

Evaluation of Hydrolytic Activity of Different Pectinases on Sugar Beet (*Beta vulgaris*) Substrate Using FT-MIR Spectroscopy

Adina CHIȘ¹, Florinela FETEA², Horea MATEI¹, Carmen SOCACIU^{2*}

¹University of Medicine and Pharmacy "I. Hațieganu", Faculty of Medicine, 6 Pasteur Street, Cluj-Napoca, Romania

²University of Agricultural Sciences and Veterinary Medicine, Faculty of Agriculture, 3-5 Mănăștur Street, Cluj-Napoca, Romania; casocaciu@usamucluj.ro (*corresponding author)

Abstract

The aim of this study was to evaluate the pectinase activity of two commercial enzymes Rohament PL and Rohapect 10L, using as substrate the sugar beet pulp, in different conditions. The method applied to check the rate of hydrolysis was Fourier-transformed infrared spectroscopy (FT-MIR), by recording the absorption spectra of different carbohydrates in the specific, MIR (middle IR) spectral range (650-4000 cm⁻¹). In order to calibrate the method, it has been recorded the FT-MIR spectrum of standard solutions of glucose, fructose, sucrose and galacturonic acid, at different concentrations, establishing also the peaks and the fingerprint regions specific to these compounds. Considering the specific peak intensities identified for glucose (at 1033 cm⁻¹), fructose (at 1063 cm⁻¹), sucrose (at 995 cm⁻¹), and based on peak area for galacturonic acid (1500-700 cm⁻¹), it has been calculated their concentrations, as a result of the Rohament PL or Rohapect 10L activity. Based on calibration curves for each sugar type, it has been calculated their concentrations in the sugar beet samples, and after their release by enzymatic treatment, establishing an optimized protocol of action for the two enzymes. Differences among the two enzymes activity were identified, specifically their optimum concentration and hydrolysis timing. FT-MIR spectroscopy proves to be an adequate method to evaluate the enzymatic activity of the different enzyme types, having certain advantages in comparison with the chromatographic methods, being a rapid, non-destructive method, at relatively low costs.

Keywords: FT-MIR, hydrolytic enzymes, Rohament PL, Rohapect 10L, sugar beet

Introduction

Sugar beet (*Beta vulgaris*) is a plant of the genus *Beta*, and a member of the family *Chenopodiaceae*. Sugar beet pulp is especially rich in polysaccharides, such as cellulose (20-24%), hemicellulose (25-36%) and pectic substances (15-25%) (Thibault *et al.*, 1990). The chemical analysis of sugar beet pulp showed that beet pectins differ from apple and citrus pectins by their higher rhamnose content, by the presence of acetyl groups and by the presence of ferulic acid, one of the major phenolic acids found in plant cell walls (Micard *et al.*, 1996). The pectin substances in plant cell walls are found mainly in two regions: a "hairy" rhamnogalacturonan region (RG) and a "smooth" homogalacturonan region (HGA). Both, RG and HGA regions, consist of a backbone of α -(1-4)-linked galacturonic acid residues, partially esterified with methyl group ("smooth" regions) which may be interrupted by "hairy" regions (which contain mainly neutral sugars) (Baciu and Jordening, 2004). In these regions, the galacturonic acid accounts for approximately 54.4-77.9%, the rhamnose for 0.9-3.2%, arabinose for 1.8-12.5% and galactose for 2.4-8.1%. Also, sugar beet pulp contains 1-2% lignin, 7-8% protein and carbohydrates that compose the structural homopolymers (cellulose, hemicelluloses) and pectins such as sucrose, glucose, fructose (Foster *et al.*, 2001).

There are two types of enzymes that catalyze the hydrolysis of the pectic substances from sugar beet pulp: pectolytic enzymes, which react with the "smooth" regions of the pectin (composed of partially methoxylated galacturonic acid) and glycan hydrolases, which hydrolyze glycosidic bonds in the "hairy" pectins regions (Sakamoto *et al.*, 1993). Under enzyme action, the cell wall polysaccharides are solubilized and depolymerized, generating different types of substances, such as monosaccharides, disaccharides, galacturonic acid, etc. (Chiș *et al.*, 2010).

Fourier-transformed infrared spectroscopy (FT-MIR), is a well established technique in chemical analysis, as well as a convenient method employed for sugar determination owing to the strong absorption bands of carbohydrates, but it is relatively uncommon compared with chromatographic or other classical methods. FT-MIR has been used to predict sugar contents in both agricultural and food products (Rambla *et al.*, 1998).

This technique, a non-destructive one was reported successful for the determination of sucrose and glucose (Fernandez and Agosin, 2007; Patz *et al.*, 2004) and the discrimination of red wines, edible oils and beer (Ramasa-mi *et al.*, 2004; Urtubia *et al.*, 2008).

The aim of this study was to evaluate, by FT-MIR spectroscopy, the activity of two different types of commercial pectinases, Rohament PL and Rohapect 10L, under dif-

ferent experimental conditions, using as substrate a sugar beet pulp. The utilization of commercial preparations, instead of purified enzymes, was intended with respect to a possible industrial application.

Materials and methods

Commercial pectinases

The commercial enzymes chosen for this study were: Rohament PL and Rohapect 10L, produced of AB Enzymes, Darmstadt, Germany. Rohament PL has higher pectolytic activity, useful for the maceration of fruit and vegetable tissues; it contains almost exclusively polygalacturonase activity with minor pectinesterase activity. Rohapect 10L is a commercial enzyme produced by *Aspergillus niger*, and has pectolytic and arabinase activity. The optimum pH (3.9) for enzyme activity was achieved by acetate buffer addition (acetic acid 1N, NaOH 1N).

Sample preparation and enzymatic treatment

Sugar beet pulp from the common beet *Beta vulgaris* was provided from Sugar Factory "Zahărul" from Luduş County (Transylvania, North of Romania) and stored at -20°C before analysis.

The unfrozen pulp was cut into small pieces and then chopped in a blender. The puree obtained was divided into 21 aliquots, 9 samples for each type of enzyme used in this study and 3 control samples (one for each incubation time). Each aliquot consisted of 5 g sugar beet puree and 7 ml acetate buffer, pH 3.9. The enzymes were added at different concentrations (24, 12 and 2.4%), using different incubation times (30 min, 3 and 24h), as presented in Tab.

Tab. 1. Experimental conditions applied for testing pectinase activity (incubation time, concentration and code)

Enzyme	Incubation time	Concentration	Code
Rohament PL	30 min	2,4%	RPL30min2,4%
		12%	RPL30min12%
		24%	RPL30min24%
	3 h	2,4%	RPL3h2,4%
		12%	RPL3h12%
		24%	RPL3h24%
	24 h	2,4%	RPL24h2,4%
		12%	RPL24h12%
		24%	RPL24h24%
Rohapect 10L	30 min	2,4%	R10L30min2,4%
		12%	R10L30min12%
		24%	R10L30min24%
	3 h	2,4%	R10L3h2,4%
		12%	R10L3h12%
		24%	R10L3h24%
	24 h	2,4%	R10L24h2,4%
		12%	R10L24h12%
		24%	R10L24h24%

1. All samples were stirred and then incubated for different time at 25°C. After incubation, samples were centrifuged for 3x15 minutes at 4000 rpm. Tab. 1 summarizes the preparation of samples and their code.

FT-MIR measurements

All analyses (on carbohydrate standards and juice supernatants obtained after enzyme hydrolysis) were done using a FT-MIR spectrometer (Shimadzu Prestige 2, spectrometer). Each spectrum was recorded in the MIR region, from 4000 to 650 cm^{-1} , and 64 scans were accumulated for each spectrum using the Horizontal Attenuated Total Reflection (HATR) device.

Calibration curves

In order to identify the carbohydrate specific fingerprint regions and to calibrate the method, different concentrations of pure, standard solutions of glucose (1, 2, 3, 4, 5, 10, 15, 20 and 25 g/100 ml), fructose (3, 4, 5, 7, 10, 15, 20 and 25 g/100 ml), sucrose (1, 2, 3, 4, 5, 10, 15, 20 and 25 g/100 ml) and galacturonic acid (20.08, 28.92 and 34.71 g/100 ml) were analyzed by FT-MIR spectrometry in the same conditions.

Data analysis

The spectra were processed using the IR solution Software Overview (Shimadzu) and OriginR 7SR1 Software (OriginLab Corporation, Northampton, USA). To calculate quantitatively the glucose, fructose, sucrose and galacturonic acid-like the mainly compound released in the sugar beet juice, it has been considered the calibration curves and it has been applied the curve factor.

Results and discussion

FT-MIR fingerprints and calibration curves for glucose, fructose, sucrose and galacturonic acid

The FT-MIR spectra (4000-900 cm^{-1}) of aqueous solutions of glucose (25%), fructose (25%) and sucrose (25%) were presented in Fig. 1A. As can be seen, the FT-MIR spectra of these solutions are very well defined and show the specific absorption bands (Fig. 1B) in the fingerprint region.

For glucose, the fingerprint region (1200-900 cm^{-1}) includes characteristic bands, with absorptions at 991, 1033, 1078, 1107 and 1149 cm^{-1} , the peak at 1033 cm^{-1} having the highest absorption. Fructose and sucrose have, also, the same fingerprint region (1200-900 cm^{-1}): fructose has specific maxima at 966, 979, 1063, 1082 cm^{-1} , and a specific absorption peak at 1063 cm^{-1} , while sucrose shows specific maxima at 995, 1053 and 1136 cm^{-1} , with a maximum absorption at 1053 and 995 cm^{-1} , in a ratio of 1:1.

The FT-MIR spectra and fingerprint region for galacturonic acid were different from glucose, fructose and sucrose spectra, as shown in Fig. 2A. The fingerprint region of galacturonic acid (Fig. 2B) is much larger than the other

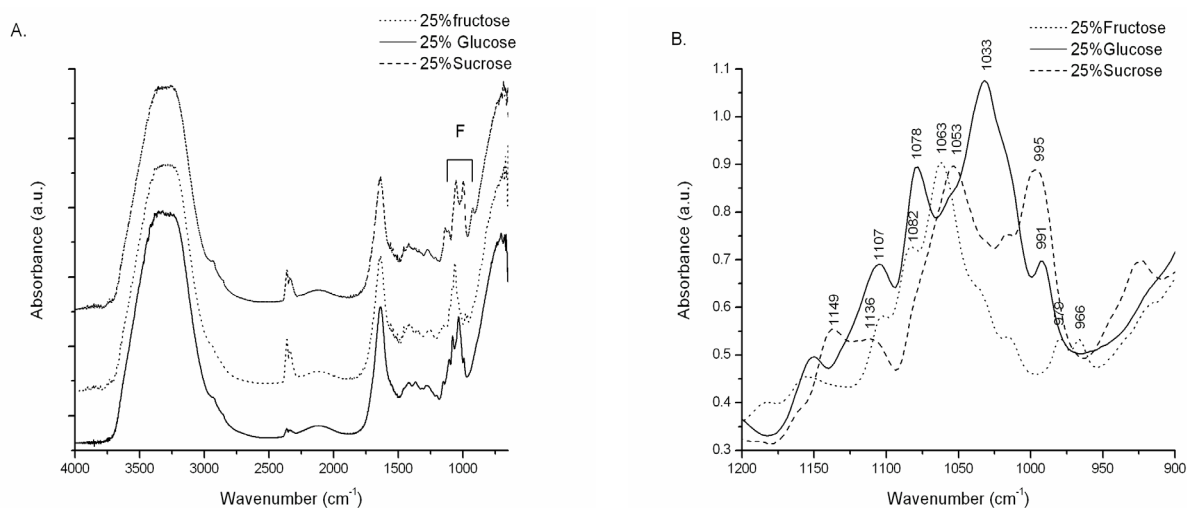


Fig. 1. (A) The integral FT-MIR spectra (4000-900 cm⁻¹) of aqueous standard solutions containing 25% glucose (solid line), 25% fructose (dot line) and 25% sucrose (dash line). (B) Detailed, specific peaks in the fingerprint region (F) (1200-900 cm⁻¹) of the normalized spectra (1200 cm⁻¹)

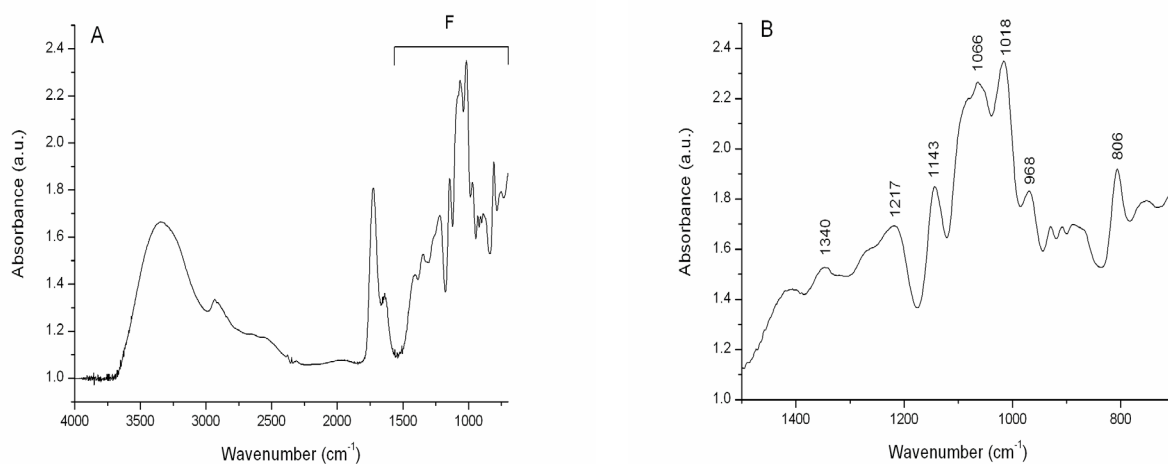


Fig. 2. (A) The integral FT-MIR spectra (4000-700 cm⁻¹) of aqueous standard solution containing 41.66% galacturonic acid. (B) Specific peaks in the fingerprint region (F) (1500-700 cm⁻¹)

carbohydrates, namely 1500-700 cm⁻¹. The specific peaks for galacturonic acid are: 1340, 1217, 1143, 1066, 1018, 968 and 806 cm⁻¹ (Fig. 2B).

The spectral signatures of sugars are somewhat different from each other, as mentioned also by other authors (Max and Chapados, 2007). The most intense peak of glucose (1033 cm⁻¹) is characteristic to the C-O and C-C stretching vibrations, while for fructose or sucrose the most intense peaks appear around 1063, or 1053 and 995 cm⁻¹, respectively. Unlike other simple molecules, sugars have endocyclic and exocyclic C-O bonds, located at 995 cm⁻¹ (exocyclic) for sucrose, and around 1080 cm⁻¹ (endocyclic) for glucose and fructose (Max and Chapados, 2007). The peak of galacturonic acid (located at 1340 cm⁻¹) is characteristic to the CH₂ bending vibrations, while the 1217, 1066 and 1018 cm⁻¹ peaks to the C-O bending vibrations, while 1143 cm⁻¹ to the C-O-C asymmetric stretching vibra-

tions and 968 cm⁻¹ to the CO symmetric stretching vibrations (Kacurakova *et al.*, 2002).

Based on these data it has been obtained the calibration curves (data not shown), based on the peak intensities for each carbohydrate (glucose, fructose and sucrose) (Chiş *et al.*, 2010) or based on peak areas (for galacturonic acid) in the fingerprint region (1500-700 cm⁻¹). The Calibration curves with pure standards were used in order to determine the glucose, fructose, sucrose and galacturonic acid concentrations in the present samples. The equations and the correlation coefficients for the calibration curves of carbohydrates were: $Abs = +3.298E-1 + 2.929E-2 \cdot c^{\wedge}1$, $R^2 = 0.999282$ for glucose, $Abs = +3.208E-1 + 2.334E-2 \cdot c^{\wedge}1$, $R^2 = 0.998039$ for fructose, $Abs = +3.501E-1 + 2.057E-2 \cdot c^{\wedge}1$, $R^2 = 0.996550$ for sucrose and $y = 2.5999x - 47.131$, $R^2 = 0.9968$ for galacturonic acid.

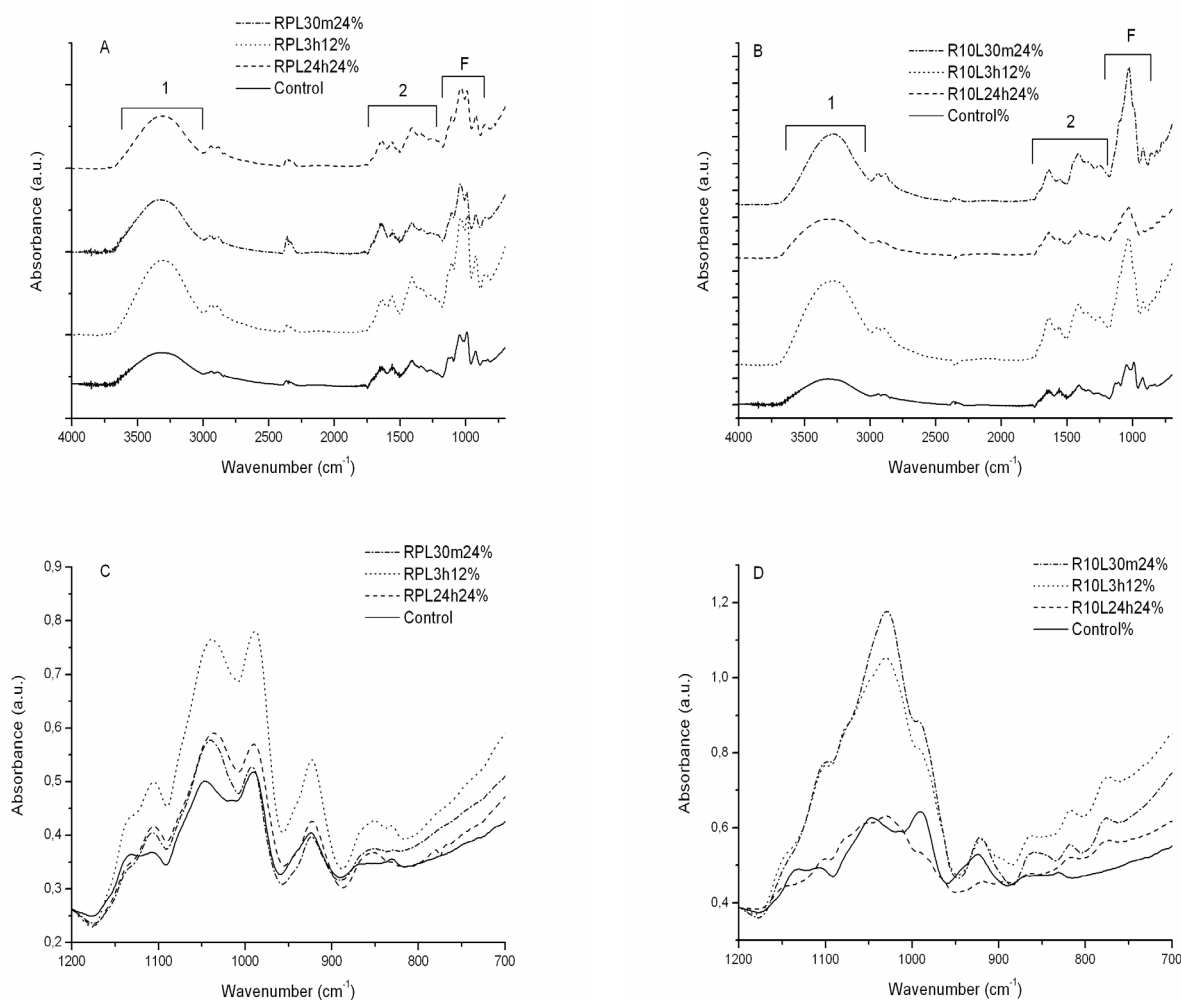


Fig. 3. The FT-MIR spectra ($4000\text{-}700\text{ cm}^{-1}$) of the sugar beet juice obtained by enzymatic treatment [RPL (A) and R10L (B)] and without enzymatic treatment (control). Three specific regions 1, 2 and F are marked. Details of peaks in the specific fingerprint region (F) ($1200\text{-}700\text{ cm}^{-1}$) in experimental samples against control [RPL (C) and R10L (D)]. For sample codes see Tab. 1.

FT-MIR spectra and fingerprint regions specific to sugar beet juice obtained with and without enzymatic treatment

In the present experiment it has been recorded a number of 21 FT-MIR spectra from the sugar beet juices obtained from puree, with or without enzymatic treatment. The specific FT-MIR spectra ($4000\text{-}700\text{ cm}^{-1}$) for both enzymes are shown (Fig. 3A and 3B), as well the fingerprint regions ($1200\text{-}700\text{ cm}^{-1}$) after enzymatic treatment, comparing with control (Fig. 3C and 3D).

The spectra of sugar beet juice obtained with/without enzymatic treatment show three absorption regions: $3700\text{-}2850\text{ cm}^{-1}$ (1), $1800\text{-}1200\text{ cm}^{-1}$ (2) and a fingerprint region, $1200\text{-}700\text{ cm}^{-1}$ (F). The first region, marked 1, correspond to water and OH absorption frequencies, and to stretching vibrations of C-H inside CH_3 or CH_2 groups. The region 2 corresponds to carbonyl-specific absorptions ($1700\text{-}1500\text{ cm}^{-1}$). The third region corresponds to the fingerprint region (F) of carbohydrates specific to sugar beet. As can be seen in Fig. 3B, this region is different in case of

sample treated with RPL and R10L. The FT-MIR spectra of samples obtained after enzymatic treatment with RPL shows two specific peaks in this region, 1039 and 991 cm^{-1} , similar peaks as for control, while in the case of samples treated with R10L, it has been observed just one peak, around 1030 cm^{-1} .

Other author (Kameoka *et al.*, 1998) noticed many peaks in this region, attributed to C=O and C-OH stretching modes, which overlapped each other. These peaks depend on the sugar structure and on the interaction between the sugar molecules and their environments.

Thus, it was possible to identify the peaks characteristic to glucose, fructose, sucrose and galacturonic acid in sugar beet juice, in accordance with peaks observed in pure carbohydrates (Fig. 1). The peaks recorded at 1033 , 1063 and 995 cm^{-1} were considered for the evaluation of hydrolytic activity and the rate of glucose, fructose and sucrose release, respectively. For galacturonic acid quantification in the samples, it has been considered the peaks area of the region $1500\text{-}700\text{ cm}^{-1}$.

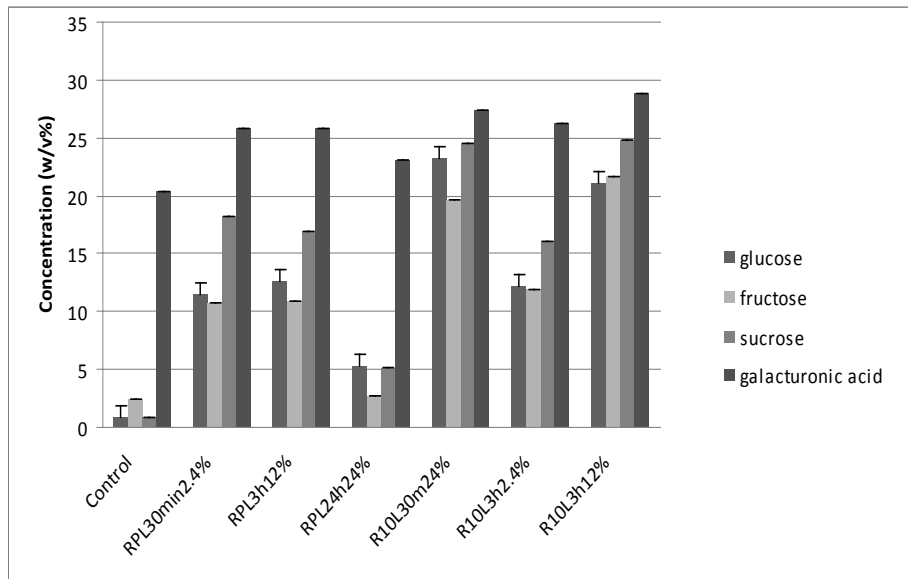


Fig. 4. Comparative representation of glucose (expressed by a peak at 1033 cm^{-1}), fructose (expressed by a peak at 1063 cm^{-1}), sucrose (expressed by a peak at 995 cm^{-1}) and galacturonic acid (expressed by a peak area in the region $1500\text{-}700\text{ cm}^{-1}$) concentrations ($x \pm \text{SD}$, expressed in g/100 ml juicereleased after enzymes treatment), against control

Quantitative evaluation of glucose, fructose, sucrose and galacturonic acid after enzymatic hydrolysis

Using the data obtained from the calibration curves of glucose, fructose, sucrose and galacturonic acid, it has been calculated the concentrations of these carbohydrates in sugar beet juice after enzymatic treatment (for the most specific samples) and without enzymatic treatment. For glucose, fructose and sucrose it has been considered the characteristic intensity of each peak (1033 cm^{-1} for glucose, 995 cm^{-1} for sucrose and 1063 cm^{-1} for fructose). The concentration of galacturonic acid was calculated based on calibration curve obtained from peak areas on fingerprint region of galacturonic acid ($1500\text{-}700\text{ cm}^{-1}$).

As can be seen in Fig. 4, after the enzymatic treatment the concentrations of all compounds (glucose, fructose, sucrose and galacturonic acid) increase considerably in comparison with the control. The higher concentration of glucose was obtained in the sample treated with a concentration of 24% R10L and incubation time of 30 min (R10 L 30 m 24%), while the lower concentration was obtained in the sample treated with RPL (24 h, 24%) (RPL 24 h 24%). Fructose, sucrose and galacturonic acid were released at higher concentrations in samples treated with R10L for three hours at a concentration of 12% (R10 L 3h 12%) (Fig. 4).

Comparing the activity of the Rohament PL at the three concentrations (2.4%, 12% and 24%) and three incubation times (30 min, 3 h and 24 h), it has been observed the optimum enzyme activity at 2.4% and incubation time of 30 min. This suggests that Rohament PL had a higher rate activity, being also very specific. In the case of

Rohapect 10 L, the optimum activity appears at 12%, and optimum time of 3 hours. Comparing these two enzymes, it has been observed that Rohament PL had higher activity, optimum in 30 min, and best affinity to the substrate, at 2.4%, while Rohapect 10 L needs a concentration of 12%. Regarding the enzymes efficiency, expressed by released hydrolysis products, the high-efficiency enzyme was Rohapect 10 L.

Conclusions

In the present reaserch it has been applied FT-MIR spectroscopy, a non-destructive tool and useful method, to measure the pectinolytic activity of two enzymes (Rohament PL or Rohapect 10L), on sugar beet as substrate. The measurements were based on the release of small, individual sugar like glucose, fructose, sucrose and galacturonic acid by enzymatic hydrolysis of pectins. It has been identified the specific peak intensities for glucose (at 1033 cm^{-1}), fructose (at 1063 cm^{-1}), sucrose (at 995 cm^{-1}), and peak area for galacturonic acid ($1500\text{-}700\text{ cm}^{-1}$), and based on these data, it has been calculated the concentrations of each sugar. It has been identified as well the differences among the two enzymes activity, specifically their optimum concentration and best hydrolysis timing.

FT-MIR spectroscopy allows accurate glucose, fructose, sucrose and galacturonic acid concentration determination. Actually, FT-infrared spectroscopy is a versatile method, to be used for the simultaneous determination of a large variety of compounds present in different biological samples. Validation of this method using HPLC analysis is also underway for a more accurate quantification.

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