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► **To cite this version:**

M Azzi, S Elkadaoui, J Zim, J Desbrieres, Y El Hachimi, et al.. Tenebrio Molitor breeding rejects as a high source of pure chitin and chitosan: Role of the processes, influence of the life cycle stages and comparison with *Hermetia illucens*. International Journal of Biological Macromolecules, 2024, 277, 10.1016/j.ijbiomac.2024.134475 . hal-04672879

HAL Id: hal-04672879

<https://univ-pau.hal.science/hal-04672879v1>

Submitted on 19 Aug 2024

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Tenebrio Molitor breeding rejects as a high source of pure chitin and chitosan: Role of the processes, influence of the life cycle stages and comparison with *Hermetia illucens*

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ARTICLE INFO

Keywords:

Tenebrio molitor
Exuviae
Chitin
Biopolymers
Degree of acetylation (DA)
Molar mass (Mv)

ABSTRACT

This work valorizes rejects from *Tenebrio Molitor* TM breeding through the production of chitin and chitosan. Two processes are proposed for extracting chitin from larval exuviae and adult. The first process P1 provides chitin with high contents compared to literature data but the characterization shows the presence of impurities in the exuviae chitin responsible for the shifts in the values of the physicochemical characteristics towards those presented by γ chitin. These impurities are removed by delipidation and pure α chitin is obtained. The effective delipidation of this chitin would be linked to its fibrous surface structure. The analysis of the results of P1 led us to develop a second extraction process P2 which provides pure chitin with improved yields using delipidation followed by deproteinization. The *N*-deacetylation of chitin according to Kurita or Broussignac process makes possible the preparation of pure, highly deacetylated chitosan samples (2 % < DA < 12 %) with high yields and controlled molar masses (Mv). A kinetic study of molecular degradation during deacetylation is carried out. A comparison with *Hermetia illucens* allows to extend the use of insects as a potential source of chitin and chitosan and confirms the role of the source and the processes in the determination of their characteristics.

1. Introduction

The interest in insect breeding is becoming more important in the world. It is carried out to diversify protein sources to ensure human and animal nutrition (aquaculture, livestock, and poultry breeding) [1,2] and produce biofertilizers for agriculture. The main insects subjected to breeding activities are the Black Soldier Fly (BSF), the mealworm, bees, grasshoppers, and locusts [3–6]. This breeding is accompanied by successive molts during the different stages of their growth cycle. Molting is the biological phenomenon that allows insects - and all arthropods - to renew their exoskeleton to grow [1,7,8]. Between the successive molts of this process, the biosynthesis of chitin takes place, which is part of the composition of the cuticles of arthropods. The chitin thus produced is composed of a chain of *N*-acetylglucosamine monomers linked together

by β 1–4 glycosidic bonds. It constitutes the second most abundant polymer in nature after cellulose. It is present in different taxonomic groups such as fungi, algae, and molluscs but especially arthropods which have more than a billion species recorded for the entire animal kingdom [9,10]. However, chitin prepared in laboratories or produced industrially is most often extracted from the exoskeletons of shrimp, crabs or mushrooms [11,12]. Chitin can then be transformed by *N*-deacetylation reaction to a high value-added polymer; chitosan. In the majority of studies, these polymers do not present an ideal structure in which all chitin monomers will be acetylated and all chitosan monomers will be deacetylated. Chitin and chitosan are distinguished by the degree of acetylation (DA) which reflects the relative proportion of *N*-acetylglucosamine monomers present in the copolymer. When the DA is >50 % the copolymer is practically insoluble in dilute acid solutions and

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<https://doi.org/10.1016/j.ijbiomac.2024.134475>

Received 12 June 2024; Received in revised form 16 July 2024; Accepted 2 August 2024

Available online 3 August 2024

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corresponds to chitin. The more the DA is lower than 50 %, the more soluble the copolymer is in dilute acid solutions and the name chitosan is attributed to it [11,13].

The interest given to these polymers, both at a scientific and economic level, is strongly linked to their chemical, biological, and physical properties which allow several applications in different fields such as cosmetics, biomedical, and water treatment... [11,14–18]. Chitosan is also used in the form of composites in several applications [19–24].

In 2017, global demand for chitinous derivatives was approximately 75,000 T, while the production did not exceed 40,000 T. This production does not currently make it possible to meet manufacturers' needs in terms of quality and quantity. In this context, it is important to explore new potential sources of chitin and chitosan to meet the needs of the market which is growing at 16 % per year [25].

This work is a continuation of our recent work on the exploration of insects as chitinous sources [26,27]. This is a study of the valorization of waste from the breeding of the insect *Tenebrio Molitor* (TM) by the production of chitin and chitosan with controlled characteristics. This insect, which belongs to the order Coleoptera, follows a life cycle characterized by complete metamorphosis, also called holometabolous [28]. This cycle consists of four distinct stages: egg, larva, pupa and adult. Females lay around 300 eggs during their life [29], from these eggs small larvae emerge which develop through several larval stages accompanied by successive molts (between 8 and 20 molts). The duration of these stages varies from four weeks to several months, depending on ambient humidity and temperature [30] and the larvae produced, commonly called mealworms, are particularly used as a source of protein in animal feed. These larvae enter the pupal stage, during which they undergo significant morphological changes. The nymph is a resting stage during which the insect transforms into an adult. This stage is of particular importance; it is distinguished from previous stages by the development of a cuticle composed mainly of chitin. This cuticle gradually hardens and darkens and gives the insect increased resistance, unlike the larval stages where the cuticle is quite flexible to allow rapid growth of the insect.

To carry out this study we collected during the breeding, the exuviae of the larval stages which we divided into two groups according to their

age (LE1 and LE2) and the adults of *Tenebrio Molitor* (ATM) (Fig. 1). This work was aimed to valorize breeding rejects from this insect by preparing chitin using process P1 and developing an optimized process P2 to provide pure chitin with improved yields. The extraction products are subjected to *N*-deacetylation reactions with KOH in a mixture of ethanol and monoethylene glycol (Broussignac process) [31] or with NaOH in an aqueous solution (Kurita process) [32]. The prepared chitin and chitosan were characterized by different spectroscopic and analytical methods (FTIR, XRD, TGA/DTG, SEM/EDX, viscometry, potentiometric titration and ^1H NMR). The physicochemical characteristics of the prepared chitin and chitosan were presented, discussed, and compared to those cited in the literature for TM [33–37] and to the results obtained for the larval exuviae and adult of the black soldier fly (BSF) [26]. The results obtained were also compared, if necessary, to the physicochemical characteristics of α and β chitin prepared respectively from the exoskeleton of the grey shrimp and the squid pens according to the reaction conditions described in our previous work carried out from crustaceans and cephalopods [38–42].

2. Experimental

2.1. Insect material

The different types of larval exuviae of *Tenebrio molitor* used include exuviae from larvae aged 25 to 35 days (LE1), exuviae from larvae aged over 60 days (LE2), and adult carcasses (ATM). These waste materials were collected from a mass rearing facility at the Laboratory of Entomology, Hassan II Agronomic and Veterinary Institute, Agadir Horticultural Complex, Morocco (X = 5,182,140.855, Y = -1,349,412.234). The strain was provided by the International Foundation for Wildlife Conservation in April 2019. The rearing was maintained in smooth plastic food containers to prevent insects from climbing. During the larval and adult stages, insects were fed the same diet consisting of 60 % wheat bran and 40 % compound poultry feed (17.3 % Protein, 11.5 % Moisture, 6.7 % Cellulose, 5.3 % Minerals, 4.3 % Lipids, 0.44 % Calcium, and 0.48 % Phosphorus). Fresh potato pieces were distributed every 4 days as a water source. Rearing environmental conditions were

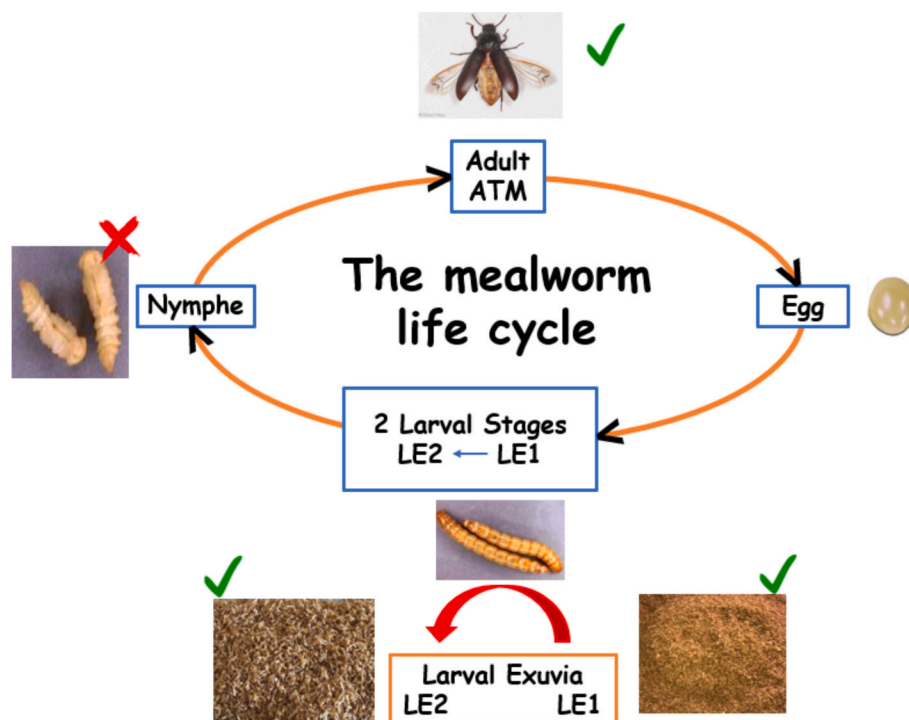


Fig. 1. The mealworm life cycle and its different stages utilized in this study: larval exuviae (LE1 and LE2) and adult *Tenebrio molitor* (ATM).

