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Characterization of Apple Pectin – A Chromatographic Approach

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<http://dx.doi.org/10.5772/52627>

1. Introduction

The apple tree belongs to the *Plantae* kingdom, *Magnoliophyta* class, *Rosales* order, *Rosaceae* family, *Pomoidae* sub-family and *Malus* genus. Originating in the mountains of Asia, the apple (*Malus domestica* Borkh) is a temperate fruit with an ancient history [1].

The center of the origin of the *Malus* cultivars is understood to be in Asia Minor, in the Caucasus, located between the Caspian and the Black Seas, the Indian Himalayas, Pakistan and Western China[2], all areas that had access to the Silk Road where marketable products were transported from the east to Europe since antiquity. This is a series of trade routes that crossed Eurasia by land and by stretches of sea or river for over 2,000 years until 1,500 BC, and which enabled the movement of important materials that promoted the exchange of ideas and innovations between different cultural groups. Over the centuries, gunpowder, the compass, the printing press, silk, mathematics and ceramics all migrated along this road, as well as stringed instruments. Resources, information and news were all spread in this fashion among many cultures for such a long period that is often difficult to identify the source of many traditions.

Whatever may have been the mechanism of transport of varieties of apples it is certain that animals, birds, cattle and bats, among others, played an important role [2]. The caravans, with their humans and camels, ate the fruit and left the seeds in fertile areas along the highway since the Neolithic period and many cultivars were perfectly defined in the Middle East around 4000 BC. These regions are characterized by a temperate climate, with low winter temperatures - important for the winter hibernation of apple trees. Commercial apples today are descendants of species from Asia, including the Gala, Golden Delicious and Fuji.

2. Geographical distribution of apple agroindustries

The cultivation of apples is found in diverse temperate climates around the world, in both hemispheres but with a greater distribution in the northern hemisphere. In the first decade of the twenty-first century, in a situation with many global changes taking place, the five biggest producers of fruit are those known by the acronym BRICS (Brazil, Russia, India, China and South Africa).

The worldwide production of apples of 68 million metric tons, contains 40% that is relative to the main producing country, China, which is characterized by having a monovarietal culture based on the Fuji variety [3]. The area under cultivation in China has been stable over the past three years, with about 2,000 ha and a yield of 16 ton/ha, and the country still imports 58,000 tons which brings the domestic market up to a total of 32,058 million tons. The consumption of fresh fruits is 25.6 million tons, with a per capita index of 19.2 kg, and 1,460,000 tons of these high quality apples are exported, leaving 4,998 million tons for industrial use, of which 87.5% are destined for use as concentrated juice [4]. These statistics place China in a prominent position in the apple agroindustry. This means that just as China had increased its production to become the largest producer as forecast [5] many other countries had decreased their production to justify decline in production in the rest of the world of -2%. The production of the European Economic Community, including the UK, demonstrates stability of production and points to a fall of 5% between 2002 and 2011, demonstrating how the largest producers such as Poland and Italy (> 2,000,000 tons), France (> 1,000,000 tons) and Germany (900,000 tons), which together account for 70% of this activity of apple production available for both the common market and for export [6]. The United States, the second largest producer, accounted for 6.2% of the volume of apples produced in 2008. In South America, the main producers are, in order, Chile, Argentina and Brazil, although none of the three produced more than 2% of the world total in 2008. In that year, production in Brazil accounted for 1.6% of the world total, positioning the country as 12th in the ranking of countries by apple production [7].

There are thousands of domesticated and commercial species of apples that act as a producer or pollinator in commercial orchards and they are ranked periodically in relation to their commercial importance. The top ten apple varieties (excluding those produced in China) in descending order are Golden Delicious, Delicious, Gala, Granny Smith, Fuji, Jonagold, Idared, Jonathan, Braeburn and McIntosh [8].

The major advances in the cultivation of apples are listed by [9] and include the development of new varieties and disposal of others in the process of applying emerging research processes. Granny Smith, Gala, Fuji, Jonagold, Pink Lady and Empire are all types of apples that have excelled more effectively than expected. A new feature has been the practice of treating all the varieties in a similar way as a means of maintaining the unit price, since the producers are moving towards producing predetermined and defined amounts of fruit. This homogenous practice spread to Australia, but in Europe there are already 10 sets of varieties and in the United States there are at least seven such sets. The dwarfing of apples is regarded as an improvement as well as the optimization of planting density

systems, with the solution of problems of overheating solved by setting branches at angles, which demonstrates the practical aspects of the solutions. The development of growth regulators and genomics shows the dependence on current scientific development [9].

3. Principal products from apples

In the apple processing industry the principal products are apple juice and fermented products such as cider and vinegar. In some countries (France, Spain, Switzerland and England), genetically improved fruit are used with special physical and chemical characteristics in order to standardize products, which are called 'industrial apples'. On the other hand, the production of apples generates a discarding process both at the time of harvest and in the classification process for the fresh market. These discarded apples can vary from between 10 to 30% of total production [10].

These discarded fruits are taken for processing to extract the juice or must. In Brazilian companies, besides the mixture of cultivars, there are fruits in different stages of ripening (from pre-maturation to senescence) and with possible phytopathological defects due to high temperatures (25-35°C) at harvest time. This occurs in summer and fruit for processing is stored externally, as in Europe, but the temperature at harvesting time in France is 7-15°C [11].

