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Journal of Food Science and Nutrition Therapy

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Dates: Received: 12 May, 2017; **Accepted:** 01 June, 2017; **Published:** 02 June, 2017

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Keywords: Prunes; *Prunus domestica*; Insoluble dietary fibers; Structural characterization

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Research Article

Investigation of Structural Features of Prunes (*Prunus domestica*) Insoluble Dietary Fibers

Abstract

Structural characteristics of dietary fibers are closely related to its biological functions in the human body. Previously, soluble dietary fibers from prunes were extracted and characterized. In this work, structural analysis of insoluble dietary fibers was conducted using monosaccharide composition, methylation, molecular weight determination and ^{13}C-NMR data. Prunes' non-cellulosic insoluble fibers were found to contain, a pectic type I arabinogalactan, a fucogalactoxyloglucan and a heteroxylan. These findings suggest that insoluble dietary fibers can be composed by some pectic polysaccharides besides cellulose and hemicellulosic polymers. This paper brings important structural features of insoluble dietary fibers from prunes that may be of biological significance.

Introduction

Dietary fibers are carbohydrate polymers composed by ten or more monomeric units, which are not hydrolyzed by endogenous enzymes in the small intestine and can be partially or totally fermented in the large intestine of humans[1,2] . Health benefits associated with dietary fiber consumption result from its low caloric content, physical effects in the stomach and small intestine and fermentation in the colon. Dietary fibers can be classified as either water soluble and mostly fermentable (such as pectin) or insoluble, less fermentable, and nonviscous (such as cellulose, lignin, and some of the hemicelluloses) [3].

Soluble fibers are generally known to increase viscosity of the stomach and small intestine content, improving satiety, reducing post-prandial glycaemia and preventing reabsorption of bile acids, thus reducing circulating blood cholesterol levels. Moreover, due to its high fermentability, soluble fibers can positively modulate the colonic microflora preventing pathologies such as infectious diseases, allergy or asthma, colon cancer, obesity, liver disease, diabetes and inflammatory bowel disease. On the other hand, insoluble fibers are known to be poorly fermentable, but are able to increase fecal bulk and decrease transit time, increasing stool frequency [4]. However, not all soluble/insoluble fibers behave in the same way. For example, the soluble dietary fiber inulin was shown to increase stool frequency [5]. Likewise, insoluble fibers, such as resistant starch, are highly fermented by the human gut microbiota [6]. This may be because carbohydrate polymers, as dietary fibers, represent the most heterogeneous and diverse group of associated molecules found in nature. Therefore, not only water solubility, but other structural features such as monosaccharide composition, linkage types between monosaccharides, size of the polymers, branching patterns, etc., also dictates their biological activities [6,7]. Thus, the knowledge about chemical structure of food dietary fibers is important to explore how it interacts with the human body and possibly produce health benefits.

Prunes, the dried fruits of plums (*Prunus domestica*), possess as high as 62.7% of carbohydrates and its consumption is related to laxative effects, reductions in cardiovascular risk and sugar metabolism control that may be associated with dietary fiber constituents [8]. We have previously carried out the isolation and characterization of soluble dietary fibers found in prunes [9] and the pectic polysaccharides homogalacturonan and rhamnogalacturonans with type I arabinogalactans side chains have been described. In this work, our objective was to further analyze the chemical structure of insoluble dietary fibers through monosaccharide composition, linkage analysis, molecular weight determination and ^{13}C-NMR data and thus expand our knowledge about their structural characteristics.

Materials and Methods

Plant material

Pitted prunes (*Prunus domestica*) from cultivar d'Agen were purchased at local market in Curitiba (Brazil) (LA VIOLETERA®).

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Extraction and purification of polysaccharides

Prunes (2 kg) were blended and exhaustively extracted with water (6 L) at 100 ºC under reflux for 2 h as previously described [9] to remove soluble dietary fibers (SDF). The residue of hot water extraction, containing the insoluble dietary fibers (IDF), was separated after centrifugation (8000 rpm, 15 min at 15 °C). To solubilize some of the polysaccharides present in the IDF, mainly hemicelluloses, the residue was submitted to alkaline extraction with KOH 10% (2 L each, 3x) at 100 ºC under reflux for 2 h. Alkaline extract was then obtained by centrifugation (8000 rpm, 15 min at 15 °C), followed by neutralization with HOAc, dialysis and lyophilization, resulting in a polysaccharide fraction named herein as PK (prunes's alkaline extract) (Figure 1). The residue remaining of this extraction contained cellulose that has not been solubilized with this treatment.

As a first step of fractionation, a freeze-thaw treatment was applied in fraction PK, to give cold-water soluble (SPK) and insoluble (PPK) fractions. In this procedure, the sample was frozen and then thaw at room temperature followed by centrifugation (8000 rpm, 15 min at 15 °C).

Fraction SPK was further fractionated by Fehling's treatment. Briefly, it was dissolved in distilled water and treated with Fehling's solutions [10] resulting, after centrifugation (8000 rpm, 15 min at 15 °C), in a xyloglucan-copper complex as the pellet (fraction PF-SPK) and a soluble fraction (SF-SPK). After neutralization with HOAc, both fractions were dialyzed against tap water and deionized with cation exchange resin. The fraction PF-SPK was later purified by ultrafiltration through a membrane with cut-off of 100 kDa (PLHK04710-Ultracel, Millipore), yielding the fractions PF-SPK-100E (eluted in 100 kDa) and PF-SPK-100R (retained in 100 kDa) (Figure 1).

Fraction PF-SPK-100R was further purified by anion exchange chromatography. It was dissolved in distilled water (50 mg/mL), centrifuged (12000 x *g*, 10 min at 10 ºC) and the supernatant applied to a DEAE-Sepharose Fast Flow column (3.0 cm×25 cm). The column was eluted with distilled water (F1) followed by 4.0 M NaCl solution (F2) at a flow rate of 1.5 mL/min. Polysaccharides in the eluted fractions were detected using phenol–sulfuric acid method [11] . The obtained fractions were concentrated and freeze-dried.

The yields were expressed as % based on the weight of dried prunes pulp that was submitted to extraction (1400 g) (Figure 1).

Sugar composition

Polysaccharides' neutral monosaccharides composition was determined by hydrolysis with 2 M TFA (8 h/100 °C), conversion into alditol acetates using successive $NaBH_4$ reductions, and acetylation with Ac_2O-pyridine (1:1, v/v, 2 mL - 100 °C, 30min). A Varian gas chromatograph and mass spectrometer (Saturn 2000R), with He as carrier gas were used for analysis. For quantitative analysis, a capillary column (30 m x 0.25 mm i.d.) of DB-225 was held at 50 ºC during injection for 1 min, then programmed at 40 ºC/min to 220 ºC and held at this for 19.75 min.

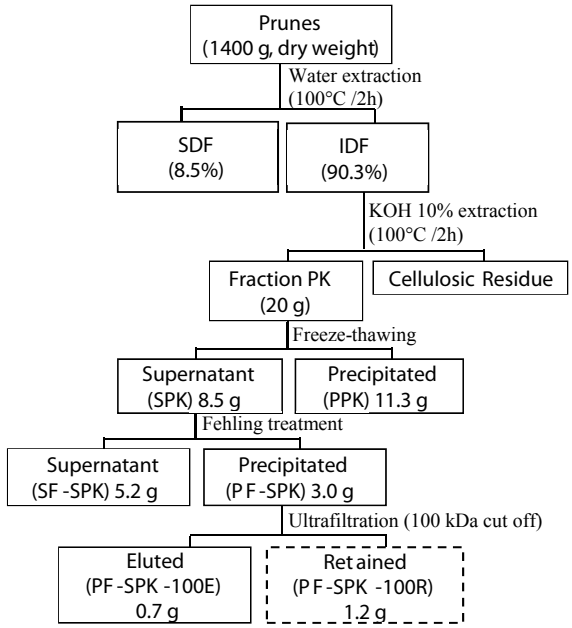

Figure 1: Scheme of extraction and fractionation of alkali soluble polysaccharides from prunes (*Prunus domestica*).

The determination of uronic acid contents was conducted according to the m-hydroxybiphenyl method [12].

Determination of homogeneity and molecular weight of polysaccharides

The homogeneity of polysaccharides was evaluated by high performance steric exclusion chromatography (HPSEC), with a Waters 2410 differential refractometer as equipment for detection. A series of four columns, with exclusion sizes of 7 x 10^6 Da (Ultrahydrogel 2000, Waters), 4 x 10^5 Da (Ultrahydrogel 500, Waters), 8 x 10^4 Da (Ultrahydrogel 250, Waters) and 5 x 10^3 Da (Ultrahydrogel 120, Waters) was used. The eluent was 0.1 M aq. $NaNO_2$ containing 200 ppm aq. NaN_3 at 0.6 mL/min. The sample, previously filtered through a membrane (0.22 µm, Millipore), was injected (250 µl loop) at a concentration of 1 mg/mL. To obtain the molecular weight, standard dextrans (487kDa, 266kDa, 124kDa, 72.2kDa, 40.2kDa, 17.2kDa and 9.4kDa, from Sigma) were employed to obtain the calibration curve. The molecular weight of the sample was calculated according to the calibration curve.

Methylation analysis of polysaccharide

Fraction PF-SPK-100R was *O*-methylated as described by Ciucanu and Kerek [13]. The per-*O*-methylated polysaccharide was further submitted to methanolysis in 3% HCl–MeOH (80 °C, 2 h) followed by hydrolysis with H_2SO_4 (0.5M, 12 h) and neutralization with $BaCO_3$. The material was then reduced an acetylated as described above for monosaccharides composition, except that $NaBD_4$ was used for reduction. The resultant partially *O*-methylated alditol acetates were analyzed with a GC-MS. For separation, a 30 m x 0.25 mm i.d. capillary column of DB-225 was held at 50 ºC during injection for 1 min, then programmed at 40 ºC/min to 210 ºC and held at this temperature for 31 min. Typical electron impact breakdown

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profiles and retention times of partially *O*-methylated alditol acetates were used for identification [14].

Nuclear magnetic resonance (NMR) spectroscopy

Spectra of ^{13}C NMR were acquired using a Bruker spectrometer (DRX 400 MHz AVANCE III NMR - Bruker Daltonics, Germany). Samples were dissolved in D_2O and placed in a 5 mm inverse gradient probe, at 70 °C for analysis. Chemical shifts were expressed as δ ppm and acetone CH_3 group's resonance was used as internal standard (δ 30.2). The spectra were handled using the Topspin® (Bruker) computer program.

Results and Discussion

In order to extract water insoluble polysaccharides from prunes, the residue of prunes' water extraction was submitted to alkaline extraction, which resulted, after dialysis, in fraction PK. This was further fractionated through freeze-thaw treatment followed by centrifugation to give rise to a supernatant fraction (SPK) and a precipitated fraction (PPK) (Figure 1). The latter presented arabinose and glucose as main monosaccharides (Table 1), but NMR analysis was not possible due to its high insolubility in different solvents.

On the other hand, SPK presented arabinose and galactose as main monosaccharides (Table 1). The ^{13}C-NMR spectrum (Figure 2A) had signals of β-D-Gal*p* at δ 104.3 (C-1), δ 74.5 (C-5), δ 73.5 (C-3), δ 72.1 (C-2) and δ 77.6 (substituted C-4) [15-17]. The anomeric signal at δ 107.6 was assigned to units of α-L-Ara*f* [17]. These data could indicate the presence of a type I arabinogalactan (AG-I) in fraction SPK, already reported for prunes' water extract [18]. However, besides the signals of an AG-I, SPK presented a diversity of other anomeric signals in the region between δ 98.0 and δ 104.0 (Figure 2A), as well as significant content of glucose and xylose according to the monosaccharide analysis (Table 1), indicating the presence of another polysaccharide.

In order to separate the different polymers present in SPK, it was treated with Fehling's solution, resulting in a precipitated fraction (PF-SPK) and a supernatant fraction (SF-SPK) (Figure 1). As previously observed for water extracts [18], the AG-I remained in the Fehling supernatant as could be seen in the ^{13}C-NMR (Figure 2B) and monosaccharide analysis (Table 1), of fraction SF-SPK. The fraction precipitated with Fehling solution PF-SPK, had glucose and xylose as main monosaccharides (Table 1). Besides, anomeric signals of β-Glc*p* and α-Xyl*p* could be seen at δ 102.3 and δ 99.4/98.9, respectively, in the ^{13}C-NMR spectrum of PF-SPK [19] (Figure 2C). These data indicate that while the AG-I present in SPK remained soluble after Fehling treatment, the fraction containing xylose and glucose was precipitated.

Once PF-SPK had a heterogeneous profile in HPSEC (data not shown), it was further filtrated with 100 kDa cutoff Milipore membrane, yelding an eluted fraction PF-SPK-100E and a retained fraction PF-SPK-100R (Figure 1). Only fraction PF-SPK-100R presented a homogeneous elution profile when analysed by HPSEC (Figure 3). The calculated molecular weight was 66 kDa.

Table 1: Monosaccharide composition of fractions obtained from alkali extraction of prunes (*Prunus domestica*).

Fraction	Neutral sugars (%)[a]						Uronic acid[b]
	Rha	Fuc	Ara	Xyl	Gal	Glc	
PPK	-	-	66.9	6.9	5.8	18.5	1.9
SPK	2.5	2.7	34.7	10.0	25.8	17.3	6.8
SF-SPK	1.5	-	45.7	4.8	37.1	4.5	6.8
PF-SPK	-	2.3	17.3	33.1	16.3	24.9	5.9
PF-SPK-100R	-	3.5	12.7	34.0	11.2	38.6	-

[a] % of peak area relative to total peak areas, determined by GC-MS.
[b] Determined using the *m*-hydroxybiphenyl method [12].

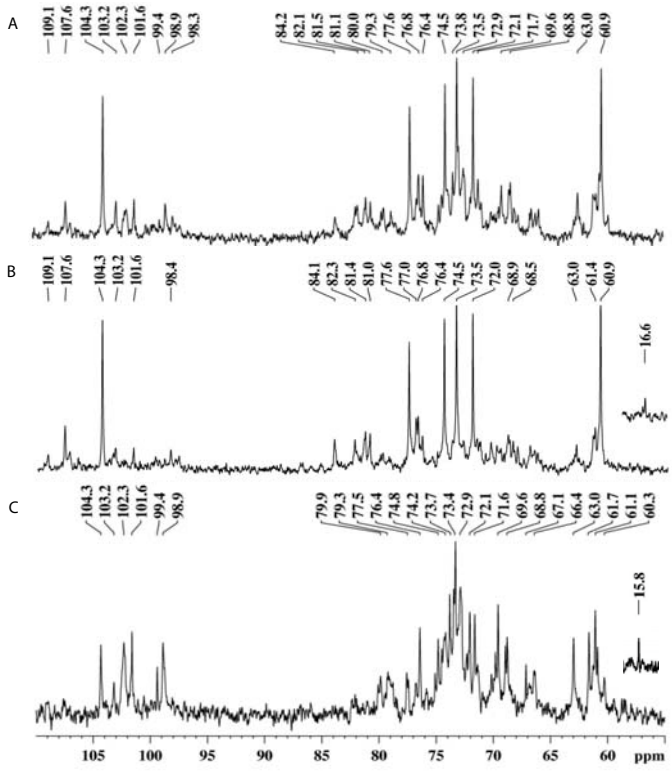

Figure 2: ^{13}C-NMR spectra of fractions SPK (A), SF-SPK (B) and PF-SPK (C) in D2O at 70 ºC.

The monosaccharide composition of PF-SPK-100R showed glucose and xylose as main sugars, and minor amounts of arabinose, galactose and fucose (Table 1). Methylation data of PF-SPK-100R is presented on Table 2. The major methylated derivatives were 2,3,6-Me_3-Glc-ol acetate (15%) and 2,3- Me_2-Glc-ol acetate (21.6%), indicating the presence of 4-*O*- and 4,6-*O*-substituted glucose units. Terminal and 2-*O*-substituted xylose units were also found, according to the derivatives 2,3,4-Me_3- and 3,4-Me_2-Xyl-ol acetates, respectively. These data suggest the presence of a xyloglucan in fraction PF-SPK-100R. However, the presence of 2,3-Me_2– and 3-Me-Xyl-ol derivatives, relative to 4-*O*- and 2,4-di-*O*-substituted xylose units were also present. This type of linkage is uncommon in xyloglucans since typically xylose units are exclusively *O*-2-linked. Although few exceptions could be found in the literature [20,21], it's most likely that these 4-*O* and 2,4-di-*O*- substituted xylose arose from the concomitant presence of a heteroxylan in fraction PF-SPK-100R.

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As the derivatives 3,4-Me_2-xylitol acetate and 2,3-Me_2-xylitol acetate have the same retention time in the conditions employed in the GC-MS, they were deuterated at C-1, so each component of the peak was detected by their fragmentation patterns. The 3,4-Me_2-derived xylitol acetate gives ions of m/z 190, 130, 117 and 88, while the 2,3-Me_2-derived xylitol acetate gives ions of m/z 189, 129, 118 and 87 [20]. From the intensity of each ion, it was observed a 1.0:1.4 ratio of 3,4-Me_2-Xyl*p* and 2,3-Me_2-Xyl*p* (Figure 4).

In addition to the methylated derivatives of glucose and xylose, 2,3,4,6-Me_4-Gal-ol acetate relative to terminal galactose from xyloglucan branches was also present. Derivatives 2,3,4-Me_3-Fuc-ol acetate, 2,3,4-Me_3-Ara-ol-acetate and 2,3,5-Me_3-Ara-ol-acetate, indicate the presence of terminal units of Fuc*p*, Ara*p* and Ara*f*, respectively. Terminal fucose units are commonly found in xyloglucans from dicotyledonous [22]. Moreover, instead of fucose, Ara*f* units were identified on side chains of xyloglucans from solanaceous plants [23]. Despite this, the concomitant presence of Fuc*p*, Ara*p* and Ara*f* units is an unusual feature in xyloglucans. Thus,

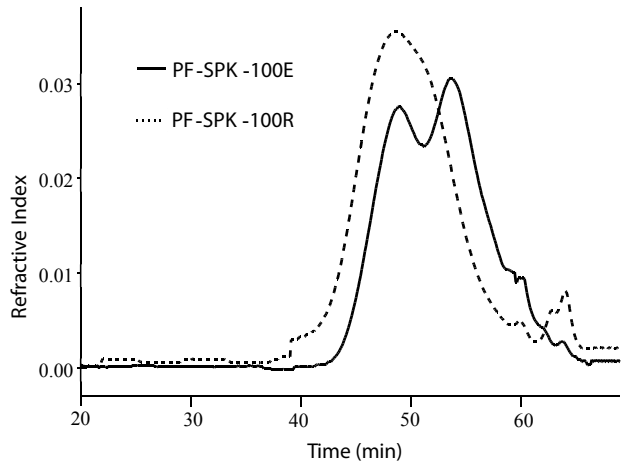

Figure 3: HPSEC elution profile of fractions PF-SPK-100E and PF-SPK-100R obtained from prunes (refractive index detector).

Table 2: Linkage types based on analysis of partially *O*-methyl alditol acetates obtained from methylated fraction PF-SPK-100R from prunes (*Prunus domestica*).

Partially O-methylalditol acetate	PF-SPK-100R[b]	Linkage type[c]
2,3,5-Me_3-Ara[a]	6.7	Ara*f*-(1→
2,3,4-Me_3-Ara	5.3	Ara*p*-(1→
2,3,4 –Me_3-Xyl	10.8	Xyl*p*-(1→
2,3,4 –Me_3-Fuc	3.6	Fuc*p*-(1→
2,3,4,6-Me_4-Gal	9.8	Gal*p*-(1→
3,4-Me_2-Xyl/2,3-Me_2-Xyl[d]	15.9	→2)-Xyl*p*-(1→/→4)-Xyl*p*-(1→
3-Me-Xyl	10.3	→2,4)-Xyl*p*-(1→
2,3,6-Me_3-Glc	15.4	→4)-Glc*p*-(1→
2,3- Me_2-Glc	22.2	→4,6)-Glc*p*-(1→

[a] 2,3,5-Me_3-Ara = 2,3,5-tri-*O*-Methylarabinitolacetate, etc.
[b] % of peak area of *O*-methylalditol acetates relative to total area, determined by GC-MS.
[c] Based on derived *O*-methylalditol acetates.
[d] The ratio of 3,4-Me_2-xylitol acetate and 2,3-Me_2-xylitol estimated by their fragmentation patterns in GC-MS was 1.0:1.4.

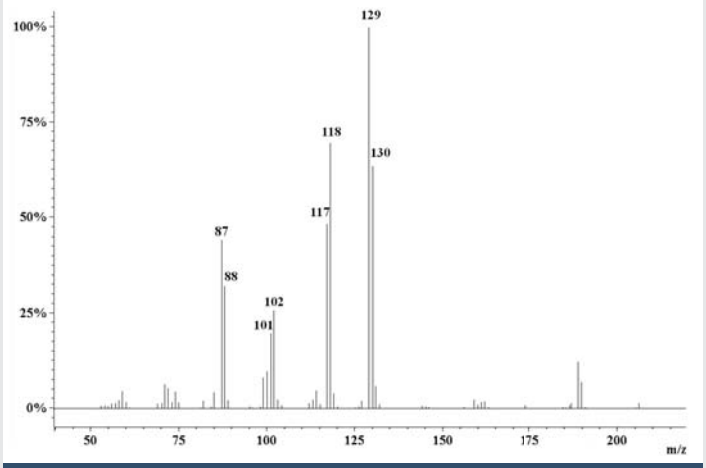

Figure 4: Fragmentation of 2,3- and 3,4-Me2-xylitol acetates mixture with deuterium at C-1 in GC-MS analysis obtained from fraction PF-SPK-100R.

these Ara*f* are likely to be side chains in the xylan, corroborating for the assumption that fraction PF-SPK-100R is composed by a mixture of a fucogalactoxyloglucan and a heteroxylan. The xyloglucan content in fraction PF-SPK-100R was estimated to be ~68% based on the sum of 4-*O* and 4,6-*O*-linked glucose, terminal and 2-*O*-substituted xylose and terminal galactose and fucose.

The presence of xylan–xyloglucan complexes has been previously identified in the cell walls of olive pulp [24]. In our research group, a xyloglucan and an acid heteroxylan were also found together in alkaline extracts from starfruit (*Averrhoa carambola* L.) and separated through anion exchange chromatography (unpublished data). In fraction PF-SPK-100R however, due to the absence of uronic acid linked to the xylan backbone, no polymers were retained after anion exchange chromatography (Fraction F1) making its separation from the xyloglucan not possible (data not shown). Moreover, the presence of the xylan and xyloglucan was also observed in the fraction PF-SPK-100E (Figure 5A), which demonstrated a similar ^{13}C-NMR spectrum as that of fraction PF-SPK-100R (Figure 5B). Attempts were also made to separate these polymers through ultrafiltration with 50, 30 and 10kDa membranes. However, all the retained and eluted fractions showed the presence of both polymers.

The ^{13}C-NMR spectrum of PF-SPK-100R (Figure 5B) is in accordance with methylation data and presented the main signals related to the xylan and xyloglucan mixture. Anomeric signals related to (1→4) and (1,4→6)-linked β-Glc*p* units that form the main chain of the xyloglucan are found at δ 102.3 and δ 103.2, respectively [25,26]. The signal at δ 98.9 was assigned to anomeric carbons of terminal and 2-*O*-substituted α-Xyl*p* units, while that at δ 99.4 and δ 15.8 can be assigned to C-1 and C-6 of terminal Fuc*p* units, respectively. In addition, the signal at δ 104.4 can be assigned to C-1 of terminal β-Gal*p* units [25]. Signals from the heteroxylan could be seen at δ 101.6 and δ 107.7 from anomeric carbons of β-Xyl*p* and α-L-Ara*f* units, respectively.

Overall, fraction SPK, which contains the insoluble dietary fibers of prunes was composed of a type I arabinogalactan,

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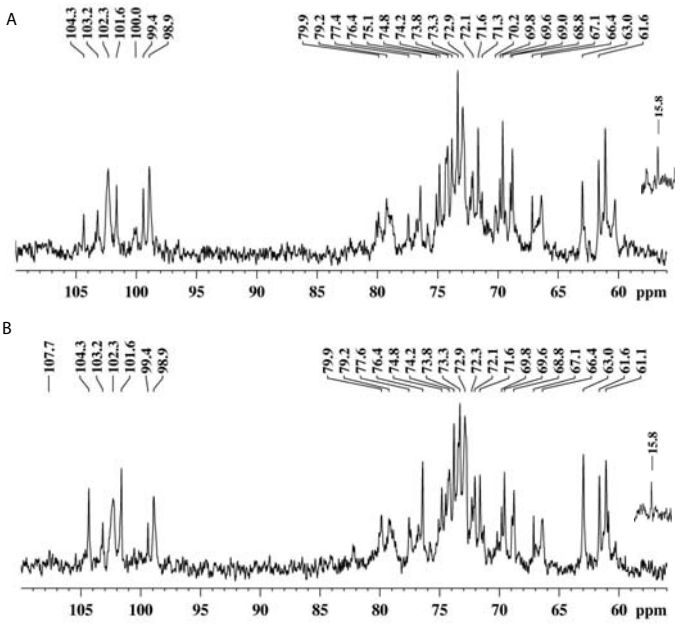

Figure 5: ^{13}C-NMR spectra of fractions PF-SPK-100E (A) and PF-SPK-100R (B) in D2O at 70 °C.

a fucogalactoxyloglucan and a heteroxylan. Despite being a soluble dietary fiber and solubilized with water [9], the type I arabinogalactan was also found herein in the fraction extracted with alkali. The presence of pectic polysaccharides associated with hemicelluloses and cellulose within the cell wall and that required harsher extraction conditions was also reported by Oechslin and others [27] in apple cellulosic residue. Thus, these findings suggest that prunes' insoluble dietary fibers are composed by some pectic polysaccharides besides cellulose and hemicellulosic polymers.

Some biological activities have already been attributed to type I arabinogalactans, such as immunological [28-30] and anti-ulcer activities [9,31]. Xyloglucan from different sources were also previously shown to display biological activities such as hypolipidemic [32], anti-tumoral [33,34], immunomodulatory [35-38] and hypoglycemic [39-41]. It's noteworthy, that some of these effects, such as hypolipidemic and hypoglycemic, were previously found in prunes, however, the responsible components were not fully resolved [8].

Conclusion

Prunes' non-cellulosic insoluble fibers were found to contain, a pectic type I arabinogalactan, a fucogalactoxyloglucan and a heteroxylan, suggesting that IDF can be composed by some pectic polysaccharides besides cellulose and hemicellulosic polymers. Moreover, this paper brings important structural features of insoluble dietary fibers from prunes that may be associated to biological functions, and provides new insights into the diversity of fruit hemicellulosic polymers.

Acknowledgement

This research was supported by Projeto Universal (Process 477971/2012-1) provided by CNPq foundation (Brazil) and by PRONEX-Carboidratos. The author Thaisa Moro Cantu-Jungles *received a fellowship* from *CNPq* foundation. The authors are grateful to the NMR Center of UFPR for recording the NMR spectra.

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Citation: Cantu-Jungles TM, Iacomini M, Cordeiro LMC (2017) Investigation of Structural Features of Prunes (*Prunus domestica*) Insoluble Dietary Fibers. J Food Sci Nutr The 3(1): 001-006.