Modification of citrus and apple pectin by power ultrasound: effects of acid and enzymatic treatment

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Abstract

Pectin-derived oligosaccharides are emerging as a new generation of functional ingredients with new or improved technological and/or bioactive properties as compared to pectin. This work addresses the impact of power ultrasound (US) on the structure of citrus and apple pectin under different experimental conditions of power, amplitude and pectin concentration in aqueous and acid media, as well as in the presence of a pectinase. Results indicated that depolymerisation of both pectin increased with time and intensity of US in aqueous media and their polydispersity decreased. In general, a higher depolymerisation was observed in pectin treated by US in the presence of nitric and citric acids than in water, and hardly any difference was detected between both types of acids. Most of the assays gave rise to high-methoxylated pectin with a degree of esterification above 50%, pointing out their suitability for potential gelling agents. Finally, US did not have any impact in assisted enzymatic hydrolysis on the degree and/or rate of depolymerisation at low and medium levels of pectin concentration (0.5 and 2%), whereas a higher diversity of pectin fragments was found at 5% which could be indicative of a more controlled depolymerisation. These findings highlight the importance of the selection of appropriate US processing conditions to diversify the applications of modified pectin, as well as the potential of US as a prospective alternative to currently used depolymerisation techniques.

Keywords: ultrasound, nitric, citric, modified pectin, depolymerisation, pectinase
1. Introduction

Pectin is usually obtained from agro-food by-products and, although it has been used in pharmaceutical formulations [1, 2], is in the food industry where more applications are found. Pectin is a fascinating carbohydrate with a complex structure that is yet to be fully unravelled, which may be composed of up to 17 different monosaccharides distributed in different domains covalently bonded [3]. It mainly consists of chains of homogalacturonan (HG; ~65%), rhamnogalacturonan-I (RG-I; 20-30%) and a small proportion of rhamnogalacturonan-II (RG-II; 1-8%). HG is a linear homopolymer of galacturonic acid (GalA) (70-120 molecules) with α-1, 4 linkages, partially methylesterified in the carboxyl group C-6 and O-acetylated in positions C2 and C3. RG-I is attached to the main HG chain and constituted by repetitions of \[\alpha-D-GalA-1, 2-\alpha-L-Rha-1, 4-\] with ramifications of galactan, arabinan and arabinogalactan. The most complex domain is RG-II, which has a chain of GalA and may contain in defined positions minor amounts of fucose, glucuronic acid and glucuronic acid methyl-esterified, among other rare carbohydrates [4]. This fine structure confers to pectin important characteristics in terms of technological properties and it is used as gelling agent, thickener, emulsifier, texturizing and stabilizer in jams, jellies, dairy products, and as substitutes of fat in ice creams, food spreads and sauces for salads, reducing the caloric intake [2]. Recently, there is a renewed interest towards pectin derivatives of lower molecular weight (Mw) with new functional groups since the tailor-made pectin can impart specific rheological properties to processed foods to broaden their applications as functional ingredients [5]. In addition, some authors have suggested that the modified pectin and derived oligosaccharides could be a new generation of bioactive ingredients such as prebiotics [6-10], besides having antimicrobial [11], and preventive activities of the progression of certain types of cancer [4, 12].

Among the different modifications that can be promoted in pectin to broaden their technological or biological uses, its depolymerisation is the most applied and it is usually carried out by strong chemical or enzymatic hydrolysis according to conventional methods. However, the option of using emergent technologies such as power ultrasound (US) (20-100 kHz) as an alternative or complement to these techniques for Mw reduction, provides a powerful and promising approach to control the extent of depolymerisation, increase its yield, decrease process time and avoid undesired reactions. Cavitation is the main involved US mechanism which can give rise to the breakage of glycosidic linkages [13, 14]. Nevertheless, Cravotto et al. [15] found that the polymer scission under sonication is not completely stochastic in nature and therefore, could occur preferentially at the midpoint of the chain. As it has been recently reviewed by Soria et al. [16], type, Mw and concentration of polysaccharide, as well as the US
intensity and frequency and the temperature and time of treatment are the most influential factors for depolymerisation.

Several studies have reported that US can modify the structural characteristics and rheological properties of pectin in aqueous and in acid media. Seshadri et al. [17] found that the optical properties of pectin gels are improved through US application which could be beneficial in clear beverages since more transparent and less turbid gels are more desirable in the food industry. Zhang et al. [18] subjected citrus pectin to power US (20 kHz, 60-544 W/cm², pulsed 2s on/1s off, 0-40 min) and found an increase in GalA and rhamnose content with increasing US intensity, whereas mannose, galactose, and arabinose amount decreased. These authors Zhang et al. [19] investigated the impact of similar US conditions (20 kHz, 121-362 W/cm², pulsed 2s on/2s off, 0-60 min) on apple pectin and they observed a diminution in the Mw, probably ascribed to the breakdown of the larger complexes and the decrease of neutral sugar side chains. In addition, a marked reduction of the esterification degree was detected giving rise to considerable decrease of viscosity. Zhang et al. [20] also found structural modifications in citrus pectin treated by acid (0.1 M HCl at 80 °C, 12 h), US-assisted acid (0.1 M HCl at 25 °C, 2.5 h) and US (25 °C, 1.5 h). Neither different concentrations of pectin nor other acids including food-grade were assayed.

On the other hand, enzymatic treatments have been increasingly explored as alternative to transform the native form of polysaccharides to more applicable functional ingredient since may enable the obtainment of well-defined and/or stereospecific structures under mild conditions and with less secondary by-products [21]. However, limited information is available on the combination of enzymes and US with this purpose. Prajapat et al. [22] have depolymerized guar gum under an enzymatic treatment with cellulase assisted by US and they have established that US undoubtedly exert a positive effect in the enzymatic depolymerisation degree of guar gum, achieving values up to 98% under the optimized parameters. To the best of our knowledge, no data have been published on the combination of US and enzymatic treatment for pectin depolymerisation.

