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1 **Sequential natural deep eutectic solvent pretreatments of apple pomace: a novel way to**
2 **promote water extraction of pectin and to tailor its main structural domains**

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7 **Abstract**

8 To establish a "green" biorefinery extraction of apple pomace pectin, a sequential pretreatment with
9 three natural deep eutectic solvents (NADES, choline chloride (CC): glycerol (G); CC: lactic acid (LA);
10 potassium carbonate (K): G) was used prior to hot water extraction. A synergistic effect of CC:G and
11 CC:LA pretreatments was observed and led to the highest recovery of pectin. The sequential
12 NADES/water extraction process also provided a mean to tailor pectin main structure. It was
13 explained as resulting from ion exchange and individual NADES components effects. The ¹³C solid
14 state NMR $T_{1\rho}^H$ and T_{HH} parameters indicated a reorganization of cellulose in the residues following
15 extraction of pectin, notably after alkaline K:G pretreatment/water extraction. Hence, sequential
16 NADES pretreatments/water extraction represents a "green" alternative to mild mineral acid to
17 extract pectin and to tailor its main structures, while the residual pomace can be further sources of
18 valuable compounds and polymers.

19 **Key words:** Natural deep eutectic solvent, Apple pomace, Sequential extraction, Pectin structural
20 domain, ¹³C CP/MAS NMR spectroscopy, VCT-CPMAS.

21

22 **1. Introduction**

23 Pectin is a family of structurally complex polysaccharides of plant cell wall. It is generally classified as
24 homogalacturonan (HG), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII) structural
25 domains. HG takes up 65% of pectin structure, followed by RGI (20–35% of pectin) and lastly by RGII
26 (10% of pectin) (Mohnen, 2008). HG backbone consists only of galacturonic acid unit (Voragen,
27 Beldman, & Schols, 2001) of which the carboxylic acid function can be esterified by methanol while
28 acetyl ester can be found on galacturonic acid at position of O-2 and/or O-3 (Atmodjo, Hao, &
29 Mohnen, 2013). According to the degree of methyl esterification, pectin is distinguished as high
30 methoxyl (HM) pectins (degree of esterification > 50%) and low methoxyl (LM) pectins (degree of
31 esterification < 50%) (Löfgren & Hermansson, 2007). RGI consists of a repeating disaccharide unit of

32 galacturonic acid and rhamnose with side chain made of arabinose and galactose linked on O-4 of the
33 rhamnosyl residues (Scheller, Jensen, Sørensen, Harholt, & Geshi, 2007). RGII is a complex structural
34 domain based on a branched HG backbone. The side chains are of four types made of 12 different
35 sugars (O'Neill, Ishii, Albersheim, & Darvill, 2004). Moreover, xylose can be found at O-2 position of
36 galacturonic acid to form xylogalacturonan (Schols, Bakx, Schipper, & Voragen, 1995).

37 Nowadays, pectin is widely applied as gelling agent, stabilizer, emulsifier and thickener in the
38 cosmetic and food industries (Güzel & Akpınar, 2019). The sources of pectin are mainly from
39 grapefruit peel, orange peel and apple pomace. Million tons of apple pomace was generated each
40 year from apple processing industry (Lu & Foo, 2000). The conventional industrial pectin extraction
41 method used is based on mild mineral acid. However, it often leads to environment related problems
42 and low extraction yield when compared with other emerging technology, such as enzymatic
43 extraction and ultrasound/microwave assisted extraction (Wikiera, Mika, Starzyńska-Janiszewska, &
44 Stodolak, 2015). Although these innovative technologies show advantages over traditional processes
45 with regard to environmental and energy saving issues (Adetunji, Adekunle, Orsat, & Raghavan,
46 2017), their scale-up and use by industry is delayed due to the expensive upfront investment and to
47 incomplete understanding of the process. In that context, there is room for new "green" and efficient
48 extraction processes compatible with industrial practices. One possibility is to take advantage of new
49 solvents, such as natural deep eutectic solvents (NADES). These solvents are composed of hydrogen
50 bond donors (HBD) and hydrogen bond acceptors (HBA), which in a definite molar ratio melt at
51 temperature far below than that of individual component to form transparent liquids (Liu et al.,
52 2018). These eutectic mixtures are naturally present in the cells of living organisms as a combination
53 of organic acids, sugars and amino acids (Choi et al., 2011). Since NADES are cheap, biodegradable,
54 eco-friendly and can be recycled, they are actively investigated as potential "green" solvent for
55 various purposes. Moreover, since NADES are formed from non-toxic metabolites, these solvents can
56 be applied in processes for food and cosmetics applications (Fernandez, Espino, Gomez, & Silva,
57 2018). However, the high viscosity of NADES can impede their use, but increasing temperature
58 and/or adding small amounts of water (5-20%) can alleviate this drawback by decreasing viscosity to
59 nearly that of water. Such adjustments render NADES usable as a water-based extractant for
60 industrial productions (Choi & Verpoorte, 2019). Based on the pH of the solvent, NADES can be
61 classified into neutral, acidic, alkaline NADES. Recently, many acidic NADESs have been tested as
62 potential solvent for pectin extraction (Benvenuto, Sanchez-Camargo, Zielinski, & Ferreira, 2020;
63 Shafie, Yusof, & Gan, 2019). Besides, in a previous work, we showed that choline chloride:Lactic acid
64 as a pretreatment of apple pomace could markedly ease subsequent hot water extraction of pectin
65 obtained in high yield (Chen & Lahaye, 2021). However, pretreatment with this solvent has to be

66 done with caution as it can lead to loss of arabinose in cell wall polysaccharides. As the pH of the
67 solvent is a known factor affecting extraction of pectin (Methacanon, Krongsin, & Gamonpilas, 2014),
68 neutral or alkaline NADESs may also have the potential to be applied in pectin extraction process.
69 Among various NADES combinations, the choline chloride : glycerol was widely used for extraction of
70 bioactive substances from agri-food waste (Grudniewska et al., 2018; Mouratoglou, Malliou, &
71 Makris, 2016; Sakti, Saputri, & Mun'im, 2019), while potassium carbonate:glycerol as emerging
72 NADES have shown the ability to isolate cellulose fibers or nanocrystals (Gan, Sam, Abdullah, Omar,
73 & Tan, 2020; Lim, Gunny, Kasim, AlNashef, & Arbain, 2019). Moreover, apart from the fact that the
74 gel like structure can be formed between dissociated carboxyl groups of HGs, certain pectin
75 structures strongly interact with cell wall cellulose (Broxterman, & Schols, 2018). The alkaline NADES
76 may represent a suitable candidate for recovering this part of pectin through its pH characteristics.
77 However, it is worth mentioning that due to its high pH, potassium carbonate : glycerol treatment
78 may degrade methyl-esterified pectin by a β -elimination mechanism. In contrast with one-step
79 extraction process, sequential extraction showed advantages in being more selective and
80 fractionating polysaccharides at the laboratory scale. Generally, sequential extraction process of
81 pectin is divided into three to four stages, and each stage uses a different extractant, such as, water,
82 chelating agent, diluted acid or concentrated alkaline solvents (Ramasamy, Gruppen, & Schols, 2013;
83 Yapo, Lerouge, Thibault, & Ralet, 2007), which allows for the recovery of pectin fractions with specific
84 properties (Gawkowska, Cybulska, & Zdunek, 2018; Guo et al., 2018).

85 Although many researches have been conducted on pectin extraction with conventional mineral acid
86 or alkaline solutions within our group (Kaya, Sousa, Crépeau, Sørensen, & Ralet, 2014; Koubala,
87 Kansci, Mbome, Crépeau, Thibault, & Ralet, 2008; Yapo et al., 2007), in the process of establishing an
88 innovative and “green” biorefinery of apple pomace, NADES as a promising green solvent was tested
89 for this purpose. As our previous result showed that abundant pectin resource still remained in
90 residual pomace after one-step NADES pretreatment following water extraction (Chen & Lahaye,
91 2021). Therefore, in present study, three types of NADES: Choline chloride:Lactic acid (acidic NADES),
92 Choline chloride:Glycerol (neutral NADES), Potassium carbonate:Glycerol (alkaline NADES) in
93 sequence prior to hot water extraction were tested as a mean to selectively extract pectin enriched
94 in specific structural domains while yielding extraction residues suitable for further recovery of
95 valuable polymers. Extractions yield and sugar composition were used to evaluate NADES
96 pretreatments efficiency while molecular weight distribution and degree of esterification assessed
97 the quality of the pectin recovered. Pectin extracted by methods from literature and NADES
98 pretreatments were also compared. The effects of NADES pretreatment/water extraction on pectin
99 yield and structural characteristic were discussed. Moreover, possible mechanisms of NADES

100 pretreatment effect were proposed. Finally, ¹³C solid-state NMR (ssNMR) spectroscopic analyses
101 were realized to assess the impact of NADES pretreatments/water extractions on the polysaccharides
102 composition, structure and organization in the residual pomace.

103

104 **2. Materials and methods**

105 2.1. Pomace

106 Dry industrial pomace was provided by IFPC (Le Rheu, France). Pomace was rehydrated to reach a
107 water content (68%, w/w) registered in fresh pomace (Chen & Lahaye, 2021) and then stored at -
108 20 °C prior use.

109

110 2.2. Chemicals

111 Choline chloride (CAS: 67-48-1, Sigma-Aldrich, France), glycerol (CAS: 56-81-5, Sigma-Aldrich, France),
112 potassium carbonate (CAS: 584-08-7, Merck, Germany), DL-lactic acid (CAS: 50-21-5, Sigma-Aldrich,
113 France), ethanol (CAS: 64-17-5, Carlo Erba reagents, France), acetone (CAS: 67-64-1, Carlo Erba
114 reagents, France), sodium acetate trihydrate (CAS: 6131-90-4, Sigma-Aldrich, France), *trans*-1,2-
115 Diaminocyclohexane-*N,N,N',N'*-tetraacetic acid monohydrate (CDTA) (CAS: 125572-95-4, Sigma-
116 Aldrich, France), sodium carbonate (CAS: 497-19-8, Merck, Germany), sodium borohydride (CAS:
117 16940-66-2, Sigma-Aldrich, France) were used in the present research.

118

119 2.3. Preparation and physiochemical properties measurement of natural deep eutectic 120 solvents

121 Both glycerol (G) and lactic acid (LA) were mixed with choline chloride (CC) in the molar ratio of 2:1
122 which is widely used ratio for polysaccharides processing (Zdanowicz, Wilpiszewska, & Szychaj, 2018).
123 Potassium carbonate (K) was mixed with G in different molar ratio as shown in **Table 1**. The NADESs
124 were mixed in an oil bath at 100 °C until a colorless transparent liquid was formed. The pH of various
125 solvents was measure using pH meter (IoLine, SCHOTT Instruments), while viscosities at 40 °C were
126 determined according to our previous research (Chen & Lahaye, 2021). The solvents were stored at
127 room temperature. The water content of prepared NADESs (CC:G=0.31% (w/w); CC:LA=0.91% (w/w);
128 K:G=0.57% (w/w)) were determined by freeze drying to constant weight prior to their use.

