

SCUOLA DI SCIENZE

Dipartimento di Chimica Industriale “Toso Montanari”

Corso di Laurea Magistrale in

Chimica Industriale

Classe LM-71 - Scienze e Tecnologie della Chimica Industriale

Obtainment of chitosan with different
molecular weight by varying chitin
decarbonation conditions and study of a
mathematical model representing the
process

Tesi di Laurea sperimentale

CANDIDATO

Matteo Verdolini

RELATORE

Prof. Daniele Caretti

CORRELATORE

Prof. Francisco Carrillo de la Fuente

Abstract

The effects of different chitin decarbonation conditions on chitosan molecular weight have been studied. The factors that could affect chitosan molecular weight and that have been studied in this work are temperature, time and hydrochloric acid concentration; the factor that proved to mostly affect the chitosan molecular weight is the temperature, followed by time and acid concentration. The effect of chitin molecular weight on the finally obtained deacetylation degree has also been studied, and proved to be almost irrelevant. The effect on chitosan molecular weight given by deacetylation carried out with different NaOH concentration has been analyzed; this factor proved to give inversely proportional effect on chitosan molecular weight.

Riassunto

Sono stati studiati gli effetti delle diverse condizioni di decarbonatazione della chitina sul peso molecolare del chitosano così ottenuto. I fattori che possono influire di più sul peso molecolare del chitosano e che sono stati studiati sono temperatura, tempo e concentrazione di acido cloridrico; il fattore che è risultato essere più influente sul peso molecolare è la temperatura, seguita da tempo e concentrazione di acido. E' stato inoltre studiato l'effetto del peso molecolare della chitina sul suo grado di deacetilazione ottenuto alla fine del processo, ed è risultato essere praticamente irrilevante. E' stato analizzato l'effetto di diverse concentrazioni di NaOH utilizzate durante il processo di deacetilazione sul peso molecolare del chitosano; questo fattore ha provato di avere un effetto inversamente proporzionale sul peso molecolare.

Index

Abstract	3
Riassunto.....	3
1 Introduction	7
1.1 <i>Procambarus Clarkii</i> (American Red Crab).....	7
1.1.1 Description	7
1.1.2 Environmental Problem	8
1.1.3 Composition of the exoskeleton	9
1.2 Chitin and Chitosan.....	11
1.2.1 Description	11
1.2.2 Properties and applications of chitin, chitosan and chitosan oligosaccharides (COS).....	12
1.2.2.a Chitooligosaccharides	13
1.2.3.b Application of COS in food industry.....	14
1.2.3.c Application of COS-based systems as carriers of anticancer drugs	14
1.2.3.d Other industrial applications	16
1.3 Chitosan obtainment methods	17
1.3.1 Deproteinization.....	17
1.3.1.a Enzymatic Deproteinization	19
1.3.1.b Chemical Deproteinization.....	20
b.1 Acid Hydrolysis	21
b.2 Basic Hydrolysis	23
1.3.2 Demineralization and its effect on molecular weight.....	24
1.3.3 Deacetylation.....	25
1.4 Determination of deacetylation degree.....	26
1.4.1 Determination of DD using FTIR.....	26

1.4.2 Determination of DD using NMR.....	27
1.5 Determination of molecular weight of a polymer	28
1.5.1 GPC.....	28
1.5.2 Molecular weight through viscosity	30
1.5.2.a Capillary viscometers.....	30
1.5.2.b Types of viscosity	31
1.5.2.c Determination of intrinsic viscosity and molecular weight	33
1.5.2.d Other ways to determine $[\eta]$: the Fedors equation	36
2 Aim of the project	37
3 Results and discussion.....	39
3.1 Response surface methodology.....	39
3.1.1 Design of experiment: general notes on the Central Composite Design	40
3.1.1.a Preliminary study: Determination of upper and lower limits	42
Acid concentration	42
Temperature	44
Time.....	44
2.1.1.b Design of Experiment	45
2.1.2 Determination of the mathematical model	46
Method of least squares	47
Optimization.....	48
3.2 Analysis of the results	49
3.2.1 Collection of the data.....	49
3.2.2 Analysis of the first response: Kinematic Viscosity	51
ECHIP analysis of kinematic viscosity	52
3.2.3 Determination of intrinsic viscosity (to be used for MW determination)	54

3.2.3.a Intrinsic viscosity using Kraemer viscosity plot: analysis of the results	55
ECHIP analysis of intrinsic viscosity obtained from inherent viscosity	56
3.2.3.b Intrinsic viscosity using Fedors equation: analysis of the results	58
Determination of the Fedors coefficient c_m	59
ECHIP7 analysis of intrinsic viscosity obtained from the Fedors equation ..	61
3.3 Determination of the molecular weight	62
3.3.1 Determination of coefficients K and α	62
3.3.2 ECHIP7 analysis of molecular weight	63
3.4 Effect of molecular weight of chitin on the deacetylation degree	67
3.5 Effect of deacetylation conditions on molecular weight	71
4 Conclusions	73
5 Experimental part	75
5.1 General Notes	75
5.2 Raw Material	75
5.3 Washing	77
5.4 Deproteinization	79
5.5 Decarbonation	81
5.6 Deacetylation	83
5.7 Measurement of flowing times for viscosimetry	85
Bibliography	87

1 Introduction

The approach to this work of thesis comes from the necessity to deal with the economical and environmental problem that the red crab of the swamps of Guadalquivir is generating as an invader species.

The invasion of this species is fought using the massive capture of this crab; this has generated a proliferation of little-small factories whose purpose is the treatment of this crab finalized to the obtainment of direct-consumption food from its abdomen. These treatments generate a big amount of solid residuals, which are mostly heads and shells of these crabs.

The solution to the problem consists in a conversion of these residuals in a high added-value product, which is the chitin, or its derivative, chitosan. Nowadays the mentioned production of chitin is estimated around 10^{11} tons/year¹, and the exoskeletons of these crabs represent the most important source of raw material.

1.1 *Procambarus Clarkii* (American Red Crab)

1.1.1 Description

The *Procambarus Clarkii* is a crustacean of the family of the *cambaridae*, with a cylindrical-shaped body. The exoskeleton of the adults is dark red but some are coffee-colored. The young ones are instead grey, with some black shapes.

It lives in many kinds of freshwater: rivers, plantations, irrigation canals ecc., avoiding strong-current rivers; it's very territorial and aggressive towards his owns species.

It's benthic and omnivorous, it usually eats insects, larvas, debris, preferring though animal matter.²



Figure 1.1 – Picture of the *Procambarus Clarkii*

1.1.2 Environmental Problem

Until the second half of the last century the native river crab (*Austropotamobius pallipes*), was widely spread in the water streams of the andalucian limestone mountains and in the rest of the national territory.

Since then, the Spanish economical dinamization has been causing an industrialization of the country, bringing harmful effects to the aquatic environments, like water contamination, shores alteration or destruction of the fluvial geomorphology, which bring a sort of decline of the associated communities.

In 1974, with economical purpose, the *American Procambarus clarkia* was introduced in the swamps of Guadalquivir. Given their debris-eater nature, these crabs are modeler of most of the fluvial communities, in which can be found bryophytes, macrophytes, macro-invertebrates, amphibious and fishes. Without any doubt, they are a key-species for the energy-fluxes of the environment, but they caused a lot of trouble.

In first place, given its voraciousness and reproductive capacity, the *Procambarus* managed to send away the native crabs from their original and environmental nest. Second, its introduction put the native crab in a risky situation. The crab plague named *afanomicosis* (derivating from a fungus, the *Aphanomyces astaci*) came together with the American crab to the Iberian Peninsula; this disease causes a 100% mortality of the affected native population, and this brought almost to the extinction of the species in just 30 years.

So that we can definitely say that the red crab is a concerning environmental problem.³

Furthermore, the growth of the American red crab causes economical losses to the rice cultivations, very common in this region.

To stop, or slow down this diseases caused by the crabs, factories working and producing food from them were born. This originated a second environmental problem, which is the generation of a solid residual, the exoskeleton of the crab, which is the great part of its body weight.

1.1.3 Composition of the exoskeleton

To take advantage of any residual it is important to know its chemical-physical properties and composition. The nature of the residual is very variable, but the concentration results are very often between typical values.

The exoskeleton is usually formed of 30-40% of proteins, 30-50% of calcic carbonate and 20-30% of chitin. At lower concentrations there are the lipid-pigments which give to the crab the typical red color.⁴

The exoskeleton is formed by an external epicuticle, followed by an exocuticle, an endocuticle and the internal layer of the epidermis. This is common between both marine and terrestrial organisms, so there could be differences in concentrations of chitin, carbonates, and proteins depending on the exoskeleton zone and the species.

A list of commercially exploited marine species and their chitin and calcium carbonate content is shown in Table 1.1.

Type	Chitin (%)	CaCO ₃ (%)	Location
Phylum Crustacea			
<i>Euphausia</i> sp. and <i>Meganyctiphanes</i> sp. (Krill)	20–30	20–25	Cuticle/exoskeleton
<i>Chionoecetes</i> sp., <i>Cancer</i> sp., and <i>Carcinus</i> sp. (Crab)	15–30	40–50	Cuticle/exoskeleton
<i>Paralithodes</i> sp. (King crab)	~35	40–50	Cuticle/exoskeleton
<i>Callinectes</i> sp. (Blue crab)	~14	40–50	Cuticle/exoskeleton
<i>Crangon</i> sp. and <i>Pandalus</i> sp. (Shrimp)	17–40	20–30	Cuticle/exoskeleton
<i>Penaeus</i> sp. (Prawn)	~40	20–30	Cuticle/exoskeleton
<i>Nephrops</i> sp. and <i>Homarus</i> sp. (Lobster)	60–75	20–30	Cuticle/exoskeleton
<i>Lepas</i> sp. (Goose Barnacle)	~59	20–30	Shell
Phylum Mollusca			
<i>Mytilus</i> sp. and <i>Pecten</i> sp., etc. (Mussels, clams, etc.)	~3	85–90	Shell
<i>Crassostrea</i> sp. (Oyster)	~6	85–90	Shell
<i>Loliginidae</i> sp. and <i>Ommastrephidae</i> sp. (Squid)	20–40	Negligible	Pen

Sources: Adapted from Tharanathan, R.N. and Kittur, F.S., *Crit. Rev. Food Sci. Nutr.*, 43, 61, 2003; Kurita, K., *Mar. Biotechnol.*, 8, 203, 2006.

Table 1.1 - List of commercially exploited marine species and their chitin and calcium carbonate content

In the cuticle we can find two different types of chitin, one is tubular (stick-shaped) and other is laminar, binded to two different types of proteins.⁴ The chitin locates itself in layer-shapes, and its principal purpose is to serve as organic matrix for structural stabilization (structural fibers).

A schematic interpretation of this organic matrix is reported in Figure 1.2⁵

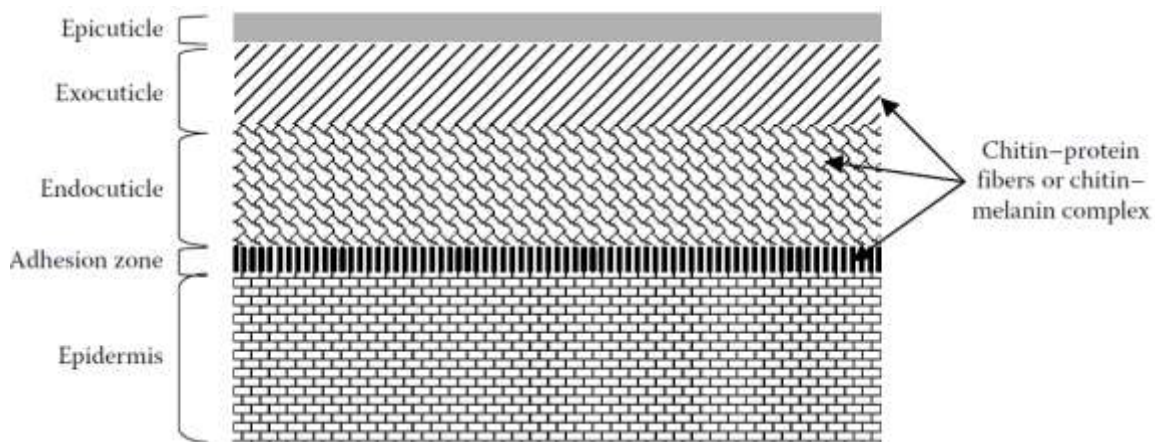


Figure 1.2 - Organic matrix of chitin

1.2 Chitin and Chitosan

1.2.1 Description

The chitin is a polysaccharide widely spread in nature, in fact is the second most abundant polymer after cellulose.

Chitin is a cationic amino polysaccharide composed of *N*-acetyl-d-glucosamine (GlcNAc, 2-acetamido-2-deoxy-d-glucose) with β (1 \rightarrow 4) glycosidic bonds between each monomer.

When chitin reaches a nitrogen content of more than 7% by weight, or when the degree of deacetylation (DD) is over 60% the term “chitosan” is preferred.

In nature, chitin is found 90-95% acetylated.

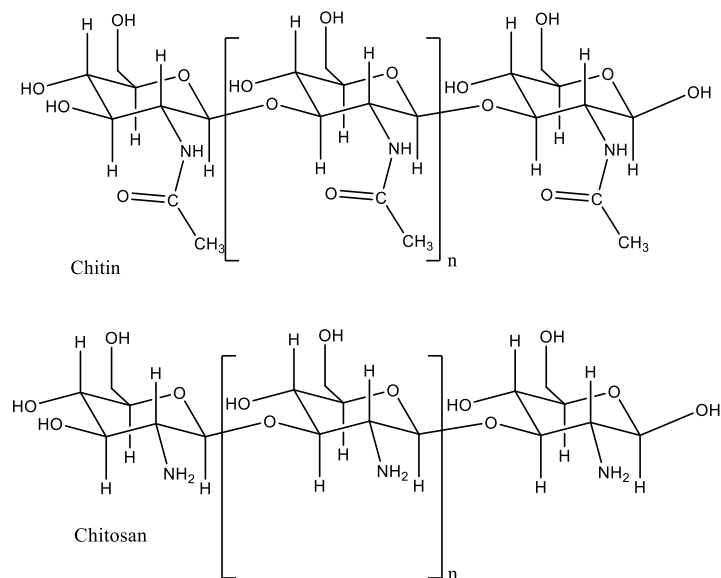


Figure 1.3 - Molecular structure of chitin and chitosan

Chitin occurs in three polymorphic solid state forms designated as α , β and γ chitin which differ in their degree of hydration, size of unit cell, and number of chitin chains per unit cells.

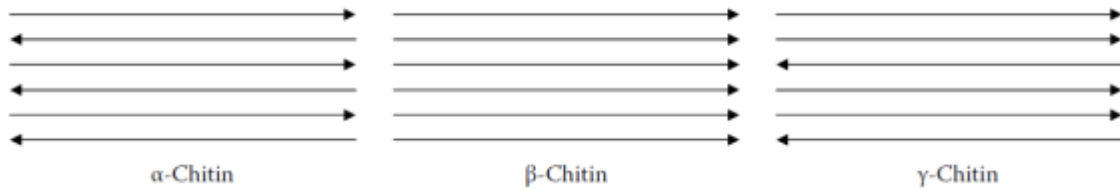


Figure 1.4 – Polymorphic solid states of chitin

In the α -chitin the chains of polysaccharide are disposed like sheets, all oriented in the same direction, they are grouped as orthorhombic compact cells.⁶ In the β -chitin the chains are disposed in anti-parallel way, grouped as monoclinic cells. The third structure can be considered as the combination of the previous ones, as the chains are oriented two in one direction, and the third in the opposite direction.⁷

The most common polymorph in nature is the α -chitin, which for this reason has been more studied than the other two.

1.2.2 Properties and applications of chitin, chitosan and chitosan oligosaccharides (COS)

Both chitin and chitosan have unusual multifunctional properties, including high tensile strength, bioactivity, biodegradability⁸, biocompatibility, nonantigenicity, and nontoxicity⁴, which made them possible to be used in many applications. Furthermore, the chemical modifications of the three reactive functional groups of chitosan have increased the applications of chitosan in different fields (Figure 1.5).⁹

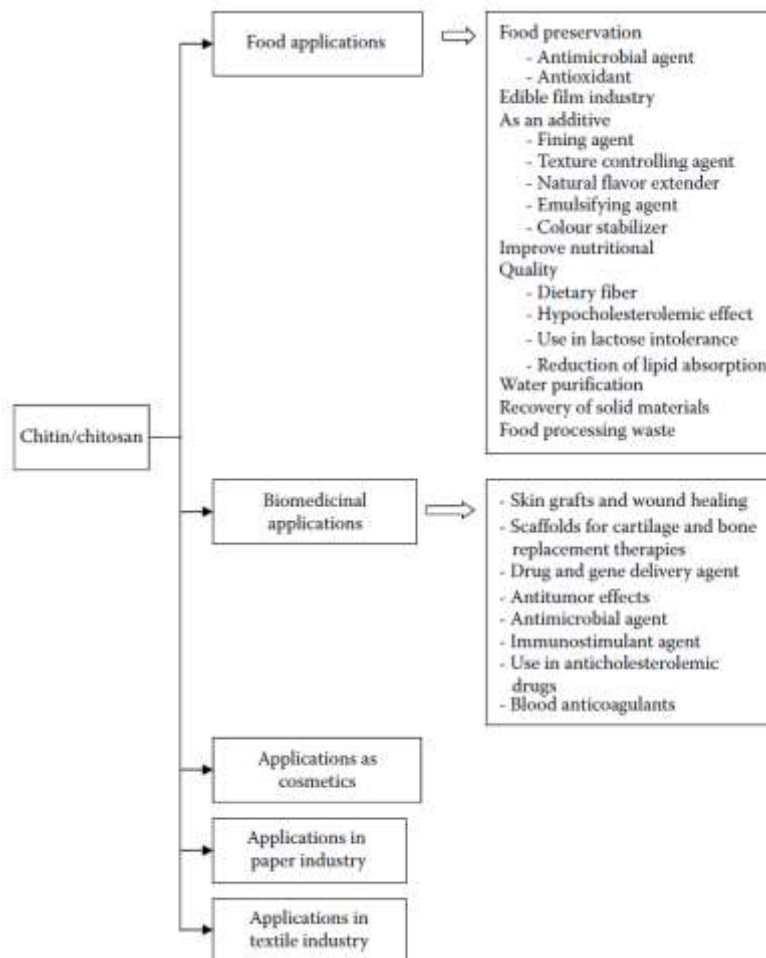


Figure 1.5 – Most exploited applications of chitosan

1.2.2.a Chitooligosaccharides

Even though chitin and chitosan have various properties that enable them to be applied in many fields, their poor solubility at neutral pH due to high molecular weight and high viscosity limit their application at certain industries, especially in the food and biomedical fields.

However, chitosan oligosaccharides (COS), which are composed of 2-10 units of d-glucosamine units are readily soluble in water. This is due to the short chain lengths and the presence of free amino groups in the d-glucose units.¹⁰

This greater solubility of COS at neutral pH has attracted attention for the application of COS in different fields where poor solubility of chitin and chitosan has been a limiting factor.

1.2.3.b Application of COS in food industry

The main utilization of chitosan in the food industry is the food preservation.

