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Food Chemistry 96 (2006) 477–484
Food Chemistry
www.elsevier.com/locate/foodchem

Kinetic of the hydrolysis of pectin galacturonic acid chains and quantification by ionic chromatography

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Received 8 October 2004; received in revised form 2 March 2005; accepted 4 March 2005

Abstract

These experiments were designed to develop a rapid, repeatable and accurate analysis method for the quantification of galacturonic acid of pectins. Different pectin hydrolysis procedures (chemical and enzymatic) were carried out with H_2SO_4, TFA and HCl at different acid concentrations (0.2, 1 and 2 M) and temperatures (80 and 100 °C). Enzymatic and combined chemical and enzymatic hydrolysis of pectin were also studied. A acid hydrolysis under drastic conditions (100 °C) is insufficient for complete hydrolysis and results in low recovery of galacturonic acid residues. Mild chemical hydrolysis (0.2, 1 and 2 M H_2SO_4 72 h at 80 °C) is also insufficient for complete depolymerization. Its main advantage is cleavage of the galacturonic acid chains into oligomeric forms without any degradation within 72 h of hydrolysis. However, enzymatic hydrolysis with VL9 for 2 h at 50 °C and combined chemical and enzymatic hydrolysis (0.2 M TFA at 80 °C for 72 h and VL9) give high recovery of this acid. Analysis of the liberated sugar residue by HPAEC allows us to determine the galacturonic acid composition of this polysaccharide accurately with high selectivity and sensitivity in one assay without the need for derivatization.

Keywords: Pectin; Galacturonic acid; Chemical and enzymatic hydrolysis; HPAEC-PAD analysis

1. Introduction

Analysis of pectic carbohydrates in plant is difficult because of the varied and complex matrix of non uronide carbohydrates associated with these materials. Sugar, starch, cellulose and other non uronide carbohydrates can be present in large quantities in samples such as fruits, vegetables, juices and jellies. Fractional extraction, precipitation, and other separation and purification techniques are commonly used to separate and/or characterize pectic material from these extraneous carbohydrates. Neutral sugars and galacturonic acid are the major constituents of pectin chains.

Several procedures are available for hydrolysis of the pectic substances to uronic acid and neutral monosaccharide components. Methods involving acids (Biermann, 1988; De Ruiter, Schols, Voragen, & Rombouts, 1992; Leitão, Alarcão Silva, Januário, & Azinheira, 1995; Marga, Freyssac, & Morvan, 1995; Nesser & Schweizer, 1984; Selvendran, March, & Ring, 1979; Quemener & Thibault, 1990; Quemener, Lahaye, & Thibault, 1993) and enzymes (Ford, 1982; Garleb, Bourquin, & Fahey, 1991; Leitão et al., 1995; Matsuhashi & Hatanaka, 1992; Rumpunen, Thomas, Badilas, & Thibault, 2002) have been reported. The differences in the results suggest that hydrolytic techniques should be significantly improved.

Acid hydrolysis of pectin to galacturonic acid requires prolonged treatment with acid to achieve complete hydrolysis and, once released, the galacturonic

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doi:10.1016/j.foodchem.2005.03.002

acid is subject to degradation, forming lactones in irreproducible amounts (Blake & Richards, 1968). The conditions recommended for acid hydrolysis have been found to be far from ideal for the quantitative analysis of the uronate content in acidic polysaccharides (Leitão et al., 1995).

Enzymatic pectin hydrolysis is a better technique for hydrolysis of this polymer without degradation. However, this procedure needs different types of enzyme activities such as pectolytic, hemicellulolytic and carbohydratases for the efficient degradation of pectin (Ford, 1982; Garleb et al., 1991; Leitão et al., 1995; Matsuhashi, Inoue, & Hatanaka, 1992).