On the basis of these investigations, the objective of this work was to study the impact of US on the structure of pectin under different experimental conditions of power, amplitude and concentration in aqueous medium and with a food-grade acid media as well as in the presence of a pectinase, in order to obtain pectin with modified functionality that can be used in the elaboration of value-added foods, including liquid foods.

2. Materials and Methods
2.1. Materials

Standard monosaccharides (glucose, mannose, rhamnose, arabinose, galactose, GalA and xylose), Pullulan Standard Set (788-0.34 kDa), apple pectin, trifluoroacetic and citric acid were purchased from Sigma (St. Louis, MO, USA). Viscozyme L was a generous gift from Novozymes (Bagsvaerd, Denmark). Other chemicals, including sulfuric acid, nitric acid and phenol were purchased from Panreac (Darmstadt, Denmark), ethanol 96% obtained from VWR (Barcelona, Spain) and citrus pectin was from Acofarma (Barcelona, Spain).

2.2. Purification of pectin

The pectin was first purified by precipitation using ethanol according to the method described previously by Zhang et al. [19] with some modifications. Citrus and apple pectins (50 g/L) were dissolved in distilled water with stirring for 45 min at 50 °C. Then, 1:3 volumes of 96% ethanol were added to the pectin solutions, kept for 10 min with stirring and centrifuged at 1700g for 10 min at 4 °C. The supernatants were discarded, and the precipitates were lyophilised and stored at -20 °C.

2.3. Ultrasound depolymerisation of pectin

The US power (W) for the different treatments was determined recording the temperature as a function of time and using the following equation [23].

\[ P = m \cdot C_p \cdot \frac{dT}{dt} \]

where \( m \) is the water mass (kg), \( C_p \) is the specific heat of water (J/(kg °C s)) and \( \frac{dT}{dt} \) is the change in temperature along with the whole time range (°C/s) determined by a polynomial curve fitting. The intensity of US treatment dissipated from a probe tip with radius \( r \) was calculated by the following equation, according to Mason et al. [23]:

\[ I = \frac{P}{\pi r^2} \]

The ultrasonic densities (UD) (for probe and bath) expressed in watts per unit volume of the sonicated solution (W/cm³), were calculated according to Jambrak et al. [24]:

\[ UD = \frac{P}{V} \]

All assays were performed in duplicate.
2.3.1. Treatments of pectin by power US in aqueous media with and without acids

US treatments (Fig. 1) were carried out using two ultrasonic processors operating at a frequency of 20 kHz, with different maxima power values of 200 and 400 W (Digital Sonifier, Branson Ultrasonics Corporation, Danbury, CT, USA). A microtip horn of 3 mm diameter was immersed 2 cm in depth with respect to the liquid surface into a 50 mL polypropylene tube (3 cm diameter, 11.5 cm height) with 25 mL of purified pectin solutions (0.5% and 2% w/v) dissolved in distilled water, nitric acid or citric acid 0.1 M. Pulsed US (2 s on/1 s off) was the operating mode. The temperatures were kept constant using an ice-water bath.

After the acid treatment the samples were washed with 70% ethanol to remove the anions from acids, until the conductivity was next to 1 Ω/cm. All sonicated samples were freeze-dried and stored at -20 °C for further analyses.

Fig. 1 shows a scheme of the assays carried out including solvent, power, amplitude, US intensity, and temperature conditions.

2.3.2. Enzymatic treatments of pectin assisted by power US

US assisted enzymatic depolymerisation of apple and citrus pectins were carried out using Viscozyme L pectinase (2 and 4 U/mL, 45 °C, pH 4.5). Twenty five millilitres of pectin in aqueous medium at different concentrations (0.5, 2 and 5% w/v) were sonicated during 30 and 60 min in i) one of the sonicator above described (200 W maxima power) with pulsed operating mode (2s on/1s off) and intensity values of 81.7 W/cm² corresponding to 30% amplitude; ii) a US bath (Sonica Sweep System EP 2200, Solec Srl., Milano, Italy), working at a constant frequency (45 kHz) was used at power density of 0.17 W/cm³. The pectin samples (25 mL) were placed in a 100 mL glass beaker. Fig. 2 shows the conditions used in each assay (concentrations 0.5, 2 and 5%). The corresponding controls without US (Control heating at 30 and 60 min, CH-30 and CH-60 respectively) were carried out.

The sonicated samples were lyophilised and stored at -20 °C until analyses.

2.4. Physico-chemical characterisation of samples
The dry matter (DM) content of samples was gravimetrically determined in an oven at 102 °C until constant weight according to the Association of Official Analytical Chemists (AOAC) [25]. Water activity (aw) measurement was carried out in an AW Sprint TH-500 instrument (Novasina, Pfäffikon, Switzerland). The pH of purified apple and citrus pectin (2%, w/v) were obtained using a pH meter (Mettler Toledo GmbH, Schwerzenbach, Switzerland). Protein content in purified pectin was determined by the Bradford’s method [26] using bovine serum albumin as standard.

The mineral composition of the apple and citrus pectin was measured in an ICP-MS Elan 6000 Perkin-Elmer Sciex instrument from the Service Interdepartmental Research (SIdI-UAM) in Madrid. A semiquantitative analysis and quantitative analysis of the elements of interest using the external calibration method and internal standards to correct instrumental drift were carried out [27].