The microbial deterioration and oxidation of foods are the major problems that arise in the increasing of the shelf life of foods. However, food preservation has been achieved successfully with the chemical preservatives. The growing consumer demand toward foods without chemical preservatives has received attention due to natural products, including chitin, chitosan, and COS. The special properties such as antimicrobial activity and antioxidative activity allow the chitin, chitosan and especially COS to get successful as natural preservatives in the food industry.

Different microorganisms, including bacteria, fungi and yeast, are responsible for the microbial deterioration of foods and they act as food pathogens as well. Though chitosan also exhibits antimicrobial property, the greater efficiency of the COS against the microorganism and greater solubility increase the possibilities of COS to be applied as a natural food preservative. The COS can act against both bacteria and fungi that are involved in the spoilage of food. However, scientists have found that the antibacterial activity of the COS is higher, compared to their antifungal activity.¹¹

1.2.3.c Application of COS-based systems as carriers of anticancer drugs

The principal modes of cancer management are surgery, radiotherapy, and chemotherapy. Recently hormonal therapy and immunotherapy are increasingly being used as well, but their applications are limited for a few cancer types such as breast neoplasia.¹² Chemotherapy, the use of cytotoxic drugs to kill cancerous cells, remains the most common approach for cancer treatment. Generally, cytotoxic drugs are highly toxic but poorly specific, and do not differentiate between normal and cancer cells. Therefore, conventional chemotherapy administration or systemic administration has been shown to produce side effects.

Most of the drug content is released soon after administration, causing drug levels in the body to rise rapidly, peak, and then decline sharply, leading to unacceptable side effects at the peaks and inadequate therapy at the troughs.¹³ Due to the short duration of action, repeated injections are often required, which can lead to the exacerbation of side effects and inconvenience, and can unfortunately lead to less patient compliance. In systemic administration, cytotoxic drugs are extensively transported to the whole body, therefore, only a small fraction of the drugs reach the tumor site and other healthy organs or tissues can be affected or damaged by the nonspecific action of the cytotoxic agents.¹²

Due to these obstacles, controlled or localized release technology has been replacing systemic administration and has some potential for cancer treatment. For its antibacterial, biocompatible, biodegradable and anticancer activity, therapeutic applications of chitosan nanoparticles possessing mucoadhesive properties, has been widely studied and formulated in anticancer drug delivery systems.

One of the solutions which use chitosan-based systems for drug delivery is the use of in situ injectable chitosan hydrogels for localized and controlled release of therapeutics. Sustained-release injectable formulations are basically designed as microparticulates (microcapsules or microspheres), implants, or gel systems.¹⁴ Drugs are commonly loaded into microspheres via a passive absorption method whereby microspheres are added to drug solution. Microspheres swell in solution and the drug molecules enter the gel matrix.¹⁵ However, the efficiency of this loading method for cytotoxic drugs is limited and a high loading capacity is unattainable. An implant requires surgery to insert it near the tumor site, which adds to the cost and the risk of this system. These problems have oriented research towards injectable in situ gelling formulations.¹⁶ Injectable in situ chitosan hydrogels that have been trialed in the treatments of cancers are summarized in Table 1.2.¹⁷

Injectable In Situ Chitosan Hydrogels in Cancer Treatments

Cancer Type	Hydrogel System	Method to Induce Gelation	Therapeutic Agent	Route of Administration	Effect
Lung cancer	Azide-chitosan-lactose (Az-CH-LA)	UV irradiation	Paclitaxel	Subcutaneously injected beneath the tumor	<ul style="list-style-type: none"> • Tumor-growth inhibition • Tumor-angiogenesis inhibition
Breast cancer	Chitosan/ β -glycerophosphate (C/GP)	Temperature	Paclitaxel	Intratumoral injection	<ul style="list-style-type: none"> • Tumor-growth inhibition • Tumor-recurrence prevention
Fibrosarcoma	Chitosan/ β -glycerophosphate (C/GP)	Temperature	Camptothecin	Intratumoral injection	<ul style="list-style-type: none"> • Tumor-growth inhibition
Cervical cancer	Chitosan/ β -glycerophosphate (C/GP)	Temperature	Doxorubicin and vaccinia virus-based vaccine expressing Sig/E7/LAMP-1 (Vac-Sig/E7/LAMP-1)	Intratumoral injection	<ul style="list-style-type: none"> • Tumor-growth inhibition
Mucin-production-associated cancers	Chitosan/glycerol monooleate (C/GMO)	pH	Paclitaxel	—	—
Osteosarcoma	Chitosan/dibasic orthophosphate	Temperature	Doxorubicin and gene encoding pigment epithelium-derived factor	Subcutaneously injected next to the tumor	<ul style="list-style-type: none"> • Tumor-growth inhibition • Metastasis - development inhibition

Table 1.2 – Some of the injectable in situ chitosan hydrogels already trialed

1.2.3.d Other industrial applications

Here are other industrial fields where the chitosan production is strongly implanted:

- Clarification, prevention of the oxidation, stabilization of color and antibacterial agent in wine. Chitosan has a good affinity with the phenolic molecules, which are the main responsible of the oxidation and dimming of wine. It also reduces the quantity of polyphenols and stabilizes the white wine in the same way an already widely utilized compound like the potassium caseinate does. Thanks to its antibacterial ability, it is also used to eliminate the microorganisms responsible of the degradations of vines, like the bacteria *Brettanomyces*.
- Purification of water effluents. Chitosan can be used as a tool to purify residual waters thanks to its high absorption ability. Other feature of the chitosan, which can be useful in water purification, is its flocculating ability.
- Deacidification of drinks such as fruit juices or coffee. To this purpose, it is common to use chitosan salts in which the amino group acts as a weak base.

1.3 Chitosan obtainment methods

Industrial techniques for chitin and chitosan extraction from different shell waste normally rely on harsh chemical processes which generate large quantities of hazardous chemical wastes. In spite of that, several procedures for the preparation of chitin and chitosan from different shellfish wastes have been developed over the years, some of which form the basis of the chemical processes used for the industrial production of chitin and derivatives.¹⁸

Schematically, the exoskeleton of the *P. Clarkii* is composed by an organic matrix made of chitin which, as said before, is a polymer having a structural purpose. Bonded to it there are proteins of different nature and an inorganic phase made by calcium and magnesium carbonate, which confers toughness and mechanical resistance to the exoskeleton.

Therefore, the production of chitosan from chitin requires the elimination of the proteins and the inorganic phase and, at last, the deacetylation to obtain chitosan.

1.3.1 Deproteinization

The deproteinization consists of the elimination of the proteins bonded to the chitin. The nature of the chitin-protein bond is vary, and there are many models describing this interaction.

Hackman, using iontophoresis and paper chromatography, determined in 1960 the presence of aspartic acid, histidine and D-Glucosamine. This allowed him to conclude that there was a peptide bond between a non-acetylated amine group of the chitin and a carboxylic group of the protein-chain (Figure 1.6)

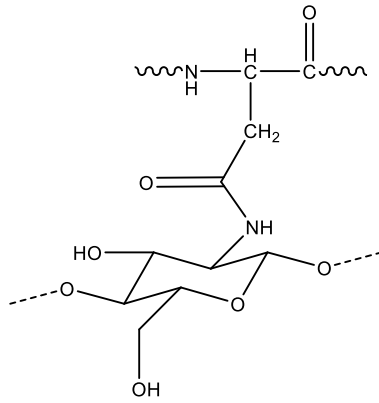


Figure 1.6 – Hackman's model of chitin-protein bond

Another model proposed by Hackman is the union between the chitin and the protein through the formation of a Schiff base, with a terminal unit N-acetylglucosamine 2-acetamide-2-deoxy-D-glucose of the chitin (Figure 1.7)

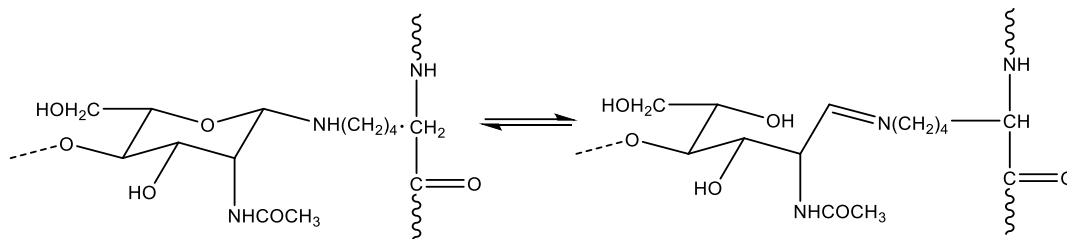


Figure 1.7 - Another Hackman's model of chitin-protein bond

Hunt, in the early 1970 determined that the protein-chitin complex can be explained with two structures formed by two actual amino acids: the N-glycosidic structure involving the amino group of the asparagine and the O-glycosidic structure involving the serine (respectively left and right structure in Figure 1.8).

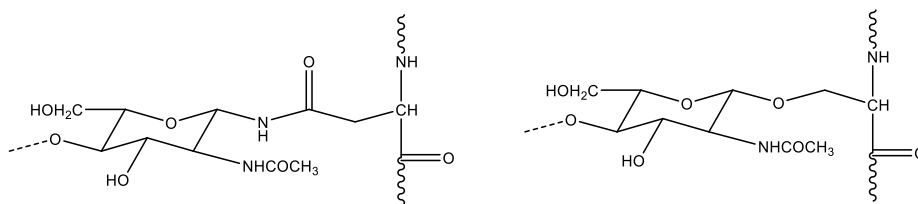


Figure 1.8 – Hunt's model of chitin-protein complex

Rudall and Kenchington formulated another possible complex chitin-protein, through the carboxylic group of the N-acetylmuramic acid and the amino group of a terminal alanine (Figure 1.9).

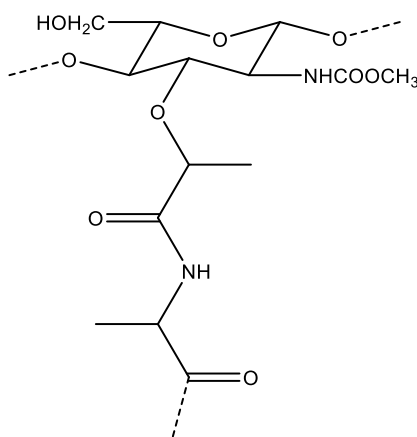


Figure 1.9 – Rudall's and Kenchington's model for the complex chitin-protein

So, as it is shown, many forms of chitin-proteins interactions are speculated; this implies that it is necessary to apply a method which can eliminate proteins independently from the nature of their bond with the chitin, to make sure that the deproteinization is effective.

Generally, there are many methods to achieve this deproteinization, but they can be assorted in two groups: chemical deproteinization and enzymatic deproteinization.¹⁹

1.3.1.a Enzymatic Deproteinization

Thanks to the advancements in biochemistry and, most of all, to the advances in chemical engineering, enzymes are being incorporated more and more in the industrial processes, and not only in the agro-alimentary industry, where fermentation processes are performed.

An example is the incorporation of enzymes in the deproteinization for chitin production. There are several enzymes which could be used in order to achieve the deproteinization of the exoskeleton of crustaceans, like for example the

proteinase SV1 (Manni, Ghorbel-Bellaaj, Jellouli, Younes, & Nasri, 2010), where the reaction was achieved at pH of 8.0, temperature of 40°C and for 3 hours, accomplishing to diminish the protein percentage from 40,83±1,22% to 10,78±0,2%.

Other enzymes used for the deproteinization were derived from tuna, papaya and other extracted from some bacteria.²⁰

A problem related to this kind of deproteinization, comparing it to the more traditional chemical deproteinization, are the too long reactiontime, the lower effectiveness and the proteic residual naturally associated to the process.²¹

The efficiency in the deproteinization of the exoskeleton of crabs of different commercial enzymes: Delvolase[®], Cytolase PCL5[®], Econase CEPi[®], Econase MP 1000[®], MaxameTM MNP[®] and Ccllupulin MG[®], was compared getting to the conclusion that the Delvolase[®] is the most effective enzyme. It managed to eliminate almost 90% of proteins, after a one-day incubation.²²

1.3.1.b Chemical Deproteinization

Hackman and Goldberg used different extraction conditions, each of them was associated to a different kind of chitin-protein interaction.²³

- Water at pH 7 for 48 hours for non-bonded water-soluble proteins.
- Sodium sulfate 0,17M at pH 7 for 48 hours for proteins connected with weak bonds like Van der Waals forces.
- Urea 7M at pH 7 for 48 hours, for proteins bonded with H-bonds.
- NaOH 0,01M at T_{amb} for 5 hours, for proteins bonded with electrostatic forces.
- NaOH 1M at 50-60°C for 5 hours, for proteins connected with covalent bonds.

Proteins linked to the chitin with electrostatic bonds like Van der Waals forces or hydrogen bonds loose their bounding ability varying the pH of the solution;

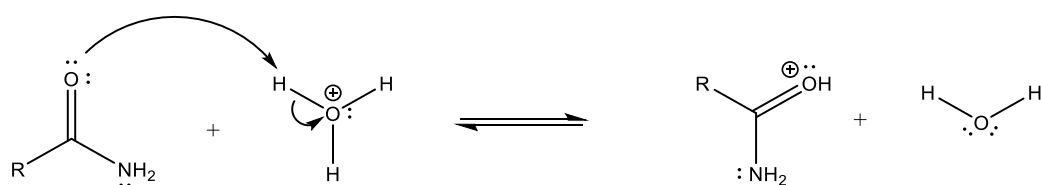
because protonating or de-protonating functional groups makes the protein change its nature.

The proteins linked through covalent bonds are almost 5% of the proteins of the exoskeleton, furthermore they are the most difficult to remove because of the bond strength. The main connection between the chitin and the protein is the peptide bond between a non-carbonated amino group of the chitin and a terminal carboxylic group of the protein. This bond is stable in water, but can be broken by heating or using strong acids or bases through hydrolysis. This hydrolysis is nothing else than an acyl nucleophilic substitution, giving an amine and a carboxylic acid. In an acid medium the amine will be protonated as an ammonium ion, in a basic medium the carboxylic acid will be deprotonated as a carboxylate ion.

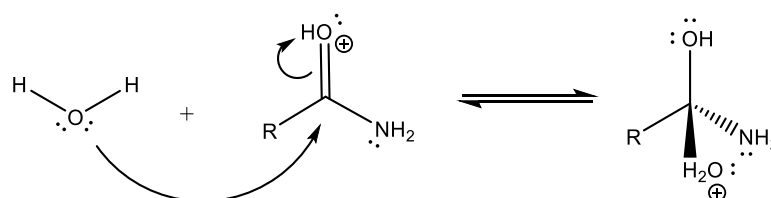
The mechanism of the hydrolysis of an amide is very similar to the mechanism of the hydrolysis of the other carboxylic derivatives.

b.1 Acid Hydrolysis

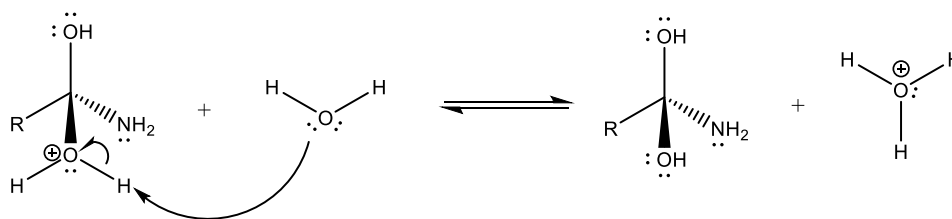
The hydrolysis in acidic medium goes through two steps: the formation of a tetrahedral intermediate and its successive dissociation. To make the nucleophilic attack possible, the carbonyl oxygen must be protonated, so that the amide results activated. The cation produced in this step is stabilized by resonance.



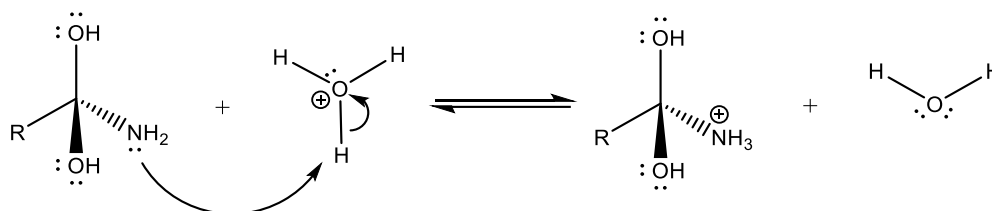
Once the amide gets O-protonated, the nucleophilic addition of the water happens:



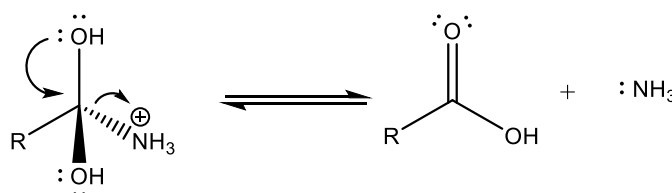
The latterly formed ion loses its proton, giving the neutral form of the tetrahedron:



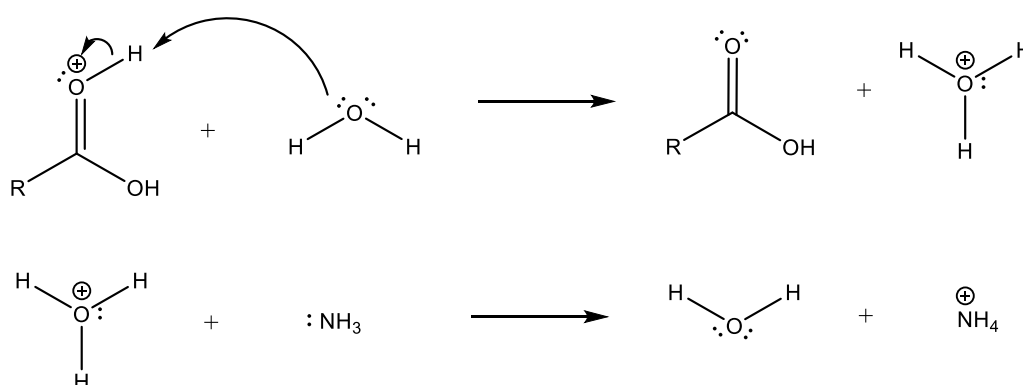
The next step is the formation of the ion ammonium with the protonation of the nitrogen:



After that, the dissociation of the ion happens, giving ammonia and the protonated form of the carboxylic acid:



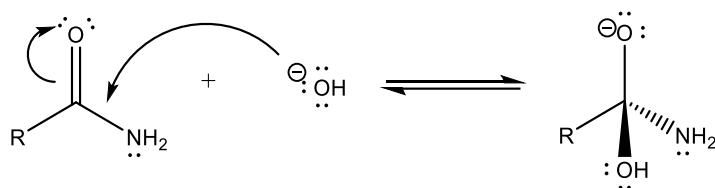
Since we are in acid medium, the most stable species are the ammonium ion and the carboxylic acid. The equilibrium constant of the last step is so high that makes the global reaction irreversible:



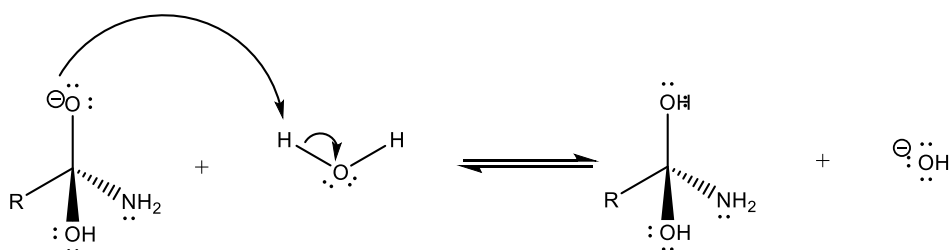
b.2 Basic Hydrolysis

Like for the acid hydrolysis, this process happens in two steps: the formation of a tetrahedral intermediate and its dissociation giving the breaking of the amide bond.

