UNIVERSIDADE BEIRA INTERIOR

Insects as alternative sources of value-added by-products

A study of different extraction approaches and characterization of chitin and chitosan from two edible insect species (*T. molitor* and *A. domesticus*) and by-products

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(Pedro Jorge Silva Navarro)

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Resumo

A quitina e o quitosano são dois biopolímeros bastante conhecidos, que têm uma grande diversidade de excelentes propriedades físico-químicas e biológicas que os tornam extremamente versáteis e capazes de serem aplicados em setores como a agricultura, biomédico, farmacêutico, alimentar, entre outros. Os crustáceos e os seus subprodutos são a principal fonte de extração de quitina e produção de quitosano, sendo que o mercado do quitosano está em constante expansão e estima-se que atingirá 4.7 biliões de dólares até 2027. No entanto a quitina, é também sintetizada noutros organismos vivos como os insetos, fungos e algas. Os insetos em particular são de especial interesse devido ao grande crescimento da popularidade da entomofagia. Esta popularidade é manifestada tanto na indústria (aumento de empresas que produzem insetos e produtos alimentares que incorporam insetos), como no mundo académico, com um vasto leque de estudos que se focam no fracionamento dos insetos (proteínas, lípidos e quitina). Desta forma, a extração de quitina e produção de quitosano a partir de insetos apresenta uma oportunidade para aumentar o valor económico dos insetos edíveis. O presente estudo teve como objetivo estudar duas espécies edíveis de insetos como fontes alternativas para a extração destes polímeros, especificamente larvas do besouro da farinha (*T. molitor)*, indivíduos adultos do grilo doméstico (*A. domesticus*), assim como os subprodutos (patas e asas) gerados pela produção dos grilos domésticos. A extração de quitina foi conseguida através de um método químico otimizado baseado na hidrólise ácida e alcalina, sendo que foi também aplicado um método alternativo numa tentativa de ter extração mais ecológica, recorrendo a sistemas de solventes eutécticos naturais. Após a extração as amostras foram caracterizadas físico-química e biologicamente. O tratamento químico resultou em rendimentos totais de quitina de 8.15%, 7.77% e 13.85% (baseado em matéria seca) para as larvas de *T. molitor*, os indivíduos adultos de *A. domesticus* e os seus subprodutos, respetivamente. Considerando a extração com os solventes eutécticos, os rendimentos resultantes foram bastante elevados em comparação com o tratamento químico, o que indica elevada impureza das amostras e por essa razão futuras análises às amostras foram abandonadas. Os resultados obtidos por FTIR-ATR das diferentes amostras de quitina extraída mostraram padrões de α-quitina, enquanto os resultados do quitosano indicaram que a deacetilação foi eficaz. Adicionalmente, os resultados de FTIR-ATR das amostras de insetos eram semelhantes às amostras comerciais de camarão, resultados esses que foram também confirmados através de análise por FT-RAMAM. O maior grau de deacetilação (%) foi encontrado no quitosano de *A. domesticus* (indivíduos adultos) (63.7%), seguido pelo quitosano de *T. molitor* (62.3%) e por último o quitosano derivado das patas e asas (62.0%). A análise de difração por raio X também confirmou os resultados obtidos previamente na

espectroscopia e adicionalmente demonstrou que o quitosano tem uma estrutura mais amorfa que a quitina, devido aos padrões obtidos e aos valores do índice de cristalinidade (CrI). O peso molecular das diferentes amostras de quitosano foram 302.58 kDa, 332.58 kDa e 245.77 kDa para as larvas de *T. molitor*, os indivíduos adultos de *A. domesticus* e os seus subprodutos respetivamente. As imagens de SEM revelaram diferentes morfologias de superfície para todas as amostras, ainda que a maioria tenha apresentado estruturas microporosas e fibrosas. Os resultados da análise termogravimétrica para as amostras de quitina demonstraram maior estabilidade térmica para a amostra comercial de quitina, no entanto no caso do quitosano a amostra termicamente mais estável foi o quitosano extraído das larvas de *T. molitor*. A partir da avaliação da atividade biológica foi encontrada capacidade das amostras de quitosano para inibir a peroxidação lipídica no teste do β-caroteno. No entanto, nenhuma das amostras demonstrou atividade para sequestrar radicais livres no teste do DPPH. Todas as amostras apresentaram baixa atividade antimicrobiana contra bactérias Gram-positivas e Gram-negativas, sendo que a atividade antimicrobiana mais elevada foi obtida contra a *Klebsiella pneumoniae*. Nenhuma atividade antifúngica foi observada durante os ensaios.

Palavras-chave: Caracterização; Economia circular; Insetos; Quitina; Quitosano; Subprodutos

Abstract

Chitin and chitosan are two well-known biopolymers, that have a wide range of physiochemical and biological properties which makes them extremely versatile, and capable of being applied in sectors like the agricultural, biomedical, pharmaceutical, food, amongst others. Crustaceans and their by-products are the main source of chitin extraction and chitosan production, and the chitosan market is in constant expansion, and it is estimated that by 2027 the market will reach 4.7 billion USD. However, chitin is also synthesized in several other living organisms such as insects, fungi, and algae. In particular insects are of special interest due to the growing popularity of entomophagy. This popularity is manifested on both the industry (increase in insect rearing companies and food products incorporating insects) and in academia, with a vast selection of studies focusing on insect fractionation (proteins, lipids, and chitin). As such, chitin extraction and chitosan production from insects presents an opportunity to increase the economic value of edible insects. The current study aimed to examine two different edible insect species as alternative sources for the extraction of these polymers, namely yellow mealworm larvae (*T. molitor*), adult house cricket (*A. domesticus*) as well as by-products (legs and wings) generated by house cricket rearing. The extraction was achieved through an optimized chemical treatment based on sequential acid and alkaline hydrolysis. In an attempt for a greener extraction, an alternative method based on natural deep eutectic solvents was also employed. After the extraction the samples were physiochemical and biologically characterized. The chemical treatment resulted in total chitin yields of 8.15%, 7.77% and 13.85% (Dry Weight basis), for *T. molitor* larvae, *A. domesticus* (adult) and *A. domesticus* by-products, respectively. Concerning the extraction with the natural deep eutectic solvents, the resultant chitin yields were very high in comparison to the chemical treatment which indicates high impurity of the samples and for that reason, further analysis of these samples were abandoned. The FTIR results of the different extracted chitin samples showed patterns of α-chitin while the chitosan results indicated the effectiveness of the deacetylation. Additionally, the FTIR results from the insect samples were similar to those from the commercial shrimp samples and were also confirmed throughout FT-RAMAN analysis. The highest degree of deacetylation was found in the chitosan from adult *A. domesticus* (63.7%), followed by *T. molitor* chitosan (62.3%) and lastly L&W chitosan (62.0%). The x-ray diffraction analysis also confirmed the obtained results from the spectroscopy and in addition demonstrated that chitosan had a more amorphous structure than chitin, due to the patterns and crystallinity index (CrI) values obtained. The molecular weight of the different chitosan samples was 302.58 kDa, 332.58 kDa and 245.77 kDa for *T. molitor* larvae, *A. domesticus* (adult) and *A. domesticus* by-products, respectively. SEM images revealed different surface

morphologies for all the samples, although the majority showed microporous and fibrous structures. The thermogravimetric analysis results for the chitin samples demonstrated higher thermal stability for the commercial chitin, however, in the case of chitosan the more thermal stable sample was the chitosan extracted from *T. molitor* larvae. From the biological activity evaluation, it was possible to conclude that all chitosan samples showed capacity to inhibit oxidative peroxidation in the β-carotene bleaching test. However, no scavenging activity in the DPPH test was observed. All samples displayed low antimicrobial activity against different Gram-positive and Gram-negative bacteria and the highest antibacterial activity was observed against *Klebsiella pneumoniae*. Non-antifungal activity was found during the assays.

Keywords: By-products; Characterization; Chitin; Chitosan; Circular economy; Insects; By-products

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List of acronyms

AD – Adult specimens of the house cricket *A. domesticus*

FTIR-ATR – Attenuated reflection mode Fourier transform infrared spectroscopy

BE – Betaine

BHT – Butylated hydroxytoluene

BHI - Brain Heart Infusion Agar

BmNPV – *Bombyx mori* nuclear polyhedrosis virus

CA – Citric acid

CAGR – Compound annual growth rate

ChCl – Choline chloride

CLSI – Clinical laboratory and Standard institute

CRI – Crystallinity index

DA – Degree of acetylation

DD – Degree of deacetylation

DES – Deep eutectic solvents

DMSO – Dimethyl sulfoxide

DSC – Differential scanning calorimetric analysis

$DTG_{máx}$ – Maximum degradation temperature

DW – Dry weight

EA – Elemental analysis

FTIR - Fourier transform infrared spectroscopy

G – Glycerol

Hac – Acetic acid

HBA – Hydrogen bond acceptor

HBD – Hydrogen bond donor

HPLC -High performance liquid chromatography

ILs – Ionic liquids

L&W – *A. domesticus* by-products (legs and wings)

MA – Malonic acid

MBC – Minimum bactericidal concentration

MHB – Müeller-Hinton Broth

MIC – Minimum inhibitory concentration

Mw – Molecular weight

NADESs – Natural deep eutectic solvents

NMR – Nuclear magnetic resonance

SDA - Sabouraud Dextrose Agar

SEM – Scanning electron microscopy

TGA – Thermogravimetric analysis

TM – Larvae from the yellow mealworm *T. molitor*

U – Urea

XRD – X-ray diffraction

1. Introduction

1.1 Insects

Insects are one of the greatest groups of animals in the world, with over a million species already identified and many more yet to be discovered [1]. These animals are one of the main bases of the ecosystems of Earth, contributing to plant pollination, soil structure and fertility maintenance, cycle nutrients, population control of other organisms and are also a part of the food chain, providing a food source for other animals [2].

Insects, as well as crustaceans (e.g., crab; lobster, shrimp), are classified as arthropods [3]. Coleoptera (e.g., beetle and mealworm), Orthoptera (e.g., house cricket), Diptera (e.g., black soldier fly), Hymenoptera (e.g., honey bee), Hemiptera, Neuroptera, and Lepidoptera (butterflies and moth), are amongst the orders that include these animals [1, 4]. Insect classification had seen significant changes over the years, with scientists such as Carl Linnaeus (1707–1778), Charles R. Darwin (1809–1882), Ernst Heinrich Philipp August Haeckel (1834–1919), and Emil Hans Willi Hennig (1913–1976) studying this topic [4].

In their development, insects go through some life stages (which might differ across species) such as embryo, nymph or larva and pupae or adult [5]. The fundamental external insect anatomy is comprised of three major body parts: the head, the thorax, and the abdomen [6]. Adult insect body size (measured in body length) fluctuates by three orders of magnitude and the tracheal system is assumed to restrict the maximum insect size [7]. Insects are the only winged invertebrates, they are cold-blooded, reproduce rapidly, have huge populations, and also suffer metamorphosis to adjust to seasonal fluctuations [8]. Regarding insects' composition, an important element is the exoskeleton (cuticle) as the development of this light however mechanically hard exoskeleton has helped these animals survive in nature. The cuticle that is connected to the epidermis is one of the largest and most significant organs on an insect's body. It is characterized as a supramolecular assembly of chitin, proteins, minerals, lipids, catechol, pigments, water, and other components that determine the insect's body form and let it grow, move, communicate, reproduce, and handle predators, pathogens, and poisonous substances. The cuticle anchors skeletal muscles and shields insects from physical damage, pathogen penetration, and desiccation [9].

For a great part of humans, insects are regarded as pests, since these animals have an active participation in the spread of diseases like malaria, Zika virus and Dengue fever

(mosquitoes), and they also contribute to the damage of farmland (e.g., desert locust), affecting people's health and the agricultural sector which has a drastic impact on the economy worldwide [10, 11]. As referred earlier, insects are more than simply a pest as they make huge contributions to the ecosystem through processes such as seed dispersion, pollination, organic matter processing, and water filtration [12]. In addition to these processes, insects have long participated and collaborated in human life, like the case of the bees (e.g., *Apis mellifera*) that produce honey [13]. Furthermore, insects have been utilized in traditional Chinese medicine, and in recent years they have been a target of pharmacological and health-related research, being tested for the treatment of cancer, AIDS, infections, and other diseases due to their composition of peptides, proteins, toxins, and other elements [14]. Moreover, one of the major uses of insects is the production of silk. Originated mainly by silkworms of the Bombycidae family, silk is one of the oldest natural protein fibers known by humankind. It has been used in the textile industry for thousands of years and it is considered a premier textile [15].

Additionally, another great and ancient role of insects is using them as food for humans or as feed (e.g., birds, reptiles). Insect consumption is practiced in various regions (mainly in Asia, Africa and Latin America), with more than two billion people worldwide currently consuming a wide range of insects. In Western countries, the consumption of insects as human food has been neglected, but in the last decade, this situation has been changing gradually, due to an increased interest in alternative food sources that can provide nutritional and environmental benefits [1].

1.2 Entomophagy and environmental issues

The practice of consuming insects is described as entomophagy [16]. Insects were used as a primary food source by both *Homo sapiens'* ancestors and early tribes to cover their biological demands. Insects were an essential element of humans' diet before they developed equipment for hunting and harvesting food, as demonstrated by ancient human coprolites [17]. In modern times, insect consumption is a traditional practice prevalent in several regions (more specifically Asia, Africa, and Latin America), with over two billion people across the world currently consuming a myriad of insects [1]. This variety of species comprises more than 2100 insect species that are considered edible [18]. Among all these species, the most consumed insects are the beetles that belong to the Coleoptera order [19].Numerous populations of Sub-Saharan Africa, South and Central America, South-East Asia, and the Australia-Papua New Guinea region, still regard edible insects as a sustainable food source [20]. For example, when staples are

insufficient in the African continent, insects that are abundant in this part of the world become an important source of food, performing an important role in food security [19].

Nevertheless, insects are not just eaten to face hunger and in some parts of the world like Latin America or Asia they simply make part of people's diet and can even assume a position of delicacies sold to tourists. In the Asian continent, species like Red palm weevils (*Rhynchophorus ferrugineus*) are extremely popular and are a highly prized delicacy, while in Latina America (more precisely in Mexico) chapulines (edible grasshoppers) are very popular, taking important participation in local small-scale markets, restaurants and even export markets. Even so, in the last few years due to globalization and the adoption of westernized food practices, the consumption of insects is decreasing in these traditional consuming regions [19].

In regions like Europe and North America, the situation is very different, with the consumption of insects being rejected, due to psychological, sociocultural, religious, and environmental factors [21]. However, in the last decade the utilization of insects as food or feed in Western countries has been gaining interest not only from scientific academia but also from the food industry. The major reason why this is happening is due to the global population growth (exceeding 9 billion by 2050) that will result in an escalated increase in the demand for food, which exacerbates current problems in the food sector, consequently requiring the need for new alternative food sources [22].

Current food practices (especially those associated with livestock production and intensive agriculture) have a notorious negative effect on issues such as climate change and environmental destruction. Livestock production emits 14.5% of human-induced greenhouse gases emissions and it also causes the acidification of the soils and water bodies [23]. Moreover, this production leads to major water waste and large use of agricultural land area (occupies 70% of global agricultural land) [24]. Relatively to plant production, the amount of water required per 1 kg of dry crop yield ranges from 300 to 2000 L, with soybeans being the greatest water consumers. However, this situation is aggravated in livestock production, with the production of 1kg of beef requiring about 43000 L of water [25]. Considering these issues, it is vital to discover new alternatives to protein-rich sources, which do not cause so severe consequences to the environment and simultaneously give the necessary nutrients to humans. Edible insects can be one of such source and are starting to gain a lot more attention in the last few years being present in numerous research studies as an alternative food source to improving global food and nutrition security [26].

Edible insects can be an outstanding alternative food source, due to some environmental advantages in comparison with traditional animal protein sources, like pigs, cattle, and chickens. Insects have a higher feed conversion efficiency which can lead to a lower use of feed compared to other animal sources. For instance, while beef requires 10 kilograms of feed to gain 1 kg of body weight, cricket requires less than 2 kg of feed to gain 1 kg of body weight, resulting in a much superior feed-to-meet conversion rate [24]. Moreover, edible insects can be reared with food waste, livestock manure and municipal biowaste, contributing to a zero-waste pathway and applying the circular economy principle [27]. In terms of greenhouse gases and ammonia emissions, the rearing of edible insects leads to lower emissions than cattle and beef production [28]. Edible insects also require less water and land to achieve the same amount of protein [19].

Another possible advantage of entomophagy in contrast with the consumption of poultry and pigs/cattle is in the field of zoonotic infections [29]. Due to the taxonomic disparity between insects and humans, it is believed that the transmission risk of zoonotic infections is lower. Still, the threat of insects being a source of hazardous infections and spreading diseases to humans has not been adequately investigated [24]. Nonetheless, extensive work has been performed on the food safety of edible insects. It is known that insects can provoke allergenic reactions in individuals allergic to crustaceans (due to cross-reactivity between insect proteins and crustacean allergens) and individuals who are constantly exposed to insects (through a primary sensitization mechanism) [30]. Edible insects can also present a high microbiological load, but adequate processing technologies can mitigate this risk and the presence of pathogens (e.g., *Salmonella, Listeria monocytogenes, Clostridium)* is extremely rare [31]. The food safety of edible insects has been further assessed by the European Food Safety Authority (EFSA), and currently, there are three species (*Tenebrio molitor* larvae, adult *Locusta migratoria* and adult *Acheta domesticus*) that are deemed as safe for consumption by humans in the EU, and there are 9 more species that are under evaluation [32].

Regarding the nutritional composition of insects, they can be excellent sources of proteins, fats, minerals, and vitamins and even though the nutritional values are extremely variable, the levels of some of these components are enough to meet human nutrition needs [33]. The variation in the nutritional levels is owing to species, life stage, environment, and the diet of the insects. The most abundant macronutrient is generally protein, with some insect species containing a higher content on a mass basis than other animals and plants (e.g., beef, fish, soy) [17]. Additionally, this protein fraction presents high quality due to amino acid composition and digestibility [33, 34]. Fat is the second most abundant macronutrient, with insects being generally rich in polyunsaturated fatty

acids but can also have high levels of some essential fatty acids (oleic, linoleic, linolenic, and palmitic acids) [35]. In terms of micronutrients, insects can present adequate levels of calcium, iron, and zinc [17]. Considering the energy levels, the average energy content of edible insect's ranges from 409.78 to 508.89 Kcal/100 g (based on dry matter) [34]. Fibers are the third most abundant group of elements present in insects [34]. In this group, the main one is chitin, and it is formed in the exoskeleton (cuticle) of these animals.

Chitin content generally contributes to roughly 5-10% of insect dry weight [36]. This polymer does not have too much interest in some food applications, with some researchers indicating that chitin affects the protein quality of edible insects [34, 37, 38]. Additionally, some authors refer that chitin is indigestible to humans, but it has also been demonstrated that the gastrointestinal tract of humans produces chitinolytic enzymes, proposing that chitin can be digested [39-41]. Furthermore, even though the role of chitin and other polysaccharides in human physiology remains in research, various insect polysaccharides present benefits for immune regulation, antioxidant, and anticancer functions [33].

Keeping the nutritional composition in mind, and once insect consumption is not well accepted by Western consumers, there is a special interest in the fractionation of edible insects, since products with processed (e.g., non-visible) insects or functional products (e.g., protein-rich products) are more accepted by consumers [42]. The fractionation of insects consists on the separation of the main elements (proteins, lipids, and chitin) turning these animals into a biorefinery [43]. The fraction with the most relevance for application in food products is the protein fraction. As such, most focus has been on optimizing protein extraction procedures and the chemical, biological and techno-functional characterization of the obtained fractions [44]. In the case of lipids, it can be useful to extract them in order to use the defatted fraction and thus increase product acceptance [45]. Due to lower ranking in sensory evaluation, the fat fraction might not be applicable for human food, but it can have other uses such as feed or biodiesel production [46-48]. As for chitin, although its direct use in food products is not a viable option, its extraction can boost the economy associated with insect rearing, not only because chitin and its deacetylated form chitosan have a wide array of applications, but also because chitin can be obtained from by-products of insect rearing [49]. Additionally, chitin removal can improve the digestibility of the protein fraction [38, 50]. Thus, insects' chitin is an undervalued fraction and increased focus should be given to its extraction and characterization to better optimize the utilization of insects as food and feed.

Therefore, it is important to review the literature on chitin and chitosan, namely how they are currently obtained, their importance and potential applications.

1.3 Chitin and Chitosan

The present section concerns chitin and chitosan, focusing on their history and structure, sources, extraction and characterization methods and ultimately, applications. Even though a general approach will be given to this topic, the main focus are insect species. Throughout this section, information with origin on a systematic review concerning chitin and chitosan from insects (part of a larger review on insects' fractionation) will be incorporated. Additionally, a supplementary table (section 8.3) that contains a summary of all the articles included in the systematic review will be used as support. The methodology applied for the systematic review followed a PRISMA methodology, consisting of a search made in three databases (PubMed, Scopus and Web Of Science) using the following *query*: (("edible insect*" OR entomophagy OR mealworm OR fly OR housefly OR butterfly OR "Clanis bilineata" OR "hermetia illucens" OR grasshopper OR silkworm OR beetle OR cockroach OR termite OR Wasp OR cricket OR moth OR cicada OR bee OR locust OR bumblebee) AND (chitin OR chitosan)). After obtaining the articles from the three databases, duplicates were removed, and a selection was made based firstly on title/abstract reading and then full-text reading (section 8.1). This selection was based on inclusion and exclusion criteria that was based on the selection of original articles that extracted chitin or produced chitosan from insects' chitin and that assessed the yield of the extraction and/or characterized the obtained chitin/chitosan.

1.3.1 History and structure

Chitin is the second most abundant polymer on Earth right after cellulose, and it is a natural linear polysaccharide that consists of variable quantities of N-acetyl-D-glucosamine (N-acetyl-2-amino- 2-deoxy-D-glucose) units linked to each other by β1-4 glycosidic bonds with a small amount of deacetylated monomer units ((2-amino-2-deoxy-D-glucose) (Fig. 1) [51]. Chitin is rarely found in its pure form in nature, but instead as nano-organized chitin-proteins, chitin-pigments or chitin-mineral composite biomaterials [51]. This polysaccharide is the main structural polymer found in the fungal cell walls, as well as on the exoskeletons of arthropods and insects [52] . Historically, chitin was first described in fungi (1811) by Henri Braconnot who named it *fungine* [53] and later in arthropods (1826) by Antoine Odier. Antoine Odier observed a substance in the exoskeleton of insects, which was identical to that discovered in the fungal, and he

named it *chitine* [54]. Chitin's ancient genesis was identified in the fossil filaments of eukaryotic creatures discovered between 810 and 715 million years ago in dolomitic shale [55]. The deacetylated form of chitin is called Chitosan (Fig. 1), and its history dates back to 1859 when Charles Rouget reported the deacetylation of chitin through its boiling in the presence of a strong alkali solution [56]. However, it was only in 1894 that the name chitosan was given to this polysaccharide by Felix Hoppe-Seyler [57]. Since their discovery, over 200 years ago, these polymers have been extensively studied, passing through some different periods: discovery (1799-1894), confusion and controversy (1894-1930) followed by exploration (1930-1950) and doubt (1950-1970), and the current period of application [52]. During these periods a great deal of information related to the formation, structure, extraction, characterization, and application of both polymers was obtained, but countless more are yet to be discovered.

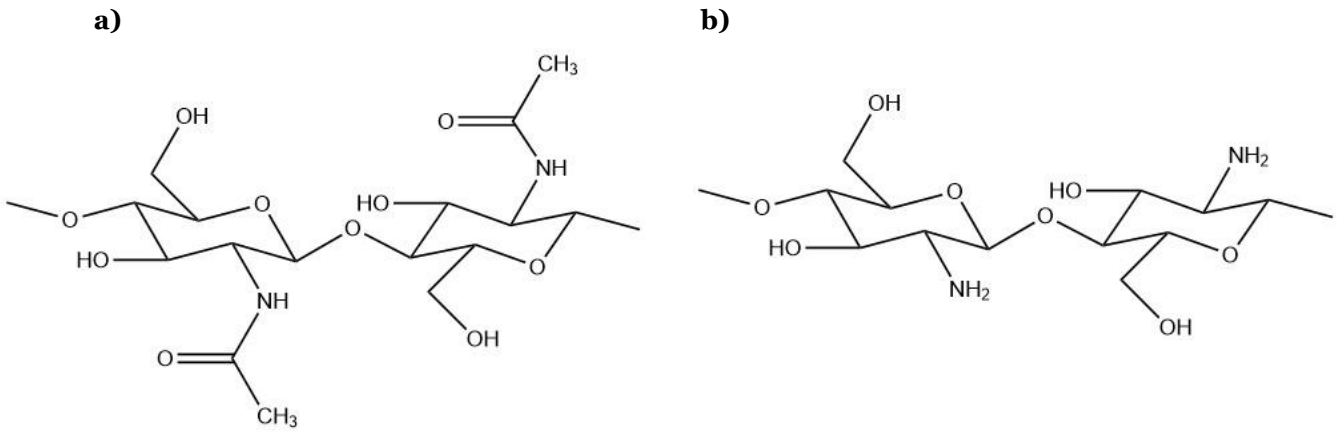

Fig. 1 - a) Chitin b) chitosan structures. Drew using ChemDraw software.

1.3.2 Biosynthesis and structure

In insects, chitin is essential for their growth and development, and besides its presence in the cuticular exoskeleton, it is also found in the internal structures of these animals. Chitin constitutes the peritrophic matrices, surrounding the gut in many insects and other invertebrates, shielding the intestinal epithelium against mechanical damage, radical oxygen species and microbial invasion [58]. The biosynthetic pathway of chitin involves a variety of enzymes which convert trehalose into chitin. The principal enzymes

are chitin synthases, members of the broad group of glycosyltransferases, an enzyme family that catalyzes the transfer of sugar fractions from activated sugar donors to specified acceptors, establishing a glycosidic bond, and in this case synthesizing chitin polymers. The active precursor that originates chitin by the reaction catalyzed with the integral membrane enzyme, chitin synthase, is called UDP-N-acetylglucosamine. After chitin formation, partial deacetylation catalyzed by chitin deacetylases occurs. Following the partial deacetylation, chitin combines with peritrophic or cuticular proteins, creating assemblies and forming, together with other elements, the peritrophic matrix or the cuticle of insects. Subsequent in the degradation pathway, chitin can be degraded with the help of endochitinases, forming low-molecular-weight chitooligosaccharides, followed by the formation of N-acetylglucosamines [59-61].

Structurally, chitin (β-1-4-linked N-acetylglucosamine) is a straight-chain polymer that is divided into three polymorphic structures, α-chitin, β-chitin, and γ-chitin (Fig. 2), due to structural differences which in turn are responsible for several variations of its physiochemical properties [51]. The α-structure is characterized by antiparallel chains contrary to β-chitin which is characterized by parallel chains. The structure of α-chitin has strong intersheet and intrasheet hydrogen bonding, while β-chitin is comprised of weak intrasheet hydrogen bonding and weak intermolecular forces. These differences between the polymorphic structures, make them differ on the molecular weight and decomposition temperature (higher molecular weight and decomposition temperature in α-chitin). In the case of β-chitin, it has been demonstrated that this form shows higher reactivity in various modifications reactions and higher affinity for solvents than α-chitin, however, all chitin is insoluble in common organic and inorganic solvents. The γ-structure is characterized by one antiparallel chain and two chains with the same direction, and it is the scarcest of the three and not much information is known about its natural distribution and structure. The most abundant allomorph is α-chitin, being found in hard structures, while β- and γ-chitin are found in flexible structures. The α-forms is found in arthropods and fungi, β-forms has been found in squid pens and γ-forms in cocoon fibers of *Ptinus* beetle and the stomach of *Loligo* [62-64].

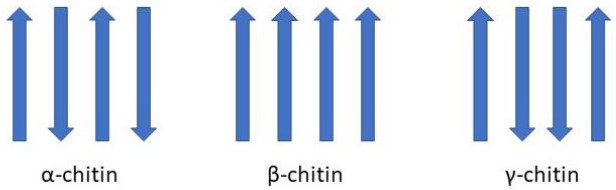

Fig. 2 Chitin polymorphic structures chains orientation.

Chitosan (β-(1-4)-linked N-acetyl-D-glucosamine and D-glucosamine units) is a product of the N-deacetylation of chitin and it is a cationic polymer that is non-toxic to humans, biodegradable and biocompatible [65]. Chitosan has different degrees of deacetylation (DD), depending on the preparation method that is used, and this degree corresponds to the ratio of glucosamine to N-acetyl glucosamine. For chitin to be converted into a chitosan structure, the polymer needs a DD of at least 50%, which makes the polymer soluble in acidic solutions and gives chitosan a higher range of applications in comparison with chitin. The glucosamine units holds a free amino group that can take a positive charge, giving chitosan a variety of features like solubility and antimicrobial properties as well as metal-binding capacity [66, 67]. The physicochemical and biological properties of chitosan are dependent on the DD, molecular weight (lower than Mw of chitin), pH and other conditions [68].

1.3.3 Sources

As referred earlier, chitin and chitosan can be found in different animal parts, such as crustacean exoskeletons, cuticles of insects, chelicerates and myriapods, mollusk shells, fungal cell walls and also in algae [69, 70]. Nowadays the main source in the industry for commercial production of chitin and chitosan is marine food waste, more concretely crustacean shells, like shells from shrimp, crab, and others. This happens because the seafood industry generates tons of shellfish waste during processing and consumption, which is discarded on the seashore. The treatment of this waste can help solve some environmental problems, while also supplying value-added products such as chitin [71]. However, it also must be considered that the seasonal availability and the applied extraction methods (exoskeletons are rich in minerals which can difficult the extraction procedures) can be some of the disadvantages associated with chitin extraction from these sources [72]. Moreover, the end products of the extraction are inconsistent on the levels of DD and can have a high Mw [73].

Regarding fungal species, chitin and chitosan can be extracted from the cell wall of different fungal species, with the Zygomycetes class having the highest amounts of these polymers [74]. These sources have a few advantages like year-long availability or particle size uniformity. As for the extraction methods, these sources do not require a harsh chemical treatment to extract chitin (demineralization step not needed), although the extraction of higher purity chitosan may need an acid treatment step, since chitosan can be linked to glucan and inorganic compounds [75, 76].

In comparison to traditional sources (crustaceans) of chitin and chitosan, the extraction of these polymers on insects presents some advantages, requiring less amount of chemicals and time, and the extracted chitin can present higher quality. Additionally, insects don't have the problem of seasonality and have a high reproductive and fertility rate [77]. The entomophagy popularity intensification results in an increase in scientific studies pertaining to the use of insects as food (including its fractionation) but also in the growth of insect breeding facilities for food and feed [19, 23, 29, 77]. As a result, members of the insect industry have a special interest in expanding the economic value of edible insects, including the extraction of chitin. Insects can be reared for the extraction of chitin but can also contribute to a circular economy since the insect waste (exuviae, legs and wings, dead animals) produced by this emerging industry, can be a good source of chitin and chitosan. Additionally, harmful, and invasive species (e.g., when a swarm occurs) that go to waste in large amounts, can also be sources of chitin and chitosan [78] (section 8.3).

Besides their advantages and disadvantages, all chitin and chitosan sources have place in the industry since it is a considerable market in an expansion phase. In 2019 the chitosan market size was appreciated at 1.7 billion USD, and by 2027 it is estimated that this market will reach a value of 4.7 billion USD with a compound annual growth rate (CAGR) of 14.5%, according to Allied Market Research in June 2020 [79]. Therefore, we can consider insects as a possible and legit source of chitin and its derivatives.

1.3.4 Extraction

The extraction of chitin can be divided into two main methods, chemical (acid-alkali treatment, deep eutectic solvents, ionic liquids) and biological (microorganisms and enzymes) (section 8.3). Moreover, other methods can be coupled to the conventional chemical method, such as the ultrasound-assisted method and microwave-assisted method to reduce the time of extraction, the consumption of energy and reagents without altering the properties of chitin and chitosan [80, 81]. Generally, chitin extraction undergoes two steps: demineralization and deproteinization while a decolorization step can be added to remove residual pigments present on the product. For the production of chitosan, a deacetylation step needs to be applied to chitin. Before starting the main treatments, the chitin sources need a pre-treatment, in order to improve the yield and the quality of the final product. This pre-treatment generally includes washing, grinding, and drying. In the case of insects, a great part of studies includes not only drying and grinding but also defatting steps too (section 8.3). Regarding drying, insects can be dried

by air-ovens, vacuum ovens, freeze-drying, or simply by air. Insects can be ground by knife mills, pestle and mortals, knives, and other tools. Methods such as oil press processes, Soxhlet, extrusion apparatus, or soaking the insects in ethanol, petroleum ether, and other chemicals are employed for defatting the insects [82-85]. These types of pre-treatments can increase the yield and purity of chitin and so of chitosan [86, 87].

1.3.4.1 Conventional chemical treatment

The most extensively used method of chitin extraction is the conventional chemical treatment, which follows a demineralization (acid treatment) and deproteinization (alkali treatment) step, and an additional decolorization step can be integrated for the purpose of eliminating residual pigments [81]. According to the performed systematic review, this process is used in 96.5% of studies extracting chitin from insects. For the commercial chitin extracted from marine sources (e.g., shrimp), this method is also the most commonly applied [88]. The industry prefers the acid-alkali treatment because it is well established, is relatively fast and allows to produce a product free of impurities. Despite these advantages, this chemical method has relevant issues such as a huge amount of water and energy consumption and the generation of toxic effluents due to the nature of the chemicals (strong alkali and acid solutions) [89].

1.3.4.1.1 Acid treatment

So, as referred previously, the method for chitosan production comprises three principal steps (demineralization, deproteinization and deacetylation of the obtained chitin) and one additional and facultative stage (decolorization). Generally, the first step is employed to remove minerals (primarily calcium, magnesium phosphates and carbonates) but also catechol. This remotion can be achieved with acid treatment, using acidic solutions of hydrochloric (most used), nitric, sulphuric, acetic, oxalic, and formic acids. The conditions of reaction vary in concentration of the solution, ratio (solid to liquid), temperature and time. In this process, the calcium carbonate is decomposed, forming calcium chloride, and a release of carbon dioxide occurs (Fig. 3) [88, 90].

Fig. 3 Demineralization chemical equation. Designed on ChemDraw software.

Regarding chitin extraction with edible insects, the most widely used chemical for demineralization is HCl (Hydrochloric acid) with quite a few conditions being applied considering molar concentration, temperature, time of reaction and solid/liquid ratio (section 8.3). Contemplating the demineralization of the yellow mealworm *T. molitor* and different cricket species in various life stages, the concentration of HCl ranged from 0.25M to 2M HCl solutions [82, 91]. Concentrations between 1 and 2 M HCl solutions are commonly used on these and other species. Still, it was also reported in the literature the use of HCl solution with much higher concentration such as 4 M on the demineralization of different grasshopper species and 6M on black soldier fly (*Hermetia illucens*) specimens [92, 93]. As for temperature, demineralization temperatures can scale from 20 to 110ºC and the reaction time can range from between 30 min to 24H [94-96]. Glab, et al. [86] sought to optimize the demineralization step on the two-spotted cricket (*Gryllus bimaculatus*), using HCl at room temperature. Different reaction conditions were tested, namely concentration (1-2 M), time (30-60 min) and extraction cycles (4-5), with the optimal extraction conditions being defined as 2 M/30 min/4 extraction cycles since the obtained sample resembled commercial chitin in X-ray diffraction (XRD) analysis.

