



HAL
open science

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

Camille Chapelle, Ghislain David, Sylvain Caillol, Claire Negrell, Graziella Durand, Myriam Desroches Le Foll, Stéphane Trombotto

► **To cite this version:**

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-02406342v1>

Submitted on 7 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

4 *Camille Chapelle¹, Ghislain David^{1*}, Sylvain Caillol¹, Claire Negrell¹, Graziella Durand²,*
5 *Myriam Desroches le Foll², Stéphane Trombotto³*

6 ¹Ingénierie et Architecture Macromoléculaire (IAM) 8 rue de l'école Normale, 34296
7 Montpellier CEDEX 5, France

8 ²CST COLAS 4, Rue Jean Mermoz CS 30504 – 78771 Magny-les-Hameaux Cedex

9 ³Ingénierie des Matériaux Polymères (IMP), CNRS UMR 5223, Université Claude Bernard Lyon
10 1, Univ Lyon, 69622 Villeurbanne, France

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_n<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 DPn of oligomers
19 only depends on the quantity of NaNO₂ 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-hydromethyl-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

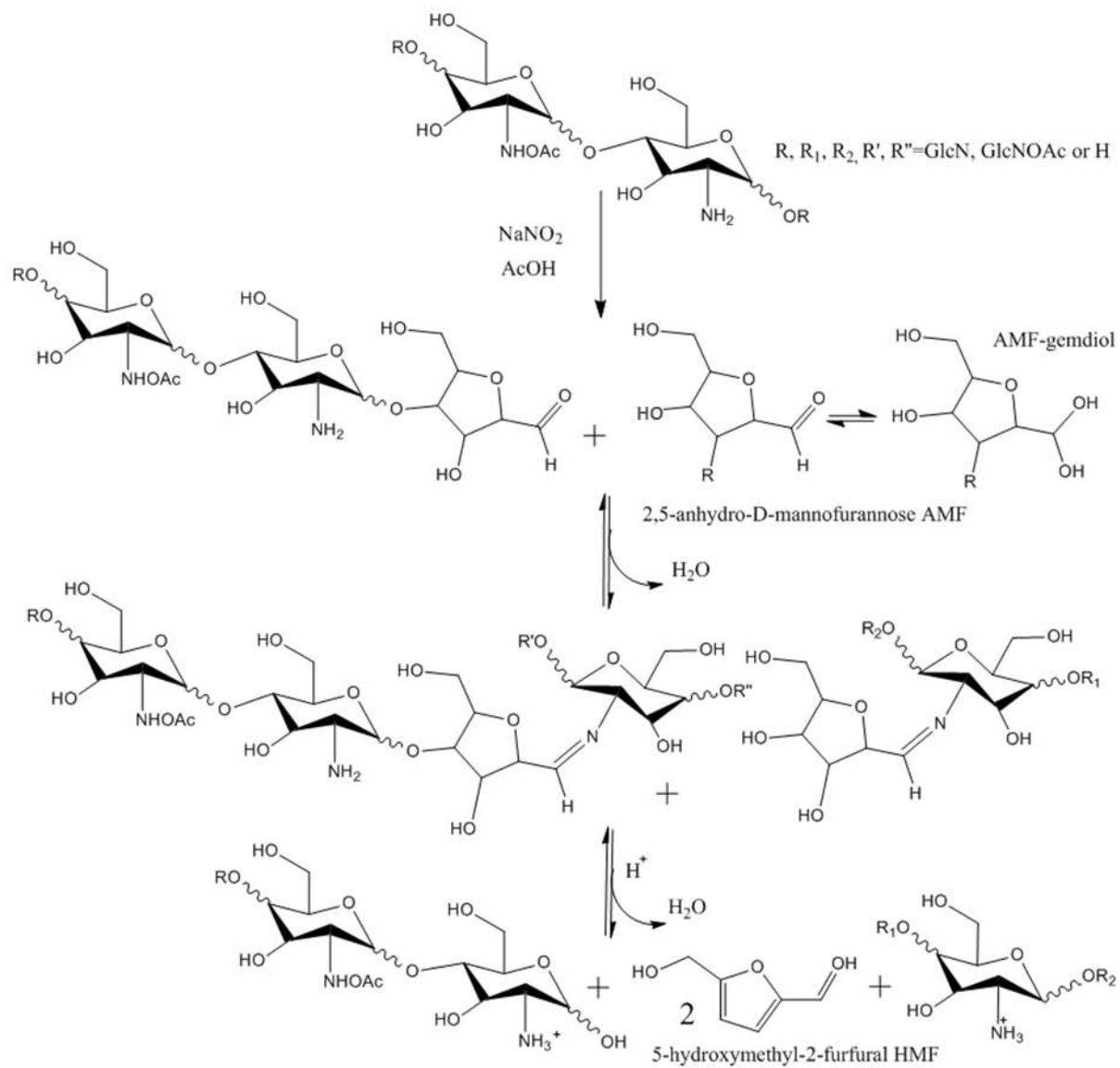
28 Chitosan is a polysaccharide constituted of β-D-glucosamine and N-acetyl-β-D-glucosamine
29 units linked by a glycosidic bond (1→4). It is the *N*-deacetylation of chitin, a polymer extracted from
30 shrimp shells, squids, insects or mushrooms, poorly processable due to its insolubility in most solvent
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 %¹. Many interesting
36 applications result from use of chitosan^{2 3 4 5}. 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 pH⁶. Hence, a flourishing interest for lowering the molar masses to obtain chitosan oligomers
39 appeared during the last decade^{7 8 9}.

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 20¹⁰, i.e. with average molar masses lower than 4,000 g/mol. COS are either composed of
44 β -D-glucosamine units or, can be a mixture of β -D-glucosamine and acetyl- β -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^{5 8}. 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 heated¹¹. 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^{9 12 13 14 15}.

56 Several ways to reduce chitosan molar masses including enzymatic, physical or chemical
57 depolymerization have been studied over the last decades^{8 16 17 18 19}. 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.²⁰ developed a depolymerization method, based on the use of sodium nitrite (NaNO₂). 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 NaNO₂ 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, Tømmeraas 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

95 performed using infrared spectroscopy (IR), ^1H 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 (NaNO_2), lithium bromide (LiBr), sodium azide (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 NaNO_2 as previously described ²¹. Briefly, 1g (w/w) of chitosan was dissolved
111 in 80 mL of AcOH (350 μL 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/ 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. ¹H
124 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}),
125 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
126 (s, 3H, H_{CH₃}), 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. ¹H NMR for D-glucosamine (D₂O, 400 MHz,
134 298 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
135 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,
136 298 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,
137 H₃), 4.0 (m, 1H, H₅), 3.8 (m, 1H, H₄ 2H, H₆) as expected²⁶. In deuterated water, AMF is 70% in
138 gem-diol and 30% of aldehyde form (SI 1 & 2).

