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Effect of extraction conditions on the saccharide (neutral and acidic) composition of the crude pectic extract from various agro-industrial residues

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Legends to Figures:

Fig S (1a): Effect of time on the production of HMF during nitric acid extraction of pectic polysaccharides

Fig S (1b): Effect of time on the production of furfural during nitric acid extraction of pectic polysaccharides

Fig S (2a): Relative abundance of log MW values derived from Size exclusion chromatograms of polysaccharides extracted from BP by enzymatic and nitric acid

Fig S (2b): Relative abundance of log MW values derived from Size exclusion chromatograms of polysaccharides extracted from PP by enzymatic and nitric acid

Fig S (2c): Relative abundance of log MW values derived from Size exclusion chromatograms of polysaccharides extracted from SBP by enzymatic and nitric acid

Fig S (2d): Relative abundance of log MW values derived from Size exclusion chromatograms of polysaccharides extracted from OH by enzymatic and nitric acid

Fig S (2e): Relative abundance of log MW values derived from Size exclusion chromatograms of polysaccharides extracted from OH by Ammonium oxalate and sodium hexametaphosphate

Fig S (3a) : HPAEC chromatogram of the monosaccharides produced during nitric acid extraction of pectin from sugar beet pulp

Fig S (3b) : HPAEC chromatogram of the monosaccharides produced during enzymatic extraction of pectin from sugar beet pulp

Fig S (1a)

Fig S (1b)

Fig S (2a)

1. EAE: Peaks of MW (I) 18479-2604 (II) 2604-507 (III) 507-33
2. NAE: Peaks of MW (I) 5371981-10554 (II) 10554-702 (III) 702-40

The highest peak is put on 100%

Fig S (2b)

1. EAE: Peaks of MW (I) 163374-30252 (II) 30252-2766 (III) 2766-540 (IV) 540-28
2. NAE: Peaks of MW (I) 1461234-6079 (II) 6079-647 (III) 647-31

The highest peak is put on 100%

Fig S (2c)

1. EAE: Peaks of MW (I) 2299596-167967 (II) 167967-5963 (III) 5963-341 (IV) 341-54
2. NAE: Peaks of MW (I) 1513202-118702 (II) 118702-2939 (III) 2939-476 (IV) 476-31

The highest peak is put on 100%

Fig S (2d)

1. EAE : Peaks of MW (I) 224565-9567 (II) 9567- 444 (III) 444-45
2. NAE: Peaks of MW (I) 295827- 9682 (II) 9682- 453 (III) 453- 49

The highest peak is put on 100%

Fig S (2e)

1. AO: Peaks of MW (I) 145352-961 (II) 961-39
2. SHMP: Peaks of MW (I) 119497-1814 (II) 1814-588 (IV) 588-29

The highest peak is put on 100%

MW: molecular weight; BP: berry pomace; PP: pressed pumpkin; SBP: sugar beet pulp; OH: onion hulls

Fig S (3a)

Fig S (3b)

Abstract α

The influence of different extraction methodologies was assessed on the composition of both neutral (arabinose, rhamnose, galactose) and acidic (galacturonic acid) pectic polysaccharides obtained from four agro-industrial residues *viz.* berry pomace (BP), onion hulls (OH), pressed pumpkin (PP) and sugar beet pulp (SBP). For acidic pectic polysaccharides, the extraction efficiency was obtained as: BP (nitric acid-assisted extraction-2h-62.9%), PP (enzymatic-assisted extraction-12h-75.0%), SBP (enzymatic-assisted extraction-48h-89.8% and nitric acid-assisted extraction-4h-76.5%) and OH (sodium hexametaphosphate-assisted extraction-0.5h-100% and ammonium oxalate-assisted extraction-0.5h-100%). For neutral pectic polysaccharides, the following results were achieved: BP (enzymatic-assisted extraction-24h-85.9%), PP (nitric acid-assisted extraction-6h-82.2%) and SBP (enzymatic assisted extraction-48h-97.5% and nitric acid-assisted extraction-4h-83.2%). Based on the high recovery of pectic sugars, SBP and OH are interesting candidates for the further purification of pectin and production of pectin-derived products.

Keywords: Berry pomace, Onion hulls, Pressed pumpkin, Sugar beet pulp, Extraction, Neutral and acidic pectic polysaccharides, Pectin, Pectin based derivatives.

1. Introduction

During the production of agricultural commodities, various agro-industrial residues are generated that can be a potential source of valuable compounds, such as antioxidant, protein, pigment and starch. Some of these residues are rich in pectin- a carbohydrate polymer. The structure of pectin is hypothesized to be made of smooth and hairy regions. The smooth region, also known as homogalacturonan, is composed of (1,4)- linked galacturonic acid residues backbone while the hairy region, recognized as rhamnogalacturonan, comprises an alternating backbone of galacturonic acid and rhamnose residues ($\rightarrow 2$)- α -L-Rha- (1 $\rightarrow$ 4)- α -D-GalA-($\rightarrow 1$)_n.¹ The hairy appearance of RG is due to the presence of branched arabinan, galactan, arabinogalactan chains at O-4 of rhamnose residues.²

Pectin is typically categorized into three classes: (1) soft pectins, extractable with dilute salt solutions; (2) chelator-soluble pectins, extractable with calcium-chelating solutions such as ethylenediaminetetraacetic acid, cyclohexanediaminetetraacetic acid and sodium hexametaphosphate, and (3) protopectin, extractable with alkali or hot dilute acid solutions.³ The widely used method for liberating pectin from plant materials is acidic extraction. The principle involves the hydrolysis of the protopectin which results in diffusion of pectin from the cell walls.⁴ The extraction with chelators, on the other hand, liberates calcium bound pectin by loosening the egg box structures formed by homogalacturonan and calcium complex.⁵

A major challenge in pectin recovery from residues concerns the extraction process used for its isolation. Previous studies have indicated that the extraction process can cause a high degree of pectin degradation, resulting in a low yield and a loss of pectin functionality *i.e.* gelling property.⁶ This can partially be due to the uncontrolled generation of pectic monomers, as a result of partial pectin hydrolysis, that fail to precipitate during purification.⁷ Knowledge on these monomers, present in the crude extract but lost during pectin

purification, aids to design a high yield recovery of minimally damaged pectic polysaccharides.