1.3.4.1.2 Alkali treatment

Demineralization is generally followed by deproteinization, in which alkali solutions such as sodium hydroxide (NaOH), potassium hydroxide (KOH), calcium hydroxide ($Ca(OH)_2$), sodium phosphate (Na3PO4), amongst other bases are used. NaOH is the most applied and preferred inorganic base among them all (section 8.3) . This step aims to cleave the bonds between chitin and proteins, and thus obtain pure chitin [97]. As it happens with demineralization, the solution concentration, reaction time and temperature and the solid to liquid ratio fluctuate depending on the source and the selected conditions by the operator. Deproteinization times in insects can fluctuate from minutes to days, and temperatures as high as 175 ° C have been described in the literature [78, 92, 98]. Once again, using a few examples with *T. molitor* and cricket species, the concentration range of NaOH solutions is between 0.25 M and 5 M [99, 100]. It is essential to mention that this step requires a careful approach to the concentration, time and temperature used, in order to produce chitin with a degree of acetylation (DA) superior to 50%. This allows to produce a product with high purity and minimizes some modifications that can occur to the chitin surface (chitin nanocrystal formation, surface deacetylation and chitin fibril hydrolysis) [100]. If the DA is lower than 50%, chitin starts to lose acetyl groups and it is converted to chitosan [86]. Concerning these previous

aspects, the concentration of NaOH solution usually applied on different insect species varies between 1 and 2M [78, 82, 101-104]. Soon, et al. [97] evaluated different NaOH solution concentrations (0.5 M, 1 M and 2 M) with a fixed temperature at 80 ºC for 20 h on the deproteinization step of the superworm (*Zophobas morio*). The authors reported that chitin yield increased with lower NaOH solution concentration. However, the sample treated with the highest NaOH concentration presented the highest DA value, which affects the yield of chitosan in the following steps. In a study using the house cricket (*Brachytrupes portentosus*), the authors invert the sequence of steps for the extraction of chitin (deproteinization followed by demineralization with oxalic acid) [105].

1.3.4.1.3 Decolorization

After deproteinization, it is possible to realize a decolorization step to eliminate residual pigments present on the product. This step is optional and depends on the source that has been used, the choice of the operator and the finality of the product. In this facultative step, distinct chemicals are utilized such as potassium permanganate (KMnO4) - oxalic acid (C2H2O4) (single or sequential treatment), methanol:chloroform:water or hydrogen peroxide [106] (section 8.3). Zlotko, et al. [107] investigated multiple approaches to the decolorization stage of chitin from *Hermetia illucens* pupa exuvia samples. The decolorization conditions that were assessed consisted of boiling water (100 ºC/24 h), H_2O_2 (80 ºC for 2.5 and 5 h) and $KMnO_4$ (80ºC/20min) followed by 4% $C_2H_2O_4$. The authors conclude that using H2O2 for a longer period and a more aggressive agent (KMnO4) results in a lower yield without boosting the advantageous properties of chitin.

The general process of insect chitin extraction is illustrated in Fig. 4.

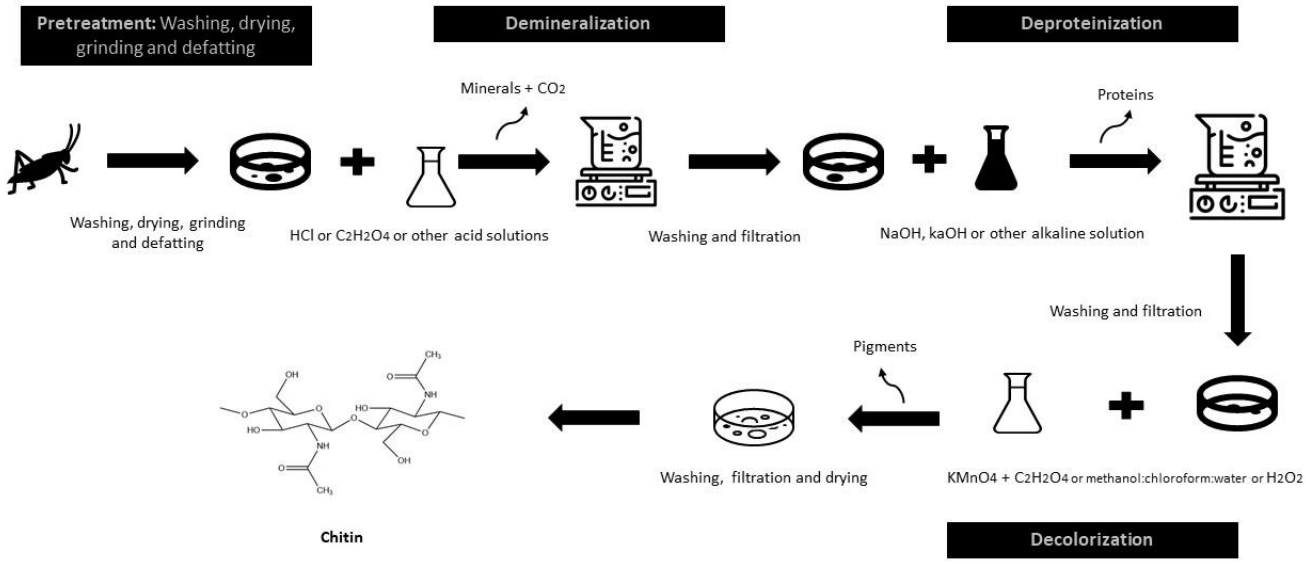

Fig. 4 General process of insect chitin extraction by the conventional chemical treatment.

1.3.4.2 Deacetylation

On the way to produce chitosan, chitin needs to be deacetylated (DD>50%), going through an *N*-deacetylation modification in which the acetyl groups are removed from chitin and are replaced by reactive amino groups (NH_2) (Fig. 5). A strong alkaline treatment (rupture of the main chain and destruction of the crystalline structure of chitin) is the choice for this step [108]. As it occurs for the deproteinization step, concentrated solutions of NaOH at high temperatures are often used and preferred (section 8.3). The reaction conditions of this step have variations in time, temperature, and concentration that affect the physicochemical characteristics of chitosan. For instance, as the reaction temperature increases, the DD raises, but the Mw suffers a reduction [109] . The concentrations of NaOH solutions generally vary between 40 and 60% (w/v or w/w) and are usually used at high temperatures (80-110 ºC) for several hours [87, 110-113]. In an investigation using the larval exuviae of *T. molitor*, the researchers studied the optimization of the deacetylation step, using different concentrations of NaOH solutions (50%, 55%, 60% (w/w)), reaction temperature (95ºC or 105 ºC) and time (3 h or 5 h). One of the conclusions the authors achieved in that study was that the chitosan treated with a 55% (w/w) NaOH solution at 105 ºC for 5 h was the most complete once it presented the higher DD [114]. In another study regarding the extraction of chitosan from the Muga silkworm (*Antheraea assamensis*) the authors

used a 40% NaOH solution (chitin to solvent ratio of 1:10), where they tested different reaction temperatures (60, 80, 100, and 110 ºC) and different times (4, 6, 8, and 10 h) to study the kinetics of the reaction. From the results obtained, the authors conclude that the deacetylation process is extremely dependent on the temperature. In fact, they stated that reaction temperatures below 80 ºC, make the conversion of chitin to chitosan negligible and also the increase in time and solution concentration result in an increase of the DD [115].

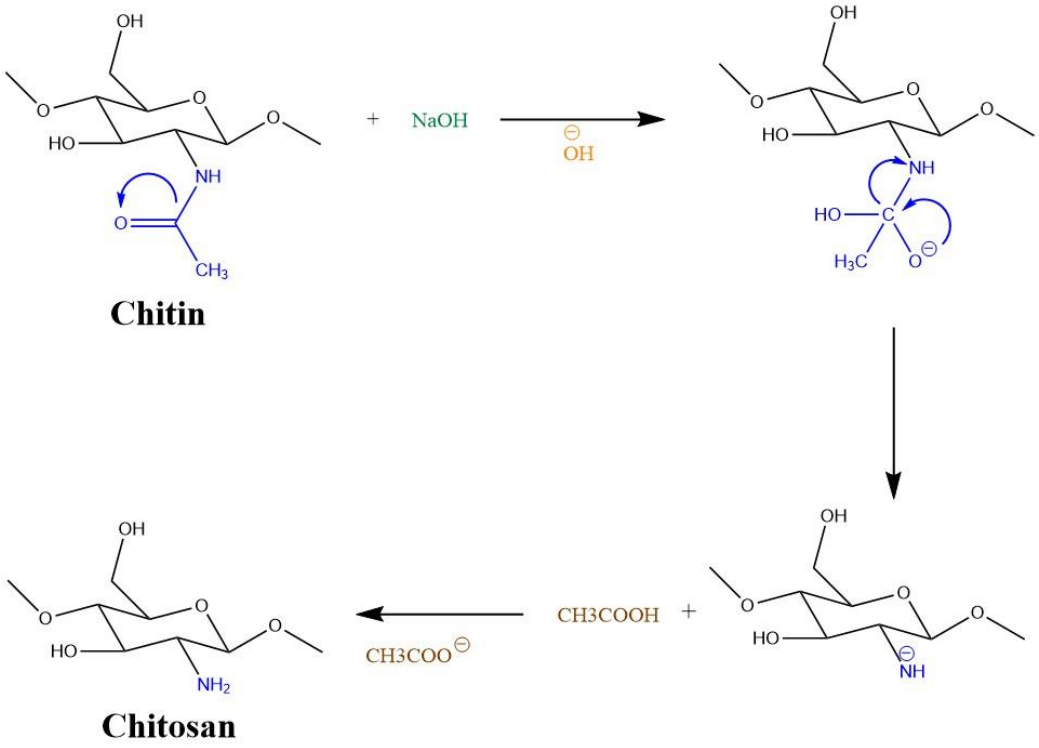

Fig. 5 Reaction pathway of chitin deacetylation into chitosan using NaOH. Design using ChemDraw software

1.3.4.3 Alternative extraction methods

1.3.4.3.1 Biological methods

As noticed before, more ecological alternatives to the conventional chemical method are needed. One of those alternatives are the biological methods that have been studied and are capturing some relevant interest. These methods utilize bacteria (e.g., *Lactobacillus, Pseudomonas aeruginosa*) and enzymes (proteolytic enzymes) in the demineralization and deproteinization steps as well as in the deacetylation step. The demineralization can occur by fermentation (production of lactic acid, which then reacts with calcium carbonate of chitin), being a more complex method (depends on glucose and carbon concentration, pH, amount of inoculation, temperature, and other parameters) or can go through an enzymatic step, using, for instance, an alcalase enzyme. With respect to deproteinization, enzymatic extraction is frequently used. Various attempts using biological methods have been applied to insects (section 8.3).

Lin et al. [116], extracted chitin by a one-step approach, based on microbial fermentation, where they used *Bacillus lichenformis* bacteria to achieve the deproteinization of *H. illucens* pupa shells powder. After 10 days of fermentation, they obtained a chitin recovery rate of 12.4%, which is a higher yield than chemical treatment with the same source (5.7-9%) [107, 117]. In another study, enzymatic hydrolysis (alcalase) was applied for the deproteinization step (only step done, after pre-treatment) using cuticles of *Tenebrio molitor* with the authors achieved a high yield of chitin (70.9% DW) . It was stated that the enzyme used was sufficient to remove proteins from the matrix, although the deproteinization was not complete. Moreover, the chitosan obtained by chemical treatment (NaOH) had a low DD (53.9%) [111]. Wu [118] tried to remove proteins from the Soyabean hawkmoth (*Clanis bilineata*) by enzymatic hydrolysis, using flavourzyme in order to produce chitosan after a posterior deacetylation step. The author analysed the effect of pH and temperature on the enzymatic hydrolysis and reported that at a pH of 6.5 and temperature of 50ºC the minimum content of insect protein was achieved.

Although the biological methods are able to produce chitin with high quality and homogeneity, the extraction by these methods is time-consuming, generally leads to lower yields, is more expensive, can suffer microbial contaminations and is still limited to laboratory scale [71, 81, 119].

1.3.4.3.2 Microwave, ultrasound and subcritical water extraction

Non-thermal and innovative technologies such as ultrasounds and microwaves are widely applied in the food and chemical industries for different applications [120, 121]. These technologies have also been applied on the extraction of chitin and chitosan production in crustacean and other sources. According to the previously mentioned systematic review, these approaches have not yet been applied on the extraction of chitin and chitosan production from insects. Regarding microwave technology, it is reported that this heating method improves reactions yields and accelerates chemical reactions, while enhancing products properties in comparison to conventional heating method. Moreover, microwave-heated processes can be more energy-efficient than conventional methods when applied on large scales [122, 123]. Concerning chitin extraction and chitosan production by microwave heating methods, it has been applied on crustacean shells, fungal biomass and even spider cuticles [124-126]. Microwave-assisted methods require less time and reduce the heating temperature compared to conventional heating methods. Furthermore, chitosan produced by microwave-assisted extractions achieved higher DD in less time than the chitosan produced by conventional heating methods, demonstrating that this technology improves the deacetylation reactions [127]. Further research in the optimization of the reaction conditions is needed to achieve a product with the desired characteristics, and good reaction rates while decreasing the production costs.

Considering the ultrasound technology, as mentioned before, this technology is widely applied in the food industry for food processing, preservation, and extraction, demonstrating effectiveness in the inactivation and killing of microorganisms [80]. Ultrasound-assisted methods as well as microwave-assisted methods have been applied in the extraction of chitin and chitosan production from crustacean shells and fungus, but also in squid pens [128-130]. For chitin extraction the employment of sonication improves the efficiency of polymer extraction in the deproteinization step, requiring less temperature and time of extraction than traditional heating methods [129]. Additionally, ultrasound assistance can increase the production yield of chitosan if applied at regular intervals, with longer deacetylation reaction times (60min) resulting in higher yields [128]. However, it was reported that longer reaction times, results in low DD and elevated degradation of β-chitosan [131].

Another studied method is the subcritical water extraction. This method utilizes water at temperatures between 100 ºC to 374 ºC under controlled pressures, sufficient to

maintain the water in the liquid state. Subcritical water extractions allow the replacement of acids or bases by a more eco-friendly solvent like water [132]. From chemical to the food industry this method has a wide range of applications such as, in coffee extraction, flavours and fragrances, the remotion of metals and organic compounds from polymers along with others. Among other applications like modifications of chitin morphology or production of oligosaccharides, the subcritical water extraction can be applied to chitin extraction in order to remove the proteins or the mineral content from the biomass and also chitosan production [133]. Although a few studies regarding these applications exist, this method was tested in some species including crustaceans (Shrimp and crabs), fungus and research with the exuviae from *H. illucens* was also reported [132, 133]. Concerning shrimp waste as a chitin source, Cortés, et al. [134] reported a 96% deproteinization efficiency and stated that the subcritical water treatment increased the crystalline domain in the α-chitin fibers. Bhavsar [133] extracted proteins from Black soldier fly exuviae using superheated water hydrolysis. The insects were pre-treated (dried, ground, and defatted) before the chitin extraction that undergone two steps. First the authors demineralized the sample with HCl and afterward the demineralized sample was subjected to a water treatment at 150 ºC in built laboratory-scale reactor for different times. The authors concluded that with the increase of reaction time, chitin became purer, and the spectrum acquired with FTIR (Fourier-transform infrared spectroscopy) technique showed great similarities with commercial one. These results make this method relevant for future studies as a greener alternative for chitin extraction.

1.3.4.3.3 Ionic liquids and Deep Eutectic Solvents

Aiming to decrease the use and manufacture of hazardous compounds in chemical processes, replacing traditional solvents with renewable sources, as well as seeking for energy consumption reduction, are the main objectives of green chemistry. In this way the improvements of production processes help diminishing long and short-term ecological impacts on Earth [135]. With this in mind, a few years ago the green chemistry came with a new type of solvents called Ionic liquids (Ils). Although the first time an Il was synthetized was in 1914, only later in 80´s scientists started to give them real attention, due to the possible electrochemical applications. Since then, Ils have been extensively studied. ILs are salts composed of cations and anions that are normally liquids at temperatures below 100 ºC, having low melting points due to the differences between the ions size, which cause weak bonds between them [136]. These liquids can be

prepared with cations of imidazole and pyridine and anions like Br^- and Cl^- for instance [137]. In addition to the low melting points, Ils have featural advantages like low vapor pressure, chemical and thermal stability, non-flammability high dissolution and tailorable ability [138]. According to their chemical properties and preparation, ILs have 3 different generations. The applications of this liquids can vary from the use in organic synthesis, catalysis of reactions, electrochemical applications, liquid-liquid extractions, biomass processing and so on [137]. Due to their non-volatility, these liquid salts were appealing for replacing traditional volatile organic compounds, therefore avoiding air pollution and climate warming produced by volatile industrial solvents, being in the earlier stages considered as alternative green solvents [139]. However, Ils were considered as a satisfactory alternative to conventional solvents used in the industry due to their properties and more environmentally friend features, they have a high cost in comparison to conventional solvents. Moreover, Ils have a weak biodegradability and dangerous toxicity, limiting their use for industrial purposes [138, 140].

In 2003, Abbott, et al. [141] introduced a new era of solvents, the deep eutectic solvents (DES) which share similar physical characteristics with the Ils differing in the compounds used and formation. Since this first report, different systems of DES have been considerable reported with the mixture of choline chloride (ChCl) – Urea (U), being the most studied [142]. Deep eutectic solvents are a mixture of 2 or more compounds, which consists of a hydrogen bond acceptor (HBA) (quaternary ammonium or phosphonium salt) and uncharged hydrogen bond donor (HBD) at a proper molar ratio that forms a eutectic mixture (homogenous combination of substances that melts or solidifies at a single temperature lower than the melting point of any of the ingredients isolated). The mixture of these compounds (ChCl-U, ChCl – Lactic acid, ChCl-Malonic acid, Betaine – U, etc.) can be prepared by heating, grinding and freeze-drying methods until a homogeneous liquid is obtained [143, 144]. If DES systems are constituted from materials of natural sources or that occur in nature, namely amino acids, organic acids, sugars, or choline derivatives, these systems are called natural deep eutectic solvents (NADES) [145, 146]. According to their composition, DES can be distributed into four different groups: Type1 (combination of organic salts and metal salts), Type 2 (combination of organic salts and metal hydrates), Type 3 (combination of organic salts and compound being hydrogen bond donors) and Type 4 (combination of metal chlorides and compounds being hydrogen bond donors) [147]. DES have some advantages in comparison to ILs such as lower toxicity, higher biodegradability, recyclability, tremendously low vapor pressure and chemical inertness with water, which facilitate the storage. In terms of preparation, they are easy to prepare, cheaper (abundance of the compounds), have high flexibility in the choice of the particular

components and achieve higher quality than Ils, that undergo multiple synthesis stages [135, 144, 145, 148]. As well as ILs, DES can be applied on a wide range of areas such as chemical and biochemical reactions, electrochemistry, polymer science and nano-chemistry [144, 149-151]. Regarding polymer science, these green solvents were recently studied in the extraction of polymers like chitin, and chitosan production amongst other related processes (e.g., chitin nanocrystal production). Studies concerning chitin extraction of crustaceans by DES are the most reported, with only one paper related to insect chitin extraction being reported. Zhu, et al. [152] assessed different DES systems, involving ChCl with different HBD, namely Glycerol (G), urea (U), thiourea and malonic acid (MA), for chitin extraction from lobster shells. The authors reported that the only mixture that showed effectiveness on the elimination of proteins and minerals, achieving a high purity chitin was the system formed by ChCl and malonic acid (molar ratio of 1:2). They also stated that the extraction by this mixture had a higher yield (20.63%) than the chitin treated with the conventional chemical treatment (16.53%). These achievements demonstrated the potential use of DES as a good option to substitute the conventional treatment in chitin commercial production. Studies concerning the extraction of chitin and chitosan production by DES were also conducted in other crustacean sources such as shrimps, where different kinds of DES and their reusability, as well as microwave assistance methods using DES as solvents, were investigated [153-155]. In 2019, Zhou, et al. [156] reported a one-step approach for the extraction of chitin from the insect *H. illucens*, using 10 different DES mixtures based on ChCl and betaine (BE). In this study the authors pertaining to understand the influence of the acidity and alkalinity of NADESs as well as their efficiency in the deproteinization and demineralization of chitin from raw material. They conclude that the mixtures of ChCl-lactic acid and BE-U resulted in the most appropriate conditions for chitin extraction among the studied ones. The authors also stated that the pH of the HBA and HBD influence the yield of the chitin extracted, which ranged between 12.01% and 26.71%.

More research is needed to investigate greener, lower-carbon footprint techniques for the extraction of chitin and chitosan from insects and other sources.

1.3.5 Characterization

1.3.5.1 Physicochemical characterization

Chitin and chitosan physicochemical characterization includes the calculation of the extraction yields, the determination of chitosan solubility, water and fat binding capacity,

the quantification of ash and moisture and the determination of the Mw of chitosan as well as the DD.

The yield of chitin extraction is obviously dependent on the chitin content of the extracted sources, which in turn is dependent on species, life stage, sex, body parts, rearing conditions and the conditions applied in the extraction method. According to the previously mentioned systematic review, the chitin yield of insects mostly varied between 5 and 20% (section 8.3).

Considering the conventional chemical treatment, the highest yield of chitin extracted from insects was reported in a study using the species *Coridius nepalensis* (adult) where the authors achieved a chitin yield of 43.97% DW (dry weight basis) (section 8.3) [157]. High extraction yields were also reported with cicada sloughs (translucent and luster shell, which can be obtained after the eclosion of the larvae of periodical cicadas), with yields of 42.6% [158] and 36.36% [98]. These results demonstrate that the waste generated from insect rearing like the case of the sloughs have a great value for the extraction of chitin and chitosan. Moreover, these polymers have also been extracted from waste of yellow mealworm *T. molitor* [114], *H. illucens* [107, 159], house fly (*M. domestica*) [87], desert locust (*Schistocerca gregaria*) [160] and other insect species waste. In fact, Song, et al. [114] reported higher extraction yield for *T. molitor* exuviae (18.01%) and rearing by-products (17.32%) than for the larvae (4.92%). Furthermore, there are also differences in chitin content among different body parts. Several studies reported that wings from Central American giant cave cockroach *(Blaberus giganteus),* the American cockroach (*P. americana*) and Mediterranean Fritillary (*Argynnis pandora*) led to higher chitin yield than other body parts [161-163]. Lastly, in a comparative study realized by Kaya, et al. [164], the authors compared the extraction yields of different body parts of males and females' adults of White-grub cockchafer (*Melolontha* sp.) and the greater yield was achieved in male elytra (40.1%), while the lowest concerned the female antennae (5.87%).

Yu, et al. [165] examined chitin extraction on larvae (different instars), pupa and adult stages of *T. molitor*, using the conventional chemical treatment. They stated that the adult specimens have the highest yield (11.79% DW) of the 3 stages and the percentage of chitin raised with the development, being 7.2% DW in larvae (instar 3-4) up to the 11.79% DW in the adult stage. Similar results were obtained in the percentage of chitin extracted from the Japanese rhinoceros beetle (*Allomyrina dichotoma*) where the chitin yield ranged from 10.53% DW in larvae to 14.20% DW in adult stage, whereas the chitin percentage obtained from the intermediate stage (Pupa) was 12.70% DW [166]. Other studies also confirm these results and can be found in section 8.3.

Chitin is insoluble in different organic solvents but chitosan is soluble in some dilute acidic solutions such as, acetic acid solutions [167]. The solubility of chitosan depends mainly on the Mw, DD, and the species. In a study regarding four different insect species, the authors tested the solubility of the produced chitosan. They reported solubilities values ranging from 94.3% to 99.3%, these values are similar to the solubility values of the chitosan produced from shrimp (91.5%) [110].

The ash content can be determined for further analysis of the efficiency of the demineralization step. Moreover, a high-quality chitosan should have an ash level of less than 1% [90]. Some studies regarding different insect species measured the ash content of chitin and chitosan. Huet, et al. [168] determine the ash content of chitin extracted from *Bombyx eri*, *H. illucens* larvae and shrimp shells by thermogravimetric analysis. The authors reported ash contents ranging between 0.3% and 1% for the *Bombyx eri* larvae and 0.2% to 0.7% for *H. illucens* while the ash content on shrimp chitin varied from 0.03% and 0.5%. The variations on the values are linked to the treatments applied to chitin by Ils and DES. Jiang, et al. [169] described an ash content of 0.87% for the silkworm (*Bombyx mori*) pupa chitin. The ash content of the extracted polymers was also determined for whole insect powder of yellow mealworms, beetles, bees, crickets, and other species [98, 99, 112, 170].

The Mw and DD of chitosan are two of the most important parameters to be identified, once they influence the physicochemical, mechanical, and biological features of the polymers, limiting their application.

The Mw of chitin and chitosan from insects can be detected by viscometry methods, high performance liquid chromatography (HPLC) and gel permeation chromatography [90, 171, 172] .The average viscosity Mw of chitosan from *T. molitor* species ranged from 9 kDa (exuviae and dead adults) to 812 kDa (adults) [99, 101]. The chitosan Mw of the exoskeleton of field crickets (*G. bimaculatus*) presented 308.3 KDa [171] while the chitin Mw of different adult orthoptera species varied from 5.2 kDa to 6.8 kDa [78]. The honeybees (*Apis mellifera*) chitin Mw was 738.806 KDa (decreased after decolorization) and the Mw of chitosan was 257 KDa (decreased to 20 KDa after increases hydrolysis time) [173, 174]. However, there have been also studies that reported chitosan with very high molecular weight such as 1950 kDa (desert locust exoskeletons) and 5900 kDa (silkworm chrysalides) [113, 160].

The degrees of acetylation and deacetylation of chitin and chitosan can be determined by a panoply of methods which include FTIR, potentiometric, conductometric and acid-base titration methods, NMR (nuclear magnetic resonance) and EA (Elemental analysis)

[95, 115, 175]. The method of extraction and the conditions (time, temperature, and concentration) employed affect this degree. The DD of chitosan produced from *T. molitor* [111], *Catharsius molossus* [176], *Brachytrupes portentosus* [105], *Bombyx mori, Hermetia illucens* [167], *Musca domestica* [83], *Allomyrina dichotoma* [166] and *Schistocerca gregaria* [112] were 88.90%, 94.9%, 80.5%, 85%, 91.3% 90.3%, 74.66% and 98% respectively. These values can be higher than the DD of fish waste, shrimp and crab shells (75%, 78% and 70% respectively) [177].

1.3.5.2 Structural characterization

1.3.5.2.1 X-ray diffraction (XRD)

The crystalline nature of chitin and chitosan (amorphous and crystalline) can be detected by XRD. It is essential to determine the CrI (crystallinity index) values of chitin and chitosan since this index helps define the applications of both polymers [90]. Besides the determination of CrI values, this technic helps identifying of the structure of chitin and in the confirmation of the presence of chitosan [95]. Chitin has a characteristic defined XRD pattern regardless of source and depending on the polymorphic structure (α, β and γ) the signals vary slightly allowing the distinguishment of the different forms [62, 178]. The CrI values for chitin extracted from several different insect species ranged between 63% and 88% [78, 96, 103, 105]. Other studies concerning insect chitin reported similar CrI values and stated that chitin was in α-form, being structurally similar to commercial chitin. Major sharp and intense peaks at 9º and 19º were obtained while minor peaks, were also generally obtained between 12º and 26º [85, 86, 110, 163, 179]. CrI values of chitosan from four different insect species calculated based on the intensity of the crystalline and amorphous region ranged from 32.9% to 64.8% and similar CrI values (49.1%) were determined for shrimp shells [110]. The two sharp diffraction peaks at theta values obtained in this study (10º and 20º) agree with the characteristic sharp peaks of chitosan [51]. Murat, et al. [175] reported that the XRD analysis peaks of chitin from larvae and adult Colorado potato beetle were similar to those reported for shrimp, crayfish, and crab. Due to broad, less resolved peaks and lower CrI, Pal, et al. [115] stated that chitosan has a more amorphous nature than chitin, referring that the deacetylation of chitin reduced the crystallinity.

1.3.5.2.2 FTIR and RAMAN

Fourier Transformed Infrared spectroscopy is the standard and most documented technic for chitin identification which allows to obtain information about the functional groups (e.g., amide and amine) present in the samples while also being a non-destructive method. In addition, through an acquired FTIR spectra it´s possible to determine the DA and DD of chitin and chitosan [180]. FTIR usually requires preparation of the sample for further analysis (usually in KBr pellets) [180], while the attenuated reflection mode (FTIR-ATR) permits placing the samples directly on the crystal prism of the apparatus. Besides this feature, FTIR-ATR provides peaks with better resolution for samples with a small chitinous presence [51]. The presence or the absence of the amide I band on chitin FTIR spectra is an important factor to distinguish between the α-form and β-form. If the amide I band is separated into two bands at nearly 1650 cm^{-1} and 1620 cm^{-1} , chitin is on the α-form, otherwise, when the amide I band, has a single band at 1656 cm^{-1}, β-form chitin is present [62, 63]. Similar to chitin extracted from crustacean sources, chitin derived from different insect species such as grasshoppers and cicadas, *H. illucens*, *C. nepalensis, Z. morio* and *T. molitor* have characteristic bands (amide I band split into two bands at approximately 1650 cm^{-1} and 1620 cm^{-1}) of α-form [92, 97, 157, 181-185].

As well as FTIR, Raman spectroscopy can be used to identify chitin and its polymorphic structures, sometimes with different information given the kind of the excitation source used. The amide I band of α-chitin in the RAMAN spectrum, is similar to the FTIR spectrum, showing two separated bands at 1622 and 1658 cm^{-1}. For β-chitin, only one band appears at 1662 cm^{-1} in the amide I vibration, while in the γ-chitin spectrum appears a broad asymmetric band at 1618cm^{-1} and a weak signal at 1657 cm^{-1} [159]. Both these methods have been successfully applied for chitin identification in different organism sources, including insects, crustaceans, fungi, mollusks, spiders, and others [51].

1.3.5.2.3 Elemental analysis (EA)

The elemental analysis of insect chitin and chitosan is an important parameter of chitin purity and can be performed by elemental analysers or scanning electron microscopes with coupled x-ray diffraction spectroscopy (SEM-EDX). Generally, the content of carbon (C), nitrogen (N) and hydrogen (H) is studied. The C and N of different chitin from insect species like *H. illucens*, *D. marrocanus*, *B. portentosus*, *P. americana*, *H. parallela* and *V. velutina* were 40.30%/5.60%, 42.30%/5.64%, 41.30%/6.022%, 45.74/6.69%, 44.36%/6.45% and 43.47%/6.85% respectively [94, 102, 103, 105, 162, 186]. Equivalent values for chitin of crustacean sources were also reported in the

literature [187]. These results indicate that chitin extracted from insects presented high purity since N values can function as measure of protein and inorganic residues (N> 6.89% - protein residues and N<6.89% inorganic residues).

1.3.5.2.4 Scanning Electron Microscopy (SEM)

SEM allows the visualization of chitin and chitosan surface morphology and their physical state. The morphology varies according to the source of chitin and chitosan and generally have different types of morphologies, which are: nanofiber and nanopore, nanofibers, nanopores without nanofibers, nanofibers without nanopores, smooth surface, and rough surface or both. Chitin extracted from the Colorado potato beetle [175], different orthoptera species [78], European hornet [188] and different cricket species [100, 105] showed nanofiber and nanopores morphology. Moreover, a morphology consisting of nanofibers without nanopores was found on chitin extracted from flies [189], and different aquatic insects [190]. Chitin extracted from the Black soldier fly showed a fibrillar surface and a honeycomb-like structure in numerous investigations [102, 116, 133, 191]. The chitin from the superworm (*Z. morio*) revealed a smooth and rough surface [97]. Additionally, the type of morphology can also vary according to the life stage, the body parts and the gender as proven in the literature [164, 192, 193].

1.3.5.2.5 Nuclear magnetic resonance (NMR)

As the highly powerful tool for structural identification of organic molecules besides other applications such as Studies of 3D structures, dynamic processes and drug design, NMR detects the absorption of electromagnetic radiation by certain nuclei (e.g., 1H; 13C; 15N) on a molecule [194-196]. This non-invasive and non-destructive technic is used on the structural elucidation of chitin and chitosan, the determination of their DA and DD and consequently allowing to determine their purity [197] . On the characterization of insect's chitin and chitosan, the most used NMR type is the ^{13}C CP/MAS NMR, followed by the ^{1}H NMR. The ^{13}C CP/MAS NMR can be useful to identify the carbon chemical shifts assignments of both polymers from insects' sources. The ^{13}C CP/MAS NMR spectrum of chitin extracted from the chrysalis's silkworm showed defined peaks C1-C7 (CH_3 peak (23ppm)) ranging from 23-105 ppm and a peak corresponding to C=O (174 ppm), with the C3 and C5 appearing as a doublet centred at 75ppm. The chitosan spectrum only showed peaks from C1-C6, without the CH_3 and C=O peaks, confirming

the deacetylation of chitin [198]. Similar results were reported for chitin extracted from cicada sloughts, Rice-field crab shells [98], elytra of *O. nasicornis* [199], blowfly [200], Black soldier fly [117] as well as for mealworm and shrimp chitin and chitosan [185].

1.3.5.2.6 Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetric Analysis (DSC)

The thermal behaviour of chitin and chitosan can be analysed by TGA and DSC. The thermal stability of both polymers on insects is generally determined in 2 mass loss stages, where the first stage corresponds to the water evaporation and second stage is due to the degradation of the polymeric structure of chitin or chitosan units, a third step could also occur owing to the thermal destruction of pyranose ring and the decomposition of the residual carbon [201]. The range of mass loss in percentage for the first stage on insects is between 0.5% for a Chitin extracted from puparia of *Hermetia illucens* using an ADF-ADL method and 9.67% from chitin of the flake from the same species using the same method. The temperature for this first step ranged from 0-150 ºC [102]. Even though this mass loss range, insect chitin and chitosan extracted using the conventional chemical method have a first step mass loss, ranging from 1.9% to 8.52% at temperatures usually between 50 and 150 ºC [102, 164, 202]. Concerning the second mass loss for chitin the percentage is between 48% and 94.5% [164, 175]. The DTGmáx (Maximum degradation temperature) reported for different insect species varies from 307 ºC to 412.7 ºC [175, 184] ,while for chitosan fluctuates between 275.2 ºC and 372 ºC [202, 203]. These results demonstrated that chitin is more thermally stable than chitosan, having a molecular disintegration at higher temperatures than chitosan. Moreover, Jang, et al. [62] reported that α-chitin have higher thermal stability than β-chitin and γ-chitin.