139 **Characterization**

140 ¹H 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 D₂O. 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 ¹H

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 μ 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 μ 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 μ m 100-60.000 g/mol, PL aquagel-OH 40 8 μ m 10.000-200.000 g/mol, TSK gel G
155 Oligo PW 7 μ 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 NaN₃, 5
157 ppm LiBr). Error were calculated by doing standard deviation on three different integrations on each
158 chromatogram and data plotted are the average value of those measurements. 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 μ L of solution
167 were filtrated on 0.45 μ 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 (M_w , M_n and \bar{D}) of chitosans and oligochitosans were determined taking in account the dn/dc
 171 determined above. DP_n were calculated according to the formula (1):

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

Chitosan	DA (%)		dn/dc	M_w (kg/mol)		M_n (kg/mol)		Dispersity		DP_n	
	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	$44 \pm 4,4$	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	M_w (g/mol)		M_n (g/mol)		Dispersity		DP_n		
CH30	0.4	0.1449 ± 0.0017	$2,500 \pm 250$	$2,750 \pm 275$	$1,850 \pm 185$	$2,500 \pm 250$	1.4 ± 0.1	1.1 ± 0.1	11 ± 1	15 ± 2
CH30	0.2	0.1510 ± 0.0045	$3,900 \pm 390$	$4,150 \pm 415$	$2,350 \pm 235$	$3,500 \pm 350$	1.6 ± 0.2	1.2 ± 0.1	14 ± 1	21 ± 3
CH30	0.1	0.1539 ± 0.0009	$7,950 \pm 795$	$7,900 \pm 790$	$5,050 \pm 505$	$8,100 \pm 810$	1.6 ± 0.2	1.2 ± 0.1	30 ± 3	48 ± 5
CH150	0.4	0.1439 ± 0.0038	$2,600 \pm 260$	$2,800 \pm 280$	$1,850 \pm 185$	$2,500 \pm 250$	1.4 ± 0.1	1.1 ± 0.1	11 ± 1	15 ± 1
CH150	0.2	0.1576 ± 0.0007	$4,400 \pm 440$	$3,450 \pm 345$	$2,850 \pm 285$	$3,000 \pm 300$	1.6 ± 0.2	1.2 ± 0.1	17 ± 2	18 ± 2
CH150	0.1	0.1512 ± 0.0015	$10,200 \pm 1020$	$11,800 \pm 1180$	$6,200 \pm 620$	$10,100 \pm 1010$	1.6 ± 0.2	1.2 ± 0.1	37 ± 3	60 ± 6
CH250	0.4	0.1366 ± 0.0034	$2,600 \pm 260$	$3,000 \pm 300$	$1,850 \pm 185$	$2,500 \pm 250$	1.5 ± 0.2	1.1 ± 0.1	11 ± 1	15 ± 1
CH250	0.2	0.1423 ± 0.0027	$4,200 \pm 420$	$3,800 \pm 380$	$2,700 \pm 270$	$3,500 \pm 350$	1.6 ± 0.2	1.2 ± 0.1	16 ± 2	21 ± 2
CH250	0.1	$0.1399 \pm$	$13,600$	$17,400$	$7,600 \pm$	$13,900$	1.8 ± 0.2	$1.2 \pm$	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

173

174 **Table 1.** DA, dn/dc, molar masses and dispersity of crude chitosan and chitosan oligomers obtained with
 175 action of NaNO₂ 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 K₂Cr₂O₇ 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 NaHCO₃ (0.200 g/L), calcium chloride CaCl₂ (0.260 g/L), magnesium chloride
 183 MgCl₂, 6 H₂O (0.148 g/L), anhydrous potassium sulfate K₂SO₄ (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.