Moreover, there is an increasing interest in pectin-based derivatives. This includes for instance the pectic oligosaccharides with a dedicated chain length that recently have gained interest as a potential prebiotic agent and are produced from the homogalacturonan and rhamnogalacturonan moieties.⁸ These type of products are in full exploration and are not expected to have the same requirements as that of pectin which is used for traditional applications. To reduce production costs, they are produced from a crude rather than from a purified pectin, and their compositional and molecular requirements, as galacturonan content or esterification degree, may be different. Therefore, also for this applications an accurate knowledge is needed on the influence of the extraction technology on the chemical composition of the crude extract.

For the production of these dedicated pectin-based derivatives, un-common agro-industrial by-products may become of value. This includes for instance by-products that are smaller in volume, different in composition or not having the required properties as the ones typically used for pectin production. Berry pomace (BP), onion hulls (OH) and pressed pumpkin (PP) are by-products generated during processing of berries, onions and pumpkin. These by-products are expected to have potential because they have not extensively been exploited for commercial purposes. Their properties can however not be assessed properly since only scanty literature is available on their pectin content/pectic saccharide composition.⁹⁻¹² Sugar beet pulp (SBP), on the other hand, a by-product of sugar beet processing, is more widely studied and contains 12-13% pectin on dry matter basis.¹³ Nevertheless, the highly-branched pectin composition makes it less suitable for typical pectin applications, necessitating the search for other types of outlets.

It has been reported that different sources of pectin are affected differently by various extraction conditions.¹⁴ The main aim of the present study is therefore to study the effect of the extraction on the composition of neutral (arabinan, galactan side chains) and acidic (galacturonic acid polysaccharide) pectic polysaccharides and their degradation into low molecular weight sugars in the crude extract. Four different wastes are assessed, being BP, OH, PP and SBP, and the effect of different extraction methods, viz., the use of acids (nitric acid), enzymes and chelators is analyzed on the recovery and composition of crude pectic polysaccharides.

2. Material and Methods

2.1 Raw material

The agro-industrial residues *i.e.* onion hulls (OH) and sugar beet pulp (SBP) were provided in a dried condition by the Institut für Getreideverarbeitung (IGV GmbH), Germany. Dried and deseeded berry pomace (BP) was provided by Eco Treasures, Belgium. Pressed pumpkin cake (PP) was procured in a wet condition from Provalor, The Netherlands, and then dried for 24 h in a vacuum oven (Thermo Scientific) to a moisture content of 7-8%. The residues were milled with a laboratory blender (Kenwood), sieved to a particle size smaller than 0.8 mm, and stored in zip-lock bags at room temperature until use. Celluclast 1.5 L (C-2730), Viscozyme L (V-2010) and standards for rhamnose, arabinose, galactose, xylose, glucose, fructose and galacturonic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Germany). The dextran oligomers (molecular mass 180-1223000 Da) and analytical grade chemicals used during extraction were also purchased from Sigma-Aldrich.

2.2 Total sugar composition of the raw material

The total sugar composition of the raw materials was determined by performing two types of hydrolysis followed by the analysis of the monosaccharides. In the first, ground BP, PP, SBP and OH residues (1 g each) were subjected to enzymatic hydrolysis at a substrate concentration of 5% (w/v) in 100 mL erlenmeyer flasks. An enzyme cocktail of Celluclast 1.5L and Viscozyme L, each at 20 U/g d.m. (based on filter paper activity, FPU/ mL), was added to the biomass suspended in 50 mmol/L citrate buffer (pH 4.8). The filter paper activity of the enzymes was analyzed using laboratory analytical procedure of National Renewable Energy Laboratory.¹⁵ The flasks were incubated in an incubator shaker (Innova, New Brunswick, USA,) at 48 °C and 150 rpm for 48 h. The hydrolyzed biomass was centrifuged at 5000 xg for 10 min. The supernatant (containing the monosaccharides) was assayed for its monosaccharide composition by high performance anion exchange chromatography with

pulsed amperometric detection (HPAEC-PAD, see Section 2.4). Fructose and glucose were corrected for the amounts of sucrose present in the enzyme cocktail. In comparison, also a seamans hydrolysis was performed. In short, it comprises of treatment of biomass with 11.5 M H₂SO₄ at 20 °C for 2 h followed by dilution of 11.5 M H₂SO₄ to 1 M and further keeping at 100 °C for 2h.¹⁶ The hydrolyzed biomass was centrifuged at 5000 xg and analyzed on HPAEC-PAD (HPAEC-PAD, see Section 2.4).

2.3 Extraction of pectin from agricultural residues

To extract intact pectic polysaccharides from the four agro-industrial residues, three types of extraction methods were investigated. All the extraction processes were carried out in 100 mL conical flasks using 1g of biomass at 5% (w/v) substrate loading. Enzymatic hydrolysis was carried out using Celluclast 1.5 L which primarily contains cellulases (endo-glucanase units) and has been found to be effective in extracting pectin from plant cell walls.¹⁷ While acid extraction was carried out using dilute nitric acid.¹⁸ For the extraction of pectin from OH, in addition to acid and enzyme extraction, chelator based extraction was carried out using ammonium oxalate¹¹ and sodium hexametaphosphate.¹⁹ The selection of extractant was based on the inefficiency of conventional extractant *i.e.* enzyme and nitric acid to release pectin from OH stated elsewhere in the article. After each extraction, the biomass was centrifuged at 5000 x g for 10 min and the supernatant was assayed for sugars on HPAEC-PAD (see Section 2.4). The extraction conditions for different agro-industrial wastes is given in Table 1.