1.3.5.3 Biological activity

1.3.5.3.1 Antioxidant activity

Cardiovascular diseases, cancer and degenerative diseases are highly influenced by the oxidative stress produced by oxygen radicals that also affect the normal aging process [204, 205]. Furthermore, lipid oxidation in food products produce undesirable chemicals that lead to rancidification and toxic end products that generate health problems [206]. To face these issues, dietary antioxidants are consumed, and synthetic

antioxidants are introduced in numerous food products to prevent or retard the deterioration caused by the lipid oxidation. Although these antioxidants are effective, some of them like the BHT (butylated hydroxytoluene) are dangerous to human health, with their use being restricted in some countries [207]. Consequently, the search for new natural alternative antioxidants became relevant and the study of the antioxidant activity of chitosan and its derivatives arose. The antioxidant activity of chitosan extracted from different insect species has been reported in several studies and the DPPH method was the most used [83, 84, 97, 175, 200, 208-210]. Chitosan extracted from the larvae of the housefly showed stronger scavenging activity than ascorbic acid on DPPH and great ferrous ions chelating ability [83, 208]. Concerning chitosan extracted from the Colorado potato beetle, Kaya, et al. [175], reported a higher scavenging radical activity on DPPH, for chitosan extracted from adult than that of larvae. Both chitosan samples showed moderate antioxidant activity. Soon, et al. [97] demonstrated that the antioxidant activity of chitosan increased with higher DD. The DD, Mw and the concentration have an effect on the antioxidant activity of chitosan [211, 212]. In addition, Fadly, et al. [213] acknowledged that carboxymethyl chitosan has better antioxidant activity than chitosan.

1.3.5.3.2 Antimicrobial activity

One of the key features of chitosan and its derivatives is the efficient antimicrobial activity against a panoply of microorganisms such as bacteria, fungi and yeast [214]. Although the precise mechanism that causes this activity is not entirely elucidated some hypotheses have been proposed. The most reasonable hypothesis relies on the interaction of polycationic chitosan with anionic groups on microbial cell membranes, changing the cell permeability and inducing intracellular components (e.g., proteinaceous material) leakage [215]. Another hypothesized mechanism is the interaction of low molecular weight chitosan with microbial DNA, which results in the inhibition of messenger RNA (mRNA), thus interfering in protein synthesis [216]. Moreover, chitosan ability to chelate metals ions and essential nutrients (vital to cell stability) as its pH value is higher than its pK_a, can also explain the inhibitory effect of chitosan on microbial growth [217]. Lastly, chitosan with a high molecular weight inhibits the development of aerobic microorganisms. It creates a thick layer on the cell surface, preventing nutrients and oxygen intake [76]. As it happens with other features the antimicrobial activity of chitosan is dependent on several factors such as, DD (the higher DD, the greater positive charge of the chitosan), Mw, the pH (amino groups of chitosan become ionized at acidic pH) of the medium, temperature and extraction source [218].

Recent discoveries on the antibacterial, antifungal, and antiviral activity of insect chitosan have been described (section 8.3). Al-Saggaf [167], reported antimicrobial activity of chitosan extracted from black soldier fly against two skin microbial pathogens (*Staphylococcus aureus* and *Candida albicans*). Chitosan extracted from the pupa and egg shells of silkworm showed similar antibacterial and antifungal activity as a commercial chitosan [96]. Moreover, the silkworm pupa chitosan showed better activity than the egg shells chitosan and all the chitosan samples revealed improve antimicrobial activity, than the standard antimicrobial agents (amoxicillin and clotrimazole) used as control (except for *Candida albicans*) [96]. The antimicrobial properties of chitosan extracted from two Orthoptera species (*Calliptamus barbarus* and *Oedaleus decorus*) were assessed against fish, clinical and food-borne pathogens. Specifically, 4 Gram-positive bacteria (*L. garvieae, S. agalactiae*, *L. monocytogenes* ATCC 7644 and *B. subtilis* RSKK 2443) 3 Gram-negative bacteria (*V. alginolyticus*, *S. enteritidis* RSKK 171 and *Y. enterocolitica* NCTC 11175) and 1 yeast (*C. albicans* ATCC 10231). The findings from this study revealed stronger antibacterial effectiveness of the extracted chitosan on Gram-negative bacteria than on Gram-positive bacteria. Additionally, the extracted chitosan had higher antimicrobial activity than the antibiotics (Amikacin, Ampicillin, Gentamicin, Erythromycin and Kanamycin) used against some of the pathogens in study. Furthermore, the insect chitosan showed weaker antimicrobial activity against fungi than against bacteria once the MBC (Minimum bactericidal concentration) values were higher and the inhibitions zones obtained were the shortest [209]. Other studies with different insect sources also confirm the previous mentioned results, proving that insect chitosan and chitooligosaccharides have antibacterial and antifungal activity [95, 116, 166, 175, 219].

Ai, et al. [208] examined the antiviral properties of chitosan extracted from the housefly, against the *Bombyx mori* nuclear polyhidrosis virus (BmNPV) (primary silkworm pathogen) on infected larvae. The results showed a decrease in larval mortality rate, proving the efficacy of chitosan in inhibiting the infection by BmNPV, therefore showing antiviral bioactivity. Moreover, chitosan derivatives and nanoparticles are also potential agents against some type of virus, including virus from the coronavirus family such as SARS-CoV-2 [76].

1.3.6 Applications

Chitin, chitosan and its derivatives have unique biochemical characteristics such as, biocompatibility, biodegradability, bioactivity, non-toxicity and well definite chemical structure, which make these polymers attractive for a wide range of industrial and medical applications. These applications are included in sectors such as food, agriculture, biomedicine, biotechnology, pharmaceuticals, and cosmetics [220] (listed below on Table 1).

Table 1 – List of potential applications of chitin, chitosan and its derivatives.

Sector	Application	Reference
Food	Food preservation	Hafsa, et al. [221]
	Edible films and coating	Dutta, et al. [222]
	Food additives	Liu, et al. [223]
	Filtration and clarification of juice	Tastan and Baysal [224]
Agriculture	Fertilizer	Yunsheng; Nge, et al [225, 226]
	Biopesticide	Kashyap, et al. [227]
	Plant seed coating	Kashyap, et al. [227]
Environment	Wastewater treatment – Flocculation and metals, dyes, and organic compounds adsorption	Simionato, et al.; Zhuang, et al.; Nechita [228-230]
Biotechnology	Enzyme immobilization	Krajewska [231]
	Biosensors	Karrat, et al. [232]
Biomedical and pharmaceutical	Wound healing	Matica, et al.; Aramwit, et al.; [217, 233]
	Tissue engineering	Yang; Sundararajan and Howard [234, 235]

Sector	Application	Reference
Biomedical and pharmaceutical	Drug carrier and gene delivery	Solairaj, et al.; Chen, et al.[236, 237]
	Cancer diagnosis and treatment	Manjusha Elizabeth, et al. [238]
Cosmetic	Skin care – Moisturizing and anti-aging agents	Morganti, et al. [239]
	Hair and oral care	Kardas, et al. [220]
Textile	Fiber production and treatments	Jagadish, et al. [240]
Pulp and paper	Papermaking	Song, et al. [241]

2. Objective

Chitin and chitosan are two well-known natural biopolymers, that have a wide range of great physiochemical and biological properties, which make them extremely versatile and capable of being applied on sectors such as the agricultural, biomedical, pharmaceutical and food. Due to this usefulness, chitosan is a target of a continuous research for new applications, better characterization, and optimization of its production. Currently the main source of chitin extraction and chitosan production are crustacean and its by-products, which present some disadvantages like the seasonality. Moreover, the method of extraction requires a high quantity of harsh chemicals that generate hazard effluents. In addition, the chitosan market is in constant expansion, and it is estimated that by 2027 the market will reach 4.7 billion USD. Concerning this information and the fact that entomophagy (insect consumption practice) acceptability and practice is rising, leading to a large spectrum of scientific studies with insects, this work aims to study different edible insect as an alternative source of chitin and chitosan extraction. Two different edible insect species (*T. molitor* larvae and adult *A. domesticus*) and the by-products (legs and wings) of *A. domesticus* rearing will be studied for the polymers' extraction. The extracted samples will be physiochemical and biologically characterized. Additionally, in an attempt to search for an alternative greener extraction method, an extraction method based on natural deep eutectic solvents (NADESs) will be studied.

3. Materials and Methods

3.1 Material

Absolute ethanol (99.5% vol), sodium hydroxide (pure), potassium permanganate (purity = 99.5%), urea (purity = 100%) and citric acid (purity = 100%) were acquired from José Manuel Gomes dos Santos, Lda. Hydrochloric acid (HCl 37%) was purchased from Chem-Lab. Choline chloride (purity > 98%), and malonic acid (purity > 99%) were purchased from TCI, and oxalic acid 2-hydrate from PanReac AppliChem ITW Reagents. Commercial chitin and chitosan powders were purchased from Sigma-Aldrich® as well as β-carotene, and butylated hydroxytoluene (BHT). Acetic acid (purity ≥99.8%) was acquired from Honeywell Fluka™. The antimicrobial studies were carried out against six bacteria, namely *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Salmonella Typhimurium* ATCC 13311, *Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 29213, *Listeria monocytogenes* LMG 16779 and one yeast (*Candida albicans* ATCC 90028). The remaining materials used, namely the instruments, are described throughout the text.

3.2 Insects' collection and pre-treatment

3.2.1 Insect species

Two different insect species and by-products from the rearing of one of the species were used in this study. Male and female adult specimens of the house cricket *Acheta domesticus* (AD) were provided frozen by Gotanbugs (insect rearing company in Santarem, Portugal). House cricket rearing by-products, namely the legs and wings (L&W) were provided dried by the same company. Larvae from the yellow mealworm *Tenebrio molitor* (TM) were provided frozen by GreenMeal - Insect Based Foods company (Matosinhos, Portugal). The crickets and larvae were submitted to a starving process of 48h to eliminate the gut content before being frozen and delivered.

3.2.2 Washing, drying and grinding

Both species were passed through water to remove residual impurities. After washing, the insects were dried on air-oven UNOX® for 7 h at 80 ºC, while the by-products were dried only for 1 h at 80 ºC. Afterwards, the samples were ground in a knife mill to obtain a powder, which was subsequently used in all experiments. Dry matter of the samples

was determined gravimetrically after drying in a stove (BINDER APT.line™) at 100 ºC for 24 h (all the samples had dry matter content above 97%).

3.2.3 Defatting

Samples were submitted to a defatting process using the Soxhlet method, following the methodology applied by Ribeiro, et al. [45] with slight modifications. Briefly, for each extraction, 20.0 ± 0.1 g of each sample was weighted to a cellulose thimble and put into the extractor. After the assembly of the extractor and round-bottom flask, 700 mL of absolute ethanol was added. The condenser was assembled to the extractor, and the extraction occurred for 6 h under constant reflux of cold water. After the extraction, the thimble containing the defatted samples was dried overnight at 100 ºC and weighed in order to calculate the amount of recovered sample (determined as a mean of four replications). The solvent used for the fat extraction was recovered with a rotary evaporator (Laborota 4000, Heidolph®). The conditions applied for recovery were bath temperature of 85 ºC, vacuum pressure of 600 mbar and a rotation of 270 rpm. The fat content on each sample was determined gravimetrically as a mean of four replications.

3.3 Chitin extraction

3.3.1 Conventional chemical treatment

Only non-defatted AD samples were used in the optimization of the demineralization and deproteinization steps in the conventional chemical treatment.

3.3.1.1 Demineralization step - Optimization

For the removal of minerals and catechol a demineralization step using an acid solvent was employed. The optimization of this step was performed based on the weight loss of the samples, which could be attributed to the removal of the previously mentioned constituents. Succinctly, 5 g of *A. domesticus* defatted powder were treated with HCl (1:20, w:v) at different concentrations and under constant stirring in a hot plate stirrer (VELP SCIENTIFICA®) at different time and temperature conditions. At the end of each reaction, the samples were filtrated by vacuum filtration through a 60-68 µm pore size paper filter and washed with distilled water until neutrality. The samples were dried on

a stove for 4 h at 100 ºC and weight for further calculations (equation 1). The conditions were evaluated on duplicated and are described in Table 2 .

$$Weight\ loss\ (\%) = \frac{Sf*100}{Si} \qquad \text{(Equation 1)}$$

Si – dry weight of the initial sample (g)

Sf – dry weight of the demineralized sample (g)

Table 2 - Demineralization step - optimization conditions tested (time, temperature, and HCl concentration)

Time (h)	Temperature (°C)	HCl concentration (mol/L)
3	Room temp.	2
3	50°C	2
3	Room temp.	1
3	50°C	1
1	50°C	1
1	100°C	1

The optimal conditions were chosen based on weight loss as well as time-cost effectiveness.

3.3.1.2 Deproteinization step – Optimization

For protein elimination, the demineralized samples were treated with a basic solution of NaOH. Optimization of this step was also performed to achieve higher weight loss which is mainly attributed to the removal of proteins, but also residual pigment and fats. The demineralized samples were treated with a 1 M NaOH solution (1:20, w:v) under constant stirring in a hot plate stirrer, at a fixed temperature of 95 ºC and different times of extraction (3 h and 5.50 h). After each reaction, the samples were filtrated by vacuum filtration through a 60-68 µm pore size paper filter and washed with distilled water until neutrality. Next, the samples were dried on a stove for 4 h at 100 ºC. The dried samples were weighed, and the percentage of weight loss was calculated Equation 2.

$$Weight\ loss\ (\%) = \frac{S2*100}{S1} \quad \text{(Equation 2)}$$

S1 – dry weight of the demineralized sample (g)

S2 – dry weight of the deproteinized sample (g)

After the conditions were optimized, a sequential extraction of demineralization followed by deproteinization was performed with non-defatted and defatted samples to assess the impact of defatting on chitin yield.

Considering the conditions that provided the better results, chitin extraction was applied to all sources (AD, L&W and TM) (Fig. 6). For each extraction, unbleached chitin yield (mean of 3 replicates) was calculated according to Equation 3:

$$Unbleached\ chitin\ yield\ (\%) = \frac{Q*100}{US} \quad \text{(Equation 3)}$$

US – dry weight of the sample before demineralization and deproteinization (g)

Q – dry weight of the extracted unbleached chitin sample (g)

3.3.1.3 Decolorization

To remove the brown color from the obtained chitin a decolorization step based on an oxidation-reduction reaction was performed according to the method described by Draczynski [173] with slight modifications. Briefly, 4 g of unbleached chitin were treated (under constant agitation at 200 rpm) with an aqueous solution of 0.5% $KMnO_4$ (oxidizing medium) for 1 hour at room temperature (*ca.* 26ºC) followed by a treatment with a 0.5% $C_2H_2O_4$ (reducing agent) solution for 1 hour at room temperature. The obtained samples were filtrated by vacuum filtration through a 60-68 µm pore size paper filter and washed with distilled water. Afterwards, the samples were dried at 100 ºC for 4 h on a stove and weighed to proceed with the yield calculations (mean of duplicates):

$$Chitin\ yield\ (\%) = \frac{DQ*100}{US} \quad \text{(Equation 4)}$$

DQ – dry weight of decolorized chitin

US – dry weight of the sample before demineralization and deproteinization (g)

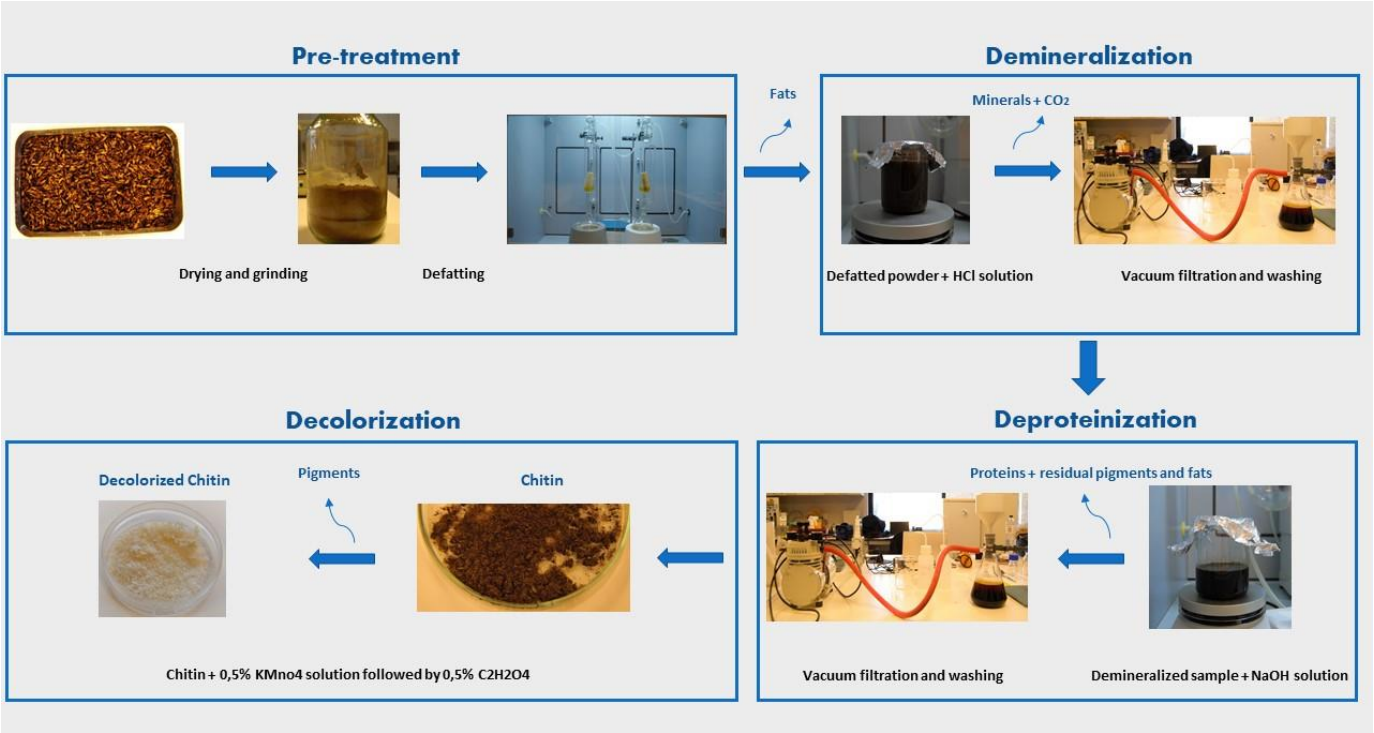

Fig. 6 – Schematic representation of the of chitin extraction process by conventional chemical method

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3.3.2 Alternative treatment – Natural Deep eutectic solvents (NADESs)

As an alternative to the conventional chemical treatment, a green chemistry approach using NADESs was applied for the extraction of chitin from the defatted L&W sample. For this experiment, 4 different DES systems were initially synthetized: choline chloride – Urea (ChCl-U), choline chloride – oxalic acid (ChCl-OA), choline chloride – citric acid (ChCl-CA) and choline chloride – malonic acid (ChCl-MA). The methodologies tested for the solvent's synthesis, chitin extraction and solvents regeneration were based on two previously described studies [152, 156].

3.3.2.1 NADESs synthesis

At first, the HBA (choline chloride (ChCl)) was mixed individually with four different HBD (urea (U), citric acid (CA), malonic acid (MA), and oxalic acid (OA)) (Fig. 8) at a molar ratio of 1:2 respectively. The components were weighed, mixed in a glass bottle, and left under magnetic stirring at 100 ºC for about 1.5 h on a heating plate until a clear

and homogeneous liquid was developed (Fig. 7). The prepared NADESs were cooled down at room temperature for further use in chitin extraction.

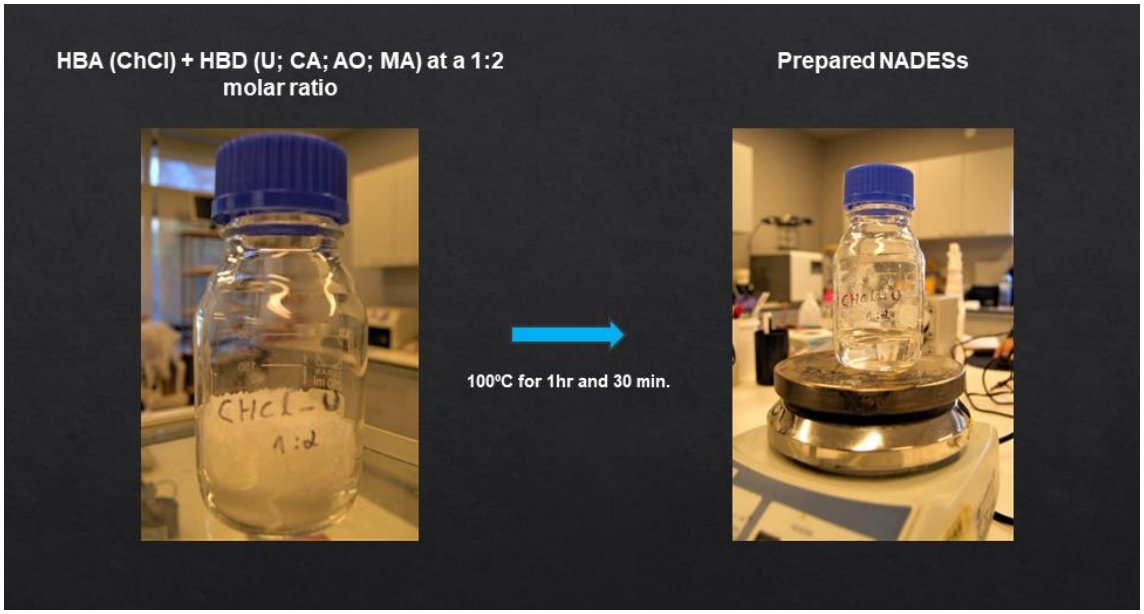


Fig. 7 – Example of NADESs preparation.

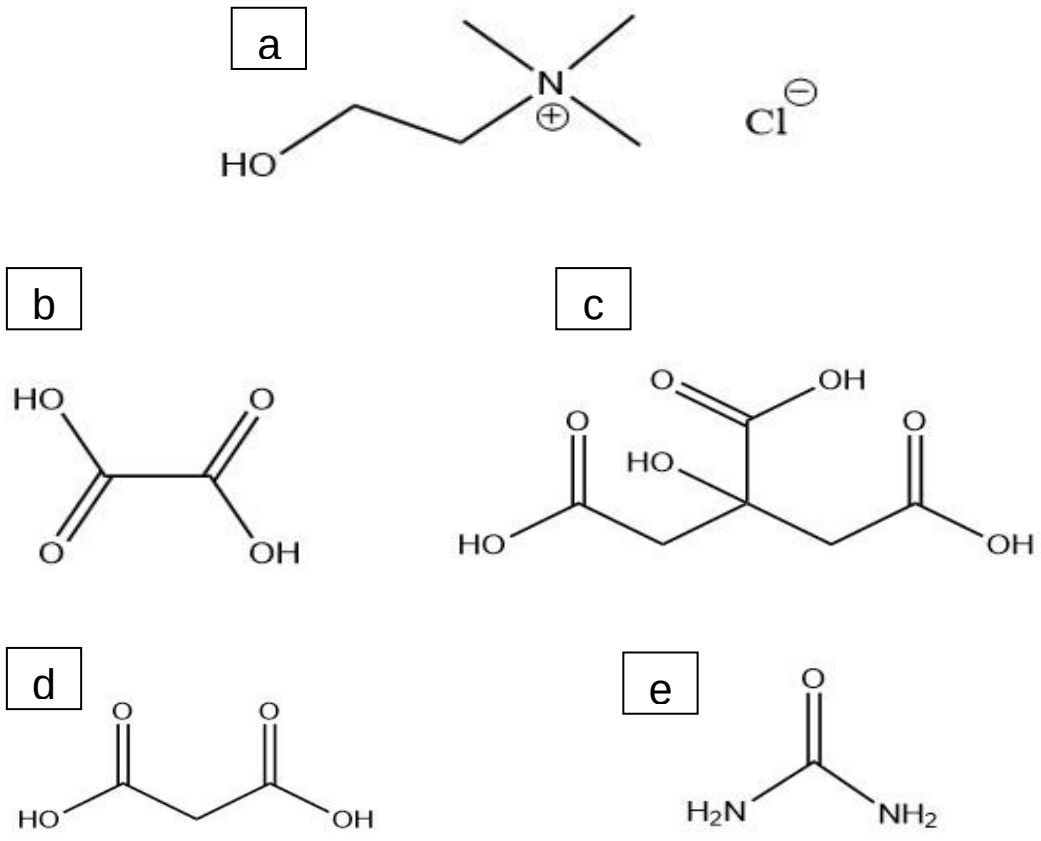


Fig. 8 - Chemical structure of the different NADESs components: HBA - a) choline chloride; HBD - b) oxalic acid; c) citric acid; d) malonic acid; e) Urea – Design in ChemDraw software

3.3.2.2 Chitin extraction: One-step approach

Two different experimental conditions were tested in an attempt to obtain chitin from L&W. The defatted L&W sample was added to the different NADESs obtained in section 3.3.2.1 in an Erlenmeyer flask at a ratio of 1:20 (w/w). One of the methodologies consisted of placing the different mixtures of NADESs and L&W into a water bath under constant agitation at different temperature (50 or 80 ºC) and time (3 or 5 h) conditions. The solid fraction obtained after the reaction was filtrated by vacuum filtration using a 60-68 µm pore size paper filter and the liquid fraction was collected and stored in the refrigerator for further regeneration (3.3.2.3). The solid fraction was washed with hot distilled water and placed on the stove at 100 ºC for 4 h. For the other approach, only the NADESs mixture ChCl-MA was used, considering the results from the first test. Succinctly, the mixture containing the NADESs, and the L&W was placed on a hot stirring plate under constant stirring at different temperature (50 or 80 ºC) and time (3 or 5 h) conditions. The filtration of the solid fraction and the recovery of the liquid fraction were conducted identically to the water bath approach. The dried samples were weighed, and the yield was calculated according to Equation 5:

$$Chitin\ yield\ (\%) = \frac{NDQ*100}{LW} \qquad \text{(Equation 5)}$$

LW – dry weight of L&W powder

NDQ – dry weight of sample obtained after NADESs treatment

3.3.2.3 NADESs regeneration

The liquid fractions collected in section 3.3.2.2 that were in the refrigerator to cool down, were mixed with absolute ethanol (twice the volume) and left in the refrigerator for 4 h. Afterwards, the mixtures (ethanol + used NADESs) were submitted to centrifugation at 9000 rpm for 15 min. The supernatant separated from the pellet was submitted to rotary evaporation (bath temperature of 85 ºC, vacuum pressure of 600 mbar and a rotation of 270 rpm) to recover the ethanol and regenerate the NADESs. The percentage of NADESs recovered was calculated according to Equation 6:

$$NADESs\ recovery\ (\%) = \frac{NRW*100}{NIW} \qquad \text{(Equation 6)}$$

NIW – dry weight of NADESs before chitin extraction

NRW – dry weight of NADESs recovered after chitin extraction and regeneration

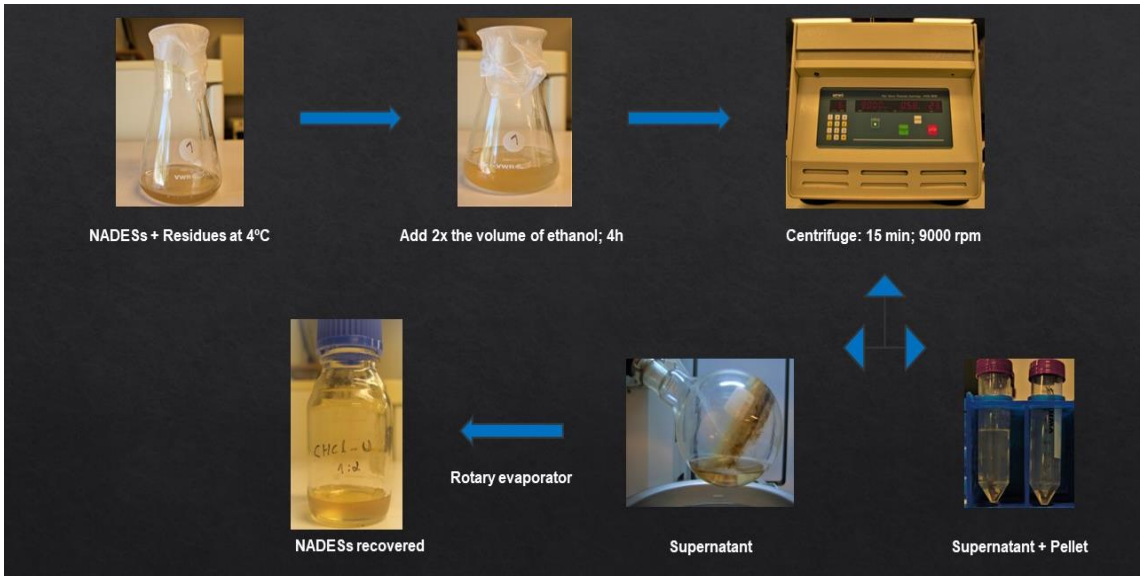


Fig. 9 - NADESs recovery process

3.4 Chitosan production

For the removal of the acetyl groups of the chitin samples, an alkaline deacetylation procedure was performed according to a previously described methodology, Song, et al. [114] with minor modifications. Five grams of the decolorized chitin samples (3.3.1.3) were homogenized with a 50% NaOH solution in a 1:50 ratio (w:v), and the reaction was carried out at 100 ºC for 4 h under constant stirring. The solid fractions were filtrated by vacuum filtration using a 60-68 µm pore size paper filter and washed with distilled water until neutrality. The samples were dried on a stove at 100 ºC for 4 h and collected for further weight analysis to calculate the reaction yield.

The yield of chitosan was calculated based on the weight of the decolorized chitin before the deacetylation reaction according to Equation 7:

$$Chitosan\ yield\ (\%) = \frac{Qs*100}{Q} \quad \text{(Equation 7)}$$

Q – dry weight of chitin

QS – dry weight of chitosan

3.5 Characterization of Chitin and Chitosan

The following characterization assays were only performed with the samples obtained by the conventional chemical treatment (section 3.3.1 and 3.4), and the commercial samples from shrimp.

3.5.1 Physicochemical properties

3.5.1.1 Color and Whiteness Index (WI)

The color parameters of the different chitin and chitosan samples were assessed using a colorimeter (Konica Minolta - Chroma Meter CR-400). The values of L* (lightness), a* (red-green) and b* (yellow-blue) were evaluated with 6 replicates. The whiteness index of the different samples was calculated consistent with Feás, et al. [186] according to Equation 8:

$$WI = 100 - [(100 - L^*)^2 + (a^*)^2 + (b^*)^2]^{1/2} \quad \text{(Equation 8)}$$

3.5.1.2 FTIR-ATR

Structural analysis was assessed with the acquisition of spectra by the Fourier transform infrared spectroscopic method with platinum Attenuated Total Reflectance (FTIR-ATR) with a Bruker spectrometer (Alpha, Bruker Optic GmbH, Ettlingen, Germany) using a diamond crystal. The samples were place directly on the crystal prism, and two spectra per sample were acquired with 64 scans per spectrum at a spectral resolution of $4cm^{-1}$ in the spectral region of 4000-400 cm^{-1}. The absorption peaks obtained on the spectra were

used to study the functional groups of the samples in order to confirm the presence or the absence of the characteristic IR bands of chitin and chitosan and possible changes in the molecular structure. Additionally, the FTIR-ATR spectra were used to estimate the degree of deacetylation (DD) of chitosan.

The DD of the different chitosan samples was calculated according to Equation 9: [242]

$$DD = 100 - \left[\frac{\left(\frac{A_{1320}}{A_{1420}}\right) - 0.03822}{0.03133}\right] \quad \text{(Equation 9)}$$

3.5.1.3 FT-RAMAN

Structural analysis was also carried out using an FT-RAMAN spectrometer (BRUKER, MultiRAM). The spectrometer was equipped with a 180° high-throughput collecting lens, an ultra-high sensitivity Liquid Nitrogen cooled Ge Diode detector, an integrated 1064 nm, diode pumped, Nd:YAG laser with a maximum output power of 5 mW. The spectra were acquired with 100 scans per spectrum at a spectral resolution of 4 cm^{-1}, with a scanner velocity of 5 kHz in 4000 to 0 cm^{-1} region, at a laser power of 453 mW. The samples were placed in a quartz cell of 5 mm of optic space with the opposite face mirrored and two measurements for each sample were performed. The spectra were taken at constant room temperature (22°C).

3.5.1.4 Molecular weight (Mw)

The average molecular weight was determined through a viscosimetric method according to Malm and Liceaga [243] with slight modifications. An Ubbelohde Dilution Viscometer (Xilem -SI analytics) with a capillary size of 0.53 mm was used in a constant-temperature water bath at 25 °C. Five different chitosan solutions ranging from 1.5 mg/ml to 5.5 mg/ml were prepared. The solvent system used for the dilution of the samples consisted of equal proportions of 0.1 M acetic acid (Hac) solution and 0.2 M NaCl solution. Firstly, the flow time of the solvent system solution (15 mL) was measured, followed by the flow time of the chitosan solutions (measured in duplicates). The measured flow times were used to calculate the relative viscosity (η_{rel}) ((Equation 10), specific viscosity (η_{sp}) (Equation 11) and reduced viscosity (η_{red}) (Equation 12)):

$$\eta_{rel} = \frac{\eta}{\eta_0} = \frac{KPt}{K_0 P_0 T_0} = \frac{t}{t_0} \quad \text{(Equation 10)}$$

t_0 – Flow time for solvent

t – Solution time

$$\eta_{Sp} = \frac{\eta}{\eta_0} - \frac{\eta_0}{\eta_0} = \eta_{rel} - 1 \quad \text{(Equation 11)}$$

$$\eta_{red} = \frac{t - t_0}{t_0 * C} = \frac{\eta_{Sp}}{C} \quad \text{(Equation 12)}$$

C – Concentration of chitosan solution

The intrinsic viscosity ([η]) which is the intercept of the reduced viscosity was determined by extrapolating the linear relationship between the reduced viscosity (η_{red}) and each chitosan solution concentration (C) when C=0 (Equation 13).

$$\lim_{C \to 0} \left(\frac{\eta_{Sp}}{C} \right) = [\eta] \quad \text{(Equation 13)}$$

Lastly, the viscosity average molecular weight (M_v) of the different chitosan samples was calculated using average intrinsic viscosity, by applying the Mark–Houwink Equation:

$$[\eta] = K M_V^{\alpha} \quad \text{(Equation 14)}$$

K and α are previous determined solvent system constants [250].

$K = 1.81 \times 10^{-3}$ and $\alpha = 0.93$

3.5.1.5 X-ray diffraction (XRD)

The XRD analysis was performed to study the crystal structures of the different samples. XRD analysis were performed using a powder X-ray Diffractometer (Rigaku DMAX III/C) equipped with a Cu-α X-ray source (λ = 1.5405 Å). The XRD peaks were measured at 40 Kv voltage, 40 mA current and 2θ with a scan angle from 5° to 50° at a scan rate of 1.2 min^{-1}.

The crystalline index values were calculated according to Equation 15 [209] :

$$CRI_{110} = [(I_{110} - I_{am})/I110] \times 100 \quad \text{(Equation 15)}$$

I_{110} - maximum intensity at 2θ ≅ 20°

I_{am} - intensity of amorphous diffraction at 2θ ≅ 16°

3.5.1.6 Scanning electron microscopy (SEM)

The surface morphologies of the different samples were examined using a Scanning electron microscope (Hitachi, FlexSEM 1000 II). The samples were fixed on a carbon tape and the surface morphology images were taken in different magnifications ranging from 140x to 10000x. The analysis was able to be carried out without coating the samples with golden.

3.5.1.7 Thermogravimetric analysis (TGA)

The thermal degradation properties of the different chitin and chitosan samples were determined by thermogravimetric analysis (TGA-DTA) using a TA instrument (Hitachi - STA7200RV). Four to six grams of each sample were placed on an aluminium (Al) heating pan and the analysis were conducted by heating the samples from 20 to 600 °C, at 10°C/min heating rate under nitrogen atmosphere (200ml/min).