maintained at a temperature of 27 ± 1 °C, relative humidity of 70 ± 5 %, and total darkness except during handling, with optimal ventilation. The exuviae were manually separated from the rearing substrate, as they had a very low density which allowed the exuviae to form a layer on the surface of the boxes. The adults used were those that had completed their cycle and had died naturally. The samples were stored at room temperature until they are used in the present trial.

The raw material composed of the LE1, LE2 larval exuviae and the ATM was washed, dried, crushed and sieved to homogenize the size so that it is neither too large (difficult access to the reaction sites) nor too small (to avoid losses during treatments). The diameter of the treated material was between 1 and 2 mm.

2.2. Extraction of chitin samples and development of reaction conditions

In this work, chitin extraction is carried out using two different processes where deproteinization constitutes the main step.

In the first process (P1) recently developed for chitin extraction from BSF [26], deproteinization is carried out with alkaline baths of 1 M NaOH at 80 °C. It is preceded by two steps of demineralization and decolorization. Demineralization uses 0.55 M HCl acid baths at room temperature while decolorization is carried out with a mixture of 33 % $\text{H}_2\text{O}_2/\text{NaHCO}_3$: 9/1 by mass.

In the second process (P2) deproteinization is carried out with alkaline baths of 1 M NaOH at 80 °C is preceded by a delipidation step using a mixture of chloroform: methanol solvents (2:1 v/v) according to the Folch method [43,44]. This process is developed in order to optimize the process, improve chitin contents and study the effectiveness of the removal of impurities by the delipidation test and its relationship with the surface structure of chitin.

For comparison purposes, we also prepared chitin from the exoskeleton of grey shrimp and squid pens under the same conditions described in our previous work [38,42] using repeated baths of 0.55 M HCl for demineralization and 0.3 M NaOH for deproteinization.

Each bath is carried out in a raw material/solution ratio: 1/10 (w/v). The number of baths during the demineralization and deproteinization steps is determined by monitoring the reactions according to the extraction principle described in the literature [38,42]. When the pH of the last demineralization bath remains acidic, this indicates that the minerals are eliminated. The deproteinization reaction is stopped when the supernatant from the last bath remains colorless.

The chitin content is expressed as a percentage of the mass of the extracted product relative to the starting mass of the raw material.

2.3. Preparation of chitosan

Chitosan samples are prepared using two processes: the Broussignac process using a glyco-potassium mixture composed of KOH (50 %), ethanol (25 %), and monoethylene glycol (25 %), at a temperature of 120 °C, the duration varies according to the source [31]. The Kurita process using a NaOH solution (50 %), at a temperature of 120 °C, once or several times for the same duration [32].

2.4. Physico-chemical characterization of chitin and chitosan

2.4.1. IR spectroscopy

The FT-IR spectrophotometer was Jasco FT-IR 4600 (Jasco, Tokyo, Japan). A Gemini sampling accessory was used to attenuate total reflectance (ATR) spectra using a standard ZnSe crystal (incidence angle = 45°). The chitin is pressed with a Minigrip device to ensure uniform contact between the samples and the ATR crystal.

2.4.2. X-ray diffraction (XRD)

The spectra were recorded using a Rigaku III diffractometer (Rigaku Corp., Tokyo, Japan) with Cu radiation (40 kV, 30 mA). Data was collected at a scan rate of 1 min^{-1} with a scan angle of 5° to 80°.

The crystallinity index I_{cr} is calculated by the area method described in the literature [13,45,46] according to the Eq. (I):

$$I_{cr} = \frac{S_c}{S_t} \times 100 \quad (I)$$

where S_c represents the area of the crystal domain and S_t the area of the total surface, respectively after correction of the baseline for amorphous regions and after correction of the general baseline [45].

2.4.3. Thermogravimetric analysis

The thermogravimetric analysis (TGA) was obtained using a TG-ATD 92-16 type thermal analyzer (Setaram, France), in a platinum crucible ranging from 30 to 500 °C at $10 \text{ °C} \cdot \text{min}^{-1}$.

2.4.4. Scanning electron microscopy (SEM)

A scanning electron microscope (TESCAN VEGA 3) (Topcon Corp., Tokyo, Japan) was used to examine the microscopic structure of chitin and chitosan samples. The samples were fixed on an SEM sample holder and covered with a thin layer of carbon by a sputtering machine (JBS E5150) for conductivity. SEM images at different magnifications (20, 50 and 100 μm) are captured to examine the surface morphology of chitin.

2.4.5. Energy dispersive X-ray spectroscopy (EDX)

An energy-dispersive X-ray spectroscope (TESCAN VEGA 3) was used to identify the elemental composition of the samples. The device was connected to the Scanning Electron Microscope to allow the collection of elementary data. Startup, analysis and reporting are done automatically using TEAM™ software. EDX spectra of several regions of the chitin sample are acquired to obtain representative elemental composition data.

2.4.6. ^1H NMR spectroscopy

^1H NMR spectra were obtained using a Bruker AC300 spectrometer (Bruker, France). The samples were dissolved at a concentration of 10 $\text{mg} \cdot \text{mL}^{-1}$ in D_2O in the presence of HCl (pH 4). The prepared chitosan solution is stirred continuously for a minimum of 24 h. The spectra were carried out at a temperature of 80 °C. The value of the degree of acetylation (DA) was determined from the integral of the $-\text{CH}_3$ signal at 1.97 ppm compared to the integral of the protons H_1 and $\text{H}_{1'}$ around 4.4 up to 5 ppm considered as internal standard [47] according to the Eq. (II):

$$DA (\%) = \frac{\left(\frac{I[\text{CH}_3]}{3}\right)}{I[\text{H}_1] + I[\text{H}_{1'}]} \times 100 \quad (II)$$

where I is the integral of each peak, H_1 represents the proton of the glucosamine unit, and $\text{H}_{1'}$ the proton of the *N*-acetylglucosamine unit.

2.4.7. Viscometry

The viscosity was measured using an Ubbelohde capillary viscometer ($0.5\text{--}3 \text{ mm}^2 \cdot \text{s}^{-1}$, 15–20 mL) at 25.0 ± 0.1 °C. The solvent used is DMAC/LiCl 5 % for chitin [48] and 0.3 M acetic acid/0.2 M sodium acetate mixture for chitosan [49]. The viscometric molar mass was calculated using the Mark Houwink (Eq. (III)) [50]. The Huggins constant k' is calculated from the reduced viscosity curves to evaluate the polymer/solvent interactions.

$$\eta = K M v^a \quad (III)$$

$[\eta]$ is the intrinsic viscosity expressed in $\text{mL} \cdot \text{g}^{-1}$ and Mv is the viscometric molar mass in $\text{g} \cdot \text{mol}^{-1}$.

K and a are constants that depend on the solvent and the temperature. They are equal to:

$K = 0.24$ and $a = 0.69$ for chitin when the intrinsic viscosity is expressed in $\text{mL} \cdot \text{g}^{-1}$ [48].

$K = 0.078$ and $a = 0.76$ for chitosan when the intrinsic viscosity is expressed in $\text{mL} \cdot \text{g}^{-1}$ [51].

2.4.8. Solubility of chitosan

Solubility tests were performed on 10 mg of chitosan sample in 2 mL of 3 % acetic acid (AcOH) solution at room temperature, with a continuous magnetic stirring, from 15 to 60 min according to samples. Solubility is visually estimated.

