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Mingrui Chen, Marc Lahaye*
UR 1268 Biopolymères Interactions Assemblages, équipe Paroi Végétale et Polysaccharides Pariétaux (PVPP), INRAE, 44300, Nantes, France
∗Corresponding author. E-mail address: marc.lahaye@inra.fr (M. Lahaye).

Abstract

Three natural deep eutectic solvents (NADES; choline chloride (CC) : lactic acid (LA), CC : oxalic acid (OA), CC : urea (U)) in the ratio 1:2 were tested as a pretreatment of apple pomace prior to hot water extraction of pectin. The pretreatment duration and temperature (for 0.5-2h, at 40-80 °C) was adjusted to limit cell wall polysaccharides losses and degradation. Extraction yields and sugar composition were used to evaluate the impact of the pretreatment on pectin extraction and the molecular weight and degree of esterification of pectin were characterized. Results showed that CC:LA and CC:U pretreatments led to close overall recovery yields with the control using water instead of NADES. Instead, CC:OA led to polysaccharides degradation and loss. The cell wall monosaccharides composition was affected after CC:LA and CC:U pretreatments at 60 °C and above, while it was preserved by CC:LA pretreatments at 40 °C for 1h. Moreover, CC:LA pretreatment facilitated hot water extraction of a large amount (33.1%-56.1% of uronic acid recovery) of high Mw HM pectin comparable with pectin obtained through classical method. CC:U pretreatment led to saponification and affected pectin composition by introducing choline. Thus, CC:LA pretreatment of apple pomace followed by water extraction offers a “green” alternative for mineral acid pectin extraction while CC:U allows functionalizing pectin in apple pomace prior to hot water extraction.

Keywords Natural deep eutectic solvents; Apple pomace; Cell wall polysaccharides; Pectin

1. Introduction

Apple is the most cultivated and consumed fleshy fruit in the world and its associated products, such as juice, cider and sauce are very popular among consumers. Apple processing generates side-streams, such as about10 million tons of pomace each year from the apple juice industry (Alongi, Melchior, & Anese, 2018) which can be valorized to extract pectin (May, 1990). Besides for food gelling ingredient, apple pomace may be source of other valuable polysaccharides, such as hemicellulose and cellulose. Hemicellulose can be applicable as film, drug carrier and stabilizing additive in food, pharmacy and other related industries (Ebringerová, 2005), while cellulose play a paramount role in paper making industry (Ververis, Georghiou, Christodoulakis, Santas, & Santas, 2004). To that end, a biorefinery approach was engaged to isolate apple pomace polysaccharides and, as a first step, pectin extraction was reconsidered. Pectin is made of approximately 65% homogalacturonan (HG), 20–35% rhamnogalacturonan I (RGI) and 10% rhamnogalacturonan II (RGII) structural domains (Mohnen, 2008). HG is formed by repeats of $[\alpha\text{-}1,4\text{-D-galacturonic acid}]_n$ units that can be partially esterified by methanol on O-6 (Voragen, Beldman, & Schols, 2001) and acetyl at O-2 and/or O-3 (Atmodjo, Hao, & Mohnen, 2013). It can be further modified at O-3 by xylose to form xylogalacturonan domains (Schols, Bakx, Schipper, & Voragen, 1995). According to the degree of methyl esterification, pectin is classified as high methoxyl (HM) pectins (degree of esterification > 50%) and low methoxyl (LM) pectins (degree of esterification < 50%) (Löfgren & Hermansson, 2007). RG-I pectic domain is built on the disaccharide repeat unit $[\alpha\text{-D-GalA-}1,2\text{-}\alpha\text{-L-Rha-}1,4]_n$ on which side-chains made of α-L-Araf and β-D-Galp are branched on O-4 of the rhamnosyl residues (Scheller, Jensen, Sørensen, Harholt, & Geshi, 2007). The minor RG-II structural domains consist of at least 8 α-1,4-D-galacturonic acid units with four types of complex side branches made of 12 different types of sugars (O'Neill, Ishii, Albersheim, & Darvill, 2004). Generally, extraction of pectin from agricultural side-streams is achieved through mild acid, mild alkali or enzyme extraction. Pectin industrial production mainly uses aqueous mineral acid which is responsible for corrosion of equipment and environmental pollution (Wikiera, Mika, Starzyńska-Janiszewska, & Stodolak, 2015). Methods using mild alkali decrease the degree of esterification and the molecular weight of pectin (Yuliarti et al., 2015) while enzymatic extraction affects pectin yield, physicochemical and rheological properties (Ptichkina, Markina, & Rumyantseva, 2008). Microwave and ultrasound techniques provide clear benefits in shortening extraction time and reducing energy consumption (Adetunji, Adekunle, Orsat, & Raghavan, 2017) but if these techniques are promising at the laboratory scale, their scaling-up and the cost of

investment in new equipment needed for their implementation refrain industry to adopt them. Hence, new solvents that can fit in classical processes of pectin extraction providing less adverse effects on the environment, equipment and polysaccharide physicochemical and rheological properties are being looked for.

Solvents, such as ionic liquids and more recently natural deep eutectic solvents (NADES) emerged to extract biopolymers and various plant molecules (Vanda, Dai, Wilson, Verpoorte, & Choi, 2018). NADES combine different cellular hydrogen bonding acceptor (HBA) and hydrogen bonding donor (HBD) metabolites (Choi et al., 2011) to form intermolecular networks at the basis of the physicochemical properties of the solvents (Yang et al., 2018). Distinct from the ionic liquids, which are analogues made of organic salts, the starting ingredients of NADES are cheap, biodegradable and eco-friendly natural non-toxic metabolites, such as, sugar, amino acid, organic acid. As deep eutectics, NADES have a lower melting temperature than that of each starting material and some of them form transparent liquids at ambient temperature, which make these solvents easy to prepare and use. High viscous NADES solutions which can impede extraction rate and time can be mitigated by high temperatures and/or a small proportion of co-solvent, such as water (Vanda et al., 2018). All of these traits, which are consistent with the concept of "green chemistry", have drawn the focus of recent researches for their extraction potential. To date, NADESs were shown to be excellent solvents for the extraction of phenolic compounds or lignin from different biomasses (Kim, Dutta, Sun, Simmons, & Singh, 2018; Ruesgas-Ramón, Figueroa-Espinoza, & Durand, 2017; Soares et al., 2017) and were tested for the extraction of polysaccharides, among which is fruit cell wall pectin (Benvenutti, Sanchez-Camargo, Zielinski, & Ferreira, 2020; Liew, Ngoh, Yusoff, & Teoh, 2018; Shafie, Yusof, & Gan, 2019). However, NADESs are less effective in extracting pectin from pomelo compared with mild organic acids in terms of yield, operational attributes and economical features (Liew et al., 2018). Since NADESs are prepared by combining different compounds in various molar ratio, they offer the possibility of being tailored for specific extraction or for pre-extraction to remove unwanted components and ease extraction or modification of valuable biopolymers (Yu et al., 2019). For various NADES combinations, the majority of NADESs are of choline chloride based (Benvenutti, Zielinski, & Ferreira, 2019; Choi & Verpoorte, 2019), while among studied HBDs, lactic acid, oxalic acid and urea were widely applied in extraction of bioactive compounds or pre-extraction of lignocellulosic matrices (Achkar, Fourmentin, & Greige-Gerges, 2019; Benvenutti et al., 2019). NADESs containing these HBDs may have the potential to extract pectin directly or indirectly. Hence, in the present study, three common

NADES: choline chloride : lactic acid (CC:LA), choline chloride : oxalic acid (CC:OA), choline chloride : urea (CC:U) were tested as a mean of pre-treating apple pomace prior to hot water extraction of pectin. The extraction yield and sugar composition analysis were used to evaluate NADES pretreatment efficiency and the molecular weight and degree of esterification of pectin recovered were characterized.

2. Materials and Methods

2.1. Materials

2.1.1. Pomace

Wet pomace from *Malus domestica* var *Kermerrien* was provided by IFPC (Le Rheu, France). The pomace was stored at -20 °C prior use.

