.75	0.2235	0.71	2.04	4.70
79.37	67.57	0.2240	1.32	2.02	4.68
98.98	77.78	0.2233	2.37	2.00	4.60
205.70	153.30	0.2232	5.87	1.98	4.33
414.54	331.94	0.2228	9.27	1.97	4.24
607.79	493.77	0.2234	12.76	1.93	4.12
805.56	682.72	0.2233	13.75	1.92	3.87
1003.32	836.60	0.2239	18.62	1.90	3.67
1202.38	1025.64	0.2225	19.86	1.88	3.53
1504.52	1277.78	0.2235	25.36	1.87	3.40
1807.27	1595.24	0.2232	23.75	1.86	3.35
Blank (0.01 M HCl)				2.12	4.23

Table 14A Adsorption capacities for Fe (II) adsorption on **chitosan L**. The capacities were measured under constant temperature of 37°C where the media is 0.01 M HCl.

Set I

Initial conc. of Fe (II) ($mg\ l^{-1}$)	**Residual conc of Fe (II) ($mg\ l^{-1}$)**	**Weight of chitosan (g)**	**Adsorption capacity (mg Fe/g chitosan)**	**pH**	
				start	**final**
10.64	6.17	0.1470	0.76	2.20	5.61
52.64	35.83	0.1447	2.91	2.15	5.37
104.20	73.27	0.1449	5.34	2.15	5.17
203.49	134.65	0.1459	11.80	2.14	5.18
406.36	230.15	0.1456	30.26	2.11	5.14
609.72	423.89	0.1471	31.59	2.08	5.06
814.76	598.63	0.1475	36.63	2.07	4.99
1052.38	794.39	0.1457	44.25	2.07	4.97
1310.24	999.07	0.1467	53.04	2.06	4.91
1757.43	1327.64	0.1473	72.97	2.03	4.65
1863.72	1400.00	0.1470	78.85	2.01	4.60
	Blank (0.01 M HCl)			2.18	5.39

Table 14A (continued).

Set II

Initial conc. of Fe (II) ($mg\ l^{-1}$)	Residual conc of Fe (II) ($mg\ l^{-1}$)	Weight of chitosan (g)	Adsorption capacity (mg Fe/g chitosan)	pH	
				start	final
10.80	9.92	0.1461	0.15	2.26	4.82
60.95	38.28	0.1459	3.89	2.26	4.40
117.74	76.68	0.1473	6.97	2.24	4.46
274.89	185.66	0.1459	15.29	2.20	4.56
451.33	304.72	0.1469	24.95	2.18	4.39
747.42	514.29	0.1469	39.67	2.16	4.14
1043.59	720.74	0.1465	55.09	2.14	3.71
1152.21	891.70	0.1461	44.58	2.12	3.53
1282.08	974.17	0.1473	52.27	2.10	3.37
1881.72	1434.91	0.1466	76.19	2.09	3.29
Blank (0.01 M HCl)				2.28	5.32

Table 14A (continued).

Set III

Initial conc. of Fe (II) ($mg\ l^{-1}$)	Residual conc of Fe (II) ($mg\ l^{-1}$)	Weight of chitosan (g)	Adsorption capacity (mg Fe/g chitosan)	pH	
				start	final
9.53	7.89	0.1456	0.28	2.06	4.85
52.12	40.57	0.1461	1.98	2.04	4.69
79.37	60.36	0.1460	3.25	2.02	4.51
98.98	75.93	0.1463	3.94	2.00	4.44
205.70	137.62	0.1461	11.65	1.98	4.40
414.54	296.34	0.1468	20.13	1.97	4.30
607.79	487.44	0.1462	20.58	1.93	4.31
805.56	633.33	0.1464	29.41	1.92	4.20
1003.32	775.67	0.1457	39.07	1.90	4.17
1202.38	988.62	0.1468	36.41	1.88	4.10
1504.52	1189.73	0.1459	53.93	1.87	3.75
1807.27	1478.96	0.1462	56.14	1.86	3.74
Blank (0.01 M HCl)				2.12	5.48

Table 15A Adsorption capacities for Fe (II) adsorption on **chitosan M.** The capacities were measured under constant temperature of 37°C where the media is 0.01 M HCl.

