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

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

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Abstract

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

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

1. Introduction

Pectin is a family of structurally complex polysaccharides of plant cell wall. It is generally classified as homogalacturonan (HG), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII) structural domains. HG takes up 65% of pectin structure, followed by RGI (20–35% of pectin) and lastly by RGII (10% of pectin) (Mohnen, 2008). HG backbone consists only of galacturonic acid unit (Voragen, Beldman, & Schols, 2001) of which the carboxylic acid function can be esterified by methanol while acetyl ester can be found on galacturonic acid at position of O-2 and/or O-3 (Atmodjo, Hao, & Mohnen, 2013). According to the degree of methyl esterification, pectin is distinguished as high methoxyl (HM) pectins (degree of esterification > 50%) and low methoxyl (LM) pectins (degree of esterification < 50%) (Löfgren & Hermansson, 2007). RGI consists of a repeating disaccharide unit of galacturonic acid and rhamnose with side chain made of arabinose and galactose linked on O-4 of the rhamnosyl residues (Scheller, Jensen, Sørensen, Harholt, & Geshi, 2007). RGII is a complex structural domain based on a branched HG backbone. The side chains are of four types made of 12 different sugars (O'Neill, Ishii, Albersheim, & Darvill, 2004). Moreover, xylose can be found at O-2 position of galacturonic acid to form xylogalacturonan (Schols, Bakx, Schipper, & Voragen, 1995).

Nowadays, pectin is widely applied as gelling agent, stabilizer, emulsifier and thickener in the cosmetic and food industries (Güzel & Akpınar, 2019). The sources of pectin are mainly from grapefruit peel, orange peel and apple pomace. Million tons of apple pomace was generated each year from apple processing industry (Lu & Foo, 2000). The conventional industrial pectin extraction method used is based on mild mineral acid. However, it often leads to environment related problems and low extraction yield when compared with other emerging technology, such as enzymatic extraction and ultrasound/microwave assisted extraction (Wikiera, Mika, Starzyńska-Janiszewska, & Stodolak, 2015). Although these innovative technologies show advantages over traditional processes with regard to environmental and energy saving issues (Adetunji, Adekunle, Orsat, & Raghavan, 2017), their scale-up and use by industry is delayed due to the expensive upfront investment and to incomplete understanding of the process. In that context, there is room for new "green" and efficient extraction processes compatible with industrial practices. One possibility is to take advantage of new solvents, such as natural deep eutectic solvents (NADES). These solvents are composed of hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA), which in a definite molar ratio melt at temperature far below than that of individual component to form transparent liquids (Liu et al., 2018). These eutectic mixtures are naturally present in the cells of living organisms as a combination of organic acids, sugars and amino acids (Choi et al., 2011). Since NADES are cheap, biodegradable, eco-friendly and can be recycled, they are actively investigated as potential "green" solvent for various purposes. Moreover, since NADES are formed from non-toxic metabolites, these solvents can be applied in processes for food and cosmetics applications (Fernandez, Espino, Gomez, & Silva, 2018). However, the high viscosity of NADES can impede their use, but increasing temperature and/or adding small amounts of water (5-20%) can alleviate this drawback by decreasing viscosity to nearly that of water. Such adjustments render NADES usable as a water-based extractant for industrial productions (Choi & Verpoorte, 2019). Based on the pH of the solvent, NADES can be classified into neutral, acidic, alkaline NADES. Recently, many acidic NADESs have been tested as potential solvent for pectin extraction (Benvenutti, Sanchez-Camargo, Zielinski, & Ferreira, 2020; Shafie, Yusof, & Gan, 2019). Besides, in a previous work, we showed that choline chloride:Lactic acid as a pretreatment of apple pomace could markedly ease subsequent hot water extraction of pectin obtained in high yield (Chen & Lahaye, 2021). However, pretreatment with this solvent has to be done with caution as it can lead to loss of arabinose in cell wall polysaccharides. As the pH of the solvent is a known factor affecting extraction of pectin (Methacanon, Krongsin, & Gamonpilas, 2014), neutral or alkaline NADESs may also have the potential to be applied in pectin extraction process. Among various NADES combinations, the choline chloride : glycerol was widely used for extraction of bioactive substances from agri-food waste (Grudniewska et al., 2018; Mouratoglou, Malliou, & Makris, 2016; Sakti, Saputri, & Mun'im, 2019), while potassium carbonate:glycerol as emerging NADES have shown the ability to isolate cellulose fibers or nanocrystals (Gan, Sam, Abdullah, Omar, & Tan, 2020; Lim, Gunny, Kasim, AlNashef, & Arbain, 2019). Moreover, apart from the fact that the gel like structure can be formed between dissociated carboxyl groups of HGs, certain pectin structures strongly interact with cell wall cellulose (Broxterman, & Schols, 2018). The alkaline NADES may represent a suitable candidate for recovering this part of pectin through its pH characteristics. However, it is worth mentioning that due to its high pH, potassium carbonate : glycerol treatment may degrade methyl-esterified pectin by a β-elimination mechanism. In contrast with one-step extraction process, sequential extraction showed advantages in being more selective and fractionating polysaccharides at the laboratory scale. Generally, sequential extraction process of pectin is divided into three to four stages, and each stage uses a different extractant, such as, water, chelating agent, diluted acid or concentrated alkaline solvents (Ramasamy, Gruppen, & Schols, 2013; Yapo, Lerouge, Thibault, & Ralet, 2007), which allows for the recovery of pectin fractions with specific properties (Gawkowska, Cybulska, & Zdunek, 2018; Guo et al., 2018).

Although many researches have been conducted on pectin extraction with conventional mineral acid or alkaline solutions within our group (Kaya, Sousa, Crépeau, Sørensen, & Ralet, 2014; Koubala, Kansci, Mbome, Crépeau, Thibault, & Ralet, 2008; Yapo et al., 2007), in the process of establishing an innovative and “green” biorefinery of apple pomace, NADES as a promising green solvent was tested for this purpose. As our previous result showed that abundant pectin resource still remained in residual pomace after one-step NADES pretreatment following water extraction (Chen & Lahaye, 2021). Therefore, in present study, three types of NADES: Choline chloride:Lactic acid (acidic NADES), Choline chloride:Glycerol (neutral NADES), Potassium carbonate:Glycerol (alkaline NADES) in sequence prior to hot water extraction were tested as a mean to selectively extract pectin enriched in specific structural domains while yielding extraction residues suitable for further recovery of valuable polymers. Extractions yield and sugar composition were used to evaluate NADES pretreatments efficiency while molecular weight distribution and degree of esterification assessed the quality of the pectin recovered. Pectin extracted by methods from literature and NADES pretreatments were also compared. The effects of NADES pretreatment/water extraction on pectin yield and structural characteristic were discussed. Moreover, possible mechanisms of NADES pretreatment effect were proposed. Finally, ^{13}C solid-state NMR (ssNMR) spectroscopic analyses were realized to assess the impact of NADES pretreatments/water extractions on the polysaccharides composition, structure and organization in the residual pomace.

2. Materials and methods

2.1. Pomace

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

2.2. Chemicals

Choline chloride (CAS: 67-48-1, Sigma-Aldrich, France), glycerol (CAS: 56-81-5, Sigma-Aldrich, France), potassium carbonate (CAS: 584-08-7, Merck, Germany), 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), sodium acetate trihydrate (CAS: 6131-90-4, Sigma-Aldrich, France), *trans*-1,2-Diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid monohydrate (CDTA) (CAS: 125572-95-4, Sigma-Aldrich, France), sodium carbonate (CAS: 497-19-8, Merck, Germany), sodium borohydride (CAS: 16940-66-2, Sigma-Aldrich, France) were used in the present research.