2.1.2. Chemicals

Choline chloride (CAS: 67-48-1, Sigma-Aldrich, France), urea (CAS: 57-13-6, Sigma-Aldrich, France), oxalic acid dihydrate (CAS: 6153-56-6, Sigma-Aldrich, France), DL-lactic acid (CAS: 50-21-5, Sigma-Aldrich, France), ethanol (CAS: 64-17-5, Carlo Erba reagents, France), acetone (CAS: 67-64-1, Carlo Erba reagents, France), nitric acid (Titripur, Germany), ammonium hydroxide solution (CAS: 1336-21-6, Sigma-Aldrich, France), acetic anhydride (CAS: 108-24-7, Sigma-Aldrich, France), 1-methylimidazole (CAS: 616-47-7, Sigma-Aldrich, France) were used in present research.

2.1.3. Preparation of NADES

Three different NADESs were prepared by separately mixing the choline chloride with lactic acid, urea and oxalic acid in a molar ratio (1:2). The molar ratio was chosen as a widely used ratio (may not be the exact ratio for eutectic point) for polysaccharides processing (Zdanowicz, Wilpiszewska, & Spychaj, 2018). The mixtures were stirred with magnetic bar and heated in the oil bath at 100 °C. The mixture was constantly stirred until the clear liquid formed. The solvents were stored at ambient temperature for later use. Due to the non-volatile property of NADES, prior to application, the water content in NADESs (CC:LA=0.91% (w/w); CC:U=0.32% (w/w); CC:OA=1.66% (w/w)) was determined by freeze drying for 24 h until constant weight was reached (Jeong et al., 2015).

2.2. Methods

2.2.1. Pretreatment of apple pomace with NADESs

Five g of thawed apple pomace (water content = 68%) were mixed with 40 mL of different NADES (1:8, w/v) and the big particles in suspension were dispersed with a Polytron mixer. The mixture was stirred with a magnetic bar and heated in water bath under different conditions as listed in Table 1. The suspension was then centrifuged at 15000 g for 20 min and the supernatant was collected as NADES supernatant fraction. The remaining pomace was washed twice with deionized water at ambient temperature to reach water pH (pH 6.5), then, the pellet was resuspended in 40 mL of deionized water at 80 °C for 10 min under constant agitation and the water extract was recovered after centrifugation as above. This operation was repeated 5 times. The pooled water washes, referred to as the water fraction, was concentrated to 10 mL with a vacuum rotary evaporator. Polymers in the NADES supernatant and water fractions were precipitated by 4 volumes of ethanol. The precipitates were recovered by centrifugation (15000 g, 20 min) and washed with 40 mL of 70% of ethanol for 10 min (3 times), followed by 40 mL of ethanol and acetone for 10 min (2 times). The ethanol and acetone washings were repeated to obtain low color solutions. The pomace residue underwent the same washing and dehydration process as above. Finally, samples were dried at 40 °C in vacuum oven over P_2O_5 powder for 12 h and stored at 4 °C for later analysis. The extraction was conducted four times and water was used as a control pretreatment condition. Fractions yield was calculated as follows:

$$\text{Yield } (\%) = \times 100$$

Where the Ws is the sample weight in each fractions and W is the initial wet weight of apple pomace.

Table 1. Extraction conditions with NADES

Extraction condition	
Time	Temperature
2 h	80 °C
1 h	80 °C, 60 °C, 40 °C
0.5 h	40 °C

The overall extraction process is summarized in Figure 1.

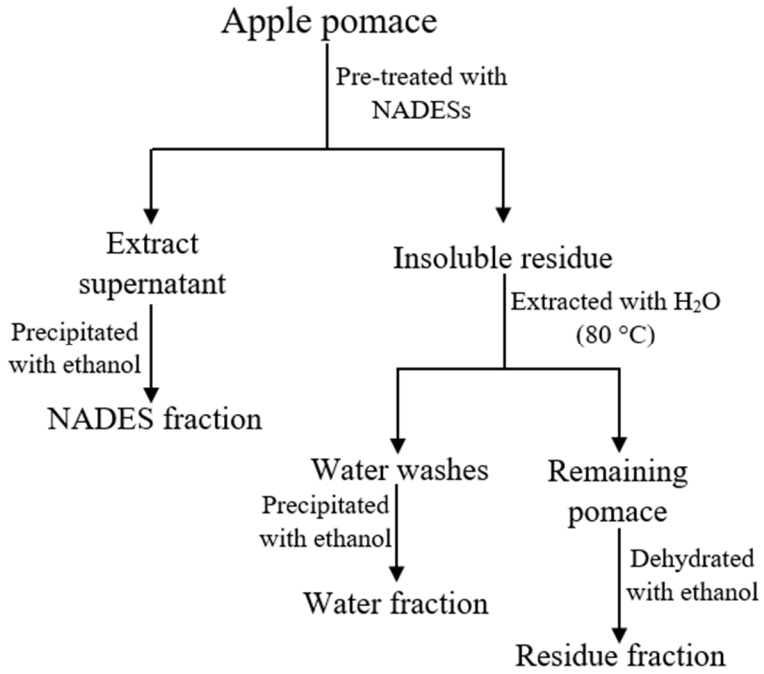

Fig. 1 Schematic representation of the extraction process with NADES

2.2.2. Neutral sugars composition and uronic acid analysis

To identify and quantify neutral sugars in different fractions, GLC (Gas-liquid chromatograph) analysis was performed according to previously established method (Ray, Vigouroux, Quemener, Bonnin, & Lahaye, 2014). Briefly, sample was dispersed in sulphuric acid (12 M, 72%) at 25 °C for 30 min, followed by further hydrolysis with sulphuric acid (2 M) at 100 °C for 2 h. The released sugars were reduced with $NaBH_4$ solution at 40 °C for 60 min, then acetylated with acetic anhydride and 1-methylimidazole at ambient temperature for 20 min. The obtained alditol acetates were analyzed by GLC (Perkin-Elmer Autosystem) equipped with DB-225 capillary column (J&W Scientific, Folsorn, CA, USA) eluted at 205 °C by hydrogen. The split injector and flame ionization detector temperatures were set at 220 °C. Both sugar standard solution and internal standard (inositol) were used for calibration. Uronic acid in the acid hydrolysate was quantified using the m-hydroxydiphenyl colorimetric acid method (Blumenkrantz & Asboe-Hansen, 1973). Galacturonic acid and glucose standard solutions were used for calibration. Sugar composition in each fraction was expressed as recovery rate and was calculated as follows:

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

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

2.2.3. Starch content

Starch content in the residue and water fraction was determined by high performance anion exchange chromatograph (HPAEC) according to established method (McCleary, Gibson, & Mugford, 1997). Briefly, 10 mg of sample was swelled in MOPS buffer (200 uL, 50 mM, pH = 7) overnight at ambient temperature. The sample was hydrolyzed by 300 uL of α-amylase (100 U/mL; Megazyme) at 100 °C for 6 min followed by 100 uL of amyloglucosidase (20 U/mL; Megazyme) in 400 uL of sodium acetate buffer (200 mM, pH = 4.5) at 50 °C for 30 min. Glucose released was quantified by HPAEC equipped with a PA1 column (4 × 250 mm, Dionex) eluted by 500 mM NaOH (eluent A, 20%) and deionized water (eluent B, 80%) at a flow rate of 1 mL min^{-1}. Rhamnose was used as an internal standard in sample and in glucose standard solutions used for calibration. Control was realized with sample without enzyme to correct for eventual free glucose.

2.2.4. FTIR spectroscopy

Infrared spectra of the dried water fractions were collected on a NICOLET IS50 spectrophotometer (Thermo scientific). Spectra were collected in the transmission mode on a ATR crystal between 400 and 4000 cm^{-1} using the Smart iTR ATR sampling accessory. Six spectra were registered for each sample. Data was further processed with R (R Core Team, 2014) using the ChemoSpec library (http://127.0.0.1:10623/library/ChemoSpec/doc/ChemoSpec.pdf) for normalization, correction and calculation of mean value.

2.2.5. Nitrogen and carbon content

The nitrogen and carbon contents of dry water fractions were analyzed using CNS Vario (Elementar, Germany).