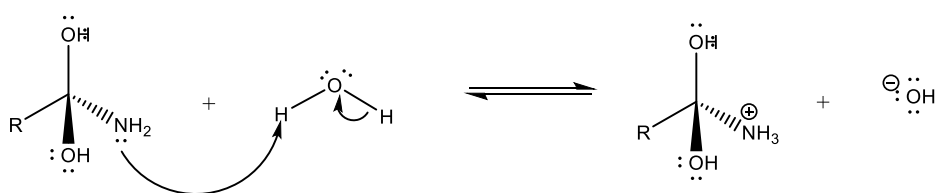
In alkaline medium, the hydroxyl ion carries out a nucleophilic attack to the carbonyl group of the amide, to give the anionic form of the tetrahedral intermediate.



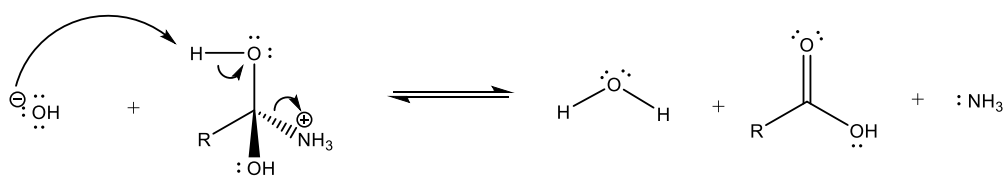
In aqueous medium, the anion interacts with water, deprotonating it to give the neutral species of the tetrahedral intermediate.



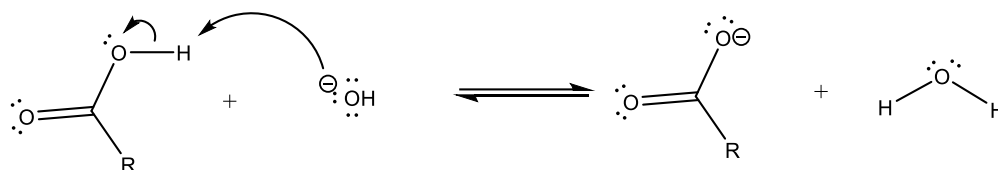
In the next step the protonation of the intermediate is accomplished. As opposed to the acid hydrolysis where the most stable form was the O-protonated form, the most stable species here is the N-protonated form.



This latter species suffer from an attack of a basis, promoting the hydrolysis of the amide bond, and giving the carboxylic acid and the corresponding amine.



Of course, this species is not stable in an alkaline solution, so the carboxylate ion irreversibly forms. This latter is so stable in basic medium, that makes the global reaction irreversible.²³

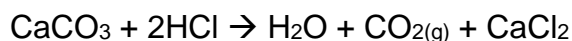


1.3.2 Demineralization and its effect on molecular weight

This step consists of the elimination of inorganic phase of the exoskeleton. The removal of calcium carbonate (approximately 95% of the inorganic matter), calcium phosphate, and other mineral salts found in shells is accomplished by extraction with dilute acids. It has been shown to be important that the amount of acid is stoichiometrically equal to or greater than all the minerals present in the shells to ensure complete demineralization.²⁴

Most of the authors coincide about the utilization of hydrochloric acid as reactant for this step, as it is a cheap and easily available acid.

The reaction of elimination of the calcic carbonate with hydrochloric acid is the following:



It is generally agreed that the processing conditions strongly affect the molecular weight and DA of chitin. Although HCl may be the cause of detrimental effects on the intrinsic properties of the purified chitin, it remains the most commonly used decalcifying agent in both laboratory and industrial scale production of chitin.

As a rule, as the acidic conditions for demineralization (pH, time, and temperature) become harsher, the molecular weight of the products thus obtained becomes lower. Indeed, chitin is an acid-sensitive material and can be degraded by several pathways: hydrolytic depolymerization, deacetylation, and heat degradation leading to physical property modifications.²⁵

Anyway, as it was said before speaking about COS, also the low molecular weight chitosan may be used for many application, so it surely could be interesting to explore different routes and conditions which can lead to a lower molecular weight polymer as well.

1.3.3 Deacetylation

Chitin can be converted to its N-deacetylated product, i.e., chitosan, by homogeneous or heterogeneous alkaline N-deacetylation. The degree of deacetylation (DD, %) is defined as the ratio of *N*-deacetylated (amino) groups to *N*-acetyl groups at the C₂ position in the backbone.²⁶

When chitin reaches a nitrogen content of more than 7% by weight, or when the degree of deacetylation (DD) is over 60% the term chitosan is preferred.

Chitin and/or chitosan has several distinctive biological properties depending on their deacetylation degree, including biocompatibility and biodegradability, cellular binding capability, acceleration of wound healing, hemostatic properties, and anti-bacterial properties.²⁶

The degree of deacetylation is defined as the molar fraction of *N*-acetylglucosamine units in the chain:

$$DD = 100 * \frac{n_{GlcN}}{n_{GlcN} + n_{GlcNAc}}$$

where, n_{GlcN} is the average number of *D*-glucosamine units and n_{GlcNAc} is the average number of *N*-acetylglucosamine units.

1.4 Determination of deacetylation degree

Several methods for determining the degree of deacetylation have been elaborated, from simple, such as pH-metric titration, UV-Vis spectroscopy, infrared spectroscopy, elemental analysis, to complex ones, which require complex and expensive equipment, such as ^1H -NMR spectroscopy and ^{13}C -NMR spectroscopy.

Anyway, the most common analytical methods for the determination of DD of chitosan are the FTIR (Fourier Transform Infrared Spectroscopy) and NMR (Nuclear Magnetic Resonance, mostly ^{13}C -NMR and ^1H -NMR).

1.4.1 Determination of DD using FTIR

Infrared (IR) spectroscopy is widely used because of its simplicity and rapidity, it is non-destructive and it is not necessary dissolve the sample; however, other problems associated with spectroscopic techniques, such as broadening of a peak and overlapping of two or more peaks which leads to incorrect results, are often detected. This technique is appropriate for qualitative study; when quantitative analysis is performed, it is necessary to carry out some complex procedures, such as statistical analysis of several absorption ratios.

Different approaches have been described by Kasaai²⁷ to calculate the DD. One of them consists of the evaluation of the absorbance ratio of the probe band (determination of the *N*-acetyl or amine content) and the absorbance of a reference band; the intensity of this band does not vary with the DD. The DD of an unknown sample can be calculated by comparing the value of the ratio with similar ratio of samples with known DD. Determination of DD can also be performed based on linear calibration plots of the absorption ratio of chitin or chitosan samples with a known DD against their DD. The DD of the unknown samples was determined from the equation of the calibration curve.

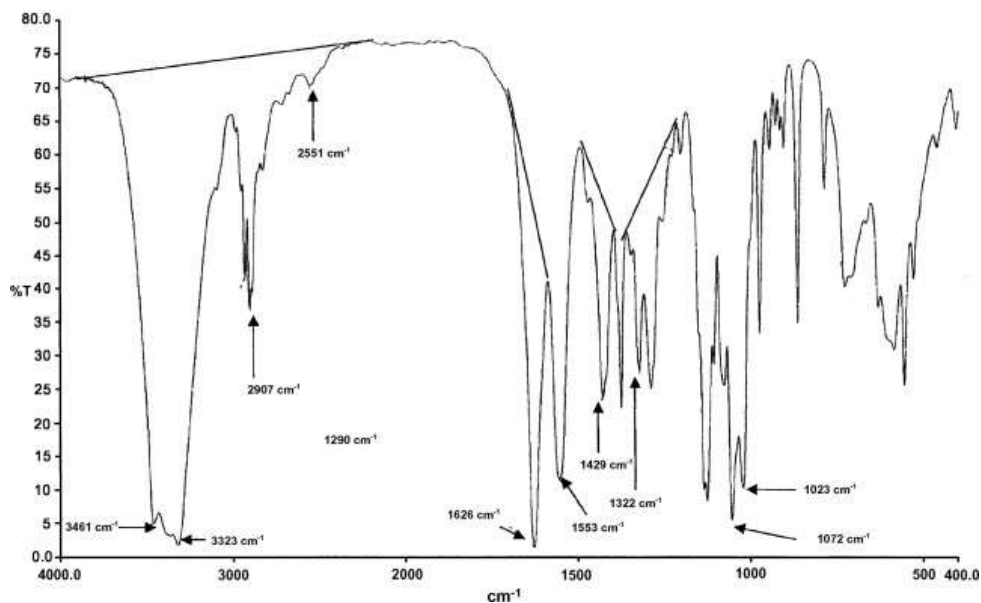


Figure 1.10 - A typical spectrum of N-acetyl d-glucosamine showing the positions of probe and reference bands and corresponding baselines²⁷

1.4.2 Determination of DD using NMR

For quantitative analysis, NMR spectroscopy either liquid-state ¹H-NMR for soluble samples or solid-state ¹³C-NMR is the preferred method because of its simplicity, quickness, and accuracy, at least in solution. Moreover, the American Standard Test Method organization has selected the ¹H-NMR as the standard method for the determination the DD in chitosan samples.^{27,28} In case of ¹³C-NMR spectroscopy, although it is not necessary to dissolve the samples, it is essential that the samples are highly pure to obtain suitable spectrum.²⁹

Fernández Cervera et al. used the solid-state ¹³C-NMR to estimate the DD of chitosans obtained from lobster chitin. The calculation was performed according the equation $DD = I_{CH_3}/I_{C_1} + I_{C_2} + I_{C_3} + I_{C_4} + I_{C_5} + I_{C_6}/6$, where (I_{C_1} ; I_{C_2} ; I_{C_3} ; I_{C_4} ; I_{C_5} ; I_{C_6}) are the relative intensities of the resonance of the ring carbon and (I_{CH_3}) is the methyl carbon obtained by ¹³C-NMR.²⁹

Abdou³⁰ applied the ¹H-NMR spectroscopy to determine the DD of chitosan samples obtained from different sources. Samples were dissolved in D₂O acidified with DCL to improve solubility. The ¹H-NMR spectra of chitosan showed the characteristic signals: the peak at 4.9 ppm that corresponds to C1 proton of

glucosamine unit in chitosan, the peaks at 3–4 ppm that correspond to C2–C6 protons of glucosamine and *N*-acetylglucosamine units; the peak at 2.06 ppm that corresponds to amide methyl protons and at 2.21 ppm for acetic acid moiety.

The DD was calculated according to the method of Lavertu³¹:

$$DD(\%) = \left[1 - \frac{\frac{1}{3} \times I_{CH_3}}{\frac{1}{6} \times I_{(H_2-H_6)}} \right] * 100$$

1.5 Determination of molecular weight of a polymer

Analytic techniques for the determination of molecular weight can be broken down into secondary (or relative) and primary (or absolute) techniques based on whether or not standards are needed to calibrate the analytic instrument.

Between the secondary techniques the most common for the molecular weight determination is surely the GPC, while among the primary techniques it is important to mention, especially for this work of thesis, the viscosimetry.

1.5.1 GPC

Gel permeation chromatography involves passing a dilute polymer solution through a tubular column packed with polymeric gel (crosslinked) beads. Under high pressure flow some of the polymer chains are forced into the pores of the gel, while others pass by the gel beads. The residence time of a given polymer chain in the packed column depends on the path it takes through the gel. For instance, a low molecular weight oligomer will easily be force into the pores of the gel and will take a circuitous path through the column, traveling a distance equivalent to hundreds of the column length. High molecular weight species can not fit into the pores of the gel, i.e. they are excluded, and can pass more directly to the exit of the column traveling a distance roughly equivalent to the column length. The selectivity of this process for molecular dimension is outstanding and the range of molecular weights which can potentially be characterized by this technique is only

limited by the ability to produce controlled spaced gels. GPC is by far the most versatile technique for the determination of molecular weight in a polymer sample.

GPC is called by different names in different fields. Organic chemists call it size exclusion chromatography (SEC), gel filtration chromatography (GFC) and variants of these such as HPSEC, HPGFC, HPGPC. There may be some minor differences but the instruments are basically all the same thing.

In GPC a sample of polymer in dilute solution is injected into the chromatograph at an instant of time, t_0 . The chromatographic column and pumps are pumping the same solvent, albeit with no polymer. A detector is used to note the overall concentration of polymer in the eluted solvent (solvent that has passed through the column) as a function of time at constant volumetric flow rate, Q . The time at constant flow rate reflects a volume of fluid which has eluted from the column, i.e. the elution volume. The time it takes the polymer to elute from the column is called the retention time, t_R , and the elution volume for this time is called the retention volume, V_R . If the right gel is inserted in the column for the molecular weight range of interest, the relation between V_R and molecular weight is linear, $V_R = V_{R,0} - kM$, where M is the molecular weight, k and $V_{R,0}$ are constants for a particular polymer/solvent/gel system. The two constants are determined by eluting two or more monodisperse standards. If monodisperse standards are not available, a reference polymer such as polystyrene is used and the molecular weight is given in terms of a polystyrene equivalent molecular weight.

The detector in a GPC must be linear with concentration. Typically a refractometer, for measurement of refraction index is used. Many other detectors can be used in a GPC and much of the recent development in liquid chromatography has focused on the use of different detectors such as spectrometers, viscometers and light scattering detectors.

The output of a GPC is considered to reflect the number of chains at a given retention volume or molecular weight. From a GPC curve all of the molecular weight distributions, noted above, can be determined. The GPC is the only simple technique to determine the modality (bimodal, trimodal etc.) of a polymer sample.

1.5.2 Molecular weight through viscosity

As mentioned before, a primary technique for the determination of molecular weight, which is important for this work of thesis, is the viscosimetry.

In fact, it is possible to obtain the viscosity average molecular weight of a polymer from its intrinsic viscosity, and using the Mark-Houwink equation:

$$[\eta] = KM^\alpha$$

Where K and α are constants for a given polymer–solvent–temperature system, $[\eta]$ is the intrinsic viscosity and M is the viscosity-average molecular weight.

Though there are many ways and instruments for the measurement of viscosity, the cheapest and easiest ones are those achievable with capillary viscometers.

1.5.2.a Capillary viscometers

Two general classes of capillary viscometer have found use, namely U-tube viscometers and suspended level viscometers. A common feature of these viscometers is that a measuring bulb, with upper and lower etched marks, is attached directly above the capillary tube. Liquid is either drawn or forced into the measuring bulb from a reservoir bulb attached to the bottom of the capillary tube. The time required for the liquid to flow back between the two etched marks is then recorded.

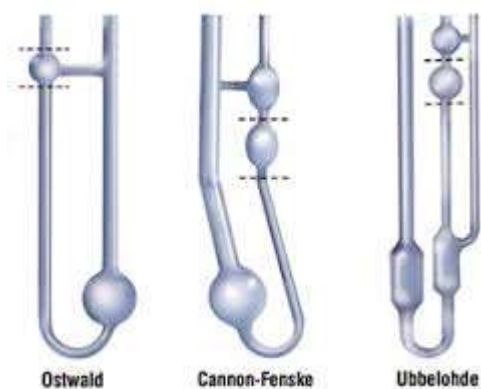


Figure 1.11 – Most common capillary viscometers: Ostwald, Cannon Fenske and Ubbelohde

In U-tube viscometer (Ostwald and Cannon-Fenske in Figure 1.11), the pressure head giving rise to flow depends upon the volume of liquid contained in the viscometer. Therefore, it is important to ensure that this volume is the same for each measurement. This is normally achieved after temperature equilibration by adjusting the liquid level to an etched mark just above the reservoir bulb. A further complication in the U-tube viscometers is the need of perfect vertical alignment of the viscometer, since slight deviations from the vertical can give rise to significant changes in the pressure head. This problem is essentially eliminated in the Cannon-Fenske viscometer having the measuring bulb position vertically above the reservoir bulb.

Most suspended level viscometers are based upon the design due to Ubbelohde (Figure 1.11), the most significant feature of which is the additional tube attached just below the capillary tube. This ensures that during measurement the liquid is suspended in the measuring bulb and capillary with atmospheric pressure acting both above and below the flowing column of liquid. These viscometers have a number of advantages in comparison to U-tube viscometers: the variation of pressure head during measurement is smaller, vertical alignment is less critical, and the volume of liquid in the viscometer need not to be constant because the position of the suspended liquid level at the bottom of the capillary is fixed. The latter feature is particularly useful since it enables solutions to be diluted directly in the viscometer. Thus, a known volume of the most concentrated solution is placed into the viscometer and its flow time determined. A known volume of solvent is then added, and through mixing and temperature equilibration, the flow time of the diluted solution is measured. This procedure is then repeated for several dilutions.³²

1.5.2.b Types of viscosity

Using a single solution with a pre-determined concentration and a single measurement is possible, with a capillary viscometer, to determine the following kinds of viscosity:

Dynamic viscosity

The dynamic (shear) viscosity of a fluid expresses its resistance to shearing flows, where adjacent layers move parallel to each other with different speeds. It can be defined through the idealized situation where a layer of fluid is trapped between two horizontal plates, one fixed and one moving horizontally at constant speed. This fluid has to be homogeneous in the layer and at different shear stresses. The plates are assumed to be very large, so that one need not consider what happens near their edges.

If the speed of the top plate is low enough, the fluid particles will move parallel to it, and their speed will vary linearly from zero at the bottom to u at the top. Each layer of fluid will move faster than the one just below it, and friction between them will give rise to a force resisting their relative motion. In particular, the fluid will apply on the top plate a force in the direction opposite to its motion, and an equal but opposite one to the bottom plate. An external force is therefore required in order to keep the top plate moving at constant speed.³³

The magnitude F of this force is found to be proportional to the speed u and the area A of each plate, and inversely proportional to their separation y :

$$F = \mu A \frac{u}{y}$$

The proportionality factor μ in the formula is the dynamic viscosity.

Kinematic viscosity

The kinematic viscosity (also called "momentum diffusivity") is the ratio of the dynamic viscosity μ to the density of the fluid ρ . It is usually denoted by the Greek letter nu (ν).

$$\nu = \frac{\mu}{\rho}$$

By using a viscometer, it is possible to determine the kinematic viscosity multiplying the flowing time (between the etched marks) of the solution through the capillary by the capillary constant.

Reduced viscosity

Reduced viscosity (η_{red}) is equal to the ratio of the relative viscosity increment η_i to the mass concentration of the polymer (usually g/ml is used as unit):

$$\eta_{red} = \frac{\eta_i}{c}$$

And the increment η_i is:

$$\eta_i = \frac{\eta - \eta_s}{\eta_s}$$

Where η_s is the viscosity of the pure solvent and η is the viscosity of the solution.