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

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

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

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

2.4. Pectin extractions

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

2.4.1. Water extraction (route A, Figure 1)

Wet apple pomace (10 g) was extracted with deionized water (400 ml) under agitation at 75 °C for 1 h. The soluble polysaccharides were recovered by centrifugation at 15000 g for 20 min. Polysaccharides were precipitated with 4 volumes of ethanol, washed with 70% ethanol for 10 min (3 times), followed by ethanol and acetone for 10 min (2 times), air dried and then dried at 40 °C in vacuum oven over P_2O_5 powder for 12 h.

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

Wet apple pomace (10 g) was extracted with HCl (pH 1.5, 400 ml) under agitation at 75 °C for 1 h. The soluble polysaccharides were recovered by centrifugation at 15000 g for 20 min. The polysaccharides were precipitated with 4 volumes of ethanol, washed with 70% ethanol for 10 min (3 times), followed by ethanol and acetone for 10 min (2 times), air dried and then dried at 40 °C in vacuum oven over P_2O_5 powder for 12 h.

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

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

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

The sequential chelating-agent extraction II was conducted according to Gawkowska et al. (2018) with some modification. In brief, wet apple pomace (10 g) was sequentially extracted under agitation 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)** 200 ml of 0.05 M sodium carbonate (Na_2CO_3) and 0.02 M sodium borohydride ($NaBH_4$) at 4 °C for 20 h and then at 20 °C for 2 h. For each step, the remaining pomace was separated by centrifugation at 15000 g for 20 min. The polysaccharide extracts were dialyzed against deionized water for 24 h and freeze dried.

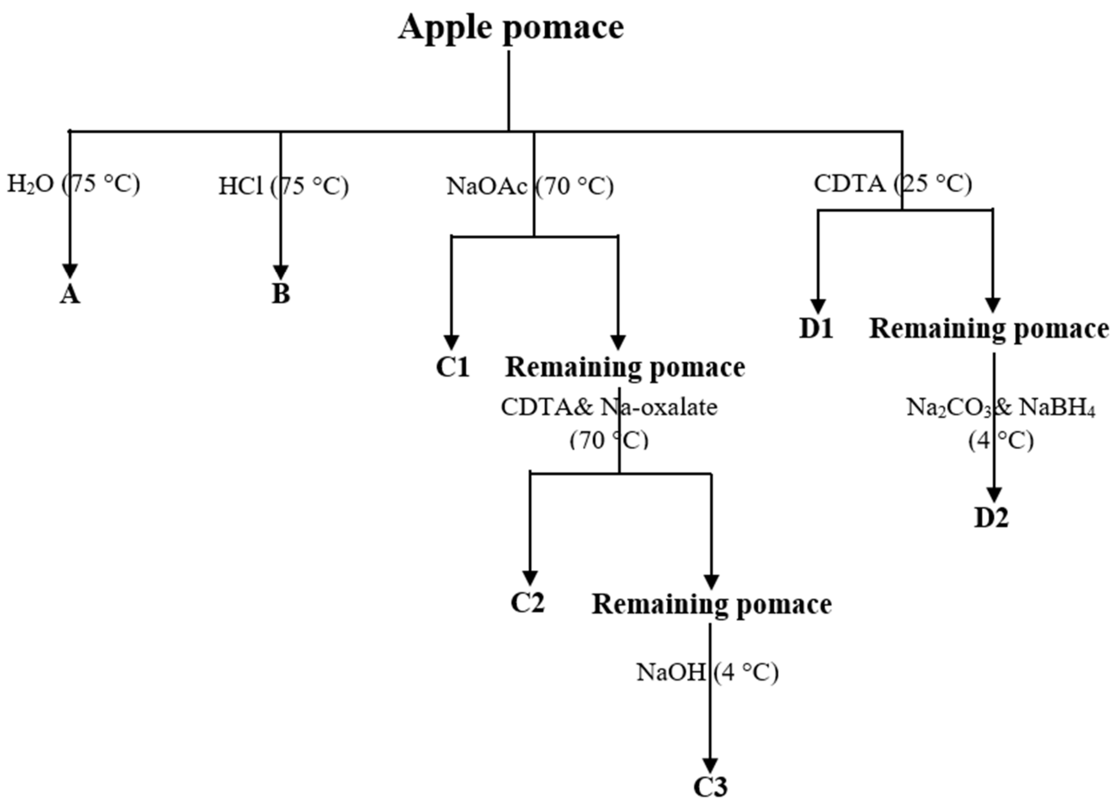


Fig. 1 Schematic representation of the extraction process with various methods

2.5. NADES pretreatments followed by pectin extraction.

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

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

(1) Wet apple pomace was mixed with CC:Glycerol at the ratio of 1:8 (w/v). The solution was agitated at 40 °C for 1 h and then centrifuged at 15000 g for 20 min to recover the remaining pellet. The pomace pellet was then resuspended in deionized water at 80 °C for 10 min under constant agitation and then centrifuged at 15000 g for 20 min. This water extraction process was repeated five times. The pooled water washes, referred to as the pectin fraction, was concentrated with a vacuum rotary evaporator and was precipitated by 4 volumes of ethanol. The precipitate was 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. The ethanol and acetone washings were repeated until the washes were colorless (at least twice). Both, the remaining pomace and pectin sample were air dried and then dried at 40 °C in vacuum oven over P_2O_5 powder for 12 h.

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

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

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

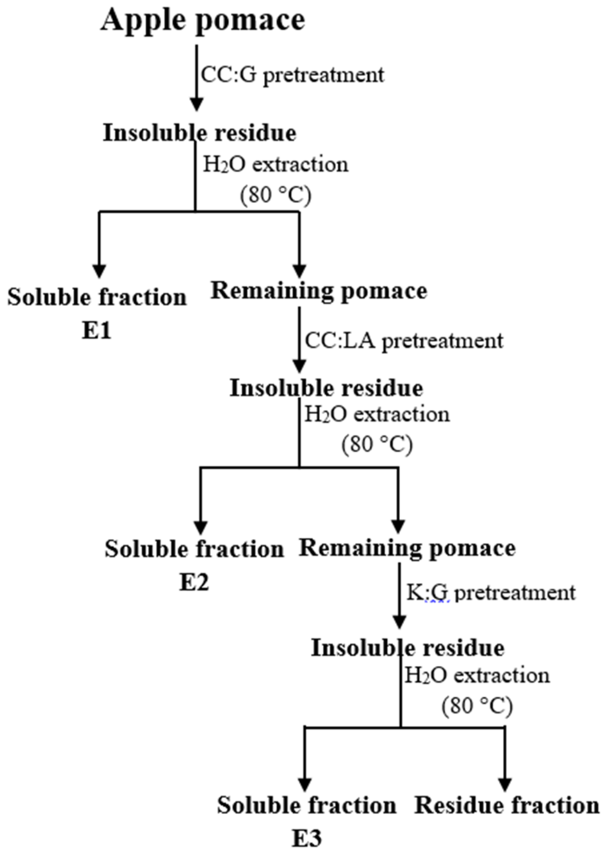


Fig. 2 Schematic representation of the sequential extraction process with NADES

2.5.2. NADESs pretreatment extraction (route F, G)

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

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

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

2.6. Extraction yield

The extraction yield was calculated as follow:

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

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

2.7. Neutral sugars composition and uronic acids content

The neutral sugar composition in each pectin fraction was determined by GLC (Gas-liquid chromatograph) analysis (Blakeney, Harris, Henry, & Stone, 1983). In brief, sample was dispersed in sulphuric acid (12 M, 72%) at 25 °C for 30 min, followed by hydrolysis (100 °C, 2 h). The released sugars were reduced and acetylated and 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. Sugar content 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.

Uronic acids 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.

