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Camille Chapelle, Ghislain David, Sylvain Caillol, Claire Negrell, Graziella Durand, et al.. Water-Soluble 2,5-Anhydro- d -mannofuranose Chain End Chitosan Oligomers of a Very Low Molecular Weight: Synthesis and Characterization. *Biomacromolecules*, 2019, 20 (12), pp.4353-4360. 10.1021/acs.biomac.9b01003 . hal-02406342

HAL Id: hal-02406342

<https://hal.science/hal-02406342>

Submitted on 7 May 2020

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Water soluble 2,5-anhydro-D-mannofuranose end chain chitosan oligomers of very low molecular weight : synthesis and characterization

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KEYWORDS: Chitosan, depolymerization, variable resources, water soluble oligomers

ABSTRACT

Five chitosan with different acetylation degree (DA), molar masses and origin were depolymerized by nitrous acid treatment in acidic media leading to water soluble 2,5 anhydro-D-mannose end chain oligomers with DP_n<20. The kinetics of the reaction was studied and the best

work conditions were found to be 3h reaction at 50°C. It was shown that the DPn of oligomers only depends on the quantity of NaNO₂ involved. Molar masses or DA do not have an impact on the depolymerization process when targeting oligomers with less than 20 units. This depolymerization process also leads to free AMF that might react with the free amines of obtained oligomers to form imines. This reaction is pH dependant and in acidic condition, leads to the formation of 5-hydromethyl-2-furfural (HMF). At the end, the oligomers were purified with dialysis to get rid of most free AMF (<5%) and other residual salts, and appeared to have no acute toxicity.

INTRODUCTION

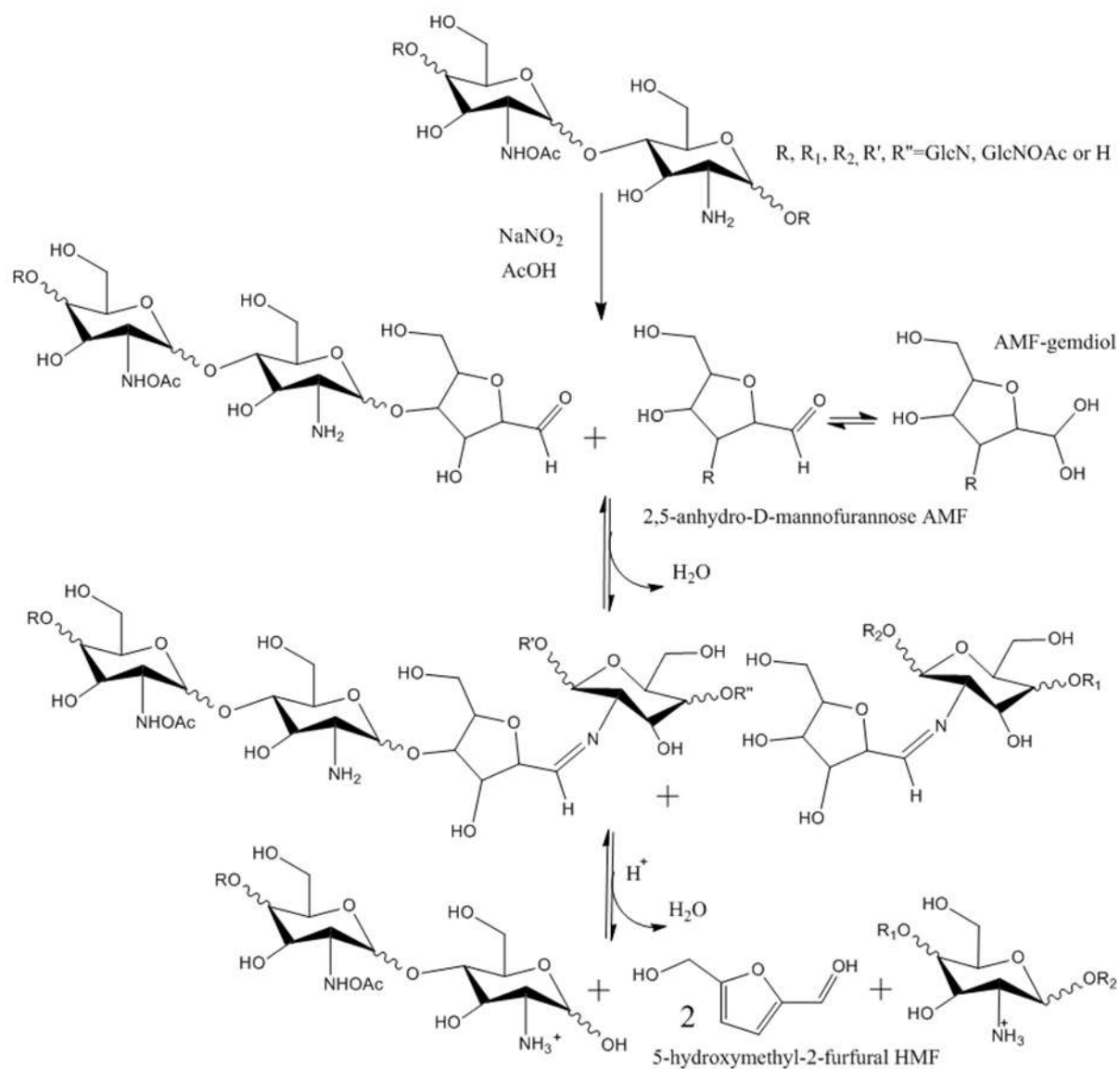
Chitosan is a polysaccharide constituted of β -D-glucosamine and N-acetyl- β -D-glucosamine units linked by a glycosidic bond (1 \rightarrow 4). It is the *N*-deacetylation of chitin, a polymer extracted from shrimp shells, squids, insects or mushrooms, poorly processable due to its insolubility in most solvent including water that leads to chitosan. Chitin and chitosan have the same chemical structure and differ only by the proportion of *N*-acetylated units along the chain, >60% for chitin and <60% for chitosan. In fine, chitosan is characterized by its source (crustacean, insects, fungi...), its *N*-acetylation degree (DA) and its molar mass. A wide range of chitosan exists with various molar masses from 100,000 g/mol up to 500,000 g/mol with acetylation degree between 0 and 30 %¹. Many interesting applications result from use of chitosan^{2 3 4 5}. Nevertheless, chitosan use is limited due to its high viscosity, its insolubility in organic solutions and poor solubility in aqueous solution owing to the pH⁶. Hence, a flourishing interest for lowering the molar masses to obtain chitosan oligomers appeared during the last decade^{7 8 9}.

ChitoOligoSaccharides (COS) have indeed recently received considerable attention as functional biomolecules with a wide range of applications in food, agriculture, medicine,

pharmaceutics and cosmetics. COS are often defined as chitosans with degree of polymerization DP less than 20¹⁰, i.e. with average molar masses lower than 4,000 g/mol. COS are either composed of β -D-glucosamine units or, can be a mixture of β -D-glucosamine and acetyl- β -D-glucosamine randomly organized. COS are defined by some unique properties such as water solubility, cell membrane penetrability, easy absorption and various biological activities. It is also worth to note that COS are easier to process, due to less chain entanglement and steric hindrance which confer them a direct solubility in water for DP<20^{5 8}. COS are insoluble in acetone, butanol, ethanol, ethyl acetate, propanol and pyridine but as opposed to chitosan, partially soluble in methanol and in dimethyl sulfoxide and dimethyl formamide when heated¹¹. Furthermore for these oligomers, the smaller are the molar masses, the better is the solubility. In addition, the utilization of COS allows to reduce the viscosity and to work with higher concentration. COS are reported to treat asthma, prevent tumor growth, malaria, control blood pressure and also have neuroprotective effects for Alzheimer disease treatment thanks to their biological activities, that include antibacterial, antifungal, antiviral, anti-tumor and antioxidant properties, which have been reported to overcome those of chitosan^{9 12 13 14 15}.