3. Results and discussion

3.1.1. Extraction of chitin samples using the process P1

The extraction of chitin from the LE1 and LE2 larval exuviae and the ATM adult stage is carried out according to a process that we described in the study recently published on the valorization of rejects from the breeding of the black soldier fly BSF through the preparation of chitin and chitosan [26]. This process is developed following numerous tests performed simultaneously on the BSF and TM. It consists of carrying out a demineralization step of the raw material using two baths of 0.55 M HCl at room temperature before a decolorization step using an H₂O₂/NaHCO₃ mixture: 9/1 by mass for 1 h at room temperature. ambient. The resulting product is then subjected to deproteinization using 1 M NaOH at a temperature of 80 °C. Although the mineral content is low in insects [52], we carried out a demineralization step to avoid the strong effervescence observed during decolorization with the presence of minerals following their reaction with the oxidant (H₂O₂) and the base (NaHCO₃). The order adopted in this process makes it possible to reduce significantly the number of deproteinization baths to 4 or 5 baths depending on the growth stages and to use a NaOH concentration of 1 M instead of 2 M [26].

The reaction conditions retained as well as the chitin contents extracted at the different stages of the TM growth cycle are presented in Table 1. They are also compared to the results of work cited in the literature mainly concerning adults [33–37] since the extraction of chitin at the larval stage is carried out in most works from the larva [34,36,37,53,54] and rarely from the exuviae [33,37].

The chitin contents that we obtained are high (>20 %) and the ATM adult presents the highest value (25 %), unlike the results obtained for the BSF where the chitin content increases progressively as the stage of the life cycle advances and drop at the adult stage (13 %). This is explained by the fact that the ATM is a beetle which has a rigid

sclerotized cuticle thanks to the elytra developed during the nymph stage to protect the wings while the black soldier adult is a dipteran which has two wings without elytra and a not very rigid exoskeleton.

It is important to note that these chitin contents are significantly higher than the values cited in the literature for exuviae and adults [33,34,37,53–55] even considering, as we will show later, that the chitins of the larval exuviae extracted with a content of around 20 and 21 % present traces of organic impurities. This was also the case for the BSF compared to other works [26]. This, in our opinion, is related to the process that uses moderate conditions and an order of treatments of demineralization, decolorization, and deproteinization steps.

In other works (Table 1), all the chitin contents cited are lower than those we obtained. For adults, for example, the values vary between 4.2 % and 14.6 % instead of 25 % in our study. In this work, demineralization is carried out using HCl treatments of 0.85 M to 2 M at temperatures varying from ambient to 60 °C for durations of 30 min to 24 h. These conditions are sometimes too severe and excessive to eliminate the few minerals present in this insect. As for the deproteinization generally carried out at 50 to 100 °C, a single continuous treatment is generally used with a NaOH solution varying from 1 M to 3 M for durations which can reach 24 h. Only the work by Khatami *et al.* [37] uses two 8-hour cycles with 1 M NaOH but the chitin contents are not cited. It is shown that the conditions of HCl and NaOH concentrations, temperature, reaction time, treatment whether it is continuous or in several stages as well as other factors could affect the quality of the chitin and the overall yield of the extraction [42,56].

3.1.2. Characterization of chitin extracted according to P1

The prepared chitin samples were characterized by IR spectroscopy (Fig. 2). The observed spectra show the characteristic bands of chitin, in particular the OH, NH, CH₃ sym, CH₃ asym, and CH₂ asym groups and those of Amides I and II (Fig. 2 A). However, these bands appear at different wavelengths depending on whether the origin of the chitin is the adult or the larval exuviae. For chitin extracted from the adult stage, the spectra showed the stretching bands of the OH groups at 3433 cm⁻¹. The deformation of CH₃ sym appears at 2875 cm⁻¹ and that of CH₃ asym and CH₂ asym, appears at 2933 cm⁻¹. These values are comparable to those observed for α chitin from adult BSF and crustacean chitin. As for the chitin from LE1 and LE2, the wavelengths observed for these bands are visibly shifted; the stretching bands of the OH groups appear at 3429 cm⁻¹ and the deformation of CH₃ sym appears at 2853 cm⁻¹ while that

Table 1

Retained conditions, the content of chitin in the different stages studied of the TM (LE1, LE2, and ATM) and comparison with other studies. DEP-DEM: deproteinization is carried out before demineralization DEM-DEP: demineralization is carried out before deproteinization.

Studies	Stages	Demineralization	Decolorization	Delipidation	Deproteinization	Chitin content %
This work	LE1	0.55 M HCl	H ₂ O ₂ /NaHCO ₃	-	1 M NaOH	20
	LE2	Room temperature 2 baths (15 min and 30 min)	Room temperature 30 min	-	T 80 °C	21
	Adult TM			-	4–5 baths 45 min	25
Y. Song <i>et al</i> 2018 [33]	LE	2 M HCl	-	-	5 % NaOH (1.2 M)	18.01
	Adult TM	T 20 °C 3 h	-	-	T 95 °C 3 h	4.92
C. Shin <i>et al</i> 2019 [34]	Adult TM	7 % HCl (0.85 M) Room temperature 24 h	-	-	10%NaOH (2.5 M) T 80 °C 24 h	8.40 ± 0.05
	Adult TM	2 M HCl T 50 °C 24 h	-	-	2 M NaOH T 50 °C 24 h	11.6
A. Mendoza <i>et al</i> 2020 [35]	Adult TM	7 % HCl (0.85 M) T 25 °C 24 h	Methanol/chloroform/H ₂ O ₂ (1/2/4)	-	10 % NaOH (2.5 M) T 80 °C 24 h	10.9
	DEP-DEM	24 h				
M. Chalghaf <i>et al</i> 2023 [36]	Adult TM (1)	1 M HCl 60 °C 30 min	Methanol/chloroform/H ₂ O ₂ (1/2/4)	-	3 M NaOH T 80 °C 2 h	14.6
	Adult TM (2)					
	DEM-DEP					
N. Khatami <i>et al</i> 2024 [37]	LE (molt)	-	Ethanol 96 %/ acetone/ diethyl ether for 1 day in each solution	-	1 M NaOH	-
	Adult TM	-		-	T 100 °C 2 cycles of 8 h	-

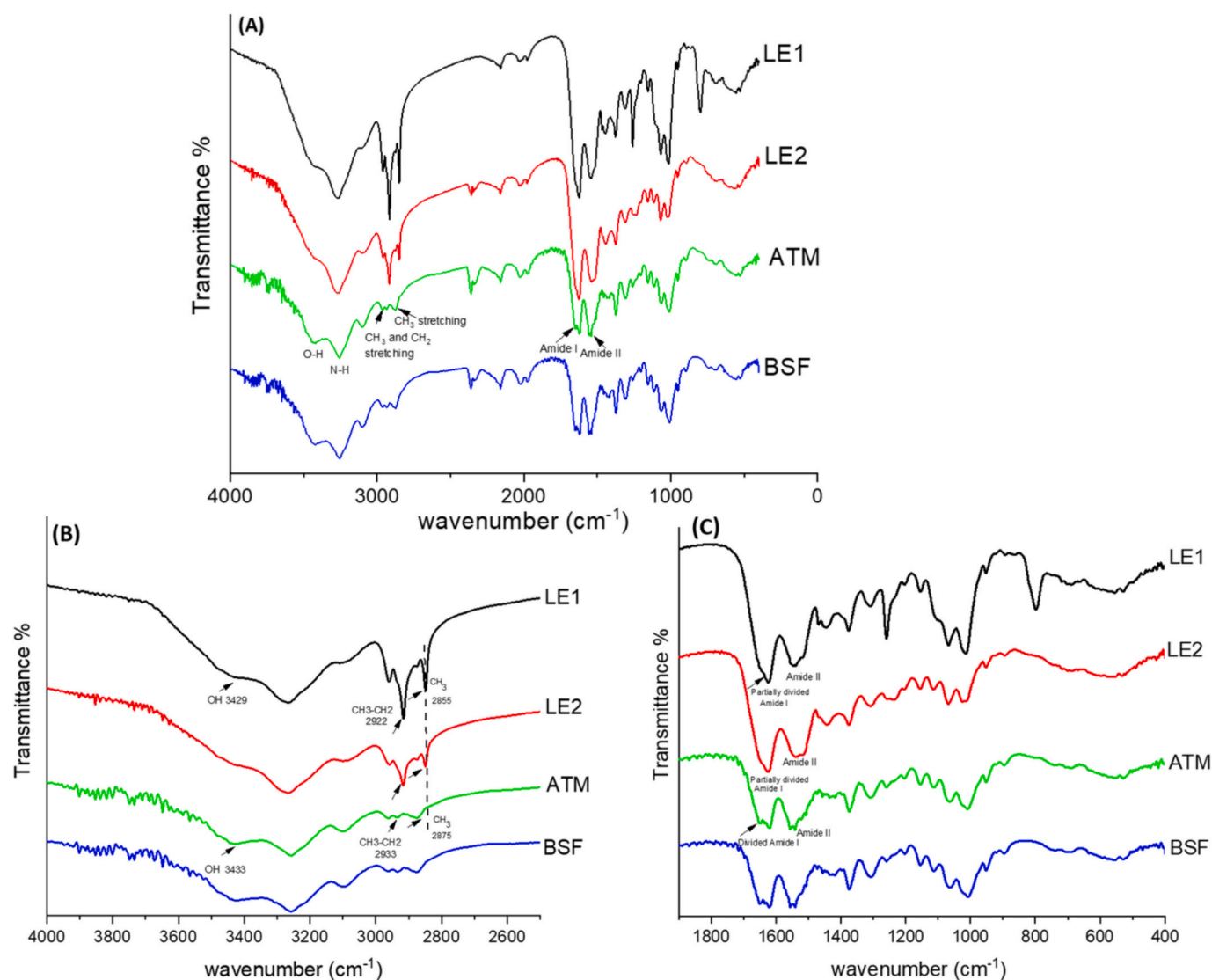


Fig. 2. FTIR spectra of all chitin samples extracted from ATM (A), the characteristics bands OH, CH₃ *sym* and CH₂ *asym* (B) and the amide bands (C) compared to the pure α chitin of BSF.