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

2.8. Pectin methylation and acetylation esterification degree

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

(0.5 mg of $CuSO_4.5H_2O$ in 0.5 mL of isopropanol solution (14 mg mL^{-1})). After centrifugation at 7400 g for 10 min, the supernatant was filtered through cartridge IC-H (Sstarpure, Maxi-Clean SPE 0.5 ml IC-H 50pk). HPLC was conducted on C18 (4 mm × 250 mm, Lichrospher 100 RP-18e (5 µm), Interchim, France) column thermostated at 25 °C. H_2SO_4 (4 mM) was used for isocratic elution at a flow rate of 1.0 mL min^{-1}. Standard solution containing methanol, acetic acid and isopropanol as internal standard was used for calibration. Due to the acetic acid peak was overestimated in HPLC analysis. The acetic acid content of different pectin extracts was re-determined by acetic acid enzymatic kit (BioSenTec, France). 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.

2.9. Molecular weight profiling

Molecular weight profile of pectin was determined through High Performance Size Exclusion Chromatography (HPSEC). The system consisted of a Shodex OHpak SB-G 6B pre-column (Shodex, Tokyo, Japan) in front of OHpak SB-805-HQ (Shodex, Tokyo, Japan) connected to pump (Jasco PU-1580, Tokyo, Japan) and injector (PerkinElmer, series 200 autosampler, Courtaboeuf, France). Pectin-rich samples (4 mg) were dissolved in 1.5 mL of distilled water, 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 0.7 mL min^{-1} and monitored by differential refractometry (Viscotek VE 3580 RI detector, Malvern Instruments, Orsay, France). Molecular weights were obtained using the OmniSEC 4.7.0 software and calibration was done in triplicate using pullulan-P108K (Viscotek, Malvern Instruments, Orsay, France).

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

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

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

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

According to the method of Larsson et al. (1997), the cellulose crystallinity was calculated from deconvoluted cellulose C_4 peak in region of 80-91 ppm. Due to spectral resolution, a simplify version was used: two crystalline cellulose C_4 peaks (cellulose I(α + β) (88.4 ppm), cellulose Iβ (87.8 ppm)) and one amorphous cellulose C_4 peak (83.3 ppm) were used. The proportion of crystalline cellulose was determined by dividing the sum peak area of two crystalline cellulose C_4 peaks by those of three cellulose C_4 peaks. Assuming the cross section of cellulose microfiber is square and all amorphous cellulose is attached on fiber surface, the lateral fiber dimension (LFD) was also estimated. The cellulosic chains width was set at 0.57 nm (Newman, 1999).

The molecular dynamic of samples was further characterized by varying contact time (τ) from 10 µs to 9000 µs. Twenty CP/MAS spectra were recorded with an accumulation of 512 scans per contact time. The evolution of carbon peak area (C_4 of crystalline cellulose and O-CH_3 of pectin methyl ester) between different groups was fitted with following formula (Kolodziejski & Klinowski, 2002):

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

Where $I(\tau)$ is the carbon peak area (C_4 of crystalline cellulose and O-CH_3 of pectin methyl ester) according to the contact time (τ), I_0 is the maximum carbon signal intensity (associated with the optimal contact time), λ is a parameter that depends on the number of protons (n) carried by carbons (λ=1/(n+1)), $T_{1\rho}^H$ is the spin-lattice proton relaxation time in the rotating frame, T_{HH} is the spin diffusion time between two nearby protons, T_{CH} is the thermal mixing time between H and C.

2.11. Statistical analysis

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

3. Results

3.1. Physicochemical properties of different NADESs

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

3.2. Effects of different extraction methods on polysaccharide yield

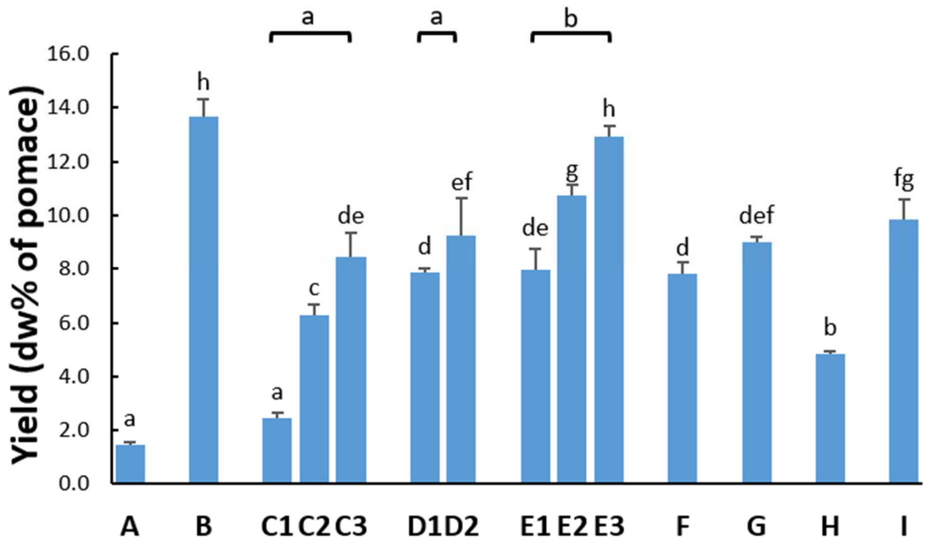


Fig. 3 Mean extraction yields (n=4) of polysaccharide extracts according to different procedures (A-I); A: water extraction; B: mineral acid extraction; C1-C3: sequential chelating agent extraction I (1: NaOAc fraction; 2: CDTA&Na-oxalate fraction; 3: NaOH fraction); D1-D2: sequential chelating-agent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

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

3.3. Monosaccharides recovery in extracts

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

The expression of monosaccharides composition as a percentage of recovery of their initial content in pomace allows better evaluating the efficiency of the different extraction methods to recover pectin related sugars (Rha, Ara, Gal, UA). From **Table S1**, these sugars were found in all extracts. Recovery of UA was the highest among them except in NaOAc treatment extract (C1) and in the water extract following K:G pretreatment (E3). Considering UA recovery, hot water extraction following non-sequential NADES pretreatments led to significantly higher recovery (E1: 23.5%; F: 27.4%; G: 23.9%) than by chelating agents extraction (C2: 10.4%; D1:8.3%). Sequential NADES pretreatments/water extraction (method E) was the most efficient in allowing extraction of 69.7% of the pomace UA content. As for the yield, CC:G pretreatment (E1) significantly improved UA recovery in the hot water extract following the CC:LA pretreatment (E2: 36.1%), compared with non-sequential CC:LA pretreatment/water extraction (F: 27.4%). However, the synergistic effect was not observed in the extract after K:G pretreatment/water extraction (E3: 10.2%). Instead, higher UA recovery was found in non-sequential K:G pretreated/water extraction group (G: 23.9%). According to extraction yield, lactic acid solution pretreatment followed by hot water extraction led to significantly lower pectin related sugar recoveries than that of CC:LA pretreatment/water extraction whether conducted sequentially or not. Although both K:G pretreatment/water extraction (G) and K_2CO_3 solution pretreatment/water extraction (I) led to comparable extraction yield, the water extract after K_2CO_3 solution pretreatment showed significantly lower UA recovery. K_2CO_3 solution had a more negative effect on the pectin galacturonic acid unit recovery. Hemicellulose and cellulose related monosaccharides (Fuc, Xyl, Man, Glc) were also found in some extracts though Fuc and Xyl may also come from HG or RG II structural domains. Glc recovery was the most represented of these sugars except in the NaOH extract (C3) and the K:G pretreatment/water extract (E3).

