Carbohydrate Polymers 117 (2015) 923–932

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Assessment of antiproliferative activity of pectic substances obtained by different extraction methods from rapeseed cake on cancer cell lines

M. Cobs-Rosas[a], J. Concha-Olmos[b,*], C. Weinstein-Oppenheimer[b,c], M.E. Zúñiga-Hansen[a,c]

[a] Escuela de Ingeniería Bioquímica, Pontificia Universidad Católica de Valparaíso, General Cruz 34, Valparaíso, Chile
[b] Escuela de Química y Farmacia, Facultad de Farmacia, Universidad de Valparaíso, Gran Bretaña 1093, Playa Ancha, Valparaíso, Chile
[c] Centro Regional de Estudio en Alimentos Saludables, R12C1001, Av. Universidad 330, Curauma, Valparaíso, Chile

ARTICLE INFO

Article history:
Received 8 January 2014
Received in revised form 2 September 2014
Accepted 2 October 2014
Available online 22 October 2014

Keywords:
Rapeseed cake
Antiproliferative activity
MCF-7
Caco-2
Pectinases

ABSTRACT

In this work the antiproliferative activity of pectic substances obtained by different extraction methods from defatted rapeseed cake was assessed on cancer cell lines. The process consisted of sequential treatment with alkalized water (pH ~ 8), EDTA (0.01 M), alkaline protease (Alkalase 2.4L) and a commercial pectinase preparation (Viscozyme L or Pectinex Ultra SP-L). Pectic extracts identification was performed using spectroscopy and chromatography techniques. FT-IR and HPLC-IR results suggest that the neutral pectic extracts produced would be arabinogalactans and β-galactans. All the pectic substances extracted (acid and neutral) from RSC exhibited antiproliferative activity, being more effective on MCF-7 cells than Caco-2. The most effective pectic extract was obtained by Alkalase 2.4 L which killed over 80% of MCF-7 cells and 60% of Caco-2 cells. At less than 10 mg/mL pectic extracts enriched in neutral sugars also exhibited antiproliferative activity (50 and 40%, respectively), which was superior to the modified citric pectins activity at the same concentration for the breast cancer cell line (61.6% for MCF-7 and 49.9% for Caco-2 cells). These results show that the antiproliferative activity depends on both the type of pectin (acid or neutral) and the extraction procedure.

1. Introduction

Rapeseed cake (RSC) is an organic waste that remains after the production of rapeseed oil. As a raw material of a large industry, rapeseed is an important energy crop where all the parts of the plant (straw, root, seed, cake and oil) have been evaluated for potential benefits (Jeong et al., 2013). Most studies about RSC are focused on rapeseed protein extraction and their biological properties (Çulcuoğlu, Ünay, & Karaosmanoğlu, 2002; Fauduet et al., 1995; He et al., 2013; Jeong et al., 2013; Pastuszewska et al., 2003; Yust et al., 2004). RSC is composed mainly of proteins (around 30–40%) and also contains high levels of carbohydrates (>30%) especially pectins (Egües, Alriols, Herseczki, Marton, & Labidi, 2010).

Pectins are a family of covalently linked galacturonic acid-rich polymers. Thus far three main pectic polysaccharides have been isolated and identified. They are homogalacturonan (HG), rhamnogalacturonan-I (RG-I), which can be substituted by arabinan or arabinogalactan, and substituted galacturonans (GS) such as rhamnogalancturonan II, apiogalacturonan and xylogalacturonan (Schols, Vierhuis, Bakx, & Voragen, 1995; Schols, Voragen, & Colquhoun, 1994; Yapo, Robert, Etienne, Wathelet, & Paquot, 2007; Yapo, 2009, 2011). In the same context, the pectin backbone has two important regions that may be distinguished, a smooth region that comprises α-1,4-D-galacturonic acid partially methylated (fraction of pectin known as acid pectin and usually used in food and pharmaceutical industry) and a hairy region (or neutral pectin, polymers mainly bond to the cellulosic material and highly water insoluble) (Schols et al., 1994; Willats, Knox, & Mikkelsen, 2006)

A wide variety of pectin extraction procedures have been documented in the literature (Combo, Aguedo, Goffin, Wathelet, & Paquot, 2012; Concha Olmos & Zúñiga Hansen, 2012; Eriksson, Andersson, & Åman, 1997; Fissore et al., 2009; Kurita, Fujiwara, & Yamazaki, 2008; Lim, Yoo, Ko, & Lee, 2012; Min et al., 2011; Nikolić & Mojovic, 2007; Siddiqui & Wood, 1972; Yapo et al., 2007; Yeoh, Shi, & Langrish, 2008). For pectin extraction from RSC

* Corresponding author. Tel.: +56 32 2508422; fax: +56 32 2508111.
E-mail addresses: jacqueline.concha@uv.cl, jacquesim@yahoo.com (J. Concha-Olmos).

http://dx.doi.org/10.1016/j.carbpol.2014.10.027
0144-8617/

usually is used cold and/or hot water or buffer (Eriksson et al., 1997; Mutter, Colquhoun, Schols, Beldman, & Voragen, 1996; Siddiqui & Wood, 1972), cold or hot solutions of chelating agents such as ethylene diamine tetraacetic acid (EDTA), ammonium oxalate or cyclohexane diaminotetraacetic acid (CDTA) (Aspinall & Kuo-Shii, 1974; Bell, 1993; Eriksson et al., 1997; Mutter et al., 1996; Siddiqui & Wood, 1972, 1977; Theander, Aman, Miksche, & Yasuda, 1977) and recently, due to high carbohydrate content, enzymatic hydrolysis using commercial enzymes (Celluclast and Alkalase) have been investigated (Jeong et al., 2013).

With regard to pectin-derived neutral oligosaccharides, there is a lack of information about enzymatic extraction processes and the biological activities of the recovered products. In recent times Concha Olmos & Zúñiga Hansen (2012) evaluated the use of pectinases for enzymatic depolymerization of sugar beet pulp, obtaining neutral pectin extracts with very promising results on antiproliferative activity in cancer cell lines.

In the last few years, evidence for the capacity of acid pectins to stop proliferation in cancer cells has been accumulated (Bergman, Djaldetti, Salman, & Bessler, 2010; Glinsky & Raz, 2009; Gunning, Bongaerts, & Morris, 2009; Jackson et al., 2007; Nangia-Makker et al., 2002; Pienta et al., 1995). Some studies performed in mice, showed that modified citrus pectin (MCP) orally administrated, reduced significantly tumor growth, angiogenesis and spontaneous metastasis. Also, there are information showing that fragments of pectin may bind to the mammalian protein galectin-3 (Gal3) and thus inhibiting its various roles in cancer progression and metastasis (Gunning et al., 2009). It has been shown for low methylated apple pectins, larch arabinogalactan and MCP that arabinogalactanes, which are part of neutral pectins, are involved in the binding to Gal3 (Bergman et al., 2010; Glinsky & Raz, 2009; Gunning et al., 2009; Kelly, 1999; Nangia-Makker et al., 2002; Pienta et al., 1995). Gunning et al. (2009) showed that the smallest fragment that can interact with Gal3 is a β1,4-galactan isolated from RGI by enzymatic treatment. Next, Gao et al. (2012) reported relevant roles for both the backbone and side chains of β1,4-galactan. These authors showed that galactan side chains positively modulated the inhibitory activity of the fragment, whereas the arabinan side chain exhibited different effect depending on their location within the molecule. Though, there are molecular data supporting the fact that neutral pectins can bind these receptors, there are fewer reports on their antiproliferative activity.

In the present study, the antiproliferative effect of acidic and neutral pectins obtained from RSC by different extraction methods were compared with the aim to explore neutral pectins as potential anticancer drugs recovered by a more environmental friendly procedure and to gain knowledge about the bioactivity of neutral pectins.