2.2.6. ^{1}H NMR spectroscopy

Five mg of the dried sample from the water fraction of CC:U treated group and control were dissolved in 0.5 ml of D_2O (99.96 atom% D, Sigma-Aldrich) and then lyophilized. This

process was repeated twice. Water pre-saturated ^{1}H NMR spectra were registered at 60 °C on a Bruker Avance III 400 MHz. Chemical shifts were referred to water assigned to 4.4 ppm.

2.2.7. Preparation of cholinium polygalacturonate and sodium pectinate

Twenty mg of polygalacturonic acid (Sigma-Aldrich) was dissolved in 2 mL of deionized water, neutralized with choline hydroxide solution (45 wt% in methanol, Sigma-Aldrich). Then, the solution was dialyzed against deionized water (MW cutoff: 6000-8000, T2-8030-23, Membrane Filtration Products, Inc.) for 24 h, prior to freeze-drying.

Twenty mg of the water fraction from pomace pretreated with CC:U at 80 °C for 2 h was dissolved in 2 mL of deionized water. The pH of solution was adjusted to 2.5-3.0 with hydrochloric acid (0.1M) before dialysis as above. Then, the solution was neutralized with sodium hydroxide (0.1M) and freeze-dried.

The freeze-dried cholinium polygalacturonate and Na pectin from CC:U treated pomace were subjected to MHDP colorimetric assay.

2.2.8. Pectin methylation and acetylation esterification degree

Methanol and acetic esters in pectin were measured by HPLC according to previous method (Levigne, Thomas, Ralet, Quemener, & Thibault, 2002). Briefly, samples were saponified for 1 h at 4 °C by NaOH (0.5 M) with $CuSO_4.5H_2O$ in isopropanol solution, then centrifuged and the supernatant was filtered through cartridge IC-H (Sstarpure, Maxi-Clean SPE 0.5 ml IC-H 50pk) prior to HPLC analysis on C18 (4 mm × 250 mm, Lichrospher 100 RP-18e (5 μm), Interchim, France) at 25 °C. H_2SO_4 (4 mM) was used for isocratic elution at 1.0 mL min^{-1}. Standard solution containing methanol, acetic acid and isopropanol as internal standard was used for calibration. The degree of methyl esterification (DM) and acetyl esterification (DA) were calculated as the number of moles of methanol and acetic acid measured per mole of uronic acid in pectin.

2.2.9. Molecular weight measurement

Molecular weight of pectin was determined by high performance size exclusion chromatography (HPSEC) using Shodex OHpak SB-G 6B pre-column and OHpak SB-805-HQ column (Shodex, Tokyo, Japan) connected to pump (Jasco PU-1580, Tokyo, Japan) and injector (PerkinElmer, series 200 autosampler, Courtaboeuf, France). Prior to injection, starch in water fractions was hydrolyzed by 500 uL of amyloglucosidase (500 U/mL) at 50 °C for 30

min. The solution was dialyzed against deionized water (MW cutoff: 6000-8000, T2-8030-23, Membrane Filtration Products, Inc.) prior to freeze-drying. Glucose content in the sample was checked by GLC to ensure the removal of starch efficiency. De-starched samples were dissolved in distilled water overnight at 4 °C, then centrifuged (10 min, 7400 g) and filtered through 0.45 μm membrane (Millex-HV, PVDF) prior to injection. Elution was performed with 50 mM $NaNO_3$ at a flow rate of 0.6 mL min^{-1} and monitored by i) differential refractometry (Viscotek VE 3580 RI detector, Malvern Instruments, Orsay, France), ii) light scattering (LS) detection and iii) differential pressure viscometry (both from Viscotek 270 dual detector, Malvern Instruments, Orsay, France). Molecular weight and viscosity were obtained using the OmniSEC 4.7.0 software and calibration was done with using pullulan-P108K (Viscotek, Malvern Instruments, Orsay, France).

2.2.10. Statistical analysis

Data was first verified for homogeneity of variance by Levene's test (P>0.05). Then, they were subjected to one-way ANOVA and Duncan's multiple range post-hoc test. Besides, independent-samples T test was carried out to compare the data in esterification degrees and molecular weight between NADES groups and control group. The SPSS 16.0 statistical software package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Differences were considered significant at P<0.05. Data are presented as mean values with their standard deviation.

3. Results and discussion

3.1. NADES pretreatment conditions of pomace affect fractions yield

Table. 2 Effect of NADES type and NADES treatment temperature and duration on the yield (% w/w) of total pomace recovery, of extraction residue, of NADES and water fractions.

Extraction Yield	**Control**	**CC:LA**	**CC:U**	**CC:OA**
Total fraction				
0.5h-40 °C	21.69 ± 0.50^{a,A}	21.13 ± 0.52^{a,B}	21.26 ± 0.60^{a,C}	
1h-40 °C	21.72 ± 0.75^{a,A}	21.34 ± 0.30^{a,B}	21.80 ± 0.85^{a,C}	
1h-60 °C	21.66 ± 0.26^{c,A}	20.58 ± 0.34^{b,B}	20.03 ± 0.36^{a,B}	
1h-80 °C	22.71 ± 0.74^{b,B}	18.54 ± 0.62^{a,A}	19.39 ± 0.67^{a,B}	
2h-80 °C	21.49 ± 0.50^{c,A}	17.65 ± 1.23^{b,A}	18.10 ± 0.09^{b,A}	8.44 ± 0.78^{a}

Residue fraction				
0.5h-40 °C	20.23 ± 0.45^{c,D}	14.71 ± 0.46^{a,E}	18.07 ± 0.47^{b,C}	
1h-40 °C	20.10 ± 0.74^{c,D}	13.43 ± 0.35^{a,D}	17.25 ± 0.46^{b,C}	
1h-60 °C	19.21 ± 0.22^{c,C}	12.46 ± 0.30^{a,C}	15.22 ± 0.24^{b,B}	
1h-80 °C	18.18 ± 0.26^{c,B}	9.95 ± 0.55^{a,B}	14.45 ± 0.83^{b,B}	
2h-80 °C	15.51 ± 0.27^{c,A}	8.48 ± 0.93^{a,A}	13.19 ± 0.86^{b,A}	7.70 ± 0.78^{a}
NADES fraction				
0.5h-40 °C	0.08 ± 0.02^{a,A}	0.26 ± 0.07^{b,A}	0.23 ± 0.04^{b,A}	
1h-40 °C	0.13 ± 0.02^{a,A}	0.27 ± 0.03^{b,A}	0.32 ± 0.03^{c,A}	
1h-60 °C	0.95 ± 0.05^{b,B}	0.29 ± 0.04^{a,A}	1.58 ± 0.04^{c,B}	
1h-80 °C	2.79 ± 0.34^{b,C}	0.57 ± 0.13^{a,B}	2.11 ± 0.53^{b,B}	
2h-80 °C	3.75 ± 0.15^{c,D}	0.69 ± 0.05^{a,C}	2.08 ± 0.62^{b,B}	0.34 ± 0.08^{a}
Water fraction				
0.5h-40 °C	1.37 ± 0.05^{a,A}	6.16 ± 0.20^{c,A}	2.96 ± 0.11^{b,A}	
1h-40 °C	1.49 ± 0.22^{a,AB}	7.64 ± 0.12^{c,B}	4.23 ± 0.45^{b,B}	
1h-60 °C	1.50 ± 0.02^{a,AB}	7.83 ± 0.25^{c,BC}	3.23 ± 0.18^{b,A}	
1h-80 °C	1.75 ± 0.27^{a,B}	8.02 ± 0.27^{c,C}	2.84 ± 0.17^{b,A}	
2h-80 °C	2.23 ± 0.27^{b,C}	8.48 ± 0.29^{d,D}	2.82 ± 0.33^{c,A}	0.49 ± 0.09^{a}

Control: water replacing for NADES; CC:LA, CC:U, CC:OA: choline chloride: lactic acid, urea and oxalic acid, Mean values (± standard deviation, n = 4) with unlike letters were significantly different (P<0.05), significant difference was analyzed in each fraction. a,d,c (row): significantly different between different groups at same condition; A,B,C: significantly different at different condition within same group (columns).