Set I

Initial conc. of Fe (II) ($mg\ l^{-1}$)	**Residual conc of Fe (II) ($mg\ l^{-1}$)**	**Weight of chitosan (g)**	**Adsorption capacity (mg Fe/g chitosan)**	**pH**	
				start	**final**
8.87	3.33	0.0752	1.84	2.13	5.69
50.23	27.99	0.0757	7.34	2.08	5.12
108.70	42.86	0.0755	21.80	2.07	4.71
280.00	177.75	0.0752	34.01	2.00	4.10
426.28	325.53	0.0754	33.42	1.98	3.87
600.00	469.16	0.0755	43.33	1.96	3.62
854.82	702.05	0.0755	50.55	1.94	3.53
1063.64	831.06	0.0760	76.54	1.92	3.32
Blank (0.01 M HCl)				2.09	5.72

Table 15A (continued)

Set II

Initial conc. of Fe (II) ($mg\ l^{-1}$)	Residual conc of Fe (II) ($mg\ l^{-1}$)	Weight of chitosan (g)	Adsorption capacity (mg Fe/g chitosan)	pH	
				start	final
9.53	3.22	0.0750	2.11	2.06	5.74
52.12	26.42	0.0755	8.52	2.04	5.24
79.37	47.75	0.0757	10.44	2.02	4.97
98.98	65.74	0.0752	11.05	2.00	4.74
205.70	135.79	0.0751	23.28	1.98	4.58
414.54	295.63	0.0752	39.56	1.97	4.39
607.79	458.95	0.0756	49.22	1.93	4.22
805.56	608.37	0.0757	65.10	1.92	4.12
1003.32	834.95	0.0753	55.92	1.90	3.87
1202.38	1011.97	0.0753	63.19	1.88	3.61
1504.52	1343.73	0.0752	53.44	1.87	3.55
Blank (0.01 M HCl)				2.12	5.49

Table 15A (continued).

Set III

Initial conc. of Fe (II) ($mg\ l^{-1}$)	Residual conc of Fe (II) ($mg\ l^{-1}$)	Weight of chitosan (g)	Adsorption capacity (mg Fe/g chitosan)	pH	
				start	final
9.53	3.22	0.0759	2.08	2.06	5.69
52.12	26.42	0.0760	8.46	2.04	5.26
79.37	47.75	0.0751	10.53	2.02	4.98
98.98	65.74	0.0752	11.04	2.00	4.50
205.70	133.04	0.0754	24.09	1.98	4.45
414.54	341.54	0.0754	24.20	1.97	4.24
607.79	455.07	0.0752	50.76	1.93	4.12
805.56	593.04	0.0753	70.55	1.92	3.87
1202.38	982.20	0.0753	73.10	1.88	3.63
1504.52	1274.83	0.0752	76.32	1.87	3.50
1807.27	1573.07	0.0750	78.03	1.86	3.32
Blank (0.01 M HCl)				2.12	5.84

Table 16A Adsorption capacities for Fe (II) adsorption on **chitosan S (125-300 μm).** The capacities were measured under constant temperature of 37°C where the media is 0.01 M HCl.

Set I

Initial conc. of Fe (II) ($mg\ l^{-1}$)	**Residual conc of Fe (II) ($mg\ l^{-1}$)**	**Weight of chitosan (g)**	**Adsorption capacity (mg Fe/g chitosan)**	**pH**	
				start	**final**
11.25	9.41	0.0580	0.79	2.03	4.76
58.68	51.75	0.0586	2.96	1.99	4.70
86.17	81.03	0.0587	2.19	1.98	4.75
112.09	96.31	0.0583	6.76	1.97	4.76
200.59	170.59	0.0580	12.92	1.95	4.79
403.16	336.27	0.0581	28.78	1.91	4.61
606.46	535.44	0.0580	30.62	1.90	4.30
800.00	727.88	0.0579	31.16	1.89	4.02
996.08	912.79	0.0580	35.87	1.88	3.93
Blank (0.01 M HCl)				2.12	4.34

Table 16A (continued).

Set II

Initial conc. of Fe (II) ($mg\ l^{-1}$)	Residual conc of Fe (II) ($mg\ l^{-1}$)	Weight of chitosan (g)	Adsorption capacity (mg Fe/g chitosan)	pH	
				start	final
9.88	8.23	0.0577	0.72	2.52	4.83
49.37	42.66	0.0583	2.88	2.51	4.91
79.76	69.38	0.0582	4.46	2.48	4.89
101.20	88.46	0.0579	5.51	2.47	4.90
203.32	163.60	0.0580	17.11	2.46	4.41
409.64	341.08	0.0580	29.56	2.44	4.57
604.33	535.00	0.0579	29.95	2.44	4.26
805.32	720.00	0.0578	36.93	2.43	3.86
1042.17	943.36	0.0580	42.56	2.42	3.91
Blank (0.01 M HCl)				2.52	4.59

Table 16A (continued).

