Pectin characterisation using size exclusion chromatography: A comparison of ELS and RI detection

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Abreviations

CCD: Centred Composite Design

ELSD: Evaporative Light Scattering Detector

HPSEC: High Performance Size Exclusion Chromatography

ICP: Industrial Citrus Pectin

LOD: Limit of Detection

LOQ: Limit of Quantification

Mw: Weight Average Molecular Weight

RID: Refraction Index Detector

RSD: Relative Standard Deviation

RSM: Response Surface Methodology

SEC: Size Exclusion Chromatograohy

Abstract

A high-performance size-exclusion chromatography (HPSEC) method coupled to Evaporative Light Scattering (ELSD) and Refractive Index (RID) detectors were evaluated and compared for the molecular mass (Mw) estimation of pectin in a wide range (0.342-805 kDa). Instrumental parameters of the ELSD were optimised by Response Surface Methodology (RSM) being 73 ºC the evaporator temperature and 0.9 mL/min the air flow rate. The linear range for the ELSD concentration response was wider (10 - 2,250 mg/L) and better (R^2=0.985) than RID (10 to 1,500 mg/L; R^2=0.875). The limits of detection (LOD) and quantitation (LOQ) for all pullulans hardly changed in ELSD (LOD: 1.22–1.99 mg/L; LOQ: 4.07–6.63 mg/L); however, RID showed huge variations (LOD: 0.49–10.41 mg/L; LOQ: 1.64–34.70 mg/L), which increased with the Mw. In general, responses of both detectors were similar for the Mw estimation although, pectin characterisation with HPSEC-ELSD exhibited better results in the lowest Mw compounds.

Keywords: Molecular mass, Pectin, Response Surface Methodology, light scattering, refraction index

1. Introduction

The complexity of pectin provides a multiplicity of structural features that determine differences in their physicochemical and technological properties (Holck, Hotchkiss, Meyer, Mikkelsen, Jorn, & Rastall, 2014; Zhang, Zhang, Liu, Ding, & Ye, 2015). Moreover, the variability of this polysaccharide may increase during its extraction from plants, storage, and processing of the food to which pectin is added as an ingredient (Novosel'skaya, Voropaeva, Semenova, & Rashidova, 2000). These reasons make the development of simple and robust analytical methodologies providing information about structural parameters of pectin necessary (Gómez-Ordóñez, Jiménez-Escrig, & Rupérez, 2012). In this regard, the weight average molecular weight (Mw) plays an important role in the structure and function of polysaccharides, influencing, for example, the gelling properties of pectin (Yapo, 2009). Among the methods for mass estimation of polymers, Size-Exclusion Chromatography (SEC) coupled with a Refractive Index Detector (RID) has been one of the most widely used (Gómez-Ordóñez et al., 2012; Zhu, Seburg, & Tsai, 2006). RID is a simple, universal and non-destructive detector system (Zhang et al., 2015); however, it has drawbacks such as low sensitivity and the lengthy time needed to stabilise the baseline, which have triggered a growing interest in the use of Evaporative Light Scattering Detector (ELSD), a semi-universal detector, which form of detection is dependent only on the mass of solute eluting. In this kind of detection, it is important to select the optimal operating conditions since the temperature of evaporation and gas flow rate can affect the ELSD signal (Dvořáčková, Šnóblová, & Hrdlička, 2014; Guiochon, Moysan, & Holley, 1988; Ma et al., 2014). Condezo-Hoyos, Pérez-Lopez, & Rupérez (2015) optimised the parameters of ELSD by response surface methodology (RSM) for the analysis of different carbohydrates including monosaccharides, oligosaccharides and

78 polysaccharides in a narrow range of Mw (up to 150 kDa). To the best of our
79 knowledge, no information is available on the comparison between RID and ELSD to
80 estimate the Mw and evaluate the abundance of the molecular species of pectin. Thus,
81 the main objective of this work was the validation and comparison of both
82 chromatographic systems HPSEC-RID and HPSEC-ELSD for the analysis of pectin and
83 pectin derived products within a wide range of Mw (0.342-805 kDa).

84 **2. Materials and methods**

85 *2.1. Reagents and standards*

86 A Pullulan Standard (Sigma, St. Louis, MO, USA), a glucan polymer composed
87 of α(1,6) linked maltotriose units, were used for calibrations. The weight average
88 molecular weight (Mw) and code of the different pullulans were P-0.3, 0.342 kDa; P-
89 1.3, 1.32 kDa, P-6, 6.20 kDa, P-10, 10 kDa; P-22, 21.7 kDa; P-49, 48.8 kDa; P-110, 113
90 kDa; P-200, 200 kDa; P-350, 348 kDa, P-800, 805 kDa). Industrial citrus pectin
91 samples, pure (ICP-4400), with maltodextrin (ICP-4030) or sugar added (ICP-4710) for
92 their standardisation, whose characteristics are presented in Table 1S, were kindly
93 provided by CEAMSA (Porriño, Pontevedra, Spain). Ammonium acetate and sodium
94 chloride were purchased from Panreac Applichem (Darmstadt, Germany).

95 *2.2. Instrumentation and chromatographic conditions*

96 Direct injection of each pullulan standards (P-0.3, P-1.3, P-6, P-10, P-22, P-49,
97 P-110, P-200, P-350, P-800 at 100 µg/L, n=3) without columns was used for a quick
98 optimisation of the ELSD parameters. Effect of air flow rate and evaporator temperature
99 on ELSD response was investigated by applying a Centred Composite Design (CCD)
100 using "Minitab® 17" software (Minitab Inc., State College, PA, USA). Nebulizer
101 temperature was established as 10 °C lower than evaporator temperature. Other

parameters of the ELSD were fixed: led intensity 100%, photomultiplier gain 1.0, data rate 40 Hz and smoothing 3.0 s. The design for the two independent variables at three levels, included thirteen experiment, four cube points, four axial points at a distance $\alpha = \pm 1.41$ from the centre, and five centre points. Response surface methodology (RSM) was used to optimise ELSD response as a function of air flow rate and evaporator temperature for each pullulan. To obtain the optimum conditions for all standards a desirability function was applied (Gamboa-Santos, Soria, Fornari, Villamiel, & Montilla, 2013).

The quadratic model for predicting the optimal point was expressed as follows:

$$y = C_0 + \sum_{i=1}^{2} C_i X_i + \sum_{i=1}^{2} C_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j<i} C_{ij} X_i X_j \qquad \text{(Equation 1)}$$

where y is response (area), C_0, C_i, C_{ii}, and C_{ij} are constant coefficients, and X_i and X_j are the independent factors. The quality of fit of the second-order model equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by F-value. The significance of the regression coefficients was tested by t-value.

Separation was achieved by HPSEC with two TSK-Gel columns G5000 PWXL (7.8 mm x 300 mm, 10 micron) and G2500 PWXL (7.8 mm x 300 mm, 6 micron) connected in series with a TSK-Gel guard column (6.0 mm x 400 mm) (Tosoh Bioscience, Stuttgart, Germany). These columns were connected at two different chromatographic systems. The HPSEC-ELSD analysis was carried out on a LC chromatograph Agilent Technologies 1220 Infinity and a detector ELSD 1260 Infinity (Agilent Technologies, Boeblingen, Germany). The HPSEC-RID analysis was done on a LC chromatograph Agilent Technologies 1220 Infinity and a detector RID 1260 Infinity (Agilent Technologies). Samples (50 µL) were eluted with two mobile phases

126 0.1 M NaCl and 0.01 M NH_4Ac, for RID and ELSD detection respectively, at flow rate
127 0.5 mL/min for 50 min at 30 °C. Before analysis, all samples and standards were
128 filtered through 0.45 µL Millipore membrane.

129 *2.3. Validation of ELSD and RID*

130 Series of pullulan standards (P-0.3, P-1.3, P-10, P-200 and P-800) were used for
131 calibration at various concentrations (ELSD: 10-2,250 mg/L; RID: 10-1,500 mg/L) and
132 injected in triplicate at the optimal conditions above selected. Standard curves of
133 pullulans for Mw estimation were obtained considering the logarithm of Mw versus the
134 corresponding elution volume.

135 Regression standard curves for quantification of the concentration of pectin by
136 ELSD were obtained considering the logarithm of detected area (mV min) versus the
137 logarithm of pullulan concentration (mg/L). In the case of RID, no logarithmic
138 transformation was needed. The linearity was evaluated by linear regression analysis
139 calculated by the least square regression method.

140 Limits of detection (LOD) and quantification (LOQ) were calculated using the
141 approach based on signal-to-noise ratio, 3/1 and 10/1 respectively, from standard
142 solutions with the lowest concentration (10 mg/L) (Ma et al., 2014).

143 The precision of the method was assessed in terms of repeatability intra- and
144 inter-day for ELSD and RID methods. Three replicates of sample ICP-4400 were
145 injected in three different days at a concentration of 1,000 mg/L. On the first day, the
146 data were used for the intra-day repeatability, whereas data from three different days
147 were used for the inter-day repeatability. The repeatability was expressed as relative
148 standard deviation (RSD) of the retention time and peak area.

3. Results and discussion

3.1. Optimisation of ELSD parameters

After performing the ANOVA analysis (Table 2S) for each pullulan, a linear significance (P>0.05) of the two parameters evaluated (air flow and evaporator temperature) was demonstrated. This result indicates a direct effect of both parameters on ELSD response. Data correlation with the RSM model was very accurate (R^2 > 92.9%). Moreover, the differences between R^2 and the R^2-adjusted were, in general, less than 1%, which indicates that the model obtained from data can explain over 92% of variation. Moreover, the models obtained for each pullulan could be used to predict the behaviour of the ELSD response for the carbohydrates analysed due to the high data correlation.

Figure 1S illustrates the optimal ELSD parameters (flow and evaporator temperature) for pullulans via RSM optimisation. It is possible to observe that the increase in the air flow rate had a great impact in the detector response. On the other hand, in most of the cases, evaporator temperatures above 85 ºC gave rise to poor responses in the ELSD (Rashan & Chen 2007). Thus, optimal conditions for ELSD quantification were set at 0.9 mL/min of air flow and 73/63 °C for evaporation/nebulisation temperatures, respectively. With these values, the individual desirabilities were higher than 0.90 and the overall 0.96, indicating the suitability of both approaches for maximisation of the detector response.

The optimised parameters allowed complete solvent evaporation as the gas flow rate was nearly twice the flow rate of mobile phase (Ma et al., 2014)**.** The utilisation of low evaporation temperature also contributes to the formation of larger droplets and higher baseline stability (Dvořáčková et al., 2014; Ma et al., 2014). Nogueira et al.,

(2005) obtained an optimal evaporator temperature of 45 ºC for the analysis of carbohydrates of beer. To determine sugars (up to tetrasaccharides), Márquez-Sillero et al., (2013) studied a nebuliser temperature between 30 - 55 ºC and 45 - 65 ºC for the evaporator temperature and established 45 ºC and 55 ºC as the optimal temperatures for the nebuliser and evaporator respectively. It is noteworthy that evaporator/nebulisation temperatures below 50 °C are employed when the mobile phase has acetonitrile in proportions ≥ to 60%. On the contrary, in the analysis of carbohydrates of higher Mw such as pullulans, dextran, pectin and maltodextrin, Condezo-Hoyos et al. (2015) found the optimal conditions for ELSD response at 1.1 mL/min of nitrogen flow and at 88 ºC and 78 ºC for the evaporator and nebuliser temperature respectively. These conditions might be explained by the flow, molarity and composition of the mobile phase (0.8 mL/min and ammonium acetate between 0.05 and 0.01 M); the nitrogen flow was higher ensuring the complete evaporation and nebulisation of samples. Similarly, Rashan & Chen (2007) established high evaporator and nebuliser temperatures (85 ºC) and 1 mL/min gas flow rate for the analysis of cellulose derivatives.

3.2 Validation of HPSEC coupled to ELSD and RID

3.2.1. Calibration curves, linearity, sensitivity, detection and quantitation limits and precision

ELSD and RID were used to set up the HPSEC analysis of pectin. Different mobile phases for each detection system were employed due to the incompatibility of using NaCl in the ELSD and the minor peaks resolution obtained with NH_4Ac 0.01 M in the RID (Figure 2S). Gómez-Ordóñez et al. (2012) established that a better reproducibility and peak shape can be observed when a low pH solution is used in RID. Those results are in accordance with the obtained pH using different mobile phases with RID (NaCl: 5.83 *vs* NH_4Ac: 6.74).

198 Table 1 shows the data obtained for the validation of the HPSEC-ELSD and
199 HPSEC-RID methods using pullulans. Retention time values provided good precision in
200 the Mw estimation, and the calibration curves obtained showed good linearity (R^2
201 ≥0.996) in the range of 0.342-805 kDa. In general, the experimental Mw were
202 reasonably close to nominal values with a variation between 1-22% for ELSD and 2-
203 27% for RID. Gómez-Ordóñez et al. (2012) and Izumi, Aikawa, Matsuda, Hasunuma &
204 Kondo (2013) determined the Mw distribution of pullulans (1-2,500 kDa and 5-788
205 kDa) using HPSEC-RID with a difference of 1-25% and 1-35%, respectively. Condezo-
206 Hoyos et al. (2015) estimated the Mw of only one pullulan (100 kDa), obtaining an
207 experimental mass of 118 kDa.

208 The linearity of the responses for ELSD and RID was evaluated (Table 1).
209 Although the RID exhibited good linear response for individual pullulans ($R^2 > 0.991$),
210 when these were considered altogether, the function of pullulan concentration had a
211 worse response in the range of 10-1,500 mg/L ($R^2 = 0.875$). However, the regression
212 curves obtained by ELSD showed better correlation values between peak area (y, mV
213 min) and concentration (x, mg/L) for all pullulans with a second order polynomial fit:

214 $y = 0.0092x^2 + 16.395x + 301.9$; $R^2 = 0.924$

215 Nevertheless, to obtain a linear relation, it was necessary to transform at $\log_{10}$ for both
216 experimental variables (Rashan & Chen, 2007; Zhu et al., 2006). Thus, the regression
217 curves for all carbohydrate standards showed better correlation (R^2=0.985) even over a
218 wide range of concentrations (10 – 2,250 mg/L) (Table 2).

219 The linear ranges reported in the literature using RID were 2.5–750 mg/L for the
220 analysis of hydroxypropyl cellulose (Zhu et al., 2006) and 500 – 2,000 mg/L for the
221 determination of polysaccharides from red seaweed (Gómez-Ordóñez et al., 2012).

Linearity data obtained by ELSD were comparable to those of the literature for different carbohydrates: oligosaccharides 15–2,000 mg/L (Zhou et al., 2014), inulin-type of oligosaccharides 40–1,180 mg/L (Yang, Hu, & Zhao, 2011), or pectin, pullulan, dextran 250–1,000 mg/L (Condezo-Hoyos et al., 2015).

The sensitivity was calculated from the slope of regression curves for all the standards (Table 1). In the case of RID, the slope values were within the range of 2.52–5.53, and were lower than those found by Gómez-Ordóñez et al. (2012) (9.13–16.27) for the quantitation of alginate, fucoidan or iota-carrageenan. On the contrary, the slopes obtained in the ELSD system ranged from 1.38 to 1.55, and were comparable to the slopes reported for oligosaccharides (1.42–2.15) (Zhu et al., 2006) and polysaccharides (1.29 – 1.44) (Condezo-Hoyos et al., 2015).

As shown in Table 1, both systems were sensitive enough with LOD and LOQ values between 0.49 and 10.41 mg/L and 1.64 and 34.70 mg/L for RID and 1.22–1.99 mg/L and 4.07–6.63 mg/L for ELSD. Even though RID seemed to allow the quantification of lower concentrations than the ELSD system, its LOQ values contained a huge error because the intercept (-100.6) of the calibration curve corresponded to an area with a value equivalent of 50 mg/L. Therefore, the quantitation could overestimate the concentration data. Moreover, LODs and LOQs for RID showed high variability among the different pullulans studied and increased with the Mw, whereas these values kept constant in the case of the ELSD system.

3.2.2 Application of the validated methods for analysis of industrial pectins

Chromatographic profiles of industrial pectins (ICP-4030, ICP-4400 and ICP-4710) are shown in Figure 1. According to the Mw distribution, a major peak (~700–430 kDa) at 25 min was found in all samples and both detectors corresponding to

molecular species included as pectin. In addition, ICP-4030 and ICP-4710 also displayed a peak around 29-35 min and other minor peaks at 35-39 min (ELSD: ~0.9-0.2 kDa; RID: ~0.8 kDa), which may be due to the addition of sugar and maltodextrin during the processing of pectins as it is described in the specification sheets. As observed, ELSD response presented a higher peak resolution allowing the elution of four well defined peaks (Figure 1a), whereas RID response was not as reliable due to the overlap of peaks 3 and 4 (Figure 1b).

Industrial citrus pectin (ICP) 4400, was used to calculate the precision of HPSEC coupled with RID and ELSD (Table 2). The intra-day and inter-day repeatability of retention times were similar (~0.6%) and good for both detectors. Furthermore, the same assays for peak areas were carried out and RSD (%) values were 3.1% and 4.4% for RID and around 2.0% for ELSD, which shows good precision for both chromatographic systems. For the analysis of hydroxypropyl-cellulose, Zhu et al. (2006) showed a precision with RSDs of 2.5% for HPSEC-ELSD and 4.5% for HPSEC-RID.

Therefore, with respect to the quantitation, as indicated in Table 3, ICP-4400 presented a higher concentration of pectin (~700 mg/g) than the others, 4030 and 4710 (~500 mg/g), thereby highlighting that the composition 4400 sample was only pectin. Moreover, peaks found in ICP-4030 and ICP-4710 corresponding to the lowest Mw compounds, reported a molecular mass analogous (~20 *vs* ~38 mg/g and ~0.2-0.9 *vs* ~0.2-0.9 mg/g) between each other for ELSD and RID, what indicated that the sugar added during the processing of pectin 4030, could have a similar structure to the maltodextrin added in the another one.

On the other hand, it is also important to highlight that the quantitation of the pure pectin (ICP-4400) was very similar with both detectors. Nonetheless, the total carbohydrates quantified in the ICP-4030 and ICP-4710 samples, with low Mw compounds, were upper in ELSD than RID (~800 *vs* ~700 mg/g), demonstrating the better suitability for quantifying samples with compounds with highly varying Mw.

Although the ELSD has been widely used to analyse different oligo- and polysaccharides (Dvořáčková et al. 2014) its utilisation to characterise acid polysaccharides such us pectin has been scarce. Coupled to HPSEC was used for qualitative study of polysaccharides containing GalA with a wide range of molecular mass (Kuang et al., 2011), similarly Xie et al. (2012) compared acid polysaccharide chromatographic profiles from two species of *Ganoderma,* without hydrolyse or treated with different enzymes, pectinase, xylanase or cellulose between others. However, as Condezo-Hoyos et al. (2015) showed, and in this work, this chromatographic system allows the determination quantitative of pectins.

ELSD have other applications related with pectins, this detector has been coupled at different chromatographic system such as HPAEC (Cameron & Grohmann, 2005), to determine Mw of pecto-oligosaccharides, similarly at pulsed amperometric detector (PAD). For the same type of compounds ELSD has been coupled to hydrophilic interaction chromatography joint to mass spectrometry detection (HILIC–ELSD–MSn), this is a valuable tool for identification of a wide range of neutral and acidic cell wall derived oligosaccharides with DP up to 15 (Leijdekkers, Sanders, Schols, & Gruppen, 2011).

On the other hand, although the commonly preferred detector for sugar is the RID, ELSD was preferred, by its sensitive for mono- and disaccharide analysis

293 (Lachowicz, Oszmianski, Seliga, & Pluta, 2017). However, it is necessary to considerer
294 that sugar analysis of salt-rich media using HPLC-ELSD has a problem of interferences
295 from salt effects on mobile phases (Epriliati, Kerven, D'Arcy, & Gidley, 2010).

296 **4. Conclusions**

297 The optimisation results of the ELSD response carried out by RSM with
298 pullulans of a wide range of Mw (0.34 - 805 kDa) indicated that the air flow rate had the
299 highest impact in the detector response. The optimal values of air flow rate and
300 evaporation temperature for all standards were 0.9 mL/min and 73 °C, reaching a
301 desirability value of 0.955. The comparison of both ELSD and RID systems showed
302 that the former had better sensitivity than the latter with lower LOD and LOQ values,
303 regardless of the Mw of the standard used. Moreover, the linear range of the pullulan
304 concentration was wider in the ELSD chromatographic system. When both methods
305 (ELSD and RID) were compared for pectin quantitation, the precision was better in the
306 ELSD (2.1 *vs* 4.4). Chromatographic profiles of analysed industrial pectins showed a
307 better resolution of peaks in the case of the ELSD system, which allowed the
308 quantitation of components with low Mw. According to the results obtained, we can
309 establish that the HPSEC-ELSD is a suitable system, better than HPSEC-RID, for
310 estimating Mw and quantitation of the concentration of different pectins.

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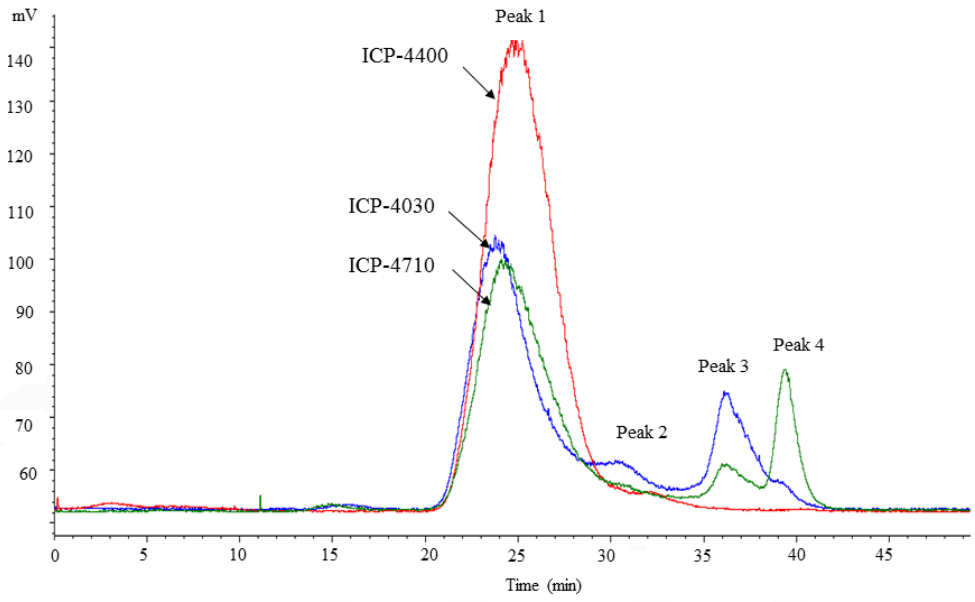
FIGURE LEGENDS

Figure. 1. Chromatographic HPSEC profiles of industrial pectins (ICP-4030, ICP-4400, ICP-4710), using a) ELS and b) RI detectors.

Table(s)

Fig. 1.

a)



b)

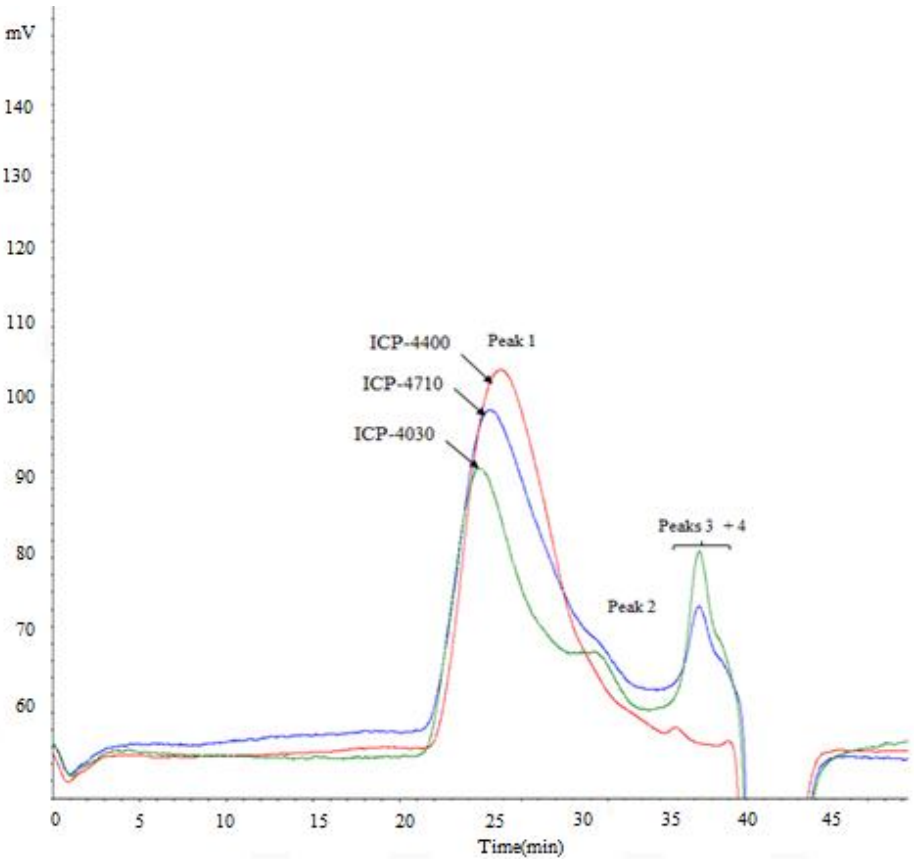


Table 1. Retention times, calibration curve equations, linearity, sensitivity, limits of detection and quantitation of the pullulans standards analysed by HPSEC using ELSD and RID at concentrations of 10-2,250 mg/L and 10-1,500 mg/L, respectively.

Detector	Code	Mw (kDa)		Retention time[b] (min)	Calibration curve[c]	Linear range (mg/L)	Linearity (R^2)	Sensitivity		LOD (mg/L)	LOQ (mg/L)	Calibration curve[d]
		Theoretical[a]	Experimental					Slope (m)	Intercept (b)			
ELSD	P-0.34	0.34	0.30	38.1 ± 0.0	log Mw = -0.465x + 11.36 R^2 = 0.997	10 – 2,250	0.9919	1.40	0.25	1.99	6.63	log (y) = 1.458 log (x) + 0.0759 R^2 = 0.985
	P-1.32	1.32	1.31	35.5 ± 0.1			0.9912	1.38	0.15	1.53	5.09	
	P-10	10.00	12.77	31.2 ± 0.1			0.9918	1.43	0.08	1.26	4.19	
	P-200	200.00	207.26	26.0 ± 0.1			0.9898	1.51	0.02	1.22	4.07	
	P-800	805.00	782.03	23.8 ± 0.2			0.9884	1.55	- 0.12	1.43	4.78	
RID	P-0.34	0.34	0.30	39.1 ± 0.1	log Mw = -0.453x + 11.35 R^2 = 0.996	10 – 1,500	0.9990	2.52	- 49.02	0.49	1.64	y = 4.5x - 100.61 R^2 = 0.875
	P-1.32	1.32	1.35	36.6 ± 0.1			0.9957	3.24	- 12.21	1.72	5.74	
	P-10	10.00	13.70	31.8 ± 0.1			0.9989	4.23	-49.50	3.58	11.92	
	P-200	200.00	222.60	26.5 ± 0.2			0.9969	5.53	-78.94	7.27	24.25	
	P-800	805.00	730.02	24.3 ± 0.2			0.9908	5.49	-195.42	10.41	34.70	

[a]Mw according to the manufacturer specifications.
[b]Data are mean values ± standard deviation with n = 9.
[c]x = elution volume (mL) R^2 = correlation coefficient.
[d]x = concentration (mg/L); y = area; R^2 = correlation coefficient.

Table 2. Intra-day and inter-day repeatability of retention time and peak area of industrial pectin (ICP-4400) analysed by ELSD and RID.

Detector	Intra-day repeatability (n = 3)				Inter-day repeatability (n=9)			
	Retention time (min)	RSD (%)	**Peak area**	**RSD (%)**	Retention time (min)	RSD (%)	**Peak area**	**RSD (%)**
ELSD	24.7 ± 0.2	0.7	19848 ± 406	2.0	24.7 ± 0.1	0.5	19766 ± 417	2.1
RID	25.8 ± 0.2	0.6	902858 ± 28341	3.1	25.9 ± 0.2	0.7	885898 ± 39291	4.4

Data are mean values ± standard deviation; RSD = relative standard deviation

Table 3. Estimation distribution and quantitation of Mw of industrial pectins (ICP-4030, ICP-4400, ICP-4710) analysed by HPSEC with ELSD and RID.

		ELSD		RID	
Industrial pectins	**Number of peaks**	**Estimated Mw (kDa)**	**Concentration (mg/g)**	**Estimated Mw (kDa)**	**Concentration (mg/g)**
4400	1	434 ± 16	732 ± 9	317 ± 11	711 ± 22
4030	1	693 ± 3	433 ± 1	603 ± 6	427 ± 2
	2	20 ± 0.1	127 ± 4	20 ± 2	124 ± 8
	3	0.9 ± 0.0	174 ± 1	0.8 ± 0.0*	166 ± 1*
	4	0.2 ± 0.0	44 ± 3		
	Total	-	778 ± 2	-	717 ± 4
4710	1	480 ± 13	534 ± 8	421 ± 7	492 ± 7
	2	38 ± 1	88 ± 6	36.4 ± 0.4	116 ± 10
	3	0.9 ± 0.0	96 ± 4	0.8 ± 0.0*	114 ± 8*
	4	0.2 ± 0.0	139 ± 4		
	Total	-	857 ± 5	-	722 ± 8

Mean value of three determinations ± SD.
*Peak corresponding to peaks 3 and 4 of ELSD.

Tabla 1S. Certificates of analysis of industrial citrus pectin (ICP) from CEAMSA.

Analysis	**ICP-4030**	**ICP-4400**	**ICP-4710**
Composition	Pectin and maltodextrin	Pectin	Pectin and sugar
pH	2.8 – 3.4 (1 % solution)	2.8 – 3.4 (1 % solution)	2.8 – 3.4 (1 % solution)
Degree of esterification (%)	71 -75	58 – 62	70 – 75
Total plate count (cfu/g)	<5000	<5000	<5000
Moulds and yeasts (cfu/g)	<300	<300	<300
Pathogenic bacteria (*E. Coli*, *Salmonella)*	Negative by tests	Negative by tests	Negative by tests
Loss on drying (%)	<12	<12	<12
Heavy metals (ppm)	<20	<20	<20
As (ppm)	<3	<3	<3
Pb (ppm)	<5	<5	<5
Hg (ppm)	<1	<1	<1
Cd (ppm)	<1	<1	<1

Table 2S. Regression coefficients, R^2, adjusted R^2, probability values, and the significance of effect of each independent variable for pullulan standards.

Source	P-0.3 kDa		P-1.3 kDa		P-6 kDa		P-10 kDa		P-22 kDa		P-49 kDa		P-110 kDa		P-200 kDa		P350 kDa		P-800 kDa	
	Coef	P-value	Coef	P-value	Coef	P-value	Coef	P-value	Coef	P-value	Coef	P-value	Coef	P-value	Coef	P-value	Coef	P-value	Coef	P-value
β	3631.32	0.000	3223.55	0.000	4114.35	0.000	4313.32	0.000	2818.43	0.000	3453.06	0.000	2697.80	0.000	3561.43	0.000	2685.21	0.000	3041.03	0.000
Linear																				
β1	-1588.29	0.000	-1581.99	0.000	-1744.18	0.000	-1841.66	0.000	-1396.76	0.000	-1467.27	0.000	-1220.69	0.000	-1530.69	0.000	-1171.91	0.000	-1516.71	0.000
β2	170.91	0.025	302.79	0.000	311.70	0.000	308.22	0.000	282.00	0.000	206.25	0.005	225.44	0.000	219.80	0.002	186.24	0.003	309.79	0.000
Quadratic																				
β11	186.70	0.023	391.68	0.000	124.78	0.115	152.87	0.074	308.69	0.000	87.62	0.241	124.39	0.048	124.33	0.088	90.43	0.150	368.74	0.000
β22	100.19	0.208	278.67	0.000	1.84	0.987	38.57	0.645	222.96	0.002	31.37	0.672	68.54	0.266	53.41	0.456	54.58	0.381	238.33	0.001
Interaction																				
β12	58.98	0.570	-67.38	0.468	-65.95	0.522	-69.67	0.528	-115.57	0.193	-24.27	0.803	-65.47	0.418	-31.63	0.737	-28.66	0.726	-144.65	0.100
Residual																				
lack of fit		0.000		0.000		0.000		0.000		0.000		0.000		0.000		0.000		0.000		0.000
Pure error																				
Total																				
R^2 (%)	93.68%		95.23%		94.86%		94.68%		94.52%		93.45%		93.68%		94.36%		92.90%		95.51%	
Adj-R^2 (%)	92.72%		94.51%		94.08%		91.51%		93.69%		92.45%		92.72%		93.51%		91.93%		94.82%	

β0 = intercept; β1,β2 = linear coefficients; β11,β22 = quadratic coefficients; β12= interaction coefficient

Figure 1S. Three dimensional surfaces for optimisation, using RSM, of ELSD parameters (air flow and evaporation temperature) in the analysis of the different pullulans standards. (a) P-0.3 kDa; (b) P-1.3 kDa; (c) P-6 kDa; (d) P-10 kDa; (e) P-22 kDa; (f) P-49 kDa; (g) P-110 kDa; (h) P-200 kDa; (i) P-350 kDa; (j) P-800 kDa.

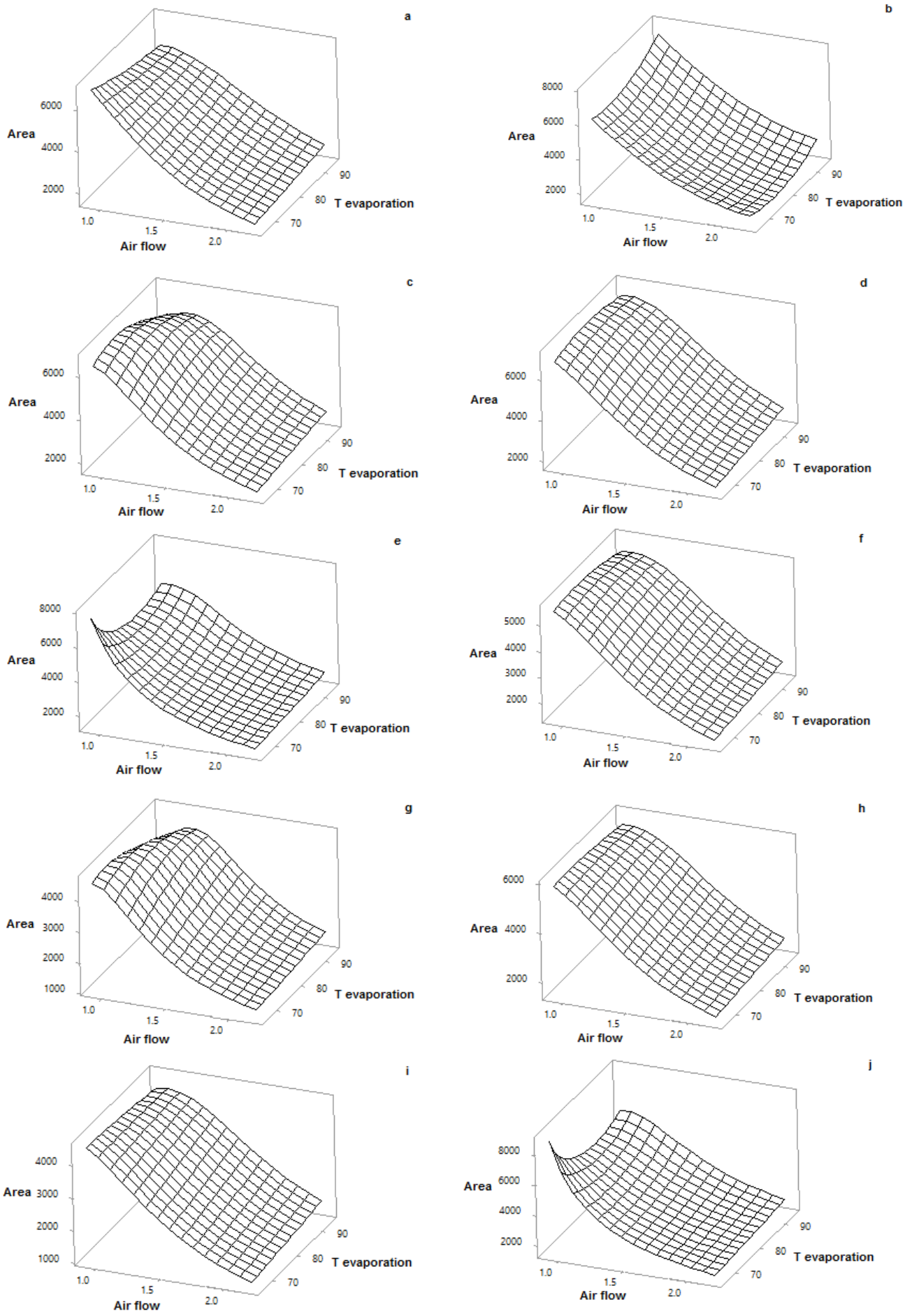
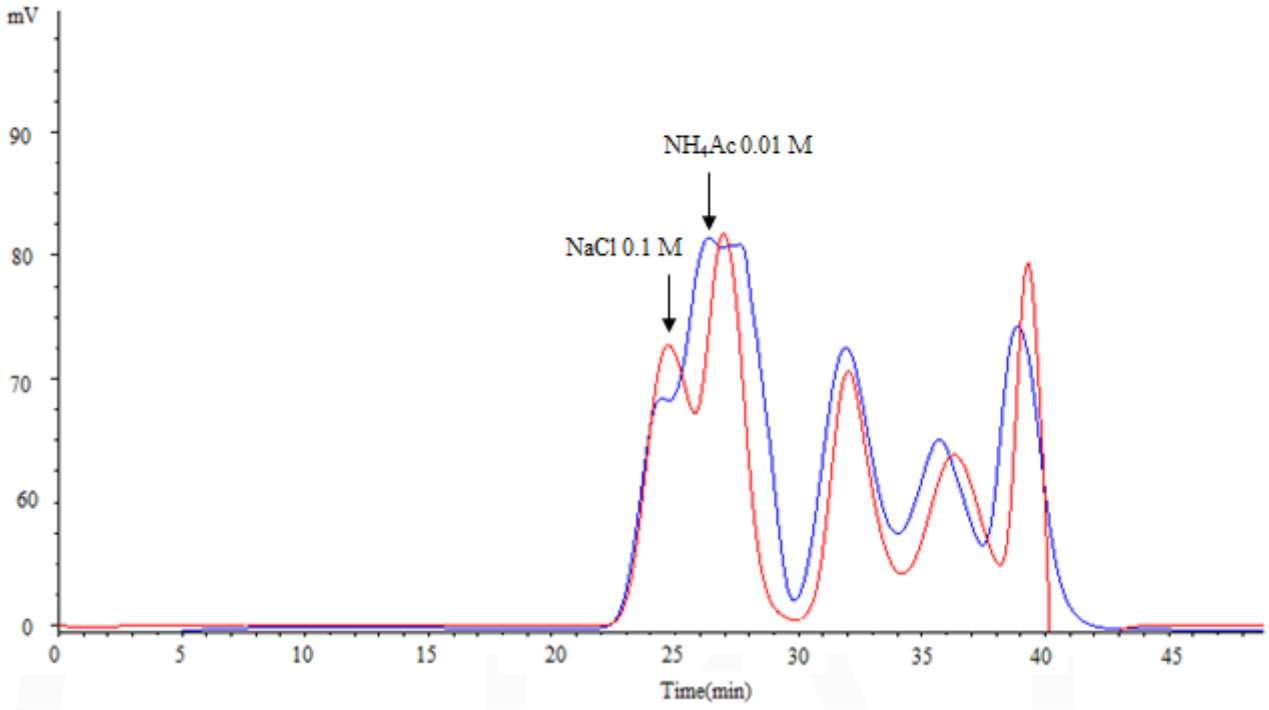


Figure 2S. Effects of mobile phase (NH_4Ac and NaCl) on the chromatographic separation of the pullulan standards detected by RID.



***Highlights (for review)**

- An optimisation of ELSD was done maximizing the detector response by RSM
- The air flow rate had the highest impact in the response ELSD
- Pectin characterisation with HPSEC-ELSD exhibited better results than HPSEC-RID