Various technologies can be used in the extraction of apple juice. The system of continuous press is the most used, but in small companies discontinuous vertical hydraulic presses are also used. The addition of pectinolytic enzymes and maceration may increase the extraction yield either by pressing or centrifuging. However, the use of enzymes should not alter the physical-chemical parameters established by international legislation or the standards of each company that will use the juice or must [10].

In the process of extracting the juice or must a by-product is produced, which is known as apple pomace. This residue can represent 20-35% of the production. The yield of pomace that is generated and its composition will depend upon the cultivars, their stage of conservation and the technology used in processing.

3.1. Apple pomace

Traditional stabilization technology takes into account that apple pomace is an agro-industrial residue with a view to the fact that its disposal creates environmental problems of high cost. However, apple pomace is an interesting material that has attracted considerable attention as a potential source of dietary sugar, fiber, phenolic compounds and pectin.

These products can be used for many purposes in the pharmaceutical, cosmetics and food industries. The commercial production of apples in Brazil, based on only two cultivars, was designed to meet highly demanding customer requirements both with respect to quality and the retail prices of table fruit, and more recently the industrial product, either as juice or in a fermented form. Industrial apple pomace has in its composition all the constituents of the

fruit in varying amounts, more or less, and is the residue from the pressing of the grated mass of unfermented juice, cider, wines, brandies and distillates or vinegars [12].

In Brazil, 70% of apples produced are sold for fresh consumption, while 30% are considered 'industrial' fruit. One third of this fraction is considered to contain fruit of low quality, which must be discarded or used for the production of distillates such as alcohol or vinegar and the remaining two thirds are fruits that can be used for the processing of apple juice [13]. On the global scene, in the 1980s, with reference to industrial fruit, 75% of the products consisted of apple juice or must and 25% was apple pomace, when 8 kg of raw material was required to produce the amount of 6 kg of apple juice necessary to make 1 kg of concentrated juice. In 1996, the yield had risen by 85% due to the use of enzymes in the preparation of the must. Since that time, the yield has increased slightly depending on the variety of the fruit and the degree of maturity, which means that currently 7 kg of apples are required for 1 kg of concentrate. But it is possible to increase this by 1% with the use of the most up-to-date enzymes [14].

The drying of apple pomace seems to be the most economic technological approach to stabilize the product because it dramatically reduces the volume of water and makes transport cheaper. The yield of dried pomace at 60°C is about 50.0 g kg⁻¹ in 10 hours, or 5% of the raw material. The appearance of the dried pomace is dependent on the adiabatic drying temperature. From 50 to 60°C, darkening enzymatic reactions are stimulated [15], while from 90 to 100°C Maillard reactions occur, with products appearing darker than those obtained in the range of 70 to 80°C. However, if the criterion of stopping the process is the time at which the temperature of the pomace starts to rise, that temperature will never rise to values higher than 52°C and thus the final temperature tends to be homogeneous.

The instability of apple pomace is related to its physical-chemical composition and the presence of some enzymes that are activated after the disintegration of plant tissues [16:17:14]. Apple pomace is composed of water (76.3%), soluble solids (23.7%) and is obtained from the epi-mesocarp (95.5%), seeds (4.1%) and stems (1.1 %). It has an average humidity of 80% and 14% of total soluble solids including glucose, fructose and sucrose. Its composition is related to the cultivar and processing [17]. The fiber content varies from 11.6 to 44.5%, and includes cellulose (12.0 to 23.2%), lignin (6.4 to 19.0%), pectin (3.5 to 18%), and hemicellulose (5.0 to 6.2%). The average dietary fibers (35.8%) and sugars (54.4%) make up 91.2% of pomace and the other components are proteins, lipids and ash [18]. The chromatic characteristics L = 51.8, a = 5.4 and b = 18.2 have been determined in samples of apple pomace [19].

The use of apple pomace as a potential source of nutrients for the production of glucosidase by *Aspergillus foetidus* was suggested by [20]. Ten years later [21] suggested its use for other technological purposes such as the recovery of phenolic compounds. Apple pomace is also recommended for biotechnological applications such as ethanol production [22], flavorings, citric acid, pectin, enzymes and molds for the extraction of dietary fiber and mineral coal [23].

The apple pectin that is derived from the extraction of apple pomace is dark brown in color compared to orange pectin. Studies are being carried out regarding the potential use of

apple pomace in the recovery of native phenolic compounds associated with darkening, for use as antioxidants, resulting in a lighter color of the pectin obtained. These points to the growing trend of industry to find alternatives that promote the 'recycling' of waste, with maximum recovery and greater commercial exploitation of components previously considered as by-products.

3.2. Pectin

Pectin refers to a family of polysaccharides and oligosaccharides, which have common characteristics but are extremely diverse in their fine structure. The pectic skeleton is primarily a homopolymer of galacturonic acid bound in $\alpha(1\rightarrow4)$, with varying degrees of methyl esterified carboxyl groups [24,25]. Pectin must consist of at least 65% galacturonic acid, according to the FAO (UN Food and Agriculture Organization) and EU (European Union) [25].