Also, the monosaccharide and polysaccharide composition of each biomass was determined by dissolving the sample (1g) at 5% (w/v) in distilled water at 48 °C for 4h.

2.4 Analysis of total free monosaccharides and pectic polysaccharides by HPAEC-PAD

All extracts were analyzed on HPAEC-PAD in two ways, *i.e.*, without further treatment to assess its monosaccharide content, and after complete hydrolysis to determine its

total saccharide content. The amount of dissolved pectic oligo- and polysaccharides, was determined by correcting the total amount of saccharides detected after hydrolysis by the amount of monosaccharides already present in the extract.

To achieve a complete hydrolysis and full recovery of the monomers of galacturonic acid and the neutral sugars, the extraction fluid was post-hydrolyzed by digestion with 5% (v/v) of Viscozyme L at 45 °C for 24 h.²⁰ Viscozyme L is a multienzyme complex composed of pectinases, hemicellulases and arabinases.²¹ The hydrolysis method is based on the work of Martinez and coworkers.²² It is worth mentioning that the commonly used acid hydrolysis was not suitable for these pectin rich extracts, even when applying the mild conditions of the seamans hydrolysis. In all cases low amounts of galacturonic acid were found due to degradation (data not given). After hydrolysis, the enzyme was inactivated by a thermal treatment at 100 °C for 5 min and the liquid was centrifuged at 5000 x g for 10 min to get a clear supernatant. Samples of the extraction fluid were adequately diluted and injected into a high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

The HPAEC-PAD used for analytical purpose is a Dionex ICS-5000 model (Thermo Scientific, Inc., USA) equipped with an ED-5000 electrochemical detector. The separation of monosaccharides was carried out with a Carbopac PA -1 (4mm X 250mm X 4 mm) column coupled to a guard column Carbopac PA- 1 (4mm X 50 mm X 4mm) column. The analyses were performed using a gradient of deionized water (eluent A and D), 250 mM sodium hydroxide (eluent B) and 1 M sodium acetate (eluent C). The mobile phase was used at a flow rate of 1 mL/min for 46 min. The monosaccharides were quantified by comparing them with the concentration of known standard solutions (ranging from 10 mg/L to 1000 mg/L) of rhamnose, arabinose, galactose, glucose, xylose, fructose and galacturonic acid.

2.5 Analysis of hydroxymethylfurfural (HMF) and furfural.

The formation of HMF and furfural in acid treated samples were determined by UPLC. The system used was from Waters coupled with a PDA UV detector, a column, Kinetex 2.6 μ biphenyl 100A with dimensions of 100 mm X 2.1 mm. The flow rate was maintained at 0.4 mL/min. The integration of the peaks were done on the basis of wavelength at 240 nm. The temperature of the column was set at 40 °C. The system was eluted isocratically with a mixture of 99% water and 1% acetonitrile.

2.6 Analysis of the methylation and acetylation degree.

Methylation and acetylation degrees were determined as previously reported.²³ Briefly, 30 mg of freeze dried crude extracts containing pectic material were incubated (2 h, room temperature) with 1 ml 0.4 M NaOH in D₂O and 0.1 ml of internal standard (TSP, 2 mg/ml in D₂O). The supernatant was centrifuged (Centrifuge eppendorf 5810R, EPPENDORF Augsburg, Germany), clarified (nylon syringe filter system, 0.4 μ m) and transferred in NMR-tubes. ¹H NMR spectra were acquired on a VARIAN-INOVA 600 MHz spectrometer, equipped with a triple resonance inverse probe (HCN), operating at 599.736 MHz for proton. Spectra were collected at 298 K, with 32K complex points, using a 90° pulse length. 128 scans are acquired with a spectral width of 7196.8 Hz, an acquisition time of 2.53 and a relaxation delay (d1) of 5s. The experiments were carried out with water suppression by low power selective water signal presaturation during 5 s of the relaxation delay. The NMR spectra were processed by MestreC software. The quantitative determination of acetic acid, methanol and ferulic acid was obtained by manual integration of the corresponding signals (1.920 ppm for acetic acid and 3.358 for methanol) and the comparison with the TSP area.

2.7 Assessment of the molecular weight.

The molecular weight (MW) of pectic polysaccharides from BP, SBP, PP and OH was determined by gel permeation chromatography against dextran standards. In this system, the

pectic polysaccharides are separated based on their different hydrodynamic volumes (radius of gyration). Since the MW estimations were done using dextran standards which have different hydrodynamic volumes as compared to pectic polysaccharides, only approximate estimations of the molecular weight were obtained. All the samples were filtered through a 0.45 µm membrane filter prior to analysis. A gel permeation chromatography system from Shimadzu, Japan was used and consisted of a solvent degasser (DGU-20 A), quaternary pump (LC-20AT), refractive index detectors (RID-10A), auto sampler (SIL-20ACHT) and column oven (CTO-20AC). An isocratic method using 0.1% sodium nitrate as eluent was applied. The flow rate was maintained at 0.6 mL/min and the temperature of the columns was maintained at 40° C. The system contained a guard column followed by two separation columns, *i.e.* Ultrahydrogel column 120 and a Ultrahydrogel column 500, both with dimension of 7.8 × 300 mm (Waters) and an exclusion limit of respectively 5x10³ and 4x10⁵. All the chromatograms were corrected for the contribution of the enzyme/ acid/ chelator by subtracting the corresponding peaks.

2.8 Statistical analysis and assessment of the extraction efficiency

All the experiments were conducted in triplicate and the mean and standard deviation were calculated using MS Excel software. Wherever necessary, the data were analyzed with one way ANOVA and LSD ($P < 0.05$) for tests of significance with JMP software (SAS Inc, Cary, NC, USA).

The percent extraction efficiency of pectic polysaccharides extracted from agro-industrial residues was determined by the following formula:

$$\text{Extraction efficiency (\%)} = \frac{(C_{total,extr} - C_{mono,extr})}{C_{total,biomass}} \times 100$$

$C_{\text{total, extr}}$ = total concentration of galactose, arabinose and galacturonic acid found in the extract after post-hydrolysis. Expressed relative to the biomass treated (in w/w DM).

