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# 1 Polymer functionalization through an enzymatic 2 process: intermediate products characterization and 3 their grafting onto gum Arabic

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11 KEYWORDS: gum Arabic, functionalization, phenol grafting, ferulic acid, oxidation product

12 ABSTRACT:

13 The modification of gum Arabic with ferulic acid oxidation products was performed in  
14 aqueous medium, at 30 °C and pH 7.5, in the presence of *Myceliophthora thermophila* laccase  
15 as biocatalyst. **First, this study aimed to investigate the structures of the oxidation products of**  
16 **ferulic acid that could possibly be covalently grafted onto gum Arabic.** HPLC analyses  
17 revealed that this reaction produced several oxidation products, whose structures were  
18 investigated using **LC-MS/MS** analyses (**liquid chromatography-mass spectrometry with mass**

19 fragmentation analyses) and NMR experiments. The chemical structure of one intermediate  
20 reaction product was fully elucidated as the 2-(4-hydroxy-3-methoxyphenyl)-4-[(4-hydroxy-  
21 3-methoxyphenyl) methylidene] cyclobutane-1, 3-dione, called by the authors  
22 cyclobutadiferulone.

23 Secondly, this study aimed to locate the grafting of the oxidation products onto gum  
24 Arabic by performing several NMR experiments. This study did not determine how much and  
25 specifically which oxidation products were grafted but some of them were undeniably present  
26 onto modified gum Arabic, close to the glucuronic acid C5 carbon or close to the galactose C6  
27 carbon.

28

## 29 1. Introduction

30 Gum Arabic (GA) is a sticky exudate of air-solidified sap from the trunks or tree  
31 branches of Acacia trees such as *Acacia senegal* and *Acacia seyal*, which essentially provides  
32 a friable gum (Idris, Williams, & Phillips, 1998; McNamee, O’Riorda, & O’Sullivan, 1998;  
33 Nussinovitch, 1997). GA is mainly composed of D-galactose (39 to 42%), L-arabinose (24 to  
34 27%), L-rhamnose (12 to 16%), D-glucuronic acid, 4-O-Methyl- $\beta$ -D-glucuronic acid (15 and  
35 16%) and a small amount of proteins (1.5 to 2.4%) (Idris *et al.*, 1998). The polysaccharides  
36 and proteins present in GA can be divided into three main fractions (Idris *et al.*, 1998;  
37 Williams *et al.*, 1990): arabinogalactan-peptide (AG), arabinogalactan protein (AGP) and  
38 glycoprotein (GP). These fractions differ in their molecular weight, protein content and  
39 chemical composition (Mahendran, Williams, Phillips, Al-Assaf, & Baldwin, 2008; Randall,

40 Phillips, & Williams, 1988; Randall, Phillips, & Williams, 1989; Sanchez *et al.*, 2008;  
41 Sanchez *et al.*, 2017; Shi *et al.*, 2017).

42 Recently, several studies are the witness of a growing interest for the modification of  
43 GA, especially aiming to improve its emulsifying properties (Pirestani *et al.*, 2017; Shi *et al.*,  
44 2017; Wang, Williams, & Senan, 2014). However, those studies are only focusing on the  
45 modification of the polysaccharide by chemical pathways. Some authors have investigated the  
46 enzymatic pathway on other polysaccharides. Previous studies demonstrated that it was  
47 possible to modify chitosan and pectin by grafting oxidation products of ferulic acid (OXF)  
48 activated by a laccase from *Myceliophthora thermophila* (Aljawish *et al.*, 2012; Karaki *et al.*,  
49 2017). Oxidation products were grafted to the amine groups (carbon C2 of glucosamine) of  
50 chitosan (by formation of a Schiff base) and onto the carboxylic acids (carbon C6 of  
51 galacturonic units) of pectin (by the formation of an ester bond), thus modifying many of their  
52 properties (Aljawish *et al.*, 2016; Karaki, Aljawish, Muniglia, Humeau, & Jasniewski, 2016).  
53 Native gum Arabic (NGA) contains both amine groups (from its arabinogalactan-protein  
54 fraction) and carboxyl groups (bore by the polysaccharides from the arabinogalactan-peptide  
55 fraction), so the OXF could be grafted onto either or both sites. Since the arabinogalactan-  
56 peptide fraction is predominant in gum Arabic (88% against 10% for the arabinogalactan-protein  
57 fraction) (Lopez-Torrez *et al.*, 2015) and that the carboxyl groups are mainly responsible for gum  
58 Arabic negative charge in solution, the grafting of oxidation products could be preferably  
59 orientated towards the carboxyl groups. However, in previous studies on other polysaccharides, it  
60 was shown that the grafting of phenolic compounds, even in very small quantities, had a  
61 significant impact on the physico-chemical properties of these polymers (Aljawish *et al.*, 2012;  
62 Karaki *et al.*, 2017). Therefore, even a grafting onto the small fraction of amine groups of gum  
63 Arabic could explain the physico-chemical changes observed in a previous study on the

64 enzymatic modification of gum Arabic with ferulic acid oxidation products in the presence of  
65 *Myceliophthora thermophila* laccase as biocatalyst (Vuillemin *et al.*, 2020). This study  
66 showed that this functionalization changed GA properties and led to an increase of its  
67 antioxidant properties and led to the apparition of a glass transition closed to 39 °C. However,  
68 the study did not prove undeniably if the oxidation products of ferulic acid were grafted on  
69 GA or only adsorbed onto it.

70 The main issue with this approach is that the laccase-catalyzed oxidation of ferulic acid  
71 is currently not well-controlled. It generates highly reactive unstable radicals that can  
72 spontaneously reorganize and may lead to the formation of phenolic oligomers. Many  
73 structural changes, including oxidative decarboxylation, may occur, thus increasing the  
74 number of possible oxidation product structures. Only a few structures have been fully  
75 elucidated, most of the others have not been completely identified yet (Ralph, Quideau,  
76 Grabber, & Hatfield, 1994). Furthermore, the existing identification of such products rely  
77 mostly on hypothesis based on the products molecular weight obtained by liquid  
78 chromatography-mass spectrometry (LC-MS) measurements (Aljawish *et al.*, 2014;  
79 Carunchio, Crescenzi, Girelli, Messina, & Tarola, 2001; Karaki *et al.*, 2017). Yet, for one  
80 molecular weight, multiple structures could be proposed. Some studies have demonstrated  
81 that several structures of the same molecular weight could coexist, based on nuclear magnetic  
82 resonance (Ralph, Quideau, Grabber, & Hatfield, 1994). Thus, this study aimed on one hand  
83 to determine the chemical structure of the oxidation products obtained from the oxidation of  
84 ferulic acid initiated by the laccase of *Myceliophthora thermophila*. The functionalization of  
85 polysaccharides through enzymatic process is a complex matter because it would require an  
86 interaction between the enzyme and polysaccharide, which are generally difficult and limited  
87 due to the steric clutter associated with the size of the polymer. As a solution, the laccase-

88 mediated functionalization is proposed. In this enzymatic pathway, only the phenolic  
89 compound (i.e. ferulic acid) is initially supported by the enzyme to form reactive species  
90 (semi-quinones). These radicals are active species which can either condense with each other  
91 or react with nucleophilic function, such as amino groups or carboxyl groups present on the  
92 polysaccharide. This mechanism has already been widely studied and although the  
93 mechanism of the non-enzymatic reaction step is still poorly understood, experimental  
94 evidence supports the hypothesis that quinones can undergo different types of reaction with  
95 amines to yield either Schiff-bases or Michael-type adducts, as well as oligomer-forming  
96 reactions with other quinones (Kumar et al., 2000, Mustafa et al., 2005, Aljawish et al., 2012).

97 The study of the grafted products is complex, which is why the study was focused on  
98 the free products that are easier to study. The assumption was made that they would not be  
99 different with or without gum Arabic since the intermediate species are formed before  
100 reacting with the polysaccharide. However, it could not be excluded that some rearrangements  
101 happen afterwards and that they could differ depending on whether the oxidation products  
102 condense with each other or react with gum Arabic.

103 Those products were expected to be grafted either on the carboxyl or on the amine  
104 group of GA. This study aimed to prove the grafting of the oxidation products onto the  
105 polymer and to locate these chemical species on GA. HPLC, LC-MS/MS and NMR analysis  
106 were used to resolve the structure of reactions products coming from the ferulic acid oxidation  
107 by the laccase. Then, NMR spectroscopies have been performed to confirm the grafting of the  
108 oxidation products on the polysaccharide.

109

## 110 **2. Experimental**

## 111 2.1. Materials

112 Gum Arabic (GA) Instantgum AA from *Acacia senegal* was a gift from Nexira (France).  
113 Ferulic acid (purity about 99%) (FA), anhydrous sodium phosphate dibasic (Na<sub>2</sub>HPO<sub>4</sub>),  
114 anhydrous potassium phosphate monobasic (KH<sub>2</sub>PO<sub>4</sub>), trifluoroacetic acid (TFA), deuterated  
115 water and deuterated methanol were purchased from Sigma-Aldrich (France). Analytical  
116 grade methanol, ethanol, formic acid and acetonitrile were purchased from Carlo Erba  
117 (Milwaukee, WI, USA).

118 The enzyme used for the oxidation was commercialized as "Novozym® 51003" from  
119 Novozymes (Bagsvaerd, Denmark). It is a fungal laccase from *Myceliophthora thermophila*,  
120 a polyphenol oxidase produced by submerged fermentation of a genetically modified  
121 *Aspergillus oryzae* (Berka *et al.*, 1997). The laccase activity was determined by following the  
122 apparition of the oxidation product of syringaldazine (the reference substrate for laccase  
123 activity determination). The laccase stock activity was 32 038 ± 1 322 LAMU.g<sup>-1</sup> (Laccase  
124 Myceliophthora Units.g<sup>-1</sup>).