Pectin is one of the most important substances found in apples because it provides approximately 10% of daily fiber requirements. Pectin is a soluble fiber that is not absorbed by the intestine, i.e. the fibers are not degraded by digestive juices, but they increase the volume of fecal material, assist in the proper functioning of the intestine, retain water and various residual substances, facilitate the elimination of toxins along with stools, promote the protection of the intestinal mucosa and help in the treatment of diarrhoea. Pectin is also highly recommended for diabetics because it reduces the absorption of glucose. The daily consumption of approximately 2 small apples provides the required dose of pectin. Pectin also assists in reducing bad cholesterol because it forms a fiber barrier in the intestinal wall preventing the absorption of cholesterol and other fats.

In the industrial sector, pectic polysaccharides promote increased viscosity and act as a protective and stabilizing colloid in foods and beverages such as jams and jellies, fruit preparations for yoghurts, concentrated fruit juices and drinks, milk and fruit-based desserts, gelled dairy products, confectionery, and dairy products that are directly acidified or fermented. Other properties include the prevention of flotation in fruit preparations, stability of bread products, protein stabilization, softness in texture, increase in volume and the control of syneresis [25].

3.2.1. History of pectin

The first citation relating to pectin is found in an English article from 1750 about the preparation of apple jelly [26]. The process of extracting liquid pectin was recorded in 1908 in Germany and the process spread rapidly to the United States, where a patent was obtained in 1913 by Douglas (U.S. Patent no. 1,082,682) [27]. As for commercial production, in the 1930s Hermann Herbstreith discovered the potential use and application of apple pomace, a hitherto discarded by-product of the production of fruit juice [28].

The content of pectic substances varies depending on the botanical source of plant material. There are four by-products of the agricultural and food industries that are high in pectic

substances (content over 15% on a dry basis): pomace from apples, citric albedo, sugar beet pulp and /sunflower rinds 29.

The cell wall, a dynamic compartment of plants, can be divided into two layers called 'primary' and 'secondary'. The primary cell wall can be classified into: [1] type I, mainly composed of cellulose, xyloglucans, pectin and extensin, generally present in dicots and some monocots (non-comelinoides) and [2] type II, comprising mainly cellulose, glucoarabinoxylans and phenolic compounds with a lower proportion of pectin, found in Poaceae and in most monocotyledonous plants. The matrix of pectin controls, among other properties, porosity. The middle lamella consists of pectin molecules that are joined by cross-linked chains with homogalacturonan layers subsequently deposited in pectin of opposing cells 30.

Most of the pectin used by the food industry originates from such raw materials and is extracted under conditions of low acidity and high temperature, resulting in chains that are primarily homogalacturonan 25.

The ratio of raw materials and solvent in the extraction of pectin can be adjusted in order to separate the solid and liquid phases, the filtration of the extract and the costs of water evaporation in the process. Thus, it is possible to control the extraction of pectin to optimize its potential use 24; 31.

3.2.2. Structure and composition of pectin

In 1934, citrus pectins were recognized as linear chains of galacturonic acid and since then it has been found that pectin is a highly complex molecule. The challenge of recent times has been to accommodate all the available information in a single structural model [25]. Pectin are formed by seventeen different monosaccharide, arranged in distinct polysaccharides from more than twenty different connections that form a network when joined together [25; 30] and they are grouped into different types of chains consisting of uronic acids, hexoses, pentoses and methyl pentoses. Several structural units may be replaced by methanol, acetic acid and phenolic acids. Sugars can exist in furanic or pyranic forms and with different anomers (α or β) with various types of linkages between monomers such as $\alpha(1\rightarrow4)$, $\alpha(1\rightarrow5)$, $\beta(1\rightarrow3)$ e $\beta(1\rightarrow4)$ e $\beta(1\rightarrow6)$ $\beta(1\rightarrow6)$ [32]. Recently there has been progress in the understanding of the very complex fine structure of pectic polymers.

Homogalacturonan (HG) is the most abundant pectic polysaccharide in the cell wall, equivalent to about 60-65% of the total pectin [25;33]. It presents units of α -D- galacturonic acid in $1\rightarrow4$ links in a linear pattern. The carboxyl groups are partly methyl-esterified. The chains may be, depending on the plant source, partially O-acetylated at C-3 or C-2 [34; 33; 30].

Rhamnogalacturonan I (RG-I) has a chain represented by the disaccharide $[\rightarrow4-\alpha\text{-D-GalA-(1}\rightarrow2)-\alpha\text{-L-Rha-(1}\rightarrow)]_n$ [34; 25; 33; 30]. In summary, a variety of different glucan chains (mainly arabinan and galactan) are linked to rhamnose units. The chain length may vary considerably and the composition of RG-I sugars may be highly heterogeneous [34]. RG-I represents 20-35% of pectin, with a high degree of cell specialization and expression

depending on the development, the type and number of simple sugars and oligosaccharides attached to this chain. The reason for this level of variation in RG-I is not known, but it suggests a functional diversity.