185

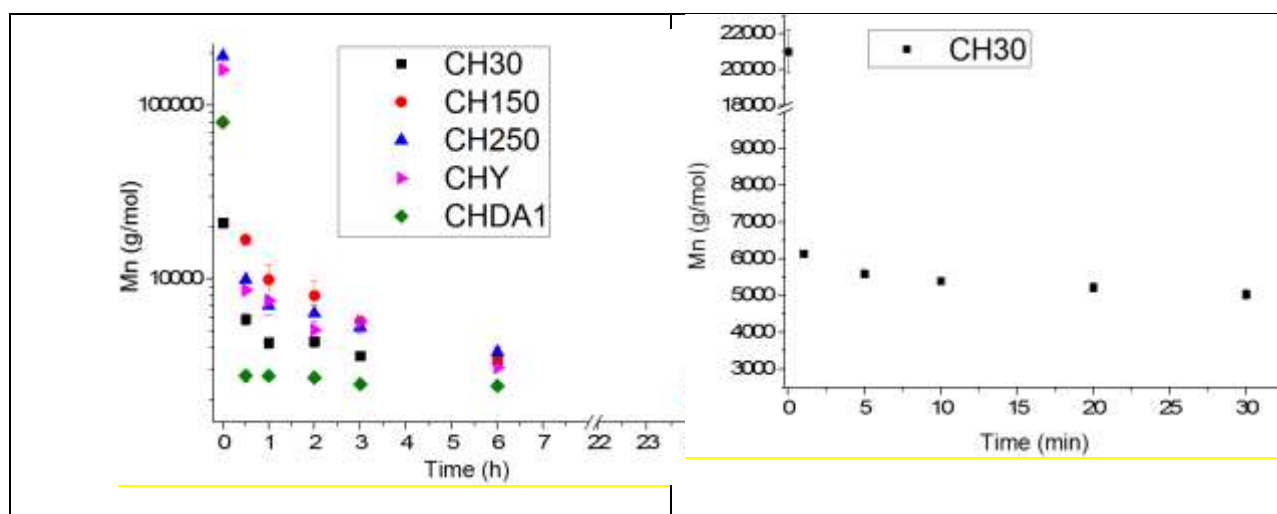
186 RESULTS AND DISCUSSION

187 Depolymerization using NaNO₂ 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.

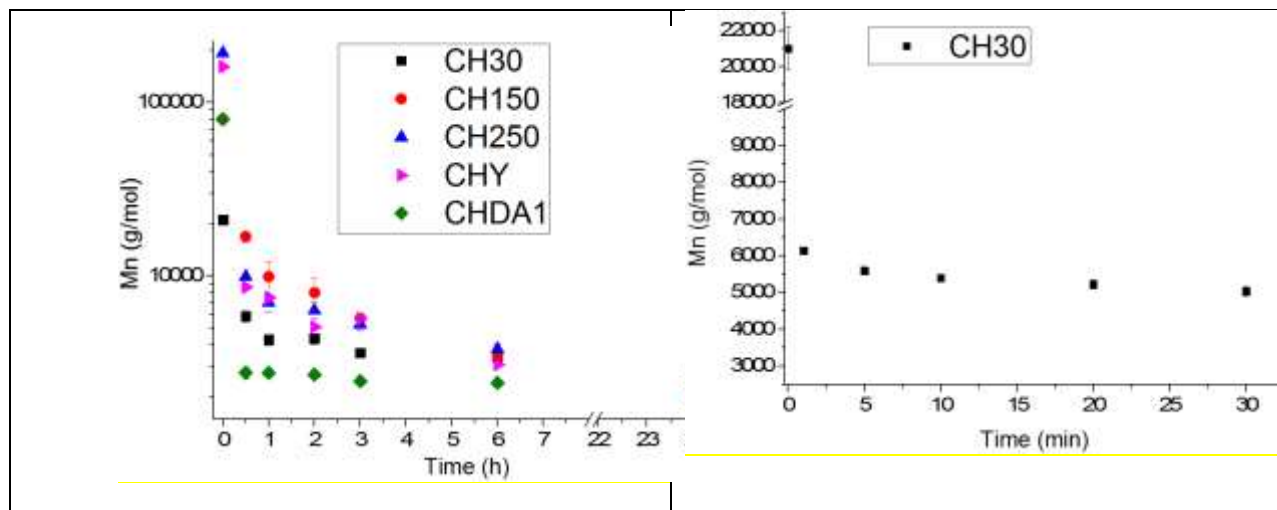
200 Mw and Mn values for each chitosan oligomer, as well as the corresponding crude ones, are
201 given in Table 1. It was especially shown that both methods afford rather close values when targeted
202 DP below 20. Above this value and also for crude chitosans, SEC using PEO/PEG standards seems to
203 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 NaNO₂ at room temperature (30 °C) and molar
207 masses decrease was followed over time (



208

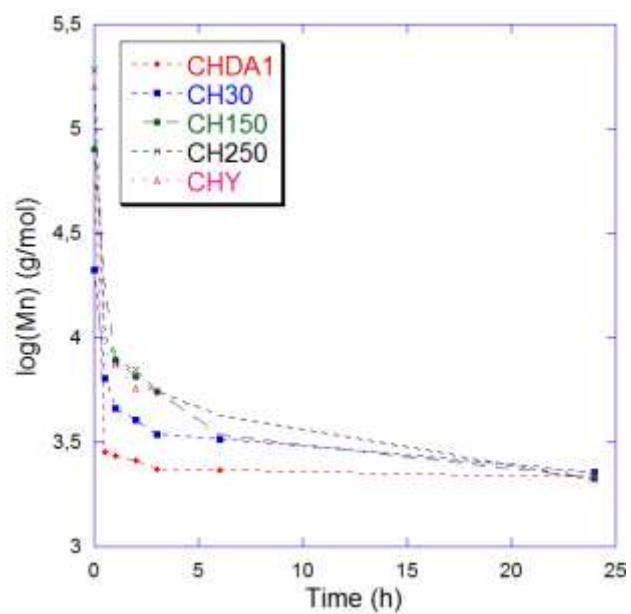
209 Figure 1 a)). Mn of CH30, CH150, CH250 and CHY showed a similar trend, i.e. a very fast
210 decrease during the first 30 min until approximately reaching 6,000 g/mol, then Mn slightly decreased
211 until stabilization. It was shown that the reaction is immediate, the most important drop of molar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



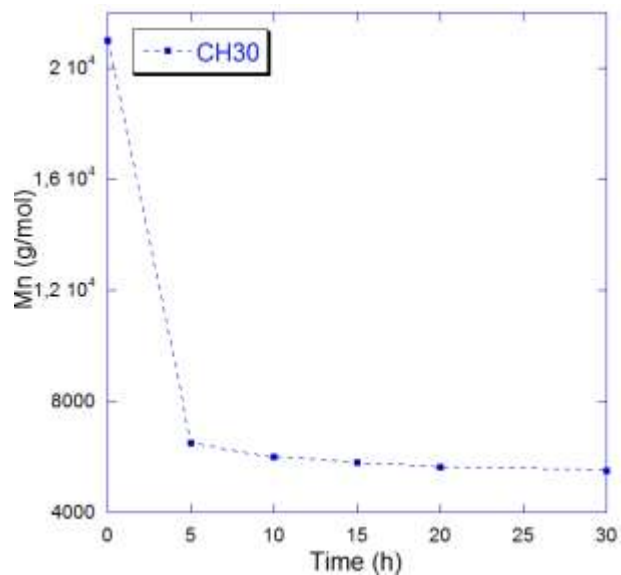
213

214 Figure 1 b)). The plateau was reached when either 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 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).

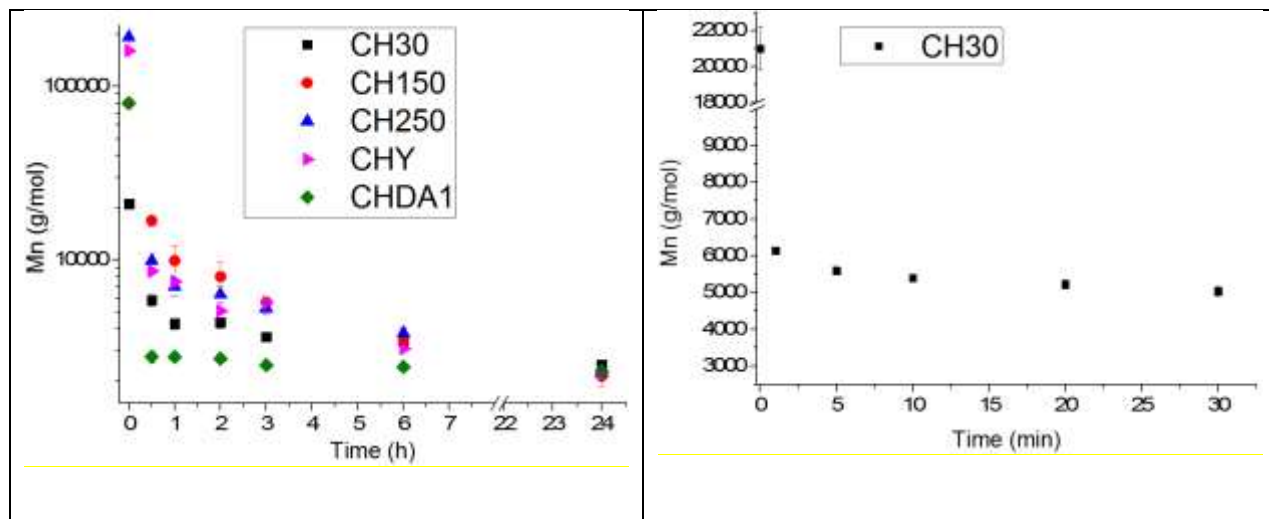
a)



b)