3.5.2 Chitosan biological properties

3.5.2.1 Evaluation of antioxidant activity

3.5.2.1.1 DPPH scavenging analysis

The radical scavenging activity assay, using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was performed to study the antioxidant activity of the different samples obtained in section 3.4 and the commercial chitosan according to Scherer and Godoy [244] with some modifications. Briefly, the different chitosan samples were dissolved in acetic acid solution (0.1 M) at different concentrations. The prepared solutions (0.1 mL) were added to different DPPH solutions (3.9 mL) in methanol (0.2, 0.1242 e 0.08 mM), that were prepared by mixing 39.4, 24.5 and 15.8 mg in 500ml of ethanol, respectively [245]. The control sample was formed by a solution of 0.1 M of acetic acid and the different DPPH solutions. The samples were left in incubation for 90 min at room temperature in the dark and the absorbance was measured by a spectrophotometer (Thermo Electron Corporation Helios-Omega UV-Vis Spectrophotometer) at 517 nm against a blank consisting of methanol. The radical scavenging activity was calculated according to Equation 16:

$$I\% = \left(\frac{Ab_{s0} - Ab_{s1}}{Ab_{s0}}\right) \times 100 \qquad \text{(Equation 16)}$$

Ab_{s0} – Absorbance of the control

Abs_1 – Absorbance in the presence the test sample at different concentrations

The IC_{50} was achieved with the help of a graph using a calibration curve in the linear range, by plotting the samples concentration *vs*. the corresponding scavenging effect.

The antioxidant activity index (AAI) was calculated according to Equation 17:

$$AAI = \frac{final\ concentration\ of\ DPPH\ in\ the\ control\ sample}{IC_{50}} \qquad \text{(Equation 17)}$$

The AAI was calculated based on the mass of DPPH, and the tested sample mass in the reaction, in that way resulted in a constant for each sample, independent of the concentration of DPPH and the sample used. The assay was realized in duplicate. For characterizing the samples antioxidant activity, it considered the following scale: AAI < 0.5 (poor antioxidant activity), 0.5 – 1 (moderate antioxidant activity), 0.5 – 1 (strong antioxidant activity) and AAI > 2.0 (Very strong antioxidant activity).

3.5.2.1.2 β-Carotene bleaching test

In order to study the capacity of the different chitosan samples to inhibit the lipid peroxidation in the phase of initiation as well as in the phase of propagation, the β-Carotene bleaching test was performed according to Luís, et al. [245] with minor adjustments. Shortly, a β-carotene solution was prepared with a concentration of 80 mg/mL in chloroform. 20 µL of this solution were mixed with linoleic acid (40 µL), Tween 40 (400 mg) and 1 mL of chloroform. For the remotion of the chloroform from the mixture, it was performed evaporation, using a rotary vacuum evaporator (Büchi Rotavapor RE 111 + Büchi 461 Water Bath + Büchi Vacuum Controller V-850), for 5 min. Then, an emulsion was formed by adding water (100 mL oxygenated water) gradually and gently stirring. Next, 5 mL of this emulsion was transferred to test tubes with the samples (section 3.4) in acetic acid 0.1 M (300 µL) at 7 different concentrations. The control was prepared by adding 5 mL of the emulsion to 300 µl of acetic acid 0.1M and sample solutions. The reference consisted of solutions of Standard butylated hydroxytoluene (BHT) in methanol at the same concentrations as the samples. Afterwards, the tubes were shaken and left at 50 ºC in a water bath (Grant GLS Aqua 18 Plus) for 2 h. The analysis was performed in duplicate. The absorbance of the different samples was obtained using a spectrophotometer (Thermo Electron Corporation Helios-Omega UV-Vis Spectrophotometer) at 470 nm, against a blank consisting only of emulsion without β-carotene. The measurements were performed at an initial time (t = 0 h) and at a final time (t = 2 h). The antioxidant activity was calculated regarding the percentage of inhibition of β-carotenes oxidation according to Equation 18:

$$\% \, Inhibition \; = \; \frac{(Abs^{t=2}{}_{sample} - Abs^{t=2}{}_{control})}{(Abs^{t=0}{}_{control} - Abs^{t=2}{}_{control})} \qquad \text{(Equation 18)}$$

$Abs^{t=0}{}_{control}$ – Absorbance of the control at initial time

$Abs^{t=2}{}_{control}$ - Absorbance of the control at final time

$Abs^{t=2}{}_{sample}$ - Absorbance of the sample at final time

The IC_{50} was achieved with the help of a graph using a calibration curve in the linear range, by plotting the samples concentration *vs*. the corresponding percentage of lipid peroxidation inhibition.

3.5.2.2 Determination of the antimicrobial activity

3.5.2.2.1 Test microorganisms, culture media and sample preparation

The antimicrobial activity of the different chitosan samples was assessed against six different bacterial strains, in which three of them are Gram-positive bacteria (*Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 29213 and *Listeria monocytogenes* LMG 16779) and the other three are Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883 and *Salmonella Typhimurium* ATCC 13311). The antimicrobial activity was also tested against a yeast strain (*Candida albicans* ATCC 90028). Freezer stock cultures were prepared with a final glycerol concentration of 20% and stored at -80 ºC until required.

Previous to microorganisms use in the antimicrobial test, the different strains were subcultured on an appropriate agar plate for 24 h. When the strains were grown from stock, they were also subcultured before use. Brain Heart Infusion Agar (BHI) was used for the growth of bacterial species and Sabouraud Dextrose Agar (SDA) was used for yeasts. For the disc diffusion assay the chitosan samples were dissolved in pure dimethyl sulfoxide (DMSO) with help of a sonicator at a temperature between 40-50 ºC, while for the MIC determination, the samples were dissolved in a culture medium containing no more than 10% DMSO. The following methodology was performed according to Luís, et al. [245] with slight modifications.

3.5.2.2.2 Disc diffusion assay

Disc diffusion assay was done to assess the antimicrobial activity of the chitosan samples. The M2-A8 and M44-A2 methods were used for bacteria and yeast as described by Clinical Laboratory and Standards Institute (CLSI). The inoculum was prepared by suspending either the bacteria or the fungi in saline solution to a cell in suspension of 0.5 McFarland (about 1-2 × 10^8 colony-forming unit/mL (CFU/mL) to non-fastidious bacteria and 1 to 5 × 10^6 CFU/mL for yeasts). For the discs (6 mm diameter) preparation, each previous dissolved chitosan sample (20 µm) was inserted (4 mg/disc) at a concentration of 200 mg/mL and positioned on the inoculated agar. Negative controls were prepared only with the DMSO solution, as the solution used to prepare the chitosan extracts. Tetracycline (30 µg/disc) and amphotericin B (25 µg/disc) were used as positive control for the bacteria and yeast strain, respectively. The non-fastidious bacteria

inoculum plates were incubated at 37 °C for 24 h and for 48 h in the case of yeasts. Afterwards, all the plates were verified for inhibition zones, and the diameters were measured in millimetres. A total of three replicates was done in each experiment.

3.5.2.2.3 Resazurin Microtiter Method

The antimicrobial activity of chitosan was determined by using resazurin microtiter assay. For this experiment a sterile 96 well plate was labelled. For the bacteria, 100 µL of extract in 10% (v/v), DMSO-stock concentration of 20 mg/ml in Müeller-Hinton Broth (MHB) (maximum [DMSO] tested was 10%) was pipetted into the first row of the plate. The other wells were filled with 50 µL of MHB. Each well had 50 µL of the test material in serial descending concentration, with each tip being discarded after use. Next, 10 µL of resazurin indicator solution (0.1% diluted in MHB) was added to each well and 30 µL of fresh MHB was pipetted into each well. After all, the bacterial suspension (10 µL, 0.5 McFarland) was added to the wells. Each plate was loosely wrapped in cling film to prevent bacteria from becoming dehydrated, as well as a set of controls. The positive control column contained a broad-spectrum antibiotic, another column was comprised by all solutions except the chitosan extracts and lastly a column with all solutions, except the bacterial suspensions, which have been replaced by the respective volume of MHB. The experiment was carried out under aseptic conditions. The plates were prepared in triplicate and put in an incubator set at 37 ºC for 18 h. The yeast inoculum was set up by transferring yeast colonies to a sterile saline solution to achieve turbidity of a 0.5 McFarland. For the visualization of the cell growth, resazurin (cell growth indicator - it goes from blue to pink color when cell growth occurs) was used, by supplementing the working suspension (inoculum diluted 1:1000 in culture media) with resazurin sterilized solution (50 µL, 20 mg/mL in water). For the broth medium preparation, the RPMI-1640 medium (5.215 g), supplemented with glutamine and phenol red, without bicarbonate and 3-(N-morpholino) propanesulfonic acid (MOPS, 35.53 g) was dissolved in 400 mL of distilled water, and pH was adjusted to 6.9 to 7.1 at 25 ºC, by adding a sodium hydroxide solution (1 mol/L). To make up a final volume of 0.5 L, water was added and then filter sterilized and stored at 4ºC until needed. The microdilution susceptibility test was performed as previously described for bacteria, differing in the final volume in the wells of the microplate, which was 200 µL. Plates preparation were done in triplicate, and these were placed in an incubator at 37ºC for 24 h. Weren´t recorded as positive, color changes from purple to pink or colorless. The Minimum Inhibitory Concentration (MIC) was obtained, by recording the lowest concentration at which a change occurred.

3.5.3 Statistical analysis

To describe the basic features of the samples, descriptive statistics were applied (mean and standard deviation). Yield, color, molecular weight, antioxidant, and microbiological activity were analysed with a One-Way ANOVA followed by a Tukey´s test of multiple comparisons. All statistical tests were applied at 95% confidence level, except when stated otherwise. All data was analysed using the software Statistical Package for Social Sciences (SPSS) - version 27 ®.

4. Results and discussion

4.1 Pre-treatment and optimizations

4.1.1 Defatting

The different insect sources were submitted to a defatting process using a Soxhlet apparatus and the values of crude fat and the recovery rate of the defatted powder were calculated (Table 3). The values obtained showed that *T. molitor* powder had the highest percentage of crude fat (46.5 ± 3.8%), followed by *A. domesticus* (31.9 ± 1.9%) and the legs and wings of *A. domesticus* (20.7 ± 2.0%). Consequently, the sample obtained from the legs and wings yielded the best recovery rate (81.0 ± 0.7%).

Table 3- Average crude fat content (mean ± s.d.) and recovered powder (mean ± s.d.) in the different insect samples after Soxhlet extraction. Means of four replicates. TM- T. molitor; AD – A. domesticus; L&W- Legs and wings

	TM	AD	L&W
Crude fat content (%)	46.5 ± 3.8	31.9 ± 1.9	20.7 ± 2.0
Recovered defatted powder (%)	58.5 ± 0.5	68.8±1.1	81.0 ± 0.7

4.1.2 Conventional chemical treatment optimization

4.1.2.1 Demineralization

As described previously in section 3.3.1.1, an optimization for the demineralization step in the chemical treatment was studied. This optimization was based on the weight loss, which indicates a loss of minerals and catechol and thus higher purity of the final product. Different conditions were tested in duplicate, and the choice was based in a commitment between the weight loss and time-cost effectiveness of the conditions. The results are listed in Table 4 and show that the conditions that led to higher weight loss were 50ºC for 3 h at different concentrations (1M HCl or 2M HCl). There were no significant differences between the conditions with HCl at different concentrations, the chosen conditions were 50 °C/3 h/1 M HCl (condition 4) since this set of conditions uses less reagent than condition 5 and allows for a less aggressive acid hydrolysis.

Table 4 – Optimization of the demineralization step – Mean of weight loss (% ± s.d.) of the AD (A. domesticus) samples following demineralization. 1- RT, 3h and 1M HCl sol., 2- RT, 3h and 2M HCl sol., 3- 50ºC, 1h and 1M HCl sol., 4- 50ºC, 3h and 1M HCl sol., 5- ., 3- 50ºC, 3h and 2M HCl sol. a, b - Homogeneous groups in accordance with the Tukey's test ($p < 0.050$). Results are the average of two replicates. RT: room temperature

	Conditions tested				
	1	**2**	**3**	**4**	**5**
Average weight loss (%)	40.6 ± 1.14 [b]	43.1 ± 0.70 [ab]	43.7± 3.81 [ab]	**48.9**± 1.27 [a]	**50.7**± 0.99 [a]

Note: The tested reactions conditions presented in the last row of the Table 2 (1 M, 1 h and 100ºC) weren't statistically analysed, due to the evaporation of the HCl solution that led to unreliable results

4.1.2.2 Deproteinization

In the optimization of the deproteinization step the only studied variables was the time of reaction (3 or 5 h), while temperature (100ºC), solution concentration (NaOH 1M) and solid to liquid ratio (1:20. w:v) remained constant. The selection of the ideal conditions to proceed with extraction of chitin were based on the weight loss values between the

initial mass (demineralized sample) and the final mass which suggests the efficiency of deproteinization. The results from the tested conditions for the deproteinization step weren't submitted to any statistical analysis due to the few variables studied but also due to problems observed with the experimental condition with 5.5 h. In this condition, there was solvent evaporation which led to unreliable results and sample degradation (it was not possible to realize the duplicate because of these issues). The samples treated with a reaction time of 3 h, revealed an average weight loss of 89.8 ± 0.01%, while the sample treated with a reaction time of 5.5 h showed a weight loss of 89.5%. Since the difference between the two tested conditions was insignificant, the conditions chosen were 1 M NaOH solution, at 95ºC for 3 h in a solid to liquid ratio of 1:20 (w:v).

4.1.2.3 Chitin extraction yield – effect of defatting

After the demineralization and deproteinization steps were optimized, the yield of unbleached chitin extraction from defatted and non-defatted samples were compared. For this experiment defatted and non-defatted powders from *A. domesticus* were submitted to the optimized chemical treatment in duplicate. The average yield (%) of chitin was calculated according to Equation 3. According to One-Way ANOVA there are significant differences ($p = 0.004$) between the extraction yields of the defatted and non-defatted samples. The average chitin yield for the extraction with the non-defatted powder was 5.20 ± 0.28% while the chitin yield for the extraction from the defatted powder was 8.70 ± 0.36%. The defatting step is not mandatory and only a few studies in the literature (section 8.3) performed it, since the fat content varies from specie to specie and the deproteinization step help on the defatting process too, however it can improve the yield and purity of chitin. Even considering the weight loss observed in the defatting step, the extraction with this source can lead to higher yields relative to the non-defatted sample (from 100g of ground and dried AD, we obtain 68.75 g of defatted sample which translates to 5.98g of unbleached chitin which is higher than the 5.20% yield from chitin extraction directly from the non-defatted sample). Considering these results and the fat content of all the insect sources (4.1.1), it was decided that all the chitin extraction procedures would be done with defatted samples.

4.2 Extraction yields

4.2.1 Conventional chemical treatment

The yield of unbleached chitin obtained was calculated (DW basis) and a comparison between species (*T. molitor* larvae and *A. domesticus* adult) and different body parts (wings and legs of *A. domesticus* and whole *A. domesticus)* were performed. The average unbleached chitin yields achieved by the conventional chemical treatment (demineralization and deproteinization) ranged from 8.70 ± 0.36% (AD) to 15.00 ± 0.16% (L&W), with significant statistical differences being observed between the wings and legs of *A. domesticus* and both adult *A. domesticus* and *T. molitor* larvae (Table 5). Decolorization led to similar yields for all the tested samples (*ca.* 90%) and the final chitin yields ranged from 7.77 ± 0.00% (AD) to 13.45 ± 0.11% (L&W) with the same statistical differences observed for the yield before decolorization (Table 5).

These results support the idea that by-products of insect rearing (as in the case of the legs and wings of *A. domesticus*) can be excellent sources of chitin and present higher yields than whole insect sources. There are no additional studies that have assessed chitin extraction from this particular source, but it has been reported that the wings and legs from white-grub cockchafer and the wings from *Argynnis pandora* and *Blaberus giganteus* are among the body parts with the highest yield content in these species [161, 163, 164].

As for the whole body sources, lower extraction yields were reported for *A. domesticus* (5.7 ± 0.10) [243]. Other studies demonstrated chitin (decolorized) extraction yields of 4.3-7.1%, 9.8%, 5%, 10.1% and 5.1% for the African crickets, Bronze glandular bush-crickets, European mole crickets and the Two-spotted crickets respectively [104, 105, 171]. Concerning this information, the chitin extraction yield value (7.77% ± 0.00) obtained from the whole body of *A. domesticus* in this study is situated between the range of values stated in the literature. Additionally, the yields of chitin extracted from *T. molitor* larvae were determined to be between 4.6% to 10.13% by different studies [165, 166]. However, it is important to note that working with by-products of *T. molitor* rearing (e.g., exuviae) can lead to higher yields (16.4-20.07%) (section 8.3). The higher content of chitin in by-products is also reflected in studies performed with cicada sloughs, *H. illucens* and *S. gregaria* exuviae, Molossus beetle waste and other insect waste generated by the insect breeding industry [98, 114, 133, 158, 160, 176, 191, 246]. The values obtained in the present study are in accordance with the range of values for the yield of chitin extracted from different insects' sources that mostly vary between 5 and 20% (section 8.3).

Nonetheless, comparisons between studies should be done carefully as extraction methods can differ and thus affect the yield values. For instance, in this study it was report the yield values as those obtained after bleaching and in relation to the defatted fraction (which is not performed by all the studies). Moreover, the type of decolorization, or the solvents used in the demineralization or deproteinization step can interfere with the final yield results.

The results from this study and others published in the literature support the idea that edible insects are valuable sources of chitin and that by-products from insect rearing can add economic value to the edible insect industry and be inserted into a circular economy perspective as these sources can present higher yields of chitin than whole body sources.

Table 5 – Representation of chitin extraction yields before and after decolorization (mean ± s.d.). Yields of unbleached chitin are relative to three replicates, while yields after decolorization are relative to duplicates. a, b – homogenous groups in each column according to Tukey's test ($p < 0.05$).

Sources	Unbleached Chitin yield (%)	Bleaching yield (%)	Chitin yield (%)
A. domesticus	$8.70^{b} \pm 0.36$	$89.36^{a} \pm 0.00$	$7.77^{b} \pm 0.00$
T. molitor	$9.01^{b} \pm 0.99$	$90.45^{a} \pm 1.41$	$8.15^{b} \pm 0.13$
Legs and wings (*A. domesticus*)	$15.00^{a} \pm 0.16$	$92.44^{a} \pm 0.70$	$13.85^{a} \pm 0.11$

4.2.1 NADESs treatment

For the NADESs treatment, preliminary studies initially performed in order to assess which NADES provided the better results. All the developed NADEs were tested under the same conditions (1:20 (w:v), water bath at 80 ºC for 5 h under constant stirring). The results are presented in Table 6 and it is possible to observe that all the NADESs treatment led to much higher yields than the conventional chemical treatment. However, these greater yields can indicate that the deproteinization and demineralization were not fully or almost fully achieved and thus the obtained samples are highly impure. Moreover, some of the NADESs systems were difficult to work with, becoming too viscous when the temperature decreased and making it impossible to filtrate (Table 6– ChCl-OA). The samples that achieved lower yields were those extracted using ChCl-MA, and it was decided to do further studies with this system in an attempt to optimize the

results and extract samples with higher purity. With this in mind, different extraction conditions (varying in temperature, time of reaction and heating method) were tested (section 3.3.2.2). Even though it was possible to obtain lower yields (particularly with the treatments with a time of reaction of 5 h), which could indicate higher purity, the yields were still too high (Table 7). Furthermore, both heating methods sometimes led to a large foam formation, which caused the reactions to be stopped. Additionally, the samples HP_80_5 were passed through the decolorization step, but the effect on color was minimal (whiteness index only increased from 32.43 to 36.00). Lastly, this sample was also submitted to deacetylation with yields being extremely low (19%) when compared to the yields of chitosan obtained from chitin extracted by the conventional chemical treatment (section 4.2.2). Considering these results, it was decided to not further characterize these samples.

Table 6 – Unbleached chitin yields (mean ± s.d.) extracted with different NADESs. Results as means of duplicates. a, b – homogenous groups in each column according to Tukey's test ($p < 0.05$). Chcl – Choline chloride; U – Urea; CA – Citric acid; OA – Oxalic acid; MA; Malonic acid

NADESs	YIELD (%)
Chcl-U	72.86 ^{b} ± 4.19
Chcl-CA	70.71 ^{b} ± 0.60
Chcl-OA	-
Chcl-MA	**39.25** ^{a} ± 17.33

Table 7 - Unbleached chitin yields (mean ± s.d.) obtained by different conditions using the NADESs system ChCl-MA. Results as means of duplicates. a, b – homogenous groups in each column according to Tukey's test ($p < 0.05$). WB - Water bath; HP - Hot heating plate; Cent. – Centrifugation; 3 or 5 – hours; 50 or 80- Degree Celsius

NADES and conditions	Average yield (%)
WB-3-50	70.39 ^{b} ± 3.03
HP-3-50	65.46 ^{ab} ± 0.30
WB-3-80	59.16 ^{ab} ± 9.00
WB-3-80 - Cent.	72.54 ^{b} ± 4.86
HP-3-80	51.72 ^{ab} ± 10.62
HP-5-80	**38.63** ^{a} ± 7.28
WB-5-80	39.25 ^{a} ± 8.22

Few studies reported the extraction of chitin using NADESs in crustacean and insect sources. Zhou, et al. [156] reported several yield values (6.51 ± 0.22% to 26.71 ± 0.29%) for chitin extracted from the prepupae of *H. illucens* (defatted powder) by different NADESs systems at different temperatures. As it occurs in the present study, the authors reported higher yields of chitin extraction than in the acid/alkali treatment (6.50 ± 0.04), yet they described that the chitin purity of the samples extracted with the NADESs was high and similar to that from the sample extracted by the conventional chemical treatment. Moreover, the yield values that the authors observed were not similar to the ones obtained in this study. It is noteworthy to refer that only two NADESs mixture were similar to the ones used in the present study (ChCl-OA and ChCl-U), furthermore the insect species and the environmental and experimental conditions were different which affects all the obtained data. Another study regarding chitin extraction from lobster shells, by one step methodology using deep eutectic solvents reported higher yield values of chitin extracted by ChCl-MA mixture (4.44 ± 2.15% (supernatant) + 16.19 ± 2.50% (pellet)) than the values obtained for the chitin extracted by conventional chemical treatment (16.53 ± 2.35%). The authors described the mixture choline chloride with malonic acid as the one that showed the best extractive ability of pure chitin. Additionally, they stated that no major differences in ash, moisture and protein content were found between the samples extracted by the mixture ChCl-MA, the commercial sample and the sample extracted by the acid/alkali treatment [152].

Concerning the previous information and the results obtained in this study, it is essential to found alternative NADESs mixtures and methodologies to improve the extraction of chitin in order to raise its purity. This will probably be a hard and defiant task due to the scarce literature regarding chitin extraction by deep eutectic solvents and even more scarce if source are insects. The starting point has to be the mixtures preparation as the mixtures in this study were prepared on heating plates as described in section 3.3.2.1. Furthermore, the mixtures need to be characterized for their acid and alkali properties which influence the chitin yield [156].

4.2.2 Chitosan production

Chitosan deacetylation was obtained by chemical deacetylation, using NaOH as solvent (section 3.4). The resulting yields from its production are presented in Table 8.

Table 8 - Chitosan production yields (mean ± s.d.). Yield relative to chitin is the yield calculated from the quantity of chitin used, while the yield relative to the defatted samples is calculated from the initial quantity of defatted samples used for chitin extraction. Results as means of duplicates. a, b – homogenous groups in each column according to Tukey's test ($p < 0.05$). n.s – nonsignificant differences. TM - T. molitor; AD – A. domesticus; L&W - Legs and wings

Sample	Yield relative to chitin (%)	Yield relative to defatted sample (%)
AD	70.57 $^{n.s}$. ± 3.21	5.48 ^{b} ± 0.25
TM	69.57 $^{n.s}$ ± 6.88	5.67 ^{b} ± 0.56
L&W	71.33 $^{n.s}$ ± 3.68	**9.88** ^{a} ± 0.51

Chitosan yields relative to chitin were not significantly different between the different sources. This result indicates that the method was equally applied to each sample and the samples have lost similar percentages of weight. Concerning the final chitosan extraction yield calculated from the defatted samples (Dw basis), yield values between 5.48% ± 0.25 (AD) and 9.88% ± 0.51 (L&W) were obtained. From these results it is possible to conclude that the deacetylation led to similar yields in relation to the chitin, however regarding the yield calculated from the defatted samples, the L&W sample showed a significant higher yield. These results were expected since the L&W showed higher chitin extraction yields, leading to higher chitosan yields. This information also suggests that the L&W samples have less lipids, minerals and/or proteins than the whole body. The yields obtained in this study for an extraction from 100g of dried insect powder are illustrated in Fig. 10.

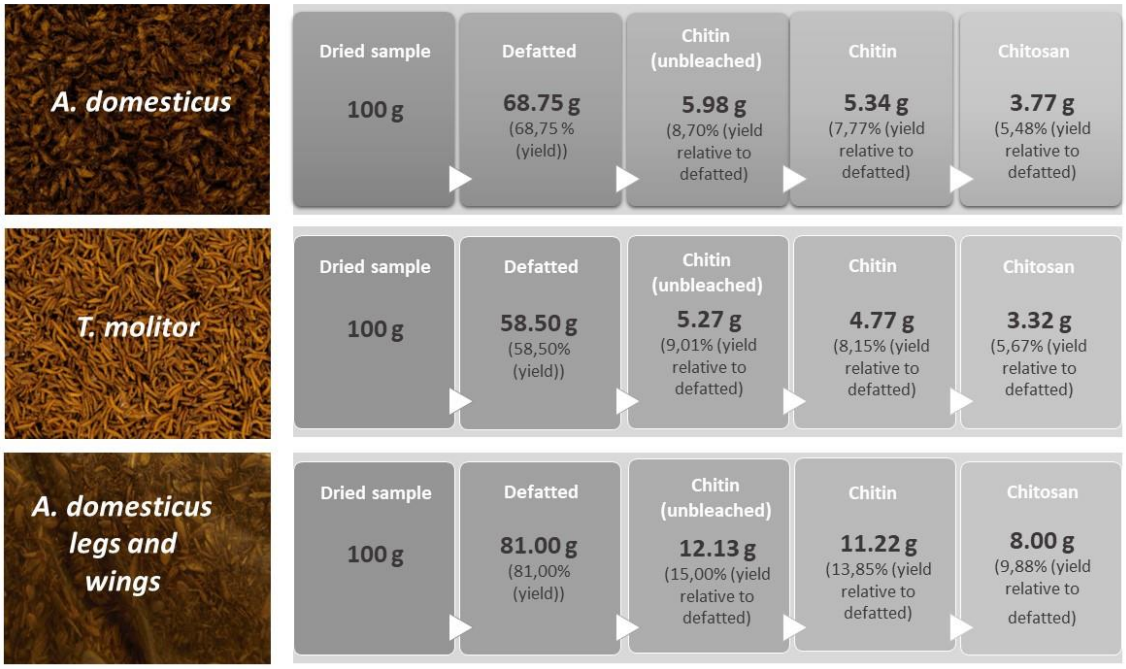
The results obtained in this study are similar to those describe in the literature. Two different studies regarding chitosan extracted from *T. molitor* larvae revealed total yields of 2.5% and 3.65%, which are similar to the values obtained for the TM chitosan yield in the present study (5.67% relative to the defatted sample and 3.32% in relation to dried powder sample) [110, 114]. Additionally, Shin, et al. [166] described that a sample of chitosan produced from *T. molitor larvae* chitin, had a 80% yield (DW basis) relative to chitin, which is a higher yield than the on obtained in this study (69.57 ± 6.88%).

Concerning different cricket species, the values for the of chitosan yield values in relation to chitin range from 41.75% for the two spotted cricket to 80.5% for the house cricket

[171, 243]. Moreover, Ibitoye, et al. [105] presented total yields values of chitin extracted from the African crickets between 2.4 to 5.8%.

As it happened in the case of chitin yields, the comparison between different chitosan yields should be carefully done since the extraction methods, as well as the environmental conditions are diverse and even in the case of the crickets there aren't other studies that have extracted chitin from *A. domesticus* through the conventional chemical treatment. This leads also to another point in which the different yields obtained by the different methods or conditions applied will also produce different characteristic outcomes. These outcomes can be different molecular weights and DD which were referred earlier as two of the most important parameters that are extremely influenced by the deacetylation procedure applied that affects other characteristic of the chitosan, consequently influencing its application.

Fig. 10 – Chitin and chitosan extraction yields obtained from 100g of dried insect powder



4.3 Physicochemical characterization

4.3.1 Color and Whiteness index (WI)

The color values (Table 9) for all the different extracted and commercial samples were evaluated as described in section 3.5.1.1. For all the insect samples, the decolorization step successfully increased the lightness (L*) and yellowness (b*) values while it decreased redness values (a*). Before decolorization the chitin samples presented L* values between 37.04-38.80 and b* values between 10.51-14.72, and after decolorization these values increased to 77.21-82.83 and 24.33-25.00, respectively. As a result, the WI (whiteness index) of the insect chitin were also increased after decolorization (from 35.20-37.26 to 66.1-70.16). The insect sample that showed higher WI values were the chitin extracted from *T. molitor* and chitin extracted from the legs and wings of *A. domesticus*, with higher values than chitin from whole *A. domesticus*. From these results, it is possible to conclude that after the treatment with potassium permanganate and oxalic acid, *A. domesticus* chitin samples were much whiter than before decolorization due to the higher average lightness values and WI (Fig. 11). However, in comparison with the commercial chitin, all the insect chitin sample showed an average L* value lower than the commercial sample (105.98 ± 1.47) which indicate that insect chitin sample are slightly darker than the commercial shrimp chitin (Fig. 12). Furthermore, the average WI value (82.85 ± 0.46) is higher in the commercial chitin sample and the differences were significant ($p<0.001$). A previous report on chitin extracted from *T. molitor* showed higher L* and WI values (82.21 ± 0.72 and 80.13 ± 0.81 respectively) than the different extracted chitins in this study [185]. However, extracted chitins in this study presented higher L* and WI values than chitin extracted from crab shells, which suggests a whiter color [247]. It´s noteworthy refer that due to the different decolorization approaches the results may differ from each other.

Table 9 - Color values results (mean ± s.d.) of the different chitin samples, before and after decolorization. a, b, c, d - Represents in each column homogeneous groups in accordance with the Tukey's within the different tested conditions ($p < 0.050$). Results are the average of six replicas

Sources	Non-decolorized chitin				Decolorized chitin			
	L*	a*	b*	WI	L*	a*	b*	WI
TM	37.04 ± 1.4[a]	4.30 ±0.16[b]	14.72 ± 0.37[a]	35.20 ± 1.42[b]	82.83 ± 0.5[b]	1.20 ± 0.24[c]	24.40 ± 0.67[b]	70.16 ± 0.82[b]
AD	38.27 ± 0.87[a]	3.89 ±0.33[c]	10.51 ± 0.13[c]	37.26 ± 0.84[a]	77.21 ± 0.51[c]	2.65 ± 0.13[a]	25.00 ± 0.14[a]	66.10 ± 0.42[c]
L&W	38.80 ± 1.23[a]	4.50 ± 0.14[a]	12.92 ± 0.77[b]	37.24 ± 1.08[a]	82.51 ± 0.15[b]	1.71 ± 0.60[b]	24.33 ± 0.19[b]	70.00 ± 0.21[b]
Commercial	-	-	-	-	105.98 ± 1.47[a]	1.72 ± 0.12[b]	15.93 ± 0.11[d]	82.85 ± 0.46[a]

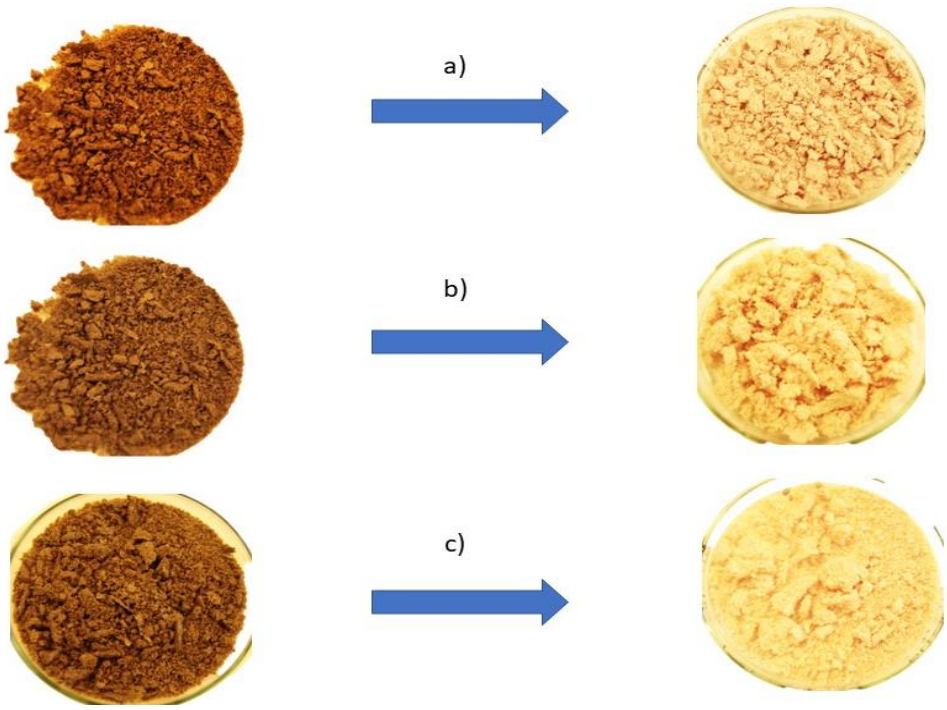


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Fig. 11 - Examples of chitin before (left) and after (right) r decolorization - a) A. domesticus; b) Legs and wings of A. domesticus; c) T. molitor

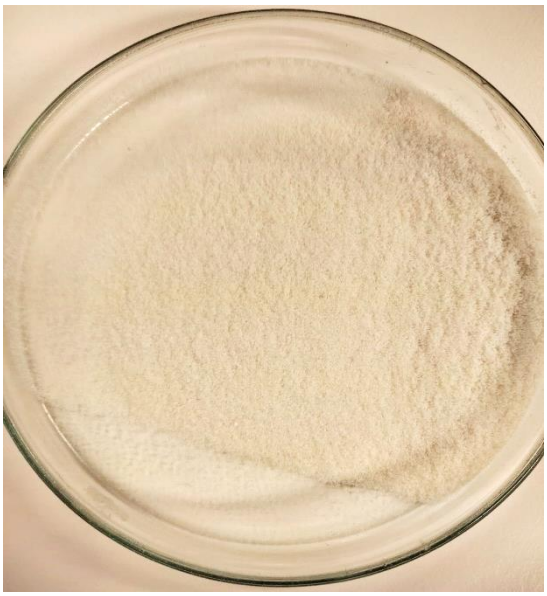

Fig. 12 - Commercial chitin sample

The average color values for chitosan are described in Table 10. Comparing the l and WI average values from the extracted insect chitosan with the commercial chitosan, the commercial sample demonstrated higher L*, b* and WI values. As for the insect samples, similarly to what occurred with chitin, chitosan from *T. molitor* showed higher values of L* and WI followed by the chitosan from the legs and wings of *A. domesticus*. From the visual analysis (Fig. 13) and confirming the b* values, it was possible to notice that the chitosan's were less yellow than the respective chitin and demonstrated a slightly grey color.