Rhamnogalacturonan II (RG-II) is the most structurally complex segment and comprises 10% of pectin. This structure, highly conserved in most plant species, consists of a homogalacturonan skeleton of approximately eight (probably more) monomeric units, containing side chains of up to 12 different types of sugars, some highly peculiar such as apiose, aceric acid, Docosaheptaenoic acid (DHA) and 3-Deoxy-D-manno-oct-2-ulosonic acid (KDO). RG-II usually exists in cell walls as cross-linked dimers by a borate diol ester apiosil between units in the side chain [34; 25].

Xilogalacturonana (XGA) is a homogalacturonan substituted with xylose linked to position 3. The degree of control of xylose may vary between 25% (watermelon) to 75% (apple). This xylose may be additionally substituted in O-4 in conjunction with another xylose in β , which is more prevalent in reproductive tissues such as fruits and seeds [25;33].

Arabinogalactan I (ARA-I) is composed of skeleton β -D-Galp; residues of α -L-Araf may be linked to galactosyl units in position 3. Arabinogalactan II (ARA-II) is primarily associated with proteins (3-8%), also called arabinogalactan-proteins (AGPs). AG-II is composed of a β -D-Galp 1 \rightarrow 3 skeleton, containing short chains [α -L-Araf (1 \rightarrow 6) β -D-Galp (1 \rightarrow 6)]_n, where n = 1, 2 or 3. The protein part is rich in proline, hydroxyproline, alanine, serine and threonine [25]. Arabinan (ARA) is composed of an α -L-Araf skeleton in 1 \rightarrow 5 links, where there may be side chains of α -L-Araf (1 \rightarrow 3) [35]. There is also another chain, not shown schematically, which is apiogalacturonan (API), HG substituted in O-2 or O-3 with D-apiof the 2-O-or D-3 apiof. It is present in aquatic monocots such as *Lemna* [33].

4. The application of chromatographic techniques

The properties and application of pectin are affected by several parameters related to pectin structure, including the composition, presence and distribution of side chains, degree of methyl-esterification (DE), degree of acetylation (DA), molar mass, and the charge distribution along their backbone. Differently from proteins, whose structure is defined in relation to a template, pectin are a mixture of heterogeneous polymers and preparations that contain pectins that have been isolated by chemical and enzymatic treatments likely to cleave covalent bonds. The conditions used for pectin extraction can also release other cell wall polymers. Preparative chromatography can be used to separate homogeneous fractions of pectic polysaccharides from a mixture.

Determining the fine structure of pectins is a challenging task which encompasses the determination of molar mass, monosaccharide composition, configuration and ring size of monosacchrides, identification of glycosidic linkages, sequence of glycosyl units in the polymer, determination of DA and DE, as well as the distribution of the acetyl and methyl esters groups along the chain. A combination of chemical, spectroscopic and chromatographic methods is commonly used to fully characterize pectic polysaccharides. In recent years, X-ray diffraction, circular dichroism, light scattering, electron and atomic force

microscopy and theoretical approaches has also been used in order to examine the fine structure and interactions of pectins. Although chromatography is primarily a separation technique, chromatography techniques are powerful tools to study the composition, DE, DA and molar mass of pectins [36].

5. Determination of molar mass by HPSEC technique with MALLS detector and RI

Size exclusion chromatography (also called gel permeation chromatography) has been widely used to analyze the molar mass distribution of polymers. The eluted fractions can be examined by different detection techniques such as: colorimetric methods, refractive index, ultraviolet, light scattering and viscosity measurements.

Size exclusion chromatography has been used to determine the molar mass of polysaccharides. In many instances, a calibration curve, previously obtained by using dextran molar mass standards, is used for this purpose. However, in size exclusion chromatography, molecules in solution are separated by their size rather than by their molar mass. Since the size is related to the conformation of the polysaccharide in a given solvent, the use of dextrans as molar mass standards is highly restricted. Experimentally, a problem in this approach is the absence of commercial molar mass standards for the different types of polysaccharides, including pectins.

The use of high performance size exclusion chromatography coupled to a differential refractometer (RI) and a multi-angle laser light scattering (MALLS) detector allows the study of the molar mass distribution of pectins as well as the determination of the absolute molar mass. While RI gives a signal proportional to the concentration, MALLS response increases with the concentration and molar mass. The MALLS instrument enables the determination of absolute molar masses of polymers from below 1000 g/mol to hundreds of millions without a calibration curve with reference standards. The molar mass is estimated directly from the angular dependence of scattered light intensity as a function of concentration, as formulated by light scattering theory. However, the molar mass calculation is only possible when the eluted fraction is detected by both RI and MALLS. An accurate determination of the refractive index increment with concentration, dn/dc , is required [37; 38]. Figure 1 displays a unimodal distribution of molar mass for a polysaccharide with $M_w 1.39 \times 10^6$ g/mol, determined by HPSEC-MALLS.