3.4. Pectin structural domains in extracts

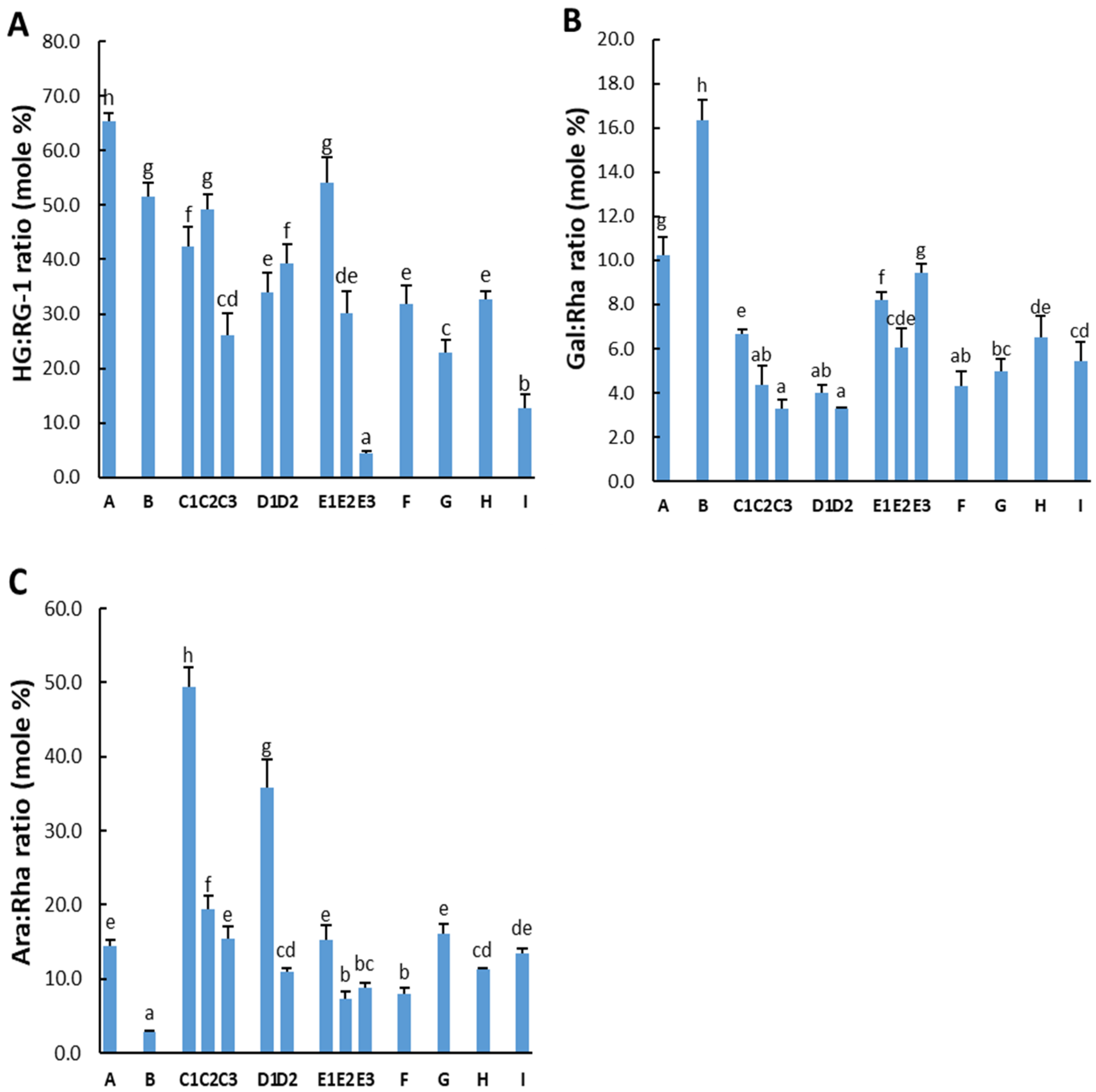


Fig. 4 Mean (n=4) molar ratio of : **A**, Homogalacturonan (HG) : rhamnogalacturonan (RGI), **B**, galactose (Gal) : rhamnose (Rha) and **C**, arabinose (Ara) : rhamnose (Rha) of pectin-rich extracts obtained by A: water extraction; B: mineral acid extraction; C1-C3: sequential chelating agent extraction I (1: NaOAc fraction; 2: CDTA&Na-oxalate fraction; 3: NaOH fraction); D1-D2: Sequential chelating-agent extraction II (1: CDTA fraction; 2: Na_2CO_3&$NaBH_4$ fraction); E1-E3: sequential NADESs pretreatment extraction (1: CC:G fraction; 2: CC:LA fraction; 3: K:G fraction); F: CC:LA pretreatment extraction. G: K:G pretreatment extraction; H: lactic acid solution pretreatment extraction; I: potassium carbonate solution pretreatment extraction. Bar: standard deviation; different letters are significantly different.

The sugar recovery data were further analysed to identify specific effects of the extraction methods with regard to pectin strutural domains. Besides UA, Rha can inform about the proportion of RGI recovered in the different extracts. According to the HG/RGI molar ratio shown in **Fig. 4A**, pectin structure profile was affected by extraction methods. The highest HG proportions were observed in the water extract (A), while all other methods led to RGI richer fraction. For water extracts after non-sequential NADES pretreatments, significantly higher HG proportion was found following CC:G pretreatment (E1) compared with that following CC:LA (F) or K:G pretreatments (G). In the opposite, extract in G fraction possessed significantly higher RGI proportions than other water extracts following non-sequential NADES pretreatments. Based on HG/RGI molar ratio, dilute acid extraction (B) showed similar effect on pectin structural domains composition as CC:G pretreatment/water extraction (E1). When apple pomace was extracted sequentially, no matter which sequential extraction methods was applied, pectin structure was remarkedly affected since HG/RGI molar ratio was significantly different for each step. HG-richer fractions were obtained after CDTA & sodium oxalate extraction (C2), while CC:LA and K:G pretreatment/water extraction (E2 and E3) led to RGI-richer fractions.