$C_{\text{mono, extr}}$ = concentration of free galactose, arabinose and galacturonic acid found in the extract by direct analysis. Expressed relative to the biomass treated (in w/w DM).

$C_{\text{total, biomass}}$ = concentration of galactose, arabinose, and galacturonic acid found after hydrolysis of the biomass. Expressed relative to the biomass treated (in w/w DM).

The extraction efficiency of rhamnose is not shown owing to its very low concentration in the biomass considered.

3. Results and Discussion

3.1 Total sugar (galacturonic acid and neutral sugar) composition of berry pomace (BP), pressed pumpkin (PP), sugar beet pulp (SBP) and onion hulls (OH)

To determine the composition of pectic polysaccharides, the biomass was hydrolyzed and analyzed for monosaccharides. In particular, these are galacturonic acid as acidic sugar, and arabinose, galactose and rhamnose as neutral sugars. In the present study, acid hydrolysis (Seamans hydrolysis) was initially used as a first choice to completely hydrolyse the biomass and determine constituent sugars. It was found that in all cases significantly low amounts of galacturonic acid were obtained (data not shown), which relates to rapid breakdown to other degradation products. Therefore second method of hydrolyzing the biomass with enzyme containing cellulolytic, pectinolytic and hemicellulolytic activities was then evaluated. Both Viscozyme and celluclast contains several activities in the form of endoglucanase, endoxylanase, pectinase, endomannase, polygalacturonase, total cellulase, cellobiohydrolase, β -D-glucosidase, β -D-mannosidase, β -D-xylosidase, α -L-arabinofuranosidase in various proportions.²⁴ Using enzyme cocktail, the obtained values of glucose and xylose were lower, which could be due to lesser β -glucosidase and β -xylosidase activity of enzyme cocktail. Therefore, to obtain the most representative estimation, the values of galacturonic acid, galactose, fructose, rhamnose and arabinose are taken from enzymatic hydrolysis while glucose and xylose were obtained from acid hydrolysis. As also stated in the review of Sluiter et al., each method has its limitations and there is still a need to develop methods for complete hydrolysis of the feedstocks.²⁵ Moreover, it is possible that the standard method like NREL and Seamans hydrolysis which are mainly developed for lignocellulosic biomass are not completely suitable for pectin containing biomass due to sensitivity of galacturonic acid moiety. Table 2 shows the constituent sugars present in various agro-industrial residues. The results are expressed as % (w/w) d.m. The galacturonic acid content of OH is the highest

(19.3 ± 0.9 %) followed by SBP (17.3 ± 0.9%), PP (5.6 ± 0.1 %) and BP (5.9 ± 0.6 %), respectively. The value of galacturonic acid obtained for SBP in the present study is slightly lower than the 21.9% (w/w) d.m. found by Leijdekkers et al.²⁶ This can be due to a different source of the sugar beet pulp starting material.

In addition to acidic sugars, some agricultural residues contain significant amounts of the pectin-related neutral sugars *i.e.* rhamnose, arabinose and galactose.²⁷ In the present study, the total neutral sugar composition calculated as the sum of the individual neutral sugars, varied from 25.2% to 44.1% for PP and SBP respectively (Table 2). Whereas, the neutral sugar content of both BP and OH are 39.1% and 36.3%, respectively. The arabinose content is the highest in SBP (16.6 ± 0.6%) while the other residues contains low amounts (0.3 ± 0.1% to 0.9 ± 0.1%). The presence of minor amount of arabinose in OH in this investigation is in line with the results obtained by Matsura et al., who also found less arabinose in OH.²⁸ The rhamnose content of SBP is the highest (1.2 ± 0.4%) followed by PP (0.6 ± 0.4%), OH (0.4 ± 0.1%) and BP (0.4 ± 0.5%), respectively. The highest galactose content of 7.0 ± 0.9 % is found in OH followed by SBP (5.7 ± 0.1%), BP (4.7 ± 0.1%), and PP (3.7 ± 0.2%).

In the residues other neutral sugars such as xylose, glucose and fructose are also found and considered to be primarily originating from the hemicellulose, cellulose and residual sucrose.⁸ The glucose content of residues range from 15.2 ± 0.3% to 26.1 ± 0.1% for PP and OH, respectively (Table 2). In case of BP and SBP, the glucose content account for 18.9 ± 0.5 and 18.8 ± 0.2, respectively. A significant amount of fructose up to 8.9 ± 0.1% and 4.3 ± 0.2 % is present respectively in BP and PP. Both OH and SBP contains negligible amount of fructose. Also, Chandrasekaran & Bahkali found that some fruit processing residues contain significant amounts of inverted sugars.²⁹ The remaining constituents of the biomasses were not analysed and can comprise of protein, ash, unhydrolysed fiber etc. There is still a need to develop methods for complete biomass hydrolysis of the feedstocks.²⁹

Thus, both SBP and OH contain pectic polysaccharides in a reasonable amount, while relatively low amounts of pectic polysaccharides are found in BP and PP. Also, based on the results, the highest rhamnogalacturonan sugars are found in SBP amongst the other residues. In the following sections the impact of different extractants on the extraction of pectic polysaccharides from the four residues are evaluated.

3.2 Effect of different methods on the extraction of pectic polysaccharides from agro-industrial residues

The effect of the hydrolysis time on the extraction of pectic polysaccharides from BP, PP, SBP and OH by both enzymatic and nitric acid treatment was evaluated. In addition, the extraction of pectic material from onion hulls also studied with the chelators mentioned earlier in the paper. In the following section, the polysaccharides relevant to pectin are described and the results are expressed as extraction efficiency and yield as % (w/w) on dry matter for all the residues.