of CH₃ *sym* and CH₂ *asym* appears at 2922 cm⁻¹ for the two larval stages (Table 2). We found that the intensity of the CH₃ *sym* and CH₃ *sym* and CH₂ *asym* bands for the chitin of the larval exuviae is very marked compared to the intensity presented by the chitin of the adult (Fig. 2 B). This would be linked to the presence of impurities of an organic nature identified in the larval exuviae of the BSF [32] which are responsible for the observed shifts in the wavelengths and the intensity of the CH₃ *sym* and CH₂ *asym* bands.

At the amide zone, the amide II band appears at 1552 cm⁻¹ for the

ATM adult stage, while it appears at 1549 cm⁻¹ for LE1 and LE2. At the amide I level, the two bands appear respectively at 1621 cm⁻¹ and 1652 cm⁻¹ for all stages. A marked division appears particularly at the adult ATM (Fig. 2 C).

The analysis of these results compared to literature data about the three structures α , β and γ highlights that the chitin of ATM presents an α structure (Table 2). Shifts in wavelengths towards the characteristic values of the γ structure [57] are observed for the OH, CH₃ *sym*, CH₃ *sym* and CH₂ *asym* groups and amide II for the chitin from LE1 and LE2. In

Table 2

The main functional groups of the chitin samples extracted from ATM, compared to α [26], β [41] and γ chitin [57].

Functional group and vibration modes	Classification	LE1	LE2	ATM	α chitin Elkadaoui et al [26]	β chitin Arrouze et al [41]	γ chitin Kaya et al [57]
O-H stretching	-	3429	3429	3433	3433	-	3430
CH ₃ <i>sym</i> . Stretch and CH ₃ <i>sym</i> . Stretch	Aliphatic compounds	2922	2922	2933	2933	2929	2918
CH ₂ <i>asym</i> . Stretch	Aliphatic compounds	2853	2853	2875	2875	2876	2850
C=O secondary amide stretch	Amide I	1652	1652	1652	1652	1640	1652
N-H bend, C-N stretch	Amide II	1549	1549	1552	1552	1552	1549

the study we carried out on the BSF [26], similar shifts were also observed for the chitin extracted from larval exuviae. We have shown that they are linked to the presence of organic impurities rather than to the γ structure [26].

The chitin samples were also characterized by XRD. The spectra show the two main crystal peaks characteristic of chitins. The first appears at 2θ between 9.21° and 9.37° and the second peak appears at 2θ between 19.09° and 19.39° . The spectra of chitin also show four minor crystal peaks at the 2θ zone between 12° and 26° . The 2θ values obtained for the main crystal peaks for ATM chitin are comparable to the values for α chitin [40] and significantly different from β chitin [41]. As for the 2θ values of chitin from LE1 and LE2 (Fig. 3), they are slightly shifted compared to ATM chitin. These results are similar to those we found for partially impure chitin in larval exuviae of BSF [26].

The crystallinity indexes of chitin samples were also determined using the area method [45,46]. The differences observed are not enormous, the calculated values are 73 % for LE1, 67 % for LE2, and 63 % for ATM.

The percentage of impurities observed in the case of chitin from larval exuviae was calculated using thermogravimetric analysis. These impurities degrade at T_{max} of 485°C with a mass loss percentage of 8.5 % for CH LE1 P1 and 4.7 % for CH LE2 P1.

The images provided by the SEM analysis at 10 and $20\ \mu\text{m}$ magnifications show that the chitin preserves the surface structure of the raw material, this was also observed for BSF [26]. The chitin from larval exuviae has a mainly fibrous surface structure with the presence of a few pores, while the adult chitin shows a compact and organized honeycomb surface structure. The images of CH LE1 P1 and CH LE2 P2 mainly show the presence of impurities in the form of granules and sheets indicated by arrows (Fig. 4). This was also observed for the chitin from BSF larval exuviae except that they have a porous (and not filamentous) structure. This porous structure would have been responsible for the persistence of impurities despite the application of several delipidation tests [26]. The structure of chitin from LE1 and LE2 of TM being filamentous led us to examine their reaction to delipidation.

EDX analysis of chitin from LE1, LE2, and ATM shows respective carbon percentages of 83 %, 78 %, and 65 %. This high percentage in the case of exuviae can be explained by the presence of carbon corresponding to the hydrocarbon chain of lipids (impurities) in addition to the carbon constituting chitin.

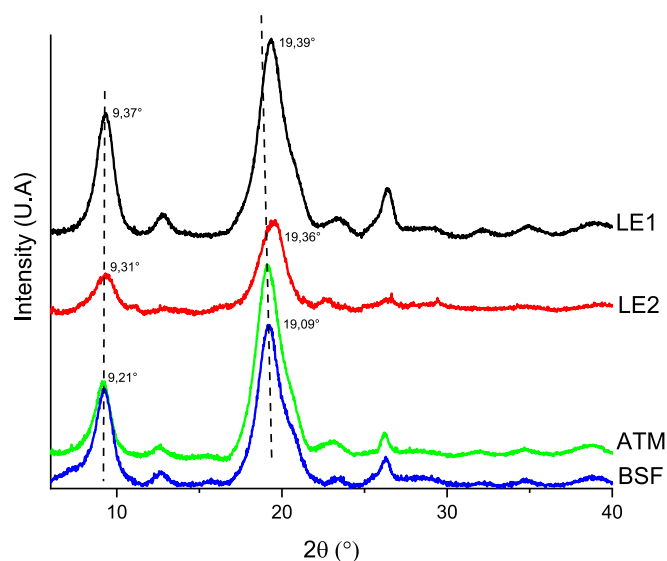


Fig. 3. X-ray diffractograms of the different chitin samples extracted from the different stages of the TM life cycle compared to the pure α chitin of BSF.

3.1.3. Delipidation test on chitin samples extracted using P1

The CH LE1 P1 and CH LE2 P1 are subjected to delipidation with the methanol/chloroform mixture (2:1, v/v) at room temperature for 24 h. The characterization of the resulting chitin samples by FTIR and XRD shows clearly that they are pure and present the same characteristics as the α chitin of ATM. The intensities of the CH_3 *sym*, CH_3 *asym*, and CH_2 *asym* bands are reduced and the shifts in the wavelength values as well as the 2θ values are not observed. The values of these characteristics become different from the values presented by chitin of physical structure γ [57] and completely comparable to the values of the α chitin of ATM and these of crustaceans (Fig. 5).

This result is particularly interesting and confirms that the differences in the physicochemical characteristics observed for the chitin from the larval exuviae of BSF and TM are linked to organic impurities rather than to the physical structure; the conclusion that had been formulated for BSF but which remained partly hypothetical since it was deduced following the study of corresponding chitosan subjected to a purification step [26] and not following the direct purification of chitin by delipidation. Indeed, the chitin delipidation tests from BSF larval exuviae were not effective. The efficiency of the delipidation of CH LE1 P1 and CH LE2 P1 would be linked to the filamentous surface structure which facilitates the elimination of lipids compared to the porous surface structure of the chitin from larval exuviae of BSF which would be at the origin of their retention. This result also led us to design a second process P2 to optimize the extraction of chitin according to the first process P1 from larval exuviae and the adult as we presented (Table 1).

3.1.4. Optimization of chitin extraction and development of the process P2

The protein content in insects is significantly higher than their mineral content [7,52,58,59]. We envisaged that deproteinization would be the main step in chitin extraction. In the first process, demineralization was only carried out to avoid the strong effervescence observed during decolorization [26]. It would be unnecessary to proceed with this step since the low mineral content could be eliminated during deproteinization by alkaline treatments as has been proven in work applied to squid pens [60] and more recently to *Tenebrio molitor* [37]. Also in the first process, deproteinization is preceded by decolorization with $\text{H}_2\text{O}_2/\text{NaHCO}_3$ to reduce both the concentration and the number of baths of the NaOH solution. We estimated that delipidation could also eliminate pigments [61]. The proposed second process P2 consists of carrying out a first step of delipidation with methanol/chloroform (2:1, v/v) followed by a deproteinization step using 1 M NaOH at a temperature of 80°C .