Inherent viscosity

Inherent viscosity is the ratio of the natural logarithm of the relative viscosity ($\eta_r = \eta/\eta_s$) to the mass concentration of the polymer:

$$\eta_{inh} = \frac{\ln(\eta_r)}{c}$$

1.5.2.c Determination of intrinsic viscosity and molecular weight

Intrinsic viscosity is a measure of a solute's contribution to the viscosity of a solution.

The most general relationship between intrinsic viscosity and dilute solution viscosity takes the form of a power series in concentration:

$$\eta_{red} = [\eta] + k_1[\eta]^2c + k_2[\eta]^3c^2 + k_3[\eta]^4c^3 + \dots$$

Where k_1 , k_2 and k_3 are dimensionless constants and $[\eta]$ is the intrinsic viscosity.

Given this definition, a large number of equations have been recommended for the evaluation of $[\eta]$ by extrapolation of experimental data. The most commonly encountered are the Huggins equation and the Kraemer equation:

$$\text{Huggins: } \eta_{red} = [\eta] + k_H[\eta]^2$$

$$\text{Kraemer: } \eta_{inh} = [\eta] + k_K[\eta]^2$$

The Huggins equation is simply a truncation of the series expansion given above. The Kraemer equation is an approximation of the Huggins equation, from which it may be derived assuming that η_i (which appears in the η_{red}) is $\ll 1$.

So, as it is possible to see from the equations, the intrinsic viscosity is nothing more than the value of reduced viscosity or inherent viscosity extrapolated to the value of null concentration, so:

$$[\eta] = \lim_{C \rightarrow 0} \eta_{red}$$

Or, as well:

$$[\eta] = \lim_{C \rightarrow 0} \eta_{inh}$$

It is now obvious that the easiest way to determine intrinsic viscosity with extrapolation of experimental data is to plot the values of reduced viscosity and/or inherent viscosity and extrapolate those values to the concentration 0. The plot that is obtained is the following.

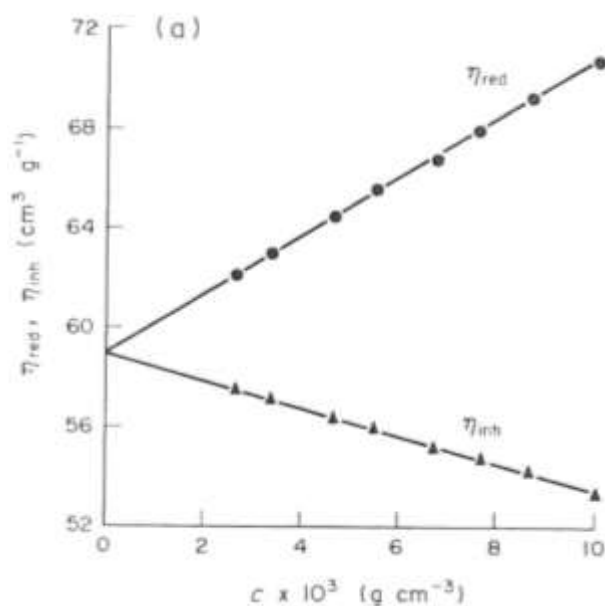


Figure 1.12 – Plot representing variation of inherent and reduced viscosity vs concentration, the interception with the y axis is the intrinsic viscosity³²

It is important to observe that the Kraemer representation has always a lower slope than the Huggins one, so the extrapolated intrinsic viscosity is supposed to bring a lower error.

Now that intrinsic viscosity is obtained, it is necessary to evaluate the coefficients K and α to be used in the Mark-Houwink equation.

In order to do that, samples of the polymer of interest with known molecular weight must be used. In fact, given the molecular weight of two or more samples (because there are two variables to determine: K and α), and extrapolated the values of intrinsic viscosity in one of the experimental methods described before, it is possible to use the Mark-Houwink equation to determine the values of K and α relative to those samples.

If the molecular weight of the samples are known to be similar or comparable to those that are still to examine, it is possible to use the K and α evaluated in this way to determine the unknown molecular weight of the polymer using the intrinsic viscosities and, again, the Mark-Houwink equation, this time knowing the value of K and α :

$$[\eta] = KM^\alpha$$

1.5.2.d Other ways to determine $[\eta]$: the Fedors equation

With the Fedors equation, it is possible to determine the intrinsic viscosity of a polymer without measuring the viscosity of samples at different concentration.

The Fedors equation is the following:

$$\frac{1}{2(\eta_r^{\frac{1}{2}} - 1)} = \frac{1}{[\eta]c} - \frac{1}{c_m[\eta]}$$

Where η_r is the relative viscosity of the solution to a given concentration c and c_m are concentration factors that must be calculated using, also in this case, samples with known intrinsic viscosity³⁴

In fact, once given the intrinsic and relative viscosity of an already known sample, it is possible to extrapolate c_m from the Fedors equation and use it to determine the intrinsic viscosity of other samples.

Of course, even after this extrapolation, it is possible to determine the molecular weight of the polymer using the obtained intrinsic viscosity and the Mark-Houwink equation.

2 Aim of the project

As mentioned before, as the acidic conditions for chitin demineralization (pH, time, and temperature) become harsher, the molecular weight of the products thus obtained becomes lower. Indeed, chitin is an acid-sensitive material and can be degraded by several hydrolytic depolymerization. So, with increasing acid concentration, temperature and time of reaction, we expect the molecular weight of the final product (chitosan) to decrease.

Within the comprehensive framework of the project, as described in the Results and Discussion, the concrete aim of this work is the determination of an empirical mathematical model that must relate the molecular weight of the finally obtained chitosan to the conditions under which its precursor, the chitin, gets demineralized.

The decarbonation reaction happens in water medium with a not so high concentration of acid. In this condition, the chitin is not soluble, so, what happens in this step is a solid-liquid heterogeneous reaction; therefore, the determination of a kinetic equation of such a process is surely difficult and for this reason, a semi-empirical approach is essential. In addition, it is not possible to study a wide range of decarbonation conditions such as acid concentration, temperature and time in all their possible values and combinations, because it would require a huge amount of time and especially a lot of experiments. What is necessary is a *technique* able to relate aimed experimental data to a representative mathematical model, which is the main tool used for this work.

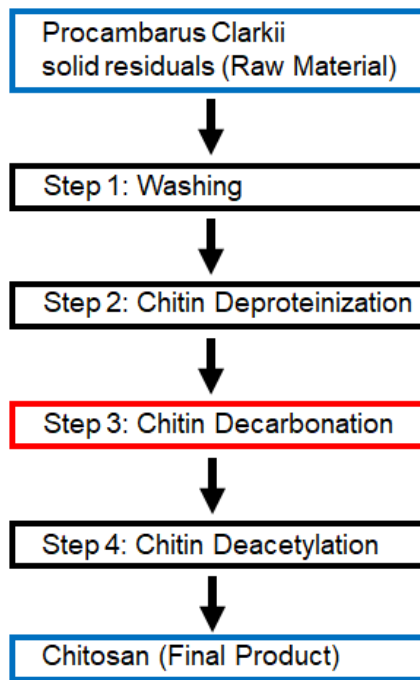


Figure 2.1 – Schematization of the production of chitosan from *Procambarus Clarkii* solid residuals

The obtained model must allow us to obtain a better understanding of the effects that variations in the decarbonation conditions (Step 3 in figure 2.1) could bring to the molecular weight of chitosan (Final Product). This mathematical model can eventually give us the instructions about the conditions of decarbonation that can lead us to the obtainment of a maximum or a minimum in our response, or rather in the molecular weight of the final product.

So, the obtainment of this model will allow whoever is interested in chitin treatment to operate varying decarbonation conditions knowing what effect these conditions will have on MW of chitosan. This can be considered like an optimization of the process, finalized to the industrial replication and utilization.

3 Results and discussion

To achieve the main target of this work, a set of experiments must be planned, in order to minimize the number of intents, although obtaining a representative number of chitosan samples with differences in molecular weight due to the utilization of different decarbonation conditions. Therefore, each of the other three steps reported in figure 2.1 must be carried out with the same conditions for every sample, in order to be sure that variations in chitosan molecular weight are due *only* to the decarbonation conditions.

The technique used to plan the experimental work and to analyze the obtained data is the Response Surface Metodology³⁵.

The details about the experimental conditions used for washing, deproteinization and deacetylation are reported in the Experimental Part, while this chapter will focus mainly on the decarbonation step and the analysis of the results with the use of the Response Surface Metodology.

3.1 Response surface methodology

Response Surface Methodology (RSM) is a set of techniques that encompasses³⁵:

1. Setting up a series of experiments (designing a set of experiments) that will yield adequate and reliable measurements of the response of interest;
2. Determining a mathematical model that best fits the data collected from the designed chosen, by conducting appropriate tests of hypotheses concerning the model's parameters;
3. Determining the optimal settings of the experimental factors that produce the maximum (or minimum) value of the response;

If discovering the best value, or values, of the response (the molecular weight MW) is beyond the available resources of the experiment, then Response Surface Methodologies are aimed at obtaining at least a better understanding of the overall process. However, in the cases examined in this work of thesis it possible to discover the maximum and the minimum value of MW obtainable, but the

relationship between the factors (acid concentration, temperature and time) and the response is too complex and unknown, so an empirical approach is necessary.

3.1.1 Design of experiment: general notes on the Central Composite Design

The design of experiment (DoE) is a statistical tool that directs and minimizes the needed experimentation, to obtain a mathematical model representative of the process with enough precision and robustness. There are many types of DoE, depending on the number of factors (in our case three: concentration, temperature, reaction time) and the resolution of the problem that we desire. In any case, the number of experiments always rises with the number of factors and with the resolution of the problem that we want.

In our case, we will use a Central Composite Design (CCD), which is the most commonly used when there are three or more factors and when there is the need to obtain a complete understanding of the influence of the factors and their synergic interaction on a single response (MW).

The CCD consists of three portions, each of them is constructed using points given by single experiments with certain conditions:

- One portion (F) corresponding to a fractional design, with 2^k points, where k is the number of factors;
- One axial portion, corresponding to experiments along the axes of the factors, at a distance (α) from the center of the design. It will consist of 2k points;
- One central portion (n_c), containing the central experiment and its repetitions.

Therefore, the minimum number of experiments is given by

$$N = F + 2k + n_c$$

Where n_c is the number of central points (1) more the number of its repetitions that must be done in order to evaluate the pure experimental error of the method (in our case we did 3 repetitions), so it becomes:

$$N = 8 + 2 \times 3 + 4 = 18$$

The value of α , which is the half length of the axis of each factor is

$$\alpha = F^{1/4} = 1,682$$

A graphic representation of our CDD is constructed like this: (Figure 3.1)

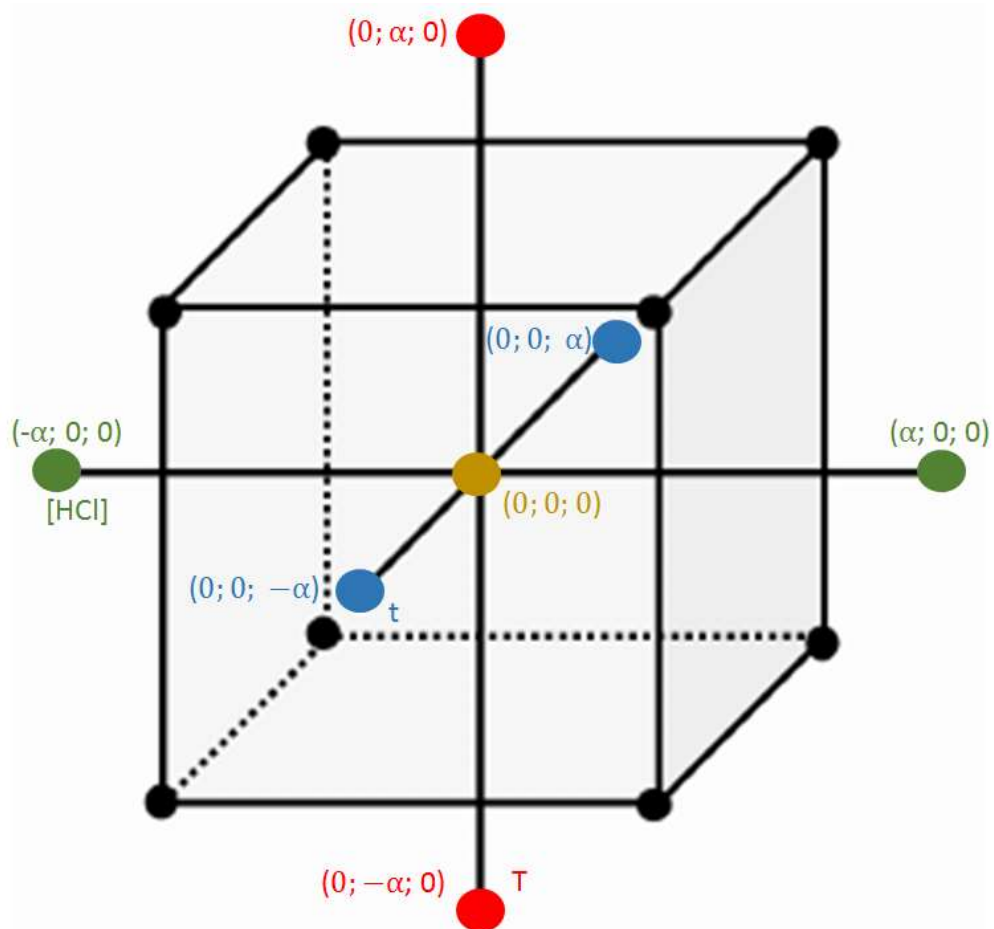


Figure 3.1 - Graphic representation of the Central Composite Design (CCD)

The yellow point is the central point, which represents the experiment with the intermediate conditions of acid concentration, temperature and time, and, as said before, must be repeated an adequate number of times in order to evaluate the pure experimental error of the method; the other colored points represent the axial portion, while the black ones represent the fractional portion. Therefore, each of the points represents a single experiment with its own conditions of acid

concentration, temperature and time; as it is shown here. There are a total of 15 experiments (and the repetitions of the central point must be added).

To fit in this kind of design and to have each of the factors not dependent from its measure unit, each factor real value must be “*codificated*” in dimensionless values between -1,682 and +1,682 ($-\alpha$ and α), for example, the maximum concentration of acid will have the +1,682 value, while minimum concentration -1,682, and the same for the other parameters. This allows us to compare the relative importance of each factor.

Anyway, before the codification, it is necessary to determine the real upper and lower limits of acid concentration, temperature and time, in order to obtain the value of $-\alpha$ and α of each factor.

3.1.1.a Preliminary study: Determination of upper and lower limits

Acid concentration

To evaluate the lower limit of acid concentration, we have calculated stoichiometrically which was the theoretical acid concentration needed to remove all the calcic carbonate present in the deproteinized matter.

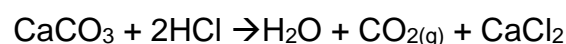
The quantities involved for the decarbonation were 35 grams of matter (with ~70% of calcic carbonate) in 350 ml of acid solution.

$$g \text{ CaCO}_3 = 35 \text{ g} * 0,70 = 24,50 \text{ g CaCO}_3$$

So:

$$\text{moles CaCO}_3 = \frac{24,50 \text{ g CaCO}_3}{100,087 \text{ g/mol}} = 0,245 \text{ moles CaCO}_3$$

The reaction of decarbonation is:



So, the minimum number of moles of HCl needed to remove all the calcic carbonate is:

$$\text{moles HCl} = 0,245 \text{ moles} * 2 = 0,490 \text{ moles HCl}$$

Which, divided by 0,350 L, that is the volume of solution used, gives 1,4 mol/L (~1,5 mol/L), which is the minimum acid concentration needed and the one used for this work. It would not make any sense using a solution less concentrated because, since the decarbonation reaction is very fast, almost all the acid would be used to remove the calcic carbonate and the acid hydrolysis of the polymeric chain would barely not happen.

To determine the upper limit of acid concentration, several experiments were carried out. As a wider range of concentration could give us a better understanding of the effect on molecular weight, a high concentration of acid (9 mol/L) was tested.

The result was the formation of black particles, permanent even after the deacetylation to chitosan and totally insoluble, and for this reason very inconvenient for any successive MW measurement or treatment. This could be explained by the occurrence of a reaction similar to the carbonization of a polysaccharide by acid treatment, in this case acting on chitin. A picture of these black particles is shown below (Figure 3.2):



Figure 3.2 – Black particles left after treatment with 9 M HCl

After several attempts varying concentration, the acidity settled for the experimental design was 3,5 mol/L of acid, which proved to give (after the successive deacetylation of chitin) soluble chitosan with no black particles.

Temperature

The lower limit of temperature was easy to settle as, to avoid the use of a refrigeration cycle which could be expensive also in an industrial context, we simply decided to put the room temperature (measured for each experiment, but always around 25 °C) as lower limit.

The upper limit was decided using the same attempts explained in the determination of the acid concentration limit. In first place, we tried 90° C as upper limit, but we assisted to the formation of the black particles already mentioned. We thought that the carbonization reaction was facilitated by the high temperature, so we lowered the temperature, and the result was the disappearance of the black particles. The actual upper limit was finally settled to 65 °C.

Time

The lower time limit was settled consulting a previous work done from the research group³⁶. In this work, it is explained and proven that the minimum time to remove all the calcic carbonate using acid concentrations very similar to the lowest one (1,8 mol/L vs the 1,5 mol/L we used), is 23,43 minutes measured at room temperature. Therefore, taking account that only a few experiments would have been done at room temperature and all the others at higher temperatures, we settled 30 minutes as lower limit, supposing that after 30 minutes all the calcic carbonate would have been dissolved.

The upper time limit was settled consulting the same work, where, after 180 minutes at room temperature, all the calcic carbonate was removed. As said before, only a few reaction were expected to be done at room temperature, and in order to minimize the needed time, we decided to set 150 minutes as upper time limit.

The limits of the design in the axial portion settled in the ways described are summarized in the table below:

	-α	+α
Conc. HCl (mol/L)	1,5	3,5
Temperature ($^{\circ}$C)	25	65
Time (min)	30	150

2.1.1.b Design of Experiment

After having settled the upper and lower limit of each variable, we proceeded with the actual construction of the whole experimental design.

A three-factor Central Composite Design is always built like shown in the Table 3.1 below. The points in Figure 3.2 are nothing else than a graphic representation of the trials reported in the table:

Trial	[HCl]	Temper.	Time
•1	-1	-1	-1
•2	1	-1	-1
•3	-1	1	-1
•4	1	1	-1
•5	-1	-1	1
•6	1	-1	1
•7	-1	1	1
•8	1	1	1
•9	0	0	0
•9	0	0	0
•9	0	0	0
•9	0	0	0
•10	α	0	0
•11	$-\alpha$	0	0
•12	0	α	0
•13	0	$-\alpha$	0
•14	0	0	α
•15	0	0	$-\alpha$

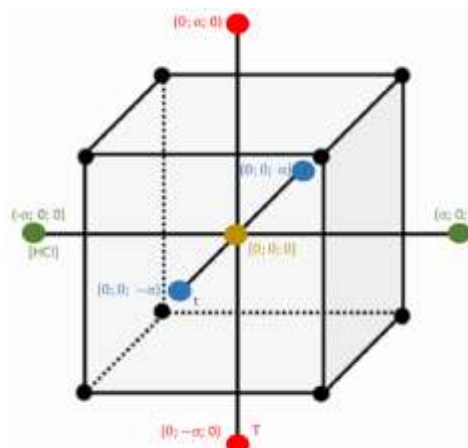


Table 3.1 – Complete set of experiment with codified values provided by this experimental design

We had to make a correspondence between the values in the table, that are dimensionless and codificated for the design and the real values of the factors. The values of $+\alpha$ and $-\alpha$ (+ 1,682 and -1,682) for every factor are respectively their upper and lower limit. While the values of -1, 0 and 1 are calculated simply using a proportion, for example: the value of the “0” of concentration is nothing else than the intermediate value of concentration between the upper and lower limit, or rather 2,5 mol/L.