125

## 126 2.2. Methods

### 127 2.2.1. **Functionalization of** gum Arabic

128 The method used to functionalize GA through a ferulic acid oxidation was adapted from  
129 previous protocols (Aljawish *et al.*, 2012; Karaki *et al.*, 2017; Vuillemin *et al.*, 2020). 1 g of  
130 GA was dispersed in 45 mL of phosphate buffer (50 mM, pH 7.4). The dispersion was stirred  
131 at 450 rpm for 1 h at 30 °C. 5 mL of ferulic acid (50 mM) dissolved in methanol was added to  
132 the batch. Once the temperature was stable, 13.5 LAMU.mL<sup>-1</sup> of Novozym® 51003 was

133 added to trigger the reaction. The batch was then stirred at 30 °C for 50 min. 150 mL of  
134 ethanol (96%) stored at -20 °C was then added to stop the reaction and separate the non-  
135 functionalized GA from the functionalized GA. The obtained mixture containing  
136 functionalized gum Arabic (FGA), non-grafted FA oxidation products (OXP) (soluble in the  
137 phosphate buffer/ethanol mixture) and non-functionalized GA (insoluble in the phosphate  
138 buffer/ethanol mixture) was then centrifuged at 12000 g for 20 min at 20 °C with a Beckmann  
139 centrifuge (Beckman Coulter Inc., Villepinte, France). The precipitate contained non-  
140 functionalized GA and a part of phosphate buffer salts (verified by FTIR measurements, data  
141 not shown), whereas the supernatant contained the functionalized GA and the ferulic acid  
142 oxidation products not grafted on GA.

143 The ethanol was then eliminated with a rotary evaporator BuchiR144 (Buchi SARL,  
144 Rungis, France) at a boiling point of 40 °C at 175 mbar. Some amount of water from the  
145 buffer solution was also evaporated by decreasing the pressure down to 72 mbar.

#### 146 2.2.2. Preparation of ferulic acid oxidation products

147 For the preparation of ferulic acid oxidation products the same protocol as for the  
148 functionalization of gum Arabic has been performed but without adding gum. Ferulic acid and  
149 OXP were soluble in the mixture of methanol and phosphate buffer. However, at the end of  
150 the reaction, 150 mL of ethanol (96%) stored at -20 °C was still added to stop the reaction and  
151 to precipitate buffer salts. The supernatant contained the OXP.

#### 152 2.2.3. Purification of the component

153 For FGA, the residual solution was dialyzed against ultrapure water with a high-grade  
154 regenerated membrane (MWCO 10 000 Da from Membrane Filtration Products Inc.) to



155 eliminate excess salts from the buffer and non-grafted FA oxidation products (the oxidation  
156 products are soluble in water up to 5% (Vuillemin *et al.*, 2020) and they have a molar mass  
157 lower than 610 Da (Figure 2)). Dialysis was stopped when the conductivity outside the  
158 membrane was equal to the one of ultrapure water, i.e. equal to  $1.0 \pm 0.2 \mu\text{S}\cdot\text{cm}^{-1}$ . The same  
159 method was used to eliminate excess salt from NGA. For the OXP, they were purified using  
160 Sephadex PD-10 pre-packed desalting columns (with Sephadex G-25 resin, GE Healthcare,  
161 Buckinghamshire, United Kingdom). 25 ml of ultrapure water was used to equilibrate the  
162 column, after what 2.5 mL of solution of OXP in ultrapure water was added. After the sample  
163 had entered the packed bed completely, 2.5 mL of ultrapure water was added again to the  
164 column. The eluate that was collected contained the OXP, while the salts remained in the  
165 column. The dialyzed FGA and NGA and the purified OXP were then freeze-dried. The  
166 powders obtained were stored in a desiccator, in the dark, at 4 °C until use.

#### 167 2.2.4. Solubilization of the products

168 The solutions were prepared by dissolving FGA, NGA or OXP in ultra-pure water. The  
169 solutions were stirred at 400 rpm overnight at 4 °C to ensure total solubilization of the  
170 products.

171

#### 172 2.2.5. Color modification during the oxidation reaction

173 During the functionalization of GA and the oxidation of ferulic acid by the laccase from  
174 *Myceliophthora thermophila*, a change of the color of the mixtures was observed. The color  
175 evolution of the reaction medium was measured with a spectrophotometer CR-5-Konica  
176 Minolta (Konica Minolta Sensing, Europe B.V.). Prior to each run, the device was calibrated

177 using two standards (white and black). Every 10 min, CIE (International Commission on  
178 Illumination) color parameters ( $L^*$ ,  $a^*$  and  $b^*$ ) were measured by placing 4 mL of the reaction  
179 mixture in the spectrophotometer tank.  $L^*$  defines lightness and ranges from 0% (black) to  
180 100% (white). The  $a^*$  parameter denotes the red/green value and  $b^*$  the blue/yellow value.  
181 The color difference ( $\Delta E$ ) between two samples was obtained using Eq.1.

$$182 \quad \Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2} \quad (\text{Eq. 1})$$

#### 183 2.2.6. Monitoring of the ferulic acid consumption during the oxidation reaction (HPLC)

184 The oxidation of ferulic acid during the enzymatic reaction was monitored by high  
185 performance liquid chromatography (HPLC) (Shimadzu LC10). 100  $\mu\text{L}$  of the reaction  
186 medium was collected and placed in 2 mL microtubes containing 900  $\mu\text{L}$  of pure methanol to  
187 stop the reaction. Prior to analysis, samples were filtered through a 0.2  $\mu\text{m}$  syringe filter and  
188 half diluted in ultra-pure water. 5  $\mu\text{L}$  was injected into the HPLC system. The separation was  
189 performed on a reversed-phase GRACE Apollo C18 chromatography column (150 x 2.1 mm -  
190 5  $\mu\text{m}$ ). The elution method consisted of a two-phase gradient at a flow rate of 0.2  $\text{mL}\cdot\text{min}^{-1}$ : a  
191 phase A (ultra-pure water/methanol/TFA 80:20:0.1) and a phase B (100% methanol). The  
192 gradient used consisted in 2 min of 20% of phase B, then the proportion of phase B was raised  
193 from 20% up to 90% during 14 min. The proportion of B was maintained at 90% for 5 min  
194 and finally at 20% for 10 min. The UV-VIS absorbance was measured between 190 and 900  
195 nm on a multichannel photo-diode-array detector (SPD-M10A VP). The results were recorded  
196 and processed by a chromatogram processing software (LC solution). The amount of AF was  
197 measured at its maximum absorption (322 nm) after making a standard ferulic acid calibration  
198 from 0.1 to 0.5 mM.

199 2.2.7. Measurement of the approximate Zeta potential

200 The approximate Zeta potential of FGA and NGA (1.00% w/v) were calculated from  
201 measurement of the electrophoretic mobility at 25 °C, at pH 5.5, using a Zetasizer Nano-ZS  
202 instrument and DTS1070 disposable zetacell (Malvern Panalytical, United Kingdom)  
203 equipped with a He/Ne ion laser ( $\lambda= 532$  nm). Measurements were collected on a detector at  
204 173°. The instrument determined the Zeta potential with the Smoluchowski equation (Jayme,  
205 Dunstan, & Gee, 1999). All measurements were made in triplicate.

206 2.2.8. Structural characterization of the modified gum Arabic: Nuclear magnetic resonance  
207 analyses

208 Native gum Arabic (NGA) and functionalized gum Arabic (FGA) were characterized by  
209 NMR. They were solubilized in deuterated water at concentrations of 5.0% and 4.5% w/v,  
210 respectively. The solutions were then analyzed by NMR to determine their molecular  
211 structure and the proximity of the atoms from each other, by using a combination of 1D and  
212 2D NMR techniques. Experiments were performed on a spectrometer operating at 9.4 Tesla  
213 (Bruker Avance III, frequencies of 400 Mhz and 100.6 MHz for  $^1\text{H}$  and  $^{13}\text{C}$ , respectively),  
214 using a Bruker 5mm BBFO probe. Pulse widths were 14.1 and 10.5  $\mu\text{s}$  for  $^1\text{H}$  and  $^{13}\text{C}$ ,  
215 respectively. All experiments were performed at room temperature. The  $^1\text{H}$ ,  $^{13}\text{C}$  decoupled  
216 from  $^1\text{H}$ ,  $^1\text{H}$ - $^{13}\text{C}$  HSQC,  $^1\text{H}$ - $^{13}\text{C}$  HMBC,  $^1\text{H}$ - $^1\text{H}$  ROESY and  $^1\text{H}$  diffusion NMR experiments  
217 were run under standard conditions. For HMBC experiment, a 50 ms waiting period was used  
218 for the evolution of long-range coupling, and a value of 3.4 ms for the low-pass  $J$  filter.  
219 ROESY was run with a spin-lock of 100ms for mixing. And diffusion measurements were  
220 done using standard stimulated echoes with bipolar gradient pulses, with  $\Delta$  300ms,  $\delta$  2.3ms,  
221 and 64 points for linear increment of the gradient between 0.96 and 47.2  $\text{G}\cdot\text{cm}^{-1}$ .

222 2.2.9. Structural characterization of the oxidation products

223 2.2.8.1. Separation and purification of oxidation products

224 Separation and fractionation of the oxidation products (OXP) solubilized in ultrapure  
225 water were performed using high-performance semi-preparative liquid chromatography  
226 (Gilson Inc., Middleton, USA). Results were processed using the Trilution @ LC software.  
227 The OXP separation was carried out using a reversed-phase column GRACE Apollo C18 (250  
228 x 22 mm - 5  $\mu\text{m}$ ). The detection was carried out at 322 nm on a UV/Vis 156 detector. The  
229 elution gradient consisted in a phase A (ultra-pure water and formic acid 100: 0.1) and a phase  
230 B (acetonitrile and formic acid 100: 0.1) at a flow rate of 10  $\text{mL}\cdot\text{min}^{-1}$ . 1 mL of sample was  
231 injected into the column at a concentration of 4% w/v. The gradient consisted in 10% of the  
232 phase B for 2 min, then 60% for 78 min, 99% for 5 min and finally 10% for 5 min. Fractions  
233 were recovered from the column outlet in glass tubes. The solvents were evaporated using a  
234 rotary evaporator BUCHI R144 (BUCHI SARL, Rungis, France). The OXP were freeze-dried  
235 and then stored at 4  $^{\circ}\text{C}$  in a desiccator until use.