Table 10 – - Color values results (mean ± s.d.) of the different chitosan samples. a, b, c - Represents in each column homogeneous groups in accordance with the Tukey's within the different tested conditions (p < 0.050). Results are the average of six replicas.

Sources	Average color values			
	L*	a*	b*	WI
TM	77.61 ± 1.19^{b}	2.93 ± 0.07^{b}	13.95 ± 0.10^{d}	73.45 ± 1.04^{b}
AD	71.20 ± 0.17^{d}	3.50 ± 0.8^{a}	15.43 ± 0.15^{c}	67.13 ± 0.20^{d}
L&W	75.03 ± 0.24^{c}	3.13 ± 0.15^{b}	16.64 ± 0.65^{b}	69.83 ± 0.24^{c}
Commercial	101.07 ± 2.37^{a}	1.87 ± 0.21^{c}	17.22 ± 0.22^{a}	82.51 ± 0.24^{a}

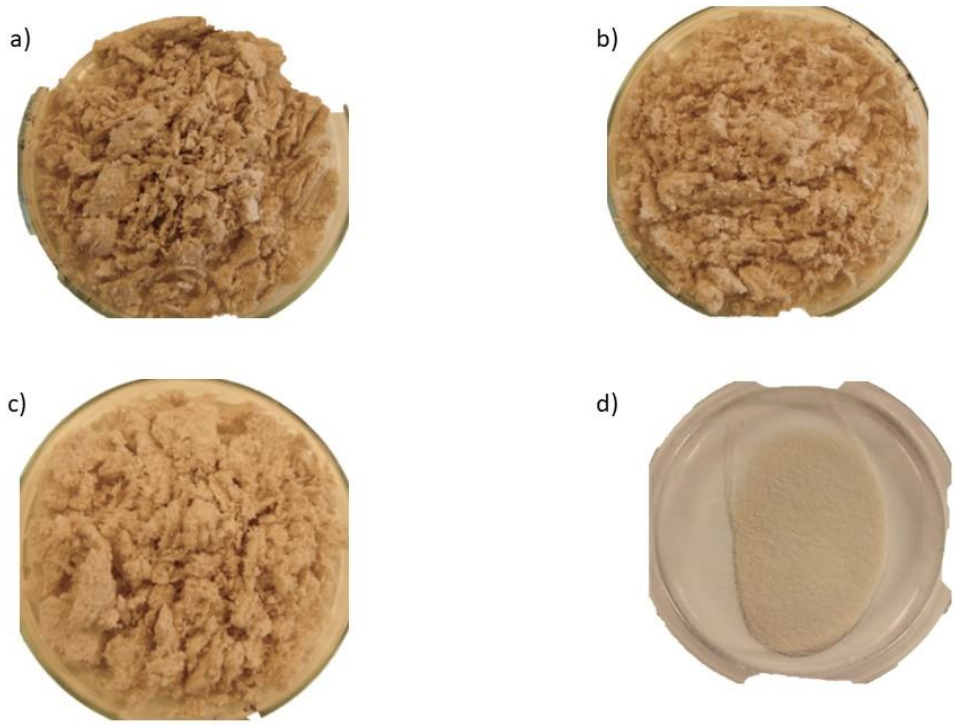

Fig. 13 - Examples of chitosan from insects - a) A. domesticus b) legs and wings; c) T. molitor – and d) commercial shrimp chitosan.

4.3.2 FTIR-ATR

Chitin chemical composition was assessed using FTIR-ATR analysis, and a list of the major characteristic peaks are present in Table 11. The chemical composition of the different extracted insect samples and the commercial one is similar, and the bands are summarized in Fig. 14 with small deviations that can be neglected. As referred in section 3.5.1.2, the different chitin allomorphic forms have different characteristic peaks in FTIR spectra. The α-chitin has three characteristic peaks around 1660-1650 cm^{-1} and 1620 cm^{-1}, and 1550 cm^{-1}, while β-chitin has only a characteristic peak at 1650 cm^{-1}. Both the insect and commercial samples presented three characteristic peaks at around 1651-1652 cm^{-1}, 1620-1622 cm^{-1}, and 1550-1551 cm^{-1}. These peaks are ascribed to the C=O secondary amide stretch (Amide I), C=O secondary amide stretch (Amide I), and N-H bend and C-N stretch (Amide II), respectively. These results indicate that the extracted chitin on this study have α-form and has close similarity to the commercial shrimp chitin. Moreover, the absence of a band at 1540 cm^{-1} suggests that the chitin did not show any protein residues, indicating that the deproteinization step was successfully applied.

The α-form was widely reported in the literature for insects' chitin, with several species from the order orthoptera and coleoptera presenting it [78, 92, 104, 175]. Other insects also presented α-form chitin, like the case of the black soldier fly (*H. illucens*), some cicada species and also wasp and bees' species, which belong to Diptera, Hemiptera and Hymenoptera orders, respectively [139, 184, 186, 192]. The obtained results indicate that the isolation of chitin from the three different insect sources was successfully achieved.

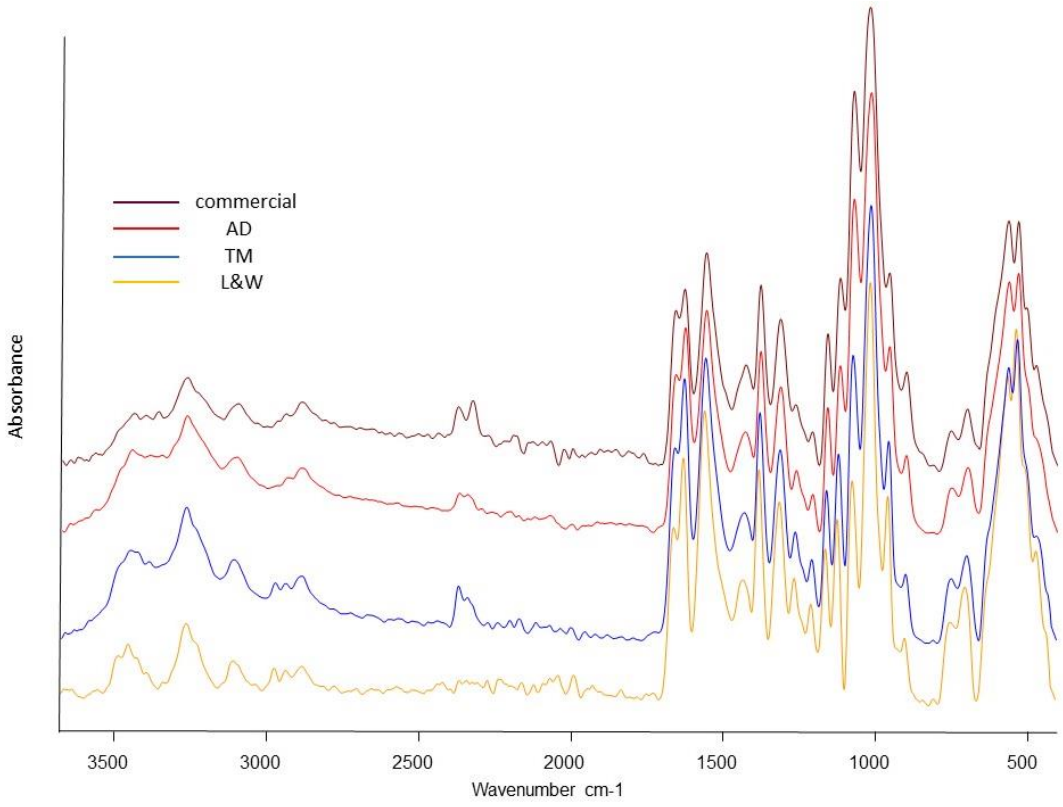

Fig. 14 – FTIR-ATR spectra of the different chitin samples

Table 11 - FTIR spectral data of the different chitin samples. Wavenumber of the different peaks attributed to functional groups characteristics of chitin.

Functional group and vibration modes	Classification	Commercial wavenumber (cm^{-1})	*A. domesticus* wavenumber (cm^{-1})	*T. molitor* wavenumber (cm^{-1})	Legs and Wings (*A.d.*) wavenumber (cm^{-1})
O-H stretching	---	3359	3436	3437	3443
N-H stretching	---	3087; 3254	3092; 3255	3097; 3254	3097; 3253
CH3 sym. stretch and CH2asym. stretch	Aliphatic compounds	2956	2957	2930	2926
CH3 sym. stretch	Aliphatic compound	2879	2882	2875	2871
C=O secondary amide stretch	Amide I	1652	1651	1651	1652
C=O secondary amide stretch	Amide I	1622	1620	1620	1620
N-H bend, C-N stretch	Amide II	1551	1550	1550	1550
CH2 ending and CH3 deformation	---	1421	1422	1422	1424
CH bend, CH3 sym. deformation	---	1373	1373	1372	1372
CH2 wagging	Amide III, components of protein	1308	1307	1306	1305
Asymmetric bridge	Saccharide rings	1153	1153	1154	1155

Functional group and vibration modes	Classification	Commercial wavenumber (cm^{-1})	*A. domesticus* wavenumber (cm^{-1})	*T. molitor* wavenumber (cm^{-1})	Legs and Wings (*A.d.*) wavenumber (cm^{-1})
oxygen stretching					
Asymmetric in-phase ring stretching mode		1111	1112	1114	1115
C-O-C asym. stretch in phase ring		1066	1066	1067	1067
C-O asym. stretch in phase ring	---	1013	1012	1008	1006
CH3 wagging	along chain	950	950	949	949
CH ring stretching	Saccharide rings	894	895	894	894

After the deacetylation of chitin from the different insect samples with NaOH (section 3.4), the chemical composition of chitosan was assessed by FTIR-ATR to confirm if the deacetylation was effective. By comparing the different FTIR spectra of the chitosan samples with the commercial shrimp chitosan sample, it is possible to see a similarity (Fig. 15). Furthermore, peaks around 1650 and 1590 cm^{-1}, corresponding to C=O in the $NHCOCH_3$ group (Amide I band) and the amine (NH_2) in the $NHCOCH_3$ group (Amide II band), are characteristic for chitosan [103] . By the data acquired from the FTIR spectra of the different obtained chitosan samples these bands were confirmed to be around 1644-1587 cm^{-1}, 1648-1584 cm^{-1} and 1656-1582 cm^{-1} for *A. domesticus*, *T. molitor* and legs and wings samples, respectively. The presence of these two bands indicates the formation of chitosan and additionally, the expected weakened band at 1644-1656 cm^{-1} (C=O) in the NHCOCH3 group (Amide I band) suggests a successful deacetylation. This band intensity is attributed to the degree of N-deacetylation, which increases when the intensity is lower [86]. The obtained results from the FTIR-ATR analysis revealed similarity to the commercial shrimp chitosan and chitosan extracted from beetles, two

Orthoptera species, muga silkworm and yellow mealworm as well as other cricket species [115, 166, 175, 209, 248, 249].

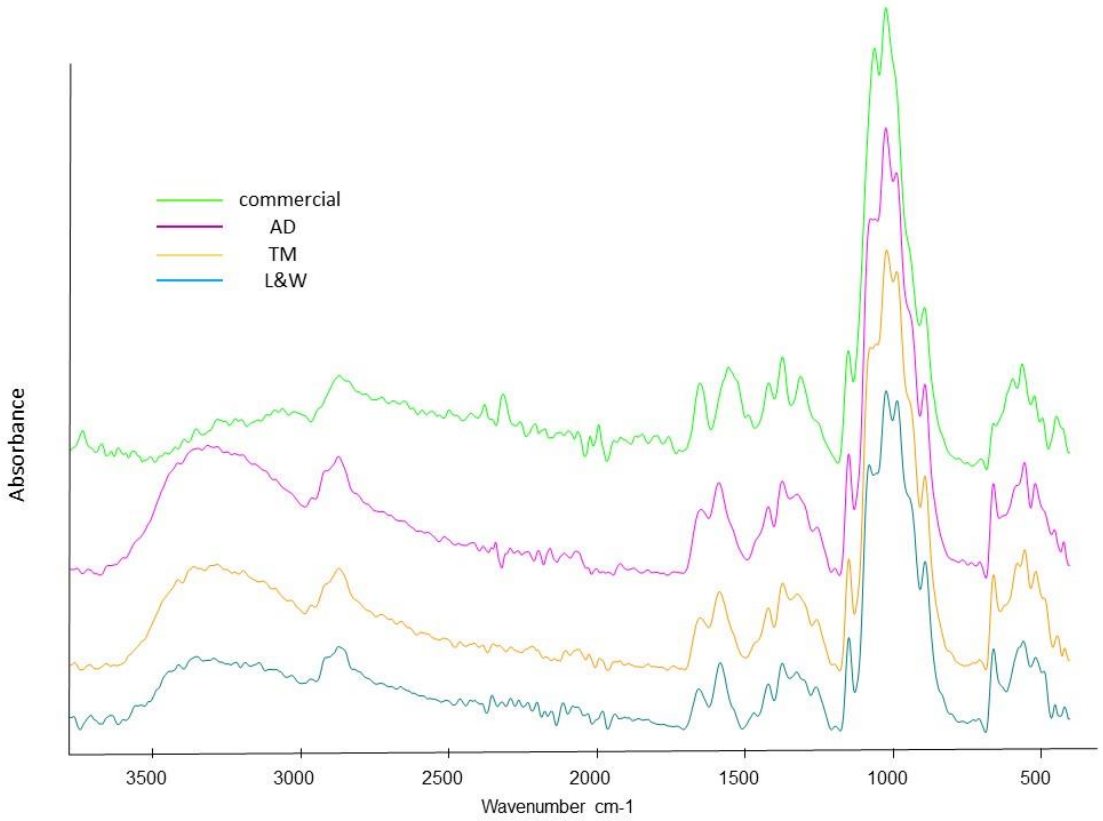

Fig. 15 – FTIR-ATR spectra of the different chitosan samples

The degree of deacetylation (DD) of the different chitosan samples was calculated with the FTIR support information as described in section 3.5.1.2. The chitosan with the highest degree of deacetylation was the chitosan from the whole *A. domesticus* (63,7%), followed by the *T. molitor* chitosan (62,3%) and lastly the L&W chitosan (62.0%). In comparison with the commercial shrimp sample (DD of 69.4%), all the insect chitosan samples showed a lower DD. All the insect samples are classified as having a low degree of deacetylation (55-69%) while the commercial shrimp chitosan, although close to a medium DD (70-85%) is also of low DD [250]. Similar results were observed for chitosan extracted from silkworm pupa (66.9%) and eggs shells (59.2%) [96] . It is noteworthy to mention that the calculation of the DD assisted by FTIR is only a rough estimation and can be perhaps unreliable, since it is dependent on the calculation method, analysis environment, samples humidity and operation conditions [242]. A study concerning chitosan production two cricket species (*A. domesticus* and *G. sigillatus*), reported DD between 72.5 – 79.4% for *A. domesticus* and 73.5-81.3% for *G. sigillatus* [243]. In the

previous mentioned study, it was possible to observe that DD increases with an increase in time of reaction. In a study performed by Chae, et al. [249], regarding chitosan production from chitin of the two-spotted field cricket (*G. bimaculatus*), the authors also achieved higher DD for the chitosan samples when the reaction times of deacetylation were increased, confirming the former mentioned results. Additionally, the authors studied the effect of the NaOH concentration and stated that when the concentration is raised, the DD also increases. Several studies for *T. molitor* larvae chitosan produced under different conditions (NaOH concentration ranging (40-60% (w/v)), time (3-9 h) and temperature (80-105 ºC)), presented DD between 75.59 to 96.19%, which were much higher than those obtained in this study [110, 114, 166, 185]. All these results demonstrate that the obtained chitosan in this study had a lower DD than chitosan extracted from similar species and is classified as chitosan with a low DD. However, like it was referred above, the DD calculation by FTIR is only a rough estimation and can vary depending on the conditions. Moreover, it is possible to increase the chitosan deacetylation degree by increasing the reaction time or NaOH concentration. As previously mentioned, (section 1.3.5.1), the DD is one of the most important parameters of chitosan and helps dictate the other physicochemical and biological features which influence its application.

4.3.3 FT-RAMAN

The obtained results from the FT-RAMAN analysis are in accordance with the results obtained from the FTIR-ATR analysis. The obtained peaks in all the spectra are very similar to each other, both insect chitin and commercial chitin peaks matched (Fig. 16). The characteristic peaks of α-chitin attributed to the amide I band are presented in all the samples and match other FT-RAMAN spectra reported in the literature for α-chitin [51]. Specifically, these two sharp peaks ascribed to the C=O secondary amide stretch (Amide I) for the commercial sample can be found at 1658 cm^{-1} and 1625 cm^{-1} and are pretty close to the corresponding peaks from the insect samples. Furthermore, the other characteristic peaks of α-chitin can also be found Fig S. 2. In this way, it is possible to confirm that the chitin extraction was successfully achieved.

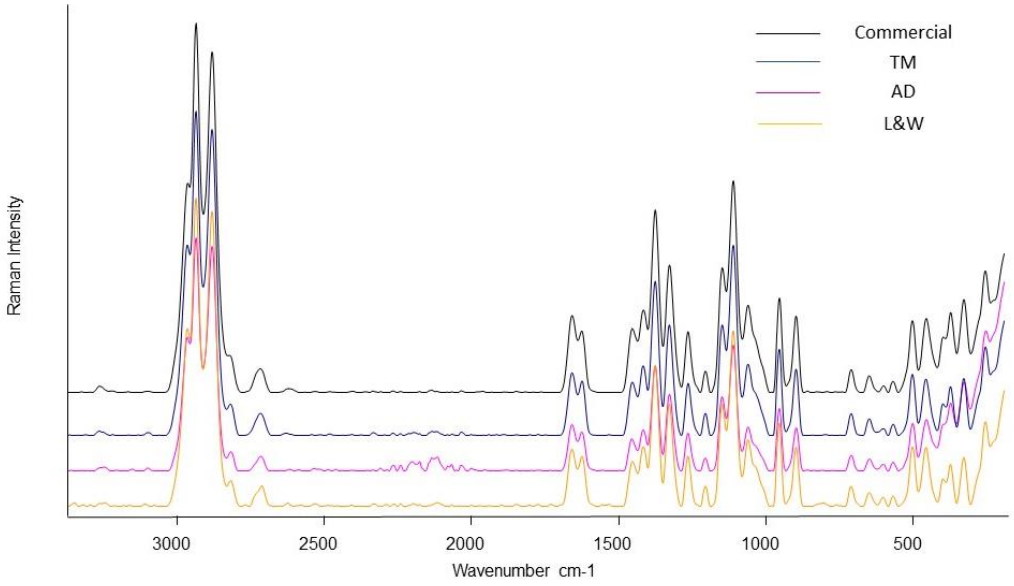

Fig. 16 - FT-RAMAN spectra of the different chitin samples

4.3.4 Molecular weight (Mw)

Molecular weight (Mw) is one of the most important parameters of chitosan and helps dictate its industrial application due to its influence in the physicochemical and biological properties. The average viscosity molecular weight of the different chitosan samples is demonstrated in Table 12. The chitosan sample which showed the lowest average molecular weight (245.77 ± 39.44 kDa) was the chitosan produced from the legs and wings of *A. domesticus* and the highest (332.58 ± 73.37 kDa) was the chitosan produced from *A. domesticus* (adult specimens). Non-significant statistical differences were found in the average molecular weight between the samples. The chitosan extracted from *T. molitor* larvae and the legs and wings of *A. domesticus* presented a medium molecular weight as well as the commercial shrimp chitosan. *A. domesticus* (adult specimens) chitosan was characterized by high molecular weight [132]. Various studies regarding chitosan extracted by conventional chemical treatment reported similar molecular weight values measured by viscosimetric methods of 308.3, 310 and 450 kDa from *G. bimaculatus* cricket's exoskeleton, larvae shells of fly *sp*. and residues of *C. molossus* respectively [171, 176, 251]. Moreover, Malm and Liceaga [243] reported a molecular weight for chitosan extracted from *A .domesticus* (adult specimens) of 344 KDa determined by viscometry, which is slightly higher than the Mw of *A. domesticus* chitosan obtained in this study, but it is in good agreement with the obtained results. Values of Mw were previously reported for chitosan extracted from the larvae of *T. molitor*, by conventional chemical treatment. These values were 96.2 KDa and 39.75 kDa which are lower than the values obtained in this study [99, 110]. Probably these values

were achieved due to the longer time of deacetylation reaction (7 and 8 h) as well as higher NaOH solution concentrations (60% (w/w)). Not only the source influences the Mw of chitosan, but also longer reaction times and higher reagent concentrations led to higher degrees of deacetylation and lower molecular weight chitosan [173]. Because the Mw of the diverse insect samples and the commercial one is similar, the biological activity is likely to be similar. Additionally, high molecular weight chitosan can be applied in wastewater treatment, papermaking industry, as a foliar treatment agent, as well as in wound healing, while low molecular weight chitosan is usually preferred in the pharmaceutical industry due to its role on drug delivery and higher antimicrobial activity [90, 132, 214, 220, 230, 241].

Table 12 - Average molecular weight (% ± s.d.) of the different chitosan samples

Sources	Average molecular weight (KDA)
T. molitor	302.58 ± 50.29
A. domesticus	332.58 ± 73.37
Legs and wings (*A. domesticus*)	245.77 ± 39.44
Commercial (shrimp)	292.38 ± 41.56

4.3.5 XRD

XRD analysis was carried out to study the crystalline structure of the different chitin and chitosan samples (3.3.1.3 and 3.4). From chitin analysis (Fig. 17) six peaks, two major and sharper peaks and 4 minor peaks, were observed for the different samples. The two major peaks were situated at about 9º and 19º and the four minor peaks around 13º, 21º, 23 and 26º. Specifically, the *T. molitor* chitin major peaks were seen at 9º and 19.05º and the minor peaks at 12.55º, 20.55º, 23º and 26.5º. For the *A. domesticus* chitin two major peaks were observed at 9.35º and 19.2º, and four minor peaks were observed at 12.6º, 20.8º, 23.15º and 26.45º. The diffractogram from the wings and legs chitin showed two major peaks at 9.25º and 19.35º and four minor peaks at 12.55º, 20.95, 23.1º and 26.3º. Similar results were obtained for the commercial chitin where the two major peaks were observed at 9.2º and 19.25º and the four minor peaks were observed at 12.75º, 20.75º, 23.15º and 26.3º. Similar peaks were found in XRD patterns of chitin from different insect species, that presented chitin in the α-form [85, 86, 110, 163, 179].

Therefore, and complementing the previous described FTIR and FT-RAMAN results (3.5.1.2), the different chitin samples extracted in this study are in the α-form. For the different chitosan samples two major and sharper peaks at around 10º and 20º were observed (Fig. 18). These are characteristic peaks of chitosan and are usually observed in chitosan extracted from different sources, like other insect species and crustaceans [166]. The chitosan from *T. molitor* showed two sharp peaks at 10.25º and 20.15º, two sharp peaks were also observed for the chitosan of *A. domesticus* and the legs and wings of *A. domesticus* at 10.3º, 19.9º, 10.15º and 20.05º respectively. The commercial chitosan sowed two major peaks at 9.9º and 19.9º. The values obtained are similar to those described in literature [103, 166, 190, 209]. Additionally, all the different diffractograms showed similarity between them, including the diffractogram from the commercial sample.

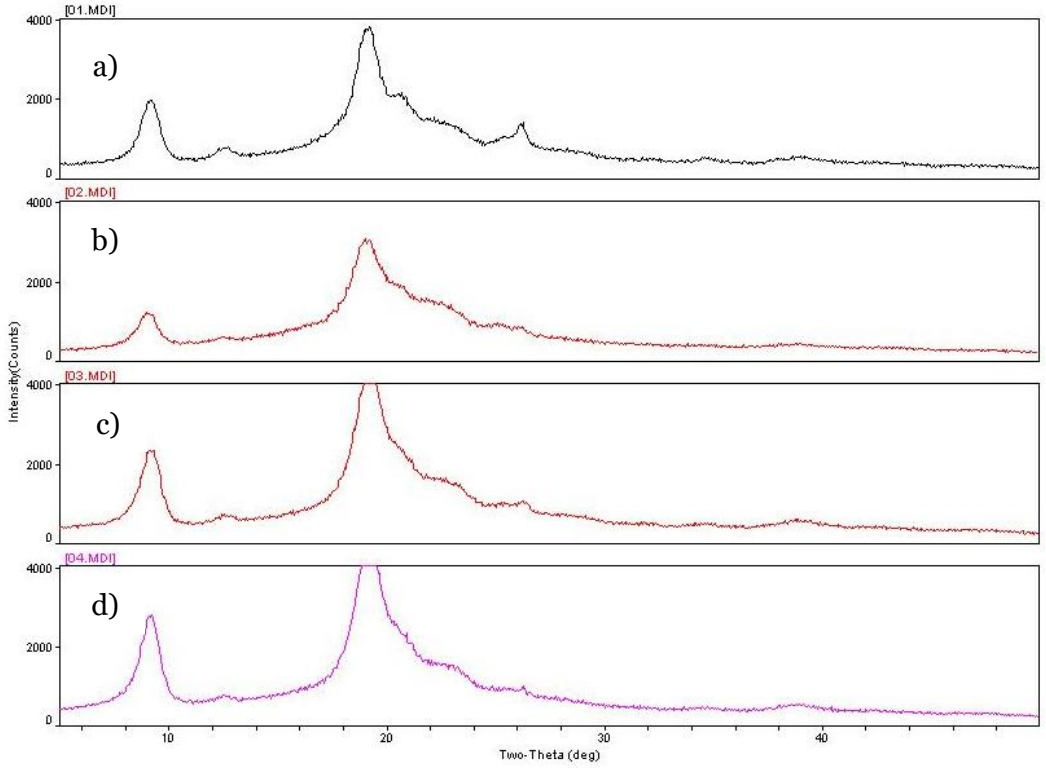

Fig. 17 - XRD chitin patterns from the different sources ((a) commercial chitin sample, (b) T. molitor larvae, (c) A. domesticus and (d) wings and legs from A. domesticus.

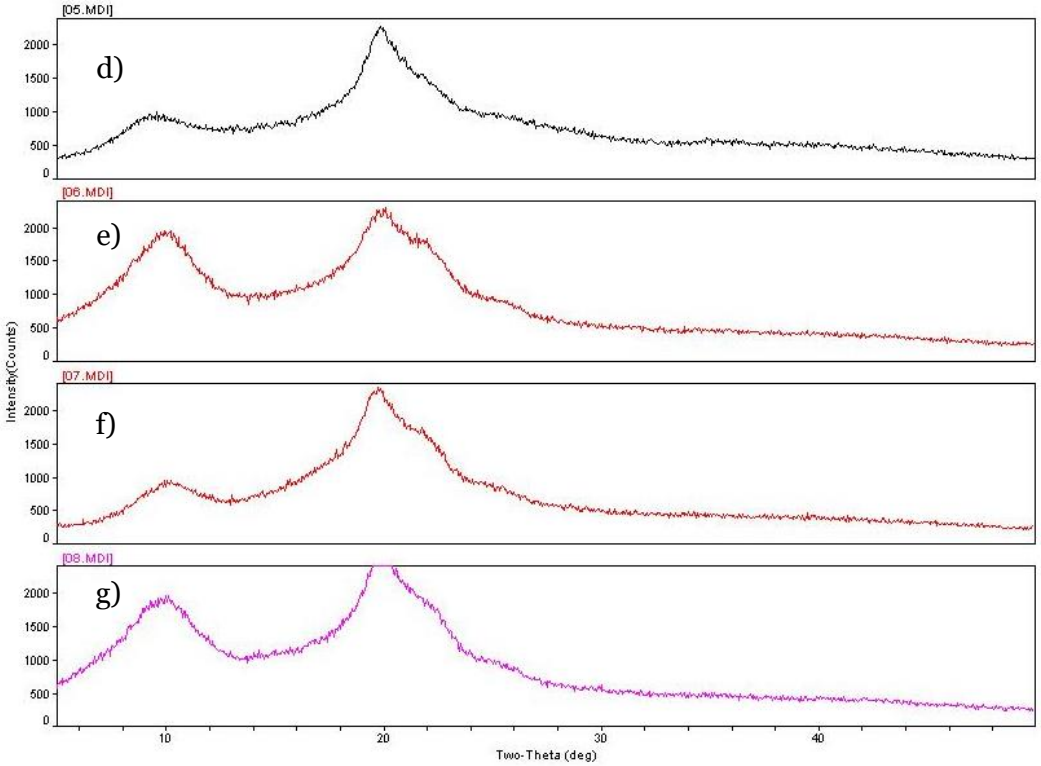

Fig. 18 - XRD chitosan patterns from the different chitin sources ((d) commercial chitosan sample, (e) T. molitor larvae, (f) A. domesticus and (g) wings and legs from A. domesticus.

The CrI values from the different samples are described in Table 13. The CrI values of chitin from *T. molitor*, *A. domesticus* and the legs and wings of *A. domesticus*, were 63.9%, 72.8% and 70.25% respectively, while the CrI value of the commercial chitin was 67.8%. These values are among the range of CrI chitin values (47-91%) reported in the literature that varies according to the source, method of extraction and calculation [105]. For the chitosan samples of *T. molitor*, *A. domesticus* and the legs and wings of *A. domesticus* the CrI values were 52.5, 61.2 and 56.8% respectively. The CrI values of the commercial chitosan was 65.6%.

From these results it´s possible to conclude that chitin from both *A. domesticus* sources have superior CrI values than those from *T. molitor* source and commercial chitin which indicates that chitin from *A. domesticus* had a more crystalline structure. Furthermore, the high and sharp peaks indicate a highly ordered crystal structure of α-chitin (Fig. 17). Additionally, comparing the CrI values of the different chitin and chitosan samples it´s possible to accomplish that chitosan presents a more amorphous structure than chitin, which is in agreement with the literature [115].

Table 13 – Crystallinity index (CrI) values (%) of insect and commercial chitin and chitosan

Sources	CrI values (%)	
	Chitin	Chitosan
TM	63.9	52.5
AD	72.8	61.2
L&W	70.25	56.8
Commercial	67.8	65.6

4.3.6 Scanning electron microscopy (SEM)

SEM analysis at different magnifications were conducted to examine the differences of the surface morphology between the extracted chitins and chitosan's and the commercial reference samples. The surface morphology photos of the different obtained chitins (3.3.1.3) and the commercial chitin sample are exhibited in Fig. 19. From the lower magnification images (140x) (Fig. 19, a-1; b-1 and d-1), it's possible to notice that the obtained chitins presented larger and more irregular flakes than the commercial chitin (Fig. 19, e-1) that presented smaller flakes. Another remarkable difference that is possible to notice at higher magnification images (850 x to 1700 x) is the presence of pores in the obtained chitins (Fig. 19, a-2 and a-3; b-2 and b-3; d-2 and d-3), while the commercial chitin sample didn't show any porosity (Fig. 19, e-2 and e-3). The presence of big pores is common to all the extracted chitin where it´s possible to observe a microfibrillar structure as well. These results are consistent with the results described in the literature for grasshoppers and different Coleoptera and Orthoptera species [103, 104]. The commercial shrimp chitin didn't present pores but instead showed a morphology that consist of irregular arranged fibers and a more regular and smoother surface. It is also noteworthy that the insect chitin samples demonstrated different and contrasting surface morphologies depending on the observed spot. For instance, chitin from *A. domesticus* (Fig. 19, a) presented a surface morphology of separated fibers and nanopores (Fig. 19, a-2), but also a surface morphology of pores and fibers that adhere to each other (Fig. 19, a-3). In addition, this chitin sample also showed some microporous smooth surface (Fig. 19, a-2). The chitin sample from the legs and wings of *A. domesticus* presented some differences too. One morphology consists of nanofibers that adhere to each other in an organized way with micropores, another consists of long, and irregular arranged nanofibers that adhere to each other without pores, and a third type that consist of a smother surface with micro and nanopores (Fig. 19, b-2). In the case of the chitin sample from *T. molitor* a lamellar morphology consisting of micro and nano fibrillar structure in a fish scale shape with large porosity is present (Fig. 19, d-2.). A similar fibrillar fish scale shape was found in chitin extracted from adult specimens of *V. crabo* species, however no porosity was observed [193]. SEM images of *T. molitor* larvae and shrimp chitin were previously taken and lamellar microstructures were also found [185]. The chitin images obtained by SEM, demonstrated that chitin surface morphology changes from species to species and between samples from the same species. Furthermore, it is remarkable to mention that this was the first time that SEM images from chitin extracted from adult specimens of *A. domesticus* and the legs and wings of adult specimens belonging to the same species were exhibited.

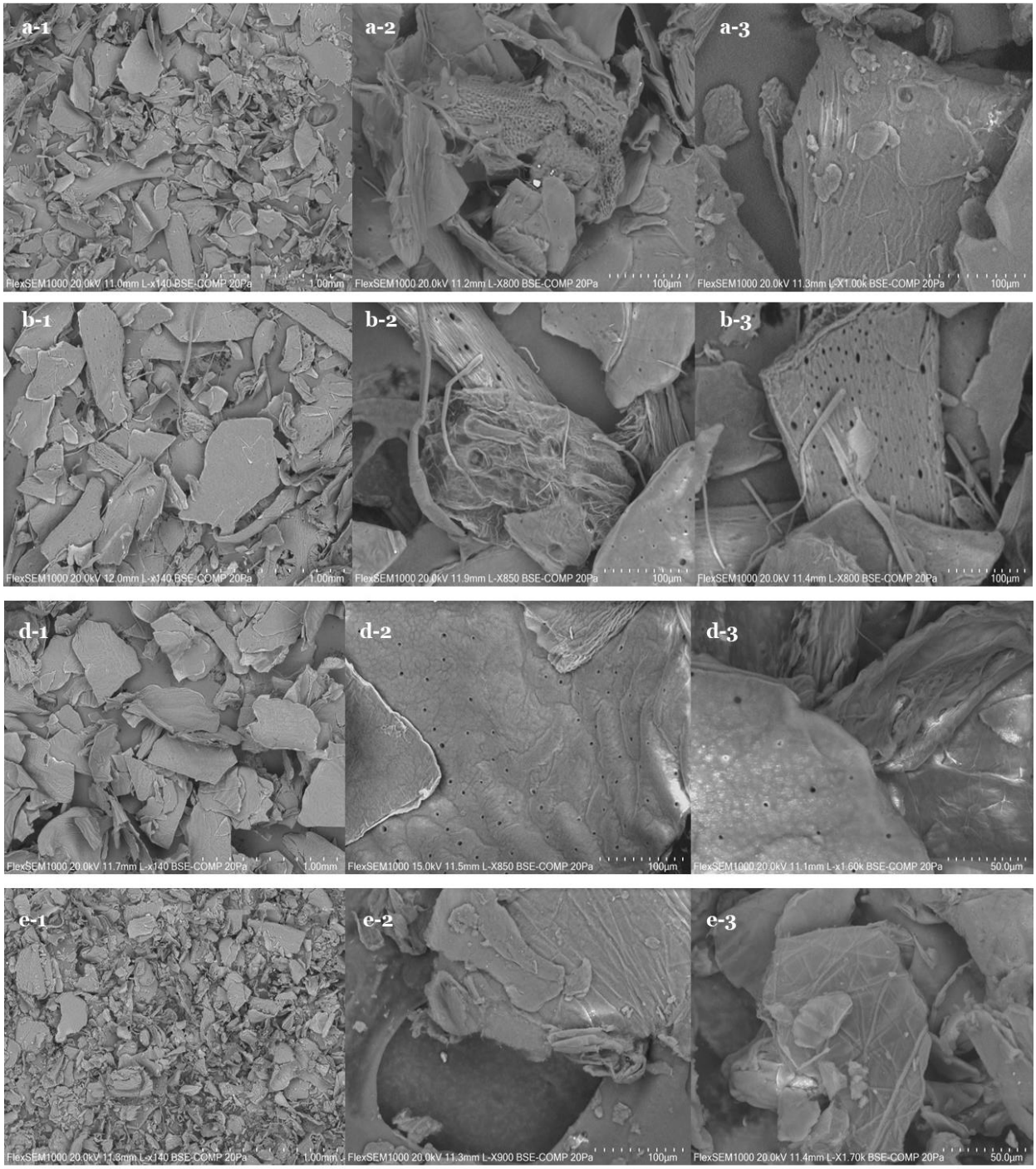

Fig. 19 - SEM images at different magnifications of chitin from: (a-1,2,3; magnification x140, x800 and x1000 respectively) - A. domesticus; (b-1,2,3; magnification x140, x850 and x800 respectively) - Wings and legs of A. domesticus; (d-1,2,3 magnification x140, x850 and x1600 respectively) - T. molitor; (e-1,2,3 magnification x140, x900 and x1700 respectively) - commercial chitin

The SEM images of chitosan (Fig. 20) revealed some major surface modifications between all the samples and also in relation to the aforementioned chitin images. Concerning the chitosan images with the lower magnifications (Fig. 20, f-1; g-1; h-1; i-1) as it happens for the chitin, the chitosan produced from the insect sources shows large flakes, while the commercial shrimp chitosan presents smaller flakes. Similar observations were reported in a comparison between chitosan produced from the cuticles of *T. molitor* larvae and commercial chitosan [111]. In addition, the commercial shrimp chitosan does not show any porosity (Fig. 20, i). *A. domesticus* chitosan exhibit larger porosity (Fig. 20, f-2) than the other samples and these were also larger than the corresponding chitin (Fig. 19, a-2, a-3). Moreover, this sample showed another structure composed of thick and tightly attached nanofibers. Chitosan derived from nymphs of *D. maroccanus* exhibited a similar surface [103]. The chitosan derived from the legs and wings of *A. domesticus* also demonstrate different types of surfaces. One type consists of irregular arranged nanofibers and micropores that adhere to each other, presenting a rough morphology. On the other hand, it is possible to observe a smoother structure, that only contain pores. A third type of morphology consisting of thick and tightly attached fibers with closed spaced porosity was revealed as well (Fig. 20, g-3). Chitosan from *T. molitor* don't suffered major modifications relatively to chitin, still being possible to observe a lamellar structure with porosity and fibrillar morphology (Fig. 20, h-2). In addition, at higher magnification, chitosan from *T. molitor* shows high porosity represented by numerous nanopores and some micropores (Fig. 20, h-3).