The correspondence between the real values and the codificated ones is reported in the table below:

	$-\alpha$	-1	0	1	$+\alpha$
Conc. HCl (mol/L)	1,5	1,9	2,5	2,9	3,5
Temperature (°C)	25	33	45	54	65
Time (min)	30	54	90	126	150

Table 3.2 – Correspondence between codificated values and real values of the factors

2.1.2 Determination of the mathematical model

The determination of the mathematical model relating molecular weight of chitosan with the conditions of decarbonation was achieved using a statistical software named “ECHIP7” (Figure 3.4).

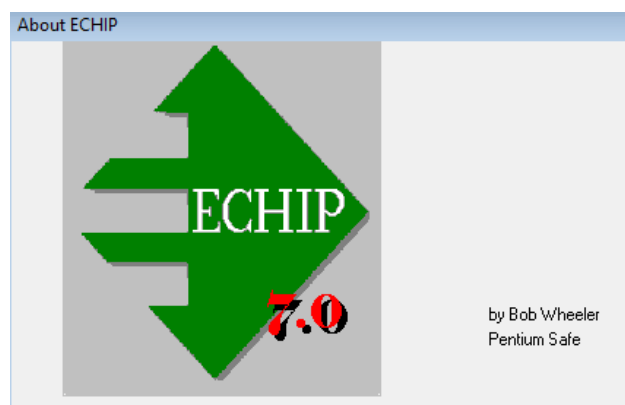


Figure 3.3 – Software ECHIP7

Inserting the codified design variables of each experiment (like for example – $\alpha;0;0, 0;0;0$ ecc..) and their respective value of the response (molecular weight, viscosity or any other one), it is possible, through the software, to obtain a statistical analysis of the experiments, including the mathematical model sought for this work. It is also possible to see 3D graphs showing the trend of the response under the simultaneous variation of two of any of the three factors.

The determination of the mathematical model comes through the method of least squares.

Method of least squares

The method of least squares is a mathematical procedure for finding the best-fitting curve to a given set of points by minimizing the sum of the squares of the offsets (the residuals i.e the difference between an observed value, and the fitted value provided by a model) of the points from the curve(Figure 3.4).

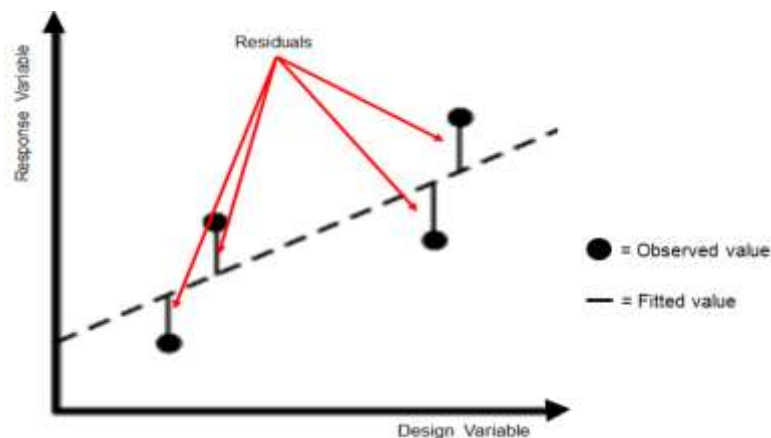


Figure 3.4 – Graphic representation of the residuals in a typical plot *Response Variable vs Design Variable*

The procedure, carried out by the software, is an algebraic procedure for fitting linear equations to data:

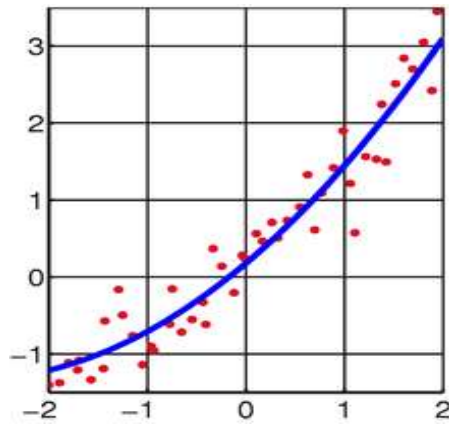


Figure 3.5 – Graphic example of a regression analysis

As an example, in the picture above (Figure 3.5), the blue curve is the result of a regression analysis done with the method of least squares on a set of experimental points (red).

Optimization

After having established the model that best fits the experimental data, the software can proceed with the optimization.

It is possible to see which are the optimal conditions to obtain a maximum or a minimum of the response. Obviously, as the graph shown are 3-axis graphs, one of the three factors must be fixed to a value, while the other two change their value in order to find the sought optimal of the response.

Below, an example of a 3D graph obtained with the software (Figure 3.6):

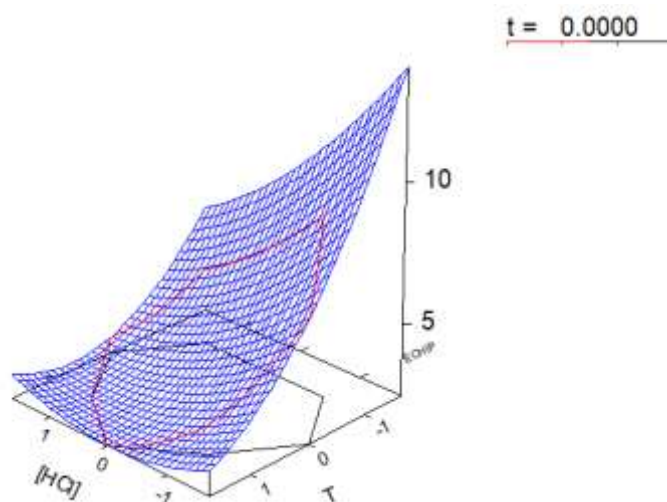


Figure 3.6 – Example of a 3D graph provided by the software ECHIP7

3.2 Analysis of the results

3.2.1 Collection of the data

The first part of the work was the application of the experimental design planned in Table 3.1. It is important to say that the order with whom the experiment had to be done is not the order of the experiment showed in the Table 3.1, indeed the order of the experiments must be random, to remove from the results every possible pattern due to the order of execution.

After the decarbonation done with the conditions described from the design, each sample had to be deacetylated to chitosan to make it soluble (chitin is not soluble in conventional solvents) and its MW measurable.

Therefore, each sample derived from the decarbonation was deacetylated under the same conditions:

- *NaOH 50% w/w*
- *Temperature 120 °C*
- *Reaction time 120 minutes*

We chose to apply the same conditions for the deacetylation to be sure that the only factors affecting the differences between the molecular weight of the chitosan samples were derived from the different decarbonation conditions.



Figure 3.7 –
Cannon-Fenske
Viscometer

At this point we had 18 chitosan samples and we had to measure their molecular weight.

To measure the molecular weight of the chitosan samples we chose to use the viscosimetry method, as it is the one of the simplest and cheapest way to measure molecular weight of a polymer.

The instructions on how to apply viscosimetry for chitosan MW calculation were found in bibliography³⁷.

We used a Cannon-Fenske viscometer (Figure 3.7), with a viscometer constant of 0,041796 cSt/s.

The solvent used to solubilize the polymer was a 0,25 M/0,25 M tampon solution of HAc/NaAc.

We decided to collect the flowing times at different concentration in all the works found in bibliography about the determination of molecular weight of chitosan³⁷, viscosities at different concentrations were required. Moreover, having data at different concentrations allows a more accurate data extrapolation and analysis regarding the viscosity properties of the polymer.

Therefore, for each sample, the flowing time of a solution containing 0,00576 g/ml of chitosan was measured. Then, each sample was diluted 20% using the tampon solution, and its flowing time was measured again; this process was repeated until we had five flowing times for each sample, one for the mother solution and four for its successive 20% dilution:

0,00576 g/ml → 0,00461 g/ml → 0,00369 g/ml → 0,00295 g /ml → 0,00236 g /ml

3.2.2 Analysis of the first response: Kinematic Viscosity

After the collection of the flowing times of each of the samples planned from the experimental design, we needed some data elaboration to reach the sought mathematical model.

The first and most standing out result obtained from the viscosimetry is the kinematic viscosity of the mother solution, in fact, as it was explained in the introduction, simply multiplying the flowing time of a solution by the constant of the capillary viscometer the kinematic viscosity of the solution is obtained.

Even if with the kinematic viscosity we could not have an actual relation of the decarbonation conditions with the molecular weight, we were sure that the variation of the kinematic viscosity was directly related with the variation of molecular weight, as a decrement of molecular weight of a polymer surely brings a decrement in the viscosity of that polymer solution.

Therefore, we multiplied the flowing times of each sample by the constant of the viscometer, and what we obtained is shown in the table below:

Trial	[HCl]	Temper.	Time	Kinematic Viscosity (cSt)
1	-1	-1	-1	12,08
2	1	-1	-1	8,45
3	-1	1	-1	5,77
4	1	1	-1	3,65
5	-1	-1	1	7,58
6	1	-1	1	3,53
7	-1	1	1	3,13
8	1	1	1	3,01
9a	0	0	0	3,79
9b	0	0	0	4,18
9c	0	0	0	3,96
9d	0	0	0	4,00
10	α	0	0	3,05
11	$-\alpha$	0	0	6,34
12	0	α	0	2,24
13	0	$-\alpha$	0	5,47
14	0	0	α	4,02
15	0	0	$-\alpha$	8,43

ECHIP analysis of kinematic viscosity

As said before, to apply the method of least squares a statistical software named ECHIP was used. It is possible to set the design variables, to set the upper and lower limit, and to set a response variable. Each value of the response, in this case the kinematic viscosity of each sample, must be associated with the correspondent trial (Experiment), defined by one set of conditions of those expected by the design.

After we set the trials and the value of kinematic viscosity associated with the respective trial, we can proceed to the analysis of the parameters that most affect the response. The software can provide a table reported below in which are reported the parameters affecting the response, their coefficients in the kinematic equation, and the value of the statistical parameter "P".

The value of P, can tell us if the examined parameter has a statistical significance or not. In fact, the smaller the p -value, the larger the significance because it tells the investigator that the hypothesis under consideration (or rather the influence of a specific parameter or combination of parameters) may not adequately explain the observation. For this case, the term $[HCl]*t$ was ignored because, according to the software analysis based on the $t_{student}$ value, it did not prove to have a statistical significance.

Coefficients	P value	Parameter
4.08		Constant
-1.14	0.0000	[HCl]
-2.03	0.0000	T
-1.49	0.0000	t
0.680	0,0025	[HCl]*T
0.770	0.0012	T*t
0.301	0.0456	[HCl] ²
0.621	0.0044	T ²
0.561	0.0001	t ²
R²		0,987
Adj R²		0,969

Therefore, according to the coefficients found from ECHIP7, the resulting mathematical model is the following:

$$v(kin) = 4.08 - 1.14*[HCl] - 2.03*T - 1.49*t + 0,680*[HCl]*T + 0.770*T*t + 0.301*[HCl]^2 - 0.621*T^2 + 0.561*t^2$$

As it is shown, the greater influence on kinematic viscosity is given by temperature, time and acid concentration in this order. As it was expected, all of them bring an adverse effect on the viscosity of the final product.

The values of the statistical parameters R^2 and the adjusted R^2 can be considered very high for a model built on experimental results.

Another information that the software can provide is a set of 3D graphs and an optimization done according to these graphs that can tell us what conditions can give us the maximum or the minimum of the viscosity value.

The 3D graphs are the following. Of course they are three, as each one of them shows one of the three factors fixed (in this case to the codificated value of 0)

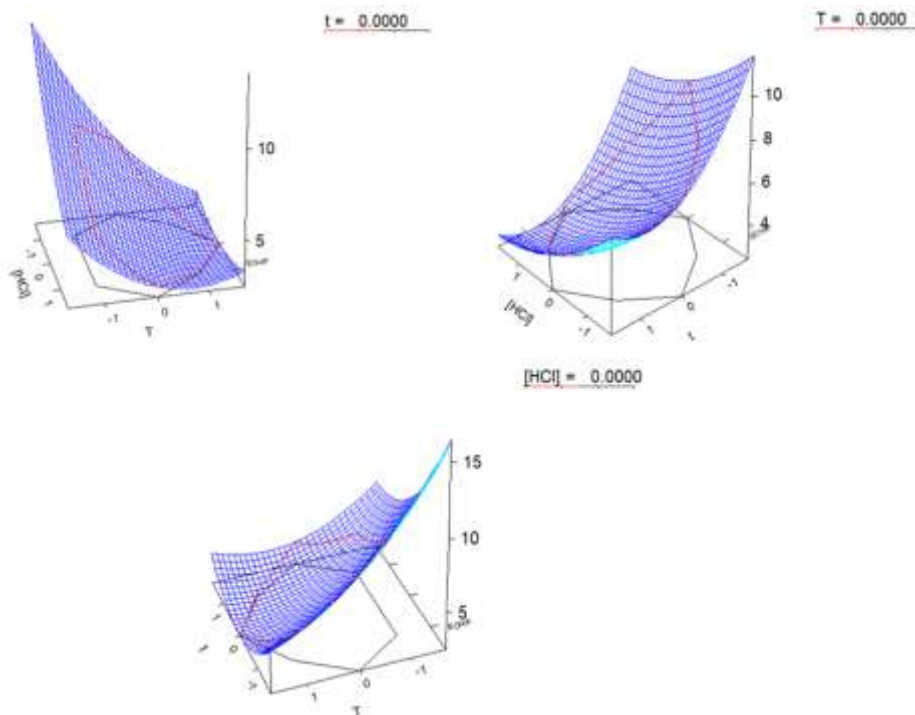


Figure 3.8 – 3D graphs of kinematic viscosity vs the three examined factors

The results of the optimization, which is due to remember that they are trustable only between the upper and lower limits of the factors, are reported in the table below:

	[HCl]	Temperature	Time
Maximum ν	-1	-1	-1
Minimum ν	0	1.682	0

3.2.3 Determination of intrinsic viscosity (to be used for MW determination)

To obtain the actual trend of the molecular weight of chitosan related to the decarbonation conditions we must first obtain the intrinsic viscosity of each chitosan sample, from which it is possible to obtain the viscosity-average molecular weight through the Mark-Houwink equation:

$$[\eta] = KM^\alpha$$

As we had already measured the flowing times of each sample at different concentration, thinking that the easiest way between those shown in the introduction to determine the intrinsic viscosity was the use of the Huggins and Kraemer plots (Figure 3.9):

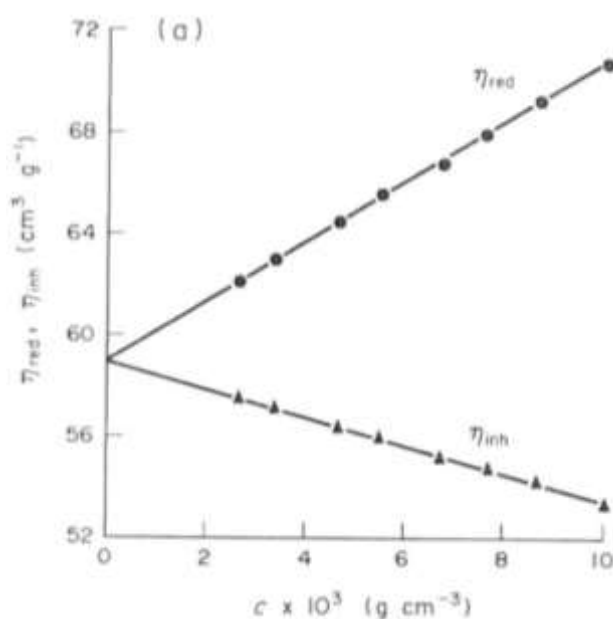


Figure 3.9 – Example of a Kraemer plot (η_{inh}) and a Huggins plot (η_{red})

We decided to use the Kraemer plot (η_{inh}) as it always has a lower slope, so it theoretically would bring a lower error on the determination of intrinsic viscosity:

$$\text{Kraemer: } \eta_{inh} = [\eta] + k_K[\eta]^2$$

3.2.3.a Intrinsic viscosity using Kraemer viscosity plot: analysis of the results

We calculated the inherent viscosity of each sample and at each concentration with the formula:

$$\eta_{inh} = \frac{\ln(\eta_r)}{c}$$

We decided to consider the density of the solutions at different concentrations constant, as we weren't able to measure the density with enough precision to influence the resulting intrinsic viscosity, so the equation becomes:

$$\eta_{inh} \sim \frac{\ln\left(\frac{t - t_0}{t_0}\right)}{c}$$

Where t_0 is the flowing time of the pure solvent, and t is the flowing time of the chitosan solution at concentration c expressed in g/ml.

Once we calculated the intrinsic viscosity of each sample at each of the five concentrations we have measured the flowing time, we plotted inherent viscosities against the concentrations in order to obtain the Kraemer plot of each sample.

The value of inherent viscosity at the point where the Kraemer plot meets the y axis represents the intrinsic viscosity of the sample.

ECHIP analysis of intrinsic viscosity obtained from inherent viscosity

The table below reports the trials with their set of conditions and their respective values of intrinsic viscosity extrapolated in the way described above:

Trial	[HCl]	Temper.	Time	$[\eta]$ (ml/g)
1	-1	-1	-1	506
2	1	-1	-1	449
3	-1	1	-1	326
4	1	1	-1	230
5	-1	-1	1	387
6	1	-1	1	229
7	-1	1	1	213
8	1	1	1	212
9a	0	0	0	239
9b	0	0	0	278
9c	0	0	0	267
9d	0	0	0	273
10	1.682	0	0	212
11	-1.682	0	0	398
12	0	1.682	0	151
13	0	-1.682	0	339
14	0	0	1.682	260
15	0	0	-1.682	395

To accomplish the statistical analysis of the results, we used the same approach used for the kinematic viscosity.

We set the trials and their set of conditions on the software ECHIP7, connected with their respective values of the response, or rather intrinsic viscosity.