2. Material and methods

2.1. Raw materials

Defatted rapeseed cake was donated by Oleotop S.A. (Temuco, Chile). The agroindustrial residue was milled into flour (60 mesh) using an IKA A-11 Basic Analytical mill. After, this was stored at 20–25 °C until used. The commercial pectinase preparations (CPP), Pectinex Ultra SP-L and Viscozyme L, both with pectinolytic activity, and Alkalase 2.4 L, as a protease, were supplied by Novozymes (Denmark).

2.2. Defatted rapeseed meal characterization

The following composition (dry matter, protein, crude fiber, fat and ash) before and after enzyme treatment of DRM, was measured according to the methods described in AOAC (1990). Dry matter was determined by weight loss after drying (60 °C for 24 h). Protein content was determined indirectly from the total amount of nitrogen, measured by Kjeldhal's method, multiplied by factor 5.53 (Tkachuk, 1969). Fat was determined by Soxhlet extraction of lipids from dry samples, using petroleum ether as solvent. Dry samples were refluxed at a boiling point of 60 °C for 5–6 h and then the dry crude lipid was gravimetrically determined. Ash content

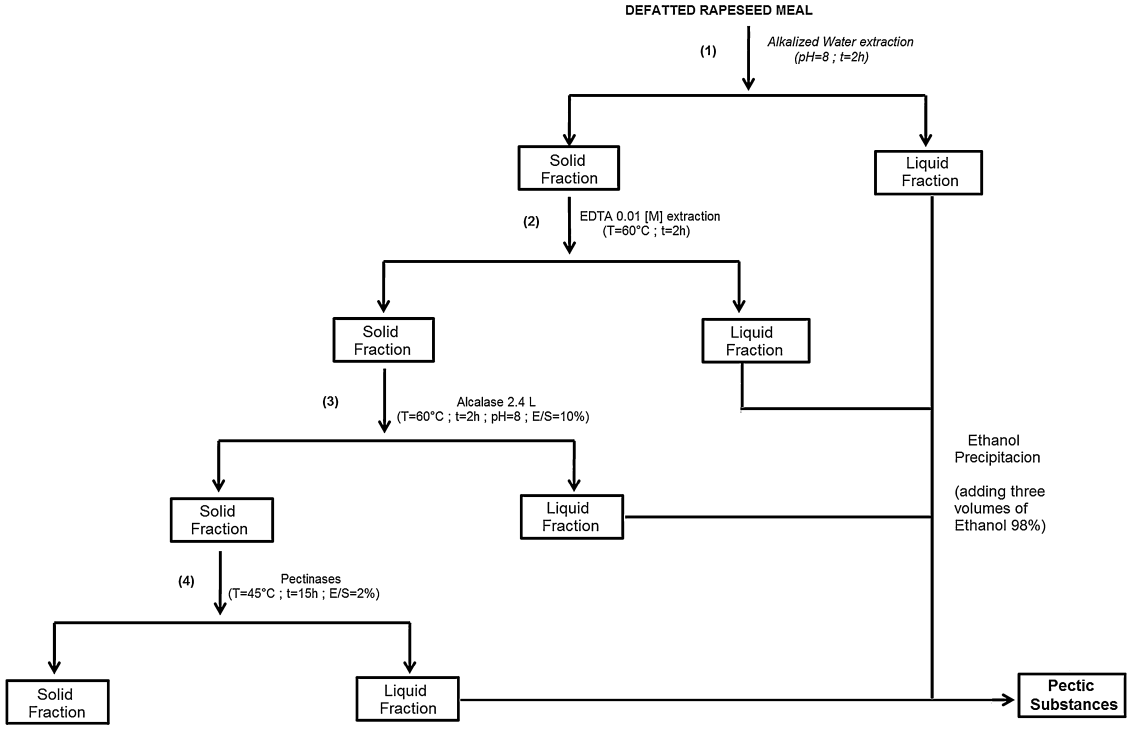

Fig. 1. Workflow of pectic substances extraction.

was determined gravimetrically after ashing at 550 °C for 6 h. The nitrogen-free extract was calculated by difference.

Cell wall composition of defatted rapeseed meal (cellulose, hemicellulose and lignin) was determined using the detergent fiber methods (Van Soest, Robertson, & Lewis, 1991). This method is based in the sequential extraction and separation of three fractions: residue insoluble in solution of sodium lauryl sulfate buffered at pH 7 with EDTA-Borate (NDF); residue insoluble in solution of cetyl-trimethyl-ammonium-bromide ($C_{19}H_{42}BrN$) in 1 N sulfuric acid (ADF); and residue insoluble in H_2SO_4 72% (w/w)(ADL). Then hemicellulose is calculated as (NDF–ADF), cellulose as (ADF–ADL), and lignin is determined as the ADL fraction free of ash.

The pectic substances content was determined according to Carbonell, Costell, & Duran, (1990). Briefly, 2 g of dry and defatted rapeseed meal were thoroughly extracted with water (40 °C, 3 h), ethanol–benzene (1:2, v/v) for 6 h, and 0.5% aqueous ammonium oxalate solution (50 °C, 1 h).

2.3. Extraction of pectic substances

The extraction of pectic substances from DRM was performed by an adaptation of the Claye's method (Concha Olmos & Zúñiga Hansen, 2012). With the purpose to obtain a purified pectic extract, the extraction process was conducted in four steps (Fig. 1): (A) Extraction of water-soluble pectins (WSP) applying an aqueous extraction (1:10 w/v ratio) for 2 h at 30 °C using alkaline water (pH 7.0–7.5) (B) Extraction of soluble pectins in ethylenediamine tetraacetic acid (ESP). The remaining solid residues from the step 1 were treated with ethylenediamine tetraacetic acid (EDTA) solution 0.01 M for 3 h at 60 °C. The two remaining liquid parts in each step were treated with three volumes of ethanol 80% (v/v) to precipitate alcohol-soluble material. Then, the mixture was centrifuged at 6000 rpm. The supernatant was discarded and the pellet was heat-dried at 25 °C, obtaining a brownish powder. (C) Since DRM exhibits an important content of protein the incorporation of a strong treatment to remove proteic material from the insoluble residues is necessary. In this case the hydrolysis was performed by Alkalase 2.4 L with an enzyme/substrate (E/S) ratio of 10%, liquid/solid (L/S) ratio of 20:1 and a hydrolysis time of 2 h. The pH (8.0) and temperature (45 °C) are the optimal values supplied by the manufacturer. The mixture was filtered and the remaining deproteinized solid, was washed three times with 80% (v/v) ethanol, once with 95% (v/v) ethanol, three times with distilled de-ionized water, and then freeze-dried. (D) Finally, the solid residue after deproteinization was treated with two commercial enzymes Pectinex Ultra SP-L and Viscozyme L. The conditions of the treatment were: an enzyme/substrate (E/S) ratio of 2%, liquid/solid (L/S) ratio of 10:1 and hydrolysis times at 3, 6, 9, 12, 15, 18 h. The pH (7.0) and temperature (45 °C) were the optimal values supplied by the manufacturer for both commercial enzymes. The mixture was filtered and the remaining liquid parts (hydrolysates), after protease and pectinase treatment, were treated with three volumes of ethanol, in order to precipitate alcohol-soluble material. The mixtures were centrifuged at 6000 rpm for 10 min, the supernatant was discarded and the remaining pellets heat-dried at 25 °C in order to obtain a powder product.

2.4. Chemical analysis of DRM's pectic substances

The reducing sugars in the hydrolysates after enzymatic treatment were determined by using a 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959), and total carbohydrates by phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), using glucose as the calibration standard for both methods.

Pectic oligosaccharides were analyzed by HPLC-IR using Perkin-Elmer Series 200 equipment with a refractive index detector and autosampler, using a BP-100 Ag+ (300 mm × 4.6 mm) column for the carbohydrates analysis (Benson Polymerics, Reno, NV, USA) and Totalchrom software. The temperature of the column was kept at 85 °C and the detector temperature at 30 °C. Samples were

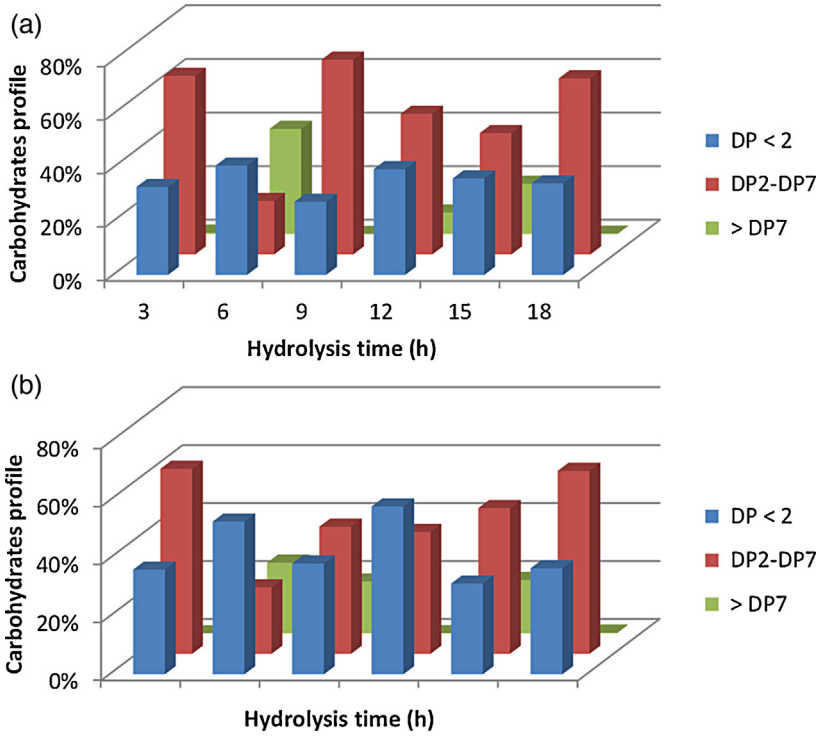

Fig. 2. DP's distribution of the neutral pectic substances extracted by (a) Pectinex Ultra SP and (b) Viscozyme L. Conditions: temperature 45 °C, L/S ratio 10:1, E/S ratio 2 wt% and 120 rpm on shaker agitation.

eluted with 0.5 mL/min^{-1} of Mili-Q water. The oligosaccharides standards used in HPLC-IR, in order to identify the degree of polymerization (DP) by comparing the retention time, were (Supelco, Sigma-Aldrich Co.): arabinose, fructose, galactose, glucose, mannose and xylose as DP1; α-lactose, maltose and sucrose as DP2; maltotriose, D-(+) raffinose and isomaltotriose as DP3; maltotetraose and stachyose as DP4; matopentaose as DP5; maltohexaose as DP6; and maltoheptaose as DP7.

2.5. FT-IR characterization

The identity of pectic polysaccharides and the degree of esterification were estimated by FTIR spectroscopy. The spectra obtained were compared against polygalacturonic acid, three standard polysaccharides (arabinogalactan, arabinan and rhamnogalaturonan) and modified citrus pectin (Pectasol^{TM}). Infrared spectra were measured on a RayLeigh WQF-510A Fourier transform infrared (FT-IR) spectrophotometer (USA), coupled to a personal computer with MAIN FTOS analysis software. Pellets were prepared by blending pectin samples with KBr at 10% (w/w) and each spectrum was recorded at a resolution of 4 cm^{-1} with 64 accumulated scans into the 500 cm^{-1}–4000 cm^{-1} range.

2.6. Anti-proliferative activity on cancer cell lines

The effect of the pectic substances on the antiproliferative activity on MCF-7 and Caco-2 cell lines (ATCC) was measured using the resazurin method, which is based on the ability of viable cells to reduce this molecule to form a fluorescent product with an excitation wavelength at 544 nm and an emission at 590 nm. The fluorescence, read in Thermo Scientific Appliskan equipment, is proportional to the cell number and that allows to measure if cells are proliferating or dying. The cells stocks were maintained in Dulbecco's Modified Eagle's medium (DMEM) with 10% Fetal Bovine Serum (FBS), 2 mM glutamine, 10 U/L penicillin and 100 μg/mL streptomycin in a humidified incubator (Thermo Forma) with a 5% CO_2 atmosphere.

For the assay, 5.000 MCF-7 or 10.000 Caco-2 cells were seeded in 96 wells plates and exposed to selected dilutions of pectic extracts or treatment-free cell culture media. Each treatment was

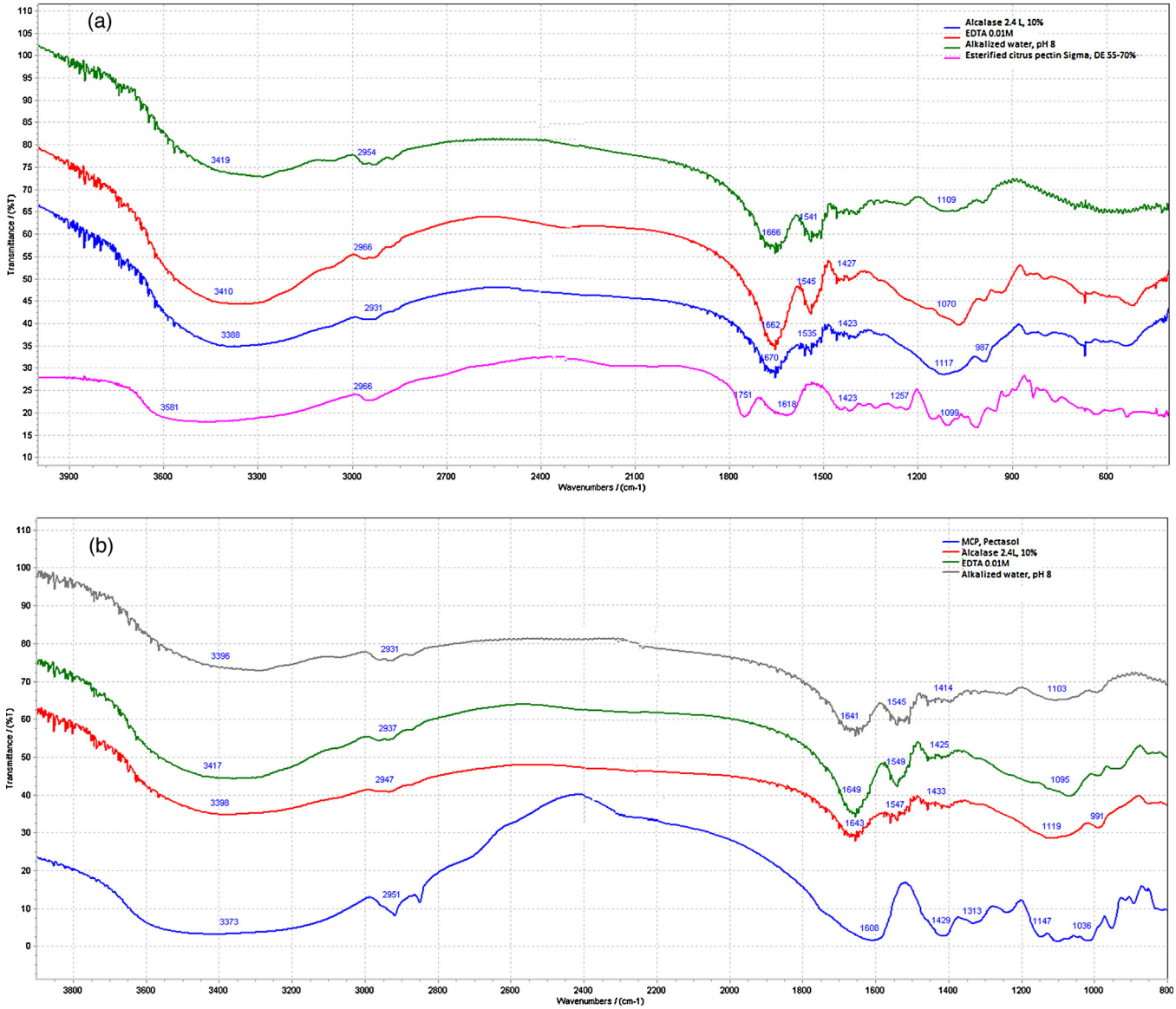

Fig. 3. FT-IR spectra of RSC pectic substances extracted by alkalized water, EDTA and Alkalase 2.4 L (a) comparative with commercial pectins; (b) comparative with modified citrus pectin

Table 1
Chemical composition (% on dry weight basis) of crude rapeseed cake[a] and defatted rapeseed meal[b].

Components	Crude rapeseed cake	Defatted rapeseed meal
Moisture content (%)		9.85 ± 0.49
Crude protein (%) ($N \times 6.25$)	31.58 ± 0.26	33.66 ± 0.67
Crude fat (%)	13.67 ± 0.13	1.87 ± 0.14
Ash (%)	5.12 ± 0.02	6.36 ± 0.10
Nitrogen free extract (%)		48.56 ± 1.69
Cellulose (%)		6.78 ± 0.30
Hemicellulose (%)		4.53 ± 0.31
Lignin (%)	10.31 ± 0.03	8.5 ± 0.74
Pectins (by oxalate ammonium method) (%)		6.04 ± 0.97

Values are mean ± standard deviation.
[a] Jeong et al., 2013.
[b] Raw material used in this research.

performed in triplicate. After 48 h of incubation, resazurin was added into fresh media at a final concentration of 4 mg/mL for 2 h, and then a sample of the supernatant was taken to read fluorescence. Survival cells were calculated from the ratio of Relative Fluorescence Units (RFU) for cells exposed to the treatment and cells exposed to cell culture media (Skehan et al., 1990).

3. Results and discussion

3.1. Characterization of DRM

Proximate composition (g/100 g dry matter) of DRM is shown in Table 1. In general, the percentage for component is very similar to the results obtained by Jeong et al. (2013) even though they might arise from a different source material. As was expected, DRM presented a high level proteins and nitrogen-free extract (which corresponds to an approximation of the carbohydrate content). In this case the high protein content represents a disadvantage for pectin extraction by enzymatic treatment with CPP. For this reason, in this study was considered the removal of protein with Alkalase 2.4 L using a ratio enzyme/substrate (E/S) of 10%, 20:1 ratio of liquid/solid (L/S) and a hydrolysis time of 2 h, providing a yield of 11.5%, corresponding to a 61.5% removal of total protein.

3.2. Extraction yield of pectic substances from DRM

A mass balance was applied in order to know how much material was released in each step of treatment, measuring weight in solid fractions and obtaining weight of liquid fraction by difference. Furthermore, the weight of soluble substances precipitated by ethanol was measured, obtaining a 2.53% yield by alkaline water, 0.18% by EDTA, 0.93% by Alkalase 2.4 L and 1.63% by CPP. It is important to mention that adding all these percentages gives a quite similar value to the 6.04% of pectin measured previously with the ammonium oxalate extraction, which indicates the success of the sequential treatment applied. Also, protein measurements with the Kjeldhal method were performed before and after every treatment carried out. The results show that protein content measured as Kjeldhal nitrogen, decreased from 33.7% to 16.3% which demonstrates the effectiveness of the extracting agents, mainly Alkalase 2.4L which allowed decreasing the protein content from 26.5% to the final 16.3%. Considering this, the protein content was no longer an undesirable material in the final product.

3.3. Characterization of pectic substances obtained by enzymatic treatment

3.3.1. Degree of polymerization (DP) pectins

The degree of polymerization (DP) of pectin extracts obtained by acid and enzymatic treatment was estimated through the ratio of anhydrogalacturonic acid (AGA) to the content of reducing groups (AGA/CHO). DP values fluctuated between 60 and 300. While molecular weight varied between 10 and 100 KDa.

The pectin oligosaccharides distribution obtained by enzymatic method is shown in Fig. 2. The analysis for each hydrolysis time examined, showed that independently of the evaluated time, the pectin extracts were mainly constituted by carbohydrates with polymerization degree lower than 3 and higher than 6. In relation to carbohydrates with pectins of DP between 3 and 6, Viscozyme L treatment produces a slight increase in the yield of this category, in comparison to Pectinex Ultra SP-L. On the other hand, the distribution of the carbohydrates, determined by HPLC-IR, showed no change according to the enzyme utilized. This indicates that there are no differences in each time of hydrolysis independently of the commercial pectinase preparation used.

Considering that biological properties of pectins depend on the oligosaccharides composition and DP distribution, pectin-derived neutral oligosaccharides obtained by Pectinex Ultra SP-L and Viscozyme L treatment at 15 h were chosen to evaluate antiproliferative activity. This condition was selected because it allowed to obtain a significant amount (mass) of extract, in contrast to lower times and also because the extracts at this time showed a higher concentration of oligomers (DP > 3). A relationship of a lower molecular weight of pectins with a higher anticancer activity, expressed as anti-apoptotic activity, was reported for prostate cancer cell lines (Jackson et al., 2007).

About the carbohydrates profile of neutral pectins and the carbohydrates with polymerization degree lower than 3, the results obtained for Pectinex Ultra SP and Viscozyme L at 15 h, temperature 45 °C, L/S ratio 10:1, E/S ratio 2 wt% and 120 rpm are shown in Fig. 3. In both cases a high presence of arabinose and galactose, two of the essential monomers in the arabinogalactan structure, can be observed. The later have demonstrated antiproliferative activity (Gunning et al., 2009).

3.3.2. Infrared analysis

In Figs. 3 and 4 the infrared spectra of pectic extracts are shown. The extracts correspond to low methoxyl pectins (Fig. 3a). The band between 3300 and 3500 cm^{-1} is due to OH stretching. Signals near 2950 cm^{-1} correspond to the stretching of the CH and CH_2 groups, and the two bands at 1590 and 1420 cm^{-1} correspond to the asymmetric and symmetric vibration of the COO (carboxylate) structure, respectively. Carbohydrates showed a high absorbance in the region between 1200 and 950 cm^{-1} which is a typical region for polysaccharides. A strong absorbance at 1075 cm^{-1} is distinctive of the type β-glycosidic linkages between the sugar units. The band at 1740 cm^{-1} is attributable to the stretching vibration of the CO group of the carboxylic acid methyl ester (or protonated carboxylic acid), representing a characteristic differential spectra. Bands with high intensities were observed at two ranges, 1610–1550 and 1410–1300 cm^{-1}, corresponding to the asymmetric and symmetric stretching of the carboxylate group, respectively. Bands between 1620 and 1430 cm^{-1} corresponds to the characteristic wavelengths for polygalacturonic acid.

When comparing the spectra of pectin extracts obtained by alkalized water, EDTA or Alkalase treatment against Pectasol it was detected that the modified pectin exhibited differences in the 800–1400 cm^{-1}. In this case, confirms that Pectasol is low esterification pectins. On the other hand, when the spectra of pectins extracted by EDTA and Alkalase 2.4 L were analyzed, both had a

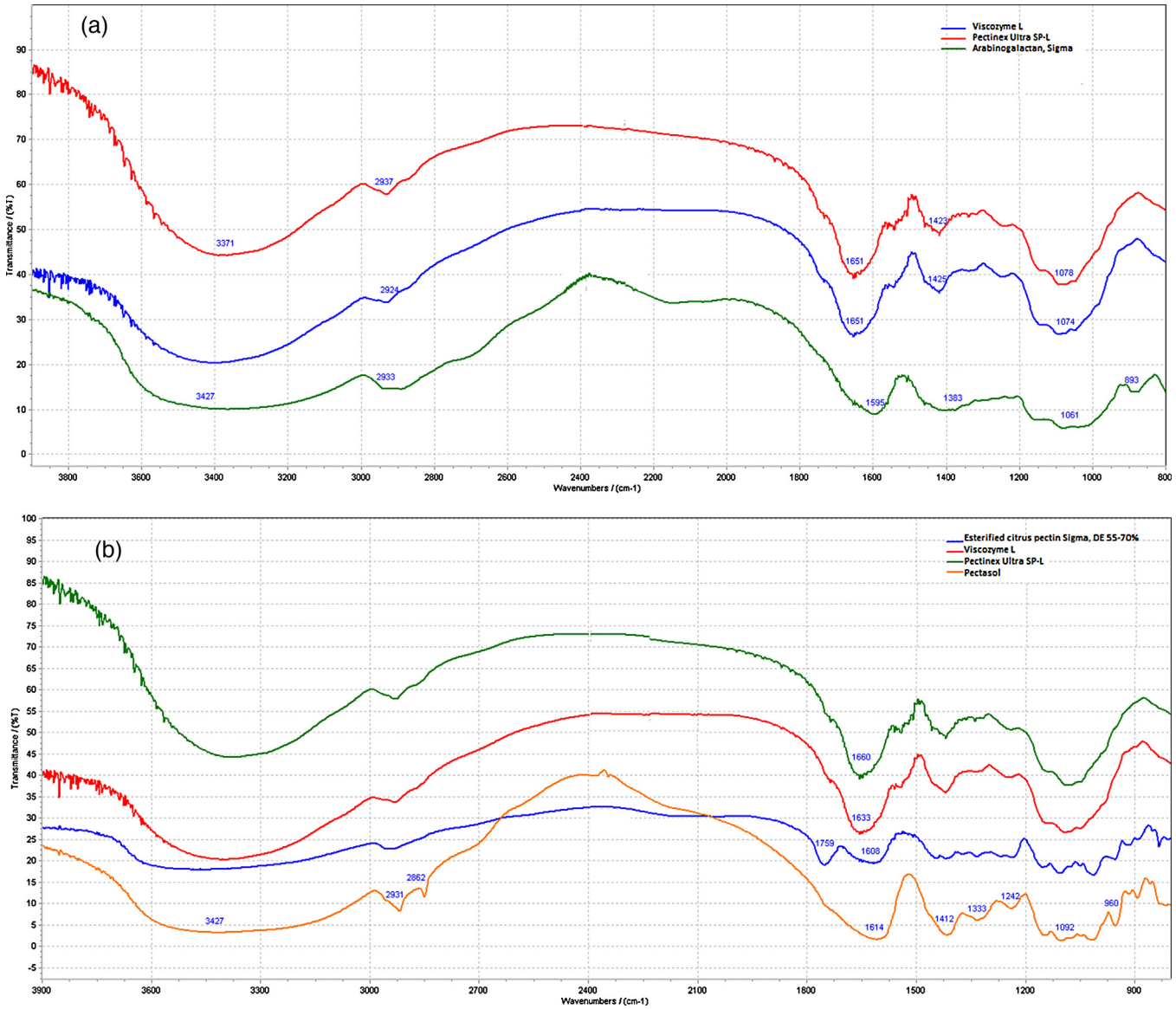

Fig. 4. FT-IR spectra of RSC neutral pectins extracted by Pectinex Ultra SP-L and Viscozyme L (a) comparative with arabinogalactan; (b) comparative with commercial pectins and modified citrus pectins.

similar shape to a β-galactan spectrum, while the spectra of pectins extracted by CPP had the same shape as the arabinogalactan (type II) spectrum (Kacuráková, 2000).

The fact that spectrograms obtained were different for the pectic extracts of the same raw material revealed that it is very important to select an appropriate treatment when the goal is to recover pectins with biological activities. On this matter, the data presented suggest that pectic substances extracted in the early treatments such as alkaline water and EDTA, would be acid pectins, mainly made up of homogalacturans, which are water-soluble and easy-to-remove from the cell matrix. The bands amide I and amide II (1660 cm^{-1} and 1590 cm^{-1}, respectively) were observed. These results are consistent with the protein value determined which indicate that small amount of protein were recovered in the extraction process (detected by Kjeldhal method, unpublished data)

In reference to CPP extracts there were no differences between neutral pectins from Pectinex Ultra SP-L and Viscozyme L (Fig. 4a–c). The shape of both, spectra and wavelength of the characteristic peaks, were practically the same, differing only for the absorbance intensity. Probably the differences might be due to the relative proportion of the mixture depending on the polymerization and esterification degree. In this case the enzymatic treatment with pectinases allowed the recovery of similar products.

Additional to the above, the carbohydrates profile for CPP extracts were examined (Fig. 5). The chromatogram showed the presence of mainly monosaccharides such as galacturonic acid, galactose and arabinose. The elevated presence of arabinose and galactose is related to the arabinogalactan structure which is in agreement with the results obtained by FT-IR.

3.4. Antiproliferative activity

The antiproliferative activity was tested on a broad spectrum of concentrations, following the manner in which a new product is usually tested, and taking advantage of the water solubility of the extracts. The results obtained (Figs. 6 and 7) showed that RSC neutral pectin obtained by CPP, within a concentration range of 2.5–20 [mg/mL], killed around 60% of the breast cancer cells and 50% of colorectal carcinoma cells with minimal differences between each extract. For these extracts there was no dose dependent

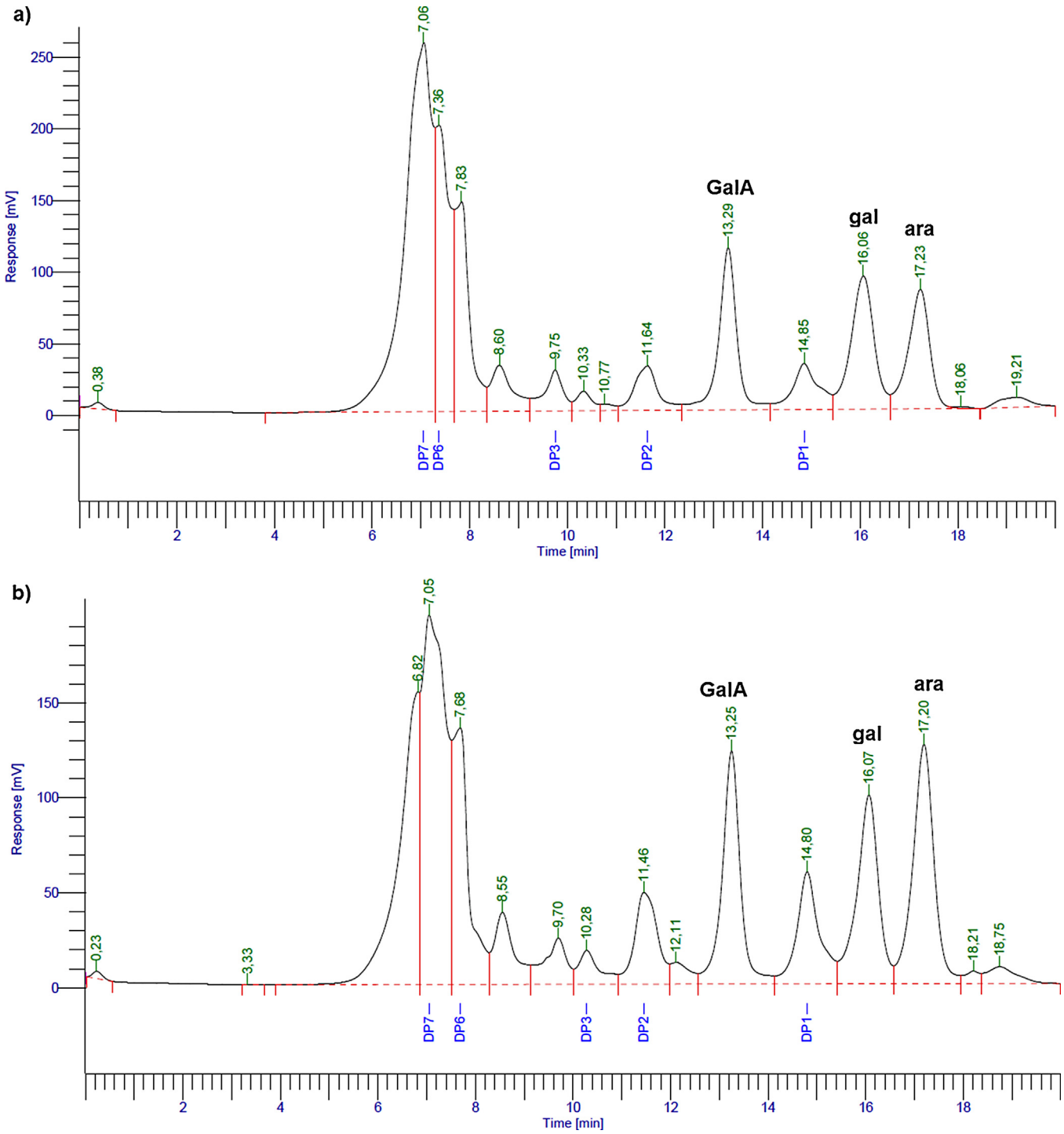

Fig. 5. Carbohydrate profiles of the neutral pectic hydrolysates extracted by (a) Pectinex Ultra SP-L and (b) Viscozyme L at 18 h. Conditions for both treatments were: 45 °C, L/S ratio 10:1, E/S ratio 2 wt % and 120 rpm on shaker agitation.

response, however, from the pharmacological point of view, the activity at lower concentrations is the most valuable for a potential clinical application. A cytotoxic effect due only to osmotic shock is marginal, because of the enrichment of this extract in neutral pectin which does not contribute to osmotic pressure and also because the estimated molarity of these extracts is 3.3×10^{-2} mM. It is also important to state that in vitro proliferation assays most of the times do not give 100% of cell death, even when applied to conventional anticancer drugs. For instance, doxorubicin *in vitro* treatment of the MCF-cells at 1 μM causes a 25% survival (Weinstein-Oppenheimer et al., 2001).

Besides, all the extracts obtained before the CPP treatment showed some antiproliferative activity. The pectic substances obtained with hydrolysates obtained after Alkalase 2.4 L treatment killed around 80% of MCF-7 cells and 60% of Caco-2 cells (Figs. 6 and 7), being the most effective as antiproliferative agent for both cell lines. This suggests that either the protease treatment delivered an active component of the extract or degraded an inhibitor.

EDTA and alkalized water treatment, both yielded acid pectins with different antiproliferative profiles. While EDTA produced pectic extract exhibiting high antiproliferative activity at the highest

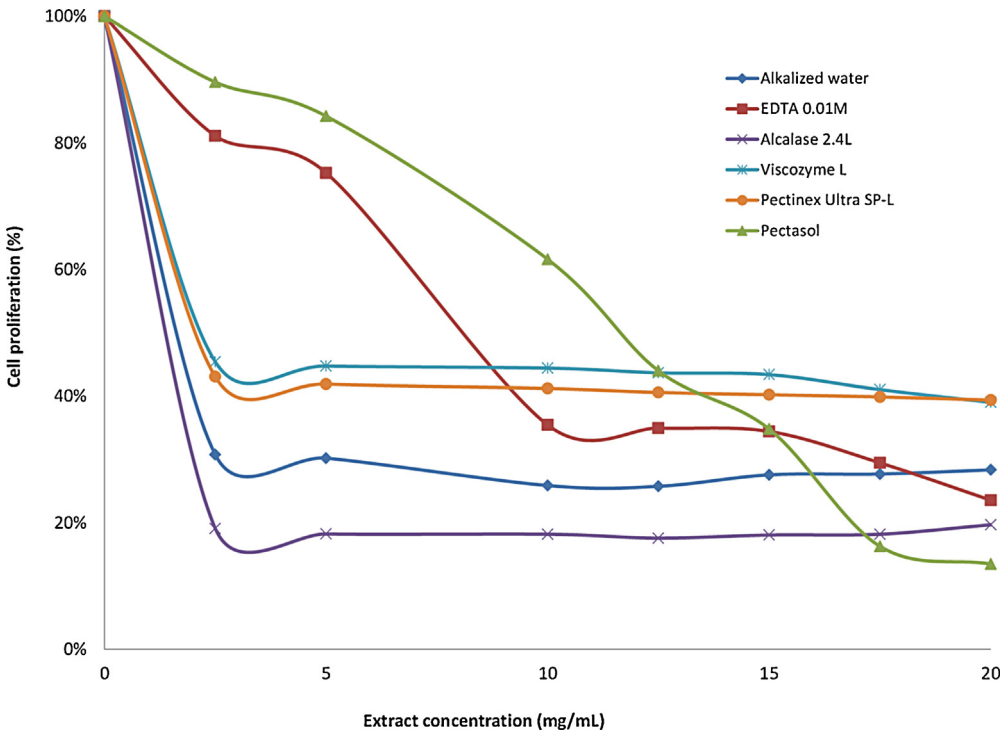

Fig. 6. Anti-proliferative activity of pectic substances extracted from RSC on MCF-7 breast cancer cell line. Percentages of proliferation are calculated in reference to the signal of cells grown of pectin free cell culture media. Each value represents a mean ± standard deviation. This is a representative result of three independent experiments.

evaluated activity, because these are charged pectins and effect of osmotic shock at 10 mg/mL cannot be overlooked, alkalized water produced pectic extract displayed an effect which was dependent on the tested cell line. For Caco-2 cells their effect was comparable to the one of EDTA, while on the MCF-7 cells this extract showed the second best response after alkalase produced extracts. This shows that different methods of extraction yield products that might differ on their activity when tested on certain cell lines. Thus, some extracts are more active in specific types of cancer, indicating particular interactions between pectin and molecular cell targets. RSC

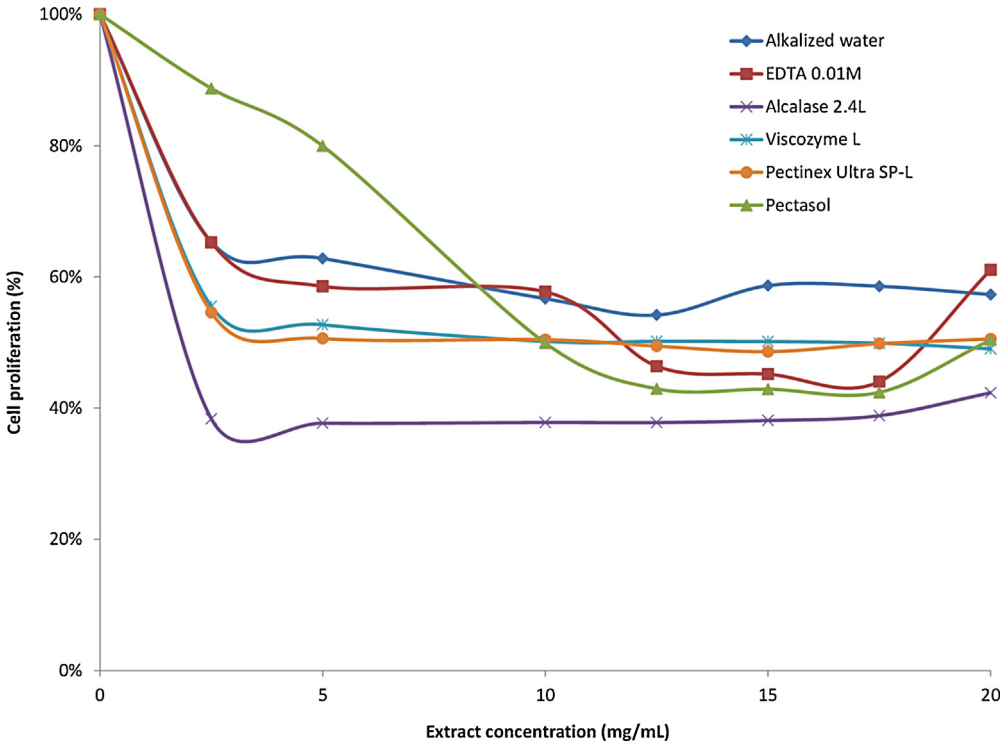

Fig. 7. Anti-proliferative activity of pectic substances extracted from RSC on Caco-2 colorectal cancer cell line. Percentages of proliferation are calculated in reference to the signal of cells grown of pectin free cell culture media. Each value represents a mean ± standard deviation. This is a representative result of three independent experiments.

pectic extracts recovered with Pectinex Ultra SP-L and Viscozyme L containarabingalactan, that allow them to interact and inhibit galectin-3 receptors on the cancer cell lines (Leclere, Cutsem, & Michiels, 2013). At less than 10 mg/mL both pectic extracts exhibited better antiproliferative activity (50 and 40%, respectively), than MCP (61.6% for MCF-7 and 49.9% for Caco-2 cells). This suggests a great potential for neutral pectins in nutraceutical or pharmaceutical industry, adding value to an agroindustrial by product.

4. Conclusion

Pectic substances extracted from Rapeseed cake exhibited antiproliferative activity on MCF-7 breast cancer cell line and Caco-2 colorectal carcinoma cell line. The bioactivity depends both on the extraction procedure and the cell line. Thus, different extracts might be more efficacious with particular cell lines that represent a type of cancer. Even though there are more scientific reports on the antiproliferative activity for acid pectins, certainly there is a promise for neutral pectins as antiproliferative, anticancer or chemopreventive products with application for the pharmaceutical and nutraceutical industry.

Acknowledgments

DIPUV Grant 37/2009
Fondecyt Grant no 1121062

References

Aspinall, G. O., & Kuo-Shii, J. (1974). Rapeseed hull pectin. *Carbohydrate Research*, *38*, 247–255. http://dx.doi.org/10.1016/S0008-6215(00)82355-1

Bell, J. M. (1993). Factors affecting the nutritional value of canola meal: A review. *Canadian Journal of Animal Science*, *73*(4), 689–697. http://dx.doi.org/10.4141/cjas93-075

Bergman, M., Djaldetti, M., Salman, H., & Bessler, H. (2010). Effect of citrus pectin on malignant cell proliferation. *Biomedicine and Pharmacotherapy=Biomédecine and Pharmacothérapie*, *64*(1), 44–47. http://dx.doi.org/10.1016/j.biopha.2009.03.004

Carbonell, E., Costell, E., & Duran, L. (1990). Determinación del contenido de pectinas en productos vegetales. *Revista de Agroquímica Y Tecnología de Los Alimentos*, *30*, 1–9.

Combo, A. M. M., Aguedo, M., Goffin, D., Wathelet, B., & Paquot, M. (2012). Enzymatic production of pectic oligosaccharides from polygalacturonic acid with commercial pectinase preparations. *Food and Bioproducts Processing*, *90*(3), 588–596. http://dx.doi.org/10.1016/j.fbp.2011.09.003

Concha Olmos, J., & Zúñiga Hansen, M. E. (2012). Enzymatic depolymerization of sugar beet pulp: Production and characterization of pectin and pectic-oligosaccharides as a potential source for functional carbohydrates. *Chemical Engineering Journal*, *192*, 29–36. http://dx.doi.org/10.1016/j.cej.2012.03.085

Çulcuoğlu, E., Ünay, E., & Karaosmanoğlu, F. (2002). Rapeseed cake as a biomass source. *Energy Sources*, *24*(4), 329–336. http://dx.doi.org/10.1080/00908310252888709

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), 350–356. http://dx.doi.org/10.1021/ac60111a017

Egües, I., Alriols, M. G., Herseczki, Z., Marton, G., & Labidi, J. (2010). Hemicelluloses obtaining from rapeseed cake residue generated in the biodiesel production process. *Journal of Industrial and Engineering Chemistry*, *16*(2), 293–298. http://dx.doi.org/10.1016/j.jiec.2010.01.036

Eriksson, I., Andersson, R., & Åman, P. (1997). Extraction of pectic substances from dehulled rapeseed. *Carbohydrate Research*, *301*(3–4), 177–185. http://dx.doi.org/10.1016/S0008-6215(97)00096-7

Fauduet, H., Coic, J. P., Lessire, M., Quinsac, A., Ribaillier, D., & Rollin, P. (1995). Rapeseed meal upgrading—Pilot scale preparation of rapeseed meal materials with high or low glucosinolate contents. *Animal Feed Science and Technology*, *56*(1–2), 99–109. http://dx.doi.org/10.1016/0377-8401(95)00811-Z

Fissore, E. N., Ponce, N. M., Wider, E. A., Stortz, C. A., Gerschenson, L. N., & Rojas, A. M. (2009). Commercial cell wall hydrolytic enzymes for producing pectin-enriched products from butternut (*Cucurbita moschata*, Duchesne ex Poiret). *Journal of Food Engineering*, *93*(3), 293–301. http://dx.doi.org/10.1016/j.jfoodeng.2009.01.024

Gao, X., Zhi, Y., Zhang, T., Xue, H., Wang, X., Foday, A. D., et al. (2012). Analysis of the neutral polysaccharide fraction of MCP and its inhibitory activity on galectin-3. *Glycoconjugate Journal*, *29*(4), 159–165. http://dx.doi.org/10.1007/s10719-012-9382-5

Glinsky, V. V., & Raz, A. (2009). Modified citrus pectin anti-metastatic properties: One bullet, multiple targets. *Carbohydrate Research*, *344*(14), 1788–1791. http://dx.doi.org/10.1016/j.carres.2008.08.038

Gunning, A. P., Bongaerts, R. J. M., & Morris, V. J. (2009). Recognition of galactan components of pectin by galectin-3. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *23*(2), 415–424. http://dx.doi.org/10.1096/fj. 08-106617

He, R., Alashi, A., Malomo, S. A., Girgih, A. T., Chao, D., Ju, X., et al. (2013). Antihypertensive and free radical scavenging properties of enzymatic rapeseed protein hydrolysates. *Food Chemistry*, *141*(1), 153–159. http://dx.doi.org/10.1016/j.foodchem.2013.02.087

Jackson, C. L., Dreaden, T. M., Theobald, L. K., Tran, N. M., Beal, T. L., Eid, M., et al. (2007). Pectin induces apoptosis in human prostate cancer cells: Correlation of apoptotic function with pectin structure. *Glycobiology*, *17*(8), 805–819. http://dx.doi.org/10.1093/glycob/cwm054

Jeong, H.-S., Kim, H.-Y., Ahn, S. H., Oh, S. C., Yang, I., & Choi, I.-G. (2013). Effects of combination processes on the extraction of pectins from rapeseed cake (*Brassica napus* L.). *Food Chemistry*, *139*(1–4), 9–15. http://dx.doi.org/10.1016/j.foodchem.2012.12.056

Kacuráková, M. (2000). FT-IR study of plant cell wall model compounds: Pectic polysaccharides and hemicelluloses. *Carbohydrate Polymers*, *43*(2), 195–203. http://dx.doi.org/10.1016/S0144-8617(00)00151-X

Kelly, G. S. (1999). Larch arabinogalactan: Clinical relevance of a novel immune-enhancing polysaccharide. *Alternative Medical Review*, *4*(2), 96–103.

Kurita, O., Fujiwara, T., & Yamazaki, E. (2008). Characterization of the pectin extracted from citrus peel in the presence of citric acid. *Carbohydrate Polymers*, *74*(3), 725–730. http://dx.doi.org/10.1016/j.carbpol.2008.04.033

Leclere, L., Cutsem, P., & Van Michiels, C. (2013). Anti-cancer activities of pH- or heat-modified pectin. *Frontiers in Pharmacology*, *4*, 128. http://dx.doi.org/10.3389/fphar.2013.00128

Lim, J., Yoo, J., Ko, S., & Lee, S. (2012). Extraction and characterization of pectin from Yuza (*Citrus junos*) pomace: A comparison of conventional-chemical and combined physical-enzymatic extractions. *Food Hydrocolloids*, *29*(1), 160–165. http://dx.doi.org/10.1016/j.foodhyd.2012.02.018

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, *31*(3), 426–428. http://dx.doi.org/10.1021/ac60147a030

Min, B., Lim, J., Ko, S., Lee, K.-G., Lee, S. H., & Lee, S. (2011). Environmentally friendly preparation of pectins from agricultural byproducts and their structural/rheological characterization. *Bioresource Technology*, *102*(4), 3855–3860. http://dx.doi.org/10.1016/j.biortech.2010.12.019

Mutter, M., Colquhoun, I. J., Schols, H. A., Beldman, G., & Voragen, A. (1996). Rhamnogalacturonase B from *Aspergillus aculeatus* is a rhamnogalacturonan [alpha]-L-rhamnopyranosyl-(1->4)-[alpha]-D-galactopyranosyluronide lyase. *Plant Physiology*, *110*, 173–177. http://dx.doi.org/10.1104/pp.110.1.73

Nangia-Makker, P., Hogan, V., Honjo, Y., Baccarini, S., Tait, L., Bresalier, R., et al. (2002). Inhibition of human cancer cell growth and metastasis in nude mice by oral intake of modified citrus pectin. *JNCI: Journal of the National Cancer Institute*, *94*(24), 1854–1862. http://dx.doi.org/10.1093/jnci/94.24.1854

Nikolić, M. V., & Mojovic, L. (2007). Hydrolysis of apple pectin by the coordinated activity of pectic enzymes. *Food Chemistry*, *101*(1), 1–9. http://dx.doi.org/10.1016/j.foodchem.2005.12.053

Pastuszewska, B., Jabłecki, G., Buraczewska, L., Dakowski, P., Taciak, M., Matyjek, R., et al. (2003). The protein value of differently processed rapeseed solvent meal and cake assessed by in vitro methods and in tests with rats. *Animal Feed Science and Technology*, *106*(1–4), 175–188. http://dx.doi.org/10.1016/S0377-8401(03)00005-1

Pienta, K. J., Nailk, H., Akhtar, A., Yamazaki, K., Replogle, T. S., Lehr, J., et al. (1995). Inhibition of spontaneous metastasis in a rat prostate cancer model by oral administration of modified citrus pectin. *JNCI Journal of the National Cancer Institute*, *87*(5), 348–353. http://dx.doi.org/10.1093/jnci/87.5.348

Schols, H. A., Vierhuis, E., Bakx, E. J., & Voragen, A. G. J. (1995). Different populations of pectic hairy regions occur in apple cell walls. *Carbohydrate Research*, *275*(2), 343–360. http://dx.doi.org/10.1016/0008-6215(95)00155-M

Schols, H. A., Voragen, A. G. J., & Colquhoun, I. J. (1994). Isolation and characterization of rhamnogalacturonan oligomers, liberated during degradation of pectic hairy regions by rhamnogalacturonase. *Carbohydrate Research*, *256*(1), 97–111. http://dx.doi.org/10.1016/0008-6215(94)84230-2

Siddiqui, I. R., & Wood, P. J. (1972). Structural investigation of water-soluble rapeseed (*Brassica campestris*) polysaccharides. *Carbohydrate Research*, *24*(1), 1–9. http://dx.doi.org/10.1016/S0008-6215(00)82254-5

Siddiqui, I. R., & Wood, P. J. (1977). Carbohydrates of rapeseed: A review. *Journal of the Science of Food and Agriculture*, *28*(6), 530–538. http://dx.doi.org/10.1002/jsfa.2740280610

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., et al. (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. *JNCI Journal of the National Cancer Institute*, *82*(13), 1107–1112. http://dx.doi.org/10.1093/jnci/82.13.1107

Theander, O., Aman, P., Miksche, G. E., & Yasuda, S. (1977). Carbohydrates, polyphenols, and lignin in seed hulls of different colors from turnip rapeseed. *Journal of Agricultural and Food Chemistry*, *25*(2), 270–273. http://dx.doi.org/10.1021/jf60210a042

Tkachuk, R. (1969). Nitrogen-to-protein conversion factors for cereals and oilseed meals. *Cereal Chemistry*, *46*, 419–423.

Van Soest, P. J., Robertson, J. B., & Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal

nutrition. *Journal of Dairy Science*, *74*(10), 3583–3597. http://dx.doi.org/10.3168/jds.S0022-0302(91)78551-2

Weinstein-Oppenheimer, C. R., Henríquez-Roldán, C. F., Davis, J. M., Navolanic, P. M., Saleh, O. a., Steelman, L. S., et al. (2001). Role of the Raf signal transduction cascade in the in vitro resistance to the anticancer drug doxorubicin. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, *7*(9), 2898–2907.

Willats, W. G., Knox, J. P., & Mikkelsen, J. D. (2006). Pectin: New insights into an old polymer are starting to gel. *Trends in Food Science and Technology*, *17*(3), 97–104. http://dx.doi.org/10.1016/j.tifs.2005.10.008

Yapo, B. M. (2009). Pectin quantity, composition and physicochemical behaviour as influenced by the purification process. *Food Research International*, *42*(8), 1197–1202. http://dx.doi.org/10.1016/j.foodres.2009.06.002

Yapo, B. M. (2011). Pectic substances: From simple pectic polysaccharides to complex pectins—A new hypothetical model. *Carbohydrate Polymers*, *86*(2), 373–385. http://dx.doi.org/10.1016/j.carbpol.2011.05.065

Yapo, B. M., Robert, C., Etienne, I., Wathelet, B., & Paquot, M. (2007). Effect of extraction conditions on the yield, purity and surface properties of sugar beet pulp pectin extracts. *Food Chemistry*, *100*(4), 1356–1364. http://dx.doi.org/10.1016/j.foodchem.2005.12.012

Yeoh, S., Shi, J., & Langrish, T. A. G. (2008). Comparisons between different techniques for water-based extraction of pectin from orange peels. *Desalination*, *218*(1–3), 229–237. http://dx.doi.org/10.1016/j.desal.2007.02.018

Yust, M., Pedroche, J., Megıías, C., Girón-Calle, J., Alaiz, M., Millán, F., et al. (2004). Rapeseed protein hydrolysates: A source of HIV protease peptide inhibitors. *Food Chemistry*, *87*(3), 387–392. http://dx.doi.org/10.1016/j.foodchem.2003.11.020