In addition to the average molar mass from HPSEC analyses coupled to light scattering detection it is also possible to obtain: a) the ratio M_w/M_n which is used as an index of polydispersity; b) the radius of gyration; c) the differential molar mass distribution plot which shows how much material (differential weight fraction) is; distribution for the same polysaccharide extracted using three different times of; extraction); d) the cumulative distribution of molar mass curve which gives for each molar mass, the weight fraction of material having molar mass less than the given molar mass (Figure 3 shows the cumulative distribution of molar mass for a same polysaccharide extracted using two different times of extraction); e) the log-log plot of root mean square radius of gyration as a function of molar

mass which gives information about the molecular conformation. Theoretical slopes of 0.33, 0.50 and 1.0 occur for spheres, random coils in theta solvents, and rigid rods, respectively. Usually, most real random coils have slopes in the range 0.55-0.6 [37]; f) using a viscometer connected in series with MALLS and RI in the HPSEC system, the intrinsic viscosity can be determined [40].

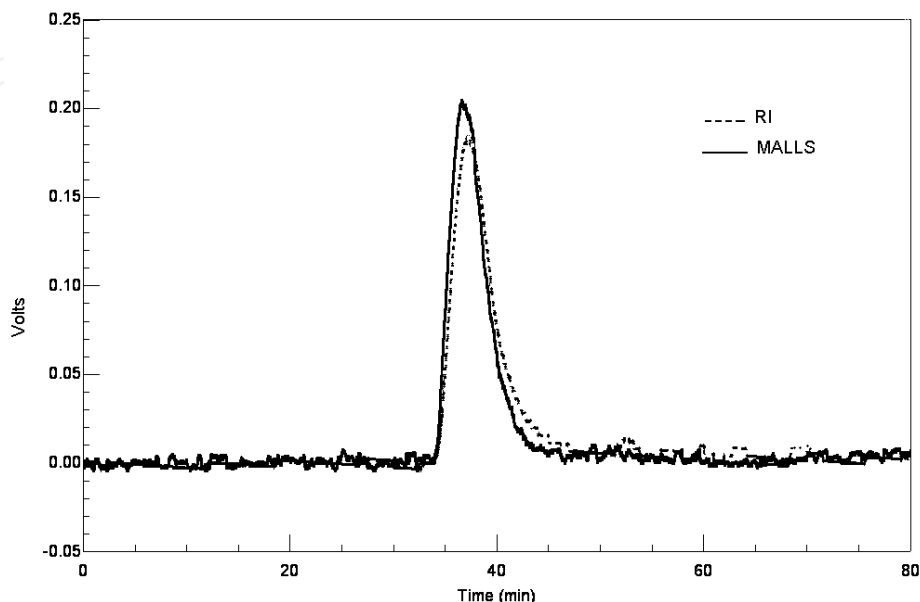


Figure 1. HPSEC elution profile of a polysaccharide isolated from seed [39].

A comparison between MALLS and LALLS detector in HPSEC has been shown to give results in very good agreement [40]

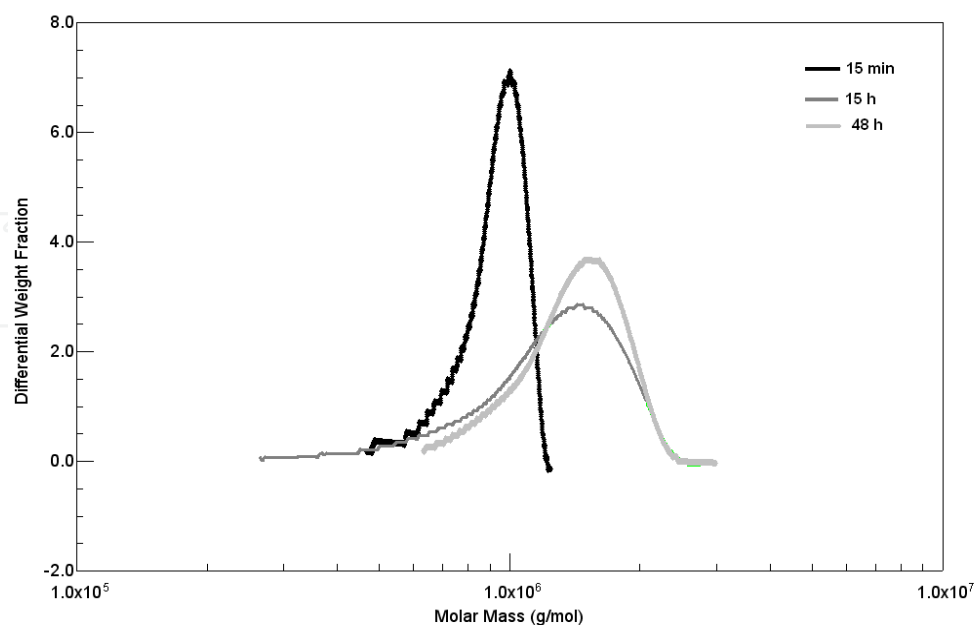


Figure 2. Differential molar mass distribution for a polysaccharide obtained after 15 min, 15 h, and 48 h of extraction [39].

HPSEC-MALLS/RI was used to compare the molar mass distribution of pectins obtained from the dried pomace of eleven apple cultivars [41]. Although the pectins were obtained under the same extraction conditions, some differences were identified among the fractions. According to the results, pectins from different variety of apple can display differences in their molar mass profiles as depicted in Figure 4 (for clarity only RI detector is shown).