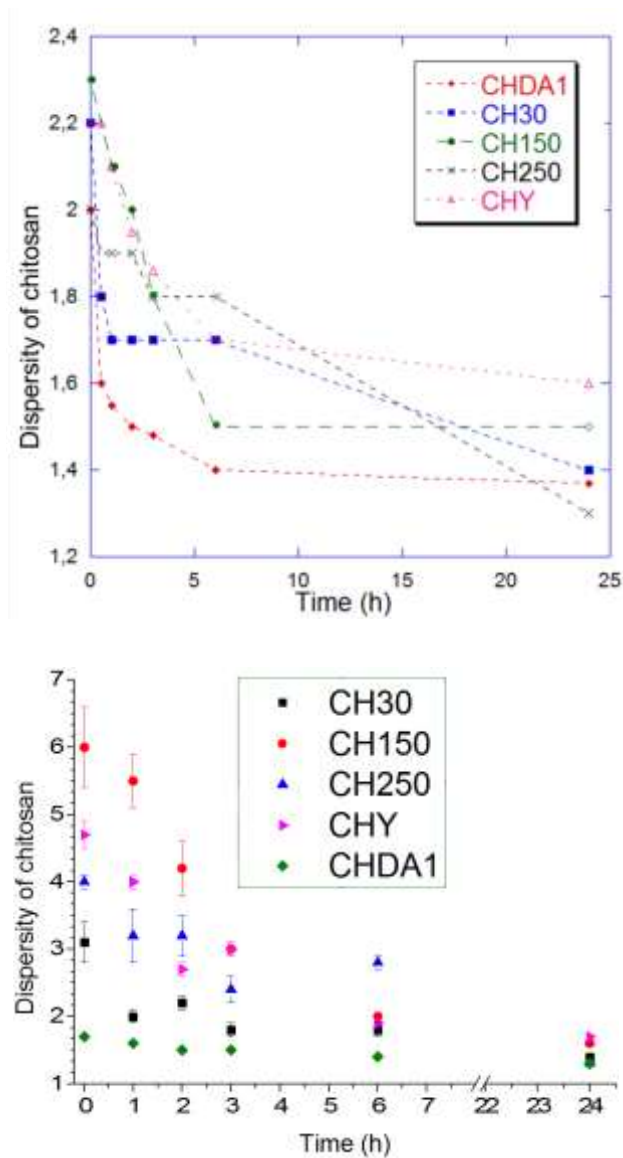
224



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 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

228



229

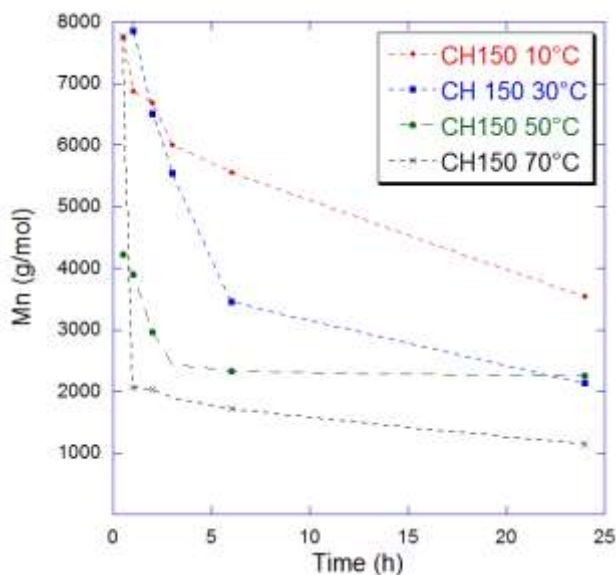
230 **Figure 2** Evolution of the dispersity as a function of time for five different chitosans (1% w/w) with 0.2eq of NaNO₂/NH₂

231

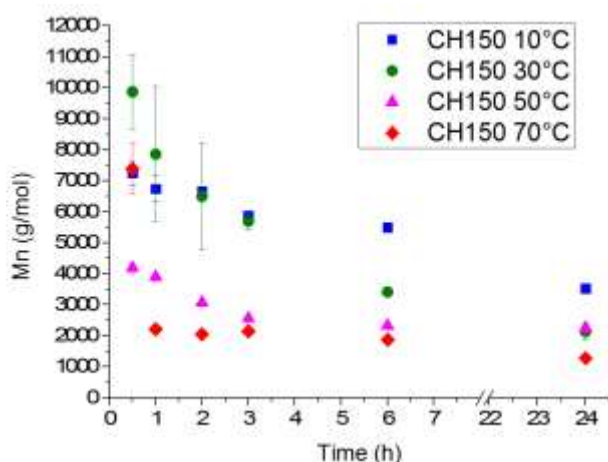
232 This reaction was carried out at room temperature, as already described in the literature²¹.
233 Nevertheless, an increase of the temperature should impact the reaction kinetics but also may lead to
234 reaction completion, until reaching lower molar masses COS, i.e. DP<20.

235 The reaction was carried out with CH150, with 0.2 eq of NaNO₂ 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, NaNO_2 could be consequently decomposed in nitrogen oxide by acetic acid
239 instead ²⁷. 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°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) ²⁸. When heated at 50°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°C during 3h.