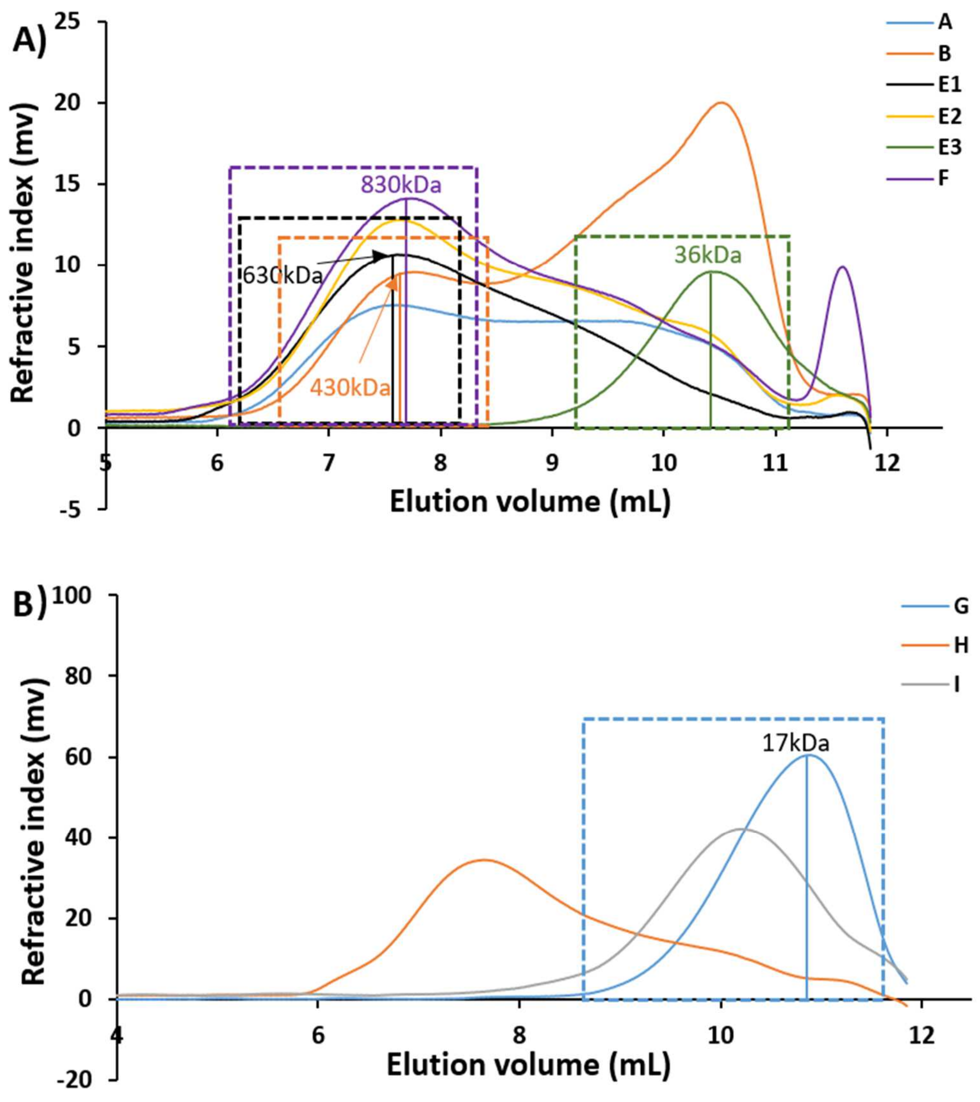
Molar ratio of Gal/Rha and Ara/Rha were also calculated to assess the effect of different methods on RGI side-chains structure assuming that galactose and arabinose were mainly constitutive of pectin side chains. As can be seen from **Fig. 4B**, the fraction extracted by dilute acid showed the highest ratio for Gal/Rha, all other methods indicated shorter/less galactose side chains to varying degrees. Close amounts of galactose side chains were observed in extracts after CDTA (D1), and water extracts following CC:LA (F) and K:G (G) pretreatments. Instead, the water extract following CC:G pretreatments demonstrated significant higher Gal/Rha molar ratio. Moreover, when CC:LA and K:G pretreatments were conducted sequentially, the amount of galactose side chain increased significantly in the extracts. For sequential extractions using chaleting agents (C and D), CDTA treatment with or without sodium oxalate led similar Gal/Rha ratio. Moreover, NaOH (C3) or Na_2CO_3 & $NaBH_4$ (D2) did not markedly change galactose side chain proportion since C2 and C3 or D1 and D2 shared close Gal/Rha ratio.

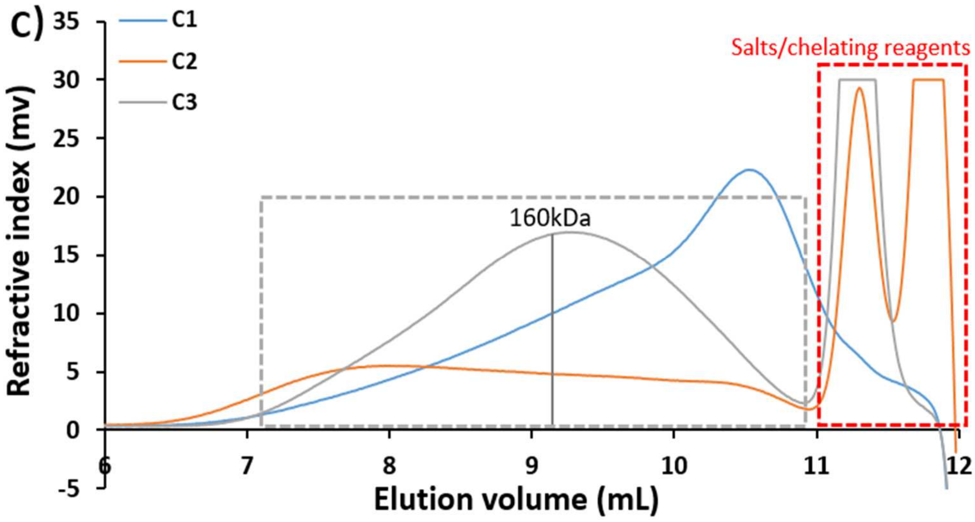
The pectin fraction showing the shortest/least arabinose side-chains was obtained by dilute acid extraction (B) (**Fig. 4C**). The water extract following CC:LA pretreatment conducted alone or sequentially (E1 and F) also led to fractions with shorter/less RGI arabinan side-chains than those recovered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

3.5. Esterification of pectin extracts

Pectin substitution by methanol and acetic acid esters was also assessed (**Table S2**). As expected due to saponification, dilute alkali in method C3 and method I had a severe effect on methyl ester group. A low DM value was also obtained in the water extract following the K:G pretreatment (E3 = 18.2 and G = 11.8) compared with the other water extracts following the CC:G or CC:LA pretreatments (E1 = 56.6, E2 = 67.8 and F = 66.6). For sequential chelating agent extractions, both CDTA (with or without sodium oxalate) and sequential Na_2CO_3 & $NaBH_4$ extraction led to low methyl esterified pectin (ie DM < 50), while for other treatments, high methyl esterified pectin was extracted. K:G pretreatment led to lower acetyl esterification of pectin in the following water extract (E3 and G). Moreover, alkaline extracting conditions favored lower DA value of pectin (C3: 1.3; I: 0.7).

3.6. HPSEC profiles from different extracts





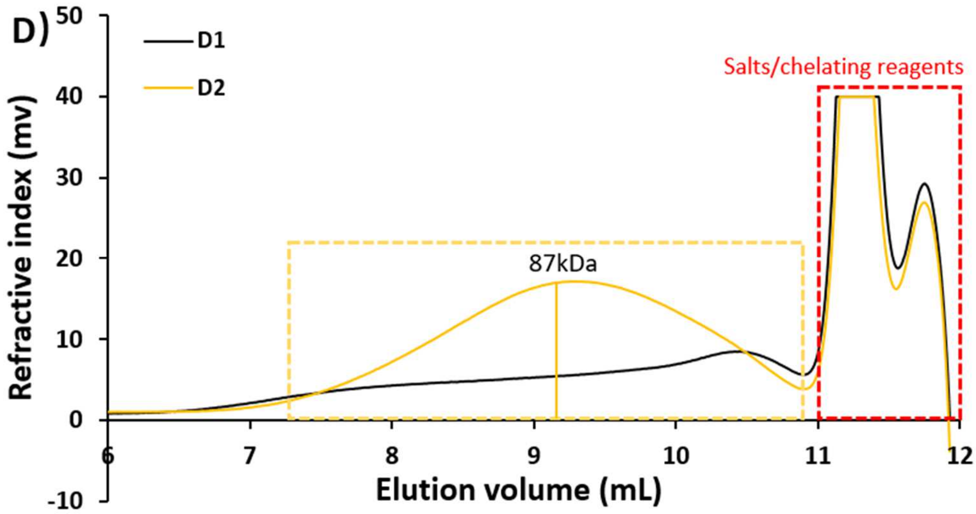


Fig. 5 HPSEC-patterns of the pectin-rich fractions. A) A: water extract; B: mineral acid extract; E1-E3: water extracts following sequential NADESs pretreatment (1: CC:G; 2: CC:LA; 3: K:G pretreatments); F: water extract following CC:LA pretreatment; B) G: water extract following K:G pretreatment; H: water extract following lactic acid solution; I: water extract following potassium carbonate solution; C) C1-C3: sequential chelating agent extracts (1: NaOAc fraction; 2: CDTA&Na-oxalate fraction; 3: NaOH fraction); D) D1-D2: sequential chelating agent extracts II (1: CDTA fraction; 2: Na_2CO_3&$NaBH_4$ fraction).