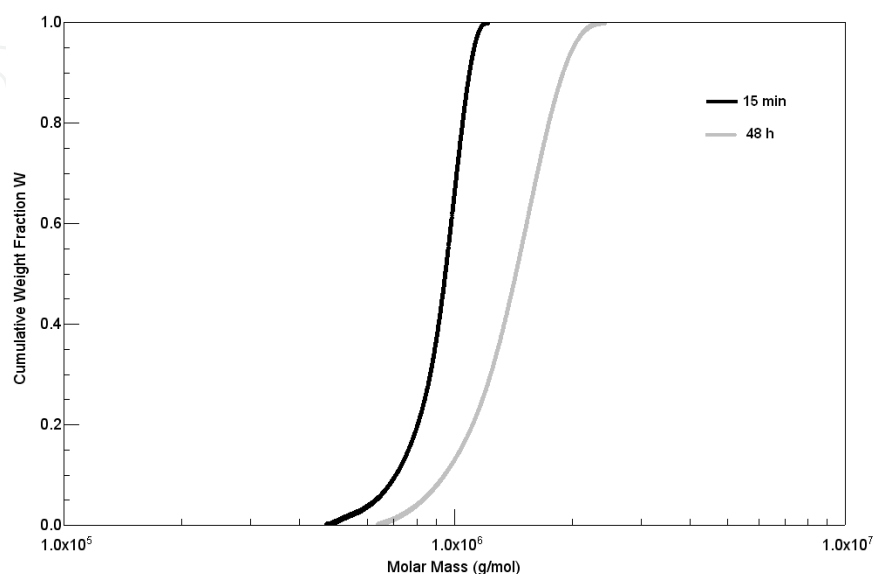


Figure 3. Cumulative molar mass distribution for a polysaccharide obtained after 15 min and 48 h of extraction [39].

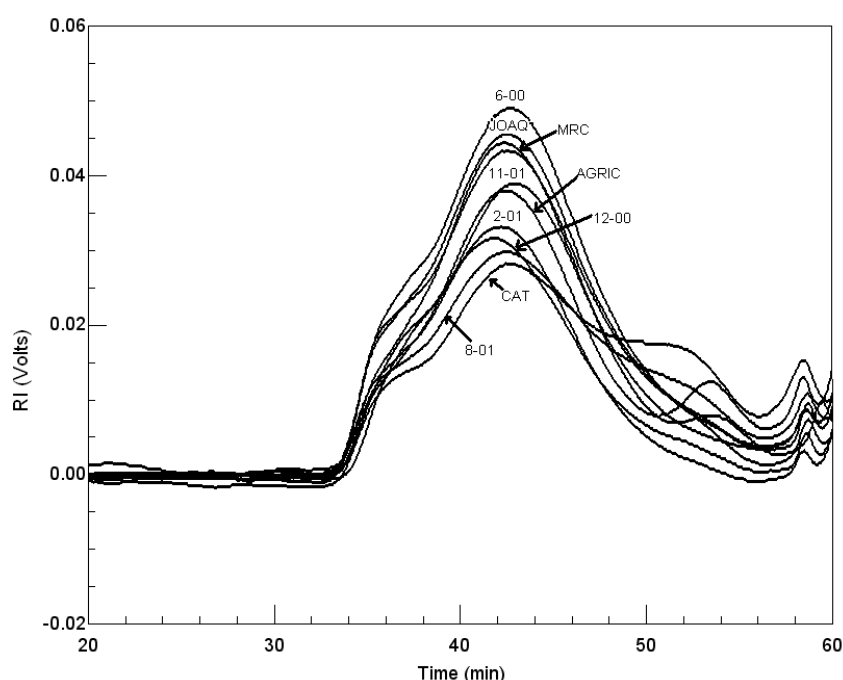


Figure 4. HPSEC elution profiles of pectins obtained from the dried pomace of eleven apple cultivars (columns: Waters Ultrahydrogel 2000/500/250/120 connected in series; eluent: 0.1M NaNO_2 solution, containing NaN_3 (0.5 g/l); flow: 1.5 mg/ml) [41].

6. Monosaccharide composition

Information on the composition of pectins can be obtained by different chromatographic methods. Regardless of the method used to determine the monosaccharide composition, the pectin under study needs to be previously hydrolyzed in order to release its monomeric units. It is well known that the glycosidic linkages with acidic monosaccharides are more resistant to hydrolysis. For pectins, the different sensitivities to acid hydrolysis are: GalA-GalA > GalA-Rha > Rha-GalA > neutral sugar-neutral sugar 42. The chosen conditions must be a compromise between maximum hydrolysis with minimal degradation.

Although the more simple chromatographic methods, such as paper chromatography (PC) and thin layer chromatography (TLC), can give only qualitative information about the composition of pectins, they can be useful to check the adequacy of the conditions of hydrolysis.