The application of these extraction conditions to LE1, LE2 and ATM allowed to obtain pure chitin samples as confirmed by FTIR, XRD, and SEM (Fig. 6). Indeed, the FTIR spectra of CH LE1 P2 and CH LE2 P2 (Fig. 6A) show that the intensities of the CH_3 *sym* and CH_3 *asym* and CH_2 *asym* bands are reduced on the one hand and that all the bands characteristic of chitin appear at wavelengths characteristics of pure α chitin on the other hand, unlike CH LE1 P1 and CH LE2 P1 prepared according to the first process (Fig. 2A). Also, the comparison of the diffractograms of the chitin extracted according to the two processes (Fig. 6B) shows that the 2θ values of the main crystal peaks of CH LE1 P2 and CH LE2 P2 are similar to the values of pure ATM chitin extracted according to the two processes and no shift is observed as is the case for CH LE1 P1 and CH LE2 P1 (Fig. 2B). The images of the SEM analysis at different magnifications of the chitin from the larval exuviae extracted according to the second process P2 demonstrate their purity in the CH LE1 P2, unlike the chitin samples provided by the first P1 process (Fig. 6C). These images also show that CH LE1 P2 maintains a fibrous surface structure observed for CH LE1 P1 and CH LE2 P1.

Therefore, this second process P2 allowed the extraction of pure chitin from the different stages of the life cycle of TM using only 2 steps instead of 3 steps for the first process P1. In the work of Khatami *et al* [37] the extraction carried out using deproteinization followed by decolorization provides impure chitin, as observed from the shifts in the

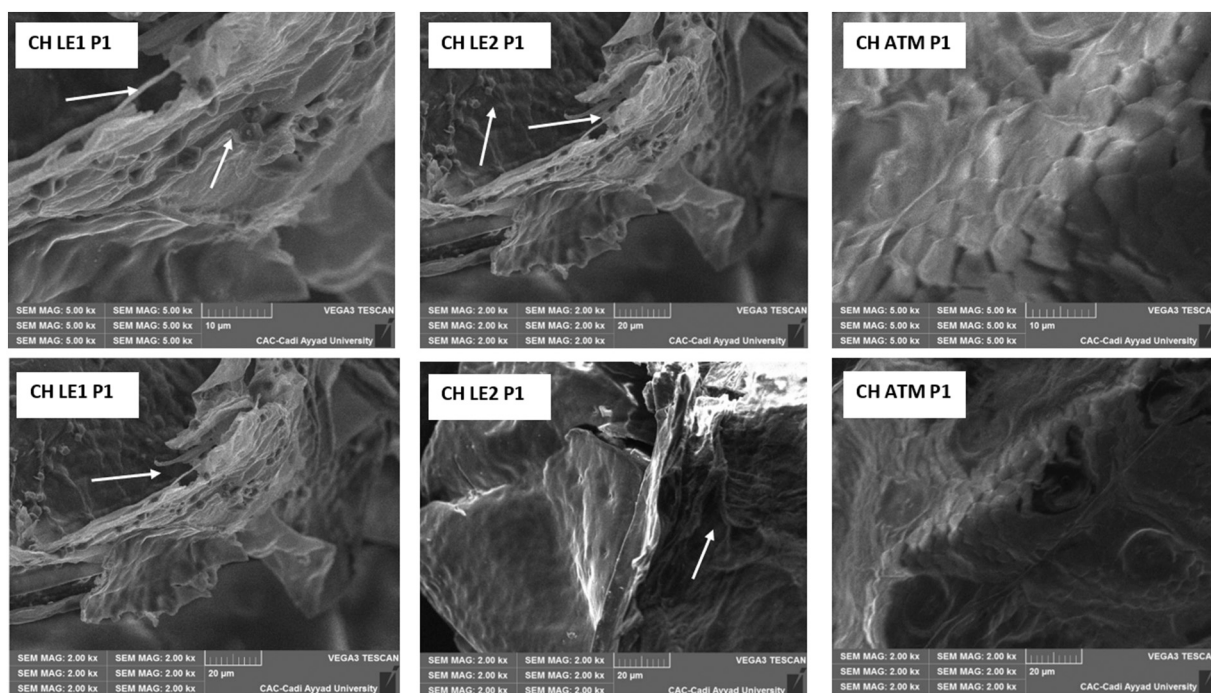


Fig. 4. SEM of CH LE1 P1, CH LE2 P1 and CH ATM P1 extracted from the studied stages CH: chitin.

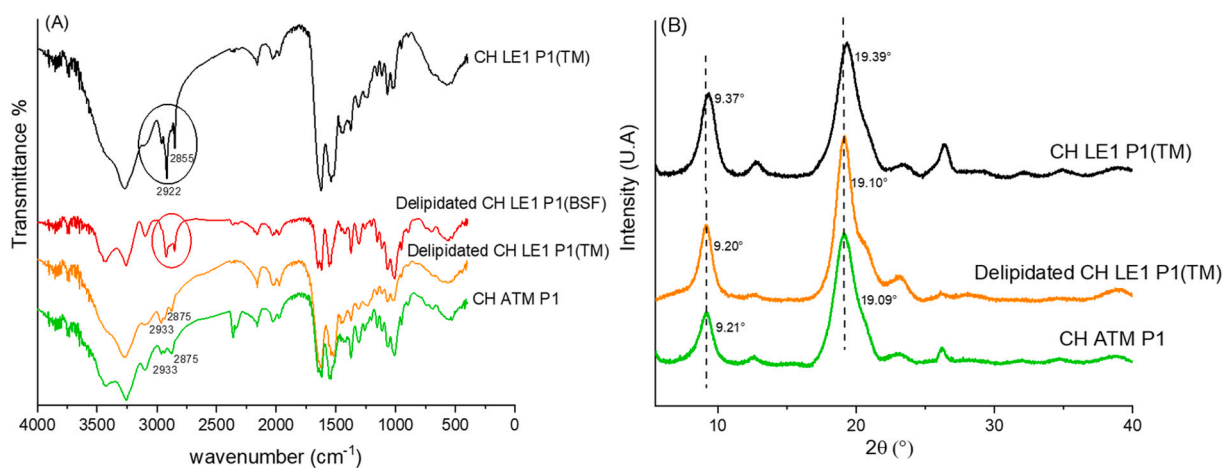


Fig. 5. FTIR (A) and XRD (B) of chitin LE1 with and without delipidation CH LE1, compared to delipidated chitin CH LE1 BSF and CH ATM.

wavelength values (FTIR) and chemical shifts (NMR).

This Process P2 requires a delipidation treatment for CH LE1 P1 and CH LE2 P1 to obtain pure chitin samples and improve the yield of the extraction by 20 to 35 %, which is carried out without using either acid (HCl) or oxidant (H₂O₂) and by reducing the losses accompanying the treatments between the different stages. The comparison of the reaction conditions of the two processes studied, the chitin contents obtained, and their characteristics concerning purity, viscosity, and crystallinity are presented in Table 3.

3.2. Preparation and characterization of chitosan samples

The *N*-deacetylation of chitin is frequently carried out with NaOH in aqueous solution at high temperatures for variable times (Kurita process) [32] and rarely with KOH in anhydrous medium at 120 °C (Broussignac process) [31]. It is shown that the Kurita process is rather effective in the case of β chitin than in the case of α chitins. It is also shown that the Broussignac process has the advantage of providing

good quality chitosan in a single, short-term step with relatively high molar masses [42,62].

To our knowledge, the work on the preparation of chitosan from TM, cited in the literature, used only NaOH in aqueous solution with concentrations varying from 50 % to 60 %, temperatures between 90 °C and 150 °C and durations of 2 h to 9 h, the DA of the resulting chitosan samples present DA values that vary between 19 % and 50 % [33–37]. The chitosan samples from recent work by Chalhaf *et al* and Khatami *et al* have a minimal value of 27 % and 19 % respectively [36,37].

