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New Method for a Two-Step Hydrolysis and Chromatographic Analysis of Pectin Neutral Sugar Chains

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A new method for the determination of the main neutral sugars in pectin has been developed. The sample preparation involves a mild chemical attack followed by an enzymatic hydrolysis. The completeness and nondestructive character of the method are demonstrated by comparison of the results obtained with different acids such as H_2SO_4, HCl, and trifluoroacetic acid (TFA) at different concentrations (2, 1, or 0.2 M) at two temperatures (80 or 100 °C). The chemical hydrolysis of pectin neutral sugar chains with strong acid (1 or 2 M) and high temperature (100 °C) shows that the liberation of the pectin sugars is not realized at the same rate for each sugar. Different optimum conditions are thus obtained. However, the chemical pectin hydrolysis with 0.2 M TFA at 80 °C is characterized by the liberation of pectin neutral sugar side chains without any degradation within 72 h of hydrolysis. Under these conditions, the liberation of some pectin sugars, essentially galactose, glucose, and rhamnose, was not complete. An enzymatic hydrolysis is necessary to obtain a complete release of all the sugars. The combination of the two treatments, a chemical hydrolysis realized with diluted acid (0.2 M) for 72 h at low temperature (80 °C) on one hand and an enzymatic hydrolysis on the other hand, allow a total liberation of pectin sugars. The quantitative analysis of the carbohydrates is realized with accuracy, high selectivity, and sensitivity with high-performance anion-exchange chromatography with pulsed-amperometric detection. The sugars can be analyzed without any derivatization with a limit of quantification of 0.1 mM.

KEYWORDS: Pectin; chemical hydrolysis; enzymatic hydrolysis; ionic chromatography

INTRODUCTION

Pectic substances, commonly known as pectin, are complex mixtures of polysaccharides. The major component consists of α-D-galacturonic acid units linked by α(1→4) glycosidic bonds. In this main chain, β-L-rhamnose units are occasionally inserted through (1→4) and (2→1) glycosidic linkages and the carboxyl groups are partially esterified by methyl alcohol or neutralized with mono- or divalent cations. Other neutral sugars such as arabinose, galactose, glucose, xylose, and mannose may occur attached as side chains. The pectin carbohydrate composition is an important characteristic for the chemical characterization of these polymers and also for the study of their functional or biological properties.

Methods available for carbohydrate analysis of pectin were mainly based on determination of their constituent sugar residues obtained after chemical or less frequently after enzymatic hydrolysis (*1*). Several chemical procedures were used for the hydrolysis of the neutral sugar chains of pectin. They differ by the nature of the acid, its concentration, the time, and the temperature (*2*−*4*). The most commonly used hydrolytic agents were sulfuric acid (H_2SO_4) (*5*−*7*), trifluoroacetic acid (TFA) (*4*, *8*), hydrochloric acid (HCl) (*9*−*11*), sulfuric acid in absolute methanol (*12*), methanolic HCl (*7*, *11*, *13*, *14*), and hydrofluoric acid (*15*). Acid concentrations, temperatures, and hydrolysis times commonly used for pectin hydrolysis vary from 1 to 2 M, from 100 to 121 °C, and from 2 to 3 h, respectively. The conditions of hydrolysis selected were drastic, to cleave, in a short time, the different glycosidic linkages. These linkages do not present the same resistance toward acid (*3*, *4*). So the kinetics of liberation and degradation of the side-chain sugars of the pectin differ. Therefore, different optimum conditions of hydrolysis were determined (*5*, *8*, *11*). Up to now, many studies have been carried out on the chemical hydrolysis of neutral sugars contained in cell walls (*16*−*21*). The cell walls were composed of pectin and other polysaccharides such as cellulose, hemicellulose, and noncellulosic polymers. The hydrolysis kinetics differs significantly from one polymer to another. The hydrolysis was realized under drastic conditions with the objective of obtaining complete hydrolysis of the polymers before determination of their sugar composition.

After chemical or enzymatic hydrolysis of pectin, many procedures were commonly applied for the quantification of the liberated monosaccharides, such as colorimetric methods, gas/liquid chromatography (GLC), and high-performance liquid chromatography (HPLC). The first methods to be used for the

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estimation of the total amount of neutral sugar were the phenol−sulfuric acid and the orcinol methods. Their main disadvantages were the lack of specificity. In fact, both uronate and non-uronate sugar residues can develop the same color. Moreover, each pectin sugar gives a different degree of color intensity for the same concentration. The second and the third methods were sensitive analytical methods used to determine the composition of each neutral sugar of pectin. These procedures have disadvantages, mainly originating from the necessity for derivatization of the monosaccharides to alditol acetates (*8*, *22*) or to trimethylsilyl ethers (*9*, *22*) or from inadequate separation of the monosaccharides by HPLC (*10*, *11*, *23*). For example, GLC analysis of the reaction products of sugar pectin after methanolysis and after derivatization to the corresponding silyl derivatives (*9*, *24*) or directly by HPLC with pulsed-amperometric detection (*11*, *25*) often results in complex chromatograms with overlapping peaks and imprecise quantification of some sugars (*11*, *12*, *22*). The effectiveness of the hydrolysis method applied cannot be assessed after derivatization, as oligomers resulting from incomplete hydrolysis often escape detection. Thus, a considerable improvement in the accuracy of monosaccharide analysis was obtained by the development of high-performance anion-exchange chromatography (HPAEC) using pellicular resin and pulsed-amperometric detection (PAD) (*18*, *26*−*29*).