2.5. Structural analysis of pectin

2.5.1. Neutral sugars and galacturonic acid analysis by Gas Chromatography (GC-FID)

Citrus and apple pectin untreated and treated with US were hydrolysed with 2 M trifluoroacetic acid (TFA) at 110 °C during 3 h. The monomers released were analysed by GC-FID in an Agilent Technologies 7890A gas chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with a flame ionisation detector. Prior to GC analysis, the samples were derivatised forming their trimethylsilyl oximes. The formation of oximes before silylation limits the possible tautomers of reducing sugars to two forms: E (syn) and A (anti). Previously 200 µL of hydrolysed samples were evaporated to remove the acid, mixed with 400 µL of phenyl-β-D-glucoside (0.5 mg/mL) used as internal standard (I.S.) and evaporated under vacuum. The formation of oximes, according to method described by Brobs et al. [28], was carried out using 250 µL of 2.5% hydroxylamine chloride in pyridine and heated at 70 °C for 30 min. Afterwards, samples were persilylated with 250 µL of hexamethyldisilazane (HMDS) and 25 µL of TFA at 50 °C for 30 min, and centrifuged at 10000 rpm for 2 min.

Analyses were carried out using a DB-5HT capillary column (15 m × 0.32 mm × 0.10 µm) (J&W Scientific, Folsom, California, USA). Injector and detector temperatures were 280 and 350 °C, respectively; oven temperature program was increasing from 150 °C to 165 °C at 1 °C/min and up to 300 °C at a heating
rate of 10 °C/min. Nitrogen was used as carrier gas, 1 mL/min flow and injections were made in split mode 1:5. Data acquisition was done using a HPChem Station software (Hewlett-Packard, Palo Alto, CA, USA). The response factors were calculated after the analysis of standard solutions (glucose, mannose, rhamnose, arabinose, galactose, GalA and xylose), over the expected concentration range in samples, (0.01-2 mg) and 0.2 mg I.S. All analyses were done in duplicate and data were expressed as mean ± standard deviation (SD).

2.5.2. Estimation and distribution of molecular weight (Mw) by Size Exclusion Chromatography (SEC)

The estimation and distribution of Mw of pectin samples were determined according to the method described by Zhang et al. [19] with some modification. The analysis was performed on a LC Agilent Technologies 1220 Infinity LC System 1260 (Agilent Technologies, Boeblingen, Germany), equipped with two consecutive TSK-GEL columns (G5000 PWXL, 7.8 x 300 mm, particle size 10 µm, G2500 PWXL, 7.8 x 300 mm, particle size 6 µm; Tosoh Bioscience, Stuttgart, Germany). Samples (20 µL) were eluted with 0.1 M NaCl at a flow rate of 0.5 mL/min for 50 min at 30 °C. The eluent was monitored with a refractive index detector (Boeblingen, Germany) at 30 °C. Pullulans of Mw 788, 473, 212, 100, 1.3, 0.34 kDa were used as standards to calibration. All the Mw values specified were weight-average.

2.5.3. Estimation of the degree of esterification by Fourier Transform Infrared Spectroscopy (FTIR)

Freeze-dried samples were analysed by FTIR analysis. KBr discs were prepared mixing the pectin samples with KBr (1:100) and pressed. FTIR spectra Bruker IFS66v (Bruker, US) were collected at absorbance mode in the frequency range of 400–4000 cm⁻¹, at a resolution of 4 cm⁻¹ (mid infrared region) with 250 co-added scans.

The degree of esterification (DE) of pectin was determined as the average of the ratio of the peak area at 1747 cm⁻¹ (COO-R) over the sum of the peak areas of 1747 cm⁻¹ (COO-R) and 1632 cm⁻¹ (COO⁻) as previously described Singthong et al. [29].

2.5.4. Nuclear Magnetic Resonance (NMR)

NMR spectra were obtained using a Bruker Avance 300 MHz (Switzerland) spectrometer. The analysed samples were aqueous apple pectin solutions (0.5%) untreated (pectin control) or treated with US
(WaH-120); the $^1$H NMR analysis was carried out at 85 °C. The enzymatic hydrolysed samples analysed were: control heating (CH-60) and sample treated with US probe (0.5UP-L60). In these samples, the $^1$H analysis was performed at 25 °C. Previously, all samples were dissolved in D$_2$O and added trimethylcyclopentane (TMCP) as internal standard ($\delta_H$= 4.79 ppm).

2.6. Intrinsic fluorescence analysis

The intrinsic fluorescence of pectinase was determined in order to assess if the US treatment affect the secondary structure of the enzyme, for this purpose the method described by Prajapat et al. [22] with some modifications was used. Fluorescence spectrophotometer (Spectrofluorometer infinite M200 Tecan, Switzerland) was used to measure the intrinsic fluorescence spectra at scanning speed 1200 nm/s over the excitation wavelength range as 300–480 nm (slit = 5 nm). The analysed samples were: citrus pectin (0.5%) and enzyme incubated samples for 60 min at 25 °C (control), 50 °C heating, 50 °C US bath and 50 °C US probe.

2.7. Statistical analysis

All experiments were carried out in duplicate and the mean values ± SD were reported. Analyses of variance (ANOVA, P < 0.05) and Tukey’s test to evaluate the differences were performed using SPSS Statistics 22.0 (BM SPSS Statistics 20.0 Inc., Chicago, IL).

3. Results and discussion

3.1. Characterisation of citrus and apple pectin

Both purified pectin had very high DM content (99%), which together with the low pH and $a_w$ values (3.03 and 0.092 for apple pectin (AP), 5.24 and 0.102 for citrus pectin (CP), respectively) indicated the stability of pectin against deterioration by microorganism, enzymatic and no enzymatic reactions. Moreover, apple and citrus pectins contained 0.86 and 0.97% of protein.

