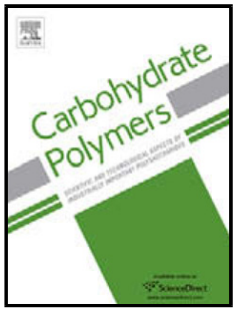

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The hydrophobic polysaccharides of apple pomace

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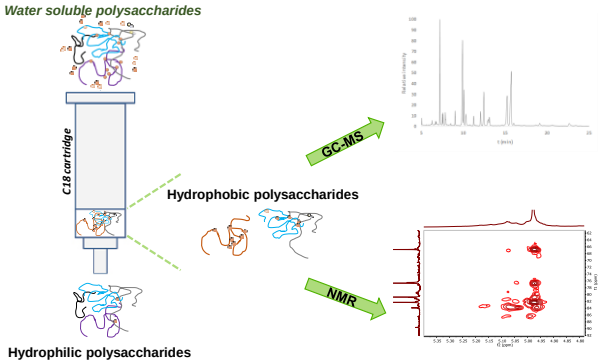
Graphical abstract:



Highlights:

- Hydrophobic polysaccharides were isolated from apple pomace;
- Pectin-polyphenol-xyloglucan and arabinan-polyphenol complexes were identified;
- Polysaccharide-polyphenol complexes are formed during apple processing;
- Oxidized polyphenols are covalently bonded to polysaccharides in apple pomace;
- Covalently bonded polyphenols modulate polysaccharides hydrophobicity.

Abstract

In this work, polysaccharides extracted with hot water from apple pomace were isolated by C18 cartridge solid-phase extraction at pH 7 (Fr7). Dialysis (12-14 kDa) of this fraction allowed to obtain 17% (w/w) of polymeric material composed by 65% of polysaccharides, mainly arabinose (58 mol%), galacturonic acid (16 mol%) and glucose (10 mol%). Folin-Ciocalteu assay showed 62 g of phloridzin equiv/kg of polyphenols. Moreover, adjusting to pH 3, it was possible to retain an additional fraction (Fr3) representing a further 4% of the polymeric material. Fr3 contained 53% of

polysaccharides composed mainly by galacturonic acid (66 mol%) and polyphenols accounted for 37 g of phloridzin equiv/kg. Precipitation with ethanol and subsequent methylation and NMR spectroscopic analysis of Fr7 dialysate allowed the identification of covalently-linked pectic-polyphenol-xyloglucan and arabinan-polyphenol complexes. These structures are possibly formed as a result of polyphenol oxidation reactions during the industrial processing of apples, conferring hydrophobic characteristics to apple pomace polysaccharides.

Key words:

Pectic polysaccharides; Xyloglucan; Arabinan; Polyphenol interaction; NMR.

1. Introduction

The production of apple juice and cider results in a solid waste composed by the pulp, skins, seeds and stalks. This solid residue, called pomace, is used for pectin extraction and animal feed, among other applications **(Joslyn & Deuel, 1963; Kennedy et al., 1999; May, 1990)**. Pectic polysaccharides are one of the most prevalent groups of polysaccharides found in apple **(Stevens & Selvendran, 1984),** alongside xyloglucans and cellulose **(Renard, Lemeunier, & Thibault, 1995)**. Homogalacturonans and type I rhamnogalacturonan substituted at the O-4 position of rhamnose with sidechains of arabinans, galactans, and arabinogalactans have also been reported as pectic polysaccharides of apple cell walls **(Renard, Voragen, Thibault, & Pilnik, 1991)**.

Apple polysaccharides have been shown to interact with polyphenols, especially with procyanidins which are polymeric compounds constituted by (+)-catechin and (-)-epicatechin units linked via C4-C8 interflavanic linkages **(Renard, Baron, Guyot, &**

Drilleau, 2001). This is a consequence of the ability of polysaccharides to establish hydrophobic interactions and hydrogen bonding with polyphenols forming non-covalent complexes. In fact, the hydrophobicity of the polysaccharides is sufficient to allow polyphenol retention either by a surface adsorption phenomenon or by encapsulation in hydrophobic cavities established by structural rearrangements of the carbohydrate residues **(Saura-Calixto, 2011)**. These mechanisms are particularly associated to pectic polysaccharides, due to their higher structural flexibility when compared to other polysaccharide structures **(Renard, Watrelot, & Le Bourvellec, 2017)**.

Polysaccharides can also form covalent bonds with polyphenols as a result of biosynthetic processes as described for arabinogalactans linked to ferulic acid derivatives in *Amaranthaceae* (e.g. spinach) **(Fry, 1983)**, as well as for arabinoxylans in *Poaceae* (bamboo) **(Ishii, 1991)**. Studies in apple and pear fruits also suggest that covalently linked polyphenols may arise from polyphenol oxidation through quinones able to randomly react with cell wall nucleophilic compounds **(Fernandes, Le Bourvellec, et al., 2019; Ferreira et al., 2002; Le Bourvellec, Guyot, & Renard, 2009)**. This results in insoluble carbohydrate/polyphenol complexes that decrease polysaccharide extractability from cell walls **(Le Bourvellec et al., 2009)**. Although these complexes can be related to the sensory properties of foods **(Soares, Mateus, & de Freitas, 2012)** and polysaccharide bioactivity **(Pérez-Jiménez, Díaz-Rubio, & Saura-Calixto, 2013)**, their structural and chemical features are in general poorly understood. This may be explained by the complexity of polyphenol oxidation reactions that yield a huge diversity of products with newly formed linkages **(Bernillon, Guyot, & Renard, 2004),** which are usually not seen in routine analyses of sugars and polyphenols **(Pérez-Jiménez et al., 2013)**.

This work aims to disclose which polysaccharide structures of apple pomace may present polyphenols covalently attached as a result of oxidation reactions during apple

processing. For this purpose, hot water extraction followed by solid-phase extraction, dialysis, and ethanol precipitation were carried out. The different fractions obtained were characterized taking advantage of the complementarity information provided by glycosidic linkage analysis using GC-MS, their sequence by NMR spectroscopic analysis and molecular weight determination by size exclusion chromatography. Evidences of the attached polyphenolic structures were obtained by alkaline fusion followed by HPLC-DAD analyses. Furthermore, the relevance of polyphenols in polysaccharides hydrophobicity was also assessed using sugar beet arabinan as a reference of polysaccharide containing covalently bonded ferulic acid derivatives.

2. Materials and Methods

2.1. Chemical reagents

Sugar beet arabinan (CAS 11078-27-6) and sugar beet debranched arabinan (CAS 9060-75-7) were purchase on Megazyme (USA). 3,4-dihydroxybenzoic acid, catechol and 3,4-dimethoxybenzoic acid (Sigma) with more than 90% of purity were used as standards. All other chemical reagents were of analytical or higher grade when available.