Set III

Initial conc. of Fe (II) ($mg\ l^{-1}$)	Residual conc of Fe (II) ($mg\ l^{-1}$)	Weight of chitosan (g)	Adsorption capacity (mg Fe/g chitosan)	pH	
				start	final
9.88	8.10	0.0582	0.76	2.52	4.85
49.37	41.58	0.0572	3.40	2.51	4.85
79.76	64.10	0.0584	6.71	2.48	4.94
101.20	84.72	0.0577	7.14	2.47	4.89
203.32	159.55	0.0583	18.76	2.46	4.93
409.64	343.98	0.0586	28.00	2.44	4.59
604.33	536.00	0.0583	29.29	2.44	4.01
805.32	728.92	0.0579	32.97	2.43	4.12
Blank (0.01 M HCl)				2.52	4.43

APPENDIX V

Washing of chitosans

All three chitosans (S, L and M) were washed to separate all other chemicals which may have been added to three chitosan during processing.

Procedure 1. Washing

2. Testing the rinsing solutions

2.1 Using 0.001 M $KMnO_4^-$

2.2 Using starch-I_3^- reaction

Table 17A Absorbance value of test solution from chitosan S, L and M.

Washing step	Absorbance					
	Reaction with 0.001 M $KMnO_4$			Starch-I_3^- reaction		
	Chitosan S	Chitosan L	Chitosan M	Chitosan S	Chitosan L	Chitosan M
1	0.650	0.000	0.645	0.000	0.000	0.170
2	0.655	0.000	0.649	0.000	0.000	0.210
3	0.653	0.016	0.640	0.000	0.000	0.127
4	0.656	0.140	0.651	0.000	0.000	0.081
5	0.648	0.429	0.647	0.000	0.000	0.049
6	-	0.539	-	-	-	0.043

Table 17A (continued)

Washing step	Absorbance					
	Reaction with 0.001 M $KMnO_4$			Starch-I_3^- reaction		
	Chitosan S	Chitosan L	Chitosan M	Chitosan S	Chitosan L	Chitosan M
7	-	0.602	-	-	-	0.050
8	-	0.593	-	-	-	0.060
9	-	0.576	-	-	-	-
10	-	0.644	-	-	-	-
11	-	0.608	-	-	-	-
12	-	0.617	-	-	-	-
13	-	0.654	-	-	-	-
14	-	0.654	-	-	-	-
15	-	0.652	-	-	-	-
Control	0.652	0.652	0.652	-	-	-

Figure 9A and 10A represented data in Table 17A. The absorbances of rinsing solution of chitosan S, L and M using MnO_4^- and I_3^--starch as a function of washing step, are shown in Figure 9A and 10A, respectively.

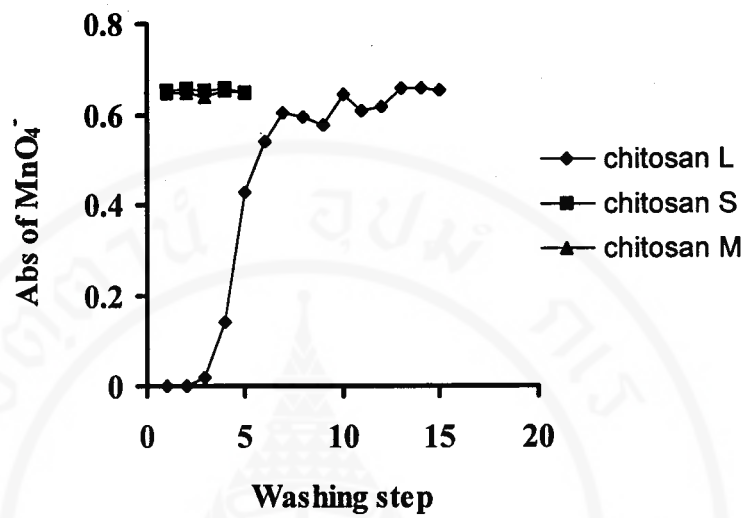


Figure 9A The plot between absorbance of rinsing solution using MnO_4^- as an oxidizing agent and washing step. The absorbance of control set is 0.652.