236 2.2.8.2. Determination of the molecular mass of the oxidation products by Liquid  
237 Chromatography-Mass Spectrometry (LC-MS/MS)

238 The mass spectra of the OXP were obtained with a UHPLC-MS system, which  
239 comprised a quaternary solvent delivery pump connected to a photodiode array and a LTQ  
240 Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped  
241 with an atmospheric pressure ionization interface operating in electrospray mode (ESI).

242 10  $\mu\text{l}$  of the mixture were separated on a C18 Alltima (150 \* 4.6 mm – 5 $\mu\text{m}$ ) column  
243 (Grace/Alltech, Darmstadt, Germany). The flow rate was set at 200  $\mu\text{l}\cdot\text{min}^{-1}$  and mobile

244 phases consisted in water modified with formic acid (0.1%) for A and acetonitrile modified  
245 with formic acid (0.1%) for B. Phenolic compounds were eluted using a linear gradient from  
246 5% to 47% of B for 60 min.

247 The spray voltage was +5.0 kV. The temperature of the heated capillary was set to  
248 300 °C. The flow rates of sheath gas, auxiliary gas, and sweep gas were set (in arbitrary  
249 units/min) to 40, 10, and 10, respectively. Capillary voltage was +36 V, tube lens was +80 V,  
250 split lens was -44 V, and the front lens was -3.25 V. Mass spectrometric parameters were  
251 optimized by infusing a standard solution of isopimpinelline (0.1 g.L<sup>-1</sup>) in mobile phase at a  
252 flow rate of 5 µl.min<sup>-1</sup>. Parent ions were detected between 100 and 1000 m/z and specific MS<sup>n</sup>  
253 fragmentation spectra (MS<sup>2</sup>, MS<sup>3</sup>) were carried out in order to obtain structural information  
254 on OXP. Data were processed using Xcalibur software (version 2.1, Thermoscientific).

#### 255 2.2.8.3. Nuclear magnetic resonance analyses

256 OXP were characterized by NMR according to the same method described for NGA and  
257 FGA. The only difference was the solvent and concentration used which in this case were a  
258 deuterated water/deuterated methanol mixture (50: 50) at a concentration of 20 mM. The  
259 structure determination was done using <sup>1</sup>H, J-modulated <sup>13</sup>C, <sup>1</sup>H-<sup>1</sup>H COSY, <sup>1</sup>H-<sup>13</sup>C HSQC,  
260 <sup>1</sup>H-<sup>13</sup>C HSQC-TOCSY, and <sup>1</sup>H-<sup>13</sup>C HMBC.

#### 261 2.2.8.4. Size-exclusion chromatography coupled to multi-detectors

262 SEC experiments were performed with a HPLC pump (LC10AD, Shimadzu) coupled to  
263 an autosampler (Autosampler VE 2001, Malvern Panalytical) and a multi-detectors system  
264 recording UV, light scattering (RALS/LALS,) intrinsic viscosity and refractive index signals  
265 (Viscotek TDA305, Malvern Panalytical). Two SEC columns (A4000-A6000, 10 or 13 µm, 8

266 mm ID x 300 mm, void volume ~6 mL, total volume ~ 12.5 mL, Malvern Panalytical) were  
267 mounted in series and equipped with a post-column nylon filter (0.22  $\mu\text{m}$ ). The columns were  
268 equilibrated with ammonium acetate buffer 350 mM, pH 5.5. The flow rate was 0.35 mL.min<sup>-1</sup>  
269 and the temperature was 30 °C. Data were processed with the Omnisec software (v5.12,  
270 Malvern Panalytical). The calibration procedure was done with  $\beta$ -lactoglobulin (Sigma) and  
271 cross-validations were performed with PEO24k and Dextran standards (Viscotek PolyCal  
272 standards, Malvern Panalytical). The refractometer was used as the concentration detector and  
273 the refractive index increment value (dn/dc) used to determine the molecular weight was  
274 0.136 mL/g (Grein *et al.*, 2013). Free-dried dialyzed samples were solubilized in the  
275 aforementioned buffer at 10 g.L<sup>-1</sup> and filtered through a 0.22  $\mu\text{m}$  PES-filter just before  
276 injection.

277

### 278 **3. Results and discussion**

#### 279 3.1. Color modification during the reaction

280 The first event that reflected a significant change during the oxidation of ferulic acid  
281 (FA) was a color change from colorless to dark red (pictures from Figure S1). Indeed, it was  
282 already shown in a previous study (Aljawish *et al.*, 2014) that the oxidation products of ferulic  
283 acid (OXP) were generally colored. Other polymers grafted with ferulic acid oxidation  
284 products were gradually modified towards yellow–orange during functionalization (Aljawish  
285 *et al.*, 2014; Karaki *et al.*, 2017). On Figure S1, the color difference of the reaction medium  
286 during the oxidation reaction of ferulic acid at different time are presented with and without  
287 GA. At this stage, it was important to evidence if the presence of GA modify the oxidation  
288 process. FGA and OXP color difference ( $\Delta E$ , between  $t_0$ , the beginning of the reaction and a

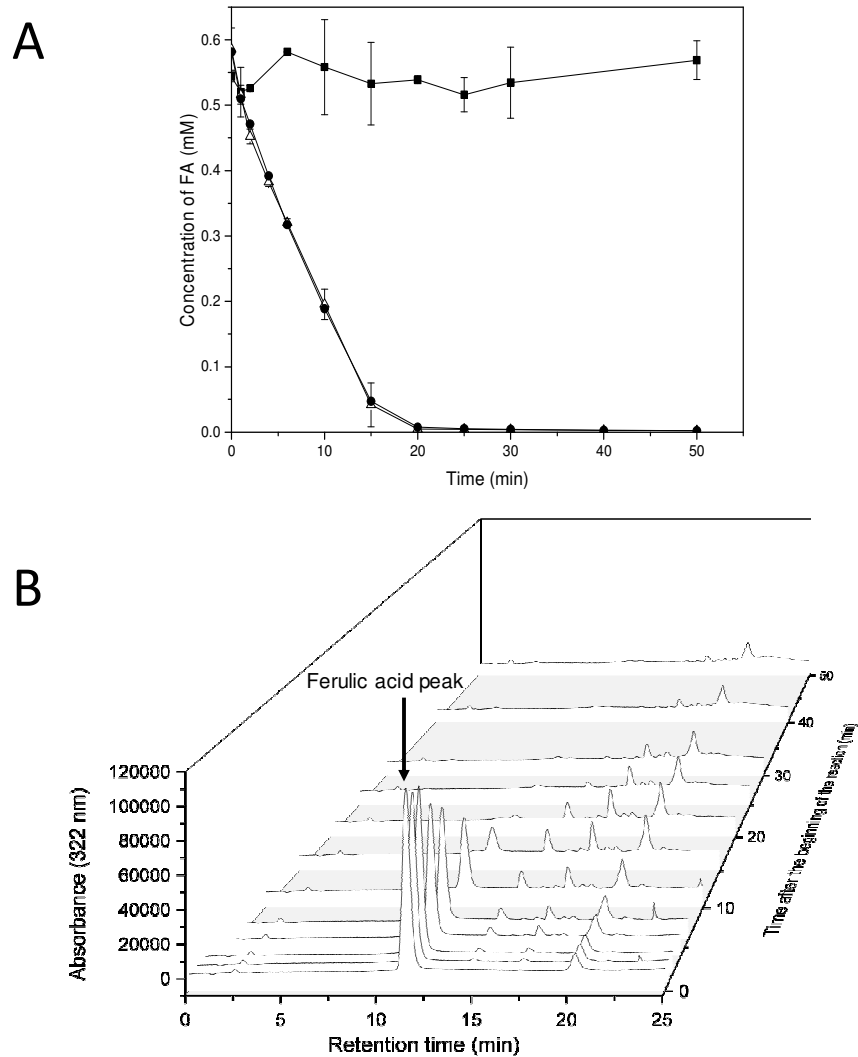
289 chosen time  $t$ ) increased upon time, meaning that changes occurred from the beginning of the  
290 reaction until its end (50 min). The values of color difference of FGA were significantly  
291 different when compared with those of OXP. The color change was more pronounced without  
292 GA (OXP). The 2 hypotheses formulated to explain this difference in coloring would be, on  
293 one hand, the presence of GA and, on the other hand, a slightly lower level of oligomerization  
294 in the presence of GA which blocks certain reaction intermediates by grafting. For the first  
295 hypothesis, GA was not soluble in the buffer (heterogeneous catalysis), so the presence of the  
296 polymer could have made the mixture a little turbid, thus changing the color parameters.  
297 However, the tendencies were close, which could indicate that the oxidation products were  
298 most probably the same with or without GA.

299

### 300 3.2. Monitoring of the ferulic acid consumption during the reaction

301 In order to verify if GA did not change the laccase activity or the ferulic acid  
302 consumption rate, the consumption of ferulic acid in the reaction mixtures was monitored by  
303 HPLC throughout the oxidation process.

304



306

307 Figure 1: (A) Ferulic acid (FA) concentration in phosphate buffer (50 mM, pH 7.4, 30 °C)  
 308 containing ferulic acid + gum Arabic + laccase ( $\Delta$ ), ferulic acid + laccase without gum Arabic  
 309 ( $\bullet$ ) and ferulic acid + gum Arabic without laccase (control) ( $\blacksquare$ ) and (B) Chromatogram at 322  
 310 nm of the oxidation products of ferulic acid at different reaction times, from the beginning of  
 311 the reaction to the end of the reaction (50 min).