Several ways to reduce chitosan molar masses including enzymatic, physical or chemical depolymerization have been studied over the last decades^{8 16 17 18 19}. Enzymatic methods are really precise but very expensive and hardly applicable on large scale. Physical depolymerizations with microwave or sonication do not lead to oligomers and are mostly used to reduce molar masses and viscosity of chitosan solutions. They are often combined with chemicals like peroxide or concentrated acid to boost the depolymerization. Those methods are hardly controllable and reproducible. Allan and al.²⁰ developed a depolymerization method, based on the use of sodium nitrite (NaNO₂). The principle is based on the action of nitrous acid, which cleaves the glycosidic linkages of chitosan. The reaction consists in the formation of a diazonium ion on a D-glucosamine unit by reaction between the free amine and the nitrous acid. A carbocation is obtained by nitrogen release. The nucleophilic

66 addition of endocyclic oxygen then leads to a 2,5-anhydro-D-mannofuranose (AMF) structure as the
 67 new chain end (Scheme 1).



68 **Scheme 1** Reaction of NaNO_2 onto chitosan and side reaction leading to HMF

69

70 Interestingly, they established an equation linking the obtained oligomer molar mass to the
71 quantity of NaNO_2 . Later on, Tømmerraas et al.²¹ investigated on the reaction mechanism with
72 detailed NMR analyses of fully *N*-acetylated and *N*-deacetylated oligomers. They especially
73 underlined the formation of AMF chain end. They also showed the presence of secondary reactions:
74 the resulting aldehyde of AMF chain end could react with the free amines of chitosan oligomers,
75 leading to the formation of a Schiff base during lyophilisation. In acidic conditions, this Schiff base
76 would then facilitate the elimination reactions leading to the formation of 5-hydroxymethyl-2-furfural
77 (HMF), another byproduct. Shirui Mao and al²², studied some factors affecting the depolymerization
78 process like chitosan concentration, reaction time and reproducibility. They studied high molecular
79 weight (600 kg/mol and 400 kg/mol) and low molar masses (150 kg/mol) chitosans as well as
80 cytotoxicity of the resulting COS with molar masses ranging from 2,800 to 49,000 g/mol but did not
81 get further on the depolymerization process to reach lower molar mass oligomers. Although nitrous
82 deamination has been studied and used many times to depolymerize chitosan^{23 24}, this reaction was
83 not investigated so far for reaching even smaller DPs, i.e. $5 < \text{DP} < 20$. Only Moussa et al. synthesized
84 oligomers of DP10 using this method but started from one single type of chitosan (DA 0%, $M_n =$
85 115,000 g/mol)²⁵. Those oligomers could be of interest as they happen to be directly water soluble
86 depending on the pH and even soluble in DMSO and exhibit better biological activities¹⁵. Besides,
87 effect of both sourcing and DA for initial chitosan onto depolymerization process was also not
88 highlighted.

89 The purpose of this paper is to obtain chitosan oligomers of different DP (average from 5 to
90 60) by nitrous deamination from different chitosans by varying the DA, the M_w and the origin
91 (marine and insect). The kinetics of the reaction was studied as well as the ideal reaction parameters
92 to lead to a robust method of chitosan depolymerization. The formation of free AMF and HMF was
93 underlined and correlated to the different reaction parameters. The molar masses of the resulting
94 oligomers were calculated using size exclusion chromatography (SEC). Structure identification was

performed using infrared spectroscopy (IR), ^1H and 2D DOSY NMR spectroscopy, and headspace chromatography.

MATERIALS AND METHODS

Materials

Three different shrimp chitosan DA around 10-15% CH30 (Mw 30 kg/mol), CH150 (Mw 150 kg/mol), CH250 (Mw 250 kg/mol) were purchased from Glentham Science, chitosan with DA equal to 1% CHDA1 (150 kg/mol) was purchased from Mathani chitosan, insect chitosan with DA equal to 20% CHY (Mw 180 kg/mol) was purchased from Ynsect and used as received. Acetic acid (AcOH), sodium acetate (NaAc), sodium nitrite (NaNO_2), lithium bromide (LiBr), sodium azide (NaN_3), sodium hydroxide (NaOH), hydrochloric acid (HCl), acetone (>98%) were purchased from Sigma and used without purification, HPLC water (>99%) was purchased from VWR.

Depolymerization of chitosan

The depolymerization of high molecular weight chitosans was performed by nitrous deamination involving NaNO_2 as previously described ²¹. Briefly, 1g (w/w) of chitosan was dissolved in 80 mL of AcOH (350 μL in 90 mL of deionized water) solution overnight (pH 4.5). Once solubilized, a freshly prepared solution (10 mL) containing the desired equivalent ratio of $\text{NaNO}_2/\text{NH}_2$ (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 eq) was added to the solution. The solution was stirred at definite temperature for a definite time. Oligomers of DP>20 (made with 0.1 eq of $\text{NaNO}_2/\text{NH}_2$) were precipitated by addition of 1M solution of NaOH and washed several times with deionized water until neutral pH. Oligomers with DP<20 did not precipitate with the addition of NaOH. The solutions were then freeze dried for 24h and the lyophilisates were dried under vacuum for 24h. The kinetics of the reaction was investigated for the five different chitosans. For that, chitosans were dissolved in deionized water with 1eq AcOH/ NH_2 , 0.2 eq of $\text{NaNO}_2/\text{NH}_2$ were added and the reaction was led at

room temperature (30°C). Samples were taken after 30 min, 1 h, 2 h, 3 h, 6 h and 24 h, freeze dried and analyzed using SEC with a PEO calibration. The same experiment was reproduced for CH150 at 10°C, 30°C, 50°C and 70°C in order to investigate influence of temperature on the reaction. Dialysis was performed using Spectra/Por dialysis membrane Biotech CE tubing MWCO: 100-500 D. ¹H NMR of 1600 g/mol oligomer, D₂O, 400 MHz, 298 K, pH 5.5: δ 8.2-8.5 (s, H_{aldehydes}), 8-7.1 (H_{imines}), 5.0 (d, 5 Hz, 1H H_{1 gemdiol}), 3.2-4.4 (m, 1H H_{2 acetyl}, 1H H₃, 1H H₄, 1H H₅, 2H H₆), 2.5 (m, 1H, H₂), 2.0 (s, 3H, H_{CH₃}), 4.8 (s, HOD). 23 % of the amines are in imines form. 51% of the aldehydes (free or chain end) have reacted with an amine to form imines, 21% are in gem diol form and 28% are in aldehyde form (SI 3).

AMF synthesis

AMF was synthesized by action of nitrous acid on D-glucosamine. Briefly, 1 g (4.6 mmol, 1 eq) of D-(+)-Glucosamine hydrochloride was dissolved in 20 mL of deionized water during 3h. Sodium nitrite (1 g, 14.5 mmol, 3.2 eq) was added and the reaction was led at room temperature for 6h. The final product was lyophilized for at least 24h. ¹H NMR for D-glucosamine (D₂O, 400 MHz, 298 K, pH 5.5) : δ 5.42 (d 4Hz, 1H, H_{1α}), 4.9 (d 8Hz, 1H, H_{1β}), 3.4-3.9 (m, 1H H₃, 1H H₄, 1H H₅, 2H H₆), 3.3 (dd 10Hz, 1H, H_{2α}), 2.9 (dd 10Hz, 1H, H_{2β}), 4.8 (HOD). ¹H NMR for AMF (D₂O, 400 MHz, 298 K, pH 5.5) : δ 8.5 (s, 1H, H₁), 5.1 (d, 5Hz, 1H, H_{1 gemdiol}), 4.2 (t 6Hz, 1H, H₂), 4.1 (t 6Hz, 1H, H₃), 4.0 (m, 1H, H₅), 3.8 (m, 1H, H₄ 2H, H₆) as expected²⁶. In deuterated water, AMF is 70% in gem-diol and 30% of aldehyde form (SI 1 & 2).

