THE USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AS SCREENING TECHNIQUE FOR PECTIN AND PECTIN SUBSTANCES OF DIETARY FIBERS

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INTRODUCTION

Pectin and polygalacturonic acid have applications as therapeutic and diagnostic pharmaceutical agents such as the magnetic resonance imaging agent Lumen Hance (Gregory, W *et al*., 1999). It has been demonstrated that modified pectin (MPC) to be effective in suppressing or preventing metastases (Eliaz, I., 2001; Eliaz, I., 2011). Determination of individual pectin contents in fresh or dried biological materials and in their fiber products is an important mixed biopolymer analysis means and food nutrition analysis means for evaluating structure, quality and variety. In the past years the analysis of water soluble pectin fraction and insoluble pectin fraction in dietary fibers has attracted the interest of the researchers due to their beneficial effects on health. It has been established that fibers in the diet can exert an antioxidant effect; preventing development of atherosclerosis. Various methods have been reported for the determination of pectin in foods, or biological materials including titration (Alfonso, Garcia, E., 2010), spectrophotometry (M. A. Monsoor *et al*., 2001), mass spectrometry (Piet J.H. *et al*., 1998; Thomas Stoll *et al*., 2003). Most of these methods are time-consuming. There is a need for a rapid analytical screening procedure to analyze the pectin. Many review articles covering different theoretical and practical aspects of chromatographic methods and its applications in different fields appeared: an HPLC (Geovana Rocha Placido Moore *et al*., 2005), high resolution size-exclusion chromatography (HR-SEC) (S. Vidal et *al*., 2001), high-performance size-exclusion chromatography (HPSEC) (Sang-Ho Yoo, *et al*., 2006; Beda Marcel Yapo *et al*., 2007). Schols and other were able to separate pectin populations present in commercial pectin compounds according to their charges, using an HPLC system equipped with an anion exchange column (MA7P column) on an analytical scale (Schols *et al*., 1989) (Piet J.H. *et al*., 1998). The HPLC method with aWAX column was found to discriminate between commercial pectins efficiently: pectins with similar DM (degree of methylesterification) or similar DS (degree of substitution: methylesters and amide groups) having different physical properties showed various populations (S. E. Guillotin *et al*., 2007).

MATERIAL AND METHODS

The insoluble pectin fraction (AIP) was submitted followed by acid hydrolysis (Ravin, Gnanasambandam, Proctor, A, 1999). Ground fibers were extracted with 0.1 N HNO_3, (1:20, ratio), at 90° C for 40 min in a rotary evaporator, cooled to room temperature in a water bath, and centrifuged (15 min). The supernatant was collected, and the sediment was extracted twice more in 0.1 N HNO_3. All the three supernatants were combined and dispersed in equal volumes of 2–propanol to precipitate the pectin, and allowed to settle for about 4 h. The precipitate was collected, centrifuged, dispersed in 2–propanol, stirred for 30 min and centrifuged. This was repeated one more time with 2–propanol and, finally, with 70% 2–propanol. The sediment was dispersed in a small amount of water and freeze dried. The pectin obtained by sequential extraction and the total pectin extracts were each subjected to the following analyses.

The water soluble pectin fraction of dietary fibers was extracted with water, with phosphate buffer (pH 4.8) or with 0.01 N Phosphoric acid. This method was developed for identifying (WSP) pectin fraction with 0.01 N Phosphoric acid. The fiber (0.2 g) were dispersed in 100 ml of 0.01 N Phosphoric acid and stirred for 1 h at room temperature. The supernatant was collected, and the sediment was extracted twice more in 0.01 N Phosphoric acid. All the three supernatants were combined, centrifuged. Combined supernatants, after centrifugation were concentrated in a rotary evaporator and then the supernatants were each subjected to the following analyses.

HPLC apparatus. An Agilent 1100 HPLC system (Agilent Technologies, Palo Alto CA-USA) operated by Windows NT based ChemStation software was used. The HPLC equipment was used with a diode array detector (DAD). System consisted of a binary pump, degasser and auto sampler. The column used was a C610H: 7.8 mm × 300 mm. The ultra-violet spectra (scanning from 190 nm to 400 nm) were recorded for all peaks.

High-performance liquid chromatography method. In addition to the gravimetric and other determinations of WSP and AIP were analyzed by the high-performance liquid chromatography method (HPLC). Solutions of samples were filtered through a 0.22 µm pore size membrane filter before injection to chromatography analysis. The HPLC (Ravin, Gnanasambandam, Proctor, A, 1999) was modified for identifying pectin. The HPLC was used to determine galacturonic acid content and pectin of the fiber. D-galacturonic acid monohydrate and polygalacturonic acid (Sigma) was used as standard. All the standard solutions underwent the same type of treatment (were filtered through a 0.22 µm disposable filter disk). Phosphoric acid (0.01 N) with a flow rate 0.70 ml/min was used as the mobile phase. Ten µl of samples were injected and the detection wavelength was 210 nm. The identification of compounds was achieved by comparing their UV spectra and retention times of separated peaks with retention times of standards. For comparison of retention times of galacturonic acid, polygalacturonic acid and pectin with pure standards of organic acids the flow rate of 0.5 ml/min and 0.7 ml/min was used.

RESULTS AND DISCUSSION

Galacturonic acid. Chromatographic analyses of organic acids, based on the method described by Chen, En, and Zhang(*et. al.*,2006) were carried out using the HPLC equipped with UV detection at wavelength 214 nm; at 265 nm (Sara, C. Cunha *et.al.*, 2002) and 210 nm, as was done in this study. The HPLC elution of galacturonic acid standard at 0.7 ml/min resulted in two peaks in the region 5÷9 min. It was observed that the area of second peak is increased from 145.550 mAU x s to 3206.94 mAU x s as the galacturonic acid content increased from 4.49 x 10^{-2}%g/ml to 100.3 x 10^{-2}%g/ml, respectively. According to the retention time of standard organic acids, the peak around 8.557 min can be attributed to galacturonic acid. Thus the retention time of this second peak may be used to identify the galacturonic acid. Data of the HPLC chromatogram of galacturonic acid is presented in the figure 1A. In general the value of retention time for standard solution having 100.3 x 10^{-2}%g/ml galacturonic acid was slightly higher (8.619 min) than for standard solution having 24.06 x 10^{-2}%g/ml galacturonic acid (8.541 min) and 4.49 x 10^{-2}%g/ml galacturonic acid (8.528 min) but these differences are less than <<0.5 min. The relative standard deviation of galacturonic acid qualification at 0.7 ml/min flow rate is 0.35%. The relationship between galacturonic acid content and peak area revealed a straight line with the correlation coefficient of 0.9997. Data were fitted to the equation *Y*= 3227.8X – 0.3211 where "Y" is the peak area and "X" is concentration in %g/ml (Fig 2A). This linearity was maintained over the concentrations range of 0.04÷1.00%g/ml. The value of LOQ was found as 0.0042 for proposed method.

Polygalacturonic acid. The chromatographic behavior of standard solutions was studied by using the flow rate 0.5 ml/min (a) and 0.7 ml/min (b) (Tab 1). The mixtures of pectin, polygalacturonic acid and other organic acids resulted in low resolution of the chromatographic separation at 0.5 ml/min that usually is used for the analysis of organic acids and neutral sugars, thereby making complexity the identification and quantification of the polygalacturonic acid and pectin at this flow rate. The polygalacturonic acid is eluted at 7.851 min by using 0.5 ml/min. The use 0.7 ml/min allowed accelerated elution of the polygalacturonic acid. The chromatograms corresponding to polygalacturonic acid showed a peak to approximately at 5.6 min (Tab 1).The change of mobile phase flow from 0.5 ml/min to 0.7 ml/min resulted in the variation of retention time for polygalacturonic acid and significantly decreased of the peak area from 23.030; 56.930; 668.29 to 15.656; 34.870; 496.92, respectively. Gregory W. White and et. al (1999) have been reported that since polygalacturonic acid is insoluble in acidic aqueous solutions, therefore for procedure optimized of the solvent system to be use a 50 mM phosphate buffer, pH 6.9. It was suggested the first, that the most successful approach to use the HPLC has been via the change pH of the solvent system. The second, using flow-rate of 0.7 ml/min, it was possible to obtain good quality chromatogram profile, injecting 10 µl into the column C610H. The peak position of polygalacturonic acid in range 5.596÷ 5.605 min at 0.7 ml/min was confirmed by HPLC analysis of this pure standard component (Fig 1B). The relative standard deviation, standard deviation, standard error and confidence interval of retention time were 0.06%; 0.323 x 10^{-2}; 0.108 x 10^{-2}; 0.248 x 10^{-2} respectively. Deviations the retention time of standard solutions of polygalacturonic acid were between 5.596 min and 5.605 min for the 0.6÷50.0 x 10^{-2} %g/ml concentrations. The fitted model was represented by the calibration equation: *Y*= 1009.8X – 14.774 where "Y" is the peak area and "X" is the concentration in %g/ml with the correlation coefficient of 0.99 (Fig 2B). The calibration graph of the area versus concentration was found to be linear over 1.7÷50.0 x 10^{-2}%g/ml range. The coefficient of correlation was 0.999 for the three linear regression analyses.

Pectin. It is expected that the HPLC method established might also be useful for the direct determination of pectin fractions. The chromatograms corresponding to pectin fractions gave two peaks around 7.980 min and 8.748 min; 5.615 min and 5.855 min (Fig 3A; B) by using 0.5 ml/min and 0.7 ml/min flow rate, respectively. The metrological characteristics of retention time of water soluble pectin fractions and insoluble pectin fractions were as follows: standard deviation 3.55 x 10^{-2}; standard error 0.725 x 10^{-2}. These values were the greater than metrological characteristics of retention time of polygalacturonic acid, but the relative standard deviation of retention time at a confidence probability of 0.95 does not exceed 0.63%. The chromatogram profile obtained for pectin solution in the presence of excipients was identical with that obtained for standard solution containing an equivalent concentration of pectin fraction indicating that the retention time and the area of peak don't change. The proposed method could be considered rugged. The variation in the statistic data of area detection (water soluble pectin fractions) were not statistically significant having average Std.dev, average Std. error and average C.I. of means: 3.77; 2.13 and 8.87, respectively. The average relative standard deviation for area detection of solutions with lower concentration (e.g. water soluble pectin fractions) is 8.93% (RSD) at 0.7 ml/min flow rate. The average relative standard deviation for area detection of solutions with higher concentration (e.g. insoluble pectin fractions) is 0.67% (RSD) at 0.7 ml/min flow rate. The results show that the proposed method was applied successfully for the assay of pectin fractions in dietary fibers and can be applied for characterizing natural products, pharmaceutical agents as well as commercial dietary fibers, etc. Each flow rate of HPLC method (e.g. 0.5 ml/min; 0.7 ml/min) relates to the generation of a slight different retention time through variety of the concentration. Generation of the slight different retention time forms the basis to the measurement of concentration range. The content of water soluble pectin fraction "X" in the solution was calculated by the calibration equation: *Y*= 1009.8X – 14.774. A more appropriate range for the concentration of pectin fractions is a 0.04÷0.19%g/ml that the retention time is generated directly in a stable range 5.553÷5.625 min closer to the retention time corresponding to standard pure solution of polygalacturonic acid (5.600 min). The deviation of value of retention time by diluted of analytes is reduced compared to initial solutions with higher concentration. Pectin solutions were diluted to 1:316.3 ratios in order to obtain detection responses within the range of the standard curve. The chromatogram for this solution is presented in the figure 3B. The relative standard deviation, std.dev, std. error and C.I. of retention time are 0.05%; 0.260 x 10^{-2}; 0.082 x 10^{-2}; 0.186 x 10^{-2} by using the dilution of initial solutions in 316.3 times, respectively. The relative standard deviation, std.dev, std. error and C.I. of retention time are 0.26%; 1.470 x 10^{-2}; 0.463 x 10^{-2}; 1.050 x 10^{-2} by using of initial solutions with higher concentration, respectively. From a comparison of the all chromatograms, it could be clearly seen that the best separations are those achieved with column (C610H) with using 0.7 ml/min flow rate. In order to optimize the respective flow rate of elution, the water soluble pectin fraction and insoluble pectin fraction with the different characteristics (molecular weigh 5÷70 KDa, galacturonic acid content 23.5÷74.7% and 0÷37.7% (DME) methoxyl content of pectin) were used. Firstly, the typical chromatogram profile of pectin solution by the HPLC method detailed here is corresponded to the chromatogram profile of polygalacturonic acid standard. Secondly, the pectin with the molecular weight 5÷70 KDa, galacturonic acid content 23.5÷74.7%, degree of esterification 0÷37.7% were eluted at the same time as polygalacturonic acid of about 5.600 min between 5.599 min and 5.644 min. Thirdly, previous studies indicated that the pectin may be detected at 210 nm as the polygalacturonic acid (S.E. Guillotin *et.al.*, 2007). The method described above can be used to investigate the pectin with heterogeneity. We suggested that the concentration of polydispersity pectin samples having two peaks must be received through summation of all area the related peaks.

Table 1 The chromatographic behavior of polygalacturonic acid standard solutions
Legend:*The flow rate was 0.5 ml/min (a) and 0.7 ml/min (b).

No.	Flow rate b*				Flow rate a*	
	Peak 1		Peak 2		Peak 1	
	Retention time, min	Area, mAU x s	Retention time, min	Area, mAU x s	Retention time, min	Area, mAU x s
1	5.602	11.210	n	n	7.858	15.030
2	5.605	11.892	n	n	7.856	16.060
3	5.603	12.910	n	n	7.854	17.530
4	5.603	12.379	n	n	7.854	17.070
5	5.599	15.656	n	n	7.849	23.030
6	5.596	34.870	n	n	7.836	56.930
7	5.598	496.92	8.009	77.033	7.847	668.29

Figure 1 HPLC chromatograms of standard galacturonic acid (A) and standard polygalacturonic acid (B) solutions.

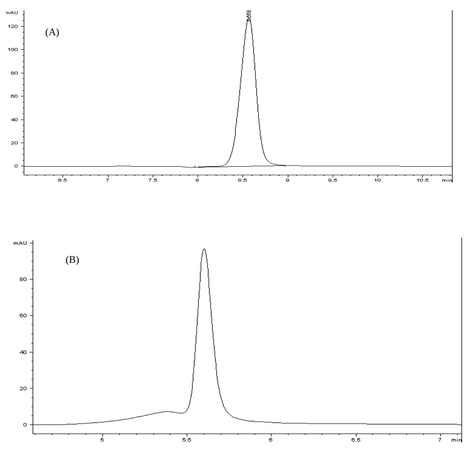


Figure 2 The calibration plots for galacturonic acid (A) and polygalacturonic acid (B).

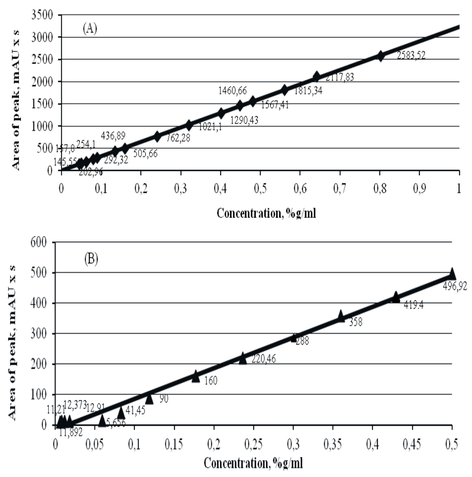
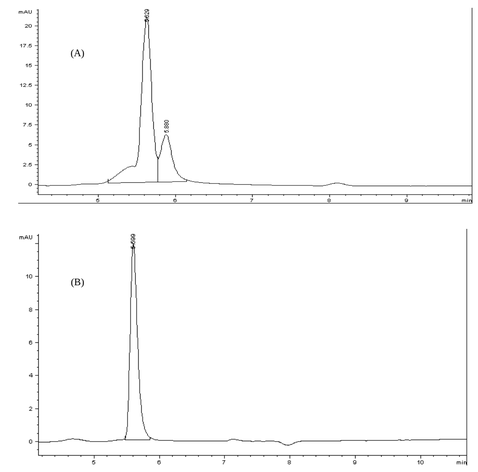


Figure 3 HPLC chromatograms of water soluble pectin solution (A) and insoluble pectin solution (was diluted to 1:316.3 ratios) (B).



CONCLUSION

In this study a rapid, accurate, precise, sensitive and selective HPLC method was developed for the determination of galacturonic acid, polygalacturonic acid and pectin compounds. Moreover, this method is simple and inexpensive and it can be employed for the routine quality control of dietary fibers. As shown here, this technique can be used for: "Targeted analysis of pectin compounds in plant tissues". These approaches are useful for characterizing natural products, pharmaceutical agents as well as dietary fibers. This can be used in a wide range of applications, including:

– Quality control
– Compositional analysis
– Product stability
– Competitive analysis
– Nutritional studies.

The retention time 8.557min and 5.600min can be used to the direct qualification of galacturonic acid and polygalacturonic acid. The retention time around 5.600 min was identified as time for the direct qualification and quantification of pectin fractions in dietary fibers. The line calibration with regression line *Y*= 1009.8X – 14.774 was used for the quantification of pectin fractions in dietary fibers. The detection limits 0.04%w/v. Good recovery results were obtained for the determination of water soluble and insoluble pectin fraction in 45 varieties of dietary fiber samples in the validation set.

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