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Ultrasound-assisted enzymatic extraction of orange peel pectin and its characterisation

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Summary Citrus processing waste (CPW) accumulates in vast amounts worldwide during fruit processing and its disposal through landfilling is environmentally damaging. However, CPW is also a renewable source of economically and medically relevant compounds such as pectin. There is a need for new methods of extracting pectin from CPW, as conventional methods are unsustainable. Therefore, this study proposed a new environmentally friendly method, which combines ultrasound cavitation with cellulase preparation, Celluclast) into one extraction procedure (UAE), and compared it to conventional acid extraction. The yield and productivity of each method were measured and the extracts were analysed for composition and structural characteristics. UAE and acid extraction pectin yields (26.9 vs. 22.1%) and productivity (0.10 vs. 0.09%/min) values were comparable. The extracts consisted mainly of galacturonic acid and galactose residues, but the ratio was different for each method; 1: 0.34 and 0.84: 1 for acid and UAE-extracted pectin, respectively. FTIR was used to estimate the degree of esterification of the pectin extracts; 8% and 55% for acid and UAE-extracted pectin, respectively. TGA revealed a higher ash content in UAE pectin, while XRD revealed similar degrees of polymer crystallinity. Viscosimetry revealed that the acid pectin had a higher molecular weight (70.22 kDa) compared to UAE pectin (197.78 kDa). In conclusion, the UAE procedure is a viable alternative to acid extraction.

Keywords citrus processing waste, pectin extraction, ultrasound-assisted enzyme extraction.

Introduction

The orange industry is an enormous global sector that produces over 76 million tons of oranges and converts over 2% of this into secondary products (FAO, 2021). Citrus processing waste (CPW) is a solid by-product of the orange juice industry and is generated in large quantities, up to 5 million tonnes in 2019 in Brazil, the largest citrus-producing nation (FAO, 2021). CPW poses a significant environmental problem, as the decomposition of this organic waste is known to generate greenhouse gases and other toxic compounds that pollute soil and water (Lin *et al*., 2013). Because of this, the EU put legislation in place to prevent the landfilling of organic waste, such as CPW, before resources have been recovered from it (Negro *et al*., 2017).

CPW is a great renewable resource that can be used to produce high-demand products, such as fuel, fertilisers and nanomaterials (Satari & Karimi, 2018). CPW is also a source of bioactive compounds, including essential oils, flavonoids, polyphenols and polysaccharides (Sharma *et al*., 2017). These bioactive compounds are seeing potential applications as antibacterial and anticancer agents or in the treatment of cardiovascular disease (Li *et al*., 2019; Addi *et al*., 2022). Pectin is one such compound that is used as a thickener in the food and cosmetics industries, and has the potential as a nutraceutical in the treatment of lifestyle diseases (Naqash *et al*., 2017; Belkheiri *et al*., 2021).

Pectin refers to a group of complex anionic heteropolysaccharides that comprise mainly galacturonic acid (GalA) and other sugar residues, including rhamnose (Rha), galactose (Gal) and arabinose (Ara) (Mohnen, 2008). Pectin fractions include homogalacturonan (HG), rhamnogalacturonan I (RG-I) and II (RG-II), and xylogalacturonan (XG) (Kaya *et al*., 2014). The galacturonic acid residues in pectin can be esterified through methylation and acetylation. Apple pomace (15%) and citrus peel (85%) are the main sources of commercial pectin.

Pectin is generally extracted from CPW by suspending the biomass residue in hot acidified water, this degrades the plant cell wall matrix and solubilises the pectin (Yapo *et al*., 2007). The technique is effective,

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but produces acidic wastewater, which is an environmentally harmful by-product (Freitas *et al*., 2020). As such, intensive research is conducted to develop new environmentally friendly and high-yield extraction processes to replace acid extraction in pectin production from CPW (Chandel *et al*., 2022).

This study aimed to develop an unconventional technique, namely ultrasound-assisted enzyme (UAE), for the extraction of pectin from CPW. This involved the use of ultrasonic cavitation to aid penetration of the enzyme cocktail, followed by a cellulase preparation (Celluclast) treatment to degrade the cell wall matrix in orange peels for the release and solubilisation of pectin. Parallel to the UAE procedure, a conventional acid extraction procedure was also conducted. Pectin extracted using both techniques was compared in terms of yield and productivity. Additionally, the effect of each technique on the composition, physical properties and structure of the pectin was also evaluated.

Materials and methods

Materials and equipment

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) unless otherwise stated and were of analytical grade. Celluclast was purchased from Novozymes (Copenhagen, Denmark). A 50 mM sodium citrate buffer (pH 5.0) was used for all applications. The dinitrosalicylic acid (DNS) reagent for quantification of reducing sugars consisted of 43.8 mM DNS, 25 mM NaOH, 105 mM Rochelle salt, 0.18 mM sodium metabisulphite and 2% (w/v) phenol prepared in dH_2O. Orange peels derived from 6 kg of commercial oranges (Freshmark, Centurion, South Africa) were provided by the Department of Consumer and Food Sciences, University of Pretoria, South Africa. The SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices, San Jose, California, USA) was used for spectrophotometric analysis.