3.2.1. *N*-deacetylation of chitin samples and assessment of their reactivity

Monitoring the deacetylation of CH LE1 P1, CH LE2 P1 and CH ATM P1 under the conditions of the Broussignac process (KOH 50 %, ethanol 25 %, monoethylene glycol 25 %, at T 120 °C) shows a reaction behavior that differs depending on the stage of the life cycle. For CH LE1 P1 and CH LE2 P1, the solubility in 3 % acetic acid of the reaction product is only observed after 24 h of reaction while the deacetylation of CH ATM P1 is significantly faster, and obtaining a completely soluble product

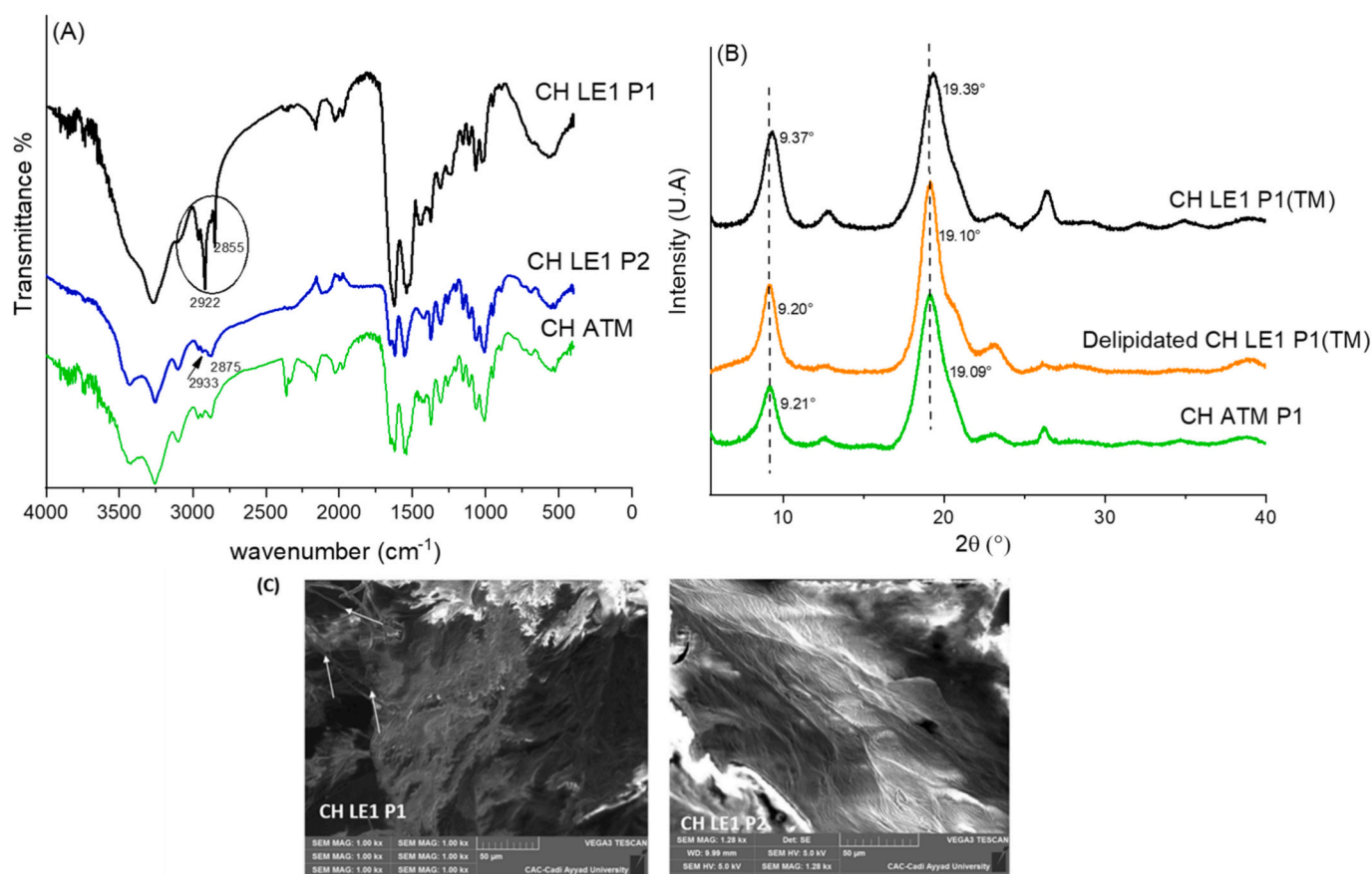


Fig. 6. FTIR, XRD and SEM of chitin LE1 extracted using the process P2 (CH LE1 P2) compared to LE1 without delipidation (CH LE1 P1).

Table 3

Comparison of the characteristics of chitin extracted from rejects at the different stages of the life cycle of TM according to two processes P1 and P2.

Process	Stage	Demineralization	Decolorization	Delipidation	Deproteinization	Chitin content %	Impurities% (determined by TGA)	Mv g.mol ⁻¹	I _{cr} %
Process 1 P1	LE1	0.55 M HCl	H ₂ O ₂ /NaHCO ₃	-	1 M NaOH	20	8.5	insoluble	73
	LE2	Room temperature 2 baths (15 min and 30 min)	Room temperature 30 min	-	T 80 °C	21	4.7	insoluble	67
	ATM				4-5 baths 45 min	25	Pure	1,751,000	63
Process 2 P2	LE1	-	-	Methanol/chloroform(2/1)	1 M NaOH	27	Pure	-	72
	LE2				T 80 °C	27.5	Pure	1,861,000	67
	ATM				4-5 baths 45 min	30	Pure	1,913,000	61

requires only 4 h of reaction. The reaction behavior of these chitin samples is generally comparable to that observed for chitin extracted using the same process at different stages of BSF [26].

As for the chitin extracted according to P2, a soluble product is obtained from CH ATM P2 after 4 h as in the case of CH ATM P1. However, the reaction behavior of CH LE1 P2 and CH LE2 P2 is significantly different from that observed for the chitin extracted from exuviae using P1; a completely soluble product is obtained after only 6 h of reaction instead of 24 h.

Following these deacetylation tests, we applied the standard Brousignac process (24 h of reaction) [31] to the extracted chitin samples to examine the purity of the resulting products and compare their physicochemical characteristics. The FTIR spectra (Fig. 7) show that all chitosan samples prepared from the different extracted chitin according to P2 as well as the chitosan prepared from CH ATM P1 are pure. Only the spectra of chitosan corresponding to CH LE1 P1 and CH LE2 P1 show the persistence of organic impurities. The intensities of the CH₃ *sym* and CH₂

asym bands appear very marked compared to pure chitosan. When an additional delipidation step is applied to these chitin samples, the intensities of these bands of their corresponding chitosan appear reduced like the other prepared chitosan, which confirms their purity.

In addition, when these partially impure chitin samples (CH LE1 P1 and CH LE2 P1) are delipidated, their deacetylation provides a fully soluble product only after 6 h of reaction instead of 24 h. This indicates that the insoluble part observed in the first case was not linked to the progress of chitin deacetylation but to the presence of organic impurities. This is comparable to the reaction behavior of partially impure chitin from larval exuviae of BSF upon deacetylation [26]. Only in this case, the delipidation was ineffective and obtaining pure chitosan requires an additional step of purification of the deacetylation product, unlike the chitin from the larval exuviae of TM where the efficiency of the delipidation makes it possible to obtain pure chitosan from 6 h.

All the chitosan samples obtained after 24 h of reaction preserve, according to SEM analysis, the surface structure presented by the

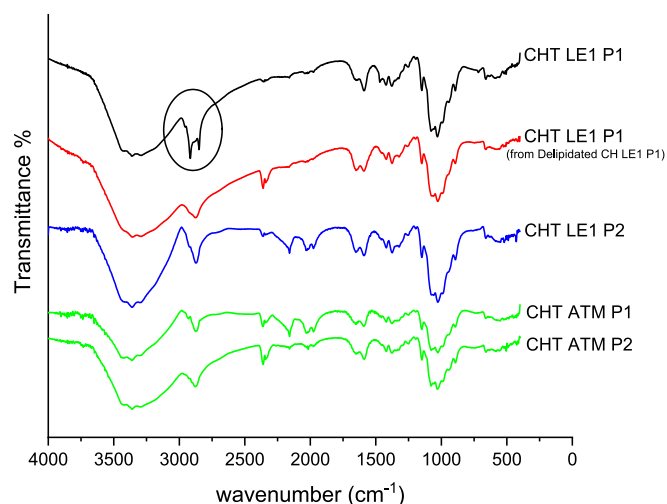


Fig. 7. Comparison of FTIR spectra of chitosan prepared from TM according to P1 and P2.

original chitin. These chitosan samples are highly deacetylated (DA between 6 % to 14 %) and have molar masses that vary depending on the starting chitin between 83,000 to 153,000 $\text{g}\cdot\text{mol}^{-1}$. The obtention of pure chitosan from CH LE1 P1 and CH LE2 P1 requires a delipidation step. The characteristics of the prepared chitosan samples are summarized in Table 4 and examples of ^1H NMR spectra are presented in Fig. 8.

When the chitin samples extracted according to the process P1 are subjected to *N*-deacetylation reactions with 50 % NaOH in an aqueous solution at 120 °C, the behavior described for α chitins, in general, is observed and the results obtained are comparable to those observed for larval exuviae and adult BSF extracted using the process P1 [26].

Indeed, monitoring the deacetylation of chitin from CH LE1 P1 and CH LE2 P1 shows that the solubility of the reaction products is very low even after 24 h. As for pure adult chitin, deacetylation provides a completely soluble product after 10 h of reaction instead of 4 h and the chitosan obtained after 24 h has a DA comparable to that obtained in the case of the Broussignac process except that the molar mass is significantly lower. It is of the order of 90,000 $\text{g}\cdot\text{mol}^{-1}$ instead of 137,000 $\text{g}\cdot\text{mol}^{-1}$.