The SEM results from this study go along with the literature regarding insect's chitin and chitosan. Different structures consisting of pores and fibers or only pores or fibers and even none of them were described. Furthermore, as previously mentioned this was the first time that the surface morphology of chitin and chitosan derived from *A. domesticus* as well as the legs and wing of the same cricket species was described. The type of surface morphology from the obtained samples, such as the large porosity, can indicate that, it could be used for absorption of metal ions, controlled drug delivery and tissue engineering [103].

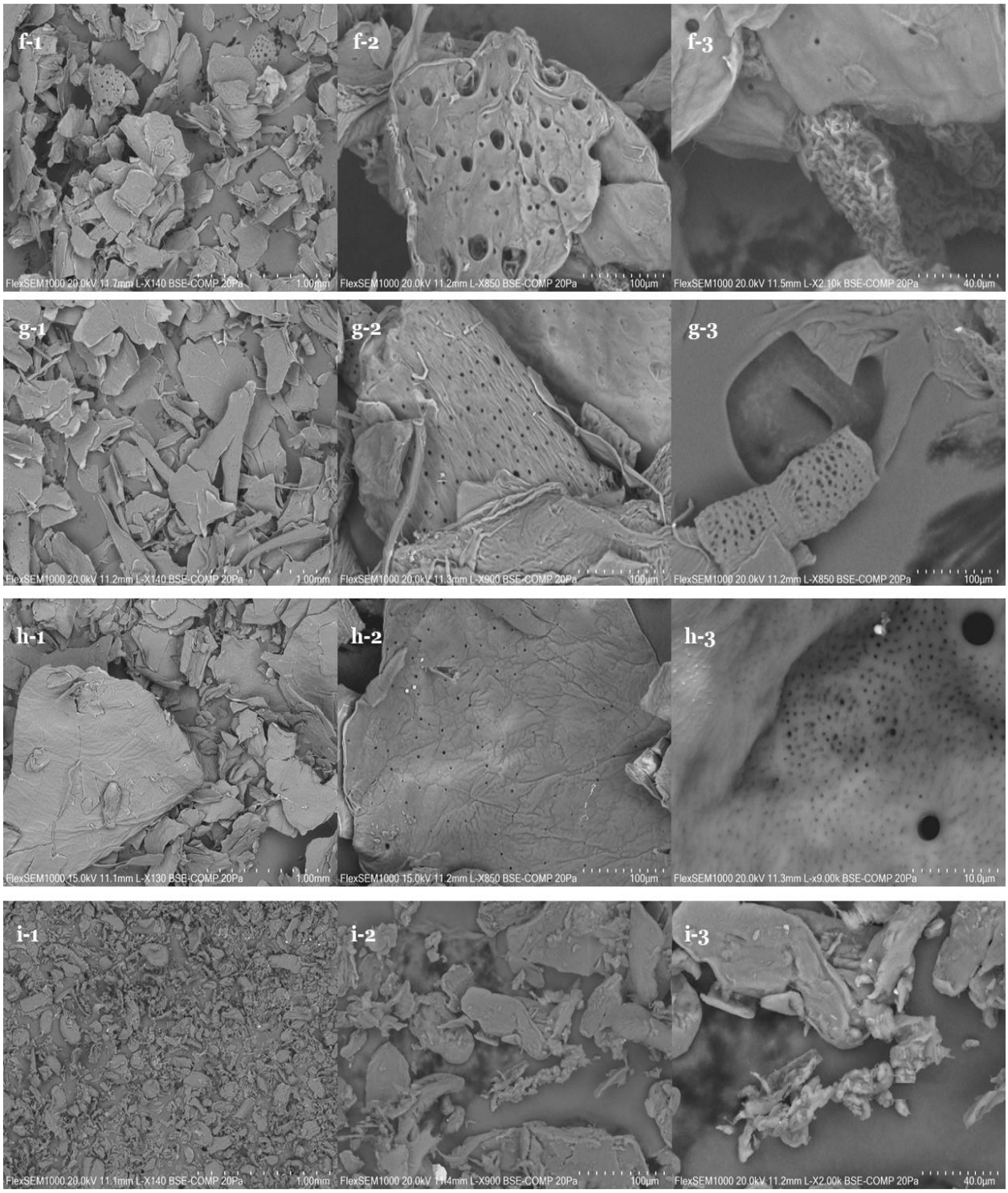

Fig. 20 - SEM images of chitosan from: (f-1,2,3 magnification x140, x850 and x2100 respectively) - A. domesticus; (g-1,2,3 magnification x140, x900 and x850 respectively) - Wings and legs of A. domesticus; (h-1,2,3 magnification x130, x850 and x9000 respectively) - T. molitor; (i-1,2,3 magnification x140, x900 and x2000 respectively) - commercial chitosan

4.3.7 Thermogravimetric analysis (TGA)

Thermal stability results are presented in Table 14 and Table 15, and were obtained by the information provided by the chitin and chitosan thermograms (section 8.4). Through DTA/TGA curves of chitin and chitosan, two distinct mass losses were found which occur with rising temperature, similar to what occurred in previous studies [78, 102, 103, 111, 163, 164, 175, 184, 189, 209]. For both chitin and chitosan, the first mass loss was observed between 50 to 100ºC and is caused by the evaporation of the water, with mass losses ranging from 2.97% (TM) to 6.22% (AD) for chitin and 5.43% (TM) to 9.04% (Commercial) for chitosan. The second mass loss ranged between 250 to 400 ºC in the case of the chitin, and 250 to 380 º C for chitosan. This second step occurs due to the decomposition of chitin and chitosan structures, caused by the depolymerization/decomposition of polymer chains through deacetylation and cleavage of glycosidic linkages [102]. Chitin's second mass loss ranged from 76.08% (L&W) to 78.68% (commercial sample), while the chitosan's second mass loss ranged from 54.95% (L & W and AD) to 57.28% (commercial sample)

Table 14 - Chitin TGA results: Weight losses in 1st and 2nd stage and maximum degradation temperatures (DTGmax)

Chitin	1º stage (50-100ºC) – Weight loss (%)	2º stage (250-400ºC) - Weight loss (%)	DTGmax peak (ºC)
TM	2.97	76.48	394
AD	6.22	76.69	394
L&W	4.89	76.08	395
Commercial	4.85	78.68	397

Table 15 - Chitosan TGA results: Weight losses in 1st and 2nd stage and maximum degradation temperatures (DTGmax)

Chitosan	1º stage (50-100ºC) – Weight loss (%)	2º stage (250-380ºC) - Weight loss (%)	DTGmax peak (ºC)
TM	5.43	55.53	314
AD	5.81	54.95	313
L&W	5.81	54.95	313
Commercial	9.04	57.28	303

The maximum degradation temperatures (DTGmax) for the chitin samples were 394 ºC, 394 ºC, 395 ºC and 397 ºC, for TM, AD, L&W, and the commercial sample respectively. The chitosan DTGmax were 314 ºC, 313 ºC, 313 ºC and 303 ºC, for TM, AD, L&W, and the commercial sample respectively. Although no major differences in the DTGmax values of chitin samples were verified, the sample that showed higher thermal stability was the commercial chitin sample (DTGmax of 397 ºC), however in the case of the chitosan samples, the sample that showed higher thermal stability was TM (DTGmax of 314 ºC). The higher thermal stability of insect chitosan in comparison with the commercial chitosan is potentially caused by the higher molecular weight and crystallinity, which also explains the higher thermal stability of the chitin samples (presence of minerals) compared to the chitosan. Once the side chain of chitin suffers deacetylation to form chitosan, the crystalline order decrease and so the thermal stability decrease too. The values for the DTGmax obtained in this study are similar to the decomposition temperature values of α-chitin and chitosan present in the literature, that were reported to be between 300-400 ºC for chitin, and between 280 to 308 ºC for chitosan [78, 190]. In a study regarding chitin extraction from *T. molitor* assisted by enzymatic deproteinization, a DTGmax value of 328.7 ◦C was reported with 70.9% of weight loss in the second stage, while chitosan showed a DTGmax value of 314.7 ºC with 66.1% of weight loss in the second stage [111]. Kabalak, et al. [104] reported a weight loss in the second stage of degradation for chitin extracted from the European mole cricket (*G. gryllotalpa*) of 70% with a DTGmax value of 374.6 °C. Additionally, Kaya, et al. [78] observed in chitin extracted from seven different Orthoptera species DTGmax values ranging from 381 to 385 ºc. The extracted insect chitin has a similar thermal behaviour as the commercial chitin and chitins extracted from other insect and crustacean sources, which supports the XRD and FTIR-ATR information, indicating that the chitin extracted in this study present α-form [158, 252, 253] .

4.4 Chitosan biological properties

4.4.1 Antioxidant properties

4.4.1.1 DPPH Scavenging activity

To evaluate the radical scavenging activity of the different chitosan samples, the 2,2-diphenyl1-picrylhydrazyl (DPPH) method was carried out as described in section 3.5.2.1.1. All the tested samples including the commercial chitosan presented poor antioxidant activity (AAI < 0.5), and high IC50 values, which indicates that the tested chitosan samples aren't able to scavenge radicals. The poor antioxidant activity can may be due to the high molecular weight of the different samples, which difficult its antioxidant activity as stated in the literature [254].

4.4.1.2 B-carotene bleaching activity

The β-carotene bleaching activity test was performed to evaluate the ability of the chitosan samples to inhibit lipid peroxidation. The results of the antioxidant activity of the different chitosan samples in terms of percentage of inhibition of linoleic acid molecules oxidation is shown in Fig. 21.

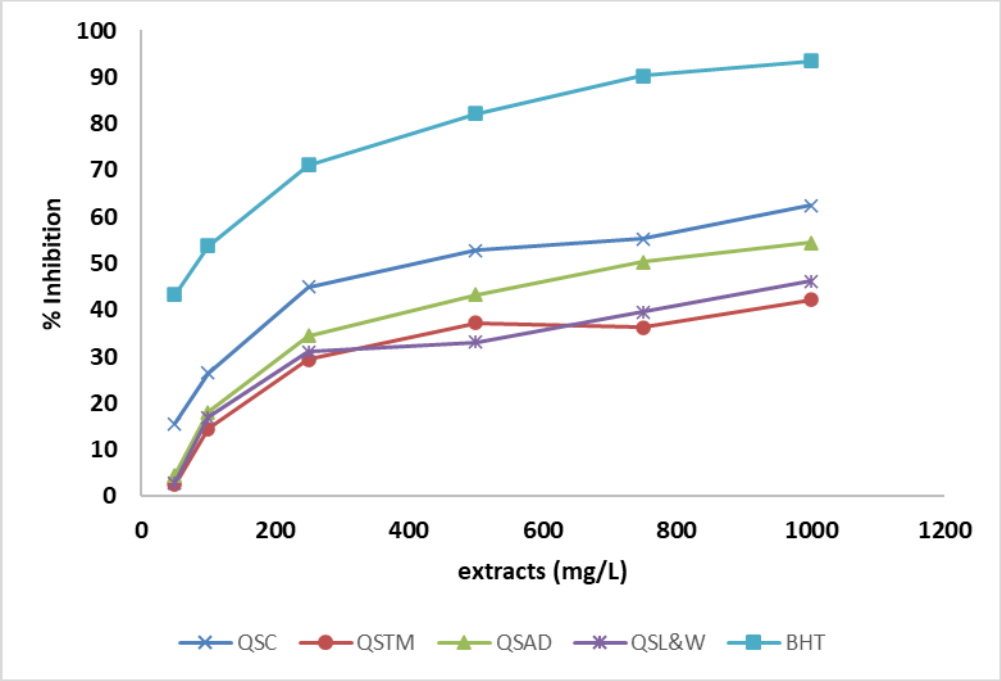

Fig. 21 - Antioxidant activity of chitosan extracts by β-carotene bleaching test. QSC – Commercial chitosan; QSTM – T. molitor chitosan; QSAD – A. domesticus chitosan; QSL&W – Legs & Wings chitosan; BHT – Control

Table 16 - IC50 (mean ± s.d.) values of the different chitosan samples in β-carotene bleaching test. Mean values from duplicates. a, b, c – homogeneous groups according to Tukey0s t test (p < 0.05). AD – A. domesticus; TM; T. molitor; L&W – legs and wings from A. domesticus

Samples	IC50 (mg/L)
BHT (control)	$76.95^{c} \pm 6.17$
AD chitosan	$776.19^{b} \pm 0.97$
TM chitosan	$1092.89^{a} \pm 18.83$
L&W chitosan	$1024.60^{a} \pm 97.07$
Commercial chitosan	$606.26^{b} \pm 6.06$

The synthetic antioxidant BHT (positive control), is a powerful antioxidant and so the difference to the chitosan samples is large as it is possible to see by the much larger percentage of inhibition (93.4% at the highest concentration tested) as well as the much lower IC50 value (76.95 mg/L) (Table 16). Nevertheless, all the chitosan samples showed capacity to inhibit the lipid peroxidation, with the commercial shrimp chitosan presenting the highest percentage of inhibition (62.3%) followed by the chitosan extracted from the AD source (54.5%). On the other hand, neither *T. molitor* nor Legs & Wings chitosan reached 50% inhibition. All the chitosan sources showed much higher values of IC50 in comparison to the BHT, with commercial chitosan (606.26 mg/L) and *A. domesticus* chitosan (776.19 mg/L) exhibiting the lowest IC50 values while both *T. molitor* and Legs & Wings chitosan had IC50 values above 1000 mg/L. The antioxidant activity was also confirmed by visualization of the different sample tubes in the experiment, since they remained the orange color of the β-carotene, demonstrating that lipid peroxidation did not occur, otherwise the color would change to yellow.

4.4.2 Antimicrobial activity

The antimicrobial activity of the different chitosan extracts was assessed by two different methodologies, the disc diffusion assay and the resazurin microtiter method. The samples were compared with large spectra antibiotics and commercial shrimp chitosan. The results from both assays are present in Table 17 and Table 18. The negative control containing pure DMSO (20 µL/disc) did not show any inhibitory effect. The chitosan extracts demonstrated small or no inhibition on all the tested organisms. Neither the commercial sample nor the chitosan extracts showed any antifungal effect on the yeast *Candida albicans* ATCC 90028 (inhibition zone diameter of 6.00 mm and Minimum Inhibition Concentration above 10 mg/mL). Additionally, the same lack of inhibitory effect was verified for the Gram-negative bacteria *Salmonella Typhimurium* ATCC 13311 and the Gram-positive bacteria *Listeria monocytogenes* LMG 16779. Although the diameter of the inhibition zones shown by the different chitosan samples against both Gram-negative and Gram-positive bacteria is similar it showed significant statistical differences and the inhibition zone diameters are slightly higher in the case of Gram-negative bacteria. The largest inhibition zone diameter (10,63 ± 0,67) was observed in the chitosan (L&W) tested against the Gram-negative bacteria *Klebsiella pneumoniae* ATCC 13883, and all the chitosan extracts demonstrated inhibition against this bacterium, which suggests higher susceptibility for this strain in comparison to the others. All the chitosan samples showed a MIC value of 5 mg/mL for this bacterium. For *Staphylococcus aureus* ATCC 29213, L&W and *A. domesticus* chitosan demonstrated higher inhibitory effects (inhibition zone diameters of 9.43 and 9.01 mm, respectively and MIC of 5 mg/mL) than commercial and *T. molitor* chitosan (which did not have any inhibitory effect). For *Bacillus cereus* ATCC 11778 only *A. domesticus* chitosan did not have an inhibitory effect but the highest inhibition zone diameter was only 8.01 mm (L&W). Lastly, for *Escherichia coli* ATCC 25922 only L&W chitosan didn´t show any inhibitory effect although the highest inhibition zone diameter observer was 8.04mm (*A. domesticus).* However, for all the tested bacteria the antibiotic (Tetracycline) demonstrated much higher inhibition zone diameters and much lower MIC values than insect or commercial. Although the efficacy of chitosan in inhibit microorganisms such as bacteria and fungus rely on several factors such as DD, Mw, chitosan concentration, methodology applied, insect source among other factors (section 1.3.5.3.2), similar conclusions were reported on literature. Kaya, et al. [209] stated that chitosan extracted from cosmopolitan Orthoptera species revealed higher antimicrobial activity against Gram-negative bacteria than Gram-positive bacteria, which are somewhat in accordance with the obtained results of the present study (better inhibitory effects were verified for

gram-negative bacterium Klebsiella *pneumonia*. Shin, et al. [166], reported similar results in the disc diffusion assay for chitosan extracted from mealworm beetle tested against *S. aureus, E. coli* and *B. cereus*, even though the authors reported an inhibitory effect against *L. monocytogenes*, which did not occur in the present study. Due to the different factors that contribute to the antimicrobial activity of chitosan, several studies reported higher antimicrobial activity than those from this study [96, 167]. Moreover, chitosan extracted from the Colorado potato beetle presented antifungal activity against *Candida Albicans,* which did not occur in this study [175]. Probably one of the main reasons for these results is due to the negative control utilized in this study (DMSO), which differs from the majority of studies that used acetic acid as negative control, achieving a better dissolution of chitosan particles. In the present study it was decide not to use an acetic acid solution for this purpose, since the acetic acid is known as an effective inhibitor of several bacteria strains [209]. Moreover, if the dissolution of the chitosan particles was better, perhaps the chitosan concentration could be raised and consequently the antimicrobial activity will improve as stated by Shin, et al. [166]. Nevertheless, Basseri, et al. [95] achieved similar results to this study, since the authors reported inhibition diameter zones between 7-10 mm for chitosan extracts of American and German cockroach, against 2 Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and one Gram-positive (*S. aureus*), and nonfungicidal activity against *C. albicans* or *C. auris*.

Table 17 - Diameter of inhibition zones (mm) of chitosan extracts (mean ± s. d.), n=3, disc diameter=6 mm. a, b, c homogeneous groups in each column according to Tukey´s t test. A, B, C homogeneous groups in each row according to Tukey`s t test. n.s. – nonsignificant

Samples (4 mg/disc)	**Gram-negative**			**Gram-positive**			***Yeast***
	Escherichia coli ATCC 25922	*Klebsiella pneumoniae* ATCC 13883	*Salmonella* Typhimurium ATCC 13311	*Bacillus cereus* ATCC 11778	*Staphylococcus aureus* ATCC 29213	*Listeria monocytogenes* LMG 16779	*Candida albicans* ATCC 90028
commercial	$7.02^{b;\,C}$ ± 0.19	$10.52^{a;\,A}$ ± 0.57	$6.00^{n.s}$ ± 0.00	$7.70^{b,a;\,B}$ ± 0.14	$6.00^{n.s;}$ ± 0.00	$6.00^{n.s}$ ± 0.00	$6.00^{n.s}$ ± 0.00
AD	$8.00^{a;\,C}$ ± 0.10	$9.63^{a;A}$ ± 0.58	$6.00^{n.s}$ ± 0,00	$6.00^{n.s}$ ±0.00	$9.05^{a;B}$ ± 0.30	$6.00^{n.s}$ ± 0.00	$6.00^{n.s}$ ± 0.00
L&W	6.0^{c} ± 0.00	$10.63^{a;\,A}$ ± 0.67	$6.00^{n.s}$ ± 0.00	$8.01^{a;\,C}$ ± 0.58	$9.39^{a;\,B}$ ± 0.40	$6.00^{n.s}$ ± 0.00	$6.00^{n.s}$± 0.00
TM	$8.19^{a;\,A}$ ± 0.17	$8.00^{b;\,A}$ ± 0.10	$6.00^{n.s}$ ± 0.00	$7.03^{b;\,B}$ ± 0.36	$6.00^{n.s}$ ± 0.00	$6.00^{n.s}$ ± 0.00	$6.00^{n.s}$ ± 0.00
DMSO (20 µL/disc)	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
Tetracycline (30 µg/disc)	23.25 ± 0.50	22.25 ± 0.50	11.50 ± 0.58	30.00 ± 0.82	30.25 ± 0.50	18.25 ± 0.60	-

Samples (4 mg/disc)	Gram-negative			Gram-positive			*Yeast*
	Escherichia coli ATCC 25922	*Klebsiella pneumoniae* ATCC 13883	*Salmonella* Typhimurium ATCC 13311	*Bacillus cereus* ATCC 11778	*Staphylococcus aureus* ATCC 29213	*Listeria monocytogenes* LMG 16779	*Candida albicans* ATCC 90028
Amphotericin B (25 µg/disc)	-	-	-	-	-	-	20.33 ± 0.58

Table 18 - MIC values (mg/mL) of chitosan extracts (Modal values). >10 = didn´t presented inhibitory activity.

Samples (4 mg/disco)	Gram-negative			Gram-positive			Yeast
	Escherichia coli ATCC 25922	*Klebsiella pneumoniae* ATCC 13883	*Salmonella* Typhimurium ATCC 13311	*Bacillus cereus* ATCC 11778	*Staphylococcus aureus* ATCC 29213	*Listeria monocytogenes* LMG 16779	*Candida albicans* ATCC 90028
commercial	10	5	>10	10	>10	>10	>10
AD	10	5	>10	>10	5	>10	>10
L&W	10	5	>10	10	5	>10	>10
TM	10	10	>10	10	>10	>10	>10
Tetracycline (µg/mL)	0.06	0.06	0.24	0.06	0.06	0.06	-
Amphotericin B (µg/mL)	-	-	-	-	-	-	0.25

5. Conclusions and future Prospects

Chitin and chitosan are two biopolymers with great physiochemical and biological properties which make them extremely versatile to be used in sectors such as agricultural, biomedical, pharmaceutical, food, amongst others. This work aimed to evaluate the viability of using different insect sources as raw material for the production of chitin and chitosan.

The extraction of chitin from the different insect sources (*T. molitor* (larvae), *A. domesticus* (adult specimens) and *A. domesticus* by-products (legs and wings)), was achieved throughout an optimized acid/alkali treatment to defatted insect samples where an additional decolorization step was added. The chitosan was produced using a strong alkali solution of NaOH.

An eco-friendly chitin extraction approach based on natural deep eutectic solvents (NADES) was also employed. However, it was unsuccessful due to the higher yields obtained which indicated high impurity and thus, further samples characterization was abandoned. Future research on the NADES preparation, production of different NADES systems as well as in the chitin extraction protocol will be necessary to improve chitin extraction in order to obtain chitin with high purity.

Afterwards, the physicochemical and biological characterization of the extracted samples was performed and a comparison with commercially available shrimp chitin and chitosan was done. The defatting step on the samples pre-treatment demonstrated that by applying this step, chitin samples achieve higher extraction yields than using the non-defatted samples. The color results showed that de decolorization step was effective in the remotion of most of the pigments. The higher chitin extraction yield was achieved on L&W, which demonstrates the potential of using insect by-products as sources for chitin extraction.

The isolation method for chitin was successfully applied and confirmed by FTIR-ATR, FT-RAMAN and XRD mainly. The results from the three analyses indicate that the chitin from all the insect sources was in α-form, which have also been reported before for chitin extracted from other insects. Moreover, spectra of the FTIR-ATR and FT-RAMAN analysis displayed similarity between the insect samples and the commercial ones. This was also verified for the X-ray diffraction analysis (XRD), which in addition showed greater amorphous structure of chitosan in relation to chitin.

The surface morphology of the extracted samples, analysed by scanning electron microscopy (SEM), revealed to be irregular in all analysed samples. Both insect chitin

and chitosan samples showed a surface composed of pores and fibers, which goes alongside with the reports on insect chitin and chitosan literature. SEM images also revealed major differences between the insect samples and the commercial shrimp samples, in which no porosity was found. The thermogravimetric analysis (TGA) results exposed the thermal degradation behaviour of both chitin and chitosan (insects and commercial), which occurred in two mass loss stages the first which is attributed to the water loss and second which is ascribed to the polymer structural decomposition. Chitin due to its higher crystalline structure have higher thermal stability (superior $DTG_{máx}$ peaks) than chitosan. Moreover, all insect chitin samples exhibited higher thermal stability than the chitosan samples.

The molecular weights of the different obtained chitosan's varied between 245.77 kDa for the L&W and 332.58 kDa for the *A. domesticus* (whole body). These results demonstrate that the obtained samples are characterized by medium and high molecular weight chitosan. Furthermore, the commercial shrimp sample also revealed medium molecular weight (292.38 kDa) and relatively close proximity to the insect's chitosan molecular weight values.

Although no significant statistical differences were observed, insect chitosan with highest DD was the chitosan from *A. domesticus* whole body (63.7%) followed by the chitosan from *T. molitor* (62.3%) and lastly the chitosan from L&W (62.0%). The DD of the commercial shrimp chitosan was slightly higher (69.4%). These results together with the FTIR-ATR spectra of chitosan showed that though insect chitin samples suffered deacetylation, the degree of deacetylation wasn´t high enough to be superior to the commercial chitosan and are characterized as low degree of deacetylation. Future optimizations to the deacetylation method will be needed to raise de DD of the insect chitosan. Those optimizations will consist mainly in raising the time of reaction and perhaps the NaOH concentration.

The antioxidant activity of the chitosan samples was evaluated by the DPPH method and β-carotene bleaching test. While in the DPPH method no antioxidant activity was observed, showing that the samples aren´t capable of scavenging radical, owing probably to their high molecular weight and low degree of deacetylation, in the β-carotene bleaching test method, antioxidant activity was found. This test revealed that all the chitosan samples are able to inhibit the lipid peroxidation and perhaps they can be good substitutes of the common utilized synthetic antioxidant like the BHT on food. However, this replacement demands further research, to ensure the food safety and also to find the appropriated chitosan dose concentration once chitosan has a weaker antioxidant activity than BHT.

The antimicrobial assays revealed that insect chitosan had antimicrobial activity against some of the studied gram positive and negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Bacillus cereus* ATCC 11778 and *Staphylococcus aureus* ATCC 29213) but non antifungal activity against *Listeria monocytogenes* LMG 16779. In comparison with the antibiotics used as control, the chitosan, showed much lower antibacterial activity, however in comparison with the commercial sample the antimicrobial activity was similar. As referred earlier the antimicrobial activity of chitosan and the mechanism of action are extremely dependent on the molecular weight and degree of deacetylation. Thus, in order to improve this activity in the future, the deacetylation reaction should be improved to decrease the values of these two factors.

In sum, the present study revealed that insect sources can be an outstanding alternative to crustacean sources for the production of chitin and chitosan. Furthermore, by-products of *A. domesticus* were studied for the first time as a source of chitin and chitosan. These by-products proved to be an alternative to be considered in the future, either due to the high yields that can be obtained, without significantly affecting the quality of the final products, or due to the fact that the circular economy principle is applied.

6. Outputs

- **Abstracted submitted and accepted for oral communication in the international conference "EFFoST 2022" taking place between 7 to 9 November in Ireland:**
  Title: "Chitin and chitosan extraction from edible insects: characterization and comparison between different species and by-products"
- **The systematic review approached during the introduction text is being included on a larger review performed for a review article which includes information of other two insects' fractions (proteins and lipids) and it will be submitted for the food science journal, "Critical Reviews in Food Science and Nutrition".**

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8. Annexes

8.1 Diagram of the obtained articles from the review search

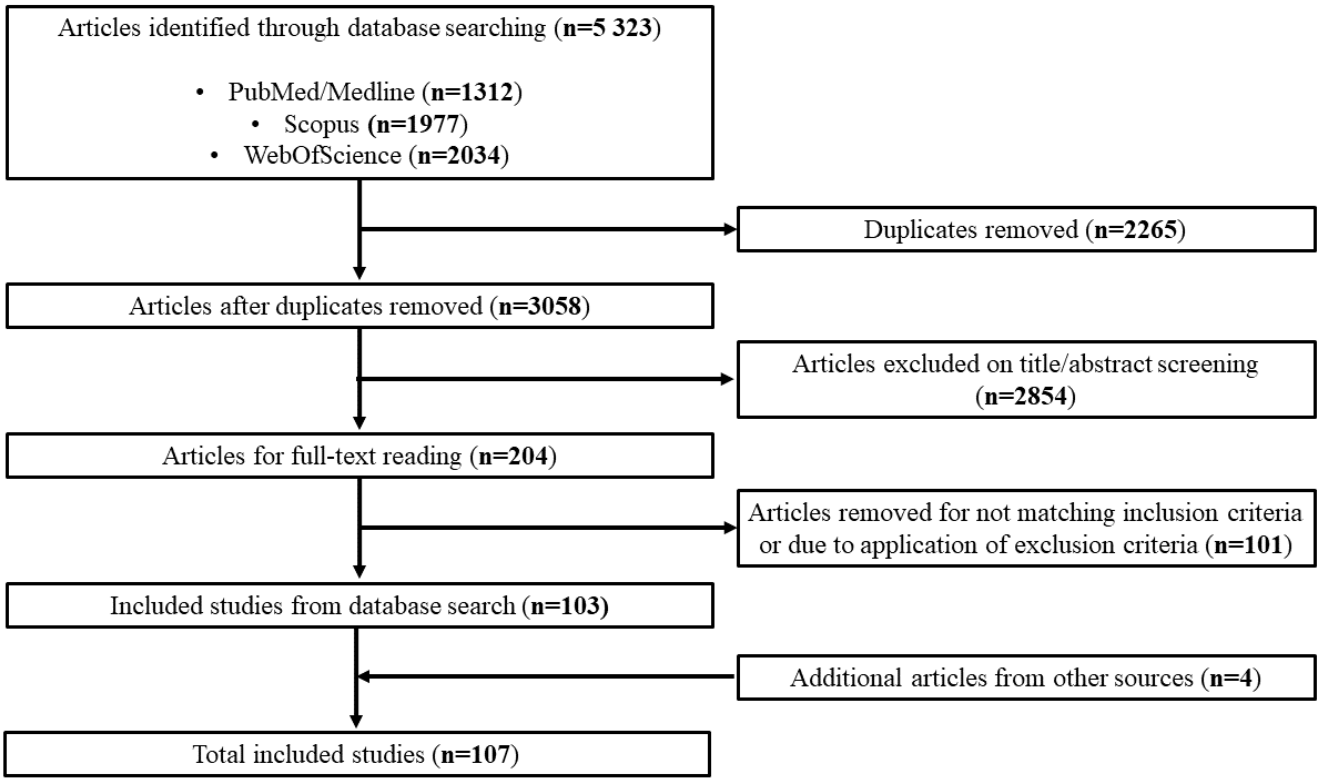

Fig S. 1 Flow chart of study inclusion/exclusion from the systematic review performed on chitin and chitosan extraction

8.2 FT-RAMAN Spectra of commercial chitosan samples

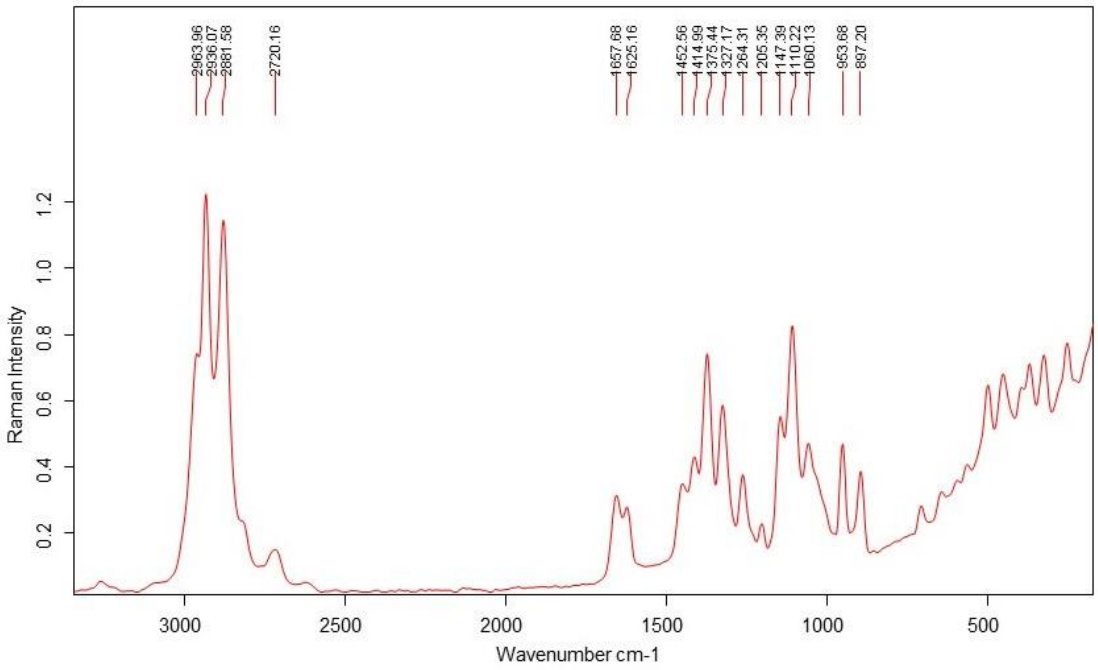

Fig S. 2 - FT-RAMAN spectrum of commercial chitin samples. Characteristic peaks of α-chitin marked on the spectrum