Extraction yields with the CC:LA, CC:OA and CC:U are depicted in Table 2. The viscosity of the NADES is provided in Table S1 and showed, as reported that it decreased with both temperature and addition of water (Yang et al., 2018; Zdanowicz et al., 2018). Moreover, consistent with other reports (Fisher & Kunz, 2014; Hou et al., 2008)), the three NADES were non-Newtonian with a shear-thinning behavior (data not shown). The initial extraction conditions were set at 80 °C for 2 h to lower viscosity of NADES (viscosity at 10 s^{-1}: from 107.6 mPas at 40 °C to 7.9 mPas at 80 °C for CC:LA; from 522.0 mPas at 40 °C to 36.4 mPas at 80 °C for CC:U; from solid at 40 °C to 37.0 mPas at 80 °C for CC:OA). As mass transfer plays an important role in determining extraction conditions, the lower viscosity at 80 °C with longer duration can facilitate the interaction between NADESs and apple pomace. Morrais et al. (2018) demonstrated that higher hardwood xylan solubilization was achieved with CC:U (1:2) at 80 °C. Moreover, the optimal condition for extracting pectin from *Averrhoa bilimbi* with CC:citric acid was at 80 °C for 2.5 h (Shafie et al., 2019). Extraction

with CC:OA for 2h at 80 °C severely affected the total yield of apple pomace with only 8.44 % that did not reach half that of control group pretreated with water. Hence, CC:OA pretreatment was not further studied. Lower losses were observed with CC:LA and CC:U pretreatments and thus, other conditions were tested. Lowering pretreatment temperature from 80 °C to 40 °C and duration from 2 h to 0.5 h significantly improved the total recovery yield of pomace after CC:LA and CC:U pretreatments. Moreover, at 40 °C for 0.5 and 1 h, no difference was observed in the total recovery yield of pomace between NADES pretreatments and control (P>0.05).

Looking at specific fractions, the yield of the NADES fraction increased with higher temperature or extended extraction time. For NADES extraction of pomace, as mentioned above, higher temperature decreased viscosity of NADESs solution. Many studies reported on the inverse relation between extraction yield of bioactive substances and viscosity of DESs (Dai, Rozema, Verpoorte, & Choi, 2016; Guo et al., 2019; Huang et al., 2017). In our study, a similar relation was observed. The significantly high yield of NADES fraction at 80 °C was partly attributed to the low viscosity of solution. In addition, when the extraction temperature was set at 40 °C, the viscosity of NADESs had a more important role in determining extraction yield than duration since the yield of extract was not significantly different at 40 °C for 0.5h or 1h. The relatively higher viscosity (viscosity at 40 °C: 107.6 mPas for CC:LA; 522.0 mPas for CC:U) impeded the mass transfer of pectin from cell wall matrix into the solvent even with extended extraction time. Nevertheless, when compared with control, the highest yield (3.75%) was obtained with the control experiment pretreated with water at 80 °C for 2 h. The yield of polymer precipitated by ethanol from CC:LA and CC:U extracts was much lower than that of control at 80 °C. Although their yields were significantly higher at temperature of 40 °C compared with control, the highest yield (0.32%) in CC:U groups still demonstrated that NADESs by themselves were not efficient in extracting polysaccharides from apple pomace. Benvenutti et al. (2020) showed that NADES (citric acid : glucose : water; 1:1:3) : water solution (1:9 w/w) offered the highest extraction yield of *Myrciaria cauliflora* pectin. Similarly, the highest pectin yield was attained from pomelo peels with NADES (Choline chloride : glucose : water; 5:2:5) at 60 °C for 2h (Elgharbawy et al., 2019). The disparity from our result may lie in different water content in NADES extraction system (water : NADES=8.5% (v/v), considering 68% of water content in raw pomace), as it affected the extraction ability of DES (Passos, Tavares, Ferreira, Freire, & Coutinho, 2016; Yiin, Yusup, Quitain, & Uemura, 2015). In contrast, the yield of the water fraction after CC:LA pretreatment was at least 6 times higher than that of corresponding NADES fraction.

Moreover, the yield of the water fraction following this pretreatment was the highest among all other solvent pretreatments and conditions tested (range from 6.16% to 8.48%, $P<0.05$). The overall cumulative yield of ethanol precipitated materials did not exceed 23% of the starting weight of raw apple pomace due to its 68% water content. It is also due to partial degradation of apple pomace by NADES as demonstrated by the extract solution colors that ranged from orange to deep red (supplementary Figure S1) from the CC:LA pretreatments. In apple, phenolic compounds are naturally present and their content is more than 10 g kg^{-1} of flesh weight (Van Buren, 1970). Phenolic compounds of apple mainly consist of hydroxycinnamic acid derivatives, flavan-3-ols, flavonols and dihydrochalcones (Guyot, Marnet, Laraba, Sanoner, & Drilleau, 1998). Condensed tannins are formed by the polymerization of flavan-3-ol compounds, such as catechin or its derivative and are degraded by acid or alkali into water insoluble reddish-colored phlobaphenes (Lachman, Martinek, Kotíková, Orsák, & Šulc, 2017). The color of the CC:LA extract was a strong indication for the degradation of phenolic compounds and their subsequent loss in the ethanol used to precipitate polysaccharides from the extract. On that account, compared with CC:LA pretreatments, the CC:U pretreatments showed lower degradation of phenolic compounds as judged from the solution color (from orange to brown; Figure S1). Such putative phenolic compounds degradation may partly explain the residue yield that was the highest from the control group, followed by the CC:U pretreated pomace and lastly, by the CC:LA pretreated pomace. NADES pH is a known factor influencing extraction efficiency: acidic NADES will break more bonds and may favor the extraction process (Hou et al., 2017). In our result, differences in extraction yield from pomace treated by CC:LA and CC:U may have resulted from acidic pH (pH 1.0) with the former and mild alkaline pH (pH 8.5) with the latter solvent, while hot water in the control replacing for NADES is known to extract cell wall polysaccharides (Fry, 1988).

3.2. NADES pretreatment affects polysaccharide sugar composition in apple pomace fractions

To assess the impact of NADES treatment on apple pomace polysaccharide composition, the recovery rate of cell wall component sugars was determined. Moreover, optimization of extraction condition was conducted not only to improve recovery yields but also to mitigate the negative effects of NADES on cell wall sugar composition. Typical component sugars for HG, RGI pectic domains are uronic acid, rhamnose, arabinose and galactose (Mohnen, 2008) while for hemicellulose they are fucose, xylose, mannose and glucose (Scheller & Ulvskov, 2010). Glucose is also typical of cellulose, but can also come from contaminating starch in

cell wall fractions. Arabinose and galactose can also arise from arabinogalactan proteins (AGP) known to be present in apple (Brillouet, Williams, Will, Müller, & Pellerin, 1996; Leszczuk, Szczuka, Wydrych, & Zdunek, 2018). Based on the sugar composition of the total material recovered (Figure 2A-supplementary Table S2), NADES pretreatments had a negative impact on the recovery of rhamnose, arabinose, fucose, galactose, glucose and uronic acids. Compared with water treatment (control), losses of neutral sugars (rhamnose, arabinose, fucose, galactose and glucose) in total fraction of CC:LA were particularly observed with pretreatment conditions of 80 °C-2h (P<0.05). Besides, the uronic acids recovery was significantly lower after CC:U pretreatment. Pretreatment at 40 °C for 1h limited such losses, except for the arabinose recovery. There was no significant difference in other neutral sugars (rhamnose, fucose, galactose and glucose) recovery between CC:LA pretreatment and control, which indicated that most of polysaccharides structure was preserved. However, overall uronic acids recovery in total fraction after 1h, 40 °C CC:U pretreatment of pomace was less than 50%. For CC:OA pretreatment, it clearly indicated that CC:OA degraded the pomace polysaccharides whether as pectin or other type of polysaccharides since both neutral monosaccharides and uronic acids recoveries in total fraction of CC:OA pretreated pomace were significantly lower than that of control pomace (supplementary Table S2).