245



246

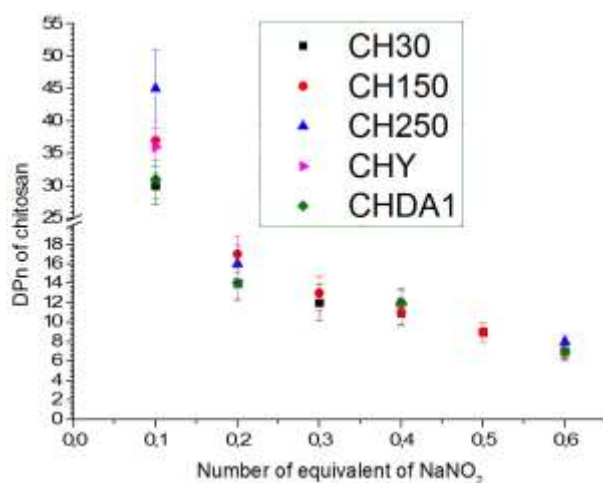
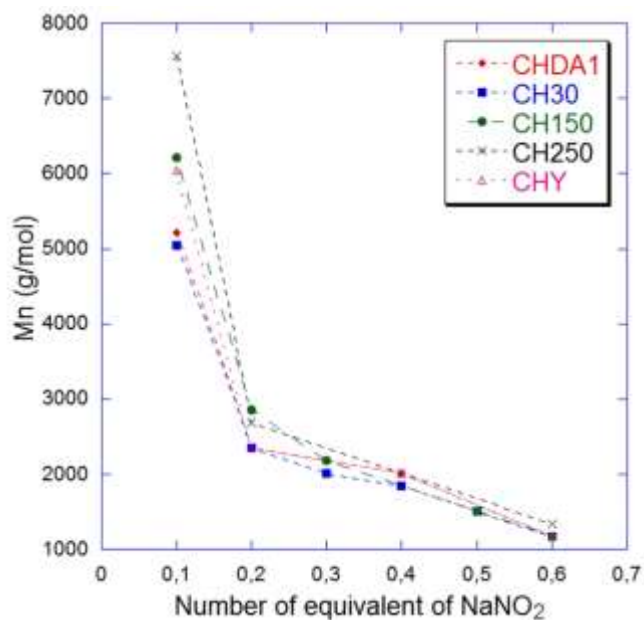
247

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

249

250 Allan et al.²⁰ already showed that the resulting Mw of COS was directly related to the content
 251 of NaNO₂. Then CH30, CH150, CH250, CHDA1 and CHY were reacted at 50°C for 3h with different
 252 NaNO₂/NH₂ 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 NaNO₂/NH₂. Above 0.2 eq NaNO₂/NH₂, Mw COS slowly decreased by increasing
257 NaNO₂ 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.²⁰, a defined number of equivalents of
259 NaNO₂/NH₂ 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.



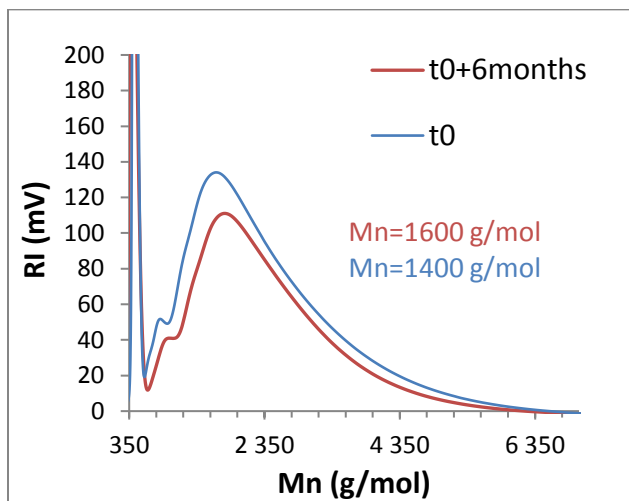
262

263

264 **Figure 4.** Evolution of Mn vs NaNO₂/NH₂ 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 NaNO₂/NH₂ were analyzed by FTIR and compared to the corresponding crude chitosans
 271 (SI 6). The carbonyl band at 1650 cm⁻¹ 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 ^1H 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 t_0 to $t_0+6\text{months}$

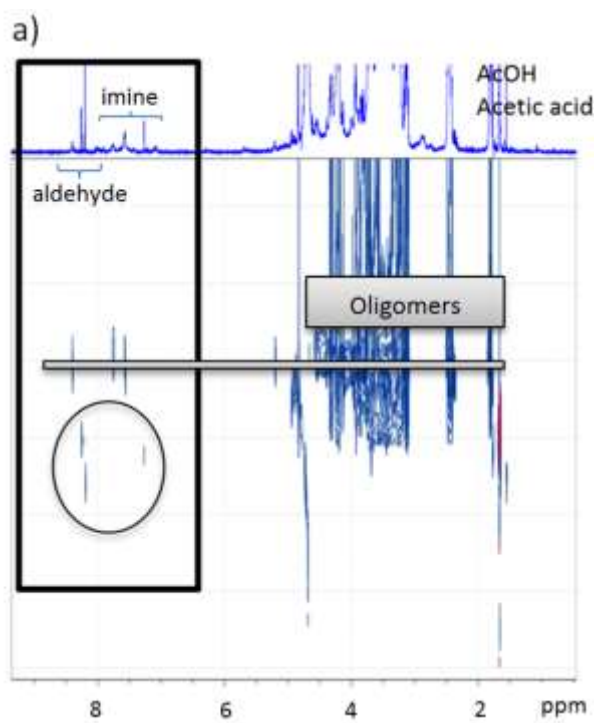
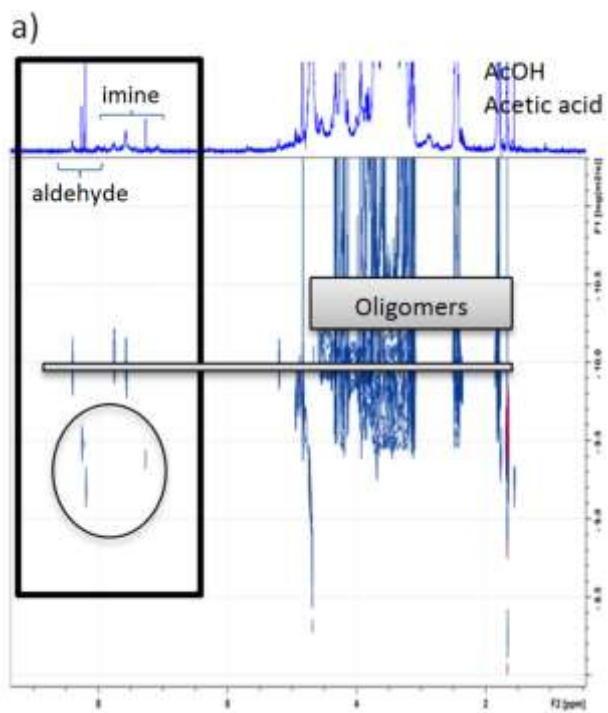
277