The introduction of the factor of repeated treatments does not show a large improvement of the molar mass; for example, the characteristics of chitosan obtained after 12 h in a single step compared with that obtained after 12 h in 3 stages of 4 h each. The introduction of the factor of repeated baths proved to be ineffective in limiting the degradation of the molar mass in the case of this insect as it was observed in the case of BSF. The results of deacetylation tests according to the Kurita process of chitin from adult ATM and BSF extracted under the same conditions [26] are presented in Table 5.

All the results of these tests allow us to conclude that the second process P2 presents the advantage of providing pure chitin samples from

Table 4

Physicochemical characteristics of chitosan samples prepared from chitin TM extracted after 24 h of reaction according to the Broussignac process (50 % KOH in 25 % ethanol and 25 % monoethylene glycol; 120 °C).

Chitin Stages and process	Chitosan			
	Trace of impurity	DA% ^1H NMR	$[\eta]$ $\text{mL}\cdot\text{g}^{-1}$	Mv $\text{g}\cdot\text{mol}^{-1}$
LE1 P1	+	5	415	83,000
LE1 P2	–	6	623	136,000
LE2 P1	+	14	605	96,000
LE2 P2	–	8	661	148,000
ATM P1	–	12	627	137,500
ATM P2	–	5	675	153,000

different stages of the life cycle while the chitin samples from larval exuviae extracted using the process P1 require an additional delipidation step before proceeding with deacetylation. These results also confirm the effectiveness of the Broussignac process compared to the Kurita process for preparing pure, highly deacetylated chitosan with controlled molar masses from α chitin.

3.2.2. Preparation of pure chitosan samples with controlled characteristics

The study of the *N*-deacetylation of chitin extracted at different stages of the life cycle of TM shows that it is possible to valorize all rejects by producing pure chitosan samples as often desired; strongly deacetylated with controlled molar masses according to the application for which they will be intended. However, the control of certain parameters especially in relation to the source of chitin and (exuviae or adult) and the extraction or deacetylation processes is decisive in obtaining chitosan with the desired characteristics.

For example, chitosan can be prepared according to the Broussignac process from LE1 and LE2, except that when the starting chitin is extracted according to the first process P1, an additional delipidation step is necessary for preparing pure chitosan after 6 h of reaction with a DA of 30 % and a molar mass of 109,000 $\text{g}\cdot\text{mol}^{-1}$. Therefore, it would be preferable to use the pure chitin provided by the second P2 process, making it possible to have for the same duration of 6 h chitosan with a significantly lower DA (11.5 %) and a molar mass almost three times greater (284,000 $\text{g}\cdot\text{mol}^{-1}$).

Also, the Kurita process makes it possible to provide highly deacetylated pure chitosan samples (DA \leq 5 %) from the chitin ATM, however, the molar mass is relatively low compared to that provided with Broussignac process in the same conditions of temperature, concentration of the alkaline agent, and duration. For example, for a DA of 5 %, the value of the molar mass of chitosan obtained after 24 h of reaction according to Kurita for chitin CH ATM P1 is 91,000 $\text{g}\cdot\text{mol}^{-1}$ instead of 137,500 $\text{g}\cdot\text{mol}^{-1}$ when the Broussignac process is used. The degradation that accompanies deacetylation with NaOH in aqueous media is greater than that which accompanies the reaction with KOH in anhydrous media [62,63]. Therefore, the choice of deacetylation process should consider the molar mass that the desired chitosan should present. For example, it is possible to obtain from the same chitin a chitosan with a DA of 5 % and a Mv of 132,000 $\text{g}\cdot\text{mol}^{-1}$ after 10 h of reaction with NaOH in an aqueous media (Table 5), while a chitosan with a DA of 6 % and a Mv of 200,000 $\text{g}\cdot\text{mol}^{-1}$ is obtained according to the Broussignac process for the same duration.

Therefore, the preparation of pure chitosan samples can be carried out from chitin extracted according to P1 and P2 processes at different stages of the life cycle of TM and the *N*-deacetylation can be done according to Kurita and Broussignac processes. However, the results of this study show that it is desirable to prepare chitosan according to the Broussignac process from a chitin prepared according to the process P2 when the starting source consists of the larval exuviae. The starting chitin samples in this case are pure and highly deacetylated chitosan is obtained from 6 h of reaction with high Mv of 280,000 $\text{g}\cdot\text{mol}^{-1}$ for LE1 and 340,000 $\text{g}\cdot\text{mol}^{-1}$ for LE2. Lower Mv can be obtained by maintaining the reaction for a longer duration. As for the preparation of chitosan samples at the adult stage, the chitin extracted according to the two processes P1 and P2 can be subject to *N*-deacetylation according to the Broussignac process with the possibility of using the Kurita process in the case where relatively low molar masses are desired.

In Table 6 we provide examples of the preparation of highly deacetylated chitosan samples (5 % \leq DA \leq 11.5 %) from chitin extracted at different stages of the life cycle of TM using the Broussignac process.

The larval exuviae can be valorized by the extraction of pure chitin samples according to the second P2 process and the preparation of chitosan samples with molar masses varying between 339,000 $\text{g}\cdot\text{mol}^{-1}$ and 136,000 $\text{g}\cdot\text{mol}^{-1}$ depending on whether the duration of the reaction in the Broussignac conditions either 6 h or 24 h.

As for adults, pure chitin can be extracted using P1 and P2 processes

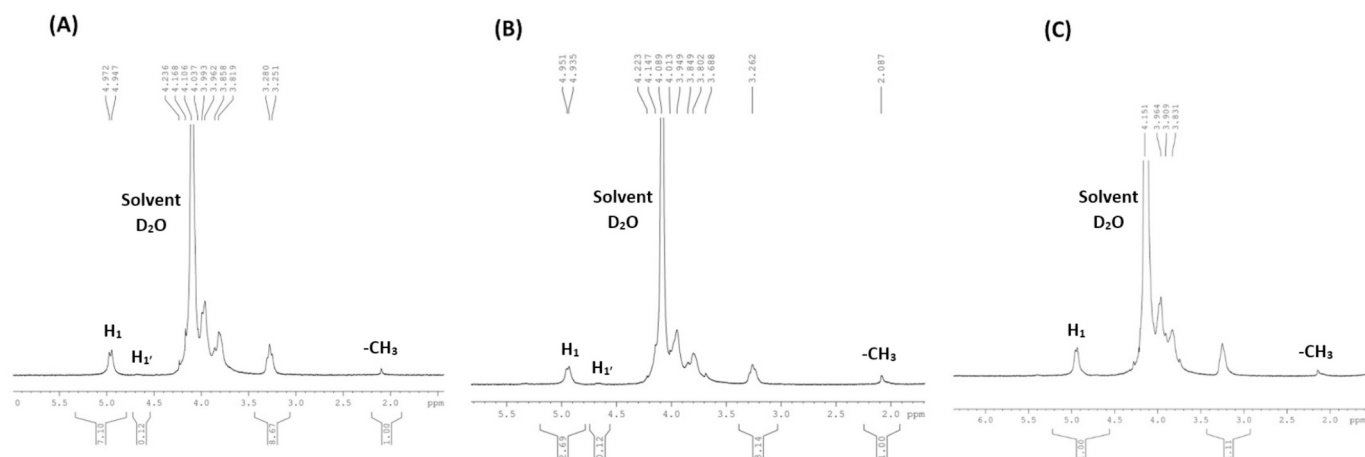


Fig. 8. ^1H NMR spectra of samples of chitosan extracted from the adult ATM DA = 5 % (A), chitosan of LE1 DA = 14 % (B), chitosan of LE2 DA = 12 % (C) after 24 h of reaction using Broussignac process.

Table 5

Physicochemical characteristics of chitosan samples prepared from CH ATM P1 according to the Kurita process (50 % NaOH, 120 °C). Comparison with the results obtained from adult BSF chitin extracted under the same conditions of the first process P1.

Chitin		Chitosan				
Stages and process	Mv g.mol ⁻¹	Duration reaction process Kurita (hour)	Yield of chitosan (wt%)	DA ¹ H NMR %	[η] mL.g ⁻¹	Mv g.mol ⁻¹
Adult P1 TM	1,751,000	10	70–75	5	607	132,000
		12		4	569	121,000
		4hx3		3	586	126,000
Adult P1 BSF	841,500	24		3	501	91,000
		10	69–72	8	471	76,000
		12		4	401	94,000
		4hx3		0	514	99,700
		24		3	214	32,000

and converted into chitosan samples with Mv varying between 284,000 g.mol⁻¹ and 137,000 g.mol⁻¹ depending on the starting chitin (CH ATM P1 or CH ATM P2) and the duration of the reaction (4 to 24 h).