After having set the design variables with their values and their respective response, the analysis started. As said before, the software provided a table with the values of the statistical parameter p , and we analyzed it in order to evaluate the relative influence of the different parameters and their synergic combinations on the intrinsic viscosity of the final product:

Coefficients	P value	Parameter
264		Constant
-48.4	0.0003	[HCl]
-80.9	0.0000	T
-53.6	0.0002	t
19.2	0.1073	[HCl]*T
30.5	0.0204	T*t
17.1	0.0843	[HCl] ²
29.1	0.8786	T ²
24.9	0.0200	t ²
R²		0.958
Adj R²		0.916

Also in this case, the term [HCl]*t was ignored because of its lack of statistical significance. The kinematic equation relating decarbonation conditions with intrinsic viscosity obtained in this way is the following:

$$[\eta] = 264 - 48.4*[HCl] - 80.9*T - 53.6*t + 19.2*[HCl]*T + 30.5*T*t + 17.1*[HCl]^2 - 29.1*T^2 + 24.9*t^2$$

The greater influence on intrinsic viscosity calculated in this way is given by temperature, time and acid concentration in this order, like it was for kinematic viscosity. The values of R² and adjusted R² are lower than for the kinematic viscosity but remain acceptably high.

The 3D graphs are very similar to those of the kinematic viscosity, as a variation on kinematic viscosity has same proportionality on the variation on intrinsic viscosity.

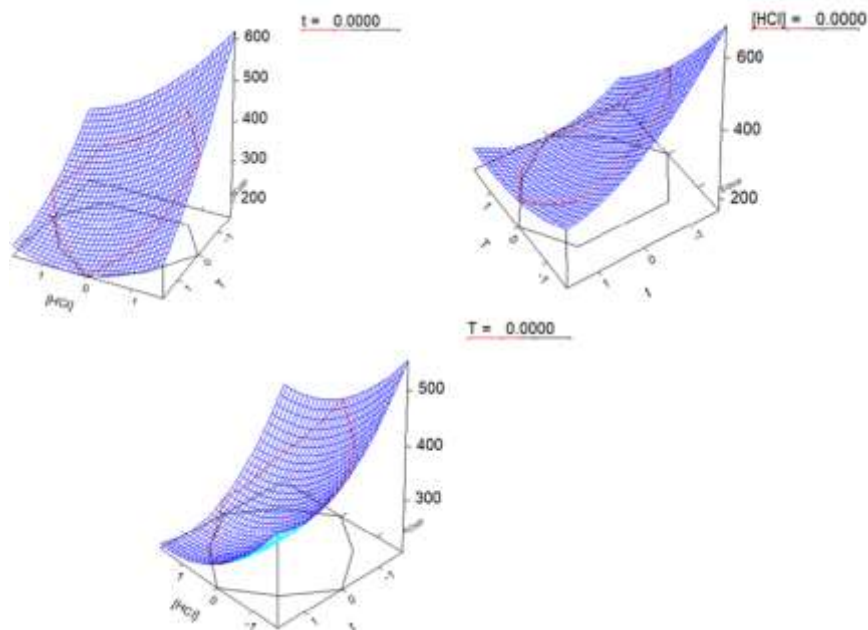


Figure 3.10 – 3D graphs of intrinsic viscosity obtained with Kraemer plot vs the three factors

And as it was expected, also the optimization gave the same results:

	[HCl]	Temperature	Time
Maximum $[\eta]$	-1	-1	-1
Minimum $[\eta]$	0	1.682	0

3.2.3.b Intrinsic viscosity using Fedors equation: analysis of the results

As it was said in the Introduction, with the Fedors equation it is possible to determine the intrinsic viscosity of a given sample without measuring its flowing time at different concentrations:

$$\frac{1}{2(\eta_r^{\frac{1}{2}} - 1)} = \frac{1}{[\eta]c} - \frac{1}{c_m[\eta]}$$

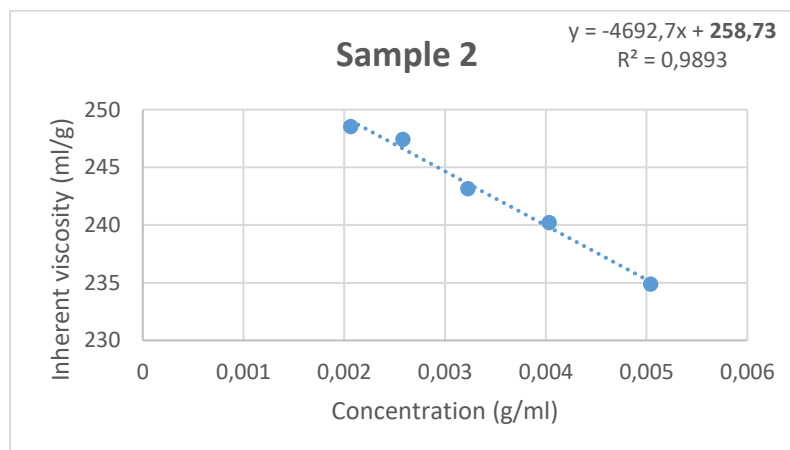
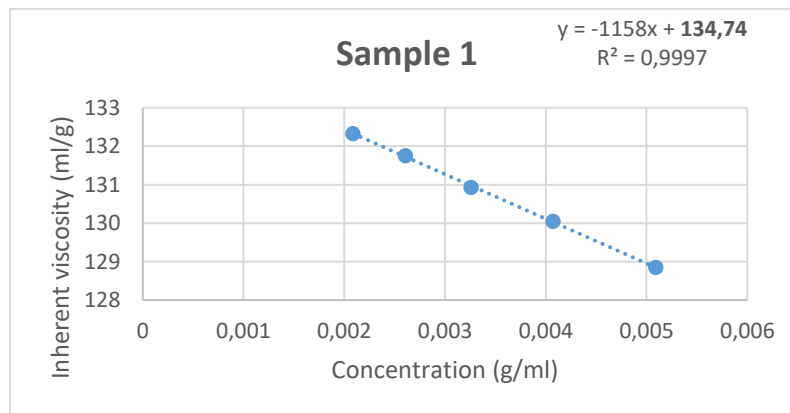
In fact, the term η_r is the relative viscosity, which is given by the flowing time of the chitosan solution with the highest concentration divided by the flowing time of the pure solvent:

$$\eta_r = \frac{t}{t_0}$$

The term “c” is the concentration in g/ml of the solution, and c_m is a coefficient that must be calculated, and it is a term that “includes” in the equation the variation of viscosity with concentration, in order to have an acceptable value of $[\eta]$. Of course, it is really hard to find this coefficient for our specific case in literature so, to extrapolate the value of c_m , we had to use a sample with a trustable value of intrinsic viscosity, and calculate c_m by the Fedors equation itself.

Determination of the Fedors coefficient c_m

We chose to use two sample of pure chitosan, already used and characterized by GPC in a previous work, with known molecular weight of 42400g/mol (sample 1) and 69900 g/mol (sample 2). We calculated their intrinsic viscosity using the Kraemer plot, as it was done for the chitosan samples prepared in this work, obtaining graphs like these:



Therefore, what we can extrapolate from the graph is:

$$[\eta]_{\text{sample1}} = 134 \text{ ml/g}$$

$$[\eta]_{\text{sample2}} = 258 \text{ ml/g}$$

The relative viscosity of the solution η_r is evaluated simply by measuring the flowing time of the solution with highest concentration and the flowing time of the pure solvent.

Now, using the Fedors equation for both the samples we can find the two c_m coefficients, which is the only unknown term:

$$C_m \text{ sample 1} = 0,036$$

$$C_m \text{ sample 2} = 0,018$$

For the calculation of the intrinsic viscosity of our chitosan samples we chose to use the average value of the c_m calculated for the two samples:

$$\text{Average } C_m = 0,027$$

Using this coefficient and the values of relative viscosity of each sample, we extrapolated all the intrinsic viscosities, which come out to be very similar to those calculated with the Kraemer plot:

Trial	[HCl]	Temper.	Time	$[\eta]$ (ml/g)
1	-1	-1	-1	647
2	1	-1	-1	512
3	-1	1	-1	327
4	1	1	-1	242
5	-1	-1	1	423
6	1	-1	1	234
7	-1	1	1	202
8	1	1	1	194
9a	0	0	0	249
9b	0	0	0	286
9c	0	0	0	272
9d	0	0	0	274
10	1,682	0	0	204
11	-1,682	0	0	416

12	0	1,682	0	136
13	0	-1,682	0	367
14	0	0	1,682	276
15	0	0	-1,682	522

ECHIP7 analysis of intrinsic viscosity obtained from the Fedors equation

The statistical analysis of the response obtained this way is very similar to the previous ones; the table provided from the software is the following:

Coefficients	P value	Parameter
270		Constant
-59.9	0.0001	[HCl]
-102.5	0.0000	T
-83.0	0.0000	t
23.3	0.0496	[HCl]*T
35.4	0.0080	T*t
15.9	0.0888	[HCl] ²
65.0	0.1351	T ²
47.2	0.0004	t ²
R²		0,979
Adj R²		0,958

The mathematical model shows the same order of factors influencing intrinsic viscosities shown for the Kraemer plot case, or rather temperature, time and acid concentration:

$$[\eta] = 270 - 59.9*[HCl] - 102.5*T - 83.0*t + 23.3*[HCl]*T + 35.4*T*t + 15.9*[HCl]^2 - 65.0*T^2 + 47.2*t^2$$

3.3 Determination of the molecular weight

It is possible to say that all the methods used to extrapolate intrinsic viscosity gave acceptable results, as the statistical analysis done by the software ECHIP7 always showed an influence on the response that was similar to the one gave on kinematic viscosity. In fact, as it was said before, the kinematic viscosity is a property measurable simply by measuring the flowing time, a parameter surely proportionally dependent from the viscosity of the sample.

To complete the analysis, it is now necessary to find the influence of the above three factors on the molecular weight of the sample. To do that, we need to obtain the molecular weight of each sample from their intrinsic viscosity with the Mark-Houwink equation.

We chose to use the intrinsic viscosity found using the Fedors equation, as it could be potentially the easiest way to find the intrinsic viscosity. In fact, once evaluated the c_m coefficient, it is possible to find the intrinsic viscosity only by measuring the flowing time of the solution of interest, without any dilution.

3.3.1 Determination of coefficients K and α

To apply the Mark-Houwink equation we must first determine the coefficients K and α , the constants for a given polymer–solvent–temperature system:

$$[\eta] = KM^\alpha$$

To achieve that, we decided to apply a mathematical extrapolation on the same samples used to determine the coefficient c_m in the Fedors equation.

The two samples have a known molecular weight, and a known intrinsic viscosity, determined using the Kraemer plot.

	Intrinsic viscosity (ml/g)	Molecular weight (Da)
Sample 1	134	42400
Sample 2	258	69900

Using “Solver” of Excel, we applied a system, with two equations and two variables, and we varied K and α until we found the values of molecular weight with the minimal difference between the latter and the experimental ones, previously measured using GPC:

	Calculated MW	Experimental MW	Difference $MW_{cal} - MW_{exp}$
Sample 1	424.364	42400	91.364
Sample 2	70029.575	69900	158.575

And the extrapolated values of K and α are:

$$K = 0.00122$$

$$\alpha = 1.306$$

3.3.2 ECHIP7 analysis of molecular weight

Once we obtained the coefficients for the Mark- Houwink equation, we applied it to the intrinsic viscosity obtained with the Fedors equation to calculate the molecular weight of the chitosan samples.

Trial	[HCl]	Temper.	Time	MW (Da)
1	-1	-1	-1	141000
2	1	-1	-1	118000
3	-1	1	-1	92300
4	1	1	-1	66400
5	-1	-1	1	102000
6	1	-1	1	65500
7	-1	1	1	58000
8	1	1	1	56300
9	0	0	0	68600
9	0	0	0	75500
9	0	0	0	72100
9	0	0	0	73100
10	1,682	0	0	59000
11	-1,682	0	0	97000
12	0	1,682	0	46300
13	0	-1,682	0	86400
14	0	0	1,682	73300
15	0	0	-1,682	97200

We set the design variable in the software and we obtain the value of the response associated to every experiment. The software provided the following statistical analysis:

Coefficients	P value	Parameter
68000		Constant
-13100	0.0001	[HCl]
-17100	0.0001	T
-15900	0.0000	t
5420	0.1259	[HCl]*T
6300	0.0153	T*t
4330	0.0340	[HCl] ²
15900	0.0871	T ²
9560	0.0005	t ²
R²		0,979
Adj R²		0,958

Therefore, we finally arrived to the determination of a mathematical model comparing the effect of chitin decarbonation condition on chitosan molecular weight:

$$MW = 68000 - 13100*[HCl] - 17100*T - 15900.6*t + 5420*[HCl]*T + 6300*T*t + 4330*[HCl]^2 - 15900.63*T^2 + 9560*t^2$$

As it was expected, the relative effect on molecular weight of the three factors is the same as it was for kinematic viscosity and intrinsic viscosity.

The relative 3D graphs and the optimization provided by ECHIP7 are reported below:

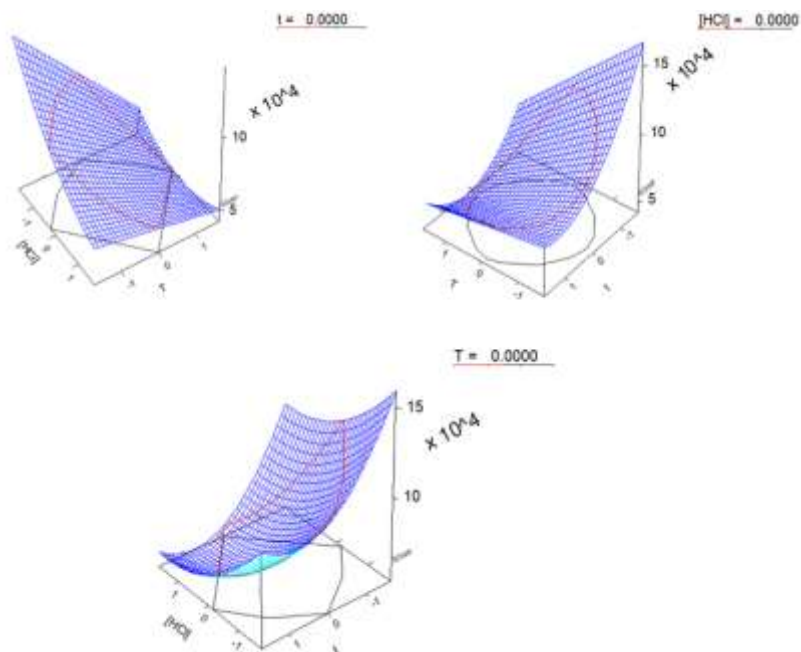


Figure 3.11 – 3D graphs of molecular weight vs the three factors

	[HCl]	Temperature	Time
Maximum <i>MW</i>	-1	-1	-1
Minimum <i>MW</i>	0	1.682	0

The equation above shows the relative influence of the three parameters on the molecular weight of chitosan, which is the main target of this work of thesis, but it is also possible to obtain a kinetic equation involving the real values of the factors and their measure unit. This equation would be built on experimental results, so it would be hard to obtain it giving the value of molecular weight with the real decarbonation conditions without a certain error on the result, but it could be useful to obtain an indication on the range of molecular weight obtainable with some given decarbonation conditions.

To do that, we must replace the codificated values of the design with the real value of acid concentration, temperature and time, associate them with their relative value of response, or rather the molecular weight, and repeat the analysis with ECHIP7:

Trial	[HCl] (mol/L)	Temper. (°C)	Time (min)	MW (Da)
1	1.9	33	54	141000
2	2.9	33	54	118000
3	1.9	54	54	92300
4	2.9	54	54	66400
5	1.9	33	126	102000
6	2.9	33	126	65500
7	1.9	54	126	58000
8	2.9	54	126	56300
9	2.5	45	90	68600
9	2.5	45	90	75500
9	2.5	45	90	72100
9	2.5	45	90	73100
10	3.5	45	90	59000
11	1.5	45	90	97000
12	2.5	3.5	90	46300
13	2.5	1.5	90	86400
14	2.5	45	150	73300
15	2.5	45	30	97200

Repeating the analysis using the data of this table gives the following table:

Coefficients	P value	Parameter
400000		Constant
-101000	0.0070	[HCl]
-2510	0.0001	t
-844	0.0057	[HCl]*T
16.7	0.0184	T*t
8140	0.1921	[HCl] ²
-61.9	0.000	T ²
7.44	0.0012	t ²
R²		0.963
Adj R²		0.965

In this way, we obtained a kinetic equation relating the real decarbonation conditions to the molecular weight of chitosan:

$$MW (Da) = 400000 - 101000*[HCl] - 2510*t + 844*[HCl]*T + 16.7*T*t + 8140*[HCl]^2 - 61.9*T^2 + 7.44*t^2$$

3.4 Effect of molecular weight of chitin on the deacetylation degree

To obtain a deeper knowledge of the process and to verify that the method used for deacetylation was effective, we decided to study the effect of the different molecular weight on the chitosan deacetylation degree (DD).

We decided to use the ¹H-NMR spectroscopy, by simple evaluation of the intensity of the chitin and chitosan characteristic peaks.