Table 1 shows the mineral composition of both pectins. A predominance of potassium (3.15 mg/g of AP and 0.63 mg/g of CP), calcium (1.70 mg/g of AP and 0.65 mg/g of CP) and sodium (4.59 mg/g of AP and
0.20 mg/g of CP) was observed. The high concentrations obtained in potassium and sodium could be due to fertilizers used to assist the growing of the plants that are still present during their processing, whereas, calcium could be derived from hardness of the water used in the treatments of the by-products [30].

3.2. Treatments of pectin by US in aqueous media with and without acids

In a first approach, a study on the effect of US severity on the structural changes of citrus and apple pectin was carried out by SEC-RID, FTIR and 1H-NMR, as can be seen, respectively, in the examples of Figs. 3, 4 and 5. Fig. 3 depicts the chromatographic profiles of citrus pectin (0.5% in water) before and after exposure to different conditions of power US in aqueous media. Respective control pectins (apple 427 kDa and citrus 438 kDa, Table 2) were the earliest in the elution profile and a displacement of the peaks to the right indicated that, after US treatment, pectin was modified to species of lower Mw in function of the severity of treatment due to its depolymerisation. Moreover, after US treatments the peaks were narrower, decreasing the polydispersity (Mw/Mn) and, consequently, increasing the homogeneity in their Mw distribution.

Other important molecular parameter for pectin was degree of esterification (DE). As it is known, FTIR spectroscopy can provide useful information of the polymeric hydrogen bond network. Fig. 4 shows the infrared spectral pattern of apple pectin (0.5% in water) before (Fig. 4a) and after (Fig. 4b) US treatment (400W, 60 min, 163.4 W/cm²). The wide and marked band of absorption that appears on 3420 cm⁻¹ corresponds to the O-H stretching (vibration) due to the inter- and intramolecular hydrogen linkages located in the main chain of GalA [31]. In addition, the reduction in the intensities of the two bands assigned to the vibrations of C-H (2920 and 2850 cm⁻¹), which includes the -CH, -CH₂, CH₃ groups and the decrease of the bond stretching vibrations band width of C-H linkages in the treated sample with respect to the control one, could indicate the depolymerisation of pectin chains [20, 31]. The band at around 1740 cm⁻¹ of strong intensity, assigned to the vibration of asymmetric tension of esters of the carbonyl groups, increased its area and the signal near 1630 cm⁻¹, belonging to the vibration of symmetrical tension of the carboxylate ions, decreased as a result of the variation of the esterification degree [31]. Despite the weak intensity and minor signal that exists around 1440 cm⁻¹, a reduction in the width band of the C-H bond of the sample sonicated for 60 min in a US 400 W in relation to the control could contribute to the reduction in the Mw as a
consequence of the depolymerisation [20]. The moderate intensity and weak bands in the range of 1300-800 cm\(^{-1}\) constitute the fingerprint region of pectin [31].

In Fig. 5 the \(^1\)H-NMR spectra pattern of apple pectin shows that all the signals corresponding to the H of GalA suffered a decrease in their intensity after US treatment in aqueous media (400 W, 70\% amplitude, 2451 W/cm\(^2\), 120 min), mainly noticeable in the case of the largest signal at 3.8 ppm derived from methyl groups binding to carboxyl groups of GalA, probably due to the demethoxylation of pectin in the C6.

In the samples analysed, these qualitative changes resulted in important quantitative modifications in the degree of depolymerisation (DD), the polydispersity (Mw/Mn) and the degree of esterification (DE), as indicated in Table 2 for apple and citrus pectin. Firstly, a statistic comparison between apple and citrus pectin behaviour under US in water and acids was carried out (results not shown) and no differences (p>0.05) were detected in both pectin subjected to identical treatments. Thus, the subsequent statistical study was focused on the comparison of each sample with its corresponding control. In general, the depolymerisation increased with time and intensity of US and the polydispersity decreased probably due to cavitation effect [18, 19]. However, other possible mechanisms should not be discarded since depolymerisation can be accompanied by secondary side reactions resulting in structural changes. Thus, after US treatment an increase in the concentration of reducing-end groups was observed (results evaluated by DNS method, not shown).

The lowest Mw (Table 2) was obtained after the treatment of pectin at 400 W, 70\% amplitude for 120 min (apple: 103 kDa; citrus: 84 kDa), and the reached DD values were around 75\%, despite the huge amount of power intensity delivered to the system (2451.5W/cm\(^2\)). It has been claimed that an increase in US intensity could derived in higher effect, but up to a certain level, since at higher intensity the large amount of bubbles that are formed during cavitation could act as a barrier for energy transmission in the system and they could not collapse in an effective way to generate the needed energy to breakdown the biomolecules [32]. Moreover, in this assay the large amount of caloric energy dissipated by controlling the temperature (below 70 °C) should be considered, since, according to the literature, the maximum mechanical effect is obtained at the lowest temperature possible [33] and according to Hernoux-Villière et al. [34] values around 60 °C appeared to be the maximum temperature for cavitation bubbles to coalesce with vapour bubbles, although other authors have found the best depolymerisation effect at 80 °C for cellulose [18, 19] and chitosan [35]. With respect to time, it has been claimed that the rate of depolymerisation can become progressively lower as the molecular weight of the polysaccharide decrease up to values close to 50 kDa.
In pectin dispersed in water at 0.5%, the effect of time was mainly noticeable when the highest power was applied (400 W). Concerning the impact of pectin concentration, when data of 0.5 and 2% of pectin in water were compared, lower depolymerisation was found in the case of the highest concentration (76 vs 65% in apple pectin and 80.8 vs 73% in citrus pectin). Kassai et al. [37] found the optimal conditions for depolymerisation of chitosan by power US with low concentrations of the polysaccharide (0.2%).