7. Determination of monosaccharide composition by GC

Gas-liquid chromatography (GC) is widely used for the analysis of carbohydrate. GC is used in the separation and analysis of complex mixtures of many components that can be vaporized without decomposition. For monosaccharides released after total hydrolysis, derivatization is necessary due to polar groups of carbohydrates which make them nonvolatile. Derivatization methods consist of the substitution of the polar groups of monosaccharides in order to increase their volatility. Many types of derivatives can be employed in the analysis of monosaccharide composition by GC, acetylated and silylated derivatives being the most popular. The advantages of acetylated derivatives include the presence of a single peak for each derivatized monosaccharide and their high stability. The derivatization reaction involves reduction with sodium borohydride followed by the acetylation itself. However, in the acetylation only the hydroxyl groups are derivatized. Complete quantification and identification of individual neutral and acidic sugars using acetylated derivatives can be accomplished by an additional step, which includes the carbodiimide-activated reduction of the carboxyl groups of uronic acids in order to give the corresponding neutral sugars [43]. Alternatively, GC can be used just to quantify the neutral monosaccharides, while titration or colorimetric method can be used to evaluate the amount of acidic units. This approach was chosen to study the composition of pectins from the dried pomace of eleven apple cultivars (Table 1) [41].

In GC analysis, compounds that have similar properties often have the same retention times. Sometimes extraneous background peaks can be a problem for the identification and quantification of monosaccharides present in minor amounts in complex mixtures. Gas chromatography associated to mass spectroscopy (GC-MS) can be used to overcome this difficulty. GC-MS combines two techniques to form a single method of analyzing mixtures of chemicals. For a pectic polymer, gas chromatography separates the monosaccharides derivatives present in the mixture and mass spectroscopy characterizes each of the derivatives individually by their mass fragments. MS has the advantages of high selectivity, specificity, and sensitivity 43.

Cultivar	Fraction (%)								
	AUA	MeO	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
1	46.90	5.88	0.65	0.12	3.33	1.31	2.23	2.51	37.3
2	51.20	6.49	0.85	0.31	4.70	3.21	0.05	4.67	28.9
3	50.90	6.34	0.82	0.32	4.70	2.46	4.10	2.94	27.5
4	51.60	6.67	0.01	0.01	3.48	3.55	9.55	5.11	24.7
5	51.60	6.70	0.96	0.23	4.23	2.50	2.99	2.37	28.5
6	51.30	6.66	1.35	0.54	5.05	3.90	4.31	3.46	23.4
7	53.40	6.93	1.08	0.33	4.88	3.00	3.25	4.38	22.8
8	50.30	6.35	2.09	0.74	8.54	3.78	3.95	3.14	21.2
9	50.40	6.30	0.34	0.00	4.79	3.86	3.43	4.31	25.4
10	48.70	6.06	0.91	0.20	3.76	2.00	2.78	3.27	32.4
11	52.90	6.72	1.08	0.35	5.03	2.90	3.96	4.68	22.4
Average	50.80	6.46	0.92	0.29	4.77	2.95	3.69	3.71	26.8
Standard Deviation	1.72	0.30	0.51	0.21	1.33	0.79	2.17	0.91	4.60
Coefficient of Variation (%)	3.39	4.62	55.57	72.00	27.86	26.82	58.92	24.44	17.21

Source: Sato et al., (2011)[41].

Table 1. Monosaccharide composition of pectins from the dried pomace of eleven apple cultivars. Neutral sugars were determined as alditol acetates by GC using a DB-210 capillary column (0.25 mm internal diameter x 30 m), film thickness 0.25 µm and flame ionization detector. Contents of uronic acids (AUA) were determined by titration.

When carboxyl-reduction of glycosyluronic acid is performed the use of sodium borodeuteride (NaBD₄) and GC-MS is preferred. The use of NaBD₄ provides an easily identified tag by MS analysis that allows the quantitative determination of the content of uronic acid as its corresponding neutral sugar [43].

GC-MS has also been used to identify and quantify unusual sugars present in RG-II. This approach is very useful, since commercial standards are not available for all unusual monosaccharides present in RG-II. The contents of DHA, KDO, aceric acid, 2-methyl-xylose and 2-methyl-fucose have been determined by GC-MS of their trimethylsilyl-esters *O*-methyl glycosides after acidic methanolysis and derivatization [43; 44].

8. Determination of monosaccharide composition by HPLC and HPAEC-PAD

High performance liquid chromatography (HPLC) is characterized by using high pressure to force chemical compounds to pass the column containing a stationary phase. Since monosaccharides released after total hydrolysis do not present enough volatility to be analyzed by GC, HPLC should be the most useful technique for the determination of monosaccharide composition. Monosaccharides cannot be detected by absorption due to the lack of chromophore in the molecular structure and are usually detected by refraction index detector. However, refraction index detector is sensitive to eluent composition and sample matrix, making the application of the gradient elution method limited and not allowing the complete separation of all monosaccharides [43].

Acid-catalysed or enzymatic hydrolysis followed by HPLC analysis to determine the GalA content in pectins has been proposed by different authors. When enzymes that release and specifically degrade pectins are used, the method can be useful for analysis of GalA in pectin samples as well as in the raw material that can be used for pectin extraction [45].

In recent years, high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) had been used to directly analyze the contents of carbohydrates. The development of this new technology had readily solved the above-mentioned problems. HPAEC-PAD can be used for simultaneous determination of monosaccharides and uronic acids. The method had been applied for the separation of GalA, GlcA, Rha, Fuc, Ara, Xyl, Man, Gal and Glc. Monosaccharides are weak acids with pK_a above 11. HPAEC uses NaOH as an eluent making sugars in their anionic form. In general, the elution sequence of monosaccharide is related to their pK_a value, and in the same conditions, the time of elution increases with the value of pK_a [46].