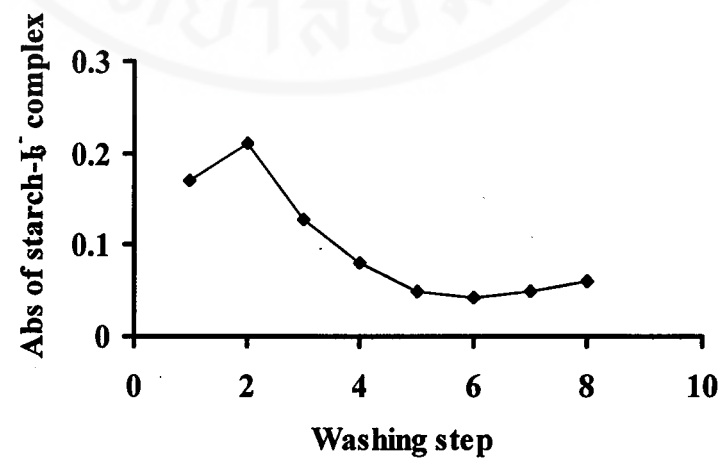


Figure 10A The plot between absorbance of rinsing solution of chitosan M using I_3^--starch reaction and washing step.

APPENDIX VI

Effect of fillers on adsorption of Fe (II)

Table 18A The effect of fillers on the average adsorptivities for Fe (II) adsorption of **chitosan S.**

Experimental condition: 0.01 M HCl as media, 37°C, 2 hours contact time.

Set I

Initial conc. of Fe (II) ($mg\ l^{-1}$)	Residual conc. of Fe (II) ($mg\ l^{-1}$)		Weight of chitosan (g)		Adsorptivity (mg Fe/g chitosan)		pH		
								final	
	Without washing	washing	Without washing	washing	Without washing	washing	start	Without washing	washing
606.42	510.97	510.12	0.2267	0.2430	10.52	9.91	2.01	4.43	4.44
1199.30	997.95	994.21	0.2276	0.2417	22.12	21.21	1.94	4.10	4.04

Table 18A (continued)

Set II

Initial conc. of Fe (II) ($mg\ l^{-1}$)	Residual conc. of Fe (II) ($mg\ l^{-1}$)		Weight of chitosan (g)		Adsorptivity (mg Fe/g chitosan)		pH		
								final	
	Without washing	washing	Without washing	washing	Without washing	washing	start	Without washing	washing
606.42	514.94	507.01	0.2282	0.2421	10.02	10.26	2.01	4.42	4.44
1199.30	1008.95	991.11	0.2275	0.2441	20.92	21.32	1.94	4.07	4.09

Table 19A The effect of ascorbic acid on the average adsorptivities for Fe (II) adsorption of **chitosan L**.

Experimental condition: 0.01 M HCl as media, 37°C, 2 hours contact time.

Set I

Initial conc. of Fe (II) ($mg\ l^{-1}$)	Residual conc. of Fe (II) ($mg\ l^{-1}$)		Weight of chitosan (g)		Adsorptivity (mg Fe/g chitosan)		pH		
								final	
	Without washing	washing	Without washing	washing	Without washing	washing	start	Without washing	washing
192.13	171.70	182.27	0.1460	0.2404	3.50	1.03	2.22	4.55	4.51
583.92	492.57	551.08	0.1470	0.2403	15.53	3.42	2.03	3.82	3.65
1187.05	944.40	1023.29	0.1469	0.2409	41.29	17.00	1.98	3.35	3.33

Table 19A (continued)

Set II

Initial conc. of Fe (II) ($mg\ l^{-1}$)	Residual conc. of Fe (II) ($mg\ l^{-1}$)		Weight of chitosan (g)		Adsorptivity (mg Fe/g chitosan)		pH		
								final	
	Without washing	washing	Without washing	washing	Without washing	washing	start	Without washing	washing
192.13	172.39	179.40	0.1466	0.2414	3.37	1.32	2.22	4.44	4.52
583.92	475.46	545.24	0.1461	0.2409	18.56	4.01	2.03	3.83	3.70
1187.05	946.60	1021.05	0.1462	0.2406	41.11	17.25	1.98	3.34	3.31

Table 20A The effect of starch on the average adsorptivities for Fe (II) adsorption of **chitosans M**.

Experimental condition: 0.01 M HCl as media, 37°C, 2 hours contact time.

Set I

Initial conc. of Fe (II) ($mg\ l^{-1}$)	Residual conc. of Fe (II) ($mg\ l^{-1}$)		Weight of chitosan (g)		Adsorptivity (mg Fe/g chitosan)		pH		
								final	
	Without washing	washing	Without washing	washing	Without washing	washing	start	Without washing	washing
606.42	497.96	444.22	0.0753	0.2001	36.01	20.27	2.01	4.58	4.64
1199.30	997.95	877.98	0.0752	0.1993	66.98	40.30	1.94	4.24	4.42

Table 20A (continued)

Set II

Initial conc. of Fe (II) ($mg\ l^{-1}$)	Residual conc. of Fe (II) ($mg\ l^{-1}$)		Weight of chitosan (g)		Adsorptivity (mg Fe/g chitosan)		pH		
								final	
	Without washing	washing	Without washing	washing	Without washing	washing	start	Without washing	washing
606.42	493.32	443.55	0.0755	0.2015	37.47	20.21	2.01	4.62	4.69
1199.30	984.00	881.65	0.0755	0.2005	71.33	39.60	1.94	4.25	4.41

Table 21A The effect of ascorbic acid for Fe (II) adsorption of **chitosan S**.

Experimental condition: 0.01 M HCl as media, 37°C, 2 hours contact time.

Set I

Initial conc. of Fe (II) ($mg\ l^{-1}$)	Residual conc. of Fe (II) ($mg\ l^{-1}$)		Weight of chitosan (g)		Adsorptivity (mg Fe/g chitosan)		pH		
								final	
	Without ascor.	With ascor.	Without ascor.	With ascor.	Without ascor.	With ascor.	start	Without ascor.	With ascor.
595.47	482.21	450.51	0.2465	0.2461	11.49	14.73	2.29	4.52	4.78
1200.79	1016.33	954.37	0.2454	0.2441	18.79	25.24	2.23	4.18	4.65

Table 21A (continued)

Set II

Initial conc. of Fe (II) ($mg\ l^{-1}$)	Residual conc. of Fe (II) ($mg\ l^{-1}$)		Weight of chitosan (g)		Adsorptivity (mg Fe/g chitosan)		pH		
								final	
	Without ascor.	With ascor.	Without ascor.	With ascor.	Without ascor.	With ascor.	start	Without ascor.	With ascor.
595.47	485.77	450.34	0.2460	0.2448	11.15	14.81	2.29	4.58	4.67
1200.79	1015.44	937.96	0.2452	0.2450	18.90	26.84	2.23	4.15	4.68

APPENDIX VII

Solubility in water, 0.01 and 0.1 M HCl

In this experiments, solubilities of all chitosans used were determined in two concentrations of hydrochloric acid, 0.01 and 0.1 M HCl. In addition, solubilities in deionized-distilled water were also investigated. The procedure are described in Section 2.4.1.3. Determination of the solubility was calculated as gram soluble amount of chitosan per 100 ml testing solutions. The results of solubility are shown in Table 22A.

Table 22A The solubilities of chitosan S, chitosan L and chitosan M in 0.01, 0.1 M HCl and in deionized-distilled water after shaking time of two hours; room temperature.

Solvent	Solubility (g/100 ml)		
	Chitosan S	**Chitosan L**	**Chitosan M**
Deionized-distilled water	0.03	0.07	0.05
0.01 M HCl	0.04	0.16	0.09
0.1 M HCl	>2	0.54	0.3

In Table 22A, it was clear that the solubilities of each chitosan in deionized-distilled water were less than in other test solution. The solubility of

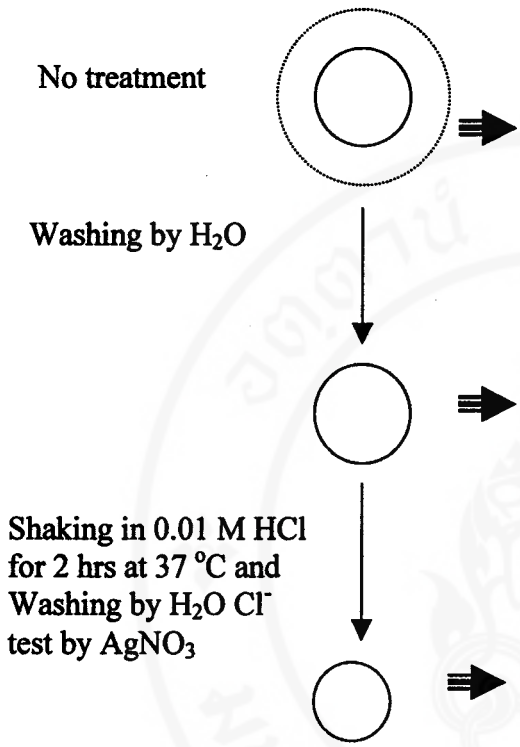
chitosan L in deionized-distilled water was greater than chitosan S and chitosan M because chitosan L contains ascorbic acid which is soluble in water.