Regarding the effect of acid, in general, higher values of DD were observed in pectin treated by US in the presence of acids than in water, and hardly any difference was detected between both types of acids, nitric and citric. In this sense, the use of citric acid could be more adequate due to its application within the growing concept of “Green Chemistry” and its consideration as food-grade ingredient. These results were different from those showed by Zhang et al. [20] for the degradation of citrus pectin by US and 0.1 M HCl, who found that US was more efficient when the pectin was prepared in distilled water than in acid and they attributed this phenomenon to solubility problems of pectin in HCl which could hinder the cavitation effect. These discrepancies with our results might be related to the different US conditions used (probe diameter, amplitude, temperature, time), as well the different acids used (nitric and citric vs hydrochloric). Moreover, those authors only assayed one US condition (181 W/cm², 25°C, 90 min, 0.1 M HCl).

As shown in Table 2, before any treatment, both pectin samples were highly methoxylated (HM) since the DE values were 70 and 83% for apple and citrus pectin, respectively. In general, most of the samples presented DE values ≥ 50% after US process, indicating their suitability for gelling properties. As it is known, DE is the main molecular parameter that affects the gelling properties of pectin [38]. DE of pectin after all US treatments in water decreased, similarly to samples subjected to hydrolysis with US and nitric. No change was detected in this parameter in the case of US treatments carried out with citric acid with respect to the corresponding control, although it seems that, in general, samples treated with acid had higher values of DE that the corresponding with water.

In the US treated samples with and without acids (nitric and citric), the monomeric composition of pectin was another structural feature evaluated, in this case by GC-FID with a previous hydrolysis with TFA. Prior to these analyses, an assessment of the potential effect of US in water or in acid on the liberation of free carbohydrates from the polysaccharide was carried out (results not shown). For this purpose the treated samples by US were directly subjected to GC-FID determination without a previous step of hydrolysis with TFA. No change was detected in any of the samples analysed, indicating that the depolymerisation of pectin
by US gives rise to molecular species of high Mw instead of the liberation of monomers, in agreement with SEC-RID data.

Fig. 6 illustrates the chromatographic profile obtained by GC-FID of TMS oxymes of the individual monosaccharides of apple pectin after hydrolysis with 2 N TFA. As observed, the monomers found were xylose, arabinose, rhamnose, galactose, mannose, glucose and GalA, although glucose was probably due to the presence of other polysaccharides (cellulose, hemicellulose) which could co-precipitated during pectin obtainment. The concentrations of these carbohydrates before and after US processing in water or acids are shown in Table 3. As expected, the most abundant carbohydrate was GalA with values respect to the total amount of carbohydrates higher than 65%, very close to the specifications to be consider as food ingredient (E440) by the FAO [12]. With the exception of xylose+arabinose the most abundant neutral sugar was galactose, followed by rhamnose and mannose. This distribution of carbohydrates, including the corresponding ratios GalA/Rha and Xyl+Ara+Gal/Rha, suggests that the pectin backbone could be formed by the smooth region of HG linked to the hairy domain mainly composed by RG I, as expected for apple pectin. Regarding the monomeric composition, no significant differences (p>0.05) were obtained in the content of GalA under the less severe conditions in water, using the US treatment with 200 W (81.7 W/cm²), however a significant increase in the content of this monomer was detected under more intense experimental conditions, in agreement with the trend found by Zhang et al. [18, 19]. One plausible explanation for this fact could be related to the formation of pectin aggregates in solution [39]. Thus, during the lowest treatment by US part of the aggregates might remain unaltered making more difficult the access of TFA during sample preparation before GC-FID, whereas under the most intense conditions the structural changes provoked by US could allow the accessibility of TFA for the liberation of GalA belonging to the backbone polysaccharide. Rhamnose, galactose and xylose+arabinose concentrations significantly decreased with the intensity of the treatments. In the case of samples subjected to US in the presence of acids, in general, lower changes were detected in the monomeric composition as compared to water. This could be due to the lower pH value of the acid samples (3.03 vs 5.24) since pectin under more acid pH could originate gels that might impair the transmission of US energy. These results, and the significant increase in the ratios GalA/Rha and Xyl+Ara+Gal/Rha, indicated that the main chain of apple pectin (HG) remained unaltered after US with and without acids, even under the most strong conditions and that the changes of Mw were mainly ascribed to alterations in the RG I region.
3.3. Treatments of pectin by pectinases assisted by US

As above indicated, the effect of power US in biochemistry and biotechnology has attracted great attention in recent years since certain experimental conditions can give significant enhancement in the enzyme activity. We evaluated the changes in pectin produced by US treatment under similar intensity conditions used for the above shown assays with acids but, in this case, in combination with a pectinase, normally employed for depolymerisation of pectin in the food industry (Viscozyme L). Fig. 7 illustrates the chromatographic profiles obtained by SEC-RID of citrus pectin (0.5, 2 and 5%) treated by the enzyme preparation at 50°C for 60 min with the assistance of US in bath and with probe. The behaviour of pectin was different depending on the concentration and ratio enzyme/pectin with a lower hydrolysis and more diversity of pectin fractions when concentration increased. At 0.5% very similar qualitative profiles were observed in all the samples analysed with a slight difference in the case of pectin treated by probe. At 2 and 5% the most different profile was that corresponding to the pectin subjected to US probe with lower hydrolysis.

Fig. 8 shows the 1H-NMR spectra for the samples of pectin at 0.5%, treated with and without US (Figs. 8a and 8b, respectively). As illustrated in the Fig. 8 the structure of pectin had important modifications, mainly in the case of the control heating. In agreement with the previous SEC profiles the sample treated by US probe was less hydrolysed, probably due to a possible denaturation of the enzyme, causing lower hydrolysis.