8.3 Supplementary table

Tab S. 1 List of studies included on the systematic review with information pertaining species, pre-treatments, extraction methods and yields, degree of acetylation and deacetylation and characterization methods on each study

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
Blattodea									
(S. C. Chen et al., 2021)	American cockroach (*Periplaneta americana*) adult	Dried, ground and defatted	Chemical treatment (NaOH and HCl) followed by bleaching (H_2O_2)	NaOH (25M/130°C/2H)	74.53% (DA)	90.85% (DDA)	Mw FTIR XRD SEM	Antioxidant Antibacterial	Chitosan films for food packaging with antioxidant and antibacterial activity and resistance to UV light
(Kamal et al., 2020)	*P. americana* adult	Dried	Alkaline treatment (NaOH)	NA	103% (DA)	NA	FTIR XRD SEM EA	ND	Chitin as a substrate for bioethanol production
(Kaya & Baran, 2015)	*P. americana* adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	Wings - 18% (Yield)/98.67% (DA) Other parts - 13% (Yield)	NA	FTIR XRD TGA SEM EA	ND	ND
(Kovaleva et al., 2016)	*P. americana* adult	Ground	Chemical treatment (NaOH and HCl) followed by defatting and bleaching (H_2O_2)	NA	38% (Yield)	NA	FTIR EA	ND	ND
(Basseri et al., 2019)	*P. americana* adult	Dried and ground	Acid treatment (HCl and oxalic acid) followed by bleaching (NaClO)	NaOH (50%/100°C/4H)	15% (Yield)	2.8% (Total yield) 32.1% (DDA)	FTIR XRD	Antibacterial Antifungal	ND
(Basseri et al., 2019)	*P. americana* nymphs	Dried and ground	Acid treatment (HCl and oxalic acid) followed by bleaching (NaClO)	NaOH (50%/100°C/4H)	8.4% (Yield)	4% (Total yield) 31.0% (DDA)	FTIR XRD	Antibacterial Antifungal	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
(Kaya et al., 2017)	Brazilian cockroach (*Blaberus giganteus*) adult	Ground	Chemical treatment (HCl and NaOH) followed by bleaching (chloroform:methanol:water ,1:2:2 OR NaClO)	NA	Dorsal proton - 21.2% (Yield) Wings – 26.9% (Yield)	NA	FTIR TGA SEM Water contact angle Optical transmittance Mechanical properties	Antibacterial Antifungal	Chitin films with antibacterial activity against pathogenic bacteria and antifungal activity
(Basseri et al., 2019)	German cockroach (*Blatella germanica)* adult	Dried and ground	Acid treatment (HCl and oxalic acid) followed by bleaching (NaClO)	NaOH (50%/100°C/4H)	6.2% (Yield)	2.8% (Total yield) 37.3% (DDA)	FTIR XRD	Antibacterial Antifungal	ND
(Basseri et al., 2019)	*B. germanica* nymph	Dried and ground	Acid treatment (HCl and oxalic acid) followed by bleaching (NaClO)	NaOH (50%/100°C/4H)	5.4% (Yield)	2.6% (Total yield) 39.2% (DDA)	FTIR XRD	Antibacterial Antifungal	ND
Coleoptera									
(Marei et al., 2019)	Beetle (*Calosoma rugosa*) exoskeleton	NA	Chemical treatment (NaOH and HCl)	NaOH (50%/100°C/8H)	NA	95% (DDA)	FTIR XRD	Antibacterial	Chitosan nanoparticles (CSNPs) enhanced drug delivery, allowed its controlled release as well as enhancing its antibacterial activity.
(Marei et al., 2016)	*C. rugosa* exoskeleton	NA	Chemical treatment (NaOH and HCl)	NaOH (50%/100°C/8H)	5% (Yield)	95% (DDA)	Ash and moisture content WBC/FBC FTIR XRD SEM	ND	ND
(M. Kaya et al., 2014)	Colorado potato beetle (*Leptinotarsa*	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching	NaOH (50%/100°C/3H)	20% (Yield)	72% (Chitin yield) 71% (DDA)	FTIR XRD TGA SEM	Antioxidant Antibacterial Antifungal	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
	decemlineata) adult		(methanol:chloroform:water , 2:1:4)				EA Mw		
(M. Kaya et al., 2014)	*L. decemlineata* larvae	Dried and ground	Chemical treatment (HCL and NaOH) followed by bleaching (methanol:chloroform:water , 2:1:4)	NaOH (50%/100°C/3H)	7% (Yield)	67% (Chitin yield) 64% (DDA)	FTIR XRD TGA SEM EA Mw	Antioxidant Antibacterial Antifungal	ND
(Murat Kaya et al., 2016)	Comb-clawed beetle (*Omophlus lepturoides*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water , 2:1:4)	NA	ND	NA	FTIR TGA XRD SEM Surface area, pore volume and size	BSA adsorption capacity	ND
(Liu et al., 2012)	Dark black chafer beetle (*Holotrichia parallela*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching ($KMnO_4$)	NA	*ca.* 15% (Yield)	NA	Nitrogen, ash and moisture content FTIR XRD SEM EA	ND	ND
(Kaya et al., 2018)	European rhinoceros beetle (*Oryctes nasicornis*) elytra	Dried	Bleaching (NaClO) followed by chemical treatment (HCL and NaOH)	NA	ND	NA	FTIR TGA DSC NMR AFM Light transmittance Contact angle Mechanical properties	ND	Natural chitin films with better properties than synthetic chitin films
(Kabalak et al., 2020)	European stag beetle (*Lucanus cervus*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching	NA	10.9% (Yield)	NA	FTIR XRD TGA	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
			(methanol:chloroform:water, 2:1:4)				SEM EA		
(Fournier et al., 2020)	Goliath beetle (*Goliathus orientalis*) adult	Dried	Chemical treatment (HCl and NaOH) followed by bleaching (H_2O_2)	NaOH (50%/95°C/Overnight)	16% (Yield)	80% (Chitin yield) 78.3% (DDA)	FTIR TGA SEM	ND	ND
(Murat Kaya et al., 2014)	Great silver water beetle (*Hydrophilus piceus*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NaOH (60%/120°C/2H)	19-20% (Yield)	74% (Chitin yield)	FTIR TGA XRD SEM	NA	ND
(Shin et al., 2019)	Japanese rhinoceros beetle (*Allomyrina dichotoma*) adult	Dried and ground	Chemical treatment (NaOH and HCl)	NaOH (55%/90°C/9H)	14.20% (Yield)	75% (Chitin yield) 74.66% (DDA)	FTIR XRD	Antibacterial	ND
(Shin et al., 2019)	*A. dichotoma* larvae	Dried and ground	Chemical treatment (NaOH and HCl)	NaOH (55%/90°C/9H)	10.53% (Yield)	83.37% (Chitin yield) 75.66% (DDA)	FTIR XRD	Antibacterial	ND
(Shin et al., 2019)	*A. dichotoma* pupae	Dried and ground	Chemical treatment (NaOH and HCl)	NaOH (55%/90°C/9H)	12.70% (Yield)	83.37% (Chitin yield) 75.67% (DDA)	FTIR XRD	Antibacterial	ND
(Ma et al., 2015)	Molossus beetle (*Catharsius molossus*) waste	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching ($KMnO_4$ and oxalic acid)	NaOH (18M/90°C/24H)	24% (Yield)	17% (Yield) 94.9% (DDA)	Moisture, protein, acid insoluble and ash content As and heavy metal content FTIR XRD TGA SEM Mw Spectrophotometry	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
							Potentiometric titration Enzyme capacity resistance Mechanical characterization Adhesion *in vitro* determination Rheological properties		
(Ma et al., 2012)	*C. molossus* residues	Ground	Chemical treatment (HCL and NaOH) followed by bleaching ($KMnO_4$ and oxalic acid)	NaOH	ND	ND	FTIR XRD	ND	ND
(Kabalak et al., 2020)	Pine chafer (*Polyphylla fullo*) adult	Dried and ground	Chemical treatment (HCL and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	11.3% (Yield)	NA	FTIR XRD TGA SEM EA	ND	ND
(Shin et al., 2019)	Superworm (*Zophobas morio*) adult	Dried and ground	Chemical treatment (NaOH and HCl)	NaOH (55%/90°C/9H)	3.90% (Yield)	83.33% (Chitin yield) 75.67% (DDA)	FTIR XRD	Antibacterial	ND
(Soon et al., 2018)	*Z. morio* larvae	Dried and ground	Chemical treatment (HCL and NaOH) followed by bleaching (glacial acetone)	NaOH (50%/90°C/30H)	4.77-5.43% (Yield) 82.39%-101.39% (DA)	65.84-75.88% (Chitin yield) 64.82%-81.06% (DDA)	FTIR XRD EA TGA DSC SEM	Antioxidant Metal chelating	ND
(Murat Kaya et al., 2014)	Water beetle (*Agabus bipustulatus*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching	NaOH (60%/120°C/2H)	14-15% (Yield)	71% (Chitin yield)	FTIR TGA XRD SEM	NA	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
			(methanol:chloroform:water , 2:1:4)						
(M. Kaya, E. Bulut, et al., 2016)	White-grub cockchafer (*Melolontha melolontha*) adult	Dried and split in different body parts	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water , 2:1:4)	NA	5.87% (Yield – Female antennae) 40.1% (Yield – Female elytra)	NA	FTIR XRD TGA SEM	BSA - Protein adsorption capacity	ND
(Saenz-Mendoza et al., 2020)	Yellow mealworm (*Tenebrio molitor*) adult	Dried and ground	Chemical treatment (HCl and NaOH)	NaOH (60%/120°C/2H) + $NaBH_4$	ND	88.55% (DDA)	FTIR XRD SEM Mw Physicochemical characterization and rheological properties Mechanical properties Water vapor permeability Solubility	ND	Chitosan films could be effective as packaging in foods sensitive to UV radiation
(Shin et al., 2019)	*T. molitor* adult	Dried and ground	Chemical treatment (NaOH and HCl)	NaOH (55%/90°C/9H)	8.40% (Yield)	78.33% (Chitin yield) 75.63% (DDA)	FTIR XRD	Antimicrobial	ND
(Yu et al., 2021)	*T. molitor* adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching ($KMnO_4$ and oxalic acid)	NA	11.79% (Yield)	NA	ND	ND	ND
(Luo et al., 2019)	*T. molitor* larvae	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching ($KMnO_4$ and oxalic acid)	NaOH (60%/100°C/8H)	ND	2.5% (Total yield) 85.9% (DDA)	Solubility WBC FBC Ash and moisture content Mw FTIR XRD	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
							TGA SEM Rheological properties		
(Shin et al., 2019)	*T. molitor* larvae	Dried and ground	Chemical treatment (NaOH and HCl)	NaOH (55%/90°C/9H)	4.60% (Yield)	80% (Chitin yield) 75.59% (DDA)	FTIR XRD	Antimicrobial	ND
(Son et al., 2021)	*T. molitor* larvae	Dried, ground and defatted	Chemical treatment (NaOH and HCl)	NaOH (50%/80°C/4H)	4.72% (Yield) 98.3% (DA)	89.4% (DDA)	FTIR XRD SEM NMR EDX	Anti-inflammatory	ND
(Song et al., 2018)	*T. molitor* larvae	Dried and ground	Chemical treatment (HCl and NaOH)	NaOH (40%-60%/95°C - 105°C/3H-5H)	4.85%-5.08% (Yield)	3.65% (Total yield) 91.90%-96.19% (DDA)	ND	ND	ND
(Yu et al., 2021)	*T. molitor* larvae	Dried and ground	Chemical treatment (HCL and NaOH) followed by bleaching ($KMnO_4$ and oxalic acid)	NA	7.19-10.13% (Yield)	NA	ND	ND	ND
(Yu et al., 2021)	*T. molitor* pupae	Dried and ground	Chemical treatment (HCL and NaOH) followed by bleaching ($KMnO_4$ and oxalic acid)	NA	9.54% (Yield)	NA	ND	ND	ND
(Paes da Silva et al., 2017)	*T. molitor* larvae exuviae	NA	Bacterial fermentation	NA	17.2%-28.6% (Yield)	NA	FTIR	ND	ND
(Song et al., 2018)	*T. molitor* larvae exuviae	Dried and ground	Chemical treatment (HCl and NaOH)	NaOH (40%-60%/95°C - 105°C/3H-5H)	16.40%-20.07% (Yield)	7.80%-10.20% (Total yield) 5.76%-50.38% (DDA)	ND	ND	ND
(Lucas et al., 2021)	*T. molitor* larvae exuviae	Dried and ground	Enzymatic treatment (Alcalase)	NaOH (40%/90°C/8H)	70.9% (Yield)	31.9% (Total yield) 53.9% (DDA)	Moisture, ash, protein, and lipids content Solubility FTIR	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
							XRD TGA SEM DSC		
(Pasquier et al., 2021)	*T. molitor* larvae exuviae	Defatted	Chemical treatment (HCL and NaOH) followed by bleaching (NaCl and NaOH)	NA	ND	NA	FTIR XRD NMR Ash content	ND	Preparation of chitin nanofibers
(Li et al., 2019)	*T. molitor* waste	Dried, ground and defatted	Chemical treatment (HCL and NaOH))	NaOH (60%/130°C/7H) followed by bleaching optional solubilization (HCl and H_2O_2)	ND	Insoluble – 73.99% (DDA) Soluble – 86.6% (DDA)	Mw Solubility FTIR XRD TGA EA SEM	ND	Chitosan improved the quality of fresh-cut apples over time in regard to their color, texture and titratable acidity
(Song et al., 2018)	*T. molitor* waste	Dried and ground	Chemical treatment (HCl and NaOH)	NaOH (50%/100°C/3H)	17.32% (Yield)	14.48% (Total yield) 5.13% (DDA)	Adsorption activity	ND	ND
Diptera									
Antonov et al. (2019)	Black soldier fly (*Hermetia illucens*) adult	Dried and ground	Chemical treatment (HCl and NaOH) and defatting	NA	21.30% (Yield)	NA	ND	ND	ND
(Brigode et al., 2020)	*H. illucens* adult	Dried, ground (chemical treatment) and defatted (ADF, ADF-ADL)	Chemical treatment (HCl and NaOH) OR ADF OR ADF-ADL	NA	ADF – *ca.* 13% (Yield) ADF-ADL – *ca.* 8% (Yield) Chemical – *ca.* 8% (Yield)	NA	FTIR XRD TGA SEM EA	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
(Khayrova et al., 2020)	*H. illucens* adult	Ground	Chemical treatment (HCl and KOH OR NaOH)	NaOH (50%/100°C/2H)	7.8-20.9% (Yield)	18% (Chitin yield)	IExC	ND	ND
(Purkayastha & Sarkar, 2020)	*H. illucens* adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching ($KMnO_4$)	NA	9% (Yield)	NA	Ash content FTIR XRD TGA SEM NMR EA Surface area analysis	ND	ND
(Soetemans et al., 2020)	*H. illucens* adult	Dried and ground	Chemical treatment (HCl and NaOH)	NaOH (50%/90°C/1-3H)	5.6-8.4% (Yield)	*ca.* 70% (DDA)	FTIR XRD TGA SEM	ND	ND
(Wang et al., 2020)	*H. illucens* adult	Dried and ground	Chemical treatment (HCl and NaOH followed by bleaching (NaClO)	NA	2.9% (Yield)	NA	FTIR XRD TGA SEM	ND	ND
(Wasko et al., 2016)	*H. illucens* adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching ($KMnO_4$)	NA	ND	NA	FTIR XRD TGA SEM EA	ND	ND
(Pasquier et al., 2021)	*H. illucens* adult	Dried, ground, defatted	Chemical treatment (HCl and NaOH) followed by bleaching (NaCl)	NA	11% (Yield)	NA	FTIR XRD NMR Ash content	ND	Preparation of chitin nanofibers
(Al-saggaf, 2021)	*H. illucens* larvae	Defatted and dried	Chemical treatment (HCl and NaOH)	NaOH (60%/25°C/35min + 60%/110°C/130min)	ND	1.56% (Total yield) 91.3% (DDA)	FTIR	Antibacterial activity	Pro-synthesized Ag-nanoparticles and Chitosan as a powerful antimicrobial

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
									against skin pathogens and high capability for fast wound healing
(D'Hondt et al., 2020)	*H. illucens* larvae	Dried and ground	Chemical treatment (HCL and NaOH)	NA	6.5-8%	NA	FTIR EA HPLC	ND	ND
(Huet et al., 2021)	*H. illucens* larvae	Dried, ground and defatted	Chemical treatment (HCl and NaOH) followed by ionic liquids OR deep eutectic solvents treatment	NaOH (50%/100°C/16H)	25.5% (Yield) Il – 95% (from chitin extracted by chemical treatment) DES – 68% (from chitin extracted by chemical treatment)	Chemical treatment – 80% (Chitin yield) IL -65% (Chitin yield) DES- 69% (Chitin yield)	FTIR XRD	ND	ND
(Ravi et al., 2020)	*H. illucens* larvae	Dried, ground and defatted	Chemical treatment (HCl and NaOH)	NA	ND	NA	FTIR	ND	ND
(Smets et al., 2020)	*H. illucens* larvae	Dried, ground and defatted	Chemical treatment (HCl and NaOH) on the pellet obtained through protein extraction	NA	3.85% (Yield)	NA	FTIR	ND	ND
(Soetemans et al., 2020)	*H. illucens* larvae	Dried and ground	Chemical treatment (HCl and NaOH)	NaOH (50%/90°C/1-3H)	7.8%-9.5% (Yield)	*ca.* 70%-85% (DDA)	FTIR XRD TGA SEM	ND	ND
(Wang et al., 2020)	*H. illucens* larvae	Dried and ground	Chemical treatment (HCl and NaOH followed by bleaching (NaClO)	NA	3.6% (Yield)	NA	FTIR XRD TGA SEM	ND	ND
(Smets et al., 2020)	*H. illucens* pupae	Dried, ground and defatted	Chemical treatment (HCl and NaOH) on the pellet	NA	6.31% (Yield)	NA	FTIR	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
			obtained through protein extraction						
(Soetemans et al., 2020)	*H. illucens* pupae	Dried and ground	Chemical treatment (HCl and NaOH)	NaOH (50%/90°C/1-3H)	10.3%-10.7% (Yield)	*ca.* 75%-90% (DDA)	FTIR XRD TGA SEM	ND	ND
(Wang et al., 2020)	*H. illucens* pupae	Dried and ground	Chemical treatment (HCl and NaOH followed by bleaching (NaClO)	NA	3.1% (Yield)	NA	FTIR XRD TGA SEM	ND	ND
(Zhou et al., 2019)	*H. illucens* prepupae	Commercial defatted	Chemical treatment (HCl and NaOH) OR NADES	NA	NADES - 12.01% - 26.71% (Yield)/56.82%-95.59% (DA) Chemical - 6.50% (Yield)/82.40% (DA)	NA	FTIR XRD TGA SEM	ND	ND
(Caligiani et al., 2018)	*H. illucens* prepuae	Ground and defatted	Chemical demineralization (HCl) of pellet obtained on protein extraction (one-step with NaOH). OR Chemical demineralization (HCl) of pellet obtained on protein extraction (Osborne fractionation). OR	NA	ND	NA	ND	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
			Pellet from enzymatic hydrolysis						
(Jayanegara et al., 2020)	*H. illucens* prepupae	Defatted	Chemical treatment (HCl and KOH)	NaOH (40%/80°C/90min)	ND	61.6% (DDA)	FTIR	ND	Chitosan reduces methane emission and ruminant feed degradation
(Smets et al., 2020)	*H. illucens* prepupae	Dried, ground and defatted on the pellet obtained through protein extraction	Chemical treatment (HCl and NaOH)	NA	4.72% (Yield)	NA	FTIR	ND	ND
(Soetemans et al., 2020)	*H. illucens* prepupae	Dried and ground	Chemical treatment (HCl and NaOH)	NaOH (50%/90°C/1-3H)	9.1%-10.9% (Yield)	*ca.* 75%-85% (DDA)	FTIR XRD TGA SEM	ND	ND
(Soetemans et al., 2020)	*H. illucens* cocoons	Dried and ground	Chemical treatment (HCl and NaOH)	NaOH (50%/90°C/1-3H)	22.4%-23.8% (Yield)	*ca.* 70%-85% (DDA)	FTIR XRD TGA SEM	ND	ND
Bhavsar et al. (2021)	*H. illucens* exuviae	Dried, ground and defatted	Chemical demineralization (HCl) followed by superheated water treatment	NaOH (50%/100°C/20H)	20% (Yield)	ND	FTIR TGA SEM DSC	ND	Chitosan as a finishing agent for polyester fabrics
(Brigode et al., 2020)	*H. illucens* flakes	Dried, ground (chemical treatment) and defatted (ADF, ADF-ADL)	Chemical treatment (HCL and NaOH) OR ADF OR	NA	ADF – *ca.* 28% (Yield) ADF-ADL – *ca.* 27% (Yield) Chemical – *ca.* 20% (Yield)	NA	FTIR XRD TGA SEM EA	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
			ADF-ADL						
(Hahn et al., 2020)	*H. illucens* larvae exoskeleton	Ground	Chemical treatment (Formic acid and NaOH)	NaOH (heterogeneous OR homogenous)	28%	Heterogeneous – 8-16% Homogeneous – 4%	NA	ND	ND
(Soetemans et al., 2020)	*H. illucens* larvae exuviae	Dried and ground	Chemical treatment (HCL and NaOH)	NaOH (50%/90°C/1-3H)	23.7%-31.1% (Yield)	*ca.* 50%-85% (DDA)	FTIR XRD TGA SEM	ND	ND
(Brigode et al., 2020)	*H. illucens* pupae exuviae	Dried, ground (chemical treatment) and defatted (ADF, ADF-ADL)	Chemical treatment (HCL and NaOH) OR ADF OR ADF-ADL	NA	ADF – *ca.* 33% (Yield) ADF-ADL – *ca.* 20% (Yield) Chemical – *ca.* 25% (Yield)	NA	FTIR XRD TGA SEM EA	ND	ND
(Khayrova et al., 2020)	*H. illucens* pupae exuviae	Ground	Chemical treatment (HCL and KOH OR NaOH)	NaOH (50%/100°C/2H)	22.2-57.0% (Yield)	29% (Chitin yield)	IExC	ND	ND
(Lin et al., 2021)	*H. illucens* pupae exuviae	Dried and ground	Enzymatic treatment followed by bleaching (H_2O_2)	NaOH (50%/95°C/4H)	12.4% (Yield)	81.5% (DDA)	FTIR XRD SEM Metal content	Antibacterial activity	ND
(Purkayastha & Sarkar, 2020)	*H. illucens* pupae exuviae	Dried and ground	Chemical treatment (HCL and NaOH) followed by bleaching ($KMnO_4$)	NA	23% (Yield)	NA	Ash content FTIR XRD TGA SEM NMR	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
							EA Surface area analysis		
(Wang et al., 2020)	*H. illucens* pupae exuviae	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (NaClO)	NA	14.1% (Yield)	NA	FTIR XRD TGA SEM	ND	ND
(Wasko et al., 2016)	*H. illucens* pupae exuviae	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching ($KMnO_4$)	NA	ND	NA	FTIR XRD TGA SEM EA	ND	ND
(Zlotko et al., 2021)	*H. illucens* pupae exuviae	Dried and ground	Acid and alkaline treatment (HCl and NaOH) followed by optional bleaching (boiling water, H_2O_2, $KMnO_4$ + oxalic acid)	NA	5.69%-7.97% (Yield)	NA	Optical and confocal microscopy FTIR XRD TGA EDX	ND	ND
(Murat Kaya et al., 2014)	Common backswimmer (*Notonecta glauca*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NaOH (60%/120°C/2H)	10-11% (Yield)	67% (Chitin yield)	FTIR TGA XRD SEM	NA	ND
(M. Kaya, B. Akyuz, et al., 2016)	Fruit fly (*Drosophila melanogaster*) adult	Dried	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NaOH (60%/150°C/48H)	7.85% (Yield)	70.91% (Chitin yield)	FTIR TGA SEM	ND	Electrospinning of chitosan to produce nanofibers
(Ai et al., 2008)	House fly (*Musca domestica*) larvae	Dried and ground	Alkaline treatment (NaOH) followed by bleaching ($KMnO_4$ and oxalic acid)	NaOH (40%/70°C/8H)	ND	90.3% (DDA)	Mw	Antioxidant Antitumor	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
(Ai et al., 2012)	*M. domestica* larvae	Dried and ground	Alkaline treatment (NaOH) followed by bleaching ($KMnO_4$ and oxalic acid)	NaOH (40%/70°C/8H)	ND	ND	ND	Antioxidant Antifungal Antiviral	ND
(Jing et al., 2007)	*M. domestica* larvae cuticles	Dried and ground	Alkaline treatment (NaOH) followed by bleaching ($KMnO_4$, oxalic acid and HCl)	NaOH (42%/70°C/4H) followed by treatment with acetic acid and H_2O_2	ND	ND	Mw	Antibacterial	ND
(Kim et al., 2016)	*M. domestica* pupae shells	Optional grinding	Chemical treatment (HCl and NaOH)	NaOH (50%/95-105°C/3-5H)	Ground – 7.71%-8.50% (Yield)	Ground - 5.87%-6.77% (Total yield)/7.60%-96.65% (DDA)	Mw	ND	ND
(Zhang et al., 2011)	*M. domestica* larvae	Dried and ground	Alkaline treatment (NaOH)	NaOH (50%/120-130°C/4-8H)	ND	*ca.* 60%-70% (Yield)/*ca.* 70-80% (DDA)	Mw Heavy metals content Aerobic bacterial count FTIR XRD TGA	ND	ND
(Gu et al., 2010)	*M. domestica* larvae shells	Dried and ground	Chemical treatment (HCl and NaOH)	NaOH (10.0 M, 11.25 M and 12.5 M NaOH for 6, 4 and 4 h at 50°C respectively)	ND	ND	NA	ND	Chitosan as an absorbable surgical haemostatic agent.
(Song et al., 2013)	Oriental blue fly (*Chrysomya megacephala*) larvae	Dried and ground	Alkaline treatment (NaOH) followed by bleaching (NaClO) and oxalic acid treatment	NaOH (67%/90°C/9H)	ND	26.2% (Total yield)	Halogen adsorption capacity Mw EA FTIR SEM NMR	Antioxidant	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
Hemiptera									
(Murat Kaya et al., 2014)	Aquatic bug (*Ranatra linearis*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NaOH (60%/120°C/2H)	15-16% (Yield)	NA	FTIR TGA XRD SEM	NA	ND
(Mol et al., 2018)	Cicada (*Cicada iodosi*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	4.97% (Yield)	NA	FTIR TGA SEM	ND	ND
(Mol et al., 2018)	Cicada (*Cicada mordoganensis*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	6.49% (Yield)	NA	FTIR TGA SEM	ND	ND
(Poerio et al., 2020)	Cicada (*Cicada orni*) slough	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (NaClO)	NA	42.6% (Yield)	NA	FTIR XRD TGA SEM	ND	ND
(Mol et al., 2018)	Cicada (*Cicadatra atra*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	6.70% (Yield)	NA	FTIR TGA SEM	ND	ND
(Mol et al., 2018)	Cicada (*Cicadatra hyalina*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	5.51% (Yield)	NA	FTIR TGA SEM	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
(Mol et al., 2018)	Cicada (*Cicadatra platyptera*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	8.84% (Yield)	NA	FTIR TGA SEM	ND	ND
(Mol et al., 2018)	Cicada (*Cicadivetta tibialis*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	5.88% (Yield)	NA	FTIR TGA SEM	ND	ND
(Wu et al., 2013)	Cicada (*Cryptotympana atrata*) larvae	Dried and ground	Chemical treatment (HCl and NaOH) followed by ethanol treatment	NaOH (55%/110°C/4H)	ND	ND	FITR	ND	Chitooligosaccharides preparation and its antibacterial activity
(Luo et al., 2019)	Cicada (species not specified) slough	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching ($KMnO_4$ and oxalic acid)	NaOH (60%/100°C/8H)	ND	28.2% (Total yield) 84.1% (DDA)	Solubility WBC FBC Ash and moisture content Mw FTIR XRD TGA SEM Rheological properties	ND	ND
(Sajomsang & Gonil, 2010)	Cicada (species not specified) slough	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (NaClO)	NA	36.6% (Yield)	NA	FTIR XRD TGA EA NMR	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
(Sharbidre et al., 2021)	Dinirodid bug (*Coridius nepalensis*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (NaClO)	NA	43.97% (Yield)/ 57.67% (DA)	NA	FTIR XRD SEM EA	ND	ND
Hymenoptera									
(Feás et al., 2020)	Asian hornet (*Vespa velutina*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by (NaClO)	NA	11.7% (Yield)/95.44% (DA)	NA	FTIR TGA SEM EA NMR	ND	ND
(Majtán et al., 2007)	Buff-tailed bumblebee (*Bombus terrestris*)	Dried and ground	Chemical treatment (HCL and NaOH) followed by bleaching (H_2O_2:33% HCl, 9:1)	NA	87.4% (DA)	NA	FTIR EA Confocal microscopy NMR	ND	ND
(Draczynski, 2008)	European honeybee (*Apis mellifera*) adult	Dried, ground and defatted	Chemical treatment (HCL and NaOH) followed by bleaching ($KMnO_4$ and oxalic acid)	NA	ND	NA	FTIR NMR Mw	ND	ND
(Kaya, Mujtaba, et al., 2015)	*A. mellifera* adult	Dried and ground	Chemical treatment (HCL and NaOH) followed by bleaching (water:methanol:chloroform, 2:2:1)	NA	6.79% (Yield – thorax)-13.25% (Yield – legs)	NA	FTIR TGA SEM	ND	ND
(Kovaleva et al., 2016)	*A. mellifera* adult	Ground	Chemical treatment (NaOH and HCl) followed by defatting and bleaching (H_2O_2)	NA	22% (Yield)	NA	FTIR EA	ND	ND
(Nemtsev et al., 2004)	*A. mellifera* adult	Dried	Alkaline treatment (NaOH) followed by optional bleaching (H_2O_2)	NaOH (50%/125°C/1H) followed by	11.4%-27.2% (Yield)	Non-discolored: 20-30% (Chitin yield)/75-80% (DDA)	ND	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
				enzymatic hydrolysis		Descolored: 16-25% (Chitin yield) /75-84% (DDA)			
(Marei et al., 2019)	*A. mellifera* adult exuviae	NA	Chemical treatment (NaOH and HCl)	NaOH (50%/100°C/8H)	ND	96% (DDA)	FTIR XRD	ND	It was predicted that chitosan-based naoparticles could be used in medicine as a carrier for antimicrobial agents
(Marei et al., 2016)	*A. mellifera* adult exuviae	NA	Chemical treatment (NaOH and HCl)	NaOH (50%/100°C/8H)	2.5% (Yield)	96% (DDA)	Ash and moisture content WBC/FBC FTIR XRD SEM	ND	ND
(Kaya, Bagriacik, et al., 2015)	European hornet (*Vespa crabro*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	8.3% (Yield)/96.85% (DA)	NA	FTIR XRD TGA SEM	ND	ND
(M. Kaya, K. Sofi, et al., 2016)	*V. crabro* adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	10.3% (Yield)/108.8% (DA)	NA	FTIR TGA SEM EA Chitinase digestive activity	ND	ND
(M. Kaya, K. Sofi, et al., 2016)	*V. crabro* larvae	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	2.2% (Yield)/109.1% (DA)	NA	FTIR TGA SEM EA Chitinase digestive activity	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
(M. Kaya, K. Sofi, et al., 2016)	*V. crabro* pupae	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	6.2% (Yield)/127.5% (DA)	NA	FTIR TGA SEM EA Chitinase digestive activity	ND	ND
(Kaya, Bagriacik, et al., 2015)	German wasp (*Vespula germanica*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	11.9% (Yield)/79.83% (DA)	NA	FTIR XRD TGA SEM	ND	ND
(Kaya, Bagriacik, et al., 2015)	Oriental hornet (*Vespa orientalis*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	6.4% (Yield)/99.82% (DA)	NA	FTIR XRD TGA SEM	ND	ND
Lepidoptera									
(Kaya, Bitim, et al., 2015)	Cardinal (*Argynnis pandora*) adult	Dried and ground (except the wings)	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	22% (Yield – wings)/104.2% (DA – wings) 8% (Yield – other body parts)/95.5% (DA – other body parts)	NA	FTIR XRD TGA SEM EA	ND	ND
(Huet et al., 2021)	Eri silkmoth (*Samia ricini)* larvae	Mechanical processing followed by defatting	Chemical treatment (HCL and NaOH) followed by ionic liquids OR deep eutectic solvents treatment	NaOH (50%/100°C/16H)	25.2% (Yield) IL - 93% (Yield chitin extracted by chemical treatment) DES - 65% (from chitin	Chemical treatment - 78% (Chitin yield) IL - 70% (Chitin yield) DES-79 (Chitin yield)%	FTIR XRD TGA SEM EA NMR	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
					extracted by chemical treatment)				
(Huet et al., 2020)	*S. ricini* larvae	Boiling, filtration and extrusion	Chemical treatment (NaOH and HCl) followed by ionic liquids treatment	NA	73.2% (Yield)/93% (DA)	NA	FTIR XRD TGA SEM EA NMR	ND	ND
(Y. M. Chen et al., 2021)	Japanese giant silkworm (*Caligula japonica*) pupae	Dried	Chemical treatment (HCl and NaOH) followed by bleaching (ethyl alcohol 70%)	NA	ND	NA	FTIR XRD TGA SEM Water contact angle EA	ND	The Ag-deposited natural chitin films exhibited surface-enhanced Raman scattering (SERS) activity
(Mehranian et al., 2017)	Mediterranean flour moth (*Ephestia kuehniella)* adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching ($KMnO_4$)	NA	9.5%-10.5% (Yield)	NA	FTIR SEM EDX Chitinase activity	ND	ND
(Pal et al., 2016)	Muga silkworm (*Antheraea assamensis*) cocoons	Dried and ground	Chemical treatment (NaOH and HCl) followed by bleaching (acetone)	NaOH (40%/50-110°C/4-10H)	8% (Yield)	7% (Total yield)	FTIR XRD TGA SEM NMR EA Potentiometric titration Conductometric titration Mw	ND	Bio composite films preparation