Characterization

¹H NMR, analyses were performed using a Bruker Avance 400 MHz NMR spectrometer at 298 K. 2D DOSY NMR was performed on a 600MHz Bruker Avance spectrometer. NMR samples were prepared by dissolving 15 mg of product in 0.5 mL of D₂O. The chemical shifts were reported in part per million relative to tetra methylsilane. Spin multiplicity is shown by s=singlet, d=doublet, t=triplet, q=quadrat and m=multiple. DA of native chitosan and oligomers were determined using ¹H

NMR. FTIR spectra were recorded on Nicolet 6700 FTIR spectrometer. GC-MS analysis were performed on GC 7890B AGILENT equipped with a capillary column Agilent 122-1364UI DB-624UI (60 m x 0.25 mm, 1.4 μ m), with FID detector and mass spectrometer 5977B AGILENT. GC is directly linked to the headspace sampler 7697A Agilent, with an injection loop (1000 μ L) and a transfer line. dn/dc were calculated using the differential refractive index (RI) detector Wyatt Optilab T-rEX. Molar masses were recorded using triple detection size exclusion chromatography (Tosoh TSK PW 2500, TSK PW 6000, TSK gel G Oligo PW) coupled with the same RI detector Wyatt Optilab T-rEX, multiangle light scattering Wyatt DAWN HELEOS II, and a Fused Silica cell, laser λ =664 nm. They were also recorded using size exclusion chromatography with RI detection (PL aquagel-OH 30 8 μ m 100-60.000 g/mol, PL aquagel-OH 40 8 μ m 10.000-200.000 g/mol, TSK gel G Oligo PW 7 μ m 7.8 mm ID x30 cm 0-6000 g/mol) calibrated using PEO/PEG standards. 10 mg/mL solutions of oligochitosans were prepared in acetate buffer (1.5% AcOH, 0.8% NaAc, 1 ppm NaN₃, 5 ppm LiBr). Error were calculated by doing standard deviation on three different integration on each chromatogram and data plotted are the average value of those measurement. The pH (correction for isotopic effect: pD=pH+0.4) were measured by use of a Mettler Toledo pH electrode.

In order to validate the PEO equivalent method for molar masses measurement, triple detection SEC was performed. To do that, variation of refractive index according to concentration, dn/dc, were measured using a differential refractometer Wyatt Optilab T-rEX. 1mg/mL chitosan/oligochitosan solutions were prepared in degassed acetate buffer (AcOH 0.2M/0.15M ammonium acetate; pH=4.5) and diluted at 80%, 60%, 40%, 20%, 10% (w/w). Refractive indexes were measured according to solution concentration (658 nm, 0.5 mL/min). After that, 3mg/mL chitosan/oligochitosan solutions were prepared in the acetate buffer from above. 100 μ L of solution were filtrated on 0.45 μ m membrane CME filter and injected in the apparatus made of Tosoh TSK PW 2500 TSK PW 6000 coupled with a differential refractometer Wyatt Optilab T-rEX, multi angular light scattering Wyatt DAWN HELEOS II, and UV cellule Fused Silica, laser λ =664 nm. Molar

170 masses (Mw, Mn and Đ) of chitosans and oligochitosans were determined taking in account the dn/dc
 171 determined above. DPn were calculated according to the formula (1):

172 (1)
$$DPn = \frac{Mn}{DA \cdot 203 + (1 - DA) \cdot 162}$$

Chitosan	DA (%)		dn/dc	Mw (kg/mol)		Mn (kg/mol)		Dispersity		DPn	
	Labeled	Measured		PEO/PEG equivalent	Triple detection	PEO equivalent	Triple detection	PEO/PEG equivalent	Triple detection	PEO/PEG equivalent	Triple detection
CH30	≥10	16 ± 2	0.1782 ± 0.0022	40 ± 4	44 ± 4,4	13 ± 1	21 ± 2	3 ± 0.3	2 ± 0.2	77 ± 6	125 ± 12
CH150	≥10	16 ± 2	0.1828 ± 0.0031	163 ± 16	150 ± 15	96 ± 10	80 ± 8	3 ± 0.3	2 ± 0.2	571 ± 60	476 ± 47
CH250	≥10	16 ± 2	0.1893 ± 0.0012	227 ± 23	370 ± 37	200 ± 20	192 ± 19	1.9 ± 0.2	2 ± 0.2	1190 ± 120	1143 ± 113
CHY	≥10	20 ± 2	0.1911 ± 0.0027	120 ± 12	240 ± 24	120 ± 12	170 ± 17	1.7 ± 0.2	1.6 ± 0.2	706 ± 70	1000 ± 100
CHDA1	≥1	1 ± 1	0.1980 ± 0.0009	100 ± 10	150 ± 15	62 ± 6	80 ± 8	2.1 ± 0.2	2 ± 0.2	383 ± 36	493 ± 30

Oligomer	Equivalent of NaNO ₂ /GlcN unit		Mw(g/mol)		Mn (g/mol)		Dispersity		DPn	
CH30	0.4	0.1449 ± 0.0017	2,500 ± 250	2,750 ± 275	1,850 ± 185	2,500 ± 250	1.4 ± 0.1	1.1 ± 0.1	11 ± 1	15 ± 2
CH30	0.2	0.1510 ± 0.0045	3,900 ± 390	4,150 ± 415	2,350 ± 235	3,500 ± 350	1.6 ± 0.2	1.2 ± 0.1	14 ± 1	21 ± 3
CH30	0.1	0.1539 ± 0.0009	7,950 ± 795	7,900 ± 790	5,050 ± 505	8,100 ± 810	1.6 ± 0.2	1.2 ± 0.1	30 ± 3	48 ± 5
CH150	0.4	0.1439 ± 0.0038	2,600 ± 260	2,800 ± 280	1,850 ± 185	2,500 ± 250	1.4 ± 0.1	1.1 ± 0.1	11 ± 1	15 ± 1
CH150	0.2	0.1576 ± 0.0007	4,400 ± 440	3,450 ± 345	2,850 ± 285	3,000 ± 300	1.6 ± 0.2	1.2 ± 0.1	17 ± 2	18 ± 2
CH150	0.1	0.1512 ± 0.0015	10,200 ± 1020	11,800 ± 1180	6,200 ± 620	10,100 ± 1010	1.6 ± 0.2	1.2 ± 0.1	37 ± 3	60 ± 6
CH250	0.4	0.1366 ± 0.0034	2,600 ± 260	3,000 ± 300	1,850 ± 185	2,500 ± 250	1.5 ± 0.2	1.1 ± 0.1	11 ± 1	15 ± 1
CH250	0.2	0.1423 ± 0.0027	4,200 ± 420	3,800 ± 380	2,700 ± 270	3,500 ± 350	1.6 ± 0.2	1.2 ± 0.1	16 ± 2	21 ± 2
CH250	0.1	0.1399 ±	13,600	17,400	7,600 ±	13,900	1.8 ± 0.2	1.2 ±	45 ± 5	83 ± 8

		0.0023	± 1360	± 1740	760	± 1390		0.1		
CHY240	0.4	0.1377 ± 0.0008	2,700 ± 270	3,100 ± 310	1,850 ± 185	2,500 ± 250	1.6 ± 0.2	1.2 ± 0.1	11 ± 1	15 ± 1
CHY240	0.2	0.1423 ± 0.0009	4,400 ± 440	5,300 ± 530	2,350 ± 235	4,350 ± 435	1.7 ± 0.2	1.2 ± 0.1	14 ± 1	22 ± 2
CHY240	0.1	0.1363 ± 0.0019	10,100 ± 101	13,100 ± 131	6,050 ± 605	10,900 ± 1090	1.7 ± 0.2	1.2 ± 0.1	36 ± 3	64 ± 6

Table 1. DA, dn/dc, molar masses and dispersity of crude chitosan and chitosan oligomers obtained with action of NaNO₂ during 3h at 50°C.

Toxicity and ecotoxicity of oligomers post dialysis (DP5 and DP10) were evaluated according to acute tests on *Daphnia magna* (NF EN ISO 6341, 2012) performed by Eurofins. This test aims at finding the concentration immobilizing 50% of the daphnia population after 24h and 48h (CE50). A solution of K₂Cr₂O₇ CE50-24h = 0,94 mg/L (this value corresponds to standard between 0.60 mg/L and 2,1 mg/L according to NF EN ISO 6341 : 2012) is used as a reference substance. Samples were prepared by dissolving oligomers in an ultra-pure water solution of anhydrous sodium hydrogen carbonate NaHCO₃ (0.200 g/L), calcium chloride CaCl₂ (0.260 g/L), magnesium chloride MgCl₂, 6 H₂O (0.148 g/L), anhydrous potassium sulfate K₂SO₄ (0.026 g/L). 100 mg/L solutions were prepared and diluted as followed: 35%, 10%, 3.5%, and 1% (w/w). Tests were performed at 20 °C.

RESULTS AND DISCUSSION

Depolymerization using NaNO₂ was investigated from 5 different chitosans. 3 of them, obtained from crustaceous, have the same DA (i.e. 16%) and only differ by their Mw: 30, 150 and 250 kg/mol. They will be labeled as CH30, CH150 and CH250. The fourth chitosan, also obtained from crustaceous and labeled CHDA1, has a Mw of 150 kg/mol with DA of 1%. The last studied chitosan, labeled CHY, has a Mw of 240 kg/mol, DA of 20% and was provided from insects.

During the course of depolymerization reaction, molecular weight evolution was followed by means of SEC using PEO/PEG standards. We have first decided to validate this method by also assessing Mw values by triple detection SEC. It was shown that dn/dc values are smaller than those of respective crude chitosans (Table 1). Moreover, even if the dn/dc values are very close for any oligomers of similar molar masses, this study shows that dn/dc values are different for all samples. Therefore, it is very important to determine dn/dc before doing further analyses. Indeed, a small variation of the dn/dc value has a significant impact on the molar masses obtained, specifically with small objects.

Mw and Mn values for each chitosan oligomer, as well as the corresponding crude ones, are given in Table 1. It was especially shown that both methods afford rather close values when targeted DP below 20. Above this value and also for crude chitosans, SEC using PEO/PEG standards seems to underestimate the Mn values (25% lower than triple detection values). Nevertheless, this work aims at synthesizing COS oligomers with DP lower than 20. Thus we have decided to use SEC with PEO/PEG standards to monitor the Mn evolution for all chitosans.

At first, each chitosan was reacted with 0.2eq of NaNO₂ at room temperature (30 °C) and molar masses decrease was followed over time (

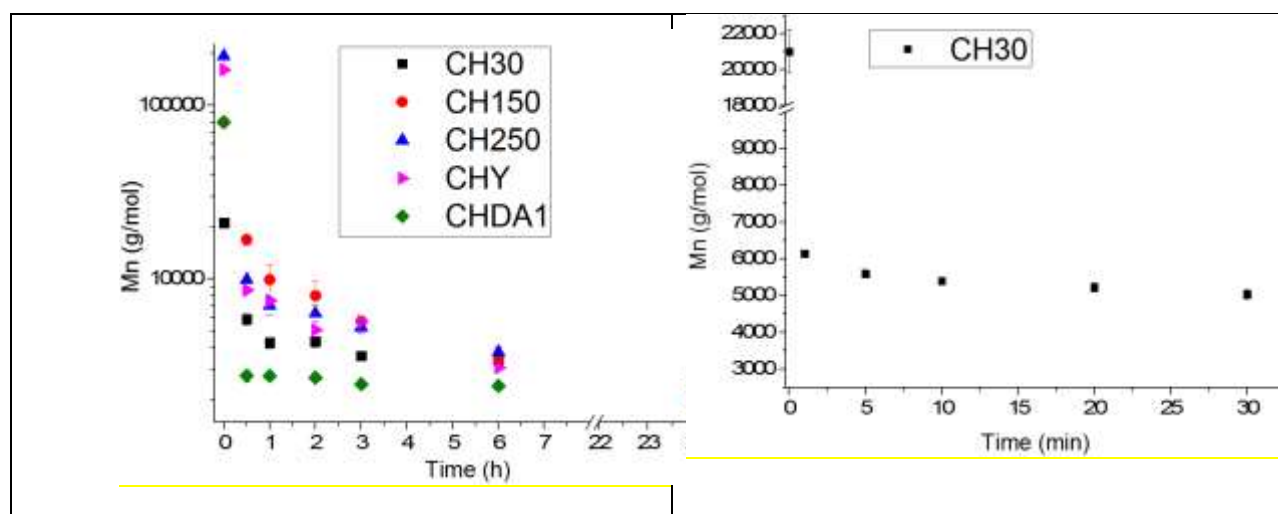


Figure 1 a)). Mn of CH30, CH150, CH250 and CHY showed a similar trend, i.e. a very fast decrease during the first 30 min until approximately reaching 6,000 g/mol, then Mn slightly decreased until stabilization. It was shown that the reaction is immediate, the most important drop of molar masses occurs during the first minutes (

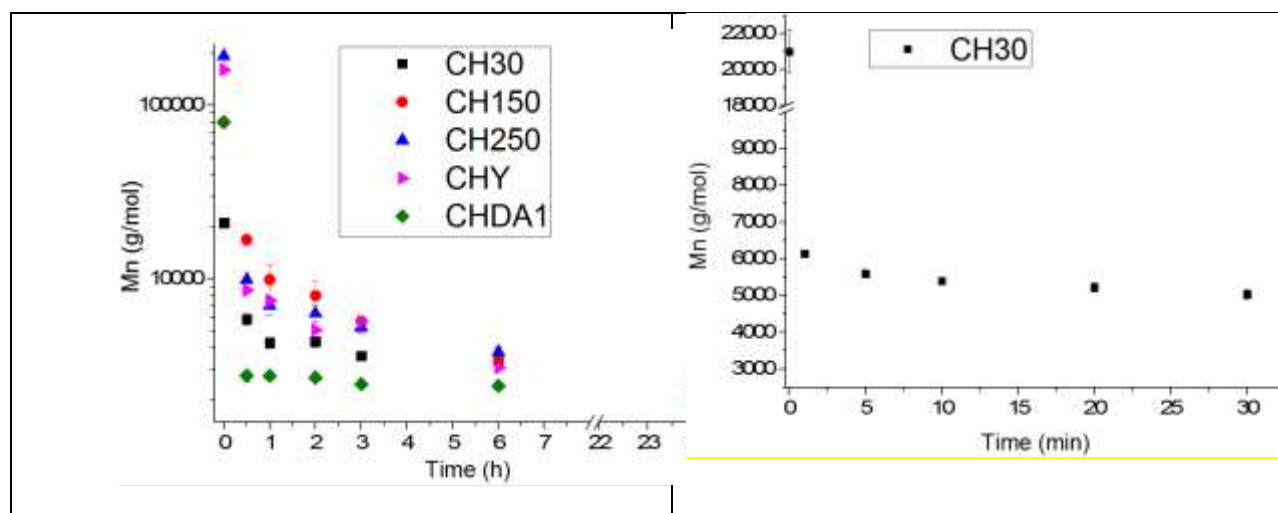
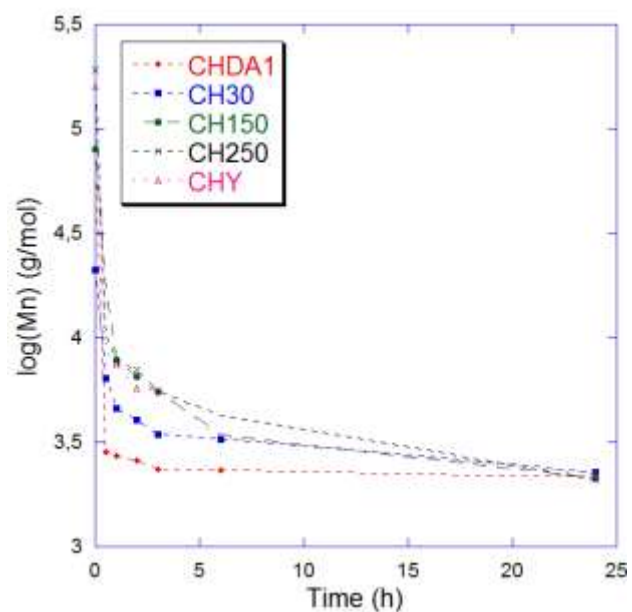
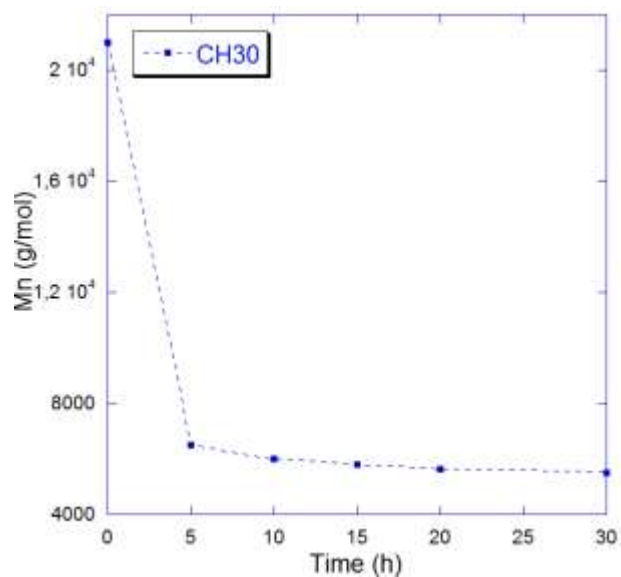


Figure 1 b)). The plateau was reached when either NaNO_2 was fully consumed or did not react anymore. CHDA1 behaves slightly differently since molar masses decrease faster to reach 2,500 g/mol after 30 min. According to the mechanism (scheme 1), this faster decrease can be easily explained by the fact that CHDA1 has more free amines available for reaction with NaNO_2 , thus leading to more glycosidic bond cleavages. Consequently, CHDA1 reaches a plateau after only 6 h, whereas 24h are needed for the four other chitosans. The kinetics of the reaction also depends on crude chitosan Mn: CH30 molar masses decreases faster than that of CH150 and CH250. This is probably due to the higher viscosity for higher Mn chitosans in solution. It was also shown that dispersity of the different chitosans decreases during the depolymerization process to reach interesting values ranging between 1.3 and 1.6 (Figure 2).

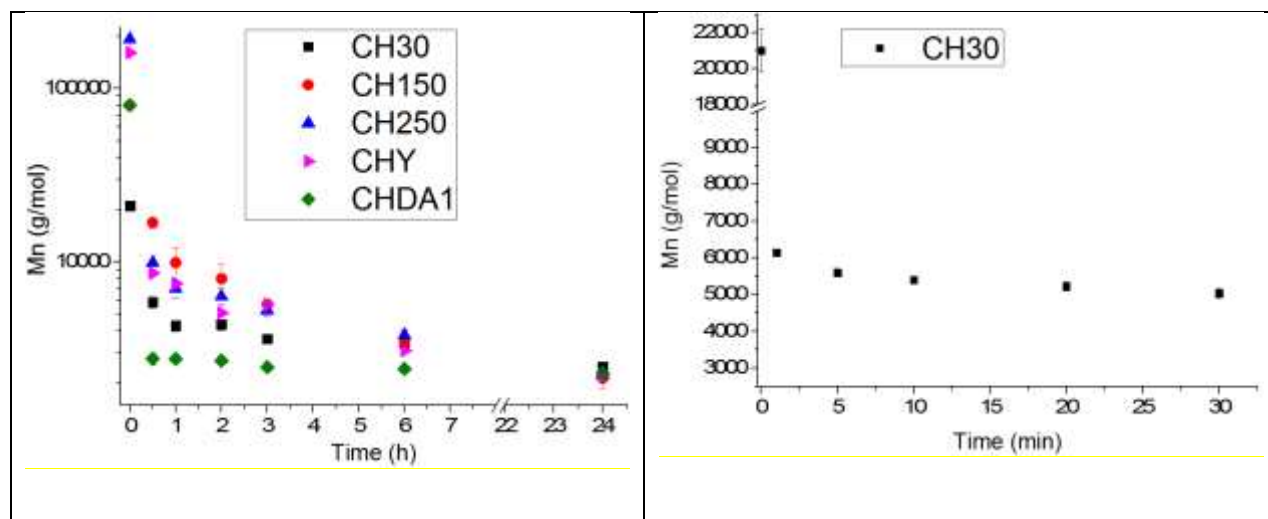
a)



b)



224



225

226 **Figure 1** a) Evolution of molar masses log (Mn) as a function of time for five different chitosans (1% w/w) with 0.2eq of
 227 NaNO₂/NH₂ b) Evolution of molar masses as a function of time for CH30 with 0.2 eq of NaNO₂/NH₂ during the first 30 min

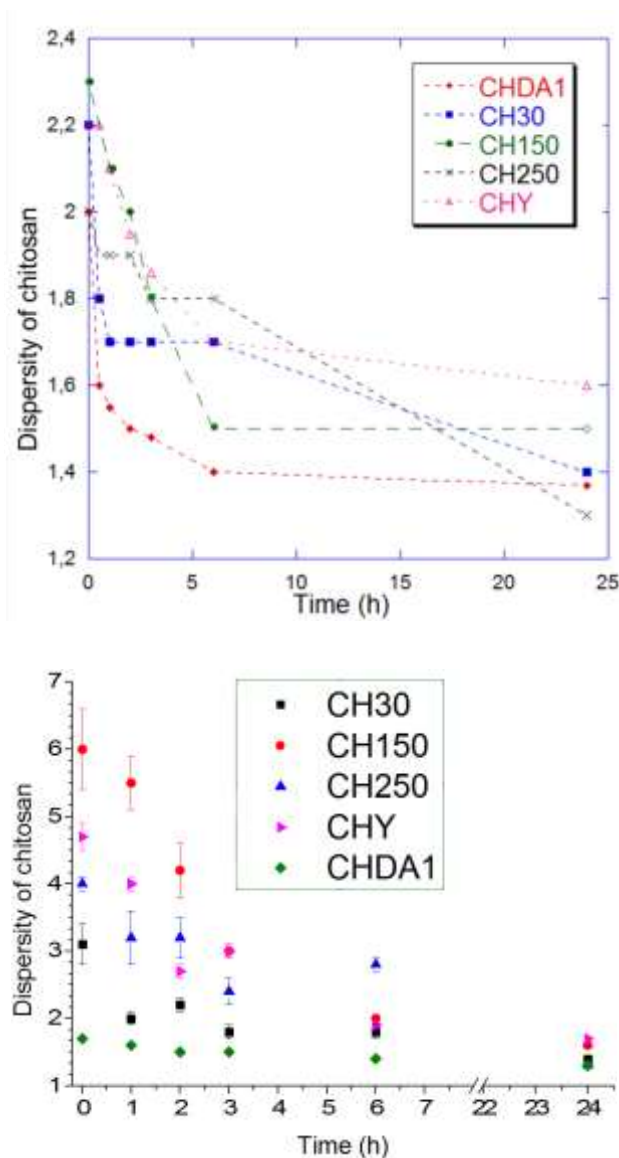


Figure 2 Evolution of the dispersity as a function of time for five different chitosans (1% w/w) with 0.2eq of $\text{NaNO}_2/\text{NH}_2$

This reaction was carried out at room temperature, as already described in the literature²¹. Nevertheless, an increase of the temperature should impact the reaction kineticss but also may lead to reaction completion, until reaching lower molar masses COS, i.e. $\text{DP} < 20$.

The reaction was carried out with CH150, with 0.2 eq of NaNO_2 at 4 different temperatures, i.e. 10, 30, 50 and 70°C (Figure 3). At 10°C, the reaction is not completed even after 24h. Indeed, at

low temperature, viscosity of crude chitosan solution is high, which therefore decreases the reaction kinetics. Furthermore, NaNO_2 could be consequently decomposed in nitrogen oxide by acetic acid instead ²⁷. With a similar $\text{NaNO}_2/\text{NH}_2$ ratio, a temperature increase leads to faster depolymerization reaction. As expected, the reaction kinetics proceeded very fast at 70°C, and lower Mn COS were obtained. This could be explained by additional acidic depolymerization, due to the presence of acetic acid (pH 4,5) ²⁸. When heated at 50°C, the plateau was reached after only 3h. The reaction was still carried out for 24h to ensure that no further degradation occurred. Consequently, the following experiments were carried out at 50°C during 3h.

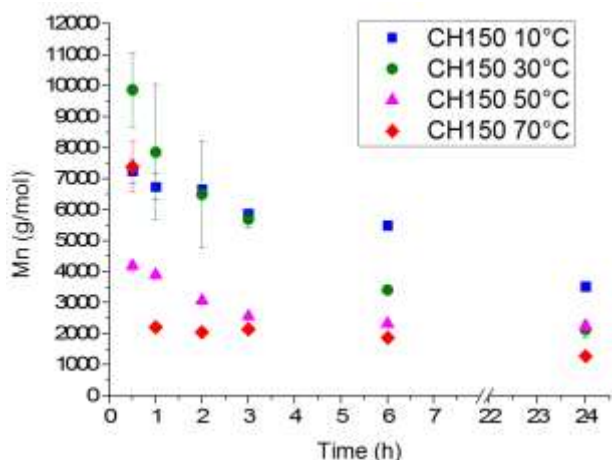
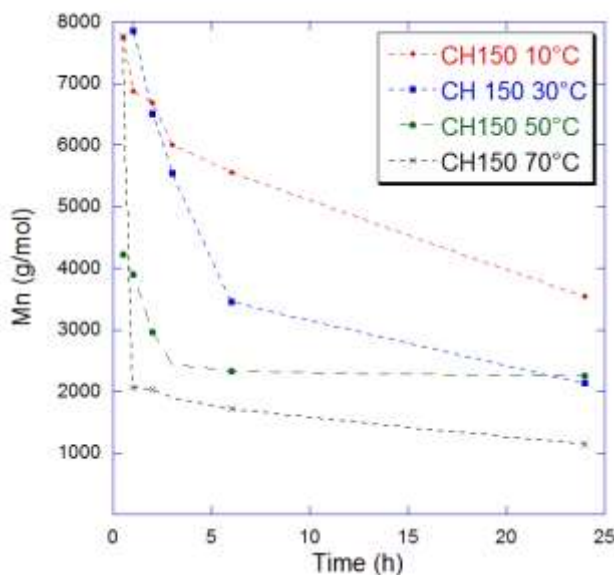


Figure 3 Evolution of Mn vs time at different temperatures for the reaction of CH150 with 0.2eq of NaNO₂/NH₂

Allan et al.²⁰ already showed that the resulting Mw of COS was directly related to the content of NaNO₂. Then CH30, CH150, CH250, CHDA1 and CHY were reacted at 50°C for 3h with different NaNO₂/NH₂ equivalents, and their Mn were assessed (Figure 4). First, we can note that the five chitosans behave similarly, i.e. the evolution of Mn changed at 0.2 eq. below this value, a strong Mn decrease was observed from crude chitosans to reach values above 2,500 g/mol, i.e. DP > 20. These

COS can be purified by precipitation into a strong base to obtain COS with DP ranging between 30 to 45 at 0.1 eq $\text{NaNO}_2/\text{NH}_2$. Above 0.2 eq $\text{NaNO}_2/\text{NH}_2$, Mw COS slowly decreased by increasing NaNO_2 content. Interestingly, the resulting COS showed DP ranging from 15 to 5 and were water soluble (pH 5.5). Thus, as already demonstrated by Allan et al.²⁰, a defined number of equivalents of $\text{NaNO}_2/\text{NH}_2$ allows obtaining COS with DP values from 5 to 20. Furthermore, this reaction is clearly not affected neither by molar masses (between 30 kg/mol and 250 kg/mol), nor DA (between 1 and 20%) or origin of crude chitosans.

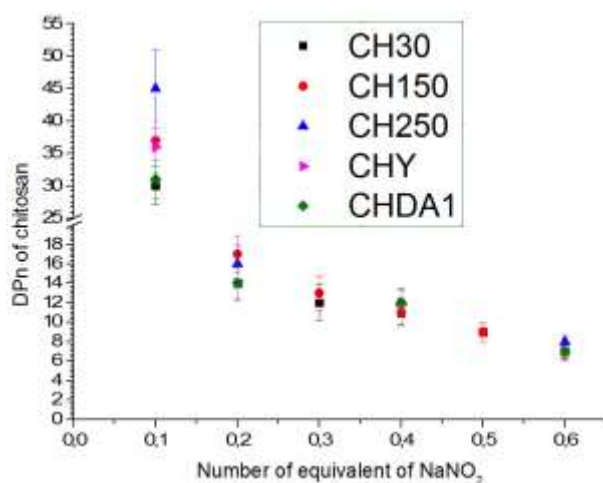
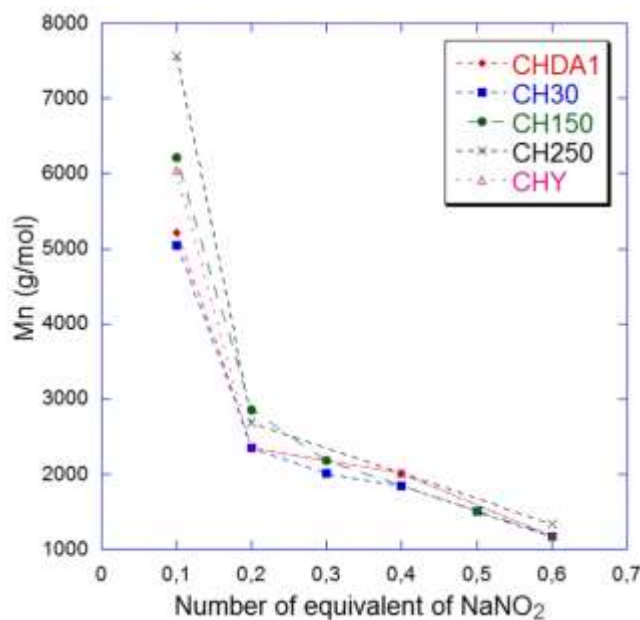


Figure 4. Evolution of Mn vs NaNO₂/NH₂ equivalents for the depolymerization of CH30, CH150, CH250, CHDA1 and CHY at 50°C for 3h.

To check their stability over time, the resulting COS with DP ranging from 5 to 20 were analyzed by SEC after 6 months (Figure 5). No decrease was observed even after 6 months, which proved that no further degradation occurred. The 10% difference between the two molar masses was explained by the change of calibration curve and evolution of the column. Finally, COS obtained with 0.2 eq. of NaNO₂/NH₂ were analyzed by FTIR and compared to the corresponding crude chitosans (SI 6). The carbonyl band at 1650 cm⁻¹ is still visible in the oligomer spectra meaning that the

acetylated units are still present. The similarity of both crude chitosan and oligomers by ^1H NMR analysis proves that no sugar units degradation occurred, as expected (SI 4 & 5). As a matter of fact, the DA is conserved during depolymerisation.

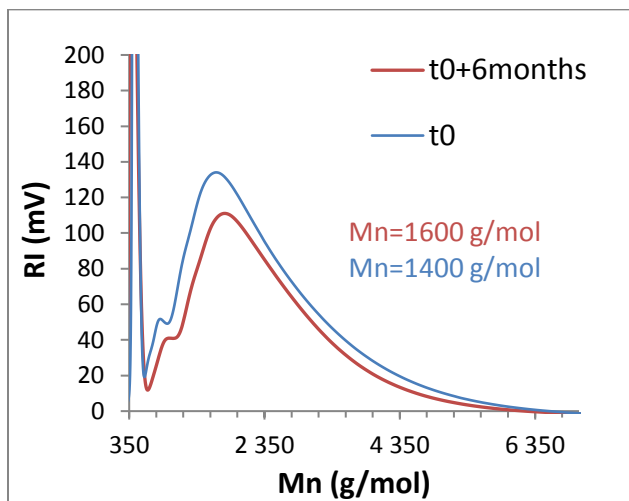


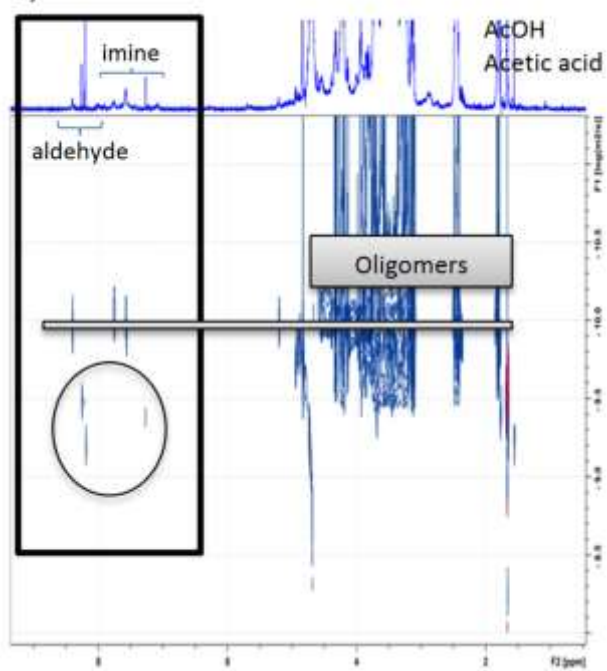
Figure 5 Evolution of Mn of oligochitosan CH30 with 0.4eq of $\text{NaNO}_2/\text{NH}_2$ from t_0 to $t_0+6\text{months}$

Tømmerraas et al.²¹ showed that nitrous deamination leads to the formation of AMF chain end of COS (reductive end), but also to the formation of free AMF (2,5-anhydro-D-mannose) when glycosidic bond breakage occurs at the non reductive polymer chain-end. Furthermore, AMF is in equilibrium with the corresponding gem diol. Both AMF chain-end and free AMF may unlikely react with amine groups of chitosan and the obtained Schiff base will undergo formation of 5-hydroxymethyl-2-furfural (HMF) when changing to acidic pH (Scheme 1 b). The presence of both AMF and HMF was first confirmed by headspace analysis. The analysis was performed by gas chromatography with static headspace coupled to a detection mass spectrometer (GC-MS). Once lyophilized, AMF, glucosamine and oligomers were characterized. AMF emissions are mainly composed of nitrogen and oxygenated compounds. The main signals from AMF are 18 and 19 (SI 7), which are identified as furfural and furanmethanol, respectively and for other signals in general as furan derivatives. Those compounds are close to the real structure of AMF. In the other way, COS mainly showed both nitrogen and oxygenated compound corresponding to

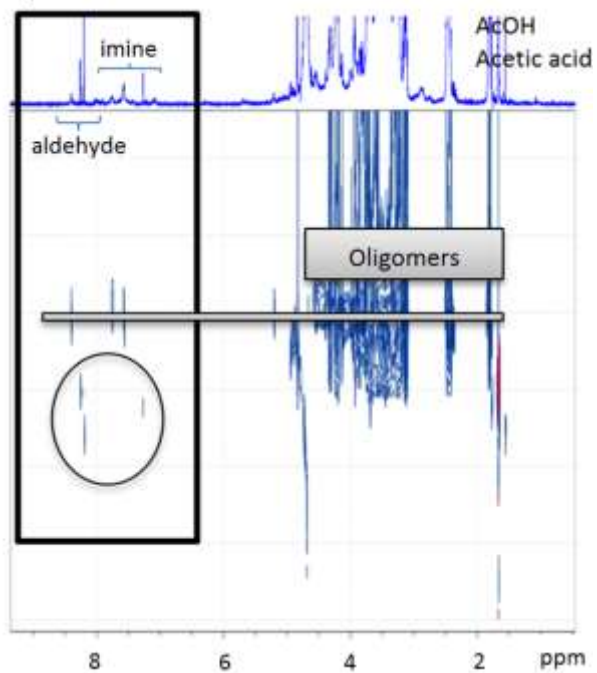
alcohol and amine functions of the sugar units and shows signals correlating with those from AMF, i.e. mainly furan derivatives (peak 18, 19, 20, 21, 23, 24, 25, 27) (SI 8). However, no correlation between glucosamine and COS emissions were noticed, meaning no D-glucosamine was left at the end of the depolymerization process (SI 9).

Then, to assess the presence of those small molecules, 2D DOSY was performed (Figure 6). This experiment allows differentiating the NMR signals of different species according to their diffusion coefficient. In ideal case of non-overlapping component lines and no chemical exchange, the 2D peaks align themselves along horizontal lines, each corresponding to one sample component. This means that on the spectrum every signal belonging to the same molecule will appear on the same line. Figure 6 a) shows 2D DOSY NMR spectrum of a 1600 g/mol COS obtained by nitrous deamination of CH30 at 50°C (0.4eq of NaNO₂). Aldehyde signals are clearly highlighted, evidencing the presence of both COS AMF chain-end and free AMF in equilibrium with the gem-diol form of both products (4,5-5 ppm). These signals were then analyzed by ¹H-¹³C 2D HSQC NMR (Figure 6 b)). The carbon signals near 180 ppm were specifically attributed to aldehydes function. Carbon signals between 140 and 180 ppm correlating to proton signals between 7 and 8 ppm are characteristic of imine groups. These imines resulted from the reaction between AMF (free or chain end) and amine functions of CH30. Then, if the pH drops, free HMF can be obtained, as already proved by Tømmeraas et al ²¹.

a)



a)



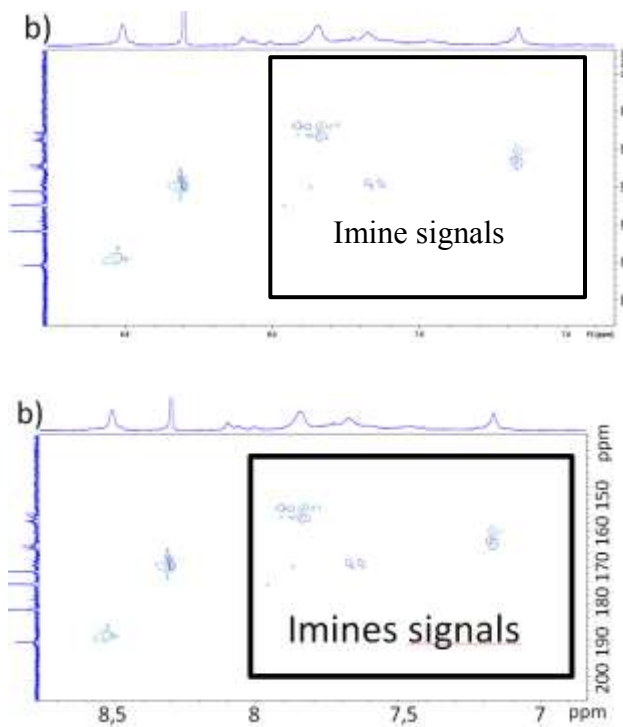


Figure 6 a) 2D DOSY NMR spectrum of a 1600 g/mol oligomer obtained from COS from CH30 + 0.4 eq NaNO₂/NH₂ (3h at 50°C) b) ¹H-¹³C 2D HSQC NMR spectrum of the aldehyde zone

Consequently, the effect of pH on COS structure was investigated. COS was solubilized in deuterated water at pH 5.5. Then, pH of the solution was either dropped to 2 with HCl or raised to 12 with NaOH. ¹H NMR spectra were then recorded straight away at pH 2, 5.5 and 12 (Figure 7). At pH 5.5 both aldehyde and imine functions are clearly visible at 8.3 and 7.9 ppm, respectively. At pH 2, the peak corresponding to imine group totally disappeared, whereas the presence of HMF was characterized by the furanic protons peaks (7.4 and 6.5 ppm) as well as by the aldehyde proton peak (9.3 ppm), in agreement with Tømmeraas et al.²¹ work. The aldehyde peaks characteristic of both AMF and COS AMF chain-end were still visible (8.2 ppm). Intensity of both aldehyde peaks allows determining that 50% of aldehydes were converted into free HMF. Finally, when increasing pH to 12, the aldehyde signal (8,1 ppm) only remained and no imines were formed.

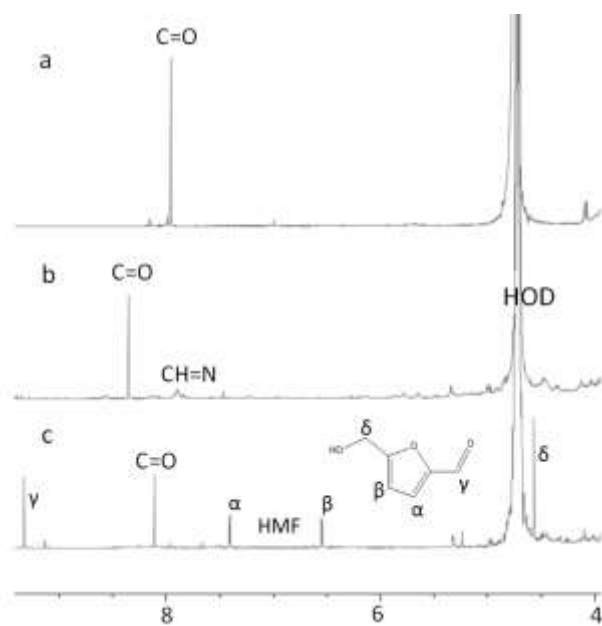
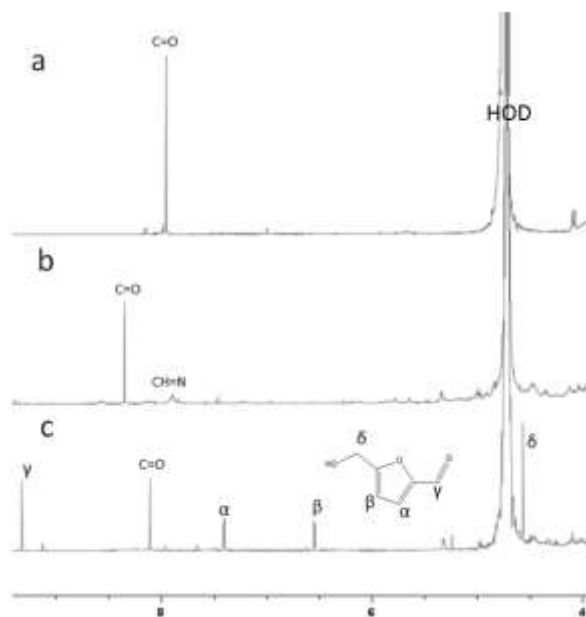


Figure 7 a) ^1H NMR spectra of COS from CH30 + 0.4 eq $\text{NaNO}_2/\text{NH}_2$ (1600 g/mol) recorded in D_2O at pH 12 (a), at pH 5.5 (b) and at pH 2 (c)

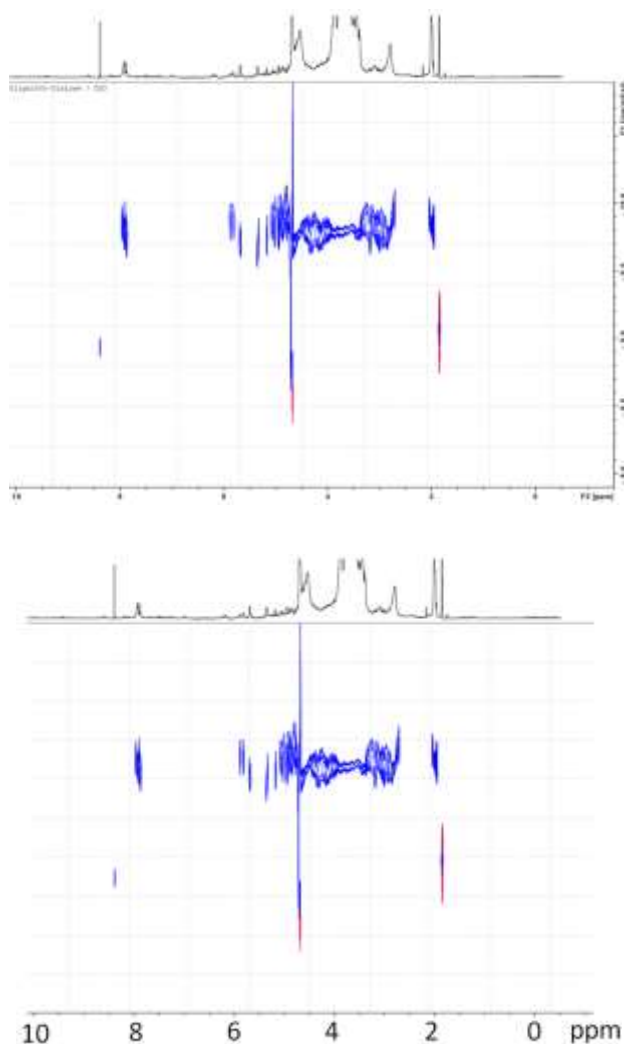


Figure 8 2D DOSY NMR of COS from CH30 + 0.4 eq NaNO₂/NH₂ (1,600 g/mol) and purified by 24h dialysis

Finally, COS were purified by dialysis during 24h against deionized water. COS were found to have the same chemical structure (¹H NMR SI 10) and a small increase (10%) in molar masses is visible due to elimination of smaller oligomers. Dialysis allows to extract not only unreacted NaNO₂ and CH₃CO₂Na but also free AMF and HMF. Furthermore, during COS dialysis, acetic acid was extracted, and then the pH increased. 2D DOSY NMR spectrum of COS was performed after dialysis (Figure 8) and almost no free aldehyde was observed, i.e. from AMF (<5%) (SI 10).

After dialysis, ecotoxicity of 2 COS, i.e. DP 5 and 10, was evaluated. The experiment was reproduced 4 times on a population of 5 daphnia. It was shown that after 48h, the daphnia used for the test are still mobiles for DP5 and DP10 at concentration of 100 mg/L and below (Figure 9 & SI 11). Those results assess that DP5 and DP10 are not acute toxic/ecotoxic and their CE50-24h and CE50-48h are above 100 mg/L.

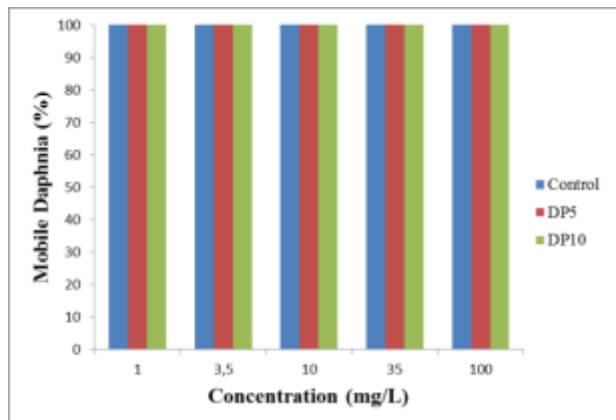


Figure 9. Mobility of daphnia after 48h against sample concentration

CONCLUSION

In this study, water soluble chitosan oligomers of different size (DP5 to DP20) were obtained by nitrous deamination. The impact of the DA, the MW and the origin (marines and insects) of crude chitosan was studied. The kinetics of the reaction was also studied. The ideal reaction parameters were determined to be a 3h reaction at 50°C. It was shown that the method is reproducible and does not depend on the chitosan nature. The relationship between the quantity of NaNO_2 and the DP of oligomers was optimized for low DP of COS. It was shown that not only AMF end chains but also free AMF was formed during the reaction. Those aldehydes react with the free amines of COS to form imines. The pH dependency of the imine formation was underlined. It is feasible to reduce imine concentration or avoid its formation by changing the pH. As well, dialysis allows decreasing the content of free AMF that might react with the free amines of COS and reduces imine formation. At

the end, the purified oligomers show no acute toxicity or ecotoxicity, which make them suitable for various interesting applications.

SUPPORTING INFORMATION

- ¹H NMR spectrum of D-(+)-Glucosamine hydrochloride
- ¹H NMR spectrum of AMF
- ¹H NMR spectrum of oligomer 1600 g/mol from CH30 + 0.4 eq NaNO₂/NH₂
- ¹H NMR spectrum of crude chitosan CH30
- ¹H NMR spectrum of chitosan oligomer from CH30 + 0.2 eq NaNO₂/NH₂
- IR spectra of both chitosan CH30 and chitosan oligomers of 1,600 g/mol
- Chromatograms from headspace chromatography of AMF, glucosamine and chitosan oligomers and their respective attribution data.
- ¹H NMR spectrum of oligomer 1600 g/mol from CH30 + 0.4 eq NaNO₂/NH₂ post dialysis
- Number of daphnia mobiles for DP5 and DP10 at 100 mg/L after 48h and inhibition percentage against control sample

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Author Contributions

The manuscript was written through contributions of all authors. The experiment were performed by C.C.. S.T. helped with triple detection chromatography. G.D. and M.DLF. helped with headspace chromatography. C.C. and Gh.D. wrote the manuscript in-depth editing from C.N., S.C. and contributions from all authors. All authors have given approval to the final version of the manuscript.

Funding Sources

This work was funded by a private funding and a CIFRE grand from the ANRT.

Notes

The authors declare no conflict of interest.

ACKNOWLEDGMENT

The authors want to thank Agnes CREPET for triple detection SEC and data analysis and Aurelien LEBRUN for performing 2D NMR analysis.

ABBREVIATIONS

SEC: size exclusion chromatography; FTIR: Fourier transform infrared spectroscopy; DA: degree of *N*-acetylation; COS: chitooligosaccharides; AMF: 2,5 anhydromannofuranose HMF: 5-hydroxymethyl-2-furfural; DP degree of polymerization

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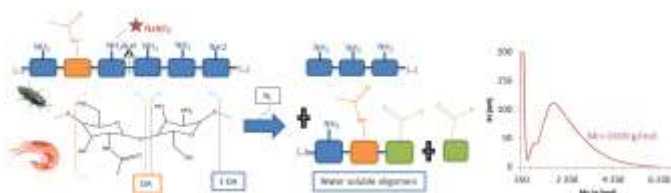
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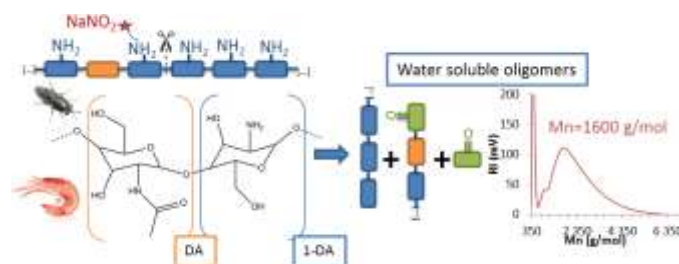
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