The modifications registered by SEC-RID were quantified by the estimation of Mw of the different fragments and their corresponding abundances (%) (Fig. 9). As observed, the depolymerisation was mainly due to the action of the pectinase and, in general, no effect was detected due to US (bath or probe). Two major fragments (~2.8 and 0.7 kDa) were found in all the treatments in the two types of pectin, apple and citrus, increasing the hydrolysis degree with the increasing of time. Hardly any difference was observed in the proportion of these fractions, with the exception of assays of US with probe at 60 min, since the smallest fractions (0.8 kDa) were slightly less abundant than in the control heating. As the behaviour of citrus and apple pectin was very similar, the subsequent assays with 2 and 5% of pectin concentration were done only with apple pectin (Fig. 10a and 10b). In this case, the increase of hydrolysis with time was more important and was more pronounced with the highest pectin concentration and a different trend was observed depending on the pectin concentration. At 2% very similar profile of molecular fragments were observed
regardless the system used (control heating, US bath and US probe). At 5% of pectin concentration lower hydrolysis was detected in US treatments than in control heating pectin samples, although the former showed higher diversity of fragments, probably due to the fact that, in more concentrated solutions more entanglements between polysaccharide chains are produced, impairing the transmission of US energy and decreasing the interaction enzyme-substrate. To test if the low hydrolysis was only due to the minor enzymatic activity, assays with 5% of pectin and less amount of enzyme (2 U/mL) were carried out. Very limited hydrolysis was found and only two types of fragments were detected, the most abundant with around 200 kDa and other less abundant with Mw of around 1 kDa, indicating that the amount of enzyme was very insufficient.

The effect of US over enzyme activity has been previously studied by different authors, so Guo et al. [40] found a decrease of US efficiency with the increase of fucoidan concentration in the range 0.05-1%. Some authors as Prajapat et al. [22] obtained a positive effect of US over enzymatic depolymerisation of a polysaccharide (guar gum). These differences with respect to our results could be explained firstly by the different experimental conditions and others factors related with the enzymes. It is known that diverse enzymes have different stereo conformations, and the same enzyme shows different stereo configurations under different surroundings, and could be affected by US; so enzymes can exhibit different intensity of activity under ultrasonication. Although the same ultrasonic parameters are used, the effect over different enzymes can be dissimilar [41]. Gamboa et al. [42] in a study on the effect of US on peroxidase and pectinmethylesterase found, under identical experimental US conditions, high values of inactivation in the former and limited effect in the latter, underlying the difficulty to identify the exact mechanism responsible for the effect of sonication on the enzymes.

3.4. Effect of US on the structure of pectinase

The possible conformational changes that could have suffered the pectinase during the modification of pectin (0.5%) were studied by the evaluation of the intrinsic fluorescence due to tryptophan (Trp) residues of the protein corresponding to samples of Fig. 11. As observed, the two samples treated with US in bath and with probe had a slight shift of the maximum of emission with respect to the control samples, although the fluorescence intensity in the former was very similar to the control heating; however, the sample treated by
probe presented the lowest intensity. These results indicated that pectinase could have suffered a molecular unfolding which would lead to the exposure of more domains decreasing the fluorescence of the enzyme. However, these slight structural modifications of pectinase did not alter its ability to modify the molecule of pectin, in agreement with the results shown in section 3.3.

4. Conclusions

According to the obtained results it is possible to conclude that, under the assayed conditions, all the US treatments gave rise to depolymerisation of citrus and apple pectin which increased with time, US power and amplitude, and decreased with pectin concentration. The presence of acids (that is, nitric and citric) increased the depolymerisation of pectin and hardly any difference was detected between both acids. Most of the assays gave rise to high-methoxylated pectin (DE>50%), indicating their suitability for gelling properties. In the assays with enzymes, the effect of Viscozyme L was kept during US treatment, even at moderately high US intensity values and a different behaviour was observed in relation to pectin concentration. At low and medium values of pectin concentration (0.5 and 2%) ultrasound-assisted enzymatic hydrolysis hardly any influence the degree and/or rate of polymerisation, however at values as high as 5% higher diversity of pectin fragments was found, indicating a higher control of depolymerisation. These results underline the importance of selection of US processing conditions to diversify the potential applications of modified pectin. In this sense, US seems to be a favourable alternative to currently used depolymerisation techniques where degradation of pectin dispersion is desirable, such is the case of using higher amount of pectin as soluble fiber in liquid foods and as stabilisation agent in acidified beverages.

Acknowledgements

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References


Table 1. Physico-chemical characteristics of apple and citrus pectin.

<table>
<thead>
<tr>
<th></th>
<th>Apple pectin</th>
<th>Citrus pectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry Matter (DM, %)</td>
<td>99.1</td>
<td>99.3</td>
</tr>
<tr>
<td>pH</td>
<td>3.03 ± 0.09</td>
<td>5.24 ± 0.32</td>
</tr>
<tr>
<td>Water activity (a_w)</td>
<td>0.092 ± 0.003</td>
<td>0.102 ± 0.005</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>0.86</td>
<td>0.97</td>
</tr>
<tr>
<td>Sodium</td>
<td>4.59</td>
<td>0.20</td>
</tr>
<tr>
<td>Potassium</td>
<td>3.15</td>
<td>0.63</td>
</tr>
<tr>
<td>Calcium</td>
<td>1.70</td>
<td>0.65</td>
</tr>
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</table>
Table 2. Effect of US treatment on the estimation and distribution of Mw of apple and citrus pectin solutions (0.5%). Samples carried out at 2% are indicated (*).

<table>
<thead>
<tr>
<th>Power (W)/ Amplitude (%)</th>
<th>Time (min)</th>
<th>Solvent</th>
<th>Samples</th>
<th>Apple Pectin</th>
<th>Citrus Pectin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mw (kDa)</td>
<td>Mw (kDa)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>Mn (kDa)</td>
<td>Mn (kDa)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mw/Mn</td>
<td>DD (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>30</td>
<td>Wa-L30</td>
<td>340 ± 0.0e</td>
<td>91 ± 2f</td>
<td>3.8 ± 0.1e</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>Wa-L60</td>
<td>316 ± 14f</td>
<td>86 ± 3g</td>
<td>3.7 ± 0.0e</td>
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<tr>
<td>300/70 [163.4]</td>
<td>30</td>
<td>Wa-M30</td>
<td>328 ± 15f</td>
<td>93 ± 2f</td>
<td>3.5 ± 0.1c</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>Wa-M60</td>
<td>266 ± 5f</td>
<td>82 ± 2e</td>
<td>3.3 ± 0.0f</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>Wa-M120</td>
<td>186 ± 5f</td>
<td>66 ± 0.7f</td>
<td>2.8 ± 0.1f</td>
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<tr>
<td></td>
<td>300/30 [163.4]</td>
<td>120</td>
<td>Wa-H120</td>
<td>103 ± 4f</td>
<td>39 ± 2e</td>
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<td></td>
<td></td>
<td>Wa-H120*</td>
<td>152 ± 13f</td>
<td>47 ± 4f</td>
<td>3.3 ± 0.0f</td>
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<tr>
<td>0</td>
<td>30</td>
<td>N-L30</td>
<td>419 ± 6f</td>
<td>118 ± 0.0f</td>
<td>3.6 ± 0.0f</td>
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<td></td>
<td>60</td>
<td>N-L60</td>
<td>351 ± 2f</td>
<td>103 ± 3e</td>
<td>3.4 ± 0.1c</td>
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<tr>
<td>400/70 [2457.7]</td>
<td>30</td>
<td>N-M30</td>
<td>285 ± 4f</td>
<td>81 ± 4f</td>
<td>3.5 ± 0.0f</td>
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<tr>
<td></td>
<td>60</td>
<td>N-M60</td>
<td>237 ± 4f</td>
<td>70 ± 3ª</td>
<td>3.4 ± 0.2f</td>
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<tr>
<td></td>
<td>120</td>
<td>N-M120</td>
<td>190 ± 18ª</td>
<td>67 ± 4ª</td>
<td>2.8 ± 0.1f</td>
</tr>
<tr>
<td>0</td>
<td>30</td>
<td>C-L30</td>
<td>456 ± 16ª</td>
<td>110 ± 3ª</td>
<td>4.2 ± 0.2ª</td>
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<tr>
<td>400/30 [163.4]</td>
<td>120</td>
<td>C-M120</td>
<td>182 ± 9ª</td>
<td>60 ± 3ª</td>
<td>3.1 ± 0.1ª</td>
</tr>
<tr>
<td>400/70 [2451.5]</td>
<td>120</td>
<td>C-H120</td>
<td>120 ± 2ª</td>
<td>45 ± 2ª</td>
<td>2.7 ± 0.1ª</td>
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Note: values with different small case superscript letters (a-h) in the same column within each solvent and each pectin indicate significant differences as estimated by Tukey’s test (P < 0.05); Mw: weight-average molar mass; Mn: number-average molar mass; Mw/Mn: polydispersity index; DD: depolymerization degree; DE: degree of esterification. *Assays carried out with 2% pectin.
Table 3. Effect of US treatment on monosaccharide composition (% expressed over total monosaccharides) of apple pectin.

<table>
<thead>
<tr>
<th>Power (W)/Amplitude (%)</th>
<th>Time (min)</th>
<th>Solvent</th>
<th>Samples</th>
<th>Xyl * Ara</th>
<th>Rha</th>
<th>Gal</th>
<th>Man</th>
<th>Glc</th>
<th>GalA</th>
<th>GalA + Xyl + Ara</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td><strong>0</strong></td>
<td>Water Control</td>
<td>16.9 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.9 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.6 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.1 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.9 ± 0.2&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>67.7 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.6 ± 2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.6 ± 2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.8 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>200/30</strong> [81.7]</td>
<td>Wa-L30</td>
<td>17.5 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.7 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.7 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.3 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.2 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66.7 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.6 ± 2.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.7 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wa-L60</td>
<td>17.0 ± 1.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.7 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.0 ± 0.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.3 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.9 ± 0.3&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>68.1 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25.2 ± 2.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.9 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>400/30</strong> [163.4]</td>
<td>Wa-M60</td>
<td>10.8 ± 1.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.6 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.1 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.1 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.1 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>78.4 ± 1.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>49.7 ± 2.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.7 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
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</tr>
<tr>
<td></td>
<td>Wa-M120</td>
<td>12.2 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.8 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.0 ± 1.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.3 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.8 ± 0.3&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>75.9 ± 1.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48.9 ± 6.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.8 ± 0.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>400/70</strong> [2451.5]</td>
<td>Wa-H120</td>
<td>10.4 ± 1.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.2 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.9 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.2 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.8 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>82.4 ± 2.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>67.3 ± 10.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.6 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wa-H120*</td>
<td>9.3 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.0 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.9 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.2 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.9 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>83.6 ± 1.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>84.4 ± 13.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.3 ± 1.3&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td><strong>0</strong> Nitric acid Control</td>
<td>17.6 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.6 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.7 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.7 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.2 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>69.1 ± 0.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>26.7 ± 2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.8 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>200/30</strong> [81.7]</td>
<td>N-L30</td>
<td>14.6 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.3 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.8 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.8 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.2 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>72.4 ± 2.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>33.4 ± 10.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.2 ± 2.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N-L60</td>
<td>12.8 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.5 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.9 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.7 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.3 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>73.7 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.6 ± 6.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.6 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>400/30</strong> [163.4]</td>
<td>N-M60</td>
<td>16.5 ± 1.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.9 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.5 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.7 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.3 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>22.5 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.3 ± 1.2&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>N-M120</td>
<td>18.2 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.0 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.7 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.7 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.2 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>68.2 ± 0.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>33.9 ± 4.1&lt;sup&gt;c&lt;/sup&gt;</td>
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<td><strong>0</strong> Citric acid Control</td>
<td>7.7 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>**</td>
<td>9.6 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.6 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.2 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>80.9 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>**</td>
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<td><strong>400/30</strong> [163.4]</td>
<td>C-M120</td>
<td>10.3 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>**</td>
<td>8.0 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.6 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.5 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td><strong>400/70</strong> [2451.5]</td>
<td>C-H120</td>
<td>10.9 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>**</td>
<td>8.6 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.7 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.4 ± 0.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>77.3 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>**</td>
<td>**</td>
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</tr>
</tbody>
</table>

Note: values with different small case superscript letters (a-d) in the same column within each solvent and each pectin indicate significant differences as estimated by Tukey’s test (P < 0.05); Xyl: xylose; Ara: arabinose; Rha: rhamnose; Gal: Galactose; Man: mannose; Glc: glucose; GalA: galacturonic acid. *Assays carried out with 2% pectin. **It was not possible to quantify due to the coelution of citric acid and rhamnose.
FIG. LEGENDS

Fig. 1. Scheme of the treatments of pectin (0.5%) by power US carried out in aqueous media with and without acids. A: Amplitude; Wa: Water; N: Nitric acid; C: Citric acid; L: Low treatment; M: Medium treatment; H: High treatment. * Treatment performed with pectin at 2%.

Fig. 2. Scheme of the assays of pectins (0.5, 2 and 5%) carried out with pectinase (4 U/mL Viscozyme L, 50 ºC for 30 and 60 min) assisted by US probe (200 W, A 30%, 81.7 W/cm²) or bath (0.17 W/cm³). A: Amplitude; UB: Ultrasound Bath; UP: Ultrasound Probe; L: Low treatment. *These treatments were also carried out with Viscozyme L (2 U/mL).

Fig. 3. SEC-RID chromatographic profiles of aqueous citrus pectin solutions (0.5%) treated in the sonicator of 400W. Wa-M30 (30%, 163.4 W/cm², 50 ºC for 30 min); Wa-M60 (30%, 163.4 W/cm², 50 ºC for 60 min); Wa-M120 (30%, 163.4 W/cm², 50 ºC for 120 min); Wa-H120 (70%, 2451.5 W/cm², 70 ºC for 120 min).

Fig. 4. FT-IR spectra of apple pectin (0.5% water). (a) Pectin control, (b) Wa-M60 (400W, 30%, 163.4 W/cm², 50 ºC for 60 min).

Fig. 5. ¹H-NMR spectra of apple pectin (0.5% water). (a) Pectin control, (b) Wa-H120 (400W, 70%, 2451.5 W/cm², 70 ºC for 120 min).

Fig. 6. Chromatographic profile obtained by GC-FID of TMS oxymes of monosaccharides of apple pectin after hydrolysis with 2N TFA of US treated. 1: Xylose 1; 2: Xylose 2 + Arabinose; 3 and 4: Rhamnose; 5: Galactose; 6: Mannose; 7: Glucose; 8: Galactose + Mannose + Glucose; 9 and 10: Galacturonic acid; 11: Standard internal.
Fig. 7. Chromatographic profiles SEC-RID of apple pectin treated by Viscozyme L (4 U/mL, 50 °C, 60 min) at (a) 0.5%, (b) 2%, (c) 5%. (Control heating at 60 min, CH-60). For labelled US samples see Fig. 2.

Fig. 8. ^1^H-NMR spectra of apple pectin (0.5%). (a) 0.5UP-L60 (Ultrasound probe, 200W, 30%, 81.7 W/cm^2^, 50 °C for 60 min); (b) CH-60 (Control heating at 60 min).

Fig. 9. Estimation of Mw (kDa) and main fragments formed (%) after treatments (control heating, US bath and US probe) of apple pectin (0.5%) with Viscozyme L.

Fig. 10. Estimation of Mw (kDa) and main fragments formed (%) after treatments (control heating, US bath and US probe) of apple pectin with Viscozyme L. Pectin concentration (a) 2%; (b) 5%. *This fragment is divided in two fractions whose weight-average Mw are 440 and 220 kDa.

Fig. 11. Fluorescence spectra of pectinase (Viscozyme L) corresponding to US and heating treatments of apple pectin (0.5%).
Figure 2.
Figure 3.
Figure 4

(a)

(b)
Figure 5

(a)  

(b)  

Figure 6.
Figure 7.

(a) 

(b)
Figure 10.

(a) Control heating | Ultrasound Bath | Ultrasound Probe

(b) Control heating | Ultrasound Bath | Ultrasound Probe

- >788-220 kDa
- 220-12 kDa
- 12-4 kDa
- 4-1.2 kDa
- 1.2-0.3 kDa

- >788 - 220 kDa
- 220 - 10 kDa
- 10 - 1 kDa
- <1 kDa
Figure 11.

Fluorescence Intensity (a.u.)

Wavelength (nm)

Control at 25°C
Control heating at 50°C
Ultrasound bath at 50°C
Ultrasound probe at 50°C