The molecular weight profile of the polymers in different extracts was analyzed by HPSEC (**Fig. 5**). Because of overlapping peaks, the estimated Mw of some peaks were provided in **Fig. 5** to give a broad view of how molecular weight distribution was influenced by various extraction methods. For both dilute acid and water fractions (trace A and B), two main peaks were observed eluting between 6 mL- 11 mL (**Fig. 5A**). Similar profiles were also found in the water extracts following the CC:G and CC:LA pretreatments, but with a minor peak eluting between 8.5mL and 10.5mL (**Fig. 5A**, trace E1 and E2). This indicated that at least two populations of polysaccharide were present. Water extract following GG:LA pretreatment in the sequential NADES pretreatments method (trace E2, **Fig. 5A**), following CC:LA pretreatment alone (F, **Fig. 5A**) and the extract obtained following dilute lactic acid pretreatment (H fraction; **Fig. 5B**) yielded close Mw distribution profiles. These profiles showed the predominant role of lactic acid in affecting Mw distribution. Alkaline NADES (E3, **Fig. 5A** and G, **Fig. 5B**) pretreatment led to polysaccharides degradation since a single peak at 36kDa or 17kDa was found eluting at approximately 10.5 mL or 11 mL, respectively (**Fig. 5A,B**). Similarly, the alkaline condition provided by NaOH and Na_2CO_3 in the sequential chelating agent methods C and D also led to polymer degradation since low molecular weight populations were found in NaOH (trace C3, **Fig. 5C**) and Na_2CO_3 & $NaBH_4$ (trace D2, **Fig. 5D**) fractions. Due to the methylesterification of pectin, degradation by a β-elimination mechanism to lower oligomers cannot be ruled out. Compared with water extracts following NADES sequential pretreatments, different molecular weight distributions profiles were observed with the extracts from sequential chelating agent extractions (methods C and D). More than one Mw populations were found in extracts after CDTA extraction (with or without sodium oxalate, trace C2 or D2, **Fig. 5C,D**). The low molecular weight substances eluted after 11 mL in these extracts are likely correspond to salts and chelating reagent (**Fig. 5C,D**).

3.7. ^{13}C NMR spectra and dynamic characterization of residual pomace

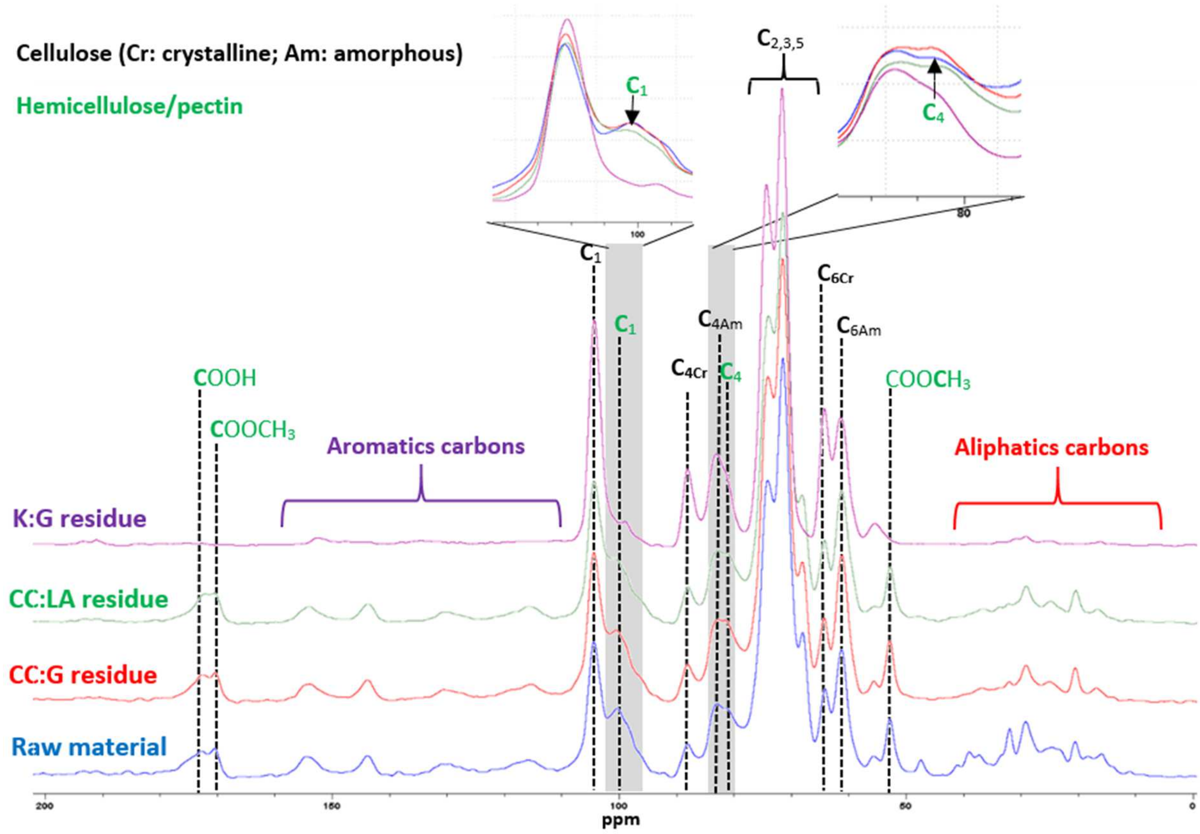


Fig. 6 ^{13}C CP/MAS spectra of residual pomaces after sequential NADES pretreatment/water extraction; peaks attribution according to (Ng et al. 2014; Phyo & Hong, 2019).

Table 2. Structural and dynamic characteristics of cellulose and pectin in apple pomace residue following sequential NADES/water extraction: crystallinity and lateral fiber dimension (LFD) of cellulose; spin-lattice proton relaxation time ($T_{1\rho}^{H}$) of crystalline cellulose (C_4 peak area), pectin methyl ester (O-CH_3 peak area) and diffusion time of nearby proton (T_{HH}) of pectin methyl ester (O-CH_3 peak area) in raw, CC:G pretreated, CC:LA pretreated and K:G pretreated/water extracted 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	—

—: not detected.

The cell wall structure in raw pomace and in the residues after the sequential NADES/water extraction was studied by ^{13}C NMR spectroscopy (**Fig. 6**). Similar spectra were observed between CC:G residue/CC:LA residue and raw apple pomace and were close to those previously published (Ng et al. 2014; Lahaye et al. 2020). The peaks of carboxyl group (whether in acidic (172.7 ppm) or methyl (170.5 ppm) forms) and methyl ester (52.7 ppm) on pectin structure disappeared in K:G residue. In contrast, the higher intensity of crystalline cellulose C_4 (87.8 ppm) peak in this residue indicated a marked impoverishment in pectin to the benefit of cellulose. In addition, the aromatic and aliphatic carbons signals (ranged from 154.3 to 115.6 ppm; from 47.5 to 16 ppm) corresponded to phenolic compounds and protein respectively that gradually disappeared in K:G residue. Moreover, the highest values of crystallinity and LFD were also observed in K:G residue. No characteristic signals for cellulose II were observed in the K:G residue. The molecular structure of both cellulose and pectin was further studied by the ^{1}H-> ^{13}C polarization transfer kinetic, and process with the two-proton reservoir model (see **Fig. S1** for experimental data along with model estimates). The $T_{1\rho}^{H}$ and T_{HH} values of crystalline cellulose C4 peak in residual pomace increased from 8.8 ms to 36.6 ms and from 0.52 ms to 1.18 ms with the successive NADESs pretreatments and water extractions **(Table 2)**. The lowest T_{HH} value (0.48 ms) of crystalline cellulose C4 peak was found in K:G residue. Since the pectin methyl ester peak was absent in the spectrum of K:G residue, the corresponding $T_{1\rho}^{H}$ and T_{HH} values could not be reported. Both CC:G and CC:LA pretreatments/water extractions led to higher $T_{1\rho}^{H}$ and T_{HH}values of methyl ester peak than that of raw apple pomace. The highest $T_{1\rho}^{H}$ value (16.8 ms) of the methyl ester peak was found in CC:G residues. Moreover, the similar trend was observed in T_{HH} value of methyl ester peak.