312

313 The consumption of ferulic acid during the reaction with or without GA was monitored by  
 314 assaying its disappearance from HPLC chromatograms at 322 nm (Figure 1). A control was  
 315 made with GA and ferulic acid without laccase to ensure the FA concentration would not



316 decrease without laccase, i.e. that FA did not adsorb onto or react with GA. The results  
317 presented on Figure 1.A indeed confirmed that FA concentration did not decrease upon time  
318 without the enzyme. Physicochemical interactions between phenolic acids and  
319 polysaccharides have already been observed by other authors (Padayachee *et al.*, 2012), but in  
320 this case, the assumption that ferulic acid would adsorb onto GA chains was ruled out.  
321 However, when laccase was added, the FA concentration decreased rapidly and similarly with  
322 or without GA ( $0.52 \pm 0.01$  mM FA.min<sup>-1</sup> for OXP and  $0.52 \pm 0.02$  mM AF.min<sup>-1</sup> for FGA).  
323 These results suggested that the presence of GA did not interfere with the laccase activity and  
324 did not affect the reaction rate. Some authors have reported the rate of reaction of phenol  
325 substrate would be slowed down by adding a polymer (Karaki *et al.*, 2017; Sun, Payne, Moas,  
326 Chu, & Wallace, 1992; Wada, Ichikawa, & Tatsumi, 1993) but another study on  
327 polysaccharide functionalization (Aljawish *et al.*, 2012), also performed under heterogeneous  
328 catalysis, demonstrated that the presence of the polymer would not change the reaction rate.

329 On Figure 1A it can be observed that almost all ferulic acid was consummated after 20  
330 min ( $0.005 \pm 0.001$  mM remained for OXP and  $0.002 \pm 0.001$  mM for FGA). This result was  
331 quite surprising because the color of the reaction still evolved between 20 and 50 min for both  
332 reactions (Figure S1). This could be explained by the reaction pathway. Laccase oxidized  
333 phenols in *ortho* or *para* position into quinones and semi-quinones (Witayakran & Ragauskas,  
334 2009) that were unstable and could then react with other molecules. Once they are formed,  
335 according to the resulting structure, the oxidation products can then react with another ferulic  
336 acid molecules or other oxidation products or be oxidized once again by the enzyme.  
337 Numerous structural changes may also occur, for example by oxidative decarboxylation, thus  
338 increasing the number of possible structures grafted onto the polysaccharide (Aljawish *et al.*,  
339 2012; Karaki *et al.*, 2016). In addition, these molecular reorganizations are often accompanied

340 by an increase in molecular mass leading to the appearance of more sustained colors, which is  
341 observed by colorimetry. In order to understand the changes that occurred after FA was  
342 consummated, and to follow the number of oxidation products formed and their modification  
343 upon time, the absorbance at 322 nm of the oxidation products of ferulic acid upon retention  
344 time, all along the reaction, was studied by HPLC. After the peak recorded at 11.4 min  
345 associated to ferulic acid (peak area used to follow its disappearance upon time) several other  
346 peaks appeared and may then sometimes disappeared related to the apparition or the  
347 disappearance of several oxidation products.

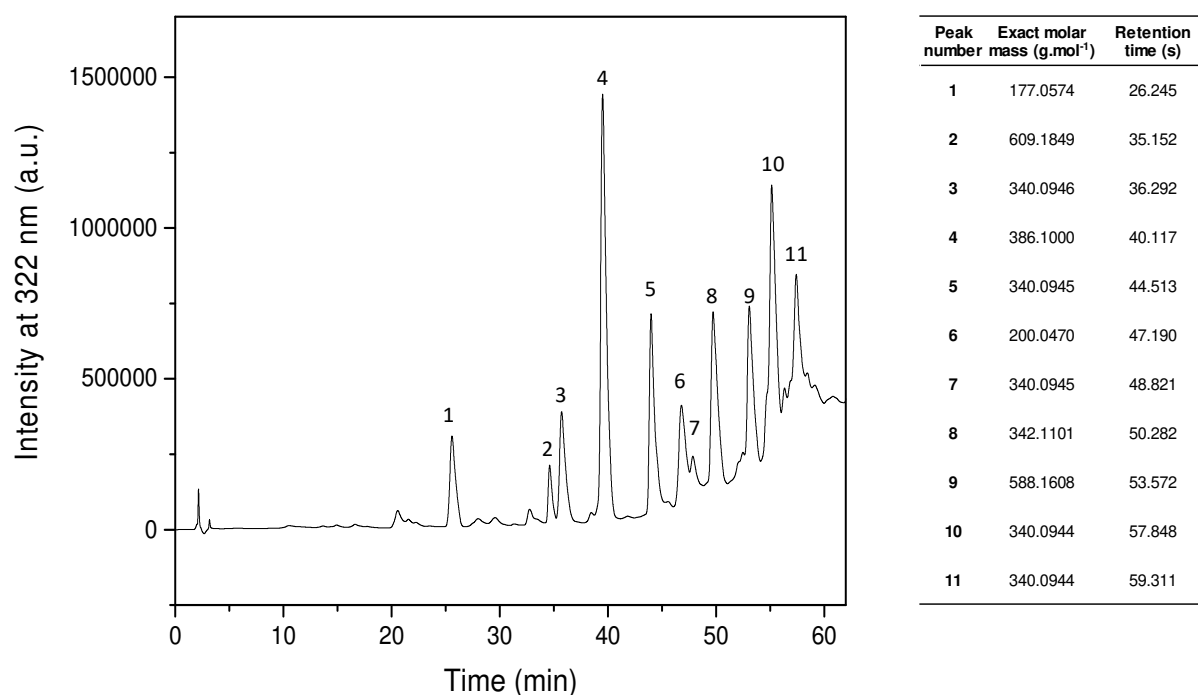
### 348 3.3.Study of the oxidation products

349 The absorbance at 322 nm of the mixtures (Figure 1.B) revealed the presence of several  
350 peaks that corresponded to different oxidation products. Their retention times were higher  
351 than that of ferulic acid (11.4 min), meaning they were more hydrophobic related to the eluent  
352 composition. Some products were formed during the reaction but disappeared before the end  
353 (for example the peak at a retention time of 14.7 min). At the end of the reaction, a massive  
354 group of peaks was observed in the range of retention time from 17.0 to 21.1 min. The  
355 oxidation products did indeed change between 20 and 50 min of reaction, thus explaining why  
356 the color of the samples would change (Figure S1) even after all FA was consummated.

357 Each observed absorbance peaks corresponded to one oxidation product which might react  
358 with the polysaccharide when GA was present in the reaction media. Their structures were  
359 thus further investigated by **LC-MS/MS**.

360 Thanks to UV screening at 322nm recorded during more than 60 minutes, 11 oxidation  
361 products were readily identified by **LC-MS/MS** analysis 50 minutes after the enzyme addition  
362 as shown in Figure 2 giving their retention time and their exact molecular mass. The peak n°1

363 corresponded to dehydrated ferulic acid (shown by its molecular mass ( $177.06 \text{ g}\cdot\text{mol}^{-1}$ ) and  
 364 retention time (26 s)). The peak n°6 has a molecular mass close to the one of ferulic acid  
 365 ( $M_{\text{FA}}=194.18 \text{ g}\cdot\text{mol}^{-1}$ ) so it would seem, therefore, that it corresponded to a molecule with  
 366 only one phenolic ring with a structure close to the one of ferulic acid. From their molecular  
 367 weights, OXP n° 2 and 9 were most probably ferulic acid derivatives trimers formed with  
 368 three derivatives of ferulic acid whereas the majority of the 7 other OXP were obviously  
 369 ferulic acid derivatives dimers as already observed in previous studies (Aljawish *et al.*, 2014;  
 370 Carunchio, Crescenzi, Girelli, Messina, & Tarola, 2001).



371

372 Figure 2: Absorbance at 322 nm and retention time of the oxidation products of ferulic acid  
 373 (at the end of the reaction) and corresponding molecular exact mass (precise until  $10^{-4} \text{ g}\cdot\text{mol}^{-1}$ ).  
 374

375 In order to investigate further their structure, the OXP were fractionated using high-  
 376 performance semi-preparative liquid chromatography. Based on the purity and quantity of the

377 OXP obtained for each peak, further **LC-MS/MS** and NMR investigations were performed  
378 only on OXP n° 3, 4, 5, 8 and 9.

379 The chosen OXP were analyzed by **LC-MS/MS**. From the exact molecular mass, formula  
380 predictions were generated. The most probable formula prediction (the one for which the  
381 exact mass was closest to the one measured) was presented in Table 1, along with neutral  
382 losses identified in MS<sup>2</sup> and MS<sup>3</sup> spectra. These OXP were also analyzed by NMR (<sup>1</sup>H and  
383 <sup>13</sup>C measurements). Due to problems of concentration and thus NMR sensitivity issues, it was  
384 not possible to fully elucidate the structure of all studied OXP. Information obtained from  
385 standard NMR measurements (<sup>1</sup>H and <sup>13</sup>C measurements) are presented in supplementary  
386 material (Table S1).

387 Table 1: Peak number from Figure 2, neutral losses from MS<sup>2</sup> and MS<sup>3</sup> spectra, most probable  
388 formula prediction.