129

130 Table 1. Characteristics of the various NADESs.

HBA	HBD	Molar ratio	pH	Viscosity at 10 s ⁻¹ (mPa.s)	Viscosity at plateau (Pa.s)
Choline chloride	glycerol	1:2	6.5	48.9	—
	lactic acid	1:2	1.0	107.3	—
Potassium carbonate	glycerol	1:2	—	—	—
	glycerol	1:3	—	—	—
	glycerol	1:4	14.0	—	27.25
	glycerol	1:5	13.6	—	13.03
	glycerol	1:6	13.1	—	1.65
	glycerol	1:7	13.0	—	1.43
	glycerol	1:25	12.5	—	0.71

131 HBA: hydrogen bond acceptor; HBD: hydrogen bond donor; The viscosity of Newtonian and non-Newtonian
 132 NASDESs was determined at 40 °C (not considering water content in apple pomace), for non-Newtonian NADES
 133 solutions, the viscosity was that determined at the plateau between 0.1 to 100 s⁻¹; —: not determined

134

135 2.4. Pectin extractions

136 Four different extraction treatments were employed in the present study and schematically
 137 represented in **Fig. 1**.

138 2.4.1. Water extraction (route A, Figure 1)

139 Wet apple pomace (10 g) was extracted with deionized water (400 ml) under agitation at 75 °C for
 140 1 h. The soluble polysaccharides were recovered by centrifugation at 15000 g for 20 min.
 141 Polysaccharides were precipitated with 4 volumes of ethanol, washed with 70% ethanol for 10 min (3
 142 times), followed by ethanol and acetone for 10 min (2 times), air dried and then dried at 40 °C in
 143 vacuum oven over P₂O₅ powder for 12 h.

144 2.4.2. Mineral acid extraction (route B, Figure 1)

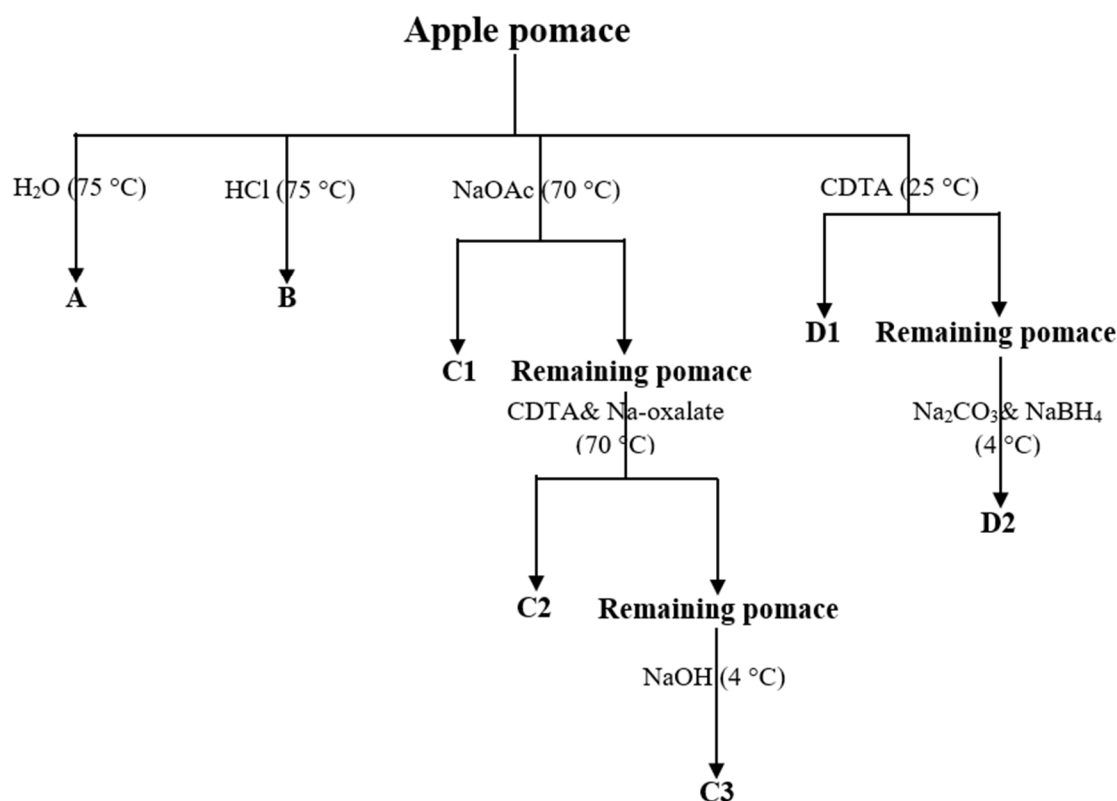
145 Wet apple pomace (10 g) was extracted with HCl (pH 1.5, 400 ml) under agitation at 75 °C for 1 h.
 146 The soluble polysaccharides were recovered by centrifugation at 15000 g for 20 min. The
 147 polysaccharides were precipitated with 4 volumes of ethanol, washed with 70% ethanol for 10 min (3
 148 times), followed by ethanol and acetone for 10 min (2 times), air dried and then dried at 40 °C in
 149 vacuum oven over P₂O₅ powder for 12 h.

150 2.4.3. Sequential chelating-agent extraction I (route C, Figure 1)

151 The sequential chelating-agent extraction I was conducted according to Vierhuis et al. (2000) with
 152 some modification. In brief, wet apple pomace (10 g) was sequentially extracted under agitation with
 153 **(1)** 0.05 M NaOAc buffer, pH 5.2 (three times, 100 ml) at 70 °C for 30 min; **(2)** 0.05 M CDTA and 0.05
 154 M Na-oxalate in 0.05 M NaOAc buffer, pH 5.2 (two times, 150 ml) at 70 °C for 30 min; **(3)** extracted
 155 with 0.05 M NaOH (two times, 150 ml) at 4 °C for 30 min. For each step, the remaining pomace was
 156 separated by centrifugation at 15000 g for 20 min. Fraction containing chelating-agent (CDTA and Na-
 157 oxalate) was first dialyzed against 0.1 M NaOAc buffer (pH 5.2) for 24 h and then dialyzed against
 158 deionized water for 24 h and freeze dried. The other two fractions were dialyzed directly against
 159 deionized water for 24 h and freeze dried.

160 2.4.4. Sequential chelating-agent extraction II (route D, Figure 1)

161 The sequential chelating-agent extraction II was conducted according to Gawkowska et al. (2018)
 162 with some modification. In brief, wet apple pomace (10 g) was sequentially extracted under agitation
 163 with **(1)** 200 ml of 0.05 M CDTA (pH 6.5) at 25 °C for 6 h and then at ambient temperature for 2 h; **(2)**
 164 200 ml of 0.05 M sodium carbonate (Na₂CO₃) and 0.02 M sodium borohydride (NaBH₄) at 4 °C for 20
 165 h and then at 20 °C for 2 h. For each step, the remaining pomace was separated by centrifugation at
 166 15000 g for 20 min. The polysaccharide extracts were dialyzed against deionized water for 24 h and
 167 freeze dried.



168

169 **Fig. 1** Schematic representation of the extraction process with various methods

170

171 2.5. NADES pretreatments followed by pectin extraction.

172 These pretreatments are represented schematically in **Fig. 2**.

173 2.5.1. Sequential NADESs pretreatment extraction (route E, Figure 2)

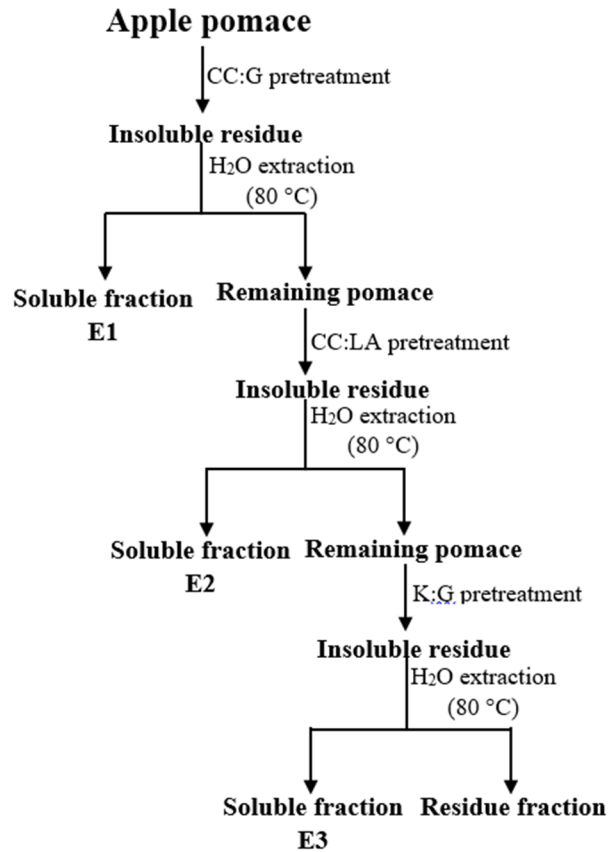
174 **(1)** Wet apple pomace was mixed with CC:Glycerol at the ratio of 1:8 (w/v). The solution was agitated
175 at 40 °C for 1 h and then centrifuged at 15000 g for 20 min to recover the remaining pellet. The
176 pomace pellet was then resuspended in deionized water at 80 °C for 10 min under constant agitation
177 and then centrifuged at 15000 g for 20 min. This water extraction process was repeated five times.
178 The pooled water washes, referred to as the pectin fraction, was concentrated with a vacuum rotary
179 evaporator and was precipitated by 4 volumes of ethanol. The precipitate was recovered by
180 centrifugation (15000 g, 20 min) and washed with 40 mL of 70% of ethanol for 10 min (3 times),
181 followed by 40 mL of ethanol and acetone for 10 min. The ethanol and acetone washings were
182 repeated until the washes were colorless (at least twice). Both, the remaining pomace and pectin
183 sample were air dried and then dried at 40 °C in vacuum oven over P₂O₅ powder for 12 h.

184 **(2)** The CC:G pretreated pomace was rehydrated (water content = 68%) and sequentially treated with
185 CC:LA at the ratio of 1:8 (w/v). The operation was as same as the first pretreatment described in (1).

186 **(3)** The CC:LA pretreated pomace was rehydrated (water content = 68%) and sequentially treated
187 with K:G at the ratio of 1:8 (w/v). The operation was as above.

188 The order of this sequential NADES pretreatments/water extraction was decided based on
189 preliminary experiments defining the order of the highest to the lowest yield of remaining pomace
190 after each NADES pretreatment/water extraction was realized alone: 57.2% for CC:G, 49.6% for
191 CC:LA, 47.3% for K:G. Furthermore, being neutral, CC:G was expected to be the least impacting on
192 the polysaccharides structure. According to our previous work (Chen & Lahaye, 2021), since only a
193 trace amount of pectin was extracted directly by NADES, the polymers in this fraction were not
194 considered in the present study.

195



196

197 **Fig. 2** Schematic representation of the sequential extraction process with NADES

198 2.5.2. NADESs pretreatment extraction (route F, G)

199 To compare the effect of sequential pretreatments, wet apple pomace was treated with CC:LA (route
200 F) or K:G (route G), respectively, following the same procedure as for routes E.

201 2.5.3. Water extraction following acid and alkaline solution pretreatments (route H, I)

202 To assess the effect of pH on extraction efficiency, lactic acid (route H) and potassium carbonate
203 solutions (route I) at the pH of CC:LA or K:G NADES pretreatments were prepared for pectin
204 extraction. The extraction procedure was as same as for routes F,G.

205

206 2.6. Extraction yield

207 The extraction yield was calculated as follow:

208
$$\text{Yield (\%)} = \frac{W_p}{W} \times 100$$

209 Where the W_p is the sample weight in each fractions and W is the initial dry weight of apple
210 pomace (for sequential extraction, the W is based on residual pomace from previous step).

211

212 2.7. Neutral sugars composition and uronic acids content

213 The neutral sugar composition in each pectin fraction was determined by GLC (Gas-liquid
214 chromatograph) analysis (Blakeney, Harris, Henry, & Stone, 1983). In brief, sample was dispersed in
215 sulphuric acid (12 M, 72%) at 25 °C for 30 min, followed by hydrolysis (100 °C, 2 h). The released
216 sugars were reduced and acetylated and the obtained alditol acetates were analyzed by GLC (Perkin-
217 Elmer Autosystem) equipped with DB-225 capillary column (J&W Scientific, Folsom, CA, USA) eluted
218 at 205 °C by hydrogen. The split injector and flame ionization detector temperatures were set at
219 220 °C. Both sugar standard solution and internal standard (inositol) were used for calibration. Sugar
220 content in each fraction was expressed as recovery rate and was calculated as follows:

$$221 \text{ Recovery rate (\%)} = \frac{(P1 \times Y1)}{(P2 \times Y2)} \times 100$$

222 Where $P1$ is the percentage of each sugar in the extracted sample, $Y1$ is the extraction yield of the
223 corresponding fraction, $P2$ is the percentage of each sugar of untreated sample, $Y2$ is the dry matter
224 percentage of the untreated sample.

225 Uronic acids in the acid hydrolysate was quantified using the m-hydroxydiphenyl colorimetric acid
226 method (Blumenkrantz & Asboe-Hansen, 1973). Galacturonic acid and glucose standard solutions
227 were used for calibration.

228 The molar sugar composition of each pectin fraction was used to evaluate pectin characteristics.
229 Assuming that all galactose and arabinose were part of RGI side-chains, the molar ratios of Gal:Rha
230 and Ara:Rha stand for the number of galactose or arabinose residues in RGI side chain. Since the
231 pectin backbone consists of HG (100% GalA) and RGI (GalA:Rha, 1:1), the molar percentage of both
232 HG and RGI can be expressed as $HG = GalA - Rha$; $RGI = 2 \times Rha$. The relative ratio between HG and
233 RGI represents the proportion of the different pectin structural domains (Huang et al., 2016).

234

235 2.8. Pectin methylation and acetylation esterification degree

236 Methanol and acetic esters in pectin were measured by HPLC according to (Levigne, Thomas, Ralet,
237 Quemener, & Thibault, 2002). Briefly, 7 mg of sample from different extracts was saponified for 1 h
238 at 4 °C by using the solution system containing 0.5 mL of NaOH (0.5 M) and 0.5 mL of $CuSO_4 \cdot 5H_2O$

239 (0.5 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 0.5 mL of isopropanol solution (14 mg mL^{-1})). After centrifugation at 7400 g
240 for 10 min, the supernatant was filtered through cartridge IC-H (Sstarpure, Maxi-Clean SPE 0.5 ml IC-
241 H 50pk). HPLC was conducted on C18 (4 mm \times 250 mm, Lichrospher 100 RP-18e (5 μm), Interchim,
242 France) column thermostated at 25 °C. H_2SO_4 (4 mM) was used for isocratic elution at a flow rate of
243 1.0 mL min^{-1} . Standard solution containing methanol, acetic acid and isopropanol as internal standard
244 was used for calibration. Due to the acetic acid peak was overestimated in HPLC analysis. The acetic
245 acid content of different pectin extracts was re-determined by acetic acid enzymatic kit (BioSenTec,
246 France). The degree of methyl esterification (DM) and acetyl esterification (DA) were calculated as
247 the number of moles of methanol and acetic acid measured per mole of uronic acid in pectin.

248

249 2.9. Molecular weight profiling

250 Molecular weight profile of pectin was determined through High Performance Size Exclusion
251 Chromatography (HPSEC). The system consisted of a Shodex OHpak SB-G 6B pre-column (Shodex,
252 Tokyo, Japan) in front of OHpak SB-805-HQ (Shodex, Tokyo, Japan) connected to pump (Jasco PU-
253 1580, Tokyo, Japan) and injector (PerkinElmer, series 200 autosampler, Courtaboeuf, France). Pectin-
254 rich samples (4 mg) were dissolved in 1.5 mL of distilled water, then centrifuged (10 min, 7400 g) and
255 filtered through 0.45 μm membrane (Millex-HV, PVDF) prior to injection. Elution was performed with
256 50 mM NaNO_3 at a flow rate of 0.7 mL min^{-1} and monitored by differential refractometry (Viscotek
257 VE 3580 RI detector, Malvern Instruments, Orsay, France). Molecular weights were obtained using
258 the OmniSEC 4.7.0 software and calibration was done in triplicate using pullulan-P108K (Viscotek,
259 Malvern Instruments, Orsay, France).

260

261 2.10. Solid state CP/MAS ^{13}C NMR spectroscopy

262 Approximately hundred mg of the residual pomaces after NADESs pretreatments followed by water
263 extraction method were rehydrated to 29-30% (w/w) with ultra-pure water. The solid-state NMR
264 spectra were registered on Bruker Advance III 400 spectrometer at a proton frequency of 400.13
265 MHz and carbon frequency of 100.62 MHz. A double resonance $^1\text{H}/\text{X}$ CP/MAS 4mm probe coupled
266 with high power level amplifier was used for CP/MAS experiment. The magic angle spinning (MAS)
267 rate was set at 12 kHz and each acquisition was acquired at ambient temperature (293 °K). The
268 experiment was conducted under a 90 ° proton pulse of $2.8 \pm 0.1 \mu\text{s}$ a contact time of 1.5 ms and a
269 8 s recycling time for an acquisition of 34 ms during which dipolar decoupling of approximately 90

270 KHz was applied. 8192 scans were accumulated for each spectrum. Chemical shifts were calibrated
271 using external glycine, assigning the carbonyl at 176.03 ppm.

272 The chemical shifts, half width and area of peak of samples were deconvoluted and determined using
273 a least-squares fitting method with the Peakfit® software (Systat software Inc., USA).

274 According to the method of Larsson et al. (1997), the cellulose crystallinity was calculated from
275 deconvoluted cellulose C₄ peak in region of 80-91 ppm. Due to spectral resolution, a simplify version
276 was used: two crystalline cellulose C₄ peaks (cellulose I(α + β) (88.4 ppm), cellulose Iβ (87.8 ppm)) and
277 one amorphous cellulose C₄ peak (83.3 ppm) were used. The proportion of crystalline cellulose was
278 determined by dividing the sum peak area of two crystalline cellulose C₄ peaks by those of three
279 cellulose C₄ peaks. Assuming the cross section of cellulose microfiber is square and all amorphous
280 cellulose is attached on fiber surface, the lateral fiber dimension (LFD) was also estimated. The
281 cellulosic chains width was set at 0.57 nm (Newman, 1999).

282 The molecular dynamic of samples was further characterized by varying contact time (τ) from 10 μs
283 to 9000 μs. Twenty CP/MAS spectra were recorded with an accumulation of 512 scans per contact
284 time. The evolution of carbon peak area (C₄ of crystalline cellulose and O-CH₃ of pectin methyl ester)
285 between different groups was fitted with following formula (Kolodziejewski & Klinowski, 2002):

$$286 \quad I(\tau) = I_0 e^{-\tau/T_{1\rho}^H} * \left\{ 1 - \lambda e^{-\tau/T_{HH}} - (1 - \lambda) e^{-3\tau/T_{2HH}} e^{-\tau^2/2T_{CH}^2} \right\}$$

287 Where $I(\tau)$ is the carbon peak area (C₄ of crystalline cellulose and O-CH₃ of pectin methyl ester)
288 according to the contact time (τ), I_0 is the maximum carbon signal intensity (associated with the
289 optimal contact time), λ is a parameter that depends on the number of protons (n) carried by
290 carbons ($\lambda=1/(n+1)$), $T_{1\rho}^H$ is the spin-lattice proton relaxation time in the rotating frame, T_{HH} is the
291 spin diffusion time between two nearby protons, T_{CH} is the thermal mixing time between H and C.

292

293 2.11. Statistical analysis

294 Data was subjected to one-way ANOVA and Duncan's multiple range tests using the SPSS 16.0
295 statistical software package (SPSS Inc., Chicago, IL, USA). Differences were considered significant at
296 $P<0.05$. Data are presented as mean values with their standard deviations.

297

298 3. Results

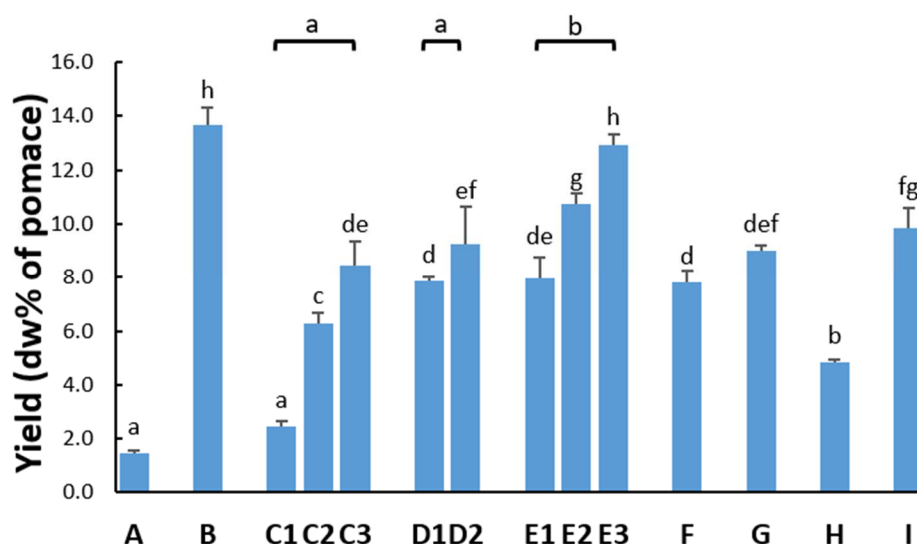
299 3.1. Physicochemical properties of different NADESs

300

301 Table 1 gathers physicochemical properties of the NADES used in this work. Since white insoluble
302 material was observed in K:G with molar ratio of 1:2 and 1:3, which indicated the hydrogen bond was
303 not successfully formed between K_2CO_3 and glycerol, these solvents were not kept for further study.
304 The pH value of K:G gradually decreased with increasing proportion of glycerol. The lowest pH value
305 (12.5) was obtained with K:G molar ratio of 1:25. A similar trend was also observed in the viscosity of
306 K:G. The molar ratio of 1:25 led to less viscous K:G solvent. Moreover, K:G was a Newtonian fluid,
307 while both CC:G and CC:LA solvents were non-Newtonian fluids, as they showed shear-thinning
308 behavior (data are not shown). From a practical point of view, solvents with lower viscosity are
309 preferable as they ease their mixing and diffusion in the substrate. Under this circumstance, the K:G
310 with molar ratio of 1:25 was chosen for pretreatment of apple pomace.

311

312 3.2. Effects of different extraction methods on polysaccharide yield



313

314 **Fig. 3** Mean extraction yields (n=4) of polysaccharide extracts according to different procedures (A-I); A: water
315 extraction; B: mineral acid extraction; C1-C3: sequential chelating agent extraction I (1: NaOAc fraction; 2:
316 CDTA&Na-oxalate fraction; 3: NaOH fraction); D1-D2: sequential chelating-agent extraction II (1: CDTA fraction;
317 2: Na_2CO_3 & $NaBH_4$ fraction); E1-E3: sequential water extraction after NADESs pretreatment extraction (1: CC:G
318 fraction; 2: CC:LA fraction; 3: K:G fraction); F: CC:LA pretreatment extraction; G: K:G pretreatment extraction; H:
319 lactic acid solution pretreatment extraction (same pH as CC:LA); I: potassium carbonate solution pretreatment
320 extraction (same pH as K:G); bars: standard deviation; mean values with unlike letters were significantly
321 different.

322

323 As shown in **Fig. 3**, the distinct extraction routes led to different extraction yields. Sequential
324 extraction methods clearly led to higher polysaccharide yields than water (A), dilute acid (B) or
325 NADES pretreatment/water extraction methods (E1, F, G). Moreover, the yield in method E (total of
326 31.6%) was significantly higher than that of methods using chelating agents (C: 17.2%; D: 17.1%).
327 Since the yield significantly differed for each step of the different methods, the results are analyzed
328 individually. Besides sequential extractions, the highest yield was achieved with dilute acid (B:13.7%),
329 while the lowest yield was obtained by extraction with water (A:1.4%). Except for the NaOAc
330 treatment (C1), all other treatments significantly improved yield when compared with only water
331 treatment (A). CC:G, CC:LA and K:G pretreatments/water extractions (E1, F, G) showed similar yields
332 as CDTA treatment (D1), while CDTA treatment with Na-oxalate led to significantly lower yield (C2).
333 The yield of water extracted polysaccharides was close following the different NADES pretreatments
334 (E1, F, G). When compared with dilute acid extraction (B), NADES pretreatments following water
335 extraction (E1, F, G) led to significantly lower extraction yields. However, these low yields could be
336 partly mitigated when CC:G, CC:LA and K:G NADES pretreatments followed by water extractions were
337 conducted sequentially. The polysaccharide extraction yields in both E2 and E3 were significantly
338 increased compared with those in F or G, respectively (**Fig. 3**). Moreover, after CC:G and CC:LA
339 pretreatment, sequential K:G pretreatment/water extraction (E3) led to similar yield as dilute acid
340 extraction (B). Besides extracts yield, the residue yield after dilute acid extraction, CC:LA. K:G
341 pretreatment/water extraction (F and G) were also calculated to explore possible losses in the
342 remaining cell wall (residue fraction: 55.7% for B; 49.6% for F; 47.3% for G).

343

344 3.3. Monosaccharides recovery in extracts

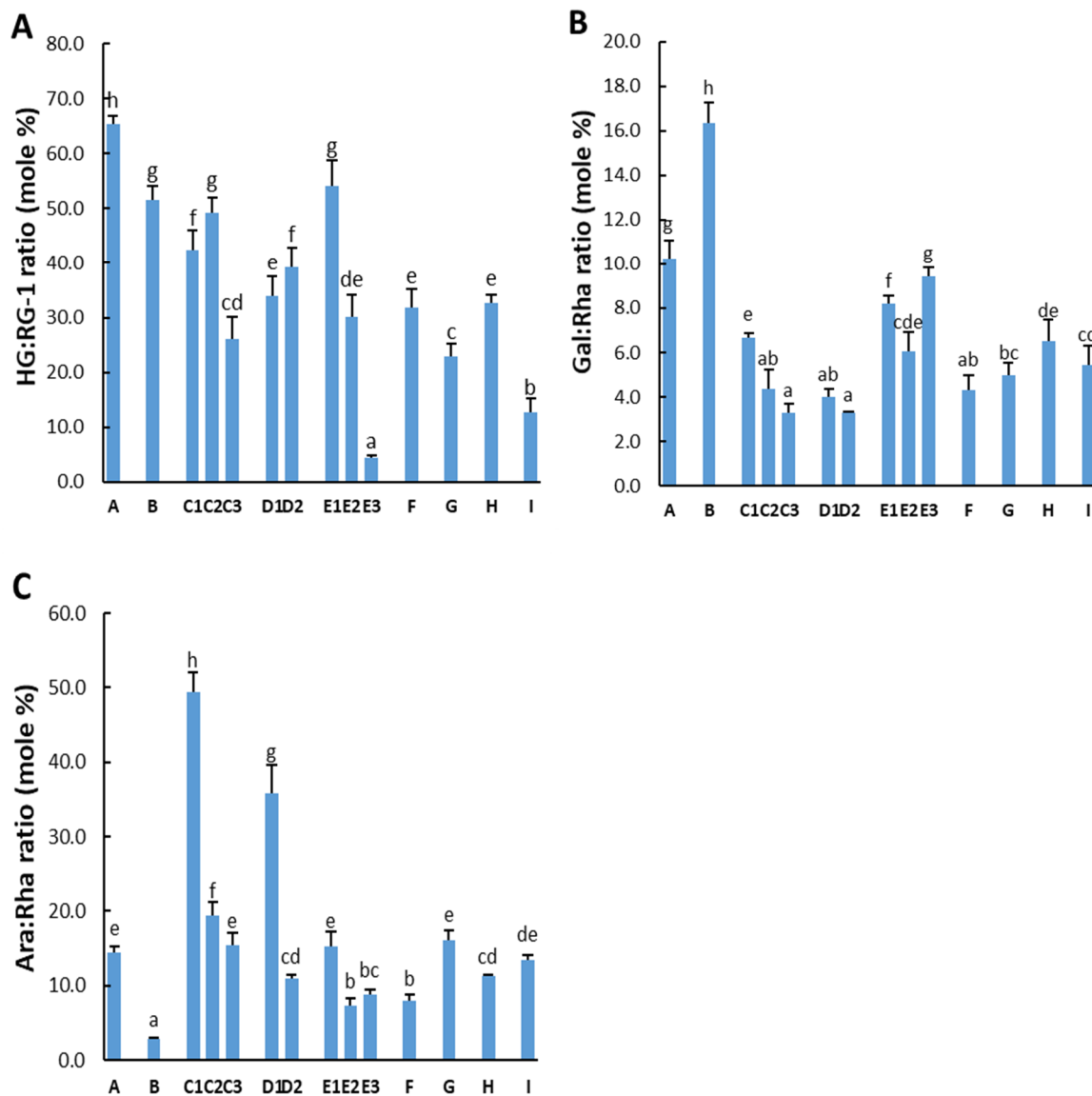
345

346 The total sugar weight percentage recovered in the raw apple pomace and in each fraction indicated
347 that non-polysaccharide substances were present in variable amount according to the exaction
348 methods (**Table S1**). With 86.7% of total sugar percentage, the water extract after CC:LA
349 pretreatment (F) was the richest in polysaccharides compared to other fractions. Water extracts
350 recovered after K:G pretreatment conducted sequentially or not (E3: 43.3%; G: 58%) were
351 significantly poorer in total sugar percentage compared to E1 (76.3%), E2 (77.4%) and F (86.7%)
352 fractions after CC:G and CC:LA pretreatments. Extracts from methods based on chelating agents (C
353 and D) contained less than 50% polysaccharide with the lowest recovery (27.6%) for the C2 fraction.
354 Fraction C1 was an exception with 73.6% total sugar. Other cell wall substances, residual salt and
355 chelating agent likely contributed to the low total sugar percentage in fractions (C2, C3, D1, D2, E3
356 and G).

357 The expression of monosaccharides composition as a percentage of recovery of their initial content
358 in pomace allows better evaluating the efficiency of the different extraction methods to recover
359 pectin related sugars (Rha, Ara, Gal, UA). From **Table S1**, these sugars were found in all extracts.
360 Recovery of UA was the highest among them except in NaOAc treatment extract (C1) and in the
361 water extract following K:G pretreatment (E3). Considering UA recovery, hot water extraction
362 following non-sequential NADES pretreatments led to significantly higher recovery (E1: 23.5%; F:
363 27.4%; G: 23.9%) than by chelating agents extraction (C2: 10.4%; D1:8.3%). Sequential NADES
364 pretreatments/water extraction (method E) was the most efficient in allowing extraction of 69.7% of
365 the pomace UA content. As for the yield, CC:G pretreatment (E1) significantly improved UA recovery
366 in the hot water extract following the CC:LA pretreatment (E2: 36.1%), compared with non-
367 sequential CC:LA pretreatment/water extraction (F: 27.4%). However, the synergistic effect was not
368 observed in the extract after K:G pretreatment/water extraction (E3: 10.2%). Instead, higher UA
369 recovery was found in non-sequential K:G pretreated/water extraction group (G: 23.9%). According
370 to extraction yield, lactic acid solution pretreatment followed by hot water extraction led to
371 significantly lower pectin related sugar recoveries than that of CC:LA pretreatment/water extraction
372 whether conducted sequentially or not. Although both K:G pretreatment/water extraction (G) and
373 K₂CO₃ solution pretreatment/water extraction (I) led to comparable extraction yield, the water
374 extract after K₂CO₃ solution pretreatment showed significantly lower UA recovery. K₂CO₃ solution
375 had a more negative effect on the pectin galacturonic acid unit recovery. Hemicellulose and cellulose
376 related monosaccharides (Fuc, Xyl, Man, Glc) were also found in some extracts though Fuc and Xyl
377 may also come from HG or RG II structural domains. Glc recovery was the most represented of these
378 sugars except in the NaOH extract (C3) and the K:G pretreatment/water extract (E3).

379

380 3.4. Pectin structural domains in extracts



381

382

383 **Fig. 4** Mean (n=4) molar ratio of : **A**, Homogalacturonan (HG) : rhamnogalacturonan (RGI), **B**, galactose
 384 (Gal) : rhamnose (Rha) and **C**, arabinose (Ara) : rhamnose (Rha) of pectin-rich extracts obtained by A: water
 385 extraction; B: mineral acid extraction; C1-C3: sequential chelating agent extraction I (1: NaOAc fraction; 2:
 386 CDTA&Na-oxalate fraction; 3: NaOH fraction); D1-D2: Sequential chelating-agent extraction II (1: CDTA fraction;
 387 2: Na₂CO₃&NaBH₄ fraction); E1-E3: sequential NADESs pretreatment extraction (1: CC:G fraction; 2: CC:LA
 388 fraction; 3: K:G fraction); F: CC:LA pretreatment extraction. G: K:G pretreatment extraction; H: lactic acid
 389 solution pretreatment extraction; I: potassium carbonate solution pretreatment extraction. Bar: standard
 390 deviation; different letters are significantly different.

391 The sugar recovery data were further analysed to identify specific effects of the extraction methods
 392 with regard to pectin structural domains. Besides UA, Rha can inform about the proportion of RGI
 393 recovered in the different extracts. According to the HG/RGI molar ratio shown in **Fig. 4A**, pectin
 394 structure profile was affected by extraction methods. The highest HG proportions were observed in
 395 the water extract (A), while all other methods led to RGI richer fraction. For water extracts after non-

396 sequential NADES pretreatments, significantly higher HG proportion was found following CC:G
397 pretreatment (E1) compared with that following CC:LA (F) or K:G pretreatments (G). In the opposite,
398 extract in G fraction possessed significantly higher RGI proportions than other water extracts
399 following non-sequential NADES pretreatments. Based on HG/RGI molar ratio, dilute acid extraction
400 (B) showed similar effect on pectin structural domains composition as CC:G pretreatment/water
401 extraction (E1). When apple pomace was extracted sequentially, no matter which sequential
402 extraction methods was applied, pectin structure was remarkably affected since HG/RGI molar ratio
403 was significantly different for each step. HG-richer fractions were obtained after CDTA & sodium
404 oxalate extraction (C2), while CC:LA and K:G pretreatment/water extraction (E2 and E3) led to RGI-
405 richer fractions.

406 Molar ratio of Gal/Rha and Ara/Rha were also calculated to assess the effect of different methods on
407 RGI side-chains structure assuming that galactose and arabinose were mainly constitutive of pectin
408 side chains. As can be seen from **Fig. 4B**, the fraction extracted by dilute acid showed the highest
409 ratio for Gal/Rha, all other methods indicated shorter/less galactose side chains to varying degrees.
410 Close amounts of galactose side chains were observed in extracts after CDTA (D1), and water extracts
411 following CC:LA (F) and K:G (G) pretreatments. Instead, the water extract following CC:G
412 pretreatments demonstrated significant higher Gal/Rha molar ratio. Moreover, when CC:LA and K:G
413 pretreatments were conducted sequentially, the amount of galactose side chain increased
414 significantly in the extracts. For sequential extractions using chaleting agents (C and D), CDTA
415 treatment with or without sodium oxalate led similar Gal/Rha ratio. Moreover, NaOH (C3) or Na₂CO₃
416 & NaBH₄ (D2) did not markedly change galactose side chain proportion since C2 and C3 or D1 and D2
417 shared close Gal/Rha ratio.

418 The pectin fraction showing the shortest/least arabinose side-chains was obtained by dilute acid
419 extraction (B) (**Fig. 4C**). The water extract following CC:LA pretreatment conducted alone or
420 sequentially (E1 and F) also led to fractions with shorter/less RGI arabinan side-chains than those
421 recovered in water extracts following other NADES pretreatments. No difference was found between
422 fractions recovered after water extraction (A) and water fractions following CC:G pretreatment (E1)
423 or K:G pretreatment (G). Significant higher amount of Ara/Rha molar ratio was found after CDTA
424 (with or without sodium oxalate) extraction when compared with NADES pretreatment/ water
425 extraction method.

426

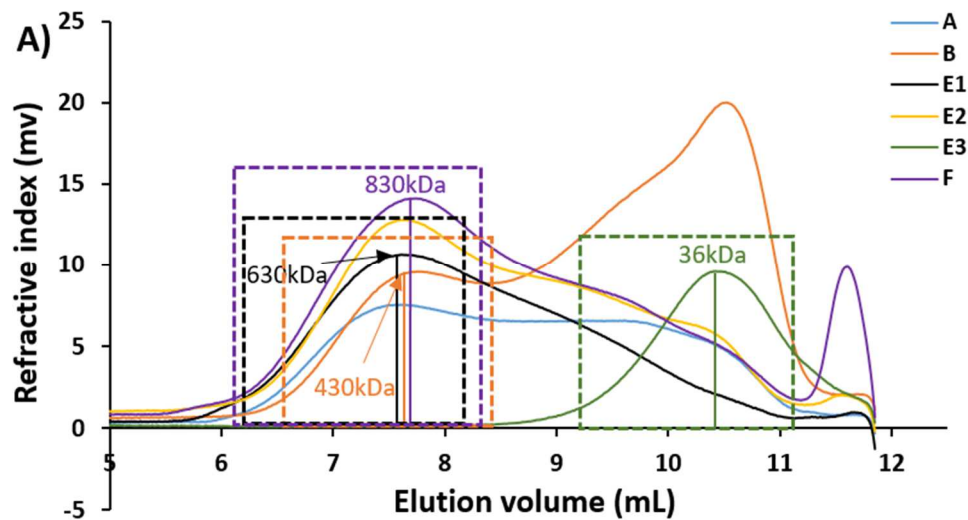
427 3.5. Esterification of pectin extracts

428

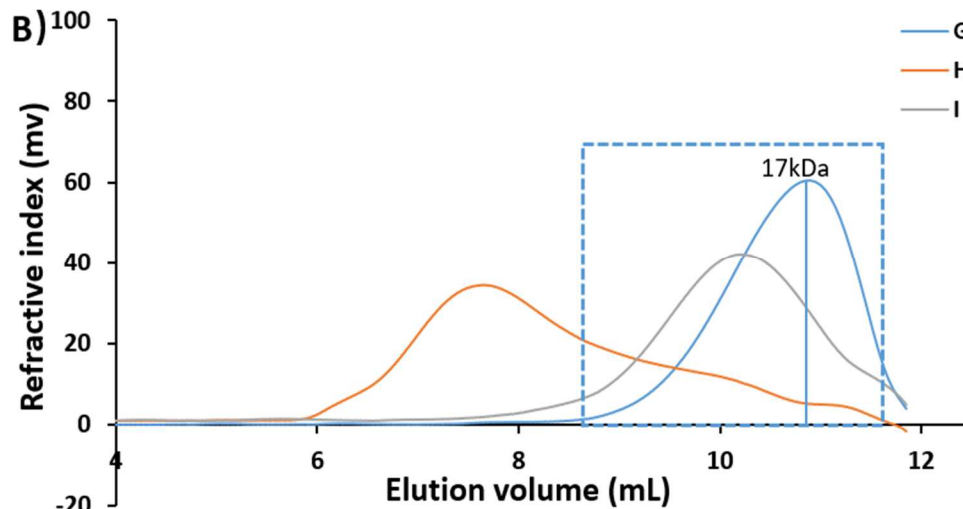
429 Pectin substitution by methanol and acetic acid esters was also assessed (**Table S2**). As expected due
 430 to saponification, dilute alkali in method C3 and method I had a severe effect on methyl ester group.
 431 A low DM value was also obtained in the water extract following the K:G pretreatment (E3 = 18.2 and
 432 G = 11.8) compared with the other water extracts following the CC:G or CC:LA pretreatments (E1 =
 433 56.6, E2 = 67.8 and F = 66.6). For sequential chelating agent extractions, both CDTA (with or without
 434 sodium oxalate) and sequential Na₂CO₃ & NaBH₄ extraction led to low methyl esterified pectin (ie DM
 435 < 50), while for other treatments, high methyl esterified pectin was extracted. K:G pretreatment led
 436 to lower acetyl esterification of pectin in the following water extract (E3 and G). Moreover, alkaline
 437 extracting conditions favored lower DA value of pectin (C3: 1.3; I: 0.7).

438

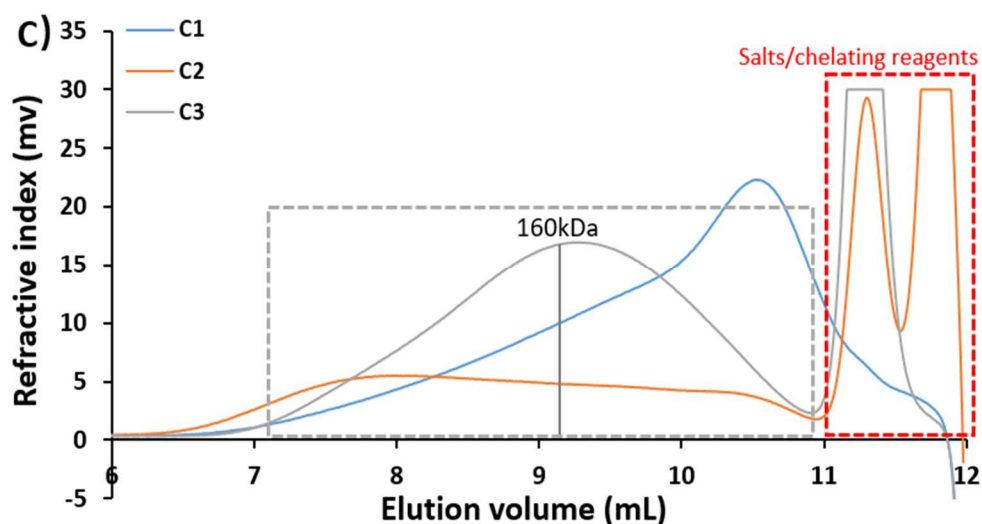
439 3.6. HPSEC profiles from different extracts



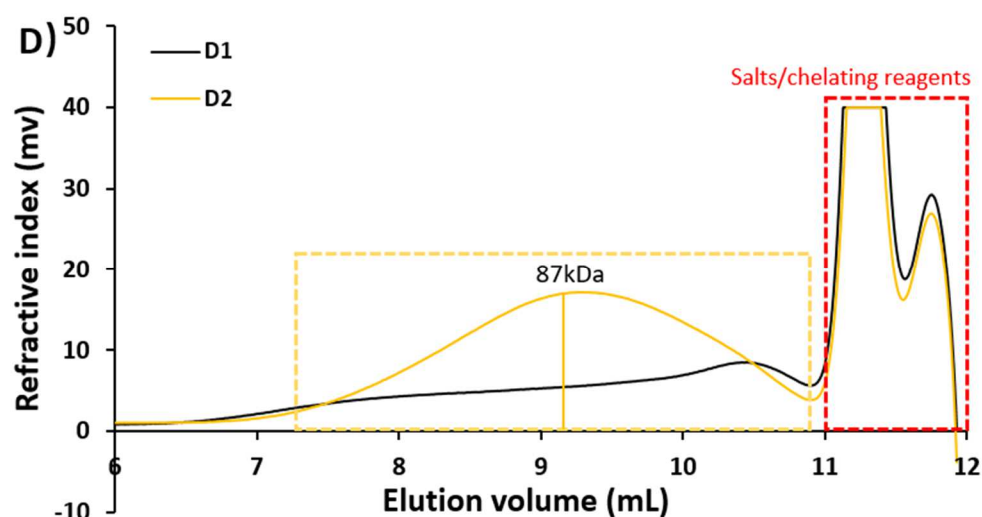
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441



442



443

444 **Fig. 5** HPSEC-patterns of the pectin-rich fractions. A) A: water extract; B: mineral acid extract; E1-E3: water
 445 extracts following sequential NADESs pretreatment (1: CC:G; 2: CC:LA; 3: K:G pretreatments); F: water extract
 446 following CC:LA pretreatment; B) G: water extract following K:G pretreatment; H: water extract following lactic
 447 acid solution; I: water extract following potassium carbonate solution; C) C1-C3: sequential chelating agent
 448 extracts (1: NaOAc fraction; 2: CDTA&Na-oxalate fraction; 3: NaOH fraction); D) D1-D2: sequential chelating
 449 agent extracts II (1: CDTA fraction; 2: Na₂CO₃&NaBH₄ fraction).

450

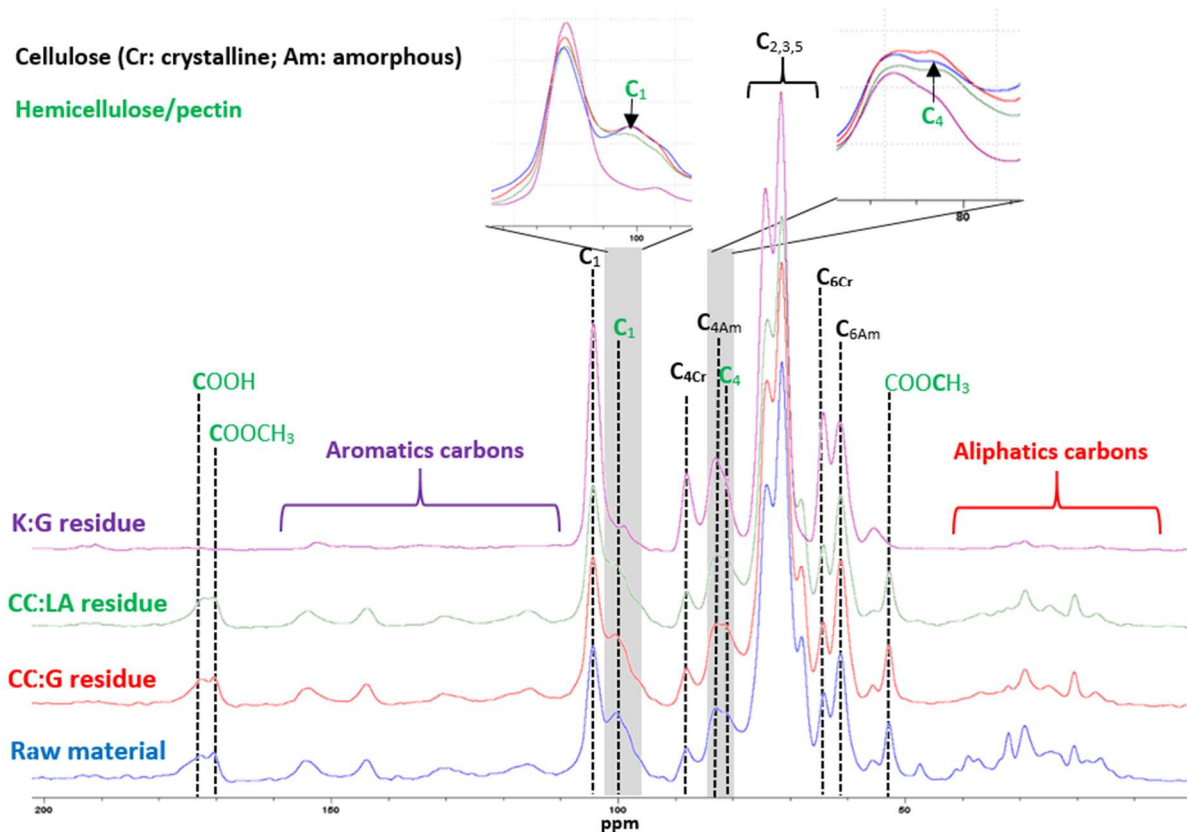
451 The molecular weight profile of the polymers in different extracts was analyzed by HPSEC (**Fig. 5**).
 452 Because of overlapping peaks, the estimated Mw of some peaks were provided in **Fig. 5** to give a
 453 broad view of how molecular weight distribution was influenced by various extraction methods. For
 454 both dilute acid and water fractions (trace A and B), two main peaks were observed eluting between
 455 6 mL- 11 mL (**Fig. 5A**). Similar profiles were also found in the water extracts following the CC:G and
 456 CC:LA pretreatments, but with a minor peak eluting between 8.5mL and 10.5mL (**Fig. 5A**, trace E1

457 and E2). This indicated that at least two populations of polysaccharide were present. Water extract
458 following GG:LA pretreatment in the sequential NADES pretreatments method (trace E2, **Fig. 5A**),
459 following CC:LA pretreatment alone (F, **Fig. 5A**) and the extract obtained following dilute lactic acid
460 pretreatment (H fraction; **Fig. 5B**) yielded close Mw distribution profiles. These profiles showed the
461 predominant role of lactic acid in affecting Mw distribution. Alkaline NADES (E3, **Fig. 5A** and G, **Fig.**
462 **5B**) pretreatment led to polysaccharides degradation since a single peak at 36kDa or 17kDa was
463 found eluting at approximately 10.5 mL or 11 mL, respectively (**Fig. 5A,B**). Similarly, the alkaline
464 condition provided by NaOH and Na₂CO₃ in the sequential chelating agent methods C and D also led
465 to polymer degradation since low molecular weight populations were found in NaOH (trace C3, **Fig.**
466 **5C**) and Na₂CO₃ & NaBH₄ (trace D2, **Fig. 5D**) fractions. Due to the methylesterification of pectin,
467 degradation by a β-elimination mechanism to lower oligomers cannot be ruled out. Compared with
468 water extracts following NADES sequential pretreatments, different molecular weight distributions
469 profiles were observed with the extracts from sequential chelating agent extractions (methods C and
470 D). More than one Mw populations were found in extracts after CDTA extraction (with or without
471 sodium oxalate, trace C2 or D2, **Fig. 5C,D**). The low molecular weight substances eluted after 11 mL
472 in these extracts are likely correspond to salts and chelating reagent (**Fig. 5C,D**).

473

474 3.7. ¹³C NMR spectra and dynamic characterization of residual pomace

475



476
477 **Fig. 6** ^{13}C CP/MAS spectra of residual pomaces after sequential NADES pretreatment/water extraction; peaks
478 attribution according to (Ng et al. 2014; Phyo & Hong, 2019).

479

480 **Table 2.** Structural and dynamic characteristics of cellulose and pectin in apple pomace residue
481 following sequential NADES/water extraction: crystallinity and lateral fiber dimension (LFD) of
482 cellulose; spin-lattice proton relaxation time ($T_{1\rho}^H$) of crystalline cellulose (C_4 peak area), pectin
483 methyl ester (O-CH₃ peak area) and diffusion time of nearby proton (T_{HH}) of pectin methyl ester (O-
484 CH₃ peak area) in raw, CC:G pretreated, CC:LA pretreated and K:G pretreated/water extracted
485 residues

	Raw material	CC:G residue	CC:LA residue	K:G residue
Crystallinity	31%	32%	34%	46%
LFD (nm)	2.6	2.6	2.7	3.6
$T_{1\rho}^H$ (ms)				
Crystalline cellulose (C_4 , 87.8 ppm)	8.8	16.2	20.1	36.6
Pectin methyl ester (O-CH ₃ , 52.7 ppm)	6.3	16.8	11.1	—
T_{HH} (ms)				
Crystalline cellulose (C_4 , 87.8 ppm)	0.52	0.96	1.18	0.48
Pectin methyl ester (O-CH ₃ , 52.7 ppm)	0.28	0.39	0.33	—

486 —: not detected.

487

488 The cell wall structure in raw pomace and in the residues after the sequential NADES/water
489 extraction was studied by ^{13}C NMR spectroscopy (**Fig. 6**). Similar spectra were observed between
490 CC:G residue/CC:LA residue and raw apple pomace and were close to those previously published (Ng
491 et al. 2014; Lahaye et al. 2020). The peaks of carboxyl group (whether in acidic (172.7 ppm) or
492 methyl (170.5 ppm) forms) and methyl ester (52.7 ppm) on pectin structure disappeared in K:G
493 residue. In contrast, the higher intensity of crystalline cellulose C_4 (87.8 ppm) peak in this residue
494 indicated a marked impoverishment in pectin to the benefit of cellulose. In addition, the aromatic
495 and aliphatic carbons signals (ranged from 154.3 to 115.6 ppm; from 47.5 to 16 ppm) corresponded
496 to phenolic compounds and protein respectively that gradually disappeared in K:G residue. Moreover,
497 the highest values of crystallinity and LFD were also observed in K:G residue. No characteristic signals
498 for cellulose II were observed in the K:G residue. The molecular structure of both cellulose and pectin
499 was further studied by the $^1\text{H} \rightarrow ^{13}\text{C}$ polarization transfer kinetic, and process with the two-proton
500 reservoir model (see **Fig. S1** for experimental data along with model estimates). The $T_{1\rho}^H$ and T_{HH}
501 values of crystalline cellulose C_4 peak in residual pomace increased from 8.8 ms to 36.6 ms and from
502 0.52 ms to 1.18 ms with the successive NADES pretreatments and water extractions (**Table 2**). The
503 lowest T_{HH} value (0.48 ms) of crystalline cellulose C_4 peak was found in K:G residue. Since the pectin
504 methyl ester peak was absent in the spectrum of K:G residue, the corresponding $T_{1\rho}^H$ and T_{HH} values
505 could not be reported. Both CC:G and CC:LA pretreatments/water extractions led to higher $T_{1\rho}^H$ and
506 T_{HH} values of methyl ester peak than that of raw apple pomace. The highest $T_{1\rho}^H$ value (16.8 ms) of
507 the methyl ester peak was found in CC:G residues. Moreover, the similar trend was observed in T_{HH}
508 value of methyl ester peak.

509

510 **4. Discussion**

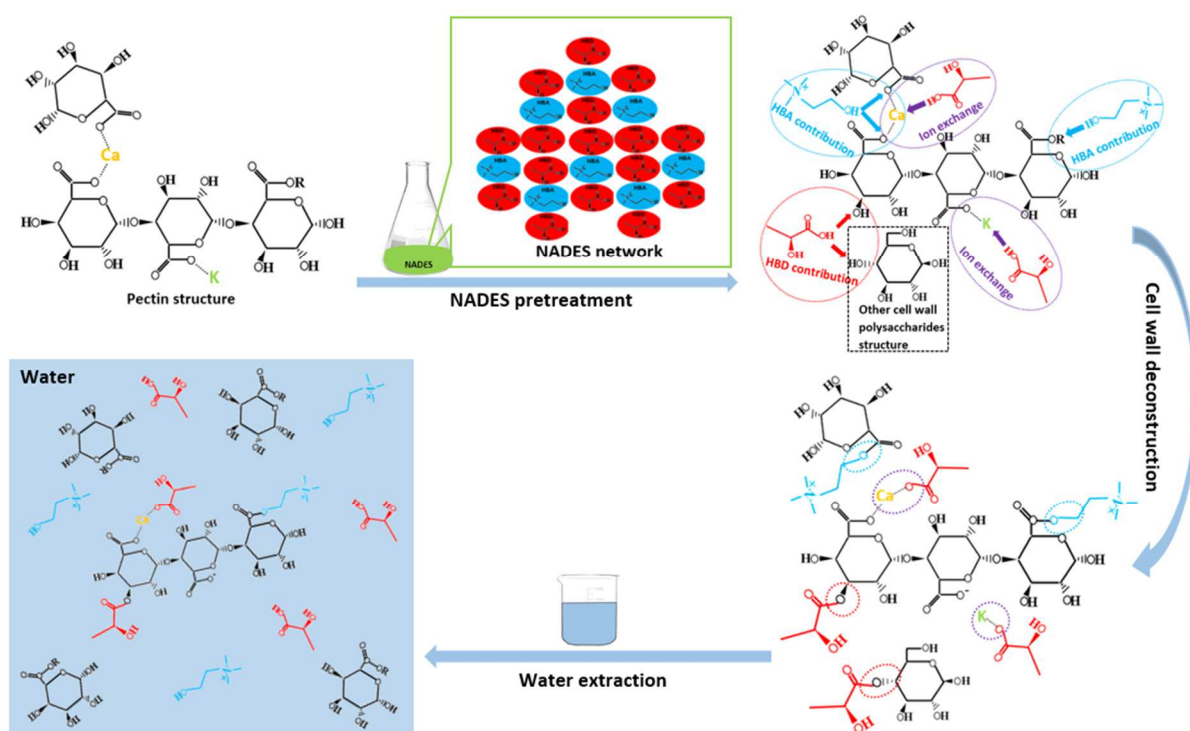
511 4.1. NADES pretreatments assist in loosening cell wall interactions for polysaccharides 512 extraction

513 Water was not suitable to extract pectin from apple pomace as previously reported (Renard, 2005).
514 The inefficiency of hot water to extract pectin was markedly improved after NADES pretreatments.
515 These pretreatments increased the subsequent water extraction yield by 5 times (yield: 1.5% for A;
516 8.0% for E1; 7.8% for F; 9.0% for G, dw%). However, none of the process taken independently
517 involving NADES pretreatments followed by hot water extraction gave higher yield than dilute acid,

518 which is widely applied in industry for pectin recovery. The possible reason for such lower yields
519 likely resulted from three aspects. First, the heat treatment applied by the industrial provider to
520 dehydrate pomace may have modified interactions between cell wall polysaccharides that reduced
521 contact area of cell wall to the extractant, making it difficult to extract polysaccharides. Although the
522 dry pomace was first rehydrated to swell the cell wall with a water amount close to that registered in
523 fresh pomace (Chen & Lahaye, 2021), irreversible microstructural/chemical modifications of the
524 pomace resulting from the dehydration process remained. When compared with our previous work,
525 CC:LA pretreatment/water extraction showed higher extraction yield in fresh pomace than in dry
526 pomace (Chen & Lahaye, 2021). CC:LA pretreatment/water extraction led to similar yield as that of
527 mineral acid extraction when fresh apple pomace was used for this purpose. Second, the pH of the
528 CC:LA solvent was lower than that of HCl solution. The strong acidity may have partly broken-down
529 pectin to oligosaccharides that were lost during polysaccharide ethanolic precipitation and resulted
530 in a low extraction yield of polysaccharides. This is evidenced by the low yield of residue after CC:LA
531 pretreatment (49.6% compared to 55.7% for the diluted acid extract). A similar result was found by
532 Liew et al. (2018) who showed that higher yield of pectin was extracted from pomelo peel using citric
533 acid (pH 1.8) compared to that treated with lactic acid–glucose–water solvent with a ratio of 6:1:6. A
534 low yield of residue (47.3%) was also found after non sequential K:G pretreatment, which indicated
535 that the alkaline pH of K:G led to cell wall polymer losses and was therefore responsible for lower
536 extraction yield. The pH (pH 6.5) of the CC:G NADES may not be appropriate to break bonds in the
537 cell wall and subsequently less pectin was released compared with that of dilute acid extraction.
538 Third, in addition to the microstructure of different pomace, the relatively higher viscosity of NADES
539 solvent compared with dilute HCl solution may have also limited the mass transfer between solvent
540 molecules and cell wall components, and therefore decreased the extraction efficiency. When NADES
541 pretreatments were applied sequentially, the hot water extraction following each NADES
542 pretreatment could access to more polysaccharides in the cell wall than any one step extraction
543 method. This led to a marked improvement in extraction yields of water extracts following sequential
544 CC:LA and K:G pretreatment (**Fig. 3**). The highest yield obtained by this sequential method compared
545 with other extraction methods represents a promising alternative process for pectin extraction.

546 To promote its application in industry, it would be necessary to understand the underlying
547 mechanism of NADES pretreatments in assisting water-soluble pectin extraction. As mentioned
548 above, the pH condition of the extraction system was paramount in determining extraction efficiency.
549 The lactic acid and potassium carbonate solution as pretreatments were used to explore the effect of
550 pH on the extraction yield. From the results shown in **Fig. 3**, potassium carbonate solution
551 pretreatment/water extraction (I) led to the same yield as K:G pretreatment/water extraction (G) did.

552 However, CC:LA pretreatment/water extraction (F) was significantly more efficient than lactic acid
 553 solution pretreatment/water extraction alone (H) in extracting more polysaccharides. These results
 554 indicated that alkaline pH can explain polymer recovery after K:G pretreatment, while acidic
 555 condition was not the only factor in determining the extraction yield. Although CC:LA pretreatment
 556 may involve hydrolysis of covalent linkages in the cell wall which allows extraction of polysaccharides,
 557 both hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA) from NADES may also play a role
 558 in influencing the extraction process. Wang et al. (2020) reported that the carboxylic HBD can
 559 interact with hydroxyl groups of cell wall polysaccharides to form esterification products and then
 560 ease cell wall deconstruction. The carboxylic group on the lactic acid may have transiently been
 561 engaged in esters with cell wall polysaccharides which helped in the deconstruction of apple due to
 562 the CC:LA pretreatment. As metallic cations, such as calcium and potassium interact with pectin
 563 (Vidot, Gaillard, Rivard, Siret, & Lahaye, 2018; Mierczyńska, Cybulska, Sołowiej, & Zdunek, 2015;
 564 Vidot, Maury, Siret, & Lahaye, 2020), ionic bonds breakage between galacturonic acid through ion
 565 exchange may subsequently promote choline chloride to react with pectin structure. Taken together,
 566 the ion exchange and effects of individual component of CC:LA contributed to the overall loosening
 567 of the cell wall structure, which facilitated polysaccharides extraction by hot water (**Fig. 7**). As for K:G
 568 NADES, the solvent can form hydrogen bonds with cell wall polysaccharides by accepting or donating
 569 the protons (Gan et al., 2020). However, the hydrolysis of cell wall caused by alkaline pH condition
 570 may have an overwhelming effect on extraction yields masking other possible contribution of the K:G
 571 solvent pretreatment compared with extraction by K_2CO_3 solution.



572

573 **Fig. 7** Hypothetical mechanism involved in CC:LA pretreatment/hot water extraction. The individual
574 components (HBA and HBD) of NADES form esterification products with cell wall polysaccharides,
575 coupled with ion exchange with metal cations to loosen the cell wall, which facilitates the
576 subsequent hot water extraction of pectin. NADES: Natural deep eutectic solvent; HBD: hydrogen
577 bond donor; HBA: hydrogen bond acceptor; Ca: calcium; K: potassium.

578

579

580 4.2. Sequential NADES pretreatment/water extraction: an efficient method for the recovery 581 of pectin enriched in HG or RGI structural domains

582 According to the uronic acid content of the fractions, CC:G and CC:LA pretreatments followed by hot
583 water extraction clearly afforded efficient pectin extraction from apple pomace (fractions E1, E2 and
584 F). While for the K:G pretreatments (fractions E3 and G), pectin were likely released from the cell wall
585 by the combined action of the dissociation of the calcium ions and their replacement by potassium,
586 by cleavage of ester linkages and by degradation of β -elimination in the alkaline pH. Part of pectin
587 are known to require basic conditions to be extracted (Santiago et al., 2018). In fact, the K:G solvent
588 in its higher pH mode could be used as an efficient extractant of hemicellulose due to the
589 observation that diluted K:G solvent (molar ratio: 1:25) used in present work co-extracted pectin.
590 NADES pretreatments allowed enriching pectin in HG or RGI structural domains. Of interest is the
591 fact that HG/RGI ratio for pectin extracted after CC:LA pretreatment was close, whether the
592 pretreatment was applied alone or after CC:G pretreatment (30.1:1 for E2 and 31.7:1 for F; **Fig. 4A**).
593 Therefore, CC:LA pretreatment appears specific in freeing RGI structure from apple pomace
594 compared to other pretreatments. The lowest HG/RGI molar ratio found in the water extract after
595 K:G pretreatment in the sequential method (E3) is likely related with the loss of methylesterified HG
596 structural domains following their degradation by β -elimination under the alkaline conditions of the
597 solvent. As a result, the extract of low molecular weight (trace E3, **Fig. 5A**) corresponded to pectin
598 enriched in RGI structure. In agreement with our results, the RGI rich fraction was also obtained
599 from carrot-based purées under hot alkaline condition (Santiago et al., 2018). Additionally, based on
600 UA recovery in E3 fraction, most of the cell wall HG might have already been extracted by hot water
601 following the first two pretreatments (E1 and E2) resulting in a lower HG/RGI molar ratio. This is
602 supported by the higher HG/RGI molar ratio observed in the water extract following the non-
603 sequential K:G pretreatment compared with that of E3 fraction. Hence, the combination of pH and
604 the synergistic effect of NADES pretreatments was responsible for the lowest HG/RGI molar ratio in
605 E3 fraction. Besides pH, other factor may exist to cause the relative higher HG proportion in CC:G
606 pretreated fraction. Actually, CC:G pretreatment did not specifically help extracting HG rich fraction

607 since a low HG/RGI molar ratio was observed in CC:G fraction when the reversed order pretreatment
608 was realized, namely, CC:LA pretreatment first, followed by CC:G (data not shown). The detail
609 explanation for this result remains to be further elucidated. Furthermore, more cell wall losses were
610 observed when apple pomace was pretreated first with CC:LA or K:G. Therefore, the order of NADES
611 pretreatment/water sequential extraction was fixed to 1) CC:G, 2) CC:LA, 3) K:G.

612 When compared with sequential NADES pretreatment/water extraction, sequential chelating agent
613 extraction possessed less ability to separate pectin rich in RGI structural domains as HG proportion
614 was increased after CDTA & Na-oxalate treatment (C2) or Na₂CO₃ & NaBH₄ treatment (D2). CDTA was
615 an effective Ca²⁺ chelating agent (Jarvis, Hall, Threlfall, & Friend, 1981), the HG bridged by Ca²⁺ was
616 released after CDTA treatment. However, the HG/RGI molar ratio was lower in CDTA fraction (D1)
617 compared with that in CDTA & Na-oxalate fraction, which indicated Na-oxalate also promoted HG
618 structural domain enrichment. Although HG is generally unbranched, the xylose-substitution on HG
619 may prevent calcium cross-links of HG chains and thus hinder the pectic network formation (Jensen
620 et al., 2008). In contrast, the neutral side chains on the RGI take part in cell wall construction and
621 development (Willats, Steele-King, Markus, & Knox, 1999; Jones, Milne, Ashford, & McQueen-Mason,
622 2003), play roles in fruit mechanical properties (Lahaye, Bouin, Barbacci, Le Gall, & Foucat, 2018) and
623 chain interactions in pectin network (Sousa, Nielsen, Armagan, Larsena, & Sørensen, 2015). The
624 influence on pectin RGI side chain of the NADES pretreatments and other extraction methods used in
625 this study was further studied. All extracts had less or shorter galactose side-chains than that of
626 dilute acid extraction. The galactose content in extracts after sequential extractions showed a
627 decreasing trend (C, D and E; **Fig. 4B**). However, the Gal/Rha ratio in E3 fraction increased. As
628 galactan side-chains can bind cellulose through hydrogen bonds (Lin, Lopez-Sanchez, Selway, &
629 Gidley, 2018; Zykwiniska, Ralet, Garnier, & Thibault, 2005), CC:G and/or CC:LA pretreatments may
630 expose residual pectin that are H-bonded to other cell wall polymers but that are labile to the
631 alkaline condition of the K:G pretreatment. Consistent with Huang et al. (2016) who reported that
632 EDTA & sodium oxalate led to shorter galactan side-chains on RGI from potato, the CDTA extraction
633 of apple pomace with or without sodium oxalate had a negative effect on the Gal/Rha molar ratio
634 (4.4:1 for C2; 4.0:1 for D1). Arabinose side-chains are known to be rapidly cleaved under mild acidic
635 conditions (Thibault, Guillon, & Rombouts, 1991). Therefore, dilute acid extraction caused severe
636 arabinan side chains losses. The pH of CC:LA is 1, which is close to that of diluted acid (pH=1.5).
637 Hence, it is reasonable that CC:LA pretreatment (E2 and F) had a similar negative impact on these
638 side chains. Moreover, the low Ara/Rha ratio observed in the extract after sequential K:G
639 pretreatment indicated a marked effect of CC:LA pretreatment on arabinan side-chain. Similarly,

640 pectin samples with very low (Ara + Gal)/Rha ratio were observed when peel of orange, lemon, lime,
641 and grapefruit was extracted by nitric acid (pH 1.6) (Kaya et al., 2014).

642

643 4.3. Extraction process affected molecular weight distribution of extract

644 The lowest molecular weight of the extracts was obtained after sequential chelating agent extraction
645 (C and D) compared to those following NADES pretreatments (**Fig. 5**). Especially for both CDTA & Na-
646 oxalate (C2) and CDTA (D1) fractions, low molecular weight compounds (except for salt and chelating
647 reagents) may have originated from other apple pomace components since they were poor in sugars
648 (27.6 % for C2 and 29.8% for D1, **Table S1**). Renard et al. (1993) have reported that pectin structure
649 was extensively degraded when apple was extracted with CDTA (pH 6.5) at 80 °C and two
650 galacturonic acid peaks occurred. These authors also showed that temperature had a lower impact
651 than pH in determining the degradation of pectin by CDTA. In our study, the low MW components in
652 C2 and D1 extracts may result from degradation of cell wall components by CDTA & Na-oxalate
653 treatment (pH 6.5, 70 °C) and CDTA treatment (pH 6.5, 25 °C). However, detailed mechanism needs
654 to be further studied.

655 The extracts from B, E1, E2, F fractions showed at least two Mw populations on their HPSEC profile
656 (**Fig. 5A**). A relatively high Glc recovery was also found in the fractions following dilute acid treatment
657 (B) or CC:G and CC:LA pretreatments (E1, E2, F). Glucose is a typical sugar of cellulose and
658 hemicellulose, but can also come from remaining starch in the pomace due to incomplete regression
659 in apple prior processing. Due to partial acid hydrolysis of starch by dilute HCl, the largest peak
660 eluting at 10.5 ml observed on the HPSEC profile of the dilute acid extract (trace B, **Fig. 5A**), which is
661 the richest in Glc, may have arisen from starch fragments. Acidic NADES were reported to be good
662 extractants of starch (María, Bruinhorst, & Kroon, 2012; Zdanowicz & Szychaj, 2011). The
663 pretreatment of apple pomace with CC:LA promoted an efficient mean of starch solubilization and
664 degradation to glucan oligomers that were most likely lost during the recovery of the fraction (F).
665 Residual starch fragments probably corresponded to the peak eluting at about 11.5 ml (trace F, **Fig**
666 **5A**). However, the absence of this peak in the water extract following the sequential CC:LA
667 pretreatment (E2), indicated that the previous pretreatment by CC:G and hot water may have
668 extracted part of the starch that was the most susceptible to acid degradation.

669

670 4.4. Cell wall cellulose aggregates following pectin extraction from pomace

671 To establish an integrated biorefinery process of apple pomace and its further use after pectin
672 extraction, the impact of sequential NADES pretreatment/water extraction on structure and
673 organization of the residual pomace polymers was investigated by CP/MAS ^{13}C NMR spectroscopy.
674 The spectra (**Fig. 6**) revealed the evolution of cell wall structure from a complex raw material to a
675 simpler one in K:G pretreated residues. NADESs pretreatment/water extraction removed pectin, as
676 judged from the decreasing intensity of the signals for C_1 (around 100.4 ppm) corresponding to the
677 overlapping chemical shift of pectin backbone galacturonic acid, rhamnose and xylose sidechain of
678 xyloglucan (Ng et al. 2014; Phyo & Hong, 2019), C_4 (80.8 ppm) corresponding to pectin backbone
679 galacturonic acid (Sinitsya, Copiková, & Pavliková, 1998) and that of the pectin methyl ester at 52.7
680 ppm. The last alkaline NADES pretreatment/water extraction with K:G was particularly efficient in
681 removing also phenolic compounds and proteins, which signal intensity markedly decreased in the
682 spectrum of the residue. Apple varieties for cider production are known to be particularly rich in
683 phenolic compounds that form insoluble complex with cell wall material in pomace (Bourvellec,
684 Guyot, & Renard, 2009). K:G pretreatment can provide a mean for extracting them. As previous
685 research suggested (Newman, Ha, & Melton, 1994), the crystallinity of cellulose and its cross-section
686 dimension can be estimated by solid-state NMR. The crystallinity and LFD up to CC:LA residue were
687 ranged from 31%-34% and 2.6 nm-2.7 nm respectively. A similar 38 % of crystallinity and 2–3 nm of
688 cross-section dimension on apple cell wall has already been reported (Lahaye, Falourd, Laillet, & Le
689 Gall, 2020 and references herein). The higher crystallinity (46%) and LFD (3.6 nm) found in K:G
690 residue indicated the influence of this pretreatment on the cellulose structure and its surrounding
691 environment, which will be discussed below. At the molecular structure level, the two-proton
692 reservoir model allowed evaluating the spin diffusion time of non-bonded proton to proton linked to
693 carbon. In our case, the non-bonded proton mostly comes from water molecule, which was used to
694 rehydrate the residual pomace. Paris et al. (2001) showed that the T_{HH} value was positively
695 correlated with number of surrounding water molecule. The longer T_{HH} value of pectin methyl ester
696 in both CC:G and CC:LA pretreated residual pomace means better hydration of pectin than that of
697 raw material. The first removal of pectin by CC:G pretreatment/water extraction made the pomace
698 residue more porous so that the water used to rehydrate the residue could better interact with the
699 pectin. Further removal of pectin by the CC:LA pretreatment/water extraction did not have a major
700 impact on this porosity and the hydration of the residual pectin. The pore opening by pectin
701 extraction to allow water diffusion was also observed with the increasing T_{HH} value for cellulose up
702 to the CC:LA residue. Further processing with K:G pretreatment/water extraction led to a more
703 hydrophobic environment, most likely due to the aggregation/rearrangement in higher crystalline
704 cellulose by the removal of the alkaline-soluble pectin. The reorganization of the cellulose was
705 evidenced by the lengthening of the $T_{1\rho}^H$ relaxation attributed to more organized cellulose (Lahaye,

706 Falourd, Laillet, & Le Gall, 2020). This ordering goes along with that of pectin as judged from the $T_{1\rho}^H$
707 of the pectin methyl ester which agrees with close pectin-cellulose interactions (Wang & Hong, 2016).
708 Although this increase in ordering was observed all along the sequential extraction process and
709 notably after the first CC:G pretreatment/water extraction, K:G pretreatment/water extraction had
710 the most dramatic effect on cellulose. This result suggest that the K:G pretreatment/water soluble
711 pectin and the minor hemicellulose associated in the pectin extract play a major role in the cellulose
712 organization and support the idea that pectin distribution in the cell wall controls cellulose bundles
713 packing.

714

715 **5. Conclusion**

716 Sequential NADES pretreatments/hot water extractions of apple pomace markedly increased pectin
717 recovery. Overall pectin yield was particularly higher than those obtained using conventional
718 sequential extractions including chelating, mild alkaline or mild acidic conditions. A synergistic effect
719 was shown between CC:G and CC:LA pretreatments in the sequential extraction method. Besides the
720 harsh acidic or alkaline conditions of NADES (CC:LA and K:G solvents, respectively), which both led to
721 cell wall polysaccharides hydrolysis, ion exchange together with the effect of individual NADES
722 components contributed to these high pectin yields. The sequential process of the different NADES
723 also provided a mean to tailor the main structure of pectin recovered (HG, RGI and RGI side chains).
724 It also induced reorganization of the cellulose fibers in the pomace. These results open the way to
725 more sustainable extractions of pectin by use of sequential pretreatments with recyclable NADES,
726 which can be part of a more integrated biorefinery process including recovery of valuable NADES
727 soluble compounds and extraction residues.

728

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