The values of the molar masses obtained for the same durations from

Table 6

Preparation of chitosan samples with controlled characteristics according to the Broussignac process (50 % KOH in 25 % ethanol and 25 % ethylene glycol, 120 °C) from different stages.

Chitin Stages and process	Chitin content %	Molar mass g.mol ⁻¹	Duration reaction process Broussignac (hour)	Average yield of chitosan (wt%)		DA ¹ H NMR %	[η] mL.g ⁻¹	k'	Mv g.mol ⁻¹
				After N-deacetylation reaction	Yield from raw material				
LE1 P2	27	1,775,000	6	76	20.5	11.5	1088	0.35	284,000
			10			9	897	0.38	234,000
			24			6	623	0.45	136,000
LE2 P2	27,5	1,861,000	6	75	21	11	1244	0.50	339,000
			10			9.5	996	0.47	272,000
			24			8	661	0.41	148,000
ATM P1	25	1,751,000	4	78	19.5	11	1026	0.32	263,000
			6			9	125	0.27	229,000
			8			8	893	0.46	219,000
			10			6	849	0.49	200,000
			24			5	627	0.52	137,500
ATM P2	30	1,912,000	4	80	24	8.5	1087	0.43	284,000
			6			8	1026	0.39	263,000
			8			7	966	0.46	243,000
			10			6	898	0.41	221,000
			24			5	675	0.42	153,000

CH ATM P2 are relatively greater than those obtained from CH ATM P1. This is linked to the differences in their starting molar masses.

It is also interesting to point out that the valorization of rejects by producing good quality chitosan can be done with yields varying from 19 % to 24 % depending on the stage of the life cycle studied (larval exuviae or adult TM). These values are significantly higher than those obtained for the majority of crustaceans studied or used for industrial production (crabs 6.5 to 12 %, shrimps 10 to 15 %) [40,64] and they are comparable to those obtained in the case of exuviae BSF (17 to 32 %) [26] or squid pen (26 %) [41,65].

3.2.3. Deacetylation of chitin samples and molecular degradation: Kinetic approach

The N-deacetylation reactions of chitin are always accompanied by more or less intense molecular degradation depending on the starting chitin (source and structure) and the deacetylation process used.

Monitoring the deacetylation of chitin extracted at different stages under the conditions of Broussignac process allowed to note that this degradation can take place with a different intensity depending on whether chitin is from larval exuviae or adult, particularly during the first hours of the N-deacetylation reaction. Indeed, although the molar masses of the chitin from the larval exuviae CH LE1 P2 and CH LE2 P2 are lower than the molar mass presented by the chitin of the adult CH

ATM P2 for example, we noted that the obtained chitosan after 6 h of reaction present Mv values higher than the Mv value of chitosan prepared from CH ATM P2 (Table 6). The same case is observed when the molar masses of the chitosan are obtained after 6 h of reaction from the CH LE1 P2 and CH ATM P1 which have comparable starting molar masses. The comparative analysis of the different molar masses obtained (Table 6) shows that the degradation intensity is relatively less significant for CH LE than for CH ATM. The chitosan resulting after 6 h of reaction, for example from CH LE P2, it represents 18.2 % of the molar mass of the starting chitin while that obtained from CH ATM P2 only represents 13.7 %. These differences will be reduced as the deacetylation progresses; after 24 h of reaction, all chitosan samples are obtained with molar masses which represent only 7.6 to 8 % of the molar masses of the different starting chitins. Monitoring of deacetylation according to the Broussignac process is carried out for 24 h for CH ATM P1, CH ATM P2, CH LE1 P2 and CH LE2 P2 allowed to determine the rate constants of molecular degradation which accompanies the *N*-deacetylation (Fig. 9). The values of these constants are respectively $2.10^{-4} \text{ min}^{-1}$ for CH ATM P1 and CH ATM P2. A value of $3.10^{-4} \text{ min}^{-1}$ is obtained for both exuviae chitin extracted using P1 and P2 processes, which confirms a slower degradation but remains very close to the overall order of α chitin degradation rates. Indeed, in previous work, it is shown that the *N*-deacetylation reaction under the conditions of the Broussignac process presents pseudo 1st-order degradation kinetics and the degradation of the polymer takes place with a rate constant of the order of $2.10^{-4} \text{ min}^{-1}$ for α chitin of crustaceans (shrimp, spider crab and squilla) [28,31]. The rate constant of degradation of β chitin of cephalopods (cuttlefish bone and squid pens) is significantly greater from 1.10^{-3} to $1.58.10^{-3} \text{ min}^{-1}$ [41,65]. This study of the kinetics of molecular degradation accompanying deacetylation reactions contributes to defining the optimal conditions to prepare chitosan with a molar mass adapted to the targeted applications.

4. Conclusion

In this work, we show that the breeding of *Tenebrio Molitor* intended to produce proteins can be optimized by the production of good-quality chitin and chitosan samples. Chitin extraction is carried out using two different processes. The first process P1 provides chitin with high contents compared to other works cited in the literature. However, impurities are observed for CH LE1 P1 and CH LE2 P1, unlike ATM which presents pure chitin. This result is also obtained when this process is applied to exuviae and adult BSF. The application of a delipidation treatment to chitin samples containing impurities is effective in the case of TM and ineffective for BSF. This would be linked to the difference in surface structure; fibrous for the chitin of TM exuviae and porous for that of BSF. The evaluation of the results of the first process P1 led us to develop a second process P2 which presents the advantage of providing pure chitin samples from all stages with significantly improved yields. This process is composed of only two steps, delipidation followed by deproteinization.

The study of the *N*-deacetylation of all the extracted chitin samples is carried out according to the Kurita (NaOH in aqueous solution) and Broussignac (KOH in anhydrous medium) processes. Pure and highly deacetylated chitosan samples ($2 \% < \text{DA} < 12 \%$) are prepared with controlled molar masses ($90,000 \text{ g.mol}^{-1} < \text{Mv} < 340,000 \text{ g.mol}^{-1}$). The Broussignac process presents the advantage of providing chitosan in a single, short-term step with relatively high molar masses. The study of the molecular degradation which accompanies the *N*-deacetylation of chitin with the glycopotassium mixture allowed us to determine the rate constants of molecular degradation. The values of these constants are comparable to the order of values obtained for α chitin of the crustaceans studied. Molecular degradation of ATM chitin is slightly faster than that of larval exuviae chitin. This kinetic study of *N*-deacetylation contributes to the development of the reaction conditions for preparing chitosan samples with controlled molar mass according to the

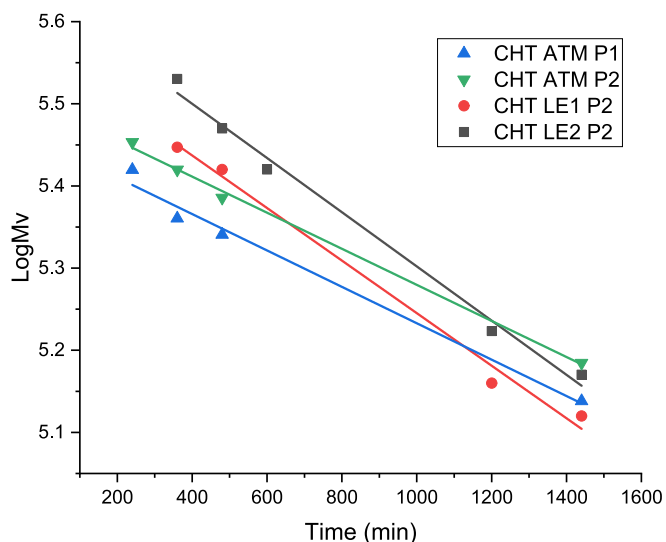


Fig. 9. Molecular degradation of chitosan samples CHT ATM P1, CH ATM P2 and CH LE2 P2 according to the deacetylation reaction of chitin under the standard conditions of the Broussignac process.

application in which it will be intended.

All the obtained results show that it is possible to valorize all rejects from TM breeding through the production of high-quality chitin and chitosan samples with yields comparable to or greater than those obtained for the crustaceans and cephalopods studied or for sources used in industrial production.

The comparison with *Hermetia illucens* makes it possible to extend the use of insects as potential sources of chitinous derivatives and confirms the role of the source and the process in the preparation of chitin and chitosan with controlled characteristics.

CRediT authorship contribution statement

M. Azzi: Writing – original draft, Software, Methodology, Data curation, Conceptualization. **S. Elkadaoui:** Writing – original draft, Software, Methodology, Data curation, Conceptualization. **J. Zim:** Writing – original draft, Resources. **J. Desbrieres:** Writing – review & editing, Validation. **Y. El Hachimi:** Supervision, Project administration. **A. Tolaimate:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to acknowledge the Center for Analysis and Characterization, Marrakech, Morocco, and the Regional Center for Analysis and Characterization, Settat, Morocco for the characterization facility.

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