Following hydrolysis, various methods are available for quantification of galacturonate. The colorimetric carbazole sulfuric reaction was first reported by Dische (1947) and later modified by others for use in pectin analysis (Bitter & Muir, 1962; Gregory, 1960; Stark, 1950). It is based on the analysis of the degradation products (furfoique derivated) obtained from galacturonic acid after pectin hydrolysis with concentrated acid at high temperature. These compounds react then with carbazole and form color complex. The coloration obtained is proportional of the galacturonic acid concentration. However, this method is subject to interference from the non uronide carbohydrates associated with pectin samples and removal of these interferences often entails considerable manipulation (Joslyn & Chen, 1967; Kinter & Van Buren, 1982; McComb & McCready, 1952; Selvendran, 1975). This has caused difficulty in obtaining reliable uronide measurements for samples containing high levels of these substances.

The *m*-hydroxydiphenyl sulfuric acid assay is similar to the carbazole method but the *m*-hydroxydiphenyl reagent has the advantages of being more specific for uronates and less sensitive to non uronate neutral sugar interference (Blumenkrantz & Asboe-Hansen, 1973; Thibault, 1979). Although the *m*-hydroxydiphenyl method has been used by many workers, this method has also proved to be influenced by neutral sugars at a high level (Kinter & Van Buren, 1982). An improved procedure, using a combination of the carbazole (Galambos, 1967) and *m*-hydroxydiphenyl methods (Blumenkrantz & Asboe-Hansen, 1973), has recently been proposed by FlisettiCozzi and Carpita (1991); neutral sugars interference are virtually eliminated by this method. Besides neutral sugar interference, Penman and Sanderson (1972), Scott (1979) and Matsuhashi et al. (1992) recognized that both the carbazole and the *m*-hydroxydiphenyl reagents react differently with galacturonates in the free and conjugated forms. As a result, these methods will overestimate the galacturonate content of pectin.

Gas liquid chromatographic (GLC) procedures are also available for quantification of uronic acids. Jones and Albersheim (1972) successfully separated and detected galacturonate and glucuronate following conversion to their alditol acetates. Recoveries of galacturonate were 84% and glucuronate 92% as corresponding alditol acetates. They indicated that the failure to achieve 100% recoveries resulted from opening of the aldono-lactone ring prior to reduction. GLC of the trimethylsilyl derivatives has also been used to identify and quantify glucuronate and galacturonate (Ford, 1982).

The HPLC technique used in the determination of acid sugars in complex mixtures of polysaccharides has not been successful due to the difficulty of releasing the units that constitute the polymer (Garleb et al., 1991; Hicks, Lim, & Haas, 1985). This technique has been used to determine the composition of neutral sugars and galacturonic acid of pectin (De Ruiter et al., 1992; Leitão et al., 1995; Matsuhashi et al., 1992; Rumpunen et al., 2002; Quigley & Englyst, 1994; Quemener & Thibault, 1990). It is a sensitive analytical method, simple, accurate, and particularly useful for routine pectin analysis. The HPLC method is superior to GLC method. Sample preparation is much less tedious for HPLC compared to GLC, which requires that the uronic acids be derived prior to separation and detection. Also, incomplete derivatization may occur which would result in lower quantification. It should be emphasized that hydrolytic procedure, not HPLC technology, is the major limitation in uronic acid analysis.

Up to now, uronic acids of pectin have been determined as anhydrogalacturonic acid by spectrophotometry, using the metadihydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973; Thibault, Renard, Axelos, Roger, & Crépeau, 1993), decarboxylation (Theander & Aman, 1982) or titrimetry (Schultz, 1965). All these methods can lead to the error in galacturonic acid quantification.

For this reason, the aim of the present work is to develop analysis method for rapid, repeatable and accuracy quantification of intact galacturonic acid without degradation of pectic substances. The HPAEC-PAD had been used as analytical technique for the quantification of galacturonic acid after pectin hydrolysis.

In this context, three different types of hydrolysis have been carried out in order to obtain the complete release of galacturonic