Peak number	Exact mass	Most probable formula prediction	Neutral loss (MS <sup>2</sup> )	Neutral loss (MS <sup>3</sup> )
3	340.0945	C <sub>19</sub> H <sub>16</sub> O <sub>6</sub>	CO <sub>2</sub>	CH <sub>3</sub> OH
4	386.1000	C <sub>20</sub> H <sub>18</sub> O <sub>8</sub>	CO <sub>2</sub>	H <sub>2</sub> O
5	340.0945	C <sub>19</sub> H <sub>16</sub> O <sub>6</sub>	CO	H <sub>2</sub> O
8	342.1101	C <sub>19</sub> H <sub>17</sub> O <sub>6</sub>	2H <sub>2</sub> O	None
9	588.1608	C <sub>32</sub> H <sub>28</sub> O <sub>11</sub>	M=386 g.mol <sup>-1</sup> = OXP 4	None

389

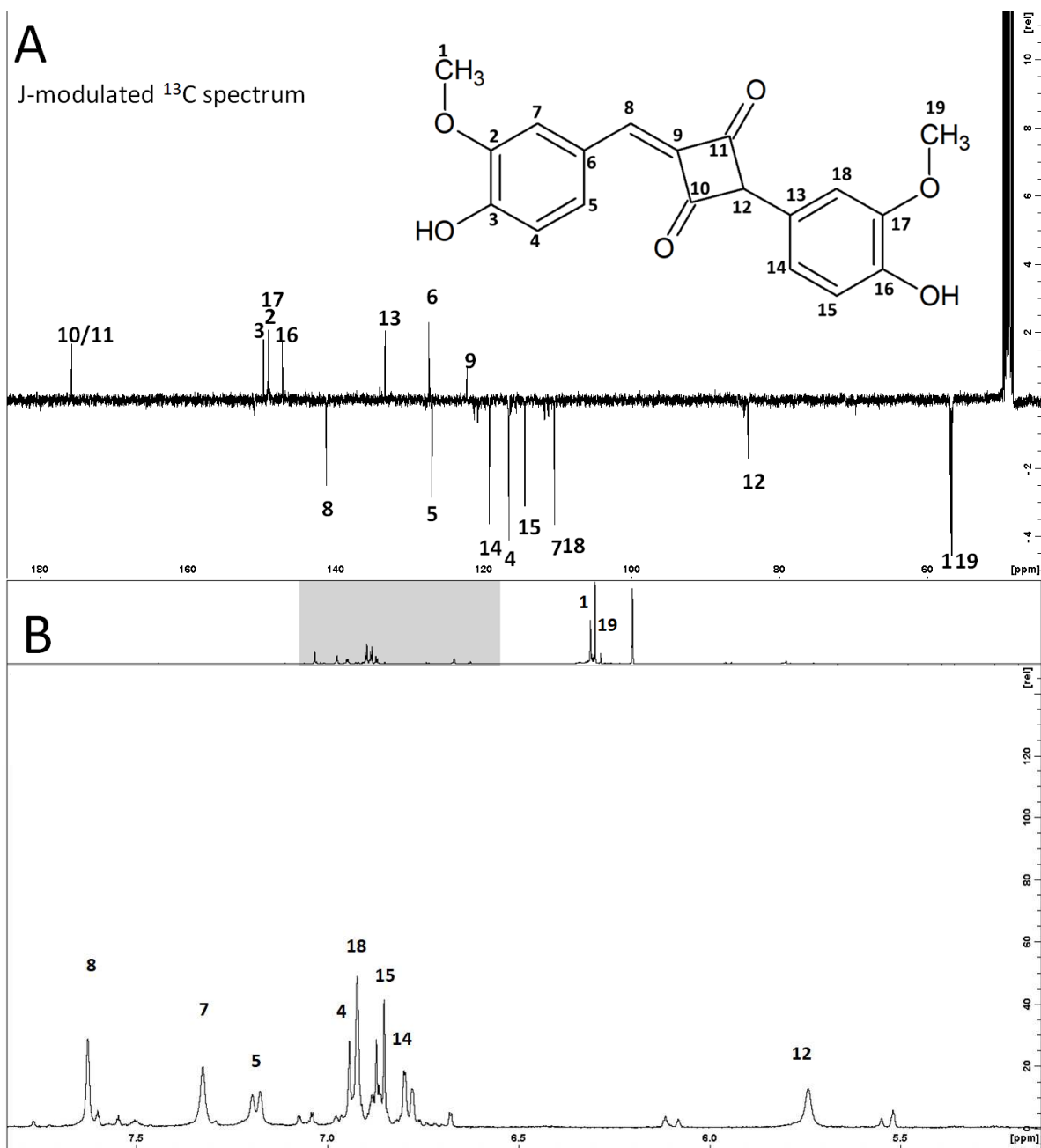
390 This preliminary study revealed four of these five OXP were ferulic acid derivatives dimers  
391 which probably kept their aromatic rings along with their methoxy groups. The fragmentation  
392 of the OXP n° 9 revealed it was probably a trimer composed of a dimer similar to OXP n° 4

393 and another ferulic acid derivative. Another interesting result was the OXP fragmentations  
394 were different, even for the ones with the same molecular mass (n° 3 and 5). These OXP had  
395 different retention times (Figure 2), meaning their hydrophobia was different. Though the  
396 most probable formulas for these products were the same, it would be difficult to presume to  
397 design their structure solely based on their molecular mass, however this approach is often  
398 available in literature.

399 From these results, it was admitted that most oxidation products were ferulic acid  
400 derivatives dimers with two aromatic rings and methoxy groups onto it, meaning the most of  
401 the oxidation product mechanism would involve forming ester bound between ferulic acids  
402 carboxyl groups and hydroxyl groups (Table S1). Further NMR experiments were conducted  
403 on the OXP n° 3 to clarify its structure.

#### 404 3.4. Determination of the structure of the oxidation product n° 3

405 Since it was the one with the highest signal/noise ratio in  $^1\text{H}$  and  $^{13}\text{C}$  measurements, the  
406 structure of the OXP 3 was investigated further.



407

408 Figure 3:  $^{13}\text{C}$  (A) and  $^1\text{H}$  (B) spectra of the OXP n<sup>o</sup>3 (**cyclobutadiferulone**) (9.4T, ambient  
 409 temperature, solvent D<sub>2</sub>O/deuterated methanol) with the peaks' attribution (the chemical shift,  
 410 J-coupling and  $^1\text{H}$ - $^1\text{H}$  COSY and  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectra of the OXP n<sup>o</sup>3 are presented in  
 411 supplementary material)

412

413  $^{13}\text{C}$  spectrum of the OXP n<sup>o</sup>3 is presented on Figure 3.A. It showed the presence of 18  
 414 signals. However, the predicted formula obtained from the molecular mass of the compound

415 contained 19 C (Table 1), which meant that two carbons were probably equivalent. <sup>1</sup>H  
416 spectrum of the OXP n°3 was presented on Figure 3.B and <sup>1</sup>H-<sup>1</sup>H COSY experiment is  
417 presented in supplementary materials (Figure S2). The characteristic signals for methoxy  
418 groups were displayed at 3.88 and 3.82 ppm. The compound spectrum also showed six  
419 aromatic proton at 6.91 (d; J=8 Hz), 6.84 (d; J=8 Hz), 7.17 (dd; J=8 and 1.7 Hz), 6.77 (dd; J=8  
420 and 1.5 Hz), 7.31 (d; J=1.7 Hz) and 6.90 (d; J=1.5 Hz), characteristics for the H-4, H-15, H-5,  
421 H-14, H-7 and H-18 of aromatic part of the compound. All chemical shifts and J-couplings  
422 are presented in supplementary materials (Table S2) These results, along with the knowledge  
423 of the substrate peaks attributions (Sajjadi *et al.*, 2012) led to presume there were two  
424 aromatic rings from ferulic acid with the methoxy and the hydroxy groups on them.  
425 Correlations obtained by <sup>1</sup>H-<sup>13</sup>C HSQC and <sup>1</sup>H-<sup>13</sup>C HMBC measurements confirmed this  
426 hypothesis and are presented in supplementary materials (Figure S3). Only one peak was  
427 observed in the aldehyde/ketone area of the carbon-13 spectrum (175.7 ppm) leading to the  
428 assumption there were C=O equivalent groups. They were both correlated to the proton in C-  
429 8. This OXP was then identified to be 2-(4-hydroxy-3-methoxyphenyl)-4-[(4-hydroxy-3-  
430 methoxyphenyl) methylidene] cyclobutane-1, 3-dione presented on Figure 3, **called by the**  
431 **authors cyclobutadiferulone**. This molecule possesses a tautomeric form that could potentially  
432 be charged in solution (Figure S4), but in NMR measurement only one form was detected. It  
433 was not possible to propose a mechanism for this OXP formation, which suggested that it did  
434 not result simply from the reaction of two oxidized ferulic acids derivatives. The most  
435 probable hypothesis was that one ferulic acid was first oxidized/modified by the enzyme and  
436 that this intermediate product would have reacted onto another ferulic acid molecule or a  
437 modified one. In order to propose a mechanism for the 2-(4-hydroxy-3-methoxyphenyl)-4-[(4-  
438 hydroxy-3-methoxyphenyl) methylidene] cyclobutane-1, 3-dione formation, further

439 investigations of the intermediate products that appear and then disappear during the reaction  
440 (Figure 1) have to be performed all along the reaction.

441 However, GA has several sites which may react with the oxidation products previously  
442 studied. The OXP could react with these functional groups to be covalently linked to the  
443 polysaccharide. The grafting of these products onto GA was thus investigated.