3.2.1 Berry pomace (BP)

The pectin composition of BP is shown in Table 2, highlighting the presence of galacturonic acid and galactose as major pectic sugars. The data in Fig 1a shows that time has a significant effect on the recovery of galacturonic acid polysaccharides. A rapid increase in the galacturonic acid polysaccharides is seen in the first 24 h with an extraction efficiency of 59.0% and a yield of 3.5% (Fig 1 a). It has been reported that cellulase breaks down the cellulosic material and β -glucanase further converts cellobiose to glucose, thereby liberating pectin during hydrolysis.¹⁶ Simultaneously, also increasing amounts of free galacturonic acid is formed during the extraction, accounting for 20.0% at 24h and additionally also doubles by the end of 48h. Celluclast 1.5L is a multi-enzyme complex composed of different enzyme sub components in different titres. In addition to the cellulase activity, also polygalacturonase and arabinase activity are present.²² These side-activities are considered to be responsible for

increased monosaccharide concentration at extended extraction times. At the optimal extraction time of 24h, the extraction efficiency of galacturonic acid comprises of 59.0% polysaccharides and 20.5% monosaccharides which indicates that 20.0% of galacturonic acid remains unextracted. Ptichkina et al., studied the recovery of pumpkin pectin and demonstrated that the use of an enzyme from *Aspergillus awamori* having higher cellulase activity (β -glucanase) gave higher pectin recovery than the enzyme prepared from *Trichoderma viridae*.³⁰ In the present study, enzyme from *Trichoderma viridae* was used which has multi-component enzyme activity as mentioned earlier and therefore could be responsible for lower activity and hence lesser yield. For the treatment with nitric acid, time has insignificant effect on the galacturonic acid polysaccharide extraction from BP (Fig 1b). The highest extraction efficiency of 62.9% with a corresponding yield of 3.7% is obtained at 2 h with a levelling off of the concentration after this time. Negligible amounts of monosaccharides are formed (Fig 1b). A comparison of the results of Table 2 with those in Fig 1b, suggests that at an ideal extraction time with nitric acid solution, still 37.1% of galacturonic acid polysaccharides remains unextracted. For neutral sugars, the enzymatic extraction at 24h results in highest extraction and yield of galactose polysaccharides *i.e.* 85.9% and 4.0% respectively (Figure 1a). Also, the presence of 0.025% (w/w) HMF is observed at the end of hydrolysis, which is likely from the degradation of monosaccharides (Fig S1 a).

Based on the results obtained it can be inferred that nitric acid extraction seems to be more effective for the extraction of galacturonic acid polysaccharides and enzymatic extraction more suitable for galactose polysaccharides extraction from BP which can be explained owing to structure of the raw material. It is very likely that the source of enzyme and the nitric acid concentration play a significant role in the extraction. Nevertheless, further

investigation with other extractants is needed to recover all the galacturonic acid polysaccharides from BP.

3.2.2 Pressed pumpkin (PP)

Pumpkin cake contains mainly galacturonic acid, galactose and smaller amount of arabinose (Table 2). It is evident from Fig 2a, that hydrolysis time in enzymatic extraction has insignificant effect on the extraction of galacturonic acid polysaccharide. An extraction efficiency of 75.0% with corresponding yield of 4.3% is obtained after 12 h of hydrolysis. Production of insignificant amounts of monosaccharides of 0.3% and 0.6% are seen at 36 and 48 h respectively with enzymatic extraction, which can probably be due to polygalacturonase activity of cellulase.²² In case of nitric acid extraction, at 6 h, an extraction of 59.2% galacturonic acid polysaccharides are obtained (Fig 2b). The extraction behavior of the polysaccharides in case of BP was different as the time of hydrolysis (with nitric acid) has insignificant effect on the yield. Differences in the structure of plant material are likely to have a significant effect on the extraction processes.³¹

For neutral sugars, the highest extraction of galactose polysaccharides is 44.3% and 82.2% at 12 h and 6 h hydrolysis with enzymatic and nitric acid extraction, respectively (Fig 2a and 2b). Small amount of arabinose polysaccharides (< 1%) are also extracted by both the extractants (Fig 2a and 2b). It should be noted that in PP, about 18.9% monosaccharides are already present in the control extraction. It is worth mentioning here that some HMF is formed in the beginning of hydrolysis which is due to presence of monosaccharides of galactose and a further increase in HMF content after 6 h is noted (Fig S1 a). Formation of furfurals were also observed in the beginning of hydrolysis and their content levelled off thereafter (Fig S1 b). Presence of furfurals indicates the degradation of pentose sugars *i.e.* arabinose also took place during hydrolysis.

Enzymatic extraction results in highest galacturonic acid polysaccharides yield, indicating the possible hydrolysis of cellulose which led to solubilization of pectin from cell wall. The extraction of galactose polysaccharides is higher with nitric acid which is in contrast with the results obtained for BP, clearly highlighting the differences in the structure of plant materials.

3.2.3 Sugar beet pulp (SBP)

The presence of both neutral and acidic sugars in sugar beet pulp indicates a substantial amount of neutral side chains and acidic backbone of pectic polysaccharides (Table 2). Fig 3a, shows that with the time of incubation in enzymatic extraction, a linear increase in galacturonic acid polysaccharides occurs. The highest extraction efficiency of 89.8% and corresponding yield of 15.5% is obtained at 48 h. Throughout the enzymatic hydrolysis, monosaccharide production is not observed. These results are in contrast to the results obtained for BP and PP, wherein a degradation of acidic polysaccharides is noted with a simultaneous increase in monosaccharides. In case of nitric acid, an extraction of galacturonic acid polysaccharides is highest at 4 h with an extraction efficiency of 76.5% and yield of 13.2%, after which it levelled off (Fig 3b).