4. Discussion

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

Water was not suitable to extract pectin from apple pomace as previously reported (Renard, 2005). The inefficiency of hot water to extract pectin was markedly improved after NADES pretreatments. These pretreatments increased the subsequent water extraction yield by 5 times (yield: 1.5% for A; 8.0% for E1; 7.8% for F; 9.0% for G, dw%). However, none of the process taken independently involving NADES pretreatments followed by hot water extraction gave higher yield than dilute acid, which is widely applied in industry for pectin recovery. The possible reason for such lower yields likely resulted from three aspects. First, the heat treatment applied by the industrial provider to dehydrate pomace may have modified interactions between cell wall polysaccharides that reduced contact area of cell wall to the extractant, making it difficult to extract polysaccharides. Although the dry pomace was first rehydrated to swell the cell wall with a water amount close to that registered in fresh pomace (Chen & Lahaye, 2021), irreversible microstructural/chemical modifications of the pomace resulting from the dehydration process remained. When compared with our previous work, CC:LA pretreatment/water extraction showed higher extraction yield in fresh pomace than in dry pomace (Chen & Lahaye, 2021). CC:LA pretreatment/water extraction led to similar yield as that of mineral acid extraction when fresh apple pomace was used for this purpose. Second, the pH of the CC:LA solvent was lower than that of HCl solution. The strong acidity may have partly broken-down pectin to oligosaccharides that were lost during polysaccharide ethanolic precipitation and resulted in a low extraction yield of polysaccharides. This is evidenced by the low yield of residue after CC:LA pretreatment (49.6% compared to 55.7% for the diluted acid extract). A similar result was found by Liew et al. (2018) who showed that higher yield of pectin was extracted from pomelo peel using citric acid (pH 1.8) compared to that treated with lactic acid–glucose–water solvent with a ratio of 6:1:6. A low yield of residue (47.3%) was also found after non sequential K:G pretreatment, which indicated that the alkaline pH of K:G led to cell wall polymer losses and was therefore responsible for lower extraction yield. The pH (pH 6.5) of the CC:G NADES may not be appropriate to break bonds in the cell wall and subsequently less pectin was released compared with that of dilute acid extraction. Third, in addition to the microstructure of different pomace, the relatively higher viscosity of NADES solvent compared with dilute HCl solution may have also limited the mass transfer between solvent molecules and cell wall components, and therefore decreased the extraction efficiency. When NADES pretreatments were applied sequentially, the hot water extraction following each NADES pretreatment could access to more polysaccharides in the cell wall than any one step extraction method. This led to a marked improvement in extraction yields of water extracts following sequential CC:LA and K:G pretreatment (**Fig. 3**). The highest yield obtained by this sequential method compared with other extraction methods represents a promising alternative process for pectin extraction.

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

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

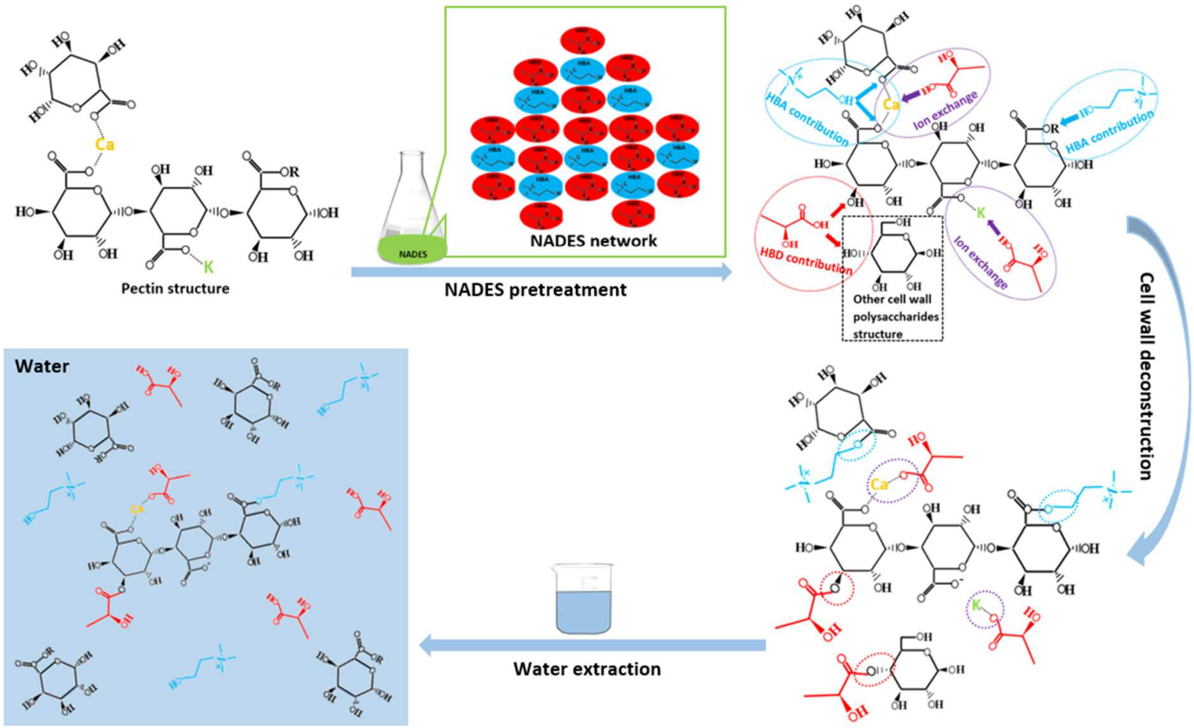


Fig. 7 Hypothetical mechanism involved in CC:LA pretreatment/hot water extraction. The individual components (HBA and HBD) of NADES form esterification products with cell wall polysaccharides, coupled with ion exchange with metal cations to loosen the cell wall, which facilitates the subsequent hot water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

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

According to the uronic acid content of the fractions, CC:G and CC:LA pretreatments followed by hot water extraction clearly afforded efficient pectin extraction from apple pomace (fractions E1, E2 and F). While for the K:G pretreatments (fractions E3 and G), pectin were likely released from the cell wall by the combined action of the dissociation of the calcium ions and their replacement by potassium, by cleavage of ester linkages and by degradation of β-elimination in the alkaline pH. Part of pectin are known to require basic conditions to be extracted (Santiago et al., 2018). In fact, the K:G solvent in its higher pH mode could be used as an efficient extractant of hemicellulose due to the observation that diluted K:G solvent (molar ratio: 1:25) used in present work co-extracted pectin. NADES pretreatments allowed enriching pectin in HG or RGI structural domains. Of interest is the fact that HG/RGI ratio for pectin extracted after CC:LA pretreatment was close, whether the pretreatment was applied alone or after CC:G pretreatment (30.1:1 for E2 and 31.7:1 for F; **Fig. 4A**). Therefore, CC:LA pretreatment appears specific in freeing RGI structure from apple pomace compared to other pretreatments. The lowest HG/RGI molar ratio found in the water extract after K:G pretreatment in the sequential method (E3) is likely related with the loss of methylesterified HG structural domains following their degradation by β-elimination under the alkaline conditions of the solvent. As a result, the extract of low molecular weight (trace E3, **Fig. 5A**) corresponded to pectin enriched in RGI structure. In agreement with our results, the RG I rich fraction was also obtained from carrot-based purées under hot alkaline condition (Santiago et al., 2018). Additionally, based on UA recovery in E3 fraction, most of the cell wall HG might have already been extracted by hot water following the first two pretreatments (E1 and E2) resulting in a lower HG/RGI molar ratio. This is supported by the higher HG/RGI molar ratio observed in the water extract following the non-sequential K:G pretreatment compared with that of E3 fraction. Hence, the combination of pH and the synergistic effect of NADES pretreatments was responsible for the lowest HG/RGI molar ratio in E3 fraction. Besides pH, other factor may exist to cause the relative higher HG proportion in CC:G pretreated fraction. Actually, CC:G pretreatment did not specifically help extracting HG rich fraction since a low HG/RGI molar ratio was observed in CC:G fraction when the reversed order pretreatment was realized, namely, CC:LA pretreatment first, followed by CC:G (data not shown). The detail explanation for this result remains to be further elucidated. Furthermore, more cell wall losses were observed when apple pomace was pretreated first with CC:LA or K:G. Therefore, the order of NADES pretreatment/water sequential extraction was fixed to 1) CC:G, 2) CC:LA, 3) K:G.