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
(Battampara et al., 2020)	Silkworm (*Bombyx mori)* eggs	Ground and defatted	Chemical treatment (HCl and NaOH)	NaOH (55%/90°C/9H)	6% (Yield)	4.4% (Total yield)/59.2% (DDA)	FTIR XRD TGA SEM	Antibacterial Antifungal Cytotoxicity	ND
(Vasquez et al., 2014)	*B. mori* larvae	Defatted	Chemical treatment (HCL and NaOH) followed by bleaching ($KMnO_4$ and H_2O_2)	NaOH	ND	ND	NA	ND	Nanoparticles based on poly(ethylene glycol)-O-chitosan trigger for the delivery of molecules
(Battampara et al., 2020)	*B. mori* pupae	Ground and defatted	Chemical treatment (HCl and NaOH)	NaOH (55%/90°C/9H)	18% (Yield)	16% (Total yield) /66.9% (DDA)	FTIR XRD TGA SEM	Antibacterial Antifungal Cytotoxicity	ND
(Jiang et al., 2019)	*B. mori* pupae	Commercial powder	Chemical treatment (NaOH and HCl) followed by bleaching (H_2O_2)	NaOH	5.2% (Yield)	ND	ND	ND	Viscose fibers (VFs) modified with silkworm pupa chitosan oligosaccharide (SPCOS) by β-glucosaccharase were prepared
(Luo et al., 2019)	*B. mori* pupae	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching ($KMnO_4$ and oxalic acid)	NaOH (60%/100°C/8H)	ND	3.1% (Total yield) 85.5% (DDA)	Solubility WBC FBC Ash and moisture content Mw FTIR XRD TGA SEM	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
							Rheological properties		
(Paulino et al., 2007)	*B. mori* pupae	Dried and ground	Chemical treatment (HCl and NaOH)	NaOH/NaHB4	NA	75% (DDA)	SEM NMR Metal adsorption capacity	ND	ND
(Paulino, Simionato, et al., 2006)	*B. mori* pupae	Dried	Chemical treatment (HCl and NaOH) followed by bleaching (Na_2CO_3) Deproteinization: closed OR open system	NaOH/NaHB4	Closed system - 2.59-4.16% (Yield) Open system – 3.23-4.23% (Yield)	73-96.75% (Chitin yield)	FTIR TGA SEM NMR DSC	ND	ND
(Paulino et al., 2008)	*B. mori* pupae	Dried and ground	Chemical treatment (HCl and NaOH)	NaOH/NaHB4	ND	80% (DDA)	XRD TGA Metal adsorption capacity	ND	Chitosan to treat battery manufacture wastewater.
(Paulino, Minasse, et al., 2006)	*B. mori* pupae	Dried and ground	Chemical treatment (HCl and NaOH)	NaOH/NaHB4	ND	ND	SEM NMR Metal adsorption capacity	ND	Chitosan for wastewater treatment.
(Rakhmanova et al., 2009)	*B. mori* pupa	Defatted	Chemical treatment (HCL and NaOH) followed by bleaching (H_2O_2)	NaOH (120°C/2H)	ND	60% (Chitin yield)/85% (DDA)	Mw FTIR XRD	ND	ND
(Rashidova et al., 2004)	*B. mori* waste	Defatted	Chemical treatment (HCl and NaOH) followed by bleaching ($KMnO_4$ and H_2O_2)	NaOH	ND	63% (DDA)	FTIR XRD SEM Optical and electron microscopy Water adsorption EA	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
(Simionato et al., 2006)	*B. mori* pupae	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (Na_2CO_3)	NaOH/$NaHB_4$	2.59-4.18% (Yield)	73-96.75% (Chitin yield)/83% (DDA)	SEM NMR Metal adsorption capacity	ND	Chitosan for textile wastewater treatment – Al removal
(Simionato et al., 2014)	*B. mori* pupae	Dried	Chemical treatment (HCl and NaOH) Deproteinization: closed OR open system	NaOH/$NaHB_4$	ND	ND	FTIR TGA SEM NMR	ND	Immobilization of dyes in textile industry effluents
(Zhu et al., 2018)	*B. mori* pupae	Dried, ground and defatted	Chemical treatment (HCl and NaOH) followed by bleaching (H_2O_2)	NaOH	ND	ND	Solubility Mw FTIR TGA SEM XPS	ND	N,O-carboxymethyl chitosan was generated
(Fadly et al., 2020)	*B. mori* pupae	Dried	Chemical treatment (HCl and NaOH)	NaOH	NA	NA	FTIR	Antioxidant	Carboxymethyl chitosan provided a significant inhibitory effect on the lipid peroxide level of human plasma.
(Zhang et al., 2000)	*B. mori* pupae exuviae	Dried and ground	Chemical treatment (HCl and NaOH)	NaOH/$NaBH_4$	ND	ND	FTIR XRD NMR Chitinase activity	ND	ND
(Wu, 2011)	Two-line velvet hawkmoth (*Clanis bilineata*) larvae (skin)	NA	Enzymatic deproteinization (flavourzyme)	NaOH (35-60%/90-140°C/1-6H)	ND	31.3% (Total yield)/ *ca.* 30-90% (DDA)	FTIR	ND	ND
(Wu et al., 2017)	*C. bilineata* larvae	Dried and ground	Chemical treatment (HCl and NaOH) followed by	NaOH	ND		FTIR	Antiaging Antioxidant	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
			water-solubilization (acetic acid and α-amylase)						
(Fukamizo et al., 1986)	Tobacco hornworm (*Manduca sexta)* larvae	Dried	Alkaline treatment (NaOH) followed by enzymatic hydrolysis	NA	ND	NA	NMR	ND	ND
Odonata									
(Murat Kaya et al., 2014)	Emperor dragonfly (*Anax imperator*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NaOH (60%/120°C/2H)	11-12% (Yield)	NA	FTIR TGA XRD SEM	NA	ND
(M. Kaya, I. Sargin, et al., 2016)	Red-veined Darter (*Sympetrum fonscolombii*) adult (corneal lenses)	ND	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	20.3% (Yield)/102% (DA)	NA	FTIR XRD TGA SEM Chitinolytic activity	ND	ND
Orthoptera									
(Ibitoye et al., 2018)	African crickets (*Brachytrupes portentosus*) adult	Dried	Chemical treatment (NaOH and oxalic acid) followed by bleaching (NaClO)	NaOH	4.3%-7.1% (Yield)/108.1% (DA)	2.4%-5.8% (Total yield)/80.5% (DDA)	FTIR XRD SEM EA	ND	ND
(Ibitoye et al., 2019)	*B. portentosus* adult	Dried	Chemical treatment (NaOH and oxalic acid) followed by bleaching (NaClO)	NaOH	ND	ND	ND	ND	Cricket chitosan a nutritional tool for growth promotion in poultry
(Kaya, Erdogan, et al., 2015)	African stick grasshopper	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching	NA	6.6% (Yield)	NA	FTIR XRD TGA	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
	(*Pyrgomorpha cognata*) adult		(methanol:chloroform:water, 2:1:4)				SEM Mw		
(Kaya, Baran, et al., 2015)	Band-winged grasshopper (*Oedaleus decorus*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NaOH (50%/130°C/2H)	16.5% (Yield)/90.64% (DA)	76% (Chitin yield) /70-75% (DDA)	FTIR XRD TGA SEM	Antimicrobial Antioxidant	ND
(Kaya, Lelesius, et al., 2015)	Black grasshopper (*Celes variabilis*) adult	Dried and ground	Chemical treatment (HCl and NaOH)	NA	Male - 9.93% (Yield)/125.21 %(DA) Female - 6.65%(Yield)/180.71%(DA)	NA	FTIR XRD TGA SEM EA Chitinase hydrolytic activity	ND	ND
(Kaya, Erdogan, et al., 2015)	Blue-winged grasshopper (*Oedipoda caerulescens*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	8.9% (Yield)	NA	FTIR XRD TGA SEM Mw	ND	ND
(Kabalak et al., 2020)	Bronze glandular bush-cricket (*Bradyporus charpentier*) adult	Dried and ground	Chemical treatment (HCL and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	9.8% (Yield)	NA	FTIR XRD TGA SEM EA	ND	ND
(Wu et al., 2020)	Bush-cricket (*Ruspolia differens*) adult	Ground	Chemical treatment (HCl and NaOH) followed by bleaching (H_2O_2 and NaOH)	NA	5% (Yield)	NA	FTIR XRD SEM NMR Mechanical properties	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
(Kaya, Lelesius, et al., 2015)	Common wart-biter (*Decticus verrucivorus)* adult	Dried and ground	Chemical treatment (HCl and NaOH)	NA	Male- 11.84%%(Yield)/108.50%(DA) Female - 10.03%(Yield)/ 115.00%(DA)	NA	FTIR XRD TGA SEM EA Chitinase hydrolytic activity	ND	ND
(Kipkoech et al., 2021)	Cricket (*Scapsipedus icipe*) adult	Dried, ground and defatted	Chemical treatment (HCl and NaOH) followed by bleaching (NaClO) on the pellet obtained through protein extraction	NaOH	ND	ND	FTIR NMR	Antibacterial	Cricket-derived chitosan may be a functional prebiotic due to its ability to stimulate the growth of specific beneficial bacteria
(Hirsch et al., 2019)	Cricket (species not specified)	Dried and defatted	Chemical treatment (HCL and NaOH)	NA	5.14% (Yield)	NA	Particle size Interfacial properties Emulsifying properties	ND	ND
(Marei et al., 2019)	Desert locust *(Schistocerca gregaria*) exoskeleton	NA	Chemical treatment (NaOH and HCl)	NaOH (50%/100°C/8H)	ND	98% (DDA)	FTIR	ND	It was predicted that CSNPs could be used in medicine as a carrier for antimicrobial agents
(Marei et al., 2016)	*S. gregaria* exoskeleton	NA	Chemical treatment (NaOH and HCl)	NaOH (50%/100°C/8H)	12.2%	98% (DDA)	Ash and moisture content WBC/FBC FTIR XRD SEM	ND	ND
(Marei et al., 2017)	*S. gregaria* adult exuviae	NA	Chemical treatment (NaOH and HCl)	NaOH (50%)	22.5% (Yield)	55% (Chitin yield)/97.80% (DDA)	FTIR XRD SEM	ND	Chitosan scaffolds with wound healing properties

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
							Elasticity Mw		
(Kabalak et al., 2020)	European mole cricket (*Gryllotalpa gryllotalpa*) adult	Dried and ground	Chemical treatment (HCL and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	10.1% (Yield)	NA	FTIR XRD TGA SEM EA	ND	ND
(Kaya, Erdogan, et al., 2015)	Grasshopper (*Aiolopus strepens*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	7.4% (Yield)	NA	FTIR XRD TGA SEM Mw	ND	ND
(Kaya, Erdogan, et al., 2015)	Grasshopper (*Duroniella fracta*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	5.7% (Yield)	NA	FTIR XRD TGA SEM Mw	ND	ND
(Kaya, Lelesius, et al., 2015)	Grasshopper (*Paracyptera labiata*) adult	Dried and ground	Chemical treatment (HCl and NaOH)	NA	Male - 7.60%(Yield)/130.00%(DA) Female - 6.8%(Yield)/162.80%(DA)	NA	FTIR XRD TGA SEM EA Chitinase hydrolytic activity	ND	ND
(Luo et al., 2019)	Grasshopper (species not specified) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching ($KMnO_4$ and oxalic acid)	NaOH (60%/100°C/8H)	ND	5.7% (Total yield) 89.7% (DDA)	Solubility WBC FBC Ash and moisture content Mw FTIR XRD	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
							TGA SEM Rheological properties		
Morgan Malm and Andrea M. Liceaga	House cricket (*Acheta domesticus*) adult	Dried and ground	Chemical treatment (HCL and NaOH) of pellet obtained from enzymatic hydrolysis	NaOH (67%/2-10H)	5.7% (Yield)	69.0%-80.5% (Chitin yield)/72.5%-79.4% (DDA)	FTIR Mw	Anti-Obesity Antimicrobial	ND
(Kaya, Lelesius, et al., 2015)	Lesser field-cricket (*Melanogryllus desertus*) adult	Dried and ground	Chemical treatment (HCl and NaOH)	NA	Male - 7.35%(Yield)/168.76%(DA) Female - 4.71%(Yield)/131.41%(DA)	NA	FTIR XRD TGA SEM EA Chitinase hydrolytic activity	ND	ND
(Kaya, Erdogan, et al., 2015)	Locust *(Aiolopus simulatrix)* adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	5.3% (Yield)	NA	FTIR XRD TGA SEM Mw	ND	ND
(Torres-Castillo et al., 2015)	Mexican katydid (*Pterophylla beltrani*) adult and nymphs	Dried and ground	Chemical treatment (HCl and NaOH)	NaOH (70%/120°C/90min)	11.8% (Yield)	58.8% (Chitin yield)	NA	Antifungal	ND
(Erdogan & Kaya, 2016)	Moroccan locust (*Dociostaurus maroccanus*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NaOH (60%/150°C/4H)	14% (Yield)/232% (DA)	81.69% (Chitin yield)/64% (DDA)	FTIR XRD TGA SEM EA Mw	ND	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
(Erdogan & Kaya, 2016)	*D. maroccanus* nymphs	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water , 2:1:4)	NaOH (60%/150°C/4H)	12% (Yield)/187% (DA)	77.38% (Chitin yield)/22% (DDA)	FTIR XRD TGA SEM EA Mw	ND	ND
(Saenz-Mendoza et al., 2020)	Plains lubber (*Brachystola magna*) adult	Dried and ground	Chemical treatment (HCl and NaOH)	NaOH (60%/120°C/2H) + $NaBH_4$	ND	89.89% (DDA)	FTIR XRD SEM Mw Physicochemical characterization and rheological properties Mechanical properties Water vapor permeability Solubility	ND	Chitosan films – Food packaging
(Tirado-Gallegos et al., 2021)	*B. magna* adult	Dried and ground	Chemical treatment (HCL and NaOH)	NaOH/NaBH4	ND	ND	Rheological properties, Mechanical properties	ND	Chitosan films – packaging material for meat products
(Kaya, Erdogan, et al., 2015)	Red-winged grasshopper (*Oedipoda miniate*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water , 2:1:4)	NA	8.1% (Yield)	NA	FTIR XRD TGA SEM Mw	ND	ND
(Kaya, Baran, et al., 2015)	Short-horned grasshopper (*Calliptamus barbarus*) adult	Dried and ground	Chemical treatment (HCL and NaOH) followed by bleaching (methanol:chloroform:water , 2:1:4)	NaOH (50%/130°C/2H)	20.5% (Yield) /87.80% (DA)	75% (Chitin yield)/70-75% (DDA)	FTIR XRD TGA SEM	Antimicrobial Antioxidant	ND

Reference	Species	Pre-treatment	Extraction method	Deacetylation	Chitin yield/DA	Chitosan yield/DDA	Characterization	Biological properties	Other
Morgan Malm and Andrea M. Liceaga	Tropical house cricket (*Gryllodes sigillatus*) adult	Dried and ground	Chemical treatment (HCL and NaOH) of pellet obtained from enzymatic hydrolysis	NaOH (67%/2-10H)	3.4% (Yield)	60.3%-65.0% (Chitin yield)/73.5%-81.3% (DDA)	FTIR Mw	Anti-Obesity Antimicrobial	ND
(Chae et al., 2018)	Two-spotted cricket (*Gryllus bimaculatus*) adult	Dried and ground	Chemical treatment (oxalic acid and NaOH) followed by bleaching (APS solution)	NaOH (50-67%/95°C/9-12H)	5.1% (Yield)	41.75% (Chitin yield)/56.47%-84.98% (DDA)	FTIR XRD Mw Color, zeta potential, and pH value	ND	Cricket chitosan could be applied to either an intact form or an active polymer for nanocapsules.
(Glab et al., 2021)	*G. bimaculatus* adult	Dried and defatted	Chemical treatment (HCl and NaOH) followed by bleaching (H_2O_2)	NaOH	ND	74.5% (DDA)	FTIR XRD	ND	Polymer capsules based on crickets chitosan with nisin for delivery systems of drugs with antibacterial or anticancer activity.
(Kim et al., 2017)	*G. bimaculatus* adult exoskeleton	Ground	Chemical treatment (HCL and NaOH)	NaOH (50%-95-105°C/3H)	10.91% (Yield)	7.50% (Total yield)	Mw	ND	ND
(Kaya, Erdogan, et al., 2015)	Wide-headed delta grasshopper (*Duroniella laticornis*) adult	Dried and ground	Chemical treatment (HCl and NaOH) followed by bleaching (methanol:chloroform:water, 2:1:4)	NA	6.5% (Yield)	NA	FTIR XRD TGA SEM Mw	ND	ND

* Chitin extraction method: Includes 2 or 3 steps: Deproteinization, demineralization, and decolorization.
* Pre-treatment: Comprises only the samples which were defatted (includes: Soxhlet extraction; oil-press; ethanol or other reagents soaking), dried (includes: air; oven; stove; freeze-dry) and/or ground (includes: crushed; milled; cut; grind). The pre-treatment can include other steps like washing, soaking in ethanol and others.

Acronyms:

FITR: Fourier-transform infrared spectroscopy

XRD: X-ray diffraction

TGA: Thermogravimetric analysis

DSC: Differential scanning calorimetry

SEM: Scanning electron microscope

SEM-EDX: Scanning Electron Microscopy - Energy Dispersive X-Ray

TEM: Transmission electron microscopy

AFM: Atomic force microscopy

XPS: X-Ray photoelectron spectroscopy

NMR: Nuclear magnetic resonance

EA: Elemental analysis

HPLC: High performance liquid chromatography

GC: Gas chromatography

IExC: Ion exchange chromatography

Supplementary table Bibliography

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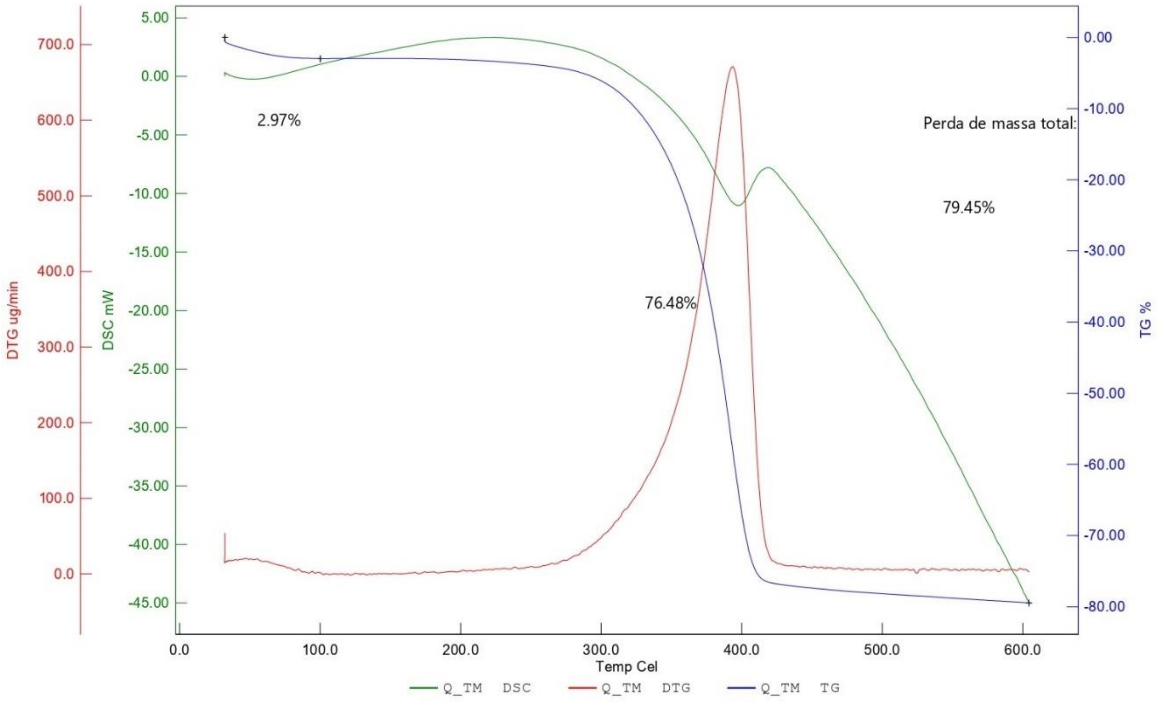
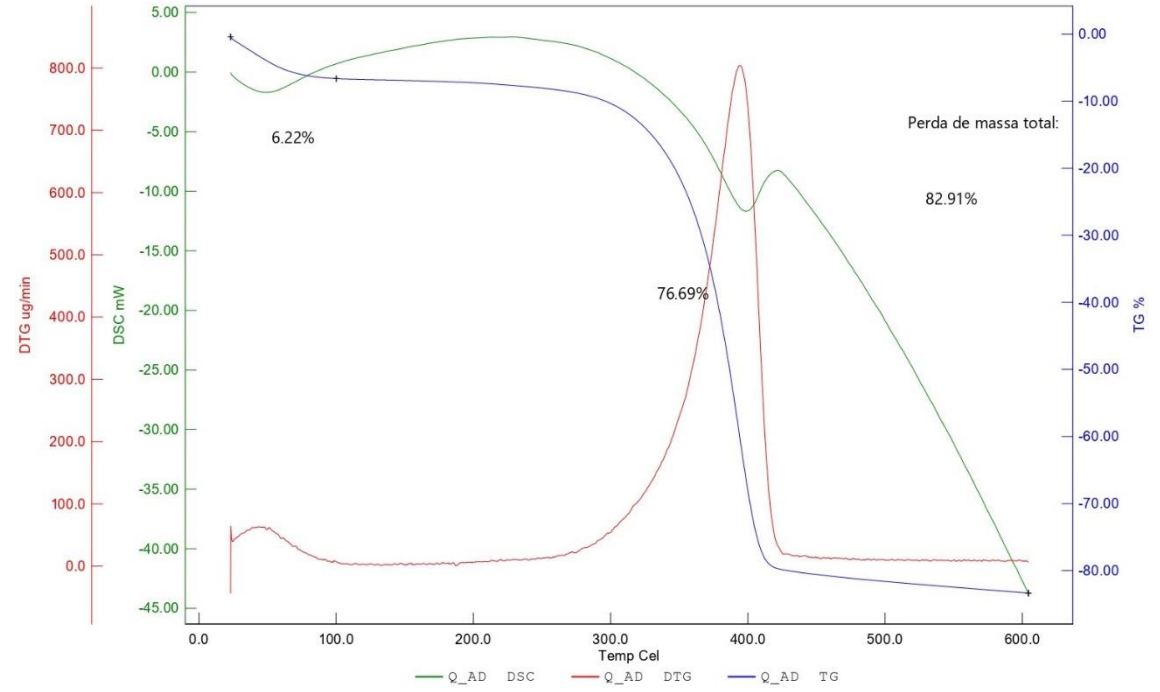
8.4 Thermograms of the different chitin and chitosan samples obtained from TGA

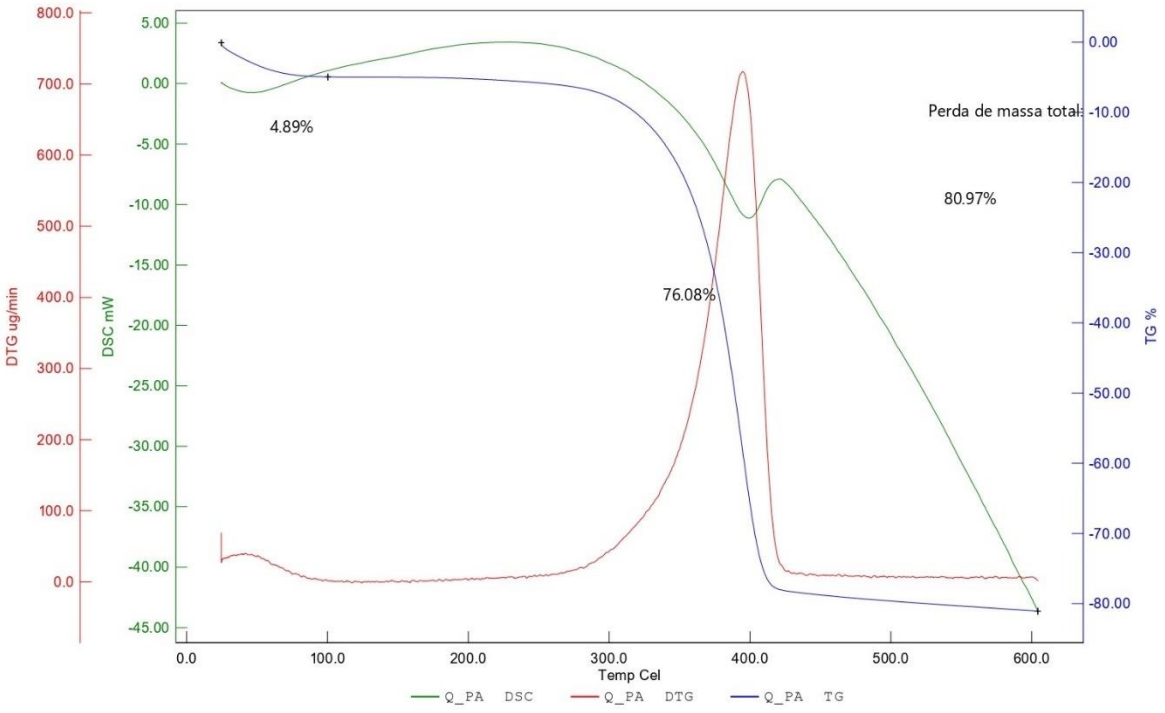
8.4.1 DTG, TG and DSC curves of *T. molitor* chitin



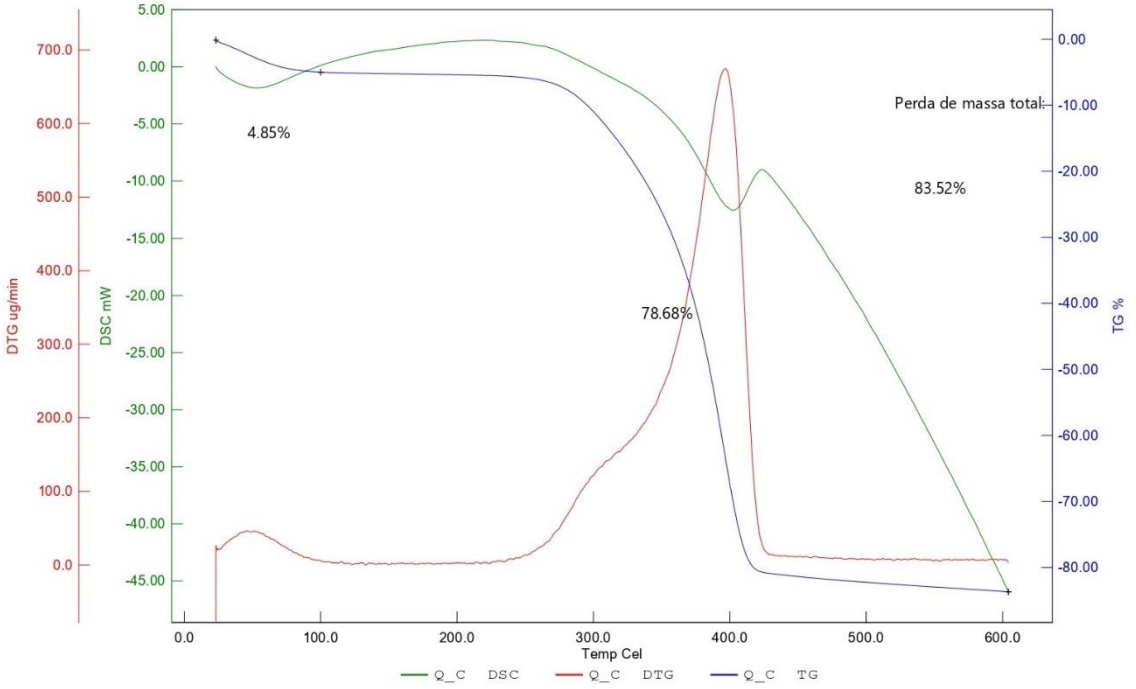
8.4.2 DTG, TG and DSC curves of *A. domesticus* chitin



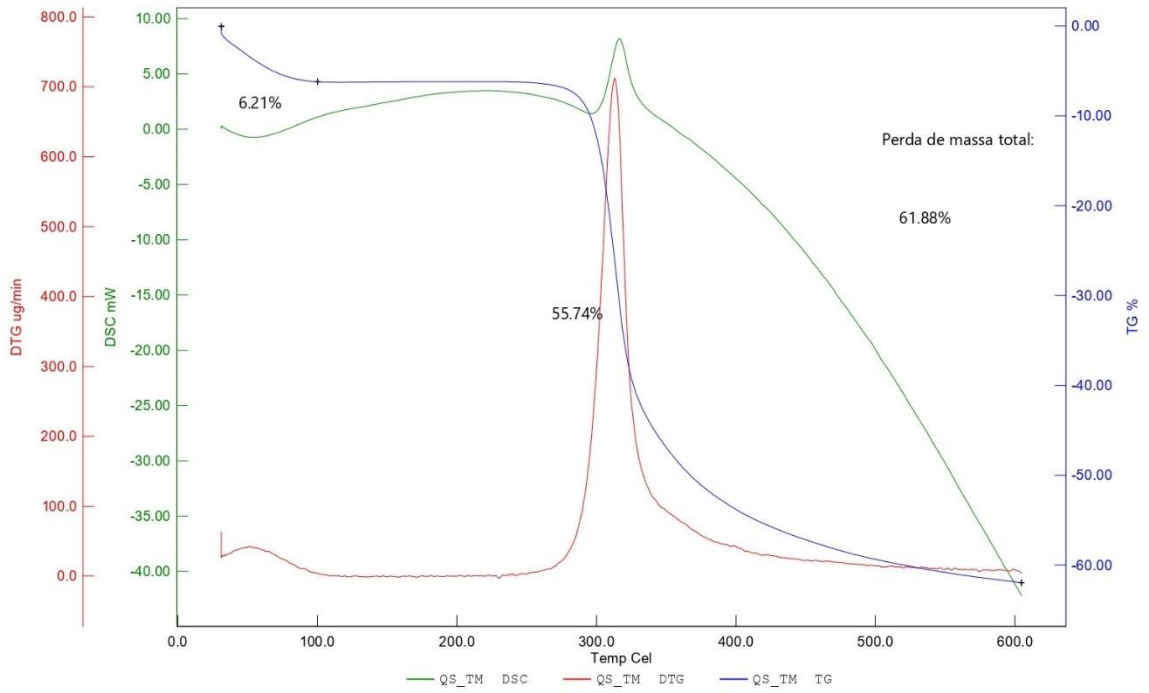
8.4.3 DTG, TG and DSC curves of *A. domesticus* legs and wings chitin



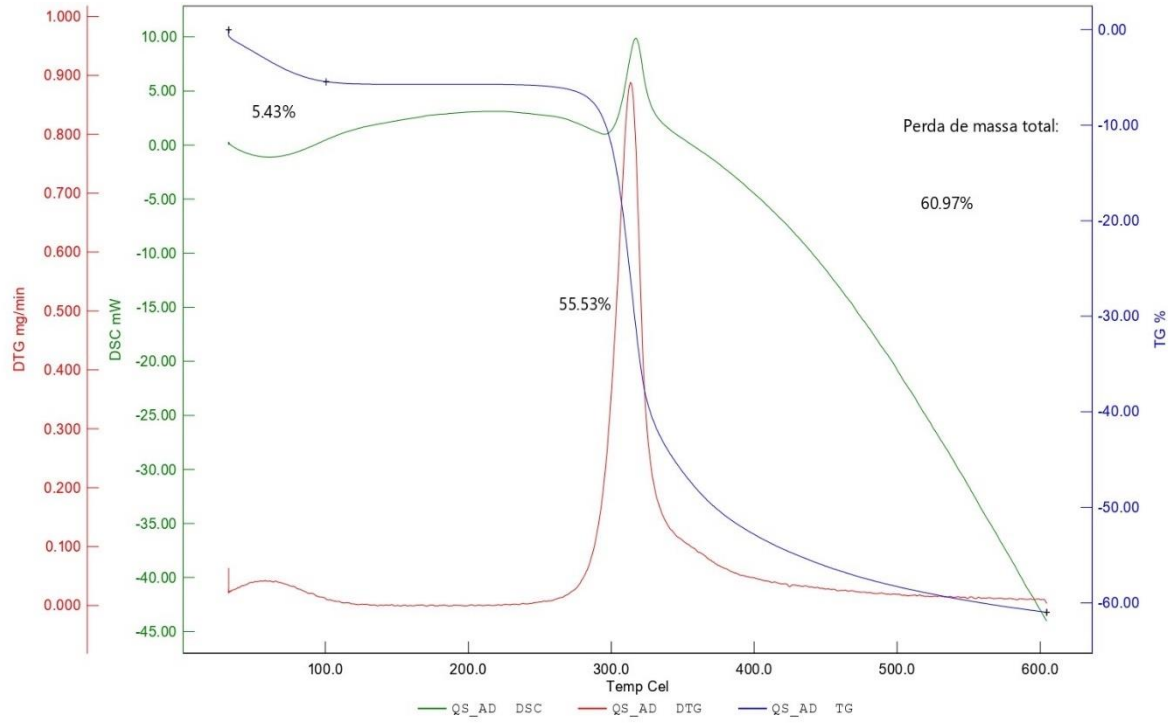
8.4.4 DTG, TG and DSC curves of commercial shrimp chitin



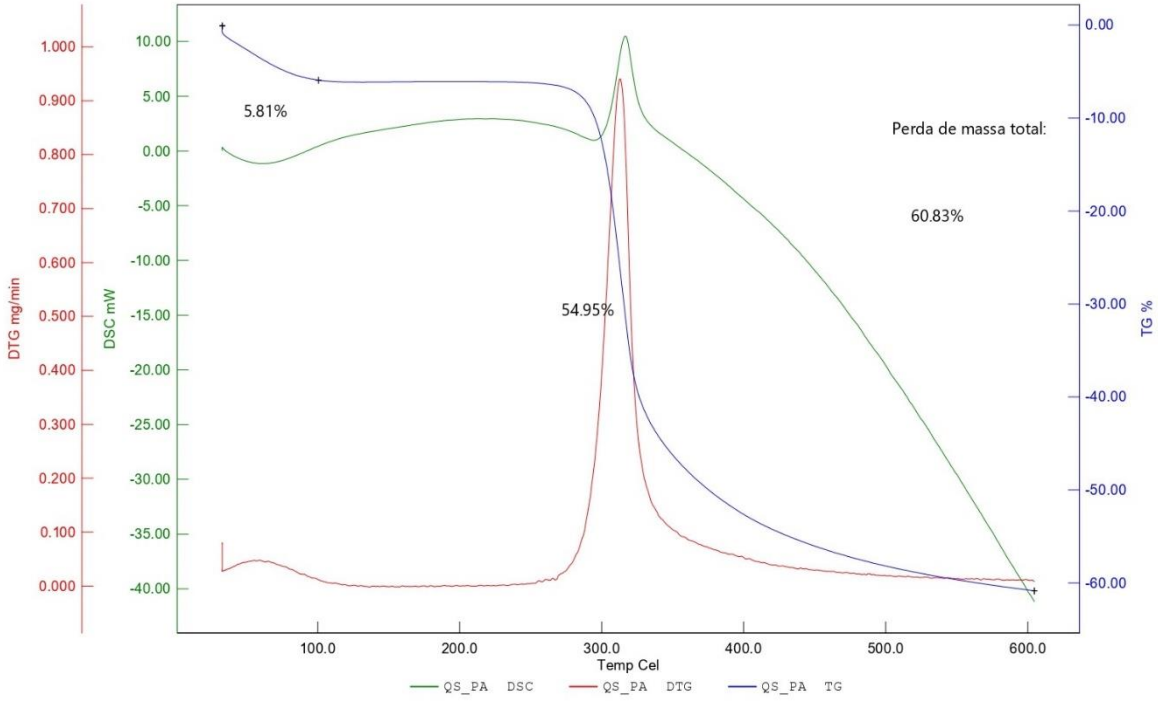
8.4.5 DTG, TG and DSC curves of *T. molitor* chitosan



8.4.6 DTG, TG and DSC curves of *A. domesticus* chitosan



8.4.7 DTG, TG and DSC curves of the legs and wings A. domesticus chitosan



8.4.8 DTG, TG and DSC curves of commercial shrimp chitosan