The order of solubility of chitosans in two concentrations of hydrochloric acid solution is chitosan S > chitosan L > chitosan M. It may be due to presence some fillers such as starch in chitosan M which is slightly soluble in the test solution. However this order is different for 0.01 M HCl.

APPENDIX VIII

Residual weight of chitosan after the contact with 0.01 M hydrochloric acid

According to the solubility data, pure chitosan can slightly solubilize to data, pure chitosan can slightly solubilize in 0.01 M HCl which is used as the solvent for Fe (II) in the adsorption studies (Section 2.4.2). Also some fillers such as ascorbic acid or starch can solubilize in the acid solvent. These cause some loss in the initial weight of chitosan taken into each flask for adsorption studies and for the experiment in determination of adsorptivity (Section 3.2.6 and 3.2.7). It is therefore necessary to find out the actual weight which is left after shaking with Fe (II) solutions.



Step	Weight of chitosan	%residual chitosan	%impurity	%solubilized chitosan
0	W_0	-	-	-
1	W_1	$(W_1/W_0)100$ $= \%W_1$	$100-\%W_1$ $= \%I_1$	-
2	W_2	$(W_2/W_0)100$ $= \%W_2$	-	$(W_2/W_1)100$ $= \%W_{2a}$

Figure 11A Schematic diagram illustrating the procedure of washing chitosans and this table shows method of calculation for weight correction.

A schematic diagram is drawn in Figure 11A to illustrate the loss of a filler or chitosan itself after washing or shaking with solvents. In 'step 0', the picture represent a chitosan with some coated filler which is washed away in 'step 1' through the procedures of washing as described in Section 3.2.6 and 3.2.7. Original weight of sample (W_0) is then reduced to W_1. Column 3 and 4 shows how to calculate % (w/w) residual chitosan ($\%W_1$) and % (w/w) impurity including filler ($\%I_1$). Some of the pure chitosan can solubilize in 0.01 M HCl (after 2 hours of contact) in 'step 2'. This

loss in weight of chitosan is used to calculate the final-residual weight as written in column 3 as $\%W_2$. $\%W_2$ is used in calculations of the adsorption capacities and the adsorptivities of chitosan with fillers. $\%W_{2a}$ were calculated based on the weight of pure chitosan (W1) after cleaning in 'step 1'. This $\%W_{2a}$ is used in calculations of the adsorptivity in Section 3.2.6 for the samples which are cleaned. Table 23A is the summary of the values of % W_1, $\%W_2$ and $\%W_{2a}$ of the three chitosans used in this work. $\%I_1$ are also shown in the same table.

Table 23A Summary of $\%W_1$, $\%I_1$, $\%W_2$ and $\%W_{2a}$ of chitosans used in this work.

	Chitosan S[a]		Chitosan L	Chitosan M
	> 300 μm	125-300 μm		
$\%W_1 = \frac{W_1}{W_0} \times 100$	98	98	62	50
$\%I_1 = 100 - \%W_1$	2	2	38	50
$\%W_2 = \frac{W_2}{W_0} \times 100$	89	58	58	30
$\%W_{2a} = \frac{W_2}{W_1} \times 100$	91	60	96	80

W_0: Original weight of sample.

W_1: Weight of pure chitosan after washing.

W_2: Weight of pure chitosan after solubilization during 2 hours shaken with 0.01 M HCl.

W_{2a}: Residual chitosan after solubilization during 2 hours shaken with 0.01 M HCl.

I_1: Impurity.

[a] Two particle sizes (125-300 μm and >300 μm) of chitosan S are studied by different ratio between weight of chitosan and volume of Fe (II) solution.

- 125-300 μm ratio is 0.1 g/25.0 ml.
- >300 μm ratio is 0.25 g/25.0 ml.

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