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^{1}H-NMR, HPSEC-RID, and HPAEC-PAD Characterization of Polysaccharides Extracted by Hydrodynamic Cavitation from Apple and Pomegranate By-Products for Their Valorization: A Focus on Pectin

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Abstract: Several chemical analytical methods were applied to characterize the chemical structure of polysaccharides extracted from discarded apples and pomegranate peels using hydrodynamic cavitation methods in a circular economy perspective. In particular, the purity of the polysaccharides and the degrees of acetylation and methylation were evaluated by proton Nuclear Magnetic Resonance (^{1}H-NMR) analysis; simple sugars and galacturonic acid were analyzed simultaneously by High-Performance Anion Exchange Chromatography—Pulsed Amperometric Detector (HPAEC-PAD); the molecular weight of the extracted polysaccharides was determined by High-Performance Size Exclusion Chromatography-Refractive Index Detector (HPSEC-RID). The results showed a negligible presence of co-precipitated proteins/tannins, easily removed by dialysis, as well as other co-precipitated molecules such as monosaccharides and organic acids. Polysaccharides from apples consisted mainly of pectic material with a prevalence of homogalacturonans. Polysaccharides from pomegranate peels showed greater compositional variability with significant amounts of arabinose and galactose, a lower content of pectin, and the presence of rhamnogalacturonans I. Both polysaccharides were highly methylated and differed in the degree of acetylation, which could lead to different properties. Polysaccharides from apples presented two main molecular weights (>805 kDa and 348–805 kDa, respectively), while those from pomegranate peel showed a major fraction at 348 kDa and minor fractions < 23 kDa. In conclusion, the research tools proposed by this study have allowed defining the macrostructure of polysaccharides in a quick and efficient way to valorize these food by-products.

Keywords: pectin; HPSEC-RID; HPAEC-PAD; sugar analysis; galacturonic acid; food by-product valorization; pomegranate peel; tannins

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1. Introduction

Plant polysaccharides include a large variety of biopolymers that are gaining interest in the scientific community [1], including cellulose, hemicellulose, and pectic polysaccharides [2]. Thanks to their chemical and physical characteristics, they have been successfully

applied in the food and pharmaceutical industries [3,4]. In fact, plant polysaccharides have found different applications due to their multiple health-promoting activities such as antimicrobial, prebiotic, anticarcinogenic, and immunostimulant [5,6]. Food applications, on the other side, mostly rely on pectic polysaccharides, which explicate thickening, gelling, and emulsifying activities [7].

The food industry produces enormous amounts of by-products such as fruit peels, seeds, and shells, which are rich in secondary metabolites and dietary fiber [8,9]. Research focused on fruit polysaccharide fractions is currently a hot topic aimed at finding new sources of active compounds, also from the perspective of circular economy [10].

In this context, polysaccharides from apple and pomegranate by-products are currently being investigated. Apple by-products are represented by defective fruits, discarded by the market due to pest attacks, infections, or mechanical damage [11], while pomegranate by-products consist essentially of the fruit peel and seeds [12].

Investigation of polysaccharides derived from food by-products requires at least two main steps: extraction and structure elucidation. As for extraction, polysaccharides are usually recovered using an acid environment, high temperatures, and long reaction times, making the process highly energetically expensive [13]. Researchers are currently investigating new greener extractive methodologies such as ultrasound-assisted extraction [14], microwave-assisted extraction [7], subcritical water-assisted extraction [15], and the use of enzymes [16], allowing for different benefits in terms of yields and/or environmental impact. In the present study, hydrodynamic cavitation (HC), with a fixed Venturi-shaped reactor, was chosen since it represents an affordable, efficient, easy-to-scale-up, and eco-friendly extraction technique [17]. In fact, HC methods applied to the extraction of natural products on a preindustrial scale [18] have shown high yields in the recovery of interesting compounds, such as starch, proteins, and phenolic compounds in water-based extracts [19]. The same technological implementation of HC was used to extract pectin from several matrices, including conifer tree parts [20] and citrus fruits such as orange [21], lemon [22], and grapefruit [23]. While HC was recently tested on discarded apples and pomegranate by-products [24–26], no study, to the authors' knowledge, focuses on the polysaccharides or pectin extracted using this technology.

Since the chemical structure of polysaccharides depends on several factors, including the extraction method [27], specific analyses are required for their characterization and their application fields. The investigation of polysaccharide structure is usually carried out by evaluating their molecular weight, the degree of esterification (especially methylation and acetylation), the content of phenols/proteins, the content of galacturonic acid (GalA), the type and content of simple sugars, along with the type of glycosidic bonds in the polymer [1]. Regarding pectic polysaccharides, the most common linkages involve GalA residues featuring an $\alpha(1\rightarrow4)$ glycosidic bond in the linear region, while the rhamnogalacturonans I (RGI) regions are characterized by GalA linked to $\alpha(1\rightarrow2)$ rhamnose as a repeating unit. Branches are characterized by different sugars; the side chains of the RGI regions are mainly constituted by arabinose and galactose linked to rhamnose with an $\alpha/\beta(1\rightarrow4)$ bond, while the rhamnogalacturonans II (RGII) regions present a wide variety of sugars linked to the GalA in the backbone with $\alpha/\beta(1\rightarrow2)$ or $\alpha/\beta(1\rightarrow3)$ glycosidic linkages [28,29]. Low molecular weight polysaccharides, smaller than 10 KDa, are associated with better beneficial effects [30], while pectin, characterized by a wide range of molecular weights (ranging 20–800 kDa), also exerts important activities towards food applications, in addition to health-promoting effects [31]. Sugar analysis is necessary to evaluate the main types of available polysaccharides: D-galacturonic acid and L-rhamnose are typical in pectic polysaccharides [32], and their ratio helps to clarify the structure of pectin; glucose, arabinose, galactose, and mannose can be indicative of other types of polysaccharides, presenting a diversified pattern of sugars, which is associated with a positive impact on health [33]. Lastly, the degree of methylation is associated with the gelling activities of the pectic polysaccharides [34], while the degree of acetylation is known for altering their emulsifying and gelling capacity [35].

Several analytical methodologies to evaluate the chemical structure of polysaccharides have been reported in the literature [36]. Briefly, volumetric acid-base titration has been used to evaluate the galacturonic acid (GalA) content, along with colorimetric methods with UV/Vis spectrophotometry. A major problem with these methods is the interference due to other uronic acids, neutral sugars, and proteins, leading to an incorrect estimation of the GalA content. Spectroscopic methods, NMR, and FT-IR spectroscopy have been implemented for general structural investigation and determination of the degree of methylation (DM) and acetylation (DA). These methods measure the methanol and acetic acid released after hydrolysis. The main advantage of these techniques is the shorter analysis time; additionally, no calibration curve is needed [37]. Chromatographic methods include High-Performance Size Exclusion Chromatography (HPSEC) for the determination of the hydrodynamic volume and the relevant molecular weight [31]. At the same time, Gas Chromatography coupled with a Flame Ionization Detector (GC-FID) or High-Performance Liquid Chromatography coupled to a Refractive Index Detector (HPLC-RID) are used for sugar analysis [36]. The limitation in the case of GC analysis is the need for the derivatization of monosaccharides to alditol acetates/trimethylsilyl esters, which may lead to inaccurate quantification [38]. This issue can be overcome by High-Performance Anion Exchange Chromatography—Pulsed Amperometric Detector (HPAEC-PAD), which allows for high sensitivity, specificity, and resolution.