2.2. Preparation and fractionation of apple pomace hot water extracts

Apple juice extraction was performed on a mixture of apples, mainly Royal Gala variety, by milling, enzymatic digestion and pressing procedures at industrial scale (Indumape S.A., Portugal) for a period of at least 3 h **(Fernandes, Ferreira, et al., 2019; Kennedy et al., 1999)**. The obtained apple pomace was transported to the lab at 4 ºC in less than 1 h, frozen in liquid nitrogen, milled, and stored at -20 ºC until freeze-drying

and further analysis. These low temperatures were adopted to minimize polyphenol-oxidase activity, optimal between 25-35 ºC **(Nicolas, Richard- Forget, Goupy, Amiot, & Aubert, 1994)**. The obtained dried pomace was then extracted with hot acidified water and fractionated as summarized in **Figure 1**.

An aqueous solution of acetic acid (2% v/v) was added to the apple pomace powder at a ratio of 1:5 (w/v) and extracted at 100 ºC for 1 h. The obtained suspension was then sequentially filtered using a glass microfibre filter (MFV3, Filter Lab) followed by a G3 sintered funnel. The obtained solid residue was then re-extracted with hot acidified water three additional times following the same procedure. The obtained four water soluble fractions were combined, concentrated under reduced pressure at 45 ºC, frozen and freeze-dried, yielding a hot water extract (HWE).

To separate hydrophilic from the hydrophobic material, the HWE was subjected to solid-phase extraction with C18 sep-pak cartridges (SPE-C18, Supelco-Discovery, 20 g) by a two-step fractionation process. First, the column was preconditioned with 20 mL of methanol followed by 20 mL of water and 20 mL of phosphate buffer (10 mM, pH 7). The sample, dissolved in the same buffer solution, was added to the cartridge and washed with 140 mL of phosphate buffer followed by water/acetic acid solution (98:2; v/v) to remove salts. The retained fraction at pH 7 (Fr7) was then eluted using methanol/water/acetic acid (70/28/2; v/v/v), concentrated under reduced pressure at 45 ºC, frozen, and freeze-dried.

The pH of the solution containing the non-retained material in the C18 cartridge at pH 7 was adjusted to 3 with acetic acid. This fraction was then submitted to solid-phase extraction using the same eluents as before, except for phosphate buffer. The use of water/acetic acid (98/2; v/v) and methanol/water/acetic acid (70/28/2; v/v/v) as eluents allowed to obtain a hydrophilic (non-retained neither at pH 7 nor at pH 3 (NrFr)) and

hydrophobic materials at pH 3 (Fr3). Both fractions were concentrated under reduced pressure at 45 ºC, frozen, and freeze-dried. The NrFr, the Fr7 and Fr3 fractions were then sequentially dialysed in a 12-14 kDa cut-off membrane (Medicell) and 1 kDa cut-off membrane (Medicell), yielding fractions with >12 kDa and 1< x <12 kDa, respectively. All fractions were concentrated under reduced pressure at 45 ºC, frozen, and freeze-dried. This procedure was also performed for sugar beet arabinan used as standard. The polysaccharide was solubilized in water/acetic acid (98:2; v/v) and submitted to solid-phase extraction. The obtained fractions were dialysed in a 12-14 kDa cut-off membrane.

The Fr7 and NrFr retained in the 12 kDa membranes were additionally suspended in water at a concentration of 10 mg/mL at 4 ºC and centrifuged at 20,000*g*, yielding a water insoluble (WPp) and soluble fractions. The soluble fraction was then rotary evaporated until 1/6 of its initial volume followed by the addition of absolute ethanol until a concentration of 50% (v/v) and centrifugation yielding a precipitate (Et50Pp). To the 50% (v/v) soluble material, absolute ethanol was added to reach 80% (v/v) and centrifuged, yielding a precipitate (Et80Pp) and a supernatant (Et80Sn). All fractions were evaporated under reduced pressure to remove ethanol, frozen, and freeze dried.

2.3. Carbohydrate, protein and polyphenol analysis

Neutral sugars analysis was performed by gas chromatography with a flame ionization detector (GC-FID) after Saemen acid hydrolysis and derivatization to alditol acetates. Free sugars were determined without the hydrolysis step. Due to epimerization of fructose during the reduction step, this sugar was determined as the sum of mannitol and sorbitol using the ratio of the epimerization reaction **(Brunton, Gormley, & Murray, 2007)**. For uronic acids, phenylphenol colorimetric method was used **(Blumenkrantz & Asboe-Hansen, 1973)**. The results were expressed as GalA, the

representative uronic acid reported to occur in apple pectins **(Renard et al., 1991; Schols, Bakx, Schipper, & Voragen, 1995; Stevens & Selvendran, 1984)**. Sugars associated to polysaccharides were estimated by difference between the total sugars determined after acid hydrolysis and free sugars. Total carbohydrates were determined by the sum of free sugars and sugars attributed to polysaccharides. Glycosidic-linkage analysis was performed as described by **Coimbra, Delgadillo, Waldron, and Selvendran (1996),** by gas chromatography–mass spectrometry (GC–MS, GCMS-QP2010 Ultra, Shimadzu) of partially methylated alditol acetates (PMAA). For arabinans, the average branching points (ABP) was determined by the ratio of total arabinose with branching points according to equation 1:

$$ABP = \frac{\text{Total Ara}}{(1\rightarrow3,5)\text{-Araf} + (1\rightarrow2,5)\text{-Araf} + (1\rightarrow2,3)\text{-Araf} + (1\rightarrow2,3,5)\text{-Araf}\times2} \quad (1)$$

The Folin-Ciocalteu method was used to determine the total polyphenol content **(Singleton & Rossi, 1965).** The results were expressed as g of phloridzin equiv (PLZE) per kg of sample, as representative of apple polyphenols, and gallic acid equiv (GAE) per kg of sample. Polyphenols bound to polysaccharides were assessed by alkaline fusion following the procedure of **Coelho et al. (2014),** using 3,4-dimethoxybenzoic acid (1 mg/mL) as an internal standard. Quantification was performed by HPLC (Dionex, Ultimate 3000) using a reversed-phase column (C18-ACE; 25 cm length, 0.45 cm internal diameter, and 5 µm particle diameter). Given the higher interaction of procyanidins towards polysaccharides than monomeric polyphenols of apple **(Le Bourvellec, Le Quere, & Renard, 2007)** and their capacity to form covalent adducts under oxidative conditions **(Le Bourvellec et al., 2009)**, procyanidin catechol (B-ring) products from alkaline fusion (3,4-dihydroxybenzoic acid and catechol) were screened and used as

fingerprints for identification and quantification of polyphenols **(Batistic & Mayaudon, 1970; Coelho et al., 2014; Gramshaw, 1968)**.

2.4. Size exclusion chromatography

Samples were suspended in a 0.1 M $NaNO_3$ aqueous solution at a concentration of about 8-10 mg/mL and stirred until complete dissolution at 20 ºC for 12h. The solution was then filtered in a 0.4 µm PVDF filter. The size exclusion chromatography (SEC) analysis was performed by injecting 100 µL of sample on a PL-GPC 110 system (Polymer Laboratories, UK) with two PLaquagel-OH MIXED 8 µm (300 mm × 7.5 mm) columns protected by a PL aquagel-OH Guard 8 µm pre-column **(Mendes, Xavier, Evtuguin, & Lopes, 2013)**. The temperature of the injector system, columns, and detector (RI) were kept at 36 ºC. $NaNO_3$ at 0.1 M was used as eluent at a flow rate of 0.9 mL/min. Pullulans (Polymer Laboratories, UK) in the range 0.7–48.0 kDa were used for columns calibration.