This work had two main objectives. The first one was to study the effect of chemical hydrolysis on the kinetics of liberation and degradation of neutral pectin sugar chains versus hydrolysis parameters such as the acid concentration, acid type, and temperature. The goal was the liberation of different pectin components without any degradation under the hydrolysis conditions. To improve the phenomenon of heteropolysaccharide hydrolysis, enzymatic pectin hydrolysis has also been studied. A combined chemical and enzymatic hydrolysis has been developed to give a nondestructive sample preparation method and complete sugar release. The second objective was to develop a sensitive and accurate method of sugar analysis by HPAEC-PAD and to compare this method to gas/liquid chromatography after derivatization of sugars to alditol acetates.

MATERIALS AND METHODS

Chemicals. 2-Deoxy-D-glucose, fucose, *myo*-inositol, L-rhamnose, L-arabinose, D-xylose, d-glucose, D-galactose, D-mannose, glucuronic acid, D-galacturonic acid, sodium borohydride, and sodium acetate trihydrate were purchased from Fluka Chemical Co. (Buchs, Germany). Sodium hydroxide natronlange 50% was purchased from JT Baker (Devenier, Holland). Sodium hydroxide pellets, acetic acid, acetic anhydride, dimethyl sulfoxide, and dichloromethane were purchased from Merck (Darmstadt, Germany).

Standard Solutions. Standard solutions containing sugars and uronic acids with concentrations from 0.1 to 1 mM were prepared from a 10 mM stock solution to confirm the linearity of the detector response and to determine the relative response factors.

Pectins. Highly methylated (HM) apple pectin (DM = 75%) was purchased from Fluka.

Enzymes. The enzyme used, VL9, was a commercial liquid preparation (Viscozyme L9) obtained from Novo Nordisk (Copenhagen, Denmark). This enzyme preparation was produced from a selected strain of *Aspergillus aculeatus*. It was a multienzyme complex containing a wide range of carbohydrases, including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase. A 1 mL sample of this solution was purified before use by ultrafiltration (1 h at 2000*g*) with the help of Centricon tubes (VivaSpin 20 mL concentrator; cutoff 30 kDa) to eliminate carbohydrates and glycerol. The retentate obtained was washed twice with 15 mL of acetate buffer (20 mM, pH 5) and finally diluted up to 100 mL in this buffer.

Chemical Hydrolysis. *H_2SO_4 Hydrolysis.* A 40 mg sample of apple pectins was hydrolyzed (for different times) with 0.2, 1, and 2 M H_2SO_4 (2.5 mL) at 80 and 100 °C. The reaction medium was neutralized with 1 mL of NH_4OH (14 M); 1 mL of *myo*-inositol, 2-deoxy-D-glucose, and glucuronic acid (2 mg/mL) was added as internal standard. This solution was adjusted to pH 7 and diluted to 25 mL. Aliquots of hydrolysate were taken and filtered through a 0.45 μm filter membrane before injection into the HPAEC-PAD instrument.

TFA and HCl Hydrolysis. A 40 mg sample of pectins was hydrolyzed respectively with (2.5 mL) 1 M TFA and HCl at 100 °C. Pectin hydrolysis was also realized with 0.2 M TFA at 80 °C. Then the hydrolysate was treated as in the case of H_2SO_4 hydrolysis.

Enzymatic Hydrolysis. The hydrolysis was optimized with a mixture of apple pectin (0.2% w/v), 2 mM 2-deoxy-D-glucose, *myo*-inositol, and glucuronic acid as internal standard and VL9 diluted 250-fold in 20 mM pH 5 sodium acetate buffer. The mixture was incubated at 50 °C. During the hydrolysis (0−72 h), aliquots of hydrolysate were taken and heated at 100 °C for 3 min to inactivate the enzymes and filtered through a 0.45 μm filter membrane before injection into the HPAEC-PAD instrument.

Combined Chemical and Enzymatic Hydrolysis. A 100 mg sample of apple pectin was subjected to treatment with 5 mL of 0.2 M TFA for 72 h at 80 °C. The final pH was adjusted to 5, and the hydrolysate was diluted to 25 mL. A 10 mL sample of this solution was mixed with 10 mL of VL9 diluted 250-fold in 20 mM pH 5 sodium acetate buffer and 2-deoxy-D-glucose, *myo*-inositol, and glucuronic acid (2mM) as internal standard. The mixture was incubated at 50 °C for 24 h and treated as described in the precedent procedure.

Analytical Methods. *HPSEC (High-Performance Size-Exclusion Chromatography).* To evaluate the polymer distribution, pectin and the hydrolysate (obtained after chemical and combined chemical and enzymatic pectin hydrolysis) were analyzed with an HPSEC system coupled on line with a differential refractive index (RI) detector. The HPSEC system consisted of a Waters 2690, a Waters RI 2410 differential refractive index detector, and a G300PWXL Progel TSK column (Tosohaas, Japan). This column (30 cm × 7.8 mm) has an exclusion limit between 1 and 50000 kDa using dextrans. All analyses were carried out at a temperature of 25 °C and at a flow rate of 0.7 mL/min. The eluant was 50 mM $NaNO_3$ containing NaN_3 (0.05% w/v) as a bactericide.

GLC analysis was carried out using a Hewlett-Packard HP 6890 series GC system, fitted with a hydrogen flame ionization detector. The monosaccharides liberated by the combined chemical and enzymatic hydrolysis were converted to alditol acetates according to the procedure of Blakeney et al. (*17*). Alditol acetate derivatives were separated in a high-performance capillary column, HP1 (length 30 m, internal diameter 0.5 mm, film thickness 0.25 μm). The injector (splitless mode) and detector temperatures were 250 and 300 °C, respectively. The oven temperature was initially at 120 °C, programmed to rise linearly at 4 °C/min until 220 °C after the separation of sugar and then reach 290 °C at a rate of 35 °C/min to condition the column. The carrier gas was helium.