444

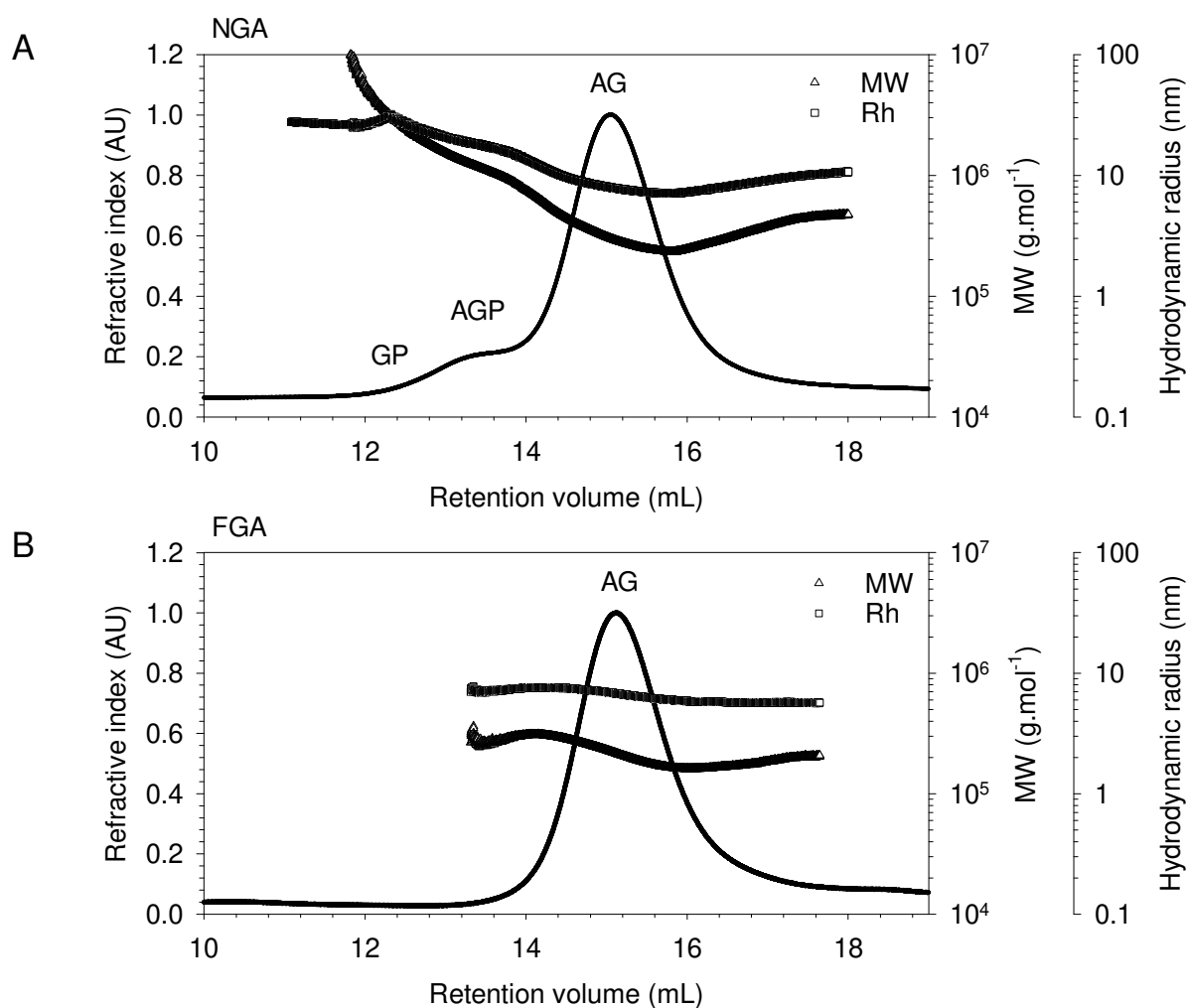
### 445 3.5. Modification of gum Arabic

446

447 The impact of OXP grafting on the molecular weight and hydrodynamic radius of GA has  
448 been determined using Size exclusion chromatography (SEC) coupled to a multi-detectors  
449 system (UV, LS, IV and RI signals were recorded). NGA was analyzed (Figure 4A) and three  
450 peaks were observed, corresponding to the expected GP, AGP and AG fractions (Picton *et al.*,  
451 2000). The proportion of each fraction was determined using RI detector (0.7%, 10.6% and  
452 88.7%, respectively). The determination of  $M_w$  using light scattering gave  $M_w$  of  $\sim 3.88 \times 10^6$   
453  $\text{g}\cdot\text{mol}^{-1}$ ,  $\sim 1.25 \times 10^6 \text{ g}\cdot\text{mol}^{-1}$  and  $\sim 3.33 \times 10^5 \text{ g}\cdot\text{mol}^{-1}$  for the respective fractions. Finally, the  
454 determination of the hydrodynamic radius ( $R_h$ ) was performed thanks to IV detector, giving  
455 values of  $\sim 28.2$ , 18.4 and 8.4 nm (see Table S1 in supporting information for a summary of  
456 the derived parameters). It is worth notifying that since no significant UV signal at 280 nm  
457 was observed, the determination or estimation of the protein content of each fraction was not  
458 possible. Nevertheless, all aforementioned results are in line with the literature (Randall *et al.*,  
459 1989; Sanchez *et al.*, 2008).



460 FGA was analyzed in the same way (Figure 4B). The first observation was the  
461 disappearance of the peaks corresponding presumably to the GP and AGP fractions. Indeed,  
462 only one peak having a retention volume really close to the one of the AG fractions in NGA  
463 was observed. It was concluded that the two fractions were eliminated during ethanol  
464 precipitation realized after the enzymatic reaction leaving only a purified and functionalized  
465 AG fraction.  $M_w$  obtained for NGA was  $\sim 2.13 \times 10^5 \text{ g.mol}^{-1}$  and  $R_h$  was 6.6 nm (see Table S3  
466 for a summary of the derived parameters). Such values indicated that a large decrease of  $M_w$   
467 of the AG fraction occurred during the purification process and/or that a sub-fraction of AG  
468 was purified that was previously observed by HIC purification (Atgié *et al.*, 2019).  
469 Surprisingly, no UV signal at 322 nm was observed, certainly due to the small amount of  
470 OXP present on FGA. It was also observed that after filtration through 0.22 PES filter just  
471 before injection, the sample was colorless and no longer orange. Interestingly, the nylon post-  
472 column filter was yellowish after unmounting meaning again that excess of OXP. Excess of  
473 unbound OXP could have been stuck onto the filters. Nevertheless, thanks to the purification  
474 procedure of FGA, a purified and likely functionalized fraction was isolated, having a  
475 remarkably small polydispersity index of  $\sim 1.04$  (Table S3).



476

477 Figure 4: Size exclusion chromatography characterization (refractive index in filled line,  $M_w$   
 478  $\Delta$ , Rh  $\square$ ) of native gum Arabic (NGA) (A) and functionalized gum Arabic (FGA) (B). AG =  
 479 arabinogalactan-peptide, AGP = arabinogalactan protein and GP = glycoprotein.

480

481 It was seen previously that the studied OXP could be charged in solution. Several  
 482 authors already studied the ferulic acid oxidation products molecular mass and suggested that  
 483 they would be mostly negatively charged dimers of ferulic acid (Adelakun *et al.*, 2012;  
 484 Aljawish *et al.*, 2014). If they were grafted onto GA, it should change its charge.  
 485 Furthermore, the FGA being a functionalized AG fraction, it should contain less amine groups

486 and more uronic acid groups, which is responsible for its negative charge. This should lead to  
487 FGA charge being lower than NGA charge in solution (Grein-Iankovski et al., 2018).

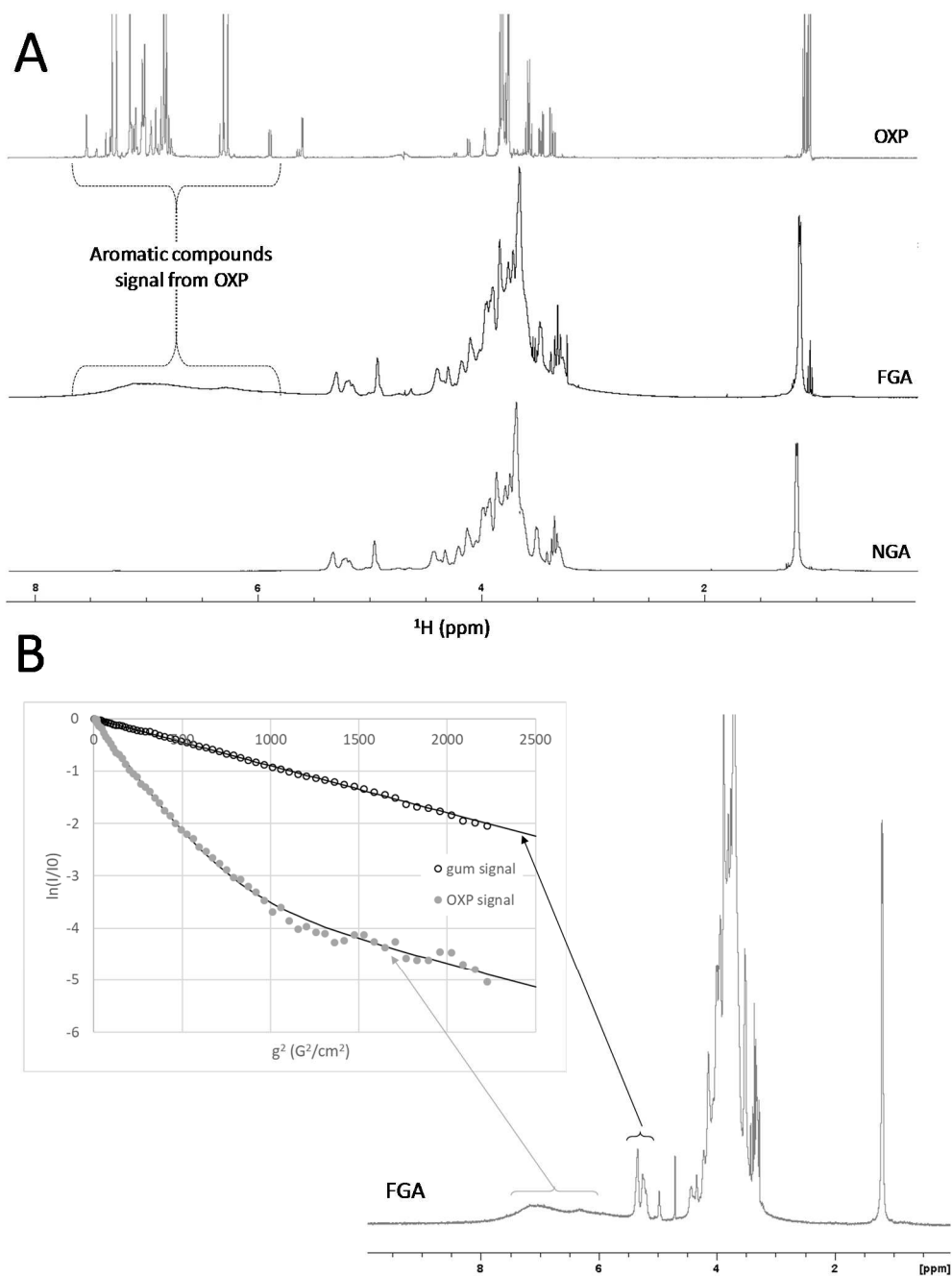
488 The Approximate Zeta potentials of NGA and FGA in ultrapure water were measured  
489 at pH 5.5, 25 °C, at 1 % w/v. At this pH, the uronic groups of NGA and FGA should be  
490 deprotonated (pKa of 3.6, (Aberkane et al., 2010)) as well as grafted ferulic acid oxidation  
491 products (pKa around 4.6 (Dempsey et al., 1979)) and the amine groups on NGA should be  
492 protonated (pKa<sub>2</sub> around 6.5 (Grein-Iankovski et al., 2018)).