Amongst the neutral sugars present in SBP, the most important neutral sugar is arabinose followed by galactose (Table 2). The enzymatic extraction of arabinose polysaccharides does not result in any monosaccharide production (Fig 3a). The highest extraction efficiency of 97.5% and yield of 16.2% is obtained at 48h. However, a continuous decline of 1.2 and 1.6 fold arabinose polysaccharide at 6h and 8h is noted with nitric acid extraction (Fig 3b). Formation of furfural arising from the degradation of arabinose monosaccharides is also observed in SBP and their concentration increases with the increase in incubation time (Fig S1 b). Also a slight production of HMF is observed in the beginning of hydrolysis and their concentration levelled off thereafter (Fig S1 a). The enzymatic

extraction of galactose polysaccharides was highest at 48 h with an extraction efficiency of 98.4% (Fig 3 a). As observed with BP, nitric acid is also not suitable for the extraction of galactose polysaccharides from SBP.

From the above results, it is clear that both enzymatic and nitric acid extraction are suitable for pectic polysaccharide extraction from SBP with enzymatic extraction giving better efficiencies and yield. The cost of nitric acid is significantly lower than enzyme, therefore nitric acid can be considered as a cheaper extractant for pectic polysaccharide extraction from SBP, since extraction times are much shorter.

3.2.4 Onion hulls (OH)

Onion hulls have a good potential for pectic polysaccharide extraction because they contain a reasonable quantity of galacturonic acid (Table 2). As evident from the results given in Fig 4a, enzymatic extraction did not facilitate the extraction of galacturonic acid polysaccharides from OH and a yield of 2.1% was obtained at 12 h without further increase. It is likely that the pectin was strongly bound through calcium cross links and could therefore not be extracted by simple enzymatic hydrolysis (Fig 4a). To the best of our knowledge, this is the first time that the extraction of pectic polysaccharides from OH with enzymes is described. With nitric acid, although, an increase in hydrolysis time has a significant effect on the extraction, but the overall yield of galacturonic acid polysaccharides remains low. The highest galacturonic acid polysaccharide yield of 3.1% is achieved at 8 h (Figure 4b). A slight increase in furfurals are observed in onion hulls (Fig S1 b).

Kalapathy & Procter³² reported that acid strength used for extracting pectin from soy hull has a significant effect on pectin extraction while a longer time and higher extraction temperature does not affect the pectin yield. It is therefore likely that further lowering of pH can help in breaking the recalcitrant bonds to liberate pectin, but at the same time further lowering of pH can damage the neutral side chains.⁸ The inefficient extraction of galacturonic

acid polysaccharides by both acid and enzyme suggests that pectic substances in OH are tightly bound within the cell wall, probably *via* Ca^{2+} cross bridges between adjacent galacturonic acid sequences within pectin polymers.¹¹ Therefore, extraction with two different chelators *i.e.* ammonium oxalate and sodium hexametaphosphate was investigated. The results in Fig 4c shows that the variation in galacturonic acid polysaccharide content is insignificant with respect to the concentration and time of ammonium oxalate extraction. The galacturonic acid polysaccharide are completely extracted with yield of 22.3% with 0.5% ammonium oxalate at 0.5h. For sodium hexametaphosphate, on the other hand, a significant interaction between extraction time and concentration of the chelator on galacturonic acid polysaccharide formation is observed (Fig 4d). Full extraction and a yield of 23.1% is obtained with 2% sodium hexametaphosphate at 0.5h with a marginal decline after 3% concentration. At sodium hexametaphosphate concentration of 4% and 5%, the pectin formed a highly viscous mass. This viscous mass may have induced diffusion limitation in the enzymatic post-hydrolysis which could be responsible for an underestimation. A lower extraction of galacturonic acid polysaccharide with sodium hexametaphosphate at 1 h in comparison to 0.5 h is observed (Fig 4d). However, it is likely that this low value is again due to an underestimation of galacturonic acid polysaccharide as explained previously. Yamaguchi et al., obtained maximum pectic polysaccharides from soy hull at sodium hexametaphosphate concentration of 2%. These results are in line with the results obtained in the present study, except that a slightly longer extraction time of 3.5 h was needed.³³ The scarce availability of literature on the extraction of pectic polysaccharide from OH restricts the discussion about this specific residue. However, the results clearly demonstrate that the sodium hexametaphosphate and ammonium oxalate treatment solubilized most of the polygalacturonic acid from OH without production of monosaccharides. These results are in line with the results obtained by Kratchanova et al., for leek.³⁴ They also found that the

extraction of pectic polysaccharides by chelators is less destructive. Furthermore, the remarkably high extraction obtained with sodium hexametaphosphate and ammonium oxalate confirms that most pectic substances are bound within the cell walls of OH *via* Ca^{2+} cross bridges between adjacent galacturonate sequences within pectin polymers.

Table 3 presents a brief summary of the results obtained at the best optimized conditions of extractants for polysaccharides extraction from all the four agro-industrial residues. Based on the detected amounts of free galacturonic acid and galactose, it is concluded that there are some losses in the quality of pectic polysaccharides during extraction at the best optimized condition. Especially, in PP, there is a loss of about 45.8% of galactose-containing polysaccharides, in BP of 20% galacturonic acid- containing polysaccharides and in SBP of 10.1% of arabinose- containing polysaccharide, respectively, with enzymatic and nitric acid extraction. In SBP, the extraction with enzymes and in OH, extractants, sodium hexametaphosphate and ammonium oxalate resulted in pectic polysaccharides without any degradation. Further studies on MW of pectic polysaccharides obtained at the optimized extraction conditions is given in section 3.4.

3.3 Degree of Methylation (DM) and Acetylation (DAc)

The degree of methylation (DM) corresponds to the percentage of carboxyl groups esterified with methanol. The degree of acetylation (DAc) on the other hand, is the percentage of galacturonosyl residues esterified with one acetyl group.³⁵ The results for the DM and DAc are shown in Table 3. As expected the SBP exhibits highest DAc (59.1%) as also found in the literature.³⁶ In general, the DAc is high in pectin extracted by enzymatic extraction for SBP, PP and BP. Low values of DAc of pectin extracted by nitric acid from these residues could be due to an increased hydrolysis of acetyl esters by nitric acid. Some exceptions are found in OH, where in the highest DAc (21.0%) is exhibited by nitric acid extracted pectin (Table 3). On the other hand, the overall trend of increased DM is found in the samples treated with

nitric acid in comparison to enzymatic extraction. This effect could partly be due to the inactivation of demethylase enzyme by nitric acid resulting in higher DM of the samples. The highest value (> 50%) of DM is found in BP followed by PP, SBP and OH (Table 3).

3.4 Molecular weight distribution of pectic polysaccharides extracted from agro-industrial residues

The chromatogram showing the molecular weight distribution of polysaccharides obtained from agro-industrial residues at their best optimized conditions is shown in Fig S2. Since, no further purification was performed, the relative abundance of log MW values derived from size exclusion chromatogram constitutes information on the crude pectin. The crude pectin also contains contaminants like degradation products of cellulose and hemicellulose. The HPSEC chromatograms (Fig S2 a) show the MW distribution of polysaccharides extracted from BP with enzymatic and nitric acid extraction at their optimized levels. With nitric acid, higher molecular weight (MW) fractions *i.e.* 40-5371981 Da are extracted in comparison to enzymatic extraction. Formation of lower MW fractions in enzymatic extraction is in agreement with the detection of higher amount of galacturonic acid monomers during extraction. Also, the presence of low molecular weight fractions in both enzymatic and nitric acid could be from the hydrolysis of cellulose. In case of PP, the higher MW fractions *i.e.* 31- 1461234 Da extracted with nitric acid show that there is less degradation of pectic polysaccharides in comparison to enzymatic extraction (Fig S2 b). In case of SBP, the fractions obtained with enzymatic extraction have higher MW 54- 2299596 Da in comparison to nitric acid (Fig S2 c). This indicates higher degradation of pectic polysaccharides with nitric acid resulting in smaller MW fractions, which is evident from the degradation of arabinose polysaccharide (Fig 3 b). From the results of Fig S2 d it can be derived that in case of OH, fractions of lower MW are formed with both enzymatic and nitric acid extraction. In comparison, pectic polysaccharides extracted by chelators, reveal the

presence of one significant peak in ammonium oxalate (961-145352 Da) and sodium hexametaphosphate (1814-119497 Da) extracted pectic polysaccharides with few smaller peaks (Fig S2 e). Therefore, both sodium hexametaphosphate and ammonium oxalate can be considered as smooth extractant due to solubilization of higher molar mass.

This study shows that out of the four residues *i.e.* sugar beet pulp (SBP), onion hulls (OH), berry waste (BW) and pressed pumpkin (PP), substantial amounts of galacturonic acid polysaccharides and both galacturonic and arabinose polysaccharides are present respectively in OH and SBP. This study provides information on the best suitable extraction method for producing a crude pectin extract causing minimal damage to pectic polysaccharides from the agro-industrial residues under study. The present research further broadens the possibilities for the production of pectic oligosaccharides and partly for producing minimally damaged pectin at an industrial scale especially useful for gelling properties.

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Declaration of Interest

Authors have no conflict of Interest.

Supporting information

Information on HMF and furfural formation during nitric acid extraction and molecular weight distribution of the pectic polysaccharides during nitric acid extraction as well as enzymatic extraction can be found in the supplementary figures. The supporting information is available free of charge via the internet at <http://pubs.acs.org>.

544   **Abbreviations:**

BP	Berry pomace
DAc	Degree of acetylation
DM	Degree of methylation
HPAEC-PAD	High performance anion exchange chromatography- pulsed amperometric detection
HPSEC	High performance size exclusion chromatography
HMF	Hydroxy methyl furfural
OH	Onion hulls
POS	Pectic oligosaccharides
PP	Pressed pumpkin
MW	Molecular weight
SBP	Sugarbeet pulp

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Legends to Figures:

Fig 1: Effect of time on the extraction of neutral and acidic pectic polysaccharides from berry pomace by (a) enzymatic and (b) chemical pretreatment

Fig 2: Effect of time on the extraction of neutral and acidic pectic polysaccharides from pressed pumpkin by (a) enzymatic and (b) chemical pretreatment

Fig 3: Effect of time on the extraction of neutral and acidic pectic polysaccharides from sugar beet pulp by (a) enzymatic and (b) chemical pretreatment

Fig 4: Effect of time on the extraction of neutral and acidic pectic polysaccharides from onion hulls by (a) enzymatic and (b) chemical pretreatment (c) ammonium oxalate and (d) sodium hexametaphosphate

Fig 1a

LSD ($p < 0.05$) values for galactose polysaccharides, galacturonic acid polysaccharides, and galacturonic acid are 0.34, 0.76 and 0.56, respectively.

Fig1b

LSD ($p < 0.05$) values for galactose polysaccharides and galacturonic acid polysaccharides are 0.05, 0.08 respectively.

Mean values having the same superscript are not significantly different ($P < 0.05$).

Control: galacturonic acid polysaccharides is 1.1% w/w on d.m.

LSD: Least significant difference Values are reported on the basis of dry matter

Fig 2a

LSD ($p < 0.05$) values for galactose polysaccharides, galacturonic acid polysaccharides, galactose and arabinose polysaccharides are 0.98, 0.85, 0.80 and 0.61, respectively.

Fig 2b

LSD ($p < 0.05$) values for galactose polysaccharides, galacturonic acid polysaccharides and galactose are 0.09, 0.20 and 0.05, respectively.

Mean values having the same superscript are not significantly different ($P < 0.05$).

Control : galactose 0.7% w/w ; galactose polysaccharides is 1.0 % w/w on d.m.

LSD: Least significant difference

Values are reported on the basis of dry matter

Fig 3a

LSD ($p < 0.05$) values for galactose polysaccharides, galacturonic acid polysaccharides and arabinose polysaccharides are 0.99, 0.99 and 0.09 respectively.

Fig 3b

LSD ($p < 0.05$) values for galacturonic acid polysaccharides, arabinose polysaccharides and arabinose are 0.06, 0.08 and 0.08, respectively.

Mean values having the same superscript are not significantly different ($P < 0.05$).