To carry out this evaluation, we used three samples involving the widest as possible range of molecular weight:

Sample (0; α; 0)	MW = 46300 Da
Sample (0;0;0) (first repetition):	MW = 75500 Da
Sample (-1; -1; -1)	MW = 141000 Da

The ¹H-NMR spectroscopy of the samples are reported in the following:

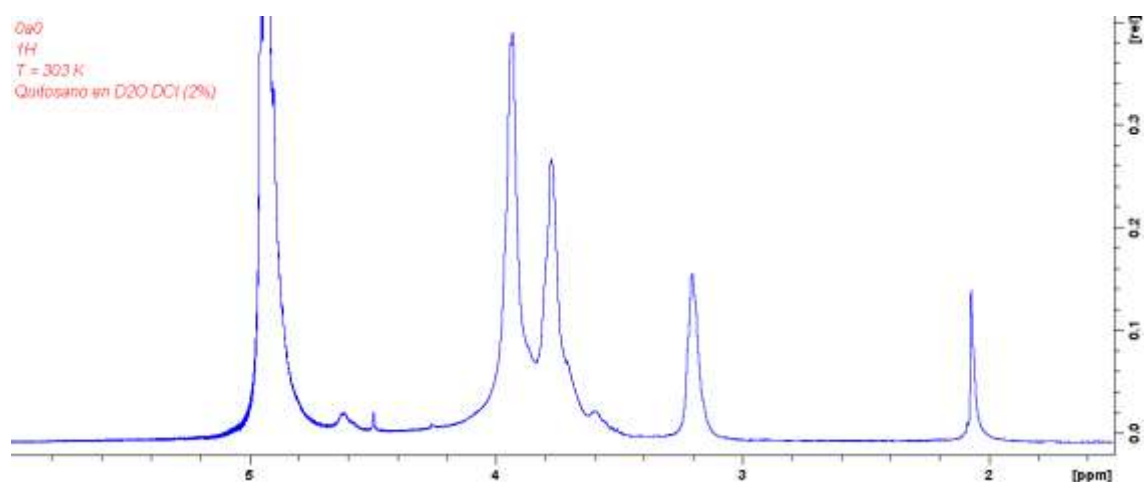


Figure 3.12 –¹H-NMR spectra of the sample (0; α; 0)

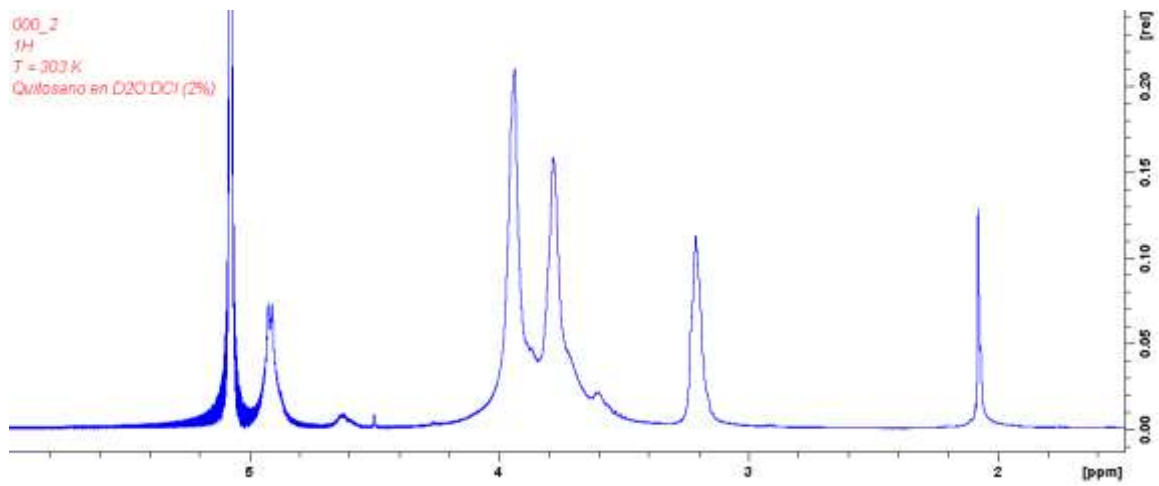


Figure 3.13 – ¹H-NMR spectra of the sample (0; 0; 0)

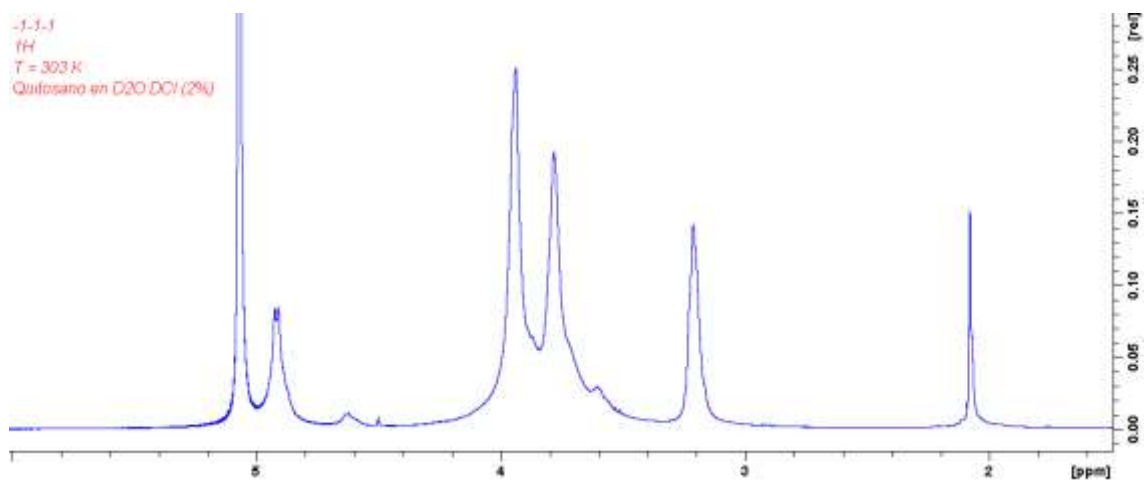
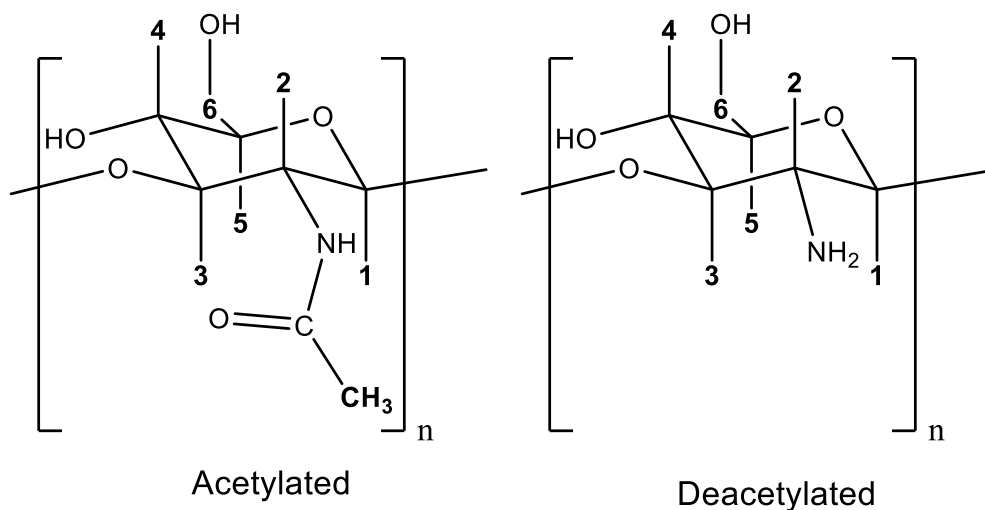


Figure 3.14 – ¹H-NMR spectra of the sample (-1; -1; -1)

Peaks assignment:

The peaks assignment was done with the indications provided on an article found on bibliography³⁸:



ppm	Group – Proton
2.0 - 2.1	CH ₃
3.2	H2 (Acetylated)
3.7	H3-H6
3.9 – 4.0	H2 (Deacetylated)
4.6	H1 (Acetylated)
4.9	H1 (Deacetylated)
5.0	Water (solvent)

There are some equations able to evaluate DD from the peaks intensity, the one reported below is the most commonly used as it minimizes the effect of the background noise:

$$DD(\%) = \left[1 - \frac{\frac{1}{3} \times I_{CH_3}}{\frac{1}{6} \times I_{(H_2-H_6)}} \right] * 100$$

We integrated the peaks using the provided ¹H-NMR software, obtaining:

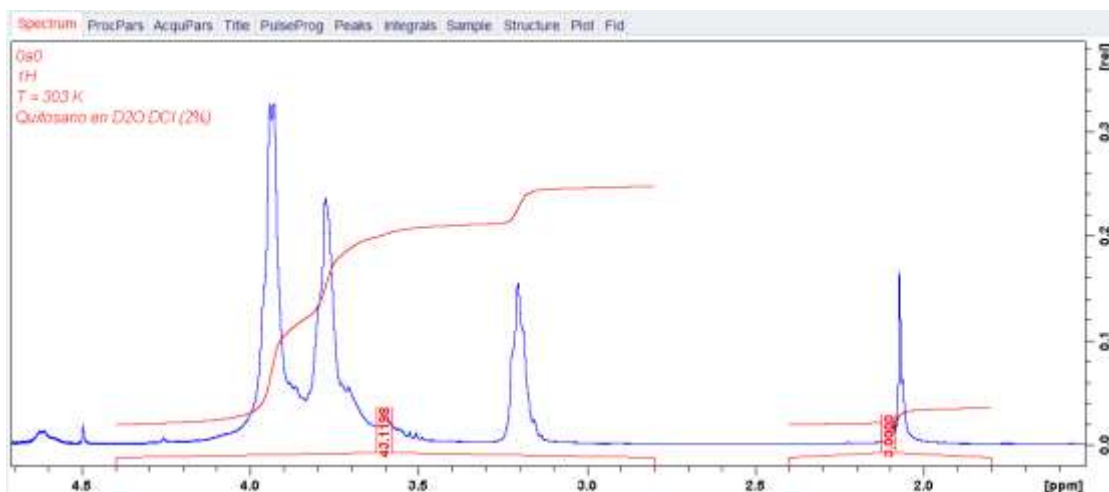


Figure 3.15 – Integrated peaks of the sample (0; α ; 0)

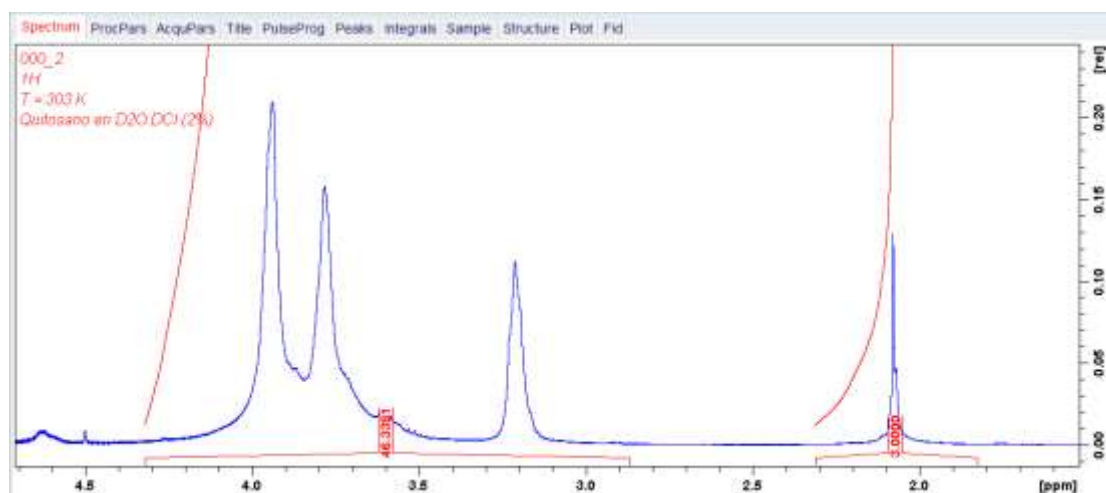


Figure 3.16 – Integrated peaks of the sample (0; 0; 0)

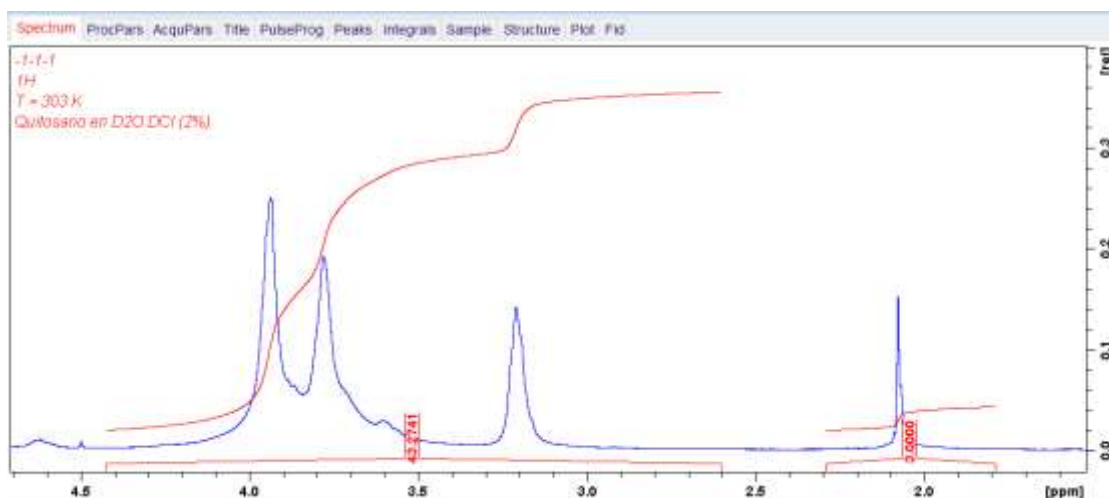


Figure 3.16 – Integrated peaks of the sample (-1; -1; -1)

The resulting deacetylation degrees, calculated using the reported formula are the following:

Sample	MW (Da)	DD (%)
(0; α ; 0)	MW = 46300 Da	86%
(0;0;0) (first repetition):	MW = 75500 Da	87%
(-1; -1; -1)	MW = 141000 Da	86%

As it is possible to see from the results, there is no influence of the molecular weight of chitin on the efficiency of its deacetylation process. The deacetylation conditions used, which resulted to give a DD > 85% can be considered effective.

3.5 Effect of deacetylation conditions on molecular weight

The last part of my thesis work was about the determination of the effect of the chitin deacetylation conditions on molecular weight of chitosan. In fact, all the data collected before were taken keeping the same exact deacetylation condition, in order to make the decarbonation conditions the only responsible factor influencing the molecular weight of chitosan.

To accomplish this evaluation, we took three portions of the same sample, and we performed three deacetylation with three different conditions on each sample:

The choice of the sample was irrelevant, so we decided to perform the three deacetylation on portions of the sample (-1; -1; -1), which is the one with highest theoretical molecular weight (141000 Da).

We used three different NaOH concentrations with the same temperature and time of reaction:

[NaOH] (% w/w)	Temperature (°C)	Reaction time (min)
50	120	120
45	120	120
40	120	120

The measurement of the molecular weight was done with the same method used for the previous chitosan samples: the Kraemer plot was used for the determination of intrinsic viscosity followed by the Mark-Houwink equation for the determination of molecular weight.

[NaOH] (%w/w)	$[\eta]$ (ml/g)	PM (Da)
50	507	116900
45	515	118300
40	532	121300

The results obtained show that there is an influence of the NaOH solution concentration on molecular weight and, as it was expected, the harsher the deacetylation conditions are, the lower the molecular weight is.

4 Conclusions

Experimental conditions for chitin decarbonation have been studied; as it was expected, acid concentration, temperature and time influence in different ways the properties of the final product, or rather chitosan. To get a deeper knowledge of these effects, the software ECHIP7 has been used, in order to obtain mathematical models comparing the relative effects of the three factors on different response variables of chitosan, such as kinematic viscosity, intrinsic viscosity obtained in two different ways and molecular weight.

The resulting mathematical models have shown the same relative influence on the response variables: temperature is the factor most affecting the response, followed by time of reaction and acid concentration. The synergic and quadratic effects of the factors have always come out to be lower than the linear ones. An optimization of the response relating the range between the upper and lower limits of the factors has been obtained. A kinetic equation giving the molecular weight and involving the real values of the factors has been derived.

The effect of the chitin molecular weight on the effectiveness of its deacetylation has been studied. The deacetylation degree, studied using $^1\text{H-NMR}$, has come out to be very similar in all the range of molecular weight used, and the deacetylation process has proved to be effective.

The effects of the deacetylation conditions on molecular weight of chitosan have been studied. The results show that there is an inversely proportional influence of the NaOH concentration used on the molecular weight of the obtained chitosan.

Therefore, it is possible to say that a deeper acquaintance of the chitosan obtainment process has been acquired. The effects of several process conditions have been studied, and the results obtained can be surely useful to those who want to work on this already exploited and effective process.

5 Experimental part

5.1 General Notes

The $^1\text{H-NMR}$ spectra were registered using a Bruker Avance 500 spectroscope. For the viscosity measurement a *Proton* Cannon-Fenske routine viscometer was used, its capillary constant was 0.041796 (25°C).

The spectrophotometer used for the determination of the protein removal (Chapter 4.3) was a *Genesys* model 10S UV-Vis

The solvents and the reactants used, where not specified, are those commercially available.

5.2 Raw Material

For the development of this study, we counted on the collaboration of the company SEAFOOD S.L. that supplied us with the raw material, or rather, the solid waste of the red crab generated in the industrial process for cooking of the *P. Clarkii*, represented in the scheme below (Figure 4.1):

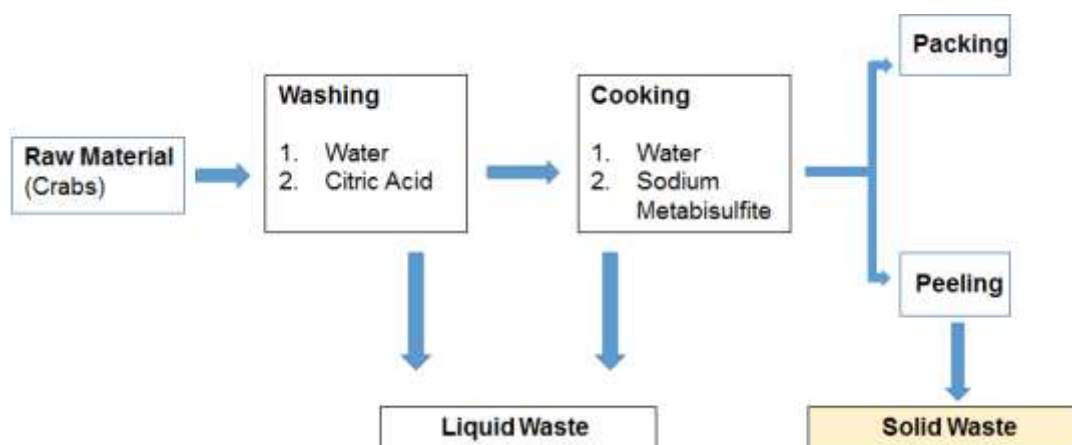


Figure 5.1 - Scheme for the industrial treatment of crabs.

The cooking and commercialization process of the *P. Clarkii* can be divided in 5 steps:

1. Reception of the raw material
2. Washing. In this first step, a conditioning of the raw material is realized, putting the crabs in a citric acid-water solution to eliminate every possible chemical or biological polluter.
3. Cooking: After the washing the crabs are put in a 90°C bath, with water and sodium metabisulfite, which acts as food preservative.
4. Packing/Peeling. After the cooking, the crabs go through two possible lines of production. One of the entire crabs, that get frozen and commercialized; the other of peeled crabs. The peeling is done by hand and, once realized, the crabs are frozen and then commercialized.
5. Pressing. The solid waste of the peeling of the *P. Clarkii* are put into an hydraulic press, where a protein-concentrated juice is extracted to be dehydrated and used for the production of flour. Therefore, the solid waste consists of heads, tweezers, torso exoskeleton and entire crabs which are not big enough to be commercialized.

As it is possible to see, is a very simple process and the reagents used are neither toxic nor dangerous. However, in the last step, after having pressed the exoskeletons, the produced solid waste is difficult to handle. This is because this waste is piled up outside of the installations, and it does not degrade within a short period of time.

A picture of the solid waste used in this work as raw material is shown below:



Figure 5.2 – Solid waste available as a raw material for this work of thesis

5.3 Washing

Once received the raw material, it gets conditioned by washing the exoskeletons with water. With this washing, all the eventual inorganic and organic pollutants are eliminated. Between the inorganic pollutants there are sand and sodium metabisulfite, while the organic ones are pigments, microorganisms (seaweed, insects, ecc..) and mostly proteins. In fact as was said in the introduction the proteins linked through covalent bonds in the exoskeleton are almost 5% of the total, the other 95% of the proteins are water-soluble, so that they can be removed with water.

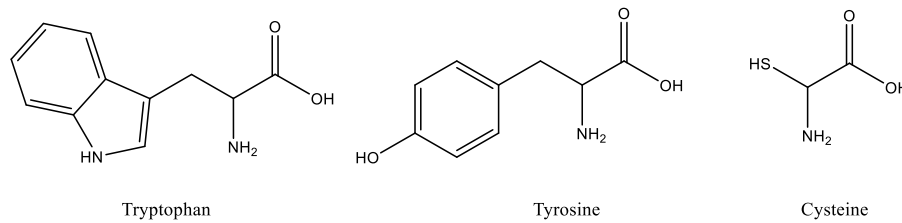
The washing was realized in a 10 L glass container, with a ratio L/S of 15 ml/g, at room temperature for 35 minutes.