493 The NGA Approximate Zeta potential was  $-25.8 \pm 0.6$  mV, which was related to the  
494 presence of the carboxylic groups of the glucuronic acid. This value decreased to  $-30.1 \pm 0.9$   
495 mV when GA was functionalized, as it was expected from the absence of the GP and AGP  
496 fractions in FGA.

497 The decrease of the charge (or the increase of the negative charge) of GA after  
498 functionalization was the first hint that the oxidation products were grafted onto it, however it  
499 was not possible to make hypothesis on where it was grafted. They could possibly be grafted  
500 onto amino groups of the proteinaceous fraction by forming a Schiff base bound as for  
501 chitosan (Aljawish *et al.*, 2012) or onto carboxyl groups of the glucuronic acids by forming an  
502 ester as for pectin (Karaki *et al.*, 2017). The negative charge of GA was expected to be stable  
503 or to increase after functionalization, whether they replace a carboxylic charge or an amine  
504 charge.

505 Zeta Potential measurements and SEC-MALS analysis evidenced a strong modification  
506 of GA by the enzymatic functionalization process attributed to the grafting of ferulic acid  
507 derivatives.

508 Therefore, Nuclear Magnetic Resonance (NMR) was used in order to get better insight of the  
509 location of the grafting onto GA.



510

511

512 Figure 5: (A)  $^1\text{H}$  NMR spectra of oxidation products (OXP), functionalized gum Arabic  
 513 (FGA) and native gum Arabic (NGA) and (B)  $^1\text{H}$  NMR spectra of functionalized gum Arabic  
 514 (FGA). In the inset: evolution of the signal (logarithmic scale, as a function of the gradient  $g$   
 515 applied) obtained by pulsed field gradient measurement, for two different areas of the spectra.  
 516 ( $\bullet$ ) Signal of OXP from 6.0 to 7.5 ppm; ( $\circ$ ) signal of gum Arabic from 5.0 to 5.5 ppm. The  
 517 diffusion coefficients were obtained from these evolutions.

518

519  $^1\text{H}$  NMR experiments were performed on three samples: NGA, FGA and OXP (Figure 5.A).

520 The signals of NGA and FGA were crowded between 3.0 and 6.0 ppm, which is typical of

521 polysaccharides and reflected the presence of the sugar residues (Nie *et al.*, 2013). Those

522 signals have not been deteriorated after functionalization, meaning that the polymer main

523 structure was preserved. On the OXP spectrum, the peaks between 6.0 and 7.5 ppm were

524 characteristic of aromatic proton (Sajjadi, Shokoohinia, & Moayedi, 2012). On the spectrum

525 of dialyzed FGA, broad signals were visible in this aromatic compound area (6.0 to 7.5 ppm),

526 which corresponded to the grafted OXP. The signal was not very intense due to the weak

527 grafting rate and to the broadening due to the loss of mobility occurring in the grafting.

528 Pulsed field gradient (PFG) is a common NMR technique to study the diffusion of polymers

529 (Callaghan & Pinder, 1985; Dixon & Larive, 1997; Oostwal, Bles, de Bleijser, & Leyte,

530 1993). Such measurement was made on the NGA and FGA samples (Figure 5.B).

531 NGA was found to have a unique diffusion coefficient (around  $1 \times 10^{-11} \text{ m}^2.\text{s}^{-1}$ , data not

532 shown). Results obtained on the FGA sample (Figure 5.B) were analyzed considering two

533 distinct areas of the  $^1\text{H}$  one-dimensional NMR spectrum. In the area corresponding to the gum

534 signal only (between 5.0 and 5.5 ppm), a unique diffusion coefficient was obtained, as shown

535 by signal monotonous evolution obtained in the inset of Figure 5.B (open symbols). The

536 obtained value of  $2 \times 10^{-11} \text{ m}^2.\text{s}^{-1}$ , slightly higher than the one obtained with NGA, indicated a

537 decrease of the polymer size and/or a decrease in medium viscosity after functionalization.

538 The evolution of the hydrodynamic radius determined from the diffusion coefficient using the

539 Stokes-Einstein equation (Gaylord and Gibbs, 1962) are in good correlation with the ones

540 obtained from SEC-multi-detectors experiments (Table S1). Indeed, the calculated  $R_h$  based

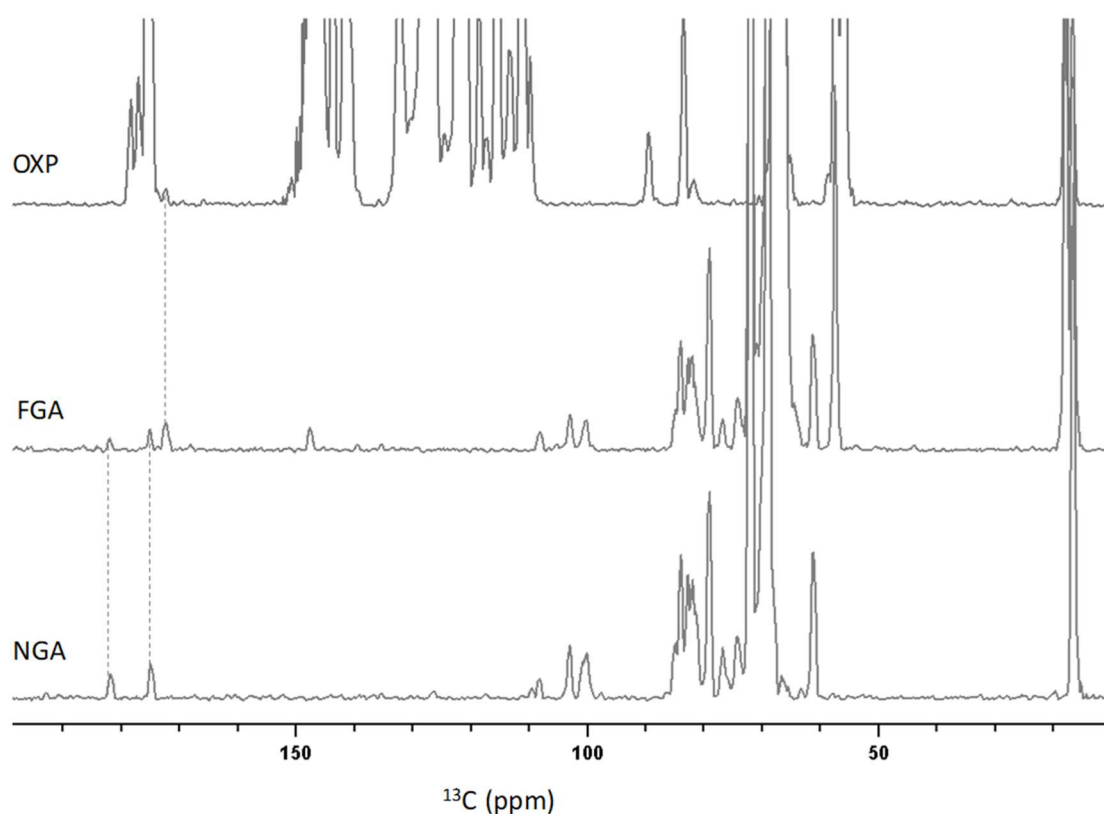
541 on the diffusion coefficient (21.8 nm for NGA and 10.9 nm for FGA) showed the same

542 tendency as the ones obtained from SEC- multi-detectors experiments (9.6 nm for NGA and  
543 6.6 nm for FGA). In both cases, the polymer size decreased after functionalization. The  
544 different values of hydrodynamic radius may be the result of the experiment being performed  
545 in different solvents. Indeed, the  $R_h$  can vary in a given solvent because of its ability to  
546 increase and expand the polysaccharide coils (Antoniou *et al.*, 2010).

547 Furthermore, in the area corresponding to OXP signals (6.0 to 7.5 ppm) on the FGA spectrum,  
548 two diffusion coefficients were obtained, as indicated by the slope break on the inset of Figure  
549 5.B (filled symbols). These two coefficients indicated that there were two types of OXP. One  
550 of the diffusion coefficients was the same as the one of the gum signal of FGA ( $2 \times 10^{-11} \text{ m}^2 \cdot \text{s}^{-1}$ ,  
551 parallel lines between gum and OXP signals from 1000 to 2500  $\text{G}^2 \cdot \text{cm}^{-2}$  in the diffusion  
552 experiment) and could therefore be attributed to a fraction of OXP linked to the gum. The  
553 other coefficient was faster (around  $8 \times 10^{-11} \text{ m}^2 \cdot \text{s}^{-1}$ ) and could be attributed to a fraction of  
554 OXP that were not directly linked to the functionalized GA. This fraction could correspond to  
555 a small fraction of non-grafted oxidation products that were not eliminated during dialysis  
556 because they were strongly adsorbed on FGA. Diffusion measurements could thus show that a  
557 fraction of OXP was grafted onto FGA, 2D NMR approaches were then performed in order to  
558 locate where they were grafted.