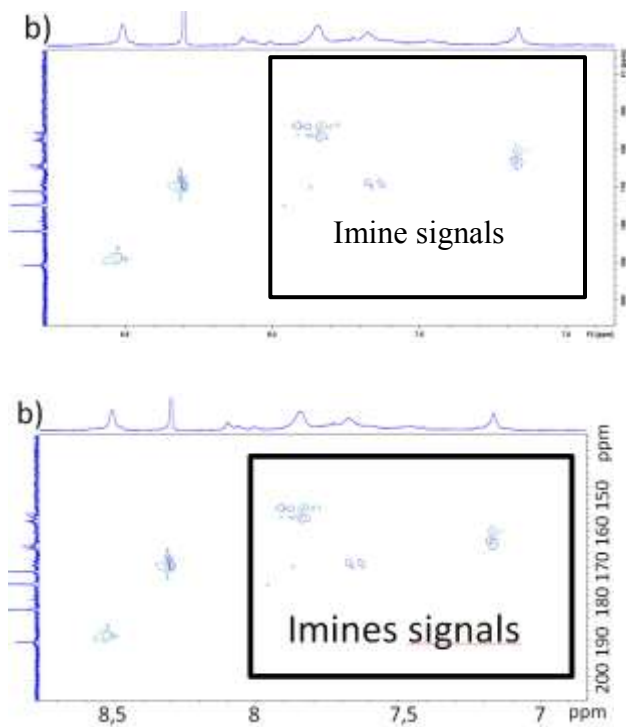
278 Tømmeraas et al.²¹ showed that nitrous deamination leads to the formation of AMF chain end of COS
279 (reductive end), but also to the formation of free AMF (2,5-anhydro-D-mannose) when glycosidic bond
280 breakage occurs at the non reductive polymer chain-end. Furthermore, AMF is in equilibrium 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 NaNO₂). 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 ¹H-¹³C 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 ²¹.

308



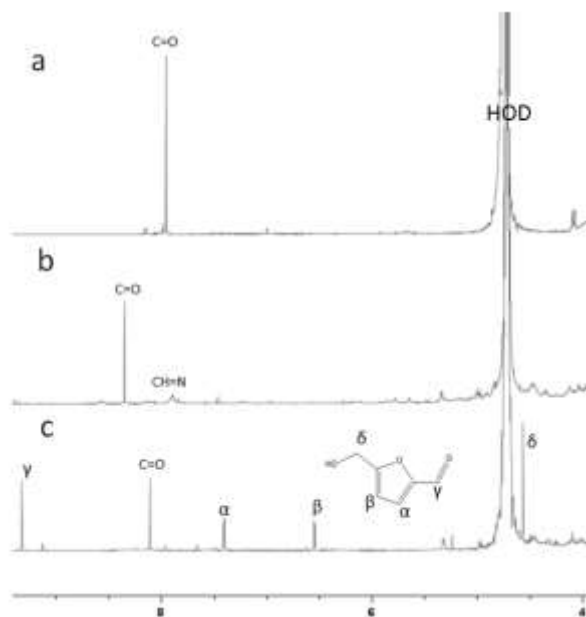


309 **Figure 6** a) 2D DOSY NMR spectrum of a 1600 g/mol oligomer obtained from COS from CH3O + 0.4 eq NaNO₂/NH₂ (3h at
 310 50°C) b) ¹H-¹³C 2D HSQC NMR spectrum of the aldehyde zone

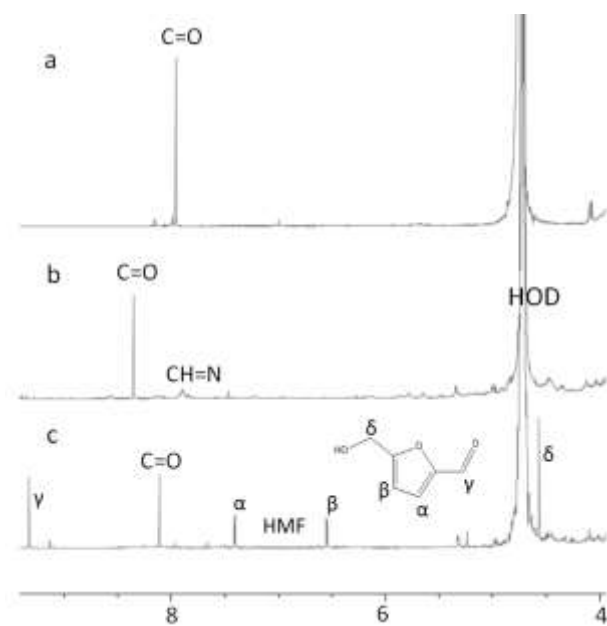
311
 312 Consequently, the effect of pH on COS structure was investigated. COS was solubilized in
 313 deuterated water at pH 5.5. Then, pH of the solution was either dropped to 2 with HCl or raised to 12 with
 314 NaOH. ¹H NMR spectra were then recorded straight away at pH 2, 5.5 and 12 (Figure 7). 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.²¹ 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



324

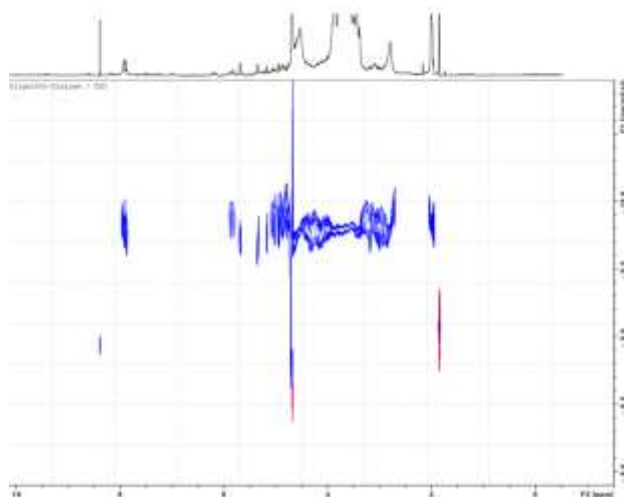


324

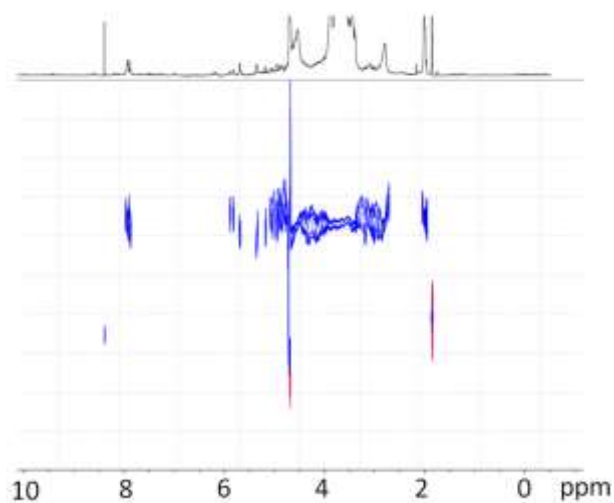
325 **Figure 7** a) ¹H NMR spectra of COS from CH3O + 0.4 eq NaNO₂/NH₂ (1600 g/mol) recorded in D₂O at pH 12 (a), at pH 5.5 (b)