2.5. NMR experiments

^{1}H and ^{13}C NMR spectra were recorded in a Bruker DRX 500 spectrometer operating at 500.13 and 125.77 MHz, respectively, using D_2O as solvent and TSS as external reference; the chemical shifts were expressed in ppm. 2D (^{1}H-^{1}H) COSYPR (homonuclear shift correlation with presaturation during relaxation delay) spectrum was recorded with 200 transients over 256 increments (zero-filled to 1 K) and 1 K data points with spectral widths of 4500 Hz. The recycle delay was 1.0 s. These data were processed in the absolute-value mode. The phase sensitive ^{1}H-detected (^{1}H,^{13}C) *g*HSQC (heteronuclear single quantum coherence, using gradient pulses for selection) spectrum was recorded with 200 transients over 256 increments (zero-filled to 1 K) and 1 K data points with spectral widths of 4500 Hz in F2 and 21739 Hz in F1. The recycle delay was

1.5 s. A cosine multiplication was applied in both dimensions. The delays were adjusted according to a coupling constant $^{1}J_{(CH)}$ of 149 Hz. The *g*HMBC (heteronuclear multiple quantum coherence, using gradient pulses for selection) spectrum was recorded with 200 transients over 256 increments (zero-filled to 1 K) and 1 K data points with spectral widths of 4500 Hz in F2 and 27777 Hz in F1. The recycle delay was 1.5 s. A sine multiplication was applied in both dimensions. The low-pass J-filter of the experiment was adjusted for an average coupling constant $^{1}J_{(CH)}$ of 149 Hz and the long-range delay utilised to excite the heteronuclear multiple quantum coherence was optimised for 7 Hz.

2.6. Statistics

All chemical analyses were performed in triplicate unless otherwise stated. The reproducibility of the results is expressed as average ± standard deviation.

3. Results and Discussion

3.1. Hot water extract fractionation

Hot water extraction has been suggested for extraction of pectin as it represents an easy industrially implementable process. Weakly acidic conditions are used to provide higher yields via β-elimination reactions **(Voragen, Coenen, Verhoef, & Schols, 2009)** while simultaneously preventing polyphenol oxidation **(Ferreira et al., 2002)**. Apple pomace acidified hot water extract contained free sugars (12%), mainly Fru (68 mol%) and Glc (32 mol%) (**Table 1**). However, it was mainly constituted by polysaccharides (72%) composed of GalA (43 mol%), Ara (23 mol%) and Gal (6 mol%), characteristic of

pectic polysaccharides. Polyphenols represented 28 g PLZE/kg of dry extract (9 g GAE/kg) and proteins only accounted for 11 g/kg of extract.

To separate hydrophilic from the hydrophobic material, a C18 solid-phase extraction was performed yielding the hydrophobic fractions retained at pH 7 (Fr7) and at pH 3 (Fr3) and a non-retained hydrophilic fraction (NrFr). Alongside with polyphenols (61-63 g PLZE/kg of dry extract) and protein (8-11%), sugar analysis revealed that a large amount of carbohydrates, ranging from 40-42% of the extract, were present in Fr7 and Fr3. Fr7 was rich in neutral sugars, namely Ara (57 mol%), Glc (8 mol%) and Gal (6 mol%). GalA accounted for 20 mol%, part of which might be de-esterified **(Schols, Posthumus, & Voragen, 1990)**, conferring negative charges to the compounds that, even so, were hydrophobically retained in the C18 matrix. This allows to infer the occurrence of hydrophobic features that surpass the effect of the negative charges and hydroxyl groups of sugar residues components of these structures. When the pH of elution was 3, the amount of GalA in the fraction retained was 70 mol%. This increase in the relative proportion of GalA from Fr7 to Fr3 may be explained by the neutralization of the negative charges of the carboxylic acids from GalA (pK_a of GalA is 3.5 according to **Kohn and Kovac (1977)**), which prevented their retention at pH 7, although not at pH 3. Given that GalA does not take part of the glycosylated polyphenols found in apple **(Guyot, Le Bourvellec, Marnet, & Drilleau, 2002; Lu & Foo, 1997)**, this data suggests that pectic polysaccharides were components of these hydrophobic fractions.

To obtain the high molecular weight material, all fractions were extensively dialysed using 12-14 kDa cut-off membranes. The polymeric material in Fr7 represented 6.1% (w/w) of the acidified hot water-soluble fraction and was composed by 65% of carbohydrates, mainly Ara (58 mol%) and GalA (16 mol%) (**Table 1**). An extra 1.9% (w/w) of material containing 45% of carbohydrates and a similar sugar composition was

obtained with a molecular weight ranging from 1-12 kDa. For the Fr3 fraction, it was also possible to recover high molecular weight material, representing 1.4% (w/w) of the acidified hot water-soluble fraction. This material was composed by 53% of carbohydrates, mainly GalA (66 mol%). The sugars composition of these polymeric fractions confirmed the occurrence of pectic polysaccharides in apple pomace with hydrophobic properties.

The polymeric material with molecular weight higher than 12 kDa and not retained in the C18 cartridges neither at pH 7 nor pH 3 (NrFr) accounted for 26% (w/w) of the hot water extracted material. On the other hand, that ranging from 1-12 kDa accounted for 2% (w/w). Both fractions were rich in carbohydrates (>80%), but the former presented a high prevalence of GalA (50 mol%), Ara (20 mol%) and Glc (18 mol%) while the latter was composed majorly by GalA (91 mol%). Both extracts approached the sugars composition of Fr3 high molecular weight material. Overall, the Fr7, Fr3, and NrFr polymeric fractions represented 37% (w/w) of the hot water-soluble material, which is in agreement with the high occurrence of pectic oligosaccharides in apple pomace extracts **(Mehrländer, Dietrich, Sembries, Dongowski, & Will, 2002)**. Based on the polymeric material recovered in Fr7, Fr3 and NrFr, it can be estimated that the amount of hydrophobic polymeric material recovered at pH 7 was 21% and 4% at pH 3. The remaining 75% corresponds to hydrophilic polymeric material. Together with the carbohydrates, Fr7, Fr3 and NrFr were also composed by a small amount of polyphenols (**Table 1**), which was higher for Fr7 and Fr3. Apple polyphenols reported in the literature correspond to single molecules or oligomeric structures with an average degree of polymerization of 5 **(Guyot et al., 2002),** which correspond to molecular weights much lower than the cut-off (12-14 kDa) of the dialysis membrane used in this study. As a result, their non-diffusion along the dialysis membrane might occur due to existing

interactions with the polysaccharides of the Fr7, Fr3 and NrFr fractions, giving rise to polysaccharide-polyphenols complexes as reported to occur in wine **(Gonçalves et al., 2018; Gonçalves, Rocha, & Coimbra, 2012).**

To characterize the type of linkages between polysaccharides and polyphenols and their possible influence on the hydrophobicity of polysaccharides isolated from apple pomace, the Fr7 and NrFr fractions of high molecular weight were submitted to water/ethanol precipitations. This was based on the principle that free polyphenols i.e. not covalently bonded to polysaccharides, remain soluble in ethanol. The resultant fractions were characterized according to the sugar and linkage composition of the polysaccharides, size exclusion chromatography and alkali fusion to identify the polyphenolic features linked to the polysaccharides.

3.2. Characterization of the polysaccharide/polyphenol complexes

The ethanol fractionation of the Fr7 high molecular weight material yielded a fraction that precipitated at 80% ethanol (Et80Pp), accounting for 3.1% (w/w), composed by 66% carbohydrates, mainly Ara (33 mol%), GalA (31 mol%) and Glc (15 mol%) (**Table 1**). Glycosidic linkage analysis (**Table 2**) revealed the occurrence of (1→5)-Ara*f* (18.8%), (1→3,5)-Ara*f* (6.8%), (1→2,5)-Ara*f* (3.8%), (1→2,3)-Ara*f* (1.3%), (1→2,3,5)-Ara*f* (3.1%), and t-Ara*f* (13.4%), characteristic of arabinans **(Renard et al., 1991)**. The occurrence of (1→4)-Glc*p* (14.6 mol %), together with (1→4,6)-Glc*p* (4.1%), (1→2)-Xyl*p* (3.8%), t-Gal*p* (2.2%), (1→2)-Gal*p* (1.6%), t-Fuc*p* (1.6%) and t-Xyl*p* (0.4%), also suggests the occurrence of xyloglucans **(Cruz et al., 2018; Renard et al., 1995)**. The remaining 6.9% of *t*-Xyl*p* could be explained by the occurrence of xylogalacturonans **(Voragen et al., 2009)**. This fraction also contained polyphenols (67 g PHLZE/kg), which represented 50% of the polyphenols in the Fr7 high molecular weight material. These

structural features suggests the presence of pectic-polyphenol-xyloglucan complexes **(Selvendran, 1985)** in which polyphenols act as linking bridges between pectic polysaccharide and xyloglucans. The average molecular weight of this fraction was 43.4 kDa, with a polydispersity of 6.4 (**Table 3**) as determined by Size Exclusion Chromatography (SEC, **Figure S4**).

The material that remained solubilized in 80% ethanol (Et80Sn), accounted for 2.7% (w/w), contained 64% of carbohydrates, mainly Ara (86 mol%) with an average molecular weight of 4.9 kDa and a polydispersity of 2.0. Glycosidic linkage analysis (**Table 2**) demonstrated the prevalence of (1→5)-Ara*f* (47%) together with (1→3,5)-Ara*f* (13%), (1→2,5)-Ara*f* (4.3%), (1→2,3)-Ara*f* (0.7%), and (1→2,3,5)-Ara*f* (2.1%), characteristic of an arabinan with an average one branching point per 4 Ara*f* units. However, t-Ara*f* only explained 8% out of the 20% of branching. The determination of 79 g PLZE/kg of polyphenols in Et80Sn may explain this difference by the occurrence of bonded polyphenols to the arabinofuranosyl terminal units, similarly to biosynthetically linked ferulic acids in *Amaranthaceae* (spinach) **(Fry, 1983)** and *Poaceae* (bamboo) **(Ishii, 1991)**, respectively**.** This difference may also result from modifications of the *t*-Ara*f* as a result of polyphenol oxidative bonding. The number of branching points associated to polyphenols covalent linkage in Et80Sn (15 in 22) was higher than those observed in Et80Pp (5 in 18), although the total polyphenolic content (PLZE equivalents) was close between fractions. As Folin–Ciocalteu method follows a structure-activity relationship, in which the reaction is proportional to the number of hydroxyl groups present in the aromatic ring, the polyphenols structural diversity should result in different polyphenol response factors **(Everette et al., 2010)**. This polyphenols structural diversity, alongside with the possible arabinofuranosyl units structural modification due to polyphenol oxidative bonding, and arabinans lability to acid hydrolysis may also explain the lower content of compounds identified in the hydrophobic fractions (about 60%) when compared with the hydrophilic ones (>80%).

To access the contribution of polyphenols on the hydrophobic properties of Fr7, the hydrophilic material not retained in the C18 cartridges (NrFr), was also submitted to ethanol fractionation. Four distinct fractions, WPp (4.0% w/w), Et50Pp (4.1% w/w), Et80Pp (16% w/w) and Et80Sn (1.2% w/w) were obtained (**Table 1**). WPp was mainly composed of Glc (92 mol %), present as 1→4-Glc*p* (88 mol%), 1→4,6-Glc*p* (4.5 mol%) and t-Glc*p* (4.7 mol %) (**Table 2**), indicative of a highly linear glucan, possibly corresponding to glucan degraded material **(Cruz et al., 2018; Renard et al., 1995)**. This fraction only contained 16 g PLZE/kg of polyphenols. Et50Pp, Et80Pp and Et80Sn were mainly constituted by GalA (51-54 mol%), Ara (23-31 mol%) and Gal (8-10 mol%) characteristic of pectic polysaccharides with arabinan and galactan as side chains (representative chromatogram of the partially methylated alditol acetates of the neutral sugars in **Figure S2,** peak assignments in **Table S1,** and mass spectra in **Figure S3**). These fractions only contained, on a weighted average, 7 g PLZE/kg of polyphenols (**Table 1**) which are 10-fold lower than that found for the Fr7 high molecular weight material. Protein, which could also contribute to the hydrophobicity of the Fr7, although absent in NrFr, accounted only for 1% in Fr7 emphasizing the relevance of polyphenols for the hydrophobic property.

To identify the possible structural polyphenolic features that may be associated to polysaccharides of Fr7, alkaline fusion was performed. As represented in **Table 3**, 3,4-dihydroxybenzoic acid (0.008-0.011 mol/kg) and catechol (0.060-0.062 mol/kg) were detected in the two fractions derived from Fr7, demonstrating the presence of a catechol ring, possibly arising from oxidized procyanidins, the most prevalent polyphenols in apple **(Guyot et al., 2002; Lu & Foo, 1997)**. To provide a more detailed structural characterization of the polysaccharide/polyphenol complexes, the Et80Sn of Fr7, containing a purified arabinan, was submitted to NMR spectroscopic analysis.

3.3. NMR spectroscopic analysis

The ^{13}C NMR spectrum of the Fr7 high molecular weight material soluble in 80% ethanol is represented in **Figure 2a** (DEPT-135 in **Figure S3**). Based on the NMR data from arabinans **(Dourado, Cardoso, Silva, Gama, & Coimbra, 2006; Xia, Liang, Yang, Wang, & Kuang, 2015)** and arabinan rich-pectins **(Renard & Jarvis, 1999; Shakhmatov, Belyy, & Makarova, 2018; Shakhmatov, Toukach, Michailowa, & Makarova, 2014)**, it was possible to identify →5)-Ara*f*-(α1→ (**A**), →3,5)-Ara*f*-(α1→ (**B**), →3)- Ara*f*-(α1→ (**C**), →2,5)-Ara*f*-(α1→ (**D**) and Ara*f*-(α1→ (**E**) residues in the characteristic chemical shifts of anomeric carbons C-1 (δ_C 107.5–106.5) from arabinofuranosyl residues. The signal at δ_C 101.3 could be attributed to Ara*f*-(β1→ (**F**), identified to occur in olive pomace pectic polysaccharides **(Cardoso, Ferreira, Mafra, Silva, & Coimbra, 2007; Cardoso, Silva, & Coimbra, 2002)**. Also, the well-defined carbon signals in the DEPT-135 at δ_C 67.0, 66.8, 62.9, and 61.1 could be attributed to the C-5 of the residues **A**, **B/D**, **F**, and **C/E** respectively (**Table 4**). Minor signals of *O*-acetyl and carboxyl groups were also observed in the ^{13}C NMR at δ_C 20.3 and 173.9, respectively. HSQC and HMBC analysis revealed that *O*-acetyl moiety at δ_C 20.3/δ_H 2.06 was correlated with δ_C 173.9 (not shown). Although no direct evidences of acetylation were determined for this fraction, Gal*p*A in apple pectin can be acetylated at O-2 and/or O-3 positions, depending on the botanical species **(Perrone et al., 2002)**. In the NMR analysis, a peak characteristic of CH_3O- groups (δ_C 57.4) was also observed suggesting that some of the GalA could be methyl esterified. No additional assignments were possible for GalA, possibly due to their lower degree of freedom when compared to the arabinose units **(Dourado et al., 2006; Schols et al., 1990)**.

From the $^{1}H–^{1}H$ COSY spectrum (**Figure 2 b**) it was possible to correlate protons for each one of the identified arabinofuranosyl units (**Table 4**), while by one-bond ^{13}C-^{1}H correlation of NMR resonances, as HSQC spectrum (**Fig. 2b** and **Table 4**), it was possible to assign the carbon signals of the residues **A-F**. Due to the overlapping nature of the spectra, some assignments might be interchangeable but agreeing with those of literature. Cross peaks of both anomeric protons and carbons of each sugar residue were observed both at intra and inter-residual level by HMBC analysis (**Figure 3d** and **Table S2**). Besides the intra-residual correlations, long-range correlations were observed, allowing to infer about 15 different sequences (**Table S2**) present along the arabinan structure.

Based on the data obtained, a putative model structure was suggested (**Fig. 3**) which includes the →5)-Ara*f*-(α1→ backbone substituted at O-2 or O-3 mainly with →3)-Ara*f*-(α1→ similarly to arabinans isolated from other sources **(Dourado et al., 2006; Shakhmatov et al., 2014; Xia et al., 2015).** In addition to the occurrence of Ara*f*-(α1→, unusual Ara*f*-(β1→, a rare structural feature in arabinans **(Cardoso et al., 2007; Cardoso et al., 2002; Shakhmatov et al., 2018)** was also shown to occur. In this model structure are also included the bonded polyphenols whose linkages to carbohydrates remain to be elucidated.

The →5)-Ara*f*-(α1→ and →3,5)-Ara*f*-(α1→ linkages of arabinans result in an helicoidal backbone that allow the orientation of the side chain outside the helix **(Pérez, Mazeau, & Hervé du Penhoat, 2000)**. It is feasible also that polyphenols bonded to the arabinan structure can additionally result in the formation of hydrophobic domains, especially considering the high flexibility of the arabinofuranose ring **(Cros, Imberty,**

Bouchemal, Du Penhoat, & Perez, 1994), explaining the isolation by retention in C18 cartridges of the hydrophobic polysaccharides herein detected. Acetyl and methyl groups associated to the GalA residues adjacent to the arabinan side chains of pectins may also contribute to the observed hydrophobicity. In fact, as shown by apple enzymatic liquefaction, apple arabinans from pectins present high degrees of acetylation and methyl-esterification of GalA **(Schols et al., 1990)**. Nevertheless, its extent should be very limited as apple pomace usually results from ripe and technologically treated fruits, where enzymes such as pectinmethylesterase, polygalacturonase, and acetylesterase are very active (**Kennedy et al., 1999**). Therefore, although methyl esterified and acetyl groups might contribute to pectic polysaccharides hydrophobicity, it is very unlikely that their contribution surpassed the one provided by the covalently linked polyphenols, which differed in more that 10-fold between the retained and non-retained fractions. To consolidate the relevance of the polyphenols bonded to the arabinan towards C18 cartridges, solid-phase extraction was performed on a sugar beet arabinan (SB Ara), which is a polysaccharide with covalently linked ferulic acid derivatives to the O-5 and O-2 positions of arabinose residues **(Levigne et al., 2004)**. The experiment was performed at pH 3 to potentiate the protonation of GalA carboxylic groups and, in this way, promoting the retention of the hydrophobic compounds. The SB Ara used presented 79% of carbohydrates, mainly Ara (74 mol%), Gal (13 mol%), and GalA (10 mol%). Polyphenols represented 7 g PLZE/kg (**Table 5**).

3.4. Contribution of polyphenols on arabinan retention in C18 cartridges

As observed for apple pomace, solid-phase extraction of SB Ara gave rise to a non-retained fraction (SB Ara NrFr) and a retained fraction (SB Ara RFr) that represented 81 and 19% of the SB Ara (**Table 5**), respectively. These two fractions presented similar

sugars content (78-82%), rich in Ara (52-77 mol%), Gal (10-23 mol%) and GalA (9-17 mol%). SB Ara RFr contained 15 g PLZE/kg while for SB Ara NrFr, polyphenols accounted 5 g PLZE/kg. This agreed with the retention of apple pomace polysaccharides with higher amounts of bonded polyphenols. However, the amount of polyphenols present in the SB Ara RFr was of the same order of magnitude as that observed for the non-retained hydrophilic material of apple pomace, thus suggesting that other structural features, in addition to polyphenols, contribute to hydrophobicity of the polysaccharides. The GalA content was 3-fold lower in SB Ara RFr than in apple pomace hydrophilic fraction suggesting that, in addition to polyphenols, the low prevalence of negative charges contribute to polysaccharides hydrophobicity. Still, as suggested by the isolation of an apple pomace hydrophobic fraction at pH 3, highly rich in GalA and polyphenols, there appears to exist a threshold limit where the effect of negative charges is negligible when compared to the hydrophobicity provided by polyphenols. It must be noted, however, that the type of phenolic structures present in SB Ara and arabinan fractions derived from apple pomace are of a completely different nature. In the case of SB Ara, the phenolic structure covalently linked to the arabinose unit result from a biosynthetic process **(Levigne et al., 2004)**. As far as we are aware, despite the extensive studies already performed for apple cell wall composition, no covalently linked polyphenols to polysaccharides, clearly associated to biosynthetic processes were identified for apple. Therefore, in the case of apple pomace derived fractions the covalently linked phenolic structures outcomes from polyphenol oxidation. The understanding of the type of linkages involved and their effect on the three-dimensional structure of the polymer is essential to unveil the hydrophobicity observed in this study, as well as in the nutritional, functional and biological properties generally attributed to polysaccharides.

4. Conclusion

In this work it was shown that apple pomace pectic polysaccharides and xyloglucans can present hydrophobic features that result on their retention in C18 cartridges. This hydrophobicity was mainly due to covalently linked polyphenols resultant from polyphenol oxidation reactions. In comparison with the Royal Gala variety used in this study, other apple varieties may present differences at quantitative level, although the main polyphenol classes are the same. In the context of polysaccharides hydrophobicity as a result of polyphenol bonding by oxidation reactions no different results are expected, regardless of the apples composing the pomace. However, several aspects still need to be elucidated to take advantage of the use of these carbohydrates. This includes the clarification of the linkages established between carbohydrates and polyphenols for a more accurate structure-property relationship, as well as the relationship between the state of oxidation of the pomace and the prevalence of chimeric structures. The present results pave the way for a deeper understanding of how carbohydrates chemistry can be tailored for several applications that, among other outcomes, may result in agro-food wastes mitigation.

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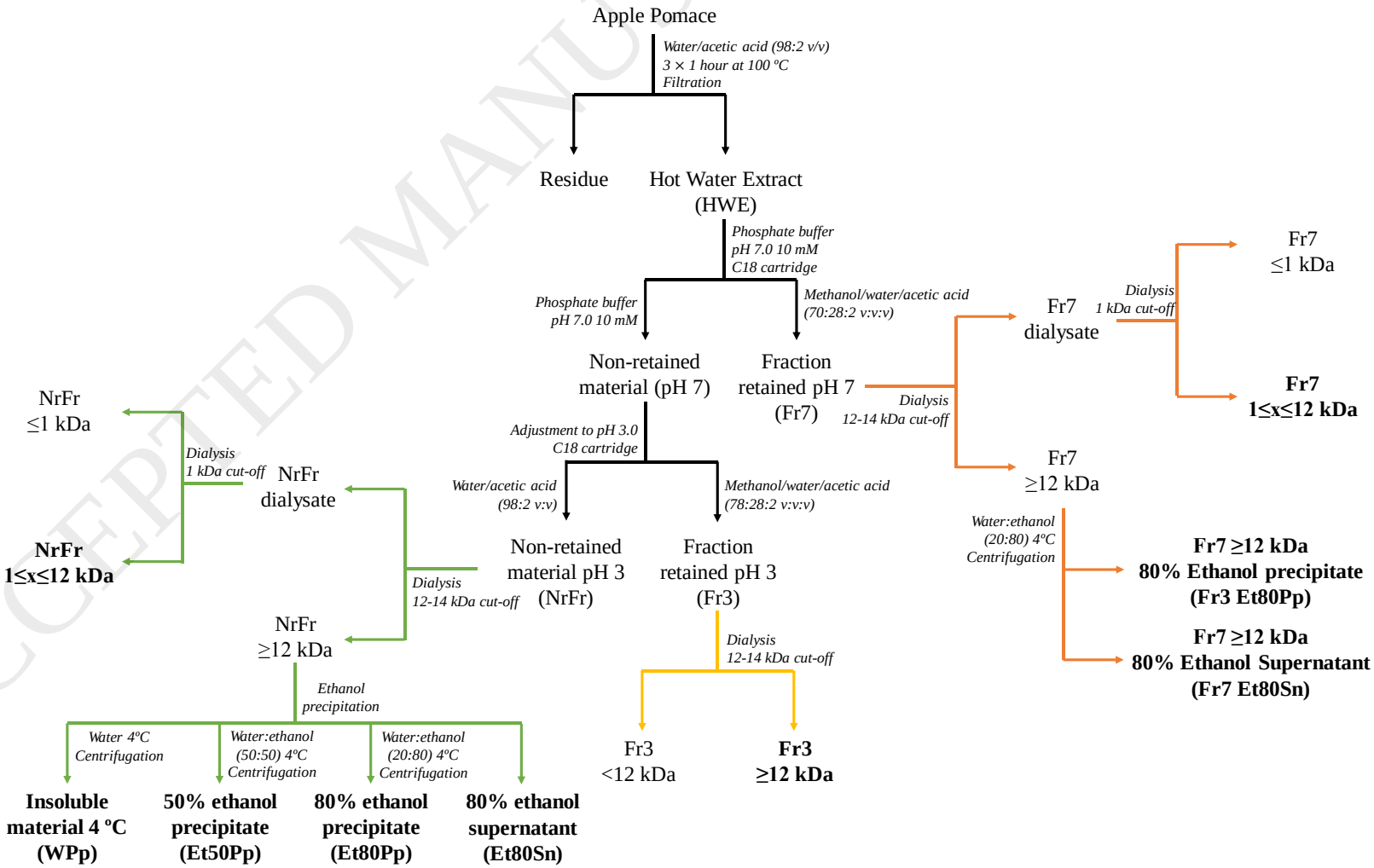

Figure 1 – Schematic representation of the fractionation procedure adopted for the different apple pomace carbohydrate fractions. In orange, yellow and green are highlighted the additional fractionation procedures adopted for the material retained in C18 cartridges at pH 7 (Fr7), pH 3 (Fr3) and non-retained (NrFr), respectively. In bold are highlighted the studied fractions.

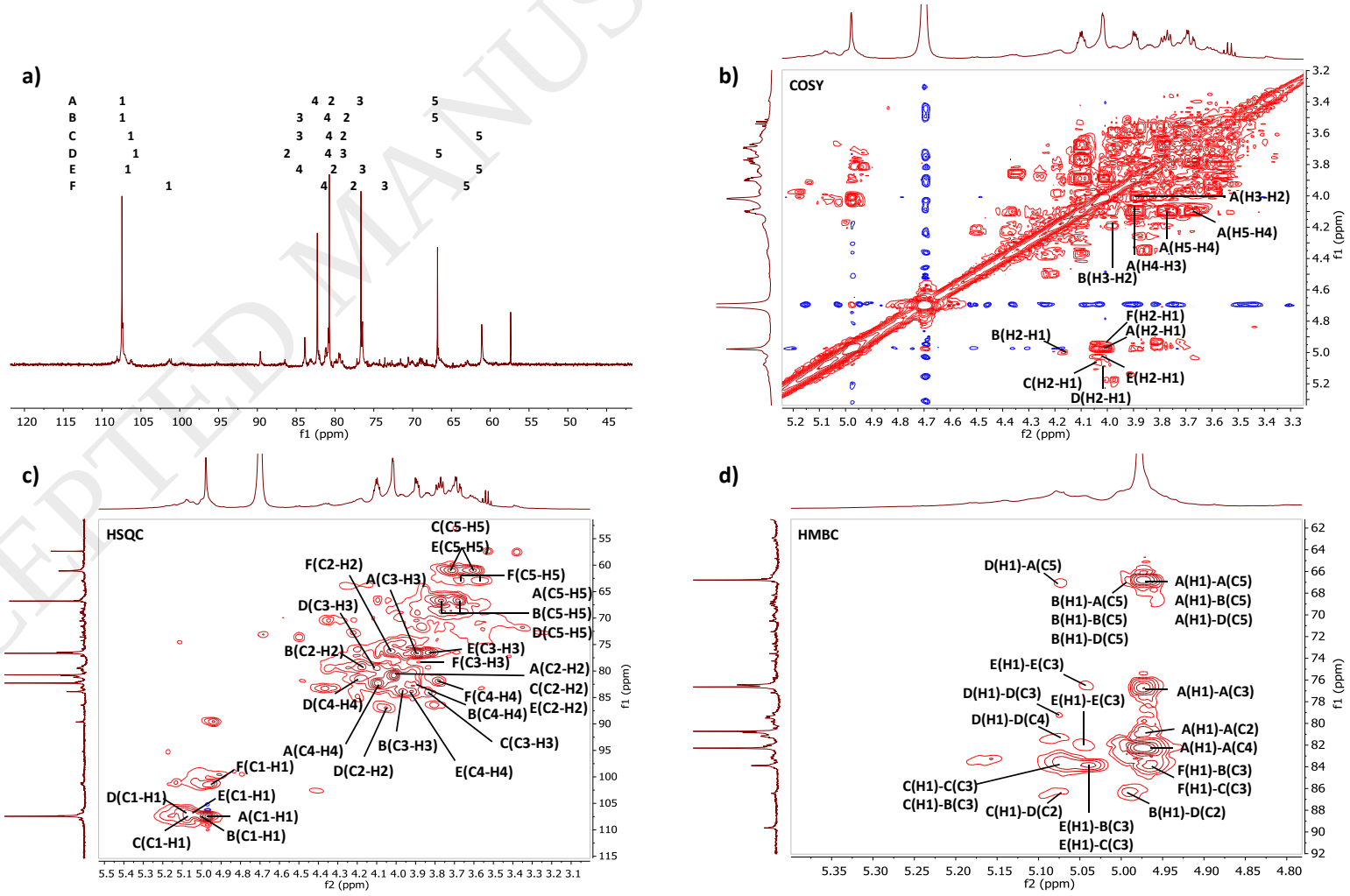

Figure 2- Representation of the key a) ^{13}C NMR spectrum, b) ^{1}H-^{1}H COSY spectrum, c) HSQC spectrum and d) HMBC spectrum of Fr7 >12-14 kDa Et80Sn fraction. Letters represent the type of arabinofuranosyl unit : A - →5)-Ara*f*-(α1→; B - →3,5)-Ara*f*-(α1→; C - →3)-Ara*f*-(α1→; D - →2,5)-Ara*f*-(α1→; E - Ara*f*-(α1→; F - Ara*f*-(β1→. Numbers represent the carbon and proton number starting from the anomeric carbon.

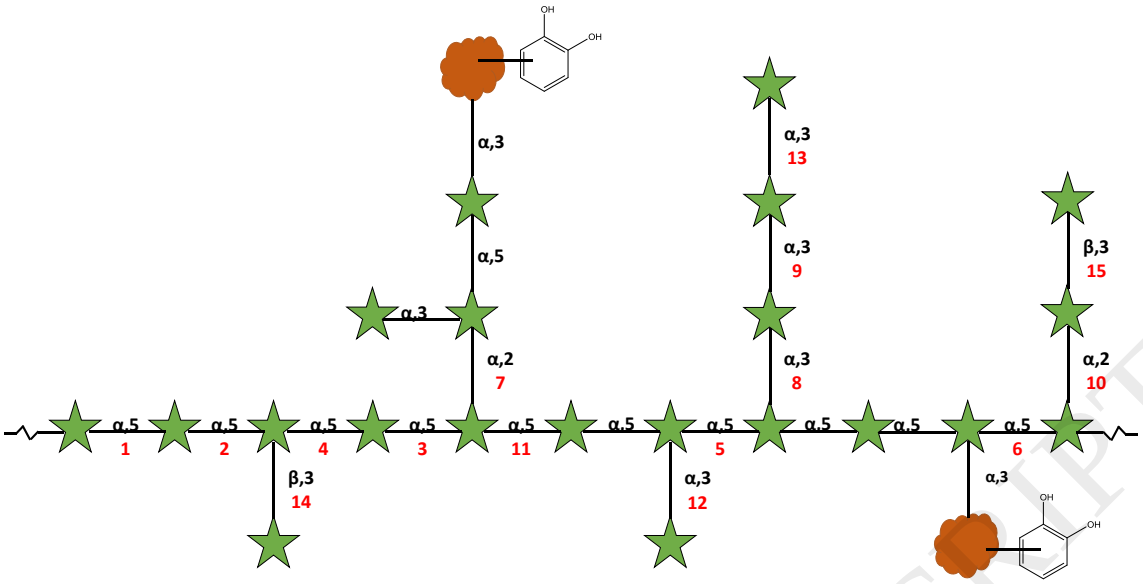

Figure 3 – Model structure representative of the isolated arabinan/polyphenol complex as provided by the complementarity between GC-MS, ^{13}C NMR and alkaline fusion analysis. Orange/brownish clouds are representative of the unknown phenolic structures with a catechol group that are attached to the arabinan. In red are shown the glycosidic sequences observed by ^{13}C NMR.

Table 1 – Yield, carbohydrate composition, total carbohydrates, protein and total polyphenols of the different dried fractions derived from the water-soluble material obtained from apple pomace. In parenthesis are presented that data in gallic acid equivalents (g GAE/kg) for total polyphenols. Data are expressed as Mean±standard deviation of three replicates. n.d means for not determined.

Fraction	**Yield (%)**		**Carbohydrate composition (molar %)**									**Total Carbohydrates (g/kg)**	**Total protein (g/kg)**	**Total PC (g PLZE/kg)**
			Rha	Fuc	Ara	Xyl	Fru*	Man	Gal	Glc	GalA			
AQ	100	Polysac.	1±0	tr	23±1	3±0	-	tr	6±0	24±2	43±2	720±24	11±0	28±1 (9±0)
		Sugars	-	-	-	-	68±4	-	-	32±4	-	116±7		
Fr7		Polysac.	2±0	1±0	57±2	4±0	-	2±0	6±0	8±0	20±2	422±5	105±1	61±3 (20±0)
>12 kDa	6.1±0.5	Polysac.	3±0	1±0	58±2	4±0	-	tr	6±0	10±0	16±2	647±13	9±1	62±2 (20±0)
Et80Pp	3.1	Polysac.	2±0	1±0	33±1	8±0	-	2±0	9±0	15±1	31±2	662±35	n.d	67±3 (25±1)
Et80Sn	2.7	Polysac.	2±0	tr	86±5	tr	-	2±0	1±0	2±0	7±0	638±23	n.d	79±8 29±3)
1<x<12 kDa	1.9±0.1	Polysac.	2±0	tr	58±2	2±0	-	1±0	5±0	9±0	23±1	448±39	92	38±2 (16±1)
Fr3		Polysac	3±0	1±0	15±1	5±0	-	tr	4±0	3±0	70±1	394±15	80±1	63±2 (18±0)
>12 kDa	1.4	Polysac.	5±0	1±0	11±1	5±0	-	1±0	6±0	5±1	66±2	531±7	29±0	37±1 (13±1)
NrFr		.												
>12 kDa	26±3	Polysac	1±0	tr	20±2	4±0	-	tr	7±1	18±1	50±4	954±38	-	8±0 (3±0)
WPp	4.0	Polysac	tr	tr	2±0	tr	-	tr	1±0	92±1	4±1	833±18	-	16±1 (5±0)
Et50Pp	4.1	Polysac	2±0	tr	23±2	4±0	-	tr	8±1	12±1	51±3	966±30	-	8±0 (3±0)
Et80Pp	16	Polysac	2±0	tr	25±1	5±0	-	tr	8±0	7±0	52±1	928±59	-	5±0 (2±0)
Et80Sn	1.2	Polysac	1±0	tr	31±1	2±0	-	tr	10±1	2±0	54±2	897±19	-	25±0 (11±0)
1<x<12 kDa	2.0±0.2	Polysac	tr	tr	1±0	tr	-	tr	6±1	1±0	91±2	806±82	37±2	8±0

														(4±0)

Table 2 – Partially methylated alditol acetates (molar%) of the different fractions obtained by ethanol precipitation of the high molecular weight material of retained fraction at pH 7 (Fr7) and non-retained fraction (NrFr).

Linkage	Fr7 >12 kDa		NrFr >12 kDa			
	Et80Pp	Et80Sn	WPp	Et50Pp	Et80Pp	Et80Sn
t-Rha*p*	1.0	0.7		0.4	0.5	
2-Rha*p*		1.9		1.9	1.8	0.8
2,4-Rha*p*	0.7	0.6		1.1	1.7	0.6
Total Rha	1.7	3.2		3.4	4.0	1.4
t-Fuc*p*	1.6	0.8		0.2	0.3	
3,4-Fuc*p*	0.4	0.3				0.1
Total Fuc*p*	2.0	1.1		0.2	0.3	0.1
t-Ara*f*	13.4	7.7	0.6	14.0	14.6	15.9
2-Ara*f*	1.0	1.5		0.1	0.1	0.1
3-Ara*f*	3.9	8.2	0.2	2.1	2.7	3.9
5-Ara*f*	18.8	47.3	0.5	21.5	26.0	37.0
3,5-Ara*f*	6.8	13.1		10.7	11.1	14.7
2,3-Ara*f*	1.3	0.7				
2,5-Ara*f*	3.8	4.3		1.9	2.4	1.1
2,3,5-Ara*f*	3.1	2.1				
Total Ara*f*	52.0	84.9	1.3	50.3	56.9	72.7
t-Xyl*p*	6.3	1.0	0.3	2.2	1.9	1.3
2-Xyl*p*	3.8		0.2			
4-Xyl*p*			0.1	1.5	3.1	0.5
2,3-Xyl*p*		0.3				
2,4-Xyl*p*					0.7	0.4
Total Xyl*p*	10.1	1.3	0.6	3.7	5.7	2.2
t-Man*p*	1.3	0.7		0.2	1.6	0.3
6-Man*p*		1.0				
Total Man*p*	1.3	1.7		0.2	1.6	0.3
t-Gal*p*	2.2	1.0		1.9	2.9	1.4
2-Gal*p*	1.6					
3-Gal*p*				0.5	0.6	0.5
4-Gal*p*	10.2	1.8	1.3	9.5	10.7	16.1
6-Gal*p*				0.2	0.3	0.4
3,4-Gal*p*				0.9	0.6	
3,6-Gal*p*				0.4	0.7	1.4
4,6-Gal*p*		0.6		0.3	1.0	
Total Gal*p*	14.0	4.9	1.3	14.8	16.8	19.8
t-Glc*p*			4.7	2.2	0.2	0.1
4-Glc*p*	14.6	1.8	87.6	24.7	14.5	3.2
6-Glc*p*		0.1				
4,6-Glc*p*	4.1	1.1	4.5	1.6		0.2
Total Glc*p*	18.7	3.0	96.8	28.5	14.7	3.5

Table 3 - Estimated alkaline fusion products, weighted-average (Mw), number-average (Mn) molecular weights and polydispersity (PD) of the different Fr7 >12-14 kDa fractions before and after 80% ethanol precipitation. Data from one replicate.

Sample	Size-exclusion chromatography			Alkaline fusion products (mol/kg)		
	Mw (kDa)	Mn (kDa)	PD (Mw/Mn)	3,4-dihydroxybenzoic acid	Catechol	Total
Fr7 >12-14 kDa	15.4	4.1	3.8	n.d.	n.d.	n.d.
Et80Pp	43.4	6.8	6.4	0.011	0.062	0.073
Et80Sn	4.9	2.5	2.0	0.008	0.060	0.068

Table 4 - ^{1}H and ^{13}C chemical shifts (δ) of Fr7 >12-14 kDa Et80Sn in D2O at 500.13 MHz and 125.77 MHz

Residue	Sugar linkage	H1-C1	H2-C2	H3-C3	H4-C4	H5-C5
A	→5)-Ara*f*-(α1→	4.97 [a,b,c]	4.02 [a,b,c]	3.88 [a,b,c]	4.11 [a,b,c]	3.65-3.70/3.76-3.78 [a,b,c]
		107.5 [b,c]	80.8 [b,c]	76.6 [b,c]	82.3 [b,c]	67.0 [b,c,d]
B	→3,5)-Ara*f*-(α1→	5.00 [a,b,c]	4.17 [a,b]	3.97 [a,b]	3.92 [a,b,c]	3.65-3.70/3.76-3.78 [a,b,c]
		107.5 [b,c]	79.2 [b]	83.9 [b,c]	82.1 [b,c]	66.8 [b,c,d]
C	→3)-Ara*f*-(α1→	5.06 [a,b,c]	4.03 [a,b]	3.82 [b,c]	3.99 [a,b]	3.57-3.61/3.67-3.72 [a,b]
		107.1 [b]	80.8 [b]	83.9 [b,c]	81.4 [b]	61.0 [b,c,d]
D	→2,5)-Ara*f*-(α1→	5.08 [a,c]	4.05 [a]	4.10 [b]	4.18 [a]	3.65-3.70/3.75-3.79 [a,b]
		106.5 [b,c]	87.0 [b,c]	79.5 [b,c]	81.4 [b,c]	66.8 [b,c,d]
E	Ara*f*-(α1→	5.03 [a,b,c]	4.02 [a,c]	3.85 [a,c]	3.95 [a,c]	3.57-3.61/3.67-3.72 [a,b,c]
		107.4 [b,c]	81.8 [b,c]	76.5 [b,c]	83.9 [b,c]	61.1 [b,c,d]
F	Ara*f*-(β1→	4.96 [a,b,c]	4.01 [a,c]	3.80 [a,c]	3.79 [a,b]	3.53-3.60/3.65-3.70 [a,b]
		101.4 [b]	76.3 [b]	74.2 [b]	82.0 [b,c]	62.9 [b,d]

[a] COSY [b]HSQC, [c]HMBC, [d]DEPT-135

Table 5 - Yield, carbohydrate composition, total carbohydrates and total polyphenols of the sugar beet arabinan (SB Ara) and its retained (RFr) and non-retained (NrFr) fraction obtained by solid-phase extraction. In parenthesis are presented that data in gallic acid equiv (g GAE/kg) for total polyphenols.

Fraction	Yield (%)	Carbohydrate composition (molar%)								Total Carbohydrates (g/kg)	Total PC (g PLZE/kg)
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA		
SB Ara	100	3	tr	74	tr	tr	13	-	10	785	6±0 (2±0
NrFr	81	2	tr	77	tr	1	10	1	9	816	5±0 (2±0)
RFr	19	5	tr	52	tr	1	23	2	17	777	15±1 (5±0)