559 HMBC spectra were obtained on the three samples. These spectra were first used in order to  
560 obtain information on the carboxyl zone before and after functionalization. Indeed, a direct  
561  $^{13}\text{C}$  measurement was not conceivable because of a lack of sensitivity, indirectly detected  $^{13}\text{C}$   
562 spectra (using  $^1\text{H}$  polarization) of the three samples were thus obtained from projections of the  
563 HMBC (Figure 6). It appeared on these spectra the additional peaks observed on the  
564 functionalized gum corresponded to phenolic groups signals. In a second step, and in order to  
565 verify if there was a correlation between the FGA carbons and the protons from OXP, HMBC

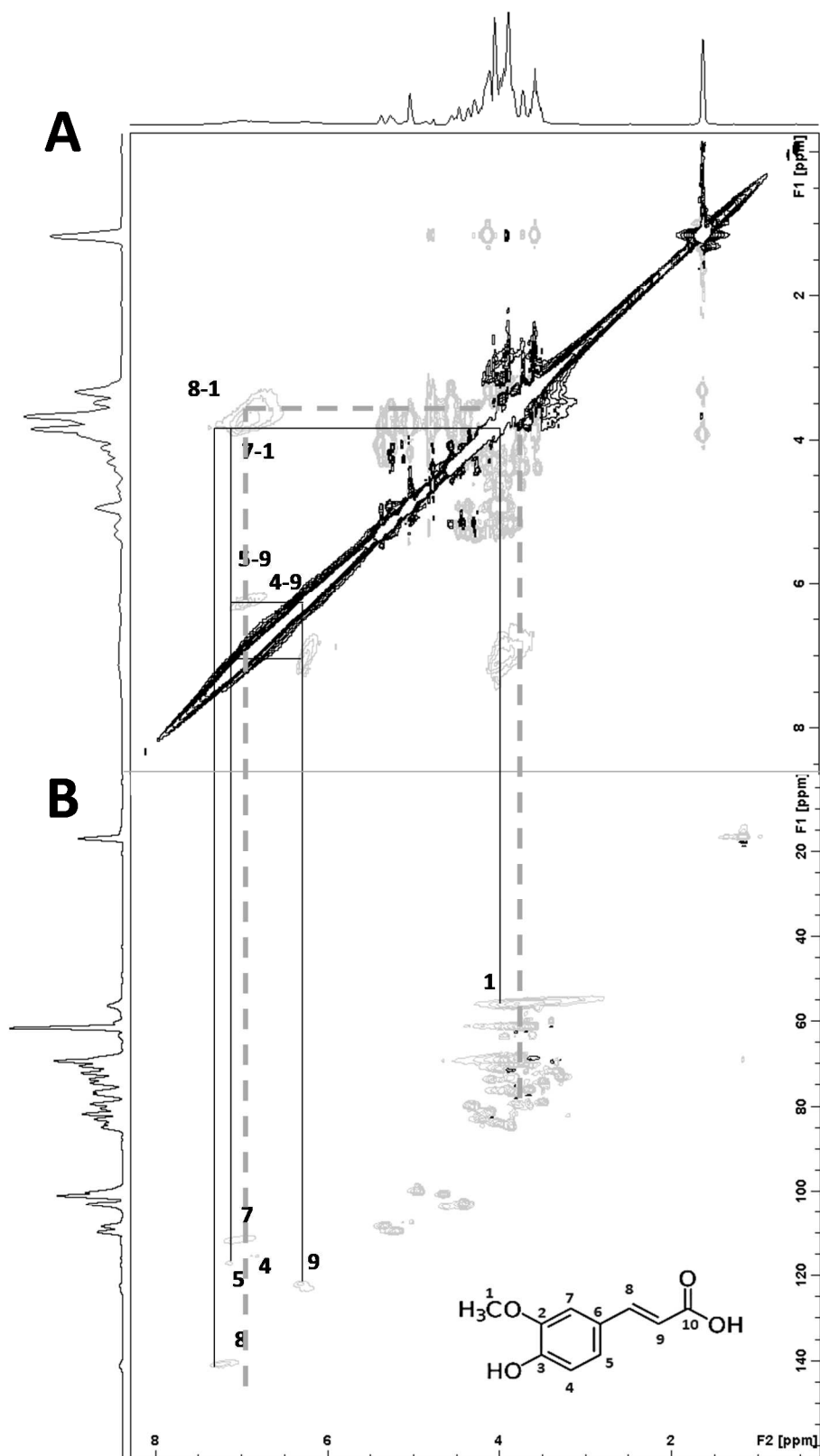
566 correlation were finely analyzed (supplementary material, Figure S5). The weak proportions  
567 of OXP did not make it possible to visualize a direct correlation between the aromatic  $^1\text{H}$  of  
568 OXP and a carbon of FGA. Even if only intra-OXP or intra-gum correlations could be  
569 observed in the HMBC, carboxyl carbon peak moved from 175.4 ppm in the OXP mixture to  
570 168.2 ppm in the FGA sample, which may indicate that OXP were possibly esterified.



571  
572 Figure 6:  $^{13}\text{C}$  NMR spectra obtained from the projections of HMBC recorded on oxidation  
573 products (OXP), native gum Arabic (NGA) and functionalized gum Arabic (FGA).  
574 ROESY experiments of FGA were recorded (Figure 7). They allowed the visualization of a  
575  $^1\text{H}$ - $^1\text{H}$  correlation by dipolar effect and thus drove to identify protons that were close in space.  
576 A  $^1\text{H}$ - $^1\text{H}$  dipolar interaction was visible between the aromatic  $^1\text{H}$  of OXP and GA but it was  
577 difficult to clearly identify the corresponding  $^1\text{H}$  due to the width of the correlation spots and  
578 their low intensity. However, a correlation was brought out between one aromatic  $^1\text{H}$  of OXP



579 and possibly the methylic protons of FGA galactose units (in C6) or the  $^1\text{H}$  in C5 of the  
580 glucuronic acid units of GA, meaning the OXP would be grafted either onto them or onto the  
581 near carbons. The results obtained in a previous study on pectin modification by grafting OXP  
582 of ferulic acid (Karaki *et al.*, 2017) with the same pathway also concluded on a grafting onto  
583 the C6 carbon of the galacturonate units of pectin.



584  
 585 Figure 7: (A) ROESY  $^1\text{H}$ - $^1\text{H}$  and (B) HSQC  $^1\text{H}$ - $^{13}\text{C}$  of functionalized gum Arabic FGA. As a  
 586 reference for OXP, the signals corresponding to ferulic acid are indicated according to the

587 numbering of the figure in the inset of (B). Intra-OXP correlations are indicated by straight  
588 lines. Gum Arabic-OXP correlation is indicated by a dotted line.

589 To summarize, the pulsed field gradient (PFG) measurements indicated that after dialyses  
590 there were still two populations of OXP in solution. One of them could be referred to as “free  
591 OXP” and the other one was strongly bound to FGA. Nevertheless, the presence of a chemical  
592 bonding between OXP and FGA was not obvious by experimental 2D NMR, due to the  
593 complexity of the spectra and especially sensitivity problems because of the low grafting rate  
594 of OXP. However, there was a set of clues proving that the grafting was conclusive, the  
595 strongest one being that the diffusion coefficient of GA was modified after functionalization  
596 and this coefficient was also measured in the spectral area corresponding to the OXP species.  
597 Although, no direct interaction between OXP and GA could be evidenced by HMBC. ROESY  
598 correlations proved the proximity of both compounds and made it possible to estimate a  
599 location for the site of the grafting: close to the C6 of galactose units or close to the C5 of the  
600 glucuronic acid units of GA, which was consistent with what was observed on other polymers  
601 (Karaki *et al.*, 2017).

602

#### 603 **4. Conclusions**

604 This study focused on the modification of gum Arabic by an enzymatic pathway. The  
605 first result was that GA did not alter the enzymatic oxidation of ferulic compounds since  
606 neither the color nor the ferulic acid consumption were modify in the presence of the  
607 polysaccharide. Furthermore, it was observed by HPLC that several oxidation products were  
608 formed which may then react with GA. The exact mass of each OXP was determined by LC-  
609 MS/MS, leading to the conclusion that the majority of the products obtained were ferulic acid

610 dimers which kept their aromatic rings and the O-CH<sub>3</sub> groups onto them. Assumptions were  
611 made about their respective raw formulas and one of them was clearly identified from NMR  
612 experiments as **cyclobutadiferulone** (2-(4-hydroxy-3-methoxyphenyl)-4-[(4-hydroxy-3-  
613 methoxyphenyl) methylidene] cyclobutane-1,3-dione). Further experiments will have to be  
614 carried out in order to make it possible for all OXP structures to be elucidated and to propose  
615 a mechanism for their formation. However, the grafting of oxidation products onto GA  
616 modified its charge (its approximate Zeta potential decreased) and only one fraction was  
617 recovered after functionalization instead of the three of the native gum. SEC-MALS analysis  
618 indeed evidenced that only AG fraction remained but with lower molecular weight and  
619 polydispersity. From NMR analysis, oxidation products were undeniably present on modified  
620 GA, probably close to the glucuronic acid C5 carbon or close to the galactose C6 carbon. In  
621 spite of the complexity of such a system due to the number and variability of the ferulic acid  
622 oxidation products, the complexity of GA with its three fractions and its high polydispersity,  
623 the combination of various well-chosen technics allowed the confirmation of the grafting of  
624 ferulic acid derivative onto GA and a better knowledge on the laccase-assisted modification of  
625 polysaccharide.

626

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