9. The use of chromatographic techniques for determining DA and DE

DE is usually determined, together with galacturonic acid content, by titration. However, the presence of acetyl can contribute to an overestimation of DE, since the acetic acid released during the saponification will be titrated. Chromatographic methods can be used for determination of DE and DA. An HPLC method for simultaneous determination of DE and DA was proposed by Voragen et al (1986) [47] and modified by Levigne et al (2002) [31]. In the former, an Aminex HPX-87H column is used while the second method uses a C18 column. The pectin is saponified and precipitated followed by methanol and acetic acid separation from the supernatant by HPLC and quantification by refractometry using an internal standard. The method allows accurate determination in a single run and within a short time, the DE and DA using low amounts of pectins (~ 5mg). This method presents some advantages when compared to that using FT-IR for DE calculation. FT-IR method requires pectins standards with different DEs in order to construct a calibration curve and the presence of acetyl can contribute to an overestimation of the DE.

Huisman et al. (2004) [48] proposed a method for DE determination using head-space GC. The approach is similar to that used in the HPLC method. Head-space GC is used for the quantification of methanol released from pectin by saponification. A lower amount of pectin is needed (~2mg) and the chromatogram shows a symmetrically shaped methanol peak which is very easy to integrate. However, the method is only applicable for the determination of DE and another approach has to be used in order to estimate DA.

10. Chromatographic techniques as an aid in the study of the fine structure of pectic polysaccharides

Chemical and enzymatic hydrolysis has been used to produce fragments of pectic polysaccharides in order to study the fine structure of pectins. The differences in the lability of glycosidic linkages to acid hydrolysis (see section 3.2) allowed the homogalacturonan region to

be isolated and its minimal length estimated. Under controlled conditions, acid hydrolysis of RG-I gives a fraction of intermediate molar mass that is rich in GalA and Rha. The neutral side chains are quickly hydrolysed to form low molar mass oligosaccharides [49–42].

RG-II was first isolated from cell walls using enzymatic treatment. The structure of RG-II was investigated using a combination of enzymatic and controlled acid hydrolysis. Using chemical fragmentation, four oligosaccharides from side chains were obtained [42].

The products from chemical and enzymatic hydrolysis are further fractionated by chromatographic techniques and structurally characterized. Usually, ion-exchange and size exclusion chromatography are used to fractionate the segments of pectic chains produced in the partial hydrolysis. The recovery of oligosaccharides with low degree of polymerization greatly facilitates the structural studies. Elucidation of the sequence of glycosyl units with the methods current available is only possible for oligosaccharides. This approach allowed the identification of xylogalacturonan in the modified hairy region of apple pectin [50].

One of the steps in the structural analysis of pectins is the determination of the glycoside linkages. Methylation analysis is one of the main tools to determine glycosidic linkages of polysaccharides and their oligomers. In this procedure, the free hydroxyl groups of pectins or their oligosaccharides are methylated. Then, the partially methylated material is hydrolysed and submitted to reactions to afford volatile derivatives. The products from this procedure are separated and analyzed using the chromatographic method of GC-MS [51].

11. Final considerations

Due to the need in recent years to seek alternatives that meet sustainable development, polysaccharides have emerged as source of sustainable products for fuel, food, materials and medicine. In this context, pectin is found in all higher plants and can be obtained from renewable sources. Although nowadays, commercial pectin is usually derived from citrus peel or apple pomace, other by-products from agroindustry can be used for pectin isolation. However, pectin preparations from non-traditional sources with a set of properties for specific applications require complete structural elucidation of the polymers. Chemical and enzymatic modification of pectins can be used to obtain new functionalities and again, an understanding of pectin fine structure–function relationships is required. Considerable progress has been made in the elucidation of the fine structure of pectin mainly due to the progress of chromatographic and spectroscopic methods; however a better understanding is necessary in order to be able to design pectins for specific industrial or biological applications either by chemical modification or genetic engineering. At the moment, apple pectin remains a well suited option for different applications.

List of abbreviation

FAO	Food and Agriculture Organization
HG	Homogalacturonan
RG-I	Rhamnogalacturonan I

RG-II	Rhamnogalacturonan II
DHA	Docosaheptaenoic acid
KDO	3-Deoxy-D-manno-oct-2-ulosonic acid
XGA	Xilogalacturonana
ARA-I	Arabinogalactan I
ARA-II	Arabinogalactan II
API	Apiogalacturonan
DE	Degree of methyl-esterification
DA	Degree of acetylation
RI	Differential refractometer
LALLS	Low angle laser light scattering
MALLS	Multi-angle laser light scattering
HPSEC	High Pressure Size Exclusion Chromatography
PC	Paper chromatography
GC	Gas-liquid chromatography
TLC	Thin layer chromatography
AUA	Uronic acids
GC-MS	Gas chromatography associated to mass spectroscopy
HPLC	High performance liquid chromatography
HPAEC-PAD	High performance anion exchange chromatography coupled with pulsed amperometric detection
FT-IR	Fourier transform infrared spectroscopy

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