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## Water-Soluble 2,5-Anhydro- d -mannofuranose Chain End Chitosan Oligomers of a Very Low Molecular Weight: Synthesis and Characterization

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

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11

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

13

14 ABSTRACT

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

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

26

## 27 INTRODUCTION

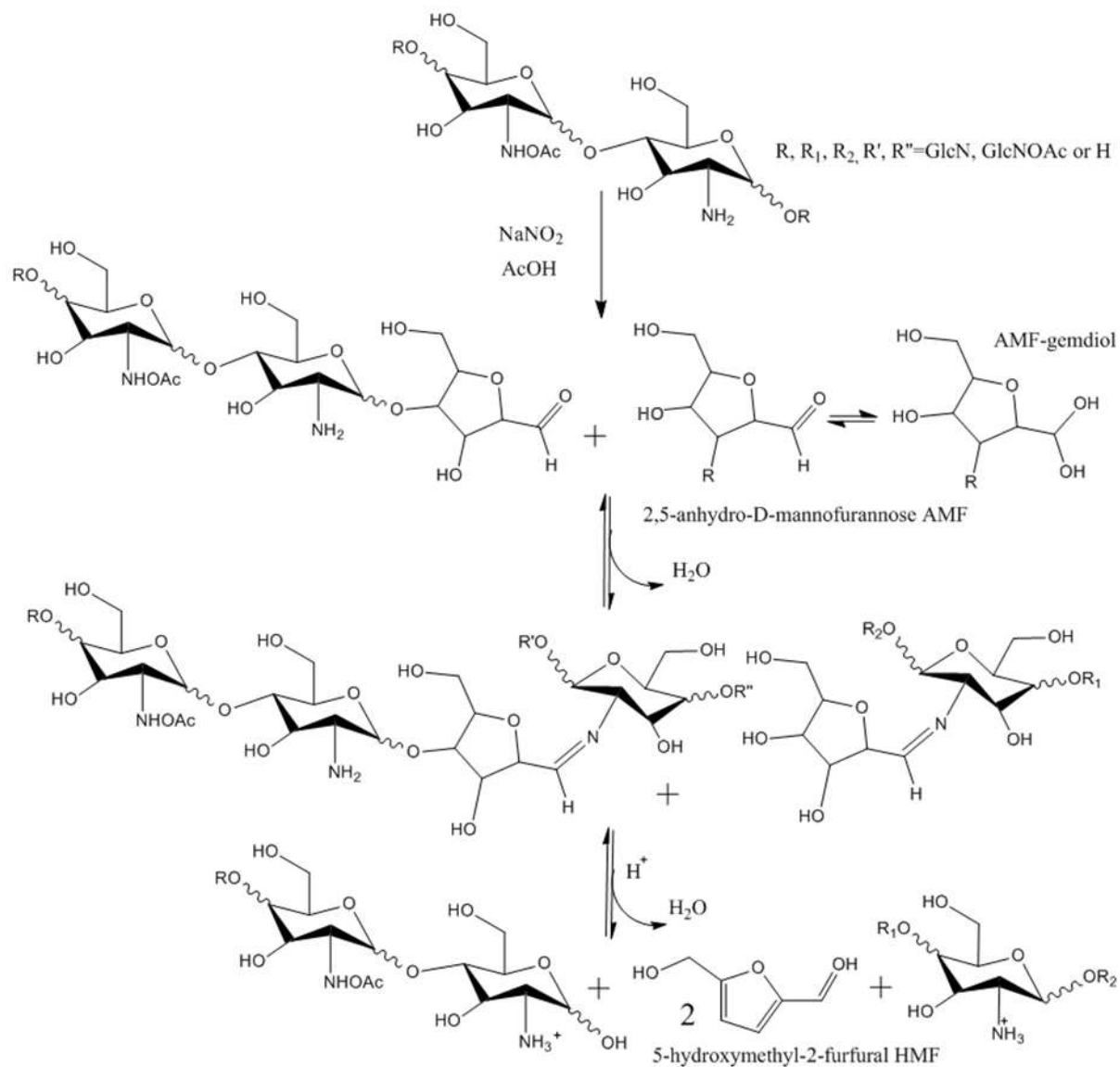
28 Chitosan is a polysaccharide constituted of β-D-glucosamine and N-acetyl-β-D-glucosamine  
29 units linked by a glycosidic bond (1→4). It is the *N*-deacetylation of chitin, a polymer extracted from  
30 shrimp shells, squids, insects or mushrooms, poorly processable due to its insolubility in most solvent  
31 including water that leads to chitosan. Chitin and chitosan have the same chemical structure and differ  
32 only by the proportion of *N*-acetylated units along the chain, >60% for chitin and <60% for chitosan.  
33 In fine, chitosan is characterized by its source (crustacean, insects, fungi...), its *N*-acetylation degree  
34 (DA) and its molar mass. A wide range of chitosan exists with various molar masses from 100,000  
35 g/mol up to 500,000 g/mol with acetylation degree between 0 and 30 %<sup>1</sup>. Many interesting  
36 applications result from use of chitosan<sup>2 3 4 5</sup>. Nevertheless, chitosan use is limited due to its high  
37 viscosity, its insolubility in organic solutions and poor solubility in aqueous solution owing to the  
38 pH<sup>6</sup>. Hence, a flourishing interest for lowering the molar masses to obtain chitosan oligomers  
39 appeared during the last decade<sup>7 8 9</sup>.

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

42 pharmaceuticals and cosmetics. COS are often defined as chitosans with degree of polymerization DP  
43 less than 20<sup>10</sup>, i.e. with average molar masses lower than 4,000 g/mol. COS are either composed of  
44  $\beta$ -D-glucosamine units or, can be a mixture of  $\beta$ -D-glucosamine and acetyl- $\beta$ -D-glucosamine  
45 randomly organized. COS are defined by some unique properties such as water solubility, cell  
46 membrane penetrability, easy absorption and various biological activities. It is also worth to note that  
47 COS are easier to process, due to less chain entanglement and steric hindrance which confer them a  
48 direct solubility in water for DP<20<sup>5 8</sup>. COS are insoluble in acetone, butanol, ethanol, ethyl acetate,  
49 propanol and pyridine but as opposed to chitosan, partially soluble in methanol and in dimethyl  
50 sulfoxide and dimethyl formamide when heated<sup>11</sup>. Furthermore for these oligomers, the smaller are  
51 the molar masses, the better is the solubility. In addition, the utilization of COS allows to reduce the  
52 viscosity and to work with higher concentration. COS are reported to treat asthma, prevent tumor  
53 growth, malaria, control blood pressure and also have neuroprotective effects for Alzheimer disease  
54 treatment thanks to their biological activities, that include antibacterial, antifungal, antiviral, anti-  
55 tumor and antioxidant properties, which have been reported to overcome those of chitosan<sup>9 12 13 14 15</sup>.

56 Several ways to reduce chitosan molar masses including enzymatic, physical or chemical  
57 depolymerization have been studied over the last decades<sup>8 16 17 18 19</sup>. Enzymatic methods are really  
58 precise but very expensive and hardly applicable on large scale. Physical depolymerizations with  
59 microwave or sonication do not lead to oligomers and are mostly used to reduce molar masses and  
60 viscosity of chitosan solutions. They are often combined with chemicals like peroxide or concentrated  
61 acid to boost the depolymerization. Those methods are hardly controllable and reproducible. Allan  
62 and al.<sup>20</sup> developed a depolymerization method, based on the use of sodium nitrite (NaNO<sub>2</sub>). The  
63 principle is based on the action of nitrous acid, which cleaves the glycosidic linkages of chitosan. The  
64 reaction consists in the formation of a diazonium ion on a D-glucosamine unit by reaction between  
65 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<sub>2</sub> 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  $\text{NaNO}_2$ . Later on, Tømmeraas et al.<sup>21</sup> 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 al<sup>22</sup>, 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<sup>23 24</sup>, 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)<sup>25</sup>. 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 activities<sup>15</sup>. 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

95 performed using infrared spectroscopy (IR),  $^1\text{H}$  and 2D DOSY NMR spectroscopy, and headspace  
96 chromatography.

## 97 MATERIALS AND METHODS

### 98 **Materials**

99  
100 Three different shrimp chitosan DA around 10-15% CH30 (Mw 30 kg/mol), CH150 (Mw 150  
101 kg/mol), CH250 (Mw 250 kg/mol) were purchased from Glentham Science, chitosan with DA equal  
102 to 1% CHDA1 (150 kg/mol) was purchased from Mathani chitosan, insect chitosan with DA equal to  
103 20% CHY (Mw 180 kg/mol) was purchased from Ynsect and used as received. Acetic acid (AcOH),  
104 sodium acetate (NaAc), sodium nitrite ( $\text{NaNO}_2$ ), lithium bromide (LiBr), sodium azide ( $\text{NaN}_3$ ),  
105 sodium hydroxide (NaOH), hydrochloric acid (HCl), acetone (>98%) were purchased from Sigma  
106 and used without purification, HPLC water (>99%) was purchased from VWR.

### 107 **Depolymerization of chitosan**

108  
109 The depolymerization of high molecular weight chitosans was performed by nitrous  
110 deamination involving  $\text{NaNO}_2$  as previously described <sup>21</sup>. Briefly, 1g (w/w) of chitosan was dissolved  
111 in 80 mL of AcOH (350 $\mu\text{L}$  in 90 mL of deionized water) solution overnight (pH 4.5). Once  
112 solubilized, a freshly prepared solution (10 mL) containing the desired equivalent ratio of  
113  $\text{NaNO}_2/\text{NH}_2$  (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 eq) was added to the solution. The solution was stirred at  
114 definite temperature for a definite time. Oligomers of DP>20 (made with 0.1 eq of  $\text{NaNO}_2/\text{NH}_2$ ) were  
115 precipitated by addition of 1M solution of NaOH and washed several times with deionized water until  
116 neutral pH. Oligomers with DP<20 did not precipitate with the addition of NaOH. The solutions were  
117 then freeze dried for 24h and the lyophilisates were dried under vacuum for 24h. The kinetics of the  
118 reaction was investigated for the five different chitosans. For that, chitosans were dissolved in  
119 deionized water with 1eq AcOH/ $\text{NH}_2$ , 0.2 eq of  $\text{NaNO}_2/\text{NH}_2$  were added and the reaction was led at

120 room temperature (30°C). Samples were taken after 30 min, 1 h, 2 h, 3 h, 6 h and 24 h, freeze dried  
121 and analyzed using SEC with a PEO calibration. The same experiment was reproduced for CH150 at  
122 10°C, 30°C, 50°C and 70°C in order to investigate influence of temperature on the reaction. Dialysis  
123 was performed using Spectra/Por dialysis membrane Biotech CE tubing MWCO: 100-500 D. <sup>1</sup>H  
124 NMR of 1600 g/mol oligomer, D<sub>2</sub>O, 400 MHz, 298 K, pH 5.5: δ 8.2-8.5 (s, H<sub>aldehydes</sub>), 8-7.1 (H<sub>imines</sub>),  
125 5.0 (d, 5 Hz, 1H H<sub>1 gemdiol</sub>), 3.2-4.4 (m, 1H H<sub>2 acetyl</sub>, 1H H<sub>3</sub>, 1H H<sub>4</sub>, 1H H<sub>5</sub>, 2H H<sub>6</sub>), 2.5 (m, 1H, H<sub>2</sub>), 2.0  
126 (s, 3H, H<sub>CH<sub>3</sub></sub>), 4.8 (s, HOD). 23 % of the amines are in imines form. 51% of the aldehydes (free or  
127 chain end) have reacted with an amine to form imines, 21% are in gem diol form and 28% are in  
128 aldehyde form (SI 3).

### 129 **AMF synthesis**

130 AMF was synthesized by action of nitrous acid on D-glucosamine. Briefly, 1 g (4.6 mmol, 1  
131 eq) of D-(+)-Glucosamine hydrochloride was dissolved in 20 mL of deionized water during 3h.  
132 Sodium nitrite (1 g, 14.5 mmol, 3.2 eq) was added and the reaction was led at room temperature for  
133 6h. The final product was lyophilized for at least 24h. <sup>1</sup>H NMR for D-glucosamine (D<sub>2</sub>O, 400 MHz,  
134 298 K, pH 5.5) : δ 5.42 (d 4Hz, 1H, H<sub>1α</sub>), 4.9 (d 8Hz, 1H, H<sub>1β</sub>), 3.4-3.9 (m, 1H H<sub>3</sub>, 1H H<sub>4</sub>, 1H H<sub>5</sub>, 2H  
135 H<sub>6</sub>), 3.3 (dd 10Hz, 1H, H<sub>2α</sub>), 2.9 (dd 10Hz, 1H, H<sub>2β</sub>), 4.8 (HOD). <sup>1</sup>H NMR for AMF (D<sub>2</sub>O, 400 MHz,  
136 298 K, pH 5.5) : δ 8.5 (s, 1H, H<sub>1</sub>), 5.1 (d, 5Hz, 1H, H<sub>1 gemdiol</sub>), 4.2 (t 6Hz, 1H, H<sub>2</sub>), 4.1 (t 6Hz, 1H,  
137 H<sub>3</sub>), 4.0 (m, 1H, H<sub>5</sub>), 3.8 (m, 1H, H<sub>4</sub> 2H, H<sub>6</sub>) as expected<sup>26</sup>. In deuterated water, AMF is 70% in  
138 gem-diol and 30% of aldehyde form (SI 1 & 2).

### 139 **Characterization**

140 <sup>1</sup>H NMR, analyses were performed using a Bruker Avance 400 MHz NMR spectrometer at  
141 298 K. 2D DOSY NMR was performed on a 600MHz Bruker Avance spectrometer. NMR samples  
142 were prepared by dissolving 15 mg of product in 0.5 mL of D<sub>2</sub>O. The chemical shifts were reported in  
143 part per million relative to tetra methylsilane. Spin multiplicity is shown by s=singlet, d=doublet,  
144 t=triplet, q=quadrat and m=multiple. DA of native chitosan and oligomers were determined using <sup>1</sup>H



145 NMR. FTIR spectra were recorded on Nicolet 6700 FTIR spectrometer. GC-MS analysis were  
146 performed on GC 7890B AGILENT equipped with a capillary column Agilent 122-1364UI DB-  
147 624UI (60 m x 0.25 mm, 1.4  $\mu$ m), with FID detector and mass spectrometer 5977B AGILENT. GC is  
148 directly linked to the headspace sampler 7697A Agilent, with an injection loop (1000  $\mu$ L) and a  
149 transfer line. dn/dc were calculated using the differential refractive index (RI) detector Wyatt Optilab  
150 T-rEX. Molar masses were recorded using triple detection size exclusion chromatography (Tosoh  
151 TSK PW 2500, TSK PW 6000, TSK gel G Oligo PW) coupled with the same RI detector Wyatt  
152 Optilab T-rEX, multiangle light scattering Wyatt DAWN HELEOS II, and a Fused Silica cell, laser  
153  $\lambda=664$  nm. They were also recorded using size exclusion chromatography with RI detection (PL  
154 aquagel-OH 30 8 $\mu$ m 100-60.000 g/mol, PL aquagel-OH 40 8  $\mu$ m 10.000-200.000 g/mol, TSK gel G  
155 Oligo PW 7 $\mu$ m 7.8 mm ID  $\times$ 30 cm 0-6000 g/mol) calibrated using PEO/PEG standards. 10 mg/mL  
156 solutions of oligochitosans were prepared in acetate buffer (1.5% AcOH, 0.8% NaAc, 1 ppm NaN<sub>3</sub>, 5  
157 ppm LiBr). Error were calculated by doing standard deviation on three different integration on each  
158 chromatogram and data plotted are the average value of those measurement. The pH (correction for  
159 isotopic effect: pD=pH+0.4) were measured by use of a Mettler Toledo pH electrode.

160 In order to validate the PEO equivalent method for molar masses measurement, triple  
161 detection SEC was performed. To do that, variation of refractive index according to concentration,  
162 dn/dc, were measured using a differential refractometer Wyatt Optilab T-rEX. 1mg/mL  
163 chitosan/oligochitosan solutions were prepared in degassed acetate buffer (AcOH 0.2M/0.15M  
164 ammonium acetate; pH=4.5) and diluted at 80%, 60%, 40%, 20%, 10% (w/w). Refractive indexes  
165 were measured according to solution concentration (658 nm, 0.5 mL/min). After that, 3mg/mL  
166 chitosan/oligochitosan solutions were prepared in the acetate buffer from above. 100 $\mu$ L of solution  
167 were filtrated on 0.45 $\mu$ m membrane CME filter and injected in the apparatus made of Tosoh TSK PW  
168 2500 TSK PW 6000 coupled with a differential refractometer Wyatt Optilab T-rEX, multi angular  
169 light scattering Wyatt DAWN HELEOS II, and UV cellule Fused Silica, laser  $\lambda=664$  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) 
$$DPn = \frac{Mn}{DA*203 + (1-DA)*162}$$

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
<b>CH30</b>	≥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
<b>CH150</b>	≥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
<b>CH250</b>	≥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
<b>CHY</b>	≥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
<b>CHDA1</b>	≥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 <sub>2</sub> /GlcN unit		Mw(g/mol)		Mn (g/mol)		Dispersity		DPn	
<b>CH30</b>	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
<b>CH30</b>	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
<b>CH30</b>	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
<b>CH150</b>	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
<b>CH150</b>	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
<b>CH150</b>	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
<b>CH250</b>	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
<b>CH250</b>	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
<b>CH250</b>	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		
<b>CHY240</b>	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
<b>CHY240</b>	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
<b>CHY240</b>	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

173

174 **Table 1.** DA, dn/dc, molar masses and dispersity of crude chitosan and chitosan oligomers obtained with  
 175 action of NaNO<sub>2</sub> during 3h at 50°C.

176 Toxicity and ecotoxicity of oligomers post dialysis (DP5 and DP10) were evaluated  
 177 according to acute tests on *Daphnia magna* (NF EN ISO 6341, 2012) performed by Eurofins. This  
 178 test aims at finding the concentration immobilizing 50% of the daphnia population after 24h and 48h  
 179 (CE50). A solution of K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> CE50-24h = 0,94 mg/L (this value corresponds to standard between  
 180 0.60 mg/L and 2,1 mg/L according to NF EN ISO 6341 : 2012) is used as a reference substance.  
 181 Samples were prepared by dissolving oligomers in an ultra-pure water solution of anhydrous sodium  
 182 hydrogen carbonate NaHCO<sub>3</sub> (0.200 g/L), calcium chloride CaCl<sub>2</sub> (0.260 g/L), magnesium chloride  
 183 MgCl<sub>2</sub>, 6 H<sub>2</sub>O (0.148 g/L), anhydrous potassium sulfate K<sub>2</sub>SO<sub>4</sub> (0.026 g/L). 100 mg/L solutions were  
 184 prepared and diluted as followed: 35%, 10%, 3.5%, and 1% (w/w). Tests were performed at 20 °C.

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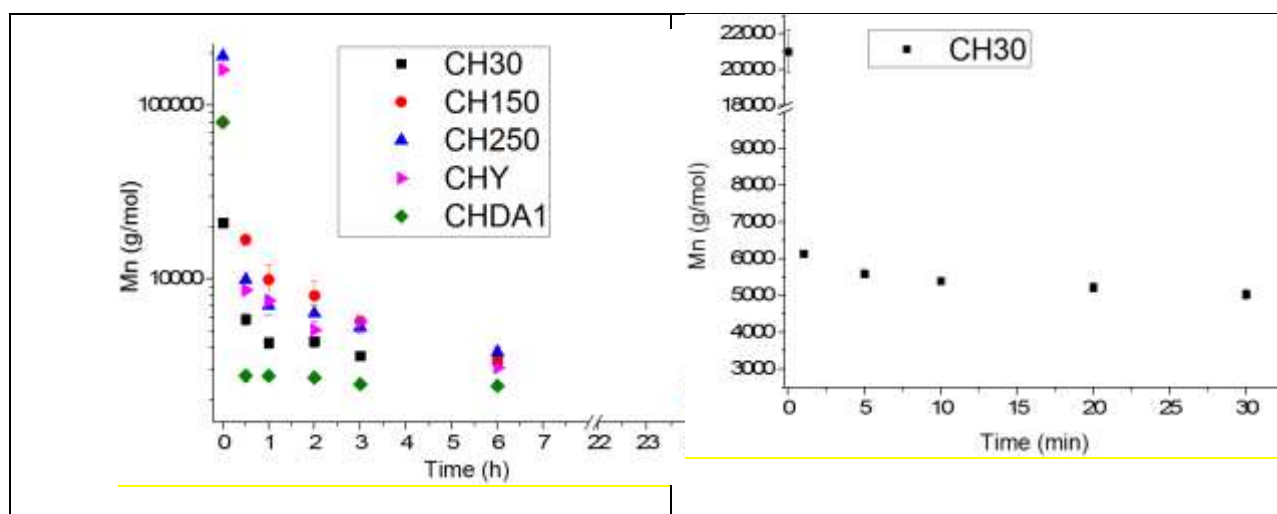
## 186 RESULTS AND DISCUSSION

187 Depolymerization using NaNO<sub>2</sub> was investigated from 5 different chitosans. 3 of them, obtained  
 188 from crustaceous, have the same DA (i.e. 16%) and only differ by their Mw: 30, 150 and 250 kg/mol.  
 189 They will be labeled as CH30, CH150 and CH250. The fourth chitosan, also obtained from  
 190 crustaceous and labeled CHDA1, has a Mw of 150 kg/mol with DA of 1%. The last studied chitosan,  
 191 labeled CHY, has a Mw of 240 kg/mol, DA of 20% and was provided from insects.

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

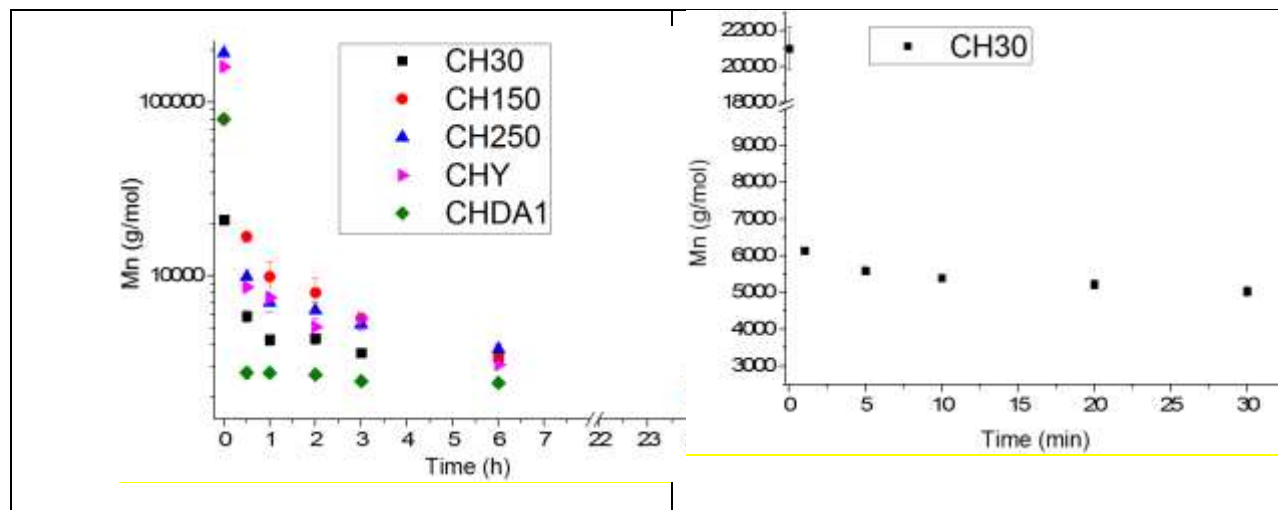
200 Mw and Mn values for each chitosan oligomer, as well as the corresponding crude ones, are  
201 given in Table 1. It was especially shown that both methods afford rather close values when targeted  
202 DP below 20. Above this value and also for crude chitosans, SEC using PEO/PEG standards seems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

206 At first, each chitosan was reacted with 0.2eq of NaNO<sub>2</sub> at room temperature (30 °C) and molar  
207 masses decrease was followed over time (



208

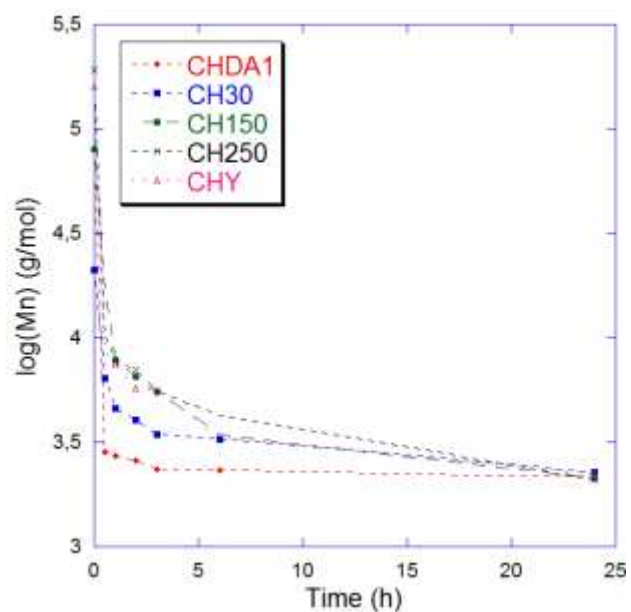
209 Figure 1 a)). Mn of CH30, CH150, CH250 and CHY showed a similar trend, i.e. a very fast  
210 decrease during the first 30 min until approximately reaching 6,000 g/mol, then Mn slightly decreased  
211 until stabilization. It was shown that the reaction is immediate, the most important drop of molar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



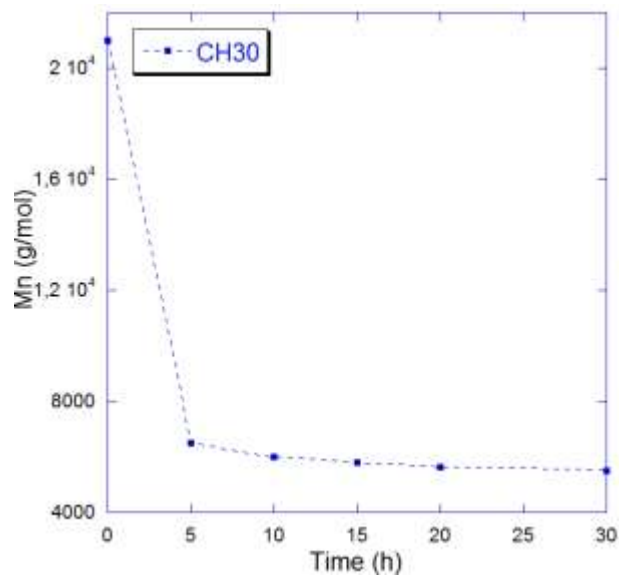
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214 Figure 1 b)). The plateau was reached when either  $\text{NaNO}_2$  was fully consumed or did not react  
215 anymore. CHDA1 behaves slightly differently since molar masses decrease faster to reach 2,500  
216 g/mol after 30 min. According to the mechanism (scheme 1), this faster decrease can be easily  
217 explained by the fact that CHDA1 has more free amines available for reaction with  $\text{NaNO}_2$ , thus  
218 leading to more glycosidic bond cleavages. Consequently, CHDA1 reaches a plateau after only 6 h,  
219 whereas 24h are needed for the four other chitosans. The kinetics of the reaction also depends on  
220 crude chitosan Mn: CH30 molar masses decreases faster than that of CH150 and CH250. This is  
221 probably due to the higher viscosity for higher Mn chitosans in solution. It was also shown that  
222 dispersity of the different chitosans decreases during the depolymerization process to reach  
223 interesting values ranging between 1.3 and 1.6 (Figure 2).

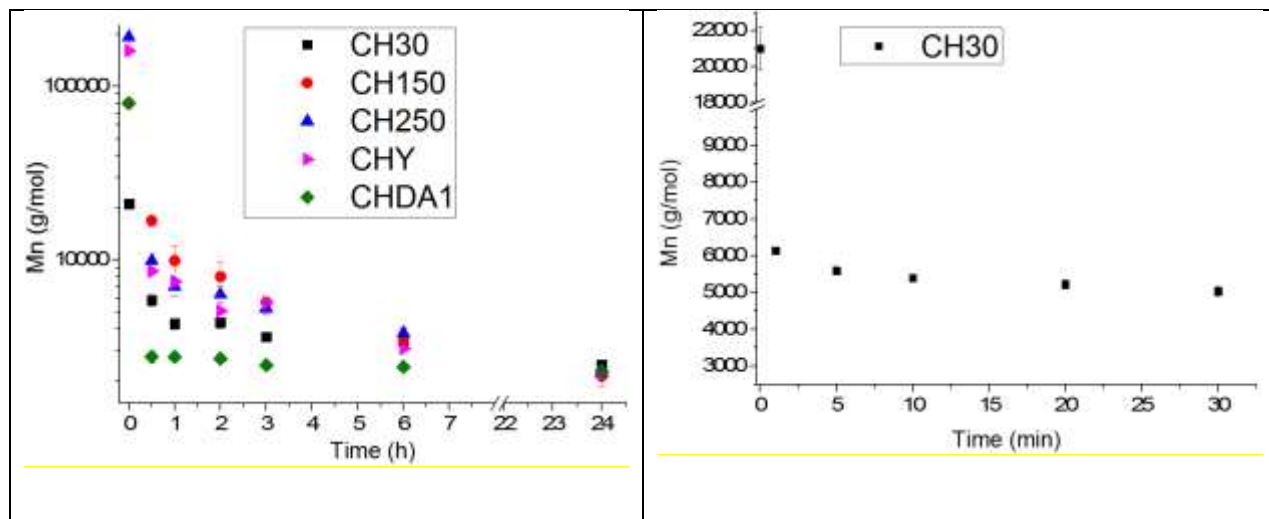
a)



b)



224

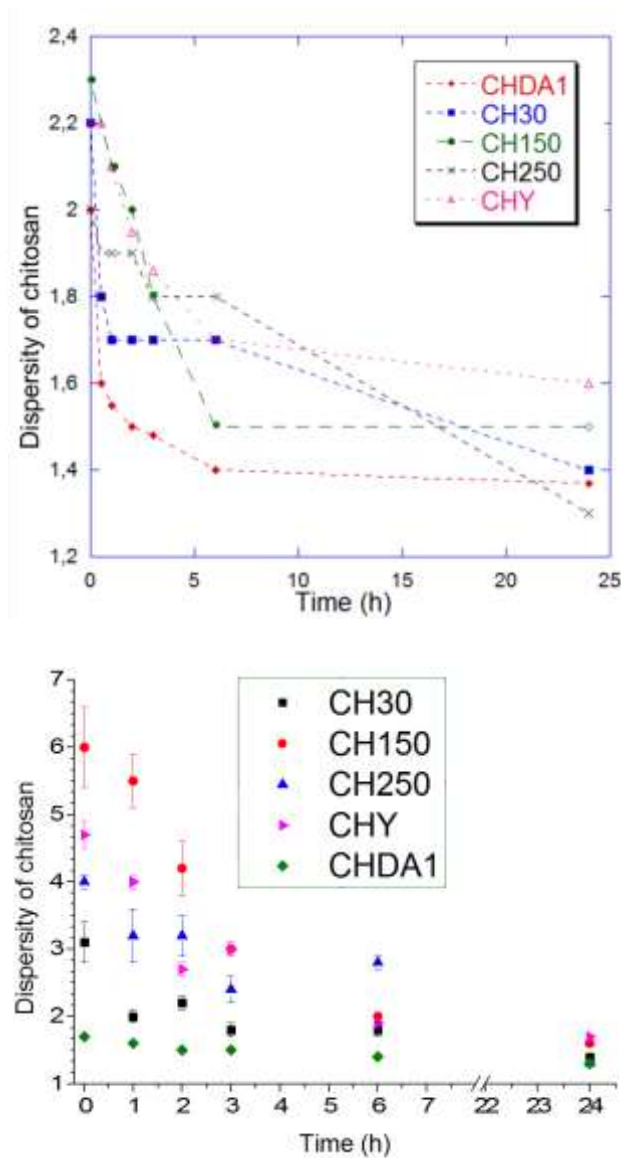


225

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 NaNO<sub>2</sub>/NH<sub>2</sub> b) Evolution of molar masses as a function of time for CH30 with 0.2 eq of NaNO<sub>2</sub>/NH<sub>2</sub> during the first 30 min

228



229

230 **Figure 2** Evolution of the dispersity as a function of time for five different chitosans (1% w/w) with 0.2eq of NaNO<sub>2</sub>/NH<sub>2</sub>

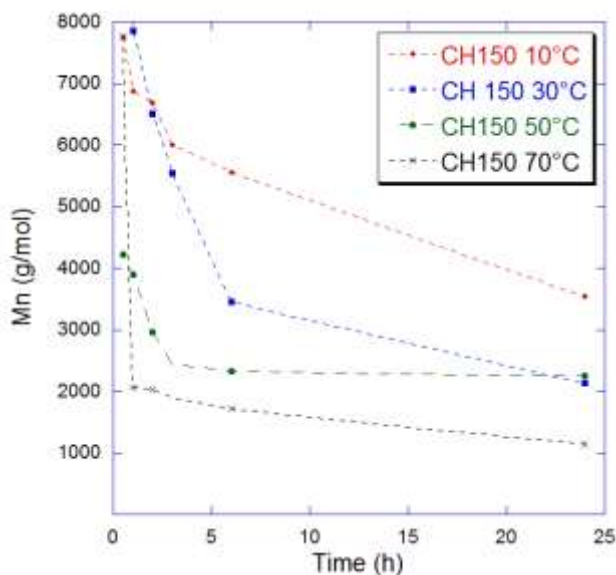
231

232 This reaction was carried out at room temperature, as already described in the literature<sup>21</sup>.  
233 Nevertheless, an increase of the temperature should impact the reaction kinetics but also may lead to  
234 reaction completion, until reaching lower molar masses COS, i.e. DP<20.

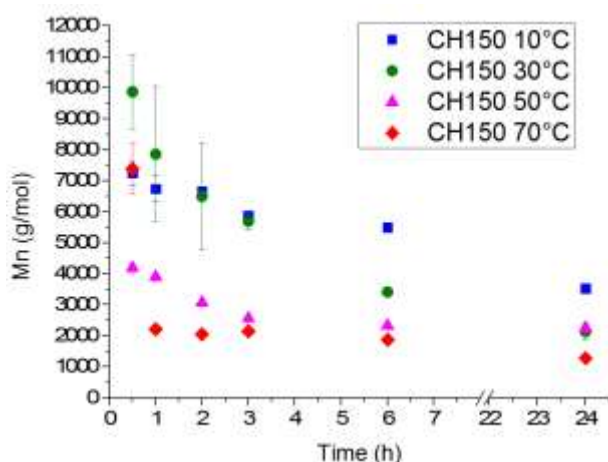
235 The reaction was carried out with CH150, with 0.2 eq of NaNO<sub>2</sub> at 4 different temperatures,  
236 i.e. 10, 30, 50 and 70°C (Figure 3). At 10°C, the reaction is not completed even after 24h. Indeed, at

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





245



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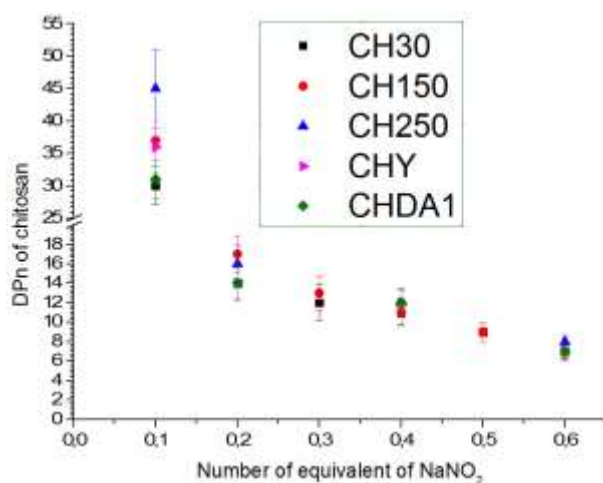
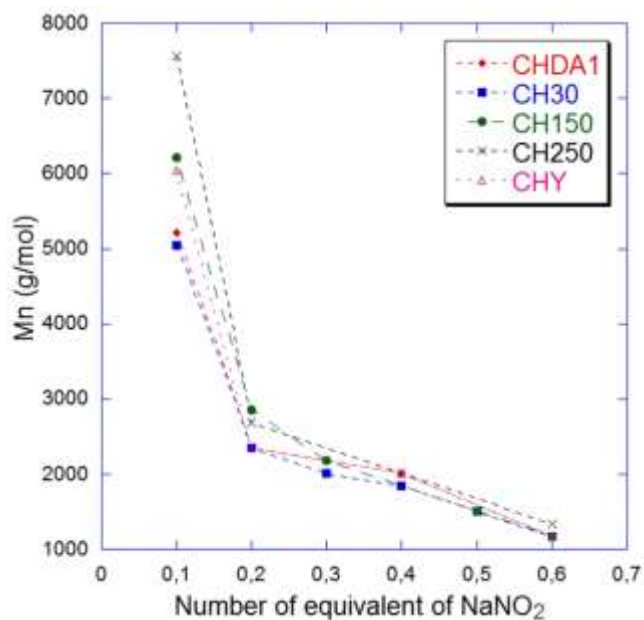
247

248 **Figure 3** Evolution of Mn vs time at different temperatures for the reaction of CH150 with 0.2eq of NaNO<sub>2</sub>/NH<sub>2</sub>

249

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

255 COS can be purified by precipitation into a strong base to obtain COS with DP ranging between 30 to  
256 45 at 0.1 eq NaNO<sub>2</sub>/NH<sub>2</sub>. Above 0.2 eq NaNO<sub>2</sub>/NH<sub>2</sub>, Mw COS slowly decreased by increasing  
257 NaNO<sub>2</sub> content. Interestingly, the resulting COS showed DP ranging from 15 to 5 and were water  
258 soluble (pH 5.5). Thus, as already demonstrated by Allan et al.<sup>20</sup>, a defined number of equivalents of  
259 NaNO<sub>2</sub>/NH<sub>2</sub> allows obtaining COS with DP values from 5 to 20. Furthermore, this reaction is clearly  
260 not affected neither by molar masses (between 30 kg/mol and 250 kg/mol), nor DA (between 1 and  
261 20%) or origin of crude chitosans.



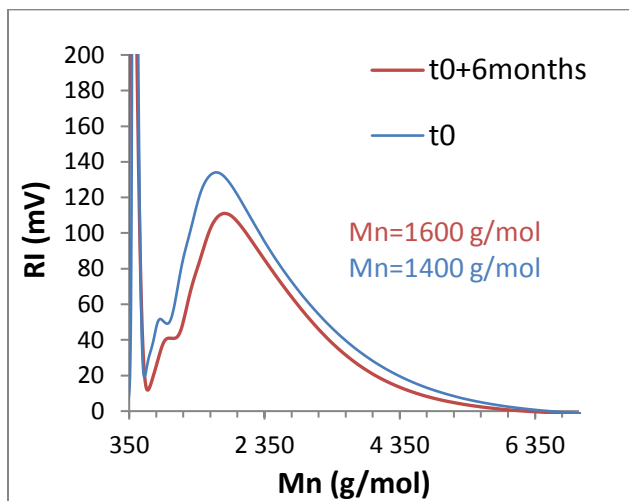
262

263

264 **Figure 4.** Evolution of Mn vs NaNO<sub>2</sub>/NH<sub>2</sub> equivalents for the depolymerization of CH30, CH150, CH250, CHDA1 and CHY at  
 265 50°C for 3h.

266 To check their stability over time, the resulting COS with DP ranging from 5 to 20 were  
 267 analyzed by SEC after 6 months (Figure 5). No decrease was observed even after 6 months, which  
 268 proved that no further degradation occurred. The 10% difference between the two molar masses was  
 269 explained by the change of calibration curve and evolution of the column. Finally, COS obtained with  
 270 0.2 eq. of NaNO<sub>2</sub>/NH<sub>2</sub> were analyzed by FTIR and compared to the corresponding crude chitosans  
 271 (SI 6). The carbonyl band at 1650 cm<sup>-1</sup> is still visible in the oligomer spectra meaning that the

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



275

276 **Figure 5** Evolution of Mn of oligochitosan CH30 with 0.4eq of  $\text{NaNO}_2/\text{NH}_2$  from t0 to t0+6months

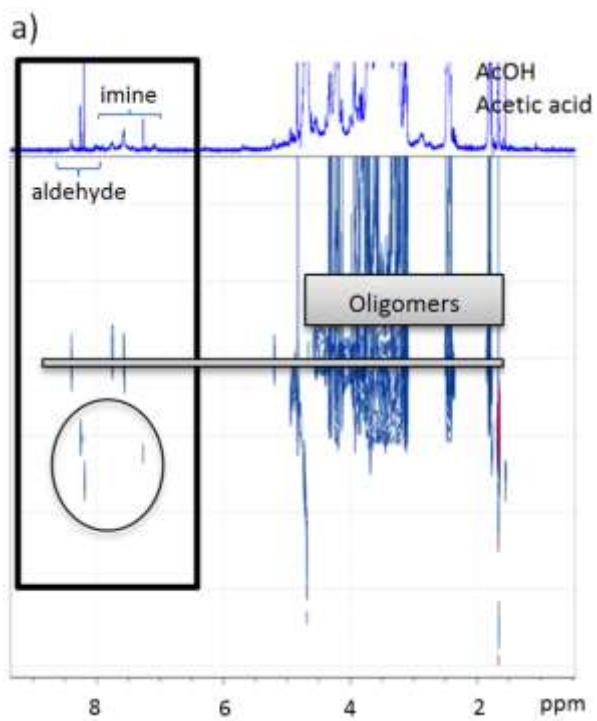
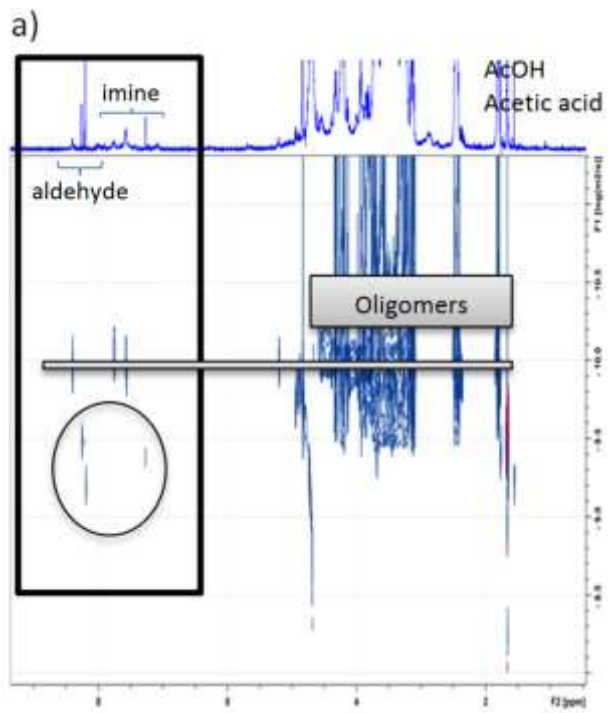
277

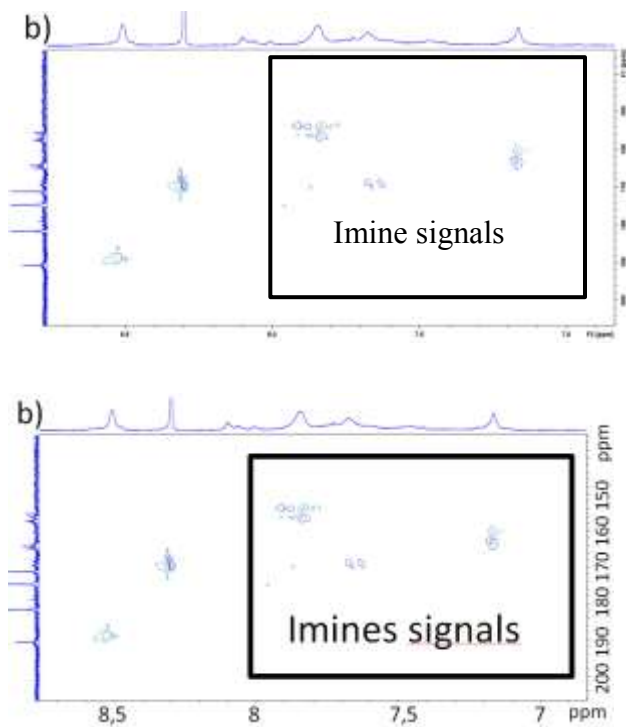
278 Tømmeraas et al.<sup>21</sup> showed that nitrous deamination leads to the formation of AMF chain end of COS  
279 (reductive end), but also to the formation of free AMF (2,5-anhydro-D-mannose) when glycosidic bond  
280 breakage occurs at the non reductive polymer chain-end. Furthermore, AMF is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

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

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

308



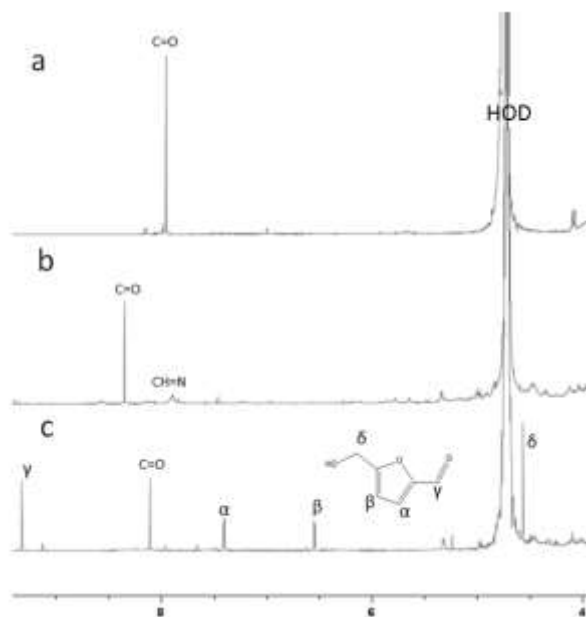


309 **Figure 6** a) 2D DOSY NMR spectrum of a 1600 g/mol oligomer obtained from COS from CH3O + 0.4 eq NaNO<sub>2</sub>/NH<sub>2</sub> (3h at  
 310 50°C) b) <sup>1</sup>H-<sup>13</sup>C 2D HSQC NMR spectrum of the aldehyde zone

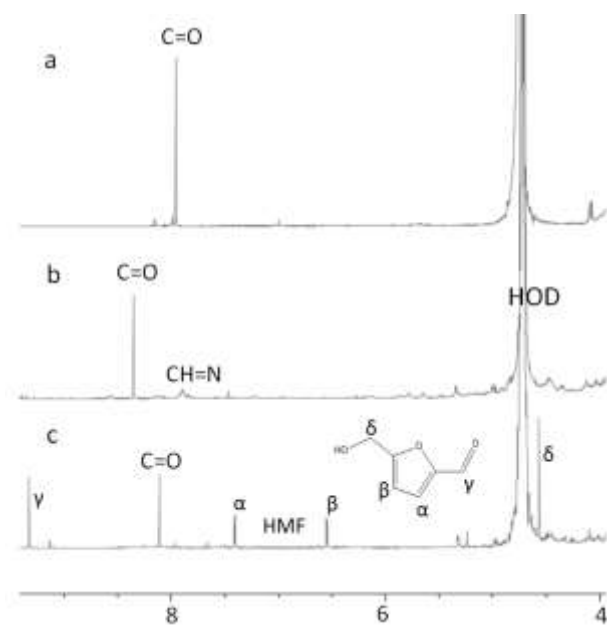
311  
 312 Consequently, the effect of pH on COS structure was investigated. COS was solubilized in  
 313 deuterated water at pH 5.5. Then, pH of the solution was either dropped to 2 with HCl or raised to 12 with  
 314 NaOH. <sup>1</sup>H NMR spectra were then recorded straight away at pH 2, 5.5 and 12 (Figure 7). At pH 5.5 both  
 315 aldehyde and imine functions are clearly visible at 8.3 and 7.9 ppm, respectively. At pH 2, the peak  
 316 corresponding to imine group totally disappeared, whereas the presence of HMF was characterized by the  
 317 furanic protons peaks (7.4 and 6.5 ppm) as well as by the aldehyde proton peak (9.3 ppm), in agreement  
 318 with Tømmeraas et al.<sup>21</sup> work. The aldehyde peaks characteristic of both AMF and COS AMF chain-end  
 319 were still visible (8.2 ppm). Intensity of both aldehyde peaks allows determining that 50% of aldehydes  
 320 were converted into free HMF. Finally, when increasing pH to 12, the aldehyde signal (8,1 ppm) only  
 321 remained and no imines were formed.

322

323



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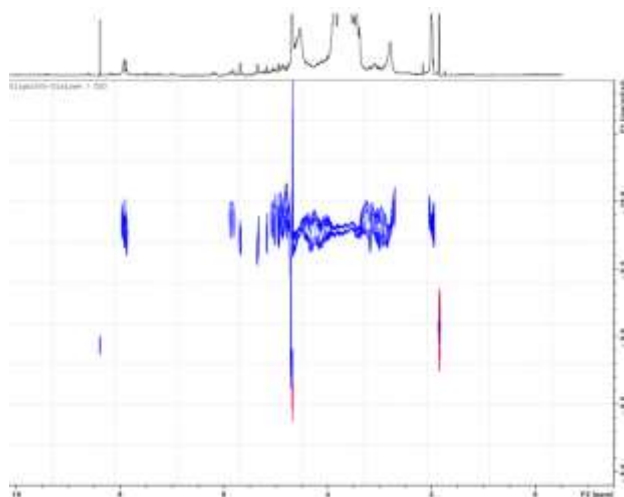
324

325 **Figure 7** a) <sup>1</sup>H NMR spectra of COS from CH3O + 0.4 eq NaNO<sub>2</sub>/NH<sub>2</sub> (1600 g/mol) recorded in D<sub>2</sub>O at pH 12 (a), at pH 5.5 (b)

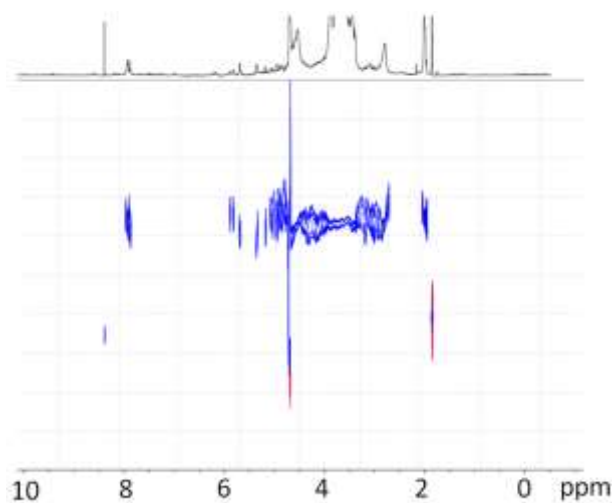
326 and at pH 2 (c)



327



328

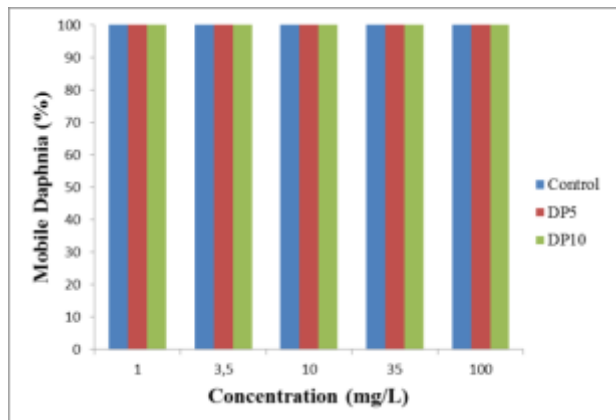


329 **Figure 8** 2D DOSY NMR of COS from CH30 + 0.4 eq NaNO<sub>2</sub>/NH<sub>2</sub> (1,600 g/mol) and purified by 24h dialysis

330

331 Finally, COS were purified by dialysis during 24h against deionized water. COS were found  
332 to have the same chemical structure (<sup>1</sup>H NMR SI 10) and a small increase (10%) in molar masses is  
333 visible due to elimination of smaller oligomers . Dialysis allows to extract not only unreacted NaNO<sub>2</sub>  
334 and CH<sub>3</sub>CO<sub>2</sub>Na but also free AMF and HMF. Furthermore, during COS dialysis, acetic acid was  
335 extracted, and then the pH increased. 2D DOSY NMR spectrum of COS was performed after dialysis  
336 (Figure 8) and almost no free aldehyde was observed, i.e. from AMF (<5%) (SI 10).

337 After dialysis, ecotoxicity of 2 COS, i.e. DP 5 and 10, was evaluated. The experiment was  
338 reproduced 4 times on a population of 5 daphnia. It was shown that after 48h, the daphnia used for the  
339 test are still mobiles for DP5 and DP10 at concentration of 100 mg/L and below (Figure 9 & SI 11).  
340 Those results assess that DP5 and DP10 are not acute toxic/ecotoxic and their CE50-24h and CE50-  
341 48h are above 100 mg/L.



342  
343  
344 **Figure 9.** Mobility of daphnia after 48h against sample concentration

## 345 346 CONCLUSION

347 In this study, water soluble chitosan oligomers of different size (DP5 to DP20) were obtained  
348 by nitrous deamination. The impact of the DA, the MW and the origin (marines and insects) of crude  
349 chitosan was studied. The kinetics of the reaction was also studied. The ideal reaction parameters  
350 were determined to be a 3h reaction at 50°C. It was shown that the method is reproducible and does  
351 not depend on the chitosan nature. The relationship between the quantity of NaNO<sub>2</sub> and the DP of  
352 oligomers was optimized for low DP of COS. It was shown that not only AMF end chains but also  
353 free AMF was formed during the reaction. Those aldehydes react with the free amines of COS to  
354 form imines. The pH dependency of the imine formation was underlined. It is feasible to reduce imine  
355 concentration or avoid its formation by changing the pH. As well, dialysis allows decreasing the  
356 content of free AMF that might react with the free amines of COS and reduces imine formation. At

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

359

360

## 361 SUPPORTING INFORMATION

- 362 • <sup>1</sup>H NMR spectrum of D-(+)-Glucosamine hydrochloride
- 363 • <sup>1</sup>H NMR spectrum of AMF
- 364 • <sup>1</sup>H NMR spectrum of oligomer 1600 g/mol from CH30 + 0.4 eq NaNO<sub>2</sub>/NH<sub>2</sub>
- 365 • <sup>1</sup>H NMR spectrum of crude chitosan CH30
- 366 • <sup>1</sup>H NMR spectrum of chitosan oligomer from CH30 + 0.2 eq NaNO<sub>2</sub>/NH<sub>2</sub>
- 367 • IR spectra of both chitosan CH30 and chitosan oligomers of 1,600 g/mol
- 368 • Chromatograms from headspace chromatography of AMF, glucosamine and chitosan oligomers  
369 and their respective attribution data.
- 370 • <sup>1</sup>H NMR spectrum of oligomer 1600 g/mol from CH30 + 0.4 eq NaNO<sub>2</sub>/NH<sub>2</sub> post dialysis
- 371 • Number of daphnia mobiles for DP5 and DP10 at 100 mg/L after 48h and inhibition percentage  
372 against control sample
- 373

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378

### 379 **Author Contributions**

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

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388 **Notes**

389 The authors declare no conflict of interest.

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393 **ABBREVIATIONS**

394 SEC: size exclusion chromatography; FTIR: Fourier transform infrared spectroscopy; DA:

395 degree of *N*-acetylation; COS: chitooligosaccharides; AMF: 2,5 anhydromannofuranose HMF: 5-

396 hydroxymethyl-2-furfural; DP degree of polymerization

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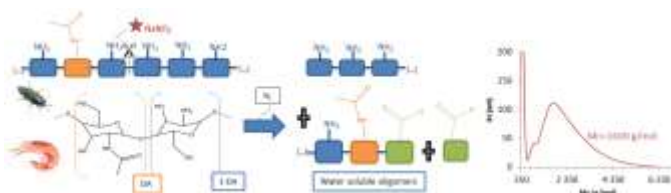
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