Application Of FTIR Spectroscopy For A Rapid Determination Of Some Hydrolytic Enzymes Activity On Sea Buckthorn Substrate

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Abstract

We investigated the efficiency of some commercial cellulosic and pectinic enzymes (Carezyme 1000 L and Pectinex Ultra SP-L, with cellulose, pectinase and hemicellulase activity, respectively) on a sea buckthorn puree substrate. The method applied to check the rate of hydrolysis was Fourier Transformed Infrared Spectroscopy (FTIR), by recording the absorption of different carbohydrates in the specific spectral range (650-4000 cm^{-1}). To calibrate the method, we used firstly, different concentrations of pure glucose, fructose and sucrose (from 1 to 25 %) and registered their IR maximal wavenumbers and peak intensity. Based on calibration curves, we calculated the release in vitro of glucose, fructose or sucrose after enzymatic action on sea buckthorn substrate. According to the IR absorption peaks registered for glucose (at 1033 cm^{-1}), fructose (at 1063 cm^{-1}) and sucrose (at 995 cm^{-1}), we were able to identify the changes in the shape and intensity of these peaks in the region 1200-900 cm^{-1}, against control. FTIR spectrometry proved to be a suitable method for accurate and direct determination of individual sugars (glucose, fructose and sucrose – like disaccharides) on sea buckthorn or other juices and a good control of enzymatic activity on cellulose, hemicellulose and pectine-rich substrates. The time used for FTIR analysis is considerably reduced compared to the classical methods. These results are promising and have a real analytical potential to supervise in situ or in vitro, as a routine procedure, the dynamics of enzymatic treatment of natural substrates rich in natural carbohydrate polymers (celluloses and pectins).

Introduction

Sea buckthorn (*Hippophae rhamnoides* L.) (SB) is a hardy, deciduous spiny shrub species that is distributed in Central Asia and Europe, and produces nutritious and delicious berries [1]. Sea buckthorn berries are an excellent source of phytochemicals such as ascorbic acid, unsaturated fatty acids, phenols, carotenoids [2], as well glucose and fructose with minor amounts of xylose and sugar alcohols (mannitol, sorbitol, and xylitol) [3]. The sugar components of sea buckthorn juice vary with the origin, climate and method of extraction. Glucose (0.5-12.5 g/100ml) and fructose (0.1-11 g/100 ml) are the major sugar components, both account for around 60-90% of the total sugar content for all origins tasted [4]. Also, sucrose is present, but its amount is very low. Many food products are made of sea buckthorn berries, such as jams, juices, and oils [5]. Juice production from sea buckthorn berries implies

many steps and the total cost of the process is high. The use of hydrolytic enzymes to obtain sea buckthorn juice may reduce the cost of the process, and, in addition, the quantity of juice will be much bigger.

Cellulases (C) (EC 3.2.1.4.), hemicellulases (HC) and pectinases (P) (EC 3.3.1.15, EC 4.2.2.2) are the main commercial hydrolytic enzymes used in food biotechnology processes, as well for animal feed, textile and laundry, paper and pulp industries, continuously optimized research by technological development [6]. Cellulases catalyze the hydrolysis of the β-1,4 glucosyl bound from the insoluble linear glucose polymer, cellulose. Three types of cellulolytic enzymes are known: endoglucanases, exoglucanases and β-glucosidases that hydrolyze the cellulose into cellobiose or glucose units [7]. Hemicellulases are enzymes that catalyze hemicelluloses (heterogeneous polymers of pentoses, hexoses and sugar acids) [8], acting either as glycoside hydrolases (xylanases, β-mannanases, α-L-arabinofuranosidases, α-D-glucuronidases, β-xylosidases) or carbohydrate esterases (acetyl xylan esterases, acetyl mannan esterases, feluric and p-coumaric acid esterases) [9]. Pectinases are responsible for the degradation of pectic substances that are complex colloidal acid polysaccharides, with a backbone of galacturonic acid residues linked by α (1-4) bound [10]. Pectinolytic enzymes are classified in three major clases: a) esterases (pectin methyl esterases); b) hydrolases (protopectinases, polygalacturonases, pectinesterases) and c) lyases (polygalacturonat lyases, polymethylgalacturonat lyases) [10]. The use of these three types of enzymes is usual in fruit juices production [11, 12]. The polysaccharides form different networks in plant cell walls, their structure and composition being analyzed in detail in several studies [13, 14, 15]. Under enzyme action, the cell wall polysaccharides are solubilized and depolymerized, generating different types of substances, such as monosaccharides, disaccharides, galacturonic acid etc.

Infrared spectrometry is relatively uncommon compared with chromatographic and classical methods [16], but can be employed as a rapid and non-destructive method to identify different functional groups and molecules which can fingerprint a food [17].

At present, FTIR spectroscopy is often applied in the analysis of plant cell wall polysaccharides. It is a simple, fast and non-destructive method for investigation of fruit juices composition and monitoring the enzymes activity. This technique coupled with chemometrics has been used to study different quality attributes in many food samples including fruits, vegetables or beverages [18], olive pulp cell-wall polysaccharides [19], must and wine analysis [20, 21, 22]. On the other hand, this technique has become an alternative method for the laborious sugar analysis [23], in food, e. g. soft drinks and fruit juices. [24].

The present paper evaluates the efficiency of FTIR spectroscopy to be used in the characterization of different carbohydrates released from a SB substrate using commercial enzyme activities (cellulases, hemicellulases and pectinases) under different conditions.

Materials and methods

Cellulases from *Aspergillus sp.* (Carezyme 1000 L – 1000 U/g) and Pectinex from *Aspergillus aculeatus* (Pectinex Ultra SP-L - 9.500 U/ml) containing pectinase and hemicellulase activity from Sigma Aldrich were used. The optimum pH (4.2) for enzymes activity was achieved by addition of acetate buffer (acetic acid 1N, NaOH 1N). SB berries were collected from Cluj County (Transylvania, North of Romania). The pulp puree obtained by blending the sea buckthorn berries was divided into four samples which were treated with enzymes as follows in Table 1.

Table 1. Type of enzymes used to hydrolyze the SB puree and their specific ratios to substate

Quantity and type of enzyme	Ratio enzyme:substrate
1 ml - Cellulase (C)	1000U C per SB sample (15 g) containing 0.45g cellulose*
0.3 ml Pectinase + Hemicellulase (PHC)	2850 U PHC per SB sample (15 g) containing 0.15g pectin
1 ml - Cellulase + 0.3 ml Pectinase + Hemicellulase (CPHC)	1000U C per SB sample (15 g) containing 0.45g cellulose* + 2850 U PHC per SB sample (15 g) containing 0.15g pectin

*according to [25].

All samples were stirred and then incubated for 24 h at 40°C. After 24 h, samples were centrifuged for 3x15 minutes at 4000 rpm. Juice supernatants were collected and filtrated, then analyzed at FTIR spectrometer (using a Shimadzu Prestige 2, Apodization: Happ-Genzel spectrometer). Each spectrum was recorded from 4000 to 500 cm^{-1} and 64 scans were accumulated for each spectrum.

In order to identify the carbohydrate specific fingerprint region and to calibrate the method, different concentrations of 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) and sucrose (1, 2, 3, 4, 5, 10, 15, 20 and 25 g/100 ml) were prepared and then analyzed by FTIR spectrometry in the same conditions. The spectra were processed using an IR solution Software Overview (Shimadzu) and Origin^{R} 7SR1 Software (OriginLab Corporation, Northampton, USA). To calculate quantitatively the glucose, fructose or sucrose – like disaccharides released in the SB juice, we considered the calibration curves and we applied the curve factor.

Results and discussion

Specific FTIR fingerprint and calibration curves of glucose, fructose and sucrose
As can be seen in Fig. 1, the FTIR spectra (4000-900 cm^{-1}) of aqueous solutions of glucose (25%), fructose (25%) and sucrose (25%) are well defined (Fig. 1A) and show intense fingerprint bands in the wavenumber range 1200-900 cm^{-1} (Fig. 1B). In this region, the specific absorption for each carbohydrate was indentified. The characteristic bands of glucose have specific maxima at 991, 1033, 1078, 1107 and 1149 cm^{-1}, the peak at 1033 cm^{-1} having the highest absorption. Fructose has specific maxima at 966, 979, 1063, 1082 cm$^{-1,}$ the maximal absorption peak at 1063 cm^{-1}. Sucrose specific maxima are located at 995, 1053 and 1136 cm^{-1}, with a maximum absorption for 1053 and 995 cm^{-1}, in a ratio 1:1. The spectral signatures among sugars are somewhat different from each other [26]. The most intense peak of glucose (1033 cm^{-1}) is characteristic to the C - O stretch vibration, 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 [26].

Based on these data we obtained the calibration curves (Fig. 2A-C), based on the peak intensities in the region, in order to determine glucose, fructose and sucrose concentrations in our samples.
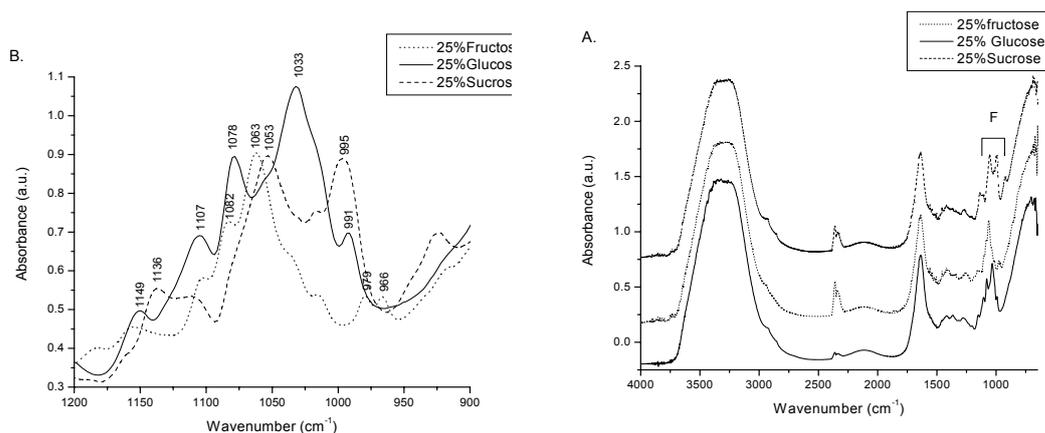
Figure 1. The integral FTIR spectra (4000-900 cm^{-1}) of aqueous standard solutions containing 25% glucose (solid line), 25% fructose (dot line) and 25% sucrose (dash line) (A), spectra are shifted vertically, and their specific peaks in the fingerprint region (1200-900 cm^{-1}) (B), in A indicated by the inserted F.
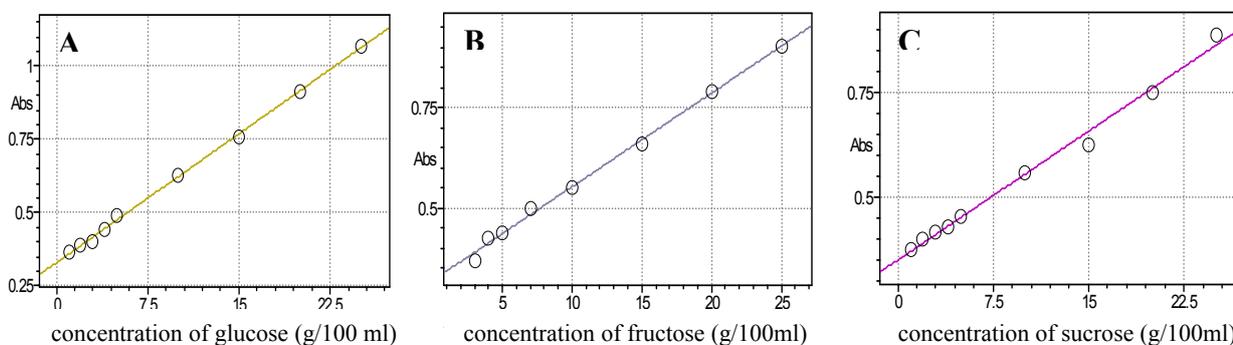

Figure 2. Calibration curves recorded for glucose (A), fructose (B) and sucrose (C). We used the maximum IR peak absorption for each solution (1033, 1063 and 995 cm^{-1} for glucose, fructose and sucrose, respectively). For details, see materials and methods.

FTIR spectra and fingerprint regions specific to sea buckthorn juice before and after enzyme hydrolysis

FTIR spectroscopy has been shown to be a powerful tool for the study of the enzyme activities on fruit substrates. The FTIR spectra and fingerprint regions specific to juice released after C, PHC and CPHC treatment, comparing with controls are shown in Fig. 3A and Fig. 3B, respectively.
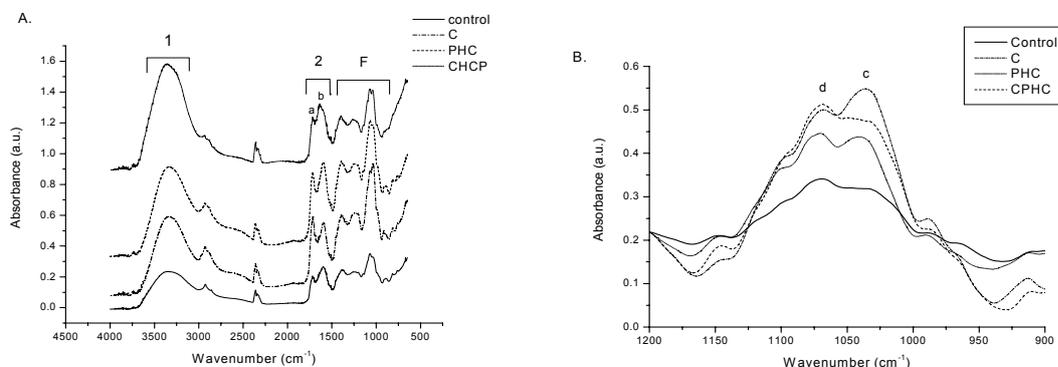

Figure 3. The FTIR spectra (4500-600 cm^{-1}) of the sea buckthorn puree before (control – solid line) and after enzymatic treatment (C – dash dot line, PHC – dash line, CPHC – short dot line) (A) spectra are shifted vertically, and their specific fingerprint region (1200-900 cm^{-1}) (B) in A indicated by the inserted F.

The spectra of SB juice without enzymatic treatment or after the enzymatic treatment show three absorption zones: 3700-2850 cm^{-1} (1), 1800-1500 cm^{-1} (2) and a fingerprint region, 1200-900 cm^{-1} (F).

The first region, marked 1, from 3700 to 3000 cm^{-1} correspond to water and OH absorption frequencies. The neighboring peaks between 2980 and 2850 cm^{-1} correspond to stretching vibrations of – C – H inside CH$_3$ or CH$_2$ groups. The region 2 corresponds to carbonyl-specific absorptions (1700-1500 cm^{-1}). The ratio between the two peaks (peak b- at 1597 cm^{-1}and peak a- at 1716 cm^{-1}) was different: while b/a was 1.33 for control, the samples treated with C had a value of b/a=0.91, while b/a for PHC and CHPC was 1.11 and 1.12, respectively. The decrease of the b/a value is associated with the yield of hydrolysis of substrates. Cellulose hydrolysis (C) seems to be more efficient than pectin hydrolysis (PHC). The association of all enzymes, CHPC, had lowest efficiency, comparing with individual enzymes.

The third region corresponds to the fingerprint region (F) of carbohydrates on sea buckthorn. Glucose, fructose and sucrose show intense and characteristic bands in the region between 1200 and 900 cm^{-1}. The ratio between the two peaks (peak c- at 1033 cm^{-1}and peak d- at 1070 cm^{-1}) was different: while d/c was 1.06 for control, the samples treated with C had a value of d/c=0.94, while d/c for PHC and CHPC was 1.03 and 1.01 , respectively. The decrease of d/c value is associated with the yield of substrate hydrolysis. Considering the decrease of the d/c ratio, we conclude that cellulose hydrolysis (C) was more efficient than PHC and CHPC hydrolysis.

There were many peaks in this region, corresponding to C=O and C–OH stretching modes, which overlapped each other [27]. These peaks depend on the sugar structure and on the interaction between the sugar molecules and their environments. Thus, we can identify the peaks characteristic to glucose, fructose, and sucrose in sea buckthorn juice, in accordance with peaks observed in puree carbohydrates (Fig. 1). The peaks recorded at 1033, 1063 and 995 cm^{-1} were considered for the identification of enzymes' hydrolytic activity and the rate of glucose, fructose and sucrose release, respectively.

Quantitative evaluation of glucose, fructose and sucrose – like disaccharides after enzymatic hydrolysis

Using the data obtained from the calibration curves (Fig. 2) of glucose, fructose and sucrose, we calculated the concentrations of these carbohydrates in sea buckthorn juice before and after enzymatic treatment with C and PHC, individually, or in combination (CPHC) (Tab.2). For each compound we considered the characteristic intensity of each peak (1033 cm^{-1} for glucose, 995 cm^{-1} for sucrose and 1063 cm^{-1} for fructose).

Table 2. Evaluation of glucose, fructose and sucrose concentrations, identified in the SB juice, before and after enzymatic treatment on SB substrate (puree)

Enzyme treatment	Wavenumber (cm^{-1})	Concentration (g/100ml)
Control	1033	0.8
	995	0.57
	1063	0.59
Cellulases (C)	1033	17.24
	995	8.8
	1063	19.71
Pectinases + Hemicellulases (PHC)	1033	18.05
	995	12.83
	1063	24.24
Cellulases + Hemicellulases + Pectinases (CPHC)	1033	5.6
	995	0.73
	1063	7.36

Under the cellulases action on sea buckthorn substrate, the concentration of the glucose, fructose and sucrose in the juice released after enzyme action, increases considerably in comparison with the untreated juice substrate (17.24 % glucose comparing with 0.8% in control, 19.71% fructose vs 0.59% for control and 8.8% sucrose vs 0.57% for control). A similar, significant increase was observed after pectinases and hemicellulases action, the glucose concentration increased to 18.05%, while fructose to 24.24% and sucrose to 12.83%. A lower hydrolytic efficiency was observed when the mixture CPHC was applied on the substrate. The final concentrations of glucose/fructose/sucrose were 5.6/7.36/0.73, inferior than the values obtained with individual enzymes. This fact suggests that enzymes activity is different if the ratios enzyme/substrate are changing. It seems that optimal hydrolytic activity may be obtained when enzymes act separately, better than in combination (Fig. 4).
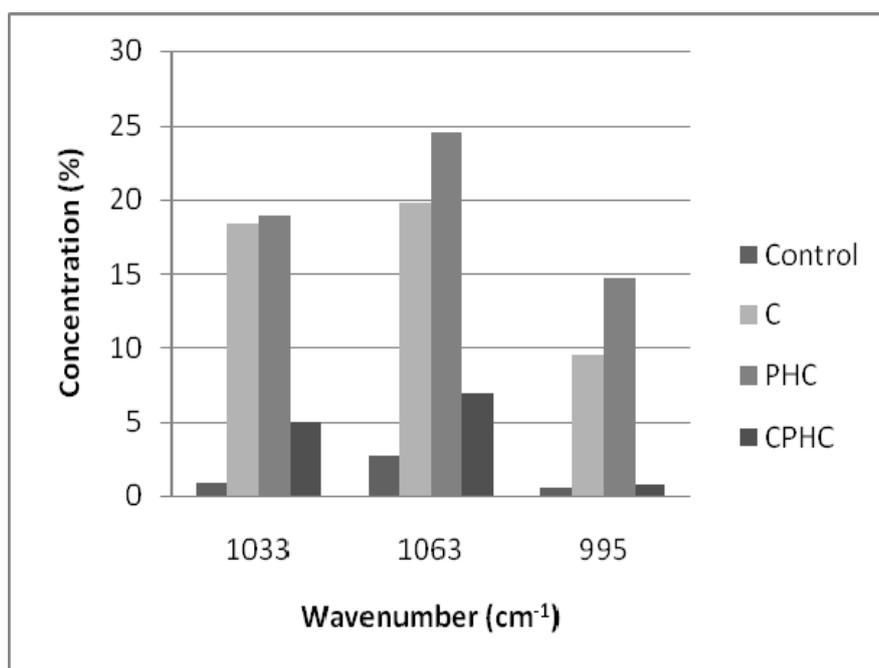

Figure 4. Comparative representation of glucose (expressed by a peak at 1033 cm^{-1}), fructose (expressed by a peak at 1063 cm^{-1}) and sucrose-like disaccharides (expressed by a peak at 995 cm^{-1}) concentrations released after enzyme (C, PHC or CPHC) treatment, against control. The data are taken from TABLE 2.

Conclusions

In this paper we applied the FTIR spectroscopy as a non-destructive tool and useful method to determine the activity of different hydrolytic enzymes on sea buckthorn substrate. According to the IR absorption peaks registered for glucose (at 1033 cm^{-1}), fructose (at 1063 cm^{-1}) and sucrose (at 995 cm^{-1}), we were able to identify changes in shape and intensity of these peaks in the region 1200-900 cm^{-1}, against control. FTIR spectrometry proved to be a suitable method for accurate and direct determination of individual sugars (glucose, fructose and sucrose) on sea buckthorn juice and a good control of enzymatic activity on cellulose, hemicellulose and pectine-rich substrates. The time of FTIR analysis is considerably reduced compared to the classical methods. These results are promising and have a real analytical potential to supervise *in situ* or *in vitro*, as a routine procedure, the dynamics of enzymatic treatment of natural substrates rich in natural carbohydrate polymers (celluloses and pectins). This method can be applied not only in laboratory, but as well *on line* in food industry.

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