The above analytical methods have never been used in combination to investigate the structure of polysaccharides extracted from food by-products. In the present study, hydrodynamic cavitation-based methods were used to extract polysaccharides from (i) apples discarded from fruit sorting operations as a conventional source of pectic polysaccharides and (ii) pomegranate peel discarded from juice production as a newly proposed source of pectic polysaccharides [39]. Taking into account the advantages/disadvantages of the analytical techniques described above, a multi-analytical approach combining spectroscopic and chromatographic methodologies was assessed to gather information on the structure of the polysaccharides extracted, with a focus on pectic polysaccharides. Specifically, HPSEC, HPAEC-PAD, and ^{1}H-NMR analysis were utilized for the evaluation of the hydrodynamic volume, for the determination of the content of galacturonic acid and simple sugars, and for the evaluation of the degree of esterification, phenolic content, and protein content, respectively.

2. Materials and Methods

2.1. Chemical and Reagents

All solvents, reagents, and standards for polysaccharides analysis were purchased from Merck (Merck, Saint Louis, USA). D_2O, H_2SO_4, maleic acid and tetramethylsilane (TMS) from Merck (Merck, Saint Louis, MO, USA) were used for ^{1}H-NMR analysis; ammonium formate from Carlo Erba Reagents (Milan, Italy) and narrow pullulan molecular weight standards of 23.8, 348 to 805 kDa (Sigma-Aldrich, Milan, Italy) were used for HPSEC analysis. HCl methanol solution, NaOH, CH_3COONa, and CF_3COOH (TFA) from Merck (Merck, Saint Louis, MO, USA) were used for HPAEC-PAD analysis.

2.2. Plant Material

Discarded apples of the Renetta variety, not complying with the fresh market criteria, were provided by Melinda Consortium S.C.A. (Cles (TN), Italy). They were treated immediately after arrival in the lab. The fresh fruits were roughly chopped with a fruit miller and extracted as described in Section 2.3.

Fresh pomegranate fruits of the Wonderful variety (Castellaneta (Taranto), Apulia, Italy) were purchased from a local greengrocer in November 2022. The fruits were processed with a manual fruit juicer, and the peels were processed as described in Section 2.3.

2.3. Extraction Using Hydrodynamic Cavitation

The extraction of the considered biomasses was performed through controlled HC. The device consisted of a closed hydraulic circuit, where the liquid-solid mixture was inserted and pumped by means of a centrifugal pump with a nominal mechanical power of 7.5 kW and a rotation speed of 2900 rpm (Lowara, Vicenza, Italy, model ESHE 50-160/75). The filling tank had a capacity of 230 L and was made of food-grade "AISI 304" stainless steel. The cavitation reactor was shaped like a circular Venturi tube. A schematic representation of the cavitation device is shown in Figure 1. The process was performed at atmospheric pressure, with no active temperature control, but monitored during the whole extraction period. Additionally, absorbed power and electrical consumption were monitored during the HC extraction. Further details were presented in a previous study [40]. The only energy source was power fed to the centrifugal pump. The Cavitation Number, a dimensionless parameter representing the ratio between the pressure drop needed to achieve vaporization and the specific kinetic energy at the cavitation inception section, describes the ability of the system to generate the cavitation yield [40]. The quantitative assessment of the Cavitation Number was performed exactly as described in a previous study [19], with the pressure downstream the HC reactor set at the atmospheric level, which resulted in fairly constant levels of the Cavitation Number in the range from 0.10 to 0.11 throughout the process, ensuring optimal cavitation yield [40].

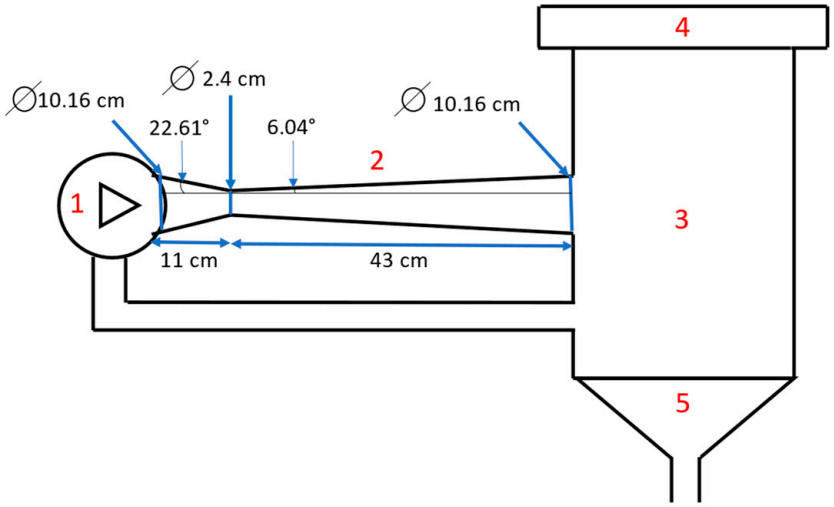

Figure 1. Schematic representation of the hydrodynamic cavitation device: (1) centrifugal pump; (2) cavitation reactor (Venturi-shaped tube); (3) main vessel; (4) lid; (5) discharge.

Apples (96.2 kg) were roughly chopped and placed in the main vessel with 49 L of tap water containing 2 L of lemon juice to delay oxidation during the process, based on preliminary trials and visual inspection of the browning of the circulating mixture. The initial and final temperatures were 24 °C and 78 °C, respectively (ΔT = 54 °C), the extraction time was 120 min, the pump power varied in the range of ~6602–6731 kW, and a total of 12.96 kWh of electricity was used.

For pomegranate peel extraction, 47.3 kg of fresh peel was placed in the main vessel. Then, 110 L of tap water was added, containing 2 L of lemon juice. The initial and final temperatures were 28 °C and 39 °C, respectively; the extraction time was 20 min; the pump power varied in the range of 7100–7339 kW; and a total of 2.56 kWh of electricity was used. Extraction parameters are summarized in Table 1.

Table 1. Extraction parameters for HC-based extraction of apples and pomegranate peel.

Extractive Conditions	Apple	Pomegranate Peel
Time (min)	120	20
Temperature range (°C)	24–78	28–39
pH [a]	3.30	3.95
Water to fresh biomass ratio (L/kg)	0.5	2.33
Energy (kWh) [a]	12.96	2.56

[a] Levels refer to the unfiltered extract after the whole HC process.

Compared with pomegranate, a longer extraction time and a higher extraction temperature were applied during apple processing, allowing for microbial inactivation due to initial matrix damage. The treatment of pomegranate peels aimed instead to achieve extraction with the lowest possible energy consumption. After processing, the mixture was discharged, then filtered with a 50 µm polypropylene filter bag, centrifuged at 4500 rpm (2722× *g*) for 30 min (NEYA 8xs, Remi Elektrotechnik Ltd., Palghar, India), and freeze-dried for 48 h (Modulyo, Edwards, Milan, Italy) to obtain the crude extract.

2.4. Isolation of Polysaccharidic Material

Purification of polysaccharides was achieved by modifying their dielectric constant by ethanol addition, resulting in precipitation of polysaccharides, as described by Baghdadi et al. [41]. An amount of five grams of extract powder was dissolved in 100 mL of ultrapure water by applying sonication for 2 min in an ultrasound bath (model DU-06, Argolab, Carpi, Italy), with a vessel capacity of 0.6 L, an ultrasound frequency of 40 KHz, an ultrasonic power of 50 W and a transduction number equal to one. Polysaccharides were recovered by precipitation as follows: initially, one volume of ethanol (100 mL) was added to the solution and placed for 45 min in an ice bath. The samples were then centrifuged (5000 rpm, 30 min, 0 °C) and the precipitate was recovered. A second volume of ethanol (100 mL) was added to the supernatant, and the mixture was set again in an ice bath for 45 min and centrifugated. The procedure was finally repeated for a third time. No additional material was recovered in the third step. The residues were then washed with three volumes of acetone, placed in an ice bath for 45 min, and centrifuged (5000 rpm, 30 min, 0 °C). The solid residue was freeze-dried, thus obtaining the crude polysaccharides.

2.5. Purification through Dialysis

The crude polysaccharides were then dialyzed to remove small molecules, such as simple sugars. Briefly, about 200 mg of material was dissolved in 10 mL deionized H_2O (dissolution ratio 1:50 *w*/*v*) with the aid of ultrasound. The solution was then added to a dialysis tube with a cutoff of 12 kDa and placed in about 1.5 L of deionized water at 4 °C for 2 days, under stirring [42]. The water was changed every 3 h during daytime. To ensure complete dialysis, the conductivity of water was measured. Dialysis was considered complete when the conductivity did not change (initial and final measures are 0 µs). Measurements were performed with HI99300 EC/TDS/Temperature meter (Hanna Instruments Srl, Woonsocket, RI, USA).

2.6. Phenols and Protein Detection by Qualitative ^{1}H-NMR

Samples were dissolved in D_2O (1 mg/0.5 mL) directly in an NMR tube and then placed in a heating bath at 50 °C to allow dissolution. ^{1}H-NMR spectra were obtained using a 400 MHz instrument Advance 400 (Bruker, Bremen, Germany). Qualitative analyses were performed before and after dialysis to assess good purification and the presence of pectin [12], as well as the presence of phenols and proteins in the samples, evaluating the presence of aromatic hydrogens [43].

2.7. High-Performance Size Exclusion Chromatography (HPSEC) Analysis

Hydrodynamic volume was estimated through HPSEC analysis following the method of Abid et al. [44] with some modifications. Briefly, samples were dissolved in ammonium formate (0.03 M) at a concentration of 2 mg/mL, centrifugated for 5 min at 14,000 rpm, and filtered with a 0.45 µm syringe filter before injection. A Varian Inc. (Palo Alto, CA, USA) HPLC system equipped with E410-series autosampler, 210-series pump, and a 356-LC Refractive Index Detector was used. Separation was performed using two columns in series (Shodex Ohpak SB-803 and SB-804 HQ; 300 mm × 8 mm I.D.; Showa Denko, Shibaura, Japan) with isocratic elution (ammonium formate 0.03 M). Methods parameters were: flow rate, 0.8 mL/min; column temperature, 45 °C; RI temperature, 45 °C; injection volume, 50 µL; analysis time, 80 min. For molecular weight estimation through hydrodynamic volume determination, pullulans with molecular weight (MW) of 805, 348, and 23.8 kDa, were used. Data analysis was carried out through the software Galaxy Chromatography data system (version 1.9.302.530) Varian Inc. (Varian Inc., Palo Alto, CA, USA).

2.8. Monosaccharides and Galacturonic Acid Analysis by High-Performance Anion Exchange Chromatography—Pulsed Amperometric Detector (HPAEC-PAD)

Double hydrolysis was performed according to the method described by Wahlström et al. [45] with some modifications: one milligram of dried polysaccharides sample was placed in screw-cup Pyrex probe. Anhydrous hydrolysis was performed by adding 1 mL of 2M HCl in methanol solution. The solution was placed in an oven at 100 °C for 5 h and let cool at room temperature. The solvent was then evaporated under vacuum. A second hydrolysis was performed by adding 1 mL of 2M TFA in water solution, and the reaction was carried out at 120 °C for 1 h. The hydrolyzed solution was let to cool down at room temperature and then freeze-dried.

The dried samples were resuspended in 8 mL water and sonicated in an ultrasound bath for 10 min at 35 °C. The samples were centrifuged at 14,000 rpm for 10 min and filtered at 0.2 µm. Chromatographic separation was performed on a Dionex ICS-6000 system (Thermo Scientific, Waltham, MA, USA) equipped with a CarboPac PA20 (6 µm, 3 × 150 mm) analytical column and a CarboPac PA20G (6 µm, 3 × 30 mm) guard column, and a pulsed amperometric detector (PAD), using an AgCl reference electrode and a gold working electrode. The column and the detector temperatures were set at 30 °C. Ultrapure water, sodium hydroxide solution (100 mM in water), and sodium acetate solution (500 mM in 100 mM NaOH) were used as the mobile phases A, B, and C, respectively, and maintained under argon atmosphere. The analyses were performed as reported by Zeppenfeld et al. [46] with minor modifications working at a 0.4 mL/min flow rate by a gradient elution as follows: 90% A/10% B/0% C (0–10 min); 60% A/0% B/40% C (10–22 min); 60% A/0% B/40% C (22–29 min) and 90% A/10% B/0% C (29–30 min). The column was reported to starting conditions by washing in 90% A and 10% B for 17 min. The injection volume was 5 µL. Thermo Scientific Chromeleon 7.2.9 software was used for data acquisition and processing.

2.9. Degree of Methylation and Acetylation through ^{1}H-NMR

Dialyzed samples were subjected to two different hydrolysis procedures to calculate the degree of acetylation and methylation according to the method described by Balli and colleagues [12] using ^{1}H-NMR. Analyses were conducted with a 400 MHz instrument, Advance 400 (Bruker, Bremen, Germany). The moles of galacturonic acids were determined after acid hydrolysis: H_2SO_4 2M was added to 5 mg of sample, and the solution obtained was kept at 100 °C for 2.5 h. After cooling, 20 µL of maleic acid was added as an internal standard. Signals of α- and β- anomeric hydrogen of galacturonic acid were determined and integral values were added up for evaluation of both the isomeric forms in the pool (see Equation (3)).

To calculate the moles of methanol, integration of the methanol's peak area obtained after milder alkaline hydrolysis was carried out: Briefly, 1 mg of dried samples was

dissolved in 0.5 mL of 0.4 M NaOH in D_2O solution. Samples were left for 30 min at room temperature and then 25 µL of maleic acid was added as an internal standard.

The number of moles for galacturonic acid (GalA), methanol, and acetic acid were calculated with the following formulas:

$$n_{MeOH} = \frac{I_{CH3}}{I_{STD}} \times \frac{n^{\circ}H_{STD}}{n^{\circ}H_{MeOH}} \times \frac{W_{STD} \times p_{STD}}{MW_{STD}} \quad (1)$$

$$n_{CH3COOH} = \frac{I_{CH3}}{I_{STD}} \times \frac{n^{\circ}H_{STD}}{n^{\circ}H_{CH3COOH}} \times \frac{W_{STD} \times p_{STD}}{MW_{STD}} \quad (2)$$

$$n_{GalA} = \frac{I_{H(\alpha+\beta)}}{I_{STD}} \times \frac{n^{\circ}H_{STD}}{n^{\circ}H_{GalA}} \times \frac{W_{STD} \times p_{STD}}{MW_{STD}} \quad (3)$$

where n_{MeOH}, number of moles of methanol; $n_{CH3COOH}$, number of moles of acetic acid; n_{GalA}, number of moles of galacturonic acid; I_{STD}, integral of the 2 protons of the maleic acid used as internal standard; I_{CH3}, integral of CH_3 group of methanol or acetic acid; I_H, integral of H group of galacturonic acid; $n^{\circ}H_{STD}$, number of protons for maleic acid as internal standard; $n^{\circ}H_{MeOH}$, number of protons for methanol; $n^{\circ}H_{CH3COOH}$, number of protons for acetic acid; $n^{\circ}H_{GalA}$, number of protons for galacturonic acid; W_{STD}, weight of maleic acid as internal standard; p_{STD}, purity degree of maleic acid as internal standard; MW_{STD}, molecular weight of maleic acid as internal standard.

To calculate degree of esterification (methylation and acetylation), the following formulas were applied:

$$DM(\%) = \frac{n_{MeOH}}{n_{GalA}} \times 100 \quad (4)$$

$$DA(\%) = \frac{n_{CH3COOH}}{n_{GalA}} \times 100 \quad (5)$$

where DM, degree of methylation; DA, degree of acetylation.

2.10. Data Analysis

Microsoft Excel (Redmond, WA, USA) was used for elaboration and analysis. Each experiment was conducted in triplicate, and the article reports mean values and respective standard deviations.

3. Results and Discussion

This study focuses on the polysaccharide fractions extracted from apple and pomegranate by-products in a pilot plant-scale HC-based extraction. HC is a physical phenomenon that occurs when negative pressure is applied to a liquid, causing the rapid formation and disruption of small cavities filled with gas. The bubble's collapse can generate a local shock wave that disrupts the plant matrix in that area, enhancing extraction [47]. Due to this peculiar mechanism of action and its advantages compared to ultrasound-assisted extraction [48,49], a preliminary extraction was conducted to chemically characterize the recovered polysaccharides and evaluate the key factors of their structure.

The logic of this research was to start with a macro-picture of the organic load of alcohol-insoluble solids, observed by ^{1}H-NMR. This analysis is meant to assess the co-presence of other compounds in addition to sugar's polymers, especially phenols and proteins, both before and after the dialysis procedure. This helped to assess the nature of the bond of any other compound that may have been detected. The following analysis of sugars was carried out by HPAEC-PAD to evaluate the relevant presence of pectic material and/or other types of polysaccharides in the recovered material. After assessing the presence of pectic polysaccharides, ^{1}H-NMR was again used to calculate the degree of acetylation and methylation, two important substitutions that happen at C6 of galacturonic acid and strongly affect the technological features of pectic polysaccharides. Lastly, analysis of the hydrodynamic volumes by HPSEC-RID was necessary to investigate any possible alterations of the molecular weights due to HC.

3.1. Polysaccharides Quality Evaluation by ^{1}H-NMR

Figure 2 reports the proton spectra of polysaccharides extracted from pomegranate peel (P-ps) before (2a) and after (2b) dialysis.

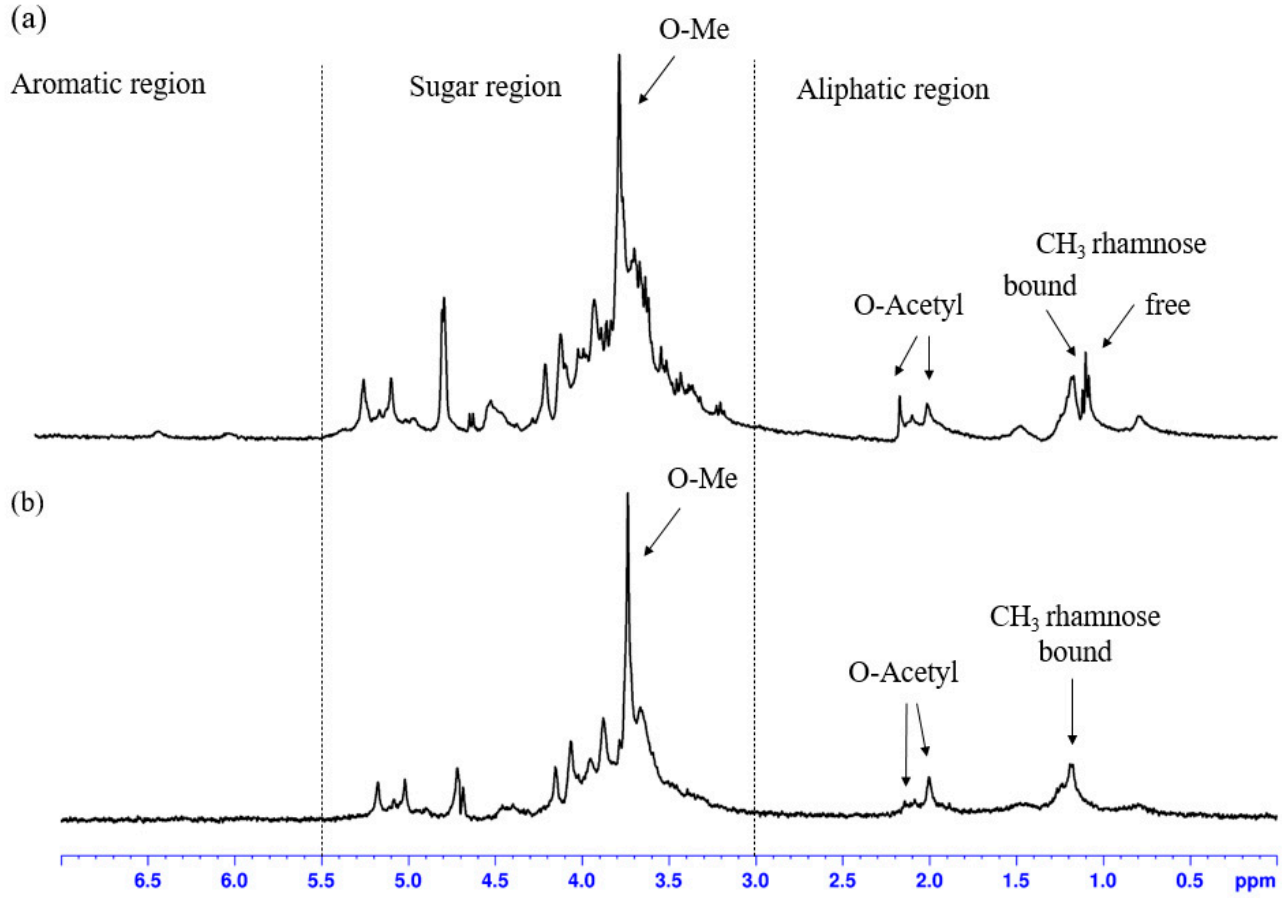

Figure 2. ^{1}H-NMR spectrum of pomegranate peel polysaccharides (**a**) before dialysis and (**b**) after dialysis.

^{1}H-NMR analysis was carried out on the polysaccharides obtained by ethanol precipitation and allowed verifying the presence of bound phenols and co-precipitated proteins. By observing the presence of peaks at values greater than 5.5 ppm, it was possible to highlight the presence of aromatic hydrogens, typical of specific amino acids such as tryptophan or phenylalanine. Yet, aromatic rings are diagnostic signals of phenols or tannins. In the case of pomegranate peel, ellagitannins are the main type of tannins present. In Figure 2a, a small signal of negligible intensity can be seen after 5.5 ppm, which disappeared after dialysis (Figure 2b). This means that any protein or tannin present in the extracted crude polysaccharide material was co-precipitated and not bound to polysaccharides since dialysis is not able to break chemical bonds.

After dialysis (Figure 2b), the signal of bound rhamnose was present at δ 1.1(9) ppm, given by hydrogens of the methyl group of rhamnose. The intensity of the peak was relatively low compared to the other signals, meaning that only a small portion of the polysaccharides extracted was rhamnose. The main peak in the dialyzed spectrum (Figure 2b) was instead at δ 3.7, given by the methyl group linked at C6 in the galacturonic acid moiety of pectic polysaccharides. Its presence confirmed that the polysaccharides extracted contained an important portion of pectic polysaccharides.

Another type of modification, which can occur on *O*-2 or *O*-3 galacturonic acid monomers of polymers, is acetylation. Acetylation affects the technological properties of polymers; it is in fact well-known that it can alter solubility [50], affecting the stabiliz-

ing/emulsifying activities of the pectic polysaccharides [51]. Hydrogens of the acetyl group generate a singlet at δ 2.0, which is of relatively low intensities in Figure 2a,b, indicating the presence of a small degree of acetylation. Quantitative evaluation of the degree of methylation and acetylation has been further discussed in Section 3.3.

Finally, through dialysis, it was possible to purify the polysaccharide fraction from free monomers that co-precipitated when ethanol was added. Indeed, many peaks in the sugars' region of the ^{1}H-NMR spectrum (between δ 3 and 5.5) disappeared after dialysis (Figure 2a,b), confirming that the dialysis process effectively removed most of the monomers. The peaks detected in the above-mentioned region are hydrogens of the α- and β- anomers of the residual sugars.

Regarding polysaccharides from apple by-products (A-ps), the ^{1}H-NMR spectra before and after dialysis are reported in Figure 3a,b, respectively. Before dialysis, polysaccharides from apples presented a higher number of signals in the spectrum, especially in the sugar region. This means that, along with polysaccharides, a large amount of simple sugars co-precipitated, leading to a higher presence in the crude extract.

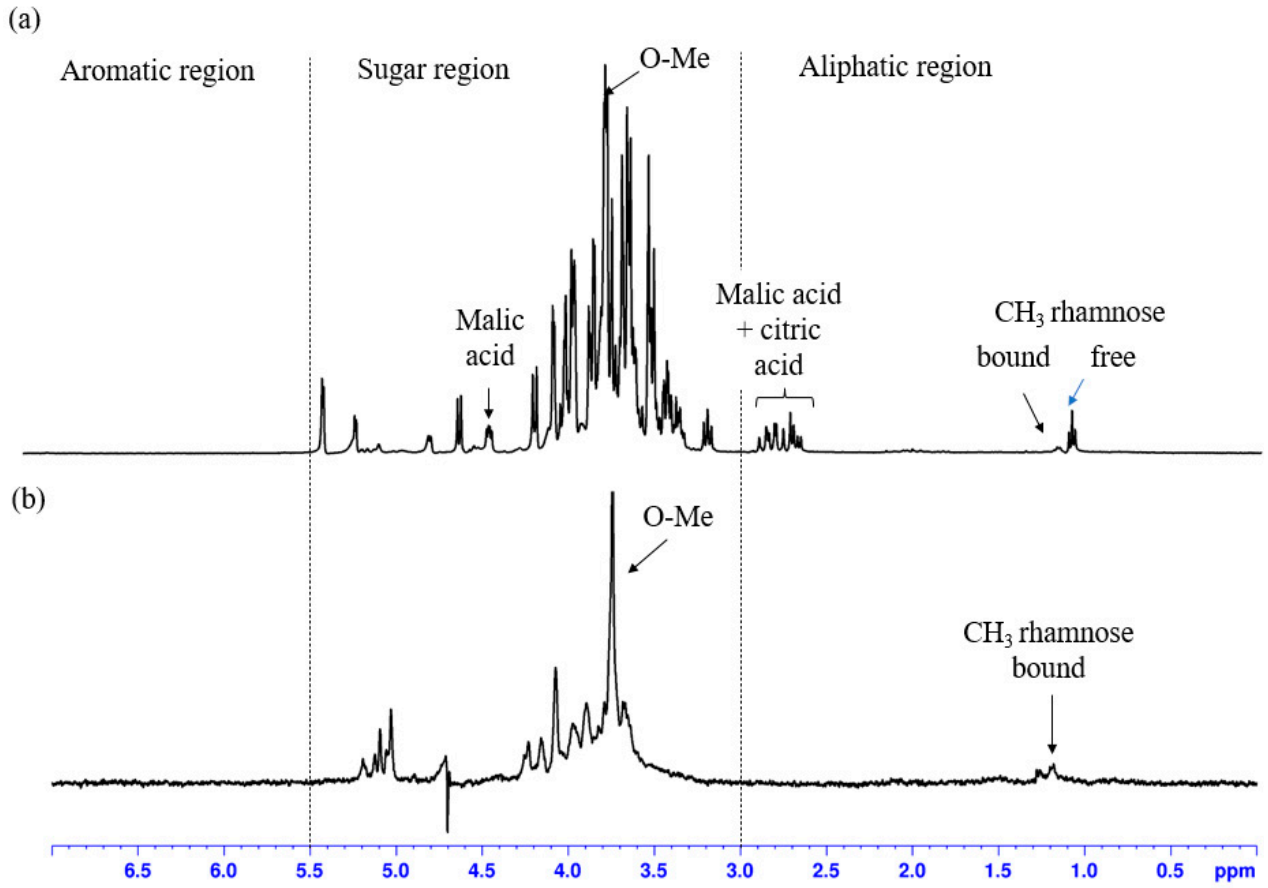

Figure 3. ^{1}H-NMR spectrum of polysaccharides from apple by-products (**a**) before dialysis and (**b**) after dialysis.

Along with simple sugars, also a small amount of organic acids co-precipitated, giving typical ^{1}H-NMR signals in the region between 2.5 and 3 ppm. More specifically, the multiplets at approx. 2.6–2.9 ppm can be attributed to the protons of the carbon of malic acid not bound to the hydroxyl, while the signal at approx. 4.36–4.38 ppm can be attributed to the proton on the carbon bound to the hydroxyl group [43]. Yet, in agreement with previous findings [52], signals (singlets) were recorded from citric acid at approx. 2.60–2.70 ppm (corresponding to α-γ-CH and α′-γ′-CH), and from acetic acid at approx. 2.0 ppm, with the latter in very small concentrations. All signals given by organic acids disappeared after dialysis (Figure 3b).

Analysis of the ^{1}H-NMR spectra also allowed excluding the presence of proteins and phenols, due to the complete absence of any kind of peak after 5.5 ppm. Concerning apple phenols, it should be noted that the extraction of the apple material started at a temperature of 24 °C, lasted 120 min, and ended at 78 °C (i.e., the majority of the process), and probably the extraction of phenolic compounds occurred well below the inactivation temperature of the polyphenol oxidase enzyme, which was determined around the level of 80 °C [53]. Therefore, it is likely that most of the phenolic compounds were degraded or destroyed by oxidation before the experimental sample was collected. Since ^{1}H-NMR analysis did not highlight a significant presence of proteins and phenols in both A-ps and P-ps, no further analysis for protein and phenol content was conducted.

After dialysis, there were far fewer peaks corresponding to protons of simple sugars, highlighting the effective cleaning of polysaccharides, and the singlet at 3.70 ppm was clearly detectable. This peak is due to the protons of -CH_3 of the methylated pectic polysaccharides, thus confirming the presence of a significant amount of pectic material in the polysaccharides extracted from apple by-products. The diagnostic signals of rhamnose (δ 1.0 for free rhamnose, δ 1.18 for bound rhamnose) were drastically reduced after dialysis, with a minor peak corresponding to the bound type. A large portion of free rhamnose was removed by dialysis, and the residual bound rhamnose portion allowed confirming the absence of significant portions of rhamnogalacturonan in the sample. Further discussions on the content of rhamnose and other neutral sugars are reported in Section 3.2. Lastly, no acetylated monomers seemed to be present. In fact, no signal of the -CH_3 of the acetyl group was present at δ 2.0 ppm. The presence of acetylated or methoxylated residues has been further discussed in Section 3.3.

3.2. Galacturonic Acid Content and Sugar Analysis by HPAEC-PAD

The content of neutral sugars and galacturonic acid was estimated simultaneously through HPAEC-PAD, one of the most innovative techniques for the detection and quantification of sugars. The quantities and percentages of the detected sugars are reported in Table 2.

Table 2. Comparison between apple and pomegranate peel polysaccharides for sugar content and composition, *Rha/GalA* ratio, molecular weight, and degree of esterification.

		Units	P-ps	A-ps
Sugars	Arabinose	% µg sugar/µg TOT	28.8 ± 1.1	18.1 ± 0.7
		µg/mg	86.6 ± 12.8	62.1 ± 10.8
	Galactose	% µg sugar/µg TOT	26.6 ± 0.3	7.4 ± 0.6
		µg/mg	79.7 ± 10.6	25.3 ± 4.7
	Galacturonic acid	% µg sugar/µg TOT	30.5 ± 3.0	65.1 ± 1.3
		µg/mg	91.5 ± 14.7	222.8 ± 28.8
	Glucose	% µg sugar/µg TOT	4.6 ± 1.2	4.4 ± 0.6
		µg/mg	13.7 ± 2.7	15.1 ± 3.6
	Mannose	% µg sugar/µg TOT	1.6 ± 0.4	0.6 ± 0.1
		µg/mg	4.9 ± 1.4	2.0 ± 0.3
	Rhamnose	% µg sugar/µg TOT	5.1 ± 0.5	3.4 ± 0.4
		µg/mg	15.2 ± 2.6	11.9 ± 3.1
	Xylose	% µg sugar/µg TOT	2.8 ± 0.6	1.08 ± 0.02
		µg/mg	8.3 ± 1.6	3.7 ± 0.5
	Rha/GalA	-	0.167	0.053
	MW	KDa	805–348	>805; 805–348
DE	DM	% mol methanol/mol GalA	84.1 ± 2.2	74.2 ± 2.3
	DA	% mol acetic acid/mol GalA	21.2 ± 1.0	3.2 ± 0.3

P-ps: pomegranate peel polysaccharides; A-ps: polysaccharides from apple by-products; Rha/GalA. ratio of the amounts of rhamnose and galacturonic acid; MW: molecular weight; DE: degree of esterification; DM: degree of methylation; DA: degree of acetylation.

Sugar analysis of A-ps revealed a significant percentage of galacturonic acid content, equal to 64.9%. According to FAO, polysaccharides could be defined as "pectin" when the amount of galacturonic acid (GalA) is equal to or greater than 65% [54], a threshold almost reached by A-ps. Therefore, it can be stated that the majority of alcohol-insoluble solids were composed of pectic polysaccharides, where homogalacturonans represented the major type of pectin. This was deduced from the low percentage of rhamnose, which should be present in equal amounts to Gal-A in the rhamnogalacturonans I (RG I) regions (Rha/GalA ratio equal to 1). In A-ps sample, rhamnose accounted only for 3.5% of the total sugars; therefore, it can be deduced that only a small fraction is constituted of RG I, assuming that all rhamnose came from RG I and not RG II regions. However, if rhamnose was somehow distributed between RG I and II regions, the amounts of the branched regions would only be lower than expected. Further considerations about the amount of rhamnogalacturonans II (RG II) were not possible since a linkage analysis would be required, which is beyond the scope of this study. However, in RG II, some particular sugars such as fucose, apiose, and xylose can be found [55], which were all monomers in low concentration or not detected at all, thus it was possible to consider the RG II amount negligible. The xylose detected could be a constituent of xylogalacturonans or xyloglucans: the former are a sub-type of homogalacturonans in which one or few GalA residues are replaced by xylose [56], while the latter are important polymers belonging to the hemicellulose family and accounting for 18% of the apple cell wall [57]. Arabinose and galactose were other sugars detected in notable quantities (i.e., 18.1% and 7.4%, respectively). The arabinose and galactose detected were probably derived from arabinogalactan polysaccharides, which are a common type of polymer in the side chains of pectin. Glucose represented a small percentage of the total sugars (4.4%), meaning that the co-presence of other types of polysaccharides (such as cellulose, which is insoluble and could not be extracted using HC in water alone) is limited. Finally, mannose was found in negligible amount (0.6%).

Sugar analysis of pomegranate peel polysaccharides highlighted low content of galacturonic acid, reaching only 30.5% of the total sugars. This means that only a part of the polysaccharides extracted was constituted of pectic material, which can derive either from lower availability of pectin in the pomegranate peel compared to the apple, or from the short time and low temperature of the respective extraction process. However, the pectic material of pomegranate peel seemed to be quite different from that of apples. The rhamnose content was slightly greater than that of apples (i.e., 5.1% vs. 3.5%) but the amount of total galacturonic acid was almost half in pomegranate peels. The *Rha/GalA* ratio was significantly greater in pomegranate peels, reaching a value of 0.167 and 0.053 for P-ps and A-ps, respectively. These data could be interpreted as a greater presence of RG I regions in the pomegranate peels. Xylose and mannose, although present in low concentrations, were more abundant in P-ps than in A-ps, indicating a slightly increased variability in the type of polysaccharides obtained, with a higher probability of xylogalacturonan portions. Mannose, found in low concentrations but doubled when compared to A-ps, also indicated the presence of more diversified side chains of the pectic polysaccharides. Glucose did not differ from A-ps (approx. 4.5% in both samples), indicating a minor presence of co-precipitated polysaccharides such as cellulose or hemicelluloses. The greatest differences were given by the percentages of arabinose and galactose, whose amounts in the P-ps samples were only slightly lower than galacturonic acid. Several studies [58,59] have highlighted how RG I regions of pectin derived from pomegranate peels are characterized especially by galactose and arabinose. The hypothesis that better described the results obtained in this study is that the low temperature and short extraction time allowed the recovery of pectic polysaccharides rich in branched regions and of galactans and arabinogalactans polysaccharides, known to be constituents of pomegranate peel [12,60]. Solid materials consisting of several types of polysaccharides were therefore obtained.

3.3. Degree of Esterification by ^{1}H-NMR

^{1}H-NMR analysis of the samples after mild alkaline hydrolysis allowed the simultaneous quantification of acetic acid and methanol esterified with the galacturonic acid residues in the pectic polysaccharides. The released methanol resulted in a single signal at δ 3.25 ppm, while hydrogens belonging to the -CH_3 of acetic acid were detected at δ 1.83 ppm, as shown in Figure 4a for pomegranate peel as an example. Galacturonic acid content was also evaluated by ^{1}H-NMR in order to obtain the number of moles with the same instrumentation. Assignments of the peaks for the estimation of GalA content are shown in Figure 4b (on P-ps as an example), where the integrals of the anomeric α- and β-hydrogens are highlighted.

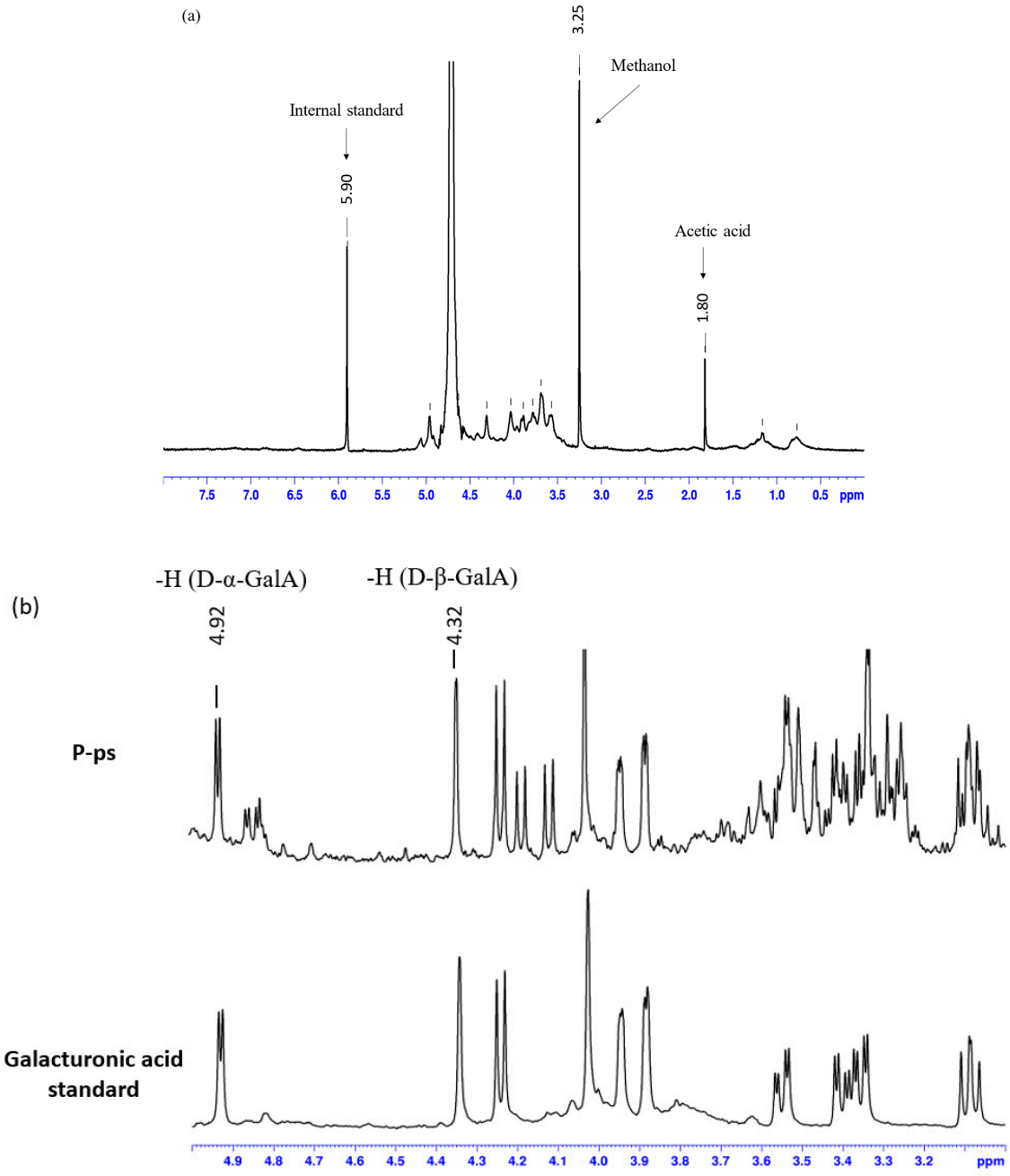

Figure 4. Pectic polysaccharides from pomegranate peel: (**a**) mild alkaline hydrolysis for methanol and acetic acid release; (**b**) acid hydrolysis for estimation of GalA content in moles, with ^{1}H-NMR spectrum of galacturonic acid standard as reference.

Regarding the degree of methylation, both types of pectic polysaccharides analyzed were classified as highly methoxylated pectin, a characteristic that confers high gelling properties at low pH, high emulsifying activity, and a high capacity to maintain emulsion stability [61]. The degree of acetylation was found strongly different among the two samples and, despite little research on the topic, the available evidence suggests that acetylation helps to increase the surface tension, thus lowering the emulsion stability [62], as well as acetylation hinders the gelling ability [63]. Table 2 reports the percentage of methoxylated galacturonic acid and acetylated galacturonic acid.

A-ps presented a degree of methylation (DM) of approx. 74%, which was consistent with the literature reporting similar extractive conditions. In fact, other studies involving high temperatures (90–100 °C) with long extractive times, between 30 min and 2.5 h, also found highly esterified pectin, with values ranging from 57% to 71% [64,65]. Regarding the degree of acetylation, which reached only 3.2% of the galacturonic acid residues, it was negligible compared to the great content of GalA. Only a few studies in the literature have investigated the degree of acetylation, and values similar to those obtained in this study have been reported for two commercial apple pectins [66]. Moreover, apple pectic polysaccharides extracted using HC were mainly composed of homogalacturonan portions and, since acetylated GalA residues are mostly found in the RGI region of pectin [67], low degree of acetylation was expected.

P-ps resulted in a highly methoxylated pectin, with 84% of esterified galacturonic acid residues. These data were in agreement with the literature, which reported slightly lower values (~78.5%) found after extraction at high temperatures, long reaction times, and low pH values [68]. Apparently, the HC-assisted extraction was able to preserve the original methyl esters present on the GalA residues. Interestingly, pomegranate peel polysaccharides also presented a high degree of acetylation, not commonly found in commercial pectin. In this study, 21% of the sugar residues were acetylated, which is slightly greater than the levels found for pomegranate peel in the literature, ranging from 12 to 18% [69,70]. This pectin obtained using HC can be considered "highly acetylated" [71], thus requiring further investigation into its possible technological applications. Indeed, it has been reported that even if acetylation hinders the gelling properties, pectin characterized by high DM and DA can be used as a gelling agent under particular conditions [71].

3.4. Molecular Weight Evaluation by HPSEC-RID

Size Exclusion Chromatography is of crucial importance in collecting information on the indicative molecular weight (MW) of polysaccharides [31]. In fact, investigation of the hydrodynamic volume can provide information on the mean MW by correlating the chromatographic data with appropriate standards. Pullulans, linear polymers of maltotriose units, are commonly used due to their similarities with apple pectin, which is mostly characterized by homogalacturonans. The chromatographic profiles of apple and pomegranate peel extracts are reported in Figure 5 and the qualitative data obtained are also reported in Table 2. In Figure 5, the elution times of pullulans indicated by arrows are highlighted without reporting the respective chromatograms.

Polysaccharides from both by-products showed a main peak with molecular weights ranging from 805 to 348 kDa. A-ps exhibited a relatively narrow peak with a visible shoulder, highlighting the presence of two fractions characterized by different molecular weights. Data recorded for A-ps align with the literature, which reports a wide range of molecular weights (approximately from 200 to 1100 kDa) when apple pectins are recovered through acidic extraction [72]. P-ps, ranging from 348 to 805 kDa, are also consistent with the literature [44]; however, they are additionally characterized fractions with smaller molecular weights (<23 kDa). Considering the elution time of raffinose (28 min), all the following peaks observed in both chromatograms are likely due to degradation products.

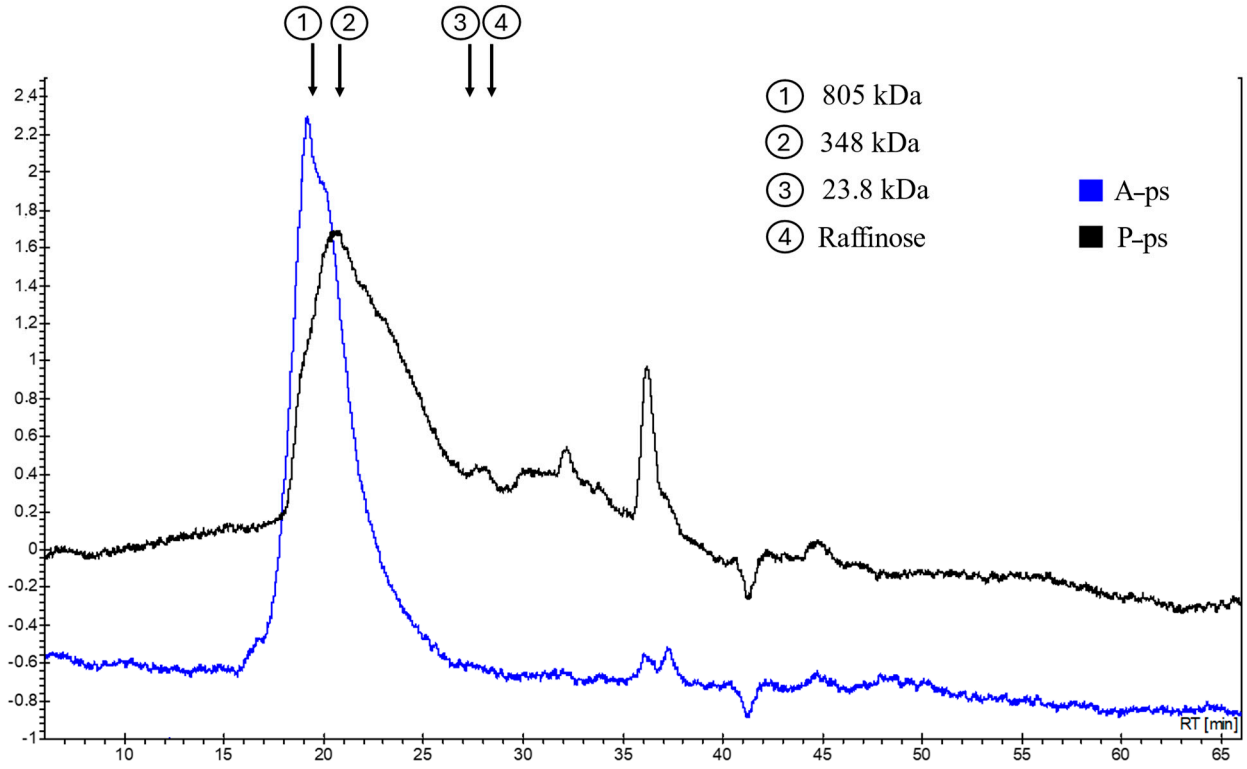

Figure 5. HPSEC-RID chromatograms. In electric blue, polysaccharides from apple by-products (A-ps); in black, pomegranate peel polysaccharides (P-ps). Arrows indicate the retention time of peaks related to pullulans used as standards, which are not shown in the chromatogram.

3.5. New Insights and Potentialities

It is worth noting that this macrocharacterization of polysaccharides achieved through these techniques could be applied both in the scientific field and at an industrial scale. In fact, key characteristics of plant polysaccharides extracted from new vegetable sources should be acknowledged, and the research tools here proposed allow their fast determination. The methodologies described are not time-consuming and very accurate; therefore, their potential applications to recover information on the chemical structure of polysaccharides could be further implemented by the scientific community. This is meant to be helpful in order to facilitate further studies on the deep analysis of the polysaccharides derived from these matrices, highlighting further key points that will address their technological application.

4. Conclusions

In this study, hydrodynamic cavitation was applied to apple and pomegranate by-products, and a multi-analytical approach was designed to efficiently determine the chemical characteristics of the complex pool of polysaccharides extracted.

A first look by ^{1}H-NMR highlighted the absence of bound/absorbed phenols and proteins, with important amounts of pectic material in the dialyzed samples. For apple polysaccharides, HPAEC-PAD showed a significantly higher content of galacturonic acid compared to other sugars, confirming the high presence of homogalacturonans. Instead, pomegranate peel polysaccharides presented a greater variability in sugar composition: galacturonic acid was the main constituent, but slightly lower contents of galactose and arabinose were observed, highlighting the presence of rhamnogalacturonans I regions and arabinogalactans. The ^{1}H-NMR analysis also pointed out that hydrodynamic cavitation efficiently preserved the pectin esterification, resulting in highly methoxylated apple pectic polysaccharides and pomegranate peel polysaccharides being highly methoxylated and

acetylated. The molecular weight of polysaccharides evaluated by HPSEC-RID was mainly between 805 and 348 kDa for both samples.

In conclusion, the techniques applied in this study allowed a fast determination of the general characteristics of pectic polysaccharides. After a proper comparison with data reported in the literature, HC can be proposed as an economically and environmentally sustainable approach to recover valuable polysaccharides with possible future applications in the food industry. Further research will be conducted to optimize the hydrodynamic cavitation process after this preliminary screening assessed that HC did not remarkably alter the structure of the tested samples. Moreover, a deeper investigation of the polysaccharides is needed to determine further conformational characteristics as well as to test their technological properties.

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