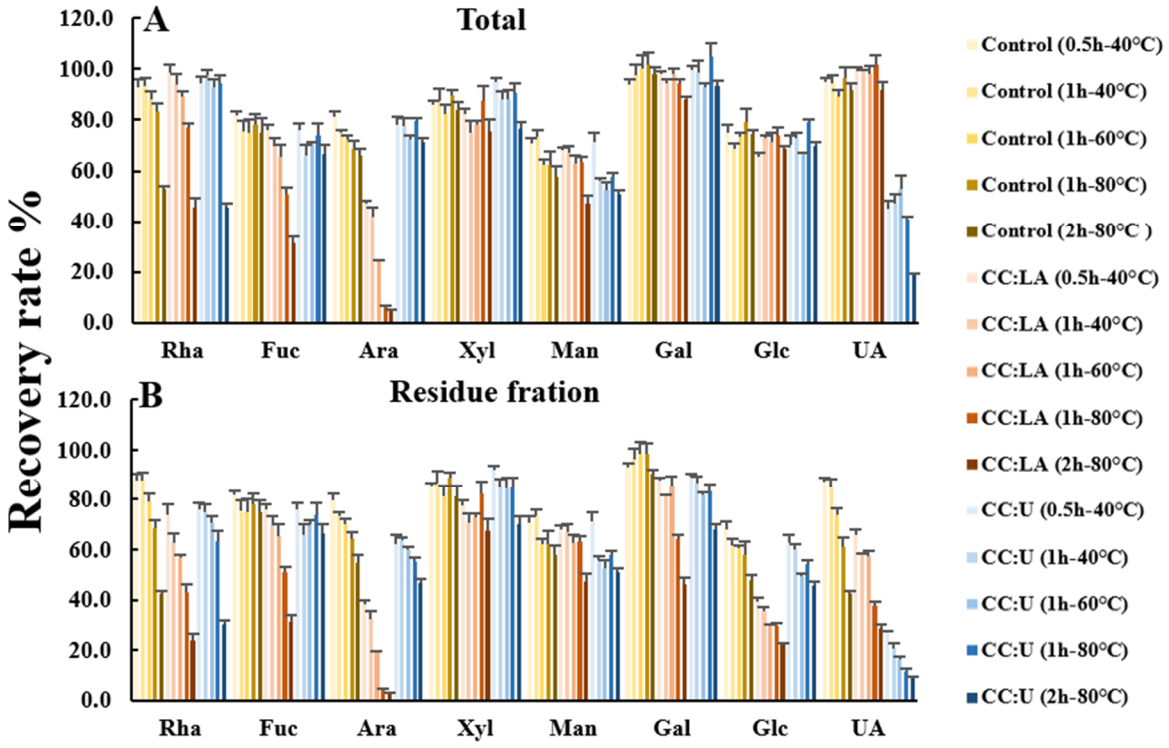

Fig. 2 Sugar recovery as % weight of individual sugars in dry raw pomace in total fraction (A) and

residue fraction (B) after CC:LA, CC:U or control pretreatment at different temperatures (40, 60, 80 °C) and time (0.5, 1, 2 h) of extraction. Means of four replicates; bar: standard deviation. Rha, Fuc, Ara, Xyl, Man, Gal, Glc, UA: rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose, uronic acids. Detailed data and statistics are provided in supplementary Table S2-3

Considering pectin-related sugars (arabinose, rhamnose, galactose and uronic acids) in detail, the recovery of uronic acids in the residue fraction of CC:LA treated pomace was much lower than that in the total fraction recovered regardless of pretreatment conditions (Figure 2A,B- supplementary Table S2-3). In fact, most of uronic acids were found in the water fraction with a highest recovery (56.1%) achieved after CC:LA pretreatment at 80 °C for 1h (Figure 3B- supplementary Table S4), which indicated that CC:LA treatment helped in the extraction of pectin. CC:LA likely loosened cell wall structures causing the extraction of pectin by hot water. As mentioned above, CC:LA had negative effects on the total recovery of pectin rhamnose and most notably side chain arabinose which is known to be rapidly cleaved under mild acidic conditions (Thibault, Guillon, & Rombouts, 1991). Similarly, the branch size of pectin obtained with CC:citric acid was found to be less than when pectin was extracted by only a citric acid solution (Shafie & Gan, 2020). The lowest recovery of total rhamnose and arabinose (45.7% for Rha; 4.8% for Ara) was observed at 80 °C-2h. Shortening the CC:LA treatment of pomace to 1 h significantly increased rhamnose recovery in both the water and residue fractions but that of arabinose remained significantly lower compared to control (Figure 2B- supplementary Table S3, Figure 3B- supplementary Table S4). This result indicated that not only pretreatment duration, but also temperature had a significant influence on pectin side-chain structure and/or AGP. The decrease in CC:LA extraction temperature from 80 °C to 60 °C for 1 h or to 40 °C for 1 h or 0.5 h, led to lesser total arabinose losses but still left a majority of the pentose hydrolyzed. Last but not least, despite the fact that uronic acids in the water fraction following CC:LA pretreatment of pomace was significantly higher than that of control regardless of the pretreatment condition, obvious reduction of uronic acids in the water fraction was noticed when the pretreatment temperature was lowered (Figure 3B- supplementary Table S4). This result means lower CC:LA pretreatment temperature may not be sufficient to break bonds in the cell wall to release pectin.

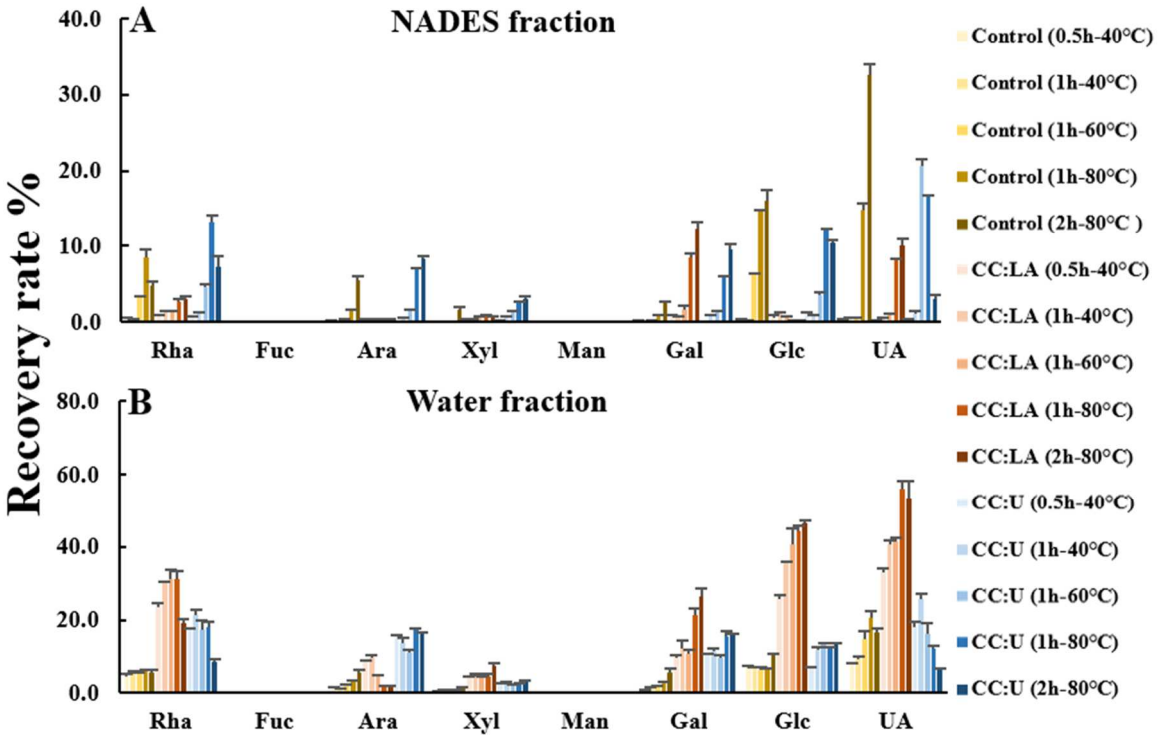

Fig. 3 Sugar recovery as % weight of individual sugars in dry raw pomace in NADES (A) and water (B) fractions after CC:LA, CC:U or control pretreatment at different temperatures (40, 60, 80 °C) and time (0.5, 1, 2 h) of extraction. Means of four replicates; bar: standard deviation. Rha, Fuc, Ara, Xyl, Man, Gal, Glc, UA: rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose, uronic acids. Detailed data and statistics are provided in supplementary Table S4-5.

3.3. Choline chloride: urea treatment functionalizes apple pomace pectin

By comparison with the CC:LA, the CC:U pretreatment of pomace had a more profound impact on uronic acids recovery. It was only 6.5% in the water fraction after 2 h treatment at 80 °C (Figure 3B- supplementary Table S4) and was also low in the NADES (CC:U) and residue fractions (Figure 3A- supplementary Table S5, Figure 2B- supplementary Table S3). More importantly, irrespective of pretreatment conditions, the total uronic acids recovery was not only much lower than the total recovery of other pectin related sugars (rhamnose, galactose and arabinose), but also significantly lower than that of control. Because the total recovery yield of fractions following CC:U treatment at 40 °C was not affected on the weight basis (Table 2), it thus implied that uronic acids quantification by colorimetry using m-hydroxydiphenyl was affected. Any modification of the uronic acids that change the

chemistry of the acid degradation product that complex with the dye may affect color development and thus uronic acids quantitative measurement. For example, amidation of pectin is known to depress the color development (Reitsma & Pilnik, 1989). Carbamate derivatives of cellulose were reported with betain:urea and CC:U solvents (Willberg-Keyriläinen, Hiltunen, & Ropponen, 2017) and similar derivatives were proposed for carrageenan extracted from the red seaweed (Das, Sharma, Mondal, & Prasad, 2016). Furthermore, Sirviö et al. (2019) have shown that sulfate groups were introduced to cellulose with DES rich in urea (sulfamic acid : urea) at 80 °C and above, which indicated that a new functional group can be added to polysaccharides in presence of high concentration of urea. To check for modifications of pectin structure extracted following NADES pretreatment, FTIR spectra of water extracts from control, CC:LA and CC:U treated apple pomace were recorded (Figure 4). The spectra were typical of pectin with -OH and -C-H stretching vibrations at 3340 cm^{-1} and 2911 cm^{-1}, C=O vibration of methyl ester at 1735 cm^{-1} and of the acidic form at 1604 cm^{-1}, –CH–O–CH– stretching at 1012 cm^{-1} (Figure 4) (Guillotin, Bakx, Boulenguer, Schols, & Voragen, 2007; Sinitsya, Čopíková, Prutyanov, Skoblya, & Machovič, 2000). Moreover, by comparing with the characteristic peaks of commercial HM pectin (Sinitsya et al., 2000), both control and CC:LA pectin were classified as HM pectin.

The spectra of water soluble pectin from the CC:U treated pomace were distinct from all others with a gradual increase in the absorption band at 1604 cm^{-1} as the extraction condition became harsher, in combination with the decrease in the C=O vibration at 1735 cm^{-1} (Figure 4). It thus appears that saponification occurred during the process. This interpretation was supported by the significantly lower degree of methyl-esterification of the pectin from the CC:U treated pomace compared to that of the control and the lowest DM value was measured for pomace treated at 80 °C for 2 h (supplementary Figure S2). In fact, the actual DM is lower than the current value considering the underestimation of uronic acid. This observation provided further clue that the pectin structure was modified after CC:U pretreatment. However, the molecular weight of pectin from CC:U treated pomace was not affected even at 80 °C for 2 h (supplementary Table S6), which indicated that CC:U pretreatment did not led to depolymerization by β-elimination. As shown in Table 3, a relatively high nitrogen content was detected in the pectin from the CC:U treated pomace, ranging from 0.54% to 3.59%. Since the typical FTIR amide I and amide II bands at about 1680 and 1595 cm^{-1} were absent from CC:U pectin (Figure 4) (Sinitsya et al., 2000), conversion to amidated pectin by ammonia that could have been produced by hydrothermal degradation of the urea (Claus-Peter, 2011) in the CC:U solvent did not occur. In fact, ^{1}H NMR spectroscopy (Figure 5)

indicated that choline was the likely source of nitrogen. Choline bound to the dissociated carboxyl group due to the electronic attraction, could have decreased the color development in the MHDP colorimetric assay of uronic acids. However, cholinium polygalacturonate and sodium pectinate from the CC:U treated pomace showed no modification in the colorimetric uronic acids content determination (supplementary Table S7). Hence, ion exchange with choline was not responsible for interfering with the MHDP colorimetric assay and more profound changes happened to the galacturonic acid structure.

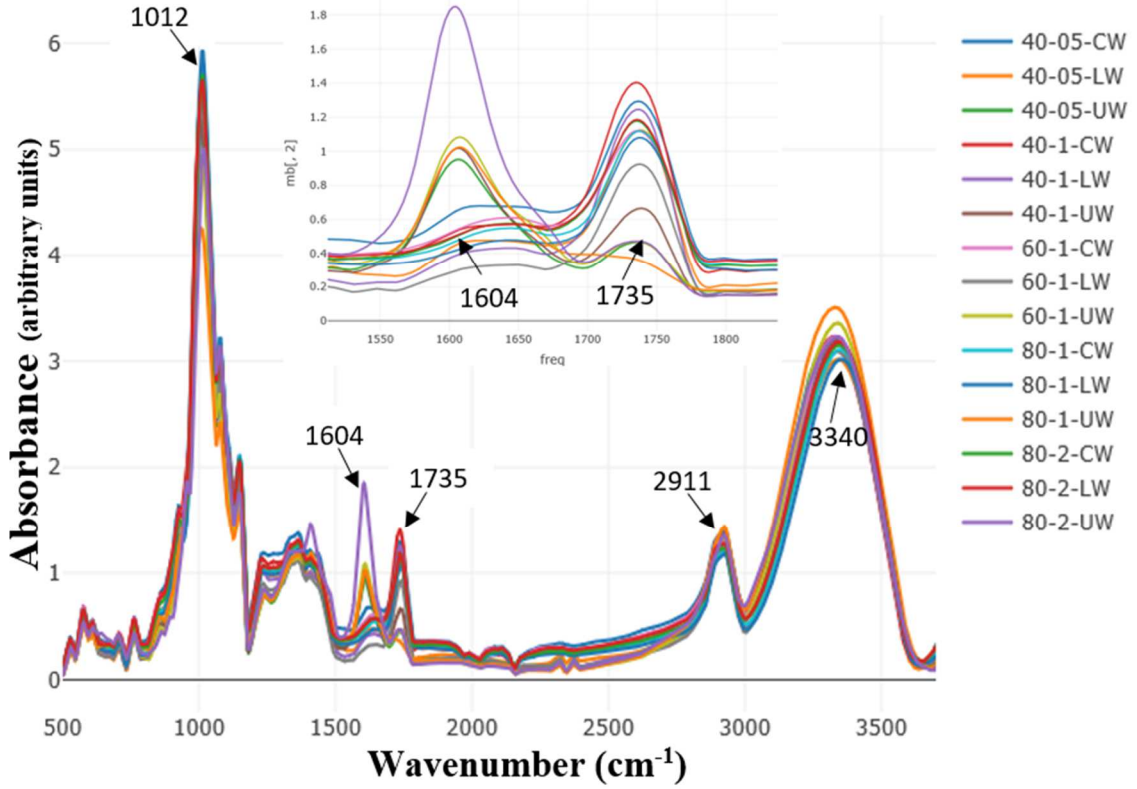

Fig. 4 FTIR spectrum of water soluble polysaccharides from control (CW), CC:LA (LW) and CC:U (UW) treated apple pomace at 80, 60 and 40 °C (80, 60, 40) for 0.5, 1.0 or 2 h (05, 1, 2).

Table. 3 Mean nitrogen and carbon contents (% of dry materials, ± standard deviation, n = 4) in dry material recovered in the water fraction after NADES pretreatment

	Control		CC:LA		CC:U	
	N %	**C %**	**N %**	**C %**	**N %**	**C %**
80 °C – 2h	0.04 ± 0.005	40.73 ± 0.89	0.06 ± 0.01	42.29 ± 0.39	3.59 ± 0.44	41.47 ± 0.99
80 °C – 1h	0.01 ± 0.001	38.42 ± 0.13	0.07 ± 0.005	42.18 ± 0.20	0.95 ± 0.13	42.37 ± 0.62
60 °C – 1h	0.01 ± 0.001	39.40 ± 0.25	0.06 ± 0.004	41.52 ± 0.36	0.54 ± 0.11	41.45 ± 0.77

40 °C – 1h	0.02 ± 0.003	40.24 ± 0.94	0.07 ± 0.02	42.08 ± 0.47	0.58 ± 0.05	41.64 ± 0.45
40 °C – 0.5h	0.01 ± 0.003	40.52 ± 0.37	0.06 ± 0.001	42.41 ± 0.09	1.47 ± 0.06	41.46 ± 0.77

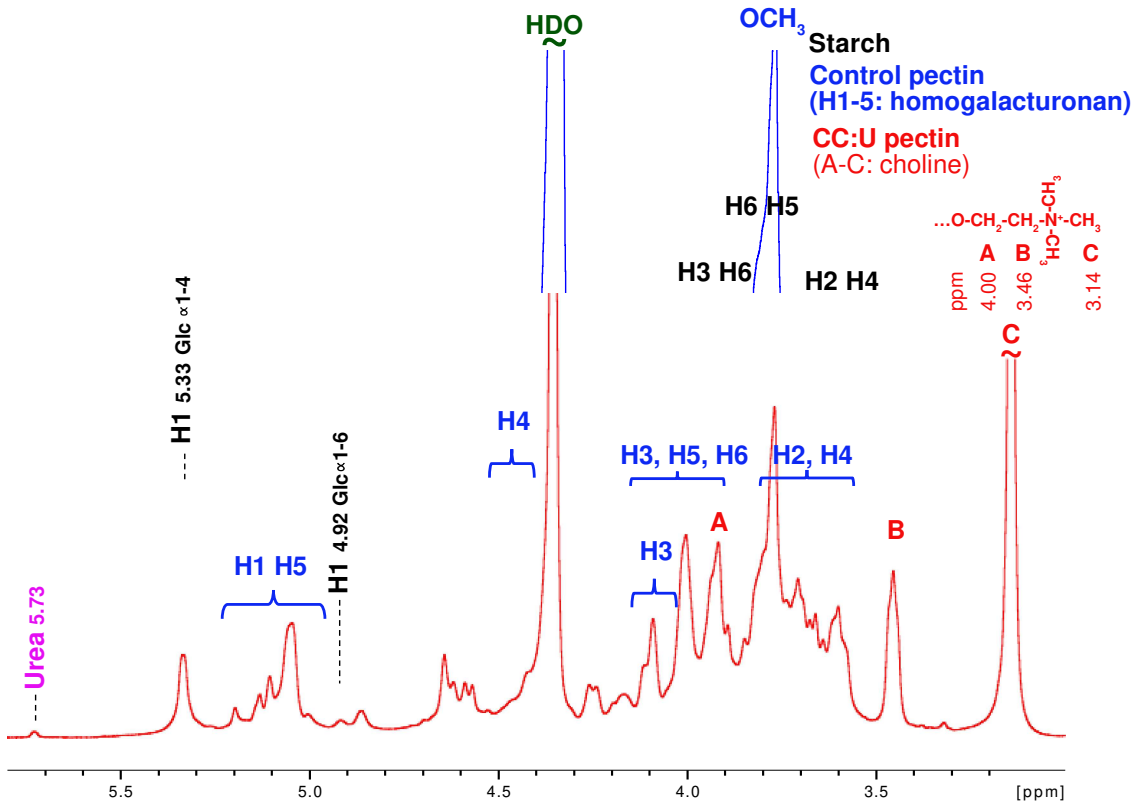

Fig. 5 ^{1}H NMR spectroscopy of water fraction from control (blue) and CC:U (red) treated apple pomace. Starch (black labels), pectin homogalacturonan (blue labels), choline (red labels) and urea (pink label) signals are within reported region of chemical shifts in the literature (Finer, Franks, & Tait, 1972; Govindaraju, Young, & Maudsley, 2000; Nilsson, Gorton, Bergquist, & Nilsson, 1996; Petersen, Meier, Duus, & Clausen, 2008).

Reducing duration of CC:U treatment of apple pomace to 1 h at 80 °C or down to 40 °C led to a significantly increased recovery of uronic acids in all fractions (Figure 2, 3-supplementary Table S2-5), while lower nitrogen content was measured in pectin (Table 3). However, when pretreatment duration was reduced to 0.5 h, the nitrogen content increased again and the uronic acids content decreased. There was a negative correlation (r=-0.75) between pectin nitrogen and uronic acids contents after CC:U treatment of apple pomace. The different impacts of temperature and time on pectin nitrogen content suggest that co-occurring mechanisms exist in the modification of uronic acids that interfere with their colorimetric determination. Thus, in the light of the previous report on carbamylation of polysaccharides in

urea rich NADES, pretreatment of apple pomace with CC:U functionalizes pectin providing new pectin derivatives. Due to the complexity of the polysaccharides in the water fraction no attempt was made to determine the degree of substitution with choline but based on nitrogen content and degree of methyl-esterification, the different levels of derivation can be achieved by varying conditions.

3.4. NADES pretreatment facilitates starch extraction

Several studies have shown that only 5 wt% to 10 wt% of starch is extracted from different types of agricultural wastes by DES (María, Bruinhorst, & Kroon, 2012; Shamsuri & Abdullah, 2010; Zdanowicz & Spychaj, 2011). In our study, a similar result was found in the CC:U extracts, which contained up to 12.4% of the pomace glucose (Figure 3A-supplementary Table S5) while the CC:LA extracts contained almost no glucose. However, in contrast with previous studies, when the NADES pretreated apple pomace was further extracted by water, high glucose contents were recovered, especially from the CC:LA treated pomace. The comparison between the content in starch and glucose in fractions shows that most if not all glucose in the water fractions of NADES treated groups originated from starch (supplementary Table S8). Moreover, between the two NADES, the CC:LA pomace pretreatment led to a better extraction of starch by hot water and the harsher the pretreatment conditions were, the more starch was extracted. In agreement with Zdanowicz et al. (2011) who reported that DES composed of citric or succinic acid led to the polymer degradation due to the acidic character of the solvents, starch in apple pomace may have been partly degraded by the lactic acid, allowing for its extraction in larger amount by the hot water.

3.5. Pectin characterization

Since a large amount of pectin was extracted with water after CC:LA pretreatment of pomace (33.1%-56.1% of uronic acid recovery), its structural characteristic was further studied. It amounted to 19.3% - 26.6% of the pomace dry weight according to the pretreatment conditions (Table 2 considering 68% water in the wet pomace), which was within the range of values (9.5%-22.0%) (Koubala et al., 2008; Rha et al., 2011; Wang, Chen, & Lü, 2014) reported for apple pomace pectin. Pectin was also extracted from the apple pomace by a conventional method using dilute nitric acid to compare its characteristics with that extracted following NADES pretreatment (SP, extraction method is provided in Supplementary Informations). SP pectin yield (19.1% dry weight basis) and sugar composition were close to that of the water fraction of CC:LA pretreated pomace at 40 °C for 0.5-1 h but contained less

uronic acids (supplementary Figure S3). As for pectin from CC:LA pretreated pomace, SP was contaminated by starch (33.2%). The esterification degree of the different pectin is shown in Table 4. The DM of pectin from CC:LA pretreated pomace was similar to that of control when pretreatment temperature was at 40 °C (P>0.05). Yet, the DM of pectin from the CC:LA treated pomace significantly decreased as the pretreatment temperature increased from 40 °C to 80 °C. The DA is not frequently reported in the study concerning pectic polysaccharides. The highest DA was obtained when pretreatment was carried out at 40 °C for 0.5 h in both pectin from CC:LA pretreated pomace and control (P<0.05). Close esterification degrees were also found in SP. The influence of extraction condition on esterification degree can be mitigated when temperature was decreased to 40 °C. Garna et al. (2007) studied the influence of different extraction conditions on the yield and chemical characteristics of apple pomace pectin. They reported that the DM of pectin ranged from 54.5% to 79.5%, with the highest value recorded for pectin extracted for 1 h at 80 °C and pH 2.0. Besides, highly acetylated pectin can be obtained from apple pomace extracted with Li Cl-DMSO, the acetyl ester content reached 3.3% of the weight (Ray et al., 2014), which is in consistent with our results (acetic acid % in CC:LA 40 °C for 0.5 h = 3.45 %).

Table. 4 Mean methyl- and acetyl-esterification degree (± standard deviation, n = 4) of extracted pectin by dilute hot mineral acid (SP), after pomace pretreatment with water (Control), or choline chloride:lactic acid (CC:LA).

	DM			DA		
	SP	**Control**	**CC:LA**	**SP**	**Control**	**CC:LA**
	80.6 ± 2.6			51.6 ± 2.2		
80 °C – 2h		64.8 ± 2.0^{A}	56.9 ± 3.6^{*A}		13.2 ± 2.9^{A}	27.9 ± 1.5^{*A}
80 °C – 1h		73.1 ± 2.1^{B}	59.1 ± 3.3^{*A}		29.9 ± 1.4^{B}	32.0 ± 2.8^{B}
60 °C – 1h		72.9 ± 1.2^{B}	60.3 ± 3.3^{*A}		31.0 ± 3.5^{B}	48.8 ± 3.9^{*C}
40 °C – 1h		75.8 ± 3.5^{B}	77.9 ± 4.3^{B}		45.4 ± 3.7^{C}	46.4 ± 0.8^{C}
40 °C – 0.5h		79.7 ± 0.7^{C}	78.3 ± 1.5^{B}		53.7 ± 3.4^{D}	54.5 ± 3.0^{D}

Mean values with unlike letters were significantly different (P<0.05), A,B,C: significantly different at different condition within same group (columns), *: significantly different between two groups at same condition (raws).

Due to the large amount of starch in the water fraction, pectin from different pretreated apple pomace was treated with amylase before Mw measurement. No glucose content was found in

554 treated pectin, which indicated that starch was totally removed. As is shown in Table 5, the
555 Mw and intrinsic viscosity of both control and CC:LA pretreated pectin were sensitive to the
556 pretreatment conditions, they both increased with the decrease in temperature or duration of
557 pretreatment. Besides, pectin from the CC:LA pretreated pomace had significantly higher Mw
558 than that of control no matter what the pretreatment conditions were. Although the sugar
559 composition analysis of extracts and residues indicated that polysaccharide structures were
560 degraded and lost during the extraction process of apple pomace, the pectin recovered in the
561 water extract after a pretreatment at 80 °C for 2 h still demonstrated high Mw close to that
562 obtained with the SP pectin. These results combined with the extraction yield and pectin
563 chemical composition, clearly showed that hot water extraction after CC:LA pretreatment is a
564 good candidate to substitute for classic industrial extraction methods of pectin.
565
566 **Table. 5** Mean molecular weight (± standard deviation, n = 4) of pectin extracted by dilute
567 mineral acid (SP), after pomace pretreatment with water (Control), or choline chloride:Lactic
568 acid (CC:LA)

	Mw (× 10^5 Da)			Viscosity ($cm^3 g^{-1}$)		
	SP	**Control**	**CC:LA**	**SP**	**Control**	**CC:LA**
	2.8 ± 0.3			8.1 ± 0.7		
80 °C – 2h		1.7 ± 0.2^{A}	$2.9 \pm 0.4^{*A}$		10.0 ± 0.5^{A}	$6.7 \pm 0.3^{*A}$
80 °C – 1h		2.5 ± 0.3^{A}	$3.5 \pm 0.7^{*AB}$		12.1 ± 0.7^{B}	$8.3 \pm 0.4^{*B}$
60 °C – 1h		2.6 ± 0.2^{A}	$3.9 \pm 0.3^{*B}$		12.9 ± 0.1^{C}	12.4 ± 1.3^{C}
40 °C – 1h		4.1 ± 0.2^{B}	$7.0 \pm 0.7^{*C}$		14.2 ± 0.5^{D}	$12.6 \pm 0.5^{*C}$
40 °C – 0.5h		5.3 ± 1.2^{C}	$8.3 \pm 0.6^{*D}$		15.8 ± 0.2^{E}	$12.7 \pm 0.1^{*C}$

569 Mean values with unlike letters were significantly different (P<0.05), A,B,C: significantly different at different
570 condition within same group (columns), *: significantly different between two groups at same condition (raws).
571
572 **4. Conclusion**
573 The present study explored the impact of apple pomace pretreatment by three different
574 NADES on the recovery of water-soluble pectin while allowing for further extractions of
575 valuable polysaccharides from the residues. Thus, one critical point in the pretreatment
576 conditions was to keep as much as possible the chemical integrity of hemicellulose and pectin.
577 Results showed that CC:LA and CC:U treatments led to overall recovery yield comparable
578 with that of control while CC:OA led to low yield due to polysaccharides degradation. The
579 polysaccharides structure was well preserved by CC:LA pretreatments when temperature was

set at 40 °C but CC:U affected pectin composition by introducing choline and decreased methyl-esterification. CC:LA facilitated extraction of a large amount of hot water-soluble pectin from the pomace and taking extraction yield and pectin structure into consideration, pretreatment at 40 °C for 1 h afforded the recovery of high Mw HM pectin of structural characteristics comparable with classical commercial pectin. Thus, CC:LA pretreatment of apple pomace followed by water extraction offers a "green" alternative to classical pectin acid extraction while CC:U pretreatment open the way to produce low esterified and choline modified pectin that remains to be further characterized either as extracted polyuronan or as semi-refined pectin for technical applications.

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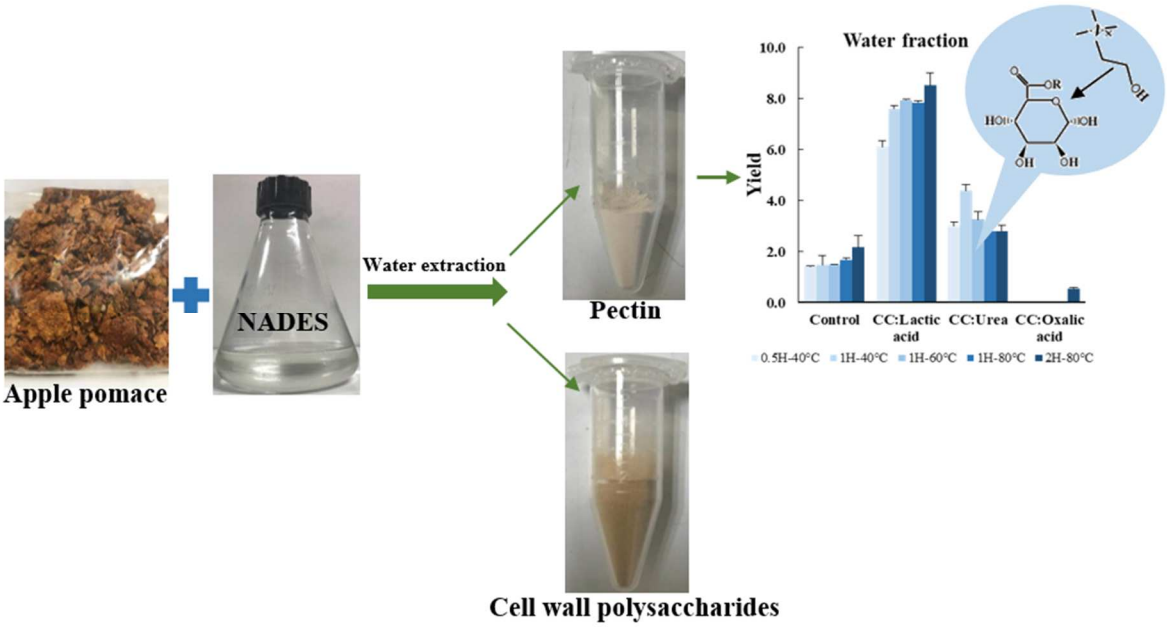
Apple pomace
NADES
Water extraction
Pectin
Cell wall polysaccharides
Water fraction
Yield
10.0
8.0
6.0
4.0
2.0
0.0
Control
CC:Lactic acid
CC:Urea
CC:Oxalic acid
0.5H-40°C
1H-40°C
1H-60°C
1H-80°C
2H-80°C