To homogenize the medium a mechanical stirrer was used .

Even if the process was tested in a previous work³⁶, it was necessary to make sure that also in our case the proteins were removed from the raw material.

In order to do that, samples of the washing water at fixed time steps were taken. Then, each sample was diluted 1/10 with water and centrifuged at 10000 rpm for 15 min. The absorbance of each sample was measured.

It was chosen to measure the absorbance at 280 nm because proteins that we know to be present in the crab shells (tryptophan, tyrosine and sulfur bridges between molecules of cysteine) all absorb at this wavelength.



The graph below shows the absorbance for the samples taken at different time:

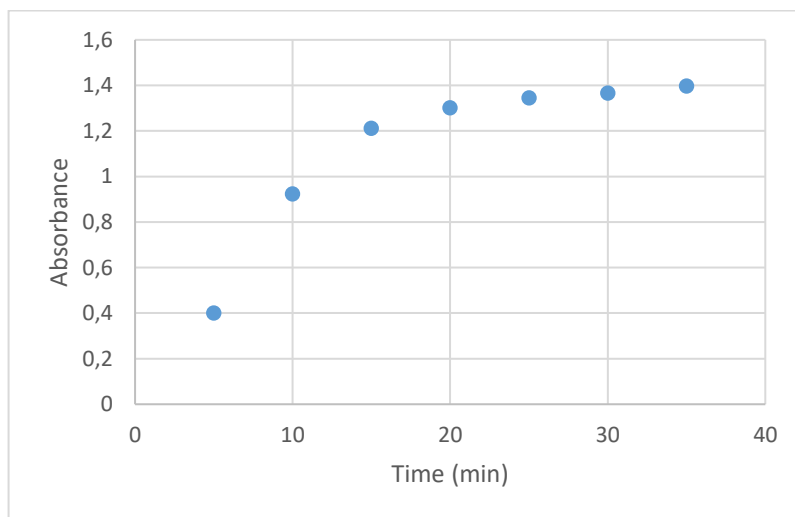


Figure 5.3 - Protein removal, absorbance at 280 nm

As it is possible to see here, after 20 minutes, the protein content is almost stationary, as the absorbance of the successive samples almost doesn't increase.

5.4 Deproteinization

After this step, the washed material was dried 24 hours at 105 °C and collected in a container.

As was said before, with the washing step, 95% of the proteins are removed, the remaining 5% consists of proteins bonded to the chitin. The nature of the bond chitin-protein is vary and, as it is shown in the introduction, many forms of chitin-proteins interactions are speculated; this implies that it is necessary to apply a method which can eliminate proteins, independently form the nature of the bond with the chitin, to make sure that the deproteinization is effective.

To achieve this, a solution of NaOH 2,5 M was used, the temperature was 60 °C and the time of reaction of 3 hours. In a previous work of my research group³⁶ is shown that the liquid/solid ratio makes a certain influence on the efficiency of the deproteinization, and the best results are given by 6 liters of NaOH solution every 0,250 Kg of washed material. After the basic treatment the product was washed with water and collected in a container placed in an oven at 105 °C.

Also in this case we had to make sure that all the proteins were removed.

To prove that, we had to compare the results of our deproteinization with those of a deproteinization done in the conditions already tested in the mentioned previous work³⁶, which was already proven to give complete removal of proteins.

So, three deproteinizations with NaOH 2,5M, with the same liquid/solid ratio at room temperature for 72 h were carried out. Each of these reactions was done in a 1 L reaction flask, and the products were washed with water and dried at 105 °C for 24h.

To compare the amount of proteins left, the three products completely deproteinized and a sample of the examined deproteinization were weighed and put in an oven at 500 °C for 1 hour to be calcined.

The calcination reaction eliminates all the organic material of the sample and, as mentioned in the introduction, the exoskeleton of the crab usually has this composition: 30-40% of proteins, 30-50% of calcic carbonate and 20-30% of chitin.

After 1 hour at 500 °C proteins and chitin are removed from the sample, and the only component left is the calcic carbonate (and a little percentage of other inorganic components). Weighing the sample known to be completely deproteinized before and after the calcification, gives us the CaCO₃/chitin ratio, which is constant for all the samples.

This ratio can be used to evaluate if the loss of weight given by the calcination of the examined sample shows an effective deproteinization or not.

These are the % of weight loss after 72 hours deproteinization:

Sample	Weight loss	Chitin/CaCO₃ w/w ratio
1	-31%	31/69
2	-30%	30/70
3	-32%	32/68
average		
	-31%	31/69

Since the reaction with NaOH 2,5 M for 3 hours at 60°C gave the weight loss after calcination of 30% we can consider the deproteinization done by this way effective, and there is no need to perform a too long 72 h deproteinization at room temperature to remove proteins.

A picture of the deproteinized matter is reported below (Figure 4.4):



Figure 5.4 - Deproteinized chitin

5.5 Decarbonation

Before proceeding with the decarbonation step, we made sure we had enough chitin to carry out all the experiments expected by the experimental design.

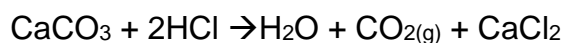
We had consider the fact that with the decarbonation, all the CaCO_3 (~70% w/w) is expected to be removed, and we would have the need to collect enough product taking account that after this step there would have been a deacetylation and some analysis of the final product (viscosimetry and $^1\text{H-NMR}$). So, for each of the experiments of the design, we chose to put 35 g of deproteinized matter, in 350 ml of acid solution.

The concentration of acid, the temperature of the system and the time of reaction were those expected by the design, and the upper and lower limits of each variable (defined $-\alpha$, -1, 1 and $+\alpha$) were previously decided by checking bibliography and making the necessary tests as described in the Results and Discussion.

	-α	-1	0	1	+α
Conc. HCl (mol/L)	1,5	1,9	2,5	2,9	3,5
Temper. (°C)	25	33	45	54	65
Time (min)	30	54	90	126	150

Each reaction was carried out in a 1 L reaction flask, the mixture was magnetically stirred (400 rpm in every case) and the temperature of the system was controlled using a probe (covered with glass to be protected from the acid) connected with a heating coat. To condense the vapour a refrigerating column was used.

Each reaction was performed under an extractor because of the large amount of CO₂ produced:



To make sure that all the inorganic matter was removed, a sample of the product of one demineralization test (the one with the mildest condition) was put for 1 hour at the temperature of 500 °C to be calcined. The container used for the calcination, after one hour, was totally empty, so it was possible to agree that the demineralization worked out well.

Each of the samples was washed accurately with water and saved in a container for the successive deacetylation.

A picture of the demineralized matter is reported below (Figure 4.5)

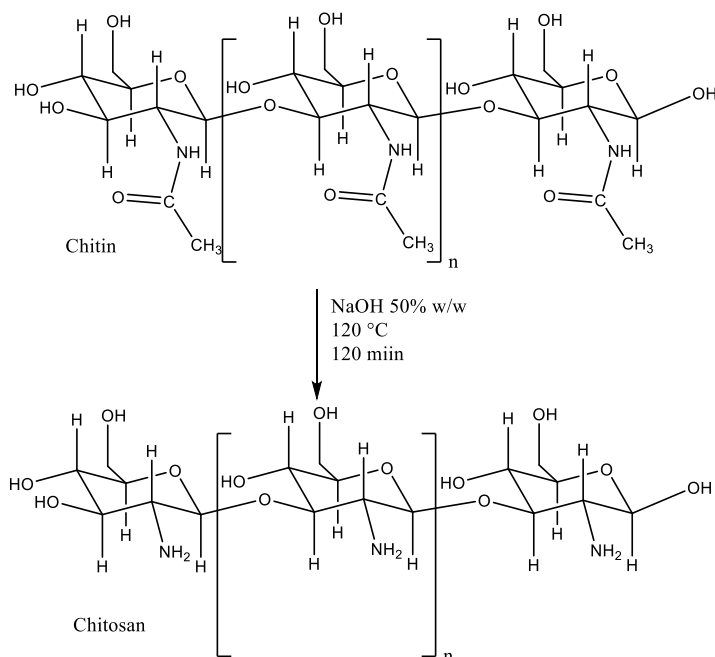


Figure 5.5 – Demineralized chitin

5.6 Deacetylation

Once we obtained the demineralized chitin, we had to take into account the fact that chitin is very hardly soluble in most of the common solvents, so the direct measurement of the molecular weight of chitin and the acquisition of the $^1\text{H-NMR}$ spectra using conventional techniques was almost impossible.

The easiest way to analyze molecular weight of the product through viscosimetry was the obtainment of chitosan, which is a water-soluble polymer.



Scheme 5.1 – Reaction of deacetylation of chitin giving chitosan as product

The deacetylation of each sample was carried out in a 1 L reaction flask, with a probe connected to a heating coat in order to control the temperature.

The weight of each demineralized sample was the weight of the matter derived from the decarbonation reaction, which was always between 8 and 9 grams.

To make sure that the differences in the molecular weight of the final product were due only to the different conditions of decarbonation, the deacetylation of each sample had to be done with the exact same conditions:

NaOH solution: 300 ml of NaOH/H₂O 50% w/w prepared by weighing the NaOH and the water with a balance

Temperature: 120°C, controlled using a probe and a heating coat

Time of reaction: 120 min

Stirring: 400 rpm, magnetic

Due to the extreme conditions and the dangerousness of the mixture used, a lot of attention must be paid, and each operation must be done under an extractor.

After the two hours, each product was washed accurately with water and saved for the next molecular weight measurement.

A picture of the chitosan obtained is reported below (Figure 4.6):



Figure 5.6 – Chitosan obtained after chitin deacetylation

5.7 Measurement of flowing times for viscosimetry

The following procedure was used for each of the products and the weights of the samples were always the same for each of the products derived from deacetylation step to facilitate the comparison of the results obtained.

Preparation of the buffer

The solution used as a solvent for the measurement of viscosity was a 0,25M / 0,25M NaAc / HAc buffer solution prepared by weighing 20,508 g of NaAc and 15,012 g of glacial HAc, putting them into a 1 L flask and filling with water.

Preparation of the chitosan solution

0,576 g of chitosan were grinded, weighed by an analytical balance, and put into a 100 ml volumetric flask. The flask was filled up to ~ 80 ml with the buffer solution and kept under stirring for 24 hours to achieve the complete solubilization of the sample. After complete solubilization, the flask was completely filled with the buffer solution.

Measurement of flowing times

For the measurement of molecular weight, the viscometer already described in the “General Notes” was used.

In first place, the flowing time of the pure solvent was measured, in order to have the value of t_0 . To do this, the viscometer already filled with the pure buffer was put in a 25°C bath for 15 minutes, after that, the flowing time of the solvent was evaluated doing five measurement by manual time recording and considering the average value.

To measure the flowing time of the examined sample, 20 ml of chitosan solution (0,00576 g/ml) prepared in the way described above were put in a becker using a 20 ml pipette, while the volumetric flask was refilled up to 100 ml with the pure buffer using another pipette to obtain a solution 20% diluted. Part of the 20 ml in

the becker were used to fill the viscometer, while the solution left in the becker was thrown away.

The diluted solution, right after the refill up to 100 ml, was put again under stirring to avoid the formation of gels.

Meanwhile, the viscometer filled with the 0,00576 g/ml solution was placed in a 25 °C water bath for 15 minutes, to homogenize the temperature of the system like it had been done for the pure solvent.

The evaluation of the average flowing time, also in this case, was done by manual recording and doing five measurements.

After that, the solution 0,00576 g/ml in the viscometer was thrown away, and the viscometer was filled with the dilute solution.

This process was repeated five times, in this way we obtained the time measurements for the mother solution (0,00576 g/ml) and for four 20% dilution of it.

Bibliography

- 1 Kurita, K. Chitin and Chitosan: functional biopolymers from marine crustaceans. *Marine biotechnology* (New York, N.Y.), **2006**, 8(3), 203-26.
- 2 Organización de las Naciones Unidas para la Alimentación y la Agricultura: Departamento de Pesca y Acuicultura: Disponible: available at: <http://www.fao.org/fisheryculturedspecies/Procambarusclarkii/es>
- 3 Junta de Andalucía. Consejería de Agricultura, Pesca y Medio Ambiente: Available at: <http://www.juntadeandalucia.es>
- 4 Tharanathan, R.N. and Kittur, F.S.: Chitin - The undisputed biomolecule of great potential. *Crit Rev Food Sci Nutr.* **2003**, 43: 61-87
- 5 Know Kin S. *Chitin, Chitosan, Oligosaccharides and Their Derivatives: Biological activities and applications*, CRC Press, Taylor & Francis group, **2011**, 4-6.
- 6 Jang, M.K., Kong, B.G., Jeong, Y.I., Lee, C.H., and Nah J.W. Physicochemical characterization of α -chitin, β -chitin and γ -chitin separated from natural resources. *Journal of Polymer Science: Part A: Polymer Chemistry*, **2004**, 42:3423-3432
- 7 Gardner, K.H. and Blackwell, J. Refinement of the structure of β -chitin. *Biopolymers*, **1975**, 14:1581-1595
- 8 Simpson, B. K, Gagne, N., and Simpson, M. V. Bioprocessing of chitin and chitosan, *Fisheries Processing: Biotechnological Applications*, **1994**, 155–173. London U.K.: Chapman and Hall.

- 9 Know Kin S. *Chitin, Chitosan, Oligosaccharides and Their Derivatives: Biological activities and applications*, CRC Press, Taylor & Francis group, **2011**, 543-557
- 10 Jeon, Y. J. and Kim, S. K. Production of chitooligosaccharides using ultrafiltration membrane reactor and their antibacterial activity. *Carbohydrate Polymers*, **2000**, 41:133–141.
- 11 Wang, Y., Zhou, P., Yu, J. Antimicrobial effects of chitooligosaccharides produced by chitosanases from *Pseudomonas* CUY8. *Asia Pacific Journal of Clinical Nutrition*, **2007**, 16:174–177.
- 12 Wong, H., Endayan, R., Rauth, A. M., Li, Y., and Wu, X. Y. Chemotherapy with anticancer drugs encapsulated in solid lipid nanoparticles, **2007**, *Adv. Drug Deliv. Rev.*, 59: 491–504.
- 13 Kumar, M. N. V. R. A review of chitin and chitosan applications. *React. Funct. Polym*, **2000**, 46: 1–27.
- 14 Martini, A. and Lauria, S. Sustained release injectable products. *Am. Pharm. Rev*, **2003**, 6: 16–20.
- 15 Sinha, V. R., Singla, A. K., Wadhawan, S. Chitosan microspheres as a potential carrier for drugs. **2004**, *Int. J. Pharm.*, 274: 1–33.
- 16 Ruel-Gariepy, E., Chenite, A., Chaput, C., Guirguis, S., and Leroux, J. C. Characterization of thermosensitive chitosan gels for the sustained delivery of drugs, **2000**, *Int. J. Pharm.*, 203: 89–98.
- 17 Know Kin S. *Chitin, Chitosan, Oligosaccharides and Their Derivatives: Biological activities and applications*, CRC Press, Taylor & Francis group, **2011**, 271-282.

- 18 Fernandez-Kim, S.O. Physiochemical and functional properties of crawfish chitosan as affected by different processing protocols. MS dissertation, **2004**, Louisiana State University, Baton Rouge, LA.
- 19 Know Kin S. *Chitin, Chitosan, Oligosaccharides and Their Derivatives: Biological activities and applications*, CRC Press, Taylor & Francis group, **2011**, 11-19.
- 20 Indra Jasmine, G., Rathnakumar, K. and Athiveerarama Pandidurai, G. Production of chitin from shrimp shell waste using protease extract from *Carcia Papaya*, *Advances in Chitin Science and Symposium*, **2006**, Hanrimwon printing Co.
- 21 Takeda, M. and Abe, E. Isolation of crustacean chitin. Decalcification by disodium ethylenediaminetetracetate and enzymic hydrolysis of incidental proteins, *Norisho Suisan Koshusho Kenkyu Hokoku*, **1962**, 11:339.
- 22 Jo, G.H., Jung, W.J., Kuk, J.H. Screening of protease-producing *Serratia Marcenscens* FS-3 and its application to deproteinization of crab shell waste for chitin extraction, *Carbohydrate Polymers*, **2008**, 74: 504-508.
- 23 Quimica Organica. Francis A. Carey. VI edition. **2016**, 110-134
- 24 Shahidi, F. and Synowiecki, J. Isolation and characterization of nutrients and value-added products from snow crab (*Chionoecetes opilio*) and shrimp (*Pandalus borealis*) processing discards. *J Agric Food Chem.*, **1991**, 39:1527–1532.
- 25 Percot, A., Viton, C. and Domard, A., Optimization of Chitin Extraction from Shrimp Shells, *Biomacromolecules*, **2003**, 4, 12-18

- 26 Nam, Y.S., Park, W.H., Ihm, D., Hudson, S.M. Effect of the degree of deacetylation on the thermal decomposition of chitin and chitosan nanofibers, *Carbohydrate Polymers*, **2010**, 80 (1): 291–295
- 27 Kasaai, M.R.: A review of several reported procedures to determine the degree of *N*-acetylation for chitin and chitosan using infrared spectroscopy. *Carbohydrate Polymers*, **2008**, 71, 497–508
- 28 Rinaudo, M. Chitin and chitosan: Properties and applications. *Progress in Polymer Science*, **2006**, 31: 603–622.
- 29 Fernández Cervera, M., Heinämäki, J., Räsänen, M., Maunu, S. L., Karjalainen, M., Nieto Acosta, O.M. and Yliruusi, J. Solid-state characterization of chitosans derived from lobster chitin. *Carbohydrate Polymers*, **2004**, 58: 401–408.
- 30 Abdou, E. S., Nagy, K. S.A. and Elsabee, M. Z. Extraction and characterization of chitin and chitosan from local sources. *Bioresource Technology*, **2008**, 99: 1359–1367
- 31 Lavertu, M., Xia, Z., Serreqi, A. N., Berrada, M., Rodrigues, A., Wang, A. D. and Gupta, A. A validated ¹H NMR method for the determination of the degree of deacetylation of chitosan. *Journal of Pharmaceutical and Biomedical Analysis*, **2003**, 32: 1149–1158
- 32 Lovell, P.A., Dilute Solution Viscometry, University of Manchester Institute of Science and Technology, **2016**, 173-194
- 33 Wikipedia, the free Enciclopedia, Viscosity, available at: <https://en.wikipedia.org/wiki/Viscosity>
- 34 Williams, P.A., Gums and Stabilizers for the Food Industry, *RSC Publishing*, **2012**, 30-31

- 35 Khuri, I.A., Cornell, J.A., Response Surfaces: Designs and Analyses, *Statistics: textbooks and monographs*, **2008** 152: 15-50
- 36 Proyecto anual **2012**, Proyecto AGR-4597M
- 37 De la Paz, N., Pèrez, D., Fernández, M., Lòpez, O.D., Evaluaciòn Viscosimètrica del Quitosano Derivado de la Quitina de Langosta, *Revista Iberoamericana de Polimero*, **2013**, 84-90
- 38 Mohammad, R., Kasaai, Determination of the degree of *N*-acetylation for chitin and chitosan by various NMR spectroscopy techniques: A review, *Carbohydrate Polymers*, **2010**, 79: 801-810