When compared with sequential NADES pretreatment/water extraction, sequential chelating agent extraction possessed less ability to separate pectin rich in RGI structural domains as HG proportion was increased after CDTA & Na-oxalate treatment (C2) or Na_2CO_3 & $NaBH_4$ treatment (D2). CDTA was an effective Ca^{2+} chelating agent (Jarvis, Hall, Threlfall, & Friend, 1981), the HG bridged by Ca^{2+} was released after CDTA treatment. However, the HG/RGI molar ratio was lower in CDTA fraction (D1) compared with that in CDTA & Na-oxalate fraction, which indicated Na-oxalate also promoted HG structural domain enrichment. Although HG is generally unbranched, the xylose-substitution on HG may prevent calcium cross-links of HG chains and thus hinder the pectic network formation (Jensen et al., 2008). In contrast, the neutral side chains on the RGI take part in cell wall construction and development (Willats, Steele-King, Markus, & Knox, 1999; Jones, Milne, Ashford, & McQueen-Mason, 2003), play roles in fruit mechanical properties (Lahaye, Bouin, Barbacci, Le Gall, & Foucat, 2018) and chain interactions in pectin network (Sousa, Nielsen, Armagan, Larsena, & Sørensen, 2015). The influence on pectin RGI side chain of the NADES pretreatments and other extraction methods used in this study was further studied. All extracts had less or shorter galactose side-chains than that of dilute acid extraction. The galactose content in extracts after sequential extractions showed a decreasing trend (C, D and E; **Fig. 4B**). However, the Gal/Rha ratio in E3 fraction increased. As galactan side-chains can bind cellulose through hydrogen bonds (Lin, Lopez-Sanchez, Selway, & Gidley, 2018; Zykwinska, Ralet, Garnier, & Thibault, 2005), CC:G and/or CC:LA pretreatments may expose residual pectin that are H-bonded to other cell wall polymers but that are labile to the alkaline condition of the K:G pretreatment. Consistent with Huang et al. (2016) who reported that EDTA & sodium oxalate led to shorter galactan side-chains on RGI from potato, the CDTA extraction of apple pomace with or without sodium oxalate had a negative effect on the Gal/Rha molar ratio (4.4:1 for C2; 4.0:1 for D1). Arabinose side-chains are known to be rapidly cleaved under mild acidic conditions (Thibault, Guillon, & Rombouts, 1991). Therefore, dilute acid extraction caused severe arabinan side chains losses. The pH of CC:LA is 1, which is close to that of diluted acid (pH=1.5). Hence, it is reasonable that CC:LA pretreatment (E2 and F) had a similar negative impact on these side chains. Moreover, the low Ara/Rha ratio observed in the extract after sequential K:G pretreatment indicated a marked effect of CC:LA pretreatment on arabinan side-chain. Similarly, pectin samples with very low (Ara + Gal)/Rha ratio were observed when peel of orange, lemon, lime, and grapefruit was extracted by nitric acid (pH 1.6) (Kaya et al., 2014).

4.3. Extraction process affected molecular weight distribution of extract

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

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

4.4. Cell wall cellulose aggregates following pectin extraction from pomace

To establish an integrated biorefinery process of apple pomace and its further use after pectin extraction, the impact of sequential NADES pretreatment/water extraction on structure and organization of the residual pomace polymers was investigated by CP/MAS ^{13}C NMR spectroscopy. The spectra (**Fig. 6**) revealed the evolution of cell wall structure from a complex raw material to a simpler one in K:G pretreated residues. NADESs pretreatment/water extraction removed pectin, as judged from the decreasing intensity of the signals for C_1 (around 100.4 ppm) corresponding to the overlapping chemical shift of pectin backbone galacturonic acid, rhamnose and xylose sidechain of xyloglucan (Ng et al. 2014; Phyo & Hong, 2019), C_4 (80.8 ppm) corresponding to pectin backbone galacturonic acid (Sinitsya, Copiková, & Pavliková, 1998) and that of the pectin methyl ester at 52.7 ppm. The last alkaline NADES pretreatment/water extraction with K:G was particularly efficient in removing also phenolic compounds and proteins, which signal intensity markedly decreased in the spectrum of the residue. Apple varieties for cider production are known to be particularly rich in phenolic compounds that form insoluble complex with cell wall material in pomace (Bourvellec, Guyot, & Renard, 2009). K:G pretreatment can provide a mean for extracting them. As previous research suggested (Newman, Ha, & Melton, 1994), the crystallinity of cellulose and its cross-section dimension can be estimated by solid-state NMR. The crystallinity and LFD up to CC:LA residue were ranged from 31%-34% and 2.6 nm-2.7 nm respectively. A similar 38 % of crystallinity and 2−3 nm of cross-section dimension on apple cell wall has already been reported (Lahaye, Falourd, Laillet, & Le Gall, 2020 and references herein). The higher crystallinity (46%) and LFD (3.6 nm) found in K:G residue indicated the influence of this pretreatment on the cellulose structure and its surrounding environment, which will be discussed below. At the molecular structure level, the two-proton reservoir model allowed evaluating the spin diffusion time of non-bonded proton to proton linked to carbon. In our case, the non-bonded proton mostly comes from water molecule, which was used to rehydrate the residual pomace. Paris et al. (2001) showed that the T_{HH} value was positively correlated with number of surrounding water molecule. The longer T_{HH} value of pectin methyl ester in both CC:G and CC:LA pretreated residual pomace means better hydration of pectin than that of raw material. The first removal of pectin by CC:G pretreatment/water extraction made the pomace residue more porous so that the water used to rehydrate the residue could better interact with the pectin. Further removal of pectin by the CC:LA pretreatment/water extraction did not have a major impact on this porosity and the hydration of the residual pectin. The pore opening by pectin extraction to allow water diffusion was also observed with the increasing T_{HH} value for cellulose up to the CC:LA residue. Further processing with K:G pretreatment/water extraction led to a more hydrophobic environment, most likely due to the aggregation/rearrangement in higher crystalline cellulose by the removal of the alkaline-soluble pectin. The reorganization of the cellulose was evidenced by the lengthening of the $T_{1\rho}^{H}$ relaxation attributed to more organized cellulose (Lahaye, Falourd, Laillet, & Le Gall, 2020). This ordering goes along with that of pectin as judged from the $T_{1\rho}^{H}$ of the pectin methyl ester which agrees with close pectin-cellulose interactions (Wang & Hong, 2016). Although this increase in ordering was observed all along the sequential extraction process and notably after the first CC:G pretreatment/water extraction, K:G pretreatment/water extraction had the most dramatic effect on cellulose. This result suggest that the K:G pretreatment/water soluble pectin and the minor hemicellulose associated in the pectin extract play a major role in the cellulose organization and support the idea that pectin distribution in the cell wall controls cellulose bundles packing.

5. Conclusion

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

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