Control: Arabinose polysaccharides is 5.5 % w/w; galactose polysaccharides is 1.4 % w/w; galacturonic acid polysaccharides is 6.6 % w/w

LSD: Least significant difference

Fig 4a

LSD ($p < 0.05$) values for galacturonic acid polysaccharides and galacturonic acid are 0.77 and 0.82, respectively.

Fig 4b

LSD ($p < 0.05$) values for galactose polysaccharides, galacturonic acid polysaccharides are 0.13, 0.01 respectively.

Fig 4c

LSD ($p < 0.05$) values for ammonium oxalate (1%) and (0.5%) are 0.39 and 0.45, respectively.

Fig 4d

LSD ($p < 0.05$) values for galacturonic acid polysaccharides (0.5 h) and (1 h) are 0.48 and 0.30 respectively.

Mean values having the same superscript are not significantly different ($P < 0.05$). The superscripts of different sugars are marked with asterisk sign to depict the difference.

Control: Did not show extraction of any monomer /polymer.

LSD: Least significant difference

Values are reported on the basis of dry matter.

Legends to Tables

Table 1: Summary of extraction conditions used for extracting pectin from agro-industrial residues.

Table 2: Galacturonic acid and neutral sugar composition of different agro-industrial residues

Table 3 : Effect of best pretreatment on the extraction efficiency and degree of acetylation & methylation of pectin extracted from various agro-industrial residues

Table 1

Extractant	Hydrolysis medium	pH during hydrolysis	Concentration of extractant	Incubation conditions	Time (h)	Neutralization	Enzyme inactivation
Nitric acid	Water	1.4	0.4% w/v	48 °C, 150 rpm	12-48	10 M NaOH to pH 4.8	-
Enzyme (Celluclast 1.5 L)	Citrate buffer 50 mmol	4.8	20 FPU/g d.m	80 °C, 125 rpm	2-8	-	100 °C, 5 min
Ammonium* Oxalate	Water	4.8	0.5 and 1% w/v	90 °C**	0.5-2	-	-
Sodium hexameta phosphate*	Water	4.8	1-5% w/v	95 °C**	0.5-1	-	-

* used only for onion hulls

** without stirring

Table 2

Residue	Galacturonic acid	Rhamnose	Arabinose	Galactose	Xylose*	Glucose*	Fructose
% (w/w) d.m							
BP	5.9 ± 0.6	0.4 ± 0.1	0.9 ± 0.1	4.7 ± 0.1	5.2 ± 0.3	18.9 ± 0.5	8.9 ± 0.1
PP	5.6 ± 0.1	0.7 ± 0.4	0.6 ± 0.4	3.7 ± 0.2	1.4 ± 0.1	15.2 ± 0.3	4.3 ± 0.2
SBP	17.3 ± 0.9	1.2 ± 0.4	16.6 ± 0.6	5.7 ± 0.1	2.1 ± 0.2	18.8 ± 0.2	0.9 ± 0.1
OH	19.3 ± 0.9	0.4 ± 0.5	0.3 ± 0.1	7.0 ± 0.9	2.9 ± 0.2	26.1 ± 0.1	N.D

Non-hydrolysed cellulose, lignin, protein, hemicellulose and other extractives make up for the remainder of the composition.

Values are mean ± SD, $n=3$.

BP: Berry pomace; PP: pressed pumpkin ; SBP: sugar beet pulp OH: onion hulls.

N.D- Not detectable

Values of glucose

* Xylose and glucose values are reported after seaman hydrolysis.

Table 3

Residue	Extraction process	Conditions	Galactose	Arabinose		alacturonic acid		Total Yield** % (w/w) d.m		DAc (%)	DM (%)	
			Extraction efficiency %									
			POLY	MONO	POLY	MONO	POLY	MONO	POLY	MONO		
BP	Enzymatic	24 h	85.9	N.D	N.D	N.D	59.0	20.0	7.6	1.1	6.4 ± 0.4	32.7 ± 1.7
	Nitric acid	2 h, pH 1.4	15.9	N.D	N.D	N.D	62.9	N.D	4.5	N.D	4.7 ± 0.7	72.5 ± 5.1
PP	Enzymatic	12 h	44.3	37.7*	N.D	N.D	75.0	N.D	6.5	0.9	58.3 ± 5.4	57.6 ± 5.8
	Nitric acid	6 h, pH 1.4	82.2	N.D	N.D	N.D	59.2	N.D	6.3	0.8	16.3 ± 0.5	72.1 ± 1.6
SBP	Enzymatic	48 h	98.4	N.D	97.5	-	89.8	N.D	37.8	N.D	59.1 ± 0.9	51.9 ± 0.9
	Nitric acid	4 h, pH 1.4	53.9	N.D	83.2	10.9	76.5	N.D	29.8	2.1	48.5 ± 0.4	62.6 ± 0.8
OH	Enzymatic	12 h	N.D	N.D	N.D	N.D	10.8	15.5	2.3	1.2	8.1 ± 0.4	29.2 ± 1.7
	Nitric acid	8 h, pH 1.4	13.4	N.D	N.D	N.D	16.3	N.D	4.1	N.D	20.6 ± 6.9	56.3 ± 3.4
	Ammonium oxalate 0.5% (w/v)	0.5 h, 95 °C	N.D	N.D	N.D	N.D	100	N.D	22.2	N.D	3.7 ± 0.8	18.6 ± 0.4
	Sodium Hexameta phosphate 2.0% (w/v)	0.5 h, 95 °C	N.D	N.D	N.D	N.D	100	N.D	22.0	N.D	4.0 ± 0.3	23.8 ± 1.3

* 18.9 % of galactose monosaccharides were already present in the control sampleBP: berry pomace; PP: pressed pumpkin; SBP: sugar beet pulp; OH: onion hulls; DAc: Degree of acetylation; DM: Degree of methylation ** Sum of galactose, arabinose and galacturonic acid polysaccharides and monosaccharides

TOC Graphic: