**Enzymatic extraction of pectin from artichoke (*Cynara scolymus*) by-products
using Celluclast[®] 1.5L**

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ABSTRACT

The aim of this study was to optimise pectin extraction from artichoke by-products with Celluclast[®] 1.5L using an experimental design analysed by response-surface methodology (RSM). The variables optimised were artichoke by-product powder concentration (2–7%, X_1), enzyme dose (2.2–13.3 U g⁻¹, X_2) and extraction time (6–24 h, X_3). The variables optimised were galacturonic acid (GalA) (R^2 93.9) and pectic neutral sugars (R^2 92.8) content and pectin yield (R^2 88.6). In the optimum extraction conditions (X_1 =6.5%; X_2 =10.1 U g⁻¹; X_3 =27.2 h), pectin yield was 175.6 mg g⁻¹ dry matter (DM). Considering 27.2 h of treatment as the + α value given by the design, the extraction time was increased up to 48 h obtaining a yield of 221.4 mg g⁻¹ DM. The enzymatic method optimised allows obtaining artichoke pectin with good yield, high GalA (720.0 mg g⁻¹ DM) and arabinose (126.6 mg g⁻¹ DM) contents and degree of methylation of 19.5%. ~~Therefore, Celluclast[®] 1.5L allowed extracting efficiently pectin from artichoke by-products.~~

Keywords: pectin extraction, artichoke, *Cynara scolymus*, by-products, Celluclast, RSM

1. Introduction

Pectins are a type of soluble fibre commonly present in vegetal cell wall and are one of the most structurally complex families of polysaccharides in nature. It is mainly composed of a linear chain of α -1,4-D-galacturonic acid (GalA) called homogalacturonan (HG) which comprises approximately 70% of pectin. This polymer is partially methylesterified at the C-6 carboxyl and may be O-acetylated at O-2 or O-3. ~~Free or esterified carboxyl groups can be partly substituted by monosaccharides as~~ ~~xylose~~. HG is the backbone that present ramified chains of rhamnogalacturonan type I (RGI), a polymer made up of alternate sequences of GalA and α -(1, 2) linked L-rhamnosyl residues, and rhamnogalacturonan II (RGII), a most complex chain consisting of 12 different types of sugars in over 20 different linkages (Garna, Mabon, Nott, Wathelet, & Paquot, 2006; Gullón et al., 2013; Mohnen, 2008).

Pectins are widely used as valuable functional food ingredients due to their technological and biological properties. The majority of pectins used are extracted from citrus peel, apple and sugar beet by-products (Santos, Espeleta, Branco, & de Assis, 2013). Functional properties, which depend on structure of pectins, are influenced by sources and conditions of extraction, location and other environmental factors (Canteri, Nogueira, de Oliveira Petkowicz, & Wosiack, 2012; Fissore et al., 2014; Gullón et al., 2013). In the last years non-traditional sources of pectins have also been studied such as fresh peas pomace, sunflower heads, bark of mango tree, red fruit pulps and sisal wastes (Santos et al., 2013). In the same way, other new source of pectin could be artichoke by-products. Artichoke (*Cynara scolymus*) is an edible vegetable widely consumed in the Mediterranean diet, harvesting in this area the 85% of world production. The industrial processing of artichoke produces a high amount of wastes (external bracts, leaves and stems), which is close to 60% of the harvested artichoke (Llorach, Espin, Tomás-

Barberán, & Ferreres, 2002). Although artichoke residues are generally used for animal feed or as manure (Machado et al., 2015), they can be used to extract bioactive compounds such as fructooligosaccharides and inulin (Lopez-Molina et al., 2005; Schütz, Muks, Carle, & Schieber, 2006; **Terkmane, Krea, & Moulai-Mostefa, 2016**); flavonoids and phenolic compounds (Llorach et al. 2002; Sałata, A., & Gruszecki 2010) and inositols (Ruiz-Aceituno, García-Sarrió, Alonso-Rodriguez, Ramos, & Sanz, 2016) as well as to isolate fractions enriched in soluble fibre (Fissore et al., 2014). However, there are scarce studies about extraction and characterisation of pectin from artichoke by-products (Femenia, Robertson, Waldron, & Selvendran, 1998; Fishman, El-Atawy, Sondey, Gillespie, & Hicks, 1991; Fissore et al., 2014).

The procedures for the extraction of commercial pectins are usually optimised to enhance the content of HG regions which generate the useful functional gelling properties of pectin (Maxwell, Belshaw, Waldron, & Morris, 2012). With this finality, the chemical extraction of pectins, employing strong acids such as hydrochloric, nitric, and sulphuric acids and high temperature (70–100 °C), has been the most widely used method for the industry (Min et al., 2011; Yapo, 2009) and although is an efficient and economical procedure, may be environmentally hazardous and could modify the structure of pectin.

Extraction of pectins can also be performed through the use of enzymes and specifically cellulases and xylanases which give rise to pectins without structural modifications. The type of enzyme could determine the yield of extraction and the composition of pectin (Dominiak et al., 2014; Wikiera, Mika, Starzyńska-Janiszewska, & Stodolak, 2015a). Among commercial enzymatic preparations with cellulase activity, Celluclast[®] 1.5L (Celluclast) led to complete pectin extraction with hardly any modification in its structure; thus, this enzyme has been used to extract pectin from

apple pomace due to its xylano-, cellulo-, and mannolytic activities (Wikiera et al., 2015a). In addition, pectin extracted with Celluclast contained more GalA of higher methylation and acetylation degree compared to pectin extracted with acids (Wikiera, Mika, Starzyńska-Janiszewska, & Stodolak, 2015b). Therefore, the aim of this study was to optimize pectin extraction from by-products (external bracts, leaves and stems) of artichoke (*Cynara scolymus*) industrialisation using the commercial enzymatic preparation Celluclast. For this purpose, a three variable central composite orthogonal design analysed by response-surface methodology (RSM) was developed.

2. Materials and Methods

2.1. Standards and samples

Analytical reference substances such as sucrose, D-arabinose, L-rhamnose, D-galactose, D-mannose, D-glucose, D-fructose, galacturonic acid (GalA), and β -phenyl glucoside were purchased from Sigma Aldrich (Steinheim, Germany). Eight commercial multienzymatic preparations were studied. Celluclast[®] 1.5L (cellulase from *Trichoderma reesei*), Pectinex Ultra SP-L[®] (pectinase from *Aspergillus aculeatus*) Viscozyme[®] L (endo-1,3(4)- β -glucanase from *A. aculeatus*), Viscozyme[®] Barley HT (amylase and cellulase from *Aspergillus* sp.) and Carezyme[®] 4500 L (cellulase from *Aspergillus* sp.) were generous gifts from Novozymes (Bagsvaerd, Denmark). Pectinase and cellulase from *A. niger* and cellulase from *T. viride* were acquired from Sigma.

Cellulase and pectinase activities were measured by quantifying the amount of reducing sugars released, using the dinitrosalicylic acid (DNS) method (Miller, 1959). To determine cellulase activity, cellulose solutions (10 mg mL⁻¹, Carboxymethyl cellulose, Sigma) dissolved in sodium acetate buffer (0.05M, pH 5) with 5 μ L mL⁻¹ or 1 mg mL⁻¹ of enzyme at 50 °C for 5 min were prepared and glucose was employed as

standard. One unit of cellulase activity was defined as the amount of the enzyme, which released 1 μmol of glucose per min. Pectinase activity was measured using polygalacturonic acid solutions (10 mg mL^{-1} , Sigma) incubated with 5 $\mu\text{L mL}^{-1}$ or 1 mg mL^{-1} of enzyme at 50 °C for 2 min. GalA was employed as a standard. One unit of pectinase activity was defined as the amount of the enzyme, which catalysed the released 1 μmol of GalA per min.

Industrial by-product of artichoke (*Cynara scolymus*) consisting in blanched external bracts, leaves and stems were kindly supplied by Riberebro[®] (La Rioja, Spain). Prior to characterisation, this product was freeze-dried, homogenised using a Wiley Mill 934 equipment (Thomas Scientific, USA) and sieving through a 500 μm sieve (Femenia et al., 1998).

2.2. Pectin extraction from artichoke by-products

Enzymatic extraction of pectin from artichoke by-products (particle size $\leq 500 \mu\text{m}$) was performed using Celluclast. The process was conducted in an orbital shaker at 50 °C, pH 5 with constant shaking (200 rpm). After hydrolysis, samples were centrifuged (1300g, 10 min, 4 °C) and supernatants were filtered through cellulose paper. The resultant precipitate was washed with distilled water (20 mL), centrifuged and filtered. The supernatants were combined and absolute ethanol at 4 °C was added to obtain a final concentration of 70%. After an hour at 4 °C, the precipitated pectins were centrifuged (1200g, 20 min), washed twice with 70% ethanol (600 mL), centrifuged again, and freeze-dried.

2.2.1. Experimental design for extraction of pectins

A three level, three variable, central composite orthogonal design was applied to determine the best conditions to extract enzymatically pectin from artichoke by-products. The factorial design consisted of 8 factorial points, 6 axial points (two axial points on the axis of each design variable at a distance of 2 from the design centre) and 3 centre points. The independent variables used in this study were artichoke by-product powder concentration (2–7% w/w, X_1), enzyme dose (2.2–13.3 U g⁻¹, X_2), and extraction time (6–24 h; X_3). Four dependent variables were taken into account to optimise pectin extraction by means of RSM: yield of pectin extraction (mg of extracted pectin / g dry matter (DM), Y_1), GalA content (mg g⁻¹ DM, Y_2), pectic neutral sugars content (arabinose, rhamnose and galactose, mg g⁻¹ DM, Y_3) and other neutral sugars content (glucose, mannose and fructose, mg g⁻¹ DM, Y_4).

The responses obtained from each set of experimental design were subjected to multiple nonlinear regressions to obtain the coefficients of the second order polynomial model. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination R-squared (R^2) and the adjusted R-squared (R^2_{adj}), which provides a measurement of how much of the variability in the observed response values could be explained by the experimental factors and their interactions (Myers, Montgomery, Vining, Borror, & Kowalski, 2004). Variables that presented R^2_{adj} values higher than 70% were submitted to simultaneous optimisation.

The overall effect of the three independent factors was used to obtain a desirability function that represents the influence of the extraction conditions on the efficiency of enzymatic extraction with Celluclast and on the final product composition. In general terms, a desirability function represents the effect of the independent variables (X_i) on the dependent variables (Y_j), determining the process efficiency

(Myers et al., 2004). For each extraction conditions, the desirability function assigns numbers between 0 (completely undesirable value) and 1 (completely desirable or ideal response). The method allows optimize extraction conditions that provide the “most desirable” response values.

Additional extractions of artichoke pectin were carried out at following conditions: artichoke by-product powder 6.5%, enzyme dose 10.1 U g⁻¹ and extraction time 36 and 48 h.

2.3. Characterisation of samples

All analyses were carried out in duplicate.

2.3.1. Artichoke by-products and extracted pectin

Dry matter (DM) was determined gravimetrically drying the samples until constant weight according to the AOAC method 950.01 (AOAC, 1990a).

pH was measured in solutions of 10% using a Mettler Toledo Five Easy Plus pH Meter.

Water activity (a_w) measurement was carried out in an AW Sprint TH-500 instrument (Novasina, Pfäffikon, Switzerland). Saturated aqueous solutions of LiCl, MgCl₂, Mg(NO₃)₂, NaCl, BaCl₂ and K₂Cr₂O₇ were used to calibrate the sensor unit.

Protein was determined following the Kjeldhal method for total nitrogen (TN) using a conversion factor (TN x 6.25) to express results as protein (AOAC, 1990b).

2.3.2. Artichoke by-product powder

Fat content was measured following AOAC 920.39 (1990c) methodology performing Soxhlet extraction using a Gerhardt soxtherm extractor (Germany).

Water soluble fraction (WSF) and insoluble fraction (WIF) determinations.

These fractions were obtained by immersing 1.5 g of powdered samples in 40 mL boiling water for 30 min. The mixture was cooled and filtered through a sintered glass filter (No 2). The residue was re-extracted twice with boiling water and then washed with 40 mL of water. Supernatant was collected and then residue was washed with 40 mL of acetone 70% and 40 mL of absolute ethanol. Water insoluble fraction (WIF) was dried and stored for further analysis. Supernatant (water soluble fraction, WSF) was partially dried at 38-40 °C in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland), freeze-dried, and redissolved in water at 10 mg mL⁻¹ for chromatographic analysis.

Alcohol soluble fraction (ASF) and insoluble fraction (AIF) determinations.

These fractions were obtained by immersing 1.5 g of powdered samples in 40 mL boiling ethanol, final concentration 80% (v/v), for 30 min. The mixture was cooled and filtered through a sintered glass filter (No 2). The residue was re-extracted twice at the same conditions. Supernatants were collected and then residue was washed first with 40 mL of 70% acetone and then with 40 mL of absolute ethanol. Alcohol insoluble fraction (AIF) was dried and stored for further analysis. Supernatant (alcohol soluble fraction, ASF) was partially dried at 38-40 °C in a rotary evaporator, freeze-dried and redissolved in water at 10 mg mL⁻¹ for chromatographic analysis.

2.4. Analytical techniques

2.4.1. ICP-MS

The ion composition of artichoke by-product **powder** and extracted pectin was determined using an ICP-MS ELAN 6000 Perkin-Elmer Sciex instrument at the Servicio Interdepartamental de Investigación (SIdI-UAM) of Madrid. Quantitative

analysis of the elements of interest using the external calibration method and internal standard to correct instrumental drift was carried out.

2.4.2. FT-IR

In order to determine the **degree of methylation**, freeze-dried samples of artichoke by-products and **extracted** pectin were analysed by FT-IR analysis. KBr discs were prepared mixing the pectin samples with KBr (1:100) and pressed. FT-IR spectra were performed in a Bruker IFS66v equipment (Bruker, US). Data were collected in absorbance mode using a frequency range of 4000–400 cm^{-1} , and resolution of 4 cm^{-1} (mid infrared region) with 250 co-added scans. **Degree of methylation** 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 described earlier (Singthong et al., 2004).

2.4.3. Gas chromatography -FID

2.4.3.1. Sample preparation

To determine the monomeric composition, artichoke by-product **powder** was hydrolysed using sulfuric acid (H_2SO_4) following the methods of Seaman (Yapo & Koffi, 2008) and trifluoroacetic acid (TFA) (Garna et al., 2006). Briefly, in the first method 35 mg of sample were dispersed in 300 μL of 72% H_2SO_4 at room temperature for 3 h, diluted with 1 M H_2SO_4 and heated at 100 $^{\circ}\text{C}$ for 2.5 h. Supernatant was dried at 38-40 $^{\circ}\text{C}$ in a rotary evaporator for chromatographic analysis. For hydrolysis with TFA 30 mg of sample were hydrolysed with 1.5 mL of TFA 2M at 110 $^{\circ}\text{C}$ under inert conditions for 4 h (Garna et al., 2006).

In order to determine the monomeric composition of extracted artichoke pectin, **powder** samples were hydrolysed with TFA following the method mentioned above. Similarly, standards of D-arabinose, L-rhamnose, D-fructose, D-galactose, D-mannose, D-glucose and GalA at 20 mg mL⁻¹ were hydrolysed to correct the obtained results.

Besides, enzymatic hydrolysis of artichoke pectin, obtained in the optimal conditions, has been carried out to determine GalA content. Samples (2% w/v) were dissolved in 0.05 M sodium acetate buffer (pH 5.0) and hydrolysed with 90 U mL⁻¹ of Viscozyme[®] L preparation (Garna et al., 2006). Then, samples were incubated in an orbital shaker at 50 °C and 750 rpm (0.94g) for 24 h and immediately immersed in boiling water for 5 min to inactivate the enzyme.

2.4.3.2. Chromatographic analyses

Monomeric composition of artichoke by-product **powder** and extracted pectins was determined by GC-FID as trimethyl silylated oximes (TMSO) formed following the method of Brobst and Lott (1966). First, samples containing between 2.5 at 5 mg of sugars were added 0.4 mL of internal standard (I.S.) solution (0.5 mg mL⁻¹ phenyl-β-glucoside). Afterwards, the mixtures were dried at 38-40 °C in a rotary evaporator. Sugar oximes were formed by adding 250 μL of hydroxylamine chloride (2.5%) in pyridine and heating the mixture at 70 °C for 30 min and then silylated with hexamethyldisilazane (250 μL) and TFA (25 μL) and kept at 50 °C for 30 min. Reaction mixtures were centrifuged at 6708 g for 2 min at room temperature. Supernatants were injected or stored at 4 °C prior to analysis.

GC-FID analysis of TMSO derivatives was performed on an Agilent Technologies 7890A gas chromatograph (Wilmington, DE, USA) using a fused silica capillary column DB-5HT, bonded, crosslinked phase (5% phenyl-methylpolysiloxane;

15 m × 0.32 mm i.d., 0.10 µm film thickness) (J&W Scientific, Folsom, California, USA). The oven temperature was initially 150 °C increased at a rate of 1 °C min⁻¹ to 165 °C, then increased at a rate of 10 °C min⁻¹ to 200 °C, then increased at a rate of 50 °C min⁻¹ to 380 °C and held for 2 min. The injector and detector temperatures were set at 280 and 385 °C, respectively. Injections were carried out in split mode (1:5) using nitrogen at 1 mL min⁻¹ as carrier gas. To study the response factors relative to the internal standard, solutions containing arabinose, rhamnose, galactose, mannose, glucose, GalA, raffinose and stachyose were prepared over the expected concentration range in samples. The identities of carbohydrates were confirmed by comparison with relative retention times of standard samples. Data acquisition and integration were performed using Agilent ChemStation Rev. B.03.01 software. All analyses were carried out in duplicate and data were expressed as mean ± standard deviation (SD).

2.4.4. High Performance Size-exclusion Chromatography with evaporative light scattering detector (HPSEC-ELSD)

The molecular weight distribution of carbohydrates present in the reaction mixtures obtained by Celluclast activity over artichoke by-product was determined by HPSEC-ELSD. Samples (0.65 mg mL⁻¹) were dissolved in water filtered using a 0.45 µm syringe filter (Symta, Spain) and analysed in an Agilent Technologies 1220 Infinity LC System (Böblingen, Germany) equipped with an evaporative light scattering detector (1260 Infinity ELSD). The nebulisation temperature was 75 °C. Synthetic air at 85 °C was used for the evaporation at a flow-rate of 1.2 mL min⁻¹. The separation of carbohydrates was carried out on a TSK-GEL G5000PW_{XL} column (300 mm x 7.8 mm, 10 µm particle size) and TSK-GEL G2500PW_{XL} column (300 mm x 7.8 mm, 6 µm particle size) in combination with a TSK-GEL PW_{XL} guard column (40 mm x 6 mm, 12

µm particle size) (Tosoh Bioscience, Montgomeryville, PA, USA) using 0.01 M NH₄Ac, as mobile phase and elution in isocratic mode at a flow rate of 0.5 mL min⁻¹ for 50 min. The column was thermostatised at 25 °C and the injection volume was 50 µL (~32 µg of total carbohydrates). Data acquisition and processing were performed employing Agilent ChemStation software.

Molecular weight of carbohydrates was calculated by the external calibration method using solutions of commercial pullulan standards (M_w 0.342-788 kDa) (Fluka Analytical) in the range 10–2250 mg L⁻¹. All analyses were performed in duplicate, obtaining relative standard deviation (RSD) values below 10% in all cases.

2.5. Statistical analyses

Experimental design, RSM and statistical analysis were performed using the software STATGRAPHICS Centurion XVI.I. (Statistical Graphics Corporation, Rockville, MD, USA). Other data not obtained in the experimental design were submitted to an analysis of variance (P < 0.05).

3. RESULTS AND DISCUSSION

3.1. Characterisation of artichoke by-product

The values of pH, dry matter (DM), a_w, protein, fat, degree of methylation, minerals and different carbohydrate fractions (AIF, ASF, WIF and WSF) found in industrial artichoke by-product powder are listed in Table 1. Protein, fat and mineral contents were similar to reported by USDA (2017) for artichokes cooked, boiled and drained.

Table 1. Characterisation of powder artichoke by-product and extracted pectin.

	Artichoke by-product powder	Extracted artichoke pectin	
	Mean $\pm$ SD	Mean $\pm$ SD	
pH	4.9	6.0	
Dry matter (DM) (%)	91.7 $\pm$ 2.5	90.5 $\pm$ 0.3	
a_w	0.20 $\pm$ 0.01	0.26 $\pm$ 0.00	
Protein (mg g ⁻¹ DM)	140.5 $\pm$ 15.8	20.7 $\pm$ 1.0	
Fat (mg g ⁻¹ DM)	2.4 $\pm$ 0.2	-	
Alcohol insoluble fraction (AIF) (mg g ⁻¹ DM)	928.2 $\pm$ 5.9	-	
Alcohol soluble fraction (ASF) (mg g ⁻¹ DM)	71.8 $\pm$ 5.9	-	
Water insoluble fraction (WIF) (mg g ⁻¹ DM)	926.5 $\pm$ 1.6	-	
Water soluble fraction (WSF)(mg g ⁻¹ DM)	73.5 $\pm$ 1.6	-	
K (mg g ⁻¹ DM)	16.9	10.6	
Na (mg g ⁻¹ DM)	3.5	9.6	
Ca (mg g ⁻¹ DM)	4.7	7.3	
Mg (mg g ⁻¹ DM)	2.4	2.9	
B (mg g ⁻¹ DM)	0.02	0.04	
Degree of methylation (%)	9.1 $\pm$ 0.5	19.5 $\pm$ 0.0	

Water and alcohol insoluble fractions (WIF and AIF), they represented 926 and 928 mg g⁻¹ DM. These values were higher than that found by Femenia et al. (1998) (64% for external bracts), however this difference could be explained by the possible leaching occurred during artichoke by-product blanching, as was indicated by Gamboa-Santos, Montilla, Soria, and Villamiel (2012) that reported a loss of weight up to 31%

of soluble solids in the blanching water of carrot samples subjected to treatment at 95 °C during 5 min. Water and alcohol soluble fractions (WSF and ASF) represented 74 and 72 mg g⁻¹ DM of the sample, respectively.

Carbohydrate composition of WSF and ASF was determined by GC-FID, and as it can be seen in Table 2 the most abundant low molecular weight carbohydrates were fructose, glucose, sucrose, kestose and nystose, most of them probably released by the spontaneous hydrolysis of inulin, occurred during extraction of WIF and AIF.

Table 2. Carbohydrate composition found in water (WSF) and alcohol soluble fractions (ASF) from artichoke by-product powder and hydrolysates of artichoke by-product powder using H₂SO₄ (Seaman hydrolysis) and TFA (2 M).

	<i>Carbohydrates (mg g⁻¹ DM)</i>			
	Water soluble fraction (WSF)	Alcohol soluble fraction (ASF)	Hydrolysates	
			H ₂ SO ₄	TFA
Arabinose	-	-	14.2 ± 4.4	72.0 ± 18.5
Rhamnose	-	-	1.1 ± 0.0	10.9 ± 2.2
Fructose	21.8 ± 0.0	18.5 ± 1.1	4.4 ± 4.4	3.3 ± 2.2
Galactose	1.1 ± 0.0	-	2.2 ± 1.1	17.4 ± 4.4
Mannose	6.5 ± 1.1	4.4 ± 1.1	1.1 ± 0.0	29.4 ± 6.5
Glucose	15.3 ± 1.1	14.2 ± 1.1	19.6 ± 6.5	15.3 ± 4.4
Galacturonic acid	-	-	2.2 ± 0.0	33.8 ± 7.6
Sucrose	6.5 ± 1.1	7.6 ± 0.0	-	-
Kestose	6.5 ± 0.0	6.5 ± 0.0	-	-
Nystose	8.7 ± 1.1	12.0 ± 1.1	-	-

Besides, artichoke by-product powder were hydrolysed with acids to know monomeric composition; the results are depicted in Table 2. As observed that hydrolysates using H₂SO₄ (Seaman hydrolysis) contained arabinose, rhamnose, galactose and galacturonic acid (GalA) as pectic monosaccharides; and fructose, mannose and glucose indicating the presence of other polysaccharides as fructans, mannans, cellulose and hemicellulose in by-products from artichoke. The same type of monosaccharides was found in TFA hydrolysates, but in higher amounts than in H₂SO₄ hydrolysates, except for fructose and glucose. GalA levels in TFA hydrolysates were about 34 mg g⁻¹ DM, fifteen times higher than in H₂SO₄ hydrolysates. Since GalA is the main constituent of pectin, the high content found in TFA hydrolysates may indicate that the artichoke by-product is a good source to obtain pectin. In addition, taking into account the results of both hydrolysis, acid hydrolysis with TFA was selected to determine the monomeric composition of pectin extracted in the different assays of the experimental design with the aim of optimise the conditions of enzymatic extraction of artichoke pectin.

3.2 Optimisation of enzymatic extraction of pectin from artichoke by-product

In order to select the best enzyme to extract pectin from artichoke by-product, different commercial preparations were tested (Table S1). According to the results obtained, Celluclast presented high values of cellulase activity although pectinase activity was not detected then it is expected that extracted pectin will not be degraded. On the other hand, Viscozyme[®]L presented the highest pectinase activity. Therefore, this enzyme was selected for enzymatic hydrolysis of pectin to determine GalA content of most accurate form.

Once Celluclast preparation was selected for pectin extraction, the experimental design was developed to optimize the extraction conditions using different variables: artichoke by-product powder concentration (X_1), enzyme dose (X_2) and extraction time (X_3). Table 3 shows the results obtained during the enzymatic extraction of pectin from artichoke by-product for the different experimental variables studied. The yields of extracted pectin (Y_1) in the 17 assays of experimental design were in range of 98.1 - 208.3 mg g⁻¹ DM. The highest pectin yield was obtained in a reaction with 4.5% of artichoke by-product powder, 7.8 U g⁻¹ of enzyme during 27.2 h. This value was close to the theoretical optimum 225.7 mg g⁻¹ DM obtained, according to the RSM analysis, after 27.2 h using 7.9% of substrate and 10.7 U g⁻¹ of enzyme.

Following with the optimisation of pectin extraction design using Celluclast, other dependent variables, as GalA content (Y_2), pectic neutral sugars (Y_3 , arabinose, rhamnose and galactose) and other neutral sugars (Y_4 , fructose, glucose and mannose) were determined (Table 3). Hydrolysis of extracted artichoke pectin using TFA was performed to obtain individual values of each monosaccharide (see Table S2). The main carbohydrate quantified was GalA (Y_2) being in the range of 30.5 – 58.1 mg g⁻¹ DM reaching the maximum value at the same conditions of substrate concentration (4.5%) and enzyme dose (7.8 U g⁻¹) which was found the highest yield of extraction but at lower time of extraction (15h). The content of neutral sugars characteristic of pectin (Y_3) was between 12.2 – 37.2 mg g⁻¹ DM for these compounds maximum content was achieved at the highest extraction time and low artichoke by-product powder concentrations (4.5%). During the obtainment of pectin using Celluclast, other polysaccharides as cellulose, hemicellulose, mannan or inulin were extracted being the monosaccharides released from these polysaccharides glucose, mannose and fructose; the amount of these

compounds (Y_4) in the different assays was between $7.3 - 21.8 \text{ mg g}^{-1} \text{ DM}$. Unlike the other parameters, the value of this variable must be minimised.

The optimisation of the experimental conditions for pectin extraction and the results obtained of the most representative dependent variables under study (yield, GalA, pectic neutral sugars and other neutral sugars contents) were related by means of RSM. The regression equations describing the changes in these studied variables are shown in Table 4. As it can be observed, the variables that presented higher regression values were content of GalA, pectic neutral sugars, and pectin yield ($R^2 = 93.9, 92.8$ and 88.6% ; and $R^2_{\text{adj}} = 86.0, 83.6$ and 73.8% , respectively). Moreover, these variables are the most important in a pectin extraction process; the extraction was aimed at maximizing pectin yield and to get a product with the highest content of pectic sugars (GalA, arabinose, rhamnose and galactose) that reflect a high purity of pectin. So, these variables were selected to optimize the enzymatic extraction process using desirability function. The corresponding three-dimensional representation of the desirability function obtained is shown in Figure 1. Maximum predicted desirability (0.96) corresponds to an artichoke by-product powder concentration of 6.5%, an enzyme dose of 10.1 U g^{-1} and an extraction time of 27.2 h corresponding to a pectin yield of $175.6 \text{ mg g}^{-1} \text{ DM}$. Among the tested conditions, the highest observed values of desirability function were 0.86, 0.80 and 0.79 (corresponding to assays 9, 1 and 12) and 0.66 ± 0.04 to the central points (assays 3, 5 and 6).

421 **Table 3.** Values of independent variables (artichoke by-product powder concentration X_1 , enzyme dose X_2 and extraction time X_3) and dependent variables
422 (pectin yield Y_1 , galacturonic acid content Y_2 , pectic neutral sugars Y_3 and other neutral sugars Y_4), and predicted and observed desirability found in each assay
423 of pectin extraction after Celluclast treatment of artichoke by-products. Data are expressed as mg g^{-1} DM artichoke by-product powder.

Run	X_1 , artichoke by-product powder concentration (%)	X_2 , Enzyme dose (U g^{-1})	X_3 , Extraction time (h)	Y_1 , Pectin yield (mg g^{-1} DM)	Y_2 , GalA (mg g^{-1} DM)	Y_3 , Pectic neutral* sugars (mg g^{-1} DM)	Y_4 , Other neutral** sugars (mg g^{-1} DM)	Predicted desirability	Observed desirability
1	7.0 (+1)	13.3 (+1)	24.0 (+1)	190.8	49.8 ± 2.4	32.6 ± 2.7	15.8 ± 1.0	0.90	0.80
2	2.0 (-1)	13.3 (+1)	6.0 (-1)	163.6	47.8 ± 0.1	21.3 ± 0.3	11.5 ± 0.7	0.34	0.27
3	4.5 (0)	7.8 (0)	15.0 (0)	176.7	52.2 ± 0.9	28.9 ± 0.5	15.0 ± 0.4	0.68	0.62
4	4.5 (0)	0.3 (-2)	15.0 (0)	106.9	30.5 ± 0.5	13.1 ± 0.1	7.3 ± 0.2	0.10	0.00
5	4.5 (0)	7.8 (0)	15.0 (0)	179.9	58.1 ± 5.1	31.2 ± 1.7	15.5 ± 1.7	0.68	0.70
6	4.5 (0)	7.8 (0)	15.0 (0)	197.4	50.6 ± 1.2	29.8 ± 0.7	16.2 ± 1.3	0.68	0.65
7	2.0 (-1)	2.2 (-1)	24.0 (+1)	133.0	45.3 ± 0.1	22.5 ± 0.4	10.0 ± 0.5	0.00	0.00
8	4.5 (0)	15.3 (+2)	15.0 (0)	187.6	51.6 ± 2.1	30.5 ± 2.1	16.4 ± 3.4	0.55	0.65
9	4.5 (0)	7.8 (0)	27.2 (+2)	208.3	57.6 ± 1.9	37.2 ± 0.7	21.8 ± 1.2	0.81	0.86
10	7.0 (+1)	2.2 (-1)	6.0 (-1)	98.1	32.2 ± 3.1	12.2 ± 0.7	8.2 ± 0.5	0.00	0.00
11	2.0 (-1)	13.3 (+1)	24.0 (+1)	167.9	52.1 ± 3.3	36.1 ± 2.8	12.6 ± 1.6	0.40	0.41
12	7.9 (+2)	7.8 (0)	15.0 (0)	185.4	48.7 ± 1.5	32.8 ± 0.1	21.5 ± 1.1	0.73	0.79
13	2.0 (-1)	2.2 (-1)	6.0 (-1)	159.2	37.4 ± 0.4	20.4 ± 0.4	9.7 ± 1.2	0.11	0.18
14	7.0 (+1)	13.3 (+1)	6.0 (-1)	129.8	44.6 ± 0.7	23.4 ± 1.1	13.4 ± 0.5	0.50	0.50
15	7.0 (+1)	2.2 (-1)	24.0 (+1)	165.8	38.6 ± 1.3	24.6 ± 1.0	13.8 ± 0.9	0.48	0.50
16	1.1 (-2)	7.8 (0)	15.0 (0)	169.0	51.6 ± 3.4	33.5 ± 0.9	11.2 ± 0.8	0.27	0.00
17	4.5 (0)	7.8 (0)	2.8 (-2)	158.1	41.9 ± 3.4	23.3 ± 1.4	12.6 ± 1.0	0.43	0.43

424

425 Y_2 , Y_3 and Y_4 : values found in TFA hydrolysates of extracted artichoke pectin (See Table S2).

426 * Σ of arabinose, rhamnose and galactose. ** Σ of fructose, glucose and mannose.

427

Table 4. Regression equations for the model fit of the different variables studied during the enzymatic extraction of pectin from artichoke by-product using Celluclast.

Variables	Fitted model equation*	R ² (%)	R ² adj (%)
Pectin yield (Y ₁ , %)	$Y_1 = 11.8809 + 1.22659X_2 + 0.0766667X_3X_1 - 0.069827X_2^2$	88.6	73.8
Galacturonic acid (Y ₂ , mg g ⁻¹)	$Y_2 = 0.593232 + 0.161405X_2 - 0.00790439X_2^2$	93.9	86.0
Neutral sugars from extracted pectin (Y ₃ , mg g ⁻¹)	$Y_3 = 0.647902 + 0.191896X_2 - 0.0110103X_2^2$	92.8	83.6
Other neutral sugars (glucose, mannose and fructose content, Y ₄ , mg g ⁻¹)	$Y_4 = 0.0661304 + 0.130052X_2 - 0.00695625X_2^2$	77.9	49.4

*(X₁) Artichoke by-product powder concentration (%), (X₂) enzyme dose (U g⁻¹), (X₃) extraction time (h)

Figure 1. Estimated response surface for the desirability function.

Taking into account that optimal extraction time (27.2 h) was the $+\alpha$ value given by the design and probably the maximum of pectin extraction may not have been reached, extraction time of pectin was increased at 36.0 and 48.0 h using the same optimised concentration of artichoke by-product powder (6.5%) and enzyme dose (10.1 U g⁻¹). According to the obtained results, pectin extracted after 48 h of hydrolysis contained more GalA and higher yields were obtained. ~~Thus, the selected conditions were as follows: raw material concentration 6.5%, enzyme dose 10.1 U g⁻¹ and extraction time 48 h.~~ Under the above mentioned conditions, the experimental yield was 22.1% (221.4 mg g⁻¹ DM), which is well matched with the maximum predictive yield (225.7 mg g⁻¹ DM).

Therefore, the use of enzymatic preparation Celluclast is a good method to extract pectin from artichoke by-products and it could be an alternative to traditional methods which use acids to extract pectin with high yield. Dominiak et al. (2014) extracted pectin from lime

peel with different cellulases obtaining yield in the range of 18-26%, while using nitric acid the pectin yields were between 13-26%; similarly, Wikiera et al. (2015a) obtained higher yield with Celluclast (19%) than in sulfuric acid (15%) using apple pomace as raw material. Liew, Chin, Yusof, and Sowndhararajan (2016) obtained higher pectin yield from passion fruit peels with Celluclast (9.2%) than with citric acid (7.7%).

3.3 Characterisation of extracted *artichoke* pectin

Finally, a general characterisation of extracted artichoke pectin was performed. Thus, the physico-chemical parameters such as pH, DM, a_w , protein and degree of methylation were determined and the results are shown in Table 1. As can be observed the protein content of extracted pectin was low enough ($20.7 \text{ mg g}^{-1} \text{ DM}$), therefore it is not necessary to use any protease during enzymatic extraction to avoid presence of high level of protein in extracted pectin. With respect to mineral content in comparison with artichoke by-product, the most abundant cation was K, although its content was lower in the extracted pectin, besides, Na content was also greater probably due to buffer used during extraction. High Ca concentrations were found, probably due to high affinity of pectin to this divalent cation. Also, it is important to point out the high content of boron $0.04 \text{ mg g}^{-1} \text{ DM}$, associated to the pectin (the content of artichoke by-product powder was $0.02 \text{ mg g}^{-1} \text{ DM}$). In the bibliography has been reported that rhamnogalacturonan II (RG-II) suffer reversible dimerization with borate contributing to wall strength of the plant cell (Caffall & Mohnen 2009), similar role have calcium in apple and citrus pectin, while, in these products boron was not detected (Muñoz-Almagro, Montilla, Moreno, & Villamiel, 2017). The artichoke pectin obtained was classified as low-methoxyl pectin with a degree of methylation of 19.5%, similar to other

pectins from sunflowers and lower than those of apple and citrus pectin previously characterised in our laboratory (72-76%).

Other parameter studied in extracted pectin was the Mw distribution analysed by HPSEC-ELSD. The pectin extracted at optimal conditions presented the profile with three fragments of 660, 105 and 4.8 kDa, with abundances of 28.5, 36.6 and 34.9% respectively (Figure 2) This size distribution non monomodal is different to that of pectins extracted from citrus or apple (Muñoz-Almagro et al. 2017).

Figure 2. HPLC-SEC-ELSD profile of a hydrolysate of artichoke by-product obtained by enzymatic hydrolysis using Celluclast® 1.5 L under optimal conditions (artichoke by-product powder concentration 6.5%; enzyme dose 10.1 U g⁻¹; extraction time 48 h).

Following in artichoke pectin characterization, a determination of monomeric composition was performed in acid hydrolysates (TFA). Pectic monosaccharides such as arabinose (126.6 ± 5.8 mg g⁻¹ DM of pectin), galactose (23.5 ± 0.4 mg g⁻¹ DM), and rhamnose (18.8 ± 2.7 mg g⁻¹ DM) were quantified. Besides, glucose (30.4 ± 4.4 mg g⁻¹ DM), mannose (26.8 ± 0.8 mg g⁻¹ DM) and fructose (4.6 ± 2.9 mg g⁻¹ DM) were also present. Taking into account that GalA suffers a high degradation during acid hydrolysis, it was determined after

enzymatic hydrolysis of pectin using Viscozyme (Babbar et al. 2016). GalA content was $720.0 \pm 44.6 \text{ mg g}^{-1} \text{ DM}$ (72.0% DM), which is higher than 65% being the limit to consider pectin as food additive (E-440) (Morris, Belshaw, Waldron, & Maxwell, 2013). The characteristic that could be highlighted of monomeric composition of here extracted artichoke pectin is the high arabinose content. This is higher than found in pectin extracted from other sources like citrus and apple (Garna, Mabon, Wathelet, & Paquot, 2004; Gómez, Gullón, Yáñez, Parajó, & Alonso, 2013; Wikiera et al., 2015a) and similar to pectin from sugar beet pulp (Leijdekkers, Huang, Bakx, Gruppen, & Schols, 2015).

The amounts of rhamnose, arabinose and galactose with respect to GalA in the purified pectin indicated possible branching along the homogalacturonan (Yuliarti, Goh, Matia-Merino, Mawson, & Brennan, 2015). Considering the monomeric composition of pectin, the degree of branching (GalA/Rha), linearity pectin backbone [GalA/(Rha+Ara+Gal)] and extent of branching of RGI [(Ara+GalA)/Rha] were 19.2, 3.2 and 23.0, respectively. The degree and extent of branching of RGI were higher and the linearity pectin backbone was lower than those obtained for pectins from other sources as gold kiwifruit (Yuliarti et al., 2015) or lime peel (Dominiak et al., 2014). Another influential factor that can modify the structure of pectin is the extraction method, thus Fishman et al. (1991) obtained artichoke pectin with a low content of arabinose (3.4%), rhamnose (1.4%) and galactose (1.8%) using ammonium oxalate; being lower than orange pectin extracted with the same method, this fact may indicate a loss of branches. The maintenance of the structure, and more specifically the high arabinose content, could confer to this pectin anticarcinogenic property (Wikiera et al., 2015a).

As it has been indicated in the introduction, few papers have been published on the production and characterisation of artichoke pectin. Thus, Fishman et al. (1991) studied the

monomeric composition of carbohydrate fraction extracted with ammonium oxalate from artichoke, found high amount of GalA and other typical monosaccharides of pectin. Femenia et al. (1998) reported GalA concentrations about 40% in the alcohol insoluble carbohydrate fraction of artichoke stems, shown to be a good source of pectic polysaccharide-rich material. Fissore et al. (2014) obtained soluble fibre extracts from artichoke by-products with a GalA content between 17 and 25% of total carbohydrates. However, no pectin separation was carried out in both cases.

4. Conclusion

Artichoke by-products (a mixture of stems, leaves and bracts) can be used as a good source of pectins. The extraction of pectin from these residues using commercial Celluclast preparation has been optimised allowing an efficient extraction giving rise to a yield of 221.4 mg g⁻¹ DM. Enzymatic extraction is an environmentally-friendly process ~~than acid extraction~~ and allows to obtain high yields of pectin under optimal conditions. The pectin extracted from artichoke by-product powder was characterised found a high GalA content (720.0 mg.g⁻¹) and due to its degree of methylation (19.5%) was classified as low-methoxylated pectin. ~~Hence this pectin could be used as a food ingredient in the industry for the production of fruit-based yoghurts, acidified milk drinks, soymilk-based drinks, ice creams or low fat spreads. These~~ characteristics would allow the use of this pectin as a food ingredient.

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Supplementary data

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Artichoke by-product

Characterization

Enzymatic extraction
With **Celluclast® 1.5 L**

Artichoke pectin

Estimated Response-Surface
Substrate concentration = 4.5%

- ☐ **Optimal conditions:**
 - ✓ Artichoke by-product powder concentration (6.5%)
 - ✓ Enzyme dose (10.1 U g⁻¹)
 - ✓ Extraction time (27.2 h, +α value)
- Desirability: 0.93**
- ☐ **Pectin yield: *175.6 mg g⁻¹ dry matter (DM)***
- ☐ **Optimal extraction time +α value**
 - increased up to 48 h:
 - Pectin yield *221.4 mg g⁻¹ DM***