326 and at pH 2 (c)

327



328

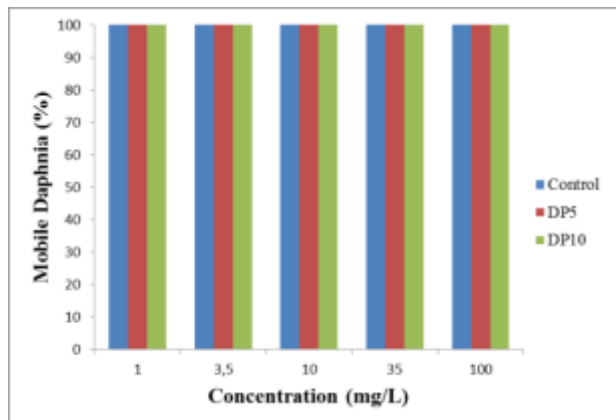


329 **Figure 8** 2D DOSY NMR of COS from CH30 + 0.4 eq NaNO₂/NH₂ (1,600 g/mol) and purified by 24h dialysis

330

331 Finally, COS were purified by dialysis during 24h against deionized water. COS were found
332 to have the same chemical structure (¹H 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 NaNO₂
334 and CH₃CO₂Na 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 NaNO₂ 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 • ¹H NMR spectrum of D-(+)-Glucosamine hydrochloride
- 363 • ¹H NMR spectrum of AMF
- 364 • ¹H NMR spectrum of oligomer 1600 g/mol from CH30 + 0.4 eq NaNO₂/NH₂
- 365 • ¹H NMR spectrum of crude chitosan CH30
- 366 • ¹H NMR spectrum of chitosan oligomer from CH30 + 0.2 eq NaNO₂/NH₂
- 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 • ¹H NMR spectrum of oligomer 1600 g/mol from CH30 + 0.4 eq NaNO₂/NH₂ 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

374 AUTHOR INFORMATION

375 **Corresponding Author**

376 **Ghislain DAVID: Ghislain.david@ensem.fr**

377 **Present Addresses**

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.

385 **Funding Sources**

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

387

388 **Notes**

389 The authors declare no conflict of interest.

390 **ACKNOWLEDGMENT**

391 The authors want to thank Agnes CREPET for triple detection SEC and data analysis and

392 Aurelien LEBRUN for performing 2D NMR analysis.

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

397 **REFERENCES**

398

399 (1) Zargar, V.; Asghari, M.; Dashti, A. A Review on Chitin and Chitosan Polymers : Structure
400 , Chemistry , Solubility , Derivatives , and Applications. **2015**, No. 3, 204–226.
401 <https://doi.org/10.1002/cben.201400025>.

402

403 (2) Gregorio Crini , Pierre-Marie Badot, E. G. *Chitine et Chitosane Du Biopolymère à*
404 *L'application*; Presses universitaires de Franche-Comté, Chapter 1,2 2009.

405

406 (3) Jiraporn Choomchit, Jantip Suesat, and P. S. Chitosan as a Thickener for Direct Printing
407 of Natural Dye on Cotton Fabric. *Adv. Mater. Res.* **2012**, 610-613, 3394–3397.

408

409 (4) Jiali Zhang*, Wenshui Xia*, Ping Liu, Qinyuan Cheng, Talba Tahirou, W. G. and B. L.
410 Chitosan Modification and Pharmaceutical / Biomedical Applications. *Mar. Drugs* **2010**,

- 411 8 (1), 1962–1987. <https://doi.org/10.3390/md8071962>.
412
- 413 (5) Akbar Ali, S. A. A Review on Chitosan and Its Nanocomposites in Drug Delivery. *Int. J.*
414 *Biol. Macromol.* **2018**, *109*, 273–286. <https://doi.org/10.1016/j.ijbiomac.2017.12.078>.
415
- 416 (6) Kumar, M. N. V. R. A Review of Chitin and Chitosan Applications Q. **2000**, *46*, 1–27.
417
- 418 (7) Vasilieva, T.; Sigarev, A.; Kosyakov, D.; Ul'yanovskii, N.; Anikeenko, E.; Chuhchin, D.;
419 Ladesov, A.; Hein, A. M.; Miasnikov, V. Formation of Low Molecular Weight Oligomers
420 from Chitin and Chitosan Stimulated by Plasma-Assisted Processes. *Carbohydrate*
421 *Polymers*. 2017, *163*, pp 54–61. <https://doi.org/10.1016/j.carbpol.2017.01.026>.
422
- 423 (8) Liang, S.; Sun, Y.; Dai, X. A Review of the Preparation , Analysis and Biological
424 Functions of Chitooligosaccharide. *Int. J. Mol. Sci.* **2018**, *19*, 2197.
425 <https://doi.org/10.3390/ijms19082197>.
426
- 427 (9) Barreteau, H.; Delattre, C.; Michaud, P. Production of Oligosaccharides as Promising
428 New Food Additive Generation. *Food Technology and Biotechnology*. 2006, *44*, pp 323–
429 333.
430
- 431 (10) Muhammad Naveed, Lucas Phil, Muhammad Sohail, Muhammad Hasnat, Mirza
432 Muhammad Faran Ashraf Baig, Awais Ullah Ihsan, Muhammad Shumzaid, Mohib
433 UllahKakar, TahirMehmood Khan, MD. Akabar, MuhammadImtiaz Hussain, Q.-G. Z.
434 Chitosan Oligosaccharide (COS): An Overview. *Int. J. Biol. Macromol.* **2019**, *129*, 827–
435 843. <https://doi.org/10.1016/j.ijbiomac.2019.01.192>.
436
- 437 (11) Inamdar, N.; Researcher, I.; Choudhari, Y. M. Chitooligosaccharides : Synthesis ,
438 Characterization and Applications Chitooligosaccharides : *Polym. Sci. Ser. A* **2011**, *53*
439 (February 2014), 583–612. <https://doi.org/10.1134/S0965545X11070066>.
440
- 441 (12) Ouyang, Q.; Zhao, S.; Li, S.; Song, C. Application of Chitosan, Chitooligosaccharide, and
442 Their Derivatives in the Treatment of Alzheimer ' S Disease. *Mar. Drugs* **2017**, *15*, 322.
443 <https://doi.org/10.3390/md15110322>.
444
- 445 (13) Sahariah, P. Antimicrobial Chitosan and Chitosan Derivatives : A Review of the Structure
446 – Activity Relationship. *Biomacromolecules* **2017**, *18*, 3846–3868.
447 <https://doi.org/10.1021/acs.biomac.7b01058>.
448
- 449 (14) Yang, Y.; Xing, R.; Liu, S.; Qin, Y.; Li, K.; Yu, H. Immunostimulatory Effects of

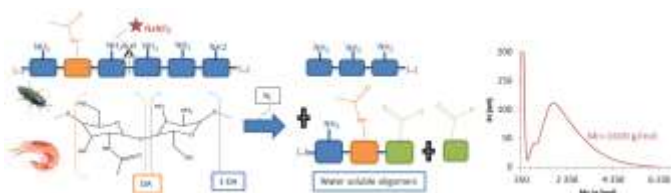
- 450 Chitooligosaccharides on RAW 264 . 7 Mouse Macrophages via Regulation of the MAPK
451 and PI3K / Akt Signaling Pathways. *Mar. Drugs* **2019**, *17*, 36.
452 <https://doi.org/10.3390/md17010036>.
453
- 454 (15) Liaqat, F.; Eltem, R. Chitooligosaccharides and Their Biological Activities : A
455 Comprehensive Review. *Carbohydr. Polym.* **2018**, *184* (November 2017), 243–259.
456 <https://doi.org/10.1016/j.carbpol.2017.12.067>.
457
- 458 (16) Xie, Y.; Hu, J.; Wei, Y.; Hong, X. Preparation of Chitooligosaccharides by the Enzymatic
459 Hydrolysis of Chitosan. *Polym. Degrad. Stab.* **2009**, *94* (10), 1895–1899.
460 <https://doi.org/10.1016/j.polymdegradstab.2009.06.021>.
461
- 462 (17) Ibrahim, K. A.; El-Eswed, B. I.; Abu-Sbeih, K. A.; Arafat, T. A.; Al Omari, M. M. H.;
463 Darras, F. H.; Badwan, A. A. Preparation of Chito-Oligomers by Hydrolysis of Chitosan
464 in the Presence of Zeolite as Adsorbent. *Marine Drugs*. *14*, 43 2016.
465 <https://doi.org/10.3390/md14080043>.
466
- 467 (18) Varum, K. M.; Ottoy, M. H.; Smidsrod, O. Acid Hydrolysis of Chitosans. *Carbohydrate*
468 *Polymers*. 2001, 46 pp 89–98. [https://doi.org/10.1016/S0144-8617\(00\)00288-5](https://doi.org/10.1016/S0144-8617(00)00288-5).
469
- 470 (19) Mecwan, M. M.; Rapalo, G. E.; Mishra, S. R.; Haggard, W. O.; Bumgardner, J. D. Effect
471 of Molecular Weight of Chitosan Degraded by Microwave Irradiation on Lyophilized
472 Scaffold for Bone Tissue Engineering Applications. *Journal of Biomedical Materials*
473 *Research*. 2011, 97 A pp 66–73. <https://doi.org/10.1002/jbm.a.33029>.
474
- 475 (20) Allan, G. G.; Peyron, M. Molecular Weight Manipulation of Chitosan II: Prediction and
476 Control of Extent of Depolymerization by Nitrous Acid. *Carbohydrate Research*. 1995,
477 277 pp 273–282. [https://doi.org/10.1016/0008-6215\(95\)00208-B](https://doi.org/10.1016/0008-6215(95)00208-B).
478
- 479 (21) Tømmeraas, K.; Vårum, K. M.; Christensen, B. E.; Smidsrød, O. Preparation and
480 Characterisation of Oligosaccharides Produced by Nitrous Acid Depolymerisation of
481 Chitosans. *Carbohydrate Research*. 2001, 333 pp 137–144.
482 [https://doi.org/10.1016/S0008-6215\(01\)00130-6](https://doi.org/10.1016/S0008-6215(01)00130-6).
483
- 484 (22) Mao, S.; Shuai, X.; Unger, F.; Simon, M.; Bi, D.; Kissel, T. The Depolymerization of
485 Chitosan: Effects on Physicochemical and Biological Properties. *International Journal of*
486 *Pharmaceutics*. 2004, 281 pp 45–54. <https://doi.org/10.1016/j.ijpharm.2004.05.019>.
487
- 488 (23) Bezrodnykh, E. A.; Blagodatskikh, I. V.; Kulikov, S. N.; Zelenikhin, P. V.; Yamskov, I. A.;
489 Tikhonov, V. E. Consequences of Chitosan Decomposition by Nitrous Acid : Approach to

- 490 Non- Branched Oligochitosan Oxime. *Carbohydr. Polym.* **2018**, *195* (April), 551–557.
 491 <https://doi.org/10.1016/j.carbpol.2018.05.007>.
 492
- 493 (24) Charuwan Suitcharit, Farisan Awae, Wae-a-risa Sengmama, K. S. Preparation of
 494 Depolymerized Chitosan and Its Effect on Dyeability of Mangosteen Dye. *Chiang Mai J.*
 495 *Sci.* **2011**, *38*, 473–484.
 496
- 497 (25) Moussa, A.; Crépet, A.; Ladavière, C.; Trombotto, S. Reducing-End “ Clickable ”
 498 Functionalizations of Chitosan Oligomers for the Synthesis of Chitosan-Based Diblock
 499 Copolymers. *Carbohydr. Polym.* **2019**, *219* (April), 387–394.
 500 <https://doi.org/10.1016/j.carbpol.2019.04.078>.
 501
- 502 (26) Claustre, S.; Pe, J. An Easy Stereospecific Synthesis of 1-Amino-2,5-Anhydro-1-Deoxy-
 503 D-Mannitol and Arylamino Derivatives. *Carbohydr. Res.* **1999**, *315*, 339–344.
 504
- 505 (27) D.L.H. Williams. *Nitrosation Reactions and the Chemistry of Nitric Oxide*; Chapter 1,
 506 2004.
 507
- 508 (28) Tsao, C. T.; Chang, C. H.; Lin, Y. Y.; Wu, M. F.; Han, J. L.; Hsieh, K. H. Kinetic Study
 509 of Acid Depolymerization of Chitosan and Effects of Low Molecular Weight Chitosan on
 510 Erythrocyte Rouleaux Formation. *Carbohydrate Research.* 2011, *346*, pp 94–102.
 511 <https://doi.org/10.1016/j.carres.2010.10.010>.
 512

513

514 BRIEFS (Word Style “BH_Briefs”). If you are submitting your paper to a journal that requires a
 515 brief, provide a one-sentence synopsis for inclusion in the Table of Contents.

516 SYNOPSIS (Word Style “SN_Synopsis_TOC”). If you are submitting your paper to a journal
 517 that requires a synopsis, see the journal’s Instructions for Authors for details.



518

519