HPAEC-PAD. The separation of different components of pectin was performed with a Dionex DX-500 Bio-LC system, using a CarboPac PA10 column (250 × 4 mm) in combination with a CarboPac guard column (25 × 4 mm), Dionex Corp., Sunnyvale, CA. All analyses are carried out at a temperature of 30 °C and a flow rate of 1 mL/min. The neutral monosaccharides were eluted isocratically using 4 mM NaOH for 35 min. But uronic acids were eluted using a gradient reaching 170 mM CH_3COONa and 100 mM NaOH. The column was washed with 500 mM NaOH for 15 min and reequilibrated with 4 mM NaOH for 20 min before the next injection. A 25 μL sample was injected. Detection was realized using a pulsed-amperometric detector with postinjection of 200 μL/min of NaOH, 900 mM. Potentials of E_1= 0.1 V, E_2 = 0.1 V, E_3 = 0.1 V, E_4 = −2 V, E_5 = −2 V, E_6 = 0.6 V, E_7 = −0.1 V, and E_8 = −0.1 V were applied for duration times of T_1 = 0 s, T_2 = 0.20 s, T_3 = 0.4 s, T_4 = 0.41 s, T_5 = 0.42 s, T_6 = 0.43 s, T_7 = 0.44 s, and T_8 = 0.50 s, respectively, at a sensitivity of 1 μC.

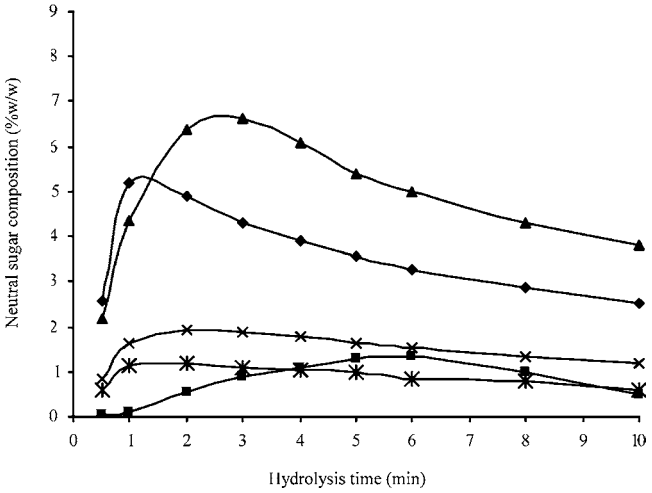

Figure 1. Chemical hydrolysis of neutral sugar chains of pectin with 1 M H_2SO_4 at 100 °C: arabinose (◆), rhamnose (■), galactose (▲), glucose (×), xylose (∗).

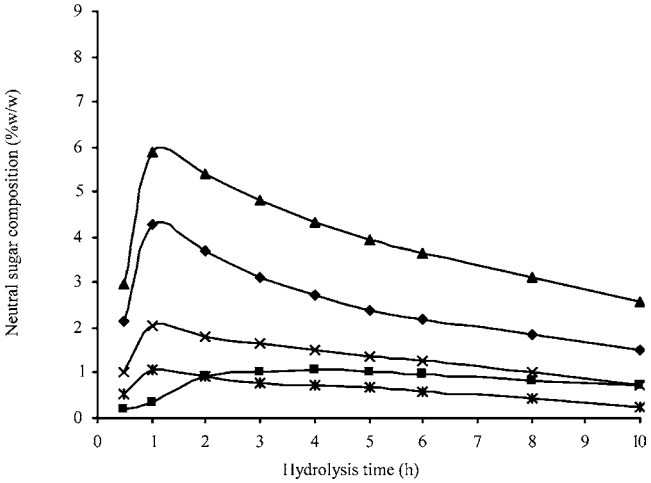

Figure 2. Chemical hydrolysis of neutral sugar chains of pectin with 2 M H_2SO_4 at 100 °C: arabinose (◆), rhamnose (■), galactose (▲), glucose (×), xylose (∗).

RESULTS AND DISCUSSION

Chemical Hydrolysis of Neutral Sugar Chains of Pectin. The results obtained after a chemical hydrolysis of neutral sugar chains of pectin with different acid types and concentrations at temperatures of 100 and 80 °C are shown.

Chemical Hydrolysis with 1 M H_2SO_4 at 100 °C. The results obtained after a chemical hydrolysis with 1 M H_2SO_4 at 100 °C of pectin neutral sugar chains are shown in **Figure 1**. The liberated sugar concentrations increase until reaching a maximal value before decreasing.

One can see that the release of the pectin sugars is not realized at the same rate for each carbohydrate. The different optima differ from one sugar to another. The arabinose side chains are the first to be hydrolyzed (*30*, *31*). The maximum concentrations are obtained after 1 h of hydrolysis for arabinose, 2 h for glucose, 2 h for xylose, 3 h for galactose, and 5 h for rhamnose. The reason for the longer time of liberation in the case of rhamnose is the strong link between this sugar and galacturonic acid in the principal building units of pectin. The most frequently used conditions of pectin hydrolysis to quantify the neutral sugars by GLC or HPLC were 1 M H_2SO_4 for 2−3 h at 100 °C (*6*, *11*, *32*, *32*−*35*). According to **Figure 1**, these conditions underestimate rhamnose and are not ideal to quantify with precision the proportion of the other neutral pectin sugars.

Chemical Hydrolysis with 2 M H_2SO_4 at 100 °C. The chemical hydrolysis of pectin with 2 M H_2SO_4 at 100 °C shows the same kinetics as that with 1 M H_2SO_4 (**Figure 2**). The liberation and degradation of sugar are faster than in the first case of hydrolysis (1 M H_2SO_4, at 100 °C). Galactose, xylose, and glucose have a maximum hydrolysis after 1 h contrary to rhamnose (3 h). The results obtained with the chemical hydrolysis at 2 M H_2SO_4 and 100 °C differ from those obtained by Selvendran et al. (*16*). These authors wrote that the yields of potato cell wall neutral sugars do not increase for periods of hydrolysis greater than 2 h except for rhamnose and glucose. The reason was the slower release of glucose from cellulose or noncellulosic polysaccharides.

Chemical Hydrolysis with HCl (1 M) and TFA (1 M) at 100 °C. Other acids such as hydrochloric acid (1 M) and trifluoroacetic acid (1 M) have been tested at 100 °C for the hydrolysis of apple pectin. The results obtained (**Figures 3** and **4**) with these acids are similar to those of the 1 M H_2SO_4 hydrolysis. But we have found that the trifluoroacetic acid causes less damage to sugars than H_2SO_4 or HCl especially in the case of rhamnose. The content of this sugar varies with the experimental conditions. This result is in agreement with those of Selvendran et al. (*16*, *37*) as well as those of Quemener and Thibault (*11*). However, Quemener and Thibault (*11*) thought that the yields

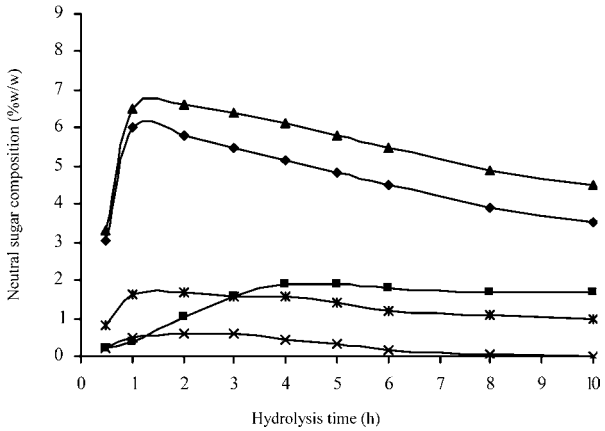

Figure 3. Chemical hydrolysis of neutral sugar chains of pectin with 1 M HCl at 100 °C: arabinose (◆), rhamnose (■), galactose (▲), glucose (×), xylose (∗).

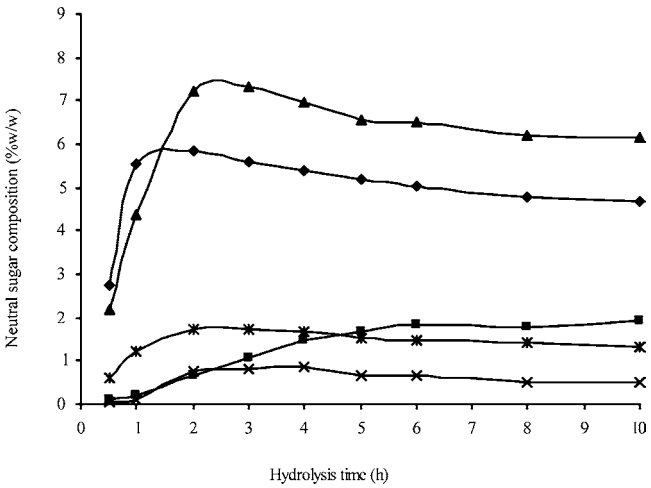

Figure 4. Chemical hydrolysis of neutral sugar chains of pectin with 1 M TFA at 100 °C: arabinose (◆), rhamnose (■), galactose (▲), glucose (×), xylose (∗).

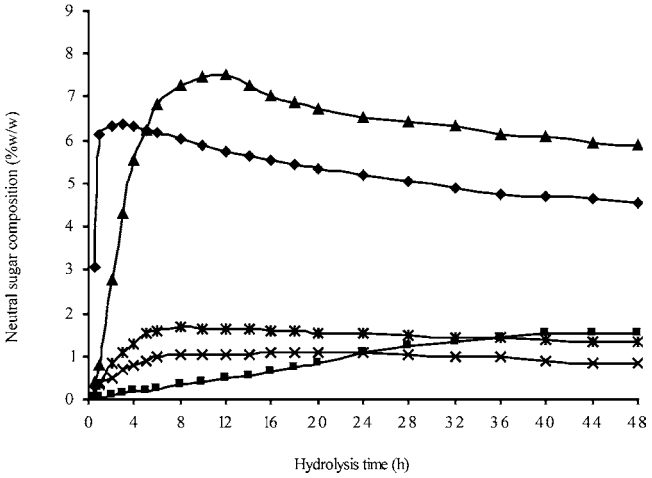

Figure 5. Chemical hydrolysis of neutral pectin sugar chains with 1 M H_2SO_4 at 80 °C: arabinose (◆), rhamnose (■), galactose (▲), glucose (×), xylose (∗).

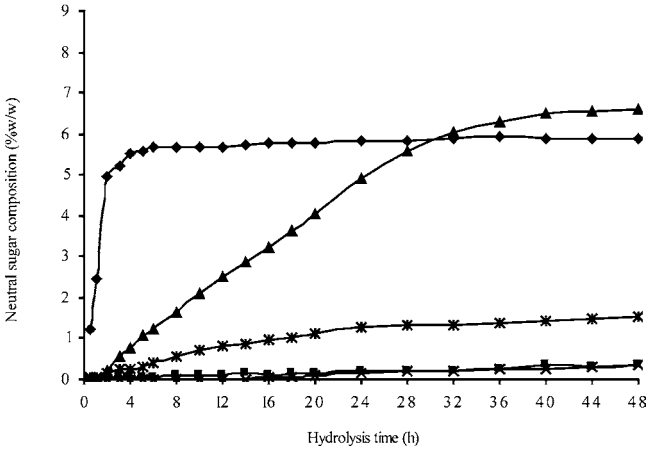

Figure 6. Chemical hydrolysis of neutral pectin sugar chains with 0.2 M H_2SO_4 at 80 °C: arabinose (◆), rhamnose (■), galactose (▲), glucose (×), xylose (∗).

of fucose, arabinose, xylose, mannose, galactose, and glucose released did not depend on the origin of the pectins nor on the conditions of hydrolysis. The maximum release for all these sugars was estimated within 1 h of hydrolysis.

Chemical Hydrolysis with 1 and 0.2 M H_2SO_4 at 80 °C. A milder chemical hydrolysis is realized at 80 °C to see the evolution of the liberation and the degradation of the pectin neutral sugar chains. The results of apple pectin hydrolysis with 1 M H_2SO_4 are represented in **Figure 5**.

Figure 5 shows the same kinetics of hydrolysis as those of the hydrolysis with 1 M H_2SO_4 at 100 °C. This hydrolysis is characterized by a slower liberation and degradation of pectin sugar side chains. Contrary to the other sugars, the content of rhamnose increases with the time of hydrolysis. However, the chemical pectin hydrolysis with 0.2 M H_2SO_4 at 80 °C is characterized by the liberation of pectin neutral sugar side chains without any degradation for the first 48 h (**Figure 6**) and even 72 h of hydrolysis (results not shown).

One can see that arabinose is always the first sugar to be released. Galactose residues are released more slowly, and xylose, which is present in significant amounts in apple pectin, is completely resistant to acid hydrolysis. These results are in agreement with those of Thibault et al. (*37*) when they used milder hydrolysis conditions (0.1 M HCl at 80 °C) for apple pectins. They highlight three fractions, each of which can be

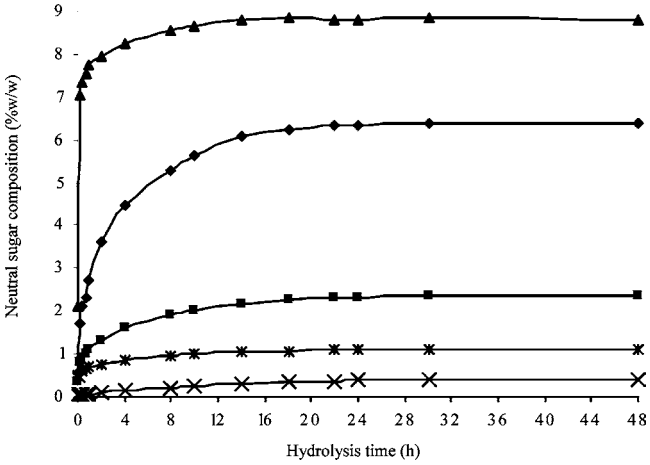

Figure 7. Enzymatic hydrolysis of pectin neutral sugar chains by VL9 (pH 5 at 50 °C): arabinose (◆), rhamnose (■), galactose (▲), glucose (×), xylose (∗).

related to one of the main structural features of pectins. The neutral side chains composed mostly by arabinans and arabinogalactans were rapidly split off and hydrolyzed to low molecular weight fragments. The rhamnose-rich backbone of the hairy regions formed a separate fraction with an intermediate size and a high rhamnose/galacturonic acid ratio. Finally, the smooth regions give galacturonans that remain insoluble. H_2SO_4 hydrolysis has been described as a superior hydrolysis procedure for carbohydrates (*18*). The removal of SO_4^{2-} ion after hydrolysis is difficult. It is inadequate for HPAEC analysis (*4*, *20*). These ions have a strong affinity for the quaternary ammonium group of the column CarboPac PA10 and consequently cause a decrease of column efficiency. For this reason we recommend the use of trifluoroacetic acid for pectin hydrolysis instead of H_2SO_4. Indeed, similar results have been obtained with 0.2 M TFA at 80 °C (results not shown).

Enzymatic Hydrolysis. Enzymatic hydrolysis of pectin neutral sugar chains by VL9 is studied. This mixture contains multiple enzyme activities, arabinase, α-L-arabinofuranosidase, β-D-galactosidase, galactanase, and xylanase (**Figure 7**) (*11*, *38*). However, the VL9 preparation requires purification as the chromatography of this solution reveals appreciable amounts of glucose, galactose, and mannose. The ultrafiltration of the solution by Centricon tubes completely removes low molecular weight sugars. In this way the purification is better than that by precipitation of proteins by ammonium sulfate used by Quemener and Thibault (*11*).

According to **Figure 7**, different plateaus are obtained for each sugar. A total of 18 h is needed for arabinose, 24 h for galactose, xylose, and rhamnose. The maximum concentrations of galactose and arabinose are higher than those obtained by a drastic chemical hydrolysis, contrary to that of xylose. However, compared to the other chemical hydrolysis, a better quantification of rhamnose is obtained.

Combined Chemical and Enzymatic Hydrolysis. The chemical pectin hydrolysis with 0.2 M TFA at 80 °C does not completely release some sugars, essentially glucose, galactose, and rhamnose. The concentrations of these sugars obtained are less than the ones reached after drastic hydrolysis. To enable us to obtain a complete hydrolysis of the sugar chains without any degradation, this chemical treatment was followed by an enzymatic hydrolysis with the VL9 enzymatic extract. This enables us to be sure to obtain a complete hydrolysis of sugar chains, which would not have been hydrolyzed by the chemical methods (0.2 M TFA at 80 °C).

Table 1. Composition of Apple Pectin Sugars (g/100 g) after Milder Chemical or Enzymatic Hydrolysis or a Combination of the Two Treatments[a]

neutral sugar	0.2 M H_2SO_4 at 80 °C	enzymatic hydrolysis with VL9	combined treatments
arabinose	5.9 (4.8)	6.4 (3.8)	6.7 (3.0)
rhamnose	0.4 (3.4)	1.7 (4.6)	1.9 (6.0)
galactose	6.6 (3.5)	8.8 (2.2)	9.2 (4.8)
glucose	0.3 (6.3)	1.1 (5.5)	1.8 (5.1)
xylose	1.5 (5.7)	0.4 (5.8)	1.8 (5.7)

[a] Values in parentheses are relative standard deviations (%); $n = 3$.

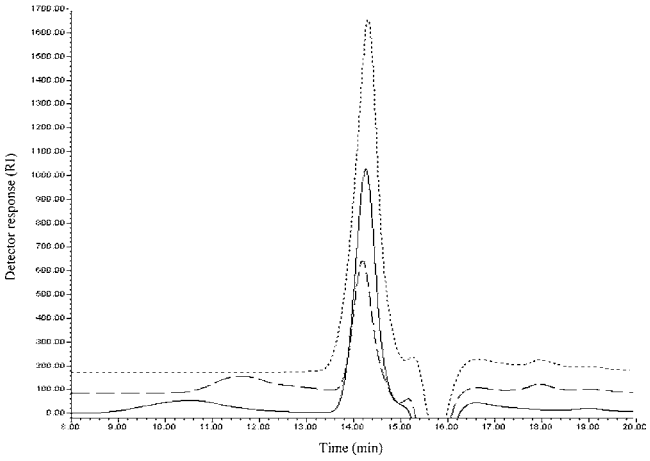

Figure 8. HPSEC of apple pectin before and after chemical or combined chemical and enzymatic hydrolysis: —, apple pectin; ---, 0.2 M, 72 h, 80 °C; •••, 0.2 M, 72 h, 80 °C, and VL9.

The combination of the two treatments, chemical hydrolysis with diluted acid (0.2 M) for 72 h at low temperature (80 °C) and enzymatic hydrolysis for 24 h, results in a better liberation of pectin sugars without degradation. Thus, a better quantification of these sugars is obtained (**Table 1**).

To prove the complete hydrolysis of pectin polysaccharide by this two-step hydrolysis, gel filtration chromatography of the reaction mixture after digestion has been used (**Figure 8**). Generally, two peaks can be observed: the first one represents a different fraction of pectin substances rich in galacturonic acid, and the second one is a peak very rich in neutral sugars and other components (ions, ...) and always eluted at the total volume of the column. After pectin chemical hydrolysis with 0.2 M TFA at 80 °C for 72 h, pectin was hydrolyzed partially. This is confirmed by the displacement of the first peak, from high molecular weight toward weaker molecular weights. Finally, combined chemical and enzymatic pectin hydrolysis allows us to hydrolyze completely the different fractions of pectin in free galacturonic acid units and neutral sugars (low molecular weight).

Table 2. Determination of the Composition of Apple Pectin Sugars after Combined Chemical and Enzymatic Hydrolysis Using Deoxyglucose, *myo*-Inositol, or Glucuronic Acid as Internal Standard and GLC or HPAEC-PAD[a]

	GLC		HPAEC-PAD	
neutral sugar	sugar compos[b] (g/100 g)	sugar compos[c] (g/100 g)	sugar compos[b] (g/100 g)	sugar compos[d] (g/100 g)
arabinose	6.8 (6.8)	10.5 (11.0)	6.1 (1.7)	6.7 (3.0)
rhamnose	1.2 (8.5)	1.6 (10.0)	1.4 (5.5)	1.9 (6.0)
galactose	9.0 (6.0)	13.0 (6.0)	8.5 (5.3)	9.2 (4.8)
glucose	2.0 (8.0)	2.7 (8.7)	1.8 (4.7)	1.8 (5.1)
xylose	1.7 (4.0)	2.6 (9.0)	1.6 (3.5)	1.8 (5.7)
mannose	0.3 (9.3)	0.4 (12.0)	0.1 (8.0)	0.1 (6.0)

[a] Values in parentheses are relative standard deviations (%); $n = 3$. [b] Deoxyglucose as internal standard. [c] *myo*-Inositol as internal standard. [d] Glucuronic acid as internal standard.

This two-step hydrolysis approach is very similar to the one published by Jones and Albersheim (*39*). However, a drastic chemical hydrolysis with 0.2 N trifluoroacetic acid for 1 h at 120 °C followed by an enzymatic hydrolysis has been used. We have seen yet that degradation of pectin neutral sugar occurs at temperatures up to 100 °C. Moreover, the enzyme preparation is secreted by the phytopathogenic fungus (*Sclerotium rotfsii* Sac.) and not commercially available.

GLC and HPAEC−PAD Assays. Once the conditions of hydrolysis are defined, it is important to choose a technique for the quantification of pectin sugars. GLC is often used (after sugar derivatization into alditol acetates) and recently HPAEC−PAD. A complete separation of derivated neutral sugars by GLC and of intact neutral sugars and uronic acids by HPAEC-PAD has been obtained (**Figures 9** and **10**). However, Jones and Albersheim (*39*) separated uronic acids and aldoses by GLC after a series of reactions. The disadvantage of this method is the necessity to separate aldonic acids from alditols with Dowex-1 (acetate form) ion-exchange resin after reduction of aldoses and uronic acids with $NaBH_4$. Moreover, the analysis of these sugar residues by GLC is realized separately.

Table 2 represents the results of the same sample obtained with three different internal standards and GLC or HPAEC-PAD. This sample is hydrolyzed with combined chemical

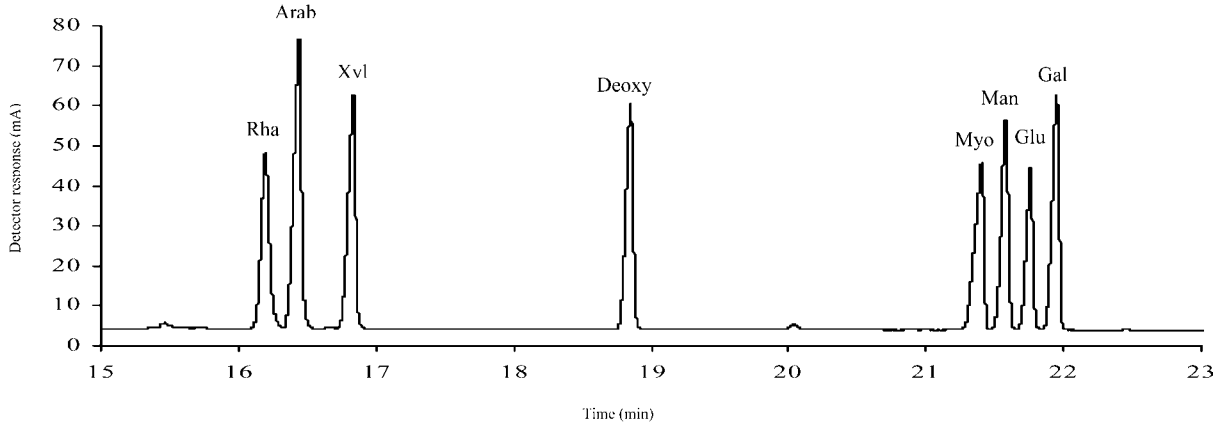

Figure 9. GLC chromatogram of a standard solution containing neutral sugars (Rha, rhamnose; Ara, arabinose; Xyl, xylose; Deoxy, deoxyglucose; Myo, *myo*-inositol; Man, mannose; Glc, glucose; Gal, galactose).

Table 3. Relative Response Factors of Neutral Sugars and Uronic Acids Using Glucuronic Acid or Deoxyglucose as Internal Standard[a]

	Deoxy	Arab	Rha	Gal	Gluc	Xyl	Man	galac acid	gluc acid
glucuronic acid as internal std		1.56 (3.6)	1.21 (2.1)	2.40 (0.1)	2.48 (2.0)	1.88 (4.0)	1.86 (3.0)	0.66 (2.1)	1.00
deoxyglucose as internal std	1.00	1.06 (4.0)	0.78 (3.3)	1.47 (4.0)	1.50 (2.5)	1.20 (6.0)	1.01 (5.8)	0.64 (3.3)	

[a] Values in parentheses are relative standard deviations (%); $n = 6$.

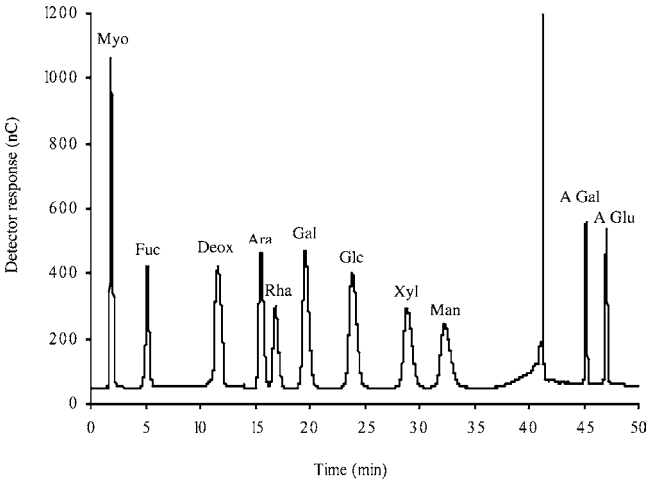

Figure 10. HPAEC-PAD chromatogram of a standard solution containing neutral sugars (Myo, *myo*-inositol; Fuc, fucose; Deoxy, deoxyglucose; Ara, arabinose; Rha, rhamnose; Gal, galactose, Glc, glucose; Xyl, xylose; Man, mannose) and uronic acids (Agal, galacturonic acid; Aglu, glucuronic acid).

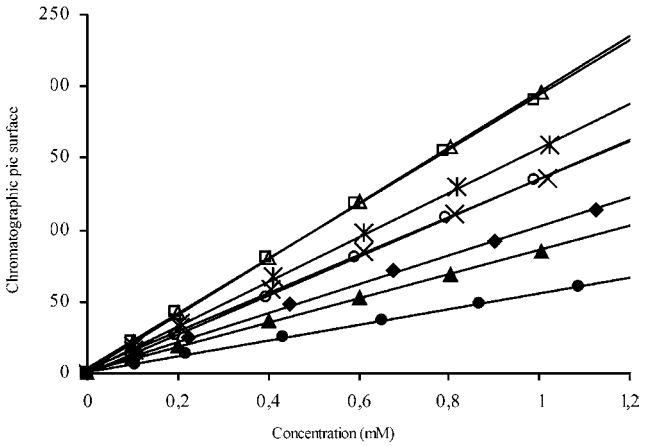

Figure 11. Intensity of the responses of ionic chromatography according to the concentration of the pectin components: rhamnose (◆), arabinose (×), galactose (△), glucose (□), xylose (∗), mannose (○), galacturonic acid (●), glucuronic acid (▲), deoxyglucose (+).

hydrolysis with diluted acid (0.2 M) for 72 h at low temperature (80 °C) and enzymatic hydrolysis for 24 h.

We observe that the use of different internal standards for sugar quantification by GLC or HPAEC-PAD gives similar values except for *myo*-inositol. This difference can be attributed to the derivatization of this compound, which is different from that of the other sugars. Moreover, in the HPAEC-PAD method, *myo*-inositol is eluted in the void volume of the column. So, the use of deoxyglucose or glucuronic acid as internal standard for sugar quantification is recommended. Selvendran et al. (*16*) analyzed the hydrolysates of the cell wall materials of potatoes and α-cellulose of parenchyma of runner beans by GLC and HPLC methods. Deoxyglucose was used as internal standard. They found that the results of GLC compare favorably with those of ion-exchange chromatography. Moreover, Quemener and Thibault (*11*) obtained the same results when they analyzed using HPLC (dimethyl L-tartrate as internal standard) or GLC (*myo*-inositol as internal standard) the composition of different neutral pectin sugars after a preliminary enzymatic hydrolysis followed by methanolysis or after hydrolysis of pectin with 2 M TFA acid for 3−4 h at 120 °C, respectively.

Compared to GLC, the main advantage of HPAEC-PAD is the determination of intact monosaccharides without any derivatization. Indeed, derivatization greatly increases the number of steps and therefore time required to assay for neutral sugar. Also, incomplete derivatization of neutral sugar occurs, which results in lower recoveries. It is emphasized that the derivatization procedure, not GLC technology, was the major limitation in neutral sugar analysis.

HPAEC-PAD has been described as a selective and sensitive technique by many authors (*4*, *20*, *29*). A complete separation of neutral sugars and uronic acids is obtained when we use CarboPac PA10. Salvador et al. (*20*), using a CarboPac PA1 column, showed the incomplete separation of arabinose and rhamnose on one hand and xylose and mannose on the other hand. With a CarboPac PA100 column arabinose and rhamnose are coeluted.

The linearity of the method has been studied on a six-point calibration curve from 0.1 to 1.0 mM ($r^2 = 0.998$) for all the pectin components (sugars and uronic acids). The calibration curves are forced through the origin (**Figure 11**).

To determine the detection and quantification limits, 10 double injections from 0.01 to 0.1 mM standard solutions containing deoxyglucose, arabinose, rhamnose, galactose, glucose, xylose, mannose, galacturonic acid, and glucuronic acid were analyzed. A 0.02 mM concentration is considered to be close to the detection limit (S/N ratio 2). A 0.1 mM concentration is considered to be the quantification limit (S/N ratio 10) for sugars and uronic acids. The method is validated with respect to the repeatability of relative response factors after six repeated injections of standard solution (day-to-day precision) (**Table 3**).

According to **Table 3**, galacturonic acid has a very low response compared to the neutral sugars, as previously reported (*4*, *20*, *29*).

Conclusions. The chemical hydrolysis of pectins with strong acid (1 or 2 M) and at high temperature (100 °C) seems to combine two simultaneous phenomena: first, the release of sugars as a product of hydrolysis of the pectin and, second, their degradation under the action of the acid and the heat. Under these conditions of hydrolysis the liberation of the different pectin sugars does not occur at the same rate. Therefore, there are different optimum parameters for each sugar. On this basis, it can be concluded that the hydrolysis conditions frequently used are not recommended for accurate quantification of the composition of pectin neutral sugars.

The use of trifluoroacetic acid for hydrolysis of pectin causes less damage to sugars than that of H_2SO_4 and HCl especially in the case of rhamnose. Also this acid has the advantage to stay in the column with high efficiency. The chemical pectin hydrolysis by 0.2 M TFA at 80 °C does not completely liberate some sugars, essentially galactose, glucose, and rhamnose. The

concentrations of the sugars obtained were less than those reached after drastic hydrolysis. The enzymatic pectin hydrolysis with VL9 shows that it contains rhamnogalacturonase, β-D-galactosidase, and α-L-arabinofuranosidase activities. Other xylanase, mannosidase, and glucosidase activities exist but in lower quantities. The combination of the two treatments, chemical hydrolysis realized with diluted acid (0.2 M) for 72 h at low temperature (80 °C) on one hand and enzymatic hydrolysis on the other hand, has for a consequence a total liberation of pectin sugars without any degradation. The determination of the carbohydrates is realized without any derivatization by HPAEC with PAD. This method is characterized by accuracy, high selectivity, and a limit of quantification of 0.1 mM for sugars and uronic acids. Finally, the combined milder chemical−enzymatic hydrolysis and HPAEC-PAD methods give, with high repeatability, a higher quantification of pectin sugars.

The determination of galacturonic acid appears to be of great importance to improve total hydrolysis of pectin. For this reason, we studied for the same sample the effect of chemical and/or enzymatic hydrolysis on the kinetics of liberation and degradation of galacturonic acid pectin chains, and we analyzed this acid by HPAEC-PAD. The results of these investigations are the subject of a future paper.

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