Determination of the substrate specificity of Celluclast

The endo-active substrate specificity of Celluclast was determined using polymeric substrates, such as citrus pectin, carboxymethylcellulose (CMC), Larchwood arabinogalactan, wheat flour arabinoxylan and linear arabinan (Megazyme, Bray, Wicklow, Ireland). The enzyme assay was performed with 1% (w/v) polysaccharide substrate and incubated at 50 °C for 30 min. The DNS reagent was then used to quantify the liberated reducing sugars, with galacturonic acid, glucose, xylose and arabinose being used as suitable standards (Miller, 1959).

The exo-activity of Celluclast was assayed with a *p*-nitrophenyl (*p*-NP)-based assay using the following chromogenic substrates; *p*-NP-glucopyranoside, *p*-NP-galactopyranoside, *p*-NP-arabinofuranoside and *p*-NP-xylopyranoside (Parry *et al*., 2001). To perform the assay, the substrates, at a concentration of 2 mM, were mixed with appropriately diluted enzyme and incubated at 50 °C for 20 min. To terminate the reaction, an equal volume of 1 M NaOH was added to the reaction volume, and the absorbance was measured at 405 nm.

Extraction of pectin from orange peels

Orange peels, consisting of both albedo and flavedo, were cut into pieces of approximately 1 cm^2 with scissors and blended into a puree until no orange peel pieces were visible. The puree was then drained and washed three times with distilled water through a cotton cloth and dried in a 50° C oven until a constant weight was reached. The dried peel residue was then further ground by blender to a fine flour, which served as the starting material for pectin extraction.

Acid extraction of pectin

About 1 g of orange peel waste flour was suspended in 20 mL of acidified dH_2O (pH 1.5) with hydrochloric acid, which served as the control extraction solution. The slurry was then incubated in an 80 °C water bath for 4 h, stirring intermittently every 15 min. The slurry was then placed in an ice bath to cool down before centrifugation with a Heraeus Megafuge 8R (Thermo Fischer Scientific, Massachusetts, USA) at 8586 ***g*** for 30 min. The pectin solution was separated from the insoluble CPW flour and decanted into a Schott bottle.

Ultrasound-assisted enzymatic extraction of pectin

Approximately 1 g of orange peel flour was suspended in 19 mL of 50 mM sodium citrate buffer (pH 5.0). The slurry was then subjected to cavitation in an ultrasonic bath (9 L digital ultrasonic cleaner, Eins Sci, Johannesburg, South Africa) at full power (40 kHz, 300 W) and 80 °C for 30 min. The slurry was then placed in an ice bath to cool to 50 °C before 1 mL of Celluclast (64.7 mg/mL) was added and then incubated for 4 h at 50 °C and 70 rpm in a Roto-Therm rotary mixer (Benchmark, Sayreville, New Jersey, USA). After extraction, the enzyme was denatured by heating the slurry to 100 °C for 5 min. The slurry was then placed in an ice bath to cool down before centrifugation with a Heraeus Megafuge 8R (Thermo Fischer Scientific, Massachusetts, USA) at 8000 rpm for 30 min. The pectin solution was separated from the insoluble CPW flour and decanted into a Schott bottle.

Pectin precipitation and washing

The pectin solution was precipitated by the addition of 40 mL of 95% (v/v) EtOH and refrigeration overnight

at 4 °C. The precipitated pectin was washed under suction in a Büchner funnel with 20 mL of EtOH to acetone at a ratio of 1:1. The pectin was collected and dried overnight at 50 °C in an oven until a constant weight was reached. The dried pectin was weighed to determine the yield and stored in a dry place for further use.

Analysis of pectin composition

Trifluoracetic acid hydrolysis of pectin

The monosaccharide composition of the extracted pectin was determined after hydrolysis of 10 mg powder with 1 mL of 2 M trifluoroacetic acid (TFA) in a dry digital bath (Eins Sci, Johannesburg, South Africa) for 2 h at 120 °C. The residual TFA was evaporated from the sample by incubating it in a hot air oven at 80 °C for 1 h. The sample was then dried and resuspended in deionised water for sugar analysis.

Monosaccharide composition of pectin

The monosaccharide composition of pectin was determined using sugar determination kits; K-URONIC, K-RHAMNOSE, K-ARGA, K-XLOSE, and K-GLUC, sourced from Megazyme (Dublin, Ireland), to quantify GalA/glucuronic acid (GlcA), Rha, Ara/Gal, xylose (Xyl) and glucose (Glc), respectively, according to the manufacturer's instructions.

Total sugar content of pectin

The sulphuric acid-UV assay was used to estimate the total sugar content of the pectin, using galacturonic acid as a suitable standard. The method was developed and optimised using previously described methods (Albalasmeh *et al*., 2013; Chen *et al*., 2023). Spectrophotometric measurements at 290 nm were made at room temperature and the total sugar was estimated and expressed as total sugar per dry mass of the pectin.

Determination of the phenolic content in pectin

The Folin-Coicalteu assay was used to detect phenolic impurities in the extracted pectin, as described previously, using gallic acid as a suitable standard (Malgas *et al*., 2016). Spectrophotometric measurements were conducted immediately after the heating step at 40 °C and phenolic concentration was expressed gallic acid equivalents per dry mass of the pectin.

Protein content determination in pectin

The Bradford assay was used to detect protein impurities in the extracted pectin, without alteration, using bovine serum albumin as a suitable standard (BSA) (Bradford, 1976). Spectrophotometric measurements were conducted at room temperature and protein concentration was estimated in terms of BSA equivalents.

Pectin structural analysis

Analysis of pectin viscosity and viscosity average molecular weight

The viscosity average molecular weight and intrinsic viscosity ([η]) of pectin was estimated by capillary viscometry. A 10 mg/mL pectin solution, prepared with a 100 mM NaCl solution, was used to prepare a range of dilutions between 0.1 and 1.0 mg/mL for the viscometrical analysis using a Cannon-Manning size 50 semi-micro viscometer (Cannon, Pennsylvania, USA). First, the relative viscosity (η_r) of the pectin was calculated using the following equation.

$$\eta_r = \frac{\eta}{\eta_s}$$

where η_r is the relative viscosity, η is the viscosity of the pectin solution (mPa s), and η_s is the viscosity of the solvent. Finally, the inherent viscosity (η_{inh}) was calculated using the following equation:

$$\eta_{inh} = \frac{\ln \eta_r}{C}$$

where η_r is the relative viscosity and C is the pectin concentration (g dL^{-1}). The values of inherent viscosity were plotted against the concentrations of the polymer solution and the intrinsic viscosity (the limit of inherent viscosity when the concentration approaches zero dilution) was determined by extrapolating the curve using the Kraemer method. The Mark-Houwink equation was used to relate the intrinsic viscosities to the viscosity average molecular weight (M). The Mark-Houwink constants for this system are $\alpha = 0.78$ and $K = 4.36 \times 10^{-5}$ L/g, which are characteristic of the solvent-polymer system (Halabalová *et al*., 2004).

$$[\eta] = KM^{\alpha}$$

Functional group analysis of pectin by Fourier-transform infrared (FTIR)

A Spectrum 100 FTIR spectrophotometer (Perkin Elmer, Waltham, USA) was used for the analysis of pectin. Each sample was pressed evenly and firmly against the sample spotting surface with a spring-loaded anvil. FTIR spectra were obtained by averaging 64 scans from 4000 to 650 cm^{-1}. Baseline and ATR corrections for penetration depth and frequency variations were performed using Spectrum One software supplied with the instrument.

Two peaks corresponding to either esterified (1740 cm^{-1}) or non-esterified (1600 cm^{-1}) galacturonic acid were identified and resolved using OriginPro, version 2022 (OriginLab Corporation, Massachusetts, USA), assuming a Gaussian distribution. The area under the resolved peaks was calculated using GraphPad Prism version 9.0.0 (GraphPad Software,

California, USA). These peaks were used to estimate the degree of esterification (DE) using the following equation (Mariana de Fátima *et al.*, 2011).

$$DE = \frac{\text{Area at } 1740\ cm^{-1} \text{ peak}}{\text{Area at } 1740\ cm^{-1} + \text{Area at } 1600\ cm^{-1} \text{ peaks}} \times 100\%$$

Glycosidic linkage analysis of pectin by nuclear magnetic resonance (NMR)

The extracted pectin was analysed by H^1 NMR spectroscopy using a Bruker Avance III 400 MHz spectrometer with a BBI probe (Bruker, Karlsruhe, Germany). For NMR analysis, 50 mg pectin was suspended in 1 mL D_2O (99.96%) (Merck, Darmstadt, Germany) and the spectra were recorded at ambient temperature. The spectra were processed and analysed using TopSpin NMR software, version 3.6.5 (Bruker, Karlsruhe, Germany).

Relative crystallinity determination of pectin by X-ray powder diffraction (XRD)

The crystallinity of pectin was determined by XRD using CU K radiation (1.5405 Å, nickel filter) on a Bruker D8® Discover, equipped with a proportional counter as described previously (Gabrielii *et al.*, 2000). Samples were scanned from 2θ of 10 to 40° with a step size of 0.02°. The determination time was 0.02^0 per second. The relative crystallinity of the polysaccharides (CrI) was estimated by inspecting the intensity and sharpness of their respective generated peaks.

Thermogravimetric analysis of pectin

Thermogravimetric analysis of pectin was performed using a thermogravimetric analyser (PerkinElmer®, Pyris Diamond model). Approximately 4 mg of each sample was placed in an aluminium crucible for analysis. Nitrogen (purity 99.99%), with a flow rate of 20 mL/min was used as a carrier gas in all experiments to minimise the mass transfer effect. The pectin was heated from 30 to 700 °C at a heating rate of 30 °C/min. In each test, a separate blank run was performed to correct the baseline, using an empty pan. Finally, the mass loss relative to the temperature increment was automatically recorded, and the derivative thermogram (DTG) was plotted using GraphPad Prism 9.0.0.

Statistical analysis

All extractions and assays in this study were performed in triplicate technical replication, with a minimum of two biological repeats, except for TGA, NMR XRD and FTIR analyses. Significant differences among means were compared using either Tukey's test or an unpaired *t*-test with $P < 0.05$. Data analysis was conducted with GraphPad Prism 9.0.0.

Results and discussion

Celluclast substrate specificity

The specific activity of Celluclast towards model substrates representing different fractions of lignocellulosic biomass was determined (Table 1). The cellulolytic activity of the preparation towards cellulose was in accordance with the activity (700 endo-glucanase units (EGU)/g or 0.7 EGU/mg) reported by the manufacturer, Novozymes (Bagsværd, Denmark) (https://biosolutions.novozymes.com/en/juice-fruit-vegetables/products/vegetables/celluclast-1.5-l). This activity is expected to be exhibited by the endo-β-1,4-glucanase (EC 3.4.1.4) fraction of the cellulase preparation.

The cellulase preparation also showed high endo-β-1,4-xylanase (EC 3.2.1.8) activity (1.72 U/mg), which was not surprising as previous studies have reported high endo-β-1,4-xylanase activities of the cellulase preparation; 438.8 U/mL and 813.9 g/mL/min per mg protein (Hu *et al.*, 2011; Gama *et al.*, 2015). The major exolytic activities exhibited by Celluclast were β-glucosidase (EC 3.2.1.21) and β-xylosidase (EC 3.2.1.37) activities (Table 1). Other studies have reported the β-glucosidase and β-xylosidase of Celluclast (Hu *et al.*, 2011; Suwannarangsee *et al.*, 2012; Gama *et al.*, 2015).

Overall, the main activities of Celluclast are ideal for the degradation of cellulosic and hemicellulosic fractions in lignocellulosic biomass, making the cellulase preparation ideal for the extraction of pectin from CPW. It is worth noting that there was a slight polygalacturonase (EC 3.2.1.15) or pectate lyase (EC 4.2.2.2) activity against citrus pectin and an α-L-arabinofuranosidase (EC 3.2.1.55) activity against *p*-NP-arabinofuranoside, suggesting that Celluclast may be able to hydrolyse some of the bonds in the pectin backbone structure and arabinan side chains in RG I or II-type pectin during extraction. This is undesirable,

Table 1 Celluclast-specific activity against model lignocellulose-derived substrates.

Substrate	Specific Activity (μmol/min/mg)	Relative activity (%)
Carboxymethyl cellulose	0.70 ± 0.03	41[a]
Citrus pectin	0.19 ± 0.12	11[a]
Wheat flour arabinoxylan	1.72 ± 0.50	100[a]
Larchwood arabinogalactan	Nd	0[a]
Sugar beet linear arabinan	Nd	0[a]
p-NP-galactopyranoside	0.28 ± 0.10	1[b]
p-NP-arabinofuranoside	7.17 ± 0.14	31[b]
p-NP-xylopyranoside	9.13 ± 0.8	39[b]
p-NP-glupyranoside	23.30 ± 9.78	100[b]

Values are represented as mean values ± SEM ($n = 2$). Where Nd = not detected, [a] = reducing sugar release and [b] = *p*-nitrophenol release.

but the unwanted activity was considered low enough to use this enzyme preparation for pectin extraction.

Pectin yield and productivity

To evaluate the success of the UAE procedure, the yield and productivity of the method were compared with that of the conventional acid extraction procedure (Fig. 1). The yield of the acid extraction procedure (22.1%) was similar to that reported in the literature (Pandharipande & Makode, 2012) and was therefore used as a benchmark for comparison with the UAE procedure. It is worth noting that the UAE extract had a higher yield (26.9%) but took 30 min longer (270 min vs. 240 min) and the resulting productivity was almost identical to the acid extraction procedure (0.1 vs. 0.09%/min). The yield and productivity of pectin extraction by the UAE method in this study were within acceptable ranges for the obtention of pectin, 17.7–26.3% and 0.06–0.11%/min, respectively, from citrus peel sources by enzymatic extraction reported in the literature (Bezus *et al.*, 2022). The extraction yield values between the acid and UAE procedure were not significantly different from each other ($P > 0.05$). In summary, the data show that the developed novel procedure is as effective as the traditional acid extraction method in fractionating pectin and is a viable alternative as a green extraction procedure.

Chemical composition of pectin

To characterise the pectin, the composition of each sample was analysed. The phenol and protein content as well as the content of individual monosaccharides of each extract were determined (Table 2). In addition, the composition of commercial pectin was also determined for comparison. No apparent protein or phenol impurities were found in the pectin samples under consideration. The total sugar content of the pectin was also assessed and it was shown that the acid extract had the highest amount of carbohydrate (77.57%), while the UAE and commercial citrus pectin were composed of a slightly lower carbohydrate fraction; 60.54% and 67.03%, respectively. The lower value of carbohydrate in the UAE pectin may be attributed to a higher ash content as determined by TGA.

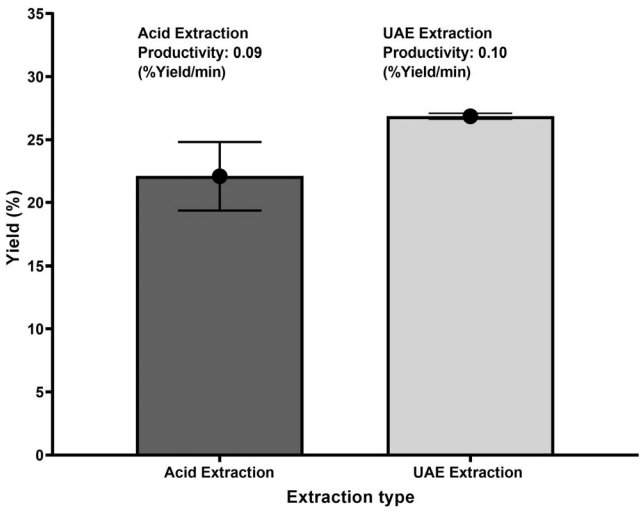

Figure 1 Comparison of the yield and productivity of acid and UAE extraction methods. Values are represented as means ± SEM, $n = 2$. The yields were not significantly different between the two extraction procedures as estimated by an unpaired *t*-test ($P < 0.05$).

The major monosaccharide residues detected in both extracted pectin samples were GalA and Ara/Gal, with almost equal amounts in the acid extract (GalA to Ara/Gal at 0.84: 1), which is comparable to literature values (Kaya *et al.*, 2014). However, the UAE extract had a significantly lower Ara/Gal content (1: 0.34), likely due to the α-L-arabinofuranosidase exolytic activity of Celluclast which may have cleaved arabinofuranosyl-linked substituents in pectin. Similar to our findings, a recent study showed that the use of an endo-α-1,5-arabinanase and endo-β-1,4-mannanase for the extraction of apple pectin led to the shortening of arabinan side chains in RG-I (Wikiera *et al.*, 2022).

No Rha was detected in the pectin samples, which is surprising as it is the second most abundant sugar residue in pectin of the RG-I type, which has a high Ara/Gal content. Its absence suggests that the pectin samples fractionated in this work consist almost exclusively of homogalacturonan (HG). However, the presence of Ara/Gal indicates otherwise, as these residues are the main components of RG-I (Kaya *et al.*, 2014).

Structural features of pectin

Intrinsic viscosity and viscosity average molecular weight of pectin

To investigate the underlying structure of the pectin, the intrinsic viscosity and viscosity average molecular weights of the extracts were determined by capillary viscometry (Table 2). The acid-extracted pectin had the highest intrinsic viscosity (0.59 g dL^{-1}) and molecular weight (197.78 kDa) and showed similar values; 0.4276 g dL^{-1} and 152 kDa, to the acid-extracted pectin of another author (Guo *et al.*, 2012). The UAE-extracted pectin had much lower values; 0.26 g dL^{-1} and 70.22 kDa, than the acid extract, indicating substantial degradation of the polysaccharide by the Celluclast enzyme cocktail during extraction.

The molecular weight of pectin affects its solubility and gelling abilities, which in turn determines its potential applications (Gawkowska *et al.*, 2018). Enzymatic extraction has been shown to lead to low Mw

Table 2 Monosaccharide composition, total sugars, intrinsic viscosity and molecular weight of the pectin samples.

Pectin sample	Monosaccharide content (%)		Total sugar (%)	η (g dL^{-1})	*M* (kDa)
	GalA	Ara/Gal			
Acid extract	20.91 ± 1.64^{a}	24.77 ± 1.49^{a}	77.57 ± 1.67^{a}	0.59 ± 0.07^{a}	197.78 ± 28.62^{a}
UAE extract	22.77 ± 2.45^{a}	7.71 ± 0.49^{b}	60.54 ± 2.81^{b}	0.26 ± 0.04^{b}	70.22 ± 11.94^{b}
Pectin control	10.68 ± 2.71^{b}	14.12 ± 2.36^{b}	67.03 ± 2.78^{ab}	0.24 ± 0.01^{b}	61.68 ± 3.60^{b}

Values are given as mean values ± SEM (n = 2). Values with different letter superscripts (a, b) were significantly different as estimated by Tukey's test ($P < 0.05$).

and viscosity pectin, leading to alterations in the functional properties of the pectin (Alba & Kontogiorgos, 2017). The low molecular weight pectin produced by the UAE extraction method may serve as a dietary additive to improve liver cholesterol levels, serve as a satiety modifier and prevent cardiac hypertrophy, and in other applications, especially where low solubility and viscosity are desirable (Yamaguchi *et al.*, 1994; Tiwary *et al.*, 1997; Li *et al.*, 2021)

Glycosidic linkage analysis of pectin by NMR

As part of the structural analysis, the pectin extracts were characterised by H^{1}-NMR (Fig. 2), revealing that the major pectin types in the samples are HG, consisting of α-1,4-linked GalA residues (5.0 ppm); RG-I, consisting of Rha and GalA residues linked by α-1,2- and α-1,4-linkages (5.16 and 3.34 ppm); RG-II which has four side chains connected to an HG backbone, each through one of the following linkages: 3-Deoxy-D-Lyxo-Heptopyran-2-ularic Acid (Dha)-β-3,2- (1.74, 2.16 and 4.00–4.10 ppm), 3-deoxy-D-manno-octulosonate (Kdo)-α-2,3- (1.74, 2.02 and 4.02 ppm) and two different apiose (Api)-β-1,2-linkages (4.11 and 4.38 ppm for a short chain and 4.31 and 5.10 for a long chain). Some other notable structures include Ara and Gal side chains (4.23–4.28 and 3.75, respectively) and the presence of methylation/acetylation (3.75–3.84 and 2.05–2.13, respectively) (du Penhoat *et al.*, 1999; Khodaei & Karboune, 2013; Kpodo *et al.*, 2017).

As per the above-named signals, small peaks for Rha/GalA residues were also evident in all samples, indicating the presence of RG-I. No peaks for Ara were visible, indicating that the Ara/Gal content measured by monosaccharide analysis is predominantly or exclusively galactose and not arabinose. The presence of RG-II is confirmed in the pectin control sample by peaks corresponding to Dha, Kdo and short-chain Api residues. RG-II in the extract samples is suggested by peaks for Dha and Kdo, but the Api peaks are not as pronounced. The types of chains in RG-II are known to vary depending on the source, so this is not unexpected. The peaks for acetylation appear in all the samples but are quite weak and the data is inconclusive. Only the ultrasound-assisted enzyme extract shows a clear peak for methylation. The chemical shift for methoxy groups (-OCH$_3$) at 3.7 ppm was observed in all pectin samples, while the acetyl group (-COCH$_3$) associated shift around 2.0 ppm was only observed in the acid-extracted and the commercial pectin.

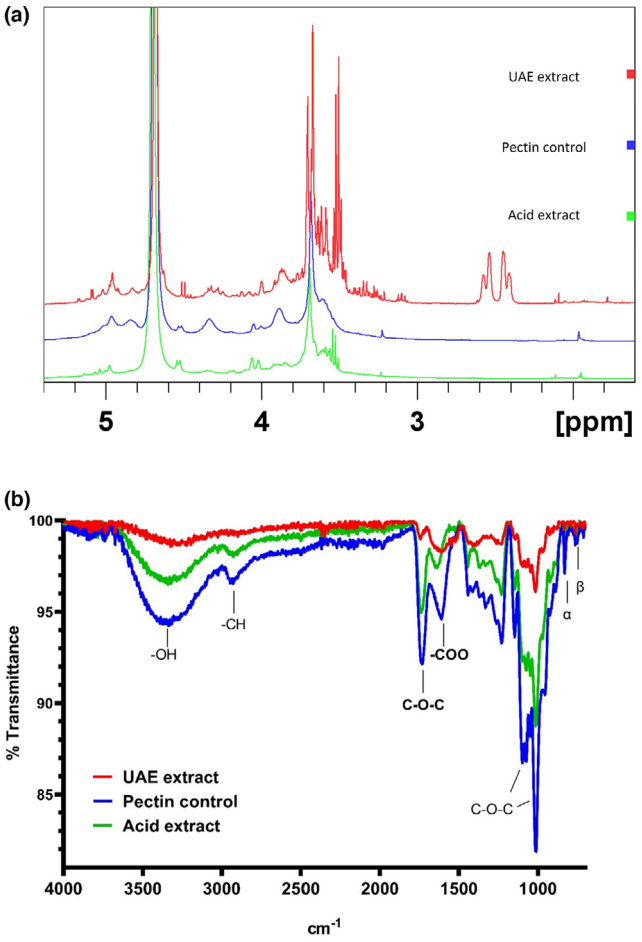

Figure 2 FTIR (a) and NMR (b) spectra of the pectin; blue is commercial citrus pectin, and green and red are acid and ultrasound-assisted enzymatic extracted pectin from orange peels, respectively.

Functional group analysis of pectin by FTIR

Further structural characterisation of pectin was performed using FTIR to identify the functional groups present in pectin (Fig. 2). The FTIR peaks of each sample were quite similar, each showing the major expected peaks for carbohydrates, such as the broad glycoside hydroxyl peak around 3300 cm^{-1}, the glycoside methyl peak at 2900 cm^{-1}, the carboxylic acid peak at 1800 cm^{-1} and the glycosidic bond/ester bond at 1200 and 1000 cm^{-1} (Bayar *et al*., 2017; Hosseini *et al*., 2019). The 1700 and 1650 cm^{-1} peaks correspond to esterified and unesterified carboxylic acids on the GalA residues, respectively, and were used to calculate the degree of esterification of the pectin extracts (Mariana de Fátima *et al*., 2011; Hosseini *et al*., 2019). The peaks at 800 and 700 cm^{-1} correspond to the conformation of the α- and β-glycosidic bonds, respectively, and can be used to identify the dominant conformation in polysaccharides (Bayar *et al*., 2017). The peak at 800 cm^{-1} was the most prominent in all pectin samples, indicating a high proportion of α-glycosidic bonds in the polysaccharide. This is to be expected as HG, the most common form of pectin consists entirely of α-linked GalA residues.

The esterification analysis showed a degree of esterification of 8% for the UAE and 55% for the acid extract. This finding corroborates the findings from NMR data regarding the esterification of the pectin samples. The low value for DE in the UAE extract suggests that Celluclast may be de-esterifying the pectin, but the evidence is inconclusive as the FTIR signal for the sample was quite weak. Celluclast has been reported to display acetyl xylan esterase (EC 3.1. 1.72) activity (0.85 U/mg) (Juhász *et al*., 2005). We suspect it may be presenting pectin esterase side activity and causing the decrease in DE of the UAE-extracted pectin. Similarly, studies have shown that apple and berry fruit pectin isolated with a combination of endo-α-1,5-arabinanase and endo-β-1,4-mannanase (Wikiera *et al*., 2022), and Celluclast (Muñoz-Almagro *et al*., 2021) respectively, exhibited a low DE.

The specific gelling conditions of pectin also depend on its DE, with low esterified pectin forming heat-stable gels in the presence of calcium, whereas highly esterified pectin gels at low pH and high sugar content (Thakur *et al*., 1997). Due to its easier gel-forming capacity, low-methyl-esterified pectin exhibits better potential than the mineral acid-extracted high-methyl-esterified pectin for application to low-sugar products in the food industry, meeting the increasing consumer demand for low-calorie or dietetic healthy diet. Dairy products are usually a natural source of calcium, which can act as a gelling catalyst for low esterified pectin.

Pectin structural arrangement as determined by XRD

The overall bulk structure of the pectin extracts was investigated by XRD (Fig. 3). Amorphous polysaccharides are those in which all possible hydrogen bonding takes randomly place between the molecules (Mazeau & Rinaudo, 2004). As a result, amorphous materials are characterised by the irregular and random arrangements of molecules, creating a loosely packed structure. Due to this structural organisation, amorphous materials do not have any symmetry and interfacial angle, therefore, generating broad X-ray scattering profiles during XRD analysis. In contrast, crystallinity is a result of certain, regular omissions of these bonds due to long linear chains, or repeating patterns in the overall structure (Mark *et al*., 2017). Crystallinity is known to affect the properties of polysaccharides, including solubility and gelling ability, both of which are reduced in crystalline materials (Mark *et al*., 2017).

As can be seen, the pectin samples showed several sharp and intense peaks at 12, 18, 21, 31, and 40° (2θ) which are due to its crystallinity (Figs 3 and 4). Overall, the order of relative *CrI* values seemed to be acid-extracted > commercial > UAE-extracted pectin. The presence of broad, flat peaks indicates a predominantly amorphous structure, but the presence of some sharp peaks suggests some ordering of the underlying pectin structure (Sami *et al*., 2010). This suggests a highly branched polysaccharide with only some regular association of the linear chains (Mark *et al*., 2017). The low relative *CrI* may indicate that the extraction solvents were not able to dissolve and remove crystalline forms of pectin from the substrate, especially considering that another study showed a higher *CrI* value (~68%) for extracted pectin (Hassan *et al*., 2021; Supreetha *et al*., 2021). From this study's data, differences in crystallinity were small, with the acid extract showing the highest crystallinity and thus a slightly more ordered structure. It's worth noting that the acid-extracted pectin has the highest DE and the lowest negative charge. Hence, it may form more

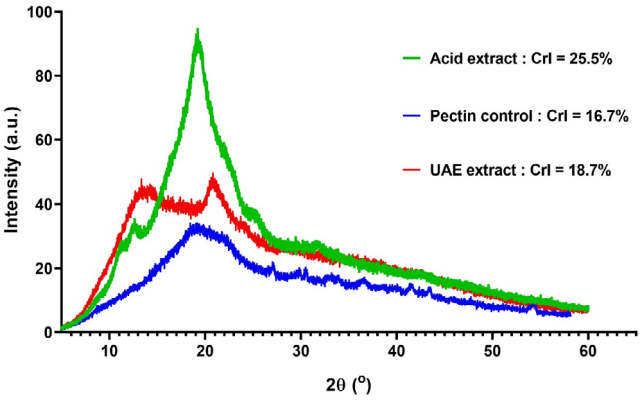

Figure 3 XRD plots for pectin; blue is commercial citrus pectin, and green and red are acid and ultrasound-assisted enzymatic extracted pectin from orange peels, respectively.

intermolecular interactions because it exhibits less charge–charge repulsion.

Thermogravimetric analysis

The thermal decomposition of the pectin extracts was characterised to confirm the purity of the polysaccharide and to determine the ash content (Fig. 4). The thermal decomposition of pectin occurs in three main steps, each of which is visible in our extracts and confirms that these extracts are indeed pectin (Ruano *et al*., 2019). First, water is evaporated between 60 and 100 °C, about 15%–20% of the sample's mass is lost, with the greatest loss around 75 °C. In the second stage, pyrolytic decarboxylation of the acidic groups with other carbons in the sugar residues takes place at 200–400 °C, producing CO_2. For the acid extract and commercial samples, the main loss was at 260 °C, but for the UAE extract it was slightly lower (220 °C), perhaps due to differences in composition (Wang *et al*., 2015) and relative *CrI*. Overall, this exothermal profile showed that the commercial and acid-extracted pectin samples are more thermally stable than the UAE-extracted sample, as their degradation occurred at a temperature 40 °C higher. The UAE sample also had an additional broad peak across 280–300 °C that could not be identified. This major exothermic peak resulted in a loss of about 40% of the mass of the samples. The final phase involves oxidation of the remaining carbonaceous material between 460 and 800 °C, the loss of which was consistent across the range. The remaining mass is non-combustible inorganic matter, ash, which was most abundant in the UAE extract at about 20% of the initial mass. The relative ash content of the pectin samples; UAE > pectin control > acid, corroborates the total sugar content of the pectin; acid > pectin control > UAE. We suspect that the higher ash content in the UAE pectin may have derived from co-precipitated buffer salts, sodium citrate, used during the extraction procedure.

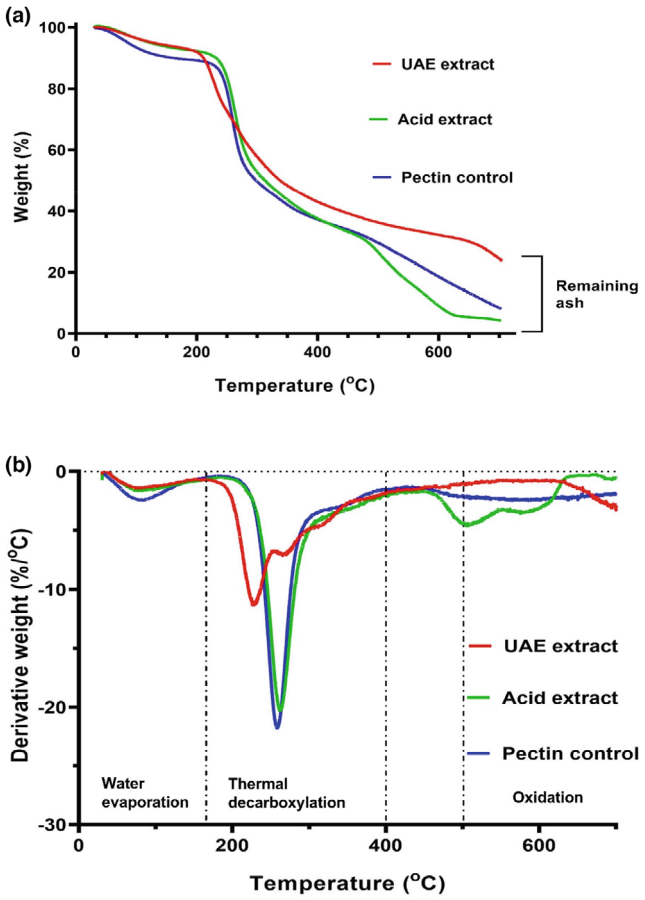

Figure 4 TGA (a) and DTG (b) plots of pectin; blue is commercial citrus pectin, and green and red are acid and ultrasound-assisted enzymatic extracted pectin from orange peels, respectively.

Conclusion

A new method for pectin extraction from orange peel, UAE using Celluclast, was compared with traditional acid extraction and proved to be an effective method that can serve as an environmentally friendly alternative to pectin extraction. The pectin extract consisted mostly of galacturonic acid and galactose, with trace amounts of other sugar residues, methyl-, and acetyl-groups as detected by NMR. In contrast, the acid-extracted pectin, although similar to UAE pectin in most aspects, did not show strong signs of esterification, instead had a higher galactose content. The underlying structures of the pectin extracts were elucidated and found to contain HG, RG-I, and, to a lesser extent, RG-II pectin in a mostly amorphous structure. The acid extract had a much higher molecular weight and DE than its counterpart, which was degraded in the extraction process. The lower molecular weight and DE of the UAE-extracted pectin allow it to serve as a dietary additive where higher solubility is preferred, or where its gelling properties are desired in low-sugar dairy products.

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Author contributions

Ryan Bosch: Conceptualization (supporting); formal analysis (equal); investigation (lead); writing – original draft (lead). **Samkelo Malgas:** Conceptualization (lead); formal analysis (equal); funding acquisition (lead); methodology (supporting); project administration (lead); resources (lead); supervision (lead); visualization (supporting); writing – original draft (supporting); writing – review and editing (lead).

Conflicts of interest

There are no conflicts to declare.

Ethical approval

Ethics approval was not required for this research.

Peer review

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Data availability statement

The study data is available from the authors upon request.

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