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Ph.D. Program

"*Applied Biology and Environmental Safeguard*"

"Use of the black soldier fly *Hermetia illucens* L. (Diptera: *Stratiomyidae*) as an alternative source of chitin and chitosan for the production of biopolymeric films in agro-food applications"

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ABSTRACT

Chitin and chitosan are natural polymer of great technological and economic interest, finding several applications in many different fields. Chitin is a structural component of the exoskeleton of arthropods and the fungal cell wall, while chitosan is its main deacetylated derivative. Currently, chitin is industrially extracted from fishery waste, mainly crustacean shells. The debate on the sustainability of this resource and the constant increase in market demand for chitin and chitosan have driven the search for alternative sources. In this context, insects are gaining great interest. In recent decades, large-scale insect breeding facilities for feed production and organic waste disposal have arisen all over the world. These farms generate large amounts of insect waste biomass (i.e., exuviae left over from moulting processes and dead insects) rich in chitin that could be exploited as a source for the extraction of this polymer.

The aim of this work was to investigate different insect biomasses (larvae, pupal exuviae and adults) generated from the farming of the dipteran *Hermetia illucens* (the most widely bred species in Europe) as sources of chitin and chitosan. Chitosan was produced with the purpose of being used for the production of coatings for the preservation of fresh fruits. From the three different sources of *H. illucens*, chitin with characteristics and purity similar to that commercially available from crustaceans was extracted. The highest yield (23%) was obtained from pupal exuviae. From the analysis of the composition of the material obtained after each step of the chitin extraction process, it was possible to calculate the efficiency of the extraction methods applied, which were suitable for removing components extraneous to chitin from the raw insect sample. The chitosan obtained from the deacetylation of chitin had some different characteristics than commercial chitosan, particularly a very low viscosity and molecular weight. This, together with the presence of impurities found in one sample, suggests the need to modify the deacetylation process or some parameters of the chitin extraction method. Chitosan produced from larvae, pupal exuviae and adults of *H. illucens* was used to produce coating solutions that were applied to cherry tomatoes, strawberries, apricots and nectarines. The preservative effect of the insect-derived chitosan coating was evaluated by investigating changes occurred in the weight, pH, titratable acidity and total soluble solids content of fruits during the storage period. Preliminary promising results were obtained from this investigation: chitosan from H. illucens had similar or, in some cases, better effects than commercial chitosan; often no different effects were observed in coated fruits than in uncoated fruits, but coating with chitosan had better effects than coating with only solvent solution (a non-optimal formulation of the solvent is hypothesised); chitosan obtained by homogeneous deacetylation always had

worse effects than heterogeneous chitosan; it was observed that bleaching treatment applied to chitin may negatively affect the performance of the resulting chitosan. Furthermore, the chitosan coating was particularly effective in reducing and delaying spontaneous mould growth on the fruit during storage.

This work has therefore highlighted the potential of *H. illucens* as a source of chitin and chitosan to be used in the agri-food sector as an alternative packaging. The results obtained are encouraging, although further studies are needed to fully optimise the extraction processes of these polymers in order to obtain the best characteristics for the desired applications.

1. INTRODUCTION

1.1 Current state of chitin purification and chitosan production from insects

What is reported in section 1.1 has been accepted and published in Journal of Chemical Technology and Biotechnology, in the form of a review titled "Current state of chitin purification and chitosan production from insects" (Hahn et al., 2020).

Summary

Chitin, and especially its deacetylated variant chitosan, has many applications, e.g., as carrier material for pharmaceutical drugs or as a flocculant in wastewater treatment. Despite its versatility and accessibility, chitin has so far been commercially extracted only from crustaceans and to a minor extent from fungi. Insects are a viable alternative source of chitin, but they have not been exploited in the past due to limited availability. Today however, for the sustainable production of animal feed, insect farming is being developed substantially. The availability of large quantities of insect biomass and chitin-rich side products such as exuviae and exoskeletons has been increasing. This review provides an overview of recently published studies of chitin extraction from insects, its subsequent conversion into chitosan and the primary analytical methods used to characterize insect-based chitin and chitosan. We have discovered a large number of research articles published over the past 20 years, confirming the increased attention being received by chitin and chitosan production from insects. Despite numerous publications, we identified several knowledge gaps, such as a lack of data concerning chitin purification degree and chitosan yield. Furthermore, analytical methods used to obtain physicochemical characteristics, structural information and chemical composition meet basic qualitative requirements but do not satisfy the need for a more quantitative evaluation. Despite the current shortcomings that need to be overcome, this review presents encouraging data on the use of insects as an alternative source of chitin and chitosan in the future.

INTRODUCTION

Chitin is an important structural component of the cell wall of fungi and yeasts and the main carbohydrate component of arthropod exoskeletons (Rinaudo, 2006). After cellulose, it is the second most abundant biopolymer present in nature (Elieh-Ali-Komi and Hamblin, 2016). The production of chitin in the biosphere is estimated to be around 1000 billion (10^{12}) tons per year (Revathi *et al*., 2012). Chitin was isolated for the first time in 1799 from the shells of molluscs (Hatchett, 1799). Chitosan, the deacetylated derivative of chitin, was obtained by Rouget in 1859 by heating chitin in alkaline medium; however, its chemical structure was determined

only in 1950 (Ruiz and Corrales, 2017). Despite this early discovery, the industrial production and commercialization of chitin and chitosan initially started in the 1970s (Crini, 2019).

Chitin is a hard, inelastic, N-acetylated aminopolysaccharide (figure 1.1a), insoluble in water and most organic solvents (Kumar, 2000; Dutta *et al*., 2004). Fungi and invertebrates use these properties and incorporate chitin microfibrils to protect and strengthen their cell matrix or as components of mechanically resilient structures such as shells, cuticles, bones (in cuttlefish) and scaffolds (in sponges) (Latgé, 2007; Merzendorfer, 2011).

Based on various orientations of its microfibrils, chitin exists in nature in three crystalline allomorphic forms: α-, β- and γ-chitin (figure 1.1b). α-chitin has antiparallel chains. It is responsible for the rigidity of the polymer and is the most abundant form (Sajomsang and Gonil, 2010). β-chitin consists of parallel chains, producing monoclinic crystals with intramolecular interactions (hydrogen bonds) in addition to intermolecular ones. β-chitin is found in the spines of diatoms, squid pens and pogonophoran tubes (Dweltz, 1961). γ-Chitin is a mixture of parallel and antiparallel chains combining the properties of both α-form and β-form (Kaya *et al*., 2015a); it is present in fungi, yeasts and insect cocoons (Rudall and Kenchington, 1973; Minke and Blackwell, 1978; Cabib *et al*., 1988; Hudson and Smith, 1998; Jang *et al*., 2004).

After being isolated from natural sources, direct application of chitin is limited to a few applications, such as the production of scaffolds to support tissue regeneration (Wang *et al*., 2001) or for biological control of plant pathogens in agriculture (Manjula and Podile, 2001), which is due to its insolubility. To widen its range of applications, chitin needs to be converted to more water-soluble derivatives with useful properties, primarily chitosan.

Chitosan (figure 1.1a) is a cationic polysaccharide obtained from chitin by alkaline hydrolysis of the acetamido groups (deacetylation process). Due to an increased number of free primary amine groups, and partially due to a lower molecular weight, chitosan is soluble in slightly acidic solutions. To define and distinguish between chitin and chitosan, several researchers have set the threshold value of degree of acetylation to 50%: when the N-acetyl group content of the polysaccharide exceeds 50%, the polymer is defined as chitin; for values below 50%, it is called chitosan (Brugnerotto *et al*., 2001; Chatelet *et al*., 2001).

The physicochemical properties of chitin and chitosan may vary among samples, being affected by many factors such as the source of chitin and parameters of the purification process. Molecular weight is an important characteristic to consider, as it affects chitosan viscosity (Chattopadhyay and Inamdar, 2010) that is crucial to many of its applications and other important features, such as antibacterial activity (Vaz *et al*., 2018). Native chitin from crustacean sources has a molecular weight exceeding 1000 kDa, while the molecular weight of

crustacean-based chitosan ranges from 100 to 1000 kDa (Hossain and Iqbal, 2014; de Queiroz Antonino *et al*., 2017).

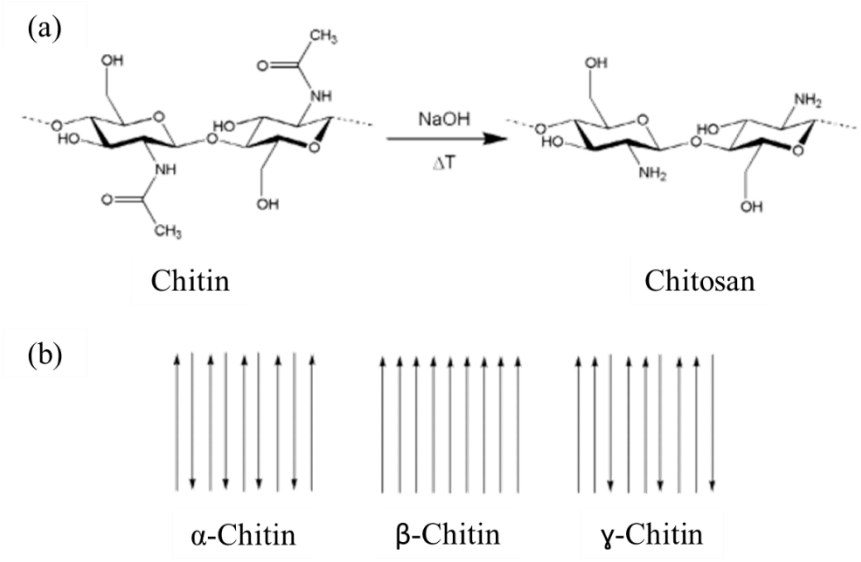


Figure 1.1 (a) Chitin and chitosan molecular structure. Chitin consists of N-acetylated-D-glucosamine (GlcNAc) and 2-amino-D-glucose (D-glucosamine, GlcN) linked by β-1,4 glycosidic bonds. Chitosan is the main deacetylated derivative of chitin. (b) Three crystalline allomorphic forms of chitin, with different microfibril orientations.

Due to its useful properties, such as biodegradability, biocompatibility, non-toxicity, adsorption capacity and antimicrobial activity (Hudson and Smith, 1998; Rinaudo, 2006), chitosan is attracting great attention for many applications within the agricultural, industrial, biotechnological and biomedical fields and in wastewater treatment (Felse and Panda, 1999; Synowiecki and Al-Khateeb, 2003; Lim and Hudson, 2003; Dutta *et al*., 2004; Rinaudo, 2006; Park and Kim, 2010; Hahn and Zibek, 2018).

The antimicrobial activity of chitosan is one of its most exploited properties. Chitosan can inhibit the growth of human pathogens, such as *Escherichia coli* (Tsai and Su, 1999; Liu *et al*., 2001), *Staphylococcus aureus* (Goy *et al*., 2016; Han *et al*., 2016), *Pseudomonas aeruginosa* (Ong *et al*., 2008) and *Aspergillus niger* (Fang *et al*., 1994). Its antimicrobial activity makes chitosan suitable to be used not only in biomedical applications but also as a natural biopolymer coating material to preserve the quality and to extend the shelf-life of fresh food (Kaku *et al*., 2006). Chitosan-based active films against contamination and microbial spoilage have been successfully used in fruit, vegetable, egg and meat packaging (Vu *et al*., 2011; Dehnad *et al*., 2014).

Chitosan has also shown excellent potential for wound dressing. The potential of chitosan as a haemostatic topical dressing for animal tissues has previously been demonstrated: chitosan

adheres to red blood cells, thus retrieving platelets for hemagglutination (Stricker-Krograd *et al*., 2018).

In cosmetics, chitosan finds application in the production of creams and lotions. It is used as a moisturizing and UV-protective agent for the skin (Casadidio *et al*., 2019). A further application for chitosan is in wastewater treatment, where it is used as a flocculating agent owing to its ability to chelate cations and adsorb waste molecules from water, such as heavy metals (Pinotti and Zaritzky, 2001; Hahn and Zibek, 2018).

Recently, chitosan has been proposed as an ecological finishing agent in the textile industry. It is used in working fabrics for hospitals or biological laboratories and for making sutures, threads and fibres in medical textiles (Bakshi *et al*., 2019; Hahn *et al*., 2019). Chitosan is also used for antistatic finishing in work wear for employees of the electronic sector (Xiao, 2018).

Three main chitin sources are available

Currently, the main commercial source of chitin and chitosan comprises waste streams from the marine food industry – mainly exoskeletons of crustaceans (Arbia *et al*., 2013). Annual world production of crustaceans for human consumption was estimated at 8 million tons in 2016 (Tacon, 2018), of which 40% comprised waste exoskeletons (Gillet, 2008) with a chitin content of 15–40% (Kurita, 2006).

However, the availability of fishery waste is highly seasonal, as commercial crustacean fishing starts in spring, after the spawning season (Pittman and McAlpine, 2003). Moreover, the sustainability of crustacean farming has been under debate for many years (Gillet, 2008). The global market for chitin and chitosan is expected to reach a volume of $4.2 billion by 2021, with a compound annual growth rate of 15.4% (Jardine and Sayed, 2018), intensifying the need for a search of other sources to satisfy the growing market.

Fungi are the second main source of chitin after crustaceans. Several research activities have focused on fungi and their commercial value as they have attracted attention as an alternative and vegan source of chitin and chitosan (Abdel-Gawad *et al*., 2017). Chitin makes up between 1 and 15% of fungal cell wall mass (Free, 2013) and its structure is comparable to that in crustaceans (Peter, 2005). Even though not all fungi contain chitin, it is widely distributed in various fungal phyla such as Basidiomycota, Ascomycota and Zygomycota (Peter, 2005). Similar to crustacean chitin, severe conditions are required to obtain chitosan from fungal chitin. In contrast, chitosan can be directly isolated from the cell wall of some fungal species without requiring the cleavage of the acetyl groups. Among them, the most investigated species for direct chitosan production include *Absidia* spp. (zygomycetes), *A. niger* (ascomycetes),

Mucor rouxii (zygomycetes), *Rhizophus oryzae* (zygomycetes) and *Lentinus edodes* (basidiomycetes) (Kim *et al*., 2001; Pochanavanich and Suntornsuk, 2002; Suntornsuk *et al*., 2002; Chatterjee *et al*., 2005). Nevertheless, the production of fungal chitin and chitosan has not yet been scaled up to the industrial level (Abdel-Gawad *et al*., 2017).

In addition to crustaceans and fungi, insects are another promising and sustainable source of chitin and chitosan, although they have not received much attention previously. Insects provide some advantages compared to crustaceans, as they are not subject to seasonality, and can be easily bred owing to their high fertility and reproductive rate; moreover, insect rearing facilities are being launched worldwide (van Huis, 2013). Notably, as bioconverters – reared for organic waste management and animal feed production – insects can be exploited as a valid alternative to crustaceans as a source for chitin and chitosan for greater ecological and economic sustainability (Jucker *et al*., 2020).

Arthropods, including centipedes (Kaya *et al*., 2016a; Bulut *et al*., 2017) and woodlice (Kaya *et al*., 2014a; Kaya and Sargin, 2016), have been investigated as sources of chitin. Notably, the body segments of large centipedes are suitable for the production of three-dimensional chitin rings (Kaya *et al*., 2016a). In addition, chitin has been extracted from poriferans (Petrenko *et al*., 2017), bryozoans (Kaya *et al*., 2015b) and tardigrades (Greven *et al*., 2016) and from guano of insectivorous bats (Kaya *et al*., 2014b).

Quo vadis insect chitin?

Economic value linked to beneficial insects has been known to humans for a long time. The commercial production of silk from *Bombyx mori* originated in China during the Neolithic period (Vainker, 2004). Humans have learned to farm several insect species and exploit them for specific applications with the course of time. Production of biocontrol insects started in the middle of the 20th century. For example, mass production of *Cochliomyia hominivorax* for biocontrol started in Florida during the late 1950s (Cáceres *et al*., 2012). Organized, large-scale production of insects for human and animal nutrition has been more recent: Protix, a Dutch company, launched the first facility in 2015.

Some industries in the domain of beneficial insect breeding are witnessing rapid growth. For example, the market for edible insects is estimated to exceed $522 million in 2023 (Kim *et al*., 2019). According to a report published in 2016, globally, more than 120 registered companies are involved in the business of farming and processing and/or marketing insects for animal and human nutrition (Ortiz *et al*., 2016). In 2019, approximately 6 kt of insect protein meal was produced in Europe (DiGiacomo and Leury, 2019) to be used for animal nutrition, from the

black soldier fly (*Hermetia illucens*), the yellow mealworm (*Tenebrio molitor*) and, to a smaller extent, the lesser mealworm (*Alphitobius diaperinus*) (Derrien and Boccuni, 2018).

In particular, the black soldier fly is processed by around 80% of all EU insect-producing companies (Derrien and Boccuni, 2018). *H. illucens* could be grown on a wide range of organic side streams and contributes to a circular economy (Schmitt *et al*., 2019; Star *et al*., 2020). Exoskeletons from *H. illucens* larvae contain up to 35% chitin (Hahn *et al*., 2018), which means that it is one of the main compounds that could be isolated from the byproducts of the insect farming industry (Ortiz *et al*., 2016).

Thus, chitin-rich byproducts from insect farming present a new and sustainable source of commercial chitin. Given the sustainability aspect and the expected rise in insect production, byproducts from insect farming present a very interesting source of chitin for the future.

The inner soft tissues of insects are covered by a hard, protective layer called the exoskeleton. The exoskeleton has several functions in insect bodies, including but not limited to: (i) as a protective covering and (ii) as a facilitator of metamorphosis. The exoskeleton is rich in chitin and is shed from the body during metamorphosis (Klowden, 2013). Chitin is contained in the procuticle, the innermost layer of the cuticle, which is in turn the outermost layer of the arthropod exoskeleton (Vincent and Wegst, 2004; Appel *et al*., 2015). In its native form, chitin is arranged into microfibers embedded in a protein matrix (Hamodrakas *et al*., 2002). To extract chitin from the arthropod cuticle, the purification process removes proteins, lipids, minerals, pigments and catechols contained therein (Mohammed *et al*., 2013). Whole insects generally contain 30–60% protein (Rumpold and Schluter, 2013), 10–25% lipid (Rumpold and Schluter, 2013), 5–25% chitin (Kramer *et al*., 1995; Kurita, 2006; Hahn *et al*., 2018), 5–10% catechols (Kramer *et al*., 1995) and 2–10% minerals such as calcium, phosphorus, potassium and magnesium salts (Finke, 2013; Rumpold and Schluter, 2013).

To date, little has been reported on the extraction methods for insect chitin and its physicochemical properties. Here, a total of 52 papers reporting chitin purification and chitosan production from 58 insect species were collected, summarized and analyzed (table 1.1 and 1.2).

CHITIN PURIFICATION

Various types of chitin purification processes can be performed, such as physical, biotechnological and chemical methods and a combination of these. Physical techniques such as crushing and stirring are used in parallel with chemicals or catalysts. Biotechnological extraction and deacetylation of chitin has been gaining interest as an environmentally friendly alternative to chemical processes. The biotechnological methods are mainly based on the use

of microbial proteases or whole microorganisms for the removal of proteins and the application of deacetylases for the deacetylation of chitin. However, the biotechnological processes developed so far produce lower yields, are time-consuming and result in products of lower purity (Mohammed *et al*., 2013; Philibert *et al*., 2017). Notably, enzymatic deacetylation of chitin using deacetylases has proven to be unsuitable for chitosan production (Martinou *et al*., 1995; Win and Stevens, 2001).

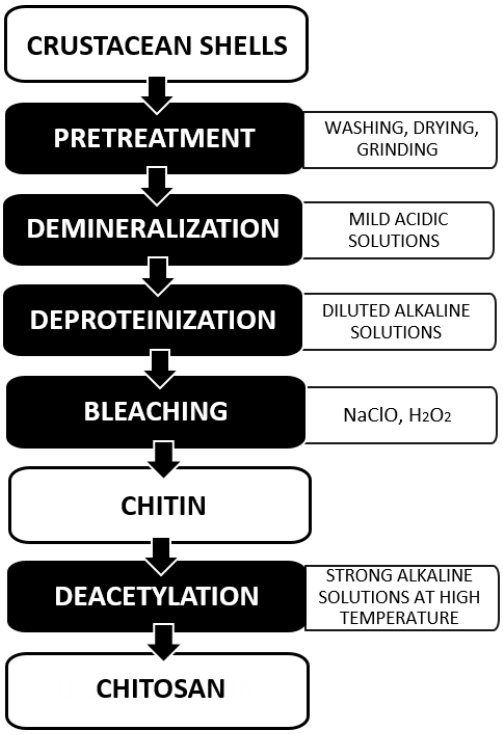


Figure 1.2. Industrial process for chitin purification and chitosan production from crustacean shells.

Chemical processes, utilizing acidic and alkaline solutions, are currently the most applied on an industrial scale to produce large amounts of chitin and chitosan from crustacean shells (Kim and Rajapakse, 2005; Philibert *et al*., 2017) (figure 1.2). Typically, chemical treatment provides pure chitin and chitosan, although it produces large volumes of waste due to the high concentrations of chemicals used (Kim and Rajapakse, 2005; Philibert *et al*., 2017; Wang *et al*., 2019). The literature reviewed here describes only chemical methods for chitin and chitosan production from insects.

Chemical methods for extracting chitin from insect exoskeletons are similar to those used for marine sources and consist of two steps: demineralization and removal of proteins. In the first step, minerals contained in the cuticle are removed with diluted acids. Various mineral acids (e.g., hydrochloric acid) or organic acids (e.g., acetic acid) can be used for this purpose. The removal of proteins is carried out with alkaline treatments, mainly applying diluted sodium

hydroxide solution. Deproteinization treatment can simultaneously extract part of the dyes and soluble lipids contained in the exoskeleton. For prawn shells, two-step purification can be performed in reverse order without affecting the properties of chitin (Mohammed *et al*., 2013). An additional step of bleaching can be performed to remove residual pigments and improve the colour of purified chitin, using reagents such as sodium hypochlorite, acetone and hydrogen peroxide.

Prior to the chitin purification process, insect samples are generally pretreated. Pretreatments include cleaning by washing with water and detergent, drying in an oven or at ambient temperature and grinding into powder to increase accessibility for the chemical agents. Solvent penetration into the particles, and thus purification efficiency, are greatly affected by corn size. Kim *et al*. (2016) obtained a higher demineralization efficiency and chitosan with a higher degree of deacetylation using ground insect samples instead of non-ground ones. A particle size lower than 200–250 μm is suitable for chitin purification (Zhang *et al*., 2000; Draczynski, 2008; Marei *et al*., 2016; Mehranian *et al*., 2017). A fat removal step may be required, especially for those insect samples rich in lipids, such as whole larvae. Larvae can be boiled and passed through an extruder or an oil press to separate unpurified chitin from the liquid fraction, containing mainly lipids and some proteins (Khayrova *et al*., 2019; Huet *et al*., 2020).

Demineralization

Crustacean shells contain a substantial amount of minerals, which can be up to 50% in crab and shrimp shells (Kurita, 2006). In contrast, insects have a much lower mineral content, generally ranging between 2 and 10% for whole insects. However, this value varies depending on the species and the stage of development (Finke, 2013; Rumpold and Schluter, 2013).

The demineralization of insect samples comprises the decomposition of minerals into their respective water-soluble salts. The solubilized salts can be separated from chitin by filtration and washing of the solid phase. Acidic treatment also releases catechol compounds and leads to a slight discoloration of biomass (Majtan *et al*., 2007). The demineralization process can be affected by the type and concentration of acid used, treatment time and temperature, particle size of the sample and the solute-to-solvent ratio (Younes and Rinaudo, 2015).

Hydrochloric acid is the most preferred reagent used for the demineralization of insect exoskeletons (table 1.1). Hydrochloric acid has also been one of the most widely used acids for the demineralization of crustacean shells on an industrial scale (Philibert *et al*., 2017). Because of its high environmental impact, hydrochloric acid has been substituted in many cases by organic acids. Furthermore, hydrochloric acid can have detrimental effects on the structure and

chemical composition of chitin (Mahmoud *et al*., 2007), as has been confirmed by investigations of Percot *et al*. (2003), who reported a lower molecular weight and a lower degree of acetylation of chitin after demineralization. As a rule of thumb, the harsher the demineralization treatment – in terms of pH, duration and temperature – the higher is the degree of hydrolysis and the worse are the aforementioned effects on chitin (Rojsitthisak *et al*., 2002). In contrast to the frequently used hydrochloric acid, only four investigations have reported the use of organic acids for the demineralization of insect biomass. Ibitoye *et al*. (2018) and Song *et al*. (2013) used oxalic acid and Badawy and Mohamed (2015) and Hahn *et al*. (2020) used acetic acid and formic acid, respectively.

In most cases, the concentration of the acidic solution used for demineralization is 1–2 mol L^{-1}. Few papers have reported the application of a higher concentration of 4 mol L^{-1} (Kaya *et al*., 2014a, 2015a, 2015c). The solute-to-solvent ratio depends on the acid concentration, as it needs two molecules of hydrochloric acid to convert one molecule of calcium carbonate, the main mineral component of the insect exoskeleton, into calcium chloride, carbon dioxide and water (Shahidi and Synowiecki, 1991).

The time required for demineralization treatment is usually short. Most protocols have reported an incubation time between 30 min and 3 h. In a few cases, the treatment lasted for up to 6 h (Kaya *et al*., 2015d, 2016c), 12 h (Ma *et al*., 2015) and 24 h (Kaya *et al*., 2015g, 2017a; Shin *et al*., 2019). The range of temperature used for demineralization varies widely from room temperature to 100 °C. High temperatures are used for very short periods (20–30 min), as reported by Kaya *et al*. (2015f) and Monter-Miranda *et al*. (2016). In contrast, longer treatment periods (12–24 h) are applied for incubations done at room temperature (Ma *et al*., 2015; Kaya *et al*., 2017a; Shin *et al*., 2019). High temperatures promote the penetration of the solvent into the chitin matrix (Truong *et al*., 2007); however, they can cause polymer degradation (de Queiroz Antonino *et al*., 2017).

The efficiency of demineralization (DME) can be evaluated by assessing the mineral content of insect samples before (MC_{BT}) and after treatment (MC_{AT}) according to the following equation (1):

$$DME\ (\%) = \frac{MC_{BT}\ (\%) - MC_{AT}\ (\%)}{MC_{BT}\ (\%)} \times 100 \quad (1)$$

From the scanty data available in the literature on the efficiency of the demineralization of insect biomass, we can observe that the highest efficiency (86–98%) was achieved by Zhou *et*

al. (2019), using natural deep eutectic solvents on *H. illucens* prepupae (table 1.1). Of the organic acids used, oxalic acid, applied by Ibitoye *et al*. (2018), resulted in a higher degree of demineralization compared to that reported by Kim *et al*. (2016, 2017) with hydrochloric acid, although a lower concentration of oxalic acid had been used. Demineralization efficiency for the *H. illucens* larval exoskeleton, reported by Hahn *et al*. (2020b), was similar to that obtained by Ibitoye *et al*. (2018), using formic acid. However, additional data on the efficiency of insect demineralization are not available. Thus, future studies should focus on assessing the suitability and optimization of the current methods. Precise evaluation of the efficiency of various acids will enable choosing acids with a lower environmental impact but guarantee good demineralization. For instance, Mahmoud *et al*. (2007) and Ameh *et al*. (2014) have reported that the efficiency of demineralization of shrimp shells using lactic or acetic acids is comparable to that obtained using hydrochloric acid. Values of demineralization efficiency for shrimp shells, using either hydrochloric acid or acetic and lactic acid, as reported by Mahmoud *et al*. (2007), are similar to those obtained by Ibitoye *et al*. (2018) with oxalic acid using insect samples. Organic acids, namely lactic, acetic and oxalic acids, can therefore be a valid alternative to hydrochloric acid for the demineralization of insect biomass. The utilization of organic acids also provides other benefits as they are less harmful to the environment, can preserve the characteristics of purified chitin, can be produced from low-cost biomass and the extracted organic salts can be used for other applications (Mahmoud *et al*., 2007).

Once the minerals have been solubilized and removed, the insect biomass is washed with distilled water until its pH is restored to neutral. After the neutralization step, insect samples are subjected to deproteinization

Protein removal

Deproteinization of insect biomass is commonly achieved using alkaline solutions. A wide range of chemicals has been tested as deproteinization reagents with crustacean samples, including sodium hydroxide, sodium carbonate, sodium bicarbonate, potassium hydroxide, potassium carbonate, calcium hydroxide, sodium sulfite, sodium bisulfite, trisodium phosphate and sodium sulfite (Younes and Rinaudo, 2015). As well as for demineralization, the efficiency of deproteinization depends on the concentration of the alkali, solid-to-solvent ratio and time and temperature of the treatment (Tharanathan and Kittur, 2003). Although high temperatures are crucial for deproteinization efficiency, they can cause undesirable side reactions if combined with very long incubation times. These include partial deacetylation of chitin and hydrolysis of the biopolymer, with consequent changes in its characteristics, primarily the

degree of acetylation and molecular weight. (Hours and Gortari, 2013; Younes and Rinaudo, 2015).

Sodium hydroxide is the most widely used base for deproteinization of crustacean biomass for the industrial production of chitin (Pighinelli, 2019). It has also been widely used for chitin purification from insects (table 1.1). Badawy and Mohamed (2015) are the only researchers which used potassium hydroxide to deproteinize insect samples. The use of potassium hydroxide has also been suggested by Fu *et al*. (2019) and Castillo *et al*. (2003) as an eco-friendlier alternative to sodium hydroxide for shrimp deproteinization, as the liquid waste generated using potassium hydroxide is suitable for use as a fertilizer, owing to its high phosphorus, potassium and nitrogen content (Fu *et al*., 2019). However, major deproteinization processes of insect samples have been performed using sodium hydroxide in low concentrations (0.5–2 mol L^{-1}). In a few cases, a concentration of 4 mol L^{-1} has been used (Kaya *et al*., 2014a, 2015a, 2015g). The incubation time required for the deproteinization of insect biomass varies greatly from a few hours to a few days; however, the treatment typically lasts for 16–20 h (table 1.1). Using the same working conditions, long deproteinization times applied to crustacean biomass have been reported to lead to a higher loss of proteins compared to shorter treatments (Paduretu *et al*., 2018). The deproteinization reaction is generally performed at 80–100 °C, with a few exceptions being 40 °C (Badawy and Mohamed, 2015) or 175 °C (Kaya *et al*., 2015d).

As well as for demineralization, the efficiency of deproteinization (DPE) can be evaluated by measuring the protein content of insect samples before (PC_{BD}) and after deproteinization (PC_{AD}), according to the following equation (2):

$$DPE\ (\%) = \frac{PC_{BD}\ (\%) - PC_{AD}\ (\%)}{PC_{BD}\ (\%)} \times 100 \qquad (2)$$

Deproteinization efficiency of insect biomass has been mentioned only by Kim *et al*. (2016, 2017). In both cases a maximum efficiency of 86–87% was achieved by applying 1.25 mol L^{-1} sodium hydroxide for 3 h at 95 °C on adult specimens of *M. domestica* (Kim *et al*., 2016) and *G. bimaculatus* (Kim *et al*., 2017). These values are similar to those obtained by Zhou *et al*. (2019), who used natural deep eutectic solvents to remove proteins from *H. illucens* prepupae. The deproteinization efficiency of sodium hydroxide on crustacean shells at both high and room temperature was 71–76% (Pighinelli, 2019).

Results from chitin purification

Information is lacking on the results obtained from chemical demineralization and deproteinization of insect samples. Most papers have reported only chitin yield as the end result of the purification process (table 1.1). Chitin yield is measured as the percentage ratio of dry weight of chitin and dry weight of the source material. Chitin yield from insect biomass varied from a minimum of approximately 2% – obtained from *Vespa crabro* larvae (Kaya *et al*., 2016b), *G. bimaculatus* adults (Kim *et al*., 2017), *Apis mellifera* adults (Marei *et al*., 2016) and *B. mori* larvae (Paulino *et al*., 2006) – to a maximum of 36% obtained from *A. mellifera* adults (Nemtsev *et al*., 2004) and cicada sloughs (Sajomsang and Gonil, 2010).

However, most authors have reported a chitin yield between 5 and 15% (table 1.1). Yields of chitin extracted from shells of crustaceans, mainly shrimps, prawns and crabs, varied from 5 to 32% (Das *et al*., 1996; Tharanathan and Kittur, 2003; Odote *et al*., 2007; Cortizo *et al*., 2008; Thirunavukkarasu and Shanmugam, 2009; Bolat *et al*., 2010; Hossain and Iqbal, 2014; Kaya *et al*., 2014a). Chitin content can vary widely depending on the species, developmental stage and body part of the crustacean (Thirunavukkarasu and Shanmugam, 2009; Bolat *et al*., 2010; Erdogan and Kaya, 2016; Kaya *et al*., 2016e). For example, Thirunavukkarasu and Shanmugam (2009) reported that the yield of chitin was higher from the cuticle of crab legs than from its carapace and claws. These findings are in accordance with the results of Kaya *et al*. (2015e) who found a higher chitin content in the legs than in other body parts of honeybees. Thus, the chitin content of a body part correlates with the mechanical load on that body part.

Only Huet *et al*. (2020) and Kaya *et al*. (2016b) have measured the degree of purification for chitin extracted from *Bombyx eri* larvae and three developmental stages of *V. crabro*, respectively (table 1.1). Using various times and temperatures for both demineralization and deproteinization treatments, they obtained similar results, that is, between 93 and 97%. Using natural deep eutectic solvents, Zhou *et al*. (2019) achieved a slightly lower degree of purification (74–91%) from *H. illucens* prepupae. The lack of quantitative assessment of the purity of insect-based chitin makes it difficult to evaluate the suitability of the methods.

After demineralization and deproteinization, chitin can be directly deacetylated to chitosan or can be bleached to improve its colour and remove the residual lipids.

Bleaching as the final step

Many kinds of pigments and structural colours are involved in the coloration of insect cuticle. They originate from the tyrosine-mediated cuticle-tanning pathway, such as melanins, or originate from 3,4-dihydroxyphenylalanine and dopamine during the process of cuticular

tanning and sclerotization (Sugumaran, 2009). During demineralization and deproteinization treatments, a small amount of pigment and lipid is removed; however, chitin retains a brownish appearance. For commercial purposes, the colour of chitin and chitosan is required to be as white as possible. Thus, an additional step of bleaching is used to remove residual pigments and improve chitin, and thus chitosan, colour.

Industrial methods for purifying chitin from crustacean waste include the use of sodium hypochlorite or hydrogen peroxide as bleaching agents (Hayes *et al*., 2008; Yeul and Rayalu, 2012). A few papers have reported the use of hydrogen peroxide combined with hydrochloric acid for bleaching insect samples (Nemtsev *et al*., 2004; Majtan *et al*., 2007). Sometimes sodium hypochlorite (Sajomsang and Gonil, 2010; Song *et al*., 2013; Kaya *et al*., 2017a; Ibitoye *et al*., 2018) or ammonium peroxidisulfate (Chae *et al*., 2018) are used, but insect decolorization is performed using a mixture of methanol–chloroform (Kaya and Baran, 2015; Erdogan and Kaya, 2016; Mol *et al*., 2018; Tan *et al*., 2018; Khayrova *et al*., 2019) or alcohol–chloroform (Kaya *et al*., 2014a, 2015a). Treatment with potassium permanganate and oxalic acid is also done frequently (Ai *et al*., 2008; Draczynski, 2008; Liu *et al*., 2012; Ma *et al*., 2015; Waśko *et al*., 2016; Mehranian *et al*., 2017; Luo *et al*., 2019). Even though they are often used, the approaches using organic solvents have no or marginal bleaching efficiency, as they do not break the bonds between chitin and tannins or catecholamines.

Bleaching treatment of insect samples is performed at room temperature for a short duration (40–90 min) (Kaya *et al*., 2015d, 2017a; Mol *et al*., 2018). Higher temperatures were applied by Chae *et al.* (2018) (50 °C for 30 min with ammonium peroxidisulfate) and Nemtsev *et al*. (2004) (75 °C for 1 h with hydrogen peroxide). A combination of potassium permanganate and oxalic acid has also been used, where the bleaching step was split into two steps: treatment with potassium permanganate at room temperature and with oxalic acid at high temperatures (60–70 °C) (Draczynski, 2008; Ma *et al*., 2015).

Evaluation of the success of the bleaching step using various reagents has not been provided. The bleaching effect of a decolorization treatment can be quantitatively evaluated by measuring the L*, a* and b* values of a sample using a colorimeter, according to the CIELab colour system. CIELab is a colour space that expresses colours as three values: L* for lightness, a* from green to red and b* from blue to yellow. From these values, the whiteness index can also be calculated (Ploydee and Chaiyanan, 2014).

Information on the effect of chitin bleaching on the yield and characteristics of chitosan has been given only by Nemtsev *et al*. (2004). Part of the chitin extracted from honeybee corpses was bleached using 3% hydrogen peroxide for 1 h at 75 °C. The yield of chitosan derived from

non-bleached chitin (20–30%) was slightly higher than that of chitosan produced from bleached chitin (16–25%); the degree of deacetylation of the two types of chitosan was similar (Nemtsev *et al*., 2004). Furthermore, chitin bleaching can greatly affect the viscosity of solubilized chitosan (Nemtsev *et al*., 2004; Yeul and Rayalu, 2012).

After the bleaching treatment is complete, chitin is dried, and characterized to investigate its suitability for a desired application. Otherwise, chitin can be deacetylated to afford chitosan.

Table 1.1 Methods for chitin purification from insects and respective yields. Where stated, "m" indicates male specimens and "f" indicates the females.

RAW MATERIAL		DEMINERALIZATION			DEPROTEINIZATION			CHITIN YIELD (dry chitin weight / dry insect weight) (%)	REFERENCE
Insect species	**Stage / Body part**	**Reagent and conc.**	**Temp. (°C)**	**Duration (h)**	**Reagent and conc.**	**Temp. (°C)**	**Duration (h)**		
Musca domestica	larvae	-	-	-	1 M NaOH	95	6	-	Ai *et al*., 2008
Shistocerca gregarea	adults	0.9 M CH_3COOH	-	-	1.8 M KOH	40	-	-	Badawy *et al*., 2015
Nezara viridula	adults							-	
Periplaneta americana	adults							-	
Blatella germanica	adults							-	
Vespa orientalis	adults							-	
Gryllus bimaculatus	adults	-	-	-	1 M NaOH	95	3 h	5.1	Chae *et al*., 2018
Apis mellifera	adults	1 M HCl	room temp.	1	1 M NaOH	80	6-64	51-77.2	Draczynski, 2008
Dociostaurus maroccanus	adults	2 M HCl	55	1	2 M NaOH	50	18	12.0, 14.0	Erdogan and Kaya, 2016
Hermetia illucens	larval exoskeleton	0.5 M CH_2O_2	room temp.	1	2 M NaOH	80	2	31.0-35.0	Hahn *et al*., 2020b
Bombyx eri	larvae	1 M HCl	80	0.6	1 M NaOH	80	24	3.3	Huet *et al*., 2020
Acheta domesticus	adults	0.1 M $C_2H_2O_4$	room temp.	3	1 M NaOH	95	-	4.3-7.1	Ibitoye *et al*., 2018
Colophon sp.	adults	2 M HCl	room temp.	6	1.5 M NaOH	10	24	-	Jang *et al*., 2004
Melolontha melolontha	adults	4 M HCl	75	-	1 M NaOH	150	18	-	Kaya *et al*., 2014a
Agabus bipustulatus	adults	1 M HCl	90	1	1 M NaOH	110	18	14.0-15.0	Kaya *et al*., 2014c
Anax imperator	adults							11.0-12.0	
Hydrophilus piceus	adults							19.0-20.0	
Notonecta glauca	adults							10.0-11.0	
Ranatra linearis	adults							15.0-16.0	
Leptinotarsa decemlineata	larvae, adults	2 M HCl	65-75	2	2 M NaOH	80-90	16	7.0, 20.0	Kaya *et al*., 2014d

Vespa crabro	adults	2 M HCl	75	2	4 M NaOH	150	18	-	Kaya *et al*., 2015c
Vespa orientalis	adults							-	
Vespula germanica	adults							-	
Ailopus simulatrix	adults	4 M HCl	75	1	2 M NaOH	175	18	5.3	Kaya *et al*., 2015d
Ailopus strepens	adults							7.4	
Duroniella fracta	adults							5.7	
Duroniella laticornis	adults							6.5	
Oedipoda miniata	adults							8.1	
Oedipoda caerulescens	adults							8.9	
Pyrgomorpha cognata	adults							6.6	
Celes variabilis	adults	4 M HCl	75	2	4 M NaOH	150	20	6.6-9.9	Kaya *et al*., 2015a
Decticus verrucivorus	adults							10.0-11.8	
Melanogryllus desertus	adults							4.7-7.3	
Apis mellifera	legs	2 M HCl	80	6	2 M NaOH	100	20	13.2	Kaya *et al*., 2015e
	thorax							6.8	
	head							8.9	
	abdomen							8.6	
	wings							7.6	
Argynnis pandora	wings	2 M HCl	50	24	2 M NaOH	50	24	22.0	Kaya *et al*., 2015f
	other body parts							8.0	
Calliptamus barbarus	adults	1 M HCl	100	0.5	1 M NaOH	80-90	21	20.5	Kaya *et al*., 2015g
Oedaleus decorus	adults							16.5	
Palomena prasina	adults	2 M HCl	100	2	2 M NaOH	140	20	10.8	Kaya *et al*., 2015b
Omophlus sp.	adults	2 M HCl	50	4	2 M NaOH	100	20	-	Kaya *et al*., 2016d

Vespa crabro	larvae, pupae, adults	1 M HCl	50	6	1 M NaOH	60	16	2.2, 6.2, 10.3	Kaya *et al*., 2016b
Drosophila melanogaster	adults	2 M HCl	40	3	3 M NaOH	70	20	7.8	Kaya *et al*., 2016c
Melontha sp.	whole body (f, m)	2 M HCl	60	20	2 M NaOH	100	20	15.7 f, 16.6 m	Kaya *et al*., 2016e
	antennae (f, m)							5.9 f, 10.5 m	
	head (f, m)							16.0 f, 15.9 m	
	eyes (f, m)							8.9 f, 8.9 m	
	thorax (f, m)							13.8 f, 17.5 m	
	abdomen (f, m)							7.1 f, 6.5 m	
	elytra (f, m)							37.9 f, 40.1 m	
	hindwings (f, m)							17.9 f, 17.3 m	
	legs (f, m)							17.8 f, 16.1 m	
Blaberus giganteus	wings	1 M HCl	room temp.	24	2 M NaOH	90	9	26.9	Kaya *et al*., 2017a
	dorsal pronotum							21.2	
Hylobius abietis	adults	2 M HCl	room temp.	-	2 M NaOH	-	2	27.9	Kaya *et al*., 2019
Periplaneta americana	wings	4 M HCl	75	2	4 M NaOH	150	20	18.0	Kaya and Baran, 2015
	other body parts							13.0	
Hermetia illucens	larvae	0.5 M HCl	room temp.	2	1.9 M NaOH	50	2	7.0 (amorphous chitin)	Khayrova *et al*., 2019
Musca domestica	adults	2 M HCl	room temp.	3	1.25 M NaOH	95	3	7.7-8.5	Kim *et al*., 2016
Gryllus bimaculatus	adults	2 M HCl	room temp.	3	1.25 M NaOH	95	3	2.4	Kim *et al*., 2017
Holotrichia parallela	adults	1 M HCl	100	-	1 M NaOH	80	-	15.0	Liu *et al*., 2012
Cicada	sloughs	1 M HCl	30	2	1 M NaOH	90	2	-	Luo *et al*., 2019
Bombyx mori	chrysalis							-	
Tenebrio molitor	larvae							-	
Grasshopper	adults							-	

Catharsius molossus	adults	1.3 M HCl	80 + room temp.	0.5 + 12	4 M NaOH	90 + room temp.	6 + 12	24.0	Ma *et al*., 2015
Bombus terrestris	adults	1 M HCl	100	0.3	1 M NaOH	85	24	-	Majtan *et al*., 2007
Schistocerca gregaria	adults	1 M HCl	room temp.	-	1 M NaOH	100	8	12.2	Marei *et al*., 2016
Apis mellifera	adults							2.5	
Calosoma rugosa	adults							5.0	
Ephestia kuehniella	adults	1 M HCl	100	0.3	1 M NaOH	85	1	9.5-10.5	Mehranian *et al*., 2017
Cicada lodosi	adults	2 M HCl	100	2	2 M NaOH	100	20	4.8	Mol *et al*., 2018
Cicada mordoganensis	adults							6.5	
Cicadatra platyptera	adults							8.8	
Cicadatra atra	adults							6.7	
Cicadatra hyalina	adults							5.5	
Cicadivetta tibialis	adults							5.9	
Brachystola magna	adults	1 M HCl	97	0.5	1 M NaOH	82	24	10.4	Monter-Miranda *et al*., 2016
Apis mellifera	adults	-	-	-	15 M NaOH	-	-	11.4-36.8	Nemtsev *et al*., 2004
Calliphora erythrocephala	larvae	-	-	-	1 M NaOH	50	2	12.2	Odote *et al*., 2007
Bombyx mori	larvae	1 M HCl	100	-	1 M NaOH	80	-	2.6-4.3	Paulino *et al*., 2006
Cicada	sloughs	1 M HCl	100	0.3	1 M NaOH	80	36	36.0	Sajomsang and Gonil, 2010
Tenebrio molitor	larvae, superworm, adults	2 M HCl	room temp.	24	3.7 M NaOH	80	24	4.6, 3.9, 8.4	Shin *et al*., 2019
Allomyrina dichotoma	larvae, pupae, adults							10.5, 12.7, 14.2	
Chrysomya megacephala	larvae	0.1 M $C_2H_2O_4$	-	3	1 M NaOH	95	6	-	Song *et al*., 2013
Hermetia illucens	larvae, prepupae, pupae	1 M HCl	100	0.5	1 M NaOH	80	24	3.8, 4.7, 6.3	Smets *et al*., 2020
Zophobas morio	larvae	1 M HCl	35	0.5	0.5-2 M NaOH	80	20	4.7-5.2	Soon *et al*., 2018

Mayfly	adults	2 M HCl	50	-	2 M NaOH	100	-	10.2	Tan *et al*., 2018
Hermetia illucens	pupal exuviae, adults	1 M HCl	-	-	1 M NaOH	80	24	-	Wasko *et al*., 2016
Clanis bilineata	larval skins	2 M HCl	room temp.	24	3.7 M NaOH	60	24	-	Wu, 2012
Cryptotympana atrata	sloughs	2 M HCl	room temp.	24	3.7 M NaOH	60	24	-	Wu *et al*., 2013
Musca domestica	larvae	-	-	-	1 M NaOH	100	3	-	Zhang A. *et al*., 2011
Bombyx mori	pupal exuviae	1 M HCl	100	-	1 M NaOH	80	3	15.0-20.0	Zhang M. *et al*., 2000
Beetle	larvae							15.0-20.0	
Hermetia illucens	prepupae	NADESs	50-80	2	NADESs	50-80	2	6.0-26.0	Zhou *et al*., 2019

CONVERSION OF CHITIN INTO CHITOSAN

Chitosan is obtained from the deacetylation of chitin (i.e., the removal of acetyl groups from the chitin polymer). The resultant chitosan has a high degree of free amino groups ($-NH_2$) that provides active sites for many chemical reactions, thus making chitosan a versatile polymer that is suitable for several modifications and applications (Yeul and Rayalu, 2012). Furthermore, chitosan has a much higher solubility than chitin and can be dissolved in slightly acidic solutions, while chitin solubilization requires strong and highly concentrated mineral or solutions of lithium chloride and organic solvents such as dimethylformamide, dimethyl-acetamide and N-methyl-2-pyrrolidone (Hu *et al*., 2007).

Chitin can be converted to chitosan by chemical deacetylation. Alternatively, enzymatic deacetylation using deacetylases has also been attempted. The enzymatic activity of various deacetylases towards chitin has been established; however, they are not efficient in converting chitin to chitosan because of the crystallinity of chitin (Martinou *et al*., 1995; Win and Stevens, 2001; Wattjes *et al*., 2019). Pretreatment of chitin, such as heating, grinding and treating with an alkaline solution, can lead to a higher, yet insufficient, enzymatic activity (Win and Stevens, 2001). Due to this limitation, on an industrial scale, chemical deacetylation is the most commonly used method for chitosan preparation from crustacean waste because of the low cost and suitability for mass production (Younes and Rinaudo, 2015). Deacetylation is performed by incubating chitin in a concentrated solution of sodium hydroxide. Chitin deacetylation can be performed either heterogeneously or homogeneously. In the heterogeneous method, chitin is usually treated with a hot concentrated solution of sodium hydroxide for a few hours. Within the frame of the homogeneous method, chitin is incubated in a concentrated sodium hydroxide solution at room temperature for a few hours, followed by dissolution in crushed ice at 0 °C (Younes and Rinaudo, 2015; Hahn *et al*., 2020b). Heterogeneous conditions result in an irregular distribution of N-acetyl-D-glucosamine / D-glucosamine units and a blockwise distribution of acetyl groups along the chitosan chain. In contrast, chitosan obtained under homogeneous conditions has a random distribution of acetyl groups along the chain. Hence, chitosans produced using these two methods can have different physicochemical properties (Younes *et al*., 2014).

Almost solely chemical heterogeneous deacetylation has been reported for chitosan production from insects with sodium hydroxide being used as the deacetylating agent. In a few cases, sodium hydroxide was combined with sodium borohydride as a ‘protecting reagent’ (Zhang *et al*., 2000; Paulino *et al*., 2006; Monter-Miranda *et al*., 2016). For heterogeneous deacetylation of insect biomass, the sodium hydroxide concentration ranges from 40 to 60% (i.e., about 15–

22 mol L^{-1}) (table 1.2). In most cases, the deacetylation step lasts from 1 to 9 h, with a few exceptions of longer incubation times of up to 2 days (Kaya *et al*., 2016c; Soon *et al*., 2018). Temperatures of heterogeneous deacetylation of insect samples range from 90 to 150 °C (table 1.2). Ideally, deacetylation should result in non-degraded chitosan with a high degree of deacetylation, enabling its solubilization in dilute acidic solutions (Yeul and Rayalu, 2012). The degree of deacetylation is defined as the proportion of glucosamine monomer residues in the chitosan chain and it can affect the solubility and performance of chitosan in many of its applications (Jaworska *et al*., 2003). Deacetylation can be optimized and adjusted according to need by adjusting various factors, including temperature, time, alkali concentration, solid-to-solvent ratio and particle size.

Assessment of chitin conversion into chitosan

Results obtained for chemical heterogeneous deacetylation of insect samples, in terms of chitosan yield, degree of deacetylation and molecular weight, are reported in table 1.2. Chitosan yield was calculated from the dry biomass of the original insect biomass or the dry weight of chitin. Chitosan yields measured from the original biomass ranged from 2 to 8%. The highest values (26–28%) were obtained by Song *et al*. (2013) from *Chrysomya megacephala* larvae and by Luo *et al*. (2019) from cicada sloughs. Chitosan yield calculated from the respective chitin dry weight ranged from 60 to 83% (table 1.2). Only Hahn *et al*. (2020b) have performed both heterogeneous and homogeneous deacetylation of the *H. illucens* larval exoskeleton, obtaining more than double the yield of chitosan with the heterogeneous method compared to the homogeneous method. Yields of chitosan produced from crustaceans with heterogeneous deacetylation varied from 4 to 15% (related to the initial dry biomass) (No and Meyers, 1989; Odote *et al*., 2007; Bolat *et al*., 2010; Hossain and Iqbal, 2014; Luo *et al*., 2019). These values are slightly higher than those obtained from insects. The primary reason for that is the presence of larger amounts of protein and fat in insect samples (de Castro *et al*., 2018). However, as with chitin, chitosan yield can be affected not only by the purification process, but also by the species and harvest time (Younes *et al*., 2014). Considering the reported data, a unique definition of 'yield' for both chitin and chitosan is needed to make the methods of measurement uniform.

The degree of deacetylation of chitosan produced from insects with heterogeneous deacetylation varied between 62 and 98% (table 1.2). A lower value has been reported only by Monter-Miranda *et al*. (2016) who obtained 57% deacetylated chitosan from *Brachystola magna* adults. The degree of deacetylation of chitosan extracted from crustaceans with the heterogeneous method normally ranges from 56 to 98% (Islam *et al*., 2016), while the average degree of

deacetylation obtained with homogeneous treatment is 48–55% (Younes and Rinaudo, 2015). At least 80–85% deacetylation is necessary a good solubility of chitosan (Islam *et al*., 2016). The degree of deacetylation can be increased or decreased by changing temperature, time and sodium hydroxide concentration (Yeul and Rayalu, 2012). Moreover, there is a correlation between temperature and rate of deacetylation: high temperatures can increase deacetylation, whereas long residence times can improve deacetylation but only up to a certain point. For instance, alkali treatment using 50% sodium hydroxide at 100 °C beyond 2 h does not deacetylate crustacean-based chitin further significantly; rather it can degrade the polymer chain (Yeul and Rayalu, 2012).

Chitosan is a biopolymer of high molecular weight, which varies depending on the source and deacetylation treatment applied. The molecular weight of crustacean-based chitosan ranges from 100 to 1000 kDa (Hossain and Iqbal, 2014; de Queiroz Antonino *et al*., 2017). Chitosan produced from insects has a molecular weight ranging from 26 to 300 kDa (table 1.2). Very low values (3 and 7 kDa) have been reported, too (Kaya *et al*., 2014d; Erdogan and Kaya, 2016; Tan *et al*., 2018). High reaction temperatures (150 °C) combined with long incubation times (4–6 h) may have caused polymer degradation. The use of standard chitosans with known molecular weights can be useful to assess the validity of the applied analysis method. However, differences in chitosan molecular weights can also be related to the insect species, as shown by Kim *et al*. (2017), who applied the same deacetylation conditions as Kaya *et al*. (2014d) and obtained a chitosan with a much higher molecular weight (308 kDa from adult crickets versus approximately 3 kDa from both adults and larvae of the Colorado potato beetle). A very high molecular weight (3290–5900 kDa) has been reported by Paulino *et al*. (2006) for chitosan produced from silkworm using 40% sodium hydroxide and sodium borohydride at 100 °C. This could be because sodium borohydride prevents oxidative cleavage of glycosidic bonds during deacetylation.

Deacetylation treatment conditions can affect chitosan molecular weight and thus its physicochemical properties or bioactivity. Notably, molecular weight has a great influence on the biological activity of chitosan: chitosan with low molecular weight (i.e., lower than 150 kDa) has good antibacterial properties (Zivanovic *et al*., 2004; Vishu Kumar *et al*., 2005). Studies carried out on bacteria potentially pathogenic to humans, such as *Salmonella typhimurium*, *Listeria monocytogenes*, *Bacillus cereus* and *E. coli*, have confirmed that low-molecular-weight chitosan has a greater effect on reducing microorganism growth and multiplication (Zivanovic *et al*., 2004; Vishu Kumar *et al*., 2005). Small chitosan chains have higher mobility, attraction and ionic interaction than long chains, facilitating an effective

binding of chitosan to the membrane surfaces of bacteria (Vishu Kumar *et al*., 2005).

To evaluate the suitability of chitin and chitosan for desired applications, they need to be characterized. Several metrics can be used to assess chitin and chitosan characteristics and properties, such as degree of deacetylation, molecular weight, viscosity, morphology and solubility. Analysis methods and the investigated characteristics vary according to the final purpose.

Table 1.2 Methods for chitin deacetylation and characteristics of respective chitosan.

RAW MATERIAL		DEACETYLATION			CHITOSAN YIELD (%)	DEACETYLATION DEGREE (%)	MOLECULAR WEIGHT (kDa)	REF.
Insect species	**Stage/Body part**	**Reagent and concentration**	**Temperature (°C)**	**Duration (h)**				
Musca domestica	larvae	15 M NaOH	70	8	-	90	-	Ai *et al*., 2008
Gryllus bimaculatus	adults	19-25 M NaOH	-	15	41.7 (from chitin)	56-85	50-190	Chae *et al*., 2018
Dociostaurus maroccanus	adults	22 M NaOH	150	4	81.7 (from chitin)	64	7	Erdogan and Kaya, 2016
Hermetia illucens	larval exoskeleton	12 M NaOH	120-140	3-6	8.0-16.0 (from initial biomass)	43-72	-	Hahn *et al*., 2019
		10 M NaOH	4	12	4.0 (from initial biomass)	34	-	
Acheta domesticus	adults	-	-	-	2.3-5.8 (from initial biomass)	80	-	Ibitoye *et al*., 2018
Agabus bipustulatus	adults	22 M NaOH	120	2	71.0 (from chitin)	74-78	-	Kaya *et al*., 2014c
Anax imperator	adults				67.0 (from chitin)		-	
Hydrophilus piceus	adults				74.0 (from chitin)		-	
Notonecta glauca	adults				69.0 (from chitin)		-	
Ranatra linearis	adults				70.0 (from chitin)		-	
Leptinotarsa decemlineata	larvae	19 M NaOH	100	3	67.0 (from chitin)	76	3	Kaya *et al*., 2014d
	adults				72.0 (from chitin)	82	3	
Calliptamus barbarus	adults	19 M NaOH	130	2	74.0-75.0 (from chitin)	70-75	-	Kaya *et al*., 2015g
Oedaleus decorus	adults				75.0-76.0 (from chitin)		-	
Drosophila melanogaster	adults	22 M NaOH	150	48	70.9 (from chitin)	-	-	Kaya *et al*., 2016c
Hylobius abietis	adults	22 M NaOH	100	4	86.2 (from chitin)	81	7	Kaya *et al*., 2019
Hermetia illucens	larvae	19 M NaOH	100	2 h	80.0 (from chitin), 32.0 (from amorphous chitin)	90	160	Khayrova *et al*., 2019

Musca domestica	adults	19 M NaOH	95-105	3-5	6.8 (from initial biomass)	33-97	-	Kim *et al*., 2016
Gryllus bimaculatus	adults	19 M NaOH	100	3	1.8 (from initial biomass)	-	308	Kim *et al*., 2017
Cicada	sloughs	22 M NaOH	100	8	28.2 (from initial biomass)	84	38	Luo *et al*., 2019
Bombyx mori	chrysalis				3.1 (from initial biomass)	85	41	
Tenebrio molitor	larvae				2.5 (from initial biomass)	86	40	
Grasshopper	adults				5.7 (from initial biomass)	90	39	
Catharsius molossus	adults	18 M NaOH	25-90	24 7	-	95	450	Ma *et al*., 2015
Schistocerca gregaria	adults	19 M NaOH	100	8	-	98	-	Marei *et al*., 2016
Apis mellifera	adults				-	96	-	
Calosoma rugosa	adults				-	95	-	
Brachystola magna	adults	15 M NaOH + $NaBH_4$ 0.25 g	105-110	-	8.1 (from initial biomass)	57	26	Monter-Miranda *et al*., 2016
Apis mellifera	adults	19 M NaOH	150	1	20.0-30.0 (from chitin) 16.0-25.0 (from bleached chitin)	75-84	200-250	Nemtsev *et al*., 2004
Calliphora erythrocephala	larvae	19 M NaOH	100-120	1-4	66.7 (from chitin)	63-81	263-413	Odote *et al*., 2007
Bombyx mori	larvae	15 M NaOH + $NaBH_4$ 1 g/L	100	-	-	78-97	3290-5900	Paulino *et al*., 2006
Tenebrio molitor	larvae, superworm, adults	21 M NaOH	90	9	80, 83.3, 78.3 (from chitin)	75, 76, 76	-	Shin *et al*., 2019
Allomyrina dichotoma	larvae, pupae, adults				83.4, 83.4, 75.0 (from chitin)	76, 76, 75	-	
Chrysomya megacephala	larvae	25 M NaOH	90	9	26.2 (from initial biomass)	88-90	501	Song *et al*., 2013
Zophobas morio	larvae	19 M NaOH	90	30	65.0-75.0 (from chitin)	64-81	-	Soon *et al*., 2018
Mayfly	adults	22 M NaOH	150	6	78.4 (from chitin)	84	4	Tan *et al*., 2018
Clanis bilineata	larval skins	21 M NaOH	110	4	-	-	-	Wu, 2012

Cryptotympana atrata	sloughs	21 M NaOH	110	4	-	-	-	Wu *et al*., 2013
Musca domestica	larvae	15 M NaOH + $NaBH_4$ 0.75 g/L	110	4	60.0-70.0 (from chitin)	83	-	Zhang M. *et al*., 2000

ANALYTICAL METHODS

As mentioned in the previous sections, multiple studies have described the extraction of insect-based chitin and its subsequent conversion to chitosan. Although the processing conditions have been stated in detail in the relevant articles, discrete and quantitative values regarding yield and degree of purification are missing. This hampers the assessment of the economic potential of chitin and chitosan derived from insects and the side streams of their cultivation, especially in comparison to production from crabs, shrimps or fungi. However, a general comparison, in terms of structure, chemical composition and purity, with commercialized sources can be made with the help of physicochemical and/or spectroscopic data. table 1.3 summarizes the main methods applied for the characterization of insect-based chitin and chitosan. Additionally, we include NMR spectroscopic data in the table. NMR, not being a method currently applied for the analysis of insect-based chitin, is promising towards validity and significance of the data. The resulting data can support performance assessment of insect-based material in prospective applications. Furthermore, the data contribute to focusing and identifying new application fields.

Table 1.3 Overview of major analytical methods applied for investigation of insect-based chitin and chitosan. Due to its importance, validity and sensitivity, NMR spectroscopy is listed as prospective powerful tool to analyse insect-based chitin and chitosan although it is not discussed here.

METHOD APPLIED	GENERAL PRINCIPLE	MAIN APPLICATIONS FOR (INSECT-BASED) CHITIN AND CHITOSAN
Infrared spectroscopy	Excitation of vibrations by irradiation with infrared beams	Determination of deacetylation degree
X-ray spectroscopy	Detection of elastic scattered X-rays	Crystallinity determination, Determination of the chitin polymorph
Thermogravimetric analysis	Mass loss or heat flow determination during heating	Degradation temperature, Moisture content, Determination of the chitin polymorph
Elemental analysis	Combustion Content determination of different elements	Determination of deacetylation degree for chitin and chitosan, Purification degree of chitin
Viscometry	Measuring the viscosity of the polysaccharide-containing solutions	Measuring viscosity-average molecular weight
Scanning electron microscopy	Reflection of electrons interacting with atoms	Determination of chitin surface morphology
Nuclear magnetic resonance spectroscopy	Investigation of the electronical environment of single atoms and the interaction with neighbouring atoms	Determination of deacetylation degree, Distribution of the acetyl groups, Determination of impurities

Infrared spectroscopy

Infrared spectroscopy is the most frequently applied method to examine insect-based chitin and chitosan; especially to determine the degree of deacetylation of the polysaccharides. The

technique, which exploits infrared light to obtain data from excited vibrational states of the functional groups in a sample, enables rapid analysis. According to the functional groups contained in the molecules, the most significant bands of insect-based chitin and chitosan occur at wavenumbers of 1310–1320 cm^{-1} (CN stretching, amide III), 1550–1560 cm^{-1} (NH bending, amide II), 1590–1600 cm^{-1} (NH2 bending), 1650–1655 cm^{-1} (CO stretching, amide I), 3100–3110 cm^{-1} (NH symmetric stretching), 3255–3270 cm^{-1} (NH asymmetric stretching) and 3430–3450 cm^{-1} (OH stretching) (Seoudi *et al*., 2005; El Knidri *et al*., 2016) (figure 1.3). The same is true for chitin and chitosan derived from crustaceans, where the wave numbers of the peaks can vary slightly among different natural sources (Badawy *et al*., 2015).

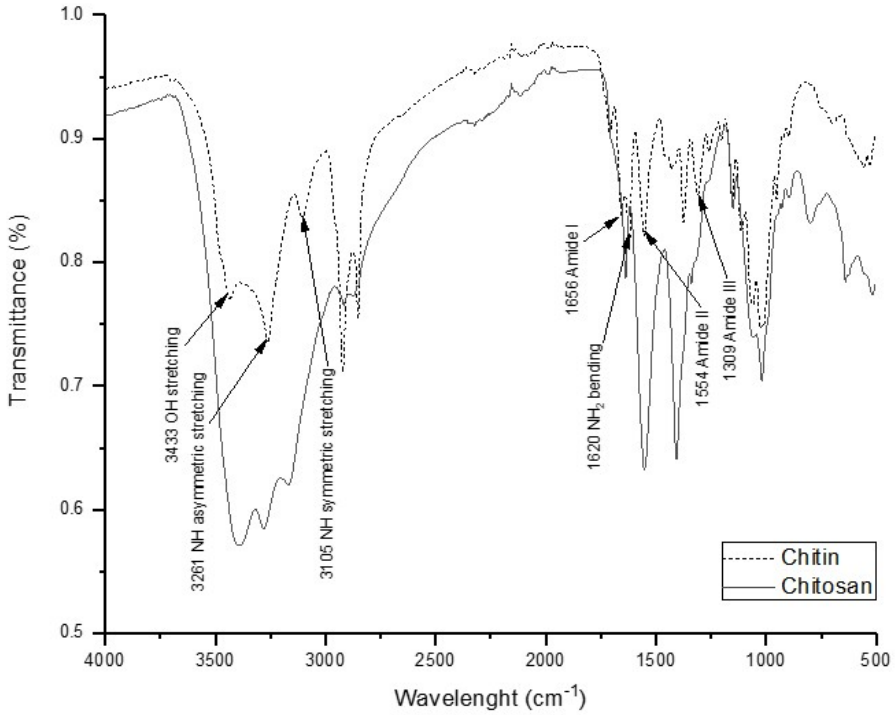


Figure 1.3 Exemplary infrared spectra of chitin and chitosan obtained from *H. illucens* pupal exuviae (own measurements).

For insect-based chitin and chitosan, infrared spectra were used to confirm the homogeneity of chitin and chitosan isolated from several insect species (Luo *et al*., 2019), to compare the spectral bands with those of commercially available chitin and chitosan (Ibitoye *et al*., 2018; Shin *et al*., 2019) or to confirm the purity of insect-based chitin after the isolation process (Kaya *et al*., 2017a) by considering the strength and position of characteristic bands.

The most practical application of this method has been for determining the degree of acetylation of investigated samples by calculating and comparing the absorption values measured at specific wavelengths (Chae *et al*., 2018). Accurate quantification is challenging (Kasaal, 2008); comparability and significance are minor due to the different bands applied for calculating the

degree of acetylation by different researchers. Further sources of errors can be individual-specific or due to inappropriate baseline settings within the spectra and impurities in the sample, e.g., the presence of proteins can lead to an overlap of the characteristic chitin and chitosan peaks in the infrared spectrum and thus to wrong values (Shigemasa *et al*., 1996; Brugnerotto *et al*., 2001). In the authors' opinion, infrared spectra used to obtain valid information or even quantitative values from insect-based chitin and chitosan are of limited conclusiveness. It is a useful auxiliary for qualitatively determining the presence of functional groups, but not to provide quantitative data subject to strong variation based on, for example, impurities or water content in samples.

Tools like partial least squares regression are hence mandatory for increasing the validity of the data and will also provide an added value to the infrared spectra of insect material (Dimzon and Knepper, 2015), although extensive and time-consuming calibration work is required. Since manual evaluation concerning the determination of the degree of acetylation is error-prone and less comparable, an automated software-assisted extraction of relevant data would thus be beneficial. Nevertheless, the identification of chitin- and chitosan-related functional groups, such as acetamido or amino groups, present in insect-based material is feasible using infrared spectroscopy.

X-ray diffraction

X-ray diffraction is the second most common analysis method used to characterize chitin from insect-based materials. Although X-ray diffraction is a powerful analytical technique for obtaining structural information, in the literature reviewed it has been exclusively used to determine the polymorphic form of chitin crystallites and the crystal structure and chitosan isolated from insects.

The X-ray pattern provides information on the periodic arrangement of atoms within a sample. The resulting diffractogram shows intensity as a function of 2θ, which is defined as the angle between the incident and diffracted beams.

X-ray diffraction measurements of insect-based chitin revealed strong significant peaks at 9–11° and 19–20°, in addition to minor peaks at 12–13°, ∼21°, ∼23° and ∼26° (Zhang *et al*., 2011; Liu *et al*., 2012; Erdogan and Kaya, 2016; Waśko *et al*., 2016; Soon *et al*., 2018). These peaks are valid for the highly symmetric orthorhombic crystal structure of insect chitin, representing the α-polymorphic form. However, there are exceptions: chitin from the cocoons of a moth (*Orgyia dubia*) is in the γ-form, exhibiting an X-ray diffraction pattern with high homology to the diffractogram obtained for α-chitin, differing from each other mainly in the

peak at 12.9° (Kaya *et al*., 2017). γ-Chitin and β-chitin are polymorphs with a lower degree of order than α-chitin, making these polymorphs more reactive. For example, β-chitin is more accessible to swelling and for enzymatic and chemical reactions than the α-form (Minke and Blackwell, 1978), and therefore more readily undergoes crystal disintegration. Thus, the conversion of β-chitin results in chitosan with lower crystallinity compared to chitosan obtained by deacetylation of α-chitin (Kurita *et al*., 1993).

Previous studies have confirmed that chitosan exists as two crystalline polymorphs, either as a hydrated polysaccharide ('tendon' form) (Clark and Smith, 1936) or as an anhydrous form ('annealed' chitosan) (Ogawa *et al*., 1984). The presence of crystallites or crystalline regions in the amorphous regions of chitosan could be due to the unreacted chitin (Mogilevskaya *et al*., 2006). The position of the X-ray peaks in the diffractogram is similar for insect-based chitosan and chitin and comparable to peak positions determined for commercially available chitosan (Chae *et al*., 2018). Main peaks for the chitosan crystal structure can be identified at 2θ of ∼10° and ∼20° (Marei *et al*., 2016; Luo *et al*., 2019; Hahn *et al*., 2020b).

The crystallinity index (CrI) for chitin and chitosan is commonly calculated using the relation of the peak intensities measured at 16° (I_{am}), which is attributable to the amorphous content of the sample, and at 20° (I_{110}) according to the equation (3) of Segal *et al.* (1959):

$$CrI\ (\%) = \frac{I_{110} - I_{am}}{I_{110}} \times 100 \qquad (3)$$

The reported crystallinity values for chitin vary greatly and cover a wide range (40–90%) but are mainly between 60 and 80% (Zhang *et al*., 2000; Erdogan and Kaya, 2016; Ibitoye *et al*., 2018; Soon *et al*., 2018; Huet *et al*., 2020). The broad range of values reported is due to the varied sources, different purification methods used and is a function of the drying, storage or preprocessing conditions (Waśko *et al*., 2016). Grinding of chitin is, for example, an effective method for decreasing its crystallinity, disturbing the overall structure of the polysaccharide (Mogilevskaya *et al*., 2006).

Hence, there is a clear need to independently determine all significant factors that affect the crystallinity of insect-based chitin and chitosan using X-ray-based investigations. The authors believe that low crystallinity values achieved for insect-based chitin and chitosan are of major importance and are highly relevant for most applications. For instance, lower crystallinity of chitosan facilitates its solubility in acidic solutions, increases its sorption ability and increases the accessibility of the primary free amino groups of chitosan (Mourya and Inamdar, 2008;

Ioelovich, 2014).

Thermogravimetric analysis

Similar to X-ray diffraction analysis, thermogravimetric analysis has also been used to determine the polymorphic form of insect-based chitin. Additionally, the temperature at which chitin and chitosan completely decompose can be measured by thermogravimetric analysis. The method records mass loss of a sample over time as temperature increases and is visualized in a thermogram. For insect chitin, thermograms exhibit two decomposition steps. One is a result of water evaporation between 50 and 110 °C, leading to a low mass loss of the sample (1–7%). A second peak at 300–400 °C is caused by the dehydration of the saccharide backbone, polymerization of the degradation products and decomposition of the acetyl function (Paulino *et al*., 2006). The amount of mass lost during this second decomposition step ranges from 50 to 95% (Kaya *et al*., 2016d, 2016e; Waśko *et al*., 2016). The maximal thermal degradation temperature (DTG_{max}), which corresponds to the temperature at which the highest mass loss is determined, is in a narrower temperature range: DTG_{max} values for chitin from different body parts of *Z. morio*, *Melolontha* sp., *A. pandora* or *H. illucens* have been evaluated to be 350–390 °C, indicating the presence of the α-form (Kaya *et al*., 2015d, 2015f, 2016e; Soon *et al*., 2018). DTG_{max} values obtained for α-chitin isolated from marine fishery waste are in the same range (Sajomsang and Gonil, 2010; Huet *et al*., 2020).

Typically, α-chitin has a higher second decomposition temperature than β-chitin (Kaya *et al*., 2016d), which is attributed to the lower crystallinity of the latter (Sagheer *et al*., 2009). Sometimes, a third decomposition peak at temperatures above 700 °C has been reported which is attributed to residual minerals not removed during demineralization (Sajomsang and Gonil, 2010).

The thermograms of insect-based chitin do not reveal large deviations to the chitosan produced. The main difference here is a shift of the decomposition peak to slightly lower temperatures during chitosan heating (<320 °C), which means that chitin is more stable (Kaya *et al*., 2014c). This is due to the increased number of N-acetyl groups in the chitin providing a higher stability than the primary amino groups of the chitosan (Mourya and Inamdar, 2008; Arora *et al*., 2011). Although several thermogravimetric analysis studies and data for insect chitin and chitosan are available, valid or useful characteristic values, such as the activation energy of degradation, have not been calculated and published yet. Hence, there is a need for substantial discussion and evaluation rather than stating superficial qualitative expressions. The DTG_{max} values obtained reveal significant differences for chitin or chitosan, but these are not application-

relevant. Processing chitin and chitosan at elevated temperatures does not significantly improve processing properties, as phase transition from crystalline to amorphous form does not occur until decomposition.

The information obtained using thermogravimetric analysis is thus limited, especially as the polymorphic form of chitin can also be identified with X-ray spectroscopy. Another option is to perform a more comprehensive thermal analysis. Newer instruments can perform calorimetric and thermogravimetric measurements simultaneously, providing additional information about the type and enthalpy values of conversion occurring during heating and purification of chitin and chitosan (Zhang *et al*., 2011; Soon *et al*., 2018).

Elemental analysis

Thermogravimetric analysis enables the determination of sample degradation temperature. In contrast, elemental analysis utilizes degradation and combustion of insect-based chitin and chitosan and the subsequent detection of carbon, hydrogen, nitrogen and oxygen to determine its molecular composition and acetyl content. Although complementary to several other methods, the determination of degree of acetylation (DA) of insect-based chitin and degree of deacetylation (DD) of insect-based chitosan via elemental analysis is of high practical relevance (Hussain *et al*., 2013; Jiang *et al*., 2017), and is calculated using the following equations (4, 5) (Xu *et al*., 1996; Abdou *et al*., 2008):

$$DA\ (\%) = \frac{\frac{C\ (\%)}{N\ (\%)} - 5.14}{1.72} \times 100 \qquad (4)$$

$$DD\ (\%) = \frac{6.89 - \frac{C\ (\%)}{N\ (\%)}}{1.72} \times 100 \qquad (5)$$

Theoretically, fully acetylated chitin contains 6.9% nitrogen and fully deacetylated chitosan contains 8.7% nitrogen (de Alvarenga, 2011). Assuming 100% purity of samples, the higher the nitrogen content the lower is the degree of acetylation and vice versa. Typically, the measured nitrogen content for insect-based chitin is lower than the theoretical value, resulting in an overestimation of the degree of acetylation. For example, Erdogan and Kaya (2016) used elemental analysis to determine the nitrogen content of chitin from *D. maroccanus* adults and nymphs at 4.6 and 5.7%, resulting in degrees of acetylation of 232 and 187%, respectively. Degrees of acetylation exceeding 100% have been determined for chitin extracted from *D.*

maroccanus and species such as *O. asellus* (169%) and *V. crabro* (127%) (Majtan *et al*., 2007; Kaya *et al*., 2014a, 2016d; Khayrova *et al*., 2019). The validity of the conclusions is hence severely limited. It can be assumed that these overestimations result from nitrogen-free impurities, such as lipids and sugars, in samples, which make up to 43% of edible insects such as *A. domesticus* and *T. molitor* (Laroche *et al*., 2019).

The overestimation of the degree of acetylation pertains to chitin from insects. It is different for insect-based chitosan and the determination of its degree of deacetylation via elemental analysis. The degree of deacetylation for chitosan obtained from the conversion of chitin from *Z. morio* and *B. mori* ranges from 52 to 95% (Zhang *et al*., 2000; Soon *et al*., 2018), which are reasonable values. It can be assumed that the validity of these values is due to a higher degree of purification of chitosan, in contrast to the insect-based chitin samples containing a significant amount of impurities. The conversion steps from chitin to chitosan involve severe conditions such as use of high temperature and sodium hydroxide concentration, followed by selective precipitation of chitosan from solution by pH neutralization. These steps provide additional purification, resulting in valid calculations of degrees of deacetylation for insect-based chitosan using elemental analysis.

However, the presence of impurities in both chitin and chitosan cannot be excluded. As already mentioned in the chitin purification and deacetylation section of this review, purity can be determined only for a minority of chitins and chitosans produced from insects. For accurate degree of acetylation of chitin or degree of deacetylation of chitosan, pre-quantification of impurities is mandatory. Nevertheless, a thorough and effective purification is crucial to provide unimpeded characterization of insect-based chitin and chitosan using various methods.

Molecular weight measurement via viscometry

The so-called viscosity-average molecular weight of insect-based chitosan can be determined by measuring the intrinsic viscosity of a solution using an Ubbelohde viscometer. Molecular weight determination is based on the fact that the viscosity of the solution, in addition to the degree of deacetylation, depends on the molar mass of chitosan. Previous studies have reported a value for insect chitosan viscosity-average molecular weight in the range of 426–450 kDa (Ai *et al*., 2008; Ma *et al*., 2015). Other authors have determined much lower values (<10 kDa) for *D. maroccanus* and *L. decemlineata* chitosan (Kaya *et al*., 2014d; Erdogan and Kaya, 2016). Odote *et al*. (2007) investigated the viscosity-average molecular weight of lobster, prawn and crab chitosan in comparison to that of chitosan from blowfly larvae and found similar values for all chitosans, which is potentially due to their similar chitin conversion conditions. The same

authors also stated that the viscosity-average molecular weight of insect chitosan decreases with higher deacetylation temperature and incubation time, confirming that the applied conversion parameters are more significant than the origin of chitin used for deacetylation. Another application for viscometry is to record the progress of chitosan hydrolysis: Nemtsev *et al*. (2004) reported a decrease of honeybee-derived chitosan viscosity-average molecular weight during enzymatic hydrolysis in slightly acidic solution from 257 to 21 kDa.

In contrast to chitosan, chitin solubility is limited to a few solvents. Nevertheless, Draczynski (2008) reported that the solubilization of honeybee chitin in dimethylacetamide–LiCl results in a viscosity-average molecular weight of 426–738 kDa that depends on the time of deproteinization (Draczynski, 2008).

More sophisticated methods and instruments are required, instead of the standard laboratory Ubbelohde viscometer, for measuring weight-average and number-average molecular weights and polydispersity or molecular weight distribution of chitin and chitosan. For example, gel permeation chromatography measurements with a multi-angle laser light scattering detector revealed a molecular weight for insect-based chitosan of approximately 308 kDa and a polydispersity index of 1.2 (Kim *et al*., 2017).

However, viscosity-average molecular weight as an outcome is important as it affects many other physicochemical or application-specific investigations, such as bioactivity, adhesion force and gelation properties.

Scanning electron microscopy

Previously described analysis methods generated information concerning structural features, chemical composition or physico-chemical properties of insect-based chitin and chitosan. Scanning electron microscopy enables visualization of the polysaccharide surface in the nanometric range using a focused beam of electrons.

Various surface morphologies have been described for insect-based chitin: rough or smooth surfaces with or without pores and/or fibrils (Marei *et al*., 2016). The range of diversity of various chitin surfaces derived from insects is shown in figure 1.4. However, most chitin surfaces extracted from insects exhibit a rough fibrillary structure with pores. The diameter of the fibrils ranges from 10 to 50 nm (Kaya *et al*., 2014a, 2015g; Erdogan and Kaya, 2016), and the chitin fibrils are surrounded by a protein matrix (Merzendorfer and Zimoch, 2003). The pore diameters range from 100 to 500 nm. The highly porous structure increases the accessible surface area and thus the adsorption capacity of the material.

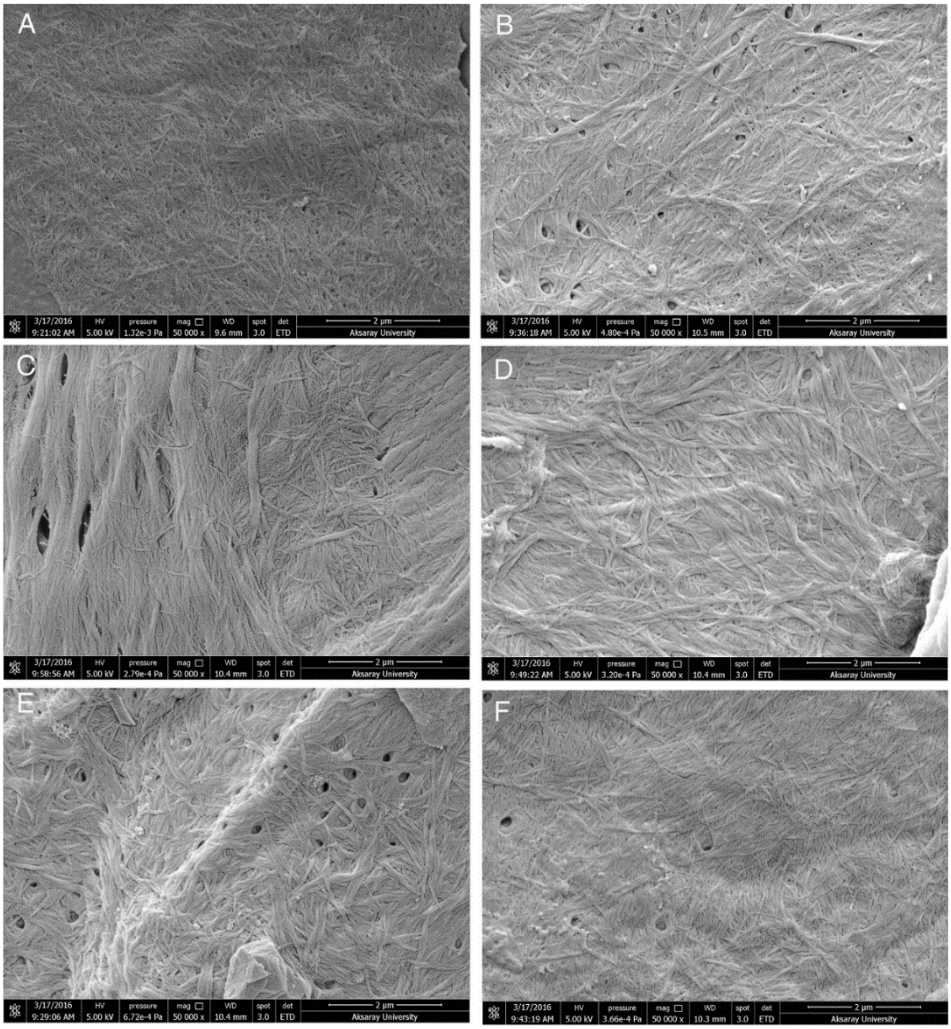


Figure 1.3 Scanning electron microscopy images of chitin extracted from (a) *Cicadatra atra*, (b) *Cicada hyalina*, (c) *Cicada lodosi*, (d) *Cicada mordoganensis*, (e) *Cicada platyptera* and (f) *Cicadivetta tibialis*. Reprinted from Mol *et al*. (2018) with permission of Wiley.

Scanning electron microscopy imaging of insect-based chitosan has been performed in only a small number of studies. Kaya *et al*. (2015g) reported chitosan with a surface morphology similar to that of the chitin from which it was derived, which suggests that the chitin structure is preserved in chitosan. The surface morphology of chitin also depends on other factors. It can be influenced by characteristics inherent to the natural source, such as species (Kaya *et al*., 2014c; Mol *et al*., 2018), sex (Kaya *et al*., 2015a) or body part (Kaya *et al*., 2015d), and by process conditions, such as the selected pretreatment procedure (Huet *et al*., 2020). Moreover, chitin content, degree of purification and washing procedure can play a major role as the surrounding matrix and matrix constituents on the surface not removed during purification mask the chitin. Due to this, the validity of this method for investigating chitin and chitosan is limited. However, knowledge of the surface structure of chitin and chitosan is important for processes in which surface architecture greatly affects functionality.

Assessment of analytical investigations

The methods described are the most frequently mentioned analytical techniques applied for the investigation of insect-based chitin and chitosan. The major conclusion from screening the analysis results is that there is a high structural and chemical homology between chitin and chitosan derived from insects and marine animals. However, despite the large amount of available data, knowledge gaps exist that need to be filled in prospective studies to assess the potential of insect-based chitin and chitosan. What has already been said for chitin yield and degree of purification also holds true here: there is a lack of valid data and/or the results were not properly evaluated or discussed to accelerate research on this topic. For example, many infrared spectra from chitins and chitosans from different sources are available, which are nearly the same as each other and provide no additional information. Similarly, thermogravimetric analysis data have limited validity as the DTG_{max} values reported are in the same temperature range. In contrast, X-ray data show strongly varying crystallinity values for chitin and chitosan. Thus, it is unclear if the data presented are a result of processing conditions, life cycle stage or body part of the insect.

Hence, the authors suggest firstly performing comprehensive studies to identify parameters that contribute significantly to the properties of insect-based chitin and chitosan. Secondly, the authors strongly believe that spectroscopic and chromatographic data would be more valid if they were related to the degree of purification of the chitin and chitosan investigated.

Therefore, it is necessary to first determine the purity of polysaccharides after extraction and deacetylation since contaminations can disrupt accurate measurements of degree of deacetylation. Elemental analysis is a tool which could be applied to, at least, estimate the degree of purification and to evaluate if the measurements performed are valid. Thirdly, there is a need to standardize the calculation methods and equations; for example, to agree on specific bands and peaks used for calculation when determining crystallinity via X-ray spectroscopy. Fourthly, we recommend using statistical software to evaluate the results and to increase the information content of a presented data set. In our opinion, infrared spectroscopy can reveal degrees of deacetylation and impurity content accurately if evaluated using multivariate data analysis or similar methods. Lastly, techniques such as titration-based methods can be applied for higher sensitivity in determining degree of deacetylation. Although revealing a precise determination method, titration is time-consuming. Another highly accurate and automatable tool, to determine the degree of deacetylation of chitin and chitosan in solid state or solubilized, is NMR spectroscopy. Several studies exhibited the potential of ^{1}H NMR, ^{13}C NMR and ^{15}N NMR spectroscopy to determine the degree of deacetylation, the distribution of acetyl groups

and the cross-linkages of the chitin and chitosan (Vårum *et al*., 1991a, 1991b; Heux *et al*., 2000). Possibly due to the need for enhanced equipment and specific expertise, comprehensive studies concerning insect-based chitin and chitosan are lacking. We further recommend performing application-based investigations, such as measurement of viscosity, adhesion, film formation and adsorption capacity. For successful commercialization of insect-based chitin and chitosan, such detailed knowledge is mandatory.

CONCLUSIONS AND FUTURE PROSPECTS

Chitin and especially chitosan are natural polymers with many useful properties and are widely used in a broad range of applications. Presently, the main commercial source of chitin and chitosan comprises waste streams from the marine fishery industry; however, their availability is limited by geography and season. The recent increase in demand for chitin and chitosan in the global market has drawn attention to alternative sources independent of marine fishery waste. Insect breeding farms, which are used for waste management through insect-mediated bio-conversion or for producing proteins and fats from larval stages, are being launched worldwide. In addition to the production of valuable compounds, insect breeding generates several side streams (dead adults, exuviae, exoskeletons, frass and residual feed) that have not yet been valorised. These side streams provide a cheap source of chitin, which is abundantly available and not regionally or seasonally limited. Furthermore, the chitin content of exuviae and exoskeletons exceeds 23% (Hahn *et al*., 2018), suggesting favourable conditions for chitin and chitosan production in the future.

In contrast, the chitin content of whole insects is generally lower (Kramer *et al*., 1995; Hahn *et al*., 2018). Due to the moderate content, insect breeding only for chitin isolation is not economically feasible without cascade usage of other compounds derived from the insect breeding.

The chitin content in insects is a function of species, type of feed and of life cycle stage. Moreover, the life cycle stage determines the complexity of the matrix in which chitin is embedded. Insects undergo sclerotization during metamorphosis to the adult stage. Sclerotization comprises cross-linkage of the insect cuticle with catecholic compounds. This makes necessary a more sophisticated purification process and at least one additional bleaching step to isolate pure chitin from insect biomass. On the contrary, decolorization is not mandatory for chitin purification from marine fishery waste. The need for bleaching arises because the chitin isolated from dead adult insects and exuviae contains these sclerotized structures with dark colour. Chitin production can be more economically feasible from side streams of insects

bred for a different purpose.

The purification of chitin begins earlier in the process and comprises fractionating the chitin-containing compounds from those with no or low chitin. Although insect breeding, for the isolation of protein and fat, is a highly automated and controlled operating process, a mechanical process for separating chitin-rich substances needs to be developed to eliminate the current practice of manual collection. Similarly, manual collection of chitin-rich material is opposed to the prospective application of insect-based chitosan in medicine and requires good manufacturing practice or laboratory-like controlled conditions. Furthermore, the raw materials used for insect breeding vary according to availability (e.g., vegetable waste from agri-food chain, cereal straw, distillers' grains and cereal meals). Although insect-mediated bioconversion is a flexible and robust process and leads to a high-quality fat and protein fraction almost independent of the varying quality of the side streams, it is challenging for an automated process to lead to chitin and chitosan with a consistent composition.

Currently, chitin purification from insect biomass is an area of focus for researchers and is mainly performed on a laboratory scale using the same methods applied for purification from crustacean shells. Most available literature on chitin and chitosan production from insects is limited to a description of the chitin extraction process and its subsequent deacetylation into chitosan. Information on the quantitative evaluation of extraction, purification, deacetylation and bleaching efficiency, and the degree of purification of products is missing. In the case of insects, it is especially important to assess the bleaching process, as insect exuviae undergo sclerotization by catecholamine linkage, which leads to dark coloration (Hopkins and Kramer, 1992; Kramer *et al*., 1995). These factors hinder accurate assessment of the economic potential of chitin and chitosan production from insects and comparison with the process chain of chitin isolation from crab shells.

A comparison of degree of deacetylation, molecular weight, stability, crystallinity and surface structure between insect- and crustacean-based chitin and chitosan, performed using various analytical methods, showed high similarity. This is encouraging regarding the performance of chitin and chitosan derived from insects for industrial applications and their use in new fields. However, for a comprehensive assessment, it is necessary to carry out application-relevant investigations and to exploit the full potential of the methods in use for characterization.

Based on current knowledge, it can be supposed that insects will be an important source of chitin and chitosan in the future, especially if future studies focus on filling the knowledge gaps highlighted in this review. The efficiency of each step of the purification process needs to be critically evaluated to optimize methods applied to crustaceans and adapt them to insect

biomass. Alternatives to traditional chemical purification methods should also be considered to make the process more environmentally friendly. Future studies should focus on these aspects to make optimal use of the side streams of insect breeding.

1.2 Chitosan as coating material for preservation of fresh food

The focus of the present PhD thesis was is the application of chitosan derived from insects to extend the shelf life of fresh fruit and vegetables, the commodities for which it has been most widely studied and applied.

Fruit and vegetables are highly perishable commodities, because of their large water content. The quick water evaporation occurring after the harvest causes shrivelling, loss of quality and shortening of shelf life. Moreover, postharvest fruit and vegetables still undertake metabolism. This further contributes to the deterioration of flavour, nutritional quality and appearance. Shrivelling, wilting, flaccidity and decay are among the major problems arising during the postharvest manipulation of fruit and vegetables, and which most affect their marketability and consumer acceptance. Postharvest deterioration is mainly due to weight loss, physiological disorders and diseases (Jianglian and Shaoying, 2013; Mahajan *et al*., 2018).

Global production of fruit and vegetables is constantly rising in response to the ever-increasing demand from a growing population. From 2007 to 2017, it was calculated that global production of the most commercialized fruits increased more than tenfold (from about 2.6 to 34.6 million tonnes) (FAOSTAT, 2019). Yet, world food production is expected to further increase by 60% by 2050 (Alexandratos and Bruinsma, 2012). It is therefore clear that postharvest losses represent a serious challenge and that efforts should focus on finding methods of processing and preserving fresh food, especially fruit and vegetables, that will minimise these qualitative and quantitative losses (Maringgal *et al*., 2020).

The losses are likely to occur due to poor handling and storage, inappropriate packaging as well as bacterial and fungal infections. In addition, variety and stage of ripeness of fruit have an impact on the nutritional quality and physico-chemical characteristics of fruit and vegetables. For instance, many fruits and vegetables, are harvested before they reach full maturity, in order to extend their shelf life. Climacteric fruits, such as tomatoes, apples, apricots, peaches, pears and several others, continue to ripen after being detached from the plant and reach their full ripeness during postharvest storage. This led to considerable losses of some important nutritive

elements compared to the product freshly picked at its peak of maturity (Goldman *et al*., 1999; Barret, 2007; Barret and Lloyd, 2012). When the fruit is harvested, there is a change of the gaseous balance between the consumption of oxygen and the production of carbon dioxide, which causes non-renewal of fruit cells and an increase in the gas transfer rate. This leads to a metabolic loss, taking the fruit to a gradual maturation and senescence (Dhall, 2013). External and internal factors affect the gas transfer rate. Among the external factors, the atmospheric composition (O2, CO2 and their ratios to the ethylene produced by the fruit) and temperature play a major role, while internal factors include the fruit species, cultivar and growth state. Contamination of the fruit pulp from the skin can also occur, with an increase in fruit spoilage leading to biochemical deteriorations such as browning, development of off-flavour and texture breakdown (Dhall, 2013; Sharif *et al*., 2017).

The food industry applies a variety of preservation or processing methods to extend the shelf life of fruits and vegetables, so that they can be consumed all year round and transported to consumers beyond the areas of production without negative effects. The main aim of preservation is to keep the food microbiologically safe, but also to maintain its quality as high as possible (Dhall, 2013). The optimal post-harvest life extension of fresh food products depends critically on three factors: *i*) reduction of dehydration, *ii*) reduction in the process of maturation and senescence, *iii*) reduction in the onset and rate of microbial growth (Erbil and Muftugil, 1986).

1.2.1 Main preservation and processing methods for fruit and vegetables

Nowadays, several different types of preservation methods can be applied to maintain the condition of fresh commodities stable for a long period. Conventional food preservation techniques, that are used comprehensively throughout the world, include drying, freezing, chilling, thermal processing and chemical preservation. Scientific advancements are contributing to the evolution of existing technologies and the development of new ones, such as irradiation, high-pressure technology and a combination of different techniques known as hurdle technology (Amit *et al*., 2017; Sharif *et al*., 2017). In traditional preservation techniques, foods often alter their qualitative status, in terms of both appearance and nutritional quality. So, the modern techniques are generally more suitable to achieve safety and quality of foods (Singh *et al*., 2018).

1.2.1.1 Chemical methods

The best way to enhance food preservation is to prevent contamination in the first place.

Chemical methods of cleaning sanitizing involve the application of mechanical washing to the product surface in the presence of sanitizers, followed by rinsing with water. Several sanitizing agents are used for fruit and vegetables to reduce the risk of microbial contamination (Ramos *et al*., 2013).

Chlorine is the most commonly used sanitizer to rinse fruit, vegetables and fresh-cut products. Although it is inactivated by organic material and it can lead to the liberation of chlorine vapours and potentially dangerous by-products, the benefits of chlorine use for industry outweigh the concerns. Alternatively, raw fruits and vegetables can be sanitized with chlorine dioxide, which has a higher antioxidant capacity (Baur *et al*., 2005; Hua and Reckhow, 2007; Rico *et al*., 2007).

Organic acids, including lactic acid, citric acid, tartaric acid and acetic acid, are also often use thanks to their antimicrobial activity against psychrophilic and mesophilic bacteria in fresh-cut fruit and vegetables. Ascorbic acid and its derivatives are particularly used as antioxidants on fruit and vegetables and in fruit juices to prevent browning and other oxidative reactions (Bari *et al*., 2005; Rico *et al*., 2007).

Other sanitizing agents used on fruit and vegetables are hydrogen peroxide, calcium-based solutions and ozone. In addition to their bacteriostatic and bactericidal efficacy, calcium treatments enable to maintain the firmness of products by interacting with pectin to form calcium pectate, that contribute to stabilise the fruit cell walls contrasting softening. Calcium treatments also reduce the loss of protein and chlorophyll (Gras *et al*., 2003; Parish *et al*., 2003).

However, the use of sanitising agents to treat the surface of products is almost always combined with other preservation methods. In the following sections the mainly used ones are described. Table 1.4 provides an overview of the characteristics, in terms of intensity and impact on food structure, of the methods described and their cost.

Table 1.4 Characteristics of different preservation treatments (modified from Heinz and Buckow, 2010).

	PROCESSING INTENSITY	IMPACT ON FOOD STRUCTURE	COST
Drying	+	++	+++
Thermal processing	+++	+++	+
Chilling	+	+	++
Freezing	+++	+++	+++
Irradiation	++	++	++
High-pressure processing	+	+	++

1.2.1.2 Drying

Drying is the process of removing water from a food by evaporation in order to obtain a solid product with a sufficiently low water content. By drying, the water content, which is required by enzymes and microorganisms to activate spoilage mechanisms in food, is reduced to the point where the activities of these microorganisms are inhibited (around 10-15% moisture) (James and Kuipers, 2003; Rayaguru and Routray, 2010).

Drying also reduces weight and volume of foods, facilitating packing, transport and storage, and can provide different flavours and smells to the product. Despite these advantages, drying also has some negative effects on food, mainly loss of aroma and degradation of nutrients such as vitamins, proteins and lipids, to a varying extent depending on the method used (Agrahar-Murugkar and Jha, 2010; Amit *et al*., 2017). This technique is commonly used for meat and fish, but it can also be applied to fruit and vegetables (Sharif *et al*., 2017).

While in drying heat can be used to remove water from the product, the high temperatures themselves can be used as a preservative agent in the thermal processing.

1.2.1.3 Thermal processing

Thermal processing is one of the most common forms of food preservation, as it efficiently reduces microbial load, degrades natural enzymes and gives better palatability to horticultural products. There are several types of heat processing employed by the food industry, essentially involving either heating unsterile foods in the final containers (canning), or heating foods prior to packaging and then packaging under sterile conditions (aseptic processing) (Barret and Lloyd, 2012). Canned fruit and vegetables can have a shelf life of multiple years.

Canning fruit and vegetables can have disadvantages, primarily a significant reduction of the biochemical, structural and mechanical properties of the raw product. Essentially, thermal treatments can improve and worsen the consistence of the finished product at the same time. As with drying, the high temperatures used for thermal processing inevitably damage vitamins, minerals, proteins and beneficial bacteria of food, with the higher the temperatures used, the worse the effect (Fellows, 2009; Orlova *et al*., 2015).

1.2.1.4 Chilling

The chilling process maintains the temperature of foods between -1 and 8 °C, reducing the rate of biochemical and microbiological changes thus extending the shelf life of fresh and processed foods. Chilling storage is extensively used for its effective short-term preservation capability. It retards microbial growth and delays postharvest metabolic activities of vegetal tissues.

Chilling also limits deteriorative chemical reactions, such as oxidative browning, oxidation of lipids and chemical changes associated with colour degradation (Fellows, 2009; Liu *et al*., 2020a).

Despite these advantages, chilling causes dehydration of surfaces of unwrapped foods, which is a major limitation of this technique. Furthermore, chilling is high capital intensive since it requires specialized equipment (Amit *et al*., 2017).

1.2.1.5 Freezing

Freezing is another easy and economical way to preserve foods. Freezing reduces the liquid water level activities, inhibiting the microbial development and decreasing the chemical reaction rates. At the same time, the process of freezing and, especially, defrosting damages the tissue structure of the product. This degradation of vegetal tissue may lead to a loss of cellular integrity and interaction of enzymes and nutrient substrates, resulting in nutrient loss in addition to deterioration of texture, colour and flavour. Frozen foods lose more nutrients during storage compared to chilling. Time and rate of freezing are the main factors affecting the quality of the frozen products (Sharif *et al*., 2017).

Three freezing methods are commonly used commercially: freezing in air, freezing by indirect contact with the refrigerant, and freezing by direct immersion in a refrigerating medium. Prior to freezing, most vegetables are exposed to a short blanching treatment with either steam or hot water to inactivate enzymes (Barret and Lloyd, 2012).

1.2.1.6 Irradiation

Irradiation is a physical process that involves the treatment of foods with ionizing radiations (IR), which can be natural, such as X-rays, gamma rays and ultraviolet radiation, or artificially generated (accelerated electrons). IR are radiations that are able to produce ions. The principal target of ionizing radiation is water that produces free radicals, which react, destroy or deactivate bacterial components (Ramos *et al*., 2013; Islam and Uddin, 2016). The effects of IR include: disinfestation of grains, fruits, and vegetables; inhibition of fruit and vegetables sprouting or alteration of their rate of maturation and senescence; inactivation of spoilage organisms and foodborne pathogens (Kanatt *et al*., 2006). Further advantages of irradiation are the absence of chemical residues left by the process and the possibility to treat already packed foods at their fresh and frozen state (Calado *et al*., 2014). IR has a minor influence on food nutritional parameters, like proteins, carbohydrates, lipids, minerals and most vitamins. Only at high dose, IR may cause loss of some vitamins but generally it has no different effect on

nutritional values than other conventional treatments (Smith and Pillai, 2004).

Nevertheless, irradiation cannot be applied to all types of food. Some fruits and vegetables, and dairy products cannot be irradiated because they lose firmness and their sensory quality worsens. An additional disadvantage is the high upfront cost of the irradiation facilities (Calado *et al*., 2014).

1.2.1.7 High-pressure processing

High pressure processing is a method where food is subjected to elevated pressures (in the range of 100–1000 MPa) for short time, typically from several seconds to several minutes, to achieve microbial and enzymatic inactivation. It is effective against microorganisms because it results in the rupture of microbial membranes (Sharif *et al*., 2017). High-pressure is the alternative to heating since it does not only change, but maintain the organoleptic properties of the treated products. The primary structure of peptides, saccharides, lipids and vitamins is rarely affected by high pressure. As the process can be operated at ambient or chilled temperatures, there is also little heat damage to nutrients or natural flavours and colours of food (Ramos *et al*., 2013). However, the high-pressure processing can change the native structure of some macromolecules, mainly starch, in a similar way to thermal treatments (Heinz and Buckow, 2010). As this method acts directly and evenly throughout the food, its efficacy depends on shape, composition and size of products (Sharif *et al*., 2017).

One of the main disadvantages of this treatment is that it is not applicable to foods in flour or powder form, and to products containing large quantities of air bubbles that would be deformed under pressure. In addition, the packaging of products treated under high pressure must have a certain degree of compressibility (at least 15%), so not all materials are suitable (Huang *et al*., 2017).

1.2.1.8 Modified atmosphere packaging

Modified atmosphere packaging (MAP) involves the modification of the internal atmosphere composition of a package by reducing the amount of oxygen (O_2) and replacing it by carbon dioxide (CO_2) and/or nitrogen (N_2). This process aims to extend the postharvest life of whole and fresh-cut fruit and vegetables by reducing their respiration rate and ethylene production, minimizing metabolic activity and delaying enzymatic browning (Ramos *et al*., 2013). As the concentration of O_2 in the package drops below around 10-12%, respiration start to slow. This suppression of respiration goes on until O_2 reaches 2-5%. Below this concentration fermentative metabolism takes place, leading to the production of off-flavours, off-odours and undesirable

volatiles. MAP must therefore balance the gases within the package to prevent the presence of oxygen below this concentration threshold. On the other hand, as the concentration of CO_2 is increased above the atmospheric level, the respiration is suppressed, as well as the ethylene production and activities and growth of microorganisms (Sivertsvik *et al*., 2002). The modification of the atmosphere in a pack can be achieved through respiration of the food product (passive MAP) or by replacing the atmosphere in the package with the required gas mixture (active MAP). For passive MAP, the food product is packed in a film with appropriate permeability characteristics. It is ensured that O_2 can enter the pack up to a concentration where it can be consumed by the product through respiration. On the other hand, the CO_2 generated by respiration must be able to move to the outside to avoid excessively high concentrations that would activate fermentative metabolism. The passively generated atmosphere cannot be further adjusted, thus active MAP is often preferred. This is achieved by placing the product under a slight vacuum and replacing the atmosphere in the package with the desired gas mixture. The modified atmosphere can also be adjusted after the package is sealed by including absorbent or adsorbent substances in the packaging material to regulate the gas exchange between the food product and the outside (Kader and Watkins, 2000; Mangaraj and Goswani, 2009).

can be achieved by introducing the desired proportion of gas mixture into the package before being sealed

MAP technology, therefore, requires the use of film to package products. Petroleum-based synthetic plastics, mainly low-density polyethylene, polyvinyl chloride, and polypropylene, have been dominating the food packaging sector due to their comparatively low cost, lighter weight, high mechanical strength, good barrier properties, ability for heat sealing and shape versatility. However, the use of these synthetic plastics presents issues related to their disposal. Petroleum-based polymer are non-degradable in nature and their source itself is limited and non-renewable (Malhotra *et al*., 2015). The packaging technology should balance food protection with environmental pollution and disposal of solid waste. Therefore, in recent decades, there has been an increasing focus on the research for bio-based packaging materials as alternatives to synthetic plastic polymers. These biodegradable alternatives include natural polysaccharides like cellulose, starch, agar and chitosan (Kumar *et al*., 2020). Natural polymeric coatings, edible in many cases, are becoming one of the most promising technologies within the modified atmosphere preservation of fresh food, mainly fruit and vegetables. The method uses biological or chemical materials as a coating layer on the surface of the product to prevent gaseous exchange, thus halting the ripening process. Edible coatings can also carry active ingredients, such as nutrients, spices, flavours, antibrowning agents and antimicrobial

compounds that can further extend the shelf life of the product and enhance its quality (Shiekh *et al*., 2013). Several types of edible coatings have been used to extend shelf-life of fresh commodities.

1.2.2 Edible coatings for fruit and vegetables preservation

Edible coatings are thin layers of edible, non-toxic material applied directly to the surface of the product, to provide a barrier to moisture, oxygen and solute movement from the food, as well as preservation of the products appearance. An ideal coating should be able to extend storage life of fresh fruits and vegetables reducing decay without affecting their quality (Kumar *et al*., 2020). The effect of coatings on fruits and vegetables depends greatly on temperature, alkalinity, thickness and type of coating, and the variety and condition of fruit and vegetables (Dhall, 2013).

Edible coatings can be produced from materials with film forming ability. Film materials must be dissolved in solvents, such as water, alcohol, acids or mixtures of other solvents. Plasticizers, antimicrobial agents, minerals, vitamins, colours, or flavours can be added in this process. The film solutions can then be applied to food by different methods including dipping, spraying and brushing followed by drying (Tian and Liu, 2020).

Edible coatings can be essentially composed of proteins (section 1.2.2.1), lipids (section 1.2.2.2) and polysaccharides (section 1.2.2.3). The concentration and combination of these components set the barrier properties of the coating.

1.2.2.1 Protein coatings

Proteins are the least developed material for coating use because of their susceptibility to moisture absorption, which makes them sensitive to temperature and humidity. Protein films are generally formed from solutions or dispersions of the protein as the solvent/carrier evaporates. The solvent/carrier is generally water, ethanol or ethanol-water mixtures (Maringgal *et al*., 2020). Generally, proteins used for coatings are denatured by heat, acid, base, or solvents in order to obtain more extended structures that are required for film formation (Wittaya, 2012). After extension, protein chains can associate through hydrogen, ionic, hydrophobic, and covalent bonding, resulting in films that are excellent oxygen barriers but are susceptible to moisture. Thus, protein films are good oxygen barriers at low relative humidity (Shalini and Singh, 2009).

The most used materials for protein-based edible coatings include gelatine, corn zein, wheat gluten, soy protein, casein, keratin, collagen and whey protein (Dhall, 2013).

1.2.2.2 Lipids

Lipid-based coatings, notably waxes, are the most historically used since the beginning of the last century, mainly for fruits and confectionery products. Lipid coatings are mainly used for their hydrophobic properties, which make them good barriers to moisture loss. They also improve food appearance by making its surface brighter. The disadvantage of the hydrophobic nature of lipids is that it leads to the formation of thicker and inelastic films. To counteract these effects, lipids are generally associated with film forming agents such as proteins or cellulose derivatives (Li *et al*., 2018; de Freitas *et al*., 2019; Bucio *et al*., 2021).

The most effective lipid compounds in providing a humidity barrier are waxes, which are used as barrier to gas and moisture, and to improve the surface appearance of fruits and vegetables. They must be removed before the consumption of the product if applied as a thick layer, according to the product label, while they are considered edible when used in thin layers (Regulation EC 1935/2004). Paraffin wax, beeswax, candelilla wax, carnauba wax, polyethylene wax and mineral waxes are the most used (Dhall, 2013).

Other lipids components used in food coating are fatty acids and monoglycerides, mainly used as emulsifiers and dispersing agents, and resins and rosins, used primarily to impart high gloss although they are mostly used in coatings for the pharmaceutical industry (Hernandez, 1991, 1994).

1.2.2.3 Polysaccharides

Polysaccharides most commonly available for edible coatings are extracted from marine and agricultural plants and animals. These coatings have been used to retard moisture loss of some foods during short-term storage. The principal polysaccharides of interest for this application are cellulose, starch, gums, and chitosan, from which tough, flexible and transparent films can be produced, resistant to fats and oils (Dhall, 2013).

Cellulose is a crystalline polymer that require treatment with alkali to swell its structure, followed by reaction with chemicals, such as chloroacetic acid, methyl chloride or propylene oxide, to obtain cellulose derivatives with enhanced film-forming properties. Despite their good barrier properties, cellulose derivatives are often too expensive for bulk use (Krochta and Mulder-Johnston, 1997). Starch and derivatives have been largely used to produce biodegradable films to replace plastic polymers, thanks to their low cost and good mechanical properties. Although additional procedures are often necessary to modify the molecular structure of starch in order to make it as suitable as possible for packaging requirements, starch-based plastics have been commercialized during the last few years and are dominating the

market of bio-based, compostable materials (Shalini and Singh, 2009; Abe *et al*., 2021). Alginates, carrageenans and pectin are also used, to a minor extent.

In addition to polysaccharides of plant origin, chitosan, a polysaccharide of animal origin, is highly promising for use as an edible coating. The use of this polymer for food preservation will be covered more extensively in the next sections.

1.2.3 Chitosan coatings

Currently, there is a lot of research about the application of chitosan in food preservation, being edible coatings its most promising application. Good success has been already achieved in extending shelf life of fruit, vegetables and meat, by preparing the chitosan film as a liquid and directly coating it on the surface of food (Tian and Liu, 2020). The physicochemical and biological properties of the chitosan solution, and consequently of the final coating, can be changed by varying the degree of deacetylation of chitosan, its molecular weight, its concentration in the solution and the inclusion of additional components.

A number of comprehensive reviews are available in the literature on the utilization of chitosan coatings in fresh foods (Elsabee and Abdou, 2013; Jianglian and Shaoying, 2013; Shiekh et al., 2013; Kerch, 2015; Wang et al., 2018; Kabanov and Novinyuk, 2020; Kumar et al., 2020; Maringgal et al., 2020; Tian and Liu, 2020). Chitosan coatings act not only as a barrier able to reduce dehydration and weight loss of fresh fruit and vegetables, but also delay maturation and senescence of these commodities during storage. Chitosan also shows antioxidant and antimicrobial activities, thus retarding microbial spoilage and growth of fungine populations (Kabanov *et al*., 2020).

The easiest and most economical way to prepare a chitosan-based coating is by solubilization of chitosan in an aqueous solution of dilute acid, mainly acetic or lactic acid, at the desired concentration. Glycerol and/or Tween 80 are then generally added to the solution to enhance its plasticity and viscosity, thus providing good adhesion to the surface of the product (Kumar *et al*., 2020). These chitosan solutions can be directly applied to food products in their liquid state. In most cases fruit and vegetables are coated with chitosan solution by dipping followed by drying under flowing air (Ramirez *et al*., 2015; Marvdashti *et al*., 2018; Sucharita *et al*., 2018; Hassan *et al*., 2020). Alternatively, products are coated by spraying the chitosan solution on their surface (Reddy *et al*., 2000; Leceta *et al*., 2015; Jiang *et al*., 2019; Vilaplana *et al*., 2020a, b). The layer-by-layer assembly technique has also been proposed for chitosan coating of lemon fruits (Chen *et al*., 2020). This method consists in the deposition by submersion or spraying and mutual attraction of alternating polyelectrolytes with opposite net charges onto

the surface of the product. The polyelectrolytes can be proteins, synthetic polymers or polysaccharides, like chitosan, that hold net charge (Kumar *et al*., 2020).

Chitosan solutions can be also used to prepare solid films for application as solid packaging. One of the most widely used methods for preparing chitosan films is the solution casting method (Xu *et al*., 2005; Vieira *et al*., 2016). It involves the casting of the solution onto a solid, flat support, followed by evaporation during which intermolecular electrostatic and hydrogen bond formation results in the polymer structure (Muxika *et al*., 2017). Other methods for the preparation of solid films, which are still very little studied for chitosan, are the extrusion (Lago *et al*., 2014; Kumar *et al*., 2020) and the application of chitosan coatings on the surface of polyethylene matrices (Lago *et al*., 2014).

For preservation of fruit and vegetables, the chitosan coating is mainly applied by dipping or spraying. Chitosan-based coatings have been effective in extending the shelf life of several fruits and vegetables, including carrots (Leceta *et al*., 2015), tomatoes (Barreto *et al*., 2016), cucumbers (Olawuyi and Lee, 2019), bell peppers (Ali *et al*., 2015), bananas (Zahoorullah *et al*., 2017), apples (Li *et al*., 2015), pomegranates (Varasteh *et al*., 2017), pears (Wang *et al*., 2016), plums (Kumar *et al*., 2017), strawberries (Jiang *et al*., 2020), papayas (Vilaplana *et al*., 2020a), mangoes (Silva *et al*., 2017), peaches (Zhang *et al*., 2019), apricots (Zhang *et al*., 2018), and also commodities of animal origin, such as fish (Remya *et al*., 2015) and meat (Tayel *et al*., 2014; Chang *et al*., 2018).

1.2.3.1 Effects of chitosan coatings on physico-chemical characteristics of fruits

Chitosan-based coatings can affect physico-chemical characteristics of the coated products during storage. The main parameters that are influenced by the chitosan coating are the weight loss of the product, its total soluble solids content, pH and titratable acidity, firmness, and activity of enzymes, mainly the polyphenol oxidase. Another major phenomenon that is affected by chitosan is the growth of fungal colonies on the product. The principal effects of the chitosan coatings are described in the following sections.

Effect on weight loss

Weight loss, caused by respiration and moisture evaporation through the skin, is a major determinant of storage life and quality of fruits. Preservative coatings act as barriers, thereby restricting water transfer and protecting fruit skin from mechanical injuries, as well as sealing small wounds and thus delaying dehydration. It is already confirmed that chitosan coatings are able to significantly reduce the weight loss of several fruits during storage (El-Ghaouth *et al*., 1991; Jiang and Li, 2001; Kittur *et al*., 2001; Jiang *et al*., 2013; Shiekh *et al*., 2013; Sucharita

et al., 2018; Andrijanto *et al*., 2019).

Effect on the total soluble solids content and titratable acidity

Sugar content, estimated from the total soluble solids content, and acidity, measured as titratable acidity, are the parameters that most affect sensorial quality of fruits and are those used to monitor the fruit maturation process. Both sugars and acidity change during the different physiological phase of development of fruits. Botanical species, variety, agricultural practices and even climate can affect the variation in sugar and acidity levels. Generally, acidity tends to decrease during storage and senescence of fruit, while sugar content tends to increase because of the acids and starch degradation into sugars occurring after fruit harvest (Barbosa-Cánovas *et al*., 2003; Vieira *et al*., 2016). Coatings, including chitosan-based ones, can maintain more stable these parameters by slowing down respiration and metabolic activity of fruits, hence retarding the ripening process. Chitosan forms a semipermeable film to gases around the productinducing the modification of the internal atmosphere by reducing O_2 and/or elevating CO_2, and suppressing ethylene evolution (Dong *et al*., 2004; Shiekh *et al*., 2013; Petriccione *et al*., 2015; Chen *et al*., 2020).

Effect on the activity of polyphenol oxidase

Browning of fruit and vegetables is an undesirable effect due to polyphenol oxidase (PPO) activity, which is able to oxidize anthocyanins and phenolic compounds in the presence of O_2. Pigment degradation is thus caused by increased PPO activity as a result of physiological stress due to water loss (Jiang *et al*., 2000; Zhang *et al*., 2000a). Water loss accelerates senescence of the fruit with consequent cell membrane degradation and release of PPO, leading to oxidation of the cellular components. Chitosan coatings can delay the increase in PPO activity by reducing dehydration, thus preventing oxidation and browning of fruits (Dong *et al*., 2004; Jiang *et al*., 2005).

Effect on fungal growth

The potential of chitosan coating to inhibit the growth of several fungi has been shown for a wide variety of harvested commodities. The antimicrobial activity of chitosan is attributable to its ability to cause severe cellular damage to the mould and interfere in the secretion of microbial growth substrates (Zhang *et al*., 2011a). The higher levels of acidity in the fruit maintained by the chitosan coating also contribute to hinder the development of mould (Dotto *et al*., 2015; Ramirez *et al*., 2015; Hassan *et al*., 2020).

Effect on firmness

Texture is a critical quality attribute in the consumer acceptability of fresh fruit and vegetables. Fruit softening is due to deterioration in the cell structure and the cell wall composition. It is a

biochemical process involving the hydrolysis of pectin and starch in the fruti cell walls by ripening-induced enzymes, mainly the cell wall hydrolases (Fisher and Bennet, 1991). The maintenance of firmness in the fruits treated with chitosan coatings could be due to their higher antifungal activity and covering of the fruit skin, thereby reducing infection, respiration and other ripening processes during storage (Asgar *et al*., 2005; Martínez-Romero *et al*., 2006).

1.2.3.2 Chitosan blending with natural active components

Chitosan is often combined with other substances to enhance the efficiency of the coating, particularly to boost its antimicrobial effect and improve the coating selective permeability to certain gases (Tian and Liu, 2020). Among natural substances, chitosan is mostly blended with other polysaccharides, proteins or natural plant and animal extracts.

Within polysaccharides, starch is one of the most used thanks to its low cost, wide availability, biodegradability and good film forming ability (Alix *et al*., 2013; Talón *et al*., 2017). Chitosan-starch films have a greater antioxidant activity and improved water vapor barrier property (Alix *et al*., 2013; Talón *et al*., 2017). Cellulose and its derivatives have also been investigated to improve mechanical properties of chitosan films. Chitosan-cellulose coatings have excellent gas barrier and antimicrobial properties (Li *et al*., 2013; Bansal *et al*., 2016; Sundaram *et al*., 2016). Alginate and pectin, as well, blended with chitosan form films with excellent gas-exchange performance and water vapor permeability property (Poverenov *et al*., 2014; Maciel *et al*., 2015; Souza *et al*., 2015).

Many proteins, of plant and animal origin, can also be blended with chitosan to form films with different predefined properties. Because of the presence of special groups, these films based on chitosan/protein have abundant functions, which promote the application in food packaging. Animal proteins are the most used, including caseinate, collagen, lysozyme and gelatine (Khwaldia *et al*., 2014; Liu *et al*., 2015; Ahmad *et al*., 2016). Proteins derived from plants, mainly proteins of legumes, also present great potential in food preservation, due to the rich sources and low cost (Ma *et al*., 2013; Abugoch *et al*., 2016).

Finally, chitosan can be blended with extracts of both animal and plant origin. Among the first ones are bee secretions, like beeswax and propolis, that show good antimicrobial activity (Pedonese *et al*., 2018; Felicioli *et al*., 2019). Many plant extracts have been used to significantly modify the chitosan film properties, from antioxidant activity, to barrier and mechanical properties and thermal stability. Citrus and thyme extracts, eugenol, tanning acid and polyphenols have been successfully used for this purpose (Iturriaga *et al*., 2014; Sun *et al*., 2017; Talón *et al*., 2017). Essential oils are also widely used in combination with chitosan to

improve antioxidant activity and barrier property, including cinnamon, olive, rosemary and oregano oils (Pelissari *et al*.,2009; Jiang *et al*., 2012; Sun *et al*., 2014).

The field of chitosan coatings is therefore very broad and subject to continuous improvement and evolution as a result of the intensive scientific research carried out. The present thesis focused on the application of pure chitosan, derived from insects, as a coating on fruit and vegetables.

2. AIM OF THE WORK

The overall aim of the present PhD project was to assess insects, specifically the dipteran *H. illucens*, as a source of chitin and chitosan alternative to the current one represented by crustacean waste. The research activities were aimed at the development of a suitable procedure for chitin and chitosan purification from different waste insect biomasses generated from the *H. illucens* farming. The main objectives were the quantitative evaluation of the efficacy of the purification procedure applied to the insect biomass and the assessment of the quality of the resulting chitin and chitosan through different characterization analyses.

Another aim of this work was to identify a valid application for the insect-derived chitosan in the agri-food sector. Assessment of the suitability of chitosan as preservative coating for fresh fruit by evaluating of the main qualitative parameters indicative of the postharvest quality of fruit was the objective.

3. MATERIALS AND METHODS

In the following sections, the methods for analysis of the insect samples, chitin and chitosan purification and characterization, and application of chitosan as preservative coating for fresh fruits are described. Unless stated otherwise, all reagents were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Insects used in this work were reared at the University of Basilicata.

3.1 Set up of the *Hermetia illucens* breeding facility

A small-scale *H. illucens* breeding facility has been set up in a shed of the Laboratory of Physiology and Molecular Biology of insects at the University of Basilicata, in Potenza. The shed has been arranged in different rearing rooms equipped with air conditioning and humidification system to meet the needs of the various development stages of the fly. A constant temperature of 27 °C±1 °C and 70% relative humidity were kept in the rearing rooms, according to the optimal parameters for *H. illucens* development (Dortmans *et al.*, 2017). *H. illucens* is a holometabolous insect, which undergoes a complete metamorphosis through five different stages of development during its life cycle: egg, larva, prepupa, pupa and adult. After hatching, larvae begin to feed and grow through five larval instars before entering the prepupal stage when they stop feeding and move away from the growing medium to pupate in dark, sheltered places. Once intrapupal development is complete, the adult emerges from the puparium, leaving behind the pupal exuviae. The adults mate two/three days after emergence and the fertile females lay eggs in small cavities from which the cycle starts again (Oliveira *et al*., 2015; Dortmans *et al*., 2017).

In the rearing shed, adult flies are kept inside flight cages, consisting of mosquito net fabric held up by an aluminium frame (figure 3.1 d). Mates occur inside the cages and females lay eggs inside wooden supports (figure 3.1 c) placed on top of attractors, consisting of boxes with decaying organic matter.

Figure 3.1 Boxes with growing larvae (a) and detail of feeding larvae inside the boxes containing the diet (b). Wooden support for egg laying (c). Dark cage for keeping pupae and light cages for adult flies where mating and egg-laying take place (d). Mechanical sieve for separation of mature larvae or pupae from residual diet and frass (e).

Eggs are collected three times per week and placed in the "nursery room" in boxes with the standard diet. As they hatch, larvae start feeding on the diet. The standard diet used as feed for *H. illucens* larvae was supplied by Mangimi Losasso S.r.l. (Balvano, Potenza, Italy) and it was composed of wheat bran (50%), alfalfa meal (30%) and corn meal (20%) with 70% moisture. The proximate composition of the standard diet, provided by the supplier, is reported in table 3.1.

Table 3.1 Proximate composition of the standard diet used as feed for *H. illucens* larvae.

Protein	13.60%
Fat	3.50%
Cellulose	13.90%
Ash	6.00%
Sodium	0.04%
Moisture	12.50%

Larvae develop in the boxes, take up the diet and increase their biomass (figure 3.1 a, b). Mature larvae (i.e., the 5th larval instar) or pupae, depending on whether they are intended for the

production of protein meal or for the maintenance of the breeding cycle, are collected by separating them from the growth substrate by mechanical sieving (figure 3.1 e). Mature larvae are then processed to produce meal, while pupae are placed in boxes at the bottom of a dark flight cage, made of a thick black fabric. This cage is connected via side openings to the adults' flight cages (figure 3.1 d). As the intrapuparial development is completed, adult flies emerge from their puparia and move spontaneously in the flight cages, attracted by light. In this step, the adults leave behind pupal exuviae which are readily collected in the boxes. Adult flies mate in the flight cages and the cycle starts again.

3.2 Insect samples collection and preparation

Three different types of insect samples were collected from the *H. illucens* farm: whole larvae, pupal exuviae and adult flies.

If the whole mature larvae number is sufficient for meal production, they were collected from their feeding containers. Larvae, together with residues of the feeding substrate and frass, were poured onto the grid of a mechanical sieve and separated by automatic oscillation of the grid. Living larvae free from substrate residues were wash several times with warm water to remove further impurities and then frozen at -20 °C. Pupal exuviae were directly taken from the boxes placed in the dark flight cage after adult's emergence. Adult dead flies were collected from the bottom of the flight cage where they fall as their life cycle ends. Pupal exuviae and adult flies did not need to be sieved from a substrate, they were only rinsed with warm water. Larvae, pupal exuviae and adults were finally dried in oven (Conlabo s.r.l., Potenza, Italy) at 60 °C over-weekend and ground into powder using a laboratory blender (Waring Commercial Stamford, USA). Particle size of the ground insects was gravimetrically determined by shaking the samples during 6 min over Retsch sieves of different mesh sizes and an average particle distribution of 0.7% > 630 µm, 33.7% between 630 and 315 µm, 46.1% between 315 and 180 µm, 8.5% between 180 and 90 µm, 6.5% between 90 and 45 µm and 4.4% < 45 µm was obtained for all insect samples.

The powdered raw samples (figure 3.2) were subjected to the process for chitin extraction.

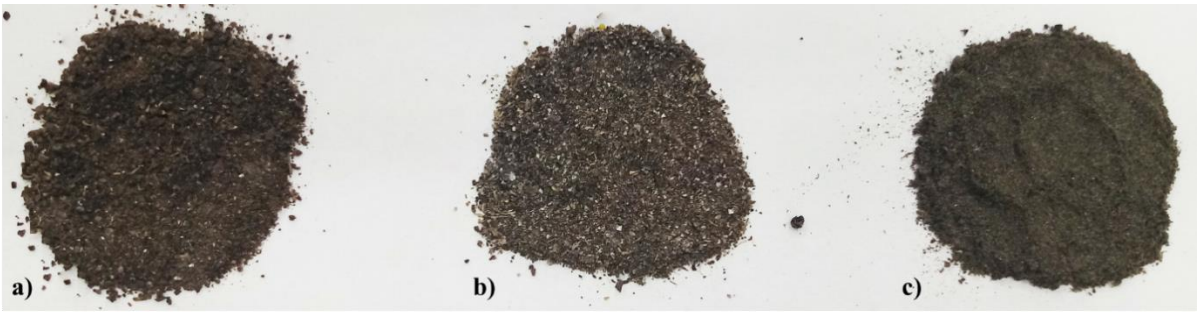


Figure 3.2 Powdered samples of larvae (a), pupal exuviae (b) and adults (c) used for chitin extraction.

3.3 Characterization of raw insect samples

Raw larvae, pupal exuviae and adults were analysed in order to determine their composition in terms of minerals, proteins, fats and fibres. First, percentage of dry matter of all the insect samples was determined. Results of composition determination were hence related to dry mass. Dried, ground insect samples were always used for analytical determinations. All the analytical determinations were performed in triplicate and the standard deviation was calculated. Mineral, protein and fibre content were determined also for the insect biomass after each step of the chitin extraction procedure.

3.3.1 Determination of dry mass

Dry mass of all insect samples was determined according to the standard method EN ISO 11465:1993. An empty ceramic crucible was dried in oven at 100 °C overnight and then weighed (*m1*) after cooling in a vacuum desiccator. Around 1 g of fresh insect sample was placed into the crucible and the filled crucible was weighed again (*m2*). Crucibles filled with fresh samples were dried in oven at 100 °C overnight and the final dry weight was wrote down (*m3*).

The dry mass of larvae, pupal exuviae and adults was calculated according to the equation (6):

$$Dry\ mass\ (\%) = \frac{m3 - m1}{m2 - m1} \times 100 \qquad (6)$$

3.3.2 Mineral content

Mineral content was determined after incineration of the insect samples, according to the standard method EN ISO 14780:2017. Empty ceramic crucibles were firstly dried overnight at

100 °C in oven and then weighed (*m0*) after cooling in a vacuum desiccator. 0.5 g of each sample were placed into the crucibles and dried again overnight at 100 °C in oven. Dried crucibles filled with the sample were weighed (*m1*). Crucibles containing samples were finally incinerated for 4 h at 550 °C in a muffle furnace and weighed (*m2*) after cooling in a vacuum desiccator. During incineration, only inorganic compounds remain in the crucibles, which constitute the minerals in the insect sample.

The mineral content of each sample was therefore calculated according to the equation (7):

$$Mineral\ (\%) = \frac{m2 - m0}{m1 - m0} \times 100 \tag{7}$$

3.3.3 Protein content

Proteins contained in insect samples were solubilized by treatment with a protein lysis buffer, and the protein concentration was subsequently photometrically determined (Olson and Markwell, 2007). Around 10 mg of each sample were incubated with 1 ml of protein lysis buffer into 1.5 ml reaction tubes for 24 h at room temperature at 200 rpm shacking. The protein lysis buffer was prepared with 5% sodium dodecyl sulphate (SDS), 50 mM acetic acid, 10 mM boric acid, 4 M urea and 10% glycerol. After incubation, samples were centrifuged at 16 000 rcf for 5 min and the supernatants were collected into new tubes. 1.5 µl of each supernatant were measured with a Nanodrop™ ND1000 spectrophotometer at a wavelength of 280 nm (A280). The 260 nm wavelength (A260) was also determined by multiplying the value of A280 by the ratio 260/280, because of possible contamination of nucleic acids and catecholamines. The protein concentration (mg/ml) of supernatants was determined according to the equation (8) by Layne (1972):

$$Protein\ in\ supernatant\ (mg/ml) = \frac{\left((1.55 \cdot A280) - (0.76 \cdot A260)\right)}{1000} \tag{8}$$

The protein content of each sample was then calculated as ratio between the protein concentration of the supernatant and the weight of the respective initial sample (equation 9):

$$Protein\ (\%) = \frac{protein\ in\ supernatant}{weight\ of\ sample} \times 100 \tag{9}$$

3.3.4 Fat content

Free lipids were removed from the raw insect samples by Soxhlet extraction using *n*-hexane as solvent. An empty 250 ml round flask was firstly dried overnight at 100 °C in oven and then weighed (*mF0*) after cooling in a desiccator. 150 ml of *n*-hexane were added into the flask and the flask was connected to the Soxhlet apparatus (Sigma-Aldrich, St. Louis, Missouri, USA) (figure 3.3). 7 g (*mS*) of sample were placed in a cellulose thimble and the thimble was inserted into the Soxhlet extractor. The flask was then heated to the boiling point of *n*-hexane and the lipid extraction started. Extraction ended after 20 solvent runs (about 3 h). At this point, the flask contained the extracted lipids and the solvent. *N*-hexane was evaporated from the flask in a rotatory evaporator at 60 °C and 335 mbar. The flask containing only lipids was finally dried at 100 °C overnight in the oven and weighed (*mF1*) again.

The fat content of each sample was determined according to the equation (10):

$$Fat\ (\%) = \frac{mF1\text{-}mF0}{mS} \times 100 \qquad (10)$$

Figure 3.3 Soxhlet extraction apparatus used for determination of the fat content of samples.

3.3.5 Fibre (ADF, ADL) and chitin content determination

In order to determine the chitin content of raw insects, the structural similarity between chitin and cellulose was exploited. Since the chitin-containing fibrous structures in insects correspond to cellulose and lignin in plants, the standard method used for fibre determination of feed (EN

ISO 13906:2008) was applied to insects, according to Hahn *et al.* (2018). The procedure consists of two steps after which different fibre components are obtained (table 3.2).

Table 3.2 Comparison between the composition of plant and insect raw samples, and fibrous residues after each step of the fibre determination procedure.

	RAW SAMPLE	ADF RESIDUE	ADL RESIDUE	ADF–ADL
PLANTS	CELLULOSE, lignin, hemicellulose, amid, sugars, minerals	CELLULOSE, lignin	Lignin	CELLULOSE
INSECTS	CHITIN, protein, lipids, catecholamine, minerals	CHITIN, catecholamine	Catecholamine	CHITIN

The first step involves the solubilization of simple sugars, soluble proteins and lipids, giving the acid detergent fibre (ADF) as solid residue. This fraction consists of cellulose and lignin in plant tissues, which correspond to chitin and catecholamine in the insect cuticles. After the following treatment with sulfuric acid, the acid detergent lignin (ADL) is obtained. ADL corresponds to the insoluble catecholic compounds linked to chitin as a result of sclerotization of the insect cuticle (Andersen, 2010). Since chitin is decomposed by the acidic treatment and is no longer in the residue, the chitin content of insect samples can be calculated as the difference between the mass percentages of ADF and ADL.

To perform the fibre determination analysis, empty glass filter crucibles (40-60 μm pore size, 50 ml volume) were firstly dried overnight at 100 °C in the oven and then weighed (*m0*). 1 g (*mS*) of each sample was incubated with 100 ml of ADF-solution into 250 ml flasks for 1 h at 100 °C, under stirring. The ADF-solution has been prepared with 2% w/v cetyl-trimethyl-ammonium bromide (CTAB) and 0.5 M sulfuric acid. After the reaction time, the content of each flask was poured into a filter crucible, placed on a filtration flask, and filtered using a vacuum pump. The solid residue was washed twice with 50 ml of deionized water at 80 °C and then twice with 50 ml of acetone, stirring for a short time the content of the crucible with a spatula. The filter crucibles containing the ADF retentates were dried overnight at 100 °C in an oven and weighed again (*m1*). The ADF content of each sample was calculated according to the equation (11):

$$ADF\ (\%) = \frac{m1 - m0}{mS} \times 100 \qquad (11)$$

The dry crucibles containing ADF were then placed into 100 ml beakers and filled with 25 ml

of 72% v/v sulfuric acid. Samples were incubated under shaking for 3 h at room temperature. At the end of the reaction, sulfuric acid was filtered off the crucibles using a vacuum pump and the solid residue was washed with deionized water until the filtrate had a neutral pH. Filter crucibles with ADL residues were dried at 100 °C overnight in oven and weighed again (*m2*). The final step was the incineration of crucibles containing ADL at 550 °C for 4 h in a muffle furnace. After cooling in a vacuum desiccator, crucibles were once again weighed (*m3*). The content of ADL was determined according to the equation (12):

$$ADL\ (\%) = \frac{m2 - m3}{mS} \times 100 \qquad (12)$$

The chitin content of each sample was finally calculated as the difference between ADF and ADL factions (equation 13):

$$Chitin\ (\%) = ADF\ (\%) - ADL\ (\%) \qquad (13)$$

3.4 Chitin extraction

As already described, chitin extraction consists of two main steps: demineralization and deproteinization. An additional step of bleaching was also performed to whiten the dark pigmented chitin. The extraction was firstly attempted trying different protocols adapted from those in the literature, which mainly refer to methods applied to crustaceans. The final extraction protocol used to produce chitin with the characteristics described in this thesis was adopted within the cooperation with Fraunhofer Institute for Interfacial Engineering and Biotechnology IGB (Stuttgart), where it had been optimised. Demineralization, deproteinization and bleaching of all *H. illucens* samples were performed according to the method reported by Hahn *et al*. (2020b).

3.4.1 Demineralization

To remove minerals, mainly calcium carbonate, insect samples were suspended in 0.5 M formic acid (solid:liquid ratio 1:10) and stirred at 350 rpm for 1 h at room temperature. The demineralized material was then filtered through a sieve cloth (40-60 μm pore size) and washed with deionized water until the filtrate reached a neutral pH. The washed demineralized samples

were dried at 60 °C overnight in oven.

Mineral content of the demineralized samples was determined (method reported in section 3.3.2) in order to assess the efficiency of the demineralization treatment (DM efficiency), according to the equation (14):

$$DM\ efficiency\ (\%) = \frac{mineral(\%)\ raw\ sample - mineral\ (\%)\ DM\ sample}{mineral\ (\%)\ raw\ sample} \times 100 \quad (14)$$

3.4.2 Deproteinization

Proteins were removed from demineralized samples by treatment with 2 M sodium hydroxide (NaOH) (solid:liquid ratio 1:10) for 2 h at 80 °C. The NaOH solution was first placed in a flask on a heating, stirring plate and heated to 80 °C. Once reached the temperature, the demineralized material was added into the flask and stirred at 350 rpm for the duration of the reaction. Temperature maintenance was verified with a thermometer. The deproteinizing solution was then discarded by filtering the suspension through a sieve cloth (40-60 μm pore size) and the solid material was washed with deionized water until the filtrate reached a neutral pH. The washed deproteinized samples were dried at 60 °C overnight in oven. After deproteinization, unbleached chitin was obtained. Protein content of the deproteinized samples was determined (method described in section 3.3.3) in order to assess the efficiency of the deproteinization treatment (DP efficiency), according to the equation (15):

$$DP\ efficiency\ (\%) = \frac{protein\ (\%)\ raw\ sample - protein\ (\%)\ DP\ sample}{protein\ (\%)\ raw\ sample} \times 100 \quad (15)$$

3.4.3 Bleaching

Part of the purified unbleached chitin was subjected to a bleaching treatment. A solution of 5% v/v hydrogen peroxide (H_2O_2) was used (solid:liquid ratio 1:20 for larvae and pupal exuviae, 1:30 for adults), according to the investigation on the optimization of the chitin bleaching treatment performed at Fraunhofer IGB (Hahn *et al.*, under review). The H_2O_2 solution was heated to 90 °C in a flask on a heating stirring plate and then the unbleached chitin was added and stirred for 1 h at 350 rpm. Temperature maintenance was verified with a thermometer. The bleached samples were filtered using filter paper (7-12 μm pore size) and washed with deionized water until the filtrate reached a neutral pH. Washed samples were finally dried at 60 °C overnight in oven. After this treatment, bleached chitin was obtained.

3.5 Chitosan production

Chitosan was obtained by deacetylation of both unbleached and bleached chitin extracted from the three developmental stages of *H. illucens*. Heterogeneous deacetylation was performed for all the chitin samples. In addition, homogeneous deacetylation was also carried out only for chitin produced from pupal exuviae. In figure 3.4, a scheme of the chitin and chitosan samples obtained from the different raw insect samples and the different deacetylation treatments is provided.

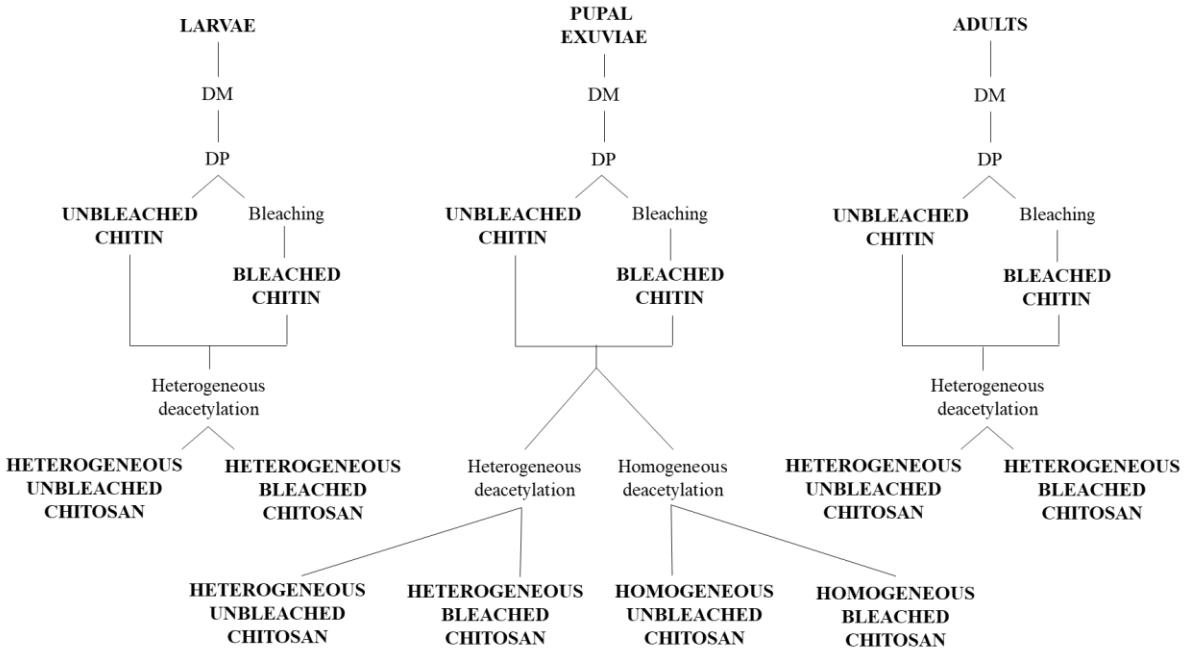


Figure 3.4 Overview of the chitin and chitosan samples obtained from the three developmental stages of *H. illucens* after demineralization (DM), deproteinization (DP), bleaching and the different deacetylation methods.

3.5.1 Heterogeneous deacetylation

Chitin samples were heterogeneously deacetylated by suspending the material in 12 M NaOH (solid:liquid ratio 1:20). The alkaline solution was poured in a glass bottle and heated to 100 °C on a heating stirring plate. When the temperature was reached (checked with a thermometer), the chitin was added into the flask and incubated for 4 h, at constant temperature, at 350 rpm. At the end of the reaction, the suspension was filtered using filter paper (7-12 μm pore size) and the solid residue was washed thoroughly with distilled water until the filtrate had a neutral pH.

After washing, the deacetylated material was suspended in 1% acetic acid and left at room temperature for 48 h, under stirring. The mixture was then centrifuged at 10 000 g for 5 min

and the supernatant, in which the solubilized chitosan was contained, was collected. The solid residual pellet, consisting of insolubilized chitosan or not-deacetylated chitin, was discarded. The supernatant was titrated with 6 M NaOH to pH 8 and allowed to stand overnight at 4 °C, in order to precipitate the solubilized chitosan. The suspension was centrifuged again at 10000 rcf for 5 min and the chitosan pellet was collected and washed thoroughly with distilled water, in order to remove the remaining acetate adsorbed by chitosan (Hahn *et al*., 2020b). The washed chitosan was finally frozen at -20 °C before lyophilization and then stored at room temperature.

3.5.2 Homogeneous deacetylation

Chitin extracted from pupal exuviae, both unbleached and bleached, was deacetylated also applying the homogeneous method. Chitin was incubated with 10 M NaOH (solid:liquid ratio 1:20) at 4 °C overnight. Then, ice from distilled water was added and the mixture was stirred until homogeneity. The addition of the ice and the resulting low temperature promote swelling and dissolving of the chitin in the alkaline solution (Nemtsev *et al*., 2002). After complete thawing of the ice, the mixture was centrifuged at 10 000 rcf for 15 min at 4 °C and the residual undissolved solid matter was discarded. Supernatant was collected and transferred into new glass bottles whose headspace was gassed with nitrogen (N_2), in order to avoid the contact with the atmospheric oxygen and thus to protect the chitosan from oxidative degradation. The mixture was incubated again for 48 h at room temperature.

After incubation, ice was added again and stirred until complete thawing, and the solution was subsequently titrated with 6 M HCl to pH 8 and left at 4 °C overnight to precipitate the chitosan. The suspension was centrifuged at 10000 rcf for 10 min to collect the chitosan pellet. Chitosan was finally washed with distilled water until a neutral pH was restored, and stored at -20 °C until lyophilization.

3.6 Chitin and chitosan characterization

The different chitin and chitosan samples were analysed in order to characterized them and assess their quality and suitability for the desired application. Figure 3.5 provides a summary of all analyses performed on chitin and chitosan, as well as, on the intermediate products of the purification process. Composition of chitin, in terms of mineral, protein and fibre content, was performed according to methods described in section 3.3.

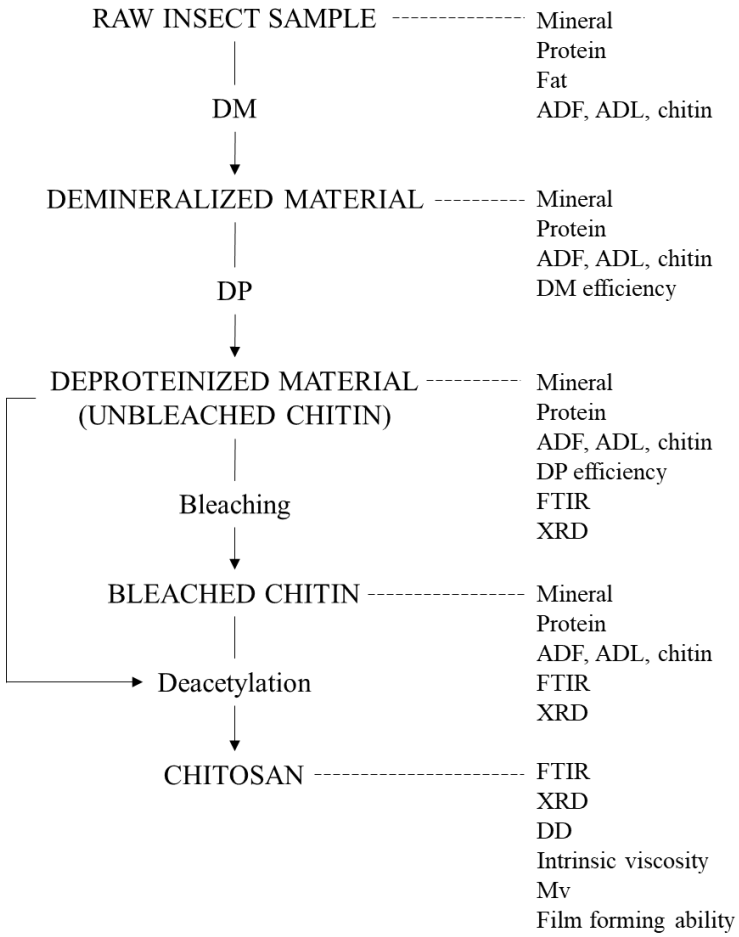


Figure 3.5 Summary of the process applied for chitin and chitosan production from *H. illucens* whole larvae, pupal exuviae and adults, consisting of demineralization (DM), deproteinization (DP), bleaching and deacetylation. The analysis performed on all the intermediate products, as well as, on all the different chitin and chitosan samples are also reported.

The yield of both chitin and chitosan was firstly calculated for all the samples, according to the following equations (16, 17):

$$Chitin\ yield\ (\%) = \frac{dry\ weight\ of\ chitin\ (g)}{dry\ weight\ of\ the\ original\ raw\ insect\ sample\ (g)} \times 100 \quad (16)$$

$$Chitosan\ yield\ (\%) = \frac{chitosan\ weight\ after\ lyophilization\ (g)}{dry\ weight\ of\ the\ original\ raw\ insect\ sample\ (g)} \times 100 \quad (17)$$

Methods applied for the Fourier-transform infrared spectroscopy (FTIR), the X-ray diffraction (XRD) analysis, and the determination of the deacetylation degree (DD), intrinsic viscosity and viscosity-average molecular weight (Mv) of chitosan, are described in the following sections.

Additionally, the film forming ability of chitosan was tested, in relation to its application as coating agent.

A sample of commercial chitin (MP Biomedicals) and one of commercial chitosan (Sigma-Aldrich), derived from crustacean shells, were always used as reference for all analyses.

3.6.1 Fourier-transform infrared spectroscopy (FTIR)

Chitin and chitosan samples were subjected to FTIR analysis, in order to identify their functional groups, which give rise to characteristic bands in terms of both intensity and frequency. This technique allows to obtain information on the bonds present within a molecule, to assess the presence of functional groups and to obtain the identity of the molecule itself, from the signals present in the fingerprint region, which is characteristic of each molecule.

The FTIR spectra were recorded using a Jasco 460Plus IR spectrometer, in the range of wavelength 400-4000 cm^{-1}, by accumulating 100 scans with a resolution of 4 cm^{-1}. The resulting spectra were processed using JASCO Spectra Manager software. For analysis, chitin and chitosan dry pulverised samples were mixed with KBr (potassium bromide) and the mixture was pressed with a hydraulic press to obtain tablets with a diameter of 1 cm.

The acetylation degree (DA) of chitin samples was estimated, attributed to the C=O stretching of amide group, by evaluating the ratio between the area of the bands centred respectively at 1660 and 2700 cm^{-1}, according to the equation (18) by Weißpflog *et al*. (2021):

$$DA\ (\%) = \frac{\frac{A1660}{A2700} \times 100}{1.33} \tag{18}$$

3.6.2 X-ray diffractometry (XRD)

The XRD analysis was applied to determine the crystallinity of chitin and chitosan. This technique is based on a radiation-matter interaction phenomenon. When X-ray radiation hits the material, the radiation is scattered by the atoms and the associated electromagnetic waves change direction. The phenomenon may or may not be related to an exchange of energy between the photons and the atoms, causing inelastic scattering and elastic scattering respectively, the latter giving rise to the diffraction phenomenon.

The directions of the diffracting beams are determined by Bragg's law (equation 19):

$$2d\ \sin\theta = n\lambda \tag{19}$$

where d is the spacing between different planes, θ is the incident angle, n is an integer indicating the diffraction order and λ is the wavelength of the beam (Bragg, 1913).

X-ray diffraction produces a diffraction spectrum showing relative intensities as a function of diffraction angle. Each crystalline plane in the sample only gives rise to diffraction at certain beam angles, so characteristic diffraction spectra correspond to characteristic structures.

From the diffractograms, the crystallinity indices (CrI) of chitin and chitosan were calculated according to the Segal method (equation 20) (Segal *et al*., 1959), by subtracting the height of the minimum intensity between major peaks (the amorphous band (Ia)) from the intensity of the highest diffraction peak, that represents the crystalline portions (Ic):

$$CrI\ (\%) = \frac{Ic - Ia}{Ic} \times 100 \qquad (20)$$

The size of the crystallites of each chitin and chitosan sample was determined as well, using the Scherrer equation (21) (Scherrer, 1912):

$$D\ (nm) = k\lambda/\beta cos\theta \qquad (21)$$

where D is the size of the crystallites (nm), k is the shape factor, which depends on the nature of the crystallites and is 0.9, λ is the wavelength, β is the width at half height, expressed in radians, of the peak analysed, while θ is the corresponding diffraction angle.

The diffractograms were recorded using an X-ray diffractometer (X'Pert PRO, Philips) with Cu Kα radiation, a voltage of 40 kV and a current of 32 mA. The scanning range was from $2\theta = 5^{o}$ to 50^{o} at a scan speed of $0.04^{o}\ s^{-1}$.

3.6.3 Determination of deacetylation degree of chitosan

The deacetylation degree (DD) of all chitosan samples was determined by potentiometric titration, according to the method of Jiang *et al*. (2003). This method exploits the pH sensitivity of the amino groups of the chitosan chain, that are subjected to protonation in acidic conditions. Hence, the concentration of deacetylated amino groups of chitosan in solution can be determined by titration (Weißpflog *et al*., 2021).

Chitosan solutions were prepared dissolving 0.25 g of chitosan in 10 ml deionized water with the addition of20 ml of 0.1 M HCl. The mixture was stirred for 2 h at room temperature until complete dissolution of the chitosan sample. The chitosan-HCl solution was then titrated with

0.1 M NaOH. 3 ml of NaOH were firstly added and the resultant pH of the solution was measured with a pH-meter. Then the titration continued by adding 1 ml of NaOH at a time, measuring the pH of the solution after each addition. The titration was finished after the addition of 7 ml NaOH.

The titrated volume of 0.1 M NaOH (V) was inserted into the function f(V) (equation 22), where V0 is the volume of 0.1 M HCl (20 ml); [NaOH] is 0.1 M; $[H^+]$ is the concentration of H^+ ions and $[OH^-]$ is the concentration of OH^- ions, both calculated by the pH value.

$$f(V) = \frac{V_0+V}{[NaOH]} \times ([H^+] - [OH^-]) \quad (22)$$

Then, V was plotted against f(V) in a diagram and the linear trend line equation was determined by fitting the data points. The intercept of the trend line on the *x*-axis corresponded to the volume consumed up to the equivalence point (all existing HCl neutralized with the corresponding amount of NaOH) of the titration of NaOH (Ve). The DD% of chitosan was therefore calculated according to the equation 23, where [HCl] is 0.1 M; VA is volume of 0.1 M HCl (20 ml); [NaOH] is 0.1 M; Ve is the consumed volume of NaOH at the equivalence point; W is the weight of the dissolved chitosan and 161 g/mol is the molecular weight of glucosamine in the chitosan chain.

$$DD\ (\%) = \frac{[HCl] \times VA - [NaOH] \times Ve}{W} \times 161\ \frac{g}{mol} \times 100 \quad (23)$$

3.6.4 Determination of chitosan intrinsic viscosity and viscosity-average molecular weight

The viscosity-average molecular weight (M_v) of chitosan was determined by measuring the intrinsic viscosity of the respective chitosan solution. This method is based on the fact that the viscosity of a chitosan solution depends on its molar mass, in addition to the DD.

Intrinsic viscosity of chitosan was determined using an Ubbelohde capillary type viscometer size 3, flow times 100-150 sec (Fisher Scientific, Waltham, Massachusetts, USA) (figure 3.6 a, c), according to the method reported by Singh *et al*. (2019). Each chitosan sample was dissolved in a solvent containing 0.2 M acetic acid and 0.15 M ammonium acetate at four different concentrations: 0.05, 0.1, 0.2 and 0.3 %. Solutions were left under stirring for 24 h at room temperature to allow chitosan dissolution and then both chitosan solution and solvent were subjected to viscosity measurement. The Ubbelohde type viscometer consists of a U-shaped

glass tube (figure 3.6 a) with a reservoir on one side and a measuring bulb with a capillary (figure 3.6 b) on the other, placed in a cooling water bath to maintain a constant temperature of 25 ± 0.1 °C (figure 3.6 c). A fixed volume (7 ml in this case) of the solution to be measured is introduced into the reservoir and aspirated into the bulb through the capillary using a rubber suction ball. The solution is then allowed to flow back down through the bulb. The viscosity of the liquid is derived by measuring the time (efflux time) it takes for the solution to pass through two calibrated marks at the top and bottom of the bulb (figure 3.6 b).

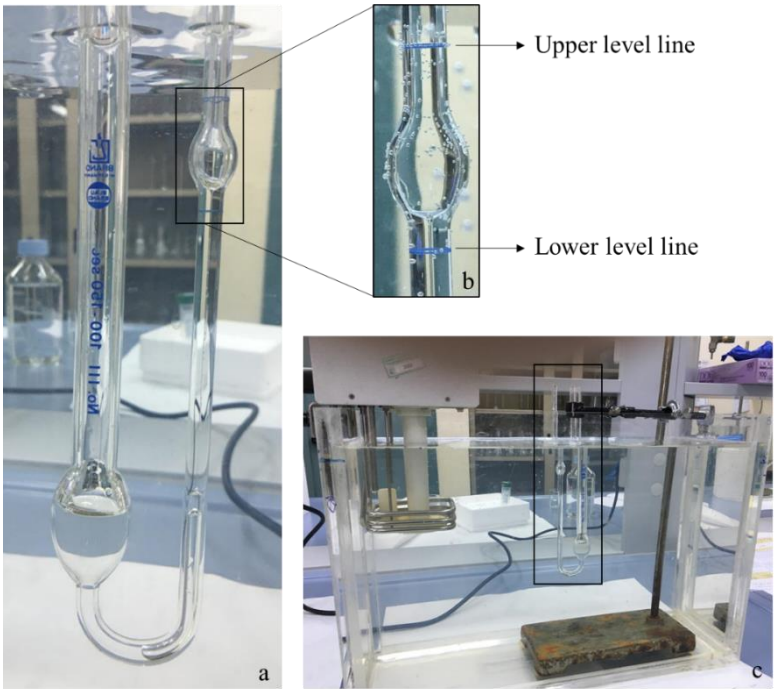


Figure 3.6 The Ubbelohde capillary type viscometer used for viscosity measurement (a) placed in the cooling water bath (c). Detail of the portion of the viscometer where the efflux times between the two level lines of the sample solutions were measured (b).

The average efflux times of solvent and samples (i.e., chitosan solutions) were then used to calculate relative and inherent viscosity using the following equations (24, 25, 26):

$$Relative\ viscosity\ (\eta_{rel}) = \frac{efflux\ time\ of\ sample\ (sec)}{efflux\ time\ of\ solvent\ (sec)} \quad (24)$$

$$Specific\ viscosity\ (\eta_{sp}) = \eta_{rel} - 1 \quad (25)$$

$$Reduced\ viscosity\ (\eta_{red}) = \frac{\eta_{sp}}{concentration\ of\ chitosan\ in\ the\ solution\ (\%)} \quad (26)$$

The reduced viscosity was plotted against chitosan concentration (0.05 to 0.3 g/dl). Intrinsic viscosity was therefore calculated from a linear equation of reduced viscosity at zero concentration.

The M_v of each chitosan sample was calculated using the following Mark–Houwink–Sakurada equation (27) (Yacob *et al*., 2013) where [η] is the intrinsic viscosity, $K=9.66\times10^{-5}$, and $\alpha=0.742$ determined in the same solvent at 25 °C (Yacob *et al*., 2013):

$$[\eta]=KM_v^{\alpha} \quad (27)$$

3.6.5 Assessment of chitosan film formation ability

The film forming ability was assessed for each chitosan produced. The ability to form films is a characteristic property of chitosan and is particularly important for its application as a coating agent. For this purpose, 0.1 g chitosan were dissolved in 10 ml of 1% acetic acid and the solution was poured into a 100 mm diameter Petri dish. The solutions were left to dry at room temperature for 3 days, leaving the lids of the Petri dishes open. The dried chitosan films were gently removed from the Petri dishes and photographically documented, visually evaluating their homogeneity and transparency.

3.7 Application of chitosan-based coatings for preservation of fresh fruits

The different chitosan samples obtained from *H. illucens* were used to prepare coating solutions for the preservation of fresh fruits. The preservative effect of these chitosan solutions was evaluated on cherry tomatoes, strawberries, apricots and nectarines.

3.7.1 Fruits

Mature, commercially available cherry tomatoes (*Lycopersicon esculentum* L.) were purchased from a local grocery store. Fresh mature strawberries (*Fragaria* x *ananassa* Duch.), apricots (*Prunus armeniaca* L.) and yellow nectarines (*Prunus persica* L.) were supplied by a local producer (APOFRUIT Italia soc. coop. agricola, Scanzano Jonico, Matera, Italy).

Fruits of similar shape and colour, and without signs of fungal infection or mechanical damage

were selected for experiments, and coated with chitosan (see section 3.7.3) within 24 h of arrival at the laboratory, keeping them at +6 °C. Prior to the tests, fruits were gently wiped with paper to remove any dust particles accumulated during transport. A total of 448 tomatoes, 288 strawberries, 192 apricots and 96 nectarines were used for experiments.

3.7.2 Preparation of coating solutions

Chitosan-based coating solutions were prepared according to Hassan *et al*. (2020). 1% (v/v) acetic acid solution was prepared and heated to 40 °C before dissolving the required amount of chitosan. 0.2% Tween-80 (v/v) and 2% glycerol (v/v) were added to improve wettability of the solution. The solutions were left under continuous stirring for 16 h to allow dissolution of chitosan.

Solutions were prepared with all the chitosan samples produced from *H. illucens*, both bleached and unbleached, as well as with commercial chitosan derived from shrimp shells (Sigma-Aldrich). All the chitosan samples were tested at two different concentrations: 0.5% and 1% (w/v). A coating treatment with the solvent solution only (1% v/v acetic acid, 0.2% v/v Tween-80 and 2% v/v glycerol), and a negative control without treatment were also carried out.

The first fruits tested were tomatoes to which only the chitosan produced from pupal exuviae was applied. This is because of the larger amount of this sample available and because it was the only chitosan obtained with two different deacetylation methods (heterogeneous and homogeneous). Possible differences in the preservative effect due to the different deacetylation were thus investigated. Due to the worse results obtained with homogeneous chitosan from pupal exuviae (see section 4.5.1), this sample was not used in the subsequent experiments with the other fruits. On strawberries and apricots, chitosan samples from larvae, pupal exuviae and adults were tested, all heterogeneously deacetylated. On nectarines, only heterogeneous chitosan from pupal exuviae was tested.

Number of coating treatments and replicates was adapted to the number of available fruits. In table 3.3 treatments applied and number of treated fruits are summarized.

Table 3.3 Overview of the treatment applied for fruits preservation and total number of treated fruits. The coating solutions used are described: solvent only, commercial chitosan, and chitosans from *H. illucens* (pupal exuviae, adults and larvae). The check mark indicates that that particular treatment has been applied to the fruit.

	COATING SOLUTIONS											
	NEGATIVE CONTROL	SOLVENT	COMMERCIAL CHITOSAN (0.5-1%)	PUPAL EXUVIAE Heterogeneous		PUPAL EXUVIAE Homogeneous		ADULTS		LARVAE		NUMBER OF TREATED FRUITS
				Bleached (0.5-1%)	Unbleached (0.5-1%)	Bleached (0.5-1%)	Unbleached (0.5-1%)	Bleached (0.5-1%)	Unbleached (0.5-1%)	Bleached (0.5-1%)	Unbleached (0.5-1%)	
TOMATOES	✔	✔	✔	✔	✔	✔	✔					448
STRAWBERRIES	✔	✔	✔	✔	✔			✔	✔	✔	✔	288
APRICOTS	✔	✔	✔	✔	✔			✔	✔	✔	✔	192
NECTARINES	✔	✔	✔	✔	✔							96

3.7.3 Application of coatings and storage conditions

In the first experiment with cherry tomatoes, two different application methods of the coating solutions were applied: dipping and spraying.

For the dipping application, cherry tomatoes were submerged in the coating solution for 5 min (figure 3.7). Then, fruits were taken out and air-dried for 30 min. This treatment was repeated three times.

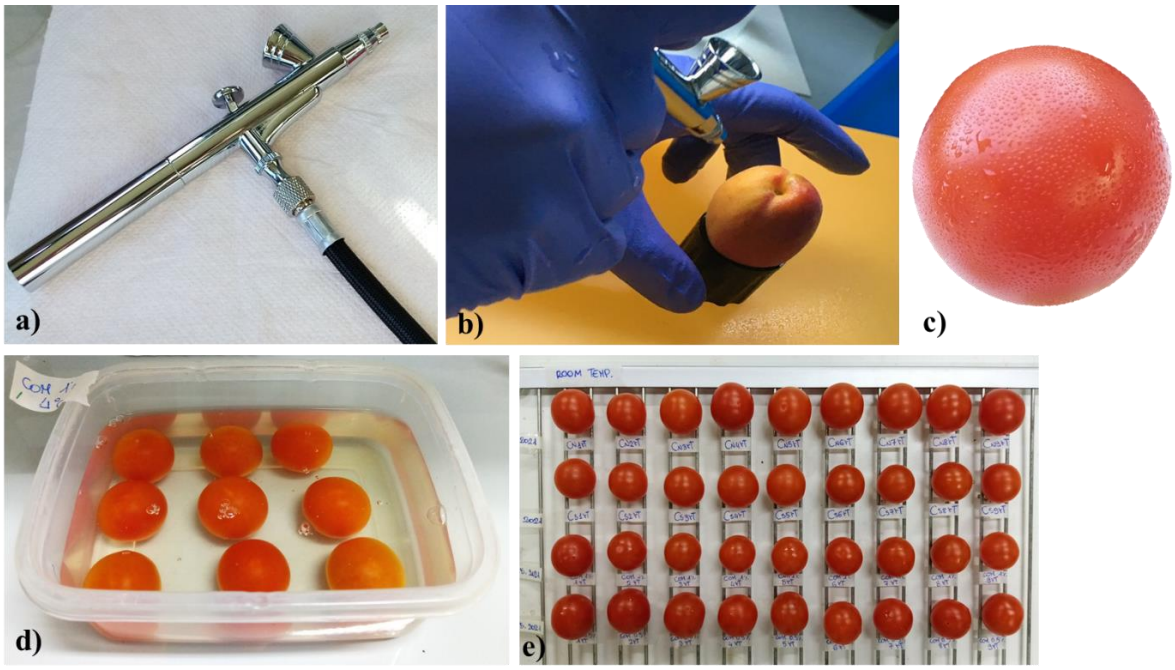


Figure 3.7 Aerograph used for spraying application of chitosan coating solutions (a, b). Tomato sprayed with chitosan solution with the droplets of the solution visible on its skin (c). Cherry tomatoes soaked in the chitosan solution for dipping application (d). Tomatoes placed on the grid for storage (e).

For the spraying application, the coating solutions were sprayed onto the fruits using an aerograph (Martellato s.r.l., Rovigo, Italy) (figure 3.7). Spraying of the fruit was repeated a second time after drying for 30 minutes at room temperature to ensure uniform surface coverage with the coating solution.

Since the best coverage was achieved by spraying, for the subsequent experiments with strawberries, apricots and nectarines, only this application method was used.

Both the dipping-coated fruits group and the spraying-coated ones were then divided into two subgroups each. A subgroup of dipped fruits and a subgroup of sprayed fruits were stored at room temperature (25 °C), while the other two subgroups were kept at refrigeration temperature (4-6 °C). The fruits were placed on grids avoiding direct exposure to sunlight and away from heat sources.

Storage duration varied, depending on the perishability of the different fruits: 30 days for

tomatoes, 9-15 days for strawberries, 14-26 for apricots and 10-21 days for nectarines.

3.8 Evaluation of the preservative effect of chitosan-based coatings

Effects of the chitosan coatings on preservation of the fresh fruits were investigated evaluating weight loss and determining changes in the total soluble solids content, pH and titratable acidity values of fruits, which are the three chemical parameters commonly examined in fruit preservation experiments (Sucharitha *et al*., 2018; Hassan *et al*., 2020; Jiang *et al*., 2020; Lin *et al*., 2020). Fruits were also regularly photographically documented in order to evaluate changes in their appearance during storage. Pictures of the fruits are shown in the supplementary materials.

3.8.1 Determination of weight loss

All the treated fruits were weighed individually at the beginning of the experiment (T_0) and thereafter at intervals of 2-3 days until the end of their storage (T_f). Weight of each fruit was measured using a precision scale with an accuracy of ±0.01 g.

Variation in the weight of fruit was then assessed by comparing their initial and final weight, and the results were expressed as percentage of weight loss, according to the equation (28):

$$Weight\ loss\ (\%) = \frac{weight\ (g)\ at\ T_0 - weight\ (g)\ at\ T_f}{weight\ (g)\ at\ T_0} \times 100 \qquad (28)$$

3.8.2 Measurement of total soluble solids, pH and titratable acidity

For chemical analysis, fruits pooled in triplicates were homogenized using a laboratory blender (International Pbi S.p.a., Milano, Italy) at room temperature for 5 min. The homogeneous pulp was then filtered through a gauze (40-60 μm pore size) to remove solid residues and skins. 5 g of the filtered pulp suspended in 25 ml of distilled water were used for the following measurements.

- Total soluble solids (TSS) were determined using a hand refractometer, according to the standard method EN ISO 2173:2003 (figure 3.8). Determination of the TSS enables an estimation of the sugar content of sweet solutions (e.g., juices, syrups, honey) in which sugar constitutes the major solid component. It is widely used in different food processings monitoring fruit development during ripening, since the sugar content

increases as ripening progresses (Barbosa-Cánovas *et al.*, 2003). A refractometer measures the refractive index of a liquid based on the principle that the refraction of a beam of light passing through a liquid increases as the amount of solids in the liquid increases. The liquid is placed on the measuring prism of the refractometer (figure 3.8) and exposed to the light. Theresult of the refraction phenomenon can be seen on the graduated scale looking into the eyepiece. A shadow line forms between the illuminated and dark areas (figure 3.8) and the result of the measurement can be read from where the line crosses the scale (Kleinhenz and Bumgarner, 2013).

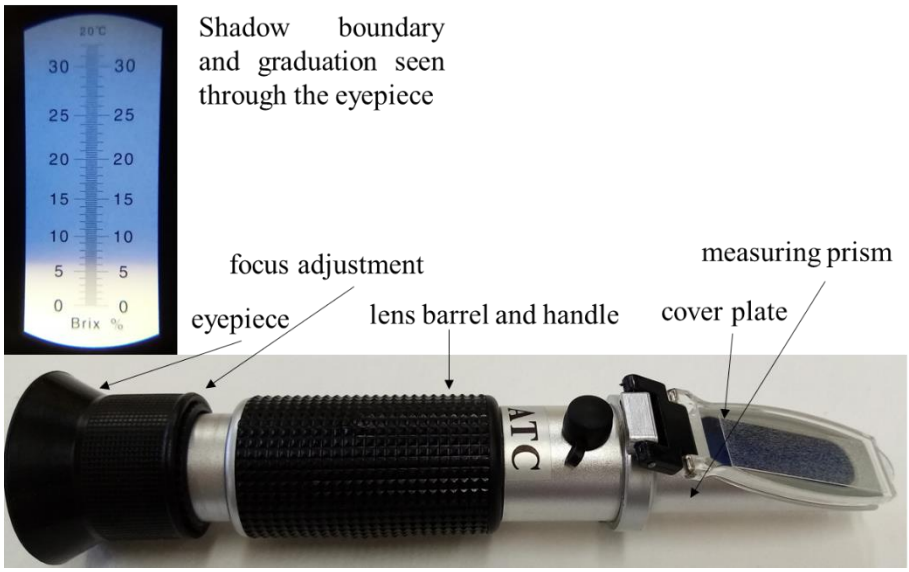


Figure 3.8 Hand refractometer used for TSS determination.

For TSS determination, few drops of the fruit pulp were placed on the surface of the measuring prism and the value of TSS was recorded by reading the intersection of the shadow line on the graduated scale visible in the eyepiece (figure 3.8). The result is expressed as degrees Brix (°Brix), a unit considered equivalent to the percentage of sucrose in a solution (i.e., 20°Brix is equivalent to a sugar content of 20%).

- The pH of the fruit pulp at room temperature was determined with a pH meter (Orion Research Inc., Boston, USA).
- The titratable acidity (TA) was determined by titrating a known amount of fruit pulp to pH 8 with 0.1 N NaOH, recording the volume of base used, according to the standard method EN ISO 750:1998.

  The TA indicates the total acid concentration in a food. Organic acids, mainly citric, malic, lactic and acetic acids, are usually the most representative food acids. These organic acids affect food's colour, flavour, microbial stability and the general quality of

food. Thus, the TA of fruits is used as an indicator of maturity and possible fermentation (Sadler and Murphy, 2010). Most foods contain not only organic acids, but also fatty acids, amino acids and other molecules that can contribute to TA. Since the titration procedure cannot discriminate between individual acids, TA is commonly expressed as content of the predominant acid of a certain food. Tables reporting the predominant acids of various foods are available in literature. However, most of the common food acids have similar equivalent weights. Hence, TA is not significatively affected by an incorrect selection of the predominant acid (Sadler and Murphy, 2010).

According to literature (table 3.4), citric acid is the predominant acid of all the tested fruits. Results of TA were, thus, expressed as percentage of citric acid applying the equation 29 (Sadler and Murphy, 2010; Saad *et al*., 2014), where V is the total volume (ml) of 0.1 N NaOH used for titration; 0.1 is the normality (mEq/ml) of NaOH; 0.064 is the equivalent weight (mg/mEq) of citric acid; 1000 is the factor relating mg to g; and W is the fresh weight of sample (g).

$$TA\ (\%\ citric\ acid) = \frac{V \cdot 0.1 \cdot 0.064}{W \cdot 1000} \times 100 \qquad (29)$$

Table 3.4 Acid composition of some commercially important fruits (modified from Sadler and Murphy, 2010)

FRUIT	PREDOMINANT ACID	ACID CONCENTRATION (% of fresh weight)
Apples	Malic	0.27-1.02
Bananas	Malic/citric (3:1 ratio)	0.25
Cherries	Malic	0.47-1.86
Cranberries	Citric	0.9-1.36
Grapefruit	Citric	0.64-2.10
Grapes	Tartaric/malic (3:2 ratio)	0.84-1.16
Lemons	Citric	4.2-8.33
Limes	Citric	4.9-8.3
Oranges	Citric	0.68-1.20
Peaches	Citric	1-2
Pears	Malic/citric	0.34-0.45
Pineapples	Citric	0.78-0.84
Raspberries	Citric	1.57-2.23
Strawberries	Citric	0.95-1.18
Tomatoes	Citric	0.2-0.6

TSS, pH and TA measurements were performed at both T_0 and T_f and the percentage variation of these parameters was evaluated.

3.8.3 Fungal decay index

With the exception of cherry tomatoes, for which no signs of moulding were observed, fungal decay of fruits was visually inspected during storage. A score from 1 to 5 was given to mouldy fruit by visual evaluation of the extent of the fruit surface covered with mould, according to the scale proposed by Babalar *et al*. (2007):

1 = no fungal decay on fruit surface;

2 = trace of decay (mould on up to 5% of the fruit surface);

3 = slight decay (mould on 5-20% of the fruit surface);

4 = moderate decay (mould on 20-50% of the fruit surface);

5 = severe decay (mould on >50% of the fruit surface).

The fungal decay index of fruits belonging to the same treatment group was calculated as the average of the score assigned to each fruit in the group. Results were then expressed as fungal decay index and percentage of mouldy fruit per each treatment.

3.9 Statistical analysis

All measurements were performed in triplicate unless stated otherwise. The data were expressed as average ± standard deviation.

The normal distribution of the data was first verified using the Shapiro-Wilk test, in order to choose appropriate statistical tests to detect significant differences ($p < 0.05$). Then, normally distributed data were analysed with the one-way Anova with Tukey's post hoc test. Data with non-normal distribution were analysed with a non-parametric test (the Mann-Whitney U test). Pairwise comparisons of percentage data were performed with the Chi-square test with Yates' correction. For pairwise comparisons of non-percentage data, the t-test with Welch's correction was used.

All statistical analyses were performed using GraphPad Prism version 6.0.0 for Windows (GraphPad Software, San Diego, California USA) and JMP®, Version *7* (SAS Institute Inc., Cary, NC, 1989–2021).

4. RESULTS

4.1 Composition of raw insects

Composition of raw *H. illucens* larvae, pupal exuviae and adults, in terms of minerals, protein, fat and fibre, was determined in relation to their dry mass. Results are reported in table 4.1.

Table 4.1 Composition of raw *H. illucens* larvae, pupal exuviae and adults used for chitin extraction and chitosan production. Data are expressed as mean ± standard deviation. Different letters in a row indicate significant differences among the different samples in the percentage of each component ($p < 0.05$) (data analyzed with one-way ANOVA and Tuckey *post-hoc* test).

	LARVAE	PUPAL EXUVIAE	ADULTS
Dry mass %	22.0±0.8^{b}	94.0±0.7^{a}	93.0±0.9^{a}
Minerals %	12.5±0.1^{b}	16.0±0.2^{a}	8.1±0.5^{c}
Protein %	38.7±1.6^{b}	30.0±2.8^{c}	49.0±0.4^{a}
ADF %	22.0±3.7^{b}	53.5±1.8^{a}	23.7±2.4^{b}
ADL %	9.5±2.2^{b}	28.0±1.2^{a}	10.8±2.4^{b}
Chitin % (ADF – ADL)	12.4±1.7^{b}	25.5±0.5^{a}	12.8±1^{b}
Fat %	23.0±0.3^{a}	5.0±0.1^{c}	19.7±0.9^{b}
Others %	13.4±0.8	23.5±1.3	10.4±0.3

Significant differences were found in the composition of the three different insect samples. Larvae were the richest in fat together with adults. On the contrary minerals and fibres were higher in pupal exuviae than in larvae and adults. The protein content of adults was higher than both that of larvae and pupal exuviae. Pupal exuviae were the insect biomass with the highest chitin content (25.5%) (table 4.1). This encouraged the primary use of this insect waste as a source of chitin.

Additional components defined in table 4.1 as "others" have been calculated by the difference between 100 and the percentage of the elements determined, on dry mass bases.

4.2 Chitin extraction

Chitin was purified from *H. illucens* larvae, pupal exuviae and adults (figure 4.1), then a part of each chitin sample was bleached. The effect of the bleaching treatment can be recognized by the change in colour of all the chitin samples. While demineralization and deproteinization led to a marginal or almost no lightening of the chitin colour, the bleaching treatment resulted in a very clear chitin. Visually, bleached chitins from larvae and pupal exuviae had a whiteness similar to commercial chitin derived from crustaceans (figure 4.1 d), while bleached chitin from

adults appeared the brightest (figure 4.1 c_1).

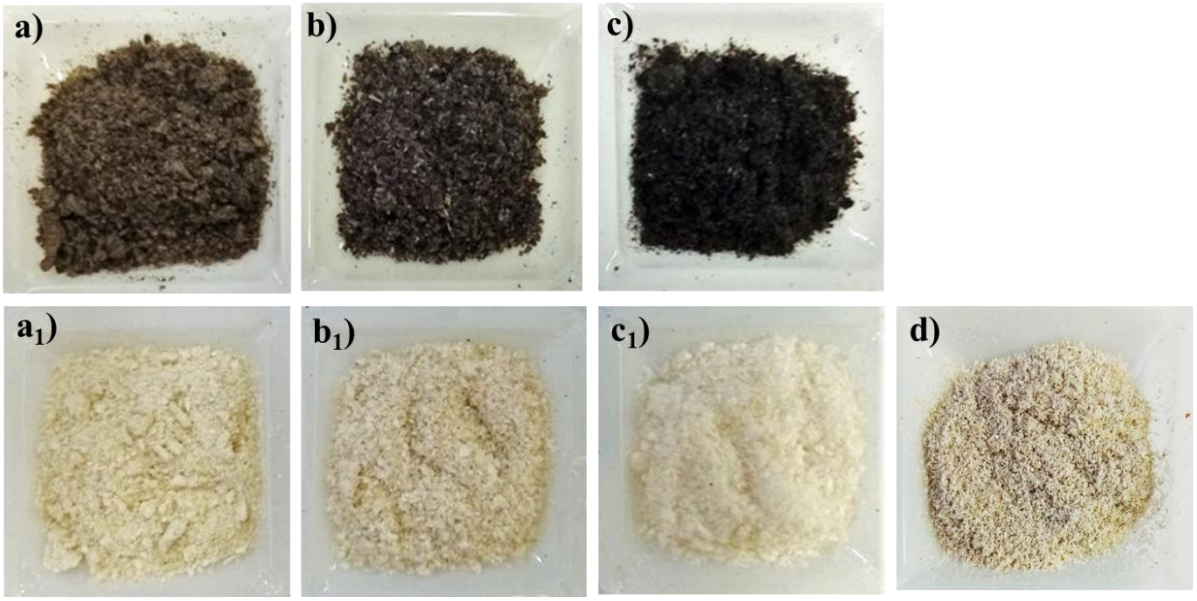


Figure 4.1 Pictures of unbleached (a, b, c) and bleached (a_1, b_1, c_1) chitins extracted from *H. illucens* larvae (a, a_1), pupal exuviae (b, b_1) and adults (c, c_1). A picture of a commercial chitin produced from crustaceans is also shown (d).

Results of the chitin extraction process are described in the next sections. They were expressed in terms of biomass recovery from one purification step to the next, final yield of chitin, efficiency of the extraction method applied and purity of the obtained chitin.

4.2.1 Biomass recovery and final chitin yield

Biomass recovery of the insect samples after each purification step and the final yield of chitin were calculated for all samples. Results are presented in table 4.2.

Significant differences were found in both biomass recovery and chitin yield among the different insect samples (larvae, pupal exuviae and adults). The highest biomass recovery was achieved with pupal exuviae in all the extraction steps, except for the bleaching treatment for which the highest biomass recovery was obtained with larvae. On the contrary, adults had the lowest percentages of biomass recovery during all the extraction procedure. Larvae gave intermediate results (table 4.2).

The highest yield of unbleached chitin was obtained from pupal exuviae (31%) and the lowest from larvae and adults (13 and 9%, respectively). Similar results were achieved with bleached chitin, for which the yields were 23, 10 and 6% from pupal exuviae, larvae and adults, respectively (table 4.2). The bleaching treatment caused the loss of 20, 26 and 32% of the biomass of the unbleached chitin purified from larvae, pupal exuviae and adults, respectively.

For all insect samples, the yield of bleached chitin was slightly lower than that of the unbleached one, although no statistical differences were found between the two values (larvae X^2=0.2, p=0.66; pupal exuviae X^2=1.24, p=0.26; adults X^2=0.29, p=0.59). Thus, the bleaching treatment did not affect chitin yield.

Generally, for all the insect samples, the highest biomass loss occurred after deproteinization, to a greater extent for larvae and adults. The lowest biomass losses for all samples, occurred after demineralization and after bleaching to a similar extent (table 4.2).

Table 4.2 Biomass recovery (%) from larvae, pupal exuviae and adults after demineralization (DM/RAW) and deproteinization (DP/DM), and yields (%) of unbleached and bleached chitin related to the original raw insect biomass. Data are expressed as mean ± standard deviation. Different letters in a row indicate significant differences in the percentage of biomass recovery and yield among the different insect samples ($p < 0.05$) (data analyzed with one-way ANOVA and Tuckey *post-hoc* test).

	LARVAE	PUPAL EXUVIAE	ADULTS
DM/RAW %	66±1^b	77±2.7^a	68±1.1^b
DP/DM %	19±1.2^b	41±0.8^a	14±0.8^c
YIELD OF UNBLEACHED CHITIN %	13±0.7^b	31±1.6^a	9±0.4^c
YIELD OF BLEACHED CHITIN %	10±0.7^b	23±1.9^a	6±0.1^c

4.2.2 Efficiency of the extraction process

Efficiency of demineralization and deproteinization were calculated in order to assess the suitability of the extraction procedure applied to the different insect samples.

Demineralization efficiency was determined as percentage ratio between the mineral content of each sample before and after the acidic treatment. Results are reported in table 4.3. A significant reduction in the mineral content occurred after demineralization with 0.5 M formic acid for 1 h at room temperature for all the three insect samples (larvae X^2=6.3, p=0.01; pupal exuviae X^2=9.5, p=0.002; adults X^2=4.1, p=0.04). The highest efficiency values were achieved with pupal exuviae and adults (85 and 87%, respectively). The demineralization efficiency of the larvae (82%) was significantly lower ($p < 0.05$), although only slightly.

Table 4.3 Mineral content (%) of larvae, pupal exuviae and adults before and after the demineralization (DM) treatment, and efficiency (%) of DM. Data are expressed as mean ± standard deviation. Different letters in a row indicate significant differences in DM efficiency among the different insect samples ($p < 0.05$) (data analyzed with one-way ANOVA and Tuckey *post-hoc* test). Asterisks indicate highly significant differences ($p < 0.0001$) between the mineral content before and after DM, within the same sample (data analyzed with Chi-Square test with Yates' correction).

	LARVAE	PUPAL EXUVIAE	ADULTS
Minerals % before DM	$12.5\pm0.1^{**}$	$16.0\pm0.2^{**}$	$8.1\pm0.5^{*}$
Minerals % after DM	2.2±0.01	2.4±0.2	1.1±0.03
DM efficiency %	82.0 ± 0.1^{b}	85.0 ± 1.5^{a}	87 ± 0.4^{a}

Efficiency of deproteinization was also determined by comparing the protein content of the insect biomass before and after the treatment with NaOH. Results are reported in table 4.4. The deproteinization treatment with 2 M NaOH for 2 h at 80 °C led to a highly significant reduction in the protein content of all insect samples (larvae X^2=37.9, p<0.001; pupal exuviae X^2=26.2, p<0.001; adults X^2=57.3, p<0.001). The highest efficiency was achieved with adults (97%). Slightly, but significant ($p < 0.05$), lower efficiency values were obtained with larvae and pupal exuviae (94 and 92%, respectively).

Table 4.4 Protein content (%) of larvae, pupal exuviae and adults before and after the deproteinization (DP) treatment, and efficiency (%) of DP. Data are expressed as mean ± standard deviation. Different letters in a row indicate significant differences in DP efficiency among the different insect samples ($p < 0.05$) (data analyzed with one-way ANOVA and Tuckey *post-hoc* test). Asterisks indicate highly significant differences ($p < 0.0001$) between the mineral content before and after DP, within the same sample (data analyzed with Chi-Square test with Yates' correction).

	LARVAE	PUPAL EXUVIAE	ADULTS
Protein % before DP	$38.7\pm1.6^{***}$	$30.0\pm2.8^{***}$	$49.0\pm0.4^{***}$
Protein % after DP	2.5±0.1	2.3±0.4	1.5±0.5
DP efficiency %	94.0 ± 0.2^{b}	92.0 ± 1.2^{b}	97.0 ± 1^{a}

4.2.3 Degree of purity of chitin

Composition of all insect samples after each chitin extraction step, as well as composition of the final chitin, were determined. A sample of commercial chitin derived from shrimp shells was analyzed and used as a comparison for chitin samples extracted from *H. illucens*. Results are reported in table 4.5.

Table 4.5 Composition of insect samples, in terms of minerals, protein and fibres, before and after each step of the chitin extraction process, in comparison with commercial chitin derived from shrimp shells. Data are expressed as mean ± standard deviation. Different letters in a row indicate significant differences among the different insect samples in the percentage of each component ($p < 0.05$) (data analyzed with one-way ANOVA and Tuckey *post-hoc* test). Same capital letters in a row, indicate no significant differences between chitin extracted from *H. illucens* and the commercial one, for each component (data analyzed with Chi-Square test with Yates' correction).

	Raw larvae	**DM larvae**	**DP larvae**	**Bleached chitin larvae**	**Commercial chitin**
Minerals %	12.5 ± 0.1^{a}	2.2 ± 0.01^{b}	2.1 ± 0.03^{b}	$1.4\pm0.02^{b\,A}$	2.2 ± 0.1^{A}
Protein %	38.7 ± 1.6^{a}	31.8 ± 2.7^{b}	2.5 ± 0.1^{c}	$1.8\pm0.02^{c\,A}$	1.7 ± 0.3^{A}
ADF %	22.0 ± 3.7^{c}	35.0 ± 3.6^{b}	85.5 ± 2.7^{a}	$88.5\pm0.8^{a\,A}$	89.2 ± 0.4^{A}
ADL %	9.5 ± 2.2^{a}	12.9 ± 3.9^{a}	8.5 ± 1.6^{a}	$3.6\pm0.6^{b\,A}$	1.2 ± 0.2^{A}
Chitin% (ADF - ADL)	12.4 ± 1.7^{d}	22.0 ± 1.9^{c}	76.9 ± 4.3^{b}	$84.0\pm1.3^{a\,A}$	88.1 ± 0.3^{A}
	Raw pupal exuviae	**DM pupal exuviae**	**DP pupal exuviae**	**Bleached chitin pupal exuviae**	**Commercial chitin**
Minerals %	16.0 ± 0.2^{a}	2.4 ± 0.2^{b}	1.9 ± 0.1^{b}	$0.5\pm0.1^{c\,A}$	2.2 ± 0.11^{A}
Protein %	30.0 ± 2.8^{a}	29.8 ± 2.8^{a}	2.3 ± 0.4^{b}	$2.1\pm0.4^{b\,A}$	$1.7\pm0.\ 3^{A}$
ADF %	53.5 ± 1.8^{c}	73.6 ± 2.8^{b}	90.5 ± 1.5^{a}	$90.7\pm0.4^{a\,A}$	89.2 ± 0.4^{A}
ADL %	28.0 ± 1.2^{a}	32.0 ± 1.8^{a}	12.7 ± 2.4^{b}	$3.9\pm0.1^{c\,A}$	1.2 ± 0.2^{A}
Chitin% (ADF - ADL)	25.5 ± 0.5^{d}	42.9 ± 3.2^{c}	77.8 ± 1.7^{b}	$86.8\pm0.4^{a\,A}$	88.1 ± 0.3^{A}
	Raw adults	**DM adults**	**DP adults**	**Bleached chitin adults**	**Commercial chitin**
Minerals %	8.1 ± 0.5^{a}	1.1 ± 0.03^{b}	1.27 ± 0.02^{b}	$0.9\pm0.1^{b\,A}$	2.2 ± 0.1^{A}
Protein %	49.0 ± 0.4^{a}	45.6 ± 2.2^{a}	1.5 ± 0.5^{b}	$1.4\pm0.1^{b\,A}$	1.7 ± 0.3^{A}
ADF %	23.7 ± 2.4^{c}	32.2 ± 1.5^{b}	84.8 ± 0.7^{a}	$86.6\pm1.2^{a\,A}$	89.2 ± 0.4^{A}
ADL %	10.8 ± 2.4^{a}	11.6 ± 1.1^{a}	11.4 ± 0.5^{a}	$0.8\pm0.01^{b\,A}$	1.2 ± 0.2^{A}
Chitin% (ADF - ADL)	12.8 ± 1^{d}	20.6 ± 0.4^{c}	73.4 ± 0.2^{b}	$85.3\pm1.2^{a\,A}$	88.1 ± 0.3^{A}

Composition of larvae, pupal exuviae and adults significantly changed during the purification process, following the same trend. Minerals were significantly reduced by the demineralization treatment, then they remained constant during the other steps. Only in pupal exuviae, the bleaching treatment induced a further decrease of the mineral content (from 1.9 to 0.5%). Proteins significantly decreased in all samples after the deproteinization step and then remained constant until the end of the purification process. In larvae, also demineralization led to a first significant reduction of the protein content (table 4.5).

With the removal of minerals and proteins, ADF percentage significantly increased progressively throughout the extraction procedure in all insect samples. In larvae and adults, the major ADF increase occurred after deproteinization (from 35 to 85.5% and from 32.2 to 84.8%, respectively), while ADF in pupal exuviae increased equally after demineralization and deproteinization. The bleaching treatment did not significantly rise the ADF content. In contrast, the other fibre component (ADL) remained constant until deproteinization and

decreased significantly after bleaching. Only in pupal exuviae, a first significant reduction in the ADL percentage occurred also after demineralization (table 4.5).

The component that denotes the increase in purity of the samples after each purification step is the chitin content. The percentage of chitin always increased significantly, with the major increment occurring after deproteinization in all insect samples. The final chitin content of the bleached chitin extracted from the three insect samples was similar, amounting to 84, 86.8 and 85.3% for larvae, pupal exuviae and adults, respectively. These values were taken as the degrees of purity of the final chitin samples. Comparing the composition of chitin purified from the three *H. illucens* samples and that of the crustacean-derived chitin, no significant differences were found (table 4.5). Hence, the purification procedure applied enabled to produce a bleached chitin with a degree of purity similar to the commercially available polymer

Comparing the composition of the three chitin samples obtained from larvae, pupal exuviae and adults, some differences were observed ($p < 0.05$, according to one-way ANOVA analysis with Tuckey *post-hoc* test). The unbleached chitins were very similar to each other. Only chitin from adults had a significantly lower residual content of minerals and proteins than the others, while chitin from larvae had a lower percentage of ADL. The degree of purity did not differ statistically between the three chitins, amounting to 73.4, 76.9 and 77.8% for chitin from larvae, pupal exuviae and adults, respectively (table 4.5).

More differences were found among the bleached. Mineral content was significantly different in all the three samples, with the highest percentage in chitin from larvae (1.44%) and the lowest in chitin from pupal exuviae (0.5%). Protein content significantly differed among the different chitins, as well, being the highest in chitin from pupal exuviae (2.1%) and the lowest in that from adults (1.4%). ADF and ADL percentages were lower in bleached chitin from adults than in the others. As already stated, the degree of purity of the bleached chitin was statistically similar (table 4.5).

4.3 Chitosan production

All the chitin samples produced from larvae, pupal exuviae and adults were heterogeneously deacetylated into chitosan. Chitin extracted from pupal exuviae was also homogeneously deacetylated. Eight different chitosan samples were thus obtained (figure 4.2). As expected, chitosans produced from unbleached chitins were darker than that from bleached chitins. Especially chitosan from unbleached adults maintained the black colour of the raw insects (figure 4.2 d). The lightest chitosans are those obtained by homogeneous deacetylation from both bleached and unbleached pupal exuviae (figure 4.2 c, c_1). All bleached chitosans appeared darker than the respective bleached chitins. The high temperatures used for heterogeneous deacetylation probably caused the darkening of the polymer. Optically, homogeneous chitosan from bleached pupal exuviae was as white as the commercial one derived from crustaceans (figure 4.2 e). All the other samples, especially those from adults and heterogeneous pupal exuviae, had a darker brownish colour.

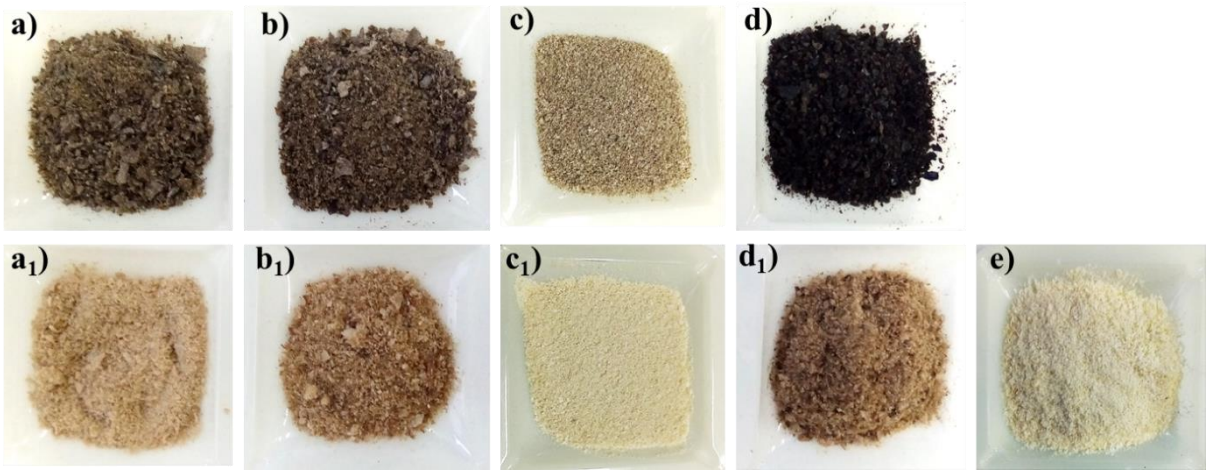


Figure 4.2 Chitosans produced by deacetylation of unbleached (a, b, c, d) and bleached (a_1, b_1, c_1, d_1) chitins from the different *H. illucens* samples: larvae (a, a_1), pupal exuviae heterogeneously deacetylated (b, b_1), pupal exuviae homogeneously deacetylated (c, c_1) and adults (d, d_1). A picture of commercial chitosan derived from shrimp shells is also showed (e).

Biomass recovery after deacetylation, as well as, the final yield related to the dry weight of the original insect sample were determined for all chitosan samples. Results are reported in table 4.6.

Table 4.6 Yields (%) related to chitin and raw insect biomass of the different chitosan samples: larvae unbleached (L. UNBL.), larvae bleached (L. BL.), pupal exuviae heterogeneous unbleached (PE. HET. UNBL), pupal exuviae heterogeneous bleached (PE. HET. BL.), pupal exuviae homogeneous unbleached (PE. HOM. UNBL.), pupal exuviae homogeneous bleached (PE. HOM. BL.), adults unbleached (ADU. UNBL.) and adults bleached (ADU. BL.). Data are expressed as mean ± standard deviation. Different letters in a row indicate significant differences in the yield among samples ($p < 0.05$) (data analyzed with one-way ANOVA and Tuckey *post-hoc* test).

	L. UNBL.	L. BL.	PE. HET. UNBL.	PE. HET. BL.	PE. HOM. UNBL.	PE. HOM. BL.	ADU. UNBL.	ADU. BL.
chitosan / chitin %	25 ± 2.5^{c}	33 ± 0.4^{b}	28 ± 4.5^{bc}	42 ± 1.5^{a}	22 ± 2.5^{c}	43 ± 3^{a}	27 ± 2^{bc}	41 ± 1^{ab}
chitosan / raw sample %	3 ± 0.4^{bc}	3 ± 0.1^{bc}	8 ± 0.8^{a}	10 ± 0.2^{a}	6 ± 0.8^{ab}	10 ± 3^{a}	2 ± 0.4^{c}	3 ± 0.3^{bc}

Significant differences were found in the biomass recovery and yields among the different chitosan samples (table 4.6). The highest biomass recovery values after deacetylation were obtained for both heterogeneous and homogeneous chitosans from bleached pupal exuviae, and for chitosan from bleached adults (42, 43 and 41%, respectively). On the contrary, the lowest values were obtained for all the unbleached chitosans, to a similar extent. For all insect samples, the bleached chitosan gave a higher percentage of biomass recovery than the respective unbleached one. On the other hand, the biomass recovery value was apparently not affected by the different types of deacetylations (table 4.6).

Comparing chitosan final yields related to the original raw insect sample, statistical differences were found as well (table 4.6). Chitosans produced from pupal exuviae, both heterogeneously and homogeneously, gave the highest yields, while larvae and adults gave the lowest yields. In contrast to what was revealed from the biomass recovery related to the weight of the deacetylated chitin, the final yield of chitosan did not appear to be affected by the bleaching treatment (table 4.6).

4.4 Chitin and chitosan characterization

All chitin and chitosan samples obtained from *H. illucens* larvae, pupal exuviae and adults were analyzed in order to characterize the polymers. Fourier-transformed infrared spectroscopy and X-ray diffractometry were performed on both, chitin and chitosan, to confirm identity of the polymers and determine their crystallinity. Further analyses were performed on the different chitosan samples in order to determine their deacetylation degree, viscosity, viscosity-average molecular weight and film forming ability.

4.4.1 Fourier-transformed infrared spectroscopy (FTIR)

FTIR was performed on all chitin and chitosan samples produced from *H. illucens*. The presence of the specific peaks identifying chitin and chitosan was investigated. The acetylation degree (DA %) of chitin was also determined.

Results of FTIR of chitin

Spectra resulting from FTIR analysis of both unbleached and bleached chitins from *H. illucens* larvae, pupal exuviae and adults are shown in figure 4.3, together with a sample of a commercial chitin derived from crustacean as reference. All the characteristic peaks were detected in all chitin samples at their specific wavelengths: 1310-1320 cm^{-1} (CN-stretching, amide III), 1550-1560 cm^{-1} (NH-bending, amide II), 1650-1655 cm^{-1} (CO-stretching, amide I), 3100-3110 cm^{-1} (NH-symmetric stretching), 3255-3270 cm^{-1} (NH-asymmetric stretching) and 3430-3450 cm^{-1} (OH-stretching). The spectra of all the chitins produced from *H. illucens* were similar to that of the commercial sample. No differences were observed in the spectra between bleached and unbleached chitins. The α-form of all chitins was assessed by observing the splitting of the amide I band into two signals at approximately 1620 and 1666 cm^{-1} (Kumirska *et al*., 2010)

DA of chitin samples, derived from the spectra, are reported in table 4.7. The DA obtained from both adult chitins was the highest. Particularly, the DA of unbleached chitin from adults (98%) was the only one significantly higher than that of the commercial sample ($X2=3.9$, $p=0.04$). The other chitin samples had a DA similar to each other (ranging from 89 to 94%) in the same range of the commercial chitin

Table 4.7 Average acetylation degree (DA), determined by FTIR, of chitin samples extracted from *H. illucens* and a commercial chitin derived from shrimp shells. Different letters indicate statistical differences in the DA among chitin samples ($p < 0.05$) (data analyzed with Chi-Square test with Yates' correction).

CHITIN SAMPLE	DA (%)
Larvae unbleached	92^{ab}
Larvae bleached	94^{ab}
Pupal exuviae unbleached	91^{ab}
Pupal exuviae bleached	89^{b}
Adults unbleached	98^{a}
Adults bleached	96^{ab}
Commercial	91^{b}

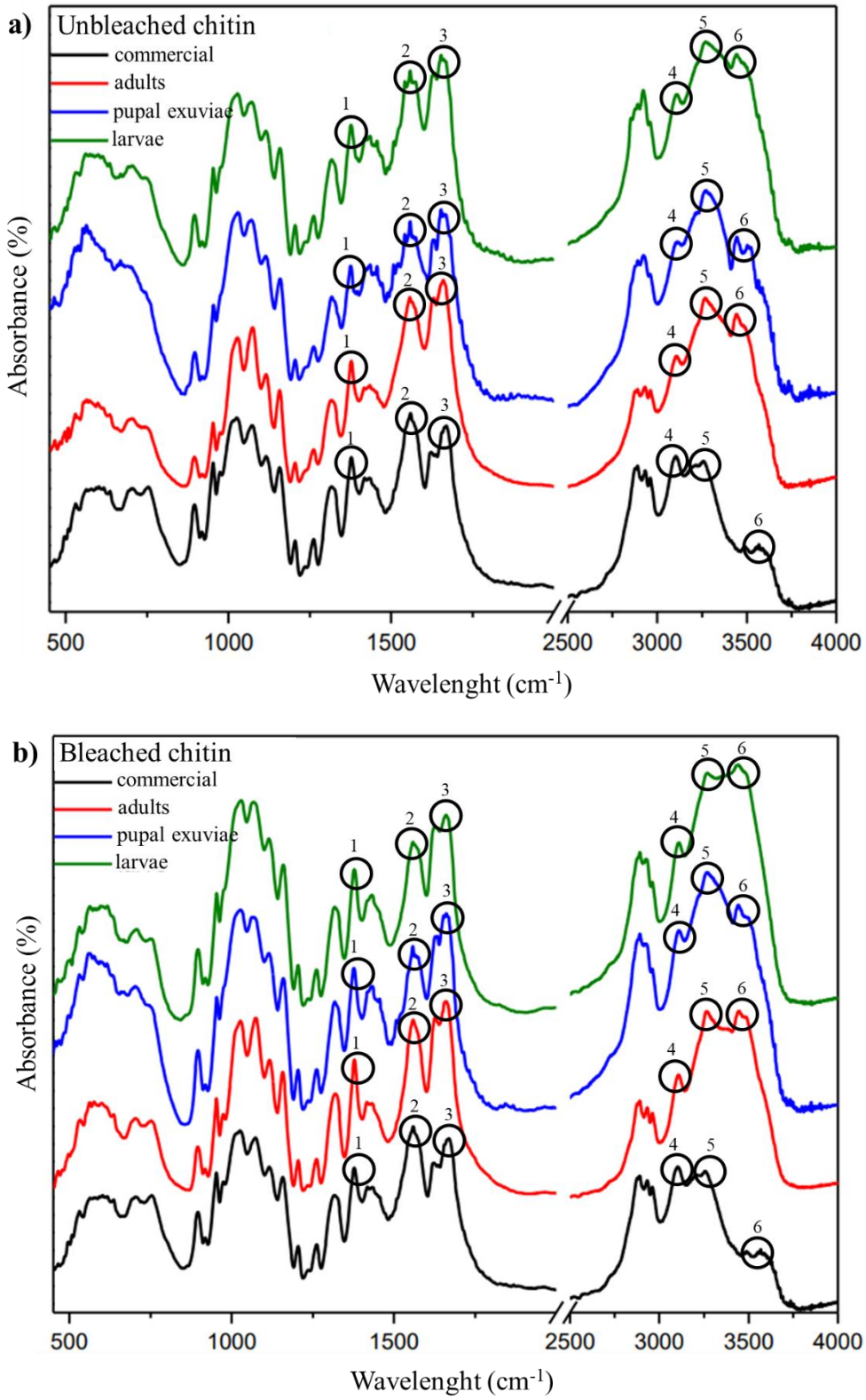


Figure 4.3. Spectra resulting from FTIR analysis of both unbleached (a) and bleached (b) chitin samples extracted from *H. illucens* larvae, pupal exuviae and adults, and commercial chitin derived from crustacean. The black circles indicate the characteristic peaks of the polymer: CN-stretching amide III (1), NH-bending amide II (2), CO-stretching amide I (3), NH-asymmetric stretching (4), NH-asymmetric stretching (5) and OH-stretching (6).

Results of FTIR of chitosan

Spectra resulting from FTIR analysis of both unbleached and bleached chitosan from *H. illucens* larvae, pupal exuviae and adults are reported in figure 4.4 and figure 4.5, together with a sample of a commercial chitosan derived from crab shells as reference. As reported for chitin, the characteristic peaks of the polymer were detected at their specific wavelengths. In the spectrum of unbleached chitosan from larvae extraneous unidentified signals were detected (figure 4.4 b). No differences were observed between spectra of unbleached chitosans and those of the bleached samples. Observing the spectra, homogeneous chitosan from pupal exuviae (figure 4.4 d and figure 4.5 d), whether bleached or not, appeared less deacetylated than the respective heterogeneous sample (figure 4.4 c and figure 4.5 c).

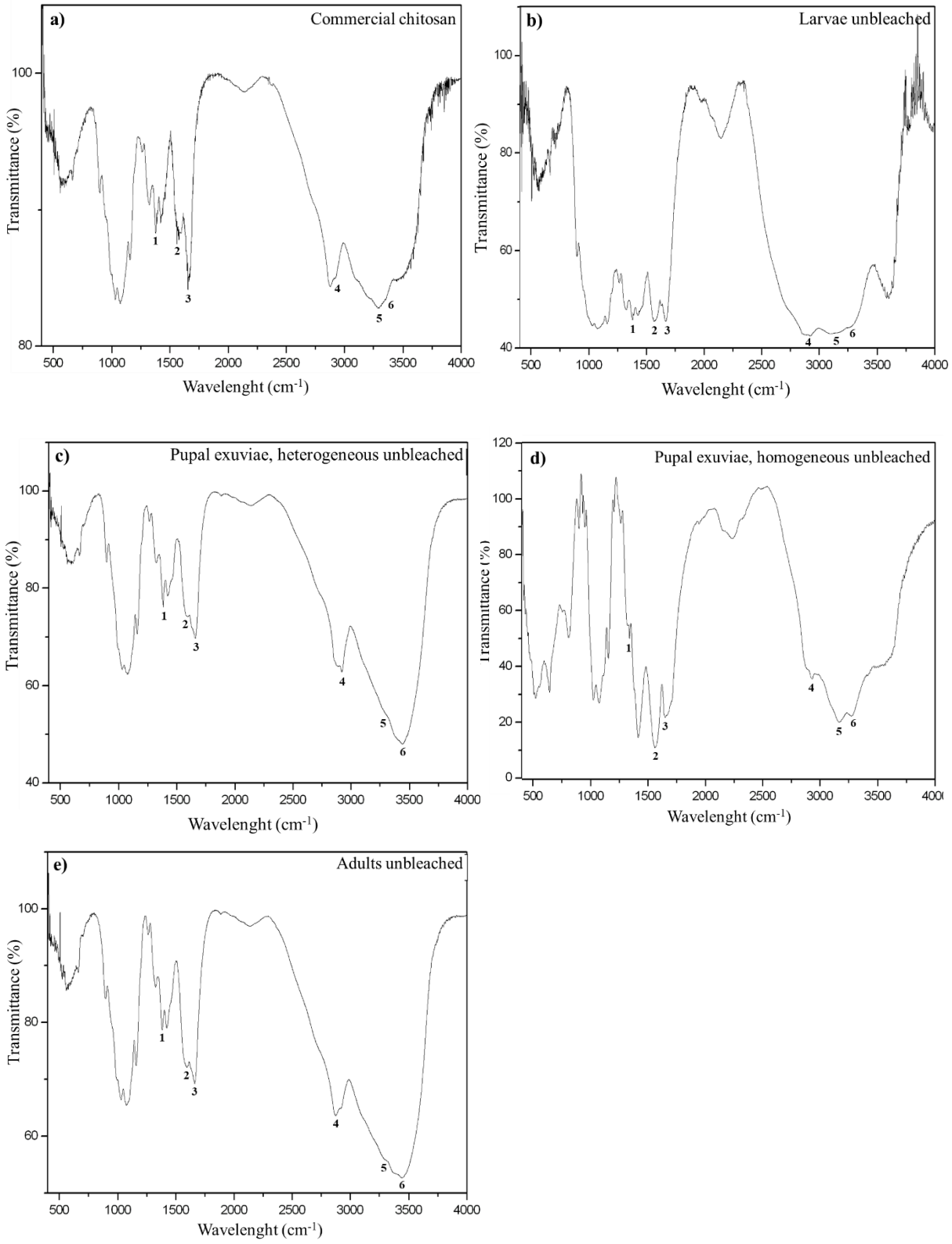


Figure 4.4 Spectra resulting from FTIR analysis of unbleached chitosan samples produced from *H. illucens* larvae (b), pupal exuviae (heterogeneous (c) and homogeneous (d)) and adults (e), and commercial chitosan (a) derived from crustacean. Numbers from 1 to 6 in each spectrum indicate the characteristic peaks of the polymer: CN-stretching amide III (1), NH-bending amide II (2), CO-stretching amide I (3), NH-asymmetric stretching (4), NH-asymmetric stretching (5) and OH-stretching (6).

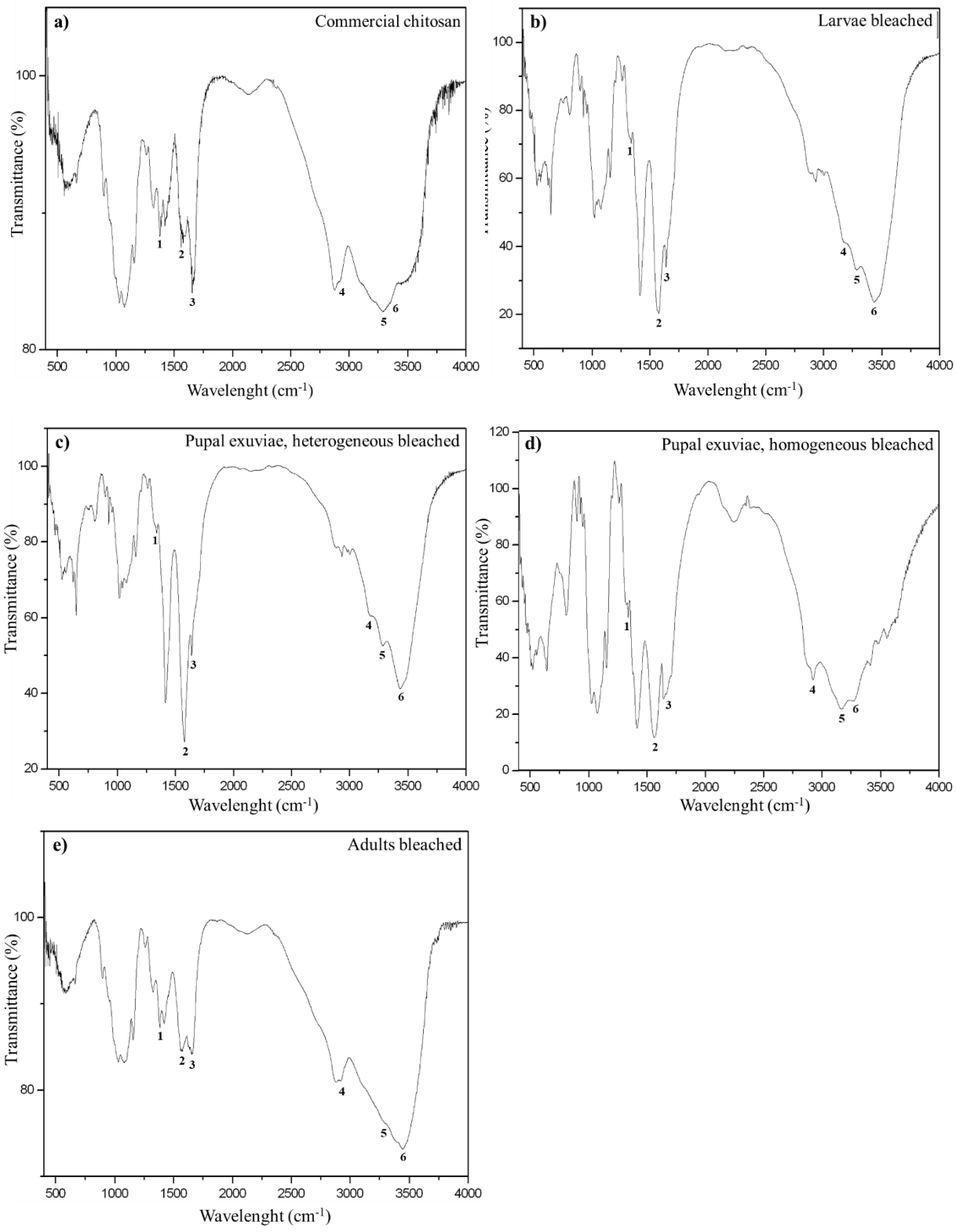


Figure 4.5 Spectra resulting from FTIR analysis of bleached chitosan samples produced from *H. illucens* larvae (b), pupal exuviae (heterogeneous (c) and homogeneous (d)) and adults (e), and commercial chitosan (a) derived from crustacean. Numbers from 1 to 6 in each spectrum indicate the characteristic peaks of the polymer: CN-stretching amide III (1), NH-bending amide II (2), CO-stretching amide I (3), NH-asymmetric stretching (4), NH-asymmetric stretching (5) and OH-stretching (6).

4.4.2 X-ray diffractometry (XRD)

XRD was performed on all chitin and chitosan samples produced from *H. illucens*. The crystallinity index (CrI %) and crystallite size of both chitin and chitosan were determined. The presence of the characteristic peaks identifying chitin and chitosan was also assess again, as well as the form of the chitin (i.e., α, β or ɣ).

Results of XRD of chitin

Spectra resulting from XRD of both unbleached and bleached chitin samples are reported in figure 4.6. Similarly to the commercial sample derived from crustaceans, all chitins extracted from larvae, pupal exuviae and adults, had the significant peaks at 9-13° and the three/four minor peaks in the range 19-26°, confirming the identity of the chitin polymer in the α form (Zhang *et al*., 2011; Liu *et al*., 2012; Waśko *et al*., 2016; Soon *et al*., 2018). No significant differences were found in the spectra between unbleached and bleached chitin.

The determination of the CrI revealed significant differences among the different samples (table 4.8). Only chitin from adults (both unbleached and bleached) were statistically as crystalline as the commercial chitin. Chitins from larvae had the second highest CrI (90% the unbleached, 84% the bleached), significantly lower than that of the commercial sample (X=4.34, p=0.037), but not different from those of chitin from adults. Both chitins from pupal exuviae were the less crystalline of all (67% the unbleached, 62% the bleached) (X=31.17, p<0.0001) (table 4.8). Generally, all the unbleached chitins had a slightly higher CrI than the respective bleached samples, although not statistically different. Crystallite size was similar among all chitins, including commercial (table 4.8).

Table 4.8 Crystallinity index (CrI) and crystallite size (nm) resulted from XRD analysis of chitin samples extracted from *H. illucens* and a commercial chitin derived from crustaceans. Different letters indicate statistical differences in the CrI among chitin samples (p < 0.05) (data analyzed with Chi-Square test with Yates' correction).

CHITIN SAMPLE	CrI (%)	Crystallite size (nm)
Larvae unbleached	90[b]	4
Larvae bleached	84[b]	5
Pupal exuviae unbleached	67[c]	4
Pupal exuviae bleached	62[c]	5
Adults unbleached	96[ab]	5
Adults bleached	93[ab]	6
Commercial	98[a]	6

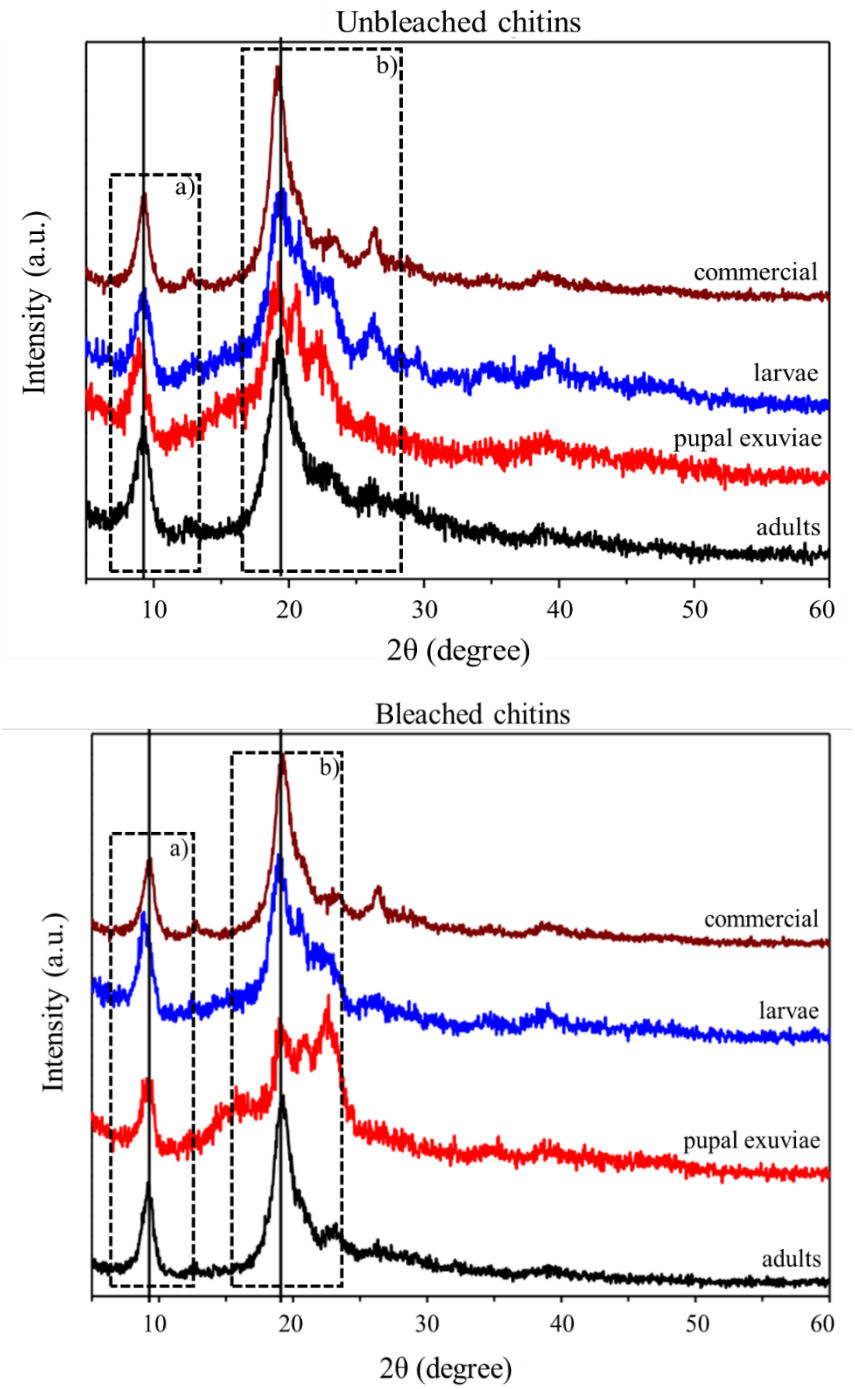


Figure 4.6 Spectra resulting from XRD analysis of unbleached (upper graph) and bleached (lower graph) chitin samples extracted from *H. illucens* and a commercial chitin derived from crustaceans. Dashed boxes highlight regions with significant peaks (a) and minor peaks (b) characteristic of α-chitin.

Results of XRD of chitosan

XRD spectra of chitosan samples showed the significant peaks at around 10 and 20°, confirming the identity of the polymer (figure 4.7, 4.8, 4.9). For a few samples, it was not possible to obtain an optimal spectrum. Bleached chitosan from larvae gave a spectrum with signals overlapping those of the polymer. The chitosan peaks at 10 and 20° are nevertheless present (figure 4.9).

Chitosan signals could not be detected in the spectra of the two samples derived from pupal exuviae by homogeneous deacetylation (figure 4.10) due to the overlapping of crystalline sodium acetate signals.

Results of the CrI determination of chitosan samples are reported in table 4.9. Although the CrI were all statistically similar, little differences occurred among the samples. Both chitosans from larvae were the samples with the lowest CrI, but still similar to all the others, including the commercial one. As for chitin, there is a tendency for bleached samples to be more crystalline than unbleached chitosans, but still no statistically different. It was no possible to determine the CrI of homogeneous chitosans from pupal exuviae, because of the failure of the signals to be detected in the spectrum.

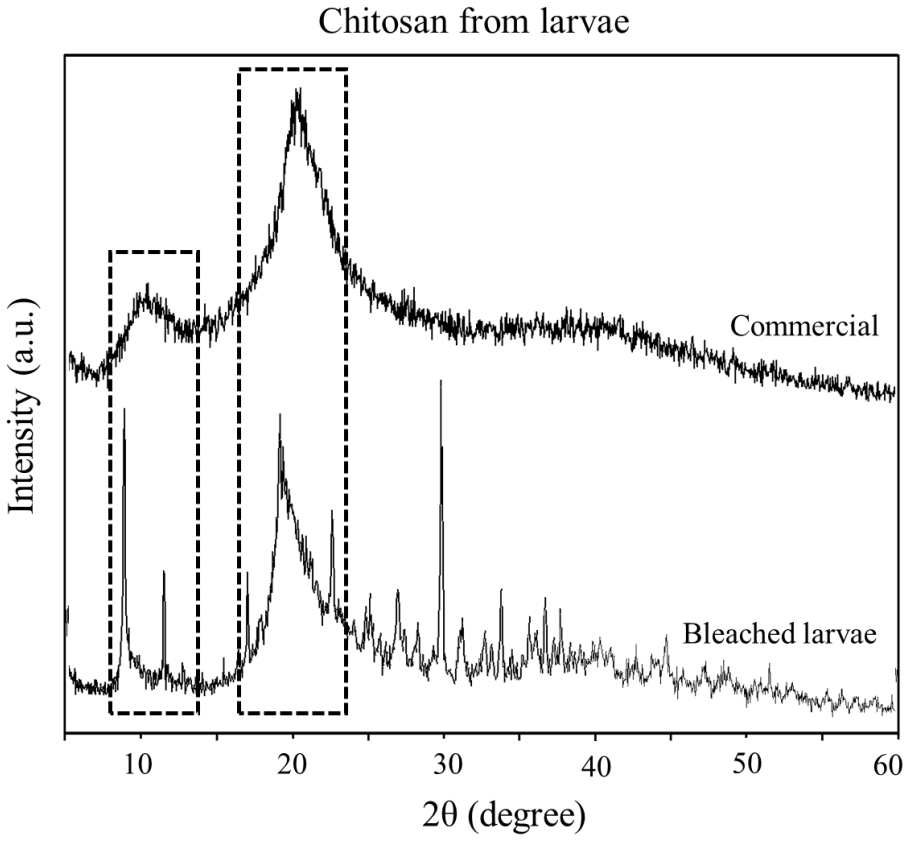


Figure 4.7 Spectra resulting from XRD analysis of chitosan samples produced from *H. illucens* bleached larvae, and a commercial chitosan derived from crustaceans. Dashed boxes highlight regions with characteristic peaks.

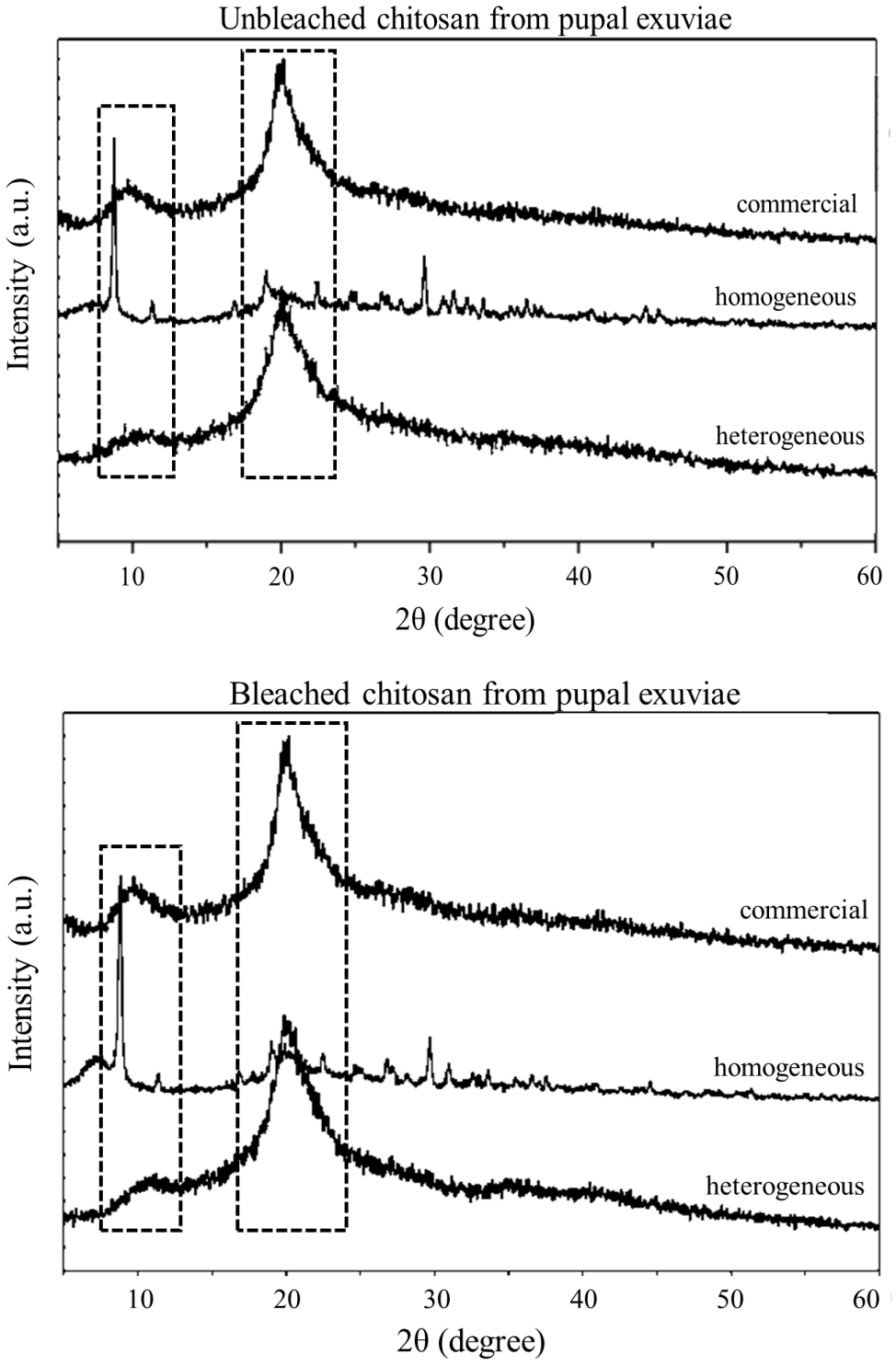


Figure 4.8 Spectra resulting from XRD analysis of unbleached (upper graph) and bleached (lower graph) chitosan samples produced from *H. illucens* pupal exuviae, both heterogeneous and homogeneous, and a commercial chitosan derived from crustaceans. Dashed boxes highlight regions with characteristic peaks.

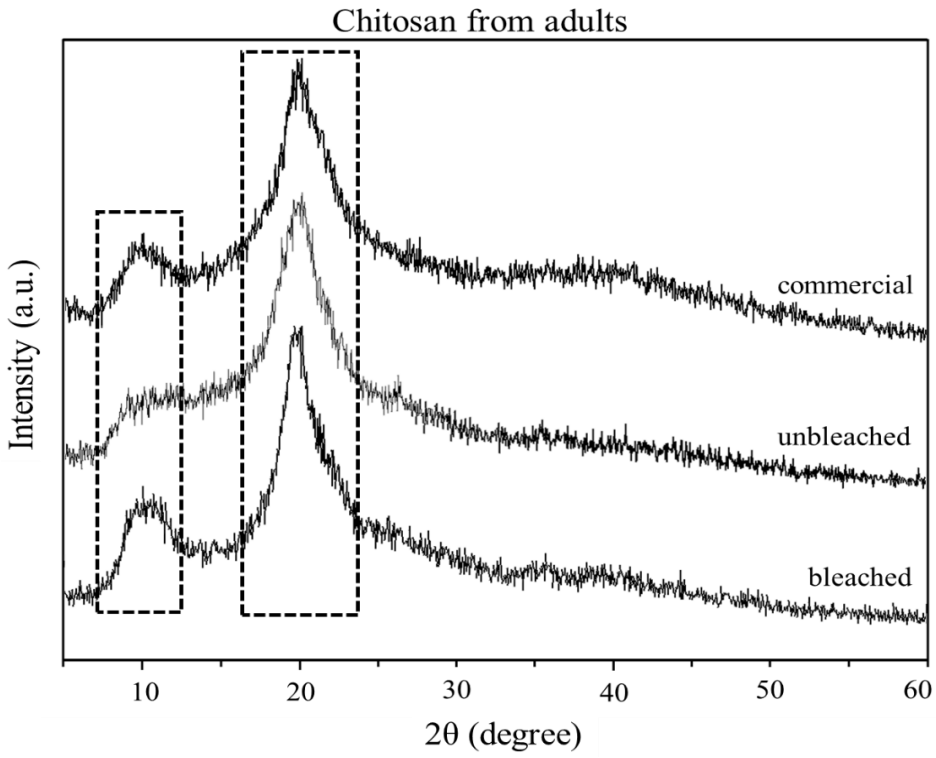


Figure 4.9 Spectra resulting from XRD analysis of chitosan samples produced from *H. illucens* adults, both unbleached and bleached, and a commercial chitosan derived from crustaceans. Dashed boxes highlight regions with characteristic peaks.

Crystallite size was similar among all chitosans, including the commercial one, and generally slightly lower than that of chitin (3-4 vs 4-6 nm) (table 4.9).

Table 4.9 Crystallinity index (CrI) and crystallite size (nm-D_{100}) resulted from XRD analysis of chitosan samples extracted from *H. illucens* and a commercial chitosan derived from shrimp shells. No statistical differences were found in the CrI among samples ($p < 0.05$), according to Chi-Square test with Yates' correction.

CHITOSAN SAMPLE	CrI (%)	Crystallite size (nm)
Larvae unbleached	74[a]	3
Larvae bleached	77[a]	3
Pupal exuviae heterogeneous unbleached	78[a]	4
Pupal exuviae heterogeneous bleached	80[a]	3
Pupal exuviae homogeneous unbleached	-	-
Pupal exuviae homogeneous bleached	-	-
Adults unbleached	79[a]	3
Adults bleached	86[a]	3
Commercial	79[a]	4

4.4.3 Potentiometric titration of chitosan

Results of the deacetylation degree (DD) determination of all chitosan samples can be seen in table 4.10. All samples had a DD much higher than 50%, the value considered as the threshold for discriminating between chitin and chitosan. In most of the samples, the DD was higher than 90%, similarly to the commercial chitosan. Only DD of unbleached chitosans from pupal exuviae, both heterogeneous and homogeneous, were significantly lower than all the others.

To confirm the correct execution of the method used for the determination of DD, a commercial chitosan (Sigma-Aldrich) with a known DD (90%), stated by the manufacturer, was used as reference. The measured DD (92%) was not statistically different from the declared one.

Table 4.10 Deacetylation degree (DD%) of chitosan samples produced from *H. illucens* and commercial chitosan derived from shrimp shells determined by potentiometric titration. Data are expressed as mean ± standard deviation. Different letters indicate significant differences in the DD among chitosan samples ($p < 0.05$) (data analyzed with one-way ANOVA and Tuckey post-hoc test).

CHITOSAN SAMPLE	DD%
Larvae unbleached	91 ± 0.3^{a}
Larvae bleached	92 ± 0.7^{a}
Pupal exuviae heterogeneous unbleached	83 ± 3.1^{b}
Pupal exuviae heterogeneous bleached	90 ± 4.2^{a}
Pupal exuviae homogeneous unbleached	62 ± 2.8^{c}
Pupal exuviae homogeneous bleached	90 ± 1.4^{a}
Adults unbleached	91 ± 0.7^{a}
Adults bleached	93 ± 1.4^{a}
Commercial	92 ± 0.7^{a}

4.4.4 Chtitosan viscometry

For all chitosan samples obtained from *H. illucens*, intrinsic viscosity (η) was determined. The viscosity-average molecular weight (M_v) for each chitosan was then derived from the η value. Results of both measurements are reported in table 4.11.

The most viscous chitosan was the commercial one (7.5 dl/g) derived from shrimp shells. All the chitosans produced from *H. illucens* had a significantly lower η, ranging from 1.2 to 2 dl/g for most samples. The lowest η values were determined for chitosan from both bleached larvae and bleached adults (0.6 and 0.9 dl/g, respectively). Generally, chitosans from unbleached chitin had a significantly higher η than the ones from bleached chitin, except for heterogeneous chitosans from pupal exuviae for which the opposite occurredWithin chitosans from pupal exuviae, the heterogeneous chitosan had higher η than the homogeneous one in the bleached

samples. The opposite result was observed for the unbleached chitosans (table 4.11). From these results it appeared that the bleaching treatment had an influence on the η of the final chitosan. In contrast, the different starting insect biomass seemed not to affect it.

Table 4.11 Intrinsic viscosity (η) and viscosity-average molecular weight (M_v) of chitosan samples obtained from *H. illucens* larvae, pupal exuviae and adults, and the commercial one derived from crustaceans. Data are expressed as mean ± standard deviation. Different letters in a column indicate significant differences in the η or in M_v among the chitosan samples ($p < 0.05$) (data analyzed with *Mann-Whitney* U test).

CHITOSAN SAMPLE	η (dl/g)	M_v (kDa)
Larvae unbleached	2.0 ± 0.1^{b}	92 ± 0.1^{b}
Larvae bleached	0.6 ± 0.01^{g}	21 ± 1.4^{h}
Pupal exuviae heterogeneous unbleached	1.2 ± 0.2^{e}	55 ± 1.7^{e}
Pupal exuviae heterogeneous bleached	1.7 ± 0.02^{c}	35 ± 4.3^{g}
Pupal exuviae homogeneous unbleached	2.0 ± 0.1^{b}	84 ± 0.2^{c}
Pupal exuviae homogeneous bleached	1.2 ± 0.1^{e}	46 ± 2^{f}
Adults unbleached	1.5 ± 0.1^{d}	62 ± 0.1^{d}
Adults bleached	0.9 ± 0.03^{f}	36 ± 0.5^{g}
Commercial	7.5 ± 0.04^{a}	376 ± 3.3^{a}

The trend in the results of the M_v determination was similar to that observed for η (table 4.11). All chitosans produced from *H. illucens* had a significantly lower M_v (ranging from 21 to 92 kDa) than the commercial chitosan from crab shells (376 kDa). Bleached chitosan from larvae was again the sample with the lowest value (21 kDa), while the unbleached chitosan from larvae had the highest M_v after the commercial sample (92 kDa). The M_v of chitosans from unbleached chitin was always significantly higher than that of the respective bleached samples. Within chitosans from pupal exuviae, the homogeneous sample always had a significantly higher M_v than the heterogeneous one (table 4.11). As observed for η, an effect of the bleaching treatment on the M_v of the final chitosan may occurred. Furthermore, from the results of chitosans from pupal exuviae, an effect of the type of deacetylation could be also supposed.

4.4.5 Chitosan film formation ability

Film forming ability was assessed for all the chitosan samples obtained from *H. illucens*. Figure 4.10 provides the photographic documentation. Film formation was observed in all the examined chitosans. Each film was uniform in surface and thickness, with no holes or damaged areas. The films were strong enough to be removed from the Petri dishes and handled without breaking. No appreciable elasticity was manually detected, but some resistance to tearing could

be determined. Films obtained from homogeneous deacetylation of pupal exuviae chitin (figure 4.10 c, c_1) were the most fragile and difficult to remove from the plates without damaging them. Optically, film of chitosan from adults (figure 4.10 d, d_1) were the most different from all other samples, including commercial ones. The unbleached chitosan film kept its dark brown colour. The bleached one was much lighter, but was still the most pigmented of all the other bleached chitosan films. The most transparent films were those obtained from homogeneous chitosan from pupal exuviae (figure 4.10 c, c_1). They appeared as transparent as the commercial films. Only a slight difference was found between the unbleached and the bleached homogeneous films (slightly more brownish the unbleached). Both the unbleached chitosan films of larvae (figure 4.10 a) and heterogeneous pupal exuviae (figure 4.10 b) were slightly brownish, more pigmented than commercial and homogeneous films, but still rather transparent. The respective bleached chitosan films (figure 4.10 a_1, b_1) are clearer than the unbleached ones, but still less transparent than the homogeneous and commercial films.

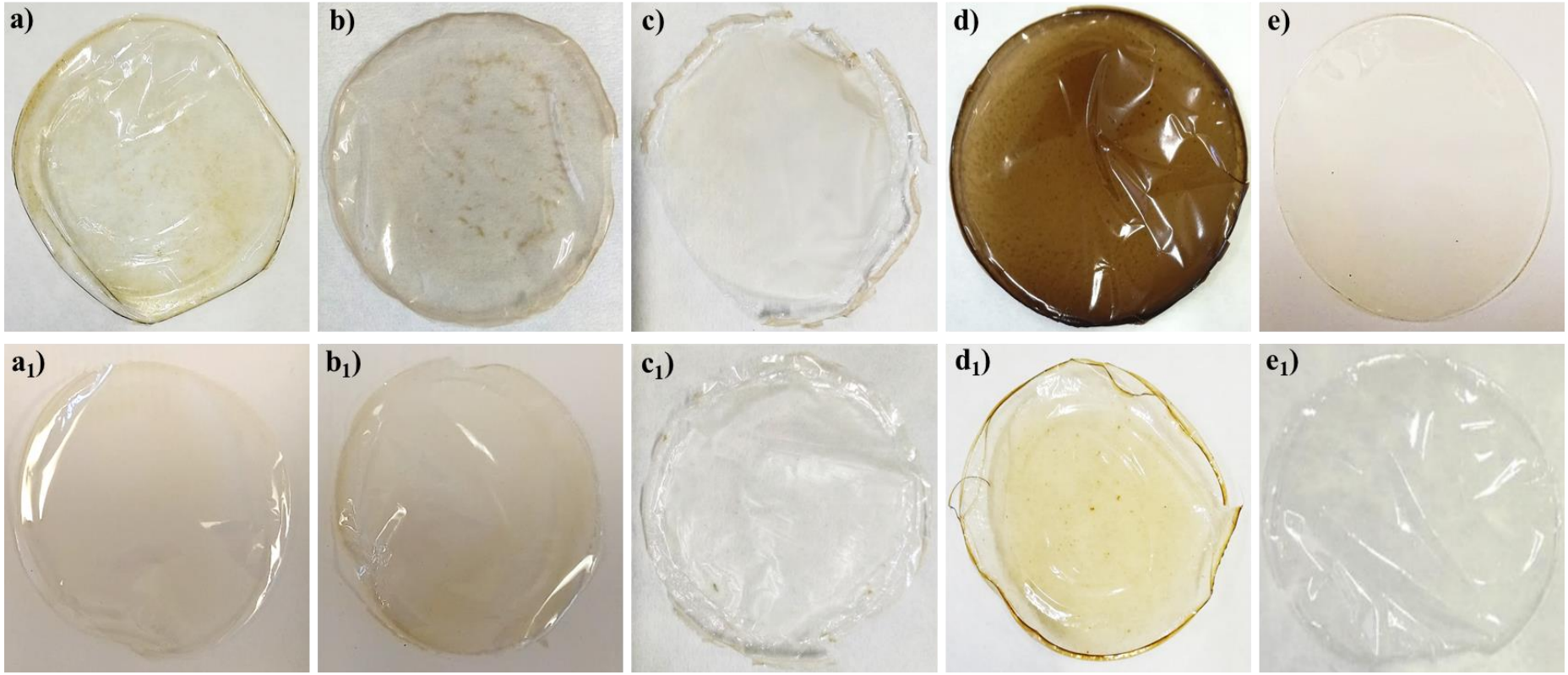


Figure 4.10 Films obtained from the different chitosan samples produced from *H. illucens*: bleached larvae (a), unbleached larvae (a_1), heterogeneous bleached pupal exuviae (b), heterogeneous unbleached pupal exuviae (b_1), homogeneous bleached pupal exuviae (c), homogeneous unbleached pupal exuviae (c_1), unbleached adults (d), bleached adults (d_1). Pictures of films obtained from commercial chitosan are also reported: chitosan from crab shells (e) and chitosan from shrimp shells (e_1) (Sigma-Aldrich).

4.5 Effects of chitosan-based coatings on preservation of fresh fruits

All chitosan samples obtained from *H. illucens* were used for the formulation of coating solutions to be applied to different fresh fruits, in order to evaluate the preservative effect of chitosan. The products selected for the storage experiments were tomatoes, one of the most widely consumed and marketed vegetables, and three different seasonal fruits available on the market (strawberries, apricots and nectarines). Effects of chitosan coatings were evaluated at the end of the storage period determining fruits weight loss, changes in total soluble solids (TSS) content, pH and titratable acidity (TA), and occurrence of fungal decay. Results obtained for each fruit are described in the following sections.

4.5.1 Tomatoes

Cherry tomatoes were coated by dipping or spraying with heterogeneous and homogeneous chitosans produced from both bleached and unbleached chitin from pupal exuviae, and stored at room or cold temperature for 30 days. Results of the determination of weight loss, TSS content, pH and TA are provided in the following sections.

4.5.1.1 *Weight loss*

Weight of tomatoes belonging to all treatments (negative control, only solvent, chitosan coatings) decreased significantly during storage, both at room and cold temperatures. Statistical differences found in the weight loss of tomatoes among treatments are described below.

Room temperature storage

- Dipping-coated fruits

  The highest weight losses occurred in treatments with 1% homogeneous bleached chitosan and solvent only, while 1% heterogeneous bleached chitosan gave the lowest loss (figure 4.11 a). Only tomatoes treated with 1% homogeneous chitosan, both unbleached and bleached, differed significantly from the negative control, having a greater weight loss. The 1% homogeneous chitosans led to a significantly higher weight loss than all heterogeneous chitosans, as well. All heterogeneous chitosan did not differ from the negative control, but gave a significantly lower weight loss than the solvent only treatment. Tomatoes treated with commercial chitosan were not different from the negative control, nor from those coated with solvent alone (figure 4.11 a).

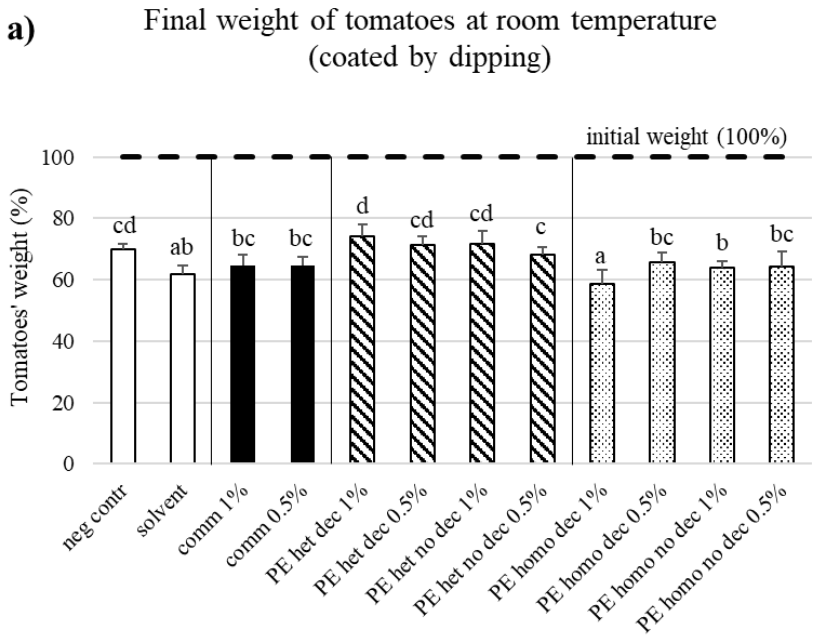
  Significant differences between chitosan concentrations were found only with the homogeneous bleached sample, for which the 1% concentration gave a greater weight

loss than the 0.5%. The 1% homogeneous chitosan was also the only treatment in which a significant difference between the unbleached and the bleached samples was detected, the latter leading to a higher weight loss.

- Spraying-coated fruits

  The highest weight losses, significantly grater than the negative control, were caused by treatment with solvent only and 1% homogeneous bleached chitosan, to an equal extent (figure 4.11 b). All other treatments with homogeneous chitosan, as well as with the heterogeneous and the commercial samples, did not differ from the negative control but led to significantly lower weight loss than the solvent alone. All homogenous chitosans gave significanlty higher weight loss than the heterogeneous ones.

  As occurred for the dipped tomatoes, a significant difference between chitosan concentrations was found only with the homogeneous bleached sample, for which the 1% concentration gave a greater weight loss than the 0.5%. A significant difference was also detected between the 1% homogeneous bleached chitosan and the respective unbleached sample, the latter leading to a lower weight loss (figure 4.11 b).



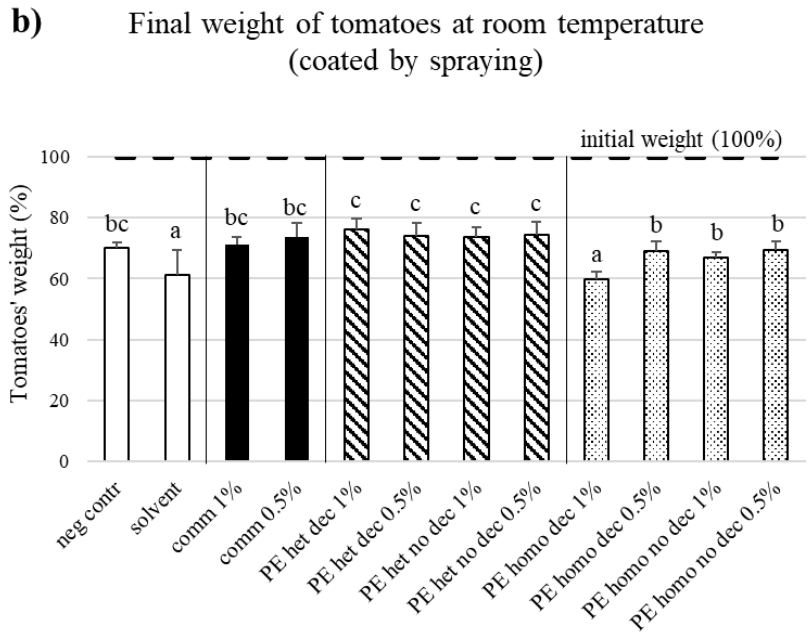


Figure 4.11 Histograms reporting the initial weight (100%) and the decreased weight (%) at the end of the storage period (30 days) of tomatoes coated by dipping (a) or spraying (b), stored at room temperature. The coating solutions prepared with commercial chitosan and chitosan (heterogeneous and homogeneous, decolorized (dec) and not decolorized (no dec)) produced from *H. illucens* pupal exuviae (PE) are provided on the x-axis. Different letters in a graph indicate significant differences ($p<0.05$) in the weight loss of tomatoes among the different treatments, according to *Mann-Whitney* U test.

Cold temperature storage

- Dipping-coated fruits

  No treatment resulted in a smaller weight loss than the negative control (figure 4.12 a). The samples that gave the best results were the 0.5% heterogeneous bleached and the 1% heterogeneous unbleached which led to a significantly lower weight loss than the solvent alone. The worst treatments were the 1% bleached chitosan samples, both heterogeneous and homogeneous, which induced a significantly greater weight loss than both the negative control and the solvent alone.

  The low chitosan concentration (0.5%) gave significant better results than the high concentration (1%) for the heterogeneous bleached samples, while the opposite occurred for the unbleached ones. The unbleached chitosan gave better results than the bleached one for treatments with 1% chitosan, both heterogeneous and homogeneous, while the opposite was true for the 0.5% heterogeneous chitosan (figure 4.12 a).

- Spraying-coated fruits

  As happened for dipping-coated tomatoes, no treatment gave a lower weight loss than the negative control (figure 4.12 b). 0.5% commercial chitosan and the 1% heterogeneous bleached gave the best results with a weight loss equal to that of the

negative control. The treatments that gave the greatest weight loss were the solvent alone and the 1% commercial chitosan.

Differences between chitosan concentrations were found only for the commercial sample, for which the higher concentration performed significantly worse. No differences were detected between bleached and unbleached chitosans (figure 4.12 b).

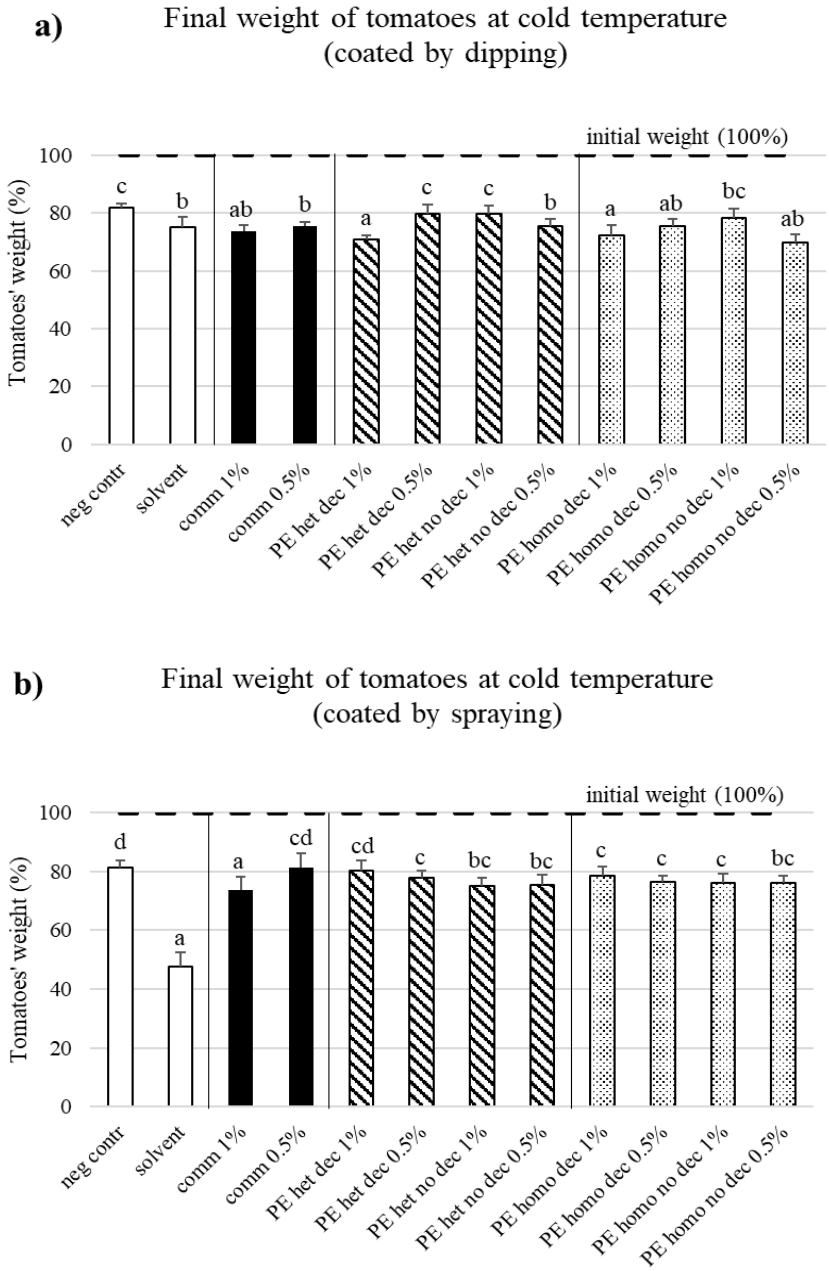


Figure 4.12 Histograms reporting the initial weight (100%) and the decreased weight (%) at the end of the storage period (30 days) of tomatoes coated by dipping (a) or spraying (b), stored at cold temperature. The coating solutions prepared with commercial chitosan and chitosan (heterogeneous and homogeneous, decolorized (dec) and not decolorized (no dec)) produced from *H. illucens* pupal exuviae (PE) are shown on the x-axis. Different letters in a graph indicate significant differences (p<0.05) in the weight loss of tomatoes among the different treatments, according to *Mann-Whitney* U test.

Comparison between storage temperatures (table 8.1, supplementary materials)

Weight loss of tomatoes belonging to the negative control was significantly greater at room temperature storage than at cold temperature. Differences were found among the other treatments, mostly in case of coating by dipping.

- Dipping-coated fruits

  All treatments, except those with the heterogeneous bleached chitosan, gave a significanlty higher weight loss at room temperature than at cold storage.

- Spraying-coated fruits

  All the homogenous chitosan and the heterogeneous bleached samples led again to a greater weight loss at room temprature than at cold one. All the other treatments were not affected by temperature.

Comparison between coating application methods (table 8.2, supplementary materials)

- Room temperature storage

  A significantly higher weight loss occurred in tomatoes coated by dipping than in the sprayed ones, for treatments with 0.5% heterogeneous unbleached chitosan and with 0.5% homogeneous samples both bleached and unbleached. No differences were observed for the other treatments.

- Cold temperature storage

  Tomatoes trated with solvent alone and 1% heterogeneous unbleached chitosan had a higher weiht loss when coated by spraying than by dipping. The opposite was observed in tomatoes treated with both heterogeneous and homogeneous 1% bleached chitosan, and with the 0.5% homogeneous unbleached one. No differences were observed for the other treatments.

4.5.1.2 *TSS content*

TSS content of tomato fruits was determined by refractometry prior to treatments and at the end of the storage period. The percentage variation in the TSS content from the beginning to the end of the experiment was then assessed. The TSS of all tomatoes increased significantly during storage, but to a different extent depending on the treatment, temperature and method of application.

Room temperature storage

- Dipping-coated fruits

  No treatment gave a lower increase in TSS than the negative control. However, all heterogeneous chitosan samples, except the 0.5% bleached one, led to a significantly

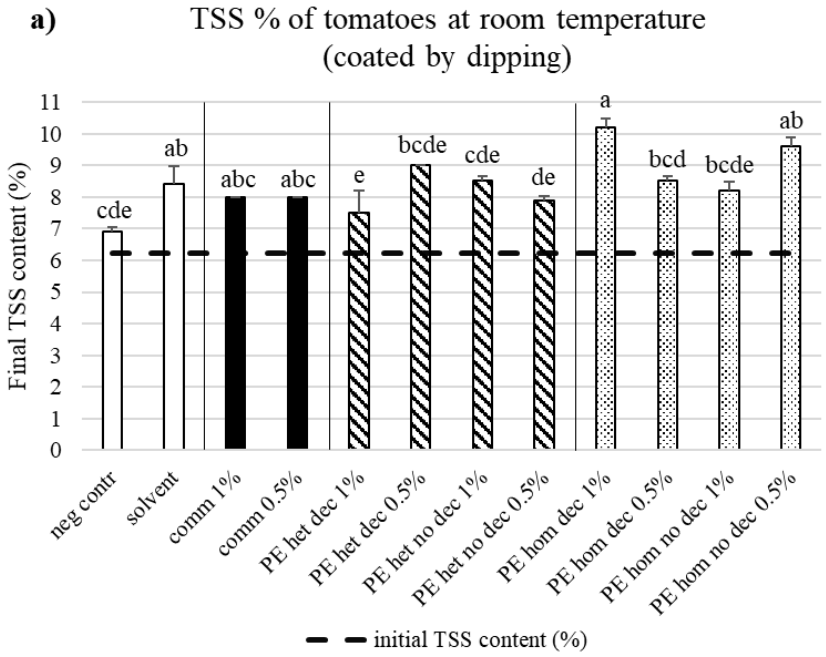
smaller increase of TSS compared to both the solvent alone anche the commercial chitosan (figure 4.13 a). Two samples of homogeneous chitosan (the 1% bleached and the 0.5% unbleached) gave a greater increase in TSS than the negative control.

Differences between chitosan concentrations and bleaching status were both found only with the homogeneous chitosan, for which the bleached sample at high concentration (1%) gave a greater TSS increased than both the bleached sample at low concentration (0.5%) and the 1% unbleached one (figure 4.13 a).

- Spraying-coated fruits

  All treatments gave a greater TSS increase than the negative control (figure 4.13 b). All homogeneous chitosan led to a significantly higher TSS increase than the solvent alone treatement, while the opposite occurred with the heterogeneous chitosan samples. Commercial chitosan had an effect similar to the heterogeneous one.

  Differences between chitosan concentrations were found only for the heterogeneous unbleached chitosan (greater TSS increase with 1% than 0.5%). Within the same sample, at 1% chitosan concentration, the unbleached samples gave a greater TSS increase than the bleached one (figure 4.13 b).



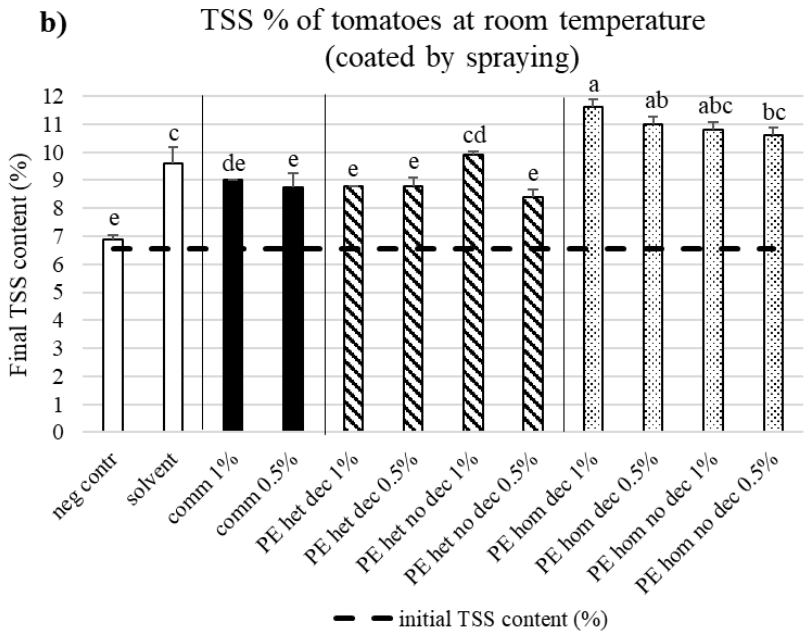


Figure 4.13 Histograms reporting the initial TSS content (%) and the increased TSS (%) at the end of the storage period (30 days) of tomatoes coated by dipping (a) or spraying (b) and stored at room temperature. The coating solutions prepared with commercial chitosan and chitosan (heterogeneous and homogeneous, decolorized (dec) and not decolorized (no dec)) produced from *H. illucens* pupal exuviae (PE) are shown on the x-axis. Different letters in a graph indicate significant differences (p<0.05) in the TSS variation of tomatoes among the different treatments, according to one-way ANOVA with Tukey's post hoc test.

Cold temperature storage

- Dipping-coated fruits

  None of the chitosan treatments differed significantly from the negative control and solvent-only treatment (figure 4.14 a). The greatest increase in TSS occurred with the 1% homogeneous bleached chitosan, and the least with the 1% heterogeneous unbleached one. The difference between these two treatments was the only significant one detected. Generally, the homogeneous chitosans, as well as, the commercial one, gave a greater TSS increase than all the heterogeneous samples.

  No differences were found between the two chitosan concentrations, nor between the bleaching conditions (figure 4.14 a).

- Spraying-coated fruits

  No treatment gave a lower increase in TSS than the negative control (figure 4.14 b). The significant highest TSS increase was observed in tomatoes coated with the solvent alone. All chitosan treatment had a similar effect to each other, giving an increase in TSS greater than or equal to the negative control, but significantly lower than the solvent alone.

  No significant differences were found due to the chitosan concentration or bleaching

condition, although all treatments with 0.5% chitosan gave a greater increase in TSS than those at 1% (figure 4.14 b).

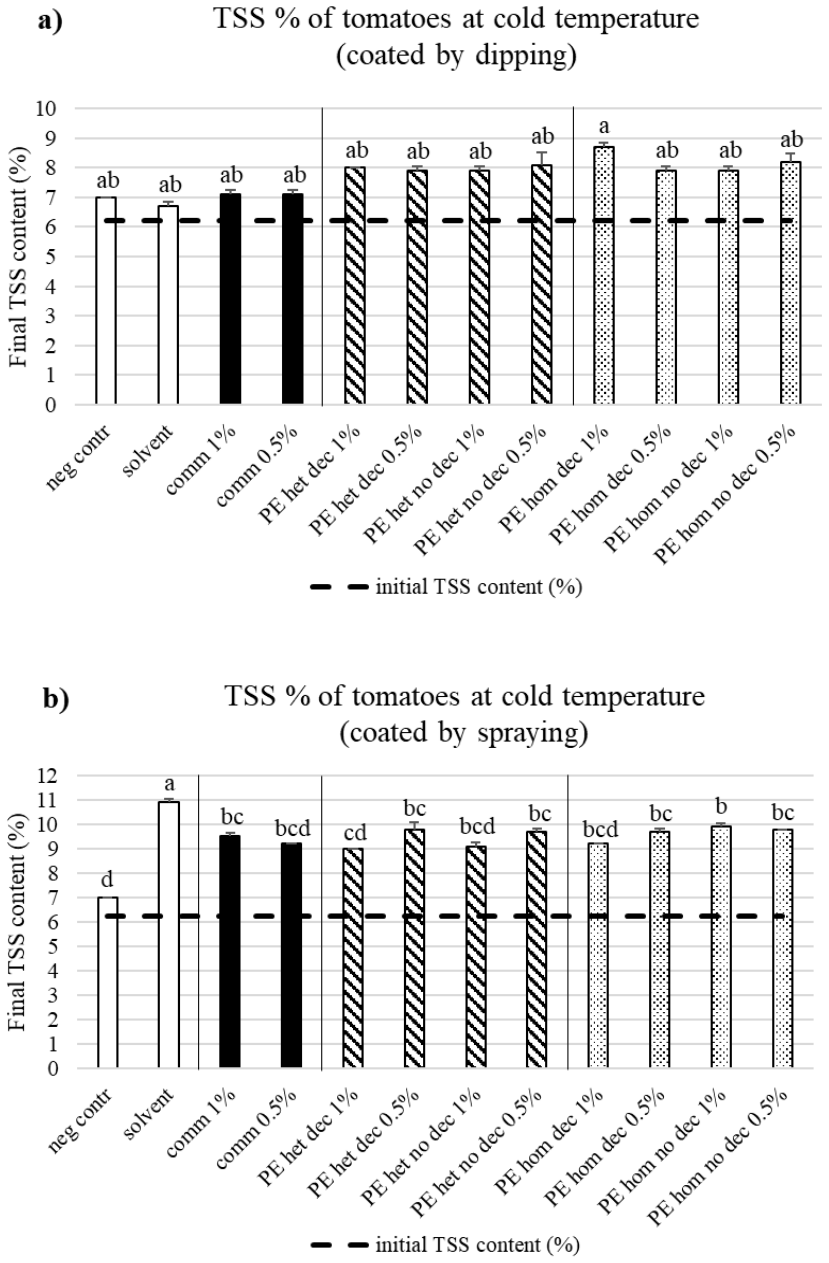


Figure 4.14 Histograms reporting the initial TSS content (%) and the increased TSS (%) at the end of the storage period (30 days) of tomatoes coated by dipping (a) or spraying (b) and stored at cold temperature. The coating solutions prepared with commercial chitosan and chitosan (heterogeneous and homogeneous, decolorized (dec) and not decolorized (no dec)) produced from *H. illucens* pupal exuviae (PE) are shown on the x-axis. Different letters in a graph indicate significant differences (p<0.05) in the TSS variation of tomatoes among the different treatments, according to one-way ANOVA with Tukey's post hoc test.

Comparison between storage temperatures (table 8.3, supplementary materials)

Increase of TSS content of tomatoes belonging to the negative control did not differ between the two storage temperatures. Differences were found among the other treatments.

- Dipping-coated fruits

  When a significant difference was detected, the TSS increase was always greater in tomatoes stored at room temperature than in those at cold temperature. Only with treatments with commercial chitosan, the 1% bleached and 0.5% unbleached heterogeneous samples, and the 1% homogeneous unbleached one the TSS variation did not differ with the storage temperature.

- Spraying-coated fruits

  In tomatoes treated with both 0.5% commercial chitosan and 1% heterogeneous bleached chitosan no differences were detected between the storage temperatures. A greater TSS increase occurred at cold temperature than at room temperature in tomatoes coated with solvent alone, 1% commercial chitosan, and both bleached and unbleached 0.5% heterogeneous chitosan. For all the other treatments the opposite difference was observed.

Comparison between coating application methods (table 8.4, supplementary materials)

- Room temperature storage

  Tomatoes coated with most of the treatments showed a significantly greater TSS increase in case of spraying coating than dipping. Only with four treatments (solvent, commercial chitosan, 0.5% heterogeneous bleached and the 0.5% homogeneous unbleached samples) no differences were observed.

- Cold temperature storage

  As happened at room temperature storage, almost all the treatments gave a higher TSS increase in sprayed tomatoes than in the dipped ones. No differences were detected for commercial chitosan and the 1% homogeneous bleached one.

4.5.1.3 *pH*

pH of tomato fruits was determined prior to treatments and at the end of the experiment. The percentage variation of the pH value from the beginning to the end of the storage period (30 days) was assessed. In most cases, pH of tomatoes subjected to all treatments increased during storage, while in a few cases it remained stable. Significant differences were detected depending on the treatment, temperature and method of application.

Room temperature storage

- Dipping-coated fruits

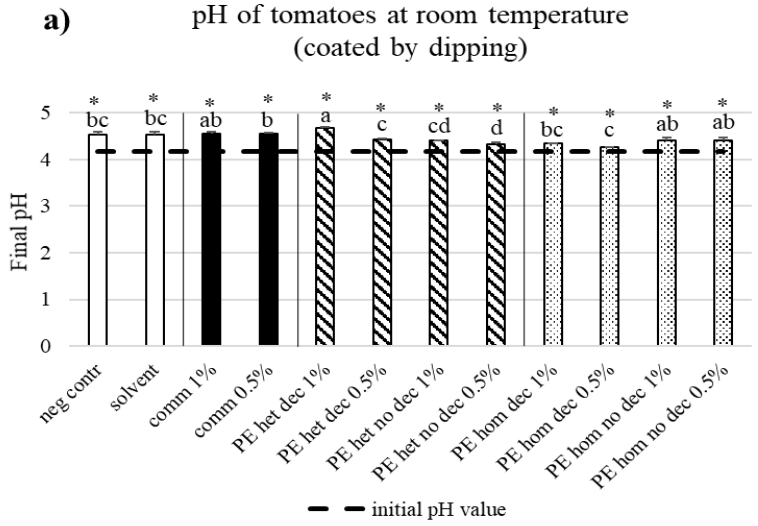
  pH of all tomatoes significantly increased during storage (figure 4.15 a). Only the 1% heterogeneous bleached chitosan gave a greater pH increase than both the negative and solvent-only control, while the 0.5% heterogeneous unbleached gave a significantly lower pH increase. All the other treatements did not differ from the negative control, nor the solvent alone.

  The only significant difference between chitosan concentrations was found for the heterogeneous bleached sample, for which the 1% concentration led to a higher pH increase than the 0.5%. Significant differences were also observed between chitosan bleaching condition for all treatments, except for the 1% homogeneous one. With both the heterogeneous chitosans the pH increased more with the bleached samples, while for the 0.5% homogeneous sample the opposite happened (figure 4.15 a).

- Spraying-coated fruits

  pH remained stable in tomatoes coated with all heterogeneous chitosan samples, except the 0.5% bleached, and the 1% homogeneous bleached (figure 4.15 b). With all the other treatments the pH value significantly increased, to the same extent as the negative control but less than the solvent.

  The only significant difference between chitosan concentrations was found for the homogeneous bleached sample, for which the higher concentration led to a higher pH increase than the low one. Statistical differences were also found between chitosan bleaching status for all treatments with the insect-based chitosans, except for the 0.5% homogeneous one. In most cases, the pH increased more with the bleached chitosan, while for the 1% homogeneous sample the opposite occurred (figure 4.15 b).



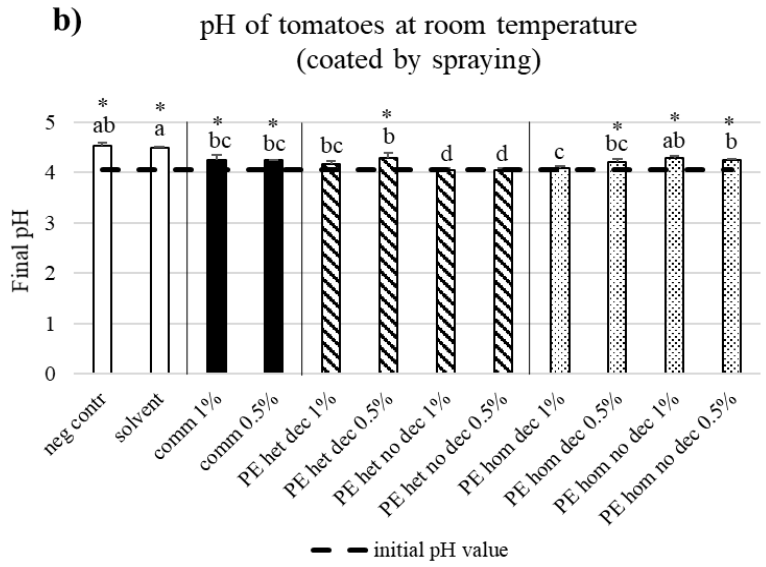


Figure 4.15 Histograms reporting the pH at the beginning and at the end of the storage period (30 days) of tomatoes coated by dipping (a) or spraying (b) and stored at room temperature. The coating solutions prepared with commercial chitosan and chitosan (heterogeneous and homogeneous, decolorized (dec) and not decolorized (no dec)) produced from *H. illucens* pupal exuviae (PE) are shown on the x-axis. Different letters in a graph indicate significant differences ($p<0.05$) in the pH variation of tomatoes among the different treatments, according to Mann-Whitney U test. Asterisks indicate a significant difference between initial and final pH value.

Cold temperature storage

- Dipping-coated fruits

  The pH increased significantly during storage only in the negative control, with solvent-only treatment, with commercial chitosan and with the 1% decolorized heterogeneous one (figure 4.16 a). Commercial chitosan gave a pH increase significantly greater than the negative control, while tomatoes coated with the heterogeneous chitosan sample did not differ from the uncoated fruits.

- Spraying-coated fruits

  Even in the case of sprayed tomatoes stored at cold temperature, the pH remained stable in most treatments. The pH is significantly increased only in the negative control and in the treatments with solvent alone and 1% commercial chitosan. In contrast, a significant decrease in pH was observed with the decolorized heterogeneous chitosan at both concentrations (figure 4.17 b).

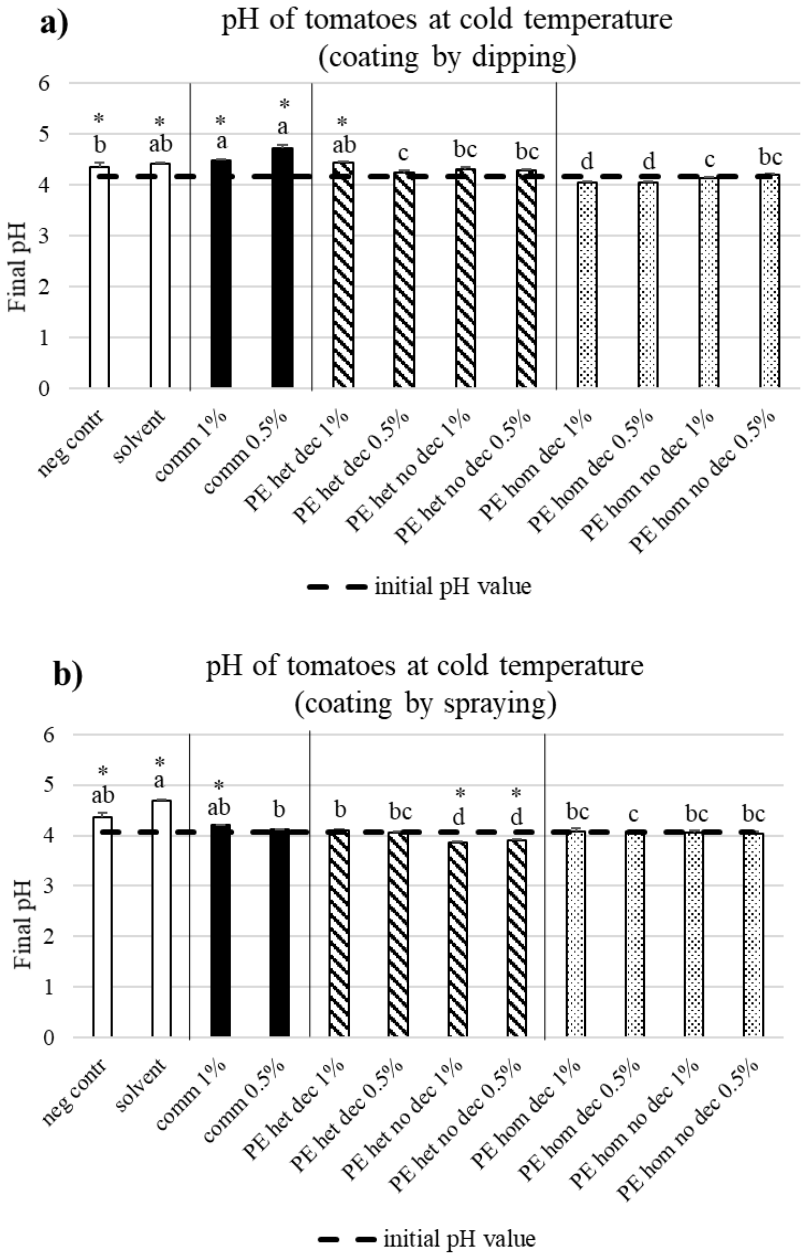


Figure 4.16 Histograms reporting the pH at the beginning and at the end of the storage period (30 days) of tomatoes coated by dipping (a) or spraying (b) and stored at cold temperature. The coating solutions prepared with commercial chitosan and chitosan (heterogeneous and homogeneous, decolorized (dec) and not decolorized (no dec)) produced from *H. illucens* pupal exuviae (PE) are shown on the x-axis. Different letters in a graph indicate significant differences (p<0.05) in the pH variation of tomatoes among the different treatments, according to Mann-Whitney U test. Asterisks indicate a significant difference between initial and final pH value.

Comparison between storage temperatures (table 8.5, supplementary materials)

Increase of pH value of tomatoes belonging to the negative control was significantly greater at room temperature than at cold storage. Differences were found also among the other treatments.

- Dipping-coated fruits

  pH of dipped tomatoes increased significantly more at room temperature than cold temperature in all treatments, with the exception of 0.5% commercial chitosan for

which the opposite occurred and the 0.5% heterogeneous unbleached sample which did not differ with temperature.

- Spraying-coated fruits

  For all treatments the pH increase was greater at room than at cold temperature, except for the solvent-only treatment, for which the opposite occurred, and the 1% commercial chitosan, for which no difference was detected.

Comparison between coating application methods (table 8.6, supplementary materials)

- Room temperature storage

  Only with solvent-alone treatment, the pH increased more in sprayed tomatoes than in the dipped ones. With the other treatements (i.e., 0.5% commercial chitosan, 1% heterogeneous bleached, the heterogeneous unbleached samples and the 1% homogeneous bleached one), when a difference occurred, the pH increase was greater in dipped tomatoes.

- Cold temperature storage

  As happened at room temperature, the solvent-only coating gave a greater pH increase in sprayed tomatoes, while for the majority of the other treatments the opposite occurred. Only for 1% commercial chitosan and the homogeneous bleached samples no differences were detected.

4.5.1.4 *TA*

The percentage variation of TA from the beginning to the end of the storage period (30 days) was assessed. TA of tomatoes exhibited different trends of variation, increasing or decreasing during storage, depending on treatment and temperature.

Room temperature storage

- Dipping-coated fruits

  All the homogeneous chitosan samples gave an increase in the TA value, while all the other treatements led to a decrease, with the exception of the solvent-alone and the 0.5% heterogeneous bleached sample for wich TA remained stable. The major decrease was observed in the negative control (figure 4.17 a).

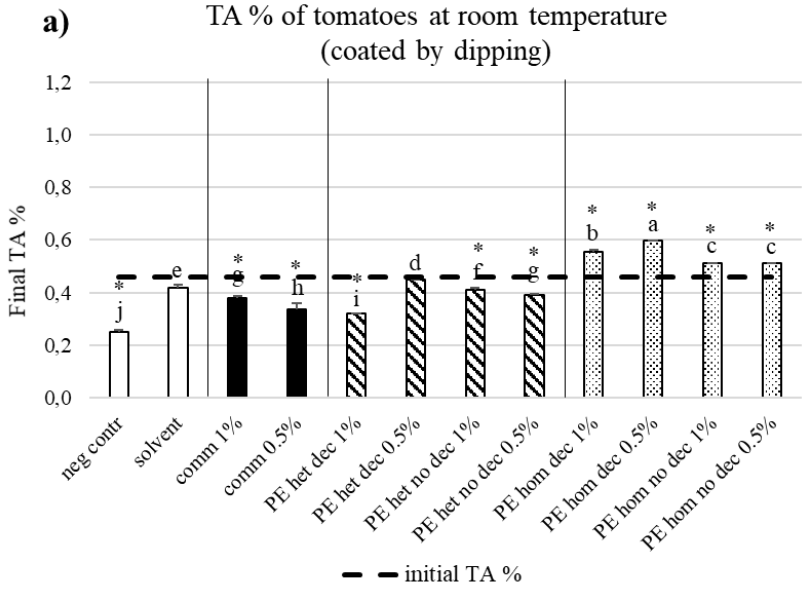
  Significant differences related to the concentration of chitosan were observed for all samples, except the homogeneous unbleached. Generally, the lower concentration gave a greater variation of TA, whether it was positive or negative. Only with the heterogeneous bleached chitosan the opposite occurred. Differences in all treatments

were also found depending on the bleaching condition of the chitosan, with a stronger TA variation observed in most cases with the bleached samples (figure 4.17 a).

- Spraying-coated fruits

  TA significantly increased during storage in all treatments except in the solvent-alone and the negative control, which gave a TA decrease (figure 4.17 b). All chitosan samples produced from *H. illucens* gave a greater TA increase than the commercial one. TA increased generally more with coating with the heterogeneous chitosan than with the homogeneous ones.

  Furthermore, the unbleached samples of all chitosans (excluding the 1% heterogeneous) led to a greater increase in TA than the respective bleached samples. Only for the 1% homogeneous chitosan, the opposite happened (figure 4.17 b)



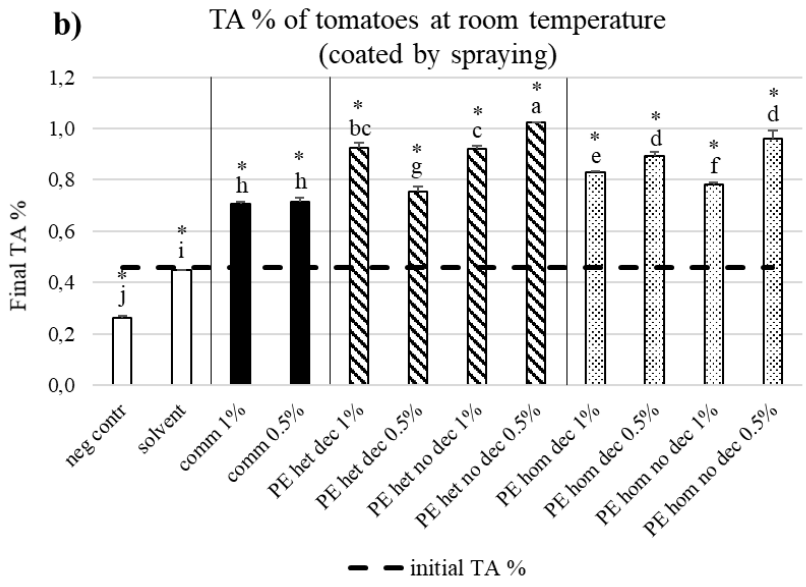


Figure 4.17 Histograms reporting the TA % at the beginning and at the end of the storage period (30 days) of tomatoes coated by dipping (a) or spraying (b) and stored at room temperature. The coating solutions prepared with commercial chitosan and chitosan (heterogeneous and homogeneous, decolorized (dec) and not decolorized (no dec)) produced from *H. illucens* pupal exuviae (PE) are shown on the x-axis. Different letters in a graph indicate significant differences (p<0.05) in the TA variation of tomatoes among the different treatments, according to Mann-Whitney U test. Asterisks indicate a significant difference between initial and final pH value.

Cold temperature storage

- Dipping-coated fruits

  TA increased significantly during storage only in fruits treated with all the homogeneous chitosans (figure 4.18 a). With all the other treatments a TA decrease was observed, to a greater extent in negative control, solvent-only and commercial chitosan.

  For all chitosan samples, excluding the homogeneous unbleached, the 0.5% concentrations led to a significantly greater variation of TA, whether positive or negative, than the 1%. Concerning differences related to the bleaching status, all bleached chitosans (excluding the 0.5% heterogeneous) gave a greater TA variation than the respective unbleached samples (figure 4.18 a).

- Spraying-coated fruits

  TA of sprayed fruits always increased significantly during storage, excluding the negative control for which a decrease was observed (figure 4.18 b). The lower increase was observed with the solvent-alone. The heterogeneous bleached chitosan samples gave a TA increase smaller than both the heterogeneous unbleached chitosan and the

homogeneous ones. Commercial chitosan had an effect similar to the heterogeneous bleached one.

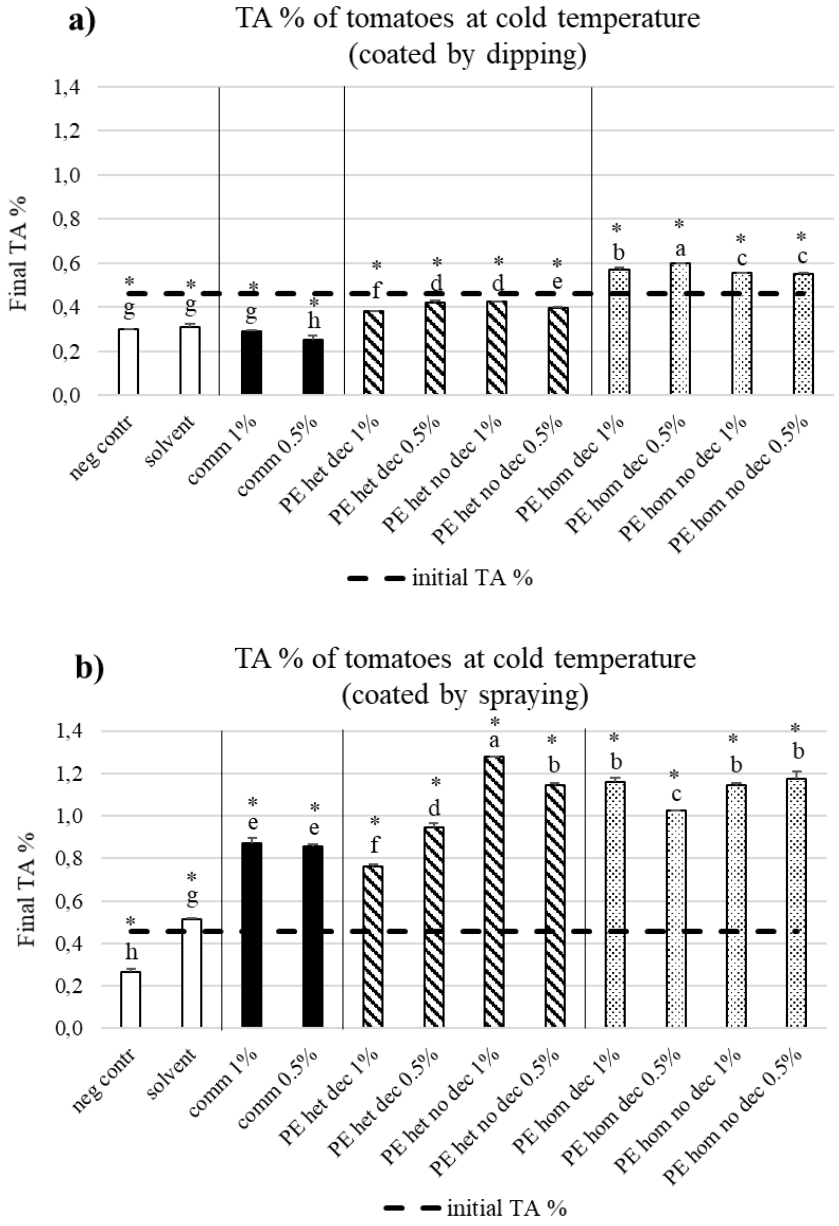


Figure 4.18 Histograms reporting the TA % at the beginning and at the end of the storage period (30 days) of tomatoes coated by dipping (a) or spraying (b) and stored at cold temperature. The coating solutions prepared with commercial chitosan and chitosan (heterogeneous and homogeneous, decolorized (dec) and not decolorized (no dec)) produced from *H. illucens* pupal exuviae (PE) are shown on the x-axis. Different letters in a graph indicate significant differences (p<0.05) in the TA variation of tomatoes among the different treatments, according to Mann-Whitney U test. Asterisks indicate a significant difference between initial and final pH value.

For both the unbleached chitosans, the 1% concentration gave a TA variation greater than the 0.5%. The opposite difference was observed for the bleached heterogeneous chitosan. A difference according to the bleaching was also observed in all but the 1%

homogeneous chitosan. In all cases, the unbleached samples gave a more pronounced TA variation than the bleached ones (figure 4.18 b).

Comparison between storage temperatures (table 8.7, supplementary materials)

Differences in the variation of TA of tomatoes belonging to the negative control were not detected between the two storage temperatures. Differences were found among the other treatments.

- Dipping-coated fruits

  A greater TA variation was observed at cold than at room temperature storage in tomatoes coated with solvent-alone, commercial chitosan and the 0.5% heterogeneous bleached sample. The opposite was detected with treatment with 1% heterogeneous bleached chitosan. No differences occurred with the other treatments.

- Spraying-coated fruits

  TA variation was greater at cold temperature with all treatments. Only with 1% heterogeneous bleached chitosan the TA value varied more at room temperature than at cold one.

Comparison between coating application methods (table 8.8, supplementary materials)

- Room temperature storage

  TA did not differ with coating application method only with the solvent-alone. In all other cases, the TA variation was greater in sprayed tomatoes than in the dipped ones, except for the 0.5% heterogeneous unbleached chitosan for wich the opposite occurred.

- Cold temperature storage

  TA variation was always greater in sprayed fruits than in the dipped ones, with the exception of the solvent-only treatment for which the opposite difference was observed.

4.5.2 Apricots

Apricots were coated by spraying with heterogeneous chitosan obtained from larvae, pupal exuviae and adults of *H. illucens*, and stored at room or cold temperature. Storage period ended after 14 days at room temperature and after 26 days at cold temperature, in accordance with the fruits' longevity at the different conditions. Results of the determination of weight loss, TSS content, pH, TA, and fungal decay assessment are provided in the following sections.

4.5.2.1 *Weight loss*

Weight of apricots belonging to all treatments decreased significantly during storage, both at room and cold temperatures. Few differences were detected among treaments.

Room temperature storage

None of the treatments gave a significantly different result from the negative control, nor from the solvent-alone (figure 4.19 a). Commercial chitosan had a similar effect to all the other insect-derived chitosans.

Only for unbleached chitosan from adults, a significant difference was found between the two concentrations (higher weight loss with the 0.5% concentration). No statistical differences were detected among chitosans according to the different bleaching status (figure 4.19 a).

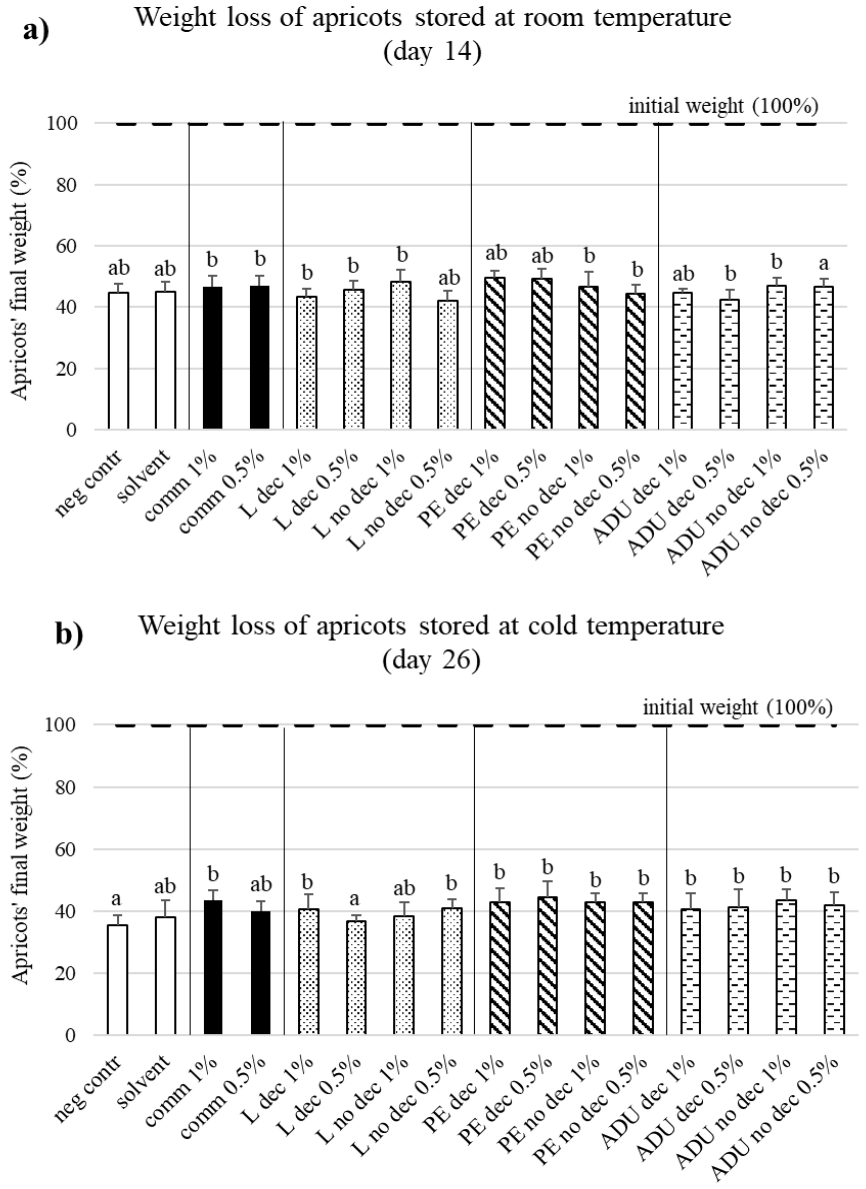


Figure 4.19 Histograms reporting the initial weight (100%) and the decreased weight (%) at the end of the storage period (14 days at room temperature, or 26 days at cold temperature) of apricots stored at room temperature (a) or cold temperature (b). The coating solutions prepared with commercial chitosan and chitosans (decolorized (dec) and not decolorized (no deco)) produced from *H. illucens* larvae (L), pupal exuviae (PE) and adults (ADU) are reported on the x-axis. Different letters in a graph indicate significant differences ($p<0.05$) in the weight loss of apricots among the different treatments, according to *Mann-Whitney* U test.

Cold temperature storage

The highest weight loss occurred in the negative control, which was similar to that of treatments with solvent-alone, 0.5% commercial chitosan, 0.5% larvae bleached and 1% larvae unbleached chitosan, but significantly greater than all the others (figure 4.19 b). Commercial chitosan performed similarly to the insect-derived chitosans.

The only sample for which a significant difference was found both between the two chitosan concentrations and between the different bleaching status was the 0.5% bleached from larvae. This one resulted in a greater weight loss than both the respective 0.5% unbleached sample and the 1% bleached (figure 4.19 b)

Comparison between storage tempearatures (table 8.9, supplementary materials)

Differences related to the different storage temperatures were also investigated, comparing the weight loss of apricots at the same day of storage (14^{th}). For all treatments, a significantly greater weight loss was observed in fruit stored at room temperature compared to those stored at cold temperature.

4.5.2.2 *TSS content*

TSS of apricots subjected to all treatments and stored at both room and cold temperature significantly increased from the beginning to the end of the storage period.

Room temperature storage

The highest TSS increase was observed in the negative control, while the lowest occurred in apricots treated with 1% unbleached chitosan from adults (figure 4.20 a). A significant difference was found only between the four treatments that gave the greatest increase in TSS (i.e., negative control, 0.5% commercial chitosan, 0.5% unbleached chitosan from pupal exuviae, and the 0.5% bleached from adults) and the three that gave the lowest increase (1% unbleached chitosan from adults and the two chitosans from larvae at 0.5% concentration).

Cold temperature storage

Under storage conditions at cold temperature, no significant differences were found among the different treatments (figure 4.20 b). Although not statistically different, the highest TSS increase occurred with 1% unbleached chitosan from larvae, while the lowest with the 1% bleached from adults.

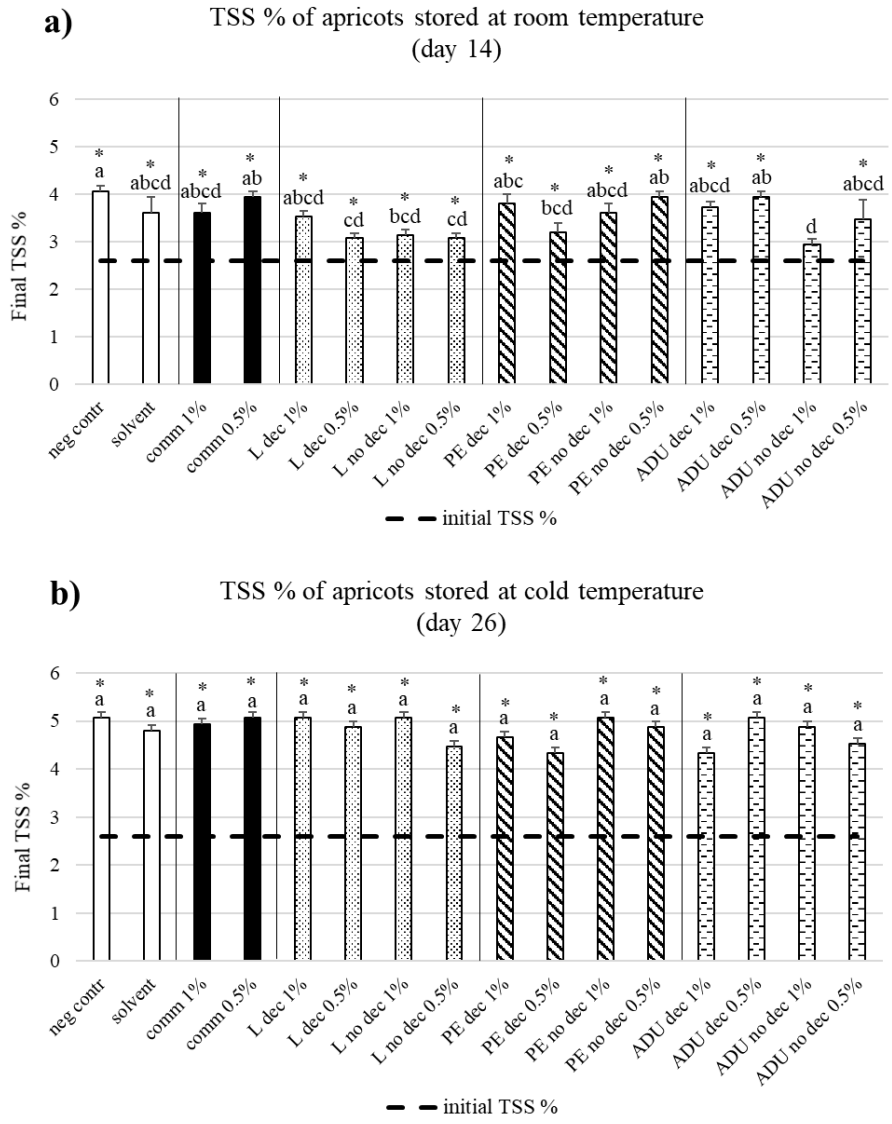


Figure 4.20 Histograms reporting the initial TSS content (%) and the decreased one at the end of the storage period (14 days at room temperature, or 26 days at cold temperature) of apricots stored at room temperature (a) or cold temperature (b). The coating solutions prepared with commercial chitosan and chitosans (decolorized (dec) and not decolorized (no deco)) produced from *H. illucens* larvae (L), pupal exuviae (PE) and adults (ADU) are reported on the x-axis. Different letters in a graph indicate significant differences ($p<0.05$) in the TSS variation of apricots among the different treatments, according to *Mann-Whitney* U test.

Comparison between storage temperatures

Given the difference in the length of the storage period (14 days at room temperature and 26 days at low temperature), the increases in TSS were greater in apricots stored at cold temperature. Despite this difference in duration, treatments with 0.5% unbleached chitosan from pupal exuviae and 1% adults bleached gave an equal increase in TSS at the two storage temperatures.

4.5.2.3 *pH*

pH of apricots was determined prior to treatments and at the end of the experiment. The percentage variation of pH from the beginning to the end of the storage period (14 days at room temperature and 26 days at cold storage) was assessed. The pH of apricots always increased significantly during storage, both at room and cold temperatures. Differences were observed among the various treatments.

Room temperature storage

pH of apricots increased the most in the negative control (figure 4.21 a). All chitosan treatements, except the samples from bleached larvae, resulted in a smaller pH increase than the negative control. Chitosan from unbleached larvae and all chitosan samples from pupal exuviae gave a lower pH increase also than the solvent-alone.

Although not significantly, almost all 0.5% chitosans gave a higher pH increase than the respective 1% samples. A significant difference related to the bleaching condition was found only for chitosan from larvae at both concentrations: the bleached samples gave a higher pH increase than the unbleached ones (figure 4.21 a).

Cold temperature storage

As happened at room temperature, the highest pH increase was detected in the negative control (figure 4.21 b). Treatments with commercial chitosan and chitosan from pupal exuviae resulted in the lowest pH increase, similarly to the solvent-only control.

Differences between chitosan concentrations were observed only for both chitosans from adults, for which the 0.5% concentration gave a greater pH increase than the 1%. As observed at room temperature, bleached chitosan from larvae at both concentrations gave a significantly higher pH increase than the respective unbleached samples (figure 4.21 b).

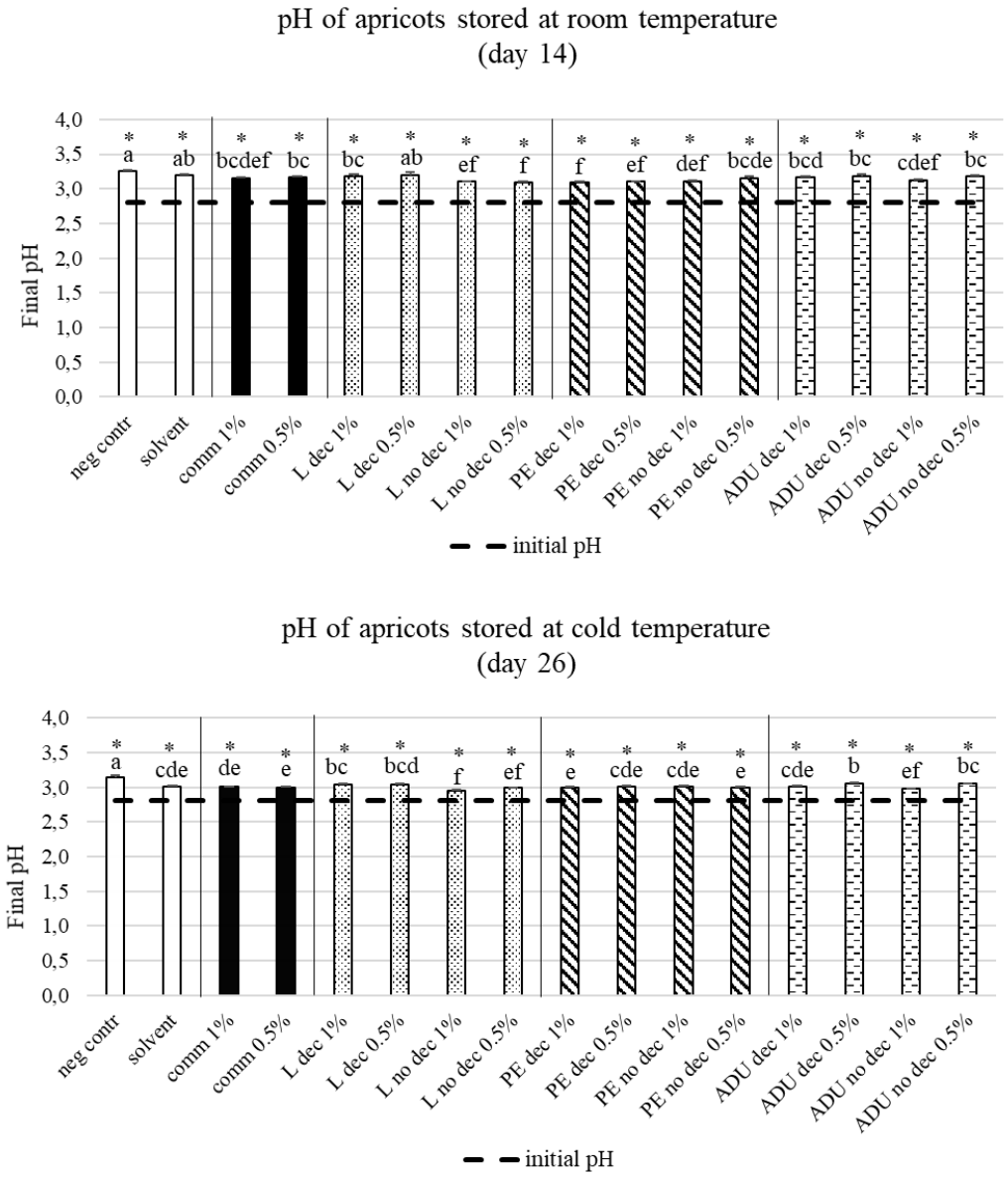


Figure 4.21 Histograms reporting the pH values at the beginning and at the end of the storage period (14 or 26 days) of apricots stored at room (a) or cold temperature (b). The coating solutions prepared with commercial chitosan and chitosans (decolorized (dec) and not decolorized (no deco)) produced from *H. illucens* larvae (L), pupal exuviae (PE) and adults (ADU) are showed on the x-axis. Different letters in a graph indicate significant differences (p<0.05) in the pH variation among the different treatments, according to one-way ANOVA with Tukey's post hoc test.

Comparison between storage temperatures

Tomatoes belonging to all treatments showed a significantly greater variation in the pH value at room temperature storage than at the cold one.

4.5.2.4 *TA*

The TA of apricots always increased significantly during storage, both at room and cold temperatures. Differences were observed among the various treatments.

Room temperature storage

TA increased the most in fruits treated with the 0.5% bleached and the 1% unbleached chitosan from adults, while the lowest increase occurred with 0.5% commercial chitosan and 1% unbleached chitosan from larvae (figure 4.22 a). Most of treatments with the insect-derived chitosans gave a significantly lower TA increase than both controls (negative and solvent-only). The 0.5% concentration of bleached chitosan from adults and the unbleached one from larvae led to a significantly higher increase in TA than the respective samples at 1% concentration. In contrast, for both commercial chitosan and the unbleached one from adults the opposite happened. Differences related to the bleaching status were also found. The TA increased more with the bleached samples of 0.5% chitosan from adults and the 1% from larvae, than with the respective unbleached samples. The opposite happened with 1% chitosan from adults and the 0.5% from larvae (figure 4.22 a).

Cold temperature storage

In contrast to what occurred at room temperature, the smallest increase in TA was found in the negative control (figure 4.22 b). Majority of chitosan treatments resulted in a TA increase grater than the negative control, but smaller than the solvent-alone treatment. Among chitosan derived from *H. illucens*, TA increased least with samples from pupal exuviae.

The 0.5% concentration gave a significantly greater TA increase than the 1% in both bleached chitosan from larvae and the commercial one. The opposite occurred with the unbleached chitosan from larvae. Significant differences in the bleaching status of chitosan were also observed: The TA increased more in both the 0.5% bleached chitosan from larvae and the 0.5% from adults than in the respective unbleached samples. The opposite was true for chitosan 1% from larvae and the 1% from adults (figure 4.22 b).

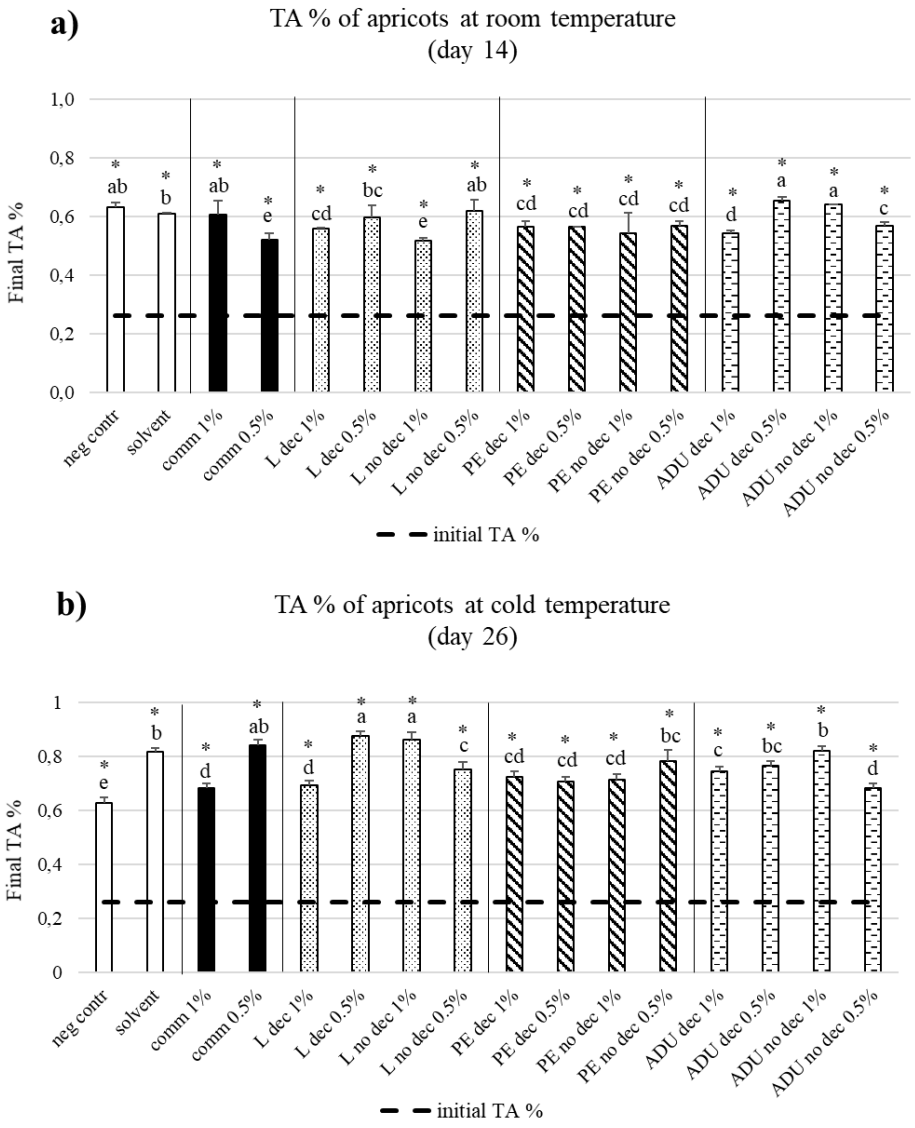


Figure 4.22 Histograms reporting the TA (%) at the beginning and at the end of the storage period (14 or 26 days) of apricots stored at room (a) or cold temperature (b). The coating solutions prepared with commercial chitosan and chitosans (decolorized (dec) and not decolorized (no deco)) produced from *H. illucens* larvae (L), pupal exuviae (PE) and adults (ADU) are showed on the x-axis. Different letters in a graph indicate significant differences ($p<0.05$) in the TA variation among the different treatments, according to one-way ANOVA with Tukey's post hoc test.

Comparison between storage temperatures

The increase in TA was significantly greater in apricots stored at cold temperature than in those at room temperature, for all treatments. Only in the negative control no significant difference was observed.

4.5.2.4 *Fungal decay*

Fungal decay occurrence of apricots was investigated per each treatment at both storage conditions. The presence of mould was only detected in apricots stored at room temperature, with a different extent among the various treatments. In all treatments, the first signs of fungal

decay were observed from the 12th day of storage. The percentage of moldy fruit observed at the end of the storage period and the respective average fungal decay index per treatment are provided in table 4.12.

Only in the negative control, 100% of the fruits were moldy at the end time (14 days). The second two treatments with the highest percentage of moldy fruits (83%) were the solvent control and the 0.5% commercial chitosan (table 4.12). The lowest percentages of mouldy apricots were found in treatments with both 1% chitosans from pupal exuviae (33%), the 1% commercial chitosan and the 1% unbleached from larvae (both 16%). In most of the chitosan treatments (9 out of 14) the percentage of mouldy fruit was ≤ 50%. For all chitosans, excluding the bleached from larvae, a lower percentage of mouldy fruit was observed with the 1% concentration compared to 0.5%. This difference was not observed between bleached and unbleached chitosans.

The highest average fungal decay index (2.7) was again observed in the negative control and in the treatment with 0.5% commercial chitosan (table 4.12). In most cases, the average index was between 1.5 and 1.8. The lowest occurrence of mouldy apricots (0.3) was found in treatments with 1% commercial chitosan and the 1% unbleached from larvae.

Table 4.12 Percentage of decayed apricots at the end (T_f) of the storage period at room temperature and average fungal decay index per each treatment. Data are expressed as mean±standard deviation. Different letters in a column indicate significant differences ($p < 0.05$) in the percentage of decayed fruits among treatments, according to the Chi-square test with Yates' correction.

TREATMENT	DECAYED FRUITS AT T_f (%) (room temperature storage)	FUNGAL DECAY INDEX
Negative control	100[a]	2.7±1.2
Solvent	83[b]	2.3±1.4
Comm 1%	16f	0.3±0.8
Comm 0.5%	83[b]	2.7±1.8
L dec 1%	67[c]	2.2±2.0
L dec 0.5%	50d	1.8±2.2
L no dec 1%	16[f]	0.3±0.8
L no dec 0.5%	67[c]	1.7±1.5
PE dec 1%	33[e]	0.8±1.3
PE dec 0.5%	50[d]	1.5±2.0
PE no dec 1%	33[e]	1.0±1.7
PE no dec 0.5%	50[d]	1.5±2.0
ADU dec 1%	50[d]	2.5±2.7
ADU dec 0.5%	67[c]	1.7±1.5
ADU no dec 1%	50[d]	1.5±2.0
ADU no dec 0.5%	67[c]	1.5±1.2

4.5.3 Strawberries

Strawberries were coated by spraying with chitosans (all produced by heterogeneous deacetylation) obtained from larvae, pupal exuviae and adults of *H. illucens*, and stored at room or cold temperature, as with apricots. Storage period was finished after 9 days at room temperature and at 15 days at cold temperature, in accordance with the fruits' longevity at the different conditions.

Results of the determination of weight loss, and fungal decay assessment are provided in the following sections. It was not possible to repeat the measurement of TSS, pH and TA at the end of the storage period as the strawberries were too dehydrated for pulp recovery. Therefore, an evaluation of the variation of these parameters during storage was not possible.

4.5.3.1 *Weight loss*

Weight of strawberries belonging to all treatments (negative control, solvent alone, chitosan coatings) decreased significantly during storage, both at room and cold temperatures.

Room temperature storage

At room temperature, strawberries had a similar weight loss in almost all treatments, included the controls (figure 4.23 a). The highest loss was found with 0.5% commercial chitosan, which was significantly greater than the losses observed with most treatments with chitosan from both larvae and adults.

No differences were detected depending on the concentration of chitosan, nor on its state of bleaching (figure 4.23 a).

Cold temperature storage

The highest weight loss occurred with the solvent-only treatment (figure 4.23 b). Coating with commercial chitosan and chitosan from adult flies had a similar effect to both the controls. Chitosan samples from larvae (excluding the 0.5% unbleached) and the 0.5% sample from unbleached pupal exuviae resulted in a smaller weight loss than the solvent-only control.

No differences were detected depending on the concentration of chitosan, nor on its state of bleaching (figure 4.23 b).

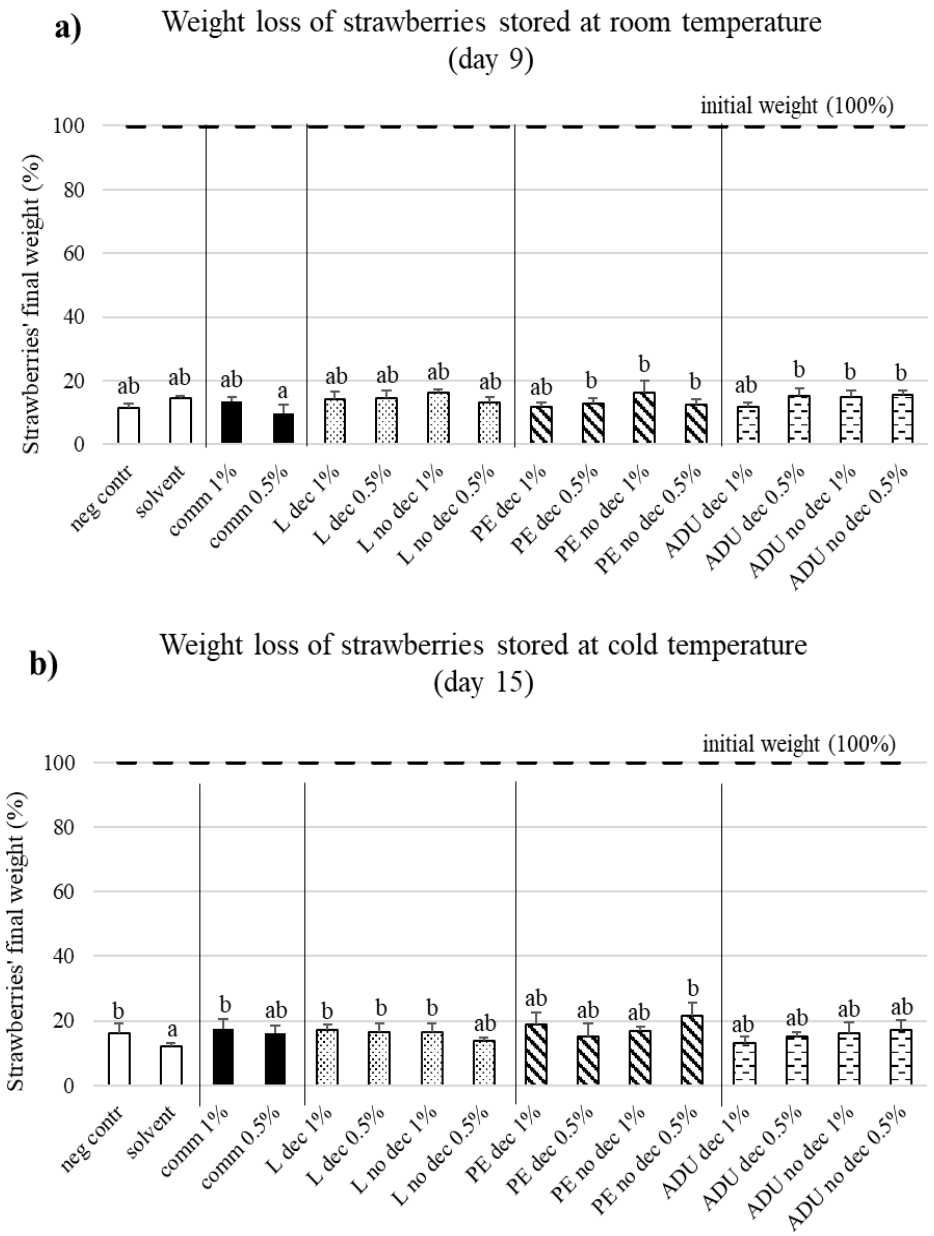


Figure 4.23 Histograms reporting the initial weight (100%) and the decreased weight (%) at the end of the storage period (9 days at room temperature, or 15 days at cold temperature) of strawberries stored at room temperature (a) or cold temperature (b). The coating solutions prepared with commercial chitosan and chitosans (decolorized (dec) and not decolorized (no deco)) produced from *H. illucens* larvae (L), pupal exuviae (PE) and adults (ADU) are showed on the x-axis. Different letters in a graph indicate significant differences ($p<0.05$) in the weight loss of strawberries among the different treatments, according to *Mann-Whitney* U test.

Comparison between storage temperatures (table 8.10, supplementary materials)

Comparing the two storage temperatures, given the same storage duration (9 days), only strawberries coated with 1% bleached chitosan from pupal exuviae, the 0.5% unbleached from pupal exuviae, the 0.5% commercial sample and the negative control lost significantly more weight at room temperature than at cold. For all the other treatments, no statistically significant differences were detected, although the weight loss at room temperature was always higher than

at cold temperature.

4.5.3.2 *Fungal decay*

Fungal decay occurrence in strawberries was investigated per each treatment at both storage conditions. The presence of mould was detected in fruit stored at both room and cold temperatures, in the latter to a much lesser extent. Differences were observed among treatments. In all treatments, the first signs of fungal decay on strawberries stored at room temperature were observed from the 3rd day of storage. The percentage of mouldy fruit observed at the end of the storage period and the respective average fungal decay index per treatment are provided in table 4.24.

Table 4.13 Percentage of decayed strawberries at the end (T_f) of the storage period at room and cold temperature, and average fungal decay index per each treatment. Data are expressed as mean±standard deviation. Different letters in a column indicate significant differences ($p < 0.05$) in the percentage of decayed fruits among treatments, according to the Chi-square test with Yates' correction.

TREATMENT	Room temperature storage		Cold temperature storage	
	DECAYED FRUITS AT T_f (%)	FUNGAL DECAY INDEX	DECAYED FRUITS AT T_f (%)	FUNGAL DECAY INDEX
Negative control	78^{ab}	3.1±2.1	22^{a}	0.4±0.9
Solvent	89^{a}	3.7±1.9	22^{a}	0.4±0.9
Comm 1%	55.5^{bc}	2.1±2.2	0^{b}	0.0
Comm 0.5%	78^{ab}	3.9±2.2	11^{a}	0.2±0.7
L dec 1%	33^{c}	1.0±1.6	0^{b}	0.0
L dec 0.5%	33^{c}	1.0±1.6	11^{a}	0.2±0.7
L no dec 1%	78^{ab}	2.7±1.7	0^{b}	0
L no dec 0.5%	55.5^{bc}	2.3±2.3	0^{b}	0
PE dec 1%	67^{b}	3.2±1.8	0^{b}	0
PE dec 0.5%	44^{c}	1.6±2.4	0^{b}	0
PE no dec 1%	67^{b}	3.0±2.4	0^{b}	0
PE no dec 0.5%	44^{c}	1.5±2.1	0^{b}	0
ADU dec 1%	67^{b}	2.2±2.3	0^{b}	0
ADU dec 0.5%	33^{c}	1.1±1.8	0^{b}	0
ADU no dec 1%	44^{c}	1.1±1.5	0^{b}	0
ADU no dec 0.5%	55.5^{bc}	1.1±1.2	0^{b}	0

The highest percentages of mouldy strawberries were observed in the solvent and negative controls (78 and 89%, respectively), and in treatments with 0.5% commercial chitosan and the 1% unbleached from larvae (both 78%). In treatments with bleached chitosan from larvae at both concentrations and the 0.5% bleached from adults, the minimum percentage of decayed fruit was detected (33%). In most treatments, a lower incidence of fungal decay occurred with

the 0.5% chitosan concentration compared to the 1%. Generally, all chitosan-based treatments gave a lower percentage of mouldy fruit than the control treatments (excluding the 0.5% commercial chitosan and the 1% unbleached from larvae). The average fungal decay index followed the same trend. The worst values were those of strawberries treated with solvent alone and 0.5% commercial chitosan (3.7 and 3.9, respectively). Decay index with most chitosan-based treatments was ≤ 2.7.

At cold storage, fungal decay occurred only in strawberries belonging to both controls and in fruit treated with 0.5% commercial chitosan and 0.5% bleached chitosan from larvae. The higher incidence of mouldy fruit was in the two controls (22%). Also the fungal decay index was slightly worse for strawberries belonging to the control treatments than those treated with the two chitosans.

4.5.4 Nectarines

Nectarines were coated by spraying with chitosans (all produced by heterogeneous deacetylation) obtained from pupal exuviae of *H. illucens*, and stored at room or cold temperature. Storage period was ended after 10 days at room temperature and at 21 days at cold temperature, in accordance with the fruits' longevity at the different conditions. Results of the determination of weight loss, TSS content, pH and TA, and fungal decay assessment are provided in the following sections.

4.5.4.1 *Weight loss*

Weight of nectarines significantly decreased during storage at both storage temperatures. Although weight losses varied, no statistically significant differences were found among the treatments.

Room temperature storage

The weight loss of nectarines stored at room temperature was not significantly different among the different treatments (figure 4.24 a). Nevertheless, the two unbleached chitosans from pupal exuviae gave a greater weight loss than the respective bleached samples and both controls (negative and solvent-only).

Cold temperature storage

A similar result was found at cold storage temperature (figure 4.24 b). Again, although not statistically different, unbleached chitosan from pupal exuviae gave a greater weight loss than bleached samples. The commercial chitosans gave the greatest weight loss, whereas the lowest loss was observed in the solvent control.

Comparison between storage temperatures (table 8.11, supplementary materials)

Comparing the weight loss of nectarines at the two storage temperatures, given the same treatment and storage duration (10 days), fruit at room temperature lost significantly more weight than those at cold temperature in all treatments.

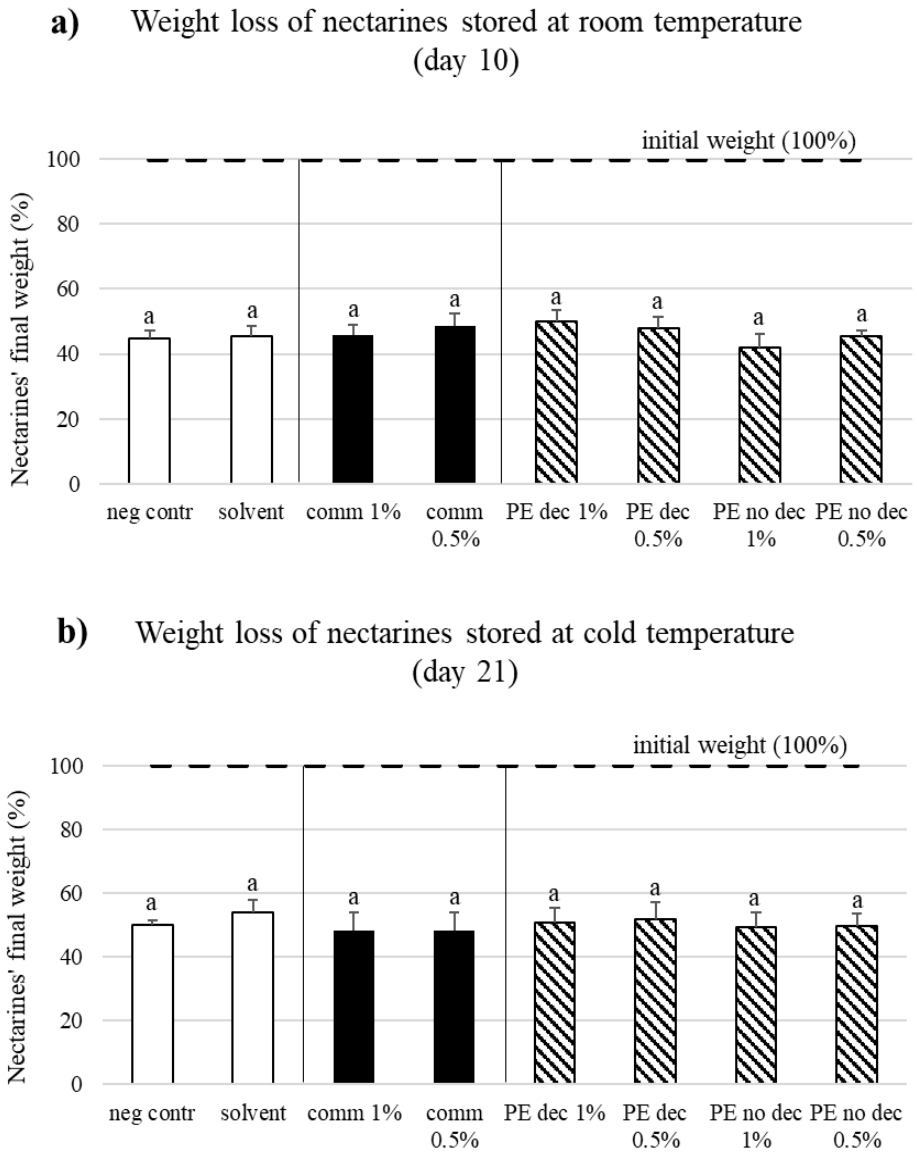


Figure 4.24 Histograms reporting the initial weight (100%) and the decreased weight (%) at the end of the storage period (10 days at room temperature, or 21 days at cold temperature) of nectarines stored at room temperature (a) or cold temperature (b). The coating solutions prepared with commercial chitosan and chitosans (decolorized (dec) and not decolorized (no dec)) produced from *H. illucens* pupal exuviae (PE) are showed on the x-axis. Different letters in a graph indicate significant differences (p<0.05) in the weight loss of nectarines among the different treatments, according to *Mann-Whitney* U.

4.5.4.2 *TSS* content

TSS content of nectarines stored at both room and cold temperature significantly increased from the beginning to the end of the storage period in all treatments.

Room temperature storage

The highest TSS increase was detected in both negative and solvent-only controls (figure 4.25 a). Both bleached and unbleached chitosans from pupal exuviae at 0.5% concentration gave the lowest increase in TSS, which was significantly lower than that observed in the control treatments. No significant differences were found between commercial chitosan and samples derived from *H. illucens*.

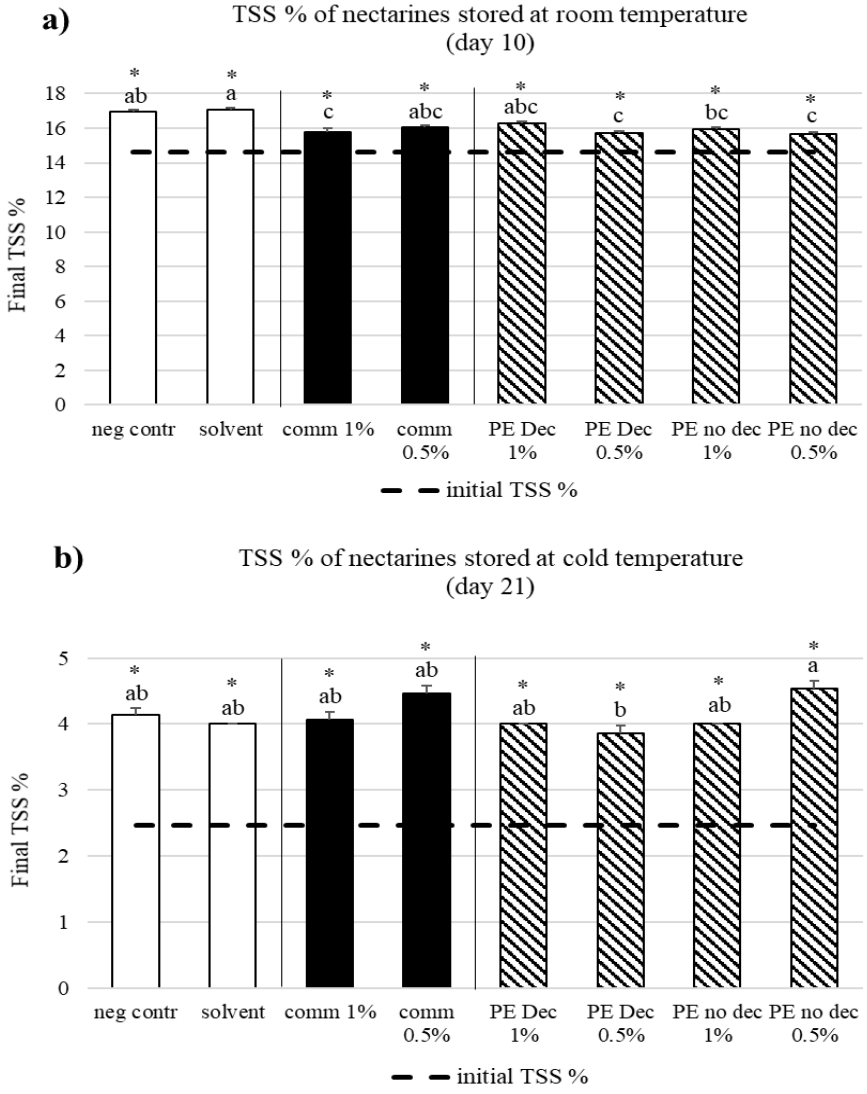


Figure 4.25 Histograms reporting the TSS content (%) at the beginning and at the end of the storage period (10 or 21 days) of nectarines stored at room (a) or cold temperature (b). The coating solutions prepared with commercial chitosan and chitosans (decolorized (dec) and not decolorized (no dec)) produced from *H. illucens* pupal exuviae (PE) are showed on the x-axis. Different letters in a graph indicate significant differences (p<0.05) in the TSS variation among the different treatments, according to one-way ANOVA with Tukey's post hoc test.

Cold temperature storage

All treatments had a similar effect to each other and similar to both negative and solvent control (figure 4.25 b). The only significant difference was detected between the 0.5% chitosan samples from both bleached and unbleached pupal exuviae, with the latter resulted in a greater weight loss. All the insect-derived chitosans performed comparable with regard to the commercial sample and both controls.

Comparison between storage temperatures

Given the same treatment, TSS always increased significantly more in nectarines stored at cold temperatures than in those at room temperature.

4.5.4.3 *pH*

Variation in pH of nectarines was assessed at the end of the storage period. It significantly increased during storage, but to a different extent depending on the treatment.

Room temperature storage

The highest pH increase was observed in the solvent-only control (figure 4.26 a). Commercial chitosan resulted in pH increase lower than the solvent-alone but greater than the negative control. All chitosans from *H. illucens* pupal exuviae, excluding the 0.5% unbleached, gave a significantly lower pH increase than both controls and commercial chitosan.

The pH increased significantly more with treatments with insect-chitosan at the 0.5% concentration than at the 1%. Statistical differences were also found between the different chitosan bleaching conditions. For chitosan at a concentration of 0.5%, the unbleached sample gave the greatest increase in pH. The opposite was true for chitosan at 1% (figure 4.26 a).

Cold temperature storage

The pH of nectarines belonging to the two controls increased significantly more than all the others (figure 4.26 b). The second largest increase was found with commercial chitosan at both concentrations. Chitosan from *H. illucens* gave the smallest increases in pH compared to all others. pH remained stable in fruits coated with 0.5% bleached and 1% unbleached chitosan from pupal exuviae.

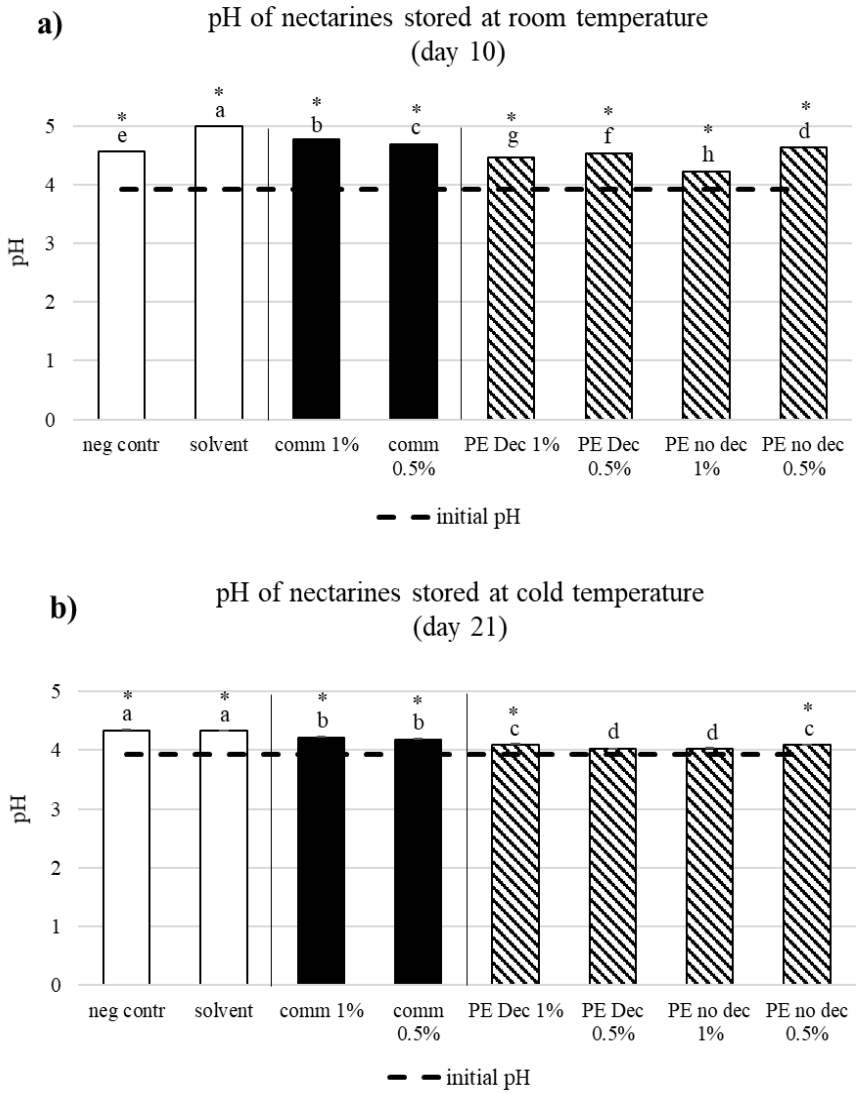


Figure 4.26 Histograms reporting the pH values at the beginning and at the end of the storage period (10 or 21 days) of nectarines stored at room (a) or cold temperature (b). The coating solutions prepared with commercial chitosan and chitosans (decolorized (dec) and not decolorized (no dec)) produced from *H. illucens* pupal exuviae (PE) are showed on the x-axis. Different letters in a graph indicate significant differences (p<0.05) in the pH variation among the different treatments, according to one-way ANOVA with Tukey's post hoc test.

Comparison between storage temperatures

Given the same treatment, nectarines stored at room temperature had a significantly greater pH increase than those at cold temperature.

4.5.4.4 *TA*

Variation in TA of nectarines was assessed at the end of the storage period. Also the TA percentage significantly increased during storage, in all treatments.

Room temperature

TA increased the most in negative control and treatment with 1% unbleached chitosan from pupal exuviae (figure 4.27 a). All other chitosans from *H. illucens* gave a lower TA increase

than these two samples, but do not significantly differ from the solvent-only control. Commercial chitosan performed similarly to most insect chitosan samples.

The 1% concentration of chitosan from *H. illucens* gave a significantly higher TA increase than the 0.5% concentration (figure 4.27 a).

Cold temperature storage

TA increased the least in both control treatments (figure 4.27 b). Chitosans from pupal exuviae gave the highest increases, while commercial chitosan gave intermediate results.

The bleached chitosan from pupal exuviae at 1% concentration gave a TA increase significantly greater than the respective sample at 0.5%. No significant differences were found between bleached and unbleached chitosans (figure 4.27 b).

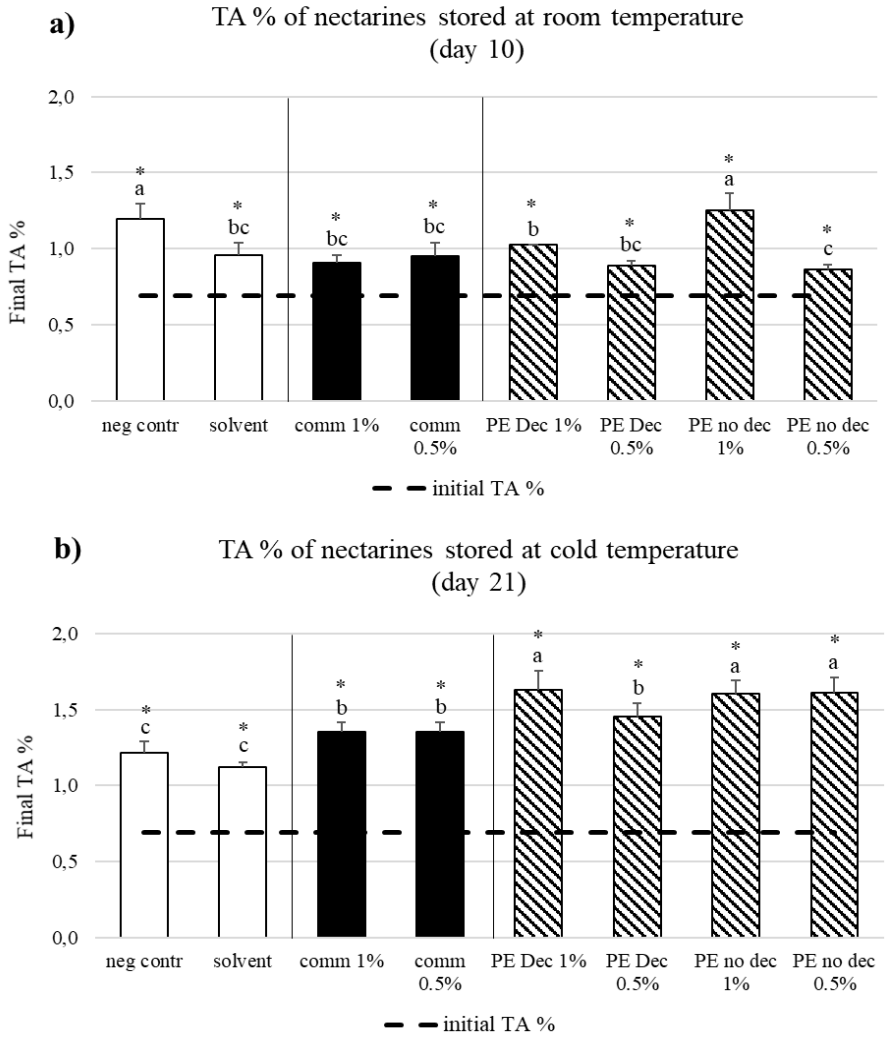


Figure 4.27 Histograms reporting the TA (%) at the beginning and at the end of the storage period (10 or 21 days) of nectarines stored at room (a) or cold temperature (b). The coating solutions prepared with commercial chitosan and chitosans (decolorized (dec) and not decolorized (no dec)) produced from *H. illucens* pupal exuviae (PE) are showed on the x-axis. Different letters in a graph indicate significant differences (p<0.05) in the TA variation among the different treatments, according to one-way ANOVA with Tukey's post hoc test.

Comparison between storage tempeatures

Given the same treatment, TA increased significantly more in fruit stored at cold temperature than in those at room temperature.

4.5.4.5 *Fungal decay*

Fungal decay occurrence in nectarines was investigated per each treatment at both storage conditions. The presence of mould was detected only in fruit stored at room temperature, to a different extent among treatments. The first signs of fungal decay were observed from the 7th day of storage.

The percentage of mouldy fruit observed at the end of the storage period and the respective average fungal decay index per treatment are reported in table 4.14. Fungal decay was detected only in four treatments: both negative and solvent controls, the 0.5% bleached and the 1% unbleached chitosan from pupal exuviae. The highest percentage of mouldy fruit was detected in the two control treatments (67% in the negative and 50% in the solvent-alone). Only 33% of the fruits treated with the two chitosans developed mould. The average fungal decay index was higher for the controls than the chitosan treatments, as well (1.8 *vs* 0.7). Hence, all chitosan treatments gave a better result than controls with regard to fungal decay of nectarines.

Table 4.14 Percentage of decayed nectarines at the end (T_f) of the storage period at room temperature and average fungal decay index per each treatment. Data are expressed as mean±standard deviation. Different letters in a column indicate significant differences ($p < 0.05$) in the percentage of decayed fruits among treatments, according to the Chi-square test with Yates' correction.

TREATMENT	DECAYED FRUITS AT T_f (%) (room temperature storage)	FUNGAL DECAY INDEX
Negative control	67a	1.8±1.8
Solvent	50b	1.8±2.2
Comm 1%	0d	0.0
Comm 0.5%	0d	0.0
PE dec 1%	0d	0.0
PE dec 0.5%	33c	0.7±1
PE no dec 1%	33c	0.7±1
PE no dec 0.5%	0d	0.0

5. DISCUSSION

5.1 Composition of raw insects

Composition of raw *H. illucens* samples in terms of mineral, protein, fat and chitin content, was compared to those reported for insects in the literature. On average, whole insects contain 30-60% protein (Rumpold and Schluter, 2013), 10-25% lipid (Rumpold and Schluter, 2013), 5-25% chitin (Kramer *et al*., 1995; Kurita, 2006; Hahn *et al.*, 2018) and 2-10% minerals (Finke, 2013; Rumpold and Schluter, 2013). All these components are reported in fairly wide ranges, as the composition of insects varies, especially depending on their species and stage of development. The percentages of the components of larvae, pupal exuviae and adults collected from *H. illucens* farm set up at the University of Basilicata are within these ranges.
Similarities were also found with regard to the average composition given for the different developmental stages of *H. illucens* (table 5.1).

Table 5.1 Composition of raw *H. illucens* larvae, pupal exuviae and adults from the farm set up at the University of Basilicata, and average composition of *H. illucens* samples reported in the literature. Fields marked with a hyphen indicate data that are not available in the literature. Data are related to the insect dry mass.

	***H. illucens* samples from University of Basilicata**			**Literature**		
	LARVAE	PUPAL EXUVIAE	ADULTS	LARVAE	PUPAL EXUVIAE	ADULTS
Minerals %	12.5	16.0	8.1	9.6-20.6	10.5-19.4	5.2
Protein %	38.7	30.0	49.0	38.8-43.0	-	-
ADF %	22.0	53.5	23.7	-	-	-
ADL %	9.5	28.0	10.8	-	-	-
Chitin % (ADF – ADL)	12.4	25.5	12.8	3.8-9.5	23.8	5.6
Fat %	23.0	5.0	19.7	36.0-40.0	8.0	27.0

Mineral content of larvae was in the range reported by Nafisah *et al*. (2019), Smets *et al*. (2020) and Soetemans *et al*. (2020) (9-20%). Pupal exuviae have a mineral content similar to that reported for *H. illucens* (Khayrova *et al*., 2020; Soetemans *et al*., 2020) but higher than that generally indicated for insects. The high ash content of pupal exuviae could be a peculiarity of this species. Mineral content of adults has been determined only by Khayrova *et al*. (2020), resulting in a value of 5.2% which is slightly lower than that determined for our flies. Protein percentage was available only for larvae (table 5.1) and it was similar to that of larvae collected from our farm (Nafisah *et al*., 2019; Smets *et al*., 2020). Chitin content usually range from 3.8 to 9.5% for larvae (Smets *et al*., 2020; Soetemans *et al*., 2020), around 24% for pupal exuviae (Soetemans *et al*., 2020) and around 6% for adult flies (Soetemans *et al*., 2020). All these values

are similar to those calculated for our insects, except for the chitin content of the adults, which was a little higher. The fat content is the datum that differs most from the literature (table 5.1). All our *H. illucens* samples had a lower lipid content than those reported for the same insect biomass (Nafisah *et al*., 2019; Smets *et al*., 2020).

Based on the few data available on the composition of larvae, pupal exuviae and adults of *H. illucens*, it can be observed that, overall, our samples were comparable to those used by other authors. It is known that differences in composition, especially in terms of lipids, may be due to different diets on which larvae are fed (Barragan-Fonseca *et al*., 2017; Mai *et al*., 2019). The larvae from our farm were fed only standard diet in order to avoid this potential influence of diet. Little variations in the insect composition can be also due to the different methods applied to determine the proximate composition of samples. While ash content is always determined by incineration using a muffle furnace, the protein percentage is often calculated from the total nitrogen content obtained with the Kjeldahl method (Smets *et al.,* 2020). The Kjeldahl method does not measure the true protein fraction and it requires the application of a conversion factor that is not suitable for all protein types and should be corrected according to the amino acid composition of the examined protein (Sáez-Plaza *et al.*, 2013). This may therefore lead to different results in the protein determination. For lipid extraction, although the standard method is Soxhlet, different solvents can be used (e.g., hexane, ethanol, chloroform, petroleum ether, acetone and others), which may have different extraction efficiency depending on the polarity of the lipids (Ramluckan *et al*., 2014). Pupal exuviae were the richest biomass in chitin (25%). This encouraged the primary use of this insect farming waste as a source of chitin. Hahn *et al*. (2020b) reported an even higher chitin content for the *H. illucens* larval exoskeletons (about 35%) compared to that of pupal exuviae. Nevertheless, larval exoskeletons are a difficult substrate to isolate and collect from the farm, being mixed with dietary waste and frass in the containers where the larvae grow and feed. Pupal exuviae, in contrast, "self-collect" in the boxes where pupae are kept when they are left behind by the emerging adults. Pupal exuviae, therefore, are the waste substrate with the highest chitin content and most readily available, without the need to incur additional costs to mechanise its harvesting, for the extraction of this polymer.

Average chitin content of the crustacean and mollusc shells most commonly used for the industrial production of chitin is provided in table 5.2. The average chitin content of insects (5-25%) is within these ranges or slightly below. The chitin content of the pupal exuviae of *H. illucens* is very close to the maximum values reported for crustacean shells. As with insects, it must again be considered that the chitin content of crustaceans varies according to species, stage

of development and even body part (Thirunavukkarasu and Shanmugam, 2009; Bolat *et al*., 2010). Hence, in terms of chitin content, insects, *H. illucens* more specifically, are sufficiently similar to crustaceans to be considered as a viable alternative.

Table 5.2 Average chitin content (%) of common crustaceans and molluscs used for the industrial production of chitin.

SOURCE	CHITIN (%)	REFERENCE
Crab shells	15-30	Kurita 2006; Antonov *et al*., 2019
Shrimp shells	10-35	Antonov *et al*., 2019
Antarctic krill (whole body)	3-5	Antonov *et al*., 2019
Clam/oyster shells	2-6	Kurita, 2006

5.2 Chitin purification from *H. illucens*

In the literature, there is a general lack of data providing a quantitative evaluation of the purification process of insect chitin. Most papers report only a qualitative assessment. Even less data is available on the extraction process of chitin from *H. illucens*.

5.2.1 Biomass recovery and chitin yield

The biomass recovery of insect samples in the intermediate steps of the chitin extraction process was rarely calculated. The values reported by Khayrova *et al.* (2019; 2020) are the only ones that can be used to compare data obtained from larvae, pupal exuviae and adults of *H. illucens*. Biomass recovery after demineralization of both larvae (66%) and pupal exuviae (77%) was similar or slightly higher than that obtained by Khayrova *et al.* (2019; 2020) (58 and 74%, respectively). In contrast, biomass recovery from adults was lower (68% vs 87%) (Khayrova *et al.* 2020). After deproteinization, 19, 41 and 14% of the biomass of larvae, exuviae and adults, respectively, was recovered (in relation to the demineralized biomass), all lower values than those reported in the literature: 46% for larvae (Khayrova *et al.* 2019), 77 and 24% for pupal exuviae and adults (Khayrova *et al.* 2020). Values reported by Hahn *et al.* (2020b) using larval exoskeletons of *H. illucens* (79% after demineralization and 34% after deproteinization) were close to those obtained from pupal exuviae. There may be a correlation between protein content of the starting sample and the biomass recovery after deproteinisation. Looking at the results of the present work, the sample with the highest protein content (adults) was the one with the lowest percentage of biomass recovered after deproteinisation. On the contrary, the pupal

exuviae with the lowest protein content gave the highest yield after deproteinisation. Final yield of bleached chitin, related to the original insect biomass, from larvae, pupal exuviae and adults was similar or higher than those obtained by other authors using the same species and developmental stage (table 5.3). In a similar range are the yield values obtained by Zhou *et al.* (2019) using natural deep eutectic solvents for the purification of chitin from *H. illucens* prepupae: 6-26%, depending on the reaction conditions used.

Table 5.3 Average yield (%) of both unbleached and bleached chitin obtained from larvae, pupal exuviae and adults of *H. illucens* (experimental data) compared to those reported in the literature for the same insect species and developmental stage.

	YIELD (%) UNBLEACHED CHITIN	YIELD (%) BLEACHED CHITIN	REFERENCE
LARVAE	13.0	10.0	Experimental data
	-	3.8	Smets *et al.*, 2020
	-	9.5	Soetemans *et al.*, 2020
	-	3.6	Wang *et al.*, 2020
PUPAL EXUVIAE	31.0	23.0	Experimental data
	-	25.0	Brigode *et al.*, 2020
	-	23.8	Soetemans *et al.*, 2020
	-	14.1	Wang *et al.*, 2020
	-	6.8	Złotko *et al.*, 2021
ADULTS	9.0	6.0	Experimental data
	-	8.0	Brigode *et al.*, 2020
	-	5.6	Soetemans *et al.*, 2020
	-	2.9	Wang *et al.*, 2020

In most cases, a yield between 5 and 15% is reported for bleached chitin extracted from insects (Hahn *et al*., 2020a). The highest values, ranging from 31 to 36%, were obtained from larval exoskeletons of *H. illucens* (Hahn *et al*., 2020b), cicada sloughs (Sajomsang and Gonil, 2010) and adults of *Apis mellifera* (Nemtsev *et al*., 2004).

The range of average chitin yields from crustaceans is wide (5-30%) (Tharanathan and Kittur, 2003; Oduor-Odeto et al., 2005; Bolat et al., 2010; Hossain and Iqbal, 2014). Only in a few instances, higher yields have been obtained from the purification of chitin from insects. In the majority of cases, these are lower than the maximum values indicated for crustaceans, but still within the average range. It should be considered that the yield of chitin can vary greatly depending on the species, the stage of development, the body part, and the extraction method used.

5.2.2 Efficiency of the purification process

Efficiency of the chitin purification process was evaluated, determining the effectivity of the demineralization and deproteinization treatments. Between 82 and 87% of the minerals were removed from larvae, pupal exuviae and adults with demineralization using formic acid. Only Hahn *et al*. (2020b) used formic acid for demineralization of *H. illucens* larval exoskeletons, removing 84% of minerals. A similar efficiency (84.1%) was achieved by Khayrova *et al.* (2020) using hydrochloric acid on *H. illucens* pupal exuviae, while only 47.5% minerals was removed from adult flies applying the same acid. The use of natural deep eutectic solvents allows a maximum demineralization efficiency of 98% on *H. illucens* prepupae to be achieved (Zhou *et al*., 2019). The use of an organic acid, with less environmental impact and less potential negative influence on the final chitin (Mahmoud *et al*., 2007), can therefore remove minerals from different types of *H. illucens* biomass with similar or higher efficiency than a mineral acid. This evidence is also supported by other examples. A treatment with oxalic acid at room temperature for 3 h removed a much higher percentage of minerals (85%) from house crickets (Ibitoye *et al*., 2018) than that achieved using hydrochloric acid under the same conditions on both houseflies (39.5%) (Kim *et al*., 2016) and the two-spotted cricket (8-21%) (Kim *et al*., 2017). Further data on the efficiency of insects' demineralisation is not available. The efficiency of organic acids in removing minerals was also demonstrated for crustaceans. Lactic acid was used on shrimp shells with an efficiency similar to hydrochloric acid (87 and 90%, respectively) (Mahmoud *et al*., 2007), while acetic acid and citric acid were slightly less efficient (73-75%) (Mahmoud et al., 2007; Ameh *et al*., 2014).

Concerning deproteinization, the use of 2 M NaOH for 2 h at 80 °C enables the removal of 92-97% of proteins from the three developmental stages of *H. illucens*. A similar result (97%) was achieved using natural deep eutectic solvents on *H. illucens* prepupae (Zhou *et al*., 2019). Treatment with 1.25 M NaOH for 3 h at 95 °C on houseflies and crickets had a slightly lower efficiency (86-87%) (Kim *et al*., 2016, 2017). No additional papers are available in which the efficiency of deproteinisation of insects was determined. For the chemical deproteinization of crustaceans (mainly crabs and shrimps), available efficiency values are between 71 and 78% using 1.25 M NaOH for 2 h at 90 °C (Cao, 2014; Younes *et al*., 2015) or 0.5 M NaOH for 2 h at room temperature (Khor and Lim, 2003).

In summary, the demineralisation and deproteinisation treatments applied in the present work were effective in removing mineral and protein components from larvae, pupal exuviae and adults of *H. illucens*. The efficiency of these treatments was comparable, or even better, to than that reported for both other insect species and crustaceans. However, there is very little data in

the literature on the efficiency of the chitin extraction process for comparison. A more in-depth study of these parameters would be recommended. It would be desirable, for instance, to always measure the removal efficiency of the components from the starting samples so that the parameters of the purification steps can be adjusted when necessary for maximum efficiency. Chitin purification from insects has not yet been fully optimised, but it has mainly been limited to applying the same treatments as for crustaceans. In the perspective of a scale-up of this process, a better adaptation of the existing protocols for crustaceans to insects would be needed.

5.2.3 Chitin purity and characteristics

Degree of purity

Purity of chitin extracted from the three developmental stages of *H. illucens*, expressed as chitin content, was similar to that determined for commercial chitin derived from crustaceans (table 4.5). The purification method applied in the present work allows the purity of chitin after each step of the purification process to be significantly increased, including the bleaching treatment (figure 4.3). Degree of purity of bleached chitin extracted from different insect species available in the literature is shown in table 5.4. No data was found on the purity of unbleached chitin. Overall, the degrees of purity of chitin do not greatly differ, mostly ranging from 85 to 97%.

Given the same insect biomass, differences in chitin purity observed in table 5.4 can be due to the different purification treatments applied. Except for Hahn *et al.* (2020b), in none of the other works an organic acid is used for demineralisation, but always HCl. Furthermore, duration of the deproteinization step is often different from the one adopted in this work. Very long times (12-16 h) were used by Kaya *et al.* (2016b) and Soetemans *et al.* (2020), while only 35 min was the reaction duration applied by Huet *et al.* (2020). In another case, completely different reagents (natural deep eutectic solvents) were used for both minerals and proteins removal (Zhou *et al.,* 2019). Different reagents and reaction durations can give different chitin purity degree.

Different methods used to calculate the degree of purity of chitin can also determined different results. For instance, Soetemans *et al.* (2020) calculated the chitin content of the purified chitin trhough determination of the glucosamine concentration by chromatography combined to mass spectrometry after hydrolysis of the polymer. Different methods have different accuracy and sensitivity and can lead to variations in the result.

Purity degree of chitin obtained from *H. illucens* in this work could be improved by acting on the efficiency of the individual extraction steps. For instance, a change in the demineralisation parameters could be tried to achieve a mineral removal of more than 90%. Various bleaching

treatments could also be tried to allow the removal of most catechol compounds. Another important factor is the particle size of the starting insect biomass. About 34% of the sample used had a particle size above 315 µm, and 46% between 315 and 180 µm. The particle size considered optimal for a good solvent penetration into the particles is below 200 µm (Draczynski, 2008; Marei *et al.*, 2016; Mehranian *et al.*, 2017). A more performing grinding tool could be searched for in order to reduce as much as possible the fraction of the starting sample with a large particle size.

However, there is very little quantitative data on the purity of chitin produced from *H. illucens* available and in general from insects. This makes it difficult to compare and accurately assess the suitability of the purification methods used.

Table 5.4 Degree of purity (%) of bleached chitin extracted from different insect species compared to a commercial sample produced from crustaceans.

SOURCE		CHITIN PURITY (%)	REFERENCE
H. illucens	Larvae	84	Experimental data
	Pupal exuviae	87	
	Adults	85	
H. illucens	Larval exoskeletons	89	Hahn *et al*., 2020b
H. illucens	Larvae	96	Soetemans *et al*., 2020
	Prepupae	94	
	Pupae	94	
	Larval exoskeletons	76	
	Pupal exuviae	97	
	Adults	96	
H. illucens	Prepupae	74-91	Zhou *et al*., 2019
B. eri	Larvae	93	Huet *et al*., 2020
V. crabro	Larvae	97	Kaya *et al*., 2016b
	Pupae	97	
	Adults	95	
Crustaceans	Commercial sample	88	Experimental data

FTIR spectra and degree of acetylation (DA)

In order to further characterize chitin produced from *H. illucens*, FTIR analysis was performed, mainly to identify chitin- and chitosan-related functional groups to confirm the identity of the polymer and the chitin form (α, β or ɣ). The degree of acetylation (DA) of chitin was also determined. All the chitin samples produced in the present work exhibited the bands at the characteristic wavelengths, hence the identity of the chitin polymer was confirmed. According to Kumirska *et al*. (2010) and Kaya *et al*. (2015a), the split of the amide I band into two peaks

around wavelengths 1650-1620 cm^{-1} confirmed the α-form of chitin produced from *H. illucens*. The spectra of the examined samples showed a structural similarity with commercial chitin derived from crustaceans, but there are some differences in peak wavelengths, probably due to the different source and extraction process applied. DA did not significantly differ between bleached and unbleached chitin samples, although unbleached chitin from both pupal exuviae and adults were slightly more acetylated than the respective bleached samples. This suggests that the bleaching treatment have a slight deacetylation effect on the chitin. It is known that the bleaching of chitin can affect characteristics of the chitosan derived from it, especially its viscosity (Nemtsev *et al.*, 2004; Yeul and Rayalu, 2012). Since viscosity also depends on the degree of deacetylation, in addition to the molecular weight, a different DA of the chitin due to bleaching may affect the final chitosan (Wang and Deshi, 1994). The results obtained for chitin from *H. illucens* were compared with those available in the literature for chitin produced from the same dipteran and also from other insect species, considering only the DA determined by FTIR. In most cases, DA of bleached chitin extracted from insects was between 80 and 100%, as well as the DA of chitin derived from crustaceans (table 5.5). In some instances, a DA higher than 100% was recorded. A DA value higher than 100% suggests that some inorganic materials remain in the chitin, namely nitrogen-free impurities (Sajomsang and Gonil, 2010), such as lipids and sugars, which can be contained in consistent percentages in insect samples (Laroche *et al.*, 2019).

In summary, the purification method applied allows to obtain chitin from the different *H. illucens* samples with a DA generally comparable to that of commercial chitin, and DA values below 100% suggest that the initial insect samples were sufficiently purified.

Table 5.5 Acetylation degree (DA) of bleached chitin purified from *H. illucens* and other insect species, and from crustaceans determined by FTIR analysis.

SOURCE		CHITIN DA (%)	REFERENCE
H. illucens	Larvae	94	Experimental data
	Pupal exuviae	89	
	Adults	96	
H. illucens	Prepupae	67	Jayanegara *et al*., 2020
H. illucens	Pupal exuviae	86	Purkayastha and Sarkar, 2020
	Adults	115	
H. illucens	Larvae	89	Smets *et al*., 2020
	Prepupae	90	
	Pupae	92	
H. illucens	Larvae	92	Soetemans *et al*., 2020
	Prepupae	78	
	Pupae	97	
	Larval exoskeletons	93	
	Pupal exuviae	90	
S. gregarea	Adults	63	Badawy and Mohamed, 2015
N. viridula	Adults	72	
P. americana	Adults	70	
B. germanica	Adults	63	
V. orientalis	Adults	59	
P. algerinum	Adults	71	
R. linearis	Adults	133	Kaya *et al*., 2014c
H. piceus	Adults	121	
N. glauca	Adults	120	
A. bipustulatus	Adults	117	
A. imperator	Adults	86	
A. aquaticus	Adults	86	
H. parallela	Adults	93	Liu *et al*., 2012
B. terrestris	Adults	87	Majtan *et al*., 2007
E. kuehniella	Adults	71	Mehranian *et al*., 2017
Crustaceans	Commercial sample	91	Experimental data
Shrimps		94	Liu *et al*., 2012
		99	Majtan *et al*., 2007
		71	Mehranian *et al*., 2017

XRD spectra and crystallinity

XRD was performed to determine chitin crystallinity. All the significant peaks were found in spectra deriving from XRD performed on all chitin samples, confirming again the α-form of the polymer. In insects, α-chitin is the most common form (Sajomsang and Gonil, 2010). Crystallinity of bleached chitins produced from larvae, pupal exuviae and adults of *H. illucens* was lower, although not significantly, than that of unbleached samples. This suggests a possible

detrimental effect of the bleaching treatment on the crystalline structure of chitin. A reduction in crystallinity due to bleaching was also reported for chitin extracted from shrimp shells (Aranday-García *et al*., 2019). Indeed, all the steps in the chitin chemical extraction process inevitably led to a partial deacetylation and hydrolysis of the polymer, thus reducing its degree of acetylation, molecular weight and even crystallinity (Pacheco *et al*., 2011; Younes and Rinaudo, 2015).

Crystallinity values of bleached chitin purified from *H. illucens*, as well as from different insect species and crustaceans, available in the literature, are provided in table 5.6. Adults' chitin was the most crystalline, similarly to the commercial one, while the lowest crystallinity was found for chitin extracted from pupal exuviae. Similar results were reported also by other authors for chitin produced from different developmental stages of *H. illucens*, with adult chitin always being more crystalline than chitin from other stages, particularly pupal exuviae (Brigode *et al*., 2020; Purkayastha and Sarkar, 2020; Wang *et al*., 2020) (table 5.6). It was inferred that the structure of chitin becomes more ordered, thus showing greater crystallinity, during the development of the dipteran from pupa to adult (Purkayastha and Sarkar, 2020), giving greater mechanical strength and providing the requisite mechanical properties for muscle attachment and locomotion (Hopkins and Kramer, 1992). The crystallinity index of crustacean-derived chitin varies widely, depending on the extraction method, pretreatment and storage conditions, but mainly ranges between 60 and 80% (Hahn *et al*., 2020a). The crystallinity indices reported for insect chitin, as well as those determined for the samples in the present work, are mostly in this range (table 5.6). Particularly lower values were found for chitin extracted from two wasp species (Kaya *et al*., 2015c), and for some samples of larvae, prepupae and adults of *H. illucens* (Wasko *et al*., 2016; Zhou *et al*., 2019; Purkayastha and Sarkar, 2020; Wang *et al*., 2020). A low crystallinity of chitin can be beneficial as it makes the polymer generally more accessible for enzymatic and chemical reactions and increases its sorption ability (Minke and Blackwell, 1978), while it is a negative factor for other purposes such as the production of nanofibrils for which a high crystallinity is required (Morganti and Morganti, 2008).

Table 5.6 Crystallinity indexes (CrI %) of bleached chitin purified from *H. illucens* and other insect species, and commercial chitin determined by XRD.

SOURCE		CHITIN CrI (%)	REFERENCE
H. illucens	Larvae	84	Experimental data
	Pupal exuviae	62	
	Adults	93	
H. illucens	Pupal exuviae	74	Brigode *et al*., 2020
	Adults	78	
H. illucens	Pupal exuviae	25	Purkayastha and Sarkar, 2020
	Adults	49	
H. illucens	Larvae	89	Soetemans *et al*., 2020
	Pupal exuviae	95	
	Adults	90	
H. illucens	Larvae	33	Wang *et al*., 2020
	Pupal exuviae	68	
	Adults	88	
H. illucens	Larvae	35	Wasko *et al*., 2016
	Adults	25	
H. illucens	Prepupae	51	Zhou *et al*., 2019
H. illucens	Pupal exuviae	60	Złotko *et al.* 2021
S. gregarea	Adults	71	Badawy and Mohamed, 2015
B. germanica	Adults	72	
V. orientalis	Adults	64	
D. maroccanus	Adults	71	Erdogan and Kaya, 2016
A. domesticus	Adults	88	Ibitoye *et al*., 2018
M. melontha	Adults	75	Kaya *et al*., 2014a
R. linearis	Adults	85	Kaya *et al*., 2014c
H. piceus	Adults	89	
N. glauca	Adults	87	
A. bipustulatus	Adults	91	
A. aquaticus	Adults	77	
L. decemlineata	Adults	76	Kaya *et al*., 2014d
P. americana	Adults	87	Kaya and Baran, 2015
V. crabro	Adults	70	Kaya *et al*., 2015c
V. orientalis	Adults	54	
V. germanica	Adults	50	
Ailopus spp.	Adults	75-76	Kaya *et al*., 2015d
Oedipoda spp.	Adults	74	
P. cognata	Adults	63	
H. parallela	Adults	89	Liu *et al*., 2012
Cicada	Sloughs	83	Sajomsang and Gonil, 2010
Z. morio	Larvae	68	Soon *et al*., 2018
B. mori	Larvae	60	Zhang *et al*., 2000
Crustaceans	Commercial chitin	98	Experimental data

In summary, spectroscopic and diffractometric characterisation of chitin extracted from *H. illucens* revealed good similarity both to chitins produced from other insects and to commercial chitin derived from crustaceans. The results of the determination of the degree of acetylation and crystallinity were useful to reveal a possible effect, wheter positive or negative, of the bleaching treatment on some chitin characteristics. Even in the case of a worsening of certain characteristics in relation to the application, this negative effect could however be compensated by a higher degree of purity of the polymer obtained after decolourisation.

5.3 Chitosan from *H. illucens*

Chitin extracted from larvae, pupal exuviae and adults of *H. illucens* (both bleached and unbleached) was deacetylated into chitosan through chemical heterogeneous deacetylation. A part of chitin from pupal exuviae was also subjected to homogeneous deacetylation. Various analyses were then performed on the different chitosan samples for its characterization.
Very few papers currently in the literature report on the production of chitosan from insects. Among these, only four have investigated chitosan production from *H. illucens*. These works all described only heterogeneous chemical deacetylation. Only in one case homogeneous deacetylation was performed on chitin from larval exoskeletons of *H. illucens* (Hahn et al., 2020b).

5.3.1 Biomass recovery and chitosan yield

Biomass recovery after deacetylation (ratio between dry weights of chitin and the respective chitosan) was always higher for bleached chitin samples than the unbleached ones. The yield of chitosan related to the original insect biomass followed the same trend, but with a smaller gap between bleached and unbleached chitosan (table 4.6). The highest yields were achieved for chitosan from *H. illucens* pupal exuviae, both heterogeneous and homogeneous (10%).
Yield of chitosan derived from *H. illucens* by heterogeneous deacetylation was reported only by Khayrova *et al*. (2019) for larvae (53% related to chitin) and Hahn *et al*. (2020b) for larval exoskeletons (47% related to chitin and 16% related to the initial biomass). Generally, yield of chitosan produced from insects ranged from 2 to 8%, related to the original biomass (60-83% in relation to the respective chitin) (Hahn *et al*., 2020a). The results obtained in the present work are in this range. The yield of bleached chitosan from pupal exuviae obtained by homogeneous deacetylation was more than double that reported by Hahn *et al*. (2020b) (10% versus 4%,

respectively). Given the same deacetylation conditions, this difference may be due to the different treatments previously applied for chitin purification. Since a higher chitin yield corresponds to a higher chitosan yield, the whole chitin purification process can affect the final chitosan yield. Likewise, chitin yield also depends on the species, developmental stage and body part of the starting source (Bolat *et al.*, 2010; Kaya *et al.*, 2015e; Erdogan and Kaya, 2016; Kaya *et al.*, 2016b). Hence, the chitosan yield can also be affected by the different starting biomass.

There are no other works reporting homogeneous deacetylation of chitin from insects for comparison. Homogeneous deacetylation was attempted also for crustacean-based chitin, in order to obtain chitosan with a more regular distribution of the residual acetamide groups than that obtained under heterogeneous conditions (Nemtsev *et al*., 2002; Younes *et al*., 2014). Applying high temperature, deacetylation proceeds more rapidly in the amorphous region of chitin than in the crystalline areas. This results in a block distribution of the acetamide and primary amine groups along the chitosan chain. Incubation of chitin in alkali at low temperature, in contrast, leads to swelling and activation of the polymer which becomes amorphous, allowing a similar rate of deacetylation in all regions of the chitin chain (Nemtsev *et al*., 2002). However, there is no information available on the chitosan yield obtained with this method.

For heterogeneous chitosan derived from crustaceans, yield usually varies from 4 to 15% (related to the original biomass) (Oduor-Odeto *et al*., 2005; Thirunavukkarasu and Shanmugam, 2009; Bolat *et al*., 2010; Hossain and Iqbal, 2014; Luo *et al*., 2019), values slightly higher than those obtained from insects. It should be considered that insect biomass generally has a higher content of proteins and lipids than crustaceans (de Castro *et al*., 2018), extraneous components to chitin and chitosan that can lower the final polymer yield. However, as reported for chitin, chitosan yield can be affected by various factors, including the source, the purification method and the deacetylation treatment applied to chitin (Younes *et al*., 2014).

The choice of the deacetylation method should be made by considering various factors, including time, energy consumption and characteristics of the final chitosan. The reaction of heterogeneous deacetylation requires high temperature but it lasts few hours. For the homogeneous deacetylation the cost of maintaining the reaction at refrigeration temperature for few days has to be borne. The great time consumption is one of the main reasons why homogeneous deacetylation is not considered suitable for the production of chitosan on an industrial scale (Lamarque *et al*., 2005). On the other hand, heterogeneous deacetylation inevitably leads to partial hydrolysis and depolymerisation of the chitosan. Reaction parameters need to be adjusted to minimised these side effects. Therefore, a choice must be made between

the two deacetylation methods according to the specific needs, taking all these factors into account.

5.3.2 Chitosan characteristics

As for chitin, FTIR and XRD analysis were performed on chitosan samples obtained from *H. illucens.* Furthermore, deacetylation degree (DD), intrinsic viscosity and viscosity average molecular weight (M_v) were determined.

It was evident how challenging the characterisation of this polymer is, given the many factors that can influence the various characteristics of chitosan. The DD, viscosity and M_v are interrelated, each influencing the other parameters in different ways. And each of these characteristics in turn influences the properties and activities of chitosan in a different way. The characteristics to be preferred are not always the same, because the most suitable chitosan qualities change according to its application.

FTIR spectra

Concerning FTIR spectra, the significant bands (already described for chitin) were detected in all chitosan samples at the characteristic wavelengths, confirming the identity of the polymer. The structural similarity between chitosan produced from *H. illucens* and the commercial one was also assessed. Extraneous signals found in the spectrum of unbleached chitosan from larvae were probably due to residual contaminants that were not removed by the chitosan purification process or by inadequate washing in the final step. A qualitative evaluation of the effectivity of the deacetylation of chitin can be done by observing the intensity of bands at 1655 (amide I) and 1590 cm^{-1} (NH_2 bending). During deacetylation, the band at 1655 cm^{-1} gradually decreased, while that at 1590 cm^{-1} increased, indicating the prevalence of NH_2 groups (Kumirska *et al*., 2010). According to this, the homogeneous chitosan from pupal exuviae appeared less deacetylated than the respective heterogeneous sample, whether bleached or not, since the band at 1590 cm^{-1} had a lower intensity than the one at 1655 cm^{-1}. With proper deacetylation, the band at 1590 cm^{-1} must have a higher intensity than that at 1650 cm^{-1} (Kumirska *et al*., 2010; Monter-Miranda *et al*., 2016; Shin *et al*., 2018; Luo *et al*., 2019).

XRD spectra and crystallinity

XRD was performed to determine chitosan crystallinity. Characteristic peaks detectable in the diffractogram are similar between chitin and chitosan, and those identified for insect-based chitosan are comparable to peaks determined for the commercial polymer derived from crustaceans (Chae *et al*., 2018). Diffractograms of chitosan differs from those of chitin for the lower intensity of the relevant peaks determining crystallinity (Kumirska *et al*., 2010). This is

due to the deacetylation treatment that reduces the amount and size of crystalline regions along the polymer chain, resulting in a lower crystallinity of chitosan comparing to chitin (Mogilevskaya *et al*., 2006). Chitosan samples produced from *H. illucens* had the main characteristic peaks at 2θ of around 10° and 20°, in accordance with the literature (Erdogan and Kaya, 2016; Marei *et al*., 2016; Luo *et al*., 2019). Only for homogeneous chitosan produced from pupal exuviae, both unbleached and bleached, it was not possible to detect the typical chitosan signals in their diffractograms. This problem may be due to something interfering with the diffractometric analysis. Bleached chitosan from larvae also gave a diffractogram with signals overlapping those of the polymer, which may be attributed to contaminants.

Crystallinity indexes (CrI) of all samples ranged from 74 to 86%, with the bleached chitosan always being slightly more crystalline than the unbleached ones, although not significantly (table 4.9). Due to the lack of studies on the effect of chitin bleaching on the crystallinity of chitosan, it was not possible to compare this datum with others in the literature. Among the few papers reporting the production of chitosan from *H. illucens*, the degree of crystallinity has never been calculated. CrI of insect-based chitosan and commercial samples available in the literature are provided in table 5.7. The crystallinity of chitosan obtained from *H. illucens* was higher than that reported for the other insects and more similar to that of commercial one. As observed for chitin, bleached chitosan from adults was the most crystalline. This was also found by Shin *et al*. (2018), but for adults and larvae of two different species (*A. dichotoma* and *T. molitor*, respectively) (table 5.7). The lower crystallinity of insect-derived chitosan can be an advantageous feature for various applications, as it facilitates its solubilisation in acidic solutions, increases its sorption capacity and increases the accessibility of free amino groups along the polymer chain (Mourya and Inamdar, 2008; Ioelovich, 2014).

Table 5.7 Crystallinity indexes (CrI) of heterogeneous chitosan produced from different insect species, and commercial chitosan determined by XRD (only chitosans derived from bleached chitin are reported).

SOURCE		CHITOSAN CrI (%)	REFERENCE
H. illucens	Larvae	77	Experimental data
	Pupal exuviae	80	
	Adults	86	
G. bimaculatus	Adults	57	Chae *et al*., 2018
A. domesticus	Adults	87	Ibitoye *et al*., 2018
Cicada	Sloughs	65	Luo *et al*., 2019
B. mori	Chrysalis	33	
T. molitor	Larvae	52	
Grasshopper	Adults	50	
C. molossus	Adults	64	Ma *et al*., 2015
S. gregarea	Adults	69	Marei *et al*., 2016
A. mellifera	Adults	59	
C. rugosa	Adults	49	
T. molitor	Larvae	58	Shin *et al*., 2018
A. dichotoma	Adults	63	
Crustaceans	Commercial sample	79	Experimental data
		94	Ibitoye *et al*., 2018
		85	Kaya *et al*., 2014d
		61	Marei *et al*., 2016

Deacetylation degree (DD)

Chitosan DD was determined by potentiometric titration. DD of all chitosan samples produced from *H. illucens* was around 90%, similarly to that of commercial chitosan, except for the two unbleached chitosan from pupal exuviae for which a lower DD was measured (83% the heterogeneous and 62% the homogeneous) (table 4.10). Thus, the homogeneous deacetylation appeared to be less effective in removing acetyl groups from the chitin chain. The same effect was observed by Hahn *et al*. (2020b) for *H. illucens* larval exoskeletons, achieving 72% DD with heterogeneous deacetylation and only 34% with the homogeneous treatment. Also for chitosan derived from crustaceans, the average DD obtained by homogeneous deacetylation is low (around 55%) (Younes and Rinaudo, 2015). Concerning heterogeneous chitosan, the DD reported by other authors for the chitosan produced from *H. illucens* is similar to that measured in the present work, ranging from 85 to 90% (Khayrova *et al*., 2019; Soetemans *et al*., 2020). In most cases, chitosan from insects has a DD between 62 and 98% (Hahn *et al*., 2020b), in accordance with the average DD reported for crustacean-derived chitosan (56-98%) (Islam *et al*., 2017). It is known that the DD of chitosan varies with the deacetylation conditions applied, primarily the temperature, but also the duration of the reaction and the NaOH concentration.

Generally, in the heterogeneous deacetylation, higher temperatures can increase the DD (Yeul and Rayalu, 2012). Comparing the results of the DD determination with those of CrI, lower crystallinity should be observed as the chitosan DD increases, since the deacetylation reaction reduces the amount and size of crystalline regions (Mogilevskaya *et al*., 2006). This relation was not detected for the chitosan samples obtained in the present work. There was rather a tendency for samples with lower CrI to also show lower DD, but the differences were not statistically significant. This tendency was also found in some works in the literature, although, even in these cases, no statistically significant differences were reported. For instance, chitosan purified from silkworm, mealworm and grasshopper had a DD of 85, 86 and 90%, which corresponded to a CrI of 33, 59 and 50%, respectively (Luo *et al*., 2019). Similarly, Marei *et al*. (2016) produced chitosan from *Schistocerca gregarea*, *A. mellifera* and *Calosoma rugosa* with DDs of 98, 96 and 95%, and respective CrIs of 69, 59 and 49%. For chitosan produced from crustaceans was instead found that as the degree of deacetylation increases, the crystallinity of chitosan decreases (Kumirska *et al*., 2010). For instance, Kumari *et al*. (2017) produced chitosan from three different marine sources (shrimp, fish and crab) with DDs of 82, 84 and 88% and respective CrIs of 78, 75 and 70%. It is not clear whether this difference is due to the different source or whether it is random, since other characteristics of chitosan, primarily molecular weight, also can have an influence on its crystallinity (Kumirska *et al*., 2010).

Viscosity

Chitosan viscosity is generally measured in order to determine its $M_v$and in many papers on the purification of insect-based chitosan, viscosity values are not reported. Nevertheless, viscosity itself is an important parameter for various applications of chitosan. In the textile industry, as an example, chitosan is used to improve the dyeability fo wool and to confer shrink-resist properties (Pascual and Julià, 2001). The viscosity of chitosan solutions is a determining factor for the felting properties of wool, as fabric shrinkage decreased as the viscosity increased. The viscosity also influences the dyeing rate of the treated fabric: the lower the viscosity of the chitosan solution, the faster was the dyeing kinetic (Pascual and Julià, 2001). Viscosity is also an important characteristic for the use of chitosan solutions as a preservative coating for food. Indeed, the viscosity of the chitosan-coating solutions can affect the adhesion, thickness and permeability of the coating. In general, the higher the viscosity, the more easily the chitosan solution can adhere to the surface of the treated product (Liu *et al*., 2020). High viscosity chitosan solutions are also able to form a thicker coating and are more effective in extending the shelf-life of the food product (Du *et al*., 1998).

For chitosan solutions, dynamic or intrinsic viscosity are generally determined (Hwang and

Shin, 2000). Data of viscosity of chitosan purified from insects available in the literature are showed in table 5.8. The intrinsic viscosity of chitosan produced from *H. illucens* is lower than that reported for both chitosan derived from other insect species and commercial chitosan. One of the main chitosan characteristics affecting the viscosity is its molecular weight: lower molecular weights correspond to lower viscosity (Chattopadhyay and Inamdar, 2010). Chitosan samples produced from *H. illucens* had also a low M_v, confirming this relation. Another factor that has degrading effect on chitosan is the free access of oxygen to chitin during the deacetylation reaction. Deacetylation in the presence of nitrogen yielded chitosan of higher viscosity and molecular weight than in air (Yeul and Rayalu, 2013). Homogeneous chitosan from bleached pupal exuviae was slightly less viscous than the respective heterogeneous sample, while the viscosity was higher in the homogeneous chitosan for the unbleached samples. The latter result is in accordance with that reported by Hahn *et al*. (2020b) for chitosan produced from *H. illucens* larval exoskeletons, for which the shear viscosity of the homogeneous sample was almost double that of the heterogeneous chitosan (509 and 277 mPa·s, respectively) (table 5.8). Except for the heterogeneous chitosan from pupal exuviae, the bleached samples were less viscous than the unbleached ones. This is in accordance with Nemtsev *et al*. (2004), who reported a higher dynamic viscosity for chitosan obtained from unbleached chitin from honeybees compared to the sample produced from bleached chitin (5-10 and 3-4.5 mPa·s, respectively). It is known that the bleaching treatment applied to chitin generally decreases the viscosity of the derived chitosan (Moorjani, 1975; Yeul and Rayalu, 2013).

Table 5.8 Intrinsic viscosity (dl/g) of chitosan produced from different insect species, and commercial chitosan (unless otherwise stated, consider chitosan produced by heterogeneous deacetylation of bleached chitin).

SOURCE		VISCOSITY	REFERENCE
H. illucens	Larvae	0.6 dl/g	Experimental data
	Pupal exuviae	1.7 dl/g	
	Pupal exuviae homogeneous	1.2 dl/g	
	Adults	0.9 dl/g	
C. molossus	Adults	5.2 dl/g	Ma *et al*., 2015
B. mori	Chrysalis	20.7-33.5 dl/g	Paulino *et al*., 2006
C. megacephala	Larvae	3.6 dl/g	Song *et al*., 2013
Crustaceans	Commercial sample	7.5 dl/g	Experimental data
		13.2 dl/g	Hossain and Iqbal, 2014
		3.7 dl/g	Ma *et al*., 2015
		2.6-4.5 dl/g	Paulino *et al*., 2006

However, viscosity of chitosan solutions is highly variable, depending on various different factors, mainly chitosan DD and molecular weight. Generally, the viscosity decreases as the DD increases, as observed from the results obtained in the present work. This finding is confirmed by other authors who reported consistently higher viscosity for samples with lower DD, given the same purification treatments applied to insect-derived chitin and chitosan (Nemtsev *et al*., 2004; Kim *et al*., 2016, 2017; Hanh *et al*., 2020b). It was reported that higher DD indicates higher ratio of amino/acetyl groups in the chitosan chain due to breakage of the *N*-acetylglucosamine chains, and thus resulted in lower viscosity (Habibaa *et al*., 2017). Highly viscous solutions were usually provided by chitosan with high molecular weight (Bajaj *et al*., 2011; Hossain and Iqbal, 2014), being the intrinsic viscosity of a polymer accurately proportional to the square-root of its molecular weight (see equation 27). Our results are in accordance with this evidence. A comparison with the literature on insect-derived chitosan is not possible, as neither molecular weight nor viscosity have ever been measured in the same work. Low viscosity was associated also with non-optimal purity of chitosan. The presence of residual substances, such as ashes, may indeed adversely affect the solubility of chitosan and lower its viscosity (Hossain and Iqbal, 2014). This could be an explanation for the very low viscosity measured in the bleached chitosan from larvae, for which the presence of contaminants was detected by XRD analysis. Concerning purification treatments, both long demineralisation times and low chitin deproteinisation temperatures can reduce the viscosity of the derived chitosan (Moorjani, 1975; Bajaj *et al*., 2011). In addition, severe deacetylation conditions can reduce the viscosity, due to the breaking of chitosan polymer during reaction with concentrated and hot NaOH for a long incubation time (more than 3 h) (Bajaj *et al*., 2011). Reducing the access of oxygen to chitin during deacetylation is also important to reduce the polymer degradation. $NaBH_4$ is thus often used in the reaction as reducing protective reagent to reduce the degradation effect of the oxygen. Another solution is performed the deacetylation under controlled atmosphere in presence of nitrogen (Nemtsev *et al*., 2004; Yeul and Rayalu, 2013).

Viscosity-average molecular weight (M_v)

Molecular weight of chitosan produced from *H. illucens* was determined via viscometry. Viscometry is a simple and rapid method, widely used for determining the M_v of chitosan (Norzita *et al*., 2011). Since it is calculated from the intrinsic viscosity value, the M_v results followed the same trend as the viscosity, i.e., M_v was lower for bleached chitosan than for the unbleached, and for the unbleached heterogeneous sample than the homogeneous one (table 4.11). M_v of all chitosan samples (from 21 to 92 kDa) was much lower than that of commercial

chitosan (376 kDa). Previous studies reported a M_v for insect chitosan in the range of 426-450 kDa (Ai *et al*., 2008; Ma *et al*., 2015). Considering the molecular weight determined by chromatographic techniques, chitosan from insects ranges from 26 to 300 kDa (Hahn *et al*., 2020a), whereas chitosan from crustaceans is reported to range from 100 to 1000 kDa (Hossain and Iqbal, 2014; de Queiroz Antonino *et al*., 2017). Much lower values of both M_v and molecular weight, between 3 and 10 kDa, have also been reported for insect chitosan, which are more similar to those obtained in the present work, although lower (Kaya *et al*., 2014d; Erdogan and Kaya, 2016; Tan *et al*., 2018).

Chitosan molecular weight varies greatly, depending mainly on the source and the deacetylation conditions applied. Extremely low molecular weight can be due to a polymer degradation caused by harsh deacetylation conditions, such as incubation time over 4 h combined with high reaction temperature (150 °C) (Hossain and Iqbal, 2014; Hahn *et al*., 2020a). Molecular weight can greatly affect physicochemical properties and biological activity of chitosan. It is generally reported that chitosan with low molecular weight (< 150 kDa) has better antibacterial properties than high-molecular weight chitosan, since it can easier cross the cell wall of bacteria (Zivanovic *et al*., 2004; Vishu Kumar *et al*., 2005). On the contrary, for application as preservative coating of fruit, high-molecular weight chitosan usually gave better results, being more effective in delaying ripening and maintaining the postharvest quality of coated fruit (Jongsri *et al*., 2016; Liu *et al*., 2020). Liquid coatings based on high-molecular weight chitosan are able to seal the fruit peel, thus creating a modified internal atmosphere with the reduction of ethylene production and occurrence of disease (Jongsri *et al*., 2016). For chitosan solid films, the mechanical and barrier properties, wettability and stability are improved by a high molecular weight (Liu *et al*., 2020).

Film forming ability

All chitosan samples produced from *H. illucens* larvae, pupal exuviae and adults were able to form uniform homogeneous films by solubilization in 1% acetic acid solution. Films made from homogeneous chitosan were the most fragile. For applications where good film forming ability is required, therefore, heterogeneous chitosan is likely to be preferred.

5.4 Effect of chitosan-based coatings on preservation of fresh fruit

Chitosan samples produced from larvae, pupal exuviae and adults of *H. illucens* were used to prepare coating solutions for the preservation of different fresh fruits. The experimental use of

chitosan-based coatings to extend the shelf life of various fresh commodities, especially fruit and vegetables, has been largely studied in recent years. So far, only the use of commercial chitosan derived from crustaceans has been tested. To the best of our knowledge, chitosan produced from insects has never been investigated for this application.

In order to assess the effect of chitosan solutions on the postharvest quality of tomatoes, apricots, strawberries and nectarines, different parameters were evaluated: weight loss, change in TSS, pH and TA, and the occurrence of fungal decay during storage. The results were compared with the available literature, taking into account the different initial source of chitosan.

5.4.1 Weight loss

Tomatoes, apricots, strawberries and nectarines have all lost weight during storage, both at room and cold temperature. Loss of weight occurs in fresh fruits and vegetables, mainly due to transpiration and respiration processes that cause moisture evaporation between the fruit tissue and surrounding air (Hernández-Munoz *et al*., 2006; Petriccione *et al*., 2015).

Tomatoes

In tomatoes, no chitosan treatment was effective in significantly reducing weight loss compared to the negative control, irrespective of coating method and storage conditions. However, at room temperature, all chitosan treatments (except the 1% homogeneous bleached) reduced the fruits weight loss significantly more than the solvent-only control. At all storage conditions, homogeneous chitosan performed worse than both the heterogeneous and the solvent control, having caused in most cases a greater weight loss than the negative control. The homogeneous samples had a lower DD than the heterogeneous ones. According to the literature, this may have negatively affected the barrier properties of the chitosan coating (Jongsri *et al*., 2016). This result deterred us from using homogeneous chitosan in subsequent experiments with the other fruits.

Concerning the method of application of the coating, with dipping all tomatoes lost significantly more weight at room temperature than at cold temperature. In sprayed tomatoes this difference was attenuated: in most cases, the weight loss did not differ between the two storage temperatures. Only in the sprayed tomatoes treated with homogeneous chitosan the weight loss was still signifycantly higher at room temperature than at cold temperature, confirming the lower barrier effect of this chitosan. This suggested a better effectiveness of the spraying coating than the dipping application. Therefore, spraying was the method of choice for subsequent experiments, also considering the shorter time required for treatment. Another data

supporting the preference for spraying is that, at the same storage temperature, weight loss was higher in tomatoes treated by dipping than by spraying. Although mostly the fruits to be treated are dipped in the coating solutions, in many cases spraying is employed, using either hand sprayers or electrostatic sprayers (Reddy *et al*., 2000; Meng *et al*., 2008; Darmawati *et al*., 2019; Jiang *et al*., 2019, 2020), but no comparison between the two application methods was provided. Generally, a reduction in weight loss is reported in tomatoes coated with chitosan solutions compared to untreated fruit, whether stored at room or cold temperature (El-Ghaouth *et al*., 1992; Barreto *et al*., 2016; Sucharita *et al*., 2018), as coatings confer a physical barrier to moisture loss, retarding dehydration and fruits shrivelling (Hernández-Munoz *et al*., 2006; Petriccione *et al*., 2015). Jongsri *et al*. (2016) compared the preservation effect of chitosan with different molecular weights, finding a better barrier action with higher molecular weight and higher viscosity chitosan solutions. In contrast, no difference in weight loss reduction of nectarines was found between high and low molecular weight chitosan by Zhang *et al*. (2019). The chitosan used in the present work had a low molecular weight and viscosity. This may have hindered the formation of a proper barrier on the surface of the treated tomatoes, thus affecting the moisture-retaining effect of the coating. However, since the solvent-only treatment caused a greater weight loss than the negative control, while the heterogeneous chitosan from pupal exuviae gave the same weight loss as the control, a non-optimal formulation of the solvent solution could be speculated. If this were true, chitosan would have mitigated the detrimental effect of the solvent, improving its performance in reducing tomato weight loss. The composition of the solvent used in this work (i.e., 1% acetic acid, 0.2% Tween-80 and 2% glycerol) is in accordance with the literature (Ramirez *et al*., 2015; Sucharita *et al*., 2018; Hassan *et al*., 2020; Jiang *et al*., 2020), but a modification of the components or their concentrations may be necessary.

Apricots

As for tomatoes, with the room temperature storage, no treatment, including solvent-alone, reduced the weight loss of the apricots compared to the negative control. At cold temperature, in contrast, almost all chitosan treatments significantly reduced the weight loss compared to both the negative and the solvent-only control.

In a few papers, chitosan has been used as a coating for apricots. Ghasemnezhad *et al*. (2010) tested crustacean derived chitosan comparing three different concentrations (0.25, 0.5 and 0.75%). They found only a slight reduction in weight loss at the lowest concentration in apricots stored at cold temperature, while chitosan at 0.5 and 0.75% had no effect. In the other available works, chitosan was used in combination with other components, including soy protein, gums

and zinc, to investigate their synergistic effect on maintaining the postharvest quality of apricots (Younas *et al*., 2014; Zhang *et al*., 2018; Ziaolhagh and Kanani, 2021). In all cases, the chitosan-compound solutions significantly reduced the weight loss compared to both the negative control and the treatment with the additional component alone. It should be noted that in all of the cited papers, the apricots were stored at a low temperature. Thus, the reason why our chitosan had no effect at room temperature could probably be due to the storage temperature itself.

Strawberries

As happened in apricots, at room temperature storage, the chitosan coating on strawberries gave no different effect from the negative controlBenhabiles *et al*. (2013) stored strawberries at room temperature and observed a significant reduction in weight loss in those coated with chitosan compared to the negative control. The chitosan used by Benhabiles *et al*. (2013) had a higher molecular weight than those used in the present work, exceeding 370 kDa. This again supports the assumption that the molecular weight of the chitosan produced from *H. illucens* was too low to ensure a proper barrier effect of the coating.

Also at cold storage temperature all chitosan treatments induced the same weight loss as the negative control. The solvent-only treatment, however, gave a greater weight loss than the negative control. As already hypothesised for tomatoes, this may be due to a suboptimal solvent composition which did not reduce the transpiration of the fruit and thus the weight loss. It is encouraging, though, that the addition of chitosan to the solvent improves its effect, making the weight loss at least no worse than in the negative control. Changing the composition of the solvent solution would maybe allow the positive effect of chitosan to be fully realised.

In experiments performed on strawberries stored at cold temperatures, coating with chitosan always significantly reduced weight loss compared to the negative control (Petriccione *et al*., 2015; Hassan *et al*., 2020; Jiang *et al*., 2020). Petriccione *et al*. (2015) observed better effectiveness in reducing strawberry weight loss of 2% chitosan compared to 1%. This might suggest investigatin higher concentration of chitosan in the coating solution.

Nectarines

In nectarines, no treatment with chitosan reduced the weight loss compared to the negative control, at both storage temperatures. However, some trends can be observed among the various treatments. Although not significantly, unbleached chitosans performed worse than the bleached ones. In the literature, there are no investigations on the differences between bleached and unbleached chitosan for this application. Furthermore, most chitosan treatments gave a slightly lower, though not significant, weight loss than the solvent-alone. Again, this confirms

what has already been discussed regarding the composition of the solvent solution.

In nectarines stored at room temperature, the weight loss reducing effect of chitosan coating was observed by Zhang *et al*. (2019). In their work, in contrast to other papers previously cited, a difference in performance between high and low molecular weight chitosan was not observed. In contrast, the reduction in weight loss given by chitosan was not significant in nectarines stored at 0 °C coated with a commercial formulation based on 10 g/l chitosan (Giacalone and Chiabrando, 2015). Ramirez *et al*. (2015) also observed no differences in weight loss between fresh-cut nectarines coated with chitosan and the negative control. Thus, for this specific fruit, the positive effect of chitosan coating does not seem so straightforward.

Final consideration on weight loss

Chitosan coating acts as a semipermeable barrier against oxygen, carbon dioxide and moisture, thereby reducing respiration and water loss and counteracting the dehydration and shrinkage of the fruit (Petriccione *et al*., 2015). This effect has been demonstrated on many other fruits, in addition to those examined in the present work, such as blueberry (Vieira *et al*., 2016), grape (Andrijanto *et al*., 2019), mango (Silva *et al*., 2017), papaya (Vilaplana *et al*., 2020a), blackberry (Vilaplana *et al*., 2020b), mandarin (Baswal *et al*., 2020), lemon (Chen *et al*., 2020) and pomegranate (Varasteh *et al*., 2017).

When comparing the results obtained with chitosan from *H. illucens* with those found in the literature, it must be considered primarily the different raw source and some different characteristics of the polymer, mainly molecular weight, that may have affected the performance of the chitosan coatings. In addition, in many papers only uncoated fruits are used as control, with no provision for a control made with fruits coated with the chitosan-free solvent solution. Thus, the effects of the solvent per se that may have influenced the effect of the chitosan coating are often not known. To conclude, the results obtained for fruit weight loss with chitosan derived from *H. illucens*, although not optimal, can be considered encouraging in light of two main observations: insect chitosan always performed similarly or better than commercial chitosan, and it had better effects than the solvent solution. By adjusting the formulation of the coating solutions and modifying some parameters in the chitosan production process, that could lead to a higher molecular weight polymer, the potential of chitosan maybe could be expressed and fully exploited.

5.4.2 TSS content

TSS are an estimation of the sugar content of fruit, used as an indicator of fruit ripening. TSS increase during ripening, due to the hydrolytic conversion of starch stored by the fruit into

sugars, mainly glucose, fructose and sucrose (Ghasemnezhad *et al*., 2011; Shah and Hashmi, 2020). As the fruit reaches full ripeness, the TSS content is expected to decrease, as a consequence of the reduction of ethylene production and respiration rate. As storage proceeds, ethylene synthesis begins again, restarting respiration and, consequently, the hydrolysis of starch into sugars. Thus, TSS increase again as a consequence of the advancement of the postharvest ripening process. This process occurs in "climacteric" fruits (e.g., tomatoes, apricots, peaches, apples, pears) in which the biochemical ripening mechanisms continue even after detachment of fruit from the plant. In "non-climacteric" fruits (such as strawberries, citrus fruits, cherries and grapes), on the other hand, there is no starch accumulation, and respiration ceases once the fruit reaches ripeness on the plant (Valero and Serrano, 2013; Lin *et al*., 2020).

Tomatoes

The TSS of tomatoes increased during storage at all temperature conditions. At room temperature, most of the chitosan treatments gave an increase in TSS significantly greater than the negative control, in both dipped and sprayed fruits. Only the heterogeneous and the commercial chitosan limited the increase in TSS to a level similar to that of the negative control. Since a greater increase in TSS was observed in the solvent-only control than in the negative control, as with the weight loss, a positive effect of heterogeneous chitosan in slowing down the post-harvest ripening processes of tomatoes stored at room temperature can be supposed. The same trend was also observed at cold temperature. The preference for heterogeneous over homogeneous chitosan for this application was confirmed also by results of TSS variation.

Our results are similar to those obtained by Sucharita *et al*. (2018) with tomatoes stored at cold temperature for 30 days. They observed a general increase in TSS during storage. Only coating with 0.25% chitosan solution significantly reduced the TSS increase, while 0.5% chitosan solution was not effective (Sucharita *et al*., 2018). We did not observe significant differences between the two chitosan concentrations, but it might be useful to test other higher and lower concentrations than 0.5 and 1%. In contrast, Barreto *et al*. (2016) reported a decrease in TSS in tomatoes stored both at room temperature for 12 days and at cold temperature for 24 days. At either condition, coating with chitosan significantly reduced the TSS decrease.

The chitosan coating can modify the internal atmosphere of the fruit, with a reduction in the oxygen level and/or an increase in the carbon dioxide level, thus reducing the respiration rate and metabolic activity and delaying both the accumulation of sugars and the starch degradation (Petriccione *et al*., 2015; Silva *et al*., 2017). Furthermore, chitosan coating reduces fruit dehydration, and this can contribute to maintaining a stable TSS level. Water loss increases the TSS concentration in fruits, which may be wrongly interpreted as a real change in TSS (Shah

and Hashmi, 2020). Actually, the heterogeneous chitosans that most reduced the variation in TSS were also the ones that most reduced the weight loss of tomatoes.

The non-uniformity in the variation of the TSS content could be due to the different physiological stage of the tomatoes in the time of the experiment. According to the literature, the increase in TSS observed both in the present work and by Sucharita *et al*. (2018) could indicate either tomatoes in the ripening stage or in advanced storage phase. Whereas the decrease in TSS could indicate the physiological phase just after ripening and before the reactivation of ethylene production and respiration (Valero and Serrano, 2013; Shah and Hashmi, 2020). Since the exact time of harvest of the tomatoes used in the present work is unknown, it is reasonable to assume that at the time of purchase, being visually ripe, the fruits were already in the phase of reactivation of the biochemical ripening mechanisms, which then led to the constant increase in TSS throughout the storage period.

Apricots

In apricots TSS increased during storage, similarly to tomatoes. At room temperature, larval chitosan, one sample from pupal exuviae (the 0.5% bleached chitosan) and one sample from adults (the 1% unbleached chitosan), were the most effective in reducing the TSS increase compared to both the negative and the solvent-only controls. At cold temperature, in contrast, there were no differences between the controls and the chitosan treatments. Ghasemnezhad *et al*. (2010) and Zhang *et al*. (2018) also found no difference in the variation of TSS between coated apricots and the control, under cold storage. In contrast, a coating based on chitosan and zinc was effective in reducing the decrease in TSS, and instead led to an increase in apricots stored at 4 °C (Younas *et al*., 2014). Also for this fruit, the different trend in the variation of TSS may be due to the different stage of development of the fruit.

However, chitosan produced from *H. illucens* larvae gave encouraging results, inducing a significantly lower increase in TSS than the controls. This result could not have been influenced by the effect of chitosan on dehydration, as chitosan from larvae was the one that reduced the weight loss the least compared to the others.

Nectarines

TSS increased during storage also in nectarines. At room temperature, only the 1% commercial chitosan and chitosan from pupal exuviae gave a TSS increase significantly smaller than both the controls, with 0.5% samples being more effective than the 1% ones. At cold storage, again, no differences were detected between coated nectarines and the controls. Thus, in the case of nectarines, only chitosan produced from pupal exuviae gave positive results.

There are conflicting results in the literature on the effect of chitosan coating on the variation

of TSS in apricots. Both Zhang *et al*. (2019) and Giacalone and Chiabrando (2013) observed a general increase in TSS of apricots stored at both room and cold temperature, respectively, in accordance to our results. While at room temperature the chitosan coating significantly reduced the increase in TSS (Zhang *et al*., 2019), at low temperature the coating actually induced a greater increase in TSS than the control (Giacalone and Chiabrando, 2013). In the work by Ramirez *et al*. (2015), the TSS content of apricots stored at 3 °C decreased slightly in the control, while it increased in the chitosan-coated fruits.

Final consideration on TSS variation

The efficacy of chitosan coatings in reducing the variation of TSS, either increasing or decreasing, has been confirmed for several different fruits, including longan fruit (Lin *et al*., 2020), blueberry (Vieira *et al*., 2016), mango (Silva *et al*., 2017; Shah and Hashmi, 2020), papaya (Vilaplana et al., 2020a), blackberry (Vilaplana *et al*., 2020b), lemon (Chen *et al*., 2020) and pomegranate (Varasteh *et al*., 2017). Silva *et al*. (2017) reported a better efficacy of 3% chitosan coating solutions than 2 or 1% in reducing the TSS variation. This, as already pointed out, suggests testing additional concentrations of chitosan in the coating solutions. Furthermore, the results by Chen *et al*. (2020) showed that a multilayer application of the chitosan coating on the fruit surface is more effective than a single layer application. Different application methods could therefore be investigated to maximise the barrier effect of chitosan coatings.

In summary, similarly to what has already discussed for weight loss, the results obtained on the variation of TSS content, although not optimal, are encouraging. Especially in apricots and nectarines stored at room temperature, significantly better results than controls were obtained with some chitosan samples from *H. illucens*, particularly from larvae and pupal exuviae. Experiments on tomatoes again showed a significantly better performance of heterogeneous chitosan than the homogeneous one. An encouraging finding for the future use of insect chitosan is that, in the present work, samples from *H. illucens* always performed similarly or better than commercial chitosan. According to literature studies, TSS results are more variable than the weight loss. It should be considered that variations in physico-chemical parameters of the fruits, such as TSS, can also be affected by cultivar, cultural practices, season and growing area (Suárez *et al*., 2008).

5.4.3 pH and TA variation

pH and TA were determined to detect variation in acidity of fruits during storage. Organic acids accumulate in the fruit cells during ripening, as they are the main substrate for the enzymes involved in the respiration process of fruits (Etienne *et al*., 2013; Shah and Hashmi, 2020). An

increase in the respiration rate accelerates glycolytic metabolism and tricarboxylic acid cycle, thus increasing the acid content of fruit (Lin *et al*., 2020). During the postharvest storage, acidity usually decreases as result of the acid metabolism that converts acid and starch into sugars (Vieira *et al*., 2016). Thus, a decrease of TA and increase in pH of fruits usually occur with storage.

5.4.3.1 *pH variation*

Tomatoes

In tomatoes stored at room temperature, pH generally increased. In fruits coated by dipping, almost all chitosan treatments did not differ from the controls. Differently, in sprayed tomatoes, pH remained stable with the majority of heterogeneous chitosan. At cold storage, in both dipped and sprayed tomatoes, pH increased only in both negative and solvent controls, and with commercial chitosan. The reduction in the pH increase mediated by some chitosan samples is a sign of a deceleration of the acidity decline due to a slowdown in the acid metabolism of the fruit (Vieira *et al*., 2016). A better efficacy of heterogeneous chitosan compared to homogeneous chitosan is confirmed and, in many cases, also compared to the commercial one. The effect of chitosan coating was more pronounced at cold temperature than at room temperature. It is known that increasing the temperature during fruit storage can modify the acid metabolism, by affecting activity of enzymes involved in the glycolysis and the tricarboxylic acid cycle (Etienne *et al*., 2013). Generally, with higher temperature the fruit respiration is stimulated and and the citrate production during ripening is decreased, thus lowering the fruit acidity. But the final effect on the fruit acidity varies depending on the fruit species and stage of ripeness (Etienne *et al*., 2013).

Apricots

Also in apricots the pH increased, both at room and cold temperature. At both storage conditions, the pH of the chitosan-coated fruit increased significantly less than the negative control and, at room temperature, also than the solvent-only control. The positive effect of chitosan in slowing down the decrease in acidity was therefore more marked and uniform in apricots than in tomatoes. Younas *et al*. (2014) also reported a small pH increase in chitosan- and zinc-coated apricots compared to the control, while Ghasemnezhad *et al*. (2010) observed no differences between coated and uncoated fruits.

Nectarines

Similarly to apricots, the pH of nectarines generally increased, but the increase was significantly smaller in coated fruits than in both negative and solvent-only controls. Again, a detrimental

effect of the higher storage temperature was observed, since at room temperature only the chitosan from pupal exuviae maintained significantly lower pH levels than the controls, whereas at cold temperature all chitosans were effective. There are no works reporting the change in pH of chitosan-coated nectarines with which to compare our results. However, these results again show the positive effect of chitosan in reducing the pH variation of the fruit during storage.

5.4.3.2 *TA variation*

Tomatoes

TA increased with all chitosan treatments, both at room and cold temperature, but only in spraying-coated tomatoes. In these samples, the negative control had a decrease in TA. TA of tomatoes treated with the solvent alone remained stable at room temperature, decreased like the negative control at cold temperature in dipped fruits and increased more than the negative control in the sprayed ones. This result suggests that chitosan coating applied to tomatoes by spraying was effective in reducing the acid metabolism and thus the conversion of acids into sugars (Khaliq *et al*., 2015; Barreto *et al*., 2016). For this parameter, no significant difference was observed between heterogeneous and homogeneous chitosan. More important, though, was the application method, which highlighted the better suitability of spraying compared to dipping.

A reduction in the TA decline of tomatoes due to chitosan coating was reported also by El-Ghaouth *et al*., (1992) and Barreto *et al*. (2016) at both room and cold storage temperature. Contrarily, no difference between coated and uncoated tomatoes was found by Sucharita *et al*. (2018). The maintenance of higher TA levels in treated tomatoes is beneficial also because high decrease in TA induces faster senescence of fruits (Khaliq *et al*., 2015; Shah and Hashmi, 2020).

Apricots

A general increase in the TA of apricots was observed. At room temperature, most of the chitosan treatments significantly reduced the TA increase compared to both negative and solvent controls. At cold temperature the opposite occurred: all chitosan treatments gave a greater TA increase than the negative control. The effect of the chitosan coating was therefore adversely affected by the higher storage temperature. According to the literature, higher storage temperatures should stimulate the respiration of the fruit and increase its metabolism, resulting in a greater drop of acidity (Etienne *et al*., 2013; Fan *et al*., 2018). It is therefore unclear why the opposite occurs in our apricots stored at room temperature. In the literature, a general decrease in the TA of apricots stored at low temperatures is reported (in no work the room

storage temperature was used). In one case, coating with chitosan alone had no effect (Ghasemnezhad *et al*., 2010), whereas coatings with both chitosan and soy protein, as well as chitosan and zinc, significantly reduced the decline in TA compared to the control (Younes *et al*., 2014; Zhang *et al*., 2018).

Nectarines

Chitosan coating was effective in maintaining significantly higher level of TA than both negative and solvent controls in nectarines stored at cold temperature, while the opposite occurred in fruit stored at room temperature. As already discussed for apricots, the reason for the minor efficacy of chitosan coating at higher storage temperature is not clear. A reduction in the TA decrease in coated nectarines stored at room temperature was obtained by Zhang *et al*. (2019), while in other cases chitosan coating had no effect on nectarines stored at cold temperature, whether TA increased (Ramirez *et al*., 2015) or decreased (Giacalone and Chiabrando, 2013).

Final consideration on pH and TA variation

An increase in the pH of other different fruits during storage is generally reported, with a positive effect of chitosan coating in reducing the alkalisation of the fruit (Bal *et al*., 2013; Vieira *et al*., 2016), except for blackberry investigated by Vilaplana *et al*. (2020b) for which pH increase more in coated fruits than in the control. A decrease in TA, significantly reduced by chitosan coating, was observed in blueberry (Vieira *et al*., 2016), mango (Silva *et al*., 2017; Shah and Hashmi, 2020) and pomegranate (Varasteh *et al*., 2017). Silva *et al*. (2017) found a better efficacy of 3 and 2% chitosan coating solutions compared to that containing 1% chitosan. In contrast, TA increased during storage of longan fruits, but to a lesser extent in coated fruits than in the control (Lin *et al*., 2020), while on lemons, chitosan coating had no effect (Chen *et al*., 2020). No change in pH and TA was observed during storage of papaya by Vilaplana *et al*. (2020a).

Concluding, also for these parameters, chitosan produced from *H. illucens* was effective in many cases in maintaining lower pH levels and higher TA values than controls. Furthermore, in most cases, chitosan from *H. illucens* had similar or better effects than commercial chitosan. In the experiments with tomatoes, it was again found that the application of the coating by spraying was better than by dipping. No significant differences were observed between the two chitosan concentrations (0.5 and 1%), but it might be useful to investigate additional concentrations. A possible effect of storage temperature on the performance of chitosan coating was also observed, particularly on apricots and nectarines.

The results of the present work concerning pH and TA are not always in accordance with those reported by other authors. However, the results reported in the literature on pH and TA variation are rather variable. As for TSS, many factors can affect these two physical parameters, including fruit variety, cultivation practices, growing area, climate, and transport conditions (Suárez *et al*., 2008; Etienne *et al*., 2013). The pH can also be affected by the presence of fungal population, as fungi and moulds can use organic acids as a growth substrate, thus increasing the pH of the fruit (Shah and Hashmi, 2020).

Concerning the data in the literature, it should also be considered, as already discussed for weight loss, that in many cases only untreated fruits are used as control, without investigating the eventual effects of the solvent solution itself.

5.4.4 Fungal decay

As an additional parameter for evaluating the effect of chitosan coating, the occurrence of mould on fruits during storage was recorded. Only in tomatoes no fungal growth was observed, whereas in other fruits it was.

In apricots stored at room temperature, mould developed in all treatments but to a different extent. In the negative control, all fruits were mouldy at the end of the storage period. The solvent-only control and the 0.5% commercial chitosan were the second treatments with the highest percentage of mouldy fruit. All chitosans from *H. illucens* performed better than both controls, keeping the percentage of mouldy fruits and their fungal decay index lower. Similar results were obtained on strawberries and nectarines. In both fruits, the significantly higher percentage of mouldy fruits was observed in the controls, while chitosan coating reduced the development of fungal populations. No clear differences were found between the two chitosan concentrations. While in apricots and nectarines the fungal growth was only observed at room temperature, in strawberries mould occurred also at low temperature.

The chitosan coating therefore appears to have a crucial effect in reducing fungal growth.

The use of chitosan coating to inhibit fruit pathogens in the postharvest storage has been largely investigated. An effective inhibition action of chitosan-based coatings against fungal growth was generally assessed in different fruits, such as strawberry (Benhabiles *et al*., 2013; Hassan *et al*., 2020; Jiang *et al*., 2020), tomato (Liu *et al*., 2007; Barreto *et al*., 2016; Sucharita *et al*., 2018), papaya (Dotto *et al*., 2015), pear (Meng *et al*., 2010), mango (Jitareerat *et al*., 2007), blueberry (Jiang et al., 2016).

Results from the present work highlighted a significant better effect of the higher chitosan concentration (1%) compared to the lower one in preventing fungal growth on apricots. This is

in accordance with the general better inhibiting effect of coatings with a higher concentration of chitosan reported in the literature (Jitareerat *et al*., 2007; Liu *et al*., 2007).

In this work, chitosan produced from *H. illucens* had generally a similar effect to the commercial sample. The major difference between these two samples was in the molecular weight (much higher in the commercial chitosan), but this did not seem to affect the final inhibiting effect of the coating. From the literature, it is not clear how the chitosan molecular weight affects its antimicrobial capacity. For example, Dotto *et al*. (2015) found a better effect of low molecular weight chitosan, while Benhabiles *et al*. (2013) reported a better fungal inhibition with high molecular weight chitosan. In contrast, the results of Jiang *et al*. (2020) showed no differences between chitosan with different molecular weights. One reason for the greater effectiveness of high-molecular-weight chitosan may be the lower oxygen permeability of its coating (Ramirez *et al*., 2015). On the other hand, low molecular weight chitosan, having a less organized and less crystalline structure, has a higher solubility in acidic solutions and is therefore able to form a more homogeneous coating. This provides a more effective barrier between the fruit and the external environment, hindering the respiratory activity of microorganisms (Souza *et al*., 2013; Dotto *et al*., 2015).

The exact mechanism of the antifungal action of chitosan is not completely known yet, but few main theories have been proposed. As a polycationic polymer, chitosan can consume the electronegative charges on the microbial cell surface changing its permeability, thus resulting in the leakage of intracellular electrolytes and protein constituents. Another mechanism may be the entry of chitosan into fungal cells with the adsorption of essential nutrients, which inhibit or slow down the synthesis of mRNA and protein (Avadi *et al*., 2004; Zhang *et al*., 2011a). Furthermore, chitosan can act as elicitor and induce resistance in the host fruit, by increasing the activities of defence-related enzymes, such as chitinase and β-1,3-glucanase, as observed in strawberries, raspberries and oranges (Fajardo *et al*., 1998; Zhang and Quantick, 1998).

When investigating the antimicrobial effect of a chitosan coating, is important to take into account the components of the solution in which the chitosan is dissolved, in particular the acid. Indeed, acetic acid alone, which was used for chitosan solubilization, is able to inhibit microbial growth (Jitareerat *et al*., 2007; Jiang *et al*., 2020). Therefore, an appropriate control must always be set up to check the effect of the solvent solution and assess the effect of the chitosan. In our case, since the solvent-only control always gave a higher presence of mould than the other treatments, the effect observed in reducing the incidence of mouldy fruits can be attributed to the chitosan itself.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

Chitin and chitosan are natural polymers that have been of great technological and commercial interest for several years now. The current industrial source of chitin and chitosan, consisting of waste from the fishing industry, mainly crustacean exoskeletons, has some shortcomings and limitations that are increasingly driving the search for alternative sources. Among arthropods, insects could provide a valid solution.

Insect farming on an industrial scale has been increasingly developed in recent decades, driven by two main issues: the search for new sources of protein for animal and human nutrition, and the need to dispose of an increasing amount of organic waste through the bioconversion process mediated by the larval stages of many insect species (van Huis, 2020). *H. illucens* has become the most important species for both feed production and waste management, being reared worldwide on a large scale (Derrien and Boccuni, 2018). The byproducts generated by the *H. illucens* farming, i.e., larval and pupal shedding, and dead adult flies, are rich in chitin and can be exploited for the purification of this useful and versatile natural polymer. The present work revealed a chitin content of pupal exuviae of 25%, comparable to the average of the range reported for crustacean exoskeletons (15-40%) (Kurita, 2006). This high chitin content and the ease of collecting this substrate from the insect farm make *H. illucens* pupal exuviae a biomass of choice for chitin extraction.

Currently, the production of chitin and chitosan from insects is only carried out on a laboratory scale for experimental purposes, in most cases using the same procedures as for crustaceans. There is a lack of numerical data providing a quantitative assessment of the effectiveness of the extraction processes used. The chitin extraction method applied in this work was effective in significantly reducing the percentage of minerals and proteins contained in the insect samples and resulted in a chitin of similar purity to the commercially available polymer. The yields of bleached chitin obtained from larvae, pupal exuviae and adults (6, 10, 23%) were similar to or higher than the mean yields reported for insects (5-15%) (Hahn *et al*., 2020a) and in the average range reported for crustaceans (5-30%) (Tharanathan and Kittur, 2003; Oduor-Odeto *et al*., 2005; Bolat *et al*., 2010; Hossain and Iqbal, 2014). Especially pupal exuviae gave a very high yield. Spectrometric and diffractometric characterization also confirmed the similarity of chitin extracted from *H. illucens* both to chitin produced from other insects and to commercial chitin derived from crustaceans. A possible detrimental effect of the bleaching treatment on the acetylation and crystallinity of chitin was revealed, although compensated by a significantly higher degree of purity of the polymer obtained after bleaching. This negative effect of bleaching is well known, and this is the reason why industrial chitin from crustaceans is often

not bleached. In contrast to crustaceans, however, chitin obtained from insects is often very dark in colour due to the catechol compounds that cross-link to the insect cuticle during the sclerotization process. A bleaching step is therefore necessary to remove these compounds. Optimisation of this step must be pursued in order to minimise its negative effects on the final product. Different bleaching reagent could be investigated, as well as, different reaction parameters, mainly temperature and reagent concentration.

With a view to expanding the scale of insect chitin production, further investigation of all process parameters is needed in order to achieve a specific optimisation for extraction from insects. The optimisation should aim at obtaining the highest possible yields of chitin of the best possible quality using the least amount of reagents and the lowest possible energy consumption. In order to reduce the environmental impact of this extraction process, enzymatic and microbiological extraction methods should be further investigated. These are already being explored for the production of chitin from crustaceans but have not yet been tested for insects. Maintaining the chemical method, the use of organic acids, with less environmental impact, instead of mineral acids could be adopted for demineralisation more widely, given their proven effectiveness both in the present work and in the literature.

In the present work, chitosan from *H. illucens* was produced from both bleached and unbleached chitin, with heterogeneous deacetylation. Homogeneous deacetylation was also tried on pupal exuviae. Also in the case of chitosan, pupal exuviae gave the highest yield (10%), relative to the initial biomass. Chitosan yields obtained from *H. illucens* were in the average range (4-15%) of those obtained from crustaceans (Oduor-Odeto *et al*., 2005; Thirunavukkarasu and Shanmugam, 2009; Bolat *et al*., 2010; Hossain and Iqbal, 2014; Luo *et al*., 2019), and also within the range reported for insects (2-8%) (Hahn *et al*., 2020a).

In general, an effect of bleaching on the final characteristics of chitosan was observed. Particularly, the decolourisation of chitin led to an increase in the degree of deacetylation and a reduction in the molecular weight and viscosity of the respective chitosan. These effects should be further investigated in order to establish whether it is worthwhile to bleach chitin if the final aim is the production of chitosan. The major differences between chitosan obtained from *H. illucens* and commercial chitosan were observed for molecular weight and viscosity. Both were much lower in the samples obtained in this work. These characteristics of chitosan can be influenced by several factors: the deacetylation conditions (especially temperature and alkali concentration), the parameters of the chitin extraction process applied previously, the source and the type of analysis used to characterize the polymer. The optimisation of all these

parameters is therefore challenging and should be studied according to the final application for which the chitosan is intended. Indeed, the optimal characteristics of this polymer change depending on its use. It is therefore not possible to define unique optimal parameters for its production.

As in the case of chitin, alternative, "greener" enzyme-based methods should also be investigated for the deacetylation step, even though these enzymatic procedures have not yet given optimal results for the production of chitosan from crustaceans (Martinou *et al.*, 1995; Win and Stevens, 2001).

The final application of the chitosan produced from *H. illucens* was as coating for the preservation of some fresh fruits. The chitosan samples obtained were used to produce coating solutions at two different concentrations of chitosan (0.5 and 1%) that were applied to the fruits. The effect of the chitosan coating was then evaluated by investigating the change in four parameters of fruits: weight loss, total soluble solids content, pH and titratable acidity. The effect of chitosan on the spontaneous growth of mould on the fruit during storage was also evaluated. Although the results obtained were sometimes non-uniform and sub-optimal, some significant evidences were drawn:

- for all physico-chemical parameters analysed, chitosan from *H. illucens* had a similar or better effect than the commercial chitosan used as a control;
- the application of the coating by spraying was significantly better than the application by dipping;
- heterogeneous chitosan from pupal exuviae always gave better results than the homogeneous one;
- in many cases the chitosan coating had no different effect than the untreated fruits, but gave better results than the coating with only the solvent solution, leading to the hypothesis that a better formulation of the solvent solution is needed to allow the chitosan to function properly.;
- there were no significant differences between the two chitosan concentrations, nor unique differences between chitosan from bleached or unbleached chitin;
- no clear significant differences were found between the chitosan obtained from the different *H. illucens* samples;
- all chitosan samples from *H. illucens* had a significant effect in reducing the occurrence of mould on the fruit compared to the controls.

Chitosan produced from insects has never been used for this application. A comparison with the literature is therefore difficult, taking into account the different source. Also in the literature,

the results obtained from preservation experiments with chitosan produced from crustaceans are often not unique and vary depending on the food product tested. Variations in the preservative effect of chitosan coatings may be due also to the storage temperature, the physiological stage of the fruit, the concentration of the applied chitosan and the method of application of the coating. The characteristics of the chitosan, especially molecular weight, viscosity and degree of deacetylation, also have an influence on the effectiveness of the protective coating.

It is also important to note that in most of the literature studies, the effect of the coating with chitosan-free solution is not investigated, so that it is often uncertain whether the observed effects are due to the chitosan itself or whether the other reagents in the solution have an influence. In this work, the results obtained with the chitosan coating were always compared with a control consisting of the solvent-only coating solution, so that the significant effects observed are attributable to the chitosan itself.

Taking into account the previous considerations, future investigations on insect-based chitosan for this application should consider the following points:

- optimization of the chitosan production steps in order to obtain a polymer with the best characteristics for this application (mainly higher viscosity, higher molecular weight). For instance, the optimal particle size of the starting samples should be ensured by using more efficient grinding equipment, the efficiency of each chitin extraction step should be further improved to obtain the greatest possible purity, the conditions of the deacetylation reaction should be adjusted in order to prevent polymer degradation as far as possible (e.g., by avoiding contact with oxygen);
- optimization of the formulation of the solvent solution, in terms of reagents and their concentrations, in order to avoid possible negative effects of the solvent that would affect the performance of the chitosan coating (as hypothesized in this work);
- assess the moisture retention capacity of chitosan as an important characteristic related to its anti-dehydration effect on fresh food;
- try solutions with different concentrations of chitosan and different application methods (e.g., multilayer coating application was found to be more effective than monolayer application) (Chen *et al.*, 2020), and establish a method to measure the real chitosan concentration of the surface of the product;
- concerning the evaluation of the antifungal effect of chitosan coating, the use of a software able to calculate the exact area of the product's surface covered by mould can make this

measurement faster and more accurate;
- test the addition of other elements to chitosan in coating solutions to enhance its preservative effects (e.g., waxes, proteins, plant extracts, essential oils, microelements);
- test the combination of other preservation methods with the chitosan coating (e.g., modified or controlled atmosphere);
- given the film-forming ability observed for chitosan, consider developing solid films for use as packaging fresh foods.

Finally, a consideration must be made about the edibility of chitosan coatings in case they are applied directly on the food. In these coating solutions plasticizing agents (i.e., Tween 80 and glycerol) and acids (generally acetic or lactic acid) for solubilization of the chitosan are generally added. Chitosan itself is known to have no toxicity for humans and is used in many oral drug delivery systems. The major questions are therefore towards the other ingredients in the solutions. Chitosan-based coatings containing glycerol and Tween 80 are classified as edible (Sharif *et al*., 2017). Among acids, both acetic and lactic acid, as well as many others, are used as food additives (E260 and E270, respectively), in accordance with current regulations (GFSA online database, http://www.fao.org/gsfaonline/index.html). The chitosan-based coating used in the present work can thus be considered edible.

Based on current knowledge, we can assume that insects have the potential to become an important alternative source of chitin and chitosan in the near future. Insects have advantages over crustaceans, especially from an ecological point of view, and the many large-scale breeding facilities now existing in several countries make it possible to have large amounts of waste insect biomass available that can be used for this purpose. This work especially highlighted the suitability of waste from *H. illucens* breeding for the production of chitin and chitosan. Pupal exuviae seemed to be the best, as it is the biomass richest in chitin, giving the highest yield of both chitin and chitosan. Compared to other insect farm waste, they are also the easiest and quickest to collect, and the most abundant. Since pupal exuviae, like other by-products of the insect farming, would have no other destination than disposal as waste, it would be advantageous to place them in a new productive cycle for the production of valuable materials (chitin and chitosan) that can be put on the market again.
There are still many aspects to be studied and optimised regarding the extraction process aimed at specific applications of the final polymer. However, the results obtained from this work are encouraging and represent a starting point for future in-depth investigations into the production

of chitin and chitosan from insects, as well as their use in the agro-food industry and for other applications, with a view to making optimal use of the waste biomass generated by insect breeding.

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7. REFERENCES

Abdel-Gawad, K. M., Hifney, A. F., Fawzy, M. A., & Gomaa, M. (2017). Technology optimization of chitosan production from *Aspergillus niger* biomass and its functional activities. *Food Hydrocolloids*, *63*, 593-601.

Abdou, E. S., Nagy, K. S., & Elsabee, M. Z. (2008). Extraction and characterization of chitin and chitosan from local sources. *Bioresource technology*, *99*(5), 1359-1367.

Abe, M. M., Martins, J. R., Sanvezzo, P. B., Macedo, J. V., Branciforti, M. C., Halley, P., ... & Brienzo, M. (2021). Advantages and disadvantages of bioplastics production from starch and lignocellulosic components. *Polymers*, *13*(15), 2484.

Abugoch, L., Tapia, C., Plasencia, D., Pastor, A., Castro-Mandujano, O., López, L., & Escalona, V. H. (2016). Shelf-life of fresh blueberries coated with quinoa protein/chitosan/sunflower oil edible film. *Journal of the Science of Food and Agriculture*, *96*(2), 619-626.

Agrahar-Murugkar, D., & Jha, K. (2010). Effect of drying on nutritional and functional quality and electrophoretic pattern of soyflour from sprouted soybean (*Glycine max*). *Journal of food science and technology*, *47*(5), 482-487.

Aguilar, A., Zein, N., Harmouch, E., Hafdi, B., Bornert, F., Offner, D., ... & Hua, G. (2019). Application of chitosan in bone and dental engineering. *Molecules*, 24(16): 3009.

Ahmad, M., Nirmal, N. P., Danish, M., Chuprom, J., & Jafarzedeh, S. (2016). Characterisation of composite films fabricated from collagen/chitosan and collagen/soy protein isolate for food packaging applications. *Rsc Advances*, *6*(85), 82191-82204.

Ai, H., Wang, F., Yang, Q., Zhu, F., & Lei, C. (2008). Preparation and biological activities of chitosan from the larvae of housefly, *Musca domestica*. *Carbohydrate polymers*, *72*(3), 419-423.

Al-Sagheer, F. A., Al-Sughayer, M. A., Muslim, S., & Elsabee, M. Z. (2009). Extraction and characterization of chitin and chitosan from marine sources in Arabian Gulf. *Carbohydrate polymers*, *77*(2), 410-419.

Alexandratos, N., & Bruinsma, J. (2012). World agriculture towards 2030/2050: the 2012 revision. Rome: *FAO ESA Working paper No. 12-03*.

Ali, A., Noh, N. M., Mustafa, M. A. (2015). Antimicrobial activity of chitosan enriched with lemongrass oil against anthracnose of bell pepper. *Food Packaging and Shelf Life, 3,* 56–61.

Alix, S., Mahieu, A., Terrie, C., Soulestin, J., Gerault, E., Feuilloley, M. G., ... & Leblanc, N. (2013). Active pseudo-multilayered films from polycaprolactone and starch-based matrix for food-packaging applications. *European Polymer Journal*, *49*(6), 1234-1242.

Ameh, A. O., Isa, M. T., Abutu, D., & Danlami, A. (2014). Kinetic modelling of the demineralization of shrimp exoskeleton using citric acid. *Leonardo Electronic Journal of Practices and Technologies*, *25*, 99-108.

Amit, S. K., Uddin, M. M., Rahman, R., Islam, S. R., & Khan, M. S. (2017). A review on mechanisms and commercial aspects of food preservation and processing. *Agriculture & Food*

Security, *6*(1), 1-22.

Andersen, S. O. (2010). Insect cuticular sclerotization: a review. *Insect biochemistry and molecular biology*, *40*(3), 166-178.

Andrijanto, E., Reksa, T., Maheda, J., Diani, R., & Wahyu, E. (2020, April). Synthesis and utilization of chitosan as edible coating material for natural fruit preservation. In *IOP Conference Series: Materials Science and Engineering* (Vol. 830, No. 2, p. 022009). IOP Publishing.

Antonov, A., Ivanov, G., Pastukhova, N., & Bovykina, G. (2019, August). Production of chitin from dead *Hermetia illucens*. In *IOP Conference Series: Earth and Environmental Science* (Vol. 315, No. 4, p. 042003). IOP Publishing.

Appel, E., Heepe, L., Lin, C. P., & Gorb, S. N. (2015). Ultrastructure of dragonfly wing veins: composite structure of fibrous material supplemented by resilin. *Journal of Anatomy*, *227*(4), 561-582.

Aranday-García, R., Saimoto, H., Shirai, K., & Ifuku, S. (2019). Chitin biological extraction from shrimp wastes and its fibrillation for elastic nanofiber sheets preparation. *Carbohydrate polymers*, *213*, 112-120.

Arbia, W., Arbia, L., Adour, L., & Amrane, A. (2013). Chitin extraction from crustacean shells using biological methods–a review. *Food Technology and Biotechnology*, *51*(1), 12-25.

Arora, S., Lal, S., Kumar, S., Kumar, M., & Kumar, M. (2011). Comparative degradation kinetic studies of three biopolymers: chitin, chitosan and cellulose. *Archives of Applied Science Research*, *3*(3), 188-201.

Asgar, A. Mahmud, T.M.M., Kamaruzaman, S. & Mohamad Zaki, A.R. (2005). Effect of chitosan coating on the retention of colour development and firmness of papaya fruit during storage. In *Proceedings of First International Symposium on Papaya. 22-24 November, Genting Highlands Malaysia, 2005*.

Avadi, M. R., Sadeghi, A. M. M., Tahzibi, A., Bayati, K. H., Pouladzadeh, M., Zohuriaan-Mehr, M. J., & Rafiee-Tehrani, M. (2004). Diethylmethyl chitosan as an antimicrobial agent: Synthesis, characterization and antibacterial effects. *European Polymer Journal*, *40*(7), 1355-1361.

Babalar, M., Asghari, M., Talaei, A., & Khosroshahi, A. (2007). Effect of pre-and postharvest salicylic acid treatment on ethylene production, fungal decay and overall quality of Selva strawberry fruit. *Food chemistry*, *105*(2), 449-453.

Badawy, R. M., & Mohamed, H. I. (2015). Chitin extration, composition of different six insect species and their comparable characteristics with that of the shrimp. *Journal of American Science*, *11*(6), 127-134.

Bajaj, M., Winter, J., & Gallert, C. (2011). Effect of deproteination and deacetylation conditions on viscosity of chitin and chitosan extracted from Crangon crangon shrimp waste. *Biochemical engineering journal*, *56*(1-2), 51-62.

Bakshi, P. S., Selvakumar, D., Kadirvelu, K., & Kumar, N. S. (2019). Chitosan as an environment friendly biomaterial–a review on recent modifications and applications. *International journal of biological macromolecules*, *4*, 1-3.

Bal, E. (2013). Postharvest application of chitosan and low temperature storage affect respiration rate and quality of plum fruits. *J. Agr. Sci. Tech.*, *15*, 1219-1230.

Bansal, M., Chauhan, G. S., Kaushik, A., & Sharma, A. (2016). Extraction and functionalization of bagasse cellulose nanofibres to Schiff-base based antimicrobial membranes. *International journal of biological macromolecules*, *91*, 887-894.

Barbosa-Cánovas, G. V., Fernández-Molina, J. J., Alzamora, S. M., Tapia, M. S., López-Malo, A., Welti Chanes, J. (2003). Basic harvest and post-harvest handling consideration for fresh fruits and vegetables. In: *Handling and preservation of fruits and vegetables by combined methods for rural areas: technical manual*, *149*. Food and Agriculture Organization of the United Nations, Rome.

Bari, M. L., Ukuku, D. O., Kawasaki, T., Inatsu, Y., Isshiki, K., & Kawamoto, S. (2005). Combined efficacy of nisin and pediocin with sodium lactate, citric acid, phytic acid, and potassium sorbate and EDTA in reducing the *Listeria monocytogenes* population of inoculated fresh-cut produce. *Journal of food protection*, *68*(7), 1381-1387.

Barragan-Fonseca, K. B., Dicke, M., & van Loon, J. J. (2017). Nutritional value of the black soldier fly (Hermetia illucens L.) and its suitability as animal feed–a review. *Journal of Insects as Food and Feed*, *3*(2), 105-120.

Barrett, D. M. (2007). Maximizing the nutritional value of fruits & vegetables. *Food technology (Chicago)*, *61*(4), 40-44.

Barrett, D. M., & Lloyd, B. (2012). Advanced preservation methods and nutrient retention in fruits and vegetables. *Journal of the Science of Food and Agriculture*, *92*(1), 7-22.

Barreto, T. A., Andrade, S. C., Maciel, J. F., Arcanjo, N. M., Madruga, M. S., Meireles, B., ... & Magnani, M. (2016). A chitosan coating containing essential oil from *Origanum vulgare* L. to control postharvest mold infections and keep the quality of cherry tomato fruit. *Frontiers in Microbiology*, *7*, 1724.

Baswal, A. K., Dhaliwal, H. S., Singh, Z., Mahajan, B. V. C., Kalia, A., & Gill, K. S. (2020). Influence of carboxy methylcellulose, chitosan and beeswax coatings on cold storage life and quality of Kinnow mandarin fruit. *Scientia Horticulturae*, *260*, 108887.

Baur, S., Klaiber, R., Wei, H., Hammes, W. P., & Carle, R. (2005). Effect of temperature and chlorination of pre-washing water on shelf-life and physiological properties of ready-to-use iceberg lettuce. *Innovative Food Science & Emerging Technologies*, *6*(2), 171-182.

Benhabiles, M. S., Drouiche, N., Lounici, H., Pauss, A., & Mameri, N. (2013). Effect of shrimp chitosan coatings as affected by chitosan extraction processes on postharvest quality of strawberry. *Journal of Food Measurement and Characterization*, *7*(4), 215-221.

Bolat, Y., Bilgin, Ş., Günlü, A., Izci, L., Koca, S. B., Çetinkaya, S., & Koca, H. U. (2010). Chitin-chitosan yield of freshwater crab (Potamon potamios, Olivier 1804) shell. *Pakistan*

Veterinary Journal, *30*(4), 227-31.

Bragg, W.L. (1913). The structure of some crystals as indicated by their diffraction of X-rays. *Proceedings of the Royal Society of London. Series A, Containing papers of a mathematical and physical character*, 89(610): 248-277.

Brigode, C., Hobbi, P., Jafari, H., Verwilghen, F., Baeten, E., & Shavandi, A. (2020). Isolation and physicochemical properties of chitin polymer from insect farm side stream as a new source of renewable biopolymer. *Journal of cleaner production*, *275*, 122924.

Brugnerotto, J., Lizardi, J., Goycoolea, F. M., Argüelles-Monal, W., Desbrieres, J., & Rinaudo, M. (2001). An infrared investigation in relation with chitin and chitosan characterization. *Polymer*, *42*(8), 3569-3580.

Bucio, A., Moreno-Tovar, R., Bucio, L., Espinosa-Dávila, J., & Anguebes-Franceschi, F. (2021). Characterization of beeswax, candelilla wax and paraffin wax for coating cheeses. *Coatings*, *11*(3), 261.

Bulut, E., Sargin, I., Arslan, O., Odabasi, M., Akyuz, B., & Kaya, M. (2017). In situ chitin isolation from body parts of a centipede and lysozyme adsorption studies. *Materials Science and Engineering: C*, *70*, 552-563.

Cabib, E., Bowers, B., Sburlati, A., & Silverman, S. J. (1988). Fungal cell wall synthesis: the construction of a biological structure. *Microbiological sciences*, *5*(12): 370-375.

Cáceres, C., Rendón, P., & Jessup, A. (2011). The FAO/IAEA spreadsheet for designing and operating insect mass-rearing facilities-procedures manual. *FAO Plant Production and Protection Paper*. Food and Agriculture Organization of the United Nations, p. 46.

Calado, T., Venâncio, A., & Abrunhosa, L. (2014). Irradiation for mold and mycotoxin control: A review. *Comprehensive Reviews in Food Science and Food Safety*, *13*(5), 1049-1061.

Cao, W., Tan, C., Zhan, X., Li, H., & Zhang, C. (2014). Ultraviolet irradiation and gradient temperature assisted autolysis for protein recovery from shrimp head waste. *Food chemistry*, *164*, 136-141.

Casadidio, C., Peregrina, D. V., Gigliobianco, M. R., Deng, S., Censi, R., & Di Martino, P. (2019). Chitin and chitosans: Characteristics, eco-friendly processes, and applications in cosmetic science. *Marine drugs*, *17*(6), 369.

Castillo, V., Matos, M., & Müller, A. J. (2003). A new route towards the preparation of a biodegradable nanocomposite based on chitin whiskers from exoskeleton of shrimp and polycaprolacton. *Revista Latinoamericana de Metalurgia y Materiales*, *23*(2), 12-19.

Chae, K. S., Shin, C. S., & Shin, W. S. (2018). Characteristics of cricket (*Gryllus bimaculatus*) chitosan and chitosan-based nanoparticles. *Food science and biotechnology*, *27*(3), 631-639.

Chang, W., Liu, F., Sharif, H. R., Huang, Z., Goff, H. D., Zhong, F. (2018). Preparation of chitosan films by neutralization for improving their preservation effects on chilled meat. *Food Hydrocolloids*, *90*, 50–61.

Chatelet, C., Damour, O., & Domard, A. (2001). Influence of the degree of acetylation on some biological properties of chitosan films. *Biomaterials*, *22*(3), 261-268.

Chatterjee, S., Adhya, M., Guha, A. K., & Chatterjee, B. P. (2005). Chitosan from Mucor rouxii: production and physico-chemical characterization. *Process Biochemistry*, *40*(1), 395-400.

Chattopadhyay, D. P., & Inamdar, M. S. (2010). Aqueous behaviour of chitosan. *International Journal of Polymer Science*, *2010:* 1-7.

Chen, F., Zhang, J., Chen, C., Kowaleguet, M. G., Ban, Z., Fei, L., & Xu, C. (2020). Chitosan-based layer-by-layer assembly: Towards application on quality maintenance of lemon fruits. *Advances in Polymer Technology*, *2020*.

Clark, G. L., & Smith, A. F. (2002). X-ray Diffraction Studies of Chitin, Chitosan, and Derivatives. *The Journal of Physical Chemistry*, *40*(7), 863-879.

Cortizo, M. S., Berghoff, C. F., & Alessandrini, J. L. (2008). Characterization of chitin from *Illex argentinus* squid pen. *Carbohydrate polymers*, *74*(1), 10-15.

Crini, G. (2019). Historical review on chitin and chitosan biopolymers. *Environmental Chemistry Letters*, 17(4): 1623-1643.

Darmawati, E., Nava, N., & Suyatma, N. E. (2019, September). Aloe Vera as A Coating Material for Tropical Fruits Using Spray Method. In *IOP Conference Series: Earth and Environmental Science* (Vol. 309, No. 1, p. 012011). IOP Publishing.

Das, N. G., Khan, P. A., & Hossain, Z. (1996). Chitin from the shell of two coastal portunid crabs of Bangladesh. *Indian Journal of Fisheries*, *43*(4), 413-415.

de Alvarenga, E. S. (2011). Characterization and properties of chitosan. *Biotechnology of biopolymers*, *91*, 48-53.

de Castro, R. J. S., Ohara, A., dos Santos Aguilar, J. G., & Domingues, M. A. F. (2018). Nutritional, functional and biological properties of insect proteins: Processes for obtaining, consumption and future challenges. *Trends in Food Science & Technology*, *76*, 82-89.

de Freitas, C. A. S., de Sousa, P. H. M., Soares, D. J., da Silva, J. Y. G., Benjamin, S. R., & Guedes, M. I. F. (2019). Carnauba wax uses in food–A review. *Food chemistry*, *291*, 38-48.

de Queiroz Antonino, R. S. C. M., Lia Fook, B. R. P., de Oliveira Lima, V. A., de Farias Rached, R. Í., Lima, E. P. N., da Silva Lima, R. J., ... & Lia Fook, M. V. (2017). Preparation and characterization of chitosan obtained from shells of shrimp (Litopenaeus vannamei Boone). *Marine drugs*, *15*(5), 141.

Dehnad, D., Mirzaei, H., Emam-Djomeh, Z., Jafari, S. M., & Dadashi, S. (2014). Thermal and antimicrobial properties of chitosan–nanocellulose films for extending shelf life of ground meat. *Carbohydrate polymers*, *109*, 148-154.

Derrien, C., & Boccuni, A. (2018). Current status of the insect producing industry in Europe. In *Edible insects in sustainable food systems*, ed. by Halloran, A., Flore, R., Vantomme, P. and Roos, N. Springer International Publishing, Cham, pp. 471-479.

Dhall, R. K. (2013). Advances in edible coatings for fresh fruits and vegetables: a review. *Critical reviews in food science and nutrition*, *53*(5), 435-450.

DiGiacomo, K., & Leury, B. J. (2019). Insect meal: a future source of protein feed for pigs? A*nimal*, *13*, 3022-3030.

Dimzon, I. K. D., & Knepper, T. P. (2015). Degree of deacetylation of chitosan by infrared spectroscopy and partial least squares. *International journal of biological macromolecules*, *72*, 939-945.

Dong, H., Cheng, L., Tan, J., Zheng, K., & Jiang, Y. (2004). Effects of chitosan coating on quality and shelf life of peeled litchi fruit. *Journal of food engineering*, *64*(3), 355-358.

Dortmans, B.M.A., Diener, S., Verstappen, B.M. & Zurbrügg, C. (2017). Waste treatment by BSF. *Black Soldier Fly Biowaste Processing. A Step-by-Step Guide*, (6-10). Eawag: Swiss Federal Institute of Aquatic Science and Technology, Dübendorf, Switzerland.

Dotto, G. L., Vieira, M. L., & Pinto, L. A. (2015). Use of chitosan solutions for the microbiological shelf-life extension of papaya fruits during storage at room temperature. *LWT-Food Science and Technology*, *64*(1), 126-130.

Draczynski, Z. (2008). Honeybee corpses as an available source of chitin. *Journal of applied polymer science*, *109*(3), 1974-1981.

Du, J., Gemma, H., & Iwahori, S. (1998). Effects of chitosan coating on the storability and on the ultrastructural changes of'Jonagold'apple fruit in storage. *Food Preservation Science*, *24*(1), 23-29.

Dutta, P. K., Dutta, J., & Tripathi, V. S. (2004). Chitin and chitosan: Chemistry, properties and applications. *Journal of Scientific and Industrial Research*, *63*:20–31.

Dweltz, N.E. (1961). The structure of β-chitin. *Biochimica et Biophysica Acta*, 51:283–294.

El Ghaouth, A., Arul, J., Ponnampalam, R., & Boulet, M. (1991). Use of chitosan coating to reduce water loss and maintain quality of cucumber and bell pepper fruits. *Journal of food processing and preservation*, *15*(5), 359-368.

El Ghaouth, A., Ponnampalam, R., Castaigne, F., & Arul, J. (1992). Chitosan coating to extend the storage life of tomatoes. *HortScience*, *27*(9), 1016-1018.

El Knidri, H., El Khalfaouy, R., Laajeb, A., Addaou, A., & Lahsini, A. (2016). Eco-friendly extraction and characterization of chitin and chitosan from the shrimp shell waste via microwave irradiation. *Process Safety and Environmental Protection*, *104*, 395-405.

Elieh-Ali-Komi, D., & Hamblin, M. R. (2016). Chitin and chitosan: production and application of versatile biomedical nanomaterials. *International journal of advanced research*, *4*(3): 411.

Elsabee, M. Z., & Abdou, E. S. (2013). Chitosan based edible films and coatings: A review. *Materials Science and Engineering: C*, *33*(4), 1819-1841.

Erbil, H. Y., & Muftugil, N. (1986). Lengthening the postharvest life of peaches by coating with hydrophobic emulsions. *Journal of Food Processing and Preservation*, *10*(4), 269-279.

Erdogan, S., & Kaya, M. (2016). High similarity in physicochemical properties of chitin and chitosan from nymphs and adults of a grasshopper. *International journal of biological macromolecules*, *89*, 118-126.

Etienne, A., Génard, M., Lobit, P., Mbeguié-A-Mbéguié, D., & Bugaud, C. (2013). What controls fleshy fruit acidity? A review of malate and citrate accumulation in fruit cells. *Journal of experimental botany*, *64*(6), 1451-1469.

Fajardo, J. E., McCollum, T. G., McDonald, R. E., & Mayer, R. T. (1998). Differential Induction of Proteins in Orange Flavedo by Biologically Based Elicitors and Challenged by *Penicillium digitatum* Sacc. 1. *Biological control*, *13*(3), 143-151.

Fan, X., Xi, Y., Zhao, H., Liu, B., Cao, J., & Jiang, W. (2018). Improving fresh apricot (*Prunus armeniaca* L.) quality and antioxidant capacity by storage at near freezing temperature. *Scientia Horticulturae*, *231*, 1-10.

Fang, S. W., Li, C. F., & Shih, D. Y. (1994). Antifungal activity of chitosan and its preservative effect on low-sugar candied kumquat. *Journal of food protection*, *57*(2), 136-140.

FAOSTAT (2019). Food and agriculture organization corporate statistical database. 2019. Retrieved from http://www.fao.org/faostat/en/#data.

Felicioli, A., Cilia, G., Mancini, S., Turchi, B., Galaverna, G., Cirlini, M., ... & Fratini, F. (2019). In vitro antibacterial activity and volatile characterisation of organic *Apis mellifera ligustica* (Spinola, 1906) beeswax ethanol extracts. *Food Bioscience*, *29*, 102-109.

Fellows, P. (2009). Processing by application of heat. In: *Food processing technology, principles and practice*, 229-352. Second edition, Woodhead Publishing Limited and CRC Press LLC, Cambridge, England.

Felse, P. A., & Panda, T. (1999). Studies on applications of chitin and its derivatives. *Bioprocess Engineering*, *20*(6), 505-512.

Finke, M. D. (2013). Complete nutrient content of four species of feeder insects. *Zoo biology*, *32*(1), 27-36.

Fischer, R. L., & Bennett, A. B. (1991). Role of cell wall hydrolases in fruit ripening. *Annual review of plant biology*, *42*(1), 675-703.

Free, S. J. (2013). Fungal cell wall organization and biosynthesis. *Advances in genetics*, *81*, 33-82.

Fu, X., Zhu, L., Li, L., Zhang, T., Li, M., & Mou, H. (2019). Eco-friendly preparation of chitooligosaccharides with different degrees of deacetylation from shrimp shell waste and their effects on the germination of wheat seeds. *Marine Life Science & Technology*, *1*(1), 95-103.

General standard for food additives (GSFA) online database. FAO/WHO Food standards (http://www.fao.org/gsfaonline/index.html).

Ghasemnezhad, M., Shiri, M. A., & Sanavi, M. (2010). Effect of chitosan coatings on some quality indices of apricot (*Prunus armeniaca* L.) during cold storage. *Caspian journal of environmental sciences*, *8*(1), 25-33.

Ghasemnezhad, M., Nezhad, M. A., & Gerailoo, S. (2011). Changes in postharvest quality of loquat (*Eriobotrya japonica*) fruits influenced by chitosan. *Horticulture, Environment, and Biotechnology*, *52*(1), 40-45.

Giacalone, G., & Chiabrando, V. (2013, June). Effect of preharvest and postharvest application of chitosan coating on storage quality of nectarines. In *VIII International Peach Symposium 1084* (pp. 675-680).

Gillett, R. (2008). *Global study of shrimp fisheries*, in FAO Fisheries Technical Paper, 475. FAO, Rome, p. 331.

Goldman, I. L., Kader, A. A., & Heintz, C. (1999). Influence of production, handling, and storage on phytonutrient content of foods. *Nutrition Reviews*, *57*(9), 46-52.

Goy, R. C., Morais, S. T., & Assis, O. B. (2016). Evaluation of the antimicrobial activity of chitosan and its quaternized derivative on E. coli and S. aureus growth. *Revista Brasileira de Farmacognosia*, *26*(1), 122-127.

Gras, M. L., Vidal, D., Betoret, N., Chiralt, A., & Fito, P. (2003). Calcium fortification of vegetables by vacuum impregnation: interactions with cellular matrix. *Journal of food engineering*, *56*(2-3), 279-284.

Greven, H., Kaya, M., & Baran, T. (2016). The presence of α-chitin in Tardigrada with comments on chitin in the Ecdysozoa. *Zoologischer Anzeiger-A Journal of Comparative Zoology*, *264*, 11-16.

Habiba, U., Siddique, T. A., Talebian, S., Lee, J. J. L., Salleh, A., Ang, B. C., & Afifi, A. M. (2017). Effect of deacetylation on property of electrospun chitosan/PVA nanofibrous membrane and removal of methyl orange, Fe (III) and Cr (VI) ions. *Carbohydrate polymers*, *177*, 32-39.

Hahn, T., Roth, A., Febel, E., Fijalkowska, M., Schmitt, E., Arsiwalla, T. & Zibek, S. (2018). New methods for high-accuracy insect chitin measurement. *Journal of the Science of Food and Agriculture*, 98(13): 5069-5073.

Hahn, T., & Zibek, S. (2018). Sewage Polluted Water Treatment via Chitosan: A review. In *Chitin-Chitosan: Myriad Functionalities in Science and Technology*. InTech, 2018.

Hahn, T., Bossog, L., Hager, T., Wunderlich, W., Breier, R., Stegmaier, T., & Zibek, S. (2019). Chitosan application in textile processing and fabric coating. *Chitin and Chitosan: Properties and Applications*, ed. by van den Broek, B. and Boeriu, C. Wiley VCH, 395-428.

Hahn, T., Tafi, E., Paul, A., Salvia, R., Falabella, P. & Zibek, S. (2020a). Current state of chitin purification and chitosan production from insects. *Journal of Chemical Technology & Biotechnology*, 95(11), 2775-2795.

Hahn, T., Roth, A., Ji, R., Schmitt, E. & Zibek, S. (2020b). Chitosan production with larval exoskeletons derived from the insect protein production. *Journal of biotechnology*, 310, 62-67.

Hahn, T., Tafi, E., Falabella, P., Salvia, R., Thomä, J., Febel, E., Fijalkowska, M., Schmitt, E. & Zibek, S. (under review). Purification of chitin from pupal exuviae of the Black Soldier Fly. *Waste and biomass valorization*.

Hamodrakas, S. J., Willis, J. H., & Iconomidou, V. A. (2002). A structural model of the chitin-binding domain of cuticle proteins. *Insect biochemistry and molecular biology*, *32*(11), 1577-1583.

Han, S. D., Sung, H. J., Lee, G. H., Jun, J. H., Son, M., & Kang, M. J. (2016). Chitosan-based film of tyrothricin for enhanced antimicrobial activity against common skin pathogens including Staphylococcus aureus. *Journal of microbiology and biotechnology*, *26*(5), 953-958.

Hassan, J., Anwar, R., Khan, A. S., Ahmad, S., Malik, A. U., Nafees, M., ... & Inam-ur-Raheem, M. (2020). Chitosan-Based Edible Coating Delays Fungal Decay and Maintains Quality of Strawberries during Storage. *International Journal of Agriculture and Biology*, *24*(3), 486-492.

Hatchett, C. (1799). XVIII. Experiments and observations on shell and bone. *Philosophical Transactions of the Royal Society of London*, (89): 315-334.

Hayes, M., Carney, B., Slater, J., & Brück, W. (2008). Mining marine shellfish wastes for bioactive molecules: Chitin and chitosan Part A: extraction methods. *Biotechnology Journal: Healthcare Nutrition Technology*, *3*(7), 871-877.

Heinz, V., & Buckow, R. (2010). Food preservation by high pressure. *Journal of Consumer Protection and Food Safety*, *5*(1), 73-81.

Hernandez, E. (1991). Edible coatings from lipids and resins. In: *Edible Coatings and Films to Improve Food Quality*, pp. 279–303. Krochta, J. M., Baldwin, E. A. and Nisperos-Carriedo, M., Eds., Technomic Publishing Company, Lancaster, PA.

Hernandez, E. (1994). Edible coating from lipids and resins. In: *Edible Coatings and Films to Improve Food Quality*, pp. 279–303. Krochta, J. M., Balwin, E. A. and Niperos-Carriedo, M. O., Eds., Technomic Publishing Company, Basel, Switzerland.

Hernández-Muñoz, P., Almenar, E., Ocio, M. J., & Gavara, R. (2006). Effect of calcium dips and chitosan coatings on postharvest life of strawberries (Fragaria x ananassa). *Postharvest Biology and Technology*, *39*(3), 247-253.

Heux, L., Brugnerotto, J., Desbrieres, J., Versali, M. F., & Rinaudo, M. (2000). Solid state NMR for determination of degree of acetylation of chitin and chitosan. *Biomacromolecules*, *1*(4), 746-751.

Hopkins, T. L., & Kramer, K. J. (1992). Insect cuticle sclerotization. *Annual review of entomology*, *37*(1), 273-302.

Hossain, M. S., & Iqbal, A. (2014). Production and characterization of chitosan from shrimp waste. *Journal of the Bangladesh Agricultural University*, *12*(1), 153-160.

Hours, R. A., & Gortari, M. C. (2013). Biotechnological processes for chitin recovery out of crustacean waste: a mini-review. *Electronic Journal of Biotechnology*, *16*(3), 14-14.

Hu, X., Du, Y., Tang, Y., Wang, Q., Feng, T., Yang, J., & Kennedy, J. F. (2007). Solubility and property of chitin in NaOH/urea aqueous solution. *Carbohydrate Polymers*, *70*(4), 451-458.

Hua, G., & Reckhow, D. A. (2007). Comparison of disinfection byproduct formation from chlorine and alternative disinfectants. *Water research*, *41*(8), 1667-1678.

Huang, H. W., Wu, S. J., Lu, J. K., Shyu, Y. T., & Wang, C. Y. (2017). Current status and future trends of high-pressure processing in food industry. *Food control*, *72*, 1-8.

Hudson, S. M., & Smith, C. (1998). Polysaccharides: chitin and chitosan: chemistry and technology of their use as structural materials. In *Biopolymers from renewable resources* (pp. 96-118). Springer, Berlin, Heidelberg.

Huet, G., Hadad, C., Husson, E., Laclef, S., Lambertyn, V., Farias, M. A., ... & Van Nhien, A. N. (2020). Straightforward extraction and selective bioconversion of high purity chitin from *Bombyx eri* larva: Toward an integrated insect biorefinery. *Carbohydrate polymers*, *228*, 115382.

Hussain, M. R., Iman, M., & Maji, T. K. (2013). Determination of degree of deacetylation of chitosan and their effect on the release behavior of essential oil from chitosan and chitosan-gelatin complex microcapsules. *International Journal of Advanced Engineering Applications*, *6*(4), 4-12.

Hwang, J. K., & Shin, H. H. (2000). Rheological properties of chitosan solutions. *Korea-Australia Rheology Journal*, *12*(3_4), 175-179.

Ibitoye, E. B., Lokman, I. H., Hezmee, M. N. M., Goh, Y. M., Zuki, A. B. Z., & Jimoh, A. A. (2018). Extraction and physicochemical characterization of chitin and chitosan isolated from house cricket. *Biomedical Materials*, *13*(2), 025009.

Iturriaga, L., Olabarrieta, I., Castellan, A., Gardrat, C., & Coma, V. (2014). Active naringin-chitosan films: Impact of UV irradiation. *Carbohydrate polymers*, *110*, 374-381.

International Organization for Standardization (1993). *Soil quality - Determination of dry matter and water content on a mass basis - Gravimetric method* (EN ISO Standard No. 11465:1993).

International Organization for Standardization (1998). *Fruit and vegetable products - Determination of titratable acidity* (EN ISO Standard No. 750:1998).

International Organization for Standardization (2003). *Fruit and vegetable products - Determination of soluble solids - Refractometric method* (EN ISO Standard No. 2173:2003).

International Organization for Standardization (2008). *Animal feeding stuffs - Determination of acid detergent fibre (ADF) and acid detergent lignin (ADL) contents* (EN ISO Standard No. 13906:2008).

International Organization for Standardization (2017). *Solid biofuels - Sample preparation* –

Ash content (EN ISO Standard No. 14780:2017).

Ioelovich, M. (2014). Crystallinity and hydrophility of chitin and chitosan. *J. Chem*, *3*(3), 7-14.

Islam, M. M., & Uddin, G. S. (2016). Irradiation to Insure Safety and Quality of Fruit Salads Consumed in Bangladesh. *Journal of Food and Nutrition Research*, *4*(1), 40-45.

Islam, S., Bhuiyan, M. R., & Islam, M. N. (2017). Chitin and chitosan: structure, properties and applications in biomedical engineering. *Journal of Polymers and the Environment*, *25*(3), 854-866.

James, I. F. & Kuipers, B. (2003). *AD03E Preservation of fruit and vegetables* (No. 3). Agromisa Foundation, Wageningen.

Jang, M. K., Kong, B. G., Jeong, Y. I., Lee, C. H., & Nah, J. W. (2004). Physicochemical characterization of α-chitin, β-chitin, and γ-chitin separated from natural resources. *Journal of Polymer Science Part A: Polymer Chemistry*, *42*(14): 3423-3432.

Jardine, A., & Sayed, S. (2018). Valorisation of chitinous biomass for antimicrobial applications. *Pure and Applied Chemistry*, *90*(2), 293-304.

Jayanegara, A., Haryati, R. P., Nafisah, A., Suptijah, P., Ridla, M., & Laconi, E. B. (2020). Derivatization of chitin and chitosan from black soldier fly (*Hermetia illucens*) and their use as feed additives: An in vitro study. *Advances in Animal Veterinary Science*, *8*(5), 472-477.

Jaworska, M., Sakurai, K., Gaudon, P., & Guibal, E. (2003). Influence of chitosan characteristics on polymer properties. I: Crystallographic properties. *Polymer International*, *52*(2), 198-205.

Jiang, Y. (2000). Role of anthocyanins, polyphenol oxidase and phenols in lychee pericarp browning. *Journal of the Science of Food and Agriculture*, *80*(3), 305-310.

Jiang, Y., & Li, Y. (2001). Effects of chitosan coating on postharvest life and quality of longan fruit. *Food Chemistry*, *73*(2), 139-143.

Jiang, X., Chen, L., & Zhong, W. (2003). A new linear potentiometric titration method for the determination of deacetylation degree of chitosan. *Carbohydrate Polymers*, 54(4): 457-463.

Jiang, Y., Li, J., & Jiang, W. (2005). Effects of chitosan coating on shelf life of cold-stored litchi fruit at ambient temperature. *LWT-food Science and Technology*, *38*(7), 757-761.

Jiang, T., Feng, L., & Zheng, X. (2012). Effect of chitosan coating enriched with thyme oil on postharvest quality and shelf life of shiitake mushroom (*Lentinus edodes*). *Journal of agricultural and food chemistry*, *60*(1), 188-196.

Jiang, H., Sun, Z., Jia, R., Wang, X., & Huang, J. (2016). Effect of chitosan as an antifungal and preservative agent on postharvest blueberry. *Journal of Food Quality*, *39*(5), 516-523.

Jiang, Y., Fu, C., Wu, S., Liu, G., Guo, J., & Su, Z. (2017). Determination of the deacetylation degree of chitooligosaccharides. *Marine drugs*, *15*(11), 332.

Jiang, Y., Yu, L., Hu, Y., Zhu, Z., Zhuang, C., Zhao, Y., & Zhong, Y. (2019). Electrostatic spraying of chitosan coating with different deacetylation degree for strawberry preservation. *International journal of biological macromolecules*, *139*, 1232-1238.

Jiang, Y., Yu, L., Hu, Y., Zhu, Z., Zhuang, C., Zhao, Y., & Zhong, Y. (2020). The preservation performance of chitosan coating with different molecular weight on strawberry using electrostatic spraying technique. *International journal of biological macromolecules*, *151*, 278-285.

Jianglian, D., & Shaoying, Z. (2013). Application of chitosan-based coating in fruit and vegetable preservation: a review. *J. Food Process. Technol*, *4*(5), 227.

Jitareerat, P., Paumchai, S., Kanlayanarat, S., & Sangchote, S. (2007). Effect of chitosan on ripening, enzymatic activity, and disease development in mango (*Mangifera indica*) fruit. *New Zealand journal of crop and horticultural science*, *35*(2), 211-218.

Jongsri, P., Wangsomboondee, T., Rojsitthisak, P., & Seraypheap, K. (2016). Effect of molecular weights of chitosan coating on postharvest quality and physicochemical characteristics of mango fruit. *Lwt*, *73*, 28-36.

Jouquand, C., Chandler, C., Plotto, A., & Goodner, K. (2008). A sensory and chemical analysis of fresh strawberries over harvest dates and seasons reveals factors that affect eating quality. *Journal of the American Society for Horticultural Science*, *133*(6), 859-867.

Jucker, C., Lupi, D., Moore, C. D., Leonardi, M. G., & Savoldelli, S. (2020). Nutrient recapture from insect farm waste: bioconversion with Hermetia illucens (L.) (Diptera: Stratiomyidae). *Sustainability*, *12*, 1-14.

Kabanov, V. L., & Novinyuk, L. V. (2020). Chitosan application in food technology: A review of rescent advances. *Food systems*, *3*(1), 10-15.

Kader, A. A., & Watkins, C. B. (2000). Modified atmosphere packaging—toward 2000 and beyond. *HortTechnology*, *10*(3), 483-486.

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., ... & Shibuya, N. (2006). Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proceedings of the National Academy of Sciences*, *103*(29), 11086-11091.

Kanatt, S. R., Chander, R., & Sharma, A. (2006). Effect of radiation processing of lamb meat on its lipids. *Food chemistry*, *97*(1), 80-86.

Kasaai, M. R. (2008). A review of several reported procedures to determine the degree of N-acetylation for chitin and chitosan using infrared spectroscopy. *Carbohydrate polymers*, *71*(4), 497-508.

Kaya, M., Baublys, V., Can, E., Šatkauskienė, I., Bitim, B., Tubelytė, V., & Baran, T. (2014a). Comparison of physicochemical properties of chitins isolated from an insect (*Melolontha melolontha*) and a crustacean species (*Oniscus asellus*). *Zoomorphology*, *133*(3), 285-293.

Kaya, M., Seyyar, O., Baran, T., & Turkes, T. (2014b). Bat guano as new and attractive chitin

and chitosan source. *Frontiers in Zoology*, *11*(1), 1-10.

Kaya, M., Baran, T., Mentes, A., Asaroglu, M., Sezen, G., & Tozak, K. O. (2014c). Extraction and characterization of α-chitin and chitosan from six different aquatic invertebrates. *Food biophysics*, *9*(2), 145-157.

Kaya, M., Baran, T., Erdoğan, S., Menteş, A., Özüsağlam, M. A., & Çakmak, Y. S. (2014d). Physicochemical comparison of chitin and chitosan obtained from larvae and adult Colorado potato beetle (*Leptinotarsa decemlineata*). *Materials Science and Engineering: C*, *45*, 72-81.

Kaya, M., & Baran, T. (2015). Description of a new surface morphology for chitin extracted from wings of cockroach (*Periplaneta americana*). *International journal of biological macromolecules*, *75*, 7-12.

Kaya, M., Lelešius, E., Nagrockaitė, R., Sargin, I., Arslan, G., Mol, A., ... & Bitim, B. (2015a). Differentiations of chitin content and surface morphologies of chitins extracted from male and female grasshopper species. *PloS one*, *10*(1): e0115531.

Kaya, M., Baublys, V., Šatkauskienė, I., Akyuz, B., Bulut, E., & Tubelytė, V. (2015b). First chitin extraction from *Plumatella repens* (Bryozoa) with comparison to chitins of insect and fungal origin. *International journal of biological macromolecules*, *79*, 126-132.

Kaya, M., Bağrıaçık, N., Seyyar, O., & Baran, T. (2015c). Comparison of chitin structures derived from three common wasp species (*Vespa crabro* Linnaeus, 1758, *Vespa orientalis* Linnaeus, 1771 and *Vespula germanica* (Fabricius, 1793)). *Archives of insect biochemistry and physiology*, *89*(4), 204-217.

Kaya, M., Erdogan, S., Mol, A., & Baran, T. (2015d). Comparison of chitin structures isolated from seven Orthoptera species. *International journal of biological macromolecules*, *72*, 797-805.

Kaya, M., Mujtaba, M., Bulut, E., Akyuz, B., Zelencova, L., & Sofi, K. (2015e). Fluctuation in physicochemical properties of chitins extracted from different body parts of honeybee. *Carbohydrate Polymers*, *132*, 9-16.

Kaya, M., Bitim, B., Mujtaba, M., & Koyuncu, T. (2015f). Surface morphology of chitin highly related with the isolated body part of butterfly (*Argynnis pandora*). *International journal of biological macromolecules*, *81*, 443-449.

Kaya, M., Baran, T., Asan-Ozusaglam, M., Cakmak, Y. S., Tozak, K. O., Mol, A., ... & Sezen, G. (2015g). Extraction and characterization of chitin and chitosan with antimicrobial and antioxidant activities from cosmopolitan Orthoptera species (Insecta). *Biotechnology and Bioprocess Engineering*, *20*(1), 168-179.

Kaya, M., & Sargin, I. (2016). Highly fibrous and porous raw material shaped chitin isolated from Oniscus sp. (Crustacea). *Food Biophysics*, *11*(1), 101-107.

Kaya, M., Mulerčikas, P., Sargin, I., Kazlauskaitė, S., Baublys, V., Akyuz, B., ... & Tubelytė, V. (2016a). Three-dimensional chitin rings from body segments of a pet diplopod species: Characterization and protein interaction studies. *Materials Science and Engineering: C*, *68*, 716-722.

Kaya, M., Sofi, K., Sargin, I., & Mujtaba, M. (2016b). Changes in physicochemical properties of chitin at developmental stages (larvae, pupa and adult) of *Vespa crabro* (wasp). *Carbohydrate polymers*, *145*, 64-70.

Kaya, M., Akyuz, B., Bulut, E., Sargin, I., Eroglu, F., & Tan, G. (2016c). Chitosan nanofiber production from *Drosophila* by electrospinning. *International journal of biological macromolecules*, *92*, 49-55.

Kaya, M., Sargin, I., Aylanc, V., Tomruk, M. N., Gevrek, S., Karatoprak, I., ... & Bulut, E. (2016d). Comparison of bovine serum albumin adsorption capacities of α-chitin isolated from an insect and β-chitin from cuttlebone. *Journal of Industrial and Engineering Chemistry*, *38*, 146-156.

Kaya, M., Bulut, E., Mujtaba, M., Sivickis, K., Sargin, I., Akyuz, B., & Erdogan, S. (2016e). Gender influences differentiation of chitin among body parts. *Archives of insect biochemistry and physiology*, *93*(2), 96-109.

Kaya, M., Mujtaba, M., Ehrlich, H., Salaberria, A. M., Baran, T., Amemiya, C. T., ... & Labidi, J. (2017). On chemistry of γ-chitin. *Carbohydrate polymers*, *176*, 177-186.

Kaya, M., Sargin, I., Sabeckis, I., Noreikaite, D., Erdonmez, D., Salaberria, A. M., ... & Tubelytė, V. (2017a). Biological, mechanical, optical and physicochemical properties of natural chitin films obtained from the dorsal pronotum and the wing of cockroach. *Carbohydrate polymers*, *163*, 162-169.

Kaya, M., Sargin, I., Mulerčikas, P., Labidi, J., Salaberria, A. M., Cakmak, Y. S., ... & Baublys, V. (2019). Conversion of waste parasitic insect (*Hylobius abietis* L.) into antioxidative, antimicrobial and biodegradable films. *Journal of Renewable Materials*, *7*(3), 215-226.

Kerch, G. (2015). Chitosan films and coatings prevent losses of fresh fruit nutritional quality: A review. *Trends in Food Science & Technology*, *46*(2), 159-166.

Khaliq, G., Mohamed, M. T. M., Ali, A., Ding, P., & Ghazali, H. M. (2015). Effect of gum arabic coating combined with calcium chloride on physico-chemical and qualitative properties of mango (*Mangifera indica* L.) fruit during low temperature storage. *Scientia Horticulturae*, *190*, 187-194.

Khayrova, A., Lopatin, S., & Varlamov, V. (2019). Black soldier fly *Hermetia illucens* as a novel source of chitin and chitosan. *International Journal of Sciences*, *8*(4), 81-86.

Khayrova, A., Lopatin, S., & Varlamov, V. (2020, April). Obtaining chitin/chitosan-melanin complexes from black soldier fly *Hermetia illucens*. In *IOP Conference Series: Materials Science and Engineering* (Vol. 809, No. 1, p. 012020). IOP Publishing.

Khor, E., & Lim, L. Y. (2003). Implantable applications of chitin and chitosan. *Biomaterials*, *24*(13), 2339-2349.

Khwaldia, K., Basta, A. H., Aloui, H., & El-Saied, H. (2014). Chitosan–caseinate bilayer coatings for paper packaging materials. *Carbohydrate polymers*, *99*, 508-516.

Kim, W. J., Lee, W. G., Theodore, K., & Chang, H. N. (2001). Optimization of culture conditions and continuous production of chitosan by the fungi, Absidia coerulea. *Biotechnology and Bioprocess Engineering*, *6*(1), 6-10.

Kim, S. K., & Rajapakse, N. (2005). Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. *Carbohydrate polymers*, *62*(4), 357-368.

Kim, M. W., Han, Y. S., Jo, Y. H., Choi, M. H., Kang, S. H., Kim, S. A., & Jung, W. J. (2016). Extraction of chitin and chitosan from housefly, *Musca domestica*, pupa shells. *Entomological Research*, *46*(5), 324-328.

Kim, M. W., Song, Y. S., Han, Y. S., Jo, Y. H., Choi, M. H., Park, Y. K., ... & Jung, W. J. (2017). Production of chitin and chitosan from the exoskeleton of adult two-spotted field crickets (*Gryllus bimaculatus*). *Entomological Research*, *47*(5), 279-285.

Kim, T. K., Yong, H. I., Kim, Y. B., Kim, H. W., & Choi, Y. S. (2019). Edible insects as a protein source: a review of public perception, processing technology, and research trends. *Food science of animal resources*, *39*, 521-540.

Kittur, F. S., Saroja, N., & Tharanathan, R. (2001). Polysaccharide-based composite coating formulations for shelf-life extension of fresh banana and mango. *European food research and technology*, *213*(4), 306-311.

Kleinhenz, M.D., & Bumgarner, N.R. (2013). Using °Brix as an Indicator of Vegetable Quality: An Overview of the Practice. *Ohioline, Ohio State University Extension* (https://ohioline.osu.edu/factsheet/HYG-1650).

Klowden, M.J. (2013) Male reproductive system, in *Physiological Systems in Insects*. Academic Press, pp. 203–214.

Kramer, K. J., Hopkins, T. L., & Schaefer, J. (1995). Applications of solids NMR to the analysis of insect sclerotized structures. *Insect Biochemistry and Molecular Biology*, *25*(10), 1067-1080.

Krotchta, J. M., & De Mulder-Johnston, C. (1997). Edible & biodegradable polymer films: Challenges and opportunities. *J Food Technol-chicago*, *51*, 2-8.

Kumar, M. N. R. (2000). A review of chitin and chitosan applications. *Reactive and functional polymers*, *46*(1): 1-27.

Kumar, P., Sethi, S., Sharma, R. R., Srivastav, M., Varghese, E. (2017). Effect of chitosan coating on postharvest life and quality of plum during storage at low temperature. *Scientia Horticulturae*, *226*, 104–109

Kumar, S., Mukherjee, A., & Dutta, J. (2020). Chitosan based nanocomposite films and coatings: Emerging antimicrobial food packaging alternatives. *Trends in Food Science & Technology*, *97*, 196-209.

Kumari, S., Annamareddy, S. H. K., Abanti, S., & Rath, P. K. (2017). Physicochemical properties and characterization of chitosan synthesized from fish scales, crab and shrimp shells. *International journal of biological macromolecules*, *104*, 1697-1705.

Kumirska, J., Czerwicka, M., Kaczyński, Z., Bychowska, A., Brzozowski, K., Thöming, J., & Stepnowski, P. (2010). Application of spectroscopic methods for structural analysis of chitin and chitosan. *Marine drugs*, *8*(5), 1567-1636.

Kurita, K., Tomita, K., Tada, T., Ishii, S., Nishimura, S. I., & Shimoda, K. (1993). Squid chitin as a potential alternative chitin source: deacetylation behavior and characteristic properties. *Journal of Polymer Science Part A: Polymer Chemistry*, *31*(2), 485-491.

Kurita, K. (2006). Chitin and chitosan: functional biopolymers from marine crustaceans. *Marine biotechnology*, *8*(3), 203-226.

Lago, M. A., Sendón, R., de Quirós, A. R. B., Sanches-Silva, A., Costa, H. S., Sánchez-Machado, D. I., ... & Paseiro, P. (2014). Preparation and characterization of antimicrobial films based on chitosan for active food packaging applications. *Food and bioprocess technology*, *7*(10), 2932-2941.

Lamarque, G., Cretenet, M., Viton, C., & Domard, A. (2005). New route of deacetylation of α- and β-chitins by means of freeze− pump out− thaw cycles. *Biomacromolecules*, *6*(3), 1380-1388.

Laroche, M., Perreault, V., Marciniak, A., Gravel, A., Chamberland, J., & Doyen, A. (2019). Comparison of conventional and sustainable lipid extraction methods for the production of oil and protein isolate from edible insect meal. *Foods*, *8*(11), 572.

Latgé, J. P. (2007). The cell wall: a carbohydrate armour for the fungal cell. *Molecular microbiology*, *66*(2): 279-290.

Layne, E. (1972). Spectrophotometric and turbidimetric methods for measuring proteins. In: Britton Chance (Hg.): *Preparation and assay of substrates*, Bd. 3. 6. printing. New York, Academic Press (Methods in Enzymology), 3: 447–454.

Leceta, I., Molinaro, S., Guerrero, P., Kerry, J. P., & De la Caba, K. (2015). Quality attributes of map packaged ready-to-eat baby carrots by using chitosan-based coatings. *Postharvest Biology and Technology*, *100*, 142-150.

Li, F., Biagioni, P., Finazzi, M., Tavazzi, S., & Piergiovanni, L. (2013). Tunable green oxygen barrier through layer-by-layer self-assembly of chitosan and cellulose nanocrystals. *Carbohydrate polymers*, *92*(2), 2128-2134.

Li, H., Wang, Y., Liu, F., Yang, Y., Wu, Z., Cai, H., Zhang, Q., Wang, Y., Li, P. (2015). Effects of chitosan on control of postharvest blue mold decay of apple fruit and the possible mechanisms involved. *Scientia Horticulturae, 186,* 77–83

Li, J., Li, Q., Lei, X., Tian, W., Cao, J., Jiang, W., & Wang, M. (2018). Effects of wax coating on the moisture loss of cucumbers at different storage temperatures. *Journal of food quality*, *2018*.

Lim, S. H., & Hudson, S. M. (2003). Review of chitosan and its derivatives as antimicrobial agents and their uses as textile chemicals. *Journal of macromolecular science, part C: Polymer reviews*, *43*(2), 223-269.

Lin, Y., Li, N., Lin, H., Lin, M., Chen, Y., Wang, H., ... & Lin, Y. (2020). Effects of chitosan treatment on the storability and quality properties of longan fruit during storage. *Food chemistry*, *306*, 125627.

Liu, X., Lin Guan, Y., Zhi Yang, D., Li, Z., & De Yao, K. (2001). Antibacterial action of chitosan and carboxymethylated chitosan. *Journal of applied polymer science*, *79*(7), 1324-1335.

Liu, J., Tian, S., Meng, X., & Xu, Y. (2007). Effects of chitosan on control of postharvest diseases and physiological responses of tomato fruit. *Postharvest Biology and Technology*, *44*(3), 300-306.

Liu, S., Sun, J., Yu, L., Zhang, C., Bi, J., Zhu, F., ... & Yang, Q. (2012). Extraction and characterization of chitin from the beetle *Holotrichia parallela* Motschulsky. *Molecules*, *17*(4), 4604-4611.

Liu, F., Antoniou, J., Li, Y., Yi, J., Yokoyama, W., Ma, J., & Zhong, F. (2015). Preparation of gelatin films incorporated with tea polyphenol nanoparticles for enhancing controlled-release antioxidant properties. *Journal of Agricultural and Food Chemistry*, *63*(15), 3987-3995.

Liu, Y., Yuan, Y., Duan, S., Li, C., Hu, B., Liu, A., ... & Wu, W. (2020). Preparation and characterization of chitosan films with three kinds of molecular weight for food packaging. *International journal of biological macromolecules*, *155*, 249-259.

Liu, D. K., Xu, C. C., Guo, C. X., & Zhang, X. X. (2020a). Sub-zero temperature preservation of fruits and vegetables: A review. *Journal of Food Engineering*, *275*, 109881.

Luo, Q., Wang, Y., Han, Q., Ji, L., Zhang, H., Fei, Z., & Wang, Y. (2019). Comparison of the physicochemical, rheological, and morphologic properties of chitosan from four insects. *Carbohydrate Polymers*, *209*, 266-275.

Ma, W., Tang, C. H., Yang, X. Q., & Yin, S. W. (2013). Fabrication and characterization of kidney bean (*Phaseolus vulgaris* L.) protein isolate–chitosan composite films at acidic pH. *Food Hydrocolloids*, *31*(2), 237-247.

Ma, J., Xin, C., & Tan, C. (2015). Preparation, physicochemical and pharmaceutical characterization of chitosan from *Catharsius molossus* residue. *International journal of biological macromolecules*, *80*, 547-556.

Maciel, V. B. V., Yoshida, C. M., & Franco, T. T. (2015). Chitosan/pectin polyelectrolyte complex as a pH indicator. *Carbohydrate polymers*, *132*, 537-545.

Mahajan, B. C., Tandon, R., Kapoor, S., & Sidhu, M. K. (2018). Natural coatings for shelf-life enhancement and quality maintenance of fresh fruits and vegetables—A review. *J. Postharvest Technol*, *6*(1), 12-26.

Mahmoud, N. S., Ghaly, A. E., & Arab, F. (2007). Unconventional approach for demineralization of deproteinized crustacean shells for chitin production. *American Journal of Biochemistry and Biotechnology*, *3*(1), 1-9.

Mai, H. C., Dao, N. D., Lam, T. D., Nguyen, B. V., Nguyen, D. C., & Bach, L. G. (2019). Purification process, physicochemical properties, and fatty acid composition of black soldier fly (Hermetia illucens Linnaeus) larvae oil. *Journal of the American Oil Chemists' Society*, *96*(11), 1303-1311.

Majtán, J., Bíliková, K., Markovič, O., Gróf, J., Kogan, G., & Šimúth, J. (2007). Isolation and characterization of chitin from bumblebee (*Bombus terrestris*). *International journal of biological macromolecules*, *40*(3), 237-241.

Malhotra, B. H. A. N. U., Keshwani, A., & Kharkwal, H. A. R. S. H. A. (2015). Natural polymer based cling films for food packaging. *International Journal of Pharmacy and Pharmaceutical Sciences*, *7*(4), 10-18.

Mangaraj, S., & Goswami, T. K. (2009). Modified atmosphere packaging of fruits and vegetables for extending shelf-life-A review. *Fresh produce*, *3*(1), 1-31.

Manjula, K., & Podile, A. R. (2001). Chitin-supplemented formulations improve biocontrol and plant growth promoting efficiency of *Bacillus subtilis* AF 1. *Canadian Journal of Microbiology*, *47*(7): 618-625.

Marei, N. H., Abd El-Samie, E., Salah, T., Saad, G. R., & Elwahy, A. H. (2016). Isolation and characterization of chitosan from different local insects in Egypt. *International journal of biological macromolecules*, *82*, 871-877.

Maringgal, B., Hashim, N., Tawakkal, I. S. M. A., & Mohamed, M. T. M. (2020). Recent advance in edible coating and its effect on fresh/fresh-cut fruits quality. *Trends in Food Science & Technology*, *96*, 253-267.

Martínez-Romero, D., Alburquerque, N., Valverde, J. M., Guillén, F., Castillo, S., Valero, D., & Serrano, M. (2006). Postharvest sweet cherry quality and safety maintenance by *Aloe vera* treatment: a new edible coating. *Postharvest Biology and Technology*, *39*(1), 93-100.

Martinou, A., Kafetzopoulos, D., & Bouriotis, V. (1995). Chitin deacetylation by enzymatic means: monitoring of deacetylation processes. *Carbohydrate Research*, *273*(2), 235-242.

Monjazeb Marvdashti, L., Abdulmajid Ayatollahi, S., Salehi, B., Sharifi-Rad, J., Abdolshahi, A., Sharifi-Rad, R., & Maggi, F. (2020). Optimization of edible *Alyssum homalocarpum* seed gum-chitosan coating formulation to improve the postharvest storage potential and quality of apricot (*Prunus armeniaca* L.). *Journal of Food Safety*, *40*(4), e12805.

Mehranian, M., Pourabad, R. F., Bashir, N. S., & Taieban, S. (2017). Physicochemical characterization of chitin from the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). *Journal of Macromolecular Science, Part A*, *54*(10), 720-726.

Meng, X., Li, B., Liu, J., & Tian, S. (2008). Physiological responses and quality attributes of table grape fruit to chitosan preharvest spray and postharvest coating during storage. *Food Chemistry*, *106*(2), 501-508.

Meng, X., Yang, L., Kennedy, J. F., & Tian, S. (2010). Effects of chitosan and oligochitosan on growth of two fungal pathogens and physiological properties in pear fruit. *Carbohydrate Polymers*, *81*(1), 70-75.

Merzendorfer, H., & Zimoch, L. (2003). Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. *Journal of Experimental Biology*, *206*(24), 4393-4412.

Merzendorfer, H. (2011). The cellular basis of chitin synthesis in fungi and insects: common principles and differences. *European journal of cell biology*, *90*(9): 759-769.

Minke, R. A. M., & Blackwell, J. (1978). The structure of α-chitin. *Journal of molecular biology*, *120*(2), 167-181.

Mogilevskaya, E. L., Akopova, T. A., Zelenetskii, A. N., & Ozerin, A. N. (2006). The crystal structure of chitin and chitosan. *Polymer Science Series A*, *48*(2), 116-123.

Mohammed, M. H., Williams, P. A., & Tverezovskaya, O. (2013). Extraction of chitin from prawn shells and conversion to low molecular mass chitosan. *Food Hydrocolloids*, *31*(2), 166-171.

Mol, A., Kaya, M., Mujtaba, M., & Akyuz, B. (2018). Extraction of high thermally stable and nanofibrous chitin from Cicada (Cicadoidea). *Entomological Research*, *48*(6), 480-489.

Monter-Miranda, J. G., Tirado-Gallegos, J. M., Zamudio-Flores, P. B., Rios-Velasco, C., Ornelas-Paz, J. D. J., Salgado-Delgado, R., ... & Hernandez-Centeno, E. (2016). Extraction and characterization of phisicochemical, morphological, and structural properties of chitin and chitosan from *Brachystola magna* (Girard). *Revista Mexicana de Ingeniería Química*, *15*(3), 749-761.

Moorjani, M. N. (1975). Parameters affecting the viscosity of chitosan from prawn waste. *Journal of food science and technology*, *12*(4), 187-189.

Morganti, P., & Morganti, G. (2008). Chitin nanofibrils for advanced cosmeceuticals. *Clinics in dermatology*, *26*(4), 334-340.

Mourya, V. K., & Inamdar, N. N. (2008). Chitosan-modifications and applications: opportunities galore. *Reactive and Functional polymers*, *68*(6), 1013-1051.

Muxika, A., Etxabide, A., Uranga, J., Guerrero, P., & De La Caba, K. (2017). Chitosan as a bioactive polymer: Processing, properties and applications. *International Journal of Biological Macromolecules*, *105*, 1358-1368.

Nafisah, A., Mutia, R., & Jayanegara, A. (2019, June). Chemical composition, chitin and cell wall nitrogen content of Black Soldier Fly (*Hermetia illucens*) larvae after physical and biological treatment. In *IOP Conference Series: Materials Science and Engineering* (Vol. 546, No. 4, p. 042028). IOP Publishing.

Nemtsev, S. V., Gamzazade, A. I., Rogozhin, S. V., Bykova, V. M., & Bykov, V. P. (2002). Deacetylation of chitin under homogeneous conditions. *Applied Biochemistry and Microbiology*, *38*(6), 521-526.

Nemtsev, S. V., Zueva, O. Y., Khismatullin, M. R., Albulov, A. I., & Varlamov, V. P. (2004). Isolation of chitin and chitosan from honeybees. *Applied Biochemistry and*

Microbiology, *40*(1), 39-43.

No, H. K., & Meyers, S. P. (1989). Crawfish chitosan as a coagulant in recovery of organic compounds from seafood processing streams. *Journal of Agricultural and Food Chemistry*, *37*(3), 580-583.

Norzita, Y., Norhashidah, T., & Maznah, M. (2011). Determination of viscosity-average molecular weight of chitosan using intrinsic viscosity measurement. *Journal of Nuclear and Related Technologies*, *10*, 40-44.

Oduor-Odeto, P. M., Struszezyk, M. H., & Peter, M. G. (2005). Characterisation of chitosan from blowfly larvae and some Crustacean species from Kenyan marin waters prepared under different conditions. *Western Indian Ocean Journal of Marine Science*, *4*(1), 99-108.

Ogawa, K., Hirano, S., Miyanishi, T., Yui, T., & Watanabe, T. (1984). A new polymorph of chitosan. *Macromolecules*, *17*(4), 973-975.

Olawuyi, I. F., & Lee, W. (2019). Influence of chitosan coating and packaging materials on the quality characteristics of fresh-cut cucumber. *Korean Journal of Food Preservation*, *26*(4), 371-380.

Oliveira F., Doelle K., List R., & O'Reilly J. R. (2015). Assessment of Diptera: Stratiomyidae, genus *Hermetia illucens* (L., 1758) using electron microscopy. *Journal of Entomology and Zoology Studies*, 3(5), 147-152.

Olson, B. J., & Markwell, J. (2007). Assays for determination of protein concentration. *Current protocols in protein science*, *48*(1), 3-4.

Ong, S. Y., Wu, J., Moochhala, S. M., Tan, M. H., & Lu, J. (2008). Development of a chitosan-based wound dressing with improved hemostatic and antimicrobial properties. *Biomaterials*, *29*(32), 4323-4332.

Orlova, N., Kuzmenko, I., & Romanenko, R. (2015). Impact of canning method to structural and mechanical properties fruits and vegetables. *Ukrainian Journal of Food Science*, *3*, 225-233.

Ortiz, J. C., Ruiz, A. T., Morales-Ramos, J. A., Thomas, M., Rojas, M. G., Tomberlin, J. K., ... & Jullien, R. L. (2016). Insect mass production technologies. In *Insects as sustainable food ingredients* (pp. 153-201). Academic Press.

Pacheco, N., Garnica-Gonzalez, M., Gimeno, M., Bárzana, E., Trombotto, S., David, L., & Shirai, K. (2011). Structural characterization of chitin and chitosan obtained by biological and chemical methods. *Biomacromolecules*, *12*(9), 3285-3290.

Paduretu, C. C., Apetroaei, M. R., Ileana, R. Ă. U., & Schroder, V. (2018). Characterization of chitosan extracted from different romanian black sea crustaceans. *UPB Scientific Bullettin, Series B*, *80*, 13-24.

Parish, M. E., Beuchat, L. R., Suslow, T. V., Harris, L. J., Garrett, E. H., Farber, J. N., & Busta, F. F. (2003). Methods to reduce/eliminate pathogens from fresh and fresh-cut produce. *Comprehensive reviews in food science and food safety*, *2*, 161-173.

Park, B. K., & Kim, M. M. (2010). Applications of chitin and its derivatives in biological medicine. *International journal of molecular sciences*, *11*(12), 5152-5164.

Pascual, E., & Julia, M. R. (2001). The role of chitosan in wool finishing. *Journal of biotechnology*, *89*(2-3), 289-296.

Paulino, A. T., Simionato, J. I., Garcia, J. C., & Nozaki, J. (2006). Characterization of chitosan and chitin produced from silkworm crysalides. *Carbohydrate Polymers*, *64*(1), 98-103.

Pedonese, F., Verani, G., Torracca, B., Turchi, B., Felicioli, A., & Nuvoloni, R. (2018). Effetto di propoli prodotta in Toscana sullo sviluppo di *Listeria monocytogenes*, *Staphylococcus aureus* e *Bacillus cereus* in latte e ricotta. *Italian Journal of Food Safety*, *7*(1s), 15.

Pelissari, F. M., Grossmann, M. V., Yamashita, F., & Pineda, E. A. G. (2009). Antimicrobial, mechanical, and barrier properties of cassava starch− chitosan films incorporated with oregano essential oil. *Journal of agricultural and food chemistry*, *57*(16), 7499-7504.

Percot, A., Viton, C., & Domard, A. (2003). Optimization of chitin extraction from shrimp shells. *Biomacromolecules*, *4*(1), 12-18.

Peter, M.G. (2005). Chitin and chitosan in fungi, in *Biopolymers Online*, pp. 123–132.

Petrenko, I., Bazhenov, V. V., Galli, R., Wysokowski, M., Fromont, J., Schupp, P. J., ... & Ehrlich, H. (2017). Chitin of poriferan origin and the bioelectrometallurgy of copper/copper oxide. *International journal of biological macromolecules*, *104*, 1626-1632.

Petriccione, M., Mastrobuoni, F., Pasquariello, M. S., Zampella, L., Nobis, E., Capriolo, G., & Scortichini, M. (2015). Effect of chitosan coating on the postharvest quality and antioxidant enzyme system response of strawberry fruit during cold storage. *Foods*, *4*(4), 501-523.

Philibert, T., Lee, B. H., & Fabien, N. (2017). Current status and new perspectives on chitin and chitosan as functional biopolymers. *Applied biochemistry and biotechnology*, *181*(4), 1314-1337.

Pighinelli, L., Broquá, J., Zanin, B. G., Zanin, B. G., Flach, A. M., Mallmann, C., ... & Silva, M. M. (2019). Methods of chitin production a short review. *American Journal of Biomedical Science and Research*, *3*, 307-314.

Pinotti, A., & Zaritzky, N. (2001). Effect of aluminum sulfate and cationic polyelectrolytes on the destabilization of emulsified wastes. *Waste Management*, *21*(6), 535-542.

Pittman, S. J., & McAlpine, C. A. (2003). Movements of marine fish and decapod crustaceans: process, theory and application. *Advances in marine biology*, *44*(1), 205-294.

Ploydee, E., & Chaiyanan, S. (2014). Production of high viscosity chitosan from biologically purified chitin isolated by microbial fermentation and deproteinization. *International Journal of Polymer Science*, *2014*.

Pochanavanich, P., & Suntornsuk, W. (2002). Fungal chitosan production and its characterization. *Letters in applied microbiology*, *35*(1), 17-21.

Poverenov, E., Danino, S., Horev, B., Granit, R., Vinokur, Y., & Rodov, V. (2014). Layer-by-layer electrostatic deposition of edible coating on fresh cut melon model: Anticipated and unexpected effects of alginate–chitosan combination. *Food and bioprocess technology*, *7*(5), 1424-1432.

Purkayastha, D., & Sarkar, S. (2020). Physicochemical structure analysis of chitin extracted from pupa exuviae and dead imago of Wild Black Soldier Fly (*Hermetia illucens*). *Journal of Polymers and the Environment*, *28*(2), 445-457.

Ramirez, M. E., Timón, M. L., Petrón, M. J., & Andrés, A. I. (2015). Effect of Chitosan, Pectin and Sodium Caseinate Edible Coatings on Shelf Life of Fresh-Cut *Prunus persica* var. Nectarine. *Journal of Food Processing and Preservation*, *39*(6), 2687-2697.

Ramluckan, K., Moodley, K. G., & Bux, F. (2014). An evaluation of the efficacy of using selected solvents for the extraction of lipids from algal biomass by the soxhlet extraction method. *Fuel*, *116*, 103-108.

Ramos, B., Miller, F. A., Brandão, T. R. S., Teixeira, P., & Silva, C. L. M. (2013). Fresh fruits and vegetables—an overview on applied methodologies to improve its quality and safety. *Innovative Food Science & Emerging Technologies*, *20*, 1-15.

Rayaguru, K., & Routray, W. (2010). Effect of drying conditions on drying kinetics and quality of aromatic Pandanus amaryllifolius leaves. *Journal of food science and technology*, *47*(6), 668-673.

Reddy, M. B., Belkacemi, K., Corcuff, R., Castaigne, F., & Arul, J. (2000). Effect of pre-harvest chitosan sprays on post-harvest infection by *Botrytis cinerea* and quality of strawberry fruit. *Postharvest Biology and Technology*, *20*(1), 39-51.

Regulation (EC) 1935/2004 of the European Parliament and of the Council of 27 October 2004 on materials and articles intended to come into contact with food and repealing Directives 80/590/EEC and 89/109/EEC

Remya, S., Mohan, C. O., Bindu, J., Sivaraman, G. K., Venkateshwarlu, G., Ravishankar, C. N. (2015). Effect of chitosan based active packaging film on the keeping quality of chilled stored barracuda fish. *Journal of Food Science and Technology*, *53*(1), 685–693.

Revathi, M., Saravanan, R., & Shanmugam, A. (2012). Production and characterization of chitinase from *Vibrio* species, a head waste of shrimp *Metapenaeus dobsonii* (Miers, 1878) and chitin of *Sepiella inermis* Orbigny, 1848. *Advances in Bioscience and Biotechnology*, 3: 392–397.

Rico, D., Martin-Diana, A. B., Barat, J. M., & Barry-Ryan, C. (2007). Extending and measuring the quality of fresh-cut fruit and vegetables: a review. *Trends in Food Science & Technology*, *18*(7), 373-386.

Rinaudo, M. (2006). Chitin and chitosan: Properties and applications. *Progress in polymer science*, *31*(7): 603-632.

Lertsutthiwong, P., How, N. C., Chandrkrachang, S., & Stevens, W. F. (2002). Effect of

Chemical Treatment on the Characteristics of Shrimp Chitosan. *Journal of metals, materials and minerals*, *12*(1), 11-18.

Rudall, K.M. & Kenchington, W. (1973). The chitin system. *Biol Rev*, 48:597–633.

Ruiz, G. A. M., & Corrales, H. F. Z. (2017). Chitosan, chitosan derivatives and their biomedical applications. In *Biological activities and application of marine polysaccharides*, ed. by Shalaby E. InTech, pp. 87–106.

Rumpold, B. A., & Schlüter, O. K. (2013). Nutritional composition and safety aspects of edible insects. *Molecular nutrition & food research*, *57*(5), 802-823.

Saad, A. G., Jaiswal, P., & Jha, S. N. (2014). Non-destructive quality evaluation of intact tomato using VIS-NIR spectroscopy. *International Journal of Advanced Research*, *2*(12), 632-639.

Sadler, G. D., & Murphy, P. A. (2010). pH and titratable acidity. In Nielsen, S., *Food analysis* (pp. 219-238). Springer, Boston, MA.

Sáez-Plaza, P., Navas, M. J., Wybraniec, S., Michałowski, T., & Asuero, A. G. (2013). An overview of the Kjeldahl method of nitrogen determination. Part II. Sample preparation, working scale, instrumental finish, and quality control. *Critical Reviews in Analytical Chemistry*, *43*(4), 224-272.

Sajomsang, W., & Gonil, P. (2010). Preparation and characterization of α-chitin from cicada sloughs. *Materials Science and Engineering: C*, *30*(3): 357-363.

Segal, L. G. J. M. A., Creely, J. J., Martin Jr, A. E., & Conrad, C. M. (1959). An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer. *Textile research journal*, *29*(10), 786-794.

Shahidi, F., & Synowiecki, J. (1991). Isolation and characterization of nutrients and value-added products from snow crab (*Chionoecetes opilio*) and shrimp (*Pandalus borealis*) processing discards. *Journal of agricultural and food chemistry*, *39*(8), 1527-1532.

Scherrer, P. (1912). Bestimmung der inneren Struktur und der Größe von Kolloidteilchen mittels Röntgenstrahlen. In *Kolloidchemie Ein Lehrbuch* (pp. 387-409). Springer, Berlin, Heidelberg.

Shigemasa, Y., Matsuura, H., Sashiwa, H., & Saimoto, H. (1996). Evaluation of different absorbance ratios from infrared spectroscopy for analyzing the degree of deacetylation in chitin. *International Journal of Biological Macromolecules*, *18*(3), 237-242.

Schmitt, E., Belghit, I., Johansen, J., Leushuis, R., Lock, E. J., Melsen, D., ... & Paul, A. (2019). Growth and safety assessment of feed streams for black soldier fly larvae: a case study with aquaculture sludge. *Animals*, *9*(4), 189.

Segal, L., Creely, J.J., Martin, A.E. & Conrad, C.M. (1959). An empirical method for estimating the degree of crystallinity of native cellulose using the X-Ray diffractometer. *Textile Research Journal*, 29(10): 786–794.

Seoudi, R., Nada, A. M. A., Abd Elmongy, S., & Hamed, S. S. (2005). Fourier transform

infrared spectroscopic and AC electrical conductivity studies of chitin and its derivatives. *Journal of applied polymer science*, *98*(2), 936-943.

Shah, S., & Hashmi, M. S. (2020). Chitosan–aloe vera gel coating delays postharvest decay of mango fruit. *Horticulture, Environment, and Biotechnology*, *61*, 279-289.

Shalini, R., & Singh, A. (2009). Biobased packaging materials for the food industry. *Journal of Food Science. & Technology*, *5*, 16-20.

Sharif, Z. I. M., Mustapha, F. A., Jai, J., Yusof, N. M., & Zaki, N. A. M. (2017). Review on methods for preservation and natural preservatives for extending the food longevity. *Chemical Engineering Research Bulletin*, 145-153.

Shiekh, R. A., Malik, M. A., Al-Thabaiti, S. A., & Shiekh, M. A. (2013). Chitosan as a novel edible coating for fresh fruits. *Food Science and Technology Research*, *19*(2), 139-155.

Shin, C. S., Kim, D. Y., & Shin, W. S. (2019). Characterization of chitosan extracted from Mealworm Beetle (*Tenebrio molitor*, *Zophobas morio*) and Rhinoceros Beetle (*Allomyrina dichotoma*) and their antibacterial activities. *International journal of biological macromolecules*, *125*, 72-77.

Silva, G. M. C., Silva, W. B., Medeiros, D. B., Salvador, A. R., Cordeiro, M. H. M., da Silva, N. M., ... & Mizobutsi, G. P. (2017). The chitosan affects severely the carbon metabolism in mango (*Mangifera indica* L. cv. Palmer) fruit during storage. *Food Chemistry*, *237*, 372-378.

Singh, V. P. (2018). Recent approaches in food bio-preservation-a review. *Open veterinary journal*, *8*(1), 104-111.

Singh, A., Benjakul, S., & Prodpran, T. (2019). Ultrasound-assisted extraction of chitosan from squid pen: Molecular characterization and fat binding capacity. *Journal of food science*, 84(2): 224-234.

Sivertsvik, M., Rosnes, J. T., & Bergslien, H. (2002). Modified atmosphere packaging. In: Ohlsson, T. and Bengtsson, N. editors, *Minimal processing technologies in the food industry*, 61-86. Woodhead Publishing Limited and CRC Press LLC, Cambridge, England.

Smets, R., Verbinnen, B., Van De Voorde, I., Aerts, G., Claes, J., & Van Der Borght, M. (2020). Sequential extraction and characterisation of lipids, proteins, and chitin from black soldier fly (*Hermetia illucens*) larvae, prepupae, and pupae. *Waste and Biomass Valorization*, *11*(12), 6455-6466.

Smith, J. S., & Pillai, S. (2004). Irradiation and food safety. *Food technology (Chicago)*, *58*(11), 48-55.

Soetemans, L., Uyttebroek, M., & Bastiaens, L. (2020). Characteristics of chitin extracted from black soldier fly in different life stages. *International Journal of Biological Macromolecules*, *165*, 3206-3214.

Song, C., Yu, H., Zhang, M., Yang, Y., & Zhang, G. (2013). Physicochemical properties and antioxidant activity of chitosan from the blowfly *Chrysomya megacephala* larvae. *International journal of biological macromolecules*, *60*, 347-354.

Soon, C. Y., Tee, Y. B., Tan, C. H., Rosnita, A. T., & Khalina, A. (2018). Extraction and physicochemical characterization of chitin and chitosan from *Zophobas morio* larvae in varying sodium hydroxide concentration. *International journal of biological macromolecules*, *108*, 135-142.

Souza, V. C., Monte, M. L., & Pinto, L. A. (2013). Effect of carp (*Cyprinus carpio*) oil incorporation on water vapour permeability, mechanical properties and transparency of chitosan films. *International journal of food science & technology*, *48*(6), 1309-1317.

Souza, M. P., Vaz, A. F., Cerqueira, M. A., Texeira, J. A., Vicente, A. A., & Carneiro-da-Cunha, M. G. (2015). Effect of an edible nanomultilayer coating by electrostatic self-assembly on the shelf life of fresh-cut mangoes. *Food and Bioprocess Technology*, *8*(3), 647-654.

Star, L., Arsiwalla, T., Molist, F., Leushuis, R., Dalim, M., & Paul, A. (2020). Gradual provision of live Black soldier fly (*Hermetia illucens*) larvae to older laying hens: Effect on production performance, egg quality, feather condition and behavior. *Animals*, *10*, 216.

Stricker-Krongrad, A. H., Alikhassy, Z., Matsangos, N., Sebastian, R., Marti, G., Lay, F., & Harmon, J. W. (2018). Efficacy of chitosan-based dressing for control of bleeding in excisional wounds. *Eplasty*, *18*, 122-130.

Suárez, M. H., Rodríguez, E. R., & Romero, C. D. (2008). Chemical composition of tomato (*Lycopersicon esculentum*) from Tenerife, the Canary Islands. *Food Chemistry*, *106*(3), 1046-1056.

Sucharitha, K. V., Beulah, A. M., & Ravikiran, K. (2018). Effect of chitosan coating on storage stability of tomatoes (*Lycopersicon esculentum* Mill). *International Food Research Journal*, *25*(1), 93-99.

Sugumaran, M. (2009). Complexities of cuticular pigmentation in insects. *Pigment cell & melanoma research 22*, 523-525.

Sun, X., Sui, S., Ference, C., Zhang, Y., Sun, S., Zhou, N., ... & Zhou, K. (2014). Antimicrobial and mechanical properties of β-cyclodextrin inclusion with essential oils in chitosan films. *Journal of agricultural and food chemistry*, *62*(35), 8914-8918.

Sun, L., Sun, J., Chen, L., Niu, P., Yang, X., & Guo, Y. (2017). Preparation and characterization of chitosan film incorporated with thinned young apple polyphenols as an active packaging material. *Carbohydrate polymers*, *163*, 81-91.

Sundaram, J., Pant, J., Goudie, M. J., Mani, S., & Handa, H. (2016). Antimicrobial and physicochemical characterization of biodegradable, nitric oxide-releasing nanocellulose–chitosan packaging membranes. *Journal of agricultural and food chemistry*, *64*(25), 5260-5266.

Suntornsuk, W., Pochanavanich, P., & Suntornsuk, L. (2002). Fungal chitosan production on food processing by-products. *Process Biochemistry*, *37*(7), 727-729.

Synowiecki, J., & Al-Khateeb, N. A. (2003). Production, properties, and some new applications of chitin and its derivatives. *Critical Reviews in Food Science and Nutrition*, *43*, 145-171.

Tacon, A. G. (2018). Global trends in aquaculture and compound aquafeed production. *The Magazine of the World Aquaculture Society*, *49*, 33-46.

Talón, E., Trifkovic, K. T., Nedovic, V. A., Bugarski, B. M., Vargas, M., Chiralt, A., & González-Martínez, C. (2017). Antioxidant edible films based on chitosan and starch containing polyphenols from thyme extracts. *Carbohydrate polymers*, *157*, 1153-1161.

Tan, G., Kaya, M., Tevlek, A., Sargin, I., & Baran, T. (2018). Antitumor activity of chitosan from mayfly with comparison to commercially available low, medium and high molecular weight chitosans. *In Vitro Cellular & Developmental Biology-Animal*, *54*(5), 366-374.

Tayel, A. A., Ibrahim, S. I. A., Al-Saman, M. A., Moussa, S. H. (2014). Production of fungal chitosan from date wastes and its application as a biopreservative for minced meat. *International Journal of Biological Macromolecules, 69*, 471–4.

Tharanathan, R. N., & Kittur, F. S. (2003). Chitin—the undisputed biomolecule of great potential. *Critical Reviews in Food Science and Nutrition*, *43*(1), 61–87.

Thirunavukkarasu, N., & Shanmugam, A. (2009). Extraction of chitin and chitosan from mud crab *Scylla tranquebarica* (Fabricius, 1798). *International Journal on Applied Bioengineering*, *4*(2), 31-33.

Tian, B., & Liu, Y. (2020). Chitosan-based biomaterials: From discovery to food application. *Polymers for Advanced Technologies*, *31*(11), 2408-2421.

Truong, T. O., Hausler, R., Monette, F., & Niquette, P. (2007). Valorisation des résidus industriels de pêches pour la transformation de chitosane par technique hydrothermo-chimique. *Revue des sciences de l'eau/journal of Water Science*, *20*(3), 253-262.

Tsai, G. J., & Su, W. H. (1999). Antibacterial activity of shrimp chitosan against *Escherichia coli*. *Journal of food protection*, *62*(3), 239-243.

Vainker, S. J. (2004). *Chinese silk: a cultural history*. British Museum Press.

Valero, D., & Serrano, M. (2013). Growth and ripening stage at harvest modulates postharvest quality and bioactive compounds with antioxidant activity. *Stewart Postharvest Review*, *3*(5).

Vaz, J. M., Taketa, T. B., Hernandez-Montelongo, J., Chevallier, P., Cotta, M. A., Mantovani, D., & Beppu, M. M. (2018). Antibacterial properties of chitosan-based coatings are affected by spacer-length and molecular weight. *Applied Surface Science*, *445*, 478-487.

Van Huis, A. (2013). Potential of insects as food and feed in assuring food security. *Annual review of entomology*, *58*, 563-583.

Van Huis, A. (2020). Insects as food and feed, a new emerging agricultural sector: a review. *Journal of Insects as Food and Feed*, *6*(1), 27-44.

Varasteh, F., Arzani, K., Barzegar, M., & Zamani, Z. Pomegranate (*Punica granatum* L.) Fruit Storability Improvement Using Pre-storage Chitosan Coating. J. *Agr. Sci. Tech.*, *19*, 389-400.

Vårum, K. M., Antohonsen, M. W., Grasdalen, H., & Smidsrød, O. (1991). Determination of the degree of N-acetylation and the distribution of N-acetyl groups in partially N-deacetylated chitins (chitosans) by high-field nmr spectroscopy. *Carbohydrate Research*, *211*(1), 17-23.

Vårum, K. M., Anthonsen, M. W., Grasdalen, H., & Smidsrød, O. (1991a). 13C-Nmr studies of the acetylation sequences in partially N-deacetylated chitins (chitosans). *Carbohydrate Research*, *217*, 19-27.

Vieira, J. M., Flores-López, M. L., de Rodríguez, D. J., Sousa, M. C., Vicente, A. A., & Martins, J. T. (2016). Effect of chitosan–Aloe vera coating on postharvest quality of blueberry (*Vaccinium corymbosum*) fruit. *Postharvest Biology and Technology*, *116*, 88-97.

Vilaplana, R., Chicaiza, G., Vaca, C., & Valencia-Chamorro, S. (2020a). Combination of hot water treatment and chitosan coating to control anthracnose in papaya (*Carica papaya* L.) during the postharvest period. *Crop Protection*, *128*, 105007.

Vilaplana, R., Guerrero, K., Guevara, J., & Valencia-Chamorro, S. (2020b). Chitosan coatings to control soft mold on fresh blackberries (*Rubus glaucus* Benth.) during postharvest period. *Scientia Horticulturae*, *262*, 109049.

Vincent, J. F., & Wegst, U. G. (2004). Design and mechanical properties of insect cuticle. *Arthropod structure & development*, *33*(3), 187-199.

Vishu Kumar, A. B., Varadaraj, M. C., Gowda, L. R., & Tharanathan, R. N. (2005). Characterization of chito-oligosaccharides prepared by chitosanolysis with the aid of papain and Pronase, and their bactericidal action against *Bacillus cereus* and *Escherichia coli*. *Biochemical Journal*, *391*(2), 167-175.

Vu, K. D., Hollingsworth, R. G., Leroux, E., Salmieri, S., & Lacroix, M. (2011). Development of edible bioactive coating based on modified chitosan for increasing the shelf life of strawberries. *Food Research International*, *44*(1), 198-203.

Wang, W., and Deshi, X. (1994). Viscosity and flow properties of concentrated solutions of chitosan with different degrees of deacetylation. *International journal of biological macromolecules*, *16*(3), 149-152.

Wang, M., Chen, L. J., Ni, J., Weng, J., & Yue, C. Y. (2001). Manufacture and evaluation of bioactive and biodegradable materials and scaffolds for tissue engineering. *Journal of Materials Science: Materials in Medicine*, *12*(10): 855-860.

Wang, J., Yang, B., Zhang, S., Cao, J., Jiang, W. (2016). Effect of thymol on antifungal ability of chitosan coating against Penicillium expansum in Yali pear. *Emirates Journal of Food and Agriculture*, *28*(10), 725–731.

Wang, H., Qian, J., & Ding, F. (2018). Emerging chitosan-based films for food packaging applications. *Journal of agricultural and food chemistry*, *66*(2), 395-413.

Wang CH, Doan CT, Nguyen AD and Wang SL, Reclamation of fishery processing waste: a mini-review. Molecules 24:2234 (2019).

Wang, H., ur Rehman, K., Feng, W., Yang, D., ur Rehman, R., Cai, M., ... & Zheng, L. (2020).

Physicochemical structure of chitin in the developing stages of black soldier fly. *International journal of biological macromolecules*, *149*, 901-907.

Waśko, A., Bulak, P., Polak-Berecka, M., Nowak, K., Polakowski, C., & Bieganowski, A. (2016). The first report of the physicochemical structure of chitin isolated from *Hermetia illucens*. *International journal of biological macromolecules*, *92*, 316-320.

Wattjes, J., Niehues, A., Cord-Landwehr, S., Hoßbach, J., David, L., Delair, T., & Moerschbacher, B. M. (2019). Enzymatic production and enzymatic-mass spectrometric fingerprinting analysis of chitosan polymers with different nonrandom patterns of acetylation. *Journal of the American Chemical Society*, *141*(7), 3137-3145.

Weißpflog, J., Vehlow, D., Müller, M., Kohn, B., Scheler, U., Boye, S., & Schwarz, S. (2021). Characterization of chitosan with different degree of deacetylation and equal viscosity in dissolved and solid state–Insights by various complimentary methods. *International Journal of Biological Macromolecules*, *171*, 242-261.

Win, N., & Stevens, W. (2001). Shrimp chitin as substrate for fungal chitin deacetylase. *Applied microbiology and biotechnology*, *57*(3), 334-341.

Wittaya, T. (2012). Protein-based edible films: Characteristics and improvement of properties. *Structure and function of food engineering*, 43-70.

Wu, S. (2012). Preparation of chitooligosaccharides from *Clanis bilineata* larvae skin and their antibacterial activity. *International journal of biological macromolecules*, *51*(5), 1147-1150.

Wu, S. J., Pan, S. K., Wang, H. B., & Wu, J. H. (2013). Preparation of chitooligosaccharides from cicada slough and their antibacterial activity. *International journal of biological macromolecules*, *62*, 348-351.

Xiao, L. (2018). Chitosan application in textile processing. *Current Trends in Fashion Technology and Textile Engineering*, *4*, 32–34.

Xu, J., McCarthy, S. P., Gross, R. A., & Kaplan, D. L. (1996). Chitosan film acylation and effects on biodegradability. *Macromolecules*, *29*(10), 3436-3440.

Xu, Y. X., Kim, K. M., Hanna, M. A., & Nag, D. (2005). Chitosan–starch composite film: preparation and characterization. *Industrial crops and Products*, *21*(2), 185-192.

Yacob, N., Talip, N., Mahmud, M., Sani, N., Samsuddin, N., & Fabillah, N. (2013). Determination of viscosity-average molecular weight of chitosan using intrinsic viscosity measurement. *Journal of Nuclear and Related Technologies*, 10: 39–44.

Yeul, V. S., & Rayalu, S. S. (2013). Unprecedented chitin and chitosan: A chemical overview. *Journal of Polymers and the Environment*, *21*(2), 606-614.

Younas, M. S., Butt, M. S., Pasha, I., & Shahid, M. (2014). Development of zinc fortified chitosan and alginate based coatings for apricot. *Pak. J. Agri. Sci*, *51*(4), 1033-1039.

Younes, I., Sellimi, S., Rinaudo, M., Jellouli, K., & Nasri, M. (2014). Influence of acetylation degree and molecular weight of homogeneous chitosans on antibacterial and antifungal

activities. *International journal of food microbiology*, *185*, 57-63.

Younes, I., & Rinaudo, M. (2015). Chitin and chitosan preparation from marine sources. Structure, properties and applications. *Marine drugs*, *13*(3), 1133-1174.

Younes, I., Hajji, S., Rinaudo, M., Chaabouni, M., Jellouli, K., & Nasri, M. (2016). Optimization of proteins and minerals removal from shrimp shells to produce highly acetylated chitin. *International journal of biological macromolecules*, *84*, 246-253.

Zahoorullah, S. M., Dakshayani, L., Rani, A. S., Venkateswerlu, G. (2017). Effect of Chitosan Coating on the Post Harvest Quality of Banana during Storage. *Asian Journal of Biotechnology and Bioresource Technology, 1*(1), 1–10.

Zhang, D., & Quantick, P. C. (1998). Antifungal effects of chitosan coating on fresh strawberries and raspberries during storage. *The Journal of Horticultural Science and Biotechnology*, *73*(6), 763-767.

Zhang, M., Haga, A., Sekiguchi, H., & Hirano, S. (2000). Structure of insect chitin isolated from beetle larva cuticle and silkworm (*Bombyx mori*) pupa exuvia. *International Journal of Biological Macromolecules*, *27*(1), 99-105.

Zhang, D., Quantick, P. C., & Grigor, J. M. (2000a). Changes in phenolic compounds in Litchi (Litchi chinensis Sonn.) fruit during postharvest storage. *Postharvest Biology and Technology*, *19*(2), 165-172.

Zhang, A. J., Qin, Q. L., Zhang, H., Wang, H. T., Li, X., Miao, L., & Wu, Y. J. (2011). Preparation and characterisation of food-grade chitosan from housefly larvae. *Czech Journal of Food Science*, *29*(6), 616-623.

Zhang, H., Li, R., & Liu, W. (2011a). Effects of chitin and its derivative chitosan on postharvest decay of fruits: A review. *International Journal of Molecular Sciences*, *12*(2), 917-934.

Zhang, L., Chen, F., Lai, S., Wang, H., & Yang, H. (2018). Impact of soybean protein isolate-chitosan edible coating on the softening of apricot fruit during storage. *LWT*, *96*, 604-611.

Zhang, W., Zhao, H., Zhang, J., Sheng, Z., Cao, J., & Jiang, W. (2019). Different molecular weights chitosan coatings delay the senescence of postharvest nectarine fruit in relation to changes of redox state and respiratory pathway metabolism. *Food chemistry*, *289*, 160-168.

Zhou, P., Li, J., Yan, T., Wang, X., Huang, J., Kuang, Z., ... & Pan, M. (2019). Selectivity of deproteinization and demineralization using natural deep eutectic solvents for production of insect chitin (*Hermetia illucens*). *Carbohydrate polymers*, *225*, 115255.

Ziaolhagh, S. H., & Kanani, S. (2021). Extending the Shelf life of Apricots by Using Gum Tragacanth-Chitosan Edible Coating. *Journal of Agricultural Science and Technology*, *23*(2), 319-331.

Zivanovic, S., Basurto, C. C., Chi, S., Davidson, P. M., & Weiss, J. (2004). Molecular weight of chitosan influences antimicrobial activity in oil-in-water emulsions. *Journal of food protection*, *67*(5), 952-959.

Złotko, K., Waśko, A., Kamiński, D. M., Budziak-Wieczorek, I., Bulak, P., & Bieganowski, A. (2021). Isolation of Chitin from Black Soldier Fly (*Hermetia illucens*) and Its Usage to Metal Sorption. *Polymers*, *13*(5), 818.

8. SUPPLEMENTARY MATERIALS

8.1 Pictures of tomatoes, apricots, strawberries and nectarines at the beginning (T_0) and at the end (T_f) of their storage period at room (RT) and cold temperature (4 °C), belonging to the different treatments: negative control, solvent-only control, commercial chitosan and chitosan from *H. illucens* larvae (L), pupal exuviae (PE) and adults (A).

8.1.1 Tomatoes

RT, coated by dipping (T_f 30 days)

Negative control

Solvent-only control

1% Commercial chitosan

0.5% Commercial chitosan

1% PE heterogeneous unbleached

0.5% PE heterogeneous unbleached

1% PE heterogeneous bleached

0.5% PE heterogeneous bleached

1% PE homogeneous unbleached

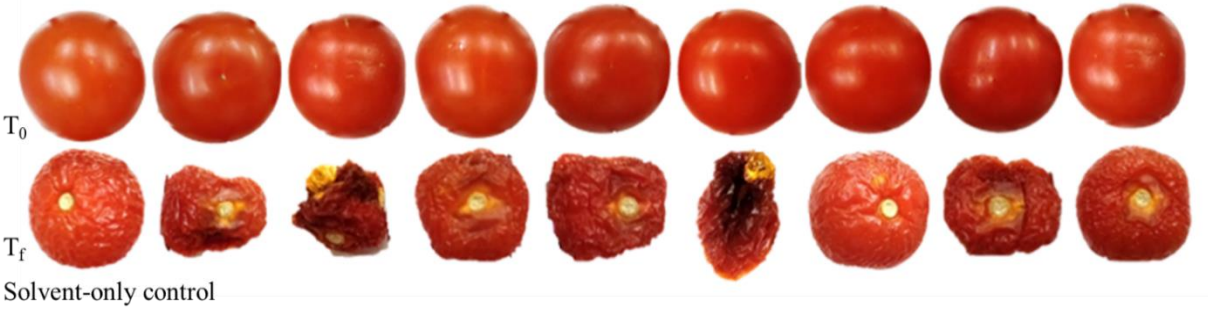
0.5% PE homogeneous unbleached

1% PE homogeneous bleached

0.5% PE homogeneous bleached

RT, coated by spraying (T_f 30 days)

Negative control



Solvent-only control

1% commercial chitosan

0.5% commercial chitosan

1% PE heterogeneous unbleached

0.5% PE heterogeneous unbleached

1% PE heterogeneous bleached

0.5% PE heterogeneous bleached

1% PE homogeneous unbleached

0.5% PE homogeneous unbleached

1% PE homogeneous bleached

0.5% PE homogeneous bleached

4 °C, coated by dipping (T_f 30 days)

Negative control

Solvent-only control

1% commercial chitosan

0.5% commercial chitosan

1% PE heterogeneous unbleached

0.5% PE heterogeneous unbleached

1% PE heterogeneous bleached

0.5% PE heterogeneous bleached

1% PE homogeneous unbleached

0.5% PE homogeneous unbleached

1% PE homogeneous bleached

0.5% PE homogeneous bleached

4 °C, coated by spraying (T_f 30 days)

Negative control

Solvent-only control

1% commercial chitosan

0.5% commercial chitosan

1% PE heterogeneous unbleached

0.5% PE heterogeneous unbleached

1% PE heterogeneous bleached

0.5% PE heterogeneous bleached

1% PE homogeneous unbleached

0.5% PE homogeneous unbleached

1% PE homogeneous bleached

0.5% PE homogeneous bleached

8.1.2 Apricots

RT (T_f 14 days)

Negative control

Solvent-only control

1% commercial chitosan

0.5% commercial chitosan

1% L unbleached

0.5% L unbleached

1% L bleached

0.5% L bleached

1% PE unbleached

0.5% PE unbleached

1% PE bleached

0.5% PE bleached

1% A unbleached

0.5% A unbleached

1% A bleached

0.5% A bleached

4 °C (T_f 26 days)

Negative control

Solvent-only control

1% commercial chitosan

0.5% commercial chitosan

1% L unbleached

0.5% L unbleached

1% L bleached

0.5% L bleached

1% PE unbleached

0.5% PE unbleached

1% PE bleached

0.5% PE bleached

1% A unbleached

0.5% A unbleached

1% A bleached

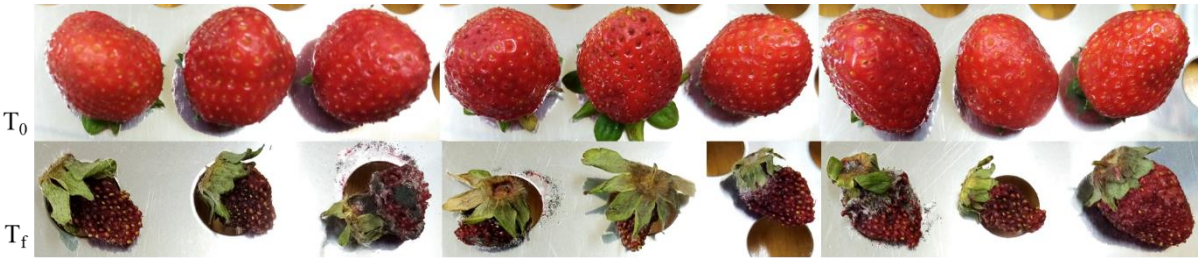
0.5% A bleached

8.1.3 Strawberries

RT (T_f 9 days)

Negative control

Solvent-only control



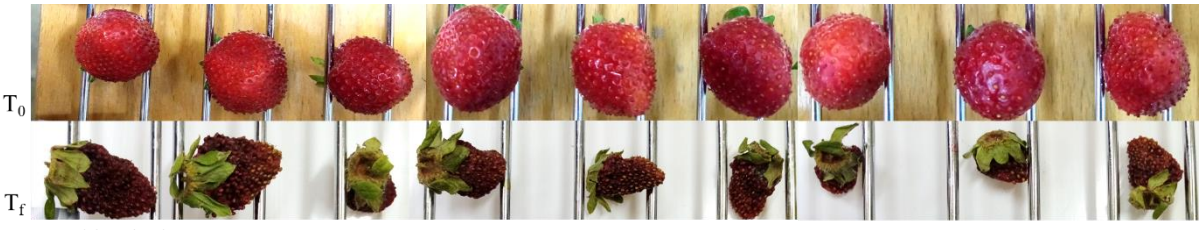
1% commercial chitosan

0.5% commercial chitosan

1% L unbleached

0.5% L unbleached

1% L bleached



0.5% L bleached

1% PE unbleached

0.5% PE unbleached

1% PE bleached

0.5% PE bleached

1% A unbleached

0.5% A unbleached

1% A bleached

0.5% A bleached

4 °C (T_f 15 days)

Negative control

Solvent-only control

1% commercial chitosan

0.5% commercial chitosan

1% L unbleached

0.5% L unbleached

1% L bleached

0.5% L bleached

1% PE unbleached

0.5% PE unbleached

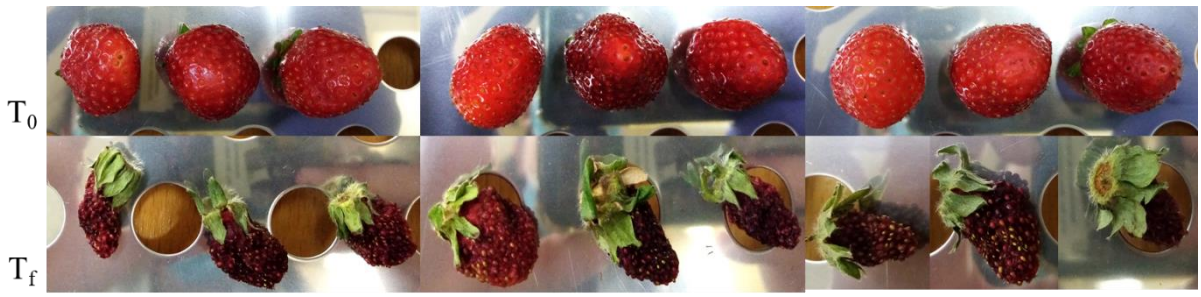
1% PE bleached

0.5% PE bleached

1% A unbleached

0.5% A unbleached

1% A bleached

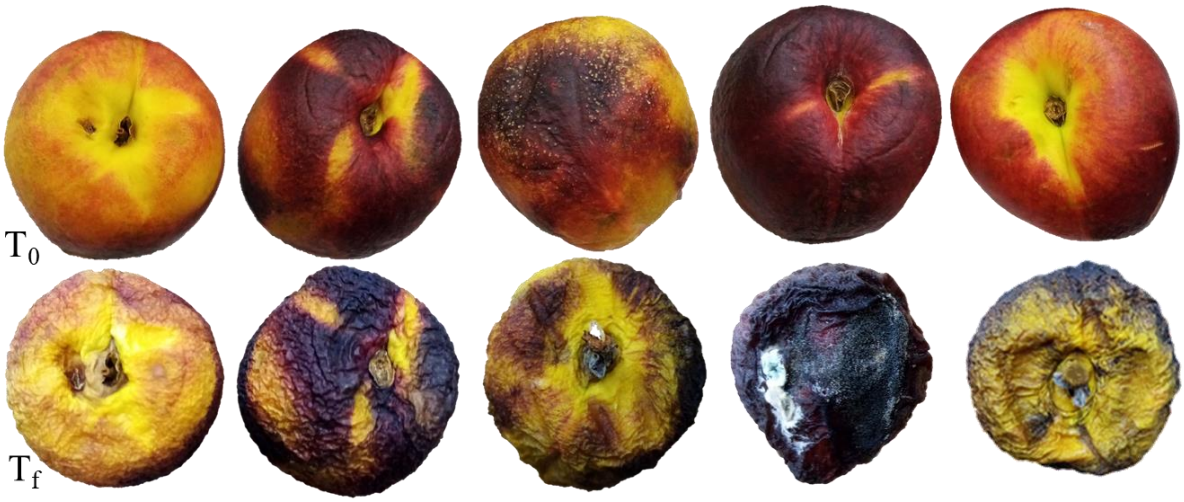


0.5% A bleached

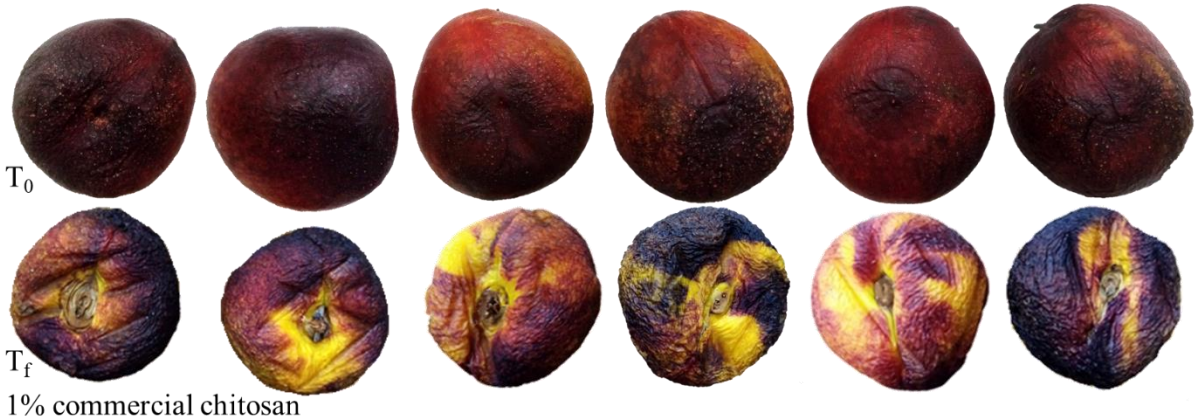
8.1.4 Nectarines

RT (T_f 10 days)

Negative control



Solvent-only control



1% commercial chitosan

0.5% commercial chitosan

1% PE unbleached

0.5% PE unbleached

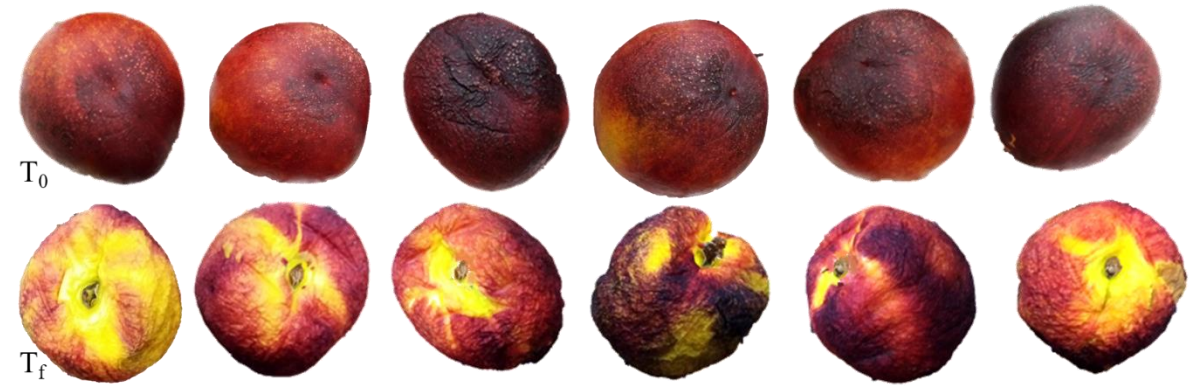
1% PE bleached

0.5% PE bleached

4 °C (T_f 21 days)

Negative control

Solvent-only control



1% commercial chitosan

0.5% commercial chitosan

1% PE unbleached

0.5% PE unbleached

1% PE bleached

0.5% PE bleached

8.2 Tables providing results of the comparison of fruits' weight loss and TSS, pH and TA variations between the two storage temperatures and the two different method of coating application.

Table 8.1 Weight loss (%) of tomatoes coated by dipping or spraying and stored at room or cold temperature for 30 days, expressed as mean ± standard deviation. *P* values resulting from the comparison of weight loss at the two different storage temperatures are reported for each treatment, given the same application method. Asterisks indicate significant differences ($p<0.05$), according to *Mann-Whitney* U test.

TREATMENT	APPLICATION METHOD	WEIGHT LOSS (%)		*P* VALUE
		Room temperature	Cold temperature	
Solvent	dipping	38.2±2.7	24.8±3.6	0.0003*
	spraying	59.3±4.3	52.6±4.6	0.5
Comm 1%	dipping	35.3±3.3	26.4±2.3	0.0003*
	spraying	28.9±2.4	30.1±4.4	0.8
Comm 0.5%	dipping	35.2±2.6	24.5±1.3	0.007*
	spraying	28.2±4.6	22±4.9	0.12
PE het dec 1%	dipping	25.7±3.9	29.3±1.5	0.14
	spraying	23.8±3.6	19.6±3.3	0.01*
PE het dec 0.5%	dipping	28.6±2.9	20.3±3.1	0.0007*
	spraying	26±4.2	22.2±2.4	0.04*
PE het no dec 1%	dipping	28.4±4.3	20.4±3.1	0.002*
	spraying	26.2±3.1	24.9±2.8	0.4
PE het no dec 0.5%	dipping	31.9±2.6	24.6±2.5	0.01*
	spraying	25.6±4.1	28.1±3.7	0.96
PE hom dec 1%	dipping	42.3±4.4	36.2±3.4	0.04*
	spraying	40.1±2.3	21.4±3.2	0.0003*
PE hom dec 0.5%	dipping	35.3±3.3	30.5±2.4	0.005*
	spraying	30.9±3.3	23.5±2.2	0.004*
PE hom no dec 1%	dipping	36.9±1.9	21.6±3.1	0.0003*
	spraying	33.1±1.9	23.8±3.1	0.0003*
PE hom no dec 0.5%	dipping	36.5±4.8	30±2.6	0.04*
	spraying	30.6±2.8	23.9±2.5	0.005*

Table 8.2 Weight loss (%) of tomatoes coated by dipping or spraying and stored at room or cold temperature for 30 days, expressed as mean ± standard deviation. *P* values resulting from the comparison of weight loss with the two different application methods are reported for each treatment, given the same storage temperature. Asterisks indicate significant differences ($p<0.05$), according to *Mann-Whitney* U test.

TREATMENT	STORAGE TEMPERATURE	WEIGHT LOSS (%)		*P* VALUE
		Dipping application	Spraying application	
Solvent	room	38.2±2.7	59.3±4.3	0.05
	cold	24.8±3.6	52.6±4.6	0.0007*
Comm 1%	room	35.3±3.3	28.9±2.4	0.14
	cold	26.4±2.3	30.1±4.4	0.45
Comm 0.5%	room	35.2±2.6	28.2±4.6	0.1
	cold	24.5±1.3	22±4.9	0.2
PE het dec 1%	room	25.7±3.9	23.8±3.6	0.35
	cold	29.3±1.5	19.6±3.3	0.0003*
PE het dec 0.5%	room	28.6±2.9	26±4.2	0.2
	cold	20.3±3.1	22.2±2.4	0.45
PE het no dec 1%	room	28.4±4.3	26.2±3.1	0.35
	cold	20.4±3.1	24.9±2.8	0.03*
PE het no dec 0.5%	room	31.9±2.6	25.6±4.1	0.01*
	cold	24.6±2.5	28.1±3.7	0.5
PE hom dec 1%	room	42.3±4.4	40.1±2.3	0.56
	cold	36.2±3.4	21.4±3.2	0.002*
PE hom dec 0.5%	room	35.3±3.3	30.9±3.3	0.04*
	cold	30.5±2.4	23.5±2.2	0.2
PE hom no dec 1%	room	36.9±1.9	33.1±1.9	0.07
	cold	21.6±3.1	23.8±3.1	0.23
PE hom no dec 0.5%	room	36.5±4.8	30.6±2.8	0.01*
	cold	30±2.6	23.9±2.5	0.009*

Table 8.3 Percentage increase of TSS of tomatoes coated by dipping or spraying and stored at room or cold temperature for 30 days, expressed as mean ± standard deviation. *P* values resulting from the comparison of TSS variation at the two different storage temperatures are reported for each treatment, given the same application method. Asterisks indicate significant differences ($p<0.05$), according to *Mann-Whitney* U test.

TREATMENT	APPLICATION METHOD	TSS VARIATION (%)		*P* VALUE
		Room temperature	Cold temperature	
Solvent	dipping	51.8±2	21.1±0.8	0.04*
	spraying	53.2±2.	73.9±3.3	0.018*
Comm 1%	dipping	44.6±1.7	28.3±1.1	0.23
	spraying	35±1.6	42.5±2	0.047*
Comm 0.5%	dipping	44.6±1.7	28.3±1.1	0.23
	spraying	31.2±1.5	38±1.8	0.21
PE het dec 1%	dipping	9.2±0.4	16.5±0.8	0.54
	spraying	32±1.5	35±1.7	0.18
PE het dec 0.5%	dipping	31.1±1.5	15±0.7	0.0038*
	spraying	32±1.5	47±2.2	0.02*
PE het no dec 1%	dipping	23.8±1.1	15±0.7	0.019*
	spraying	48.5±2.3	36.5±1.7	0.015*
PE het no dec 0.5%	dipping	15.1±0.7	17.9±0.9	0.59
	spraying	26±1.2	45.5±2.1	0.0028*
PE hom dec 1%	dipping	62.8±2.8	38.8±1.7	0.001*
	spraying	74±3.5	38±1.8	0.0001*
PE hom dec 0.5%	dipping	35.6±1.6	26.1±1.1	0.0059*
	spraying	65±3.1	45.5±2.1	0.0009*
PE hom no dec 1%	dipping	30.8±1.4	26.1±1.1	0.31
	spraying	62±2.9	48.5±2.3	0.0087*
PE hom no dec 0.5%	dipping	53.2±2.4	30.8±1.4	0.0074*
	spraying	59±2.8	47±2.2	0.0052*

Table 8.4 Percentage increase of TSS of tomatoes coated by dipping or spraying and stored at room or cold temperature for 30 days, expressed as mean ± standard deviation. *P* values resulting from the comparison of weight loss with the two different application methods are reported for each treatment, given the same storage temperature. Asterisks indicate significant differences ($p<0.05$), according to *Mann-Whitney* U test.

TREATMENT	STORAGE TEMPERATURE	TSS VARIATION (%)		*P* VALUE
		Dipping application	Spraying application	
Solvent	room	51.8±2	53.2±2.4	0.87
	cold	21.1±0.8	73.9±3.3	0.003*
Comm 1%	room	44.6±1.7	35±1.7	0.26
	cold	28.3±1.1	42.5±2	0.2
Comm 0.5%	room	44.6±1.7	31.2±1.5	0.21
	cold	28.3±1.1	38±1.8	0.36
PE het dec 1%	room	9.2±0.4	32±1.5	0.021*
	cold	16.5±0.8	35±1.6	0.0019*
PE het dec 0.5%	room	31.1±1.5	32±1.5	0.48
	cold	15±0.7	47±2.2	0.001*
PE het no dec 1%	room	23.8±1.5	48.5±2.3	0.0008*
	cold	15.1±0.7	36.5±1.7	0.0012*
PE het no dec 0.5%	room	15±0.7	26±1.2	0.0064*
	cold	17.9±0.9	45.5±2.1	0.0015*
PE hom dec 1%	room	62.8±2.8	74±3.5	0.015*
	cold	38.8±1.7	38±1.8	0.9
PE hom dec 0.5%	room	35.6±1.6	65±3.1	0.0002*
	cold	26.1±1.5	45.5±2.1	0.0004*
PE hom no dec 1%	room	30.8±1.4	62±2.9	0.0011*
	cold	26.1±1.1	48.5±2.3	0.0007*
PE hom no dec 0.5%	room	53.2±2.4	59±2.8	0.1
	cold	30.8±1.4	47±2.2	0.007*

Table 8.5 Percentage variation of pH of tomatoes coated by dipping or spraying and stored at room or cold temperature for 30 days, expressed as mean ± standard deviation. *P* values resulting from the comparison of pH variation at the two different storage temperatures are reported for each treatment, given the same application method. Asterisks indicate significant differences ($p<0.05$), according to *Mann-Whitney* U test.

TREATMENT	APPLICATION METHOD	pH VARIATION (%)		*P* VALUE
		Room temperature	Cold temperature	
Solvent	dipping	6.9±0.2	4.2±0.1	0.12
	spraying	10.4±0.3	15.7±0.5	0.049*
Comm 1%	dipping	7.2±0.2	5.8±0.2	0.049*
	spraying	5.1±0.1	4.1±0.1	0.82
Comm 0.5%	dipping	7.5±0.2	11.1±0.3	0.049*
	spraying	5±0.1	2±0.1	0.049*
PE het dec 1%	dipping	11.1±0.3	5.5±0.2	0.049*
	spraying	2.9±0.1	1.5±0	0.51
PE het dec 0.5%	dipping	5.3±0.2	0.9±0	0.049*
	spraying	6.1±0.2	0.3±0	0.046*
PE het no dec 1%	dipping	4.4±0.1	2.1±0.1	0.049*
	spraying	0	-4.2±0.1	0.037*
PE het no dec 0.5%	dipping	2.5±0.1	1.7±0.1	0.82
	spraying	0	-3.1±0.1	0.037*
PE hom dec 1%	dipping	6.9±0.2	0	0.037*
	spraying	1.2±0	1.2±0	0.51
PE hom dec 0.5%	dipping	4.9±0.1	0	0.037*
	spraying	4.3±0.1	1.2±0	0.049*
PE hom no dec 1%	dipping	8.5±0.2	2.1±0.1	0.049*
	spraying	6.4±0.2	0.5±0	0.049*
PE hom no dec 0.5%	dipping	8.4±0.2	3.5±0.1	0.049*
	spraying	5.2±0.1	0	0.037*

Table 8.6 Percentage variation of pH of tomatoes coated by dipping or spraying and stored at room or cold temperature for 30 days, expressed as mean ± standard deviation. *P* values resulting from the comparison of pH variation with the two different application methods are reported for each treatment, given the same storage temperature. Asterisks indicate significant differences ($p<0.05$), according to *Mann-Whitney* U test.

TREATMENT	STORAGE TEMPERATURE	pH VARIATION (%)		*P* VALUE
		Dipping application	Spraying application	
Solvent	room	6.9±0.2	10.4±0.3	0.049*
	cold	4.2±0.1	15.7±0.5	0.049*
Comm 1%	room	7.2±0.2	5.1±0.1	0.51
	cold	5.8±0.2	4.1±0.1	0.12
Comm 0.5%	room	7.5±0.2	5±0.1	0.049*
	cold	11.1±0.3	2±0.1	0.049*
PE het dec 1%	room	11.1±0.3	2.9±0.1	0.049*
	cold	5.5±0.2	1.5±0	0.049*
PE het dec 0.5%	room	5.3±0.2	6.1±0.2	0.82
	cold	0.9±0	0.3±0	0.046*
PE het no dec 1%	room	4.4±0.1	0	0.037*
	cold	2.1±0.1	-4.2±0.1	0.049*
PE het no dec 0.5%	room	2.5±0.1	0	0.037*
	cold	1.7±0.1	-3.1±0.1	0.049*
PE hom dec 1%	room	6.9±0.2	1.2±0	0.049*
	cold	0	1.2±0	0.12
PE hom dec 0.5%	room	4.9±0.1	4.3±0.1	0.51
	cold	0	1.2±0	0.12
PE hom no dec 1%	room	8.5±0.2	6.4±0.2	0.13
	cold	2.1±0.1	0.5±0	0.049*
PE hom no dec 0.5%	room	8.4±0.2	5.2±0.1	0.12
	cold	3.5±0.1	0	0.037*

Table 8.7 Percentage variation of TA of tomatoes coated by dipping or spraying and stored at room or cold temperature for 30 days, expressed as mean ± standard deviation. *P* values resulting from the comparison of TA variation at the two different storage temperatures are reported for each treatment, given the same application method. Asterisks indicate significant differences ($p<0.05$), according to *Mann-Whitney* U test.

TREATMENT	APPLICATION METHOD	pH VARIATION (%)		*P* VALUE
		Room temperature	Cold temperature	
Solvent	dipping	-6.9±2.5	-33.5±4.4	0.046*
	spraying	-5.6±0	8.3±0.7	0.034*
Comm 1%	dipping	-15.7±1.4	-36.3±1.1	0.043*
	spraying	54±0.9	88.6±4.5	0.043*
Comm 0.5%	dipping	-24.3±4.1	-44.8±3.4	0.043*
	spraying	56.6±2.7	87.6±1.3	0.043*
PE het dec 1%	dipping	-32±0	-18.4±0	0.025*
	spraying	101.1±3.1	66.6±1.5	0.043*
PE het dec 0.5%	dipping	-4.7±0	-10.4±1.9	0.034*
	spraying	63.1±3.7	104.7±4	0.043*
PE het no dec 1%	dipping	-12.8±1.1	-9.3±0	0.3
	spraying	100.3±1.7	179.1±0	0.034*
PE het no dec 0.5%	dipping	-17.7±1.2	-16.6±1.4	0.48
	spraying	12.3±0	149.1±1.8	0.034*
PE hom dec 1%	dipping	17.5±1.4	20.2±2	0.23
	spraying	80.1±1.2	153.6±3.8	0.043*
PE hom dec 0.5%	dipping	27±0	27.2±0.3	0.5
	spraying	95.3±3	123.3±0	0.034*
PE hom no dec 1%	dipping	8.2±1.1	17.9±0	0.034*
	spraying	70.2±1.3	150.1±1.8	0.043*
PE hom no dec 0.5%	dipping	8.9±0	17.5±0.7	0.034*
	spraying	110.9±5.4	154.7±6	0.043*

Table 8.8 Percentage variation of TA of tomatoes coated by dipping or spraying and stored at room or cold temperature for 30 days, expressed as mean ± standard deviation. *P* values resulting from the comparison of TA variation with the two different application methods are reported for each treatment, given the same storage temperature. Asterisks indicate significant differences ($p<0.05$), according to *Mann-Whitney* U test.

TREATMENT	STORAGE TEMPERATURE	pH VARIATION (%)		*P* VALUE
		Dipping application	Spraying application	
Solvent	room	-6.9±2.5	-5.6±0	0.48
	cold	-33.5±4.4	8.3±0.7	0.046*
Comm 1%	room	-15.7±1.4	54±0.9	0.043*
	cold	-36.3±1.1	88.6±4.5	0.043*
Comm 0.5%	room	-24.3±4.1	56.6±2.7	0.043*
	cold	-44.8±3.4	87.6±1.3	0.043*
PE het dec 1%	room	-32±0	101.1±3.1	0.034*
	cold	-18.4±0	66.6±1.5	0.034*
PE het dec 0.5%	room	-4.7±0	63.1±3.7	0.034*
	cold	-10.4±1.9	104.7±4	0.043*
PE het no dec 1%	room	-12.8±1.1	100.3±1.7	0.043*
	cold	-9.3±0	179.1±0	0.025*
PE het no dec 0.5%	room	-17.7±1.2	12.3±0	0.034*
	cold	-16.6±1.4	149.1±1.8	0.043*
PE hom dec 1%	room	17.5±1.4	80.1±1.2	0.043*
	cold	20.2±2	153.6±3.8	0.043*
PE hom dec 0.5%	room	27±0	95.3±3	0.034*
	cold	27.2±0.3	123.3±0	0.034*
PE hom no dec 1%	room	8.2±1.1	70.2±1.3	0.043*
	cold	17.9±0	150.1±1.8	0.034*
PE hom no dec 0.5%	room	8.9±0	110.9±5.4	0.034*
	cold	17.5±0.7	154.7±6	0.043*

Table 8.9 Weight loss (%) of apricots coated by spraying and stored at room or cold temperature for 14 days, expressed as mean ± standard deviation. *P* values resulting from the comparison of weight loss with the two different storage temperatures are reported for each treatment. Asterisks indicate significant differences ($p<0.05$), according to *Mann-Whitney* U test.

TREATMENT	WEIGHT LOSS (%) AT 14 DAYS		*P* VALUE
	Room temperature	Cold temperature	
Negative control	54.2±3.1	47.7±3	0.025*
Solvent	54.9±3.1	44.7±5.5	0.006*
Comm 1%	53.2±3.3	39.2±3.4	0.0039*
Comm 0.5%	53.2±3.4	42.6±3.3	0.0039*
L dec 1%	55.8±2.5	42.5±4.6	0.0065*
L dec 0.5%	53±2.9	45.6±1.9	0.0039*
L no dec 1%	51.6±3.8	44.1±4.5	0.0039*
L no dec 0.5%	57.8±3	41.2±3	0.0039*
PE dec 1%	50.4±2.3	40±4.6	0.016*
PE dec 0.5%	49.7±3.2	38.2±4.9	0.0039*
PE no dec 1%	53.4±5	40±3.1	0.0039*
PE no dec 0.5%	53.7±2.9	40.4±2.7	0.0039*
ADU dec 1%	53.2±1.2	41.6±5.1	0.0039*
ADU dec 0.5%	57.4±3.1	41.7±6.1	0.01*
ADU no dec 1%	53±2.7	39.1±3.6	0.025*
ADU no dec 0.5%	53.2±2.6	41.2±4.3	0.0039*

Table 8.10 Weight loss (%) of strawberries coated by spraying and stored at room or cold temperature for 9 days, expressed as mean±standard deviation. *P* values resulting from the comparison of weight loss with the two different storage temperatures are reported for each treatment. Asterisks indicate significant differences (*p*<0.05), according to *Mann-Whitney* U test.

TREATMENT	WEIGHT LOSS (%) AT 9 DAYS		*P* VALUE
	Room temperature	Cold temperature	
Negative control	88.6±1.4	64.7±3	0.02*
Solvent	85.5±0.8	77.3±1	0.9
Comm 1%	86.4±1.2	72.4±3.3	0.054
Comm 0.5%	90.3±2.7	73.8±2.4	0.01*
L dec 1%	85.9±2.6	70.5±1.6	0.5
L dec 0.5%	85.4±2.4	72.1±2.7	0.6
L no dec 1%	83.8±1.2	71.7±2.6	0.6
L no dec 0.5%	86.9±1.7	74.9±1.1	0.14
PE dec 1%	88.3±1.6	58±3.6	0.002*
PE dec 0.5%	87.1±1.5	68.3±4.1	0.3
PE no dec 1%	83.7±3.8	71.5±1.1	0.7
PE no dec 0.5%	87.8±1.6	67.5±4.1	0.01*
ADU dec 1%	88.3±1.3	70.1±2	0.4
ADU dec 0.5%	84.8±2.4	76.4±1.5	0.7
ADU no dec 1%	85.2±2.1	66.7±3.2	0.3
ADU no dec 0.5%	84.3±1.2	67.2±2.9	0.12

Table 8.11 Weight loss (%) of nectarines coated by spraying and stored at room or cold temperature for 10 days, expressed as mean±standard deviation. *P* values resulting from the comparison of weight loss with the two different storage temperatures are reported for each treatment. Asterisks indicate significant differences (*p*<0.05), according to *Mann-Whitney* U test.

TREATMENT	WEIGHT LOSS (%) AT 9 DAYS		*P* VALUE
	Room temperature	Cold temperature	
Negative control	52.9±2.3	36.6±1.5	0.004*
Solvent	53.5±3.1	32.4±3.9	0.004*
Comm 1%	54.3±3.3	37.9±5.7	0.004*
Comm 0.5%	51.3±3.6	38.1±5.9	0.004*
PE dec 1%	50.1±3.8	36.1±4.6	0.004*
PE dec 0.5%	51.9±3.4	34.4±5.2	0.004*
PE no dec 1%	58.2±4.4	36.9±4.5	0.004*
PE no dec 0.5%	54.5±1.8	37.1±3.9	0.006*

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Current state of chitin purification and chitosan production from insects

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Abstract

Chitin, and especially its deacetylated variant chitosan, has many applications, e.g. as carrier material for pharmaceutical drugs or as a flocculant in wastewater treatment. Despite its versatility and accessibility, chitin, the second most abundant polysaccharide on Earth, has so far been commercially extracted only from crustaceans and to a minor extent from fungi. Insects are a viable alternative source of chitin, but they have not been exploited in the past due to limited availability. Today however, for the sustainable production of animal feed, insect farming is being developed substantially. The availability of large quantities of insect biomass and chitin-rich side products such as exuviae and exoskeletons has been increasing. This review provides an overview of recently published studies of chitin extraction from insects, its subsequent conversion into chitosan and the primary analytical methods used to characterize insect-based chitin and chitosan. We have discovered a large number of research articles published over the past 20 years, confirming the increased attention being received by chitin and chitosan production from insects. Despite numerous publications, we identified several knowledge gaps, such as a lack of data concerning chitin purification degree and chitosan yield. Furthermore, analytical methods used to obtain physicochemical characteristics, structural information and chemical composition meet basic qualitative requirements but do not satisfy the need for a more quantitative evaluation. Despite the current shortcomings that need to be overcome, this review presents encouraging data on the use of insects as an alternative source of chitin and chitosan in the future.

Keywords: chitin; chitosan; insects; purification; analysis

INTRODUCTION

Chitin is an important structural component of the cell wall of fungi and yeasts and the main carbohydrate component of arthropod exoskeletons.[1] After cellulose, it is the second most abundant biopolymer present in nature.[2] The production of chitin in the biosphere is estimated to be around 1000 billion (10^{11}) tons per year.[3] Chitin was isolated for the first time in 1799 from the shells of mollusks.[4] Chitosan, the deacetylated derivative of chitin, was obtained by Rouget in 1859 by heating chitin in alkaline medium; however, its chemical structure was determined only in 1950.[5] Despite this early discovery, the industrial production and commercialization of chitin and chitosan initially started in the 1970s.[6]

Chitin is a hard, inelastic, *N*-acetylated aminopolysaccharide (Fig. 1(a)) with high hydrophobicity, making it insoluble in water and most organic solvents.[7,8] Fungi and invertebrates use these properties and incorporate chitin microfibrils to protect and strengthen their cell matrix or as components of mechanically resilient structures such as shells, cuticles, bones (in cuttlefish) and scaffolds (in sponges).[9,10]

Based on various orientations of its microfibrils, chitin exists in nature in three crystalline allomorphic forms: α-, β- and γ-chitin (Fig. 1(b)). α-Chitin has antiparallel chains. It is responsible for the rigidity of the polymer and is the most abundant form.[11] β-Chitin consists of parallel chains, producing monoclinic crystals with intramolecular interactions (hydrogen bonds) in addition to intermolecular ones.[12] β-Chitin is found in the spines of diatoms, squid pens and pogonophoran tubes. γ-Chitin is a mixture of parallel and antiparallel chains combining the properties of both α-form and β-form[13]; it is present in fungi, yeasts and insect cocoons.[14–18]

After being isolated from natural sources, direct application of chitin is limited to a few applications, such as the production of scaffolds to support tissue regeneration[19] or for biological control of plant pathogens in agriculture,[20] which is due to its insolubility.

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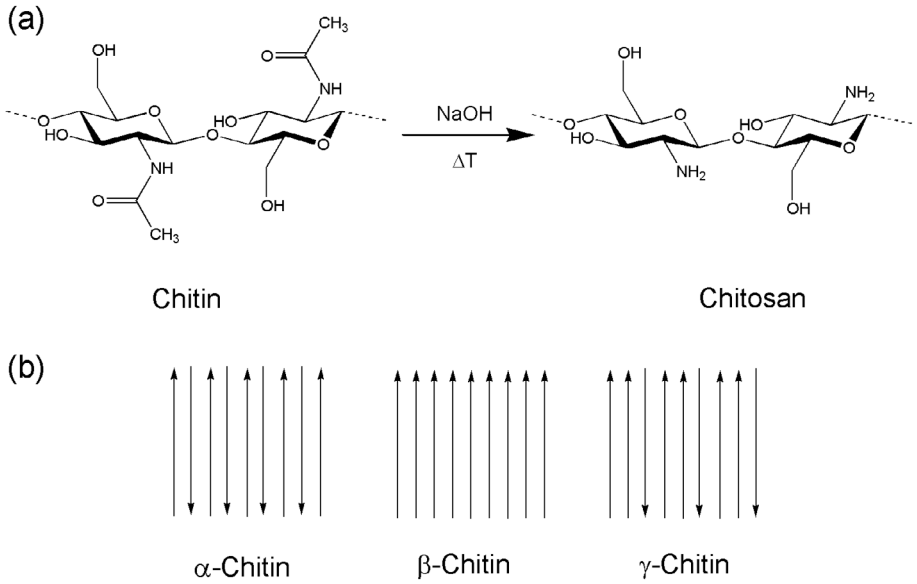

Figure 1. (a) Chitin and chitosan molecular structure. Chitin consists of *N*-acetylated-D-glucosamine (GlcNAc) and 2-amino-D-glucose (D-glucosamine, GlcN) linked by *β*-1,4 glycosidic bonds. Chitosan is the main deacetylated derivative of chitin. (b) Three crystalline allomorphic forms of chitin, with different microfibril orientations.

To widen its range of applications, chitin needs to be converted to more water-soluble derivatives with useful properties, primarily chitosan. Chitosan (Fig. 1(a)) is a cationic polysaccharide obtained from chitin by alkaline hydrolysis of the acetamido groups (deacetylation process). Due to an increased number of free primary amine groups, and partially due to a lower molecular weight, chitosan is soluble in slightly acidic solutions. To define and distinguish between chitin and chitosan, several researchers have set the threshold value of degree of acetylation to 50%: when the *N*-acetyl group content of the polysaccharide exceeds 50%, the polymer is defined as chitin; for values below 50%, it is called chitosan.[21, 22]

The physicochemical properties of chitin and chitosan may vary among samples, being affected by many factors such as the source of chitin and parameters of the purification process. Molecular weight is an important characteristic to consider as it affects chitosan viscosity[23] that is crucial to many of its applications and other important features such as antibacterial activity.[24] Native chitin from crustacean sources has a molecular weight exceeding 1000 kDa, while the molecular weight of crustacean-based chitosan ranges from 100 to 1000 kDa.[25, 26]

Due to its useful properties, such as biodegradability, biocompatibility, nontoxicity, adsorption capacity and antimicrobial activity,[1, 17] chitosan is attracting great attention for many applications within the agricultural, industrial, biotechnological and biomedical fields and in wastewater treatment.[1, 8, 27–31]

The antimicrobial activity of chitosan is one of its most exploited properties. Chitosan can inhibit the growth of human pathogens, such as *Escherichia coli*,[32, 33] *Staphylococcus aureus*,[34, 35] *Pseudomonas aeruginosa*[36] and *Aspergillus niger*.[37] Its antimicrobial activity makes chitosan suitable to be used not only in biomedical applications but also as a natural biopolymer coating material to preserve the quality and to extend the shelf-life of fresh food.[38] Chitosan-based active films against contamination and microbial spoilage have been successfully used in fruit, vegetable, egg and meat packaging.[39, 40]

Chitosan has also shown excellent potential for wound dressing. The potential of chitosan as a hemostatic topical dressing for animal tissues has previously been demonstrated: chitosan adheres to red blood cells, thus retrieving platelets for hemagglutination.[41] In cosmetics, chitosan finds application in the production of creams and lotions. It is used as a moisturizing and UV-protective agent for the skin.[42] A further application for chitosan is in wastewater treatment, where it is used as a flocculating agent owing to its ability to chelate cations and adsorb waste molecules from water, such as heavy metals.[31, 43]

Recently, chitosan has been proposed as an ecological finishing agent in the textile industry. It is used in working fabrics for hospitals or biological laboratories and for making sutures, threads and fibers in medical textiles.[44, 45] Chitosan is also used for antistatic finishing in work wear for employees of the electronic sector.[46]

Three main chitin sources are available

Currently, the main commercial source of chitin and chitosan comprises waste streams from the marine food industry – mainly exoskeletons of crustaceans.[47] Annual world production of crustaceans for human consumption was an estimated 8 million tons in 2016,[48] of which 40% comprised waste exoskeletons[49] with a chitin content of 15–40%.[50] However, the availability of fishery waste is highly seasonal, as commercial crustacean fishing starts in spring, after the spawning season.[51] Moreover, the sustainability of crustacean farming is currently under debate.[49]

The global market for chitin and chitosan is expected to reach a volume of $4.2 billion by 2021, with a compound annual growth rate of 15.4%,[52] intensifying the need for a search of other sources to satisfy the growing market.

Fungi are the second main source of chitin after crustaceans. Several research activities have focused on fungi and their commercial value as they have attracted attention as an alternative and vegan source of chitin and chitosan.[53] Chitin makes up between 1 and 15% of fungal cell wall mass[54] and its structure is comparable to that in crustaceans.[55] Even though not all fungi

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contain chitin, it is widely distributed in various fungal phyla such as Basidiomycota, Ascomycota and Zygomycota.[55] Similar to crustacean chitin, severe conditions are required to obtain chitosan from fungal chitin. In contrast, chitosan can be directly isolated from the cell wall of some fungal species without requiring the cleavage of the acetyl groups. Among them, the most investigated species for direct chitosan production include *Absidia* spp. (zygomycetes), *A. niger* (ascomycetes), *Mucor rouxii* (zygomycetes), *Rhizophus oryzae* (zygomycetes) and *Lentinus edodes* (basidiomycetes).[56–59] Nevertheless, the production of fungal chitin and chitosan has not yet been scaled up to the industrial level.[53]

In addition to crustaceans and fungi, insects are another promising and sustainable source of chitin and chitosan, although they have not received much attention previously. Insects present some advantages compared to crustaceans, as they are not subject to seasonality, and can be easily bred owing to their high fertility and reproductive rate; moreover, insect rearing facilities are being launched worldwide.[60] Notably, as bioconverters – reared for organic waste management and animal feed production – insects can be exploited as a valid alternative to crustaceans as a source for chitin and chitosan for greater ecological and economic sustainability.[61]

Arthropods, including centipedes[62, 63] and woodlice,[64, 65] have been investigated as sources of chitin. Notably, the body segments of large centipedes are suitable for the production of three-dimensional chitin rings.[62] In addition, chitin has been extracted from poriferans,[66] bryozoans[67] and tardigrades[68] and from guano of insectivorous bats.[69]

Quo vadis insect chitin?

Economic value linked to beneficial insects has been known to humans for a long time. The commercial production of silk from *Bombyx mori* originated in China during the Neolithic period.[70] Humans have learned to farm several insect species and exploit them for specific applications with the course of time. Production of biocontrol insects started in the middle of the 20th century. For example, mass production of *Cochliomyia hominivorax* for biocontrol started in Florida during the late 1950s.[71] Organized, large-scale production of insects for human and animal nutrition has been more recent: Protix, a Dutch company, launched the first facility in 2015.

Some industries in the domain of beneficial insect breeding are witnessing rapid growth. For example, the market for edible insects is estimated to exceed $522 million in 2023.[72] According to a report published in 2016, globally, more than 120 registered companies are involved in the business of farming and processing and/or marketing insects for animal and human nutrition.[73] In 2019, approximately 6kt of insect protein meal was produced in Europe[74] to be used for animal nutrition, from the black soldier fly (*Hermetia illucens*), the yellow mealworm (*Tenebrio molitor*) and, to a smaller extent, the lesser mealworm (*Alphitobius diaperinus*).[75] In particular, the black soldier fly is processed by around 80% of all EU insect-producing companies.[75] *H. illucens* could be grown on a wide range of organic side streams and contributes to a circular economy.[76, 77] Exoskeletons from *H. illucens* larvae contain up to 35% chitin,[78] which means that it is one of the main compounds that could be isolated from the byproducts of the insect farming industry.[73]

Thus, chitin-rich byproducts from insect farming present a new and sustainable source of commercial chitin. Given the sustainability aspect and the expected rise in insect production, byproducts from insect farming present a very interesting source of chitin for the future.

The inner soft tissues of insects are covered by a hard, protective layer called the exoskeleton. The exoskeleton has several functions in insect bodies, including but not limited to: (i) as a protective covering and (ii) as a facilitator of metamorphosis. The exoskeleton is rich in chitin and is shed from the body during metamorphosis.[79] Chitin is contained in the procuticle, the innermost layer of the cuticle, which is in turn the outermost layer of the arthropod exoskeleton.[80, 81] In its native form, chitin is arranged into microfibers embedded in a protein matrix.[82] To extract chitin from the arthropod cuticle, the purification process remove proteins, lipids, minerals, pigments and catechols contained therein.[83] Whole insects generally contain 30–60% protein,[84] 10–25% lipid,[84] 5–25% chitin,[50, 78, 85] 5–10% catechols[85] and 2–10% minerals such as calcium, phosphorus, potassium and magnesium salts.[84, 86]

To date, little has been reported on the extraction methods for insect chitin and its physicochemical properties. Here, a total of 52 papers reporting chitin purification and chitosan production from 58 insect species were collected, summarized and analyzed (Tables 1 and 2).

CHITIN PURIFICATION

Various types of chitin purification processes can be performed, such as physical, biotechnological and chemical methods and a combination of these. Physical techniques such as crushing and stirring are used in parallel with chemicals or catalysts. Biotechnological extraction and deacetylation of chitin has been gaining interest as an environmentally friendly alternative to chemical processes. The biotechnological methods are mainly based on the use of microbial proteases or whole microorganisms for the removal of proteins and the application of deacetylases for the deacetylation of chitin. However, the biotechnological processes developed so far produce lower yields, are time-consuming and result in products of lower purity.[83, 134] Notably, enzymatic deacetylation of chitin using deacetylases has proven to be unsuitable for chitosan production.[135, 136]

Chemical processes, utilizing acidic and alkaline solutions, are currently the most applied on an industrial scale to produce large amounts of chitin and chitosan from crustacean shells[134, 137] (Fig. 2). Typically, chemical treatment provides pure chitin and chitosan, although it produces large volumes of waste due to the high concentrations of chemicals used.[134, 137, 138] The literature reviewed here describes only chemical methods for chitin and chitosan production from insects.

Chemical methods for extracting chitin from insect exoskeletons are similar to those used for marine sources and consist of two steps: demineralization and removal of proteins. In the first step, minerals contained in the cuticle are removed with diluted acids. Various mineral acids (e.g. hydrochloric acid) or organic acids (e.g. acetic acid) can be used for this purpose. The removal of proteins is carried out with alkaline treatments, mainly applying diluted sodium hydroxide solution. Deproteinization treatment can simultaneously extract part of the dyes and soluble lipids contained in the exoskeleton. For prawn shells, two-step purification can be performed in reverse order without affecting the properties of chitin.[83]

An additional step of bleaching can be performed to remove residual pigments and improve the color of purified chitin, using

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Table 1. Methods for chitin purification from insects and respective yields

Raw material		Demineralization			Deproteinization				
Insect species	Stage/ body part	Reagent and conc.	Temp. (°C)	Duration (h)	Reagent and conc.	Temp. (°C)	Duration (h)	Chitin yield (dry chitin weight/dry insect weight) (%)	Ref.
Musca domestica	Larvae	—	—	—	1 M NaOH	95	6	—	87
Shistocerca gregarea	Adults							—	
Nezara viridula	Adults							—	
Periplaneta americana	Adults	0.9 M CH_3COOH	—	—	1.8 M KOH	40	—	—	88
Blatella germanica	Adults							—	
Vespa orientalis	Adults							—	
Gryllus bimaculatus	Adults	—	—	—	1 M NaOH	95	3	5.1	89
Apis mellifera	Adults	1 M HCl	Room temp.	1	1 M NaOH	80	6–64	51.0–77.2	90
Dociostaurus maroccanus	Adults	2 M HCl	55	1	2 M NaOH	50	18	12.0, 14.0	91
Hermetia illucens	Larval exoskeleton	0.5 M CH_2O_2	Room temp.	1	2 M NaOH	80	2	31.0–35.0	92
Bombyx eri	Larvae	1 M HCl	80	0.6	1 M NaOH	80	24	3.3	93
Acheta domesticus	Adults	0.1 M $C_2H_2O_4$	Room temp.	3	1 M NaOH	95	—	4.3–7.1	94
Colophon sp.	Adults	2 M HCl	Room temp.	6	1.5 M NaOH	10	24	—	18
Melolontha melolontha	Adults	4 M HCl	75	—	1 M NaOH	150	18	—	64
Agabus bipustulatus	Adults							14.0–15.0	
Anax imperator	Adults							11.0–12.0	
Hydrophilus piceus	Adults	1 M HCl	90	1	1 M NaOH	110	18	19.0–20.0	95
Notonecta glauca	Adults							10.0–11.0	
Ranatra linearis	Adults							15.0–16.0	
Leptinotarsa decemlineata	Larvae, adults	2 M HCl	65–75	2	2 M NaOH	80–90	16	7.0, 20.0	96
Vespa crabro	Adults							—	
Vespa orientalis	Adults	2 M HCl	75	2	4 M NaOH	150	18	—	97
Vespula germanica	Adults							—	
Ailopus simulatrix	Adults							5.3	
Ailopus strepens	Adults							7.4	
Duroniella fracta	Adults							5.7	
Duroniella laticornis	Adults							6.5	
Oedipoda miniata	Adults	4 M HCl	75	1	2 M NaOH	175	18	8.1	98
Oedipoda caerulescens	Adults							8.9	
Pyrgomorpha cognata	Adults							6.6	
Celes variabilis	Adults							6.6–9.9	
Decticus verrucivorus	Adults	4 M HCl	75	2	4 M NaOH	150	20	10.0–11.8	13
Melanogryllus desertus	Adults							4.7–7.3	

Table 1. Continued

Raw material		Demineralization			Deproteinization				
Insect species	Stage/ body part	Reagent and conc.	Temp. (°C)	Duration (h)	Reagent and conc.	Temp. (°C)	Duration (h)	Chitin yield (dry chitin weight/dry insect weight) (%)	Ref.
Apis mellifera	Legs Thorax Head Abdomen Wings	2 M HCl	80	6	2 M NaOH	100	20	13.2 6.8 8.9 8.6 7.6	99
Argynnis pandora	Wings Other body parts	2 M HCl	50	24	2 M NaOH	50	24	22.0 8.0	100
Calliptamus barbarus	Adults	1 M HCl	100	0.5	1 M NaOH	80–90	21	20.5	101
Oedaleus decorus	Adults							16.5	
Palomena prasina	Adults	2 M HCl	100	2	2 M NaOH	140	20	10.8	67
Omophlus sp.	Adults	2 M HCl	50	4	2 M NaOH	100	20	—	102
Vespa crabro	Larvae, pupae, adults	1 M HCl	50	6	1 M NaOH	60	16	2.2, 6.2, 10.3	103
Drosophila melanogaster	Adults	2 M HCl	40	3	3 M NaOH	70	20	7.8	104
Melontha sp.	Whole body (f, m) Antennae (f, m) Head (f, m) Eyes (f, m) Thorax (f, m) Abdomen (f, m) Elytra (f, m) Hindwings (f, m) Legs (f, m)	2 M HCl	60	20	2 M NaOH	100	20	15.7 f, 16.6 m 5.9 f, 10.5 m 16.0 f, 15.9 m 8.9 f, 8.9 m 13.8 f, 17.5 m 7.1 f, 6.5 m 37.9 f, 40.1 m 17.9 f, 17.3 m 17.8 f, 16.1 m	105
Blaberus giganteus	Wings dorsal pronotum	1 M HCl	Room temp.	24	2 M NaOH	90	9	26.9 21.2	106
Hylobius abietis	Adults	2 M HCl	Room temp.	—	2 M NaOH	—	2	27.9	107
Periplaneta americana	Wings other body parts	4 M HCl	75	2	4 M NaOH	150	20	18.0 13.0	108
Hermetia illucens	Larvae	0.5 M HCl	Room temp.	2	1.9 M NaOH	50	2	46.0 (chitin), 7.0 (amorphous chitin)	109
Musca domestica	Adults	2 M HCl	Room temp.	3	1.25 M NaOH	95	3	7.7–8.5	110
Gryllus bimaculatus	Adults	2 M HCl	Room temp.	3	1.25 M NaOH	95	3	2.4	111
Holotrichia parallela	Adults	1 M HCl	100	—	1 M NaOH	80	—	15.0	112
Cicada	Sloughs							—	
Bombyx mori	Chrysalis	1 M HCl	30	2	1 M NaOH	90	2	—	113
Tenebrio molitor	Larvae							—	
Grasshopper	Adults							—	
Catharsius molossus	Adults	1.3 M HCl	80 + room temp.	0.5 + 12	4 M NaOH	90 + room temp.	6 + 12	24.0	114

Table 1. Continued

Raw material		Demineralization			Deproteinization				
Insect species	Stage/ body part	Reagent and conc.	Temp. (°C)	Duration (h)	Reagent and conc.	Temp. (°C)	Duration (h)	Chitin yield (dry chitin weight/dry insect weight) (%)	Ref.
Bombus terrestris	Adults	1 M HCl	100	0.3	1 M NaOH	85	24	—	115
Schistocerca gregaria	Adults							12.2	
Apis mellifera	Adults	1 M HCl	Room temp.	—	1 M NaOH	100	8	2.5	116
Calosoma rugosa	Adults							5.0	
Ephestia kuehniella	Adults	1 M HCl	100	0.3	1 M NaOH	85	1	9.5–10.5	117
Cicada lodosi	Adults							4.8	
Cicada mordoganensis	Adults							6.5	
Cicadatra platyptera	Adults	2 M HCl	100	2	2 M NaOH	100	20	8.8	118
Cicadatra atra	Adults							6.7	
Cicadatra hyalina	Adults							5.5	
Cicadivetta tibialis	Adults							5.9	
Brachystola magna	Adults	1 M HCl	97	0.5	1 M NaOH	82	24	10.4	119
Apis mellifera	Adults	—	—	—	15 M NaOH	—	—	11.4–36.8	120
Calliphora erythrocephala	Larvae	—	—	—	1 M NaOH	50	2	12.2	121
Bombyx mori	Larvae	1 M HCl	100	—	1 M NaOH	80	—	2.6–4.3	122
Cicada	Sloughs	1 M HCl	100	0.3	1 M NaOH	80	36	36.0	11
Tenebrio molitor	Larvae, superworm, adults	2 M HCl	Room temp.	24	3.7 M NaOH	80	24	4.6, 3.9, 8.4	123
Allomyrina dichotoma	Larvae, pupae, adults							10.5, 12.7, 14.2	
Chrysomya megacephala	Larvae	0.1 M $C_2H_2O_4$	—	3	1 M NaOH	95	6	—	124
Hermetia illucens	Larvae, prepupae, pupae	1 M HCl	100	0.5	1 M NaOH	80	24	3.8, 4.7, 6.3	125
Zophobas morio	Larvae	1 M HCl	35	0.5	0.5–2 M NaOH	80	20	4.7–5.2	126
Mayfly	Adults	2 M HCl	50	—	2 M NaOH	100	—	10.2	127
Hermetia illucens	Pupal exuviae, adults	1 M HCl	—	—	1 M NaOH	80	24	—	128
Clanis bilineata	Larval skins	2 M HCl	Room temp.	24	3.7 M NaOH	60	24	—	129
Cryptotympana atrata	Sloughs	2 M HCl	Room temp.	24	3.7 M NaOH	60	24	—	130
Musca domestica	Larvae	—	—	—	1 M NaOH	100	3	—	131
Bombyx mori	Pupal exuviae	1 M HCl	100	—	1 M NaOH	80	3	15.0–20.0	132
Beetle	Larvae							15.0–20.0	
Hermetia illucens	Prepupae	NADESs	50–80	2	NADESs	50–80	2	6.0–26.0	133

reagents such as sodium hypochlorite, acetone and hydrogen peroxide.

Prior to the chitin purification process, insect samples are generally pretreated. Pretreatments include cleaning by washing with water and detergent, drying in an oven or at ambient temperature and grinding into powder to increase accessibility to the chemical agents. Solvent penetration into the particles and thus purification efficiency are greatly affected by corn size. Kim *et al.*[110] obtained a higher demineralization efficiency and chitosan with a higher degree of deacetylation using ground insect samples instead of non-ground ones. A particle size lower than 200–250 μm is suitable for chitin purification.[90, 116, 117, 132] A fat removal step may be required, especially for those insect samples rich in lipids, such as whole larvae. Larvae can be boiled and passed through an extruder or an oil press to separate unpurified chitin from the liquid fraction, containing mainly lipids and some proteins.[93, 109]

Demineralization

Crustacean shells contain a substantial amount of minerals, which can be up to 50% in crab and shrimp shells.[50] In contrast, insects have a much lower mineral content, generally ranging between 2 and 10% for whole insects. However, this value varies depending on the species and the stage of development.[84, 86]

The demineralization of insect samples comprises the decomposition of minerals into their respective water-soluble salts. The solubilized salts can be separated from chitin by filtration and washing of the solid phase. Acidic treatment also releases catechol compounds and leads to a slight discoloration of biomass.[115] The demineralization process can be affected by the type and concentration of acid used, treatment time and temperature, particle size of the sample and the solute-to-solvent ratio.[139]

Hydrochloric acid is the most preferred reagent used for the demineralization of insect exoskeletons (Table 1). Hydrochloric acid has also been one of the most widely used acids for the demineralization of crustacean shells on an industrial scale.[134] Because of its high environmental impact, hydrochloric acid has been substituted in many cases by organic acids. Furthermore, hydrochloric acid can have detrimental effects on the structure and chemical composition of chitin,[140] as has been confirmed by investigations of Percot *et al.*,[141] who reported a lower molecular weight and a lower degree of acetylation of chitin after demineralization. As a rule of thumb, the harsher the demineralization treatment – in terms of pH, duration and temperature – the higher is the degree of hydrolysis and the worse are the aforementioned effects on chitin.[142]

In contrast to the frequently used hydrochloric acid, only four investigations have reported the use of organic acids for the demineralization of insect biomass. Ibitoye *et al.*[94] and Song *et al.*[124] used oxalic acid and Badawy and Mohamed[88] and Hahn *et al.*[92] used acetic acid and formic acid, respectively.

In most cases, the concentration of the acidic solution used for demineralization is 1–2 mol L^{-1}. Few papers have reported the application of a higher concentration of 4 mol L^{-1}.[13, 64, 98] The solute-to-solvent ratio depends on the acid concentration, as it needs two molecules of hydrochloric acid to convert one molecule of calcium carbonate, the main mineral component of the insect exoskeleton, into calcium chloride, carbon dioxide and water.[143]

The time taken for demineralization treatment is usually short. Most protocols have reported an incubation time between 30 min and 3 h. In a few cases, the treatment lasted for up to 6 h,[99, 103] 12 h[114] and 24 h.[101, 123, 144] The range of temperature used for demineralization varies widely from room temperature to 100 °C. High temperatures are used for very short periods (20–30 min), as reported by Kaya *et al.*[100] and Monter-Miranda *et al.*[119] In contrast, longer treatment periods (12–24 h) are applied for incubations done at room temperature.[106, 114, 123] High temperatures promote the penetration of the solvent into the chitin matrix[145]; however, they can cause polymer degradation.[25]

The efficiency of demineralization (DME) can be evaluated by assessing the mineral content of insect samples before (MC_{BT}) and after treatment (MC_{AT}) according to the following equation:

$$\mathrm{DME}\,(\%) = \frac{\mathrm{MC_{BT}}(\%) - \mathrm{MC_{AT}}(\%)}{\mathrm{MC_{BT}}(\%)} \times 100 \qquad (1)$$

From the scanty data available in the literature on the efficiency of the demineralization of insect biomass, we can observe that the highest efficiency (86–98%) was achieved by Zhou *et al.*,[133] using natural deep eutectic solvents on *H. illucens* prepupae (Table 1). Of the organic acids used, oxalic acid, used by Ibitoye *et al.*,[94] resulted in a higher degree of demineralization compared to that reported by Kim *et al.*[110,111] with hydrochloric acid, although a lower concentration of oxalic acid had been used. Demineralization efficiency for the *H. illucens* larval exoskeleton, reported by Hahn *et al.*,[92] was similar to that obtained by Ibitoye *et al.*,[94] using formic acid. However, additional data on the efficiency of insect demineralization are not available. Thus, future studies should focus on assessing the suitability and optimization of the current methods. Precise evaluation of the efficiency of various acids will enable choosing acids with a lower environmental impact but guarantee good demineralization. For instance, Mahmoud *et al.*[140] and Ameh *et al.*[146] have reported that the efficiency of demineralization of shrimp shells using lactic or acetic acids is comparable to that obtained using hydrochloric acid. Values of demineralization efficiency for shrimp shells, using either hydrochloric acid or acetic and lactic acid, as reported by Mahmoud *et al.*,[140] are similar to those obtained by Ibitoye *et al.*[94] with oxalic acid using insect samples. Organic acids, namely lactic, acetic and oxalic acids, can therefore be a valid alternative to hydrochloric acid for the demineralization of insect biomass. The utilization of organic acids also provides other benefits as they are less harmful to the environment, can preserve the characteristics of purified chitin, can be produced from low-cost biomass and the extracted organic salts can be used for other applications.[140]

Once the minerals have been solubilized and removed, the insect biomass is washed with distilled water until its pH is restored to neutral. After the neutralization step, insect samples are subjected to deproteinization.

Protein removal

Deproteinization of insect biomass is commonly achieved using alkaline solutions. A wide range of chemicals has been tested as deproteinization reagents with crustacean samples, including sodium hydroxide, sodium carbonate, sodium bicarbonate, potassium hydroxide, potassium carbonate, calcium hydroxide, sodium sulfite, sodium bisulfite, trisodium phosphate and sodium sulfide.[139] As well as for demineralization, the efficiency of deproteinization depends on the concentration of the alkali, solid-to-solvent ratio and time and temperature of the treatment.[147] Although high temperatures are crucial for deproteinization efficiency, they can cause undesirable side reactions if combined

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Table 2. Methods for chitin deacetylation and characteristics of respective chitosan

Raw material		Deacetylation						
Insect species	Stage/body part	Reagent and concentration	Temperature (°C)	Duration (h)	Chitosan yield (%)	Deacetylation degree (%)	Molecular weight (kDa)	Ref.
Musca domestica	Larvae	15 M NaOH	70	8	—	90	—	87
Gryllus bimaculatus	Adults	19–25 M NaOH	—	15	41.7 (from chitin)	56–85	50–190	89
Dociostaurus maroccanus	Adults	22 M NaOH	150	4	81.7 (from chitin)	64	7	91
Hermetia illucens	Larval exoskeleton	12 M NaOH	120–140	3–6	8.0–16.0 (from initial biomass)	43–72	—	92
		10 M NaOH	4 °C	12	4.0 (from initial biomass)	34	—	
Acheta domesticus	Adults	—	—	—	2.3–5.8 (from initial biomass)	80	—	94
Agabus bipustulatus	Adults				71.0 (from chitin)		—	
Anax imperator	Adults				67.0 (from chitin)		—	
Hydrophilus piceus	Adults	22 M NaOH	120	2	74.0 (from chitin)	74–78	—	95
Notonecta glauca	Adults				69.0 (from chitin)		—	
Ranatra linearis	Adults				70.0 (from chitin)		-	
Leptinotarsa decemlineata	Larvae	19 M NaOH	100	3	67.0 (from chitin)	76	3	96
	Adults				72.0 (from chitin)	82	3	
Calliptamus barbarus	Adults	19 M NaOH	130	2	74.0–75.0 (from chitin)	70–75	—	101
Oedaleus decorus	Adults				75.0–76.0 (from chitin)		—	
Drosophila melanogaster	Adults	22 M NaOH	150	48	70.9 (from chitin)	—	—	104
Hylobius abietis	Adults	22 M NaOH	100	4	86.2 (from chitin)	81	7	107
Hermetia illucens	Larvae	19 M NaOH	100	2	80.0 (from chitin), 32.0 (from amorphous chitin)	90	160	109
Musca domestica	Adults	19 M NaOH	95–105	3–5	6.8 (from initial biomass)	33–97	—	110
Gryllus bimaculatus	Adults	19 M NaOH	100	3	1.8 (from initial biomass)	—	308	111
Cicada	Sloughs	22 M NaOH	100	8	28.2 (from initial biomass)	84	38	113
Bombyx mori	Chrysalis				3.1 (from initial biomass)	85	4	
Tenebrio molitor	Larvae				2.5 (from initial biomass)	86	40	
Grasshopper	Adults				5.7 (from initial biomass)	90	39	
Catharsius molossus	Adults	18 M NaOH	25–90	247	—	95	450	114
Schistocerca gregaria	Adults				—	98	—	
Apis mellifera	Adults	19 M NaOH	100	8	—	96	—	116
Calosoma rugosa	Adults				—	95	—	
Brachystola magna	Adults	15 M NaOH + 0.25 g $NaBH_4$	105–110	—	8.1 (from initial biomass)	57	26	119
Apis mellifera	Adults	19 M NaOH	150	1	20.0–30.0 (from chitin) / 16.0–25.0 (from bleached chitin)	75–84	200–250	120
Calliphora erythrocephala	Larvae	19 M NaOH	100–120	1–4	66.7 (from chitin)	63–81	263–413	121
Bombyx mori	Larvae	15 M NaOH +1 g/L $NaBH_4$	100	—	—	78–97	3290–5900	122

Table 2. Continued

Raw material		Deacetylation						
Insect species	Stage/body part	Reagent and concentration	Temperature (°C)	Duration (h)	Chitosan yield (%)	Deacetylation degree (%)	Molecular weight (kDa)	Ref.
Tenebrio molitor	Larvae, superworm, adults				80, 83.3, 78.3 (from chitin)	75, 76, 76	—	
Allomyrina dichotoma	Larvae, pupae, adults	21 M NaOH	90	9	83.4, 83.4, 75.0 (from chitin)	76, 76, 75	—	123
Chrysomya megacephala	Larvae	25 M NaOH	90	9	26.2 (from initial biomass)	88–90	501	124
Zophobas morio	Larvae	19 M NaOH	90	30	65.0–75.0 (from chitin)	64–81	—	126
Mayfly	Adults	22 M NaOH	150	6	78.4 (from chitin)	84	4	127
Clanis bilineata	Larval skins	21 M NaOH	110	4	—	—	—	129
Cryptotympana atrata	Sloughs	21 M NaOH	110	4	—	—	—	130
Musca domestica	Larvae	15 M NaOH + $NaBH_4$ 0.75 g/L	110	4	60.0–70.0 (from chitin)	83	—	132

with very long incubation times. These include partial deacetylation of chitin, hydrolysis of the biopolymer (lowering its molecular weight) and change of characteristics.[139, 148]

Sodium hydroxide is the most widely used base for deproteinization of crustacean biomass for the industrial production of chitin.[149] It has also been widely used for chitin purification from insects (Table 1). Badawy and Mohamed[88] are the only researchers to have used potassium hydroxide to deproteinize insect samples. The use of potassium hydroxide has also been suggested by Fu *et al.*[150] and Castillo *et al.*[151] as a more eco-friendly alternative to sodium hydroxide for shrimp deproteinization, as the liquid waste generated using potassium hydroxide is suitable for use as a fertilizer, owing to its high phosphorus, potassium and nitrogen content.[150] However, major deproteinization processes of insect samples have been performed using sodium hydroxide in low concentrations (0.5–2 mol L^{-1}). In a few cases, a concentration of 4 mol L^{-1} has been used.[13, 64, 97] The incubation time required for the deproteinization of insect biomass varies greatly from a few hours to a few days; however, the treatment typically lasts for 16–20 h (Table 1). Using the same working conditions, long deproteinization times applied to crustacean biomass have been reported to lead to a higher loss of proteins compared to shorter treatments.[152] The deproteinization reaction is generally performed at 80–100 °C, with a few exceptions being 40 °C[88] or 175 °C.[98]

As well as for demineralization, the efficiency of deproteinization (DPE) can be evaluated by measuring the protein content of insect samples before (PC_{BD}) and after deproteinization (PC_{AD}), according to the following equation:

$$\mathrm{DPE}\,(\%) = \frac{\mathrm{PC_{BD}}(\%) - \mathrm{PC_{AD}}(\%)}{\mathrm{PC_{BD}}(\%)} \times 100 \qquad (2)$$

Deproteinization efficiency of insect biomass has been mentioned only by Kim *et al.*[110,111] In both cases a maximum efficiency of 86–87% was achieved by applying 1.25 mol L^{-1} sodium hydroxide for 3 h at 95 °C on adult specimens of *M. domestica*[110] and *G. bimaculatus*.[111] These values are similar to those obtained by Zhou *et al.*,[133] who used natural deep eutectic solvents to remove proteins from *H. illucens* prepupae. The deproteinization efficiency of sodium hydroxide on crustacean shells at both high and room temperature was 71–76%.[149]

Results from chitin purification

Information is lacking on the results obtained from chemical demineralization and deproteinization of insect samples. Most papers have reported only chitin yield as the end result of the purification process (Table 1). Chitin yield is measured as the percentage ratio of dry weight of chitin and dry weight of the source material. Chitin yield from insect biomass varied from a minimum of approximately 2% – obtained from *Vespa crabro* larvae,[103] *G. bimaculatus* adults,[111] *Apis mellifera* adults[116] and *B. mori* larvae[122] – to a maximum of 36% obtained from *A. mellifera* adults[120] and cicada sloughs.[11]

However, most authors have reported a chitin yield of between 5 and 15% (Table 1). Yields of chitin extracted from shells of crustaceans, mainly shrimps, prawns and crabs, varied from 5 to 32%.[26, 64, 121, 147, 153–156] Chitin content can vary widely depending on the species, developmental stage and body part of the crustacean.[91, 103, 153, 156] For example, Thirunavukkarasu and Shanmugam[156] reported that the yield of chitin was higher from

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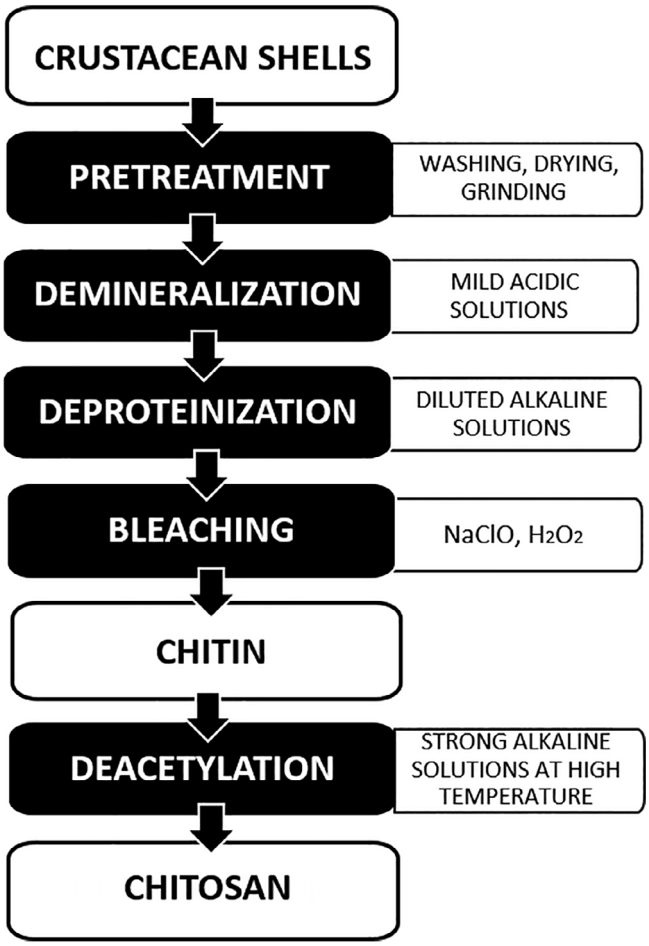

Figure 2. Industrial process for chitin purification and chitosan production from crustacean shells.

the cuticle of crab legs than from its carapace and claws. These findings are in accordance with the results of Kaya *et al.*[99] who found a higher chitin content in the legs than in other body parts of honeybees. Thus, the chitin content of a body part correlates with the mechanical load on that body part.

Only Huet *et al.*[93] and Kaya *et al.*[103] have measured the degree of purification for chitin extracted from *Bombyx eri* larvae and three developmental stages of *V. crabro*, respectively (Table 1). Using various times and temperatures for both demineralization and deproteinization treatments, they obtained similar results, that is, between 93 and 97%. Using natural deep eutectic solvents, Zhou *et al.*[133] achieved a slightly lower degree of purification (74–91%) from *H. illucens* prepupae. The lack of quantitative assessment of the purity of insect-based chitin makes it difficult to evaluate the suitability of the methods.

After demineralization and deproteinization, chitin can be directly deacetylated to chitosan or can be bleached to improve its color and remove the residual lipids.

Bleaching as the final step

Many kinds of pigments and structural colors are involved in the coloration of insect cuticle. They originate from the tyrosine-mediated cuticle-tanning pathway, such as melanins, or originate from 3,4-dihydroxyphenylalanine and dopamine during the process of cuticular tanning and sclerotization.[157] During demineralization and deproteinization treatments, a small amount of pigment and lipid is removed; however, chitin retains a brownish appearance. For commercial purposes, the color of chitin and chitosan is required to be as white as possible. Thus, an additional step of bleaching is used to remove residual pigments and improve chitin, and thus chitosan, color.

Industrial methods for purifying chitin from crustacean waste include the use of sodium hypochlorite or hydrogen peroxide as bleaching agents.[158, 159] A few papers have reported the use of hydrogen peroxide combined with hydrochloric acid for bleaching insect samples.[115, 120] Sometimes sodium hypochlorite[11, 94, 106, 124] or ammonium peroxidisulfate[89] are used, but insect decolorization is performed using a mixture of methanol–chloroform[91, 108, 109, 118, 127] or alcohol–chloroform.[64, 97] Treatment with potassium permanganate and oxalic acid is also done frequently.[87, 90, 112–114, 117, 128]

Even though they are often used, the approaches using organic solvents have no or marginal bleaching efficiency, as they do not break the bonds between chitin and tannins or catecholamines.

Bleaching treatment of insect samples is performed at room temperature for a short duration (40–90 min).[99, 103, 106, 118] Higher temperatures were applied by Chae *et al.*[89] (50 °C for 30 min with ammonium peroxidisulfate) and Nemtsev *et al.*[120] (75 °C for 1 h with hydrogen peroxide). A combination of potassium permanganate and oxalic acid has also been used, where the bleaching step was split into two steps: treatment with potassium permanganate at room temperature and with oxalic acid at high temperatures (60–70 °C).[90, 114]

Evaluation of the success of the bleaching step using various reagents has not been provided. The bleaching effect of a decolorization treatment can be quantitatively evaluated by measuring the L^*, a^* and b^* values of a sample using a colorimeter, according to the CIELab color system. CIELab is a color space that expresses colors as three values: L^* for lightness, a^* from green to red and b^* from blue to yellow. From these values, the whiteness index can also be calculated.[160]

Information on the effect of chitin bleaching on the yield and characteristics of chitosan has been given only by Nemtsev *et al.*[120] Part of the chitin extracted from honeybee corpses was bleached using 3% hydrogen peroxide for 1 h at 75 °C. The yield of chitosan derived from non-bleached chitin (20–30%) was slightly higher than that of chitosan produced from bleached chitin (16–25%); the degree of deacetylation of the two types of chitosan was similar.[120] Furthermore, chitin bleaching can greatly affect the viscosity of solubilized chitosan.[120, 159]

After the bleaching treatment is complete, chitin is dried, and characterized to investigate its suitability for a desired application. Otherwise, chitin can be deacetylated to afford chitosan.

CONVERSION OF CHITIN INTO CHITOSAN

Chitosan is obtained from the deacetylation of chitin (i.e. the removal of acetyl groups from the chitin polymer). The resultant chitosan has a high degree of free amino groups (—NH_2) that provides active sites for many chemical reactions, thus making chitosan a versatile polymer that is suitable for several modifications and applications.[159] Furthermore, chitosan has a much higher solubility than chitin and can be dissolved in slightly acidic solutions, while chitin solubilization requires strong and highly concentrated mineral acids[159] or solutions of lithium chloride and organic solvents such as dimethylformamide, dimethylacetamide and *N*-methyl-2-pyrrolidone.[161]

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Chitin can be converted to chitosan by chemical deacetylation. Alternatively, enzymatic deacetylation using deacetylases has also been attempted. The enzymatic activity of various deacetylases towards chitin has been established; however, they are not efficient in converting chitin to chitosan because of the crystallinity of chitin.[135, 136, 162] Pretreatment of chitin, such as heating, grinding and treating with an alkaline solution, can lead to a higher, yet insufficient, enzymatic activity.[136] Due to this limitation, on an industrial scale, chemical deacetylation is the most commonly used method for chitosan preparation from crustacean waste because of the low cost and suitability for mass production.[139] Deacetylation is performed by incubating chitin in a concentrated solution of sodium hydroxide. Chitin deacetylation can be performed either heterogeneously or homogeneously. In the heterogeneous method, chitin is usually treated with a hot concentrated solution of sodium hydroxide for a few hours. Within the frame of the homogeneous method, chitin is incubated in a concentrated sodium hydroxide solution at room temperature for a few hours, followed by dissolution in crushed ice at 0 °C.[92, 139] Heterogeneous conditions result in an irregular distribution of *N*-acetyl-D-glucosamine and D-glucosamine units and a blockwise distribution of acetyl groups along the chitosan chain. In contrast, chitosan obtained under homogeneous conditions has a random distribution of acetyl groups along the chain. Hence, chitosans produced using these two methods can have different physicochemical properties.[163]

Almost solely chemical heterogeneous deacetylation has been reported for chitosan production from insects with sodium hydroxide being used as the deacetylating agent. In a few cases, sodium hydroxide was combined with sodium borohydride as a 'protecting reagent'.[119, 122, 132] For heterogeneous deacetylation of insect biomass, the sodium hydroxide concentration ranges from 40 to 60% (i.e. about 15–22 mol L^{-1}) (Table 2). In most cases, the deacetylation step lasts from 1 to 9 h, with a few exceptions of longer incubation times of up to 2 days.[104, 126] Temperatures of heterogeneous deacetylation of insect samples range from 90 to 150 °C (Table 2). Ideally, deacetylation should result in non-degraded chitosan with a high degree of deacetylation, enabling its solubilization in dilute acidic solutions.[159] The degree of deacetylation is defined as the proportion of glucosamine monomer residues in the chitosan chain and it can affect the solubility and performance of chitosan in many of its applications.[164] Deacetylation can be optimized and adjusted according to need by adjusting various factors, including temperature, time, alkali concentration, solid-to-solvent ratio and particle size.

Assessment of chitin conversion into chitosan

Results obtained for chemical heterogeneous deacetylation of insect samples, in terms of chitosan yield, degree of deacetylation and molecular weight, are reported in Table 2. Chitosan yield was calculated from the dry biomass of the original insect biomass or the dry weight of chitin. Chitosan yields measured from the original biomass ranged from 2 to 8%. The highest values (26–28%) were obtained by Song *et al.*[124] from *Chrysomya megacephala* larvae and by Luo *et al.*[113] from cicada sloughs. Chitosan yield calculated from the respective chitin dry weight ranged from 60 to 83% (Table 2). Only Hahn *et al.*[92] have performed both heterogeneous and homogeneous deacetylation of the *H. illucens* larval exoskeleton, obtaining more than double the yield of chitosan with the heterogeneous method compared to the homogeneous method. Yields of chitosan produced from crustaceans with heterogeneous deacetylation varied from 4 to 15% (related to the initial dry biomass).[26, 113, 121, 153, 156, 165] These values are slightly higher than those obtained from insects. The primary reason for that is the presence of larger amounts of protein and fat in insect samples.[166] However, as with chitin, chitosan yield can be affected not only by the purification process, but also by the species and harvest time.[163] Considering the reported data, a unique definition of 'yield' for both chitin and chitosan is needed to make the methods of measurement uniform.

The degree of deacetylation of chitosan produced from insects with heterogeneous deacetylation varied between 62 and 98% (Table 2). A lower value has been reported only by Monter-Miranda *et al.*[119] who obtained 57% deacetylated chitosan from *Brachystola magna* adults. The degree of deacetylation of chitosan extracted from crustaceans with the heterogeneous method normally ranges from 56 to 98%,[167] while the average degree of deacetylation obtained with homogeneous treatment is 48–55%.[139] At least 80–85% deacetylation is necessary for the good solubility of chitosan.[167] The degree of deacetylation can be increased or decreased by changing temperature, time and sodium hydroxide concentration.[159] Moreover, there is a correlation between temperature and rate of deacetylation: high temperatures can increase deacetylation, whereas long residence times can improve deacetylation but only up to a certain point. For instance, alkali treatment using 50% sodium hydroxide at 100 °C beyond 2 h does not deacetylate crustacean-based chitin further significantly; rather it can degrade the polymer chain.[159]

Chitosan is a biopolymer of high molecular weight, which varies depending on the source and deacetylation treatment applied. The molecular weight of crustacean-based chitosan ranges from 100 to 1000 kDa.[25, 26] Chitosan produced from insects has a molecular weight ranging from 26 to 300 kDa (Table 2). Very low values (3 and 7 kDa) have been reported, too.[91,96,127] High reaction temperatures (150 °C) combined with long incubation times (4–6 h) may have caused polymer degradation. The use of standard chitosans with known molecular weights can be useful to assess the validity of the applied analysis method. However, differences in chitosan molecular weights can also be related to the insect species, as shown by Kim *et al.*,[111] who applied the same deacetylation conditions as Kaya *et al.*[96] and obtained a chitosan with a much higher molecular weight (308 kDa from adult crickets *versus* approximately 3 kDa from both adults and larvae of the Colorado potato beetle). A very high molecular weight (3290–5900 kDa) has been reported by Paulino *et al.*[122] for chitosan produced from silkworm using 40% sodium hydroxide and sodium borohydride at 100 °C. This could be because sodium borohydride prevents oxidative cleavage of glycosidic bonds during deacetylation.

Deacetylation treatment conditions can affect chitosan molecular weight and thus its physicochemical properties or bioactivity. Notably, molecular weight has a great influence on the biological activity of chitosan: chitosan with low molecular weight (i.e. lower than 150 kDa) has good antibacterial properties.[168, 169] Studies carried out on bacteria potentially pathogenic to humans, such as *Salmonella typhimurium*, *Listeria monocytogenes*, *Bacillus cereus* and *E. coli*, have confirmed that low-molecular-weight chitosan has a greater effect on reducing microorganism growth and multiplication.[168, 169] Small chitosan chains have higher mobility, attraction and ionic interaction than long chains, facilitating an effective binding of chitosan to the membrane surfaces of bacteria.[168]

To evaluate the suitability of chitin and chitosan for desired applications, they need to be characterized. Several metrics can

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be used to assess chitin and chitosan characteristics and properties, such as degree of deacetylation, molecular weight, viscosity, morphology and solubility. Analysis methods and the investigated characteristics vary according to the final purpose.

ANALYTICAL METHODS

As mentioned in the previous sections, multiple studies have described the extraction of insect-based chitin and its subsequent conversion to chitosan. Although the processing conditions have been stated in detail in the relevant articles, discrete and quantitative values regarding yield and degree of purification are missing. This hampers the assessment of the economic potential of chitin and chitosan derived from insects and the side streams of their cultivation, especially in comparison to production from crabs, shrimps or fungi. However, a general comparison, in terms of structure, chemical composition and purity, with commercialized sources can be made with the help of physicochemical and/or spectroscopic data. Table 3 summarizes the main methods applied for the characterization of insect-based chitin and chitosan. Additionally, we include NMR spectroscopic data in the table. NMR, not being a method currently applied for the analysis of insect-based chitin, is promising towards validity and significance of the data.

The resulting data can support performance assessment of insect-based material in prospective applications. Furthermore, the data contribute to focusing and identifying new application fields.

Infrared spectroscopy

Infrared spectroscopy is the most frequently applied method to examine insect-based chitin and chitosan; especially to determine the degree of deacetylation of the polysaccharides. The technique, which exploits infrared light to obtain data from excited vibrational states of the functional groups in a sample, enables rapid analysis. According to the functional groups contained in the molecules, the most significant bands of insect-based chitin and chitosan occur at wavenumbers of 1310–1320 cm^{-1} (CN stretching, amide III), 1550–1560 cm^{-1} (NH bending, amide II), 1590–1600 cm^{-1} (NH_2 bending), 1650–1655 cm^{-1} (CO stretching, amide I), 3100–3110 cm^{-1} (NH symmetric stretching), 3255–3270 cm^{-1} (NH asymmetric stretching) and 3430–3450 cm^{-1} (OH stretching)[170, 171] (Fig. 3). The same is true for chitin and chitosan derived from crustaceans, where the wavenumbers of the peaks can vary slightly among different natural sources.[88]

For insect-based chitin and chitosan, infrared spectra were used to confirm the homogeneity of chitin and chitosan isolated from several insect species,[113] to compare the spectral bands with those of commercially available chitin and chitosan[94, 123] or to confirm the purity of insect-based chitin after the isolation process[106] by considering the strength and position of characteristic bands.

The most practical application of this method has been for determining the degree of acetylation of investigated samples by calculating and comparing the absorption values measured at specific wavelengths.[89] Accurate quantification is challenging[172]; comparability and significance are minor due to the different bands applied for calculating the degree of acetylation by different researchers. Further sources of errors can be individual-specific or due to inappropriate baseline settings within the spectra and impurities in the sample, e.g. the presence of proteins can lead to an overlap of the characteristic chitin and chitosan peaks in the infrared spectrum and thus to wrong values.[22, 173] In the authors' opinion, infrared spectra used to obtain valid information or even quantitative values from insect-based chitin and chitosan are of limited conclusiveness. It is a useful auxiliary for qualitatively determining the presence of functional groups, but not to provide quantitative data subject to strong variation based on, for example, impurities or water content in samples.

Tools like partial least squares regression are hence mandatory for increasing the validity of the data and will also provide an added value to the infrared spectra of insect material,[174] although extensive and time-consuming calibration work is required. Since manual evaluation concerning the determination of the degree of acetylation is error-prone and less comparable, an automated software-assisted extraction of relevant data would thus be beneficial. Nevertheless, the identification of chitin- and chitosan-related functional groups, such as acetamido or amino groups, present in insect-based material is feasible using infrared spectroscopy.

X-ray diffraction

X-ray diffraction is the second most common analysis method used to characterize chitin from insect-based materials. Although X-ray diffraction is a powerful analytical technique for obtaining structural information, in the literature reviewed it has been exclusively used to determine the polymorphic form of chitin crystallites and the crystal structure and crystal content of chitin and chitosan isolated from insects.

The X-ray pattern provides information on the periodic arrangement of atoms within a sample. The resulting diffractogram shows intensity as a function of 2θ, which is defined as the angle between the incident and diffracted beams.

X-ray diffraction measurements of insect-based chitin revealed strong significant peaks at 9–11° and 19–20°, in addition to minor peaks at 12–13°, ~21°, ~23° and ~26°.[91, 112, 126, 128, 131] These peaks are valid for the highly symmetric orthorhombic crystal structure of insect chitin, representing the α-polymorphic form. However, there are exceptions: Chitin from the cocoons of a moth (*Orgyia dubia*) is in the γ-form, exhibiting an X-ray diffraction pattern with high homology to the diffractogram obtained for α-chitin, differing from each other mainly in the peak at 12.9°.[144] γ-Chitin and β-chitin are polymorphs with a lower degree of order than α-chitin, making these polymorphs more reactive. For example, β-chitin is more accessible to swelling and for enzymatic and chemical reactions than the α-form,[14] and therefore more readily undergoes crystal disintegration. Thus, the conversion of β-chitin results in chitosan with lower crystallinity compared to chitosan obtained by deacetylation of α-chitin.[175]

Previous studies have confirmed that chitosan exists as two crystalline polymorphs, either as a hydrated polysaccharide ('tendon' form)[176] or as an anhydrous form ('annealed' chitosan).[177] The presence of crystallites or crystalline regions in the amorphous regions of chitosan could be due to the unreacted chitin.[178] The position of the X-ray peaks in the diffractogram is similar for insect-based chitosan and chitin and comparable to peak positions determined for commercially available chitosan.[89] Main peaks for the chitosan crystal structure can be identified at 2θ of ~10° and ~20°.[91, 113, 116]

The crystallinity index for chitin and chitosan is commonly calculated using the relation of the peak intensities measured at 16° (I_{am}), which is attributable to the amorphous content of the

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Table 3. Overview of major analytical methods applied for investigation of insect-based chitin and chitosan. Due to its importance, validity and sensitivity, NMR spectroscopy is listed as prospective powerful tool to analyze insect-based chitin and chitosan although it is not discussed here

Method applied	General principle	Main applications for (insect-based) chitin and chitosan
Infrared spectroscopy	Excitation of vibrations by irradiation with infrared beams	Determination of deacetylation degree
X-ray spectroscopy	Detection of elastic scattered X-rays	Crystallinity determination Determination of chitin polymorph
Thermogravimetric analysis	Mass loss or heat flow determination during heating	Degradation temperature Moisture content Determination of chitin polymorph
Elemental analysis	Combustion and content determination of different elements	Determination of deacetylation degree for chitin and chitosan Purification degree of chitin
Viscometry	Measuring the viscosity of the polysaccharide-containing solutions	Measuring viscosity-average molecular weight
Scanning electron microscopy	Reflection of electrons interacting with atoms	Determination of chitin surface morphology
NMR spectroscopy	Investigation of the electronic environment of single atoms and the interaction with neighboring atoms	Determination of deacetylation degree Distribution of acetyl groups Determination of impurities

sample, and at 20° (I_{110}) according to the equation of Segal *et al*.[179]:

$$\text{Crystallinity index}\,(\%) = \frac{I_{110} - I_{am}}{I_{110}} \times 100 \qquad (3)$$

The reported crystallinity values for chitin vary greatly and cover a wide range (40–90%) but are mainly between 60 and 80%.[91, 93, 94, 126, 132] The broad range of values reported is due to the varied sources, different purification methods used and is a function of the drying, storage or preprocessing conditions.[128] Grinding of chitin is, for example, an effective method for decreasing its crystallinity, disturbing the overall structure of the polysaccharide.[178]

Hence, there is a clear need to independently determine all significant factors that affect the crystallinity of insect-based chitin and chitosan using X-ray-based investigations. The authors believe that low crystallinity values achieved for insect-based chitin and chitosan are of major importance and are highly relevant for most applications. For instance, lower crystallinity of chitosan facilitates its solubility in acidic solutions, increases its sorption ability and increases the accessibility of the primary free amino groups of chitosan.[180, 181]

Thermogravimetric analysis

Similar to X-ray diffraction analysis, thermogravimetric analysis has also been used to determine the polymorphic form of insect-based chitin. Additionally, the temperature at which chitin and chitosan completely decompose can be measured by thermogravimetric analysis. The method records mass loss of a sample over time as temperature increases and is visualized in a thermogram. For insect chitin, thermograms exhibit two decomposition steps. One is a result of water evaporation between 50 and 110 °C, leading to a low mass loss of the sample (1–7%). A second peak at 300–400 °C is caused by the dehydration of the saccharide backbone, polymerization of the degradation products and decomposition of the acetyl function.[122] The amount of mass lost during this second decomposition step ranges from 50 to 95%.[102, 103, 105, 128] The maximal thermal degradation temperature (DTG_{max}), which corresponds to the temperature at which the highest mass loss is determined, is in a more narrow temperature range: DTG_{max} values for chitin from different body parts of *Z. morio*, *Melolontha* sp., *A. pandora* or *H. illucens* have been evaluated to be 350–390 °C, indicating the presence of the α-form.[98, 100, 103, 126] DTG_{max} values obtained for α-chitin isolated from marine fishery waste are in the same range.[11, 93] Typically, α-chitin has a higher second decomposition temperature than β-chitin,[102] which is attributed to the lower crystallinity of the latter.[182] Sometimes, a third decomposition peak at temperatures above 700 °C has been reported which is attributed to residual minerals not removed during demineralization.[11]

The thermograms of insect-based chitin do not reveal large deviations to the chitosan produced. The main difference here is

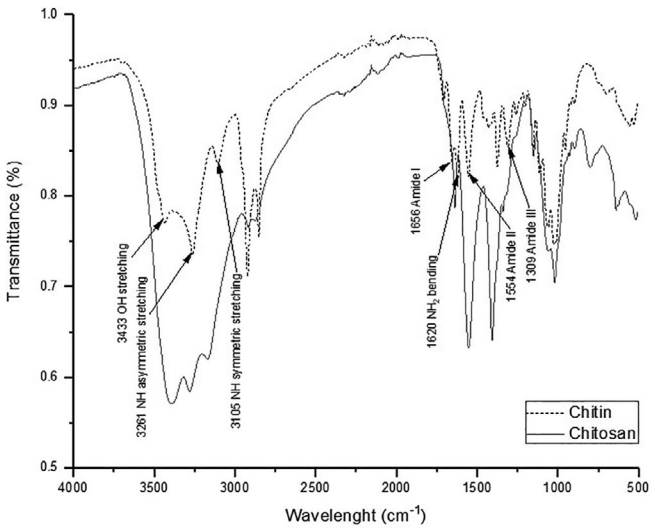

Figure 3. Exemplary infrared spectra of chitin and chitosan obtained from *H. illucens* pupal exuviae (own measurements).

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a shift of the decomposition peak to slightly lower temperatures during chitosan heating (<320 °C), which means that chitin is more stable.[95] This is due to the increased number of *N*-acetyl groups in the chitin providing a higher stability than the primary amino groups of the chitosan.[181, 183]

Although several thermogravimetric analysis studies and data for insect chitin and chitosan are available, valid or useful characteristic values, such as the activation energy of degradation, have not been calculated and published yet. Hence, there is a need for substantial discussion and evaluation rather than stating superficial qualitative expressions. The DTG_{max} values obtained reveal significant differences for chitin or chitosan, but these are not application-relevant. Processing chitin and chitosan at elevated temperatures does not significantly improve processing properties, as phase transition from crystalline to amorphous form does not occur until decomposition.

The information obtained using thermogravimetric analysis is thus limited, especially as the polymorphic form of chitin can also be identified with X-ray spectroscopy. Another option is to perform a more comprehensive thermal analysis. Newer instruments can perform calorimetric and thermogravimetric measurements simultaneously, providing additional information about the type and enthalpy values of conversion occurring during heating and purity of chitin and chitosan.[126, 131]

Elemental analysis

Thermogravimetric analysis enables the determination of sample degradation temperature. In contrast, elemental analysis utilizes degradation and combustion of insect-based chitin and chitosan and the subsequent detection of carbon, hydrogen, nitrogen and oxygen to determine its molecular composition and acetyl content. Although complementary to several other methods, the determination of degree of acetylation (DA) of insect-based chitin and degree of deacetylation (DD) of insect-based chitosan via elemental analysis is of high practical relevance,[184, 185] and is calculated using the following equations[186, 187]:

$$DA\,(\%) = \left(\frac{\frac{C(\%)}{N(\%)} - 5.14}{1.72}\right) \times 100 \qquad (4)$$

$$DD\,(\%) = \left(\frac{6.89 - \frac{C(\%)}{N(\%)}}{1.72}\right) \times 100 \qquad (5)$$

Theoretically, fully acetylated chitin contains 6.9% nitrogen and fully deacetylated chitosan contains 8.7% nitrogen.[188] Assuming 100% purity of samples, the higher the nitrogen content the lower is the degree of acetylation and vice versa. Typically, the measured nitrogen content for insect-based chitin is lower than the theoretical value, resulting in an overestimation – according to Eqn (4) – of the degree of acetylation. For example, Erdogan and Kaya[91] used elemental analysis to determine the nitrogen content of chitin from *D. maroccanus* adults and nymphs at 4.6 and 5.7%, resulting in degrees of acetylation of 232 and 187%, respectively. Degrees of acetylation exceeding 100% have been determined for chitin extracted from *D. maroccanus* and species such as *O. asellus* (169%) and *V. crabro* (127%).[64, 103, 109, 115] The validity of the conclusions is hence severely limited. It can be assumed that these overestimations result from nitrogen-free impurities, such as lipids and sugars, in samples, which make up to 43% of edible insects such as *A. domesticus* and *T. molitor*.[189]

The overestimation of the degree of acetylation pertains to chitin from insects. It is different for insect-based chitosan and the determination of its degree of deacetylation via elemental analysis. The degree of deacetylation for chitosan obtained from the conversion of chitin from *Z. morio* and *B. mori* ranges from 52 to 95%,[126, 132] which are reasonable values. It can be assumed that the validity of these values is due to a higher degree of purification of chitosan, in contrast to the insect-based chitin samples containing a significant amount of impurities. The conversion steps from chitin to chitosan involve severe conditions such as use of high temperature and sodium hydroxide concentration, followed by selective precipitation of chitosan from solution by pH neutralization. These steps provide additional purification, resulting in valid calculations of degrees of deacetylation for insect-based chitosan using elemental analysis.

However, the presence of impurities in both chitin and chitosan cannot be excluded. As already mentioned in the chitin purification and deacetylation section of this review, purity can be determined only for a minority of chitins and chitosans produced from insects. For accurate degree of acetylation of chitin or degree of deacetylation of chitosan, pre-quantification of impurities is mandatory. Nevertheless, a thorough and effective purification is crucial to provide unimpeded characterization of insect-based chitin and chitosan using various methods.

Molecular weight measurement via viscometry

The so-called viscosity-average molecular weight of insect-based chitosan can be determined by measuring the intrinsic viscosity of a solution using an Ubbelohde viscometer. Molecular weight determination is based on the fact that the viscosity of the solution, in addition to the degree of deacetylation, depends on the molar mass of chitosan. Previous studies have reported a value for insect chitosan viscosity-average molecular weight in the range of 426–450 kDa.[87, 114] Other authors have determined much lower values (<10 kDa) for *D. maroccanus* and *L. decemlineata* chitosan.[91, 96] Odote *et al.*[121] investigated the viscosity-average molecular weight of lobster, prawn and crab chitosan in comparison to that of chitosan from blowfly larvae and found similar values for all chitosans, which is potentially due to their similar chitin conversion conditions. The same authors also stated that the viscosity-average molecular weight of insect chitosan decreases with higher deacetylation temperature and incubation time, confirming that the applied conversion parameters are more significant than the origin of chitin used for deacetylation. Another application for viscometry is to record the progress of chitosan hydrolysis: Nemtsev *et al.*[120] reported a decrease of honeybee-derived chitosan viscosity-average molecular weight during enzymatic hydrolysis in slightly acidic solution from 257 to 21 kDa.

In contrast to chitosan, chitin solubility is limited to a few solvents. Nevertheless, Draczynski reported that the solubilization of honeybee chitin in dimethylacetamide–LiCl results in a viscosity-average molecular weight of 426–738 kDa that depends on the time of deproteinization.[90]

More sophisticated methods and instruments are required, instead of the standard laboratory Ubbelohde viscometer, for measuring weight-average and number-average molecular weights and polydispersity or molecular weight distribution of chitin and chitosan. For example, gel permeation chromatography measurements with a multi-angle laser light scattering detector revealed a molecular weight for insect-based chitosan of approximately 308 kDa and a polydispersity index of 1.2.[111]

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However, viscosity-average molecular weight as an outcome is important as it affects many other physicochemical or application-specific investigations, such as bioactivity, adhesion force and gelation properties.

Scanning electron microscopy

Previously described analysis methods generated information concerning structural features, chemical composition or physicochemical properties of insect-based chitin and chitosan. Scanning electron microscopy enables visualization of the polysaccharide surface in the nanometric range using a focused beam of electrons.

Various surface morphologies have been described for insect-based chitin: rough or smooth surfaces with or without pores and/or fibrils.[116] The range of diversity of various chitin surfaces derived from insects is shown in Fig. 4. However, most chitin surfaces extracted from insects exhibit a rough fibrillary structure with pores. The diameter of the fibrils ranges from 10 to 50 nm,[64, 91, 101, 108] and the chitin fibrils are surrounded by a protein matrix.[190] The pore diameters range from 100 to 500 nm. The highly porous structure increases the accessible surface area and thus the adsorption capacity of the material.

Scanning electron microscopy imaging of insect-based chitosan has been performed in only a small number of studies. Kaya *et al.*[101] reported chitosan with a surface morphology similar to that of the chitin from which it was derived, which suggests that the chitin structure is preserved in chitosan. The surface morphology of chitin also depends on other factors. It can be influenced by characteristics inherent to the natural source, such as species,[95, 118] sex[13] or body part,[99] and by process conditions, such as the selected pretreatment procedure.[93] Moreover, chitin content, degree of purification and washing procedure can play a major role as the surrounding matrix and matrix constituents on the surface not removed during purification mask the chitin. Due to this, the validity of this method for investigating chitin and chitosan is limited. However, knowledge of the surface structure of chitin and chitosan is important for processes in which surface architecture greatly affects functionality.

Assessment of analytical investigations

The methods described are the most frequently mentioned analytical techniques applied for the investigation of insect-based chitin and chitosan. The major conclusion from screening the analysis results is that there is a high structural and chemical homology between chitin and chitosan derived from insects and marine animals. However, despite the large amount of available data, knowledge gaps exist that need to be filled in prospective studies to assess the potential of insect-based chitin and chitosan. What has already been said for chitin yield and degree of purification also holds true here: there is a lack of valid data and/or the results were not properly evaluated or discussed to accelerate research on this topic. For example, many infrared spectra from chitins and chitosans from different sources are available, which are nearly the same as each other and provide no additional information. Similarly, thermogravimetric analysis data have limited validity as the DTG_{max} values reported are in the same temperature range. In contrast, X-ray data show strongly varying crystallinity values for chitin and chitosan. Thus, it is unclear if the data presented are a result of processing conditions, life cycle stage or body part of the insect.

Hence, the authors suggest firstly performing comprehensive studies to identify parameters that contribute significantly to the properties of insect-based chitin and chitosan. Secondly, the authors strongly believe that spectroscopic and chromatographic data would be more valid if they were related to the degree of purification of the chitin and chitosan investigated. Therefore, it is necessary to first determine the purity of polysaccharides after extraction and deacetylation since contaminations can disrupt accurate measurements of degree of deacetylation. Elemental analysis is a tool which could be applied to, at least, estimate the degree of purification and to evaluate if the measurements performed are valid. Thirdly, there is a need to standardize the calculation methods and equations; for example, to agree on specific bands and peaks used for calculation when determining crystallinity via X-ray spectroscopy. Fourthly, we recommend using statistical software to evaluate the results and to increase the information content of a presented data set. In our opinion, infrared spectroscopy can reveal degrees of deacetylation and impurity content accurately if evaluated using multivariate data analysis or similar methods. Lastly, techniques such as titration-based methods can be applied for higher sensitivity in determining degree of deacetylation. Although revealing a precise determination method, titration is time-consuming. Another highly accurate and automatable tool, to determine the degree of deacetylation of chitin and chitosan in solid state or solubilized, is NMR spectroscopy. Several studies exhibited the potential of ^{1}H NMR, ^{13}C NMR and ^{15}N NMR spectroscopy to determine the degree of deacetylation, the distribution of acetyl groups and the cross-linkages of the chitin and chitosan.[191–193] Possibly due to the need for enhanced equipment and specific expertise, comprehensive studies concerning insect-based chitin and chitosan are lacking. We further recommend performing application-based investigations, such as measurement of viscosity, adhesion, film formation and adsorption capacity. For successful commercialization of insect-based chitin and chitosan, such detailed knowledge is mandatory.

CONCLUSIONS AND FUTURE PROSPECTS

Chitin and especially chitosan are natural polymers with many useful properties and are widely used in a broad range of applications. Presently, the main commercial source of chitin and chitosan comprises waste streams from the marine fishery industry; however, their availability is limited by geography and season. The recent increase in demand for chitin and chitosan in the global market has drawn attention to alternative sources independent of marine fishery waste. Insect breeding farms, which are used for waste management through insect-mediated bioconversion or for producing proteins and fats from larval stages, are being launched worldwide. In addition to the production of valuable compounds, insect breeding generates several side streams (dead adults, exuviae, exoskeletons, frass and residual feed) that have not yet been valorized. These side streams provide a cheap source of chitin, which is abundantly available and not regionally or seasonally limited. Furthermore, the chitin content of exuviae and exoskeletons exceeds 23%,[78] suggesting favorable conditions for chitin and chitosan production in the future.

In contrast, the chitin content of whole insects is generally lower.[78, 85] Due to the moderate content, insect breeding only for chitin isolation is not economically feasible without cascade usage of other compounds derived from the insect breeding.

The chitin content in insects is a function of species, type of feed and of life cycle stage. Moreover, the life cycle stage determines the complexity of the matrix in which chitin is embedded. Insects

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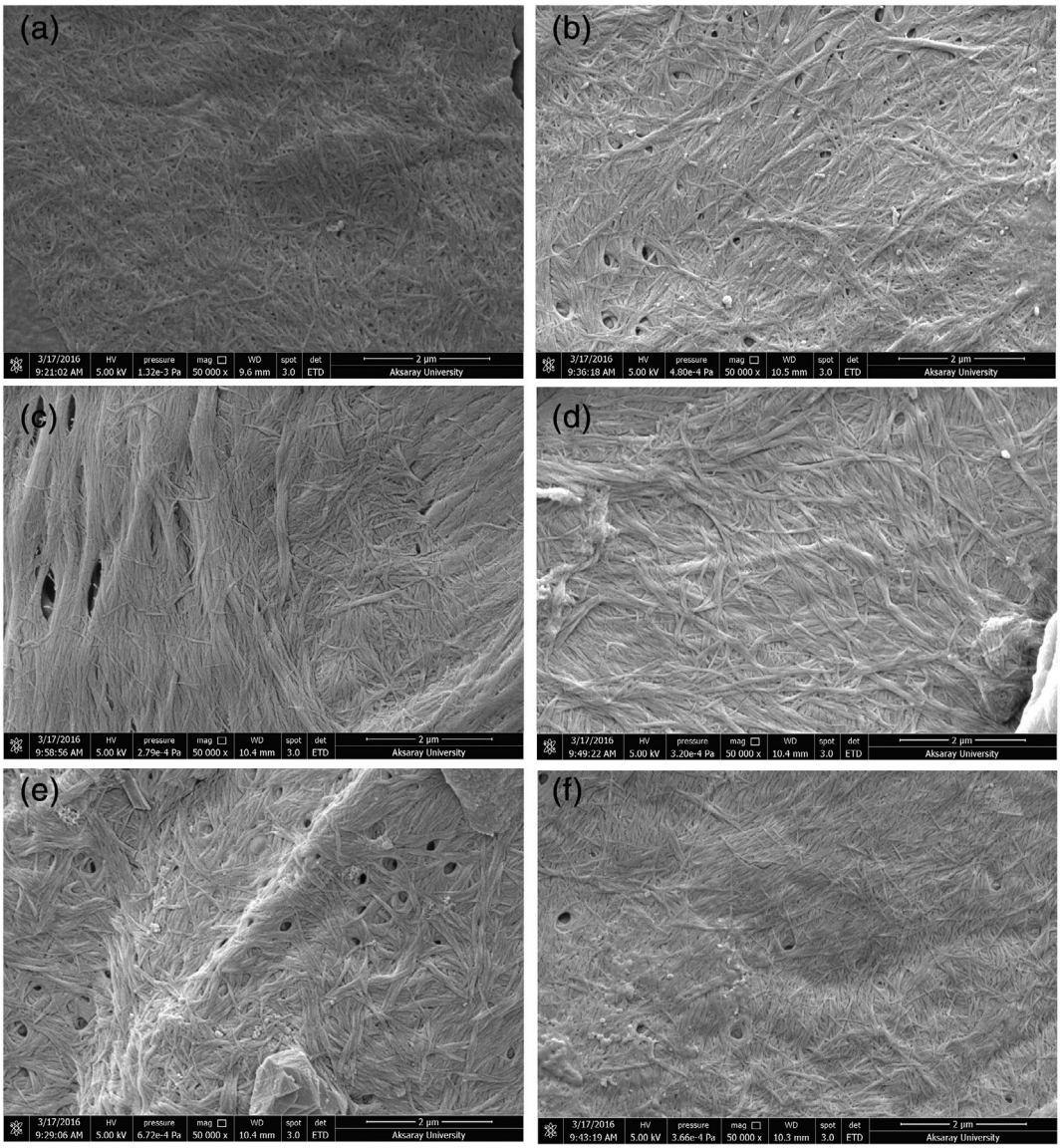

Figure 4. Scanning electron microscopy images of chitin extracted from (a) *Cicadatra atra*, (b) *Cicada hyalina*, (c) *Cicada lodosi*, (d) *Cicada mordoganensis*, (e) *Cicada platyptera* and (f) *Cicadivetta tibialis*. Reprinted from Mol *et al.*[118] with permission of Wiley.

undergo sclerotization during metamorphosis to the adult stage. Sclerotization comprises cross-linkage of the insect cuticle with catecholic compounds. This makes necessary a more sophisticated purification process and at least one additional bleaching step to isolate pure chitin from insect biomass. On the contrary, decolorization is not mandatory for chitin purification from marine fishery waste. The need for bleaching arises because the chitin isolated from dead adult insects and exuviae contains these sclerotized structures with dark colour. Chitin production can be more economically feasible from side streams of insects bred for a different purpose.

The purification of chitin begins earlier in the process and comprises fractionating the chitin-containing compounds from those with no or low chitin. Although insect breeding, for the isolation of protein and fat, is a highly automated and controlled operating process, a mechanical process for separating chitin-rich substances needs to be developed to eliminate the current practice of manual collection. Similarly, manual collection of chitin-rich material is opposed to the prospective application of insect-based chitosan in medicine and requires good manufacturing practice or laboratory-like controlled conditions. Furthermore, the raw materials used for insect breeding vary according to availability (e.g. vegetable waste from agri-food chain, cereal straw, distillers' grains and cereal meals). Although insect-mediated bioconversion is a flexible and robust process and leads to a high-quality fat and protein fraction almost independent of the varying quality of the side streams, it is challenging for an automated process to lead to chitin and chitosan with a consistent composition.

Currently, chitin purification from insect biomass is an area of focus for researchers and is mainly performed on a laboratory scale using the same methods applied for purification from crustacean shells. Most available literature on chitin and chitosan production from insects is limited to a description of the chitin extraction process and its subsequent deacetylation into chitosan. Information on the quantitative evaluation of extraction, purification, deacetylation and bleaching efficiency, and the degree of purification of products is missing. In the case of insects, it is

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especially important to assess the bleaching process, as insect exuviae undergo sclerotization by catecholamine linkage, which leads to dark coloration.[85, 194] These factors hinder accurate assessment of the economic potential of chitin and chitosan production from insects and comparison with the process chain of chitin isolation from crab shells.

A comparison of degree of deacetylation, molecular weight, stability, crystallinity and surface structure between insect- and crustacean-based chitin and chitosan, performed using various analytical methods, showed high similarity. This is encouraging regarding the performance of chitin and chitosan derived from insects for industrial applications and their use in new fields. However, for a comprehensive assessment, it is necessary to carry out application-relevant investigations and to exploit the full potential of the methods in use for characterization.

Based on current knowledge, it can be supposed that insects will be an important source of chitin and chitosan in the future, especially if future studies focus on filling the knowledge gaps highlighted in this review. The efficiency of each step of the purification process needs to be critically evaluated to optimize methods applied to crustaceans and adapt them to insect biomass. Alternatives to traditional chemical purification methods should also be considered to make the process more environmentally friendly. Future studies should focus on these aspects to make optimal use of the side streams of insect breeding.

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REFERENCES

1 Rinaudo M, Chitin and chitosan: properties and applications. *Prog Polym Sci* **31**:603–632 (2006).
2 Elieh-Ali-Komi D and Hamblin MR, Chitin and chitosan: production and application of versatile biomedical nanomaterials. *Int J Adv Res* **4**:411–427 (2016).
3 Revathi M, Saravanan R and Shanmugam A, Production and characterization of chitinase from *Vibrio* species, a head waste of shrimp *Metapenaeus dobsonii* (Miers, 1878) and chitin of *Sepiella inermis* Orbigny, 1848. *Adv Biosci Biotechnol* **03**:392–397 (2012).
4 Hatchett C, XVIII. Experiments and observations on shell and bone. *Philos Trans R Soc Lond* **89**:315–334 (1799).
5 Ruiz GAM and Corrales HFZ, Chitosan, chitosan derivatives and their biomedical applications, in *Biological Activities and Application of Marine Polysaccharides*, ed. by Shalaby E. InTech, pp. 87–106 (2017).
6 Crini G, Historical review on chitin and chitosan biopolymers. *Environ Chem Lett* **17**:1623–1643 (2019).
7 Kumar MNR, A review of chitin and chitosan applications. *React Funct Polym* **46**:1–27 (2000).
8 Dutta PK, Dutta J and Tripathi VS, Chitin and chitosan: chemistry, properties and applications. *J Sci Ind Res* **63**:20–31 (2004).
9 Latgé J-P, The cell wall: a carbohydrate armour for the fungal cell. *Mol Microbiol* **66**:279–290 (2007).
10 Merzendorfer H, The cellular basis of chitin synthesis in fungi and insects: common principles and differences. *Eur J Cell Biol* **90**: 759–769 (2011).
11 Sajomsang W and Gonil P, Preparation and characterization of α-chitin from cicada sloughs. *Mater Sci Eng C* **30**:357–363 (2010).
12 Dweltz NE, The structure of β-chitin. *Biochim Biophys Acta* **51**:283–294 (1961).
13 Kaya M, Lelesius E, Nagrockaite R, Sargin I, Arslan G, Mol A *et al*., Differentiations of chitin content and surface morphologies of chitins extracted from male and female grasshopper species. *PLoS One* **10**:e0115531 (2015).
14 Minke R and Blackwell J, The structure of α-chitin. *J Mol Biol* **120**: 167–181 (1978).
15 Rudall KM and Kenchington W, The chitin system. *Biol Rev* **48**:597–633 (1973).
16 Cabib E, Bowers B, Sburlati A and Silverman SJ, Fungal cell wall synthesis – the construction of a biological structure. *Microbiol Sci* **5**: 370–375 (1988).
17 Hudson SM and Smith C, Polysaccharides: chitin and chitosan: chemistry and technology of their use as structural materials, in *Biopolymers from Renewable Resources*, ed. by Kaplan DL. Springer, Berlin, pp. 96–118 (1998).
18 Jang M-K, Kong B-G, Jeong Y-I, Lee CH and Nah J-W, Physicochemical characterization of α-chitin, β-chitin, and γ-chitin separated from natural resources. *J Polym Sci A Polym Chem* **42**:3423–3432 (2004).
19 Wang M, Chen LJ, Ni J, Weng J and Yue CY, Manufacture and evaluation of bioactive and biodegradable materials and scaffolds for tissue engineering. *J Mater Sci Mater Med* **12**:855–860 (2001).
20 Manjula K and Podile AR, Chitin-supplemented formulations improve biocontrol and plant growth promoting efficiency of *Bacillus subtilis* AF 1. *Can J Microbiol* **47**:618–625 (2001).
21 Chatelet C, Damour O and Domard A, Influence of the degree of acetylation on some biological properties of chitosan films. *Biomaterials* **22**:261–268 (2001).
22 Brugnerotto J, Lizardi J, Goycoolea FM, Argüelles-Monal W, Desbrières J and Rinaudo M, An infrared investigation in relation with chitin and chitosan characterization. *Polymer* **42**:3569–3580 (2001).
23 Chattopadhyay D and Inamdar M, Aqueous behaviour of chitosan. *Int J Polym Sci* **2010**:1–7 (2010).
24 Vaz JM, Taketa TB, Hernandez-Montelongo J, Chevallier P, Cotta MA, Mantovani D *et al*., Antibacterial properties of chitosan-based coatings are affected by spacer-length and molecular weight. *Appl Surf Sci* **445**:478–487 (2018).
25 de Queiroz Antonino RSCM, Lia Fook BRP, de Oliveira Lima VA, de Farias Rached RI, Lima EPN, da Silva Lima RJ *et al*., Preparation and characterization of chitosan obtained from shells of shrimp (*Litopenaeus vannamei* Boone). *Mar Drugs* **15** (2017).
26 Hossain MS and Iqbal A, Production and characterization of chitosan from shrimp waste. *J Bangladesh Agril Univ* **12**:153–160 (2014).
27 Lim SH and Hudson SM, Review of chitosan and its derivatives as antimicrobial agents and their uses as textile chemicals. *J Macromol Sci Polym Rev* **C43**:223–269 (2003).
28 Felse PA and Panda T, Studies on applications of chitin and its derivatives. *Bioprocess Eng* **20**:505–512 (1999).
29 Park BK and Kim MM, Applications of chitin and its derivatives in biological medicine. *Int J Mol Sci* **11**:5152–5164 (2010).
30 Synowiecki J and Al-Khateeb NA, Production, properties and some new applications of chitin and its derivatives. *Crit Rev Food Sci Nutr* **43**:145–171 (2003).
31 Hahn T and Zibek S, Sewage polluted water treatment via chitosan: a review, in *Chitin-Chitosan – Myriad Functionalities in Science and Technology*. InTech, (2018).
32 Liu X, Lin Guan Y, Zhi Yang D, Li Z and De Yao K, Antibacterial action of chitosan and carboxymethylated chitosan. *J Appl Polym Sci* **79**: 1324–1335 (2001).
33 Tsai GJ and Su WH, Antibacterial activity of shrimp chitosan against *Escherichia coli*. *J Food Prot* **62**:239–243 (1999).
34 Goy RC, Morais STB and Assis OBG, Evaluation of the antimicrobial activity of chitosan and its quaternized derivative on *E. coli* and *S. aureus* growth. *Rev Bras Farmacogn* **26**:122–127 (2016).
35 Han SD, Sung HJ, Lee GH, Jun JH, Son M and Kang MJ, Chitosan-based film of tyrothricin for enhanced antimicrobial activity against common skin pathogens including *Staphylococcus aureus*. *J Microbiol Biotechnol* **26**:953–958 (2016).
36 Ong SY, Wu J, Moochhala SM, Tan MH and Lu J, Development of a chitosan-based wound dressing with improved hemostatic and antimicrobial properties. *Biomaterials* **29**:4323–4332 (2008).

Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

37 Fang SW, Li CF and Shih DYC, Antifungal activity of chitosan and its preservative effect on low-sugar candied kumquat. *J Food Prot* **57**: 136–140 (1994).
38 Kaku H, Nishizawa Y, Ishii-Minami N, Akimoto-Tomiyama C, Dohmae N, Takio K *et al*., Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proc Natl Acad Sci USA* **103**:11086–11091 (2006).
39 Dehnad D, Mirzaei H, Emam-Djomeh Z, Jafari S-M and Dadashi S, Thermal and antimicrobial properties of chitosan–nanocellulose films for extending shelf life of ground meat. *Carbohydr Polym* **109**:148–154 (2014).
40 Vu KD, Hollingsworth RG, Leroux E, Salmieri S and Lacroix M, Development of edible bioactive coating based on modified chitosan for increasing the shelf life of strawberries. *Food Res Int* **44**:198–203 (2011).
41 Stricker-Krongrad AH, Alikhassy Z, Matsangos N, Sebastian R, Marti G, Lay F *et al*., Efficacy of chitosan-based dressing for control of bleeding in excisional wounds. *Eplasty* **18**:122–130 (2018).
42 Casadidio C, Peregrina DV, Gigliobianco MR, Deng S, Censi R and Di Martino P, Chitin and chitosans: characteristics, eco-friendly processes, and applications in cosmetic science. *Mar Drugs* **17**:369 (2019).
43 Pinotti A and Zaritzky N, Effect of aluminum sulfate and cationic polyelectrolytes on the destabilization of emulsified wastes. *Waste Manag* **21**:535–542 (2001).
44 Hahn T, Bossog L, Hager T, Wunderlich W, Breier R, Stegmaier T *et al*., Chitosan application in textile processing and fabric coating, in *Chitin and Chitosan: Properties and Applications*, ed. by van den Broek B and Boeriu C. Wiley VCH, (2019).
45 Bakshi PS, Selvakumar D, Kadirvelu K and Kumar NS, Chitosan as an environment friendly biomaterial – a review on recent modifications and applications. *Int J Biol Macromol* **4**:1–3 (2019).
46 Xiao L, Chitosan application in textile processing. *Curr Trends Fashion Technol Text Eng* **4**:32–34 (2018).
47 Arbia W, Arbia L, Adour L and Amrane A, Chitin extraction from crustacean shells using biological methods – a review. *Food Technol Biotechnol* **51**:12–25 (2013).
48 Tacon AGJ, Global trends in aquaculture and compound aquafeed production. *Mag World Aquacult Soc* **49**:33–46 (2018).
49 Gillett R, Global study of shrimp fisheries, in *FAO Fisheries Technical Paper 475*. FAO, Rome, p. 331 (2008).
50 Kurita K, Chitin and chitosan: functional biopolymers from marine crustaceans. *Mar Biotechnol (NY)* **8**:203–226 (2006).
51 Pittman SJ and McAlpine CA, Movements of marine fish and decapod crustaceans: process, theory and application. *Adv Mar Biol* **44**: 205–294 (2003).
52 Jardine A and Sayed S, Valorisation of chitinous biomass for antimicrobial applications. *Pure Appl Chem* **90**:293–304 (2018).
53 Abdel-Gawad KM, Hifney AF, Fawzy MA and Gomaa M, Technology optimization of chitosan production from *Aspergillus niger* biomass and its functional activities. *Food Hydrocoll* **63**:593–601 (2017).
54 Free SJ, Fungal cell wall organization and biosynthesis. *Adv Genet* **81**: 33–82 (2013).
55 Peter MG, Chitin and chitosan in fungi, in *Biopolymers Online*, pp. 123–132 (2005).
56 Chatterjee S, Adhya M, Guha AK and Chatterjee BP, Chitosan from *Mucor rouxii*: production and physico-chemical characterization. *Process Biochem* **40**:395–400 (2005).
57 Suntornsuk W, Pochanavanich P and Suntornsuk L, Fungal chitosan production on food processing by-products. *Process Biochem* **37**: 727–729 (2002).
58 Pochanavanich P and Suntornsuk W, Fungal chitosan production and its characterization. *Lett Appl Microbiol* **35**:17–21 (2002).
59 Kim WJ, Lee WG, Theodore K and Chang HN, Optimization of culture conditions and continuous production of chitosan by the fungi, *Absidia coerulea*. *Biotechnol Bioprocess Eng* **6**:6–10 (2001).
60 van Huis A, Potential of insects as food and feed in assuring food security. *Annu Rev Entomol* **58**:563–583 (2013).
61 Jucker C, Lupi D, Moore CD, Leonardi MG and Savoldelli S, Nutrient recapture from insect farm waste: bioconversion with *Hermetia illucens* (L.) (Diptera: Stratiomyidae). *Sustainability* **12**:1–14 (2020).
62 Kaya M, Mulerčikas P, Sargin I, Kazlauskaitė S, Baublys V, Akyuz B *et al*., Three-dimensional chitin rings from body segments of a pet diplopod species: characterization and protein interaction studies. *Mater Sci Eng C* **68**:716–722 (2016).
63 Bulut E, Sargin I, Arslan O, Odabasi M, Akyuz B and Kaya M, In situ chitin isolation from body parts of a centipede and lysozyme adsorption studies. *Korean J Couns Psychother* **70**: 552–563 (2017).
64 Kaya M, Baublys V, Can E, Šatkauskienė I, Bitim B, Tubelytė V *et al*., Comparison of physicochemical properties of chitins isolated from an insect (*Melolontha melolontha*) and a crustacean species (*Oniscus asellus*). *Zoomorphology* **133**:285–293 (2014).
65 Kaya M and Sargin I, Highly fibrous and porous raw material shaped chitin isolated from *Oniscus* sp. (Crustacea). *Food Biophys* **11**: 101–107 (2016).
66 Petrenko I, Bazhenov VV, Galli R, Wysokowski M, Fromont J, Schupp PJ *et al*., Chitin of poriferan origin and the bioelectrometallurgy of copper/copper oxide. *Int J Biol Macromol* **104**:1626–1632 (2017).
67 Kaya M, Baublys V, Satkauskiene I, Akyuz B, Bulut E and Tubelyte V, First chitin extraction from *Plumatella repens* (Bryozoa) with comparison to chitins of insect and fungal origin. *Int J Biol Macromol* **79**: 126–132 (2015).
68 Greven H, Kaya M and Baran T, The presence of α-chitin in tardigrada with comments on chitin in the Ecdysozoa. *Zool Anz J Comp Zool* **264**:11–16 (2016).
69 Kaya M, Seyyar O, Baran T and Turkes T, Bat guano as new and attractive chitin and chitosan source. *Front Zool* **11**:59 (2014).
70 Vainker SJ, *Chinese Silk: A Cultural History*. British Museum Press, (2004).
71 Cáceres C, Rendón P and Jessup A, *Joint FAO/IPoNTiF and Agriculture, the FAO/IAEA Spreadsheet for Designing and Operating Insect Mass-Rearing Facilities: Procedures Manual, in FAO Plant Production and Protection Paper*. Food and Agriculture Organization of the United Nations, p. 46 (2012).
72 Kim T-K, Yong HI, Kim Y-B, Kim H-W and Choi Y-S, Edible insects as a protein source: a review of public perception, processing technology, and research trends. *Food Sci Anim Resour* **39**:521–540 (2019).
73 Ortiz JC, Ruiz AT, Morales-Ramos JA, Thomas M, Rojas MG, Tomberlin JK *et al*., Insect mass production technologies, in *Insects as Sustainable Food Ingredients*. Academic Press, pp. 153–201 (2016).
74 DiGiacomo K and Leury BJ, Review: insect meal: a future source of protein feed for pigs? *Animal* **13**:3022–3030 (2019).
75 Derrien C and Boccuni A, Current status of the insect producing industry in Europe, in *Edible Insects in Sustainable Food Systems*, ed. by Halloran A, Flore R, Vantomme P and Roos N. Springer International Publishing, Cham, pp. 471–479 (2018).
76 Schmitt E, Belghit I, Johansen J, Leushuis R, Lock EJ, Melsen D *et al*., Growth and safety assessment of feed streams for black soldier fly larvae: a case study with aquaculture sludge. *Animals (Basel)* **9**:189 (2019).
77 Star L, Arsiwalla T, Molist F, Leushuis R, Dalim M and Paul A, Gradual provision of live black soldier fly (*Hermetia illucens*) larvae to older laying hens: effect on production performance, egg quality, feather condition and behavior. *Animals (Basel)* **10**:216 (2020).
78 Hahn T, Roth A, Febel E, Fijalkowska M, Schmitt E, Arsiwalla T *et al*., New methods for high-accuracy insect chitin measurement. *J Sci Food Agric* **98**:5069–5073 (2018).
79 Klowden MJ, Male reproductive system, in *Physiological Systems in Insects*. Academic Press, pp. 203–214 (2013).
80 Appel E, Heepe L, Lin CP and Gorb SN, Ultrastructure of dragonfly wing veins: composite structure of fibrous material supplemented by resilin. *J Anat* **227**:561–582 (2015).
81 Vincent JF and Wegst UG, Design and mechanical properties of insect cuticle. *Arthropod Struct Dev* **33**:187–199 (2004).
82 Hamodrakas SJ, Willis JH and Iconomidou VA, A structural model of the chitin-binding domain of cuticle proteins. *Insect Biochem Mol Biol* **32**:1577–1583 (2002).
83 Mohammed MH, Williams PA and Tverezovskaya O, Extraction of chitin from prawn shells and conversion to low molecular mass chitosan. *Food Hydrocoll* **31**:166–171 (2013).
84 Rumpold BA and Schluter OK, Nutritional composition and safety aspects of edible insects. *Mol Nutr Food Res* **57**:802–823 (2013).
85 Kramer KJ, Hopkins TL and Schaefer J, Applications of solids NMR to the analysis of insect sclerotized structures. *Insect Biochem Mol Biol* **25**:1067–1080 (1995).
86 Finke MD, Complete nutrient content of four species of feeder insects. *Zoo Biol* **32**:27–36 (2013).
87 Ai H, Wang F, Yang Q, Zhu F and Lei C, Preparation and biological activities of chitosan from the larvae of housefly, *Musca domestica*. *Carbohydr Polym* **72**:419–423 (2008).

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88 Badawy RM and Mohamed HI, Chitin extraction, composition of different six insect species and their comparable characteristics with that of the shrimp. *J Am Sci* **11**:127–134 (2015).
89 Chae KS, Shin CS and Shin WS, Characteristics of cricket (*Gryllus bimaculatus*) chitosan and chitosan-based nanoparticles. *Food Sci Biotechnol* **27**:631–639 (2018).
90 Draczynski Z, Honeybee corpses as an available source of chitin. *J Appl Polym Sci* **109**:1974–1981 (2008).
91 Erdogan S and Kaya M, High similarity in physicochemical properties of chitin and chitosan from nymphs and adults of a grasshopper. *Int J Biol Macromol* **89**:118–126 (2016).
92 Hahn T, Roth A, Ji R, Schmitt E and Zibek S, Chitosan production with larval exoskeletons derived from the insect protein production. *J Biotechnol* **310**:62–67 (2020).
93 Huet G, Hadad C, Husson E, Laclef S, Lambertyn V, Araya Farias M *et al*., Straightforward extraction and selective bioconversion of high purity chitin from Bombyx eri larva: toward an integrated insect biorefinery. *Carbohydr Polym* **228**:115382 (2020).
94 Ibitoye EB, Lokman IH, Hezmee MNM, Goh YM, Zuki ABZ and Jimoh AA, Extraction and physicochemical characterization of chitin and chitosan isolated from house cricket. *Biomed Mater* **13**:025009 (2018).
95 Kaya M, Baran T, Mentes A, Asaroglu M, Sezen G and Tozak KO, Extraction and characterization of alpha-chitin and chitosan from six different aquatic invertebrates. *Food Biophys* **9**:145–157 (2014).
96 Kaya M, Baran T, Erdogan S, Mentes A, Ozusaglam MA and Cakmak YS, Physicochemical comparison of chitin and chitosan obtained from larvae and adult Colorado potato beetle (*Leptinotarsa decemlineata*). *Korean J Couns Psychother* **45**:72–81 (2014).
97 Kaya M, Bagriacik N, Seyyar O and Baran T, Comparison of chitin structures derived from three common wasp species (*Vespa crabro* Linnaeus, 1758, *Vespa orientalis* Linnaeus, 1771 and *Vespula germanica* (Fabricius, 1793)). *Arch Insect Biochem Physiol* **89**: 204–217 (2015).
98 Kaya M, Erdogan S, Mol A and Baran T, Comparison of chitin structures isolated from seven Orthoptera species. *Int J Biol Macromol* **72**:797–805 (2015).
99 Kaya M, Mujtaba M, Bulut E, Akyuz B, Zelencova L and Sofi K, Fluctuation in physicochemical properties of chitins extracted from different body parts of honeybee. *Carbohydr Polym* **132**:9–16 (2015).
100 Kaya M, Bitima B, Mujtabaa M and Koyuncub T, Surface morphology of chitin highly related with the isolated body part of butterfly (*Argynnis pandora*). *Int J Biol Macromol* **81**:443–449 (2015).
101 Kaya M, Baran T, Asan-Ozusaglam M, Cakmak YS, Tozak KO, Mol A *et al*., Extraction and characterization of chitin and chitosan with antimicrobial and antioxidant activities from cosmopolitan Orthoptera species (Insecta). *Biotechnol Bioprocess Eng* **20**:168–179 (2015).
102 Kaya M, Sargin I, Aylanc V, Tomruk MN, Gevrek S, Karatoprak I *et al*., Comparison of bovine serum albumin adsorption capacities of α-chitin isolated from an insect and β-chitin from cuttlebone. *J Ind Eng Chem* **38**:146–156 (2016).
103 Kaya M, Sofi K, Sargin I and Mujtaba M, Changes in physicochemical properties of chitin at developmental stages (larvae, pupa and adult) of *Vespa crabro* (wasp). *Carbohydr Polym* **145**:64–70 (2016).
104 Kaya M, Akyuz B, Bulut E, Sargin I, Eroglu F and Tan G, Chitosan nanofiber production from *Drosophila* by electrospinning. *Int J Biol Macromol* **92**:49–55 (2016).
105 Kaya M, Bulut E, Mujtaba M, Sivickis K, Sargin I, Akyuz B *et al*., Gender influences differentiation of chitin among body parts. *Arch Insect Biochem Physiol* **93**:96–109 (2016).
106 Kaya M, Sargin I, Sabeckis I, Noreikaite D, Erdonmez D, Salaberria AM *et al*., Biological, mechanical, optical and physicochemical properties of natural chitin films obtained from the dorsal pronotum and the wing of cockroach. *Carbohydr Polym* **163**:162–169 (2017).
107 Kaya M, Sargin I, Mulerčikas P, Labidi J, Salaberria AM, Cakmak Y *et al*., Conversion of waste parasitic insect (*Hylobius abietis* L.) into antioxidative, antimicrobial and biodegradable films. *J Renewable Mater* **7**: 215–226 (2019).
108 Kaya M and Baran T, Description of a new surface morphology for chitin extracted from wings of cockroach (*Periplaneta americana*). *Int J Biol Macromol* **75**:7–12 (2015).
109 Khayrova A, Lopatin S and Varlamov V, Black soldier fly *Hermetia illucens* as a novel source of chitin and chitosan. *Int J Sci* **8**:81–86 (2019).
110 Kim M-W, Han YS, Jo YH, Choi MH, Kang SH, Kim SA *et al*., Extraction of chitin and chitosan from housefly, *Musca domestica*, pupa shells. *Entomol Res* **46**:324–328 (2016).
111 Kim M-W, Song Y-S, Han YS, Jo YH, Choi MH, Park Y-K *et al*., Production of chitin and chitosan from the exoskeleton of adult two-spotted field crickets (*Gryllus bimaculatus*). *Entomol Res* **47**:279–285 (2017).
112 Liu SF, Sun J, Yu LN, Zhang CS, Bi J, Zhu F *et al*., Extraction and characterization of chitin from the beetle *Holotrichia parallela* Motschulsky. *Molecules* **17**:4604–4611 (2012).
113 Luo Q, Wang Y, Han Q, Ji L, Zhang H, Fei Z *et al*., Comparison of the physicochemical, rheological, and morphologic properties of chitosan from four insects. *Carbohydr Polym* **209**:266–275 (2019).
114 Ma J, Xin C and Tan C, Preparation, physicochemical and pharmaceutical characterization of chitosan from Catharsius molossus residue. *Int J Biol Macromol* **80**:547–556 (2015).
115 Majtan J, Bilikova K, Markovic O, Grof J, Kogan G and Simuth J, Isolation and characterization of chitin from bumblebee (*Bombus terrestris*). *Int J Biol Macromol* **40**:237–241 (2007).
116 Marei NH, El-Samie EA, Salah T, Saad GR and Elwahy AH, Isolation and characterization of chitosan from different local insects in Egypt. *Int J Biol Macromol* **82**:871–877 (2016).
117 Mehranian M, Pourabad RF, Bashir NS and Taieban S, Physicochemical characterization of chitin from the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). *J Macromol Sci A* **54**:720–726 (2017).
118 Mol A, Kaya M, Mujtaba M and Akyuz B, Extraction of high thermally stable and nanofibrous chitin from *Cicada* (Cicadoidea). *Entomol Res* **48**:480–489 (2018).
119 Monter-Miranda JG, Tirado-Gallegos JM, Zamudio-Flores PB, Rios-Velasco C, Ornelas-Paz JD, Salgado-Delgado R *et al*., Extraction and characterization of phisicochemical, morphological, and structural properties of chitin and chitosan from *Brachystola magna* (Girard). *Rev Mex Ing Quim* **15**:749–761 (2016).
120 Nemtsev SV, Zueva OY, Khismatullin MR, Albulov AI and Varlamov VP, Isolation of chitin and chitosan from honeybees. *Appl Biochem Microbiol* **40**:39–43 (2004).
121 Odote O, Struszczyk M and Peter G, M, Characterisation of chitosan from blowfly larvae and some crustacean species from Kenyan Marin waters prepared under different conditions. *West Indian Ocean J Mar Sci* **4**:99–107 (2007).
122 Paulino AT, Simionato JI, Garcia JC and Nozaki J, Characterization of chitosan and chitin produced from silkworm crysalides. *Carbohydr Polym* **64**:98–103 (2006).
123 Shin C-S, Kim D-Y and Shin W-S, Characterization of chitosan extracted from mealworm beetle (*Tenebrio molitor*, *Zophobas morio*) and rhinoceros beetle (*Allomyrina dichotoma*) and their antibacterial activities. *Int J Biol Macromol* **125**:72–77 (2019).
124 Song C, Yu H, Zhang M, Yang Y and Zhang G, Physicochemical properties and antioxidant activity of chitosan from the blowfly Chrysomya megacephala larvae. *Int J Biol Macromol* **60**:347–354 (2013).
125 Smets R, Verbinnen B, Van De Voorde I, Aerts G, Claes J and Van Der Borght M, Sequential extraction and characterisation of lipids, proteins, and chitin from black soldier fly (*Hermetia illucens*) larvae, prepupae, and pupae. *Waste Biomass Valorization* (2020).
126 Soon CY, Tee YB, Tan CH, Rosnita AT and Khalina A, Extraction and physicochemical characterization of chitin and chitosan from *Zophobas morio* larvae in varying sodium hydroxide concentration. *Int J Biol Macromol* **108**:135–142 (2018).
127 Tan G, Kaya M, Tevlek A, Sargin I and Baran T, Antitumor activity of chitosan from mayfly with comparison to commercially available low, medium and high molecular weight chitosans. *In Vitro Cell Dev Biol Anim* **54**:366–374 (2018).
128 Wasko A, Bulak P, Polak-Berecka M, Nowak K, Polakowski C and Bieganowski A, The first report of the physicochemical structure of chitin isolated from *Hermetia illucens*. *Int J Biol Macromol* **92**: 316–320 (2016).
129 Wu S, Preparation of chitooligosaccharides from *Clanis bilineata* larvae skin and their antibacterial activity. *Int J Biol Macromol* **51**: 1147–1150 (2012).
130 Wu SJ, Pan SK, Wang HB and Wu JH, Preparation of chitooligosaccharides from cicada slough and their antibacterial activity. *Int J Biol Macromol* **62**:348–351 (2013).
131 Zhang AJ, Qin QL, Zhang H, Wang HT, Li X, Miao L *et al*., Preparation and characterisation of food-grade chitosan from housefly larvae. *Czech J Food Sci* **29**:616–623 (2011).
132 Zhang M, Haga A, Sekiguchi H and Hirano S, Structure of insect chitin isolated from beetle larva cuticle and silkworm (*Bombyx mori*) pupa exuvia. *Int J Biol Macromol* **27**:99–105 (2000).

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133 Zhou P, Li J, Yan T, Wang X, Huang J, Kuang Z *et al.*, Selectivity of deproteinization and demineralization using natural deep eutectic solvents for production of insect chitin (*Hermetia illucens*). *Carbohydr Polym* **225**:115255 (2019).
134 Philibert T, Lee BH and Fabien N, Current status and new perspectives on chitin and chitosan as functional biopolymers. *Appl Biochem Biotechnol* **181**:1314–1337 (2017).
135 Martinou A, Kafetzopoulos D and Bouriotis V, Chitin deacetylation by enzymatic means: monitoring of deacetylation processes. *Carbohydr Res* **273**:235–242 (1995).
136 Win N and Stevens W, Shrimp chitin as substrate for fungal chitin deacetylase. *Appl Microbiol Biotechnol* **57**:334–341 (2001).
137 Kim S and Rajapakse N, Enzymatic production and biological activities of chitosan oligosaccharides (COS): a review. *Carbohydr Polym* **62**:357–368 (2005).
138 Wang CH, Doan CT, Nguyen AD and Wang SL, Reclamation of fishery processing waste: a mini-review. *Molecules* **24**:2234 (2019).
139 Younes I and Rinaudo M, Chitin and chitosan preparation from marine sources. Structure, properties and applications. *Mar Drugs* **13**:1133–1174 (2015).
140 Mahmoud NS, Ghaly AE and Arab F, Unconventional approach for demineralization of deproteinized crustacean shells for chitin production. *Am J Biochem Biotechnol* **3**:1–9 (2007).
141 Percot A, Viton C and Domard A, Optimization of chitin extraction from shrimp shells. *Biomacromolecules* **4**:12–18 (2003).
142 Rojsitthisak P, How N, Chandrkrachang S and Stevens W, Effect of chemical treatment on the characteristics of shrimp chitosan. *J Met Mater Miner* **12**:11–18 (2002).
143 Shahidi F and Synowiecki J, Isolation and characterization of nutrients and value-added products from snow crab (*Chionoecetes opilio*) and shrimp (*Pandalus borealis*) processing discards. *J Agric Food Chem* **39**:1527–1532 (1991).
144 Kaya M, Mujtaba M, Ehrlich H, Salaberria AM, Baran T, Amemiya CT *et al.*, On chemistry of gamma-chitin. *Carbohydr Polym* **176**: 177–186 (2017).
145 Truong TO, Hausler R, Monette F and Niquette P, Valorisation des résidus industriels de pêches pour la transformation de chitosane par technique hydrothermo-chimique. *Rev Des Sci De L'Eau* **20**: 253–262 (2007).
146 Ameh AO, Isa MT, Abutu D and Danlami A, Kinetic modelling of the demineralization of shrimp exoskeleton using citric acid. *Leonardo Electron J Pract Technol* **25**:99–108 (2014).
147 Tharanathan RN and Kittur FS, Chitin – the undisputed biomolecule of great potential. *Crit Rev Food Sci Nutr* **43**:61–87 (2003).
148 Hours RA and Gortari MC, Biotechnological processes for chitin recovery out of crustacean waste: a mini-review. *Electron J Biotechnol* **16**: 14 (2013).
149 Pighinelli L, Methods of chitin production a short review. *Am J Biomed Sci* **3**:307–314 (2019).
150 Fu X, Zhu L, Li L, Zhang T, Li M and Mou H, Eco-friendly preparation of chitooligosaccharides with different degrees of deacetylation from shrimp shell waste and their effects on the germination of wheat seeds. *Marine Life Sci Technol* **1**:95–103 (2019).
151 Castillo V, Matos M and Muller AJ, A new route towards the preparation of a biodegradable nanocomposite based on chitin whiskers from exoskeleton of shrimp and polycaprolactone. *Rev Latinoam de Metal y Mater* **23**:12–19 (2003).
152 Paduretu CC, Apetroaei MR, Ileana RĂU and Schroder V, Characterization of chitosan extracted from different Romanian Black Sea crustaceans. *UPB Sci Bull Ser B* **80**:13–24 (2018).
153 Bolat Y, Bilgin Ş, Günlü A, Izci L, Koca SB, Çetinkaya S *et al.*, Chitin-chitosan yield of freshwater crab (*Potamon potamios*, Olivier 1804) shell. *Pakistan Vet J* **30**:227–231 (2010).
154 Cortizo SM, Berghoff CF and Alessandrini JL, Characterization of chitin from *Illex argentinus* squid pen. *Carbohydr Polym* **74**:10–15 (2008).
155 Das NG, Khan PA and Hossain Z, Chitin from the shell of two coastal portunid crabs of Bangladesh. *Indian J Fish* **43**:413–415 (1996).
156 Thirunavukkarasu N and Shanmugam A, Extraction of chitin and chitosan from mud crab *Scylla tranquebarica* (Fabricius, 1798). *Int J Appl Bioeng* **3**:31–33 (2009).
157 Sugumaran M, Complexities of cuticular pigmentation in insects. *Pigment Cell Melanoma Res* **22**:523–525 (2009).
158 Hayes M, Carney B, Slater J and Bruck W, Mining marine shellfish wastes for bioactive molecules: chitin and chitosan—part A: extraction methods. *Biotechnol J* **3**:871–877 (2008).
159 Yeul VS and Rayalu SS, Unprecedented chitin and chitosan: a chemical overview. *J Polym Environ* **21**:606–614 (2012).
160 Ploydee E and Chaiyanan S, Production of high viscosity chitosan from biologically purified chitin isolated by microbial fermentation and deproteinization. *Int J Polym Sci* **2014**:1–8 (2014).
161 Hu X, Du Y, Tang Y, Wang Q, Feng T, Yang J *et al.*, Solubility and property of chitin in NaOH/urea aqueous solution. *Carbohydr Polym* **70**: 451–458 (2007).
162 Wattjes J, Niehues A, Cord-Landwehr S, Hoßbach J, David L, Delair T *et al.*, Enzymatic production and enzymatic-mass spectrometric fingerprinting analysis of chitosan polymers with different nonrandom patterns of acetylation. *J Am Chem Soc* **141**:3137–3145 (2019).
163 Younes I, Sellimi S, Rinaudo M, Jellouli K and Nasri M, Influence of acetylation degree and molecular weight of homogeneous chitosans on antibacterial and antifungal activities. *Int J Food Microbiol* **185**:57–63 (2014).
164 Jaworska M, Sakurai K, Gaudon P and Guibal E, Influence of chitosan characteristics on polymer properties: I. Crystallographic properties. *Polym Int* **52**:198–205 (2003).
165 No HK and Meyers SP, Crawfish chitosan as a coagulant in recovery of organic compounds from seafood processing streams. *J Agric Food Chem* **37**:580–583 (1989).
166 de Castro RJS, Ohara A, Aguilar JGS and Domingues MAF, Nutritional, functional and biological properties of insect proteins: processes for obtaining, consumption and future challenges. *Trends Food Sci Technol* **76**:82–89 (2018).
167 Islam S, Bhuiyan MAR and Islam MN, Chitin and chitosan: structure, properties and applications in biomedical engineering. *J Polym Environ* **25**:854–866 (2016).
168 Vishu Kumar AB, Varadaraj MC, Gowda LR and Tharanathan RN, Characterization of chito-oligosaccharides prepared by chitosanolysis with the aid of papain and pronase, and their bactericidal action against *Bacillus cereus* and *Escherichia coli*. *Biochem J* **391**:167–175 (2005).
169 Zivanovic S, Basurto CC, Chi S, Davidson PM and Weiss J, Molecular weight of chitosan influences antimicrobial activity in oil-in-water emulsions. *J Food Prot* **67**:952–959 (2004).
170 El Knidri H, El Khalfaouy R, Laajeb A, Addaou A and Lahsini A, Eco-friendly extraction and characterization of chitin and chitosan from the shrimp shell waste via microwave irradiation. *Process Saf Environ Prot* **104**:395–405 (2016).
171 Seoudi R, Nada AMA, Abd Elmongy S and Hamed SS, Fourier transform infrared spectroscopic and AC conductivity studies of chitin and its derivatives. *J Appl Polym Sci* **98**:936–943 (2005).
172 Kasaal MR, A review of several reported procedures to determine the degree of N-acetylation for chitin and chitosan using infrared spectroscopy. *Carbohydr Polym* **71**:497–508 (2008).
173 Shigemasa Y, Matsuura H, Sashiwa H and Saimoto H, Evaluation of different absorbance ratios from infrared spectroscopy for analyzing the degree of deacetylation in chitin. *Int J Biol Macromol* **18**: 237–242 (1996).
174 Dimzon IKD and Knepper TP, Degree of deacetylation of chitosan by infrared spectroscopy and partial least squares. *Int J Biol Macromol* **72**:939–945 (2015).
175 Kurita K, Tomita K, Tada T, Ishii S, Nishimura S-I and Shimoda K, Squid chitin as a potential alternative chitin source: deacetylation behavior and characteristic properties. *J Polym Sci A Polym Chem* **31**:485–491 (1993).
176 Clark GL and Smith AF, X-ray diffraction studies of chitin, chitosan, and derivatives. *J Phys Chem* **40**:863–879 (1936).
177 Ogawa K, Hirano S, Miyanishi T, Yui T and Watanabe T, A new polymorph of chitosan. *Macromolecules* **17**:973–975 (1984).
178 Mogilevskaya EL, Akopova TA, Zelenetskii AN and Ozerin AN, The crystal structure of chitin and chitosan. *Polym Sci Ser A* **48**:116–123 (2006).
179 Segal L, Creely JJ, Martin AE and Conrad CM, An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer. *Text Res J* **29**:786–794 (1959).
180 Ioelovich M, Crystallinity and hydrophilicity of chitin and chitosan. *Res Rev J Chem* **3**:7–14 (2014).
181 Mourya VK and Inamdar NN, Chitosan-modifications and applications: opportunities galore. *React Funct Polym* **68**:1013–1051 (2008).
182 Sagheer FAA, Al-Sughayer MA, Muslim S and Elsabee MZ, Extraction and characterization of chitin and chitosan from marine sources in Arabian Gulf. *Carbohydr Polym* **77**:410–419 (2009).

183 Arora S, Lal S, Kumar S, Kumar M and Kumar M, Comparative degradation kinetic studies of three biopolymers: chitin, chitosan and cellulose. *Arch Appl Sci Res* **3**:188–201 (2011).
184 Hussain R, Iman M and Maji TK, Determination of degree of deacetylation of chitosan and their effect on the release behavior of essential oil from chitosan and chitosan-gelatin complex microcapsules. *IJAERS* **1**:4–12 (2013).
185 Jiang Y, Fu C, Wu S, Liu G, Guo J and Su Z, Determination of the deacetylation degree of chitooligosaccharides. *Mar Drugs* **15**:332 (2017).
186 Xu J, McCarthy SP, Gross RA and Kaplan DL, Chitosan film acylation and effects on biodegradability. *Macromolecules* **29**:3436–3440 (1996).
187 Abdou ES, Nagy KSA and Elsabee MZ, Extraction and characterization of chitin and chitosan from local sources. *Bioresour Technol* **99**: 1359–1367 (2008).
188 de Alvarenga ES, Biotechnology of biopolymers, in *Characterization and Properties of Chitosan*, ed. by Elnashar M. IntechOpen, (2011).
189 Laroche M, Perreault V, Marciniak A, Gravel A, Chamberland J and Doyen A, Comparison of conventional and sustainable lipid extraction methods for the production of oil and protein isolate from edible insect meal. *Foods* **8**:572 (2019).
190 Merzendorfer H and Zimoch L, Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. *J Exp Biol* **206**:4393–4412 (2003).
191 Vårum KM, Antohonsen MW, Grasdalen H and Smidsrød O, Determination of the degree of N-acetylation and the distribution of N-acetyl groups in partially N-deacetylated chitins (chitosans) by high-field n.m.r. spectroscopy. *Carbohydr Res* **211**: 17–23 (1991).
192 Vårum KM, Anthonsen MW, Grasdalen H and Smidsrød O, ^{13}C-N.m.r. studies of the acetylation sequences in partially N-deacetylated chitins (chitosans). *Carbohydr Res* **217**:19–27 (1991).
193 Heux L, Brugnerotto J, Desbrières J, Versali MF and Rinaudo M, Solid state NMR for determination of degree of acetylation of chitin and chitosan. *Biomacromolecules* **1**:746–751 (2000).
194 Hopkins TL and Kramer KJ, Insect cuticle sclerotization. *Annu Rev Entomol* **37**:273–302 (1992).

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cosmetics

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Review

Insect Chitin-Based Nanomaterials for Innovative Cosmetics and Cosmeceuticals

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Abstract: Chitin and its derivatives are attracting great interest in cosmetic and cosmeceutical fields, thanks to their antioxidant and antimicrobial properties, as well as their biocompatibility and biodegradability. The classical source of chitin, crustacean waste, is no longer sustainable and fungi, a possible alternative, have not been exploited at an industrial scale yet. On the contrary, the breeding of bioconverting insects, especially of the Diptera *Hermetia illucens*, is becoming increasingly popular worldwide. Therefore, their exoskeletons, consisting of chitin as a major component, represent a waste stream of facilities that could be exploited for many applications. Insect chitin, indeed, suggests its application in the same fields as the crustacean biopolymer, because of its comparable commercial characteristics. This review reports several cosmetic and cosmeceutical applications based on chitin and its derivatives. In this context, chitin nanofibers and nanofibrils, produced from crustacean waste, have proved to be excellent cosmeceutical active compounds and carriers of active ingredients in personal care. Consequently, the insect-based chitin, its derivatives and their complexes with hyaluronic acid and lignin, as well as with other chitin-derived compounds, may be considered a new appropriate potential polymer to be used in cosmetic and cosmeceutical fields.

Keywords: chitin; chitosan; nanofibers; nanofibrils; insects; personal care

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1. Introduction

In the cosmetics and cosmeceuticals fields, there is a constant search and high demand for innovative and safe materials and new formulations to be used in personal care for hair, nails, lips, and skin in general. Among new materials, the natural-derived polymers, including chitin, are receiving particular and increasing attention because of their low environmental impact and no negative effects on human health [1]. Due to their excellent biological properties, chitin and its derivatives are among the most widely used polymers for several cosmetic/cosmeceuticals applications. Specifically, they are considered of great utility because they act both as carriers and active ingredients [2].

A cosmetic product is defined as a substance applied externally to the human body or in the oral cavity for the purpose of cleaning, protecting, perfuming, improving the appearance or modifying the odour [3]. The term "cosmeceutical" combines the concepts of cosmetic and pharmaceutical, highlighting the additional therapeutic effect of these new body care products [4,5]. In both cosmetics and cosmeceuticals, the use of nanomaterials is constantly growing, since nanocosmeceuticals are considered more efficient carriers, having a better ability of active ingredients than traditional materials [6]. Chitin nanomaterials, mainly nanofibers and nanofibrils, are already being produced from commercially available chitin, mainly derived from crustaceans, for biomedical applications, especially for drug delivery and wound healing [7–9]. The use in cosmetics and cosmeceuticals of chitin and

its derivatives produced from a more sustainable and growing source, such as insects, could provide a further benefit.

2. Chitin

2.1. Structure and Properties

Chitin and chitosan, its deacetylated derivative, are among the most widely used polysaccharide biopolymers [10,11]. Polysaccharide biopolymers have gained a lot of attention for their physico-chemical properties as biomaterials (i.e., materials of a non-pharmacological nature, which can interact with biological systems efficiently, improving or restoring all the functions of an organ or tissue). Polysaccharide biopolymers have a rather low cost and their similarity to some of the most important biological macromolecules makes them of particular interest for various applications [10,11].

After cellulose, chitin is the second most widespread natural polysaccharide. In 1811, Henri Braconnot discovered chitin as a "material particularly resistant to usual chemicals" in fungi and gave it the name "fungine" [12]. After some years, the French chemist August Odier discovered the same material in both insect cuticle and non-animal tissues, and he gave the name "chitin" to this substance [13]. The physiologist Charles Rouget discovered that chitin, boiled in potassium hydroxide, became its deacetylated and soluble derivative named chitosan [11].

Chitin is composed of β-(1-4)-linked N-Acetyl-D-glucosamine. It is structurally identical to cellulose, but with acetamide groups (-$NHCOCH_3$) at the C-2 positions instead of the hydroxyl group (-OH) (Figure 1). Chitin is a white, hard, inelastic and nitrogenous polysaccharide, with low chemical reactivity, having the same role of collagen in animals and cellulose in plants [14–16]. Chitosan is a linear polymer of α-(1-4)-linked 2-amino-2-deoxy-β-D-glucopyranose and is easily derived by *N*- deacetylation of chitin (Figure 1). Chitin is hydrophobic, it is insoluble in water and common organic solvents and turns out to be soluble only in hexafluoroisopropanol, hexafluoroacetone, chloroalcohols when they are conjugated with aqueous acid solutions and dimethylacetamide in 5% lithium chloride [17,18] and in ionic liquids [19,20]. The amino groups in chitosan improve its solubility at pH values below 6.5, which is their pka value. Therefore, the solubility problem of chitin can be overcome by deacetylating it into chitosan, which is thus soluble at acid pH [11,21]. Like polysaccharides with numerous hydrogen bonds, chitin and chitosan degrade before melting [22]. Different parameters affect the polymer properties, such as deacetylation degree, molecular weight and first of all polydispersity and crystallinity. Other characteristics, such as the purity, the moisturizing capacity and the content of heavy metals, endotoxin and proteins, must be determined for example for applications in food and medical fields [22]. As previously reported, the degree of deacetylation (DD) has a major effect on the solubility of chitin. The lowest deacetylation degree in chitin can be less than 10% [23]. Generally, the more chitin is deacetylated, the more soluble it becomes in acidic solution. A DD of 50% is the commonly proposed threshold above which chitin is defined as chitosan. The molecular weight (MW), as well as the DD, greatly affect many properties of chitin and chitosan, particularly their antimicrobial activity [24]. Chitin has a molecular weight exceeding 1000 kDa, while the chitosan range from 100 to 1000 kDa [10,25,26].

Both chitin and cellulose have a crystalline structure [27] which, reducing the solubility, represents the major limiting factor in its utilization. There are three polymorphic crystalline structures of chitin: α, β, γ [28] (Figure 2). α-chitin for its strong inter-sheet and intra-sheet hydrogen bonding has a compact and crystalline structure, which makes it a robust and recalcitrant material [18]. It is the most common form, mainly present in crustaceans, fungi, yeasts and insects, with an antiparallel orientation of the chains [29]. β-chitin has weak intra-sheet hydrogen bonds, which characterize a weak intermolecular force. Moreover, it has a parallel arrangement of the chains, is less crystalline, with less packaging possibility, being more flexible and more reactive [30]. β-chitin is mainly found in the pen of the *Loligo* squids. It is interesting to remember that α-chitin can be transformed into β-chitin, but not

the reverse [13]. γ-chitin is a mix of forms α and β, combining chains arranged in both parallel and antiparallel ways and can be found in cocoon fibers of the *Ptinus* beetle and also in the stomach of the *Loligo* squid [13,28].

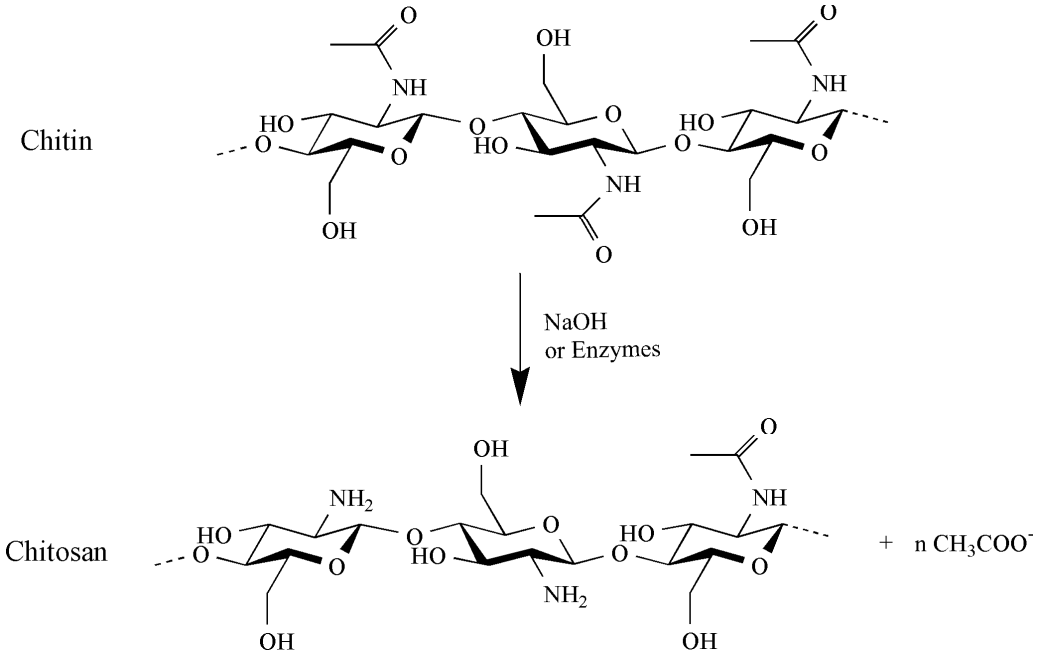

Figure 1. Chemical structure of chitin and chitosan. Chitosan is obtained through deacetylation of chitin using NaOH or enzymes.

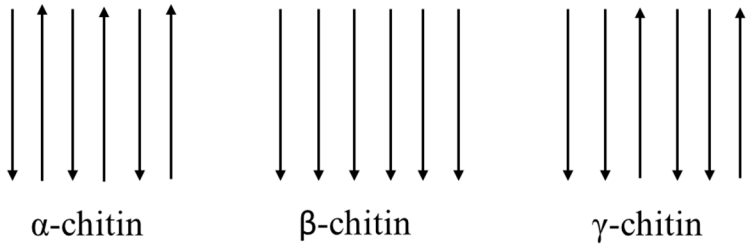

Figure 2. Representation of the three allomorphic forms of chitin in nature.

The most important biological property of chitin and chitosan is their non-toxicity. However, there are many other important properties, such as biodegradability and biocompatibility, but also hemostaticity, immunostimulating activity and bioadhesivity, which are mostly specific to chitosan [18,22]. Both chitin and chitosan can be hydrolyzed in order to obtain oligosaccharides, which are structurally simpler and with a lower MW, and make them water-soluble, widening their applications. This hydrolysis can be carried out with specific enzymes, such as chitinases and chitosanases, but also with non-specific enzymes, including papain, pepsin and lysozyme, which are able to produce chitosan hydrolysates with no residual proteins [22,31].

Properties of chitin and chitosan can vary with their source, method of preparation, MW and DD [22]. In addition, several authors have reported that both chitin and chitosan show analgesic and antimicrobial activity, properties that seem better in chitosan [22,32], so that is receiving the most attention and is among the most studied properties [22].

2.2. Chitin Fields of Application

Chitin fields of application range from cosmetics, agriculture, tissue engineering to biomedical and pharmaceutical areas [15]. However, the poor solubility of chitin makes its

use difficult. Therefore, to make it suitable for more application, it is necessary to modify its structure by adding functional chemical groups [27]. Among chitin derivatives there are carboxymethyl chitin, alkyl chitin, benzyl chitin and hydroxybutyl chitin [27,33,34].

Chitin and its derivatives, for example, can be used for the following applications [10,35] (Figure 3):

- Agriculture, thanks to their antimicrobial and fungistatic properties [16]. They also stimulate plants to produce defense agents [36]. They can be used to detect the presence of mold in plant systems [37].
- Food and nutrition, used as a food additive, acting like a colour stabiliser, a thickener, an emulsifier and a natural flavor extender [38]. They are also useful for film formation, food preservation, fruit deacidification and water purification [39,40].
- Biomedical applications, used to produce fibers, nanofibers and nanoparticles for drug delivery, but also to produce tissue engineering systems for wounds repair [41], also because chitin sutures resist degradation caused by bile, urine and pancreatic juice [42].
- Tissue engineering, as a bone substitute for bone restoration through modification with hydroxyapatite or bioactive glass ceramics [43].
- Wound dressing, thanks to the antimicrobial, blood clotting, swelling, cell attachment and cytocompatibility aspects of chitin-based composites [44].
- Wastewater treatments, thanks to their non-toxicity. They can bind to water pollutants, especially heavy metals [45].

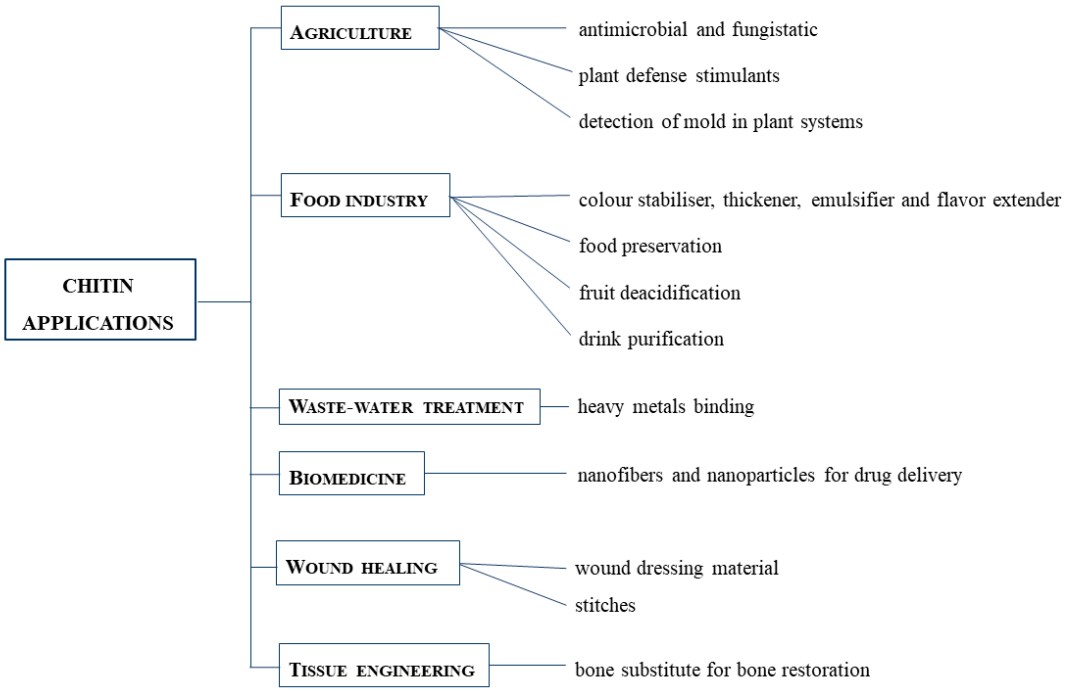

Figure 3. Chitin applications in different fields: agriculture, food industry, waste-water treatment, biomedicine, wound healing and tissue engineering.

Moreover, it should be emphasized that all these activities are notably increased when chitin and chitosan are used in their micro/nano size, as reported in many papers of this report.

2.3. Chitin Sources

It has been estimated that up to 10^{10} to 10^{12} tons of chitin are annually synthesized in nature, being a major structural component of the exoskeleton of arthropods and the cell wall of fungi and yeasts [35]. The main source for the commercial production of chitin

is the exoskeleton of crustaceans, especially crabs, shrimps and prawns. In crustaceans, chitin is deposited together with calcium carbonate in a complex protein network that enables the shell to be formed [46]. Approximately 60% of the body weight of crustaceans consists of waste exoskeleton, which can be recovered for the production of chitin and chitosan [47,48]. The chitin content of this crustacean waste widely ranges from 15% to 40% [47,49]. However, this source presents some limitations, such as seasonality and a generally poor sustainability [50,51]. The geographical limitation to coastal areas, for instance, results in high CO_2 emissions to transport the raw material to other areas [50,51]. The growing market of chitin makes it necessary to search for alternative sources in order to cope with the huge demand for chitin, avoiding further depletion of marine resources [52]. Alternative sources for the production of chitin and chitosan include fungi. Fungi can be a valid, cheap source of chitin and chitosan. The average yields of chitin and chitosan isolated from fungi are around 8.5–19.6%, correlating to 1–4% of dry biomass, respectively. The species of fungi that have been mostly investigated for chitin and chitosan production are *Pleurotus sajor-caju, Lentinula edodes, Agaricus bisporus, Auricula-judae, Trametes versicolor, Armillaria mellea, Pleurotus ostreatus*, and *Pleurotus eryngii* [53]. Although fungi as an alternative source of chitin are promising, chitin production from this origin has not yet been prepared on an industrial scale [54]. Insects can be an alternative, renewable, sustainable and non-seasonal source of chitin and chitosan [55–62]. Insect farms are spreading worldwide for the production of animal feed combined to waste management. These farms generate a huge amount of insect waste, mainly consisting in exuviae and adults dead at the end of their life cycle, that are rich in chitin and suitable for further purification processing [63].

3. Insect-Based Chitin

There is currently a strong focus on insects as a new source of valuable and useful molecules, such as lipids, (antimicrobial) peptides, polymers, proteins and vitamins, for several applications [64–66]. Among the natural polymers, chitin is becoming increasingly important. Chitin is found in the cuticle of insects, of which it can constitute up to 25–60% of the dry weight [67], embedded in a sclerotized protein matrix together with lipoproteins and other materials, both organic and inorganic [68]. Within the cuticle, chitin is embedded in the procuticle (the innermost layer) in the form of nanofibers arranged in fibre bundles, forming an asymmetrical sheet structure responsible for the elasticity and stiffness of the cuticle [69,70].

The usefulness of insects for the production of molecules with high market value, combined with their ease of breeding on a wide range of organic substrates, has led to the increasing development of their large-scale farming. Insect farms are often aimed at both feed production and waste disposal, since the larvae of many species can exploit different organic wastes, bioconverting them into a valuable biomass with high protein and lipid content [53,71]. Among these species with bioconverting ability, *Hermetia illucens* (Diptera: Stratiomyidae) is the most bred [72,73]. Larval stages of *H. illucens* are able to feed on organic material, including waste of animal and vegetable origin, converting them into valuable products that can be re-placed on the market [74]. Within the bioconversion process, which corresponds to the life cycle of the larva, *H. illucens* is able to reduce organic matter with zero value on up to 70–75% (wet weight). At the same time, the conversion into larval biomass is very quick at optimal temperature and humidity conditions of the environment and substrate (around 15 days) [73,75]. Larval biomass could be used for pet food and insectivorous animals or can be transformed into larval meal with high protein content for aquaculture, in accordance with European regulation 893/2017. Even insect frass could be considered a precious element, as they are comparable to soil improver for organic agriculture [76].

The chitin-rich insect waste biomass, generated in huge amounts from the breeding facilities of these species, is the only by-product of a zero-waste process. Particularly, during its life cycle, *H. illucens* produces larval and pupal exuviae consisting of the exoskeletons

shed by the insect as it moults from one developmental stage to the next one. If properly exploited, this biomass could be a valuable source of chitin and its derivatives, making insect breeding a total circular process and economy. Studies on insect chitin in the above-described applications are still at the beginning, but the scientific world is making many efforts to validate the usage of this polymer and its derivatives in several fields. There are ongoing projects aimed at extracting chitin from *H. illucens* to produce nanofibers for use in the biomedical and cosmetic sectors, particularly for wound healing and formulations of anti-aging products, respectively (unpublished data).

3.1. Chitin Extraction from Insects

A purification process is required to isolate chitin from the insect biomass, since chitin in the cuticle is also bound to proteins, minerals and pigments. Methods for chitin extraction from insects are almost the same used for the industrial purification from crustacean waste, involving two main steps: demineralization and deproteinization (Figure 4). Minerals, chiefly calcium carbonate, are removed using acidic solutions, while for protein removal an alkaline treatment is required. An additional bleaching step is often performed to whiten the raw chitin [77–79]. The current state of chitin production from insects has been reviewed by Hahn et al. [80]. The average yield of chitin extracted from insects ranges between 5% and 15% [80], depending mainly on the insect species, the developmental stage and the body parts of the specimens used [81–84]. For chitin extracted from crustaceans, a slightly higher average yield ranging from 5 to 32% has been reported [26,85–89].

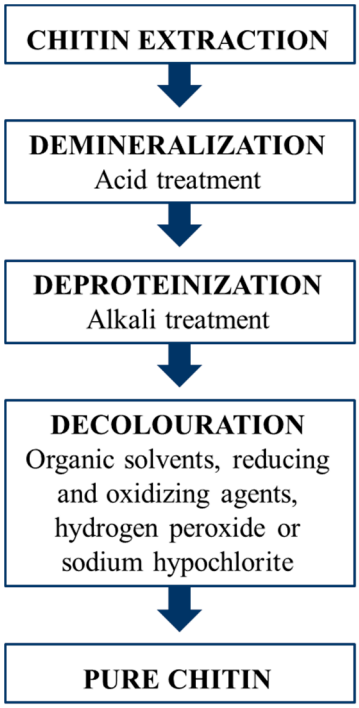

Figure 4. Schematic representation of the chitin extraction process applied for both crustaceans and insects, involving demineralization, removal of proteins and bleaching.

Characteristics of insect-based chitin are generally similar to the ones of the commercial crustacean-derived chitin [57,90,91]. For instance, the degree of acetylation (i.e., the content of *N*-acetyl groups) of insect chitin, one of the factors that has a major impact on the performance of the polymer, commonly ranges from 80 to 99% [92], similarly to that of crustacean chitin [90,93–95]. Furthermore, chitin extracted from insects has always been determined in the α-form, as the one derived from crustaceans [55,58,92,96,97]. The suitability of chitin for different applications is also greatly affected by its surface morphology. Generally, for biomedical applications, like drug delivery and tissue engineering, a porous

structure of the chitin surface is preferred [84]. Within insects, the surface morphology of chitin is mostly related to the species and, within the species, to the body part and developmental stage [55,84,98]. Nonetheless, a similarity in the morphology was observed between insect and crustacean chitin, a combination of nanofibers and pores being the most common morphology [83,87]. All these considerations support and legitimate the use of insects as a source of chitin alternative to crustaceans.

3.2. Insect Chitin Derivatives

Chitosan is the main derivative of chitin and is produced by deacetylation of chitin (i.e., partial removal of the acetyl groups from the polymer chain). Chitosan greatly widens the range of applications of chitin, being much more soluble and reactive thanks to the higher content of free amino groups [99]. Chitin can be deacetylated by chemical or enzymatic treatment. Enzymatic deacetylation involves the use of deacetylases but this does not generally result in an efficient deacetylation because of the crystalline structure of chitin [100–102]. Hence, chemical deacetylation, using high concentrated sodium hydroxide solutions at high temperature, is the most performed for chitosan production from both crustaceans and insects [46]. The average yield of chitosan produced from insects ranges from 2 to 8%, related to the initial insect dry biomass [80]. Slightly higher yields are generally obtained from crustacean chitin, ranging from 4 to 15%, related to the original dry biomass, depending on the species and the applied treatment [26,60,86,88,89,103]. It should be noted that crustaceans have a lower protein and fat content than insects, which contributes to a higher yield of chitosan [104].

One of the most important characteristics of chitosan is the degree of deacetylation (DD) (the proportion of glucosamine residues), which can affect its solubility and performance [105]. DD of insect-derived chitosan ranges from around 60 to 98%, depending on the deacetylation parameters applied, entirely similarly to that of commercial chitosan produced from crustaceans [80,106]. The molecular weight (MW) also plays a pivotal role in determining the chitosan performance in several applications, since it greatly affects its biological activity [107,108]. Insect-derived chitosan generally has a MW varying from 26 to 300 kDa [80], lower than that of commercial chitosan (100–1000 kDa) [25,103]. It is worth noting that a low MW of chitosan is often related to a greater antimicrobial activity [107,108]. These values are, however, highly variable, depending on both the chitosan source and the applied deacetylation method [83,109].

Overall, the aforementioned characteristics confirm the similarity between chitin and chitosan derived from insects and those produced from crustaceans. This supports the potential for insect-based chitin and chitosan to be used in the well-established applications for the commercial polymers. Chitosan produced from *H. illucens* is currently under investigation as a preservative coating for the shelf-life extension of fruits and vegetables. Moreover, tests are ongoing on the production of chitosan nanoparticles derived from *H. illucens* for applications in drug delivery (unpublished data).

4. Application of Chitin and Its Derivatives in Cosmetics and Cosmeceuticals

In recent years, in response to the desire to maintain youth and beauty, the goal of cosmetology has been the continuous and constant search for innovative compounds for the development of new formulations, including cosmeceuticals, to treat various skin damages and prevent, slow down and/or reduce the undesirable effects of aging [6,110]. These new compounds include biopolymers, such as starch, cellulose, chitin and their derivatives, which have a low environmental impact and no side effects for humans [1,111]. Chitin and its derivatives, thanks to their antioxidant, humectant, antimicrobial, biocompatibility, biodegradability, cleansing and protective properties, are used in cosmetic and cosmeceutical products for hair, nail, oral, lip and, especially, skin care [15]. They are excellent moisturizing and anti-aging agents, which can protect the skin from external hazards, improving important skin functions such as heat regulation, protection, secretion, excretion, sensation and absorption [10,112,113]. Furthermore, due to their specific biological and

technological properties, chitin and its derivatives can act as both active ingredients and carriers [2]. Its poor solubility often limits the use of chitin, though [27]. This problem is overcome by converting chitin into its more soluble derivatives. Further, chitin and its derivatives can, in turn, be processed into various conformations, such as micro- and nanoparticles, nanofibers, hydrogels, membranes and films (or complexed with surfactants or polyelectrolytes) in order to broaden the spectrum of possible applications [2,114–116].

Nanotechnology is one of the leading innovative sectors of the personal care industry. Nanomaterial-based cosmeceuticals are of particular interest due to their improved properties compared to conventional materials. Nanomaterials are used for their ability to release the active ingredients in a targeted and controlled manner at their site of action (mainly the skin), thus leading to a prolongation of the effects, their defined use, and an increased efficacy and bioavailability [6,110]. In Figure 5 the main classes of nanocosmeceuticals are reported, according to their respective roles in skin, hair, nail, lip and oral care [6,10,110].

Figure 5. Nanocosmeceutical applications in personal care.

Within the cosmetics and cosmeceutical research, chitosan remains the most focused and used chitin derivative. Particularly, chitosan-based drug delivery systems are attracting interest as vehicles because they are able to release their active ingredients at the desired rate and body site [117]. For instance, chitosan nanoparticles (CNPs) can be retained in the skin matrix, thanks to the positive charge of these systems, that allow them to interact with skin lipids [118]. CNPs are widely used for topical and transdermal delivery of active ingredients [119]. For example, retinol, a derivative of vitamin A, can be included in the chitin-based nanosystems for the treatment of acne or skin wrinkles [120]. CNPs can be used for sunscreen products, including natural substances like annatto and saffron [121]; chitin nanogels and clobetasol propionate are used to treat psoriasis, avoiding adverse effects such as skin atrophy or steroid acne [118].

Besides chitosan, there are other chitin derivatives that can be solubilized also in water at physiological and alkaline pH, maintaining their characteristics, such as oligosaccharides, complexes with proteins, carotenoids or glucans, and by-products obtained by chemical alkylation and carboxymethylation [2,10,34,85]. All these derivatives can be suitable molecules for cosmeceutical purposes. For instance, the carboxymethylated derivatives of chitin and chitosan (CM-chitin and CM-chitosan respectively), and particularly the 6-O-carboxymethylates derivatives of the same (6-O-CM-chitin and 6-O-CM-chitosan), can promote moisture absorption and retention of the stratum corneum (the outermost layer of the epidermis) comparable to hyaluronic acid (HA), through the formation of a moisturizing film on the skin [122,123]. In addition, other studies have demonstrated that CM-chitin and CM-chitosan, along with chitin oligomers, are promising antioxidants (ascribed to the

carboxymethyl groups) and inhibitors of metalloproteases and myeloperoxidases (ascribed to the acetyl groups), alleviating oxidative damage to the skin [114,124,125]. Another form of carboxylated chitin, known as 6-Oxychitin [126] or 6-Carboxychitin [127], was found to have even better properties than the previously mentioned derivatives, when compared to HA. In addition to its regenerative properties for which it is used in the biomedical field [128], 6-Oxychitin has shown to be a valid substitute of HA for its excellent moisture absorption and retention abilities in the formulation of cosmetics and cosmeceuticals [127].

Clinical studies performed by Gautier et al. reported that the presence of chitin-glucan complexes in skin care lotions did not alter the functionality of the epidermal barrier but increased its hydration degree, also showing a reduction in the signs of aging with a final effect of skin rejuvenation [129]. Concerning the application in wound healing, water-soluble chitin (WSC) has been shown to be more effective than chitin and chitosan in accelerating the regenerative process of the skin [130]. Also, the studies performed by Chilarski et al. and Blasinska et al. have highlighted the efficacy and mode of action in the wound healing process of dibutyrylchitin (DBC), a water-soluble derivative of chitin obtained by its reaction with the butyric anhydride [131,132]. Particularly, Chilarski et al. applied DBC non-woven fabrics on non-infected wounds and left them there until the healing process ended, with a positive outcome [132]. Blasinska et al. reported that DBC dressing materials, subcutaneously implanted in rats, contributed to the wound reparative process through an increase in the number of cells and in the amount of glycosaminoglycans, thus contributing to the modification of the extracellular matrix (ECM) and to the increase in the weight of the granulation tissue [131].

To date, there are no examples of applications with insect chitin and its derivatives (including different formulations) in cosmetic and cosmeceutical products, as this field is still unexplored, even the potentiality of chitin from insects is really promising. The physical characteristics of these biopolymers, such as degree of acetylation, the α-form and the surface morphology, are very similar to those of crustaceans and this analogy represents the starting point to replicate the same or similar experiments, in order to validate the use of insect chitin in cosmetic and cosmeceutical fields [133].

5. Chitin-Based Nanomaterials in Personal Care

5.1. Innovation of Chitin Nanofibers and Nanofibrils

Chitin nanofibers and nanofibrils have the same typical microstructure of chitin found in crustacean and insects' exoskeleton [69,111,134]. Nanofibers are defined by Ifuku et al. as fibers with a diameter of less than 100 nm and an aspect ratio (length/width ratio) of more than 100 [135]. As nanofibrillar material, chitin has a highly ordered crystalline architecture, organized into several increasing structural levels. At the molecular level there are the polysaccharide chains, arranged in anti-parallel orientation (α-chitin), forming nanofibers or nanofibrils with a diameter of approximately 2–5 nm and a length of about 300 nm, each containing 18–25 chitin chains. These fibrils, stabilized by strong hydrogen bonds and wrapped with proteins, assemble into bundles of fibers or fibrils of larger diameter (approximately 50–300 nm). For the next level of chitin hierarchical organization, these bundles of fibrils, embedded into a protein–mineral matrix, align to form branched planes (chitin–protein layers). A twisted plywood-like structure, also called "Bouligand", is created by helicoidal stacking of these planar layers [134–138]. The structural organization of nanofibers and nanofibrils confers relevant morphological characteristics and mechanical properties (high mechanical strength) and unique optical properties [115,135,138,139].

Thanks to their properties, such as very high surface area (surface/volume ratio) and high porosity, chitin nanofibers represent good candidates as environmentally friendly nanomaterials usable in various applications [134,138]. The main challenges related to these nanofibers and nanofibrils are to develop new preparation methods (optimizing also the existing ones) and to improve their properties through chemical modification, in order to increase their uses in cosmetic, cosmeceutical and biomedical fields. Recently, Ifuku et al. managed to isolate chitin nanofibers with uniform structure, high aspect

ratio and a width of 10–20 nm from crabs, shrimps and prawns shells [7,8,140], and from mushrooms [141]. On the other hand, Morganti et al. isolated α-chitin nanocrystals from crustacean waste using a patented industrial process free of environmental impact [142,143]. Chitin nanofibrils (CNs), with an average size of 240 × 5 × 7 nm, represent the crystal-sugar molecular portion of the α-chitin separated from the protein portion. Despite their small size, CNs exhibits a large surface area (400 m^2/g) that allows them to interact with different cellular components. Moreover, the size factor and predominantly electropositive surface allow CNs to remain stably suspended in aqueous solution, ready to link other active molecules [144].

5.2. Cosmetic and Cosmeceutical Applications of Chitin-Based Nanomaterial

Recently, chitin nanofibers and nanofibrils have aroused great interest for their application in the biomedical and biotechnological fields as transdermal carriers of drugs and cosmetics, and dressing for wound healing and tissue engineering [9,145,146].

CNs are eco- and bio-compatible as they are natural compounds recognized and metabolized by human enzymes. In addition, they are of great biological value, being able to support hemostasis and regulate the inflammatory response, cell proliferation and the following collagen synthesis in the healing process [1,111]. When CNs come into contact with the stratum corneum, they are hydrolyzed by skin enzymes into smaller units (disaccharides and tetrasaccharides). The latter may undergo the process of re-polymerization once they reach the epidermis' inner layers and the dermis. The complete hydrolysis of CNs, on the other hand, results in the release of glucosamine and its acetylated derivative, N-acetylglucosamine (NAG), molecules that are involved in several cellular metabolic pathways and are fundamental for one's body health, particularly for the skin [1]. Glucosamine can, in turn, be further catabolized to supply the cellular metabolism with glucose and glutamic acid, its precursors, or can be used to limit the water loss, by retaining water at the dermal extra cellular matrix (ECM) level. Indeed, glycosaminoglycans, synthesized from glucosamine and N-acetylglucosamine, are involved in the maintenance of hydration in the dermis [1,111,144,147], and, on the other hand, represents the monosaccharide unit responsible for the structural stability of ECM because of the similarity between chitin and HA [148]. N-acetylglucosamine is not only able to reduce skin hyperpigmentation and thus reduce the appearance of spots through the inhibition of melanin production, but it is also effective in reducing wrinkles and the appearance of acne, and increasing skin exfoliation and hydration [149,150]. During the wound healing process, N-acetylglucosamine controls collagen synthesis, promoting tissue granulation and the proper repair of the skin lesions [5,144]. Therefore, CNs as cosmeceutical active compounds are able to promote skin health and beauty, keeping the skin perfectly hydrated by binding many water molecules, and contributing to the maintenance of the skin barrier integrity [1,113]. As a consequence, CNs contribute to two beneficial effects: the increasing of the skin capacity to fix water molecules (active strategy), protecting itself against environmental hazards, and a reduction of the continuous and unconscious loss of water from the body, known as *perspiratio insensibilis* (passive strategy) [111,144].

Chitin acts as an excellent cosmeceutical and cosmetic carrier as well, by loading the active ingredients ensuring their penetration through the skin, and targeting and releasing them at the right dose and site, thus improving their bioavailability and efficacy with minimal side effects [1,111]. Morganti et al. underlined how the efficacy of a cosmetic product depends not only on the active ingredients bound to the CNs, but also on the type of formulation in which these compounds are integrated. For an emulsion, the most common skin delivery system, the type created (micellar or lamellar, micro or nano-emulsions) is a crucial factor to obtain a transcutaneous, transdermal or transfollicular penetration of the active molecules [144,148]. In this way, it is possible to formulate multifunctional cosmetics capable of performing their action at the level of specific skin layers. For example, sunscreens have to act on the stratum corneum, antioxidants and anti-agings on the epidermis and dermis, deodorants on sweat glands, anti-hair loss or shampoos or

similar products on hair, whitening products on melanocytes [144,148]. On the other hand, the combination of positively charged CNs with electronegative polymers, such as HA and lignin (LG), may produce nano/micro-capsules/particles that can load and entrap various active ingredients, both hydro- and lipo-soluble, which can be embedded into emulsions or bound to non-woven tissue fibers. Thus, it is possible to obtain cosmetics and cosmeceuticals that can be used for UV protection, for hair care, but mainly to moisturize, rejuvenate, and whiten the skin, and finally to repair it in case of lesions [148,151,152]. The CN-HA and CN-LG complexes can easily encapsulate many active compounds such as drugs, vitamins, amino acids, antioxidants (lutein, melatonin, vitamin C, α-lipoic acid), immunomodulants (ectoine, ß-glucane) and normalizing agents (creatine, nicotinamide, urea), protecting them from the oxidative phenomena, increasing in their effectiveness and reducing side effects [148,151,152].

Specifically in the case of sunscreens, the main challenge involved the development of multifunctional emulsions in which CNs may act not only as active carriers but as boosters of antioxidant and immunomodulant compounds (such as lutein, melatonin and ectoine), to increase their photoprotective effect against UV-induced damages [144,148]. CNs combined with urocanic acid, a natural shielding agent against UVB radiation, show higher efficacy than conventional products [153]. In addition, Ito et al. demonstrated that the protective effect of CNs nanocrystals against UV radiation is attributable to their abilities to reduce the production of TGF-β (transforming growth factor beta), a multifunctional cytokine. TGF-β regulates the inflammatory processes to maintain the skin hydration, increasing the density of the granular epithelial layer (i.e., the epidermis layer that marks the transition between the innermost portion, consisting of living and active cells, and the outer part, consisting of dead cells) [154].

CNs have proven to have a repairing and anti-aging effect, not only on the skin, but also on the scalp [152]. This polymer is able to penetrate into the hair scales, bind to keratin and repair its fibers in depth, improving shine. The efficacy of shampoos in reducing dandruff and greasy hair and conditioners containing complexes of CNs with metal ions as zinc and other ionic compounds has also been verified by in vitro and in vivo studies [155]. Zinc is a mineral that plays an important role in the sebum regulation, and in the growth and repair of hair tissue. The combined treatment of shampoo and conditioner containing these complexes was found to achieve a stronger, brighter and more manageable hair [155]. The same complexes, thanks to their sebum-regulating and anti-inflammatory properties, are also effective in the treatment of seborrheic dermatitis [156].

Morganti et al. investigated the beneficial effects of both CNs alone and complexed with HA and LG as functional agents in various formulations to obtain skin aging and repairing effectiveness [113,151,152]. Anti-aging cosmetics, in fact, may promote skin renewal through a series of cellular processes including the differentiation and proliferation of keratinocytes and fibroblasts, the increase of their activity, the production of collagen, lipids and other ECM constituents [157]. CNs-based emulsions enriched with antioxidants and immunomodulants, such as lutein, melatonin and ectoine [158,159], as well as α-lipoic acid, urea, creatine and nicotinamide [157], have been effective in reducing wrinkles, spots and roughness, and increasing hydration and elasticity, resulting in younger-looking skin within weeks. CN-HA complexes have shown a greater moisturizing and rejuvenating effect compared to the vehicle alone (CNs). Moreover, they are excellent carriers, enabling the proper delivery of the active ingredients and their interaction with the epidermis layers. Thanks to these properties, once injected, these complexes could represent a valid support to the fillers activity in cosmetic surgery, as demonstrated by the complex of phosphatidylcholine and linoleic acid complexed with HA and CNs, loading, in turn, caffeine, cholesterol, amino acids, vitamins, creatine and melatonin [160]. Among the various molecules tested, the combination consisting of melatonin, vitamin E and ß-glucane (MEB) resulted to be efficient in mitigating the signs of both aging and photoaging. When complexed to form CN-HA nanoparticles (CN-HA-MEB) and administered both in form of capsule (orally) and in form of emulsion (topically) [161], it resulted in a greater increase of

the anti-inflammatory activity and collagen production compared to the CN-HA complexes applied topically and hydrocortisone alone [162,163]. Another objective of cosmeceuticals for skin care is to counteract the spots appearance on the skin face, especially when exposed to UV rays and other environmental hazards. In this case, formulations containing whitening agents, such as undecylenoyl phenylalanine, arbutin and diacetyl boldine, complexed to CN-HA, help women with hyperpigmented skin by inhibiting the synthesis and transfer of melanin to keratinocytes [164].

In addition to HA, CN can be combined with another electronegative polymer, such as LG, to form nanoparticles incorporated into emulsions or biofunctional fabrics in order to repair skin damages related to aging, injuries and wounds [151,165]. LG is a natural polyphenolic polymer representing 15–25% of the plant cell wall, having antioxidant, antimicrobial and photoprotective properties. These beneficial properties, combined with the rejuvenating activity of CNs, confer to CN-LG complexes an anti-inflammatory and cicatrizing effect [165,166]. Indeed, these complexes, combining with glycyrrhetinic acid, an anti-inflammatory agent, act on the production of cytokines enhancing their bio-effectiveness [151].

The innovative and relevant aspect of the CN-LG complexes, compared to CN-HA complexes, is their use for the production of smart non-woven tissues that represent a valid alternative delivery system to the emulsion. Their composition (including the presence of CN and LG biopolymers) is biocompatible, biodegradable and free of preservatives and other chemical components that may cause irritation and sensitization. Moreover, they are able to mimic the structure and function of the ECM, promoting skin protection, health and repair [151,165,167]. Nowadays, these smart tissues, impregnated with active ingredients, are the basic materials for the formulation of beauty masks, a category of cosmeceuticals widely used by women for their bio-nature and convenience of use. Formulated as a lotion, cream, gel, sheet or beauty masks, they complement the skin care routine by improving hydration, treating possible skin spots and other disorders and slowing the appearance of various signs of aging and photoaging, depending on the used ingredients [167,168]. For example, vitamin C, melatonin and ß-glucane complexed to CN-LG nanoparticles increased the bio-effectiveness of the mask against photo-aging, maintaining the effect until one month [165]. The presence of poly(ethylene) oxide, on the other hand, in CN-LG non-woven fabric, has shown an in vitro potential. Medications obtained by incorporating CN-LG non-woven fabrics in poly(ethylene) oxide alone [166], and in a chitosan and poly(ethylene) oxide matrix [169], have shown antibacterial activity and shown to promote the repair of injured and burned skin also.

Preparing dressings for wound care, it is important to consider the biochemical and physiological processes that characterize the steps sequence of the healing process, such as hemostasis, inflammation, proliferation and remodeling [170]. Therefore, it has been observed that chitosan has increased its hemostatic properties through the introduction of CNs into the formulation [171]. The synergistic effect of the two biopolymers has been exploited for the design of a new medical material [171,172]. It includes two layers, one external and a non-resorbable one, made of aliphatic copolyamide nanofibers which confers mechanical support, and one internal, made of chitosan nanofibers and CNs. The latter, in contact with the skin damage, is gradually resorbed, facilitating re-epithelialization. The efficacy of these dressings in the treatment of skin injuries and burns, and therefore their potential application in medical practice, has been evaluated and confirmed through in vivo tests on mice [171,172]. Similar effects, controlling also the other phases of the skin regenerative process, besides hemostasis, were observed using three different formulations, such as spray, gel and gauze, based on CN and chitosan glycolate, each acting differently on the wound. The spray was more effective on large superficial wounds, including abrasions, while the gel took longer to aesthetically improve wounds in more delicate areas, such as the face; on the other hand, gauze was the best device in promoting complete healing of deeper wounds, requiring even longer cicatrization times [173,174]. Izumi et al. studied the biomedical efficacy of a promising functional derivative known as superficially

deacetylated CNs (SDACNFs), obtained by chemical deacetylation of the CNs surface. The results showed that SDACNFs not only accelerated the early stages of the regenerative process, but also promoted the proliferative (fibroblast migration and collagen synthesis) and final remodeling (re-epithelialization and scar tissue formation) stages, compared with CNs and chitosan in nanofibrillar form [175].

All these applications with CNs and chitin nanofibrils, alone or complexed with other bioactive molecules, including HA and LG, prepared in different formulations for skin aging and repair process, could be reproduced with insect chitin. Although no studies are currently in an advanced phase, insects could be an alternative, innovative and really promising source to extract chitin and transform it into nanofibers or nanofibrils and perform the same experiments performed with chitin from crustacean. As well as the fact that insect chitin and chitosan have the same characteristics as those of crustaceans, both CNs and nanofibers from insects have the same microstructure of those produced from crustacean, allowing to hypothesize very similar properties (antimicrobial, antioxidant, moisturizing and anti-inflammatory characteristics, biocompatibility, biodegradability and wound dressing).

6. Conclusions

Chitin and its derivatives are versatile materials useful for many applications: agriculture, food, the textile sector and wastewater purification are just to report some of the most widespread. However, the biomedical, pharmaceutical and cosmetic fields, as related to personal care, are among those of greatest interest for their use. Nanomaterials based-systems, in which chitin and its derivatives are used (such as nanofibers, nanoparticles), seem to be particularly effective for the delivery of active ingredients, ensuring a site-specific action of cosmetic and cosmeceutical products. Recently, the conventional industrial source of chitin (crustacean waste from the fishing industry) has become no longer sustainable and no longer sufficiently able to cope with the ever-increasing demand for chitin and related products. With zero environmental impact and easy reproducibility, insects are emerging as the most promising source of chitin alternative to crustaceans, although they have still received little attention, especially on an industrial scale. Insect species able to bioconvert organic waste into valuable products, first and foremost *H. illucens*, are the most advantageous solution and are already widely bred worldwide. Indeed, *H. illucens* breeding facilities, in addition to producing proteins and lipids, generate a large amount of chitin-rich insect waste, chiefly exuviae of larvae and pupae, and dead adults that could be exploited for chitin extraction, in the frame of a zero-waste production process. Due to the great sustainability of insect breeding, especially of *H. illucens* larvae, insect chitin could be the best alternative to crustacean source. Moreover, yields and characteristics of insect chitin, and particularly from *H. illucens*, are comparable to the commercially available chitin derived from crustaceans.

This preludes to its potential use in the same applications already consolidated for crustacean chitin, including cosmetics and cosmeceuticals, also in the form of nanofibers, nanofibrils or nanoparticles, and thus develop increasingly innovative and effective products. However, there are still some points to further investigate for the optimal use of this biopolymer, especially as cosmeceutical. The main challenges for the scientific community regarding insect chitin (and its derivatives) include the optimization and scale-up of its purification process from insect waste, and the development of suitable procedures to improve characterization, not only chemical–physical but also biological, with the support of in vitro and in vivo tests. Moreover, the critical points of the cosmetic production with the addition of chitin and its derivatives will be analyzed and evaluated, related to their properties, solubility and methodology of incorporation in the final formulations, in order to improve their bio-effectiveness.

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References

1. Morganti, P.; del Ciotto, P.; Morganti, G.; Fabien-Soule, V. Application of Chitin Nanofibrils and Collagen of Marine Origin as Bioactive Ingredients. In *Marine Cosmeceuticals: Trends and Prospects*; Kim, S.K., Ed.; CRC Press: Boca Raton, FL, USA, 2012; pp. 267–289.
2. Aranaz, I.; Acosta, N.; Civera, C.; Elorza, B.; Mingo, J.; Castro, C.; Gandía, M.D.L.L.; Caballero, A.H. Cosmetics and cosmeceutical applications of chitin, chitosan and their derivatives. *Polymers* **2018**, *10*, 213. [CrossRef]
3. Regulation (CE) No. 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. *Off. J. Eur. Union* **2009**, *342*, 59–209.
4. Elsner, P.; Maibach, H.I. (Eds.) . *Cosmeceuticals: Drugs vs. Cosmetics*; Marcel Dekker, Inc.: New York, NY, USA, 2000; Volume 23.
5. Morganti, P. Cosmeceuticals. *Clin. Dermatol.* **2008**, *26*, 317. [CrossRef] [PubMed]
6. Kaul, S.; Gulati, N.; Verma, D.; Mukherjee, S.; Nagaich, U. Role of Nanotechnology in Cosmeceuticals: A Review of Recent Advances. *J. Pharm.* **2018**, *2018*, 1–19. [CrossRef] [PubMed]
7. Ifuku, S.; Nogi, M.; Abe, K.; Yoshioka, M.; Morimoto, M.; Saimoto, H.; Yano, H. Preparation of chitin nanofibers with a uniform width as α-chitin from crab shells. *Biomacromolecules* **2009**, *10*, 1584–1588. [CrossRef]
8. Ifuku, S.; Nogi, M.; Abe, K.; Yoshioka, M.; Morimoto, M.; Saimoto, H.; Yano, H. Simple preparation method of chitin nanofibers with a uniform width of 10–20 nm from prawn shell under neutral conditions. *Carbohydr. Polym.* **2011**, *84*, 762–764. [CrossRef]
9. Kamble, P.; Sadarani, B.; Majumdar, A.; Bhullar, S. Nanofiber based drug delivery systems for skin: A promising therapeutic approach. *J. Drug Deliv. Sci. Technol.* **2017**, *41*, 124–133. [CrossRef]
10. Casadidio, C.; Peregrina, D.V.; Gigliobianco, M.R.; Deng, S.; Censi, R.; Di Martino, P. Chitin and chitosans: Characteristics, eco-friendly processes, and applications in cosmetic science. *Mar. Drugs* **2019**, *17*, 369. [CrossRef]
11. Bakshi, P.S.; Selvakumar, D.; Kadirvelu, K.; Kumar, N.S. Chitosan as an environment friendly biomaterial—A review on recent modifications and applications. *Int. J. Biol. Macromol.* **2020**, *150*, 1072–1083. [CrossRef]
12. Crini, G. Historical review on chitin and chitosan biopolymers. *Environ. Chem. Lett.* **2019**, *17*, 1623–1643. [CrossRef]
13. Khoushab, F.; Yamabhai, M. Chitin Research Revisited. *Mar. Drugs* **2010**, *8*, 1988–2012. [CrossRef]
14. Pillai, C.K.S.; Paul, W.; Sharma, C.P. Chitin and chitosan polymers: Chemistry, solubility and fiber formation. *Prog. Polym. Sci.* **2009**, *34*, 641–678. [CrossRef]
15. Dutta, P.K.; Dutta, J.; Tripathi, V.S. Chitin and chitosan: Chemistry, properties and applications. *J. Sci. Ind. Res.* **2004**, *63*, 20–31.
16. Goy, R.C.; De Britto, D.; Assis, O.B.G. A review of the antimicrobial activity of chitosan. *Polimeros* **2009**, *19*, 241–247. [CrossRef]
17. Austin, P.R. Chitin solutions and purification of chitin. *Methods Enzym.* **1988**, *161*, 403–407. [CrossRef]
18. Rinaudo, M. Chitin and chitosan: Properties and applications. *Prog. Polym. Sci.* **2006**, *31*, 603–632. [CrossRef]
19. Singh, S.K. Solubility of lignin and chitin in ionic liquids and their biomedical applications. *Int. J. Biol. Macromol.* **2019**, *132*, 265–277. [CrossRef]
20. Jaworska, M.M.; Kozlecki, T.; Gorak, A. Review of the application of ionic liquids as solvents for chitin. *J. Polym. Eng.* **2012**, *32*, 67–69. [CrossRef]
21. Roy, J.C.; Salaün, F.; Giraud, S.; Ferri, A.; Chen, G.; Guan, J. Solubility of Chitin: Solvents, Solution Behaviors and Their Related Mechanisms. *Solubility Polysacch.* **2017**, 10. [CrossRef]
22. Aranaz, I.; Mengíbar, M.; Harris, R.; Paños, I.; Miralles, B.; Acosta, N.; Galed, G.; Heras, Á. Functional Characterization of Chitin and Chitosan. *Curr. Chem. Biol.* **2009**, *3*, 203–230.
23. Rasul, R.M.; Tamilarasi Muniandy, M.; Zakaria, Z.; Shah, K.; Chee, C.F.; Dabbagh, A.; Rahman, N.A.; Wong, T.W. A review on chitosan and its development as pulmonary particulate anti-infective and anti-cancer drug carriers. *Carbohydr. Polym.* **2020**, *250*, 116800. [CrossRef]
24. Vaz, J.M.; Taketa, T.B.; Hernandez-Montelongo, J.; Chevallier, P.; Cotta, M.A.; Mantovani, D.; Beppu, M.M. Antibacterial properties of chitosan-based coatings are affected by spacer-length and molecular weight. *Appl. Surf. Sci.* **2018**, *445*, 478–487. [CrossRef]

25. De Queiroz Antonino, R.S.C.M.; Lia Fook, B.R.P.; De Oliveira Lima, V.A.; De Farias Rached, R.Í.; Lima, E.P.N.; Da Silva Lima, R.J.; Peniche Covas, C.A.; Lia Fook, M.V. Preparation and Characterization of Chitosan Obtained from Shells of Shrimp (*Litopenaeus vannamei* Boone). *Mar. Drugs* **2017**, *15*, 141. [CrossRef]
26. Hossain, M.; Iqbal, A. Production and characterization of chitosan from shrimp waste. *J. Bangladesh Agric. Univ.* **2014**, *12*, 153–160. [CrossRef]
27. Ravi Kumar, M.N.V. A review of chitin and chitosan applications. *React. Funct. Polym.* **2000**, *46*, 1–27. [CrossRef]
28. Jang, M.K.; Kong, B.G.; Il Jeong, Y.; Lee, C.H.; Nah, J.W. Physicochemical characterization of α-chitin, β-chitin, and γ-chitin separated from natural resources. *J. Polym. Sci. Part A Polym. Chem.* **2004**, *42*, 3423–3432. [CrossRef]
29. Minke, R.; Blackwell, J. The structure of α-chitin. *J. Mol. Biol.* **1978**, *120*, 167–181. [CrossRef]
30. Pighinelli, L. Methods of Chitin Production a Short Review. *Am. J. Biomed. Sci. Res.* **2019**, *3*, 307–314. [CrossRef]
31. Zargar, V.; Asghari, M.; Dashti, A. A Review on Chitin and Chitosan Polymers: Structure, Chemistry, Solubility, Derivatives, and Applications. *ChemBioEng Rev.* **2015**, *2*, 204–226. [CrossRef]
32. Okamoto, Y.; Kawakami, K.; Miyatake, K.; Morimoto, M.; Shigemasa, Y.; Minami, S. Analgesic effects of chitin and chitosan. *Carbohydr. Polym.* **2002**, *49*, 249–252. [CrossRef]
33. Azuma, K.; Nishihara, M.; Shimizu, H.; Itoh, Y.; Takashima, O.; Osaki, T.; Itoh, N.; Imagawa, T.; Murahata, Y.; Tsuka, T.; et al. Biological adhesive based on carboxymethyl chitin derivatives and chitin nanofibers. *Biomaterials* **2015**, *42*, 20–29. [CrossRef] [PubMed]
34. Jayakumar, R.; Prabaharan, M.; Nair, S.V.; Tokura, S.; Tamura, H.; Selvamurugan, N. Novel carboxymethyl derivatives of chitin and chitosan materials and their biomedical applications. *Prog. Mater. Sci.* **2010**, *55*, 675–709. [CrossRef]
35. Barikani, M.; Oliaei, E.; Seddiqi, H.; Honarkar, H. Preparation and application of chitin and its derivatives: A review. *Iran. Polym. J.* **2014**, *23*, 307–326. [CrossRef]
36. El Hadrami, A.; Adam, L.R.; El Hadrami, I.; Daayf, F. Chitosan in plant protection. *Mar. Drugs* **2010**, *8*, 968–987. [CrossRef] [PubMed]
37. Shahidi, F.; Arachchi, J.K.V.; Jeon, Y.J. Food applications of chitin and chitosans. *Trends Food Sci. Technol.* **1999**, *10*, 37–51. [CrossRef]
38. Da Silva, S.B.; De Souza, D.; Lacerda, L.D. Food applications of chitosan and its derivatives. *Chitin Chitosan Prop. Appl.* **2010**, *55*, 675–709.
39. Duan, C.; Meng, X.; Meng, J.; Khan, M.I.H.; Dai, L.; Khan, A.; An, X.; Zhang, J.; Huq, T.; Ni, Y. Chitosan as A Preservative for Fruits and Vegetables: A Review on Chemistry and Antimicrobial Properties. *Chitosan A Preserv. Fruits Veg. A Rev. Chem. Antimicrob. Prop.* **2019**, *4*, 11–21. [CrossRef]
40. Al-Manhel, A.J.; Al-Hilphy, A.R.S.; Niamah, A.K. Extraction of chitosan, characterisation and its use for water purification. *J. Saudi Soc. Agric. Sci.* **2018**, *17*, 186–190. [CrossRef]
41. Jayakumar, R.; Menon, D.; Manzoor, K.; Nair, S.V.; Tamura, H. Biomedical applications of chitin and chitosan based nanomaterials—A short review. *Carbohydr. Polym.* **2010**, *82*, 227–232. [CrossRef]
42. Yang, Y.M.; Hu, W.; Wang, X.D.; Gu, X.S. The controlling biodegradation of chitosan fibers by N-acetylation in vitro and in vivo. *J. Mater. Sci. Mater. Med.* **2007**, *18*, 2117–2121. [CrossRef]
43. Tao, F.; Cheng, Y.; Shi, X.; Zheng, H.; Du, Y.; Xiang, W.; Deng, H. Applications of chitin and chitosan nanofibers in bone regenerative engineering. *Carbohydr. Polym.* **2020**, *230*, 115658. [CrossRef] [PubMed]
44. Matica, M.A.; Aachmann, F.L.; Tøndervik, A.; Sletta, H.; Ostafe, V. Chitosan as a wound dressing starting material: Antimicrobial properties and mode of action. *Int. J. Mol. Sci.* **2019**, *20*, 5889. [CrossRef] [PubMed]
45. Sarode, S.; Upadhyay, P.; Khosa, M.A.; Mak, T.; Shakir, A.; Song, S.; Ullah, A. Overview of wastewater treatment methods with special focus on biopolymer chitin-chitosan. *Int. J. Biol. Macromol.* **2019**, *121*, 1086–1100. [CrossRef]
46. Younes, I.; Rinaudo, M. Chitin and chitosan preparation from marine sources. Structure, properties and applications. *Mar. Drugs* **2015**, *13*, 1133–1174. [CrossRef] [PubMed]
47. Xu, Y.; Gallert, C.; Winter, J. Chitin purification from shrimp wastes by microbial deproteination and decalcification. *Appl. Microbiol. Biotechnol.* **2008**, *79*, 687–697. [CrossRef] [PubMed]
48. Gortari, M.C.; Hours, R.A. Biotechnological processes for chitin recovery out of crustacean waste: A mini-review. *Electron. J. Biotechnol.* **2013**, *16*, 14.
49. Kurita, K. Chitin and chitosan: Functional biopolymers from marine crustaceans. *Mar. Biotechnol.* **2006**, *8*, 203–226. [CrossRef]
50. Pittman, S.J.; McAlpine, C.A. Movements of marine fish and decapod crustaceans: Process, theory and application. *Adv. Mar. Biol.* **2003**, *44*, 205–294. [CrossRef]
51. Gillett, R. Global study of shrimp fisheries. In *Food and Agriculture Organization of the United Nations Fisheries*; Technical Paper No. 475; FAO: Rome, Italy, 2008; p. 331.
52. Abidin, N.A.Z.; Kormin, F.; Abidin, N.A.Z.; Anuar, N.A.F.M.; Bakar, M.F.A. The potential of insects as alternative sources of chitin: An overview on the chemical method of extraction from various sources. *Int. J. Mol. Sci.* **2020**, *21*, 1–25.
53. Philibert, T.; Lee, B.H.; Fabien, N. Current Status and New Perspectives on Chitin and Chitosan as Functional Biopolymers. *Appl. Biochem. Biotechnol.* **2017**, *181*, 1314–1337. [CrossRef]
54. Abdel-Gawad, K.M.; Hifney, A.F.; Fawzy, M.A.; Gomaa, M. Technology optimization of chitosan production from *Aspergillus niger* biomass and its functional activities. *Food Hydrocoll.* **2017**, *63*, 593–601. [CrossRef]

55. Waśko, A.; Bulak, P.; Polak-Berecka, M.; Nowak, K.; Polakowski, C.; Bieganowski, A. The first report of the physicochemical structure of chitin isolated from Hermetia illucens. *Int. J. Biol. Macromol.* **2016**, *92*, 316–320. [CrossRef] [PubMed]
56. Nemtsev, S.V.; Zueva, O.Y.; Khismatullin, M.R.; Albulov, A.I.; Varlamov, V.P. Isolation of chitin and chitosan from honeybees. *Appl. Biochem. Microbiol.* **2004**, *40*, 39–43. [CrossRef]
57. Zhang, M.; Haga, A.; Sekiguchi, H.; Hirano, S. Structure of insect chitin isolated from beetle larva cuticle and silkworm (*Bombyx mori*) pupa exuvia. *Int. J. Biol. Macromol.* **2000**, *27*, 99–105. [CrossRef]
58. Badawy, R.; Mohamed, H. Chitin extration, Composition of Different Six Insect Species and Their Comparable Characteristics with That of the Shrimp. *J. Am. Sci.* **2015**, *11*, 127.
59. Meky, N.; Badawy, R.; Mohamed, H.; Samir, S. Extraction of chitin from six different insect species as alternative source for biological applications. *Afr. J. Biol. Sci.* **2017**, *13*, 197–205. [CrossRef]
60. Luo, Q.; Wang, Y.; Han, Q.; Ji, L.; Zhang, H.; Fei, Z.; Wang, Y. Comparison of the physicochemical, rheological, and morphologic properties of chitosan from four insects. *Carbohydr. Polym.* **2019**, *209*, 266–275. [CrossRef] [PubMed]
61. Song, C.; Yu, H.; Zhang, M.; Yang, Y.; Zhang, G. Physicochemical properties and antioxidant activity of chitosan from the blowfly *Chrysomya megacephala* larvae. *Int. J. Biol. Macromol.* **2013**, *60*, 347–354. [CrossRef]
62. Ai, H.; Wang, F.; Yang, Q.; Zhu, F.; Lei, C. Preparation and biological activities of chitosan from the larvae of housefly, Musca domestica. *Carbohydr. Polym.* **2008**, *72*, 419–423. [CrossRef]
63. Cortes Ortiz, J.A.; Ruiz, A.T.; Morales-Ramos, J.A.; Thomas, M.; Rojas, M.G.; Tomberlin, J.K.; Yi, L.; Han, R.; Giroud, L.; Jullien, R.L. Insect Mass Production Technologies. In *Insects as Sustainable Food Ingredients*; Elsevier: Amsterdam, The Netherlands, 2016; pp. 153–201.
64. Ganguly, S.; Neog, P.; Gogoi, M.; Bordoloi, P.L.; Para, P.A. Edible Insects as Sources of Novel Bioactive Compounds. In *Recent Research Trends in Veterinary Sciences and Animal Husbandry*; AkiNik Publications: Delhi, India, 2018; pp. 55–69.
65. Moretta, A.; Salvia, R.; Scieuzo, C.; Di Somma, A.; Vogel, H.; Pucci, P.; Sgambato, A.; Wolff, M.; Falabella, P. A bioinformatic study of antimicrobial peptides identified in the Black Soldier Fly (BSF) *Hermetia illucens* (Diptera: Stratiomyidae). *Sci. Rep.* **2020**, *10*, 16875. [CrossRef]
66. Manniello, M.D.; Moretta, A.; Salvia, R.; Scieuzo, C.; Lucchetti, D.; Vogel, H.; Sgambato, A.; Falabella, P. Insect antimicrobial peptides: Potential weapons to counteract the antibiotic resistance. *Cell. Mol. Life Sci.* **2021**, *1*, 3.
67. Davies, R.G. Insect structure and function. In *Outlines of Entomology*; Springer: Dordrecht, The Netherlands, 1988; pp. 7–96. [CrossRef]
68. Gunderson, S.; Schiavone, R. The insect exoskeleton: A natural structural composite. *JOM* **1989**, *41*, 60–63. [CrossRef]
69. Vincent, J.F.V.; Wegst, U.G.K. Design and mechanical properties of insect cuticle. *Arthropod Struct. Dev.* **2004**, *33*, 187–199. [CrossRef] [PubMed]
70. Chandran, R.; Williams, L.; Hung, A.; Nowlin, K.; La Jeunesse, D. SEM characterization of anatomical variation in chitin organization in insect and arthropod cuticles. *Micron* **2016**, *82*, 74–85. [CrossRef] [PubMed]
71. Jantzen da Silva Lucas, A.; Menegon de Oliveira, L.; da Rocha, M.; Prentice, C. Edible insects: An alternative of nutritional, functional and bioactive compounds. *Food Chem.* **2020**, *311*, 126022. [CrossRef]
72. Derrien, C.; Boccuni, A. Current status of the insect producing industry in Europe. In *Edible Insects in Sustainable Food Systems*; Springer: Cham, Switzerland, 2018; pp. 471–479.
73. Scala, A.; Cammack, J.A.; Salvia, R.; Scieuzo, C.; Franco, A.; Bufo, S.A.; Tomberlin, J.K.; Falabella, P. Rearing substrate impacts growth and macronutrient composition of *Hermetia illucens* (L.) (Diptera: Stratiomyidae) larvae produced at an industrial scale. *Sci. Rep.* **2020**, *10*, 1–8. [CrossRef]
74. Bava, L.; Jucker, C.; Gislon, G.; Lupi, D.; Savoldelli, S.; Zucali, M.; Colombini, S. Rearing of hermetia illucens on different organic by-products: Influence on growth, waste reduction, and environmental impact. *Animals* **2019**, *9*, 289. [CrossRef]
75. Jucker, C.; Lupi, D.; Moore, C.D.; Leonardi, M.G.; Savoldelli, S. Nutrient recapture from insect farm waste: Bioconversion with *Hermetia illucens* (L.)(Diptera: Stratiomyidae). *Sustainability* **2020**, *12*, 362. [CrossRef]
76. Schmitt, E.; de Vries, W. Potential benefits of using Hermetia illucens frass as a soil amendment on food production and for environmental impact reduction. *Curr. Opin. Green Sustain. Chem.* **2020**, *25*, 100335. [CrossRef]
77. Mohammed, M.H.; Williams, P.A.; Tverezovskaya, O. Extraction of chitin from prawn shells and conversion to low molecular mass chitosan. *Food Hydrocoll.* **2013**, *31*, 166–171. [CrossRef]
78. El Knidri, H.; Belaabed, R.; Addaou, A.; Laajeb, A.; Lahsini, A. Extraction, chemical modification and characterization of chitin and chitosan. *Int. J. Biol. Macromol.* **2018**, *120*, 1181–1189. [CrossRef] [PubMed]
79. Kumari, S.; Rath, P.; Sri Hari Kumar, A.; Tiwari, T.N. Extraction and characterization of chitin and chitosan from fishery waste by chemical method. *Environ. Technol. Innov.* **2015**, *3*, 77–85. [CrossRef]
80. Hahn, T.; Tafi, E.; Paul, A.; Salvia, R.; Falabella, P.; Zibek, S. Current state of chitin purification and chitosan production from insects. *J. Chem. Technol. Biotechnol.* **2020**, *95*, 2775–2795. [CrossRef]
81. Kaya, M.; Lelešius, E.; Nagrockaite, R.; Sargin, I.; Arslan, G.; Mol, A.; Baran, T.; Can, E.; Bitim, B. Differentiations of Chitin content and surface morphologies of chitins extracted from male and female grasshopper species. *PLoS ONE* **2015**, *10*, e115531. [CrossRef] [PubMed]
82. Kaya, M.; Bulut, E.; Mujtaba, M.; Sivickis, K.; Sargin, I.; Akyuz, B.; Erdogan, S. Gender Influences Differentiation of Chitin Among Body Parts. *Arch. Insect Biochem. Physiol.* **2016**, *93*, 96–109. [CrossRef] [PubMed]

83. Kaya, M.; Baran, T.; Erdoğan, S.; Menteş, A.; Aşan Özüsağlam, M.; Çakmak, Y.S. Physicochemical comparison of chitin and chitosan obtained from larvae and adult Colorado potato beetle (*Leptinotarsa decemlineata*). *Mater. Sci. Eng. C* **2014**, *45*, 72–81. [CrossRef] [PubMed]
84. Erdogan, S.; Kaya, M. High similarity in physicochemical properties of chitin and chitosan from nymphs and adults of a grasshopper. *Int. J. Biol. Macromol.* **2016**, *89*, 118–126. [CrossRef] [PubMed]
85. Tharanathan, R.N.; Kittur, F.S. Chitin—The Undisputed Biomolecule of Great Potential. *Crit. Rev. Food Sci. Nutr.* **2003**, *43*, 61–87. [CrossRef]
86. Thirunavukkarasu, N.; Shanmugam, A. Extraction of Chitin and Chitosan From Mud Crab Scylla Tranquebarica (Fabricius, 1798). *Int. J. Appl. Bio-Eng.* **2009**, *3*, 31–33. [CrossRef]
87. Kaya, M.; Baublys, V.; Can, E.; Šatkauskienė, I.; Bitim, B.; Tubelytė, V.; Baran, T. Comparison of physicochemical properties of chitins isolated from an insect (*Melolontha melolontha*) and a crustacean species (*Oniscus asellus*). *Zoomorphology* **2014**, *133*, 285–293. [CrossRef]
88. Oduor-Odeto, P.M.; Struszezyk, M.H.; Peter, M.G. Characterisation of Chitosan from Blowfly Larvae and Some Crustacean Species from Kenyan Marin Waters Prepared Under Different Conditions. *West. Indian Ocean J. Mar. Sci.* **2007**, *4*, 99–108. [CrossRef]
89. Bolat, Y.; Bilgin, Ş.; Günlü, A.; Izci, L.; Koca, S.B.; Çetinkaya, S.; Koca, H.U. Chitin-Chitosan Yield of Freshwater Crab (Potamon potamios, Olivier 1804) Shell. *Pak. Vet. J.* **2010**, *30*, 227–231.
90. Liu, S.; Sun, J.; Yu, L.; Zhang, C.; Bi, J.; Zhu, F.; Qu, M.; Jiang, C.; Yang, Q. Extraction and characterization of chitin from the beetle Holotrichia parallela motschulsky. *Molecules* **2012**, *17*, 4604–4611. [CrossRef] [PubMed]
91. Kaya, M.; Baran, T.; Asan-Ozusaglam, M.; Cakmak, Y.S.; Tozak, K.O.; Mol, A.; Mentes, A.; Sezen, G. Extraction and characterization of chitin and chitosan with antimicrobial and antioxidant activities from cosmopolitan Orthoptera species (Insecta). *Biotechnol. Bioprocess Eng.* **2015**, *20*, 168–179. [CrossRef]
92. Kaya, M.; Bağriaçik, N.; Seyyar, O.; Baran, T. Comparison of chitin structures derived from three common wasp species (vespa crabro linnaeus, 1758, vespa orientalis linnaeus, 1771 and vespula germanica (FABRICIUS, 1793)). *Arch. Insect Biochem. Physiol.* **2015**, *89*, 204–217. [CrossRef] [PubMed]
93. Draczynski, Z. Honeybee corpses as an available source of chitin. *J. Appl. Polym. Sci.* **2008**, *109*, 1974–1981. [CrossRef]
94. Sajomsang, W.; Gonil, P. Preparation and characterization of α-chitin from cicada sloughs. *Mater. Sci. Eng. C* **2010**, *30*, 357–363. [CrossRef]
95. Ibitoye, E.B.; Lokman, I.H.; Hezmee, M.N.M.; Goh, Y.M.; Zuki, A.B.Z.; Jimoh, A.A. Extraction and physicochemical characterization of chitin and chitosan isolated from house cricket. *Biomed. Mater.* **2018**, *13*, 25009. [CrossRef]
96. Majtán, J.; Bíliková, K.; Markovič, O.; Gróf, J.; Kogan, G.; Šimúth, J. Isolation and characterization of chitin from bumblebee (*Bombus terrestris*). *Int. J. Biol. Macromol.* **2007**, *40*, 237–241. [CrossRef]
97. Kaya, M.; Mujtaba, M.; Bulut, E.; Akyuz, B.; Zelencova, L.; Sofi, K. Fluctuation in physicochemical properties of chitins extracted from different body parts of honeybee. *Carbohydr. Polym.* **2015**, *132*, 9–16. [CrossRef]
98. Kaya, M.; Sofi, K.; Sargin, I.; Mujtaba, M. Changes in physicochemical properties of chitin at developmental stages (larvae, pupa and adult) of Vespa crabro (wasp). *Carbohydr. Polym.* **2016**, *145*, 64–70. [CrossRef] [PubMed]
99. Yeul, V.S.; Rayalu, S.S. Unprecedented Chitin and Chitosan: A Chemical Overview. *J. Polym. Environ.* **2013**, *21*, 606–614. [CrossRef]
100. Win, N.N.; Stevens, W.F. Shrimp chitin as substrate for fungal chitin deacetylase. *Appl. Microbiol. Biotechnol.* **2001**, *57*, 334–341. [CrossRef] [PubMed]
101. Wattjes, J.; Niehues, A.; Cord-Landwehr, S.; Hoßbach, J.; David, L.; Delair, T.; Moerschbacher, B.M. Enzymatic Production and Enzymatic-Mass Spectrometric Fingerprinting Analysis of Chitosan Polymers with Different Nonrandom Patterns of Acetylation. *J. Am. Chem. Soc.* **2019**, *141*, 3137–3145. [CrossRef]
102. Martinou, A.; Kafetzopoulos, D.; Bouriotis, V. Chitin deacetylation by enzymatic means: Monitoring of deacetylation processes. *Carbohydr. Res.* **1995**, *273*, 235–242. [CrossRef]
103. Cortizo, M.S.; Berghoff, C.F.; Alessandrini, J.L. Characterization of chitin from Illex argentinus squid pen. *Carbohydr. Polym.* **2008**, *74*, 10–15. [CrossRef]
104. De Castro, R.J.S.; Ohara, A.; Aguilar, J.G.D.S.; Domingues, M.A.F. Nutritional, functional and biological properties of insect proteins: Processes for obtaining, consumption and future challenges. *Trends Food Sci. Technol.* **2018**, *76*, 82–89. [CrossRef]
105. Jaworska, M.; Sakurai, K.; Gaudon, P.; Guibal, E. Influence of chitosan characteristics on polymer properties. I: Crystallographic properties. *Polym. Int.* **2003**, *52*, 198–205. [CrossRef]
106. Islam, S.; Bhuiyan, M.A.R.; Islam, M.N. Chitin and Chitosan: Structure, Properties and Applications in Biomedical Engineering. *J. Polym. Environ.* **2017**, *25*, 854–866. [CrossRef]
107. Zivanovic, S.; Basurto, C.C.; Chi, S.; Davidson, P.M.; Weiss, J. Molecular weight of chitosan influences antimicrobial activity in oil-in-water emulsions. *J. Food Prot.* **2004**, *67*, 952–959. [CrossRef]
108. Vishu Kumar, A.B.; Varadaraj, M.C.; Gowda, L.R.; Tharanathan, R.N. Characterization of chito-oligosaccharides prepared by chitosanolysis with the aid of papain and Pronase, and their bactericidal action against *Bacillus cereus* and *Escherichia coli*. *Biochem. J.* **2005**, *391*, 167–175. [CrossRef] [PubMed]
109. Kim, M.W.; Song, Y.S.; Han, Y.S.; Jo, Y.H.; Choi, M.H.; Park, Y.K.; Kang, S.H.; Kim, S.A.; Choi, C.; Jung, W.J. Production of chitin and chitosan from the exoskeleton of adult two-spotted field crickets (*Gryllus bimaculatus*). *Entomol. Res.* **2017**, *47*, 279–285. [CrossRef]

110. Lohani, A.; Verma, A.; Joshi, H.; Yadav, N.; Karki, N. Nanotechnology-Based Cosmeceuticals. *ISRN Dermatol.* **2014**, 1–14. [CrossRef]
111. Morganti, P.; Morganti, G. Chitin nanofibrils for advanced cosmeceuticals. *Clin. Dermatol.* **2008**, *26*, 334–340. [CrossRef] [PubMed]
112. Sajna, K.V.; Gottumukkala, L.D.; Sukumaran, R.K.; Pandey, A. White Biotechnology in Cosmetics. In *Industrial Biorefineries and White Biotechnology*; Elsevier: Amsterdam, The Netherlands, 2015; pp. 607–652.
113. Morganti, P.; Muzzarelli, R.A.A.; Muzzarelli, C. Multifunctional use of innovative chitin nanofibrils for skin care. *J. Appl. Cosmetol.* **2006**, *24*, 105–114.
114. Ngo, D.H.; Kim, S.K. *Antioxidant Effects of Chitin, Chitosan, and Their Derivatives*, 1st ed.; Elsevier: Amsterdam, The Netherlands, 2014; Volume 73, pp. 15–31.
115. Azuma, K.; Ifuku, S.; Osaki, T.; Okamoto, Y.; Minami, S. Preparation and biomedical applications of chitin and chitosan nanofibers. *J. Biomed. Nanotechnol.* **2014**, *10*, 2891–2920. [CrossRef] [PubMed]
116. Hernández-Rivas, M.; Guzmán, E.; Fernández-Peña, L.; Akanno, A.; Greaves, A.; Léonforte, F.; Ortega, F.; Rubio, R.G.; Luengo, G.S. Deposition of Synthetic and Bio-Based Polycations onto Negatively Charged Solid Surfaces: Effect of the Polymer Cationicity, Ionic Strength, and the Addition of an Anionic Surfactant. *Colloids Interfaces* **2020**, *4*, 33. [CrossRef]
117. Wang, J.J.; Zeng, Z.W.; Xiao, R.Z.; Xie, T.; Zhou, G.L.; Zhan, X.R.; Wang, S.L. Recent advances of chitosan nanoparticles as drug carriers. *Int. J. Nanomed.* **2011**, *6*, 765–774.
118. Panonnummal, R.; Jayakumar, R.; Sabitha, M. Comparative anti-psoriatic efficacy studies of clobetasol loaded chitin nanogel and marketed cream. *Eur. J. Pharm. Sci.* **2017**, *96*, 193–206. [CrossRef]
119. Li, J.; Cai, C.; Li, J.; Li, J.; Li, J.; Sun, T.; Wang, L.; Wu, H.; Yu, G. Chitosan-based nanomaterials for drug delivery. *Molecules* **2018**, *23*, 2661. [CrossRef]
120. Ahmed, T.A.; Aljaeid, B.M. Preparation, characterization, and potential application of chitosan, chitosan derivatives, and chitosan metal nanoparticles in pharmaceutical drug delivery. *Drug Des. Dev.* **2016**, *10*, 483–507. [CrossRef] [PubMed]
121. Ntohogian, S.; Gavriliadou, V.; Christodoulou, E.; Nanaki, S.; Lykidou, S.; Naidis, P.; Mischopoulou, L.; Barmpalexis, P.; Nikolaidis, N.; Bikiaris, D.N. Chitosan nanoparticles with encapsulated natural and Uf-purified annatto and saffron for the preparation of UV protective cosmetic emulsions. *Molecules* **2018**, *23*, 2107. [CrossRef] [PubMed]
122. Muzzarelli, R.A.A. Carboxymethylated chitins and chitosans. *Carbohydr. Polym.* **1988**, *8*, 1–21. [CrossRef]
123. Chen, L.; Du, Y.; Wu, H.; Xiao, L. Relationship between molecular structure and moisture-retention ability of carboxymethyl chitin and chitosan. *J. Appl. Polym. Sci.* **2002**, *83*, 1233–1241. [CrossRef]
124. Kong, C.S.; Kim, J.A.; Ahn, B.; Byun, H.G.; Kim, S.K. Carboxymethylations of chitosan and chitin inhibit MMP expression and ROS scavenging in human fibrosarcoma cells. *Process Biochem.* **2010**, *45*, 179–186. [CrossRef]
125. Ngo, D.N.; Kim, M.M.; Kim, S.K. Chitin oligosaccharides inhibit oxidative stress in live cells. *Carbohydr. Polym.* **2008**, *74*, 228–234. [CrossRef]
126. Muzzarelli, R.A.A.; Muzzarelli, C.; Cosani, A.; Terbojevich, M. 6-Oxychitins, novel hyaluronan-like regiospecifically carboxylated chitins. *Carbohydr. Polym.* **1999**, *39*, 361–367. [CrossRef]
127. Sun, L.; Du, Y.; Yang, J.; Shi, X.; Li, J.; Wang, X.; Kennedy, J.F. Conversion of crystal structure of the chitin to facilitate preparation of a 6-carboxychitin with moisture absorption-retention abilities. *Carbohydr. Polym.* **2006**, *66*, 168–175. [CrossRef]
128. Muzzarelli, R.A.A. Nanochitins and Nanochitosans, Paving the Way to Eco-Friendly and Energy-Saving Exploitation of Marine Resources. In *Polymer Science: A Comprehensive Reference*; Elsevier: Amsterdam, The Netherlands, 2012; Volume 10, pp. 153–164.
129. Gautier, S.; Xhauflaire-Uhoda, E.; Gonry, P.; Piérard, G.E. Chitin-glucan, a natural cell scaffold for skin moisturization and rejuvenation. *Int. J. Cosmet. Sci.* **2008**, *30*, 459–469. [CrossRef]
130. Cho, Y.W.; Cho, Y.N.; Chung, S.H.; Yoo, G.; Ko, S.W. Water-soluble chitin as a wound healing accelerator. *Biomaterials* **1999**, *20*, 2139–2145. [CrossRef]
131. Blasinska, A.; Drobnik, J. Effects of nonwoven mats of di-o-butyrylchitin and related polymers on the process of wound healing. *Biomacromolecules* **2008**, *9*, 776–782. [CrossRef]
132. Chilarski, A.; Szosland, L.; Krucińska, I.; Kiekens, P.; Błasińska, A.; Schoukens, G.; Cisło, R.; Szumilewicz, J. Novel dressing materials accelerating wound healing made from dibutyrylchitin. *Fibres Text. East. Eur.* **2007**, *15*, 77–81.
133. Soetemans, L.; Uyttebroek, M.; Bastiaens, L. Characteristics of chitin extracted from black soldier fly in different life stages. *Int. J. Biol. Macromol.* **2020**, *165*, 3206–3214. [CrossRef] [PubMed]
134. Chen, P.Y.; Lin, A.Y.M.; McKittrick, J.; Meyers, M.A. Structure and mechanical properties of crab exoskeletons. *Acta Biomater.* **2008**, *4*, 587–596. [CrossRef] [PubMed]
135. Ifuku, S.; Saimoto, H. Chitin nanofibers: Preparations, modifications, and applications. *Nanoscale* **2012**, *4*, 3308–3318. [CrossRef] [PubMed]
136. Raabe, D.; Sachs, C.; Romano, P. The crustacean exoskeleton as an example of a structurally and mechanically graded biological nanocomposite material. *Acta Mater.* **2005**, *53*, 4281–4292. [CrossRef]
137. Raabe, D.; Romano, P.; Sachs, C.; Fabritius, H.; Al-Sawalmih, A.; Yi, S.B.; Servos, G.; Hartwig, H.G. Microstructure and crystallographic texture of the chitin-protein network in the biological composite material of the exoskeleton of the lobster Homarus americanus. *Mater. Sci. Eng. A* **2006**, *421*, 143–153. [CrossRef]
138. Ling, S.; Kaplan, D.L.; Buehler, M.J. Nanofibrils in nature and materials engineering. *Nat. Rev. Mater.* **2018**, *3*, 1–15. [CrossRef]
139. Ifuku, S. Chitin and chitosan nanofibers: Preparation and chemical modifications. *Molecules* **2014**, *19*, 18367–18380. [CrossRef]

140. Ifuku, S.; Nogi, M.; Yoshioka, M.; Morimoto, M.; Yano, H.; Saimoto, H. Fibrillation of dried chitin into 10-20 nm nanofibers by a simple grinding method under acidic conditions. *Carbohydr. Polym.* **2010**, *81*, 134–139. [CrossRef]
141. Ifuku, S.; Nomura, R.; Morimoto, M.; Saimoto, H. Preparation of chitin nanofibers from mushrooms. *Materials* **2011**, *4*, 1417–1425. [CrossRef] [PubMed]
142. Mavi, S. Preparation of Chitin and Derivatives Thereof for Cosmetic and Therapeutic Use. International Patent PCT/IB2005/053576, 2 November 2005.
143. Morganti, P. Spray-Dried Chitin Nanofibrils, Method for Production and Uses Thereof. International Patent PCT/IB2006/054403, 23 November 2006.
144. Morganti, P. Chitin Nanofibrils in Skin Treatment. *J. Appl. Cosmetol.* **2009**, *25*, 251–270.
145. Jayakumar, R.; Prabaharan, M.; Nair, S.V.; Tamura, H. Novel chitin and chitosan nanofibers in biomedical applications. *Biotechnol. Adv.* **2010**, *28*, 142–150. [CrossRef] [PubMed]
146. Zhang, Y.; Chwee, T.L.; Ramakrishna, S.; Huang, Z.M. Recent development of polymer nanofibers for biomedical and biotechnological applications. *J. Mater. Sci. Mater. Med.* **2005**, *16*, 933–946. [CrossRef]
147. Jain, T.; Kumar, H.; Dutta, P.K. D-glucosamine and N-acetyl d-glucosamine: Their potential use as regenerative medicine. In *Chitin and Chitosan for Regenerative Medicine*; Springers: New Delhi, India, 2016; pp. 279–295.
148. Morganti, P.; Carezzi, F.; Del Ciotto, P.; Morganti, G.; Nunziata, M.L.; Gao, X.; Tishenko, G.; Yudin, V. Chitin Nanofibrils: A Natural Multifunctional Polymer. *Nanobiotechnology* **2014**, *1*, 1–31.
149. Bissett, D.L.; Robinson, L.R.; Raleigh, P.S.; Miyamoto, K.; Hakozaki, T.; Li, J.; Kelm, G.R. Reduction in the appearance of facial hyperpigmentation by topical N-acetyl glucosamine. *J. Cosmet. Dermatol.* **2007**, *6*, 20–26. [CrossRef] [PubMed]
150. Bissett, D.L.; Farmer, T.; McPhail, S.; Reichling, T.; Tiesman, J.P.; Juhlin, K.D.; Hurley, G.J.; Robinson, M.K. Genomic expression changes induced by topical N-acetyl glucosamine in skin equivalent cultures in vitro. *J. Cosmet. Dermatol.* **2007**, *6*, 232–238. [CrossRef]
151. Danti, S.; Trombi, L.; Fusco, A.; Azimi, B.; Lazzeri, A.; Morganti, P.; Coltelli, M.B.; Donnarumma, G. Chitin nanofibrils and nanolignin as functional agents in skin regeneration. *Int. J. Mol. Sci.* **2019**, *20*, 2669. [CrossRef]
152. Morganti, P.; Li, Y.H. Innovation in cosmetic and medical science. The role of chitin nanofibrils composites. *J. Appl. Cosmetol.* **2015**, *33*, 9–24.
153. Ito, I.; Yoneda, T.; Omura, Y.; Osaki, T.; Ifuku, S.; Saimoto, H.; Azuma, K.; Imagawa, T.; Tsuka, T.; Murahata, Y.; et al. Protective effect of chitin urocanate nanofibers against ultraviolet radiation. *Mar. Drugs* **2015**, *13*, 7463–7475. [CrossRef]
154. Ito, I.; Osaki, T.; Ifuku, S.; Saimoto, H.; Takamori, Y.; Kurozumi, S.; Imagawa, T.; Azuma, K.; Tsuka, T.; Okamoto, Y.; et al. Evaluation of the effects of chitin nanofibrils on skin function using skin models. *Carbohydr. Polym.* **2014**, *101*, 464–470. [CrossRef] [PubMed]
155. Morganti, P.; Palombo, M.; Cardillo, A.; Del Ciotto, P.; Morganti, G.; Gazzaniga, G. Anti-dandruff and anti-oily efficacy of hair formulations with a repairing and restructuring activity. The positive influence of the Zn-chitin nanofibrils complexes. *J. Appl. Cosmetol.* **2012**, *30*, 149–159.
156. Morganti, P.; Fabrizi, G.; Palombo, M.; Cardillo, M.; Cardillo, A.; Del Ciotto, P.; Carezzi, F.; Morganti, G. Activity of chitin nanofibrils block-copolymers entrapping Zn/AI/SA/allantoin on seborrheic dermatitis. A randomized double-blind placebo controlled study. *J. Appl. Cosmetol.* **2014**, *32*, 3–19.
157. Morganti, P.; Palombo, M.; Palombo, P.; Fabrizi, G.; Cardillo, A.; Carezzi, F.; Morganti, G.; Ruocco, E.; Dzierzgowski, S. Cosmetic Science in Skin Aging: Achieving the Efficacy by the Chitin Nano-Structured Crystallites. *SÖFW J.* **2010**, *136*, 14–24.
158. Morganti, P.; Morganti, G.; Fabrizi, G.; Cardillo, A. A new sun to rejuvenate the skin. *J. Appl. Cosmetol.* **2008**, *26*, 159–168.
159. Morganti, P.; Fabrizi, G.; Palombo, P.; Palombo, M.; Ruocco, E.; Cardillo, A.; Morganti, G. Chitin-nanofibrils: A new active cosmetic carrier. *J. Appl. Cosmetol.* **2008**, *26*, 113–128.
160. Morganti, P.; Palombo, P.; Palombo, M.; Fabrizi, G.; Cardillo, A.; Svolacchia, F.; Guevara, L.; Mezzana, P. A phosphatidylcholine hyaluronic acid chitin-nanofibrils complex for a fast skin remodeling and a rejuvenating look. *Clin. Cosmet. Investig. Dermatol.* **2012**, *5*, 213–220. [CrossRef] [PubMed]
161. Morganti, P.; Fabrizi, G.; Palombo, P.; Palombo, M.; Guarneri, F.; Cardillo, A.; Morganti, G. New chitin complexes and their anti-aging activity from inside out. *J. Nutr. Health Aging* **2012**, *16*, 242–245. [CrossRef] [PubMed]
162. Morganti, P.; Palombo, M.; Tishchenko, G.; Yudin, V.E.; Guarneri, F.; Cardillo, M.; Del Ciotto, P.; Carezzi, F.; Morganti, G.; Fabrizi, G. Chitin-hyaluronan nanoparticles: A multifunctional carrier to deliver anti-aging active ingredients through the skin. *Cosmetics* **2014**, *1*, 140–158. [CrossRef]
163. Morganti, P.; Palombo, M.; Fabrizi, G.; Guarneri, F.; Svolacchia, F.; Cardillo, A.; Del Ciotto, P.; Carezzi, F.; Morganti, G. New insights on anti-aging activity of chitin nanofibril-hyaluronan block copolymers entrapping active ingredients: In vitro and in vivo study. *J. Appl. Cosmetol.* **2013**, *31*, 1–29.
164. Morganti, P.; Del Ciotto, P.; Carezzi, F.; Guarneri, F.; Yeo, Y.J. Skin lightening efficacy of new formulations enhanced by chitin nanoparticles delivery system. Note I. *J. Appl. Cosmetol.* **2014**, *32*, 57–71.
165. Morganti, P.; Palombo, M.; Carezzi, F.; Nunziata, M.L.; Morganti, G.; Cardillo, M.; Chianese, A. Green nanotechnology serving the bioeconomy: Natural beauty masks to save the environment. *Cosmetics* **2016**, *3*, 41. [CrossRef]

166. Morganti, P.; Fusco, A.; Paoletti, I.; Perfetto, B.; Ciotto, P.; Del Palombo, M.; Chianese, A.; Baroni, A.; Donnarumma, G. Anti-inflammatory, immunomodulatory, and tissue repair activity on human keratinocytes by green innovative nanocomposites. *Materials* **2017**, *10*, 843. [CrossRef]
167. Morganti, P.; Morganti, G.; Colao, C. Biofunctional Textiles for Aging Skin. *Biomedicines* **2019**, *7*, 51. [CrossRef]
168. Morganti, P.; Yudin, V.E.; Morganti, G.; Colte, M.B. Trends in surgical and beauty masks for a cleaner environment. *Cosmetics* **2020**, *7*, 68. [CrossRef]
169. Donnarumma, G.; Fusco, A.; Morganti, P.; Palombo, M. Advanced medications made by green nanocomposites. *Int. J. Res. Pharm. Nano Sci.* **2016**, *5*, 261–270.
170. Velnar, T.; Bailey, T.; Smrkolj, V. The wound healing process: An overview of the cellular and molecular mechanisms. *J. Int. Med. Res.* **2009**, *37*, 1528–1542. [CrossRef]
171. Maevskaia, E.N.; Shabunin, A.S.; Dresvyanina, E.N.; Dobrovol'skaya, I.P.; Yudin, V.E.; Paneyah, M.B.; Fediuk, A.M.; Sushchinskii, P.L.; Smirnov, G.P.; Zinoviev, E.V.; et al. Influence of the Introduced Chitin Nanofibrils on Biomedical Properties of Chitosan-Based Materials. *Nanomaterials* **2020**, *10*, 945. [CrossRef] [PubMed]
172. Dobrovolskaya, I.P.; Yudin, V.E.; Popryadukhin, P.V.; Lebedeva, I.O.; Shabunin, A.S.; Stoyanovsky, R.G.; Asadulaev, M.S.; Ivan, E.M.; Morganti, P. In vivo study of the nanofiber-based composite wound dressing intended for treatment of deep skin wounds. *J. Appl. Cosmetol.* **2016**, *34*, 1–8.
173. Mattioli-Belmonte, M.; Zizzi, A.; Lucarini, G.; Giantomassi, F.; Biagini, G.; Tucci, G.; Orlando, F.; Provinciali, M.; Carezzi, F.; Morganti, P. Chitin Nanofibrils Linked to Chitosan Glycolate as Spray, Gel, and Gauze Preparations for Wound Repair. *J. Bioact. Compat. Polym.* **2007**, *22*, 525–538. [CrossRef]
174. Muzzarelli, R.A.A.; Morganti, P.; Morganti, G.; Palombo, P.; Palombo, M.; Biagini, G.; Mattioli Belmonte, M.; Giantomassi, F.; Orlandi, F.; Muzzarelli, C. Chitin nanofibrils/chitosan glycolate composites as wound medicaments. *Carbohydr. Polym.* **2007**, *70*, 274–284. [CrossRef]
175. Izumi, R.; Komada, S.; Ochi, K.; Karasawa, L.; Osaki, T.; Murahata, Y.; Tsuka, T.; Imagawa, T.; Itoh, N.; Okamoto, Y.; et al. Favorable effects of superficially deacetylated chitin nanofibrils on the wound healing process. *Carbohydr. Polym.* **2015**, *123*, 461–467. [CrossRef] [PubMed]

sustainability

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Review

Lipids from *Hermetia illucens*, an Innovative and Sustainable Source

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Abstract: The exponential increase of global demand for proteins and lipids can no longer be satisfied by classical sources. High amounts of CO_2 produced by intensive livestock breeding and its effects on the environment are the main factors that prevent the use of animals as primary sources for proteins and lipids, calling for the use of new sustainable sources, such as insects. The massive breeding of bioconverter insects as a feed source has been a major topic in recent years, with both economic and scientific aspects related to rearing and subsequent processing optimization. The larvae of *Hermetia illucens* (Diptera: Stratiomyidae) (also known as Black Soldier Fly) can be used for the eco-sustainable production of proteins and lipids with high biological and economic value. Lipids can be obtained from BSF bioconversion processes and are present in high quantities in the last instar larvae and prepupae. Fats obtained from BSF are used as animal feed ingredients, in the formulation of several products for personal care, and in biodiesel production. To enable the use of insect-derived lipids, it is important to understand how to optimize their extraction. Here, we summarize the published information on the composition, the extraction methods, and the possible applications of the BSF lipid component.

Keywords: bioconversion; fats; sustainability; animal feed; biodiesel; Black Soldier Fly

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1. Introduction

Insects are the greatest example of biodiversity on earth and their role in the ecosystem is extremely varied [1]. Although insects are often considered pests, they provide important ecological services, above all pollination of wild flora and crops, and are a source of useful biomolecules [2]. Insects also act as reliable indicators of environmental quality [3], and they can be used as a tool for biological control of pests [4]. Moreover, insects could represent innovative and alternative models in biological, medical and environmental studies [5], and they are one of the best sources of inspiration for biomimicry, to develop useful innovations for improving the quality of life through the use of biotechnology. Indeed, insects can represent a new source of genes, molecules and mechanisms of interest to agriculture, forestry, biomedicine and industry, as well as in other technological applications [6].

Bioconverter species represent an important group of insects from an ecological point of view, as they are able to consume many animal and plant decaying organic substrates during their larval development, when they are growing rapidly and accumulating biomass. Thus, several food waste and by-products can potentially be recovered and used as feed for these insects, rather than having to be disposed of in a conventional way, acquiring a new value.

In recent years, among bioconverter insects, particular attention has been given to *Hermetia illucens* (Diptera: Stratiomyidae) (also known as Black Soldier Fly—BSF). BSF larvae are able to feed on various organic substrates of vegetable and animal origin, even in decomposition, bioconverting them into larval biomass in just 14 days under optimal growth conditions (27 °C of temperature and 70% of humidity) [7–9]. The wide variety of organic materials that BSF is able to feed on includes manure [10], waste from the agri-food chain [11,12], distilled grain waste such as spent malt [13–15], and many others [11]. These insects have a potentially great economic role from the perspective of a circular economy, as they are able to consume organic waste material and convert it into insect biomass that can in turn be used as feed for other livestock and food for humans [16,17].

In many countries, especially in tropical regions, insects represent an important component of the human diet. More than 2000 species are recognized as edible (e.g., bees, caterpillars, termites, cicadas, crickets, ants) and have high nutritional value [18]. Another important environmental reason for replanting meat with insects as an alternative protein source is the reduction in emissions of greenhouse gases, land use and pollution [18]. The ever-increasing world population and, consequently, the growing demand for food and agricultural land and over-production of food waste, combined with the depletion of natural resources and climate change, lead to a worldwide realization that new alternative sources of human food and animal feed are needed. In this scenario, insects appear to be a valid and sustainable source of nutrients and an innovative and marketable solution for waste management.

Considering that one third of global agricultural and food production is wasted [19] and that insects can be grown on former foodstuffs, they represent the key solution for reducing food waste, converting them into valuable products, including animal feed, human food, lubricants, pharmaceuticals and biofuel [20].

Insect Rearing

Insects have been identified by the United Nations Food and Agricultural Organization as a potential feed for animals [21–23], if fed on authorized substrates [24]. Recently, the EU authorized the use of insect proteins in feed for farmed fish [24]. This authorization regards only seven species of insect: BSF, the common housefly (*Musca domestica*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*), banded cricket (*Gryllodes sigillatus*), and field cricket (*Gryllus assimilis*) [25].

As a consequence, the breeding of these insect species for the production of feed has been increasing. Insect farming is more eco-sustainable, requiring fewer natural resources (in terms of soil, water, fertilization) than conventional crops such as soy that are widely used as feed for livestock and whose production competes with the production of food for human consumption [26–28]. Furthermore, insect breeding farms emit lower levels of greenhouse gases and ammonium (NH_3) compared to conventional livestock [29,30]. Finally, insects could be used to fight malnutrition in developing countries [31]. For insect farming there are also fewer animal welfare constraints than for other conventionally farmed animal species [27,32]. Insects present few risks regarding the transmission of infections and zoonoses [33], being taxonomically distant from common farmed species. However, insects are potential vectors of medically relevant pathogens and the risk of infections could rise with the improper use of waste products and with unhygienic handling of insects [27]. An obvious example is the common housefly, which can be a vector and reservoir for foodborne diseases and can be responsible for transmission of bacterial diseases. Moreover, housefly larvae, generally raised on substrates containing manure of different animal species [34], can pick up and transport different pathogens. This does not occur in BSF, since the adult stage does not have functioning mouthparts and, thus, they are not associated with transmissions of diseases, or to the accumulation of pesticides or mycotoxins [35–37]. Larval treatments at suitable temperatures during the drying process ensure the destruction of pathogenic organisms present in the larvae [38].

According to Regulation (EU) 2017/1017 [39], insects can be fed with waste produced by humans only for research purposes [40]. The great diversity of insect species, the

different types of food on which they feed, and the different ecological niches determine an enormous variability in their composition [1,41,42]. Furthermore, bioconverter insects can transform abundant quantities of organic waste into a biomass rich in proteins and lipids that is suitable for animal nutrition [21,43,44] (Figure 1). Proteins, lipids, and chitin, the three main components of insects, can be properly isolated from these organisms to be used as food ingredients or for other applications in order to obtain high added value marketable products [45]. Many insects can be also used entirely in the form of powder rich in protein and/or lipid [46]. Recently EFSA approved the use of whole dried *T. molitor* larvae also for human consumption [47].

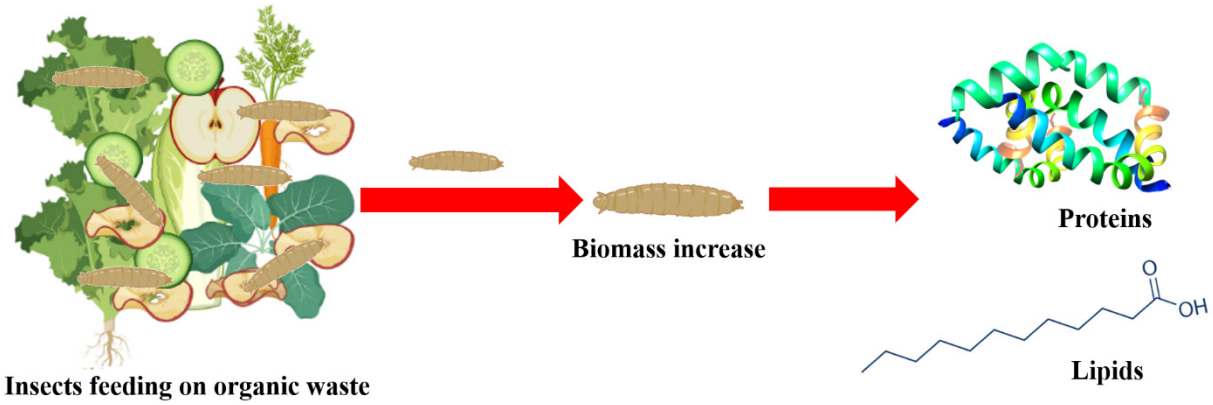

Figure 1. Insects can transform organic waste into animal biomass, which is rich in proteins and lipids.

Among the bioconverter species, BSF is considered one of the most interesting insects worldwide for the bioconversion of organic waste and as a promising and sustainable source of proteins, lipids, and bioactive compounds (i.e., chitin and antimicrobial peptides) [43,48–51] (Figure 2).

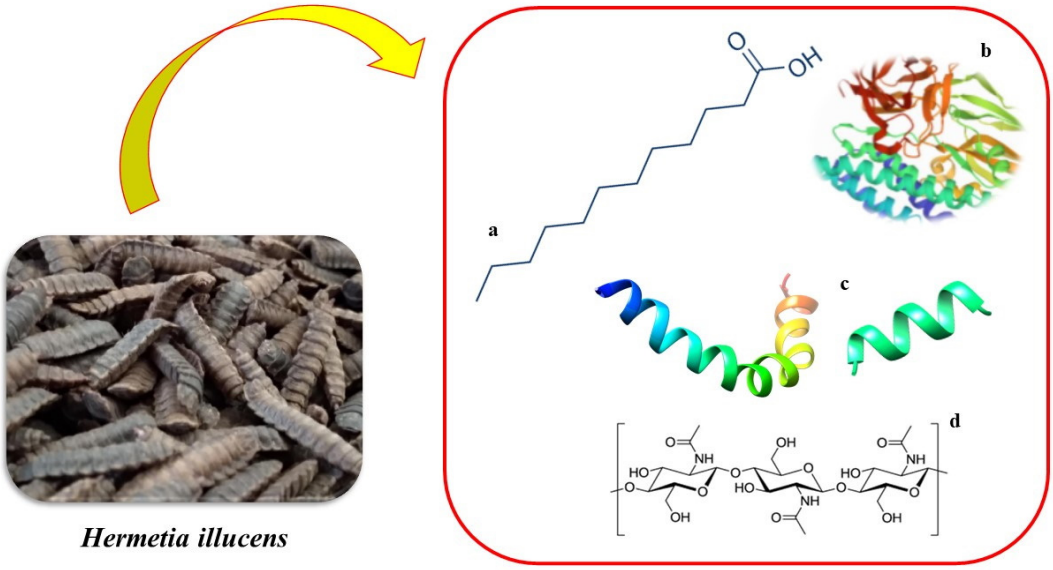

Figure 2. BSF is a sustainable source of lipids (**a**), proteins (**b**), antimicrobial peptides (**c**) and chitin (**d**).

The importance of BSF is correlated to its ability to convert and recover nutrients from different types of organic compounds and to reduce organic waste biomass by 50–60%, thereby transforming it into high protein biomass [17].

This review is focused on the lipid fraction extractable from BSF, with the aim of investigating its composition, the lipid extraction methods currently available, and the possible applications of BSF lipids.

2. Lipids from the Black Soldier Fly

BSF larvae (BSFL) are particularly rich in lipids, ranging from 15–49% of their weight. The composition of this fat, with more saturated than unsaturated fatty acids, is influenced by the type of growing substrates [52].

Fasakin et al. [53] reported that BSFL and the resulting whole meal have a fat content that exceeds the diet requirements of most animals to which they are fed [53]. This high fat content, together with other intrinsic factors of the lipids themselves, may influence the digestibility and/or the palatability of the meal. This lipid surplus of BSFL can be isolated and used for other purposes, e.g., food ingredients [54,55].

Lauric acid is among the most abundant fatty acids in BSFL, reaching a content up to 50% of the isolated fats [21,43,45,56]. However, the fatty acid composition of BSFL lipids can be modified according to the different feeding substrates [21,43].

2.1. Larval Biomass Lipid Content Based on Different Diet

Lipid content in both BSF last instar larvae and prepupae can reach 15–49% of the total dry weight, depending on the food substrate administered to the larvae [43,49]. As reported by Barragan-Fonseca et al. [57], feeding substrates can affect the larval body composition in terms of both protein and fat content. For instance, larvae have higher protein and lipid content when grown on swine manure instead of cow manure [58]. Substrates affect mainly the macronutrient levels (proteins, lipids, and carbohydrates) [58] and to a lesser extent the micronutrient concentrations (minerals and vitamins) [21,58,59] that are very important for the needs of some wild animals in captivity [58].

Lipid content in BSFL is affected by the composition of the rearing substrate [21,60,61] but the resultant crude fat content in BSFL is much higher than other insects, soybean, or fish meal [62,63]. Substrates with high amounts of proteins, lipids and, in general, substrates complete and balanced in all nutrients, are the best for the development of the BSF and its bioconversion performances [64]. Macronutrients in the substrate can affect the protein content of BSFL, larval weight, bioconversion rate, and development time [65–67].

The carbohydrate content of the feed substrate affects the lipid content of the BSFL [65,67] because of the conversion of carbohydrate into lipids by larvae [65,68]. Low protein and high carbohydrate substrates allow a higher lipid accumulation in BSF larval biomass [69] than a balanced diet (a mix of ingredients with a final equilibrated ratio of proteins, lipids and carbohydrates) [21,57,65,70]. Moreover, the lipid composition can be directly affected by the lipid composition of the feeding substrate [65,71–73].

Makkar et al. [62] reported a lipid content ranging between 15-and 25% for larvae fed on chicken manure, 28% on pig manure, 35% on cattle manure, and 42–49% on oil-rich food waste. Several studies utilized alternative substrates not suitable for human consumption (e.g., biogas digestate and many types of manure) to feed BSFL. The effect of the use of these substrates, particularly on the lipid content of BSFL, has been investigated by Spranghers et al. [21]. They demonstrated how lipid components in BSF prepupae vary as a function of substrates, rearing them on four different organic substrates: vegetable waste, chicken feed, restaurant waste, and biogas digestate. The lipid contents of these substrates were rather low (2.1–6.2%) with the exception of restaurant waste (13.9%). The authors found high fat content (mostly rich in short chain fatty acids) in larvae reared on energy rich substrates as restaurant waste (in which lipid component was around 38.6%), vegetable waste (lipid component = 37.1%), and chicken feed (lipid component = 33.6%). A lower amount of lipids was found in larvae reared on digestate (21.8%). These differences could be related to the higher uptake of fatty acids in larvae fed on high-energy substrates (rich on lipids and fatty acids). Restaurant waste was rich both in fat and non-fibre carbohydrates; vegetable waste and chicken feed were rich in non-fibre carbohydrates; while non-fibre carbohydrates were almost absent in the biogas, because of the fermentation process performed by microorganisms, transforming most of the carbohydrates in methane [21].

Concerning the different types of manure, the influence of this substrate on the lipid content of the BSFL feeding on it depends greatly on the origin of the manure. [36,74]. For

example, a lipid content of 15–35% was found in larvae fed on poultry manure [36,74], 28–34% in larvae fed on swine manure [36,75], 14% with horse manure [76] and 10% with sheep manure [76].

Meneguz et al. [77] compared performances and composition of BSFL grown on fruit waste, on a mix of vegetables and fruit waste, and winery and brewery by-products.

Larvae reared on fruit waste showed higher fat content than larvae reared on vegetable-fruit waste, probably as a consequence of the higher non-structural carbohydrate level of fruit waste (sugars, starch, pectins: energy and reserve substances contained in the plant cell) [78], which insects are able to convert into fats [21,79]. Nonetheless, the influence of the non-structural carbohydrates on the lipids content of BSFL should be further investigated, since the higher non-structural carbohydrate content in the brewery by-product did not lead to higher larval fat content [77].

Scala et al. [13] investigated on an industrial scale the influence of six different diets on BSF larval development, growth, final larval yield, substrate reduction, protein, and lipid content. The tested by-products were apple, banana, and spent grain, alone or mixed in 1:1 ratio (*w*/*w*). This study detected the highest percentage of lipids in larvae fed with apple and mixed apple and banana. Larvae fed on spent grain, on a mix of apple and spent grain, and on a mix of banana and spent grain had the lowest percentage of lipids. Low percentage of lipid content in BSFL corresponds to a high percentage of proteins [13].

In Table S1 the lipid content of BSF biomass reared on different organic waste is reported.

2.2. Fatty Acids Composition in Black Soldier Fly Lipids

The lipid fraction of BSFL is a mix of triglycerides, saturated, and unsaturated fatty acids [43,80]. A study performed by Surendra et al. [81] on larvae fed on a mix of organic waste showed that the concentration of the short chain saturated fatty acids (lauric acid—palmitic acid) (67% of total fatty acids) in the BSF prepupae fat was higher than coconut oil and palm kernel oil (55–57% of total fatty acids), which is consistent with the results obtained by other authors [45,62,80,82,83]. Moreover, they found that the concentration of unsaturated fatty acids (28% of total fatty acids) was higher than in coconut oil (10%) and higher than palm kernel oil (18%) [78]. Meneguz et al. [77] affirmed that C18:1 (n-9) (oleic acid) was the main represented monounsaturated fatty acid in BSFL, while C18:2 (n-6) (linoleic acid) and C18:3 (n-3) (alpha-linolenic acid) were the main represented polyunsaturated n-6 and polyunsaturated n-3 fatty acids, respectively. Ushakova et al. [80] showed that azelaic and sebacic acids, the esters of lauric acid, are also present in the lipid fraction of BSF. The esters, including the identified azelaic acid dibutyl ether, could guarantee the elasticity of the fat component of larvae at low temperatures [80]. However, in BSF, azelaic acid is present in very low quantities (0.83%), as reported in Ushakova et al. [80], and for this reason the melting point of other fatty acids present in higher amounts (lauric acid, palmitic acid, linolenic acid etc.) would have a bigger influence on this property. Since azelaic acid can inhibit the reproduction of lipophilic microorganisms in skin sebaceous glands, it can help to protect insect lipids from infections [84]. One of the most abundant fatty acids in BSFL is lauric acid [21,43,45,56]. The latter could also be converted into monolaurin (or glycerol monolaurate), which is an antiviral, antibacterial, and antiprotozoal glyceride for animals and humans [85], and this could explain a hypothetical anti-bacterial activity of BSF lipids [86]. Spranghers et al. [21] found that lipids of BSF prepupae were mostly composed of lauric acid, even when BSFL feed on substrate with an extremely low percentage of the above-mentioned acid. It means that the fatty acids composition of the rearing diet did not directly affect the larval fatty acids composition, which, on the contrary, was more influenced by carbohydrates [21,67]. The conversion of carbohydrates into lipids by insects has been well documented [79,87,88]. As demonstrated for other Diptera species, the BSF larval fatty acid profile is composed mainly of saturated fats like lauric acid, myristic acid, palmitic acid, and stearic acid [21,80]. As reported by Bennett and Lee [89], insects with less adaptation to low temperatures show a high presence of saturated fatty acids. Indeed, BSF has difficulty with cold adaptation, demonstrated by the lowest survival rate

at about 16 °C [90]. Currently, lauric acid is mainly extracted from vegetable matrices (coconut oil and palm kernel oil) and, for its antimicrobial properties, it is commercially used as a primary component for the preparation of several products for personal care (soaps, detergents, and shampoos) [91–94]. The use of lauric acid obtained from alternative sources, such as insect lipids, may replace the main common sources (palm kernel oil, coconut oil) and contribute to the conservation of tropical forests [43]. The high content of the aforementioned acid in BSF prepupae could be an interesting extra element to support the proposal of the inclusion of BSFL or prepupae in poultry and pig feed considering the faster and more efficient absorption and metabolism of short chain fatty acids (such as lauric acid) compared to long chain fatty acids and their nutraceutical potential [21,95]. In addition, Skrivanova et al. [96] showed that, among the valuable short chain fatty acids, lauric acid had the highest activity against the pathogen *Clostridium perfringens* and the lowest impact on the beneficial bacteria Lactobacilli. In light of beneficial effects on intestinal health and microbial growth inhibition, lauric acid could improve the performance and welfare of farm animals [21]. Considering that the use of antibiotics for non-medical applications was banned by the EU (Regulation 1831/2003/EC) [97], lauric acid could be used as an alternative to in-feed antibiotics for controlling microbial infections [97–99]. Concerning toxic elements that could influence the nutritional value of animal feed, the noxious erucic acid in BSFL fatty acids profile never exceeded 1.3%, regardless of the administered diet [43]. The admitted concentration of erucic acid in food and feed was limited by the EFSA (European Food Safety Authority) in 2016 to a tolerable daily intake (TDI) of 7 mg/kg body weight [100]. On the other hand, the low amount of polyunsaturated fatty acids (PUFA) n-3 in the BSFL could limit their application as animal feed ingredients [77]. Indeed, the use of full-fat insect meals with a high fat content, as BSF meal, corresponds to a decrease in the nutritional quality of animal products [101–103]. However, BSFL can be enriched in PUFA n-3, modulating the feeding substrate. Crude fat of BSFL frequently contains some non-triacylglycerol components including gummy matter, oxidized intermediates, fatty acids, odorous residue, colour compounds, and pigments which can negatively affect taste, nutritional value and appearance. Hence, it is necessary to remove some of these components during the extraction process to get lipids as pure as possible and to avoid undesirable characteristics (with less proteins, less turbidity, density, and viscosity) before their application in the cosmetic and/or food applications [49]. Anyway, depending on the projected aims (animal feed, biodiesel production), the qualitative fatty acid composition of lipids in BSFL can be modified according to the different organic substrates selected for the insect feed [21,43], as reported in Table S2.

3. Lipid Extraction

The lipid extraction and subsequent fractionation require the use of organic solvents and techniques not used in the purification of water-soluble molecules such as proteins and carbohydrates [104–106]. Polarity and solubility of lipids in non-polar solvents are the main characteristics to separate complex lipid mixtures [104]. Lipids that contain fatty acids bound with ester or amide bonds can be hydrolyzed with acids, alkalis, or with hydrolytic enzymes. Neutral lipids such as triacylglycerols, pigments, and waxes can be extracted with ether, chloroform and benzene [106]. Membrane lipids are more easily extracted by polar organic solvents such as methanol and ethanol since they reduce the hydrophobic interactions between the lipid molecules and weaken the hydrogen bonds and the electrostatic interactions that bind lipids to membrane proteins [106].

The procedure to extract lipids from animals or plants involves several steps:

- sample reduction, drying or hydrolysis;
- sample homogenization with organic solvent;
- separation of the organic and aqueous phase (organic phase contains lipids);
- removal of non-lipid contaminants (if necessary);
- drying of the extract to remove the organic solvent.

It is not possible to use a single standard method for the extraction of all types of lipids [107] since each matrix has different characteristics and therefore different approaches must be used [108].

In the lipid extraction procedure, the first aspect to consider concerns the size of the sample [109]. Small particle size increases the surface that allows better contact with the organic solvent, raising its extractive yield [110]. Even if lipids are not soluble in water, the presence of this molecule is also not completely negligible as it influences the extraction capacity of the solvent [110,111]. Organic solvents, such as diethyl ether or hexane, do not easily penetrate the tissues and a complete lipid extraction does not always occur [112]. On the other hand, more water usage in the extraction may lead to higher costs, as the solute separation requires a lot of energy by using water as a solvent [109,113]. A common treatment is acid or basic hydrolysis, which makes lipids more accessible to the organic solvent that is required to split lipids from proteins or carbohydrates and to break down emulsified fats [114].

The type of solvent and the extraction method depend on the chemical structure of the sample and the type of lipid that must be extracted. An important characteristic of the solvent is the high solubility for lipid compounds and the poor solubility for amino acids, proteins and carbohydrates. Solvents tend to deactivate enzymes and also prevent unwanted reactions. The solvent must easily penetrate the sample particles and must have a low boiling point to easily evaporate. The most commonly used solvents are alcohols (such as ethanol, methanol, and n-butanol), acetonitrile, acetone, halogenated hydrocarbons (chloroform, dichloromethane), ethers, hydrocarbons (benzene, hexane), or a mixture of them [115].

3.1. Traditional Lipid Extraction Methods

The methods described below (Figure 3) can be applied for lipid extraction from different cell typologies (both animals and vegetables) [116].

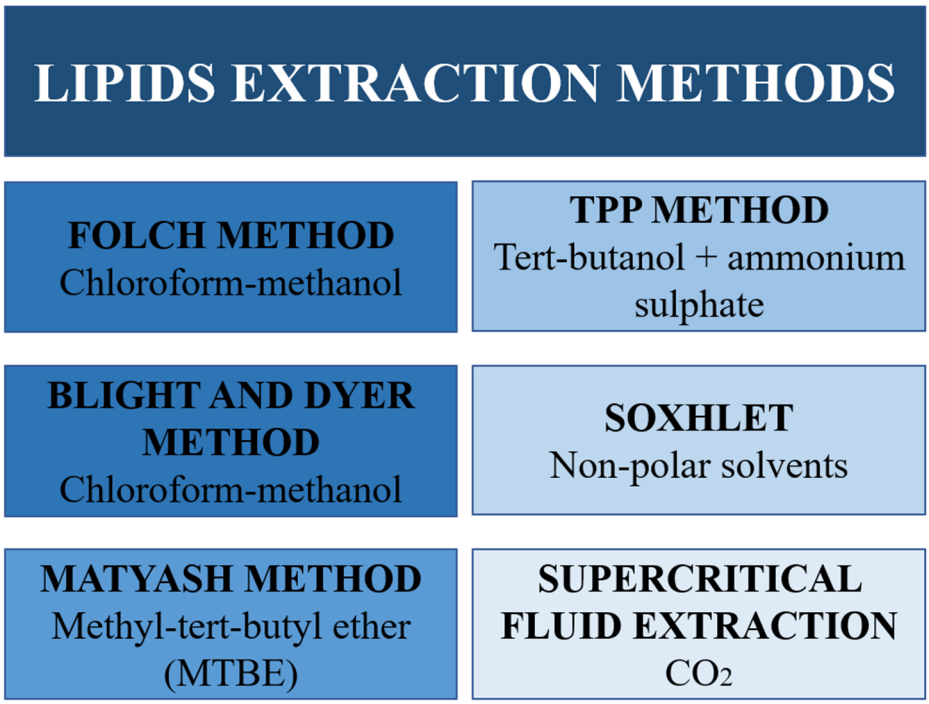

Figure 3. Several methods for lipid extraction have been developed so far: the Folch method, the Bligh and Dyer method, the Matyash method, the TPP method, the Soxhlet method, and the Supercritical fluid extraction method.

The Folch method is a traditional method used for the extraction of lipids from brain tissue. It consists of two steps in which the tissue, in a 1:20 ratio with the solvent, is homogenized with a 2:1 v/v mixture of chloroform-methanol. The filtrate is then treated with water, with the possible addition of salts, obtaining two phases where the lower one contains lipids while the upper phase contains non-lipid substances [117]. A modified Folch method has been adopted by several extraction protocols.

The Matyash et al. [118] method consists in a modification of the Folch/Bligh and Dyer methods and allows a better recovery of almost all the main classes of lipids. Methyl-tert-butyl ether (MTBE) is used as a solvent for the extraction and allows for the obtaining of a more accurate lipid profile. Solvents containing chlorine, including chloroform, should be avoided [119], as they lead to the formation of a protein interphase between the polar and the lipid phase. MTBE is an appropriate alternative to chloroform [120]. In this protocol, the sample is mixed with 7.5 volumes of methanol and 25 volumes of MTBE, and the extracted lipids can be directly used for subsequent investigations or stored in a chloroform/methane/water mixture (60/30/4.5, $v/v/v$).

The TPP method (or three-phase partitioning) was initially designed by Dennison and Lovrien Rex [121] for the extraction of proteins. The method involves the use of a solution containing tert-butanol and water. The subsequent addition of ammonium sulphate induces the formation of two phases: a lower aqueous phase containing the salt and an upper one containing the alcohols. If proteins are present in the aqueous solution, they precipitate at the interface of the two phases. The top layer of tert-butanol contains membrane lipids and other low molecular weight molecules [122].

The Soxhlet method allows for the determination of the raw lipid content by extracting the lipids from the sample with consecutive gravimetric measurements. It is a system of continuous extraction in which the sample is placed into a thimble inside an extractor called a "Soxhlet extractor". The solvent is generally a non-polar liquid such as petroleum ether and hexane. The flask containing the solvent is heated and the solvent that evaporates is conveyed into the extraction pocket which is connected to a cooling system and to the distillation flask. When completed, the solvent has evaporated and the flask contains the dry lipid residue [123,124].

The supercritical fluid extraction method involves a supercritical fluid, carbon dioxide (CO_2), as a solvent. A supercritical fluid is formed every time a substance is heated beyond its critical temperature, above which the substance cannot be condensed in its liquid state through the simple application of a pressure [125]. The CO_2 is heated in a pressurization chamber and the lipid component is contained in a new formed layer which is separated from the aqueous component. The CO_2 is subsequently removed by expansion at room pressure. The supercritical fluid extraction method is particularly advantageous for processing food products [126] and has several advantages, including reduced oxidation of solutes, the possibility of extraction of components sensitive to high temperatures, the modulation of extractive conditions, extracts that are removed without solvent, and desirable solvent conditions such as non-toxicity and non-explosiveness.

3.2. Industrial Extraction Method

The industrial extraction of fats from fresh or dried larvae follows different methodologies compared to traditional extraction [127].

The BSFL dried biomass is pressed to obtain fat and partially defatted meals. This extraction involves a screw press, which works at 100 °C and is typically used in oil extractions of nuts and seeds. During the process, the fat from larvae squeezes out and a press cake is produced. BSFL protein meal is yielded from the press cake and BSFL fat from the press liquid after further filtration processes. There are two different methods for fractioning BSFL using a screw press: dry processing and wet processing. The first one involves a drying process of BSFL before pressing while in the second method, fresh BSFL are directly pressed. The press cake can be grinded into BSFL protein meal directly and the crude BSFL fat can be further refined by a filtration or decanting step. Fat refining

separates the fat from solids, which can account for up to 40% of the mass of crude BSFL fat. For the wet process, a further drying and grinding of the press cake is required to reduce its moisture content and particle size. The separation of the press liquid is more difficult and advanced equipment would be needed. However, the processing time for fresh larvae takes more time. The microwave-dried larvae are very light and easy to press, whereas wet larvae are heavy and not only fat but also have water which has to be released and therefore increases the processing time.

4. Black Soldier Fly Lipid Applications

Currently, the study and the research of new materials that have low environmental impact and are safe for humans is of crucial importance. Research is driven not only by the reduction of fossil resources, but also by the impact of human activities on the planet, such as waste production and the excessive depletion of natural resources. The industry is using vegetable oils as an alternative to fossil fuels, but their environmental impact is still a serious problem [128]. Since the demand for vegetable oils and biofuels contributes to tropical deforestation, habitat fragmentation and loss of biodiversity, the use of insect-derived fats could be the most sustainable choice to consider. Insects are organisms with high potential thanks to their countless applications in several sectors. This review focused on the usage of BSFL-derived lipids in biodiesel production and animal feed (Figure 4).

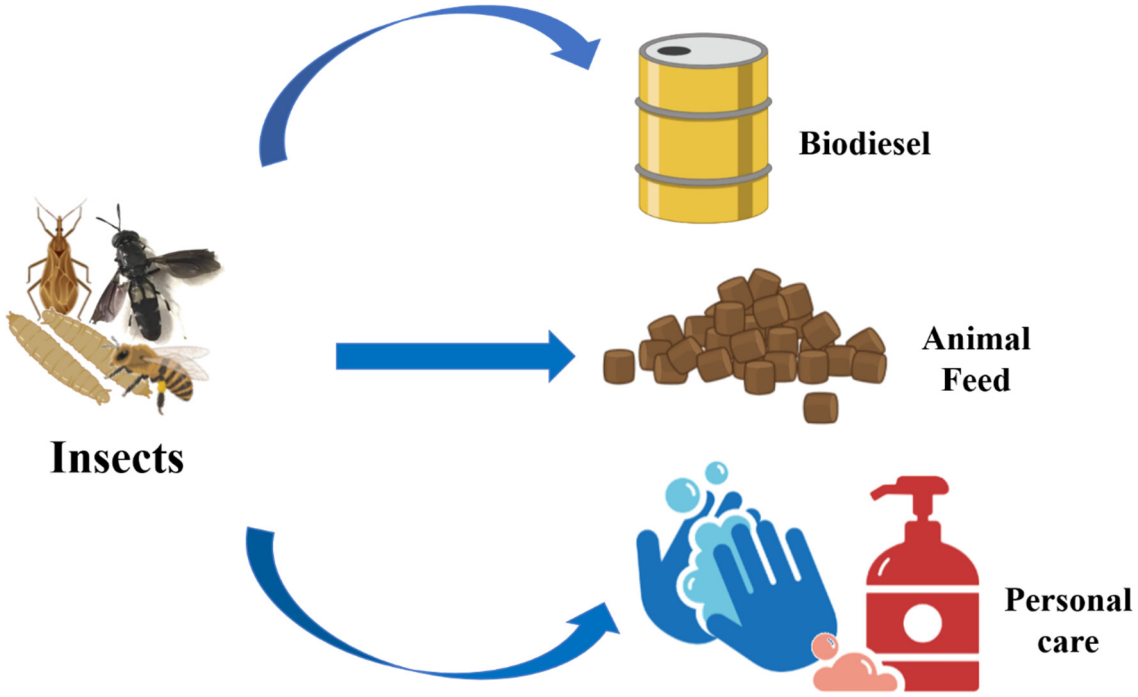

Figure 4. Main applications of insect lipids: biodiesel production, animal feed and personal care.

4.1. Biodiesel Production

Since the last century, fossil fuels have been used to meet the energy demands for economic development. Currently, climate issues and the reduction of non-renewable fossil resources have motivated the development of renewable energies such as solar, wind, and biomass. Among renewable energies with low environmental impact, liquid biofuels, such as bioethanol and biodiesel, can be used in current engines [129] and regular diesel engines [130]. In addition, biodiesel is predominantly used as an additive to traditional fuel in order to minimize the release of particulates, carbon monoxide, and hydrocarbons [131]. The main obstacle preventing biodiesel usage as a primary fuel is represented by the production cost. It has been shown that 75% of biodiesel cost derives from the feedstock, such as vegetable oil, starch, or animal fats [132]. The biodiesel production

process involves two steps: an acid-catalysed esterification of free fatty acids of the crude oil as pre-treatment, and their alkaline-catalysed transesterification [132–134]. The latter process is used to convert the free fatty acids of the raw lipid extract into biodiesel and to reduce its acidity. The use of seed oils for biodiesel production is not a sustainable option, since they are an important food resource, especially for developing countries. A promising alternative to seed oils are microalgae, thanks to their high rate of lipid accumulation and fast growth [135–137], but their use is limited by high production costs and the less than optimal lipid composition [138] and, therefore, it is still insufficiently competitive. Thus, the biodiesel market needs to find new and innovative sources, particularly cheap and non-food-materials.

BSFL were investigated for biodiesel production by Li et al. [82]. Lipid production and the amount of biodiesel derived by BSFL fed for 10 days on different types of manure were evaluated. Samples of crude lipid extract were treated to esterify free fatty acids with methanol using H_2SO_4 (1% w/w) as a catalyst. The acid-catalysed esterification was performed in different conditions: using methanol in different ratios (from 6:1 to 12:1), at different exposure times (ranging from 30 to 120 min), and at different temperatures (between 55 °C and 85 °C). In the pre-treatment of acid-catalysed esterification of free fatty acids, the highest conversion rate of fatty acids was obtained with the ratio between methanol and insect fat at 8:1, and at a temperature of 75 °C for 1 h. The best result in terms of lipids production was obtained by feeding larvae on poultry manure: a crude fat yield of 30.1% was obtained from the larvae with a respective biodiesel yield of 93%. Similar crude fat yields around 29% were obtained from larvae fed on both dairy and pig manure. The respective biodiesel yield was slightly higher from larvae fed on pig manure than from those fed on dairy manure (96% vs. 93%).

Characteristics of the biofuel produced by BSFL, such as iodine number, saponification value, melting point, peroxide value and acid value, density, viscosity, explosion point and cetane index, are similar to those of biodiesel produced with rapeseed oil, and are in accordance with the European biodiesel standard, EN14214 [68,132,138,139]. Furthermore, the biofuel produced from BSFL fat has greater oxidative stability than that produced by rapeseed oil because of the higher content of saturated ester methyl fatty acids (up to 67.6%) [132,138].

Zheng et al. [140] evaluated the biodiesel yield from both the organic waste fraction from restaurants and the BSFL fed on it. A yield of 3.3% fat and 2.7% biodiesel was obtained from restaurant waste. By contrast, BSFL raised on this waste for seven days produced about 39% lipids and a respective biodiesel yield of 25%. This investigation demonstrated that it is possible to perform a double extraction: directly from the food residue, with a lower yield, and also from the larvae fed with the same organic substrate obtaining a higher quantity of extracted lipids with a better fatty acid profile. In addition, the weight of the restaurant organic waste was reduced by 61.8%.

Another interesting study was undertaken by Surendra et al. [81] concerning the evaluation of the growth of larval biomass on human food waste. Lipid extraction from BSF prepupae was performed mechanically using a press. The larvae provided a lipid yield of 15–20% of dry product, i.e., only about 40% of the fat contained in the prepupae of BSF. This low fat yield was probably related to the low fibre content in BSF prepupae, which did not offer a sufficient back pressure during extraction. A subsequent extraction was carried out on the crude fat obtained with the Soxhlet method using petroleum ether as a solvent. The content of short chain saturated fatty acids (C12:0, C16:0) in the fat obtained from BSF prepupae (67%) was higher than in soybean oil (13%) and in palm oil (37%). Hence, food residues used in this study can be considered an optimal substrate for the larval development and the subsequent lipid extraction and biodiesel production. Considering that a third of the food produced globally is not consumed by humans but rather thrown away, its use as a feeding substrate for the biodiesel production from livestock can contribute to the reduction of greenhouse gas emissions and economic losses [22,141].

Another unconventional raw material for biodiesel production was studied by Li et al. [68]. They investigated the accumulation of lipids and the following biodiesel production from BSFL fed on glucose and xylose from lignocelluloses. Xylose and glucose, in individual or mixed form, were effectively metabolized into lipids stored in BSFL. The addition of 6% xylose to the standard larval diet led to a yield of 34.6% of extracted lipids, encouraging the use of BSF for the conversion of lignocellulose into biodiesel [68].

Wong et al. [142] analysed new conditions to increase the biodiesel yield from fats extracted from BSFL. Larvae reared on a substrate containing coconut endosperm with the addition of microorganisms to promote fermentation were tested. The amount of total fat and lipid composition for the fifth and sixth larval instars were evaluated. The lipid content in the fifth instar was 34%, while that in the sixth instar was 26%. No differences in terms of composition in fatty acid methyl esters (FAME) were found. The yield in terms of FAME was 25% in the sixth instar and 33% in the fifth instar, reaching 38.5% with the addition of 0.5% of yeast powder to the substrate. The addition of yeast had positive effects on the conversion rate of the food substrate thanks to an in-situ fermentation. The protein content of the larvae was also improved, while no changes were found for the quantity and composition of lipids.

As described in a paper by Nguyen et al. [133], a direct transesterification technique with fewer steps was proposed to reduce energy costs. In this method, the key element is methanol, which is simultaneously used as a solvent for extraction of fat and as a reagent for the transesterification of the extracted fatty acids into methyl ester fatty acids. Recently, to reduce methanol usage [143], cosolvents such as hexane, pentane, chloroform, acetone and petroleum ether, which increase extraction efficiency because of their ability to dissolve long-chain triglycerides, have been proposed [133,143,144]. Among the solvents used, hexane allowed the highest yield (63.37%) and was found to be the perfect co-solvent for lipid extraction and biodiesel production from BSFL. Subsequently, several experiments were performed with different solvents, methanol volume ratios (*v*:*v*), different solvent dosages, temperature conditions and reaction times, with the aim to establish their effect on biodiesel yield. The results revealed that the biodiesel yield increased with the solvent dosage, reaction time, temperature and with lower hexane: methanol volume ratios, most likely because an excess of solvent decreases the collision between grease and methanol [143]. Sulfuric acid catalyses the transesterification reaction of acylglycerols and the esterification reaction of free fatty acids [139,143,145], but large quantities of sulfuric acid (higher than 1.2 mL) can cause polymerization of unsaturated fatty acids, leading to a reduction of the biodiesel yield [98,143,146]. The amount of biodiesel increased with temperature but there was no substantial growth above 120 °C; therefore, 120 °C was chosen as the optimal temperature for biodiesel production. The highest biodiesel yield (94.14%) on total fat content was obtained at 120 °C with a 1:2 hexane: methanol ratio (*v*/*v*), a solvent dosage of 12 mL, and a reaction time of 90 min. Total lipid percentage, extracted from BSFL using hexane as a solvent, was 30.2%. These results showed a concordance with the optimal characteristics of biofuels, these being water content, density values, acid value, sulphur content, and viscosity comparable to the European standard for biodiesel (EN1421), the international standard of the American Society for Test and Materials (ASTM) D6751 [147,148], and to the biodiesel obtained from rapeseed oil. BSFL fat can be generally considered an optimal source to produce biodiesel with low viscosity and high oxidative stability thanks to a lipid profile rich in short-chain saturated fatty acids and low polyunsaturated fatty acids [81,101]. The process also leads to a considerable reduction in time, going from 49 h of traditional processes to 1.5 h [133].

Energy consumption analysis of lipid extraction from BSF biomass was carried out by Feng et al. [149]. The main process that shows high energy cost for insect lipid extraction is the dehydration of larvae, since the larvae can contain up to 70% water. The costs include the energy necessary to heat the homogenized larval biomass and to evaporate the contained water. The homogenization treatment before drying is used to break cell-cell bonds and to increase the flow of water [98,150]. The presence of water acts as a barrier to

the transfer of the lipid component to the solvent, reducing its extraction efficiency [110,111]. Hence, to reduce the costs there is a need to reduce drying costs. The combined use of dehydration techniques reduces the energy cost by pre-treatment of insect biomass. For example, an initial centrifugation process reduces the amount of water present in the homogenized larvae from 70% to 30%, and a subsequent heating reduces the percentage to 10% [149]. The combination mode has shown lower energy consumption. The mechanical process, rather than thermal alternatives such as centrifuge, vacuum filtration and vacuum drying, has a lower energy cost but requires longer times, and there is no existing evaluation on a large-scale production. Indeed, industrial-scale research on biorefinery and available data on massive extraction are lacking [151].

Other costs are related to the solvent used in the process. In general, the fat yield increases as the volume of solvent increases [133], but there are significant costs associated with the extraction processes.

It can be concluded that a promising alternative is represented by BSFL lipids, and these have become the current focus of several research works [138,152].

Further knowledge is needed to improve the conversion rate of food residues into biomass, to increase the lipid yield and the transesterification rate of the fat extracted into biodiesel.

4.2. Animal Feed

Insects can be used as an ingredient in poultry, pig and aquaculture feeds [21,23,62]. In 2050, the demand for animal products is expected to increase by 60–70% [62]. To date, the cost of soy and fish feed is very high and their availability in the future may be limited [153]. When processed, insects provide the protein fraction and the fat fraction, both of great interest in the production of feed for aquaculture. Both living larvae and larval meal can be used as feed, even if meal is preferred for its easier transport and storage. The use of live insects can be economically sustainable but there is a high risk of escape and colonization of the surrounding ecosystems [154].

BSF is one of the promising species whose larvae have a high nutritional value and, therefore, they are ingredients of animal feedstuff, particularly for aquaculture [36]. It is known that diets containing BSFL are as palatable as those containing soy [62].

For many years, BSF meals such as fish feed have been tested for different species of fish. To include BSF meal as a substitute for soybean and fish meal in the diets of fish species, different features were analysed (growth performance, nutrient digestibility, and meat quality). The fish species studied are reported in Table S3.

Belghit et al. [155] evaluated the effect of dietary meal and fat from BSF on body composition, growth performance and nutrient digestibility of Atlantic salmon. It has been shown that it is possible to add up to 600 g of insect meal per kg of diet, in combination with insect fat, in Atlantic salmon diets without any negative impact on food intake, growth performance or feed conversion ratio (the ratio between the taken food and the gained weight).

St-Hilaire et al. [156] and Barroso et al. [157] demonstrated how to enrich BSFL in omega 3 fatty acids by means of dietary modifications. Specifically, prepupae that included fish offal in their diet were enriched in omega 3 fatty acids, especially α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These BSF prepupae may be used to reduce animal waste and recycle omega 3 fatty acids, and, simultaneously, to produce high-quality animal foodstuff, as they are a suitable replacement for fish meal and fish oil in animal diets.

The intake of ω3 could be improved by adding ω3 to the diet [158,159] and, in general, the content of EPA and DHA in insect meal can be increased by nutritional management [157]. The bioaccumulation of the two fatty acids in insect larvae has nutritional relevance for humans [157,160] and adds a great nutritional value to promote the use of insect meal as food. Moreover, according to Barroso et al. [157], the larvae fed with fish offal need short times of feeding to get enrichment of molecules such as EPA and DHA;

specifically, the time was valued between 30 min and 3 h before harvesting. However, if the aim is to get larvae with high values of ω3 to feed animals, the enrichment of the feeding substrate is not convenient; providing ω3 directly to the animals could be simpler and cheaper.

Meals obtained from BSFL find application in the feeding of growing pigs because of the high amino acid, lipid and calcium contents [161]. A study by Nekrasov et al. [162] focused on the use of BSFL fats applied to the feeding of farmed pigs. Pigs fed with a standard diet (administered *ad libitum*) with the addition of 0.3% and 0.9% BSFL showed a higher average daily weight gain (between 8.2% and 9.1%) than the control group fed with a standard substrate. Pigs fed with supplemented BSFL also showed an increase of total protein concentration, leukocytes and bifid bacteria, a reduction in bilirubin level and no variations of lactobacilli in the large intestine when compared to the control group. Moreover, in the experimental group, where no type of antibiotic was used, survival of pigs was 100% compared to the control group in which pigs' survival was 98%, using antibiotics when necessary. This study demonstrated the possibility to use BSFL as a dietary supplement for growing pigs and that BSF can be a source of valuable biomolecules with a positive impact on animal immune system [162].

Yu et al. [163] showed that inclusion of BSFL in feedstuff for pigs had a beneficial effect on growth performance and meat quality. Four groups of pigs, weaned at 21 days, were fed with a supplementation (0%, 1%, 2% and 4%) of BSFL meal with full fat content. Two experimental feeding phases were tested: days 1–14 and days 15–28. Results revealed no mortality and no increase of diarrhoea manifestation induced by insect meal. Generally, a 2% supplementation of the BSFL meal had a significant positive effect on the growth performance and organ weight of the pigs.

A similar study was performed by Heugten et al. [164], who evaluated the impact of supplementing the diet with increasing amounts of BSFL fat on the growth and performance of 21 day old, weaned pigs. Zero, 2%, 4%, and 6% of supplemental insect fat was used as a replacement for equal quantities of corn oil. The addition of insect lipids led to an increase of both the weight and the gain/feed ratio (that means the weight gain in relation to the percentage of BSFL fat in diet) of the pigs.

Furthermore, BSF meal as a poultry feed has been largely investigated [16,165–181]. Most of the studies showed that it is possible to replace a considerable part of the soybean meal in poultry diets with BSF meal without negatively affecting performance and the quality of the final products [182]. The results of the effect of BSF feed on poultry are not univocal because different factors are involved, including the animal growth stage, the breed (broiler or layer), the method of feed administration, the inclusion rate of BSF meal in the standard diet, the feeding substrate of BSFL used as meal, BSF-feeding duration and the method of processing the BSFL [182]. For example, between the treatment containing up to 15% of BSF biomass and the control diets in terms of apparent assimilation of nutrients, feeding preference, carcass traits, productive performance, index of mortality, and sensory and meat quality characteristics while feeding chicken, no significant difference was observed [165,177,183]. Similarly, BSF meal in the diets of layers have not shown consistent results [16,167,184,185]. Moreover, replacement in a short period did not affect the performance of laying hens [16,184,186], and in a longer period negatively affected their performance [16,167,184]. On the other hand, the use of lipids only as additives to the poultry diet has recently been studied. Kim et al. [186] have performed a study on farmed chickens comparing standard substrate with an integration of three sources of fats: corn oil, coconut oil, and BSFL fat. The aim of the study was to observe the effect of the fat addition on growth performance, carcass characteristics, composition of body fatty acids, production of volatile fatty acids and serum parameters in chickens. During a feeding period of 30 days, 50 g of fat per kg of standard diet were added. The feed conversion ratio was lower with both the coconut oil and BSFL fat than with the corn oil. Insect fat changed the fatty acids composition of chickens, showing a significant increase of branched-chain fatty acids and short-chain fatty acids in the half feeding period. The fatty acid composition

of abdominal fat was influenced by dietary fat sources. Particularly, chickens fed diets containing coconut oil or BSFL fat showed higher content of saturated fatty acids such as lauric and myristic acids, than those fed with an addition of corn oil. On the other hand, polyunsaturated fatty acids were higher with corn oil compared to both coconut oil and insect fat. Finally, a significant increase of the total antioxidant capacity in chickens fed with oil derived from BSFL compared to corn oil was observed. The supplement of BSFL fat improved the colour of the chicken breast, an important feature that highlights good nutrition in terms of quality of poultry feed, and this can be of additional value for the chicken meat market.

Overall, these studies suggest that the fat extracted from BSFL can be used as a functional lipid ingredient and that it has a positive effect on gut health, enriches medium-chain fatty acids in edible tissues, and increases antioxidant capacity in broilers [161,164,187,188].

BSFL fat is a highly energetic food supplement in farmed animals that could provide a reduction in production costs through supplements made of insect flours or insect fats.

5. Conclusions

BSF contributes to the creation of a circular economy by bioconversion of different kinds of waste into biomass rich in proteins, lipids, chitin and several bioactive compounds. Lipids from BSF larvae can be used as a food and feed source, with a beneficial effect on animal growth performance and meat quality and a positive impact on animal immune systems. Moreover, they have applications in biodiesel production as innovative energy-sources in accordance with European biodiesel standards. The biodiesel production from BSFL fats needs further evaluation of results, techno-economical approaches, and evaluation of substrate accessibility; the large amount of needed fat for the biodiesel production is a limiting factor and a challenge for insect breeders. Despite the progress achieved so far, additional studies in the field are required for setting up standard protocols for insect fractions isolation, for improving the current procedures, and for investigating new and alternative applications of biomolecules from insect species. The main challenge in this regard is the implementation of BSFL breeding at an industrial scale, as there is a significant difference between benchtop and industrial scale results, and to deepen the knowledge on which applications fit better for this insect reared on different substrates and its derived valuable molecules.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/su131810198/s1. Table S1: Proximate lipid content of BSF biomass reared on different organic wastes (Unit: % of dry matter); Table S2: Fatty acids profile of the BSF biomass reared on different organic waste (Unit: % of dry matter); Table S3: Fish species fed with BSF. References [189–221] are cited in the Supplementary Materials.

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References

1. Scudder, G.G.E. *The Importance of Insects in Insect Biodiversity: Science and Society*; Foottit, R.G., Adler, P.H., Eds.; Wiley Blackwell: Hoboken, NJ, USA, 2017.
2. Pires, C.S.S.; Maués, M.M. Insect pollinators, major threats and mitigation measures. *Neotrop. Entomol.* **2020**, *49*, 469–471. [CrossRef]
3. Mauricio da Rocha, J.R.; De Almeida, J.R.; Lins, G.A.; Durval, A. Insects as indicators of environmental changing and pollution: A review of appropriate species and their monitoring. *Holos Environ.* **2010**, *10*, 250. [CrossRef]

4. Cusumano, A.; Volkoff, A.N. Influence of parasitoid-associated viral symbionts on plant-insect interactions and biological control. *Curr. Opin. Insect Sci.* **2021**, *44*, 64–71. [CrossRef]
5. Adamski, Z.; Bufo, S.A.; Chowański, S.; Falabella, P.; Lubawy, J.; Marciniak, P.; Pacholska-Bogalska, J.; Salvia, R.; Scrano, L.; Słocińska, M.; et al. Beetles as model organisms in physiological, biomedical and environmental studies–A review. *Front. Physiology* **2019**, *10*, 319. [CrossRef]
6. Snell-Rood, E. Bring biologists into biomimetics. *Nature* **2016**, *529*, 277–278. [CrossRef]
7. Diener, S.; Zurbrügg, C.; Tockner, K. Conversion of organic material by black soldier fly larvae: Establishing optimal feeding rates. *Waste Manag. Res. J. Sustain. Circ. Econ.* **2009**, *27*, 603–610. [CrossRef] [PubMed]
8. Kalová, M.; Borkovcová, M. Voracious larvae *Hermetia illucens* and treatment of selected types of biodegradable waste. *Acta. Univ. Agric. Silvic. Mendel. Brun.* **2013**, *61*, 77–83. [CrossRef]
9. Zhou, F.; Tomberlin, J.K.; Zheng, L.; Yu, Z.; Zhang, J. Developmental and Waste Reduction Plasticity of Three Black Soldier Fly Strains (Diptera: Stratiomyidae) Raised on Different Livestock Manures. *J. Med. Entomol.* **2013**, *50*, 1224–1230. [CrossRef]
10. Sheppard, C. House Fly and Lesser Fly Control Utilizing the Black Soldier Fly in Manure Management Systems for Caged Laying Hens. *Environ. Entomol.* **1983**, *12*, 1439–1442. [CrossRef]
11. Nguyen, T.T.X.; Tomberlin, J.K.; Vanlaerhoven, S. Ability of Black Soldier Fly (Diptera: Stratiomyidae) Larvae to Recycle Food Waste. *Environ. Entomol.* **2015**, *44*, 406–410. [CrossRef]
12. Jucker, C.; Erba, D.; Leonardi, M.G.; Lupi, D.; Savoldelli, S. Assessment of Vegetable and Fruit Substrates as Potential Rearing Media for *Hermetia illucens* (Diptera: Stratiomyidae) Larvae. *Environ. Entomol.* **2017**, *46*, 1415–1423. [CrossRef]
13. Scala, A.; Cammack, J.A.; Salvia, R.; Scieuzo, C.; Franco, A.; Bufo, S.A.; Tomberlin, J.K.; Falabella, P. Rearing substrate impacts growth and macronutrient composition of *Hermetia illucens* (L.) (Diptera: Stratiomyidae) larvae produced at an industrial scale. *Sci. Rep.* **2020**, *10*, 19448. [CrossRef]
14. Webster, C.D.; Rawles, S.D.; Koch, J.F.; Thompson, K.R.; Kobayashi, Y.; Gannam, A.L.; Twibell, R.G.; Hyde, N.M. Bio-Ag reutilization of distiller's dried grains with solubles as a substrate for black soldier fly larvae, *Hermetia illucens*, along with poultry by-product meal and soybean meal, as total replacement of fish meal in diets for. *Aquac. Nutr.* **2016**, *22*, 976–988. [CrossRef]
15. Ruhnke, I.; Normant, C.; Campbell, D.L.M.; Iqbal, Z.; Lee, C.; Hinch, G.N.; Roberts, J. Impact of on-range choice feeding with black soldier fly larvae (*Hermetia illucens*) on flock performance, egg quality, and range use of free-range laying hens. *Anim. Nutr.* **2018**, *4*, 452–460. [CrossRef]
16. Engel, M.S. Insect evolution. *Curr. Biol.* **2015**, *25*, R868–R872. [CrossRef] [PubMed]
17. Ojha, S.; Bußler, S.; Schlüter, O.K. Food waste valorisation and circular economy concepts in insect production and processing. *Waste Manag.* **2020**, *118*, 600–609. [CrossRef]
18. Poshadri, A. Insects as an Alternate Source for Food to Conventional Food Animals. *Int. J. Pure Appl. Biosci.* **2018**, *6*, 697–705. [CrossRef]
19. FAO. *Global Food Losses and Food Waste–Extent, Causes and Prevention. SAVE FOOD: An Initiative on Food Loss and Waste Reduction*; FAO: Rome, Italy, 2011.
20. Fowles, T.M.; Nansen, C. Insect-Based Bioconversion: Value from Food Waste. In *Food Waste Management*; Springer International Publishing: Cham, Switzerland, 2020; pp. 321–346.
21. Spranghers, T.; Ottoboni, M.; Klootwijk, C.; Ovyn, A.; Deboosere, S.; De Meulenaer, B.; Michiels, J.; Eeckhout, M.; De Clercq, P.; De Smet, S. Nutritional composition of black soldier fly (*Hermetia illucens*) prepupae reared on different organic waste substrates. *J. Sci. Food Agric.* **2017**, *97*, 2594–2600. [CrossRef] [PubMed]
22. FAO. *Agriculture Organization of the United Nations. Food Wastage Footprint: Impacts on Natural Resources*; Summary Report; Natural Resources Management and Environment Department: Rome, Italy, 2013. Available online: http://www.fao.org/3/i3347e/i3347e.pdf (accessed on 1 January 2021).
23. Xiao, X.; Jin, P.; Zheng, L.; Cai, M.; Yu, Z.; Yu, J.; Zhang, J. Effects of black soldier fly (*Hermetia illucens*) larvae meal protein as a fishmeal replacement on the growth and immune index of yellow catfish (*Pelteobagrus fulvidraco*). *Aquac. Res.* **2018**, *49*, 1569–1577. [CrossRef]
24. Commission Regulation (EU) 2017/893 of 24 May 2017 Amending Annexes I and IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council and Annexes X, XIV and XV to Commission Regulation (EU) No 142/2011 as Regards the Provisions on Processed Animal Protein (Text with EEA Relevance). Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32017R0893 (accessed on 1 January 2021).
25. Gasco, L.; Biancarosa, I.; Liland, N.S. From waste to feed: A review of recent knowledge on insects as producers of protein and fat for animal feeds. *Curr. Opin. Green Sustain. Chem.* **2020**, *23*, 67–79. [CrossRef]
26. Marone, P.A. Food safety and regulatory concerns. In *Insects as Sustainable Food Ingredients*; Dossey, A.T., Morales-Ramos, J., Rojas, M.G., Eds.; Academic Press: Cambridge, MA, USA, 2016; pp. 203–221.
27. Van Huis, A.; Van Itterbeeck, J.; Klunder, H.; Mertens, E.; Halloran, A.; Muir, G.; Vantomme, P. *Edible Insects: Future Prospects for Food and Feed Security*; Food and Agriculture Organization of the United Nations: Quebec City, QC, Canada, 2013; Volume 97, ISBN 9789251075951.
28. Van Huis, A.; Oonincx, D.G.A.B. The environmental sustainability of insects as food and feed. A review. *Agron. Sustain. Dev.* **2017**, *37*, 43. [CrossRef]

29. Cadinu, L.A.; Barra, P.; Torre, F.; Delogu, F.; Madau, F.A. Insect Rearing: Potential, Challenges, and Circularity. *Sustainability* **2020**, *12*, 4567. [CrossRef]
30. Madau, F.A.; Arru, B.; Furesi, R.; Pulina, P. Insect Farming for Feed and Food Production from a Circular Business Model Perspective. *Sustainability* **2020**, *12*, 5418. [CrossRef]
31. Tao, J.; Li, Y.O. Edible insects as a means to address global malnutrition and food insecurity issues. *Food Qual. Saf.* **2018**, *2*, 17–26. [CrossRef]
32. Mather, J.A. Ethics and care: For animals, not just mammals. *Animals* **2019**, *9*, 1018. [CrossRef] [PubMed]
33. EFSA Scientific Committee. Risk profile related to production and consumption of insects as food and feed. *EFSA J.* **2015**, *13*, 4257. [CrossRef]
34. Wanaratana, S.; Amonsin, A.; Chaisingh, A.; Panyim, S.; Sasipreeyajan, J.; Pakpinyo, S. Experimental assessment of houseflies as vectors in avian influenza subtype H5N1 transmission in chickens. *Avian Dis.* **2013**, *57*, 266–272. [CrossRef]
35. Pezzi, M.; Leis, M.; Chicca, M.; Falabella, P.; Salvia, R.; Scala, A.; Whitmore, D. Morphology of the antenna of *Hermetia illucens* (Diptera: Stratiomyidae): An ultrastructural investigation. *J. Med Entomol.* **2017**, *54*, 925–933. [CrossRef]
36. Newton, L.; Sheppard, C.; Watson, D.W.; Burtle, G.; Dove, R. *Using the Black Soldier Fly,* Hermetia illucens*, as a Value-Added Tool for the Management of Swine Manure*; Animal Poultry Waste Management Center, North Carolina State University: Raleigh, NC, USA, 2005; p. 17.
37. Purschke, B.; Scheibelberger, R.; Axmann, S.; Adler, A.; Jäger, H. Impact of substrate contamination with mycotoxins, heavy metals and pesticides on the growth performance and composition of black soldier fly larvae (*Hermetia illucens*) for use in the feed and food value chain. *Food Addit. Contam. Part A* **2017**, *34*, 1410–1420. [CrossRef]
38. Romdhana, M.H.; Lecomte, D.; Ladevie, B.; Sablayrolles, C. Monitoring of pathogenic microorganisms contamination during heat drying process of sewage sludge. *Process. Saf. Environ. Prot.* **2009**, *87*, 377–386. [CrossRef]
39. Regulation (EU) 2017/1017 of 15 June 2017 Amending Regulation (EU) No 68/2013 on the Catalogue of Feed Materials (Text with EEA Relevance). Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32017R1017&from=EN (accessed on 1 January 2021).
40. Skrivervik, E. Insects' contribution to the bioeconomy and the reduction of food waste. *Heliyon* **2020**, *6*, e03934. [CrossRef] [PubMed]
41. Carpenter, F.M.; Burnham, L. The geological record of insects. *Annu. Rev. Earth Planet. Sci.* **1985**, *13*, 297. [CrossRef]
42. Lowman, M.D.; Rinker, H.B. *Forest Canopies*; Lowman, M.D., Rinker, H.B., Eds.; Elsevier: Amsterdam, The Netherlands, 2004.
43. Müller, A.; Wolf, D.; Gutzeit, H.O. The black soldier fly, *Hermetia illucens*—A promising source for sustainable production of proteins, lipids and bioactive substances. *Z. Naturforsch.* **2017**, *72*, 351–363. [CrossRef]
44. Ramos-Elorduy, J. *Ecological Implications of Minilivestock—Potential of Insects, Rodents, Frogs and Sails*; Paoletti, M.G., Ed.; CRC Press: Boca Raton, FL, USA, 2005; pp. 263–291.
45. Caligiani, A.; Marseglia, A.; Leni, G.; Baldassarre, S.; Maistrello, L.; Dossena, A.; Sforza, S. Composition of black soldier fly prepupae and systematic approaches for extraction and fractionation of proteins, lipids and chitin. *Food Res. Int.* **2018**, *105*, 812–820. [CrossRef]
46. Montowska, M.; Kowalczewski, P.Ł.; Rybicka, I.; Fornal, E. Nutritional value, protein and peptide composition of edible cricket powders. *Food Chem.* **2019**, *289*, 130–138. [CrossRef]
47. EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA); Turck, D.; Castenmiller, J.; De Henauw, S.; Hirsch-Ernst, K.I.; Kearney, J.; Maciuk, A.; Mangelsdorf, I.; McArdle, H.J.; Naska, A.; et al. Safety of dried yellow mealworm (*Tenebrio molitor* larva) as a novel food pursuant to Regulation (EU) 2015/2283. *EFSA J.* **2021**, *19*, e06343. [PubMed]
48. Triunfo, M.; Tafi, E.; Guarnieri, A.; Scieuzo, C.; Hahn, T.; Zibek, S.; Salvia, R.; Falabella, P. Insect Chitin-Based Nanomaterials for Innovative Cosmetics and Cosmeceuticals. *Cosmetics* **2021**, *8*, 40. [CrossRef]
49. Moretta, A.; Salvia, R.; Scieuzo, C.; Di Somma, A.; Vogel, H.; Pucci, P.; Sgambato, A.; Wolff, M.; Falabella, P. A bioinformatic study of antimicrobial peptides identified in the Black Soldier Fly (BSF) *Hermetia illucens* (Diptera: Stratiomyidae). *Sci. Rep.* **2020**, *10*, 16875. [CrossRef]
50. Moretta, A.; Scieuzo, C.; Petrone, A.M.; Salvia, R.; Manniello, M.D.; Franco, A.; Lucchetti, D.; Vassallo, A.; Vogel, H.; Sgambato, A.; et al. Antimicrobial Peptides: A New Hope in Biomedical and Pharmaceutical Fields. *Front. Cell. Infect. Microbiol.* **2021**, *11*. [CrossRef]
51. Manniello, M.D.; Moretta, A.; Salvia, R.; Scieuzo, C.; Lucchetti, D.; Vogel, H.; Sgambato, A.; Falabella, P. Insect antimicrobial peptides: Potential weapons to counteract the antibiotic resistance. *Cell. Mol. Life Sci.* **2021**, *1*, 3. [CrossRef]
52. Mai, H.C.; Dao, N.D.; Lam, T.D.; Nguyen, B.V.; Nguyen, D.C.; Bach, L.G. Purification Process, Physicochemical Properties, and Fatty Acid Composition of Black Soldier Fly (*Hermetia illucens Linnaeus*) Larvae Oil. *J. Am. Oil Chem. Soc.* **2019**, *96*, 1303–1311. [CrossRef]
53. Fasakin, E.A.; Balogun, A.M.; Ajayi, O.O. Evaluation of full-fat and defatted maggot meals in the feeding of clariid catfish *Clarias gariepinus fingerlings*. *Aquac. Res.* **2003**, *34*, 733–738. [CrossRef]
54. Delicato, C.; Schouteten, J.J.; Dewettinck, K.; Gellynck, X.; Tzompa-Sosa, D.A. Consumers' perception of bakery products with insect fat as partial butter replacement. *Food Qual. Prefer.* **2020**, *79*, 103755. [CrossRef]
55. Smetana, S.; Leonhardt, L.; Kauppi, S.-M.; Pajic, A.; Heinz, V. Insect margarine: Processing, sustainability and design. *J. Clean. Prod.* **2020**, *264*, 121670. [CrossRef]

56. Leong, S.Y.; Kutty, S.R.M.; Tan, C.K.; Tey, L.H. Comparative study on the effect of organic waste on lauric acid produced by *Hermetia illucens* larvae via bioconversion. *J. Eng. Sci. Technol.* **2015**, *8*, 52–63.
57. Barragán-Fonseca, K.; Pineda-Mejia, J.; Dicke, M.; van Loon, J.J.A. Performance of the Black Soldier Fly (Diptera: Stratiomyidae) on Vegetable Residue-Based Diets Formulated Based on Protein and Carbohydrate Contents. *J. Econ. Entomol.* **2018**. [CrossRef] [PubMed]
58. Barragan-Fonseca, K.B.; Dicke, M.; van Loon, J.J.A. Nutritional value of the black soldier fly (*Hermetia illucens L.*) and its suitability as animal feed—A review. *J. Insects Food Feed* **2017**, *3*, 105–120. [CrossRef]
59. Tschirner, M.; Simon, A. Influence of different growing substrates and processing on the nutrient composition of black soldier fly larvae destined for animal feed. *J. Insects Food Feed* **2015**, *1*, 249–259. [CrossRef]
60. Henry, M.; Gasco, L.; Piccolo, G.; Fountoulaki, E. Review on the use of insects in the diet of farmed fish: Past and future. *Anim. Feed Sci. Technol.* **2015**, *203*, 1–22. [CrossRef]
61. Leong, S.Y.; Kutty, S.R.M.; Malakahmad, A.; Tan, C.K. Feasibility study of biodiesel production using lipids of *Hermetia illucens* larva fed with organic waste. *Waste Manag.* **2016**, *47*, 84–90. [CrossRef]
62. Makkar, H.P.S.; Tran, G.; Heuzé, V.; Ankers, P. State-of-the-art on use of insects as animal feed. *Anim. Feed Sci. Technol.* **2014**, *197*, 1–33. [CrossRef]
63. Pieterse, E.; Pretorius, Q. Nutritional evaluation of dried larvae and pupae meal of the housefly (*Musca domestica*) using chemical- and broiler-based biological assays. *Anim. Prod. Sci.* **2014**, *54*, 347. [CrossRef]
64. Liu, C.; Wang, C.; Yao, H. Comprehensive Resource Utilization of Waste Using the Black Soldier Fly (*Hermetia illucens L.*) (Diptera: Stratiomyidae). *Animals* **2019**, *9*, 349. [CrossRef] [PubMed]
65. Gold, M.; Binggeli, M.; Kurt, F.; de Wouters, T.; Reichlin, M.; Zurbrügg, C.; Mathys, A.; Kreuzer, M. Novel Experimental Methods for the Investigation of *Hermetia illucens* (Diptera: Stratiomyidae) Larvae. *J. Insect Sci.* **2020**, *20*. [CrossRef] [PubMed]
66. Nguyen, T.T.X.; Tomberlin, J.K.; Vanlaerhoven, S. Influence of Resources on *Hermetia illucens* (Diptera: Stratiomyidae) Larval Development. *J. Med. Entomol.* **2013**, *50*, 898–906. [CrossRef]
67. Oonincx, D.G.A.B.; van Broekhoven, S.; van Huis, A.; van Loon, J.J.A. Feed Conversion, Survival and Development, and Composition of Four Insect Species on Diets Composed of Food By-Products. *PLoS ONE* **2015**, *10*, e0144601. [CrossRef] [PubMed]
68. Li, W.; Li, M.; Zheng, L.; Liu, Y.; Zhang, Y.; Yu, Z.; Ma, Z.; Li, Q. Simultaneous utilization of glucose and xylose for lipid accumulation in black soldier fly. *Biotechnol. Biofuels* **2015**, *8*, 117. [CrossRef]
69. Pimentel, A.C.; Montali, A.; Bruno, D.; Tettamanti, G. Metabolic adjustment of the larval fat body in *Hermetia illucens* to dietary conditions. *J. Asia. Pac. Entomol.* **2017**, *20*, 1307–1313. [CrossRef]
70. Danieli, P.P.; Lussiana, C.; Gasco, L.; Amici, A.; Ronchi, B. The Effects of Diet Formulation on the Yield, Proximate Composition, and Fatty Acid Profile of the Black Soldier Fly (*Hermetia illucens L.*) Prepupae Intended for Animal Feed. *Animals* **2019**, *9*, 178. [CrossRef]
71. Lalander, C.; Diener, S.; Zurbrügg, C.; Vinnerås, B. Effects of feedstock on larval development and process efficiency in waste treatment with black soldier fly (*Hermetia illucens*). *J. Clean. Prod.* **2019**, *208*, 211–219. [CrossRef]
72. Ewald, N.; Vidakovic, A.; Langeland, M.; Kiessling, A.; Sampels, S.; Lalander, C. Fatty acid composition of black soldier fly larvae (*Hermetia illucens*)—Possibilities and limitations for modification through diet. *Waste Manag.* **2020**, *102*, 40–47. [CrossRef] [PubMed]
73. Somroo, A.A.; ur Rehman, K.; Zheng, L.; Cai, M.; Xiao, X.; Hu, S.; Mathys, A.; Gold, M.; Yu, Z.; Zhang, J. Influence of Lactobacillus buchneri on soybean curd residue co-conversion by black soldier fly larvae (*Hermetia illucens*) for food and feedstock production. *Waste Manag.* **2019**, *86*, 114–122. [CrossRef] [PubMed]
74. Arango Gutiérrez, G.P.; Vergara Ruiz, R.A.; Mejía Vélez, H. Compositional, microbiological and protein digestibility analysis of the larva meal of *Hermetia illuscens* L. (Diptera: Stratiomyiidae) at Angelópolis-Antioquia, Colombia. *Rev. Fac. Nac. Agron. Medellín* **2004**, *57*, 2491–2500.
75. Yang, S.Y.; Li, W.J.; Liu, C.X.; Hu, W.F. Effects of fermented swine manure on the conversion ratio of *Hermetia illucens* and nutritional components detection of *Hermetia illucens* larva and sandworm. *J. Anhui Agricul. Sci.* **2016**, *44*, 69–70.
76. Julita, U.; Suryani, Y.; Kinasih, I.; Yuliawati, A.; Cahyanto, T.; Maryeti, Y.; Permana, A.D.; Fitri, L.L. Growth performance and nutritional composition of black soldier fly, *Hermetia illucens* (L), (Diptera: Stratiomyidae) reared on horse and sheep manure. In *IOP Conference Series: Earth and Environmental Science*; IOP Publishing: Bristol, UK, 2018; Volume 187, p. 012071.
77. Meneguz, M.; Schiavone, A.; Gai, F.; Dama, A.; Lussiana, C.; Renna, M.; Gasco, L. Effect of rearing substrate on growth performance, waste reduction efficiency and chemical composition of black soldier fly (*Hermetia illucens*) larvae. *J. Sci. Food Agric.* **2018**, *98*, 5776–5784. [CrossRef]
78. Arrese, E.L.; Soulages, J.L. Insect Fat Body: Energy, Metabolism, and Regulation. *Annu. Rev. Entomol.* **2010**, *55*, 207–225. [CrossRef]
79. Inagaki, S.; Yamashita, O. Metabolic shift from lipogenesis to glycogenesis in the last instar larval fat body of the silkworm, *Bombyx mori*. *Insect Biochem.* **1986**, *16*, 327–331. [CrossRef]
80. Ushakova, N.A.; Brodskii, E.S.; Kovalenko, A.A.; Bastrakov, A.I.; Kozlova, A.A.; Pavlov, D.S. Characteristics of lipid fractions of larvae of the black soldier fly *Hermetia illucens*. *Dokl. Biochem. Biophys.* **2016**, *468*, 209–212. [CrossRef] [PubMed]
81. Surendra, K.C.; Olivier, R.; Tomberlin, J.K.; Jha, R.; Khanal, S.K. Bioconversion of organic wastes into biodiesel and animal feed via insect farming. *Renew. Energy* **2016**, *98*, 197–202. [CrossRef]

82. Li, Q.; Zheng, L.; Cai, H.; Garza, E.; Yu, Z.; Zhou, S. From organic waste to biodiesel: Black soldier fly, *Hermetia illucens*, makes it feasible. *Fuel* **2011**, *90*, 1545–1548. [CrossRef]
83. Kroeckel, S.; Harjes, A.G.E.; Roth, I.; Katz, H.; Wuertz, S.; Susenbeth, A.; Schulz, C. When a turbot catches a fly: Evaluation of a pre-pupae meal of the Black Soldier Fly (*Hermetia illucens*) as fish meal substitute—Growth performance and chitin degradation in juvenile turbot (*Psetta maxima*). *Aquaculture* **2012**, *364*, 345–352. [CrossRef]
84. Mashkovskii, M.D. *Lekarstvennye Sredstva*; Novaya Volna: Moscow, Russia, 2012.
85. Diclaro, J.W., II; Kaufman, P.E. Black Soldier Fly Hermetia Illucens Linnaeus (Insecta: Diptera: Stratiomyidae). Available online: https://edis.ifas.ufl.edu/pdf/IN/IN83000.pdf (accessed on 1 January 2021).
86. Almeida, C.; Rijo, P.; Rosado, C. Bioactive Compounds from *Hermetia illucens* Larvae as Natural Ingredients for Cosmetic Application. *Biomolecules.* **2020**, *10*, 976. [CrossRef] [PubMed]
87. Briegel, H. Metabolic relationship between female body size, reserves, and fecundity of *Aedes aegypti*. *J. Insect Physiol.* **1990**, *36*, 165–172. [CrossRef]
88. Venkatesh, K.; Morrison, P.E. Studies of weight changes and amount of food ingested by the stable fly, *Stomoxys calcitrans* (Diptera: Muscidae). *Can. Entomol.* **1980**, *112*, 141–149. [CrossRef]
89. Bennett, V.; Lee, R. Modeling seasonal changes in intracellular freeze-tolerance of fat body cells of the gall fly *Eurosta solidaginis* (Diptera, Tephritidae). *J. Exp. Biol.* **1997**, *200*, 185–192. [CrossRef]
90. Holmes, L.A.; VanLaerhoven, S.L.; Tomberlin, J.K. Lower temperature threshold of black soldier fly (Diptera: Stratiomyidae) development. *J. Insects Food Feed* **2016**, *2*, 255–262. [CrossRef]
91. Nakatsuji, T.; Kao, M.C.; Fang, J.-Y.; Zouboulis, C.C.; Zhang, L.; Gallo, R.L.; Huang, C.-M. Antimicrobial Property of Lauric Acid Against Propionibacterium Acnes: Its Therapeutic Potential for Inflammatory *Acne vulgaris*. *J. Invest Dermatol.* **2009**, *129*, 2480–2488. [CrossRef]
92. Park, K.-M.; Lee, S.J.; Yu, H.; Park, J.-Y.; Jung, H.-S.; Kim, K.; Lee, C.J.; Chang, P.-S. Hydrophilic and lipophilic characteristics of non-fatty acid moieties: Significant factors affecting antibacterial activity of lauric acid esters. *Food Sci. Biotechnol.* **2018**, *27*, 401–409. [CrossRef] [PubMed]
93. Zeiger, K.; Popp, J.; Becker, A.; Hankel, J.; Visscher, C.; Klein, G.; Meemken, D. Lauric acid as feed additive—An approach to reducing *Campylobacter* spp. in broiler meat. *PLoS ONE* **2017**, *12*, e0175693. [CrossRef]
94. Zhao, L.; Hu, Y.; Xu, D.; Cai, K. Surface functionalization of titanium substrates with chitosan–lauric acid conjugate to enhance osteoblasts functions and inhibit bacteria adhesion. *Colloids Surf. B Biointerfaces* **2014**, *119*, 115–125. [CrossRef] [PubMed]
95. Mohana Devi, S.; Kim, I. Effect of medium chain fatty acids (MCFA) and probiotic (*Enterococcus faecium*) supplementation on the growth performance, digestibility and blood profiles in weanling pigs. *Vet. Med.* **2014**, *59*, 527–535. [CrossRef]
96. Skřivanová, E.; Marounek, M.; Benda, V.; Březina, P. Susceptibility of *Escherichia coli*, Salmonella sp and Clostridium perfringens to organic acids and monolaurin. *Veterinární Med.* **2006**, *51*, 81–88. [CrossRef]
97. Regulation (EC) 1831/2003/EC on Additives for Use in Animal Nutrition, Replacing Directive 70/524/EEC on Additives in Feeding-Stuffs. Available online: https://ec.europa.eu/jrc/sites/default/files/EC-1831-2003.pdf (accessed on 1 January 2021).
98. Kim, S.A.; Rhee, M.S. Highly enhanced bactericidal effects of medium chain fatty acids (caprylic, capric, and lauric acid) combined with edible plant essential oils (carvacrol, eugenol, β-resorcylic acid, trans -cinnamaldehyde, thymol, and vanillin) against *Escherichia coli* O1. *Food Control.* **2016**, *60*, 447–454. [CrossRef]
99. Spranghers, T.; Michiels, J.; Vrancx, J.; Ovyn, A.; Eeckhout, M.; De Clercq, P.; De Smet, S. Gut antimicrobial effects and nutritional value of black soldier fly (*Hermetia illucens* L.) prepupae for weaned piglets. *Anim. Feed Sci. Technol.* **2018**, *235*, 33–42. [CrossRef]
100. EFSA Panel on Contaminants in the Food Chain (CONTAM); Knutsen, H.K.; Alexander, J.; Barregård, L.; Bignami, M.; Brüschweiler, B.; Ceccatelli, S.; Dinovi, M.; Edler, L.; Grasl-Kraupp, B.; et al. Erucic acid in feed and food. *EFSA J.* **2016**, *14*, e04593.
101. Bovera, F.; Loponte, R.; Marono, S.; Piccolo, G.; Parisi, G.; Iaconisi, V.; Gasco, L.; Nizza, A. Use of *Tenebrio molitor* larvae meal as protein source in broiler diet: Effect on growth performance, nutrient digestibility, and carcass and meat traits. *J. Anim. Sci.* **2016**, *94*, 639–647. [CrossRef]
102. Gasco, L.; Henry, M.; Piccolo, G.; Marono, S.; Gai, F.; Renna, M.; Lussiana, C.; Antonopoulou, E.; Mola, P.; Chatzifotis, S. *Tenebrio molitor* meal in diets for European sea bass (*Dicentrarchus labrax* L.) juveniles: Growth performance, whole body composition and in vivo apparent digestibility. *Anim. Feed Sci. Technol.* **2016**, *220*, 34–45. [CrossRef]
103. Renna, M.; Schiavone, A.; Gai, F.; Dabbou, S.; Lussiana, C.; Malfatto, V.; Prearo, M.; Capucchio, M.T.; Biasato, I.; Biasibetti, E.; et al. Evaluation of the suitability of a partially defatted black soldier fly (*Hermetia illucens* L.) larvae meal as ingredient for rainbow trout (*Oncorhynchus mykiss Walbaum*) diets. *J. Anim. Sci. Biotechnol.* **2017**, *8*, 1–13. [CrossRef]
104. Kates, M. Techniques of lipidology: Isolation, analysis and identification of lipids. In *Laboratory Techniques in Biochemistry and Molecular Biology*; Newport Somerville: Somerville, MA, USA, 1986.
105. Lee, A.G. Lipid–protein interactions in biological membranes: A structural perspective. *Biochim. Biophys. Acta Biomembr.* **2003**, *1612*, 1–40. [CrossRef]
106. Nelson, D.L.; Cox, M.M. *Lehninger Principles of Biochemistry*; Freeman, W.H.: New York, NY, USA, 2018.
107. Gil, A.; Zhang, W.; Wolters, J.C.; Permentier, H.; Boer, T.; Horvatovich, P.; Heiner-Fokkema, M.R.; Reijngoud, D.J.; Bischoff, R. One- vs two-phase extraction: Re-evaluation of sample preparation procedures for untargeted lipidomics in plasma samples. *Anal. Bioanal. Chem.* **2018**, *410*, 5859–5870. [CrossRef]

108. Pomeranz, Y. *Food Analysis: Theory and Practice*; Pomeranz, Y., Ed.; Springer Science & Business Media: Berlin/Heidelberg, Germany, 2013. [CrossRef]
109. Wongkittipong, R.; Prat, L.; Damronglerd, S.; Gourdon, C. Solid-liquid extraction of andrographolide from plants—Experimental study, kinetic reaction and model. *Sep. Purif. Technol.* **2004**, *40*, 147–154. [CrossRef]
110. Mohamad, M.; Ali, M.W.; Ripin, A.; Ahmad, A. Effect of Extraction Process Parameters on the Yield of Bioactive Compounds from the Roots of *Eurycoma longifolia*. *J. Teknol.* **2013**, *60*. [CrossRef]
111. Dong, T.; Knoshaug, E.P.; Pienkos, P.T.; Laurens, L.M.L. Lipid recovery from wet oleaginous microbial biomass for biofuel production: A critical review. *Appl. Energy* **2016**, *177*, 879–895. [CrossRef]
112. Pernet, F.; Tremblay, R. Effect of ultrasonication and grinding on the determination of lipid class content of microalgae harvested on filters. *Lipids* **2003**, *38*, 1191–1195. [CrossRef] [PubMed]
113. Ma, Y.-Q.; Chen, J.-C.; Liu, D.-H.; Ye, X.-Q. Simultaneous extraction of phenolic compounds of citrus peel extracts: Effect of ultrasound. *Ultrason. Sonochem.* **2009**, *16*, 57–62. [CrossRef] [PubMed]
114. Carpenter, D.E.; Ngvainti, J.N.; Lee, S. *Lipid Analysis in: Methods of Analysis for Nutrition Labeling*; Sulivan, D.M., Carpenter, D.E., Eds.; AOAC Press: Rockville, MD, USA, 1993; pp. 85–104.
115. Christie, W.W. *Lipid Analysis*; Pergamon Press: Oxford, UK, 1982.
116. Ranjith Kumar, R.; Hanumantha Rao, P.; Arumugam, M. Lipid Extraction Methods from Microalgae: A Comprehensive Review. *Front. Energy Res.* **2015**, *2*. [CrossRef]
117. Folch, J.; Lees, M.; Stanley, G.S. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497–509. [CrossRef]
118. Matyash, V.; Liebisch, G.; Kurzchalia, T.V.; Shevchenko, A.; Schwudke, D. Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. *J. Lipid Res.* **2008**, *49*, 1137–1146. [CrossRef]
119. Alfonsi, K.; Colberg, J.; Dunn, P.J.; Fevig, T.; Jennings, S.; Johnson, T.A.; Kleine, H.P.; Knight, C.; Nagy, M.A.; Perry, D.A.; et al. Green chemistry tools to influence a medicinal chemistry and research chemistry based organisation. *Green Chem.* **2008**, *10*, 31–36. [CrossRef]
120. Salem, M.; Bernach, M.; Bajdzienko, K.; Giavalisco, P. A Simple Fractionated Extraction Method for the Comprehensive Analysis of Metabolites, Lipids, and Proteins from a Single Sample. *J. Vis. Exp.* **2017**. [CrossRef]
121. Dennison, C.; Lovrien, R. Three Phase Partitioning: Concentration and Purification of Proteins. *Protein Expr. Purif.* **1997**, *11*, 149–161. [CrossRef]
122. Dennison, C. Three-phase partitioning. In *Methods in Protein Biochemistry*; Tschesche, H., Ed.; de Gruyter: Berlin/Heidelberg, Germany, 2011; pp. 1–12.
123. Gómez-González, S.; Ruiz-Jiménez, J.; Priego-Capote, F.; Luque de Castro, M.D. Qualitative and Quantitative Sugar Profiling in Olive Fruits, Leaves, and Stems by Gas Chromatography−Tandem Mass Spectrometry (GC-MS/MS) after Ultrasound-Assisted Leaching. *J. Agric. Food Chem.* **2010**, *58*, 12292–12299. [CrossRef]
124. Isengard, H.D.; Morlock, G.; Breithaupt, D. *Food Analysis in Food Science and Technology*; Campbell-Platt, G., Ed.; Wiley: Hoboken, NJ, USA, 2017; pp. 31–75.
125. Skoog, D.A.; West, D.M.; Holler, F.J.; Crouch, S.R. *Fundamentals of Analytical Chemistry*; Nelson Education: Toronto, ON, USA, 2013.
126. Brunner, G. Supercritical fluids: Technology and application to food processing. *J. Food Eng.* **2005**, *67*, 21–33. [CrossRef]
127. Eawag-Aquatic Research. Fractioning of Black Soldier Fly Larvae into Protein Meal and Fat. Available online: https://www.eawag.ch/fileadmin/Domain1/Abteilungen/sandec/schwerpunkte/swm/Practical_knowhow_on_BSF/bsf_factsheet_fractioning.pdf (accessed on 1 January 2021).
128. Fitzherbert, E.; Struebig, M.; Morel, A.; Danielsen, F.; Bruhl, C.; Donald, P.; Phalan, B. How will oil palm expansion affect biodiversity? *Trends Ecol. Evol.* **2008**, *23*, 538–545. [CrossRef] [PubMed]
129. Demirbas, A. The importance of bioethanol and biodiesel from biomass. *Energy Sources Part B Econ. Plan. Policy* **2008**, *3*, 177–185. [CrossRef]
130. Islam, M.M.; Hasanuzzaman, M.; Pandey, A.K.; Rahim, N.A. Modern energy conversion technologies. In *Energy for Sustainable Development*; Hasanuzzaman, M., Rahim, N.A., Eds.; Academic Press: Cambridge, MA, USA, 2020; pp. 19–39.
131. Bozbas, K. Biodiesel as an alternative motor fuel: Production and policies in the European Union. *Renew. Sustain. Energy Rev.* **2008**, *12*, 542–552. [CrossRef]
132. Li, Q.; Zheng, L.; Qiu, N.; Cai, H.; Tomberlin, J.K.; Yu, Z. Bioconversion of dairy manure by black soldier fly (Diptera: Stratiomyidae) for biodiesel and sugar production. *Waste Manag.* **2011**, *31*, 1316–1320. [CrossRef] [PubMed]
133. Nguyen, H.C.; Liang, S.H.; Li, S.Y.; Su, C.H.; Chien, C.C.; Chen, Y.J.; Huong, D.T.M. Direct transesterification of black soldier fly larvae (*Hermetia illucens*) for biodiesel production. *J. Taiwan Inst. Chem. Eng.* **2018**, *85*, 165–169. [CrossRef]
134. Van Gerpen, J. Biodiesel processing and production. *Fuel Process. Technol.* **2005**, *86*, 1097–1107. [CrossRef]
135. Balat, M. Potential alternatives to edible oils for biodiesel production–A review of current work. *Energy Convers. Manag.* **2011**, *52*, 1479–1492. [CrossRef]
136. Mata, T.M.; Martins, A.A.; Caetano, N.S. Microalgae for biodiesel production and other applications: A review. *Renew. Sustain. Energy Rev.* **2010**, *14*, 217–232. [CrossRef]

137. Pienkos, P.T.; Darzins, A. The promise and challenges of microalgal-derived biofuels. *Biofuels Bioprod. Biorefin.* **2009**, *3*, 431–440. [CrossRef]
138. Lee, A.F.; Bennett, J.A.; Manayil, J.C.; Wilson, K. Heterogeneous catalysis for sustainable biodiesel production via esterification and transesterification. *Chem. Soc. Rev.* **2014**, *43*, 7887–7916. [CrossRef]
139. Zheng, L.; Hou, Y.; Li, W.; Yang, S.; Li, Q.; Yu, Z. Exploring the potential of grease from yellow mealworm beetle (*Tenebrio molitor*) as a novel biodiesel feedstock. *Appl. Energy* **2013**, *101*, 618–621. [CrossRef]
140. Zheng, H.; Yang, X.; Xi, Y. Fat body remodeling and homeostasis control in *Drosophila*. *Life Sci.* **2016**, *167*, 22–31. [CrossRef]
141. Gustavsson, J.; Cederberg, C.; Sonesson, U.; Van Otterdijk, R.; Meybeck, A. Global Food Losses and Food Waste. 2011. Available online: http://www.fao.org/fileadmin/user_upload/suistainability/pdf/Global_Food_Losses_and_Food_Waste.pdf (accessed on 1 January 2021).
142. Wong, C.Y.; Aris, M.N.M.; Daud, H.; Lam, M.K.; Yong, C.S.; Hasan, H.A.; Chong, S.; Show, P.L.; Hajoeningtijas, O.D.; Ho, Y.C.; et al. In-situ yeast fermentation to enhance bioconversion of coconut endosperm waste into larval biomass of *Hermetia illucens*: Statistical augmentation of larval lipid content. *Sustainability* **2020**, *12*, 1558. [CrossRef]
143. Zhang, Y.; Li, Y.; Zhang, X.; Tan, T. Biodiesel production by direct transesterification of microalgal biomass with co-solvent. *Bioresour. Technol.* **2015**, *196*, 712–715. [CrossRef]
144. Rahimi, M.; Mohammadi, F.; Basiri, M.; Parsamoghadam, M.A.; Masahi, M.M. Transesterification of soybean oil in four-way micromixers for biodiesel production using a cosolvent. *J. Taiwan Inst. Chem. Eng.* **2016**, *64*, 203–210. [CrossRef]
145. Talebian-Kiakalaieh, A.; Amin, N.A.S.; Mazaheri, H. A review on novel processes of biodiesel production from waste cooking oil. *Appl. Energy* **2013**, *104*, 683–710. [CrossRef]
146. Sulaiman, S.; Abdul Aziz, A.R.; Aroua, M.K. Reactive extraction of solid coconut waste to produce biodiesel. *J. Taiwan Inst. Chem. Eng.* **2013**, *44*, 233–238. [CrossRef]
147. Ishak, S.; Kamari, A. A review of optimum conditions of transesterification process for biodiesel production from various feedstocks. *Int. J. Environ. Sci. Technol.* **2019**, *16*, 2481–2502. [CrossRef]
148. Su, C.H.; Nguyen, H.C.; Bui, T.L.; Huang, D.L. Enzyme-assisted extraction of insect fat for biodiesel production. *J. Clean. Prod.* **2019**, *223*, 436–444. [CrossRef]
149. Feng, W.; Xiong, H.; Wang, W.; Duan, X.; Yang, T.; Wu, C.; Yang, F.; Xiong, J.; Wang, T.; Wang, C. Energy consumption analysis of lipid extraction from black soldier fly biomass. *Energy* **2019**, *185*, 1076–1085. [CrossRef]
150. Yap, B.H.J.; Dumsday, G.J.; Scales, P.J.; Martin, G.J.O. Energy evaluation of algal cell disruption by high pressure homogenisation. *Bioresour. Technol.* **2015**, *184*, 280–285. [CrossRef] [PubMed]
151. Ravi, H.K.; Degrou, A.; Costil, J.; Trespeuch, C.; Chemat, F.; Vian, M.A. Larvae mediated valorization of industrial, agriculture and food wastes: Biorefinery concept through bioconversion, processes, procedures, and products. *Processes* **2020**, *8*, 857. [CrossRef]
152. Lai, E.P.C. Biodiesel: Environmental Friendly Alternative to Petrodiesel. *J. Pet. Environ. Biotechnol.* **2014**, *5*. [CrossRef]
153. Hua, K.; Cobcroft, J.M.; Cole, A.; Condon, K.; Jerry, D.R.; Mangott, A.; Praeger, C.; Vucko, M.J.; Zeng, C.; Zenger, K.; et al. The Future of Aquatic Protein: Implications for Protein Sources in Aquaculture Diets. *One Earth* **2019**, *1*, 316–329. [CrossRef]
154. Khusro, M.; Andrew, N.R.; Nicholas, A. Insects as poultry feed: A scoping study for poultry production systems in Australia. *Worlds. Poult. Sci. J.* **2012**, *68*, 435–446. [CrossRef]
155. Belghit, I.; Liland, N.S.; Waagbø, R.; Biancarosa, I.; Pelusio, N.; Li, Y.; Krogdahl, Å.; Lock, E.J. Potential of insect-based diets for Atlantic salmon (*Salmo salar*). *Aquaculture* **2018**, *491*, 72–81. [CrossRef]
156. St-Hilaire, S.; Sheppard, C.; Tomberlin, J.K.; Irving, S.; Newton, L.; McGuire, M.A.; Mosley, E.E.; Hardy, R.W.; Sealey, W. Fly prepupae as a feedstuff for rainbow trout, Oncorhynchus mykiss. *J. World Aquac. Soc.* **2007**, *38*, 59–67. [CrossRef]
157. Barroso, F.G.; Sánchez-Muros, M.-J.; Segura, M.; Morote, E.; Torres, A.; Ramos, R.; Guil, J.-L. Insects as food: Enrichment of larvae of *Hermetia illucens* with omega 3 fatty acids by means of dietary modifications. *J. Food Compos. Anal.* **2017**, *62*, 8–13. [CrossRef]
158. Belghit, I.; Liland, N.S.; Gjesdal, P.; Biancarosa, I.; Menchetti, E.; Li, Y.; Waagbø, R.; Krogdahl, Å.; Lock, E.J. Black soldier fly larvae meal can replace fish meal in diets of sea-water phase Atlantic salmon (*Salmo salar*). *Aquaculture* **2019**, *503*, 609–619. [CrossRef]
159. Belghit, I.; Waagbø, R.; Lock, E.J.; Liland, N.S. Insect-based diets high in lauric acid reduce liver lipids in freshwater Atlantic salmon. *Aquac. Nutr.* **2019**, *25*, 343–357. [CrossRef]
160. Cardoso, C.; Afonso, C.; Bandarra, N.M. Dietary DHA and health: Cognitive function ageing. *Nutr. Res. Rev.* **2016**, *29*, 281–294. [CrossRef]
161. Yu, M.; Li, Z.; Chen, W.; Rong, T.; Wang, G.; Ma, X. Hermetia illucens larvae as a potential dietary protein source altered the microbiota and modulated mucosal immune status in the colon of finishing pigs. *J. Anim. Sci. Biotechnol.* **2019**, *10*, 1–16. [CrossRef]
162. Nekrasov, R.; Zelenchenkova, A.; Chabaev, M.; Ivanov, G.; Antonov, A.; Pastukhova, N. PSIII-37 Dried Black Soldier Fly larvae as a dietary supplement to the diet of growing pigs. *J. Anim. Sci.* **2018**, *96*, 314. [CrossRef]
163. Yu, M.; Li, Z.; Chen, W.; Rong, T.; Wang, G.; Li, J.; Ma, X. Use of *Hermetia illucens* larvae as a dietary protein source: Effects on growth performance, carcass traits, and meat quality in finishing pigs. *Meat Sci.* **2019**, *158*, 107837. [CrossRef] [PubMed]
164. van Heugten, E.; Martinez, G.; McComb, A.; Koutsos, E. 285 Black soldier fly (*Hermetia illucens*) larvae oil improves growth performance of nursery pigs. *J. Anim. Sci.* **2019**, *97*, 118. [CrossRef]
165. Cullere, M.; Schiavone, A.; Dabbou, S.; Gasco, L.; Zotte, A.D. Meat quality and sensory traits of finisher broiler chickens fed with black soldier fly (*Hermetia illucens* L.) larvae fat as alternative fat source. *Animals* **2019**, *9*, 140. [CrossRef]

166. Brede, A.; Wecke, C.; Liebert, F. Does the optimal dietary methionine to cysteine ratio in diets for growing chickens respond to high inclusion rates of insect meal from *Hermetia illucens*? *Animals* **2018**, *8*, 187. [CrossRef]
167. Cutrignelli, M.I.; Messina, M.; Tulli, F.; Randazzi, B.; Olivotto, I.; Gasco, L.; Loponte, R.; Bovera, F. Evaluation of an insect meal of the Black Soldier Fly (*Hermetia illucens*) as soybean substitute: Intestinal morphometry, enzymatic and microbial activity in laying hens. *Res. Vet. Sci.* **2018**, *117*, 209–215. [CrossRef] [PubMed]
168. Dabbou, S.; Gai, F.; Biasato, I.; Capucchio, M.T.; Biasibetti, E.; Dezzutto, D.; Meneguz, M.; Plachà, I.; Gasco, L.; Schiavone, A. Black soldier fly defatted meal as a dietary protein source for broiler chickens: Effects on growth performance, blood traits, gut morphology and histological features. *J. Anim. Sci. Biotechnol.* **2018**, *9*, 1–10. [CrossRef] [PubMed]
169. Lee, J.; Kim, Y.-M.; Park, Y.-K.; Yang, Y.-C.; Jung, B.-G.; Lee, B.-J. Black soldier fly (*Hermetia illucens*) larvae enhances immune activities and increases survivability of broiler chicks against experimental infection of *Salmonella Gallinarum*. *J. Vet. Med. Sci.* **2018**, *80*, 736–740. [CrossRef]
170. Kawasaki, K.; Hashimoto, Y.; Hori, A.; Kawasaki, T.; Hirayasu, H.; Iwase, S.I.; Hashizume, A.; Ido, A.; Miura, C.; Miura, T.; et al. Evaluation of black soldier fly (*Hermetia illucens*) larvae and pre-pupae raised on household organic waste, as potential ingredients for poultry feed. *Animals* **2019**, *9*, 98. [CrossRef] [PubMed]
171. Kierończyk, B.; Sypniewski, J.; Rawski, M.; Czekała, W.; Swiatkiewicz, S.; Józefiak, D. From Waste to Sustainable Feed Material: The Effect of *Hermetia illucens* Oil on the Growth Performance, Nutrient Digestibility, and Gastrointestinal Tract Morphometry of Broiler Chickens. *Ann. Anim. Sci.* **2020**, *20*, 157–177. [CrossRef]
172. Mbhele, F.G.T.; Mnisi, C.M.; Mlambo, V. A nutritional evaluation of insect meal as a sustainable protein source for jumbo quails: Physiological and meat quality responses. *Sustainability* **2019**, *11*, 6592. [CrossRef]
173. Mwaniki, Z.; Neijat, M.; Kiarie, E. Egg production and quality responses of adding up to 7.5% defatted black soldier fly larvae meal in a corn-soybean meal diet fed to Shaver White Leghorns from wk 19 to 27 of age. *Poult. Sci.* **2018**, *97*, 2829–2835. [CrossRef]
174. Mwaniki, Z. Complete Replacement of Soybean Meal with Defatted Black Soldier Fly Larva Meal (BSFLM) in Laying Hen Feeding Programs: Impact on Egg Production and Quality. Ph.D. Thesis, University of Guelph, Guelph, ON, Canada, 2019.
175. Nery, J.; Gasco, L.; Dabbou, S.; Schiavone, A. Protein composition and digestibility of black soldier fly larvae in broiler chickens revisited according to the recent nitrogen-protein conversion ratio. *J. Insects Food Feed* **2018**, *4*, 171–177. [CrossRef]
176. Onsongo, V.O.; Osuga, I.M.; Gachuiri, C.K.; Wachira, A.M.; Miano, D.M.; Tanga, C.M.; Ekesi, S.; Nakimbugwe, D.; Fiaboe, K.K.M. Insects for Income Generation Through Animal Feed: Effect of Dietary Replacement of Soybean and Fish Meal With Black Soldier Fly Meal on Broiler Growth and Economic Performance. *J. Econ. Entomol.* **2018**, *111*, 1966–1973. [CrossRef]
177. Pieterse, E.; Erasmus, S.W.; Uushona, T.; Hoffman, L.C. Black soldier fly (*Hermetia illucens*) pre-pupae meal as a dietary protein source for broiler production ensures a tasty chicken with standard meat quality for every pot. *J. Sci. Food Agric.* **2019**, *99*, 893–903. [CrossRef] [PubMed]
178. Schiavone, A.; Cullere, M.; De Marco, M.; Meneguz, M.; Biasato, I.; Bergagna, S.; Dezzutto, D.; Gai, F.; Dabbou, S.; Gasco, L.; et al. Partial or total replacement of soybean oil by black soldier fly larvae (*Hermetia illucens* L.) fat in broiler diets: Effect on growth performances, feed-choice, blood traits, carcass characteristics and meat quality. *Ital. J. Anim. Sci.* **2017**, *16*, 93–100. [CrossRef]
179. Schiavone, A.; Dabbou, S.; De Marco, M.; Cullere, M.; Biasato, I.; Biasibetti, E.; Capucchio, M.T.; Bergagna, S.; Dezzutto, D.; Meneguz, M.; et al. Black soldier fly larva fat inclusion in finisher broiler chicken diet as an alternative fat source. *Animal* **2018**, *12*, 2032–2039. [CrossRef] [PubMed]
180. Schiavone, A.; Dabbou, S.; Petracci, M.; Zampiga, M.; Sirri, F.; Biasato, I.; Gai, F.; Gasco, L. Black soldier fly defatted meal as a dietary protein source for broiler chickens: Effects on carcass traits, breast meat quality and safety. *Animal* **2019**, *13*, 2397–2405. [CrossRef] [PubMed]
181. Secci, G.; Bovera, F.; Nizza, S.; Baronti, N.; Gasco, L.; Conte, G.; Serra, A.; Bonelli, A.; Parisi, G. Quality of eggs from Lohmann Brown Classic laying hens fed black soldier fly meal as substitute for soya bean. *Animal* **2018**, *12*, 2191–2197. [CrossRef]
182. Surendra, K.C.; Tomberlin, J.K.; van Huis, A.; Cammack, J.A.; Heckmann, L.H.L.; Khanal, S.K. Rethinking organic wastes bioconversion: Evaluating the potential of the black soldier fly (*Hermetia illucens* L.) (Diptera: Stratiomyidae) (BSF). *Waste Manag.* **2020**, *117*, 58–80. [CrossRef] [PubMed]
183. Moula, N.; Scippo, M.L.; Douny, C.; Degand, G.; Dawans, E.; Cabaraux, J.F.; Hornick, J.-L.; Medigo, R.C. Leroy, P. Performances of local poultry breed fed black soldier fly larvae reared on horse manure. *Anim. Nutr.* **2018**, *4*, 73–78. [CrossRef]
184. Marono, S.; Loponte, R.; Lombardi, P.; Vassalotti, G.; Pero, M.E.; Russo, F.; Gasco, L.; Parisi, G.; Piccolo, G.; Nizza, S.; et al. Productive performance and blood profiles of laying hens fed *Hermetia illucens* larvae meal as total replacement of soybean meal from 24 to 45 weeks of age. *Poult. Sci.* **2017**, *96*, 1783–1790. [CrossRef]
185. Maurer, V.; Holinger, M.; Amsler, Z.; Früh, B.; Wohlfahrt, J.; Stamer, A.; Leiber, F. Replacement of soybean cake by *Hermetia illucens* meal in diets for layers. *J. Insects Food Feed* **2016**, *2*, 83–90. [CrossRef]
186. Kim, Y.B.; Kim, D.H.; Jeong, S.B.; Lee, J.W.; Kim, T.H.; Lee, H.G.; Lee, K.W. Black soldier fly larvae oil as an alternative fat source in broiler nutrition. *Poult. Sci.* **2020**, *99*, 3133–3143. [CrossRef]
187. Kozłowski, K.; Ognik, K.; Stępniowska, A.; Juśkiewicz, J.; Zduńczyk, Z.; Kierończyk, B.; Benzertiha, A.; Benzertiha, A.; Józefiak, D. Growth performance, immune status and intestinal fermentative processes of young turkeys fed diet with additive of full fat meals from *Tenebrio molitor* and *Hermetia illucens*. *Anim. Feed Sci. Technol.* **2021**, *278*, 114994. [CrossRef]

188. Jankowski, J.; Kozłowski, K.; Zduńczyk, Z.; Stępniowska, A.; Ognik, K.; Kierończyk, B.; Józefiak, D.; Juśkiewicz, J. The effect of dietary full-fat *Hermetia illucens* larvae meal on gut physiology and growth performance in young turkeys. *Anim. Feed Sci. Technol.* **2021**, *275*, 114879. [CrossRef]
189. Giannetto, A.; Oliva, S.; Ceccon Lanes, C.F.; de Araújo Pedron, F.; Savastano, D.; Baviera, C.; Parrino, V.; Lo Paro, G.; Spanò, N.C.; Cappello, T.; et al. *Hermetia illucens* (Diptera: Stratiomydae) larvae and prepupae: Biomass production, fatty acid profile and expression of key genes involved in lipid metabolism. *J. Biotechnol.* **2020**, *307*, 44–54. [CrossRef]
190. Chun, C.Y.; Yoong, L.S.; Kim, L.P.; Hock, T.L.; Ling, L.J. Comparison of *Hermetia illucens* larvae and pre-pupae as potential aqua feed derived from the biotransformation of organic waste. *AIP Conference Proceedings.* **2019**, *2157*, 020008.
191. Liland, N.S.; Biancarosa, I.; Araujo, P.; Biemans, D.; Bruckner, C.G.; Waagbø, R.; Torstensen, B.E.; Lock, E.-J. Modulation of nutrient composition of black soldier fly (*Hermetia illucens*) larvae by feeding seaweed-enriched media. *PLoS ONE* **2017**, *12*, e0183188. [CrossRef]
192. Salomone, R.; Saija, G.; Mondello, G.; Giannetto, A.; Fasulo, S.; Savastano, D. Environmental impact of food waste bioconversion by insects: Application of Life Cycle Assessment to process using *Hermetia illucens*. *J. Clean. Prod.* **2017**, *140*, 890–905. [CrossRef]
193. Wang, S.Y.; Wu, L.; Li, B.; Zhang, D. Reproductive Potential and Nutritional Composition of *Hermetia illucens* (Diptera: Stratiomyidae) Prepupae Reared on Different Organic Wastes. *J. Econ. Entomol.* **2020**, *113*, 527–537. [CrossRef] [PubMed]
194. Gao, Z.; Deng, W.; Zhu, F. Reference gene selection for quantitative gene expression analysis in black soldier fly (*Hermetia illucens*). *PLoS ONE* **2019**, *14*, e0221420. [CrossRef]
195. Zheng, L.; Li, Q.; Zhang, J.; Yu, Z. Double the biodiesel yield: Rearing black soldier fly larvae, *Hermetia illucens*, on solid residual fraction of restaurant waste after grease extraction for biodiesel production. *Renew. Energy* **2012**, *41*, 75–79. [CrossRef]
196. Bondari, K.; Sheppard, D.C. Soldier fly, *Hermetia illucens* L., larvae as feed for channel catfish, *Ictalurus punctatus* (Rafinesque), and blue tilapia, *Oreochromis aureus* (Steindachner). *Aquac. Res.* **1987**, *18*, 209–220. [CrossRef]
197. Hu, J.; Wang, G.; Huang, Y.; Sun, Y.; He, F.; Zhao, H.; Li, N. Effects of substitution of fish meal with black soldier fly (*Hermetia illucens*) larvae meal, in yellow catfish (*Pelteobagrus fulvidraco*) diets. *Israeli J. Aquacul.* **2017**, *69*, 1–9.
198. Fawole, F.J.; Adeoye, A.A.; Tiamiyu, L.O.; Ajala, K.I.; Obadara, S.O.; Ganiyu, I.O. Substituting fishmeal with *Hermetia illucens* in the diets of African catfish (*Clarias gariepinus*): Effects on growth, nutrient utilization, haemato-physiological response, and oxidative stress biomarker. *Aquaculture* **2020**, *518*, 734849. [CrossRef]
199. Muin, H.; Taufek, N.M.; Kamarudin; Razak, S. A. Growth performance, feed Utilization and body composition of nile tilapia, *Oreochromis niloticus* (*Linnaeus*, 1758) fed with different levels of black soldier fly, *Hermetia illucens* (*Linnaeus*, 1758) maggot meal diet. *Iran. J. Fish. Sci.* **2017**, *16*, 567–577.
200. Devic, E.; Leschen, W.; Murray, F.; Little, D.C. Growth performance, feed utilization and body composition of advanced nursing Nile tilapia (*Oreochromis niloticus*) fed diets containing Black Soldier Fly (*Hermetia illucens*) larvae meal. *Aquac. Nutr.* **2018**, *24*, 416–423. [CrossRef]
201. Toriz-Roldan, A.; Ruiz-Vega, J.; García-Ulloa, M.; Hernández-Llamas, A.; Fonseca-Madrigal, J.; Rodríguez-González, H. Assessment of Dietary Supplementation Levels of Black Soldier Fly, *Hemertia illucens*, Pre-Pupae Meal for Juvenile *Nile Tilapia*, *Oreochromis niloticus*. *Southwest. Entomol.* **2019**, *44*, 251. [CrossRef]
202. Borgogno, M.; Dinnella, C.; Iaconisi, V.; Fusi, R.; Scarpaleggia, C.; Schiavone, A.; Monteleone, E.; Gasco, L.; Parisi, G. Inclusion of *Hermetia illucens* larvae meal on rainbow trout (*Oncorhynchus mykiss*) feed: Effect on sensory profile according to static and dynamic evaluations. *J. Sci. Food Agric.* **2017**, *97*, 3402–3411. [CrossRef]
203. Cardinaletti, G.; Randazzo, B.; Messina, M.; Zarantoniello, M.; Giorgini, E.; Zimbelli, A.; Bruni, L.; Parisi, G.; Olivotto, I.; Tulli, F. Effects of graded dietary inclusion level of full-fat *Hermetia illucens* prepupae meal in practical diets for rainbow trout (*Oncorhynchus mykiss*). *Animals* **2019**, *9*, 251. [CrossRef] [PubMed]
204. Rimoldi, S.; Gini, E.; Iannini, F.; Gasco, L.; Terova, G. The effects of dietary insect meal from *Hermetia illucens* prepupae on autochthonous gut microbiota of rainbow trout (*Oncorhynchus mykiss*). *Animals* **2019**, *9*, 143. [CrossRef] [PubMed]
205. Bruni, L.; Belghit, I.; Lock, E.J.; Secci, G.; Taiti, C.; Parisi, G. Total replacement of dietary fish meal with black soldier fly (*Hermetia illucens*) larvae does not impair physical, chemical or volatile composition of farmed Atlantic salmon (*Salmo salar* L.). *J. Sci. Food Agric.* **2020**, *100*, 1038–1047. [CrossRef]
206. Mancini, S.; Medina, I.; Iaconisi, V.; Gai, F.; Basto, A.; Parisi, G. Impact of black soldier fly larvae meal on the chemical and nutritional characteristics of rainbow trout fillets. *Animal* **2018**, *12*, 1672–1681. [CrossRef] [PubMed]
207. Dumas, A.; Raggi, T.; Barkhouse, J.; Lewis, E.; Weltzien, E. The oil fraction and partially defatted meal of black soldier fly larvae (*Hermetia illucens*) affect differently growth performance, feed efficiency, nutrient deposition, blood glucose and lipid digestibility of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **2018**, *492*, 24–34. [CrossRef]
208. Huyben, D.; Vidaković, A.; Werner Hallgren, S.; Langeland, M. High-throughput sequencing of gut microbiota in rainbow trout (*Oncorhynchus mykiss*) fed larval and pre-pupae stages of black soldier fly (*Hermetia illucens*). *Aquaculture* **2019**, *500*, 485–491. [CrossRef]
209. Rumpold, B.A.; Speckmann, H.; Schlüter, O.; Kloas, W.; Prochnow, A. Potentials of a biogenic residue-based production of *Hermetia illucens* as fish meal replacement in aquafeed for *Oncorhynchus mykiss* in Germany. *J. Insects Food Feed* **2018**, *4*, 5–18. [CrossRef]
210. Sealey, W.M.; Gaylord, T.G.; Barrows, F.T.; Tomberlin, J.K.; McGuire, M.A.; Ross, C.; St-Hilaire, S. Sensory Analysis of Rainbow Trout, *Oncorhynchus mykiss*, Fed Enriched Black Soldier Fly Prepupae, *Hermetia illucens*. *J. World Aquac. Soc.* **2011**, *42*, 34–45. [CrossRef]

211. Terova, G.; Rimoldi, S.; Ascione, C.; Gini, E.; Ceccotti, C.; Gasco, L. Rainbow trout (*Oncorhynchus mykiss*) gut microbiota is modulated by insect meal from *Hermetia illucens* prepupae in the diet. *Rev. Fish Biol. Fish.* **2019**, *29*, 465–486. [CrossRef]
212. Magalhães, R.; Sánchez-López, A.; Leal, R.S.; Martínez-Llorens, S.; Oliva-Teles, A.; Peres, H. Black soldier fly (*Hermetia illucens*) pre-pupae meal as a fish meal replacement in diets for European seabass (*Dicentrarchus labrax*). *Aquaculture* **2017**, *476*, 79–85. [CrossRef]
213. Lock, E.R.; Arsiwalla, T.; Waagbø, R. Insect larvae meal as an alternative source of nutrients in the diet of Atlantic salmon (*Salmo salar*) postsmolt. *Aquac. Nutr.* **2016**, *22*, 1202–1213. [CrossRef]
214. Biancarosa, I.; Sele, V.; Belghit, I.; Ørnsrud, R.; Lock, E.J.; Amlund, H. Replacing fish meal with insect meal in the diet of Atlantic salmon (*Salmo salar*) does not impact the amount of contaminants in the feed and it lowers accumulation of arsenic in the fillet. *Food Addit. Contam.Part A Chem. Anal. Control. Expo. Risk Assess.* **2019**, *36*, 1191–1205. [CrossRef]
215. Stenberg, O.K.; Holen, E.; Piemontese, L.; Liland, N.S.; Lock, E.J.; Espe, M.; Belghit, I. Effect of dietary replacement of fish meal with insect meal on in vitro bacterial and viral induced gene response in Atlantic salmon (*Salmo salar*) head kidney leukocytes. *Fish Shellfish. Immunol.* **2019**, *91*, 223–232. [CrossRef]
216. Zarantoniello, M.; Randazzo, B.; Truzzi, C.; Giorgini, E.; Marcellucci, C.; Vargas-Abúndez, J.A.; Zimbelli, A.; Annibaldi, A.; Parisi, G.; Tulli, F.; et al. A six-months study on Black Soldier Fly (*Hermetia illucens*) based diets in zebrafish. *Sci. Rep.* **2019**, *9*, 1–12. [CrossRef]
217. Caimi, C.; Renna, M.; Lussiana, C.; Bonaldo, A.; Gariglio, M.; Meneguz, M.; Dabbou, S.; Schiavone, A.; Gai, F.; Elia, A.C.; et al. First insights on Black Soldier Fly (*Hermetia illucens* L.) larvae meal dietary administration in Siberian sturgeon (*Acipenser baerii Brandt*) juveniles. *Aquaculture* **2020**, *515*, 734539. [CrossRef]
218. Li, S.; Ji, H.; Zhang, B.; Tian, J.; Zhou, J.; Yu, H. Influence of black soldier fly (*Hermetia illucens*) larvae oil on growth performance, body composition, tissue fatty acid composition and lipid deposition in juvenile Jian carp (*Cyprinus carpio var. Jian*). *Aquaculture* **2016**, *465*, 43–52. [CrossRef]
219. Zhou, J.S.; Liu, S.S.; Ji, H.; Yu, H.B. Effect of replacing dietary fish meal with black soldier fly larvae meal on growth and fatty acid composition of Jian carp (*Cyprinus carpio var. Jian*). *Aquac. Nutr.* **2018**, *24*, 424–433. [CrossRef]
220. Guerreiro, I.; Castro, C.; Antunes, B.; Coutinho, F.; Rangel, F.; Couto, A.; Serra, C.R.; Peres, H.; Pousão-Ferreira, P.; Matos, E.; et al. Catching black soldier fly for meagre: Growth, whole-body fatty acid profile and metabolic responses. *Aquaculture* **2020**, *516*, 734613. [CrossRef]
221. Vongvichith, B.; Morioka, S.; Sugita, T.; Phousavanh, N.; Phetsanghanh, N.; Chanthasone, P.; Pommachan, P.; Nakamura, S. Evaluation of the efficacy of aquaculture feeds for the climbing perch *Anabas testudineus*: Replacement of fishmeal by black soldier fly *Hermetia illucens* prepupae. *Fish. Sci.* **2020**, *86*, 145–151. [CrossRef]

Lipids from the bioconverter insect *Hermetia illucens*, an innovative and sustainable source: extraction, composition, and applications

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SUBSTRATE	DEVELOPMENTAL STAGE	LIPID CONTENT (%)	REFERENCES
Chicken feed	Prepupal	33.6	[21]
Biogas Digestate	Prepupal	21.8	[21]
Vegetable waste	Prepupal	37.1	[21]
Restaurant waste	Prepupal	38.6	[21]
Cafeteria waste	Prepupal	31.8 ±0.3	[81]
Vegetable and fruit waste (7:3)	Larval	26.3	[77]
Vegetable and fruit waste (17:3)	Larval	33.0±0.2	[189]
Vegetable and fruit waste (17:3)	Prepupal	30.8±0.2	[189]
Fruit waste	Larval	40.7	[189]
	Prepupal	47.4	[77]
Fruit waste	Prepupal	46.8	[189]
Apple	Larval	36.1 ±1.5	[13]

Banana	Larval	27.9±3.9	[13]
Apple and Banana (1:1)	Larval	33.4±1.8	[13]
Spent Grain	Larval	22.5±1.5	[13]
Brewery by-products	Larval	29.9±0.7	[77]
Apple and Spent Grain (1:1)	Larval	20.1±4.3	[13]
Banana and spent grain (1:1)	Larval	23.1±2.3	[13]
Winery by-produts	Larval	32.2	[77]
Brown algae (Ascophyllum nodosum)	Larval	8.1±0.9	[191]
Food waste	Prepupal	35.0	[192]
	Prepupal	37.2	[193]
Pig manure	Prepupal	36.5±3.9	[193]
Poultry manure	Prepupal	36.2±3.5	[193]
Cow manure	Prepupal	35.7±2.9	[193]
Fermented maize stover	Prepupal	30.5	[194]
Gainsville diet + 6% xylose (from lignocelluloses)	Larval	34.60	[68]
Cricket waste	Larval	38.7	[12]
Locust waste	Larval	25.8	[12]

Table S1: Proximate lipid content of BSF biomass reared on different organic wastes (Unit: % of dry matter)

SUBSTRATE	DEVELOPMENTAL STAGE	Capric C10:0	Lauric (C12:0)	Myristic (C14:0)	Palmitic (C16:0)	Palmitoleic (C16:1n-7)	Stearic (C18:0)	Oleic (C18:1n9)	Linoleic (C18:2n6)	Linolenic (C18:3n3)	SFA	MUFA	PUFA	REFERENCES
Chicken feed	Prepupal	1.4	57.4	7.3	9.7	NA	1.4	7.5	NA	0.7	77.4	10.0	12.6	[21]
Biogas Digestate	Prepupal	1.2	43-7	6.9	10.1	NA	1.8	7.9	NA	0.8	64.8	19.1	9.6	[21]
Vegetable waste	Prepupal	1.6	60.9	9.5	8.7	NA	1.1	5.7	NA	1.4	82.8	9.5	7.0	[21]
Restaurant waste	Prepupal	2.0	57.6	7.1	10.3	NA	1.0	8.0	NA	1.1	78.3	12.0	9.4	[21]
Cafeteria waste	Prepupal	NA	44.9	8.3	13.5	2.4	2.1	12.0	9.9	0.1	69.9	14.9	12.5	[81]
Vegetable and fruit waste (7:3)	Larval	NA	52.1	10.4	13.9	NA	2.6	8.5	7.0	1.7	78.9	12.3	8.8	[77]
Vegetable and fruit waste (17:3)	Larval	0.5	28.1	3.8	5.8	1.6	0.7	4.3	1.3	10.3	73.5	14.9	11.6	[189]
Vegetable and fruit waste (17:3)	Prepupal	1.2	61.9	9.1	7.9	2.4	1.1	5.3	2.4	4.7	84.5	8.4	7.1	[189]
Fruit waste	Larval	NA	57.4	9.6	13.1	NA	1.8	9.3	4.1	0.7	81.9	13.3	4.8	[77]
Brewery by-productS	Larval	NA	32.4	6.7	20.4	NA	1.8	9.2	23.6	2.5	61.3	12.7	26.0	[77]
Winery by-produts	Larval	NA	34.7	6.6	18.9	NA	2.8	12.5	17.6	0.4	61.0	19.0	18.0	[77]
Brown algae (*Ascophyllum nodosum*)	Larval	NA	23.9	6.7	16.6	2.5	4.1	17.9	18.6	1.6	52.5	22.2	24.2	[191]
Food waste	Prepupal	1.4	41.1	0.4	12.2	3.2	2.4	14.1	13.8	NA	NA	NA	NA	[192]
	Prepupal	2.3	56.2	9.3	10.3	2.4	1.9	7.1	10.3	NA	NA	NA	NA	[193]
Restaurant waste and Rice straw	Larval	3.8	27.8	8.1	14.2	4.5	7.6	22.5	1.8	2.1	NA	NA	NA	[195]
Pig manure	Prepupal	2.1	56.4	8.5	11.0	2.2	1.5	7.1	10.2	NA	NA	NA	NA	[193]
Poultry manure	Prepupal	1.6	56.9	8.2	8.4	1.9	1.4	7.0	10.7	NA	NA	NA	NA	[193]
Cow manure	Prepupal	1.7	54.2	7.0	7.9	2.1	1.2	6.3	11.0	NA	NA	NA	NA	[193]

Dairy manure	Larval	3.1	35.6	7.6	14.8	3.8	3.6	23.6	2.1	NA	NA	NA	NA	[82]
Fermented maize stover	Prepupal	0.8	22.4	4.4	13.1	1.2	1.9	23.3	24.0	1.3	45.4	24.9	25.4	[194]
Gainsville diet + 6% xylose	Larval	NA	26.9	NA	19.2	NA	7.9	28.8	NA	NA	NA	NA	NA	[68]
Soybean curd residue + Bacteria (*Lactobacillus buchneri*)	Larval	1.5	39.6	6.5	13.1	2.7	2.1	11.5	17.5	1.4	NA	NA	NA	[73]
Soybean curd residue	Larval	1.7	36.9	5.9	13.2	3.2	1.7	12.1	17.0	1.2	NA	NA	NA	[73]

Table S2: Fatty acids profile of the BSF biomass reared on different organic waste (Unit: *: % of dry matter)

TYPE OF BSF FEED ADMINISTERED TO FARMED FISH	SPECIES FED WITH BSF	REFERENCES
Larval meal/Prepupal meal	Channel catfish (*Ictalurus punctatus*)	[196]
Larval meal	Yellow catfish (*Pelteobagrus fulvidraco*)	[23,197]
Partially defatted larval meal	African catfish (*Clarias gariepinus*)	[198]
Larval meal Prepupal meal	Nile tilapia (*Oreochromis niloticus*)	[199,200] [201]
Larval meal	Blue tilapia (*Oreochromis aureus*)	[196]
Prepupal meal Partially defatted larval meal Partially defatted larval meal/oil Larval meal/Prepupal meal Larval meal Prepupal meal Partially defatted prepupal meal	Rainbow trout (*Oncorhynchus mykiss*)	[103,202-204] [205,206] [207,208] [181,209] [156,210] [211]
Prepupal meal	European seabass (*Dicentrarchus labrax*)	[212]
Larval meal/oil	Atlantic salmon (*Salmo salar*)	[155,159]

Partially defatted larval meal Larval meal Larval meal/partially defatted		[158,205, 213] [214,215] [215]
Prepupal meal	Zebrafish (*Danio rerio*)	[216]
Highly defatted meal	Siberian sturgeon (*Acipenser baerii*)	[217]
Oil Larval meal	Jian carp (*Cyprinus carpio var. Jian*)	[218] [219]
Partially defatted larval meal	Meagre (*Argyrosomus regius*)	[220]
Highly defatted meal	Climbing perch (*Anabas testudineus*)	[221]

Table S3: Fish species fed with BSF.

Waste and Biomass Valorization

Purification of chitin from pupal exuviae of the Black Soldier Fly

--Manuscript Draft--

Manuscript Number:	WAVE-D-21-00221R1	
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Abstract:

Purpose
Chitin purification from remains (pupal exuviae) of Hermetia illucens farming was optimized performing demineralization, deproteinization and bleaching under different conditions. The optimal parameters to obtain high-purity chitin were determined.

Methods
Dried and ground pupal exuviae, whose composition was initially determined, were demineralized using six different acids. Proteins were removed with a NaOH treatment in which temperature, molarity and duration were varied in a randomized experiment. Bleaching was carried out testing ten different chemicals, including NaOCl, H 2 O 2 , solvent mixtures and enzymes. The efficiency of each step was determined to assess the optimal conditions for each of them.

Results
The highest demineralization efficiency (89.6%) was achieved using 0.5 M formic acid for 2 h at 40 °C, confirming the validity of organic acids as a more sustainable alternative to inorganic acids. The treatment with 1.25 M NaOH at 90 °C for 4 h showed the highest deproteinization efficiency, removing 96% proteins. Temperature and NaOH concentration were the significant parameters for deproteinization efficiency. The most efficient bleaching treatment was with 6% NaOCl at 60 °C for 1 h (66.5% efficiency). H2O2 could also be a valid alternative to avoid environmental risk related to chlorine-containing compounds. At the end of the purification process 16.5% of the original biomass was retained with a chitin content of 84.7%, corresponding to a chitin yield of 13.9% related to the initial biomass.

Conclusion
This investigation shows an optimized method for extraction of high-purity chitin from H. illucens pupal exuviae, supporting the validity of insect-farming remains as sourceof this versatile biopolymer.

Purification of chitin from pupal exuviae of the Black Soldier Fly

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Abstract

Purpose

Chitin purification from remains (pupal exuviae after metamorphosis to adult flies) of *Hermetia illucens* farming was optimized performing demineralization, deproteinization and bleaching under different conditions. The optimal parameters to obtain high-purity chitin were determined.

Methods

Dried and ground pupal exuviae, whose composition was initially determined, were demineralized using six different acids. Proteins were removed with a NaOH treatment in which temperature, molarity and duration were varied in a randomized experiment. Bleaching was carried out testing ten different chemicals, including NaOCl, H_2O_2, solvent mixtures and enzymes. The efficiency of each step was determined to assess the optimal conditions for each of them.

Results

The highest demineralization efficiency (89.6%) was achieved using 0.5 M formic acid for 2 h at 40 °C, confirming the validity of organic acids as a more sustainable alternative to inorganic acids. The treatment with 1.25 M NaOH at 90 °C for 4 h showed the highest deproteinization efficiency, removing 96% of the proteins. Temperature and NaOH concentration were the significant parameters for deproteinization efficiency. The most efficient bleaching treatment was with 6% NaOCl at 60 °C for 1 h (66.5% efficiency). H_2O_2 could also be a valid alternative to avoid environmental risk related to chlorine-containing compounds. At the end of the purification process 16.5% of the original biomass was retained with a chitin content of 84.7%, corresponding to a chitin yield of 13.9% related to the initial biomass.

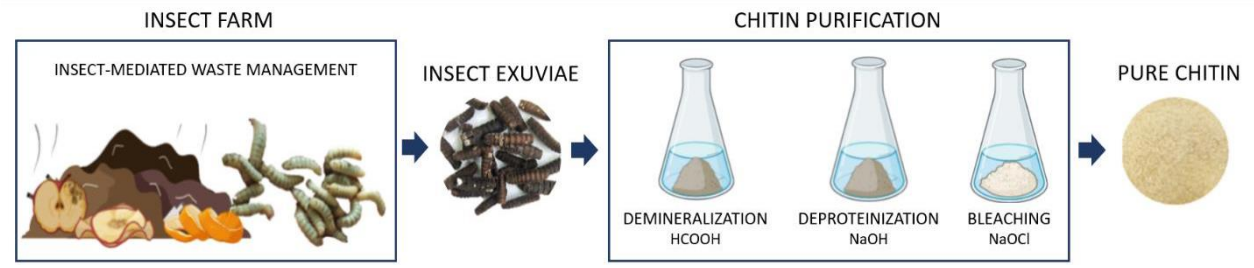
Conclusion

This investigation shows an optimized method for extraction of high-purity chitin from *H. illucens* pupal exuviae, supporting the validity of insect-farming remains as source of this versatile biopolymer.

Keywords

Hermetia illucens, pupal exuviae, chitin, demineralization, deproteinization, bleaching

Graphical Abstract



Statement of Novelty

The industrial production of insect-based proteins and lipids by the conversion of agro-industrial waste streams gained momentum in the last decade. To create a higher value of this process, a valorization of the side streams thereof is required. To this end, we describe the production of pure chitin from one of the major insect processing waste streams, the pupal exuviae. Chitin, as a parental polysaccharide of the acid-soluble chitosan, is currently the focus of comprehensive research for the substitution of synthetic polymers in various application fields. Since we addressed the decentralized extraction of this versatile natural polysaccharide from an insect processing waste stream, our investigations thus provided the basis for a prospective commercialization of this novel approach.

1. Introduction

Chitin is the Earth's second most abundant polysaccharide after cellulose, being the main structural component of arthropod's exoskeleton and the cell wall of fungi [1]. Chitin is a polymer of increasing commercial potential. It is used in agriculture for the biological control of pathogens [2], in tissue regeneration [3], for wound dressing, for adsorption of dyes and heavy metals from waste water, as moisturizing agent in cosmetics, and as additive in paper production [4]. Furthermore, chitin can be converted into chitosan, a polymer with increased solubility and reactivity provided by free amino groups, which can find a wider range of applications [5]. [6]

On an industrial scale, chitin is mostly obtained from waste streams of the fishing industry (mainly crustacean's exoskeleton), whose chitin content ranges between 15% and 40% [7]. Nevertheless, in recent years it has become necessary to look for alternative sources to cope with the huge increase in demand expected for chitin and chitosan [8]. Moreover, crustacean waste does not have a constant availability throughout the year, its supply is becoming no more sustainable without worsening the depletion of marine resources and the chitin quality is not consistent since the raw materials composition varies [9,10].

Alternative sources of chitin are fungi and insects. Fungi, as for example the species *Mucor rouxii*, can incorporate chitin (as well as chitosan) up to 40% of the cell wall's dry weight [11]. Although offering a non-seasonable source of chitin and chitosan, the content varies greatly with the fungal life cycle [12]. Insect exuviae can contain up to 35% chitin of the dry weight, depending on the species [13]. This is a chitin-rich substrate whose availability is increasing, as insect farming for feed production and waste management is growing worldwide. Bioconversion of organic waste and agricultural by-products by insects is one of the technologies that has received the most attention in recent years [14]. Insect waste deriving from this process can be a cost-free source of chitin available throughout the year with low environmental impact [15].

The black soldier fly (*Hermetia illucens* L.) is one of the most commonly bred species for the insect-based bioconversion. The larval stages of this fly can consume a wide variety of organic substrates converting them into valuable products, such as proteins and fat for animal feed, lipids for biodiesel and cosmetics production, and frass for soil improvement [16].

The life cycle of *H. illucens* is divided in five developmental stages, including egg, larvae, pre-pupae, pupae and adult. During transition from larvae to pupae, several larval exoskeletons are shed. Pupal exuviae are released by metamorphosis from pupae to adult flies [17]. As chitin is one of the major components of these by-products of the life cycle [18], they are suitable for a prospective decentralized chitin isolation. The larval exoskeletons are commonly a component of the insect frass, which is a commercial product. The chitin presence in the frass supports growth of beneficial microbes in soil and acts as a biostimulant [19]. Pupal exuviae are easier to collect from *H. illucens* farming, since they self-collect in the containers used for the development of pupae into adults, and the one that is most readily available. Hence, a process for chitin purification from pupal exuviae should be developed.

To date, the majority of studies focused on the optimization of chitin extraction from fishery waste, for which several methods are available in literature. Purification of chitin from insects still remains on laboratory scale, mainly applying the protocols reported for crustaceans that involve sequential steps to remove minerals, proteins, pigments and catecholic compounds becoming noticeable by the dark colour of the insect cuticle. A comprehensive review of methods for chitin and chitosan purification from insects is provided by Hahn et al. [20]. Chitin purification from pupal exuviae has already been in the focus of a study. However, the objective of Brigode et al. [21] was to obtain physico-chemical data rather than to optimize chitin purification. Furthermore, in most cases whole insects at different developmental stages of their life cycle (f. e. pupae, adult) are used [22-27] for chitin purification and investigation instead of side streams from insect farming.

The present work was performed to optimize systematically the chitin purification from side streams of *H. illucens* breeding, namely pupal exuviae for the first time. Purification steps, i.e. demineralization, deproteinization and bleaching, were carried out under different conditions

in order to determine the optimal parameters. The efficiency of demineralization was investigated in relation to the acid used, molarity and final washing volume. The deproteinization step with alkali was optimized concerning temperature, molarity and incubation time. The final bleaching treatment was evaluated concerning the chemical used, duration and temperature.

2. Materials and methods

2.1 Material

Pupal exuviae are side streams of the black soldier fly (*H. illucens)* cultivation, manually collected and provided by Protix (Dongen, Netherlands). Insects were reared on exclusively plant origin by-products from the food processing industry. The raw biomass used was initially crushed with an ultra-centrifugal mill (ZM 100, Retsch, Germany) equipped with a ring sieve (Hole diameter: 0.5 mm). Material was stored at -18 °C until usage.

If not stated otherwise, desalted water was used. Reagents were of practical grade and purchased either from Carl Roth GmbH (Germany) or Merck KgaA (Germany).

2.2 Analytical Methods

The basic analyses were performed as triplicates. The chitin content of both the raw material, the demineralized pupal exuviae and the purified chitin was measured as acid detergent fiber (ADF). The value of the acid detergent lignin (ADL) was measured and deducted from ADF to consider the catechol content within the biomass according to Hahn et al. [18]. The lipid content of the raw material was estimated by Soxhlet extraction with n-hexane as solvent according to Matissek et al. [28]. The moisture and ash contents were determined at 105 °C and 550 °C according to DIN EN 15935:2009 and 12880:2000. Determination of different metal ion contents in the raw material was performed via Inductively Coupled Plasma Optical Emission Spectrometry (Activa M, Horiba Scientific, Japan) after biomass hydrolysis with sulfuric acid.

A quantification of the proteins in the supernatant via Bradford assay or similar is aggravated by the presence of the catecholic compounds. Hence, the deproteinization conditions were assessed by the amino acid content in the biomass before and after the alkaline treatment. The amino acids were thus measured in the supernatant after total hydrolysis of the biomass. The decomposition was performed with 120 mg sample suspended in 6 mL 6 M HCl incubated for 45 min in a microwave oven at 110 °C. After incubation, water was added up to a volume of 10 mL. The supernatant was removed and D-Norvalin was added as internal standard.

Automated precolumn Fluorenylmethoxycarbonyl (Fmoc) derivatization enables the detection of the amino acids in the pmol/µL-range. Separation of the sample was performed with a 1290 Infinity UHPLC-System and a Zorbax-Eclipse-RRHD-column (Agilent Technologies, US) applying a gradient with variable amount of 10 mM Na_2HPO_4-$Na_2B_4O_7$-buffer pH 8.2 (mobile phase A) together with Acetonitrile/MetOH/water (45/45/10 v/v/v, Mobile Phase B). Volumetric flow rate was adjusted to 0.8 mL/min and the temperature set up to 40 °C. Derivatized amino acids were quantified with a fluorescence detector (266/305 ex/em).

The protein content of the raw material, the demineralized pupal exuviae and the deproteinized samples was determined by summarizing the contents of all amino acids in the biomass detected in the supernatant after total hydrolysis (see Supplementary Material). To perform this, we have to assume indefinite long protein chains, considering the loss of water during peptide bond formation by subtraction of the molecular weight (18 mol/g) from each amino acid. Furthermore, we hypothesize that some amino acids undergo partial or total degradation during hydrolysis [29] so that the real protein content should be slightly higher.

2.3 Purification investigations

First, demineralization investigation was carried out (step 1). Identification of a suitable demineralization agent resulted in the production of a larger batch to carry out the deproteinization experiments (step 2).

Step 1: Demineralization

For demineralization (DM), a suitable agent needs to be identified from different organic and inorganic acids. For this purpose, H_3PO_4, H_2SO_4, HNO_3, HCl, CH_3COOH, HCOOH were applied. Thus, 1 g of homogenized pupal exuviae were suspended in 15 mL of the respective acid (all 0.5 M with exception of 0.25 M H_2SO_4) using 50 mL reaction vessels of a parallel synthesis apparatus (Synthesis 1, Heidolph, Germany). The investigations were performed for 2 h at 500 rpm either at 40 or 70 °C. Water incubation without acid addition was carried out at 70 °C to set-up a benchmark. After incubation, the suspension was centrifuged (10 min, 4696 *g*) and the supernatant was decanted. Then, the residual biomass was washed with 15

mL water and again centrifuged. Wash water was decanted, biomass dried at 105 °C overnight and the ash content was finally measured. All experiments were carried out in triplicates. Demineralization efficiency (*DME*) was calculated according to the following equation:

$$DME\,[\%] = \left(\frac{AC_{Dem}}{AC_{RM}} \cdot 100\right) \quad (1)$$

whereby AC_{Dem} and AC_{RM} is the ash content after and before demineralization, respectively.

Step 2: Deproteinization

Deproteinization experiments were performed in order to determine the quantitative effect of different conditions for protein removal. For deproteinization (DP) investigations, a larger amount of demineralized pupal exuviae were previously prepared. This was performed in a 3 L-shaking flask with 200 g grinded pupal exuviae suspended in 800 mL 1 M formic acid incubated for 3 h at room temperature (RT) and a shaking frequency of 120 rpm. Biomass was then separated by centrifugation (4696 *g*, 10 min) and subsequently washed with 1.8 L water. After another centrifugation step, the demineralized biomass was dried overnight at 105 °C.

DP was performed in a parallel reaction system (Tornado™ IS6, R. B. Radley Co. Ltd, UK). 50 mL of the sodium hydroxide (NaOH) solution were heated in a 50 mL reaction vessel to the temperature fixed before. Reached the desired temperature, 5 g of demineralized pupal exuviae were added and the suspension stirred (300 rpm). Reaction was finished after the predefined incubation time by cooling on ice. The deproteinized material was separated with a glass funnel equipped with an integrated filter membrane (pore size 100-150 µm) under vacuum. Subsequent washing of the biomass with hot water (~250 mL) removed dyes and adsorbed proteins. The product was dried in an oven at 105 °C overnight and further analyzed concerning the amino acid content. The amino acid content was utilized to determine the protein content, and deproteinization efficiency (*DPE*) was calculated according to the following equation:

$$DPE\,[\%] = \left(\frac{P_{Dep}}{P_{Dem}} \cdot 100\right) \quad (2)$$

whereas P_{Dep} and P_{Dem} are the protein content in percentage after and before the reaction, respectively. The different conditions applied for the approaches and the certain results are provided in Table 1.

Temperature (°C), NaOH concentration (mol/L) and incubation time (h) were varied in randomized experiments in order to identify the most relevant deproteinization parameters. Response surface methodology (RSM) and linear regression were performed with Design-Expert® (Version 7.1.5, Stat-Ease Inc. Minneapolis, USA). Results were analyzed with the statistical software to determine the optimum conditions for the DP process and to assess validity and relevance of each parameters for deproteinization efficiency.

2.4 Bleaching

According to the results obtained by the evaluation of the DP experiments and to provide a sufficient amount of raw material for bleaching trials, a larger amount of demineralized pupal exuviae was subjected to DP at previously determined suitable conditions. Hence, 75 g of demineralized pupal exuviae were incubated for 2 h at 90 °C in 2.5 M NaOH at a liquid/solid-ratio of 10 and stirred at 500 rpm in a stainless-steel reactor (Versoclave, Büchi, Germany). Afterwards, supernatant was removed via filtration, the residue washed with 4 L 80 °C hot water and the deproteinized pupal exuviae subsequently dried at 105 °C.

Bleaching was carried out with agents and protocols identified in other studies originally applied for bleaching of cellulose or chitin derived by crab shells or insects. The individual approaches and the respective conditions for bleaching are listed in Table 2.

All experiments were carried out in duplicates and the bleaching solutions were separated from the biomass by centrifugation for 5 min at RT and 4696 *g* after the bleaching process. The samples were additionally washed with 250 mL of water at 80 °C (methods 1 and 2) or 40 °C (method 3). The wash solution was removed by further centrifugation under the already mentioned conditions. Subsequently, the bleached insect chitin was dried overnight at 105 °C.

Method 1

For bleaching experiments I-VI (Table 2), 0.5 g of the insect chitin were weighed into a 50 mL glass reactor vessel and incubated with 10 mL of the bleaching solution for 60 min (except experiment IV 90 min) at 200 rpm. The bleaching reactions were carried out in the parallel synthesis system (Tornado ™, Radleys) which was also used for DP. Experiment IV had to be finished after five min due to strong foaming. The sample was washed and dried under the abovementioned conditions.

Method 2

The bleaching with solvent mixtures (experiments VII-VIII, cf. Table 2) was carried out in 15 mL reaction vessels in a table shaker. For this purpose, 0.5 g of sample were weighed into a vessel and these were each filled with 10 mL of bleaching solution. The incubation took place at RT for 2 h (experiment VII) or 1 h (experiment VIII), at 500 rpm.

Method 3

As an alternative to the bleaching with chemicals, the use of laccase was attempted to remove pigments from insect chitin. Laccase can cleave lignin [39], thus it was applied to unbleached chitin due to the structural similarity between monomeric lignin constituents and the catecholic compounds. In these experiments (IX and X), a batch was carried out only with laccase and citrate buffer (pH 5), and an approach with an additional 3 mM ABTS 2,2'-azino-bis- (3-ethylbenzthiazolin-6-sulfonic acid) as a mediator. ABTS is a diammonium salt which supports the electron transfer of the oxidation as a mediator. Experiments were carried out in 100 mL shaking flasks in the shaker. 1 g of insect chitin was added to a flask with 20 mL of citrate buffer with or without ABTS and 0.42 mg of laccase (5.6 kU/g measured with ABTS). The batches were incubated at room temperature for 24 h at 160 rpm (Table 2).

2.5 Bleaching evaluation

In order to quantitatively assess the bleaching effect of the different treatments, the bleached chitin was colorimetrically measured in a spectrophotometer. The spectrophotometer measures the reflectance values by illuminating the surface of the sample over the entire spectrum of visible light. A pure white standard with 100% reflection and a black one with 0% reflection were used for calibration.

The CIELab system is a colour space defined by the International Commission on Illumination (French: Commission Internationale de l'Eclairage, CIE) in 1976. The internationally used CIELab colour space was utilized to relate reflectance spectra to colour and evaluate differences among samples. Using the device-independent 3D colour model, colour differences are determined numerically. CIELab describes optical colour perception in a three-dimensional space and divides it into two colour axes (red-green and blue-yellow) and one lightness axis (white-black) [http://cie.co.at/]. The lightness axis has a scale of 0-100, where 0 means pure black and 100 means pure white. Within this colour space, each colour is represented by a colour point with L^*, a^* and b^* as coordinates. The colour difference between a bleached sample and a reference sample can be measured as the distance between their respective colour points. With this method, the increase in lightness due to the bleaching treatment and, thus, the BE can be determined.

Preparation of the bleached samples

The bleached insect chitin, previously dried and ground, was pressed into tablet-shaped discs (Ø 13 mm) using a laboratory press. Approximatively 200 mg of bleached chitin was transferred into the pressing device and pressed at 10 bar. The pressed pellets were then measured in the spectrophotometer and evaluated according to the lightness axis of the CIELab colour space.

Colorimetric measurement of the bleached samples using a spectrophotometer

For the quantitative evaluation of the bleaching effect, chitin samples were measured colorimetrically using a spectrophotometer. This determines the coordinates in the 3D CIELab colour space through remission and transmission measurement via double-beam spectrophotometry. First, unbleached insect chitin was measured as a standard and its colour was converted into coordinates. The bleached samples were then measured, also converted, and the distance to the coordinates of the standard was generated as difference (D) on the individual axes (DL*, Da*, Db*). The values of lightness distances (DL*) were used to assess the bleaching effect. Since the bleaching tests involved the brightening of the samples, the absolute lightness values

L* were defined as a significant criterion for the evaluation of the bleaching effect. The higher the L* value, the brighter the sample and the more effective the bleaching treatment. Based on the L* values of the purified insect chitin as a reference sample (L* standard), the bleaching effect was calculated as percentage increase in lightness, using the following equation:

$$\text{Bleaching effect [\%]} = \frac{DL^*sample}{L^*standard} \times 100 \quad (3)$$

3. Results and Discussion

Pupal exuviae arose from the transformation of the pupae in *H. illucens* imago. A major challenge of the chitin purification thereof is the sclerotized structure of the cuticles, meaning the cross-linking of proteins with catechol derivatives and chitin, resulting in water extrusion, hardening and browning of the cuticle [40,41]. The three consecutive steps of the chitin purification process from pupal exuviae were investigated: demineralization (step 1), deproteinization (step 2) and bleaching (step 3). As is already shown for marine shell wastes, the order of the process steps has no significant effect on quality or quantity of the chitin extracted [42].

3.1 Characterization of the raw material

The metal ion contents of the pupal exuviae fresh mass were as follows: Ca^{2+} 40 mg/kg, Mg^{2+} 5 mg/kg, Mn^{2+} 0.8 mg/kg, $Fe^{2+/3+}$ 0.15 mg/kg, Zn^{2+} 0.05 mg/kg, Cu^{2+} 8 µg/kg, Ni^{2+} 4 µg/kg. The pupal exuviae have a moisture content of 8.7%, and an ash content of 15.8% on a wet mass base. Lipid content was determined to be 1.2% related to wet mass. Khayrova et al. [43] have determined the ash and lipid content of *H. illucens* pupal exuviae, obtaining 10.5% and 8.9% respectively. At the best of our knowledge, there is no other work in the literature with which to compare our data. In most cases, the composition of larvae or prepupae of *H. illucens*, the stages most commonly intended for animal feed production, is determined.

3.2 Demineralization as the first step of purification

Identification of an acid suitable for demineralization

Previous investigations identified the major amount of the ash in the Black soldier fly as calcium and magnesium salts (not shown), whereby the main counter anion is carbonate [44]. The application of an acid results in the degradation of the carbonates and the formation of carbon dioxide which is the driving force of the reaction. Various acids were investigated at different temperatures concerning the demineralization efficiency, the results are reported in Fig. 1.

Treatment with acids was mandatory as indicated by the non-significant demineralization of pupal exuviae resulted from incubation with water at both 40 and 70 °C. The solubility of

calcium carbonate (14 mg/L at 20 °C) (GESTIS substance database, 2020, https://gestis-database.dguv.de/data?name=001650) is too low to allow its removal with water only. Furthermore, calcium carbonate is an “inverse solubility salt” whose solubility decreases with increasing temperature [45].

At both temperatures, sulfuric acid and phosphoric acid showed the lowest DME, resulting in residual ash contents > 8% (Fig. 1). The use of phosphoric acid leads to the formation of heavily soluble calcium phosphates at slightly low pH values and an inverse solubility as already described for calcium carbonate [45]. The application of sulphuric acid at the given pH value leads to the formation of calcium salts with low solubility remaining in the biomass. What has just been said also applies to the magnesium salts. Hence both phosphoric and sulfuric acid are not suitable for demineralization of *H. illucens* pupal exuviae.

The use of nitric, hydrochloric, acetic and formic acids resulted in a much lower mineral content (1.6%-5.2%) at both temperatures, with the highest DME being achieved treating with both 0.5 M formic acid or hydrochloric acid (Fig. 1). The salts formed as a result of the use of these acids (nitrates, chlorides, acetates and formates) have a higher solubility than calcium phosphate or sulphate. Comparing the mineral content after the 40 °C and 70 °C treatments with all acids, it is shown that the treatment at 70 °C did not result in the removal of more minerals as could be expected. With sulfuric and acetic acid only the DME was slightly higher at 70 °C than 40 °C. Since it showed the highest DME at both 40 °C and 70 °C, formic acid was the acid of choice for DM of pupal exuviae.

These results are in line with studies on DM of shrimp shells with organic acids, such as lactic or acetic acid, reporting that the DME was comparable to that obtained using hydrochloric acid [46,47]. Furthermore, the costs of demineralisation can be reduced by using organic acids [46] and salts of formic acid exhibit lower corrosiveness than mineral acids, which can reduce material wear on an industrial scale.

Formic acid has already been used for DM of *H. illucens* larval exoskeleton, obtaining a DME of 84%-86% [20]. Similar values were achieved by Zhou et al. [48] using natural deep eutectic

solvents for DM of prepupae. [49] and [50] performed DM of *H. illucens* larvae, prepupae and pupae with hydrochloric acid, but DME of these treatments was not been measured.

3.3 Investigation of the deproteinization as second step of chitin purification

As is the case for chitin purification from crab shells, proteins are the second compound to be separated after demineralization in a deproteinization step. The protein content of samples was deduced from the mass sum of all amino acids quantified.

The amino acids content of raw pupal exuviae was 317.4 ± 30.9 mg/g, so the protein content of the pupal exuviae biomass was calculated to 31.7 ± 3.1% (w/w). This protein content is slightly lower than in the exoskeleton of other insects. For instance, exoskeleton of honeybees (*Apis mellifera*) contains around 40% proteins [31]. Cocoons of the fungus gnat (*Rhynchosciara americana*) have 38% protein [51], while cocoons of silkworm (*Bombyx mori*) have 48% proteins [52].

Deproteinization was carried out with the demineralized pupal exuviae. The deproteinization efficiency (DPE) was calculated for all the experiments. Results are reported in Table 3. The highest DPE resulted from incubation with 1.25 M NaOH solution for 4 h at 90 °C (experiment 2), during which 96% of the proteins was removed from the sample. The use of NaOH leads to partial or total hydrolysis of the peptide bonds, whereby the extent of the hydrolysis increases with the incubation temperature and alkali concentration. Indeed, the DPE raised sharply as the temperature was raised. With a temperature of 90 °C, the DPE was higher than 79% independent of incubation time and NaOH molarity.

Even incubating for 4 h, the lowest NaOH concentration (0.5 M) and a temperature of 70 °C (experiment 14), resulted in a minimal DPE of 20%. The DPE raised to 79% by increasing only the NaOH concentration to 1.25 M (experiment 3).

As already presumed by a superficial investigation of the results, temperature and NaOH concentration are significant parameters for DPE ($p < 0.005$). The dependency of the DPE in function of both parameters is illustrated in Fig. 2. The incubation time has lower significance. Thus, a linear model with two main effects was generated:

$$DPE\ (\%) = -86.5 + 1.65 \cdot temperature\ (°C) + 20 \cdot c(NaOH)\ (mol/L)$$

The F-value of 23.3 implies that the model is valid. Independent from the incubation time and according to the model, 90 °C and 2 M should result in a maximum DPE. Although providing a valid model, a R^2 of 0.78 indicates that there is significant proportion of variation in the data which cannot be reasoned by the model. It has to be further stated that the optimum temperature and NaOH concentration are at the upper limit of the range applied for investigation. This implies, that higher values would be more efficient. Nevertheless, 100 °C is the limit for the water temperature conducting the deproteinization with common equipment and 2 M NaOH is a suitable concentration to deproteinize the samples in an appropriate time span.

A quantitative evaluation of the DPE of insect biomass was only performed by few researchers. The DPE achieved within out treatment was confirmed by other studies: Kim and coworkers [53,24] applied DP conditions similar to ours (1.25 M NaOH for 3 h at 95 °C) on *Musca domestica* pupae and *Gryllus bimaculatus* adults achieving 87% DPE. A DPE of 97% was achieved by Zhou et al. [48] using natural deep eutectic solvents on *H. illucens* prepupae.

The use of NaOH is also accompanied by the solubilization of catechols that are incorporated into the insect carapaces due to the sclerotization process. Part of these catechols is known to be tightly bound to chitin [54] and they are not removed by the DP conditions applied. Thus, the use of bleaching reagents in a separate step is necessary to remove the residual coloration of the biomass. In the cocoons of the tobacco hawkmoth (*Manduca sexta*), 4% of catechols is present after sclerotization. By the time the adult hawkmoths hatch from the cocoons, the proportion of catechol derivatives increases up to 17% [55]. A similar catechol content can be expected for the *H. illucens* breeding waste.

3.4 Bleaching as the final process step

The bleaching treatment was applied at the end of the purification process, to increase chitin lightness and purification degree by removing redox-sensitive and colour-intensive contaminants, mainly catecholic compounds. All bleaching experiments were carried out with

the unbleached insect chitin, which was first demineralized and deproteinized with the process conditions identified to be optimal. The images of the resulting bleached samples are shown in Fig. 3. Bleaching effects of the different treatments were determined by colorimetric measurement using a spectrophotometer. The lightness values (L*) of bleached samples and the unbleached insect chitin as standard were first determined. With the knowledge of the L* value of the standard chitin (L* = 47.9), the DL* of each bleached sample (i.e. the difference of lightness between the standard and the bleached samples) and, consequently, the bleaching effect of the respective treatment were calculated. Lightness and bleaching effect values of each treatment are shown in Fig. 4.

The best result was achieved using NaOCl solution, which led to an increase in L* by about 38, compared to the unbleached chitin, corresponding to a bleaching effect of 66.5%. The H_2O_2-containing solutions also led to very good bleaching. The use of H_2O_2 with NaOH resulted in a significantly higher bleaching effect (62.5%) than the use of H_2O_2 alone or with $MgSO_4$ additive without lye. This makes clear that alkaline-soluble cleavage products are formed during oxidative bleaching [56]. These products remain in solution through the NaOH and can be removed during the separation of the solution. Less effective (7.7-13.9% bleaching effect) were reducing agents and solvent mixtures (experiments I, V, VII, VIII). The enzymatic approaches could not lead to a significant lightening. Only a bleaching effect of 0.3% was observed using laccase. The use of ABTS as mediator for laccase even resulted in a slight, reddish colouration of the solid based on a colour change from green to red when incubating the ABTS solution with laccase, citrate buffer and chitin. This dyeing was transferred to the solid and could not be alleviated by the washing step following the incubation.

Due to the environmental risk of chlorine-containing bleaching agents, it is desirable to identify a chlorine-free alternative to the bleaching of insect chitin. For instance, a treatment with potassium permanganate ($KMnO_4$) and oxalic acid ($C_2H_2O_4$) was proposed by Wasko et al. [57]. The use of 1% $KMnO_4$ for 1 h at room temperature followed by 4% $C_2H_2O_4$ for 1 h at 60 °C for bleaching of *H. illucens* adults and pupal exuviae chitin led to a white-gray product [57]. According to our results, the use of H_2O_2 can also be a valid alternative to chlorine-containing

compounds, being its bleaching effect almost equal to that of NaOCl. Furthermore, combining bleaching with deproteinization using a mixture of H_2O_2 and NaOH, as done in the bleaching experiment IV, could be considered. The percentage used of NaOH (1.8%) corresponds to a concentration of about 0.5 M. According to the results of our deproteinization experiments, the use of 0.5 M NaOH at high temperature results in a DPE of 80%. Thus, a combination of H_2O_2 and an increased NaOH concentration could lead to a highly efficiency deproteinization and bleaching.

A comparison with literature values is problematic and not very significant, because of different evaluation methods and chitin sources. Crustacean shells, the major chitin source, has a faint red coloration which is mainly due to non-covalently bound carotenes [58] not contaminated with cross-linked catechols as in the exuviae of insects. However, some “lessons” can be learnt: Kaya et al. [59] proposed the use of a bleaching treatment with NaOCl as the first step in the purification process of chitin from crustacean, to reduce the time required for subsequent demineralization and deproteinization. This option could also be investigated for chitin isolation from insects.

3.5 Degree of purification within processing of the pupal exuviae

In the three purification steps DM, DP and bleaching, different components of the sample material are removed and the chitin is enriched. To evaluate the purification, a wet chemical method (ADF, ADL, ADF-ADL) was performed after each step to determine the respective chitin content and to assess the purification. The results of fibre analysis during purification are shown in Table 3.

The results indicated a total loss of 83.5% biomass after the three extraction steps, with the highest losses occurring after both DM and DP to an equal extent. The lower loss in bleaching than with DM and DP was due to the proportionally lower presence of chromophores. The original biomass contained approx. 21.4% minerals and 31.7% proteins. The catechols were measured as ADL with a proportion of almost 13.0%.

Furthermore, the fibre contents of the samples are reported in Table 3. It can be seen that the ADF was enriched by DM and DP from originally 32.7% to 90.8% by removing minerals and

proteins. Considering the values of ADL, an increase of 5.5% (from 12.6% to 18.1%) occurred after DM compared to the raw sample (Table 3). Just like the ADF, it was initially enriched when the minerals were removed. However, a decrease to 11.4% was obtained by DP using NaOH. This can be explained by the fact that the splitting of the peptide bonds by means of NaOH also causes a part of the catechols to be dissolved and removed, indicated by the browning of the DP solution. The further decline of the ADL value after bleaching supports the assumption that the ADL relates to the catechol content of the insect cuticle. The aim of bleaching was to separate or disintegrate the catechols leading to darkening. The effectiveness of this treatment is illustrated by the lightening of the samples and an ADL value of only 4.2% after bleaching.

In order to evaluate the accumulation of chitin during purification, the values of ADF-ADL could be considered. Here, a stepwise increase through the individual purification steps can be seen. Starting from 20.0% in the dried raw sample, the value was increased to 84.7% after DM, DP and bleaching (Table 3). This result shows that the methods applied can be successfully used for the recovery and purification of chitin from insect remains of *H. illucens*. The chitin content of our final product can be assumed as a degree of purity of the chitin obtained from *H. illucens* pupal exuviae. A purity of 84.7% is in accordance with the average value (82.5%) obtained by Zhou et al. [48] purifying chitin from *H. illucens* prepupae with natural deep eutectic solvents. A slightly higher purity (93%) was achieved by chemical purification from *Bombyx eri* larvae using HCl and NaOH for DM and DP, respectively, without performing bleaching [60].

A biomass retention of 16.5% containing 84.7% chitin corresponds to a chitin yield of 13.9%, referring to the whole insect biomass applied for the extraction. This value is comparable to the average yield of 5-15% chitin obtained in most cases from different insect species [13]. Chitin has been purified from *H. illucens* pupal exuviae only by Brigode et al. [21] and Wang et al. [27] with a yield of 25 and 14%, respectively, using HCl for DM and NaOH for DP. Recently, also Złotko et al. [61] reported the isolation of chitin from pupal exuviae of this insect, using the same chemicals for DM and DP, but achieving a much lower chitin yield: 8% without bleaching, and 6-7% applying different discolouration treatments. Nevertheless, it should be

noted that there is no uniformity in the calculation of the chitin yield, as it refers sometimes to the total biomass retained at the end of the extraction process, and sometimes to the chitin content of this final biomass obtained.

However, chitin enrichment is accompanied by loss of the biopolymer during the purification processes. On the one hand, this is done by filtration or sample transfer. On the other hand, a partial degradation of the chitin can also be assumed. From the initial value of the chitin content of 20.0%, the yield of biomass, the total masses and the chitin content after purification (ADF-ADL = 84.7%), the loss of chitin amounts to 27.6% and is thus within an acceptable range.

4. Conclusions

This investigation provides an optimization of chitin extraction from side streams of insect farming. Pupal exuviae of the widely bred fly species *H. illucens* were used, being them a readily available waste biomass rich in chitin easy to collect from the insect-breeding facilities. Steps of the extraction process were carried out under different conditions in order to assess the optimal parameters.

DM was optimized concerning acid applied and temperature. Formic and hydrochloric acid were the most effective, particularly formic acid gave the best results at both tested temperatures. This encourages the use of organic acids instead of the most applied inorganic acids in a frame of a greater environmental sustainability.

Efficiency of DP was investigated in relation to temperature, molarity and duration, highlighting the significance of temperature and alkali molarity as parameters for an efficient protein removal. The optimized DP procedure successfully removed proteins from the insect sample but, at the same time, the use of NaOH inevitably leads to partial deacetylation of the final chitin [62]. For instance, 23% deacetylation was observed after treating chitin with 2% NaOH for 0.5 h at 60 °C [63]. Hence, the reaction parameters of DP should be properly chosen depending on the desired final product.

The bleaching experimental series shows that oxidative agents lead to a significant lightening of the insect chitin, especially solutions with NaOCl and H_2O_2. Although the bleaching effect is slightly higher using NaOCl, the use of H_2O_2 can be a viable alternative to reduce the environmental risk of chlorine-containing compounds while still maintaining a high bleaching effect. The possibility of bleaching and deproteinizing at the same time using a combination of H_2O_2 and NaOH can be also considered, thus saving time and energy.

Appling the optimized extraction method, a chitin with around 85% purity was obtained from *H. illucens* pupal exuviae with a yield of 13.9% related to the original insect biomass. This is in line with average yields and purity of chitins generally obtained from insects. Particularly, the purification degree of chitin extracted from pupal exuviae is similar to that experimentally determined of commercial chitin from fishery waste (unpublished data).

Currently, insect-based chitin is produced only on a laboratory scale. This study provides a starting point, on one hand, for the scale-up of chitin purification processes to exploit waste biomass generated from the insect farms. On the other hand, it provides the basis for further research activities fully unfolding the potential of the chitin and its derivatives isolated from insects. This was already confirmed by researchers applying chitosan obtained from *H. illucens* pupal exuviae for advanced applications, such as 3D films [64].

Declarations

Conflicts of interest/Competing interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Availability of data

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

FIGURE CAPTIONS

Fig. 1 DME as function of different acids and temperature of the acid solution. Upper bar graph represents the approaches performed at 40 °C, the lower graph provides the results of DME achieved at 70 °C. The application of water only at 70 °C results in a DME of 5.4% ± 2.7%.

Fig. 2: DPE (%) in function of NaOH concentration (mol/L) and Temperature (°C). The chitin applied was previously demineralized at optimum conditions.

Fig. 3 Reagents and concentrations used for the different bleaching methods, and the resultant respective bleached chitin. The chitin used was previously demineralized and deproteinized at optimum conditions.

Fig. 4 Absolute lightness values L* of the differently bleached samples in comparison to the unbleached insect chitin. The values in the column are the bleaching effects [%] with regard to the raw chitin. The raw chitin has a L* of 47.9.

CITATIONS

1. Elieh-Ali-Komi D, Hamblin MR (2016) Chitin and chitosan: production and application of versatile biomedical nanomaterials. Int J Adv Res 4 (3):411-427

2. Manjula K, Podile AR (2001) Chitin-supplemented formulations improve biocontrol and plant growth promoting efficiency of Bacillus subtilis AF 1. Canadian journal of microbiology 2001 v.47 no.7 (no. 7):pp. 618-625. doi:10.1139/w01-057

3. Wang M, Chen LJ, Ni J, Weng J, Yue CY (2001) Manufacture and evaluation of bioactive and biodegradable materials and scaffolds for tissue engineering. Journal of Materials Science: Materials in Medicine 12 (10):855-860. doi:10.1023/A:1012899318688

4. Felse PA, Panda T (1999) Studies on applications of chitin and its derivatives. Bioprocess Eng 20 (6):505-512. doi:10.1007/s004490050622

5. Yeul VS, Rayalu SS (2012) Unprecedented Chitin and Chitosan: A Chemical Overview. Journal of Polymers and the Environment 21 (2):606-614. doi:10.1007/s10924-012-0458-x

6. Bolat Y, Bilgin Ş, Günlü A, Izci L, Koca SB, Çetinkaya S, Koca HU (2010) Chitin-chitosan yield of freshwater crab (Potamon potamios, Olivier 1804) shell. Pak Vet J 30 (4):227-231

7. Kurita K (2006) Chitin and chitosan: functional biopolymers from marine crustaceans. Mar Biotechnol (NY) 8 (3):203-226. doi:10.1007/s10126-005-0097-5

8. Jardine A, Sayed S (2018) Valorisation of chitinous biomass for antimicrobial applications. Pure Appl Chem 90 (2):293-304. doi:10.1515/pac-2017-0707

9. Gillett R (2008) Global study of shrimp fisheries, vol 475. FAO fisheries technical paper. Rome: Food and Agriculture Organization of the United Nations,

10. Pittman SJ, McAlpine CA (2003) Movements of marine fish and decapod crustaceans: process, theory and application. Advances in marine biology 44 (1):205-294

11. White SA, Farina PR, Fulton I (1979) Production and isolation of chitosan from Mucor rouxii. Applied and environmental microbiology 38 (2):323-328

12. Abo Elsoud MM, El Kady EM (2019) Current trends in fungal biosynthesis of chitin and chitosan. Bulletin of the National Research Centre 43 (1):59. doi:10.1186/s42269-019-0105-y

13. Hahn T, Tafi E, Paul A, Salvia R, Falabella P, Zibek S (2020) Current state of chitin purification and chitosan production from insects. Journal of Chemical Technology and Biotechnology 95 (11):2775-2795. doi:10.1002/jctb.6533

14. Fowles TM, Nansen C (2020) Insect-Based Bioconversion: Value from Food Waste. In: Närvänen E, Mesiranta N, Mattila M, Heikkinen A (eds) Food Waste Management: Solving the Wicked Problem. Springer International Publishing, Cham, pp 321-346. doi:10.1007/978-3-030-20561-4_12

15. van Huis A (2013) Potential of insects as food and feed in assuring food security. Annu Rev Entomol 58:563-583. doi:10.1146/annurev-ento-120811-153704

16. Jucker C, Lupi D, Moore CD, Leonardi MG, Savoldelli S (2020) Nutrient Recapture from Insect Farm Waste: Bioconversion with Hermetia illucens (L.) (Diptera: Stratiomyidae). Sustainability 12 (1):1-14. doi:10.3390/su12010362

17. Tomberlin JK, Sheppard DC, Joyce JA (2002) Selected life-history traits of black soldier flies (Diptera : Stratiomyidae) reared on three artificial diets. Ann Entomol Soc Am 95 (3):379-386. doi:10.1603/0013-8746(2002)095[0379:slhtob]2.0.co;2

18. Hahn T, Roth A, Febel E, Fijalkowska M, Schmitt E, Arsiwalla T, Zibek S (2018) New methods for high-accuracy insect chitin measurement. J Sci Food Agric 98 (13):5069-5073. doi:10.1002/jsfa.9044

19. Schmitt E, de Vries W (2020) Potential benefits of using Hermetia illucens frass as a soil amendment on food production and for environmental impact reduction. Current Opinion in Green and Sustainable Chemistry 25:100335. doi:https://doi.org/10.1016/j.cogsc.2020.03.005

20. Hahn T, Roth A, Ji R, Schmitt E, Zibek S (2020) Chitosan production with larval exoskeletons derived from the insect protein production. Journal of Biotechnology 310:62-67

21. Brigode C, Hobbi P, Jafari H, Verwilghen F, Baeten E, Shavandi A (2020) Isolation and physicochemical properties of chitin polymer from insect farm side stream as a new source of renewable biopolymer. Journal of Cleaner Production 275:122924. doi:https://doi.org/10.1016/j.jclepro.2020.122924

22. Badawy RM, Mohamed HI (2015) Chitin extration, Composition of Different Six Insect Species and Their Comparable Characteristics with That of the Shrimp. J Am Sci 11 (6):127-134

23. Kaya M, Bagriacik N, Seyyar O, Baran T (2015) Comparison of chitin structures derived from three common wasp species (Vespa crabro LINNAEUS, 1758, Vespa orientalis LINNAEUS, 1771 and Vespula germanica (FABRICIUS, 1793)) Archives of Insect Biochemistry and Physiology 89 (4):204-217. doi:10.1002/arch.21237

24. Kim M-W, Song Y-S, Han YS, Jo YH, Choi MH, Park Y-K, Kang SH, Kim S-A, Choi C, Jung W-J (2017) Production of chitin and chitosan from the exoskeleton of adult two-spotted field crickets (Gryllus bimaculatus). Entomological Research 47 (5):279-285. doi:10.1111/1748-5967.12239

25. Ibitoye EB, Lokman IH, Hezmee MNM, Goh YM, Zuki ABZ, Jimoh AA (2018) Extraction and physicochemical characterization of chitin and chitosan isolated from house cricket. Biomed Mater 13 (2):1-12. doi:10.1088/1748-605X/aa9dde

26. Shin C-S, Kim D-Y, Shin W-S (2019) Characterization of chitosan extracted from Mealworm Beetle (Tenebrio molitor, Zophobas morio) and Rhinoceros Beetle (Allomyrina dichotoma) and their antibacterial activities. Int J Biol Macromol 125:72-77. doi:https://doi.org/10.1016/j.ijbiomac.2018.11.242

27. Wang H, Rehman KU, Feng W, Yang D, Rehman RU, Cai M, Zhang J, Yu Z, Zheng L (2020) Physicochemical structure of chitin in the developing stages of black soldier fly. Int J Biol Macromol 149:901-907. doi:10.1016/j.ijbiomac.2020.01.293

28. Matissek R, Steiner G, Fischer M (2010) Fette und Fettbegleitstoffe. In: Lebensmittelanalytik. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 29-83. doi:10.1007/978-3-540-92205-6_2

29. Urbat F, Müller P, Hildebrand A, Wefers D, Bunzel M (2019) Comparison and Optimization of Different Protein Nitrogen Quantitation and Residual Protein Characterization Methods in Dietary Fiber Preparations. Frontiers in Nutrition 6 (127). doi:10.3389/fnut.2019.00127

30. Houg GW, Kindron RR (1969) Bleaching Of Wood Pulps With Thiourea Dioxide. Canada Patent,

31. Nemtsev SV, Zueva OY, Khismatullin MR, Albulov AI, Varlamov VP (2004) Isolation of Chitin and Chitosan from Honeybees. Appl Biochem Micro+ 40 (1):39-43

32. Candido RG, Gonçalves AR (2016) Synthesis of cellulose acetate and carboxymethylcellulose from sugarcane straw. Carbohyd Polym 152:679-686. doi:https://doi.org/10.1016/j.carbpol.2016.07.071

33. Moral A, Aguado R, Mutjé P, Tijero A (2016) Papermaking potential of Citrus sinensis trimmings using organosolv pulping, chlorine-free bleaching and refining. Journal of Cleaner Production 112:980-986. doi:https://doi.org/10.1016/j.jclepro.2015.09.008

34. Yen M-T, Mau J-L (2007) Selected physical properties of chitin prepared from shiitake stipes. LWT - Food Science and Technology 40 (3):558-563. doi:https://doi.org/10.1016/j.lwt.2005.10.008

35. No HK, Meyers SP, Lee KS (1989) Isolation and Characterization of Chitin from Crawfish Shell Waste. J Agr Food Chem 37 (3):575-579. doi:DOI 10.1021/jf00087a001

36. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37 (8):911-917. doi:10.1139/o59-099

37. Rodriguez-Couto S (2012) Laccases for Denim Bleaching: An Eco-Friendly Alternative. The Open Textile Journal 5 (1)

38. Tian L, Branford-White C, Wang W, Nie H, Zhu L (2012) Laccase-mediated system pretreatment to enhance the effect of hydrogen peroxide bleaching of cotton fabric. Int J Biol Macromol 50 (3):782-787. doi:10.1016/j.ijbiomac.2011.11.025

39. Christopher LP, Yao B, Ji Y (2014) Lignin Biodegradation with Laccase-Mediator Systems. Frontiers in Energy Research 2 (12). doi:10.3389/fenrg.2014.00012

40. Gonil P, Sajomsang W (2012) Applications of magnetic resonance spectroscopy to chitin from insect cuticles. Int J Biol Macromol 51 (4):514-522. doi:10.1016/j.ijbiomac.2012.06.025

41. Hopkins TL, Kramer KJ (1992) Insect Cuticle Sclerotization. Annu Rev Entomol 37:273-302

42. Younes I, Rinaudo M (2015) Chitin and Chitosan Preparation from Marine Sources. Structure, Properties and Applications. Mar Drugs 13 (3):1133-1174. doi:10.3390/md13031133

43. Khayrova A, Lopatin S, Varlamov V (2020) Obtaining Chitin/Chitosan-Melanin Complexes from Black Soldier Fly Hermetia Illucens. IOP Conference Series: Materials Science and Engineering 809:012020. doi:10.1088/1757-899x/809/1/012020

44. Barros-Cordeiro KB, Bao SN, Pujol-Luz JR (2014) Intra-puparial development of the black soldier-fly, Hermetia illucens. J Insect Sci 14

45. Bott TR (1995) Chapter 8 - Crystallisation and Scale Formation. In: Bott TR (ed) Fouling of Heat Exchangers. Elsevier Science B.V., Amsterdam, pp 97-135. doi:https://doi.org/10.1016/B978 044482186-7/50010-5

46. Mahmoud NS, Ghaly AE, Arab F (2007) Unconventional Approach for Demineralization of Deproteinized Crustacean Shells for Chitin Production. American Journal of Biochemistry and Biotechnology 3 (1):1-9. doi:10.3844/ajbbsp.2007.1.9

47. Alewo A, David A, Isa M, Rabiu U (2014) Kinetics of Demineralization of Shrimp Shell Using Lactic Acid. Leonardo El J Pract Technol 13:13-22

48. Zhou P, Li J, Yan T, Wang X, Huang J, Kuang Z, Pan M (2019) Selectivity of deproteinization and demineralization using natural deep eutectic solvents for production of insect chitin (Hermetia illucens). Carbohyd Polym 225:1-9. doi:https://doi.org/10.1016/j.carbpol.2019.115255

49. Khayrova A, Lopatin S, Varlamov V (2019) Black Soldier Fly Hermetia illucens as a Novel Source of Chitin and Chitosan. Int J Sci 8 (04):81-86. doi:10.18483/ijSci.2015

50. Smets R, Verbinnen B, Van De Voorde I, Aerts G, Claes J, Van Der Borght M (2020) Sequential Extraction and Characterisation of Lipids, Proteins, and Chitin from Black Soldier Fly (Hermetia illucens) Larvae, Prepupae, and Pupae. Waste and Biomass Valorization. doi:10.1007/s12649-019-00924-2

51. Terra WR, de Bianchi AG (1974) Chemical composition of the cocoon of the fly, Rhynchosciara americana. Insect Biochemistry 4 (2):173-183. doi:https://doi.org/10.1016/0020-1790(74)90005-5

52. Rumpold BA, Schluter OK (2013) Nutritional composition and safety aspects of edible insects. Mol Nutr Food Res 57 (5):802-823. doi:10.1002/mnfr.201200735

53. Kim M-W, Han YS, Jo YH, Choi MH, Kang SH, Kim SA, Jung WJ (2016) Extraction of chitin and chitosan from housefly,Musca domestica, pupa shells. Entomological Research 46 (5):324-328. doi:10.1111/1748-5967.12175

54. Xu R, Huang X, Hopkins TL, Kramer KJ (1997) Catecholamine and histidyl protein cross-linked structures in sclerotized insect cuticle. Insect Biochem Molec 27 (2):101-108. doi:https://doi.org/10.1016/S0965-1748(96)00083-5

55. Kramer KJ, Hopkins TL, Schaefer J (1995) Applications of solids NMR to the analysis of insect sclerotized structures. Insect Biochem Molec 25 (10):1067-1080. doi:https://doi.org/10.1016/0965-1748(95)00053-4

56. Sun R, Lu Q, Sun XF (2001) Physico-chemical and thermal characterization of lignins from Caligonum monogoliacum and Tamarix spp. Polymer Degradation and Stability 72 (2):229-238. doi:https://doi.org/10.1016/S0141-3910(01)00023-4

57. Wasko A, Bulak P, Polak-Berecka M, Nowak K, Polakowski C, Bieganowski A (2016) The first report of the physicochemical structure of chitin isolated from Hermetia illucens. Int J Biol Macromol 92:316-320. doi:10.1016/j.ijbiomac.2016.07.038

58. Vilasoa-Martínez M, Calaza-Ramos C, López-Hernández J, Lage-Yusty MA, Losada PP, Rodríguez-Bernaldo de Quirós A (2008) Determination of vitamin E and carotenoid pigments by high performance liquid chromatography in shell of Chionoecetes opilio. Analytica Chimica Acta 617 (1):225-229. doi:https://doi.org/10.1016/j.aca.2008.03.001

59. Kaya M, Baran T, Karaarslan M (2015) A new method for fast chitin extraction from shells of crab, crayfish and shrimp. Nat Prod Res 29 (15):1477-1480. doi:10.1080/14786419.2015.1026341

60. Huet G, Hadad C, Husson E, Laclef S, Lambertyn V, Araya Farias M, Jamaly A, Courty M, Alayoubi R, Gosselin I, Sarazin C, Van Nhien AN (2020) Straightforward extraction and selective bioconversion of high purity chitin from Bombyx eri larva: Toward an integrated insect biorefinery. Carbohydr Polym 228:1-12. doi:https://doi.org/10.1016/j.carbpol.2019.115382

61. Złotko K, Waśko A, Kamiński DM, Budziak-Wieczorek I, Bulak P, Bieganowski A (2021) Isolation of Chitin from Black Soldier Fly (Hermetia illucens) and Its Usage to Metal Sorption. Polymers-Basel 13 (5):818

62. Xiaofei H, Li K, Xing R, Liu Q, Hu L, Li P (2016) The production of fully deacetylated chitosan by compression method. The Egyptian Journal of Aquatic Research 42. doi:10.1016/j.ejar.2015.09.003

63. Mizami AMA, B. M. (2007) A new process of deproteinization of chitin from shrimp head waste.8

64. Bilican I, Pekdemir S, Onses MS, Akyuz L, Altuner EM, Koc-Bilican B, Zang L-S, Mujtaba M, Mulerčikas P, Kaya M (2020) Chitosan Loses Innate Beneficial Properties after Being Dissolved in Acetic Acid: Supported by Detailed Molecular Modeling. ACS Sustainable Chemistry & Engineering 8 (49):18083-18093. doi:10.1021/acssuschemeng.0c06373

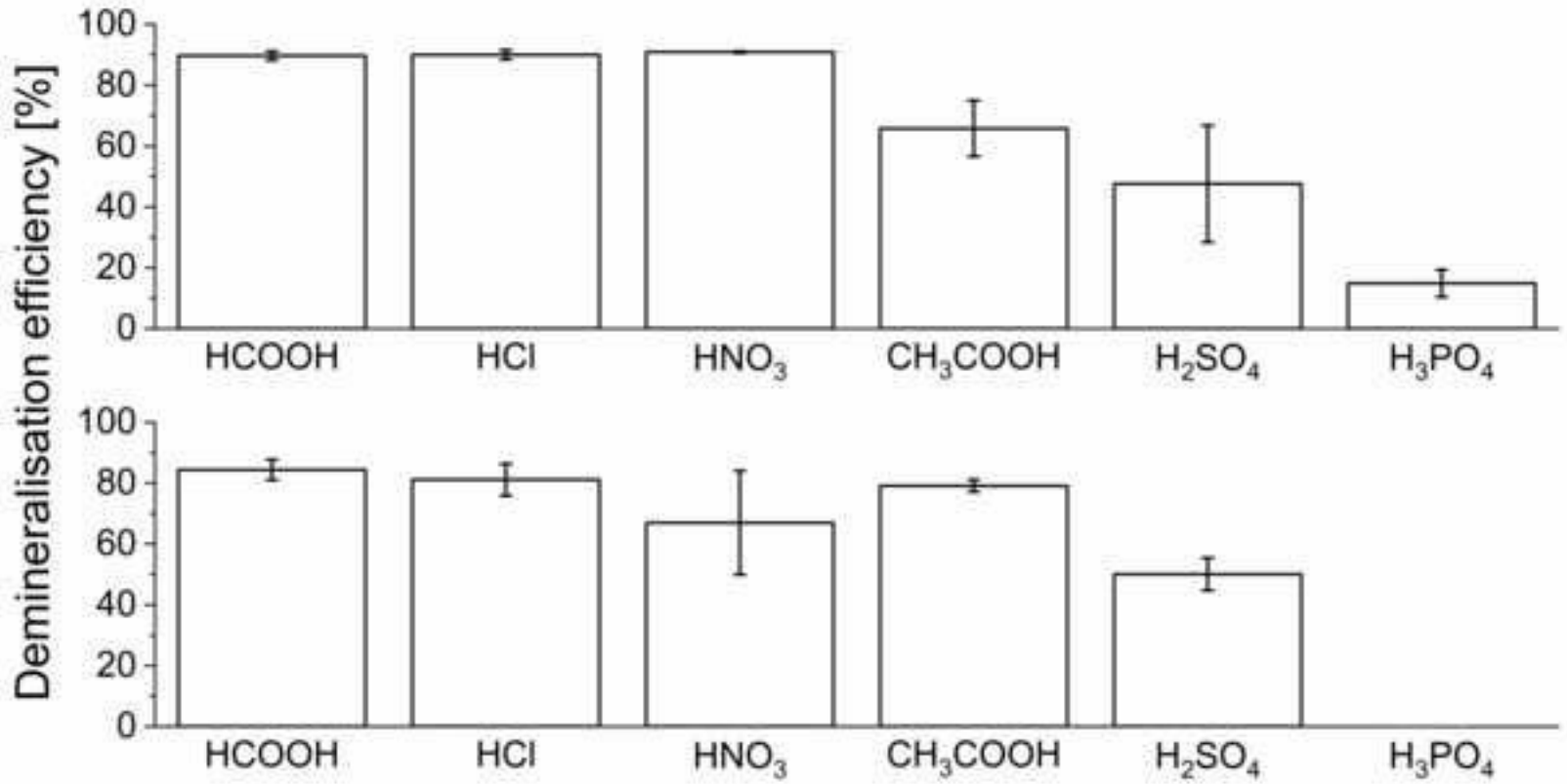
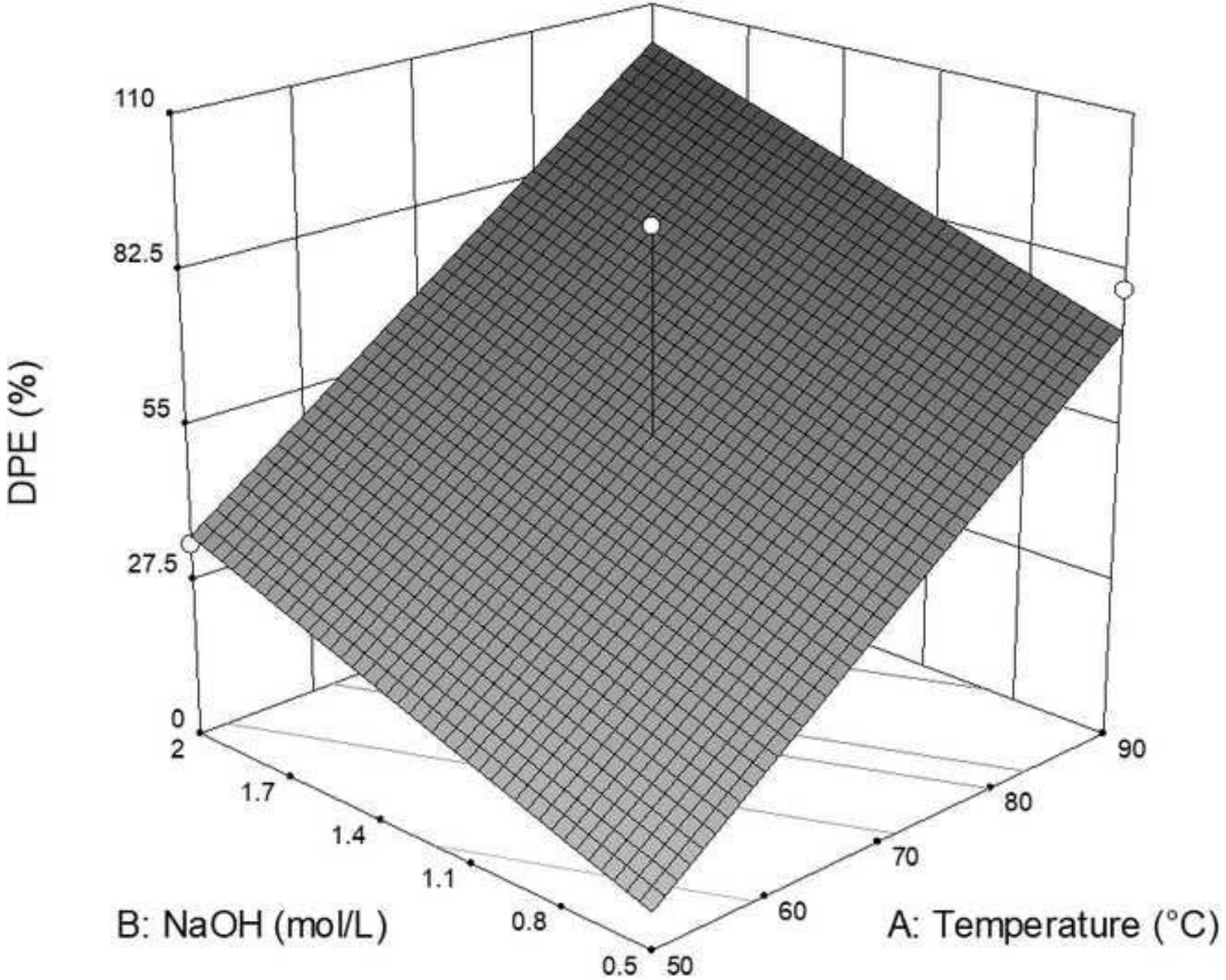


Figure 2

Click here to access/download;Figure;Figure 2.jpg



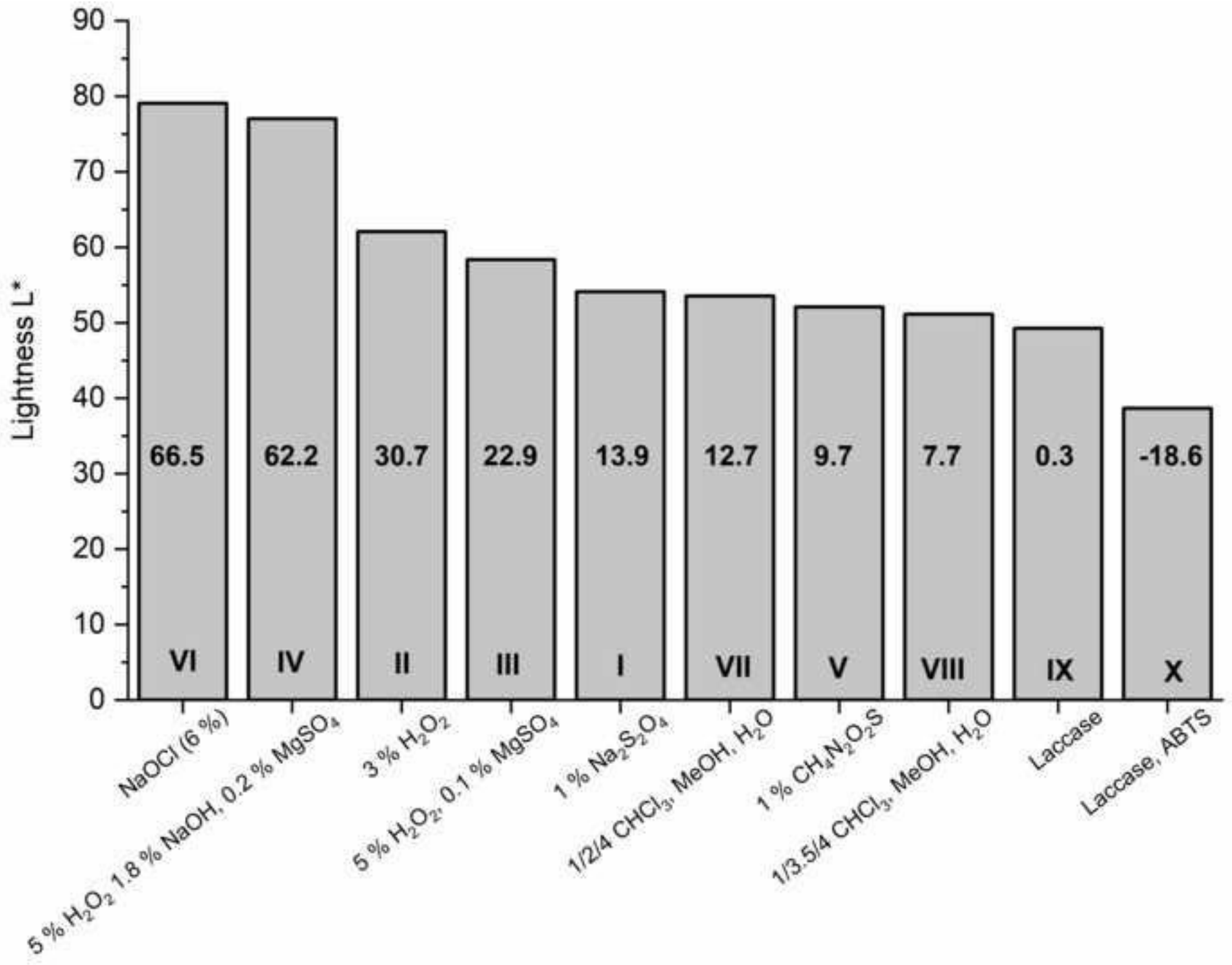


Table 1. Deproteinization efficiency as a function of different parameter combinations (NaOH concentration, incubation time, incubation temperature)

Experiment	c (NaOH) (mol/L)	Time (h)	Temperature (°C)	DP efficiency (%)
1	1,25	1	50	31
2	1,25	4	90	96
3	1,25	2,5	70	79
4	1,25	2,5	70	91
5	2	2,5	50	35
6	1,25	4	50	15
7	2	2,5	90	95
8	2	4	70	86
9	2	2,5	90	92
10	2	2,5	90	93
11	2	1	70	45
12	0,5	1	70	23
13	0,5	2,5	50	0
14	0,5	4	70	20
15	0,5	2,5	90	80
16	1,25	1	90	93

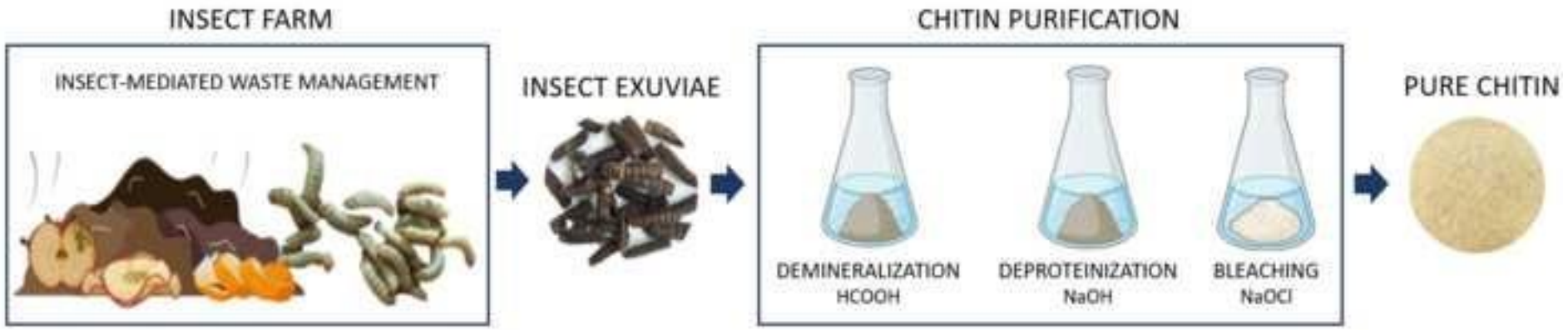
Table 2. Main contents of the different bleaching solutions, incubation conditions, methods applied and the respective study. All quantities in experiment I-VI refer to weight per volume, shares of trials VII and VIII refer to volume per volume

Trial	Bleaching solution	Conditions	Method	Adapted from
I	1% $Na_2S_2O_4$ (sodium dithionite)	70 °C, 60 min	1	[30]
II	3% H_2O_2; pH 7.5	90 °C, 60 min	1	[31]
III	5% H_2O_2; 0.1% $MgSO_4$	70 °C, 60 min	1	[32]
IV	5% H_2O_2; 1.8% NaOH; 0.2% $MgSO_4$	85 °C, 90 min	1	[33]
V	1 % $CH_4N_2O_2S$ (thiourea dioxide)	70 °C, 60 min	1	[30]
VI	6% NaOCl	60 °C, 60 min	1	[34,35]
VII	1/2/4 $CHCl_3$/MeOH/H_2O	RT, 120 min	2	[36]
VIII	1/3.5/4 $CHCl_3$/MeOH/H_2O	RT, 60 min	2	[36]
IX	Laccase	RT, 24 h	3	[37,38]
X	Laccase, 3 mM ABTS	RT, 24 h	3	[37,38]

Table 3. Representation of the fiber content after each purification step: demineralization (DM), deproteinization (DP) and bleaching

	Biomass recovery [%]	ADF [%]	ADL [%]	ADF-ADL [%]
Raw material	100	32.7 ± 0.4	12.6 ± 0.2	20.0 ± 0.5
After DM	61.4 ± 0.2	55.5 ± 0.5	18.1 ± 1.8	37.4 ± 1.9
After DP	22.5 ± 0.8	90.8 ± 0.3	11.4 ± 0.2	79.4 ± 0.3
After bleaching	16.5 ± 0.8	88.9 ± 0.4	4.2 ± 0.1	84.7 ± 0.4

Graphical abstract



	70 °C		
Acid	Demineralization efficiency [%]	Standard deviation [%]	Final pH
HCOOH	84.7	3.3	3.2
HCl	81.1	5.2	0.6
CH_3COOH	79.1	2.0	4.2
HNO_3	66.9	17.0	0.6
H_2SO_4	50.1	5.4	0.8
H_2O	5.4	2.7	%
H_3PO_4	0.0	0.0	1.7

40 °C		
Demineralization efficiency [%]	Standard deviation [%]	Final pH
89.7	1.5	3.5
90.0	1.6	0.9
65.8	9.2	4.3
90.9	0.3	0.9
47.6	19.2	1.1
%	%	%
14.9	4.5	1.8

Design of Experiments and results

	Factor values		
Number/Sample	Temperature [°C]	c(NaOH) [mol/L]	Time [h]
1	50	1.25	1
2	90	1.25	4
3	70	1.25	2.5
4	70	1.25	2.5
5	50	2	2.5
6	50	1.25	4
7	90	2	2.5
8	70	2	4
9	90	2	2.5
10	90	2	2.5
11	70	2	1
12	70	0.5	1
13	50	0.5	2.5
14	70	0.5	4
15	90	0.5	2.5
16	90	1.25	1
Raw material (wet biomass)			
Demineralized PE			

Amino acid data

Amino acid	**Abbreviation**	**M_w [g/mol]**	**M_w-H_2O [g/m**
Aspartic acid	Asp	133.11	115.11
Glutamic acid	Glu	147.13	129.13
Asparagine	Asn	132.12	114.12
Serine	Ser	105.09	87.09
Histidine	His	155.15	137.15
Glycine	Gly	75.07	57.07
Threonine	Thr	119.12	101.12
Arginine	Arg	174.2	156.2
Alanine	Ala	89.09	71.09
Cysteine	Cys	121.16	103.16
Valine	Val	117.15	99.15
Methionine	Met	149.21	131.21
Tryptophan	Trp	204.23	186.23
Phenylalanine	Phe	165.19	147.19
Isoleucine	Ile	131.17	113.17
Leucine	Leu	131.17	113.17
Lysine	Lys	146.19	128.19

Asp	Asp-H_2O	Glu	Glu-H_2O	Asn	Asn-H_2O
7344.30	6351.16	12236.998	10739.91	< 214	0.00
387.45	335.05	1955.5968	1716.35	< 221	0.00
1873.46	1620.12	7202.6157	6321.44	< 221	0.00
501.98	434.10	2915.1128	2558.48	< 221	0.00
7004.16	6057.01	12630.658	11085.41	< 225	0.00
9393.18	8122.97	16944.364	14871.38	336.23506	290.43
763.06	659.88	2031.6828	1783.13	< 221	0.00
1204.06	1041.24	3053.4125	2679.86	< 204	0.00
901.92	779.96	2617.4074	2297.19	< 221	0.00
816.02	705.68	2446.707	2147.37	< 221	0.00
6348.85	5490.32	10581.301	9286.78	< 218	0.00
8807.42	7616.42	14319.2	12567.38	279.52943	241.45
11659.26	10082.62	20759.847	18220.07	< 314	0.00
8616.62	7451.42	16416.537	14408.12	< 321	0.00
1668.79	1443.13	6225.8958	5464.21	< 221	0.00
541.89	468.61	3024.0679	2654.10	< 221	0.00
11196.5883	9682.51	21141.201	18554.77	534.93041	462.05
15053.92385	13018.23	23008.177	20193.34	820.55831	708.77

Ser	Ser-H_2O	His	His-H_2O	Gly	Gly-H_2O	Thr
18150.4	15041.57	10799.446	9546.53	51442.569	39107.86	5374.6382
< 438	0.00	1294.566	1144.37	1132.2414	860.76	< 496
752.81423	623.87	4832.9561	4272.25	11389.664	8658.69	693.7851
< 437	0.00	1853.4139	1638.39	4606.8741	3502.26	< 198
13947.414	11558.48	9711.4852	8584.79	40031.83	30433.15	4927.0094
17060.527	14138.37	13502.948	11936.38	48468.247	36846.71	7514.7495
< 437	0.00	1389.0971	1227.94	1248.71	949.30	< 198
617.97262	512.13	1913.8836	1691.84	2547.7834	1936.89	< 456
< 436	0.00	1601.7163	1415.89	1428.1253	1085.69	< 494
< 437	0.00	1717.8841	1518.58	1273.6004	968.22	< 198
10512.207	8711.66	8292.7112	7330.62	37153.345	28244.86	3635.5024
18877.85	15644.42	12385.539	10948.61	54000.451	41052.43	5817.2361
23952.469	19849.85	19455.667	17198.48	61806.585	46986.84	10994.214
13574.285	11249.26	12781.69	11298.80	48624.906	36965.81	5767.1709
< 435	0.00	4486.0388	3965.58	13657.646	10382.87	< 197
< 437	0.00	2050.1675	1812.31	3760.9153	2859.14	< 495
16089.613	13333.76	15879.758	14037.44	47827.546	36359.64	9127.7026
24159.318	20021.27	16472.599	14561.50	50773.196	38598.99	12679.485

Amino acid content [mg/kg]

Thr-H_2O	Arg	Arg-H_2O	Ala	Ala-H_2O	Cys2	Cys2-H_2O
4562.49	n.a.	0.00	42656.744	34038.25	21976.039	18711.19
0.00	< 726	0.00	996.92447	795.50	< 601	0.00
588.95	1198.509	1074.67	6765.5011	5398.58	4417.5257	3761.24
0.00	< 724	0.00	2959.0956	2361.23	814.27742	693.31
4182.50	n.a.	0.00	43612.606	34800.99	17879.212	15223.01
6379.21	n.a.	0.00	52049.075	41532.93	20701.002	17625.58
0.00	n.a.	0.00	n.a.	0.00	1929.4431	1642.80
0.00	n.a.	0.00	n.a.	0.00	997.52998	849.33
0.00	n.a.	0.00	n.a.	0.00	2593.0502	2207.82
0.00	n.a.	0.00	n.a.	0.00	1945.6803	1656.62
3086.15	n.a.	0.00	37108.969	29611.37	17888.241	15230.69
4938.20	n.a.	0.00	46261.514	36914.70	24992.078	21279.16
9332.90	n.a.	0.00	51550.143	41134.80	19984.898	17015.86
4895.70	n.a.	0.00	48990.943	39092.67	19439.787	16551.74
0.00	1349.9042	1210.42	7284.9951	5813.11	2712.995	2309.94
0.00	< 724	0.00	2173.3811	1734.26	1099.1917	935.89
7748.43	5920.0891	5308.37	38815.028	30972.73	23507.729	20015.33
10763.51	n.a.	0.00	45461.235	36276.12	31243.794	26602.09

Val	Val-H_2O	Met	Met-H_2O	Trp	Trp-H_2O	Phe
14387.493	12176.87	848.11709	745.80	< 822	0.00	3313.9255
3472.4962	2938.95	< 249	0.00	< 340	0.00	365.66734
13774.275	11657.87	< 248	0.00	< 340	0.00	1537.1782
5728.7345	4848.52	< 248	0.00	< 340	0.00	800.37919
19419.199	16435.46	641.24036	563.88	1477.2223	1347.03	2945.5245
32507.711	27512.93	1110.8404	976.83	2307.3253	2103.97	3974.2699
2899.7329	2454.19	900.69338	792.04	1605.6335	1464.12	368.72002
24671.041	20880.36	< 228	0.00	1801.4617	1642.69	576.18575
5359.1143	4535.69	1106.3686	972.90	3466.0541	3160.57	458.22966
5850.3406	4951.44	503.69795	442.93	2050.8396	1870.09	442.39449
14054.505	11895.04	487.82146	428.97	1101.9963	1004.87	2553.0263
19034.858	16110.17	871.97831	766.79	917.50508	836.64	3744.5228
40884.779	34602.87	1416.5421	1245.66	1278.4301	1165.75	5848.6512
31794.325	26909.15	1089.2102	957.81	2372.0798	2163.01	3911.1194
13107.205	11093.29	< 247	0.00	< 338	0.00	1420.853
4992.0135	4224.99	< 248	0.00	< 340	0.00	452.93917
30595.637	25894.64	1327.4435	1167.31	< 850	0.00	6216.2524
32539.979	27540.24	< 248	0.00	1245.366	1135.60	7117.0275

Phe-H_2O	i-Leu	i-Leu-H_2O	Leu	Leu-H_2O	Lys	Lys-H_2O
2952.82	5624.0653	4852.29	21819.433	18825.23	994.68747	872.21
325.82	1062.1607	916.40	831.23842	717.17	< 731	0.00
1369.68	4543.1435	3919.70	5653.5507	4877.73	1816.6906	1593.01
713.17	935.24984	806.91	2557.2744	2206.35	2679.6799	2349.74
2624.56	6460.0029	5573.52	19929.124	17194.32	2022.9151	1773.84
3541.21	11357.839	9799.24	26125.492	22540.38	3295.814	2890.01
328.54	739.84146	638.32	1101.7194	950.53	< 122	0.00
513.40	1240.2435	1070.05	1912.9167	1650.41	1583.5681	1388.59
408.30	1428.2567	1232.26	1887.4859	1628.47	< 727	0.00
394.19	1574.2213	1358.20	1695.1197	1462.50	< 730	0.00
2274.83	4751.6199	4099.57	16405.22	14153.99	1727.9561	1515.20
3336.50	6940.5311	5988.11	24010.778	20715.86	1742.0828	1527.58
5211.35	16437.55	14181.88	34165.571	29477.15	3042.7913	2668.14
3484.94	10573.043	9122.14	23684.917	20434.72	2140.4325	1876.89
1266.03	4051.0512	3495.14	6069.1124	5236.27	1301.6991	1141.42
403.58	1154.1789	995.79	1838.2963	1586.03	< 729	0.00
5538.90	13687.134	11808.90	25579.673	22069.46	6324.237	5545.55
6341.52	14167.661	12223.48	29349.657	25322.11	7168.9966	6286.30

Total protein content (% w/w)	Demineralization efficiency [%]
17.85	31
0.98	96
5.57	79
2.21	91
16.74	35
22.11	15
1.29	95
3.59	86
1.97	92
1.75	93
14.24	45
20.05	23
26.84	-3
20.69	20
5.28	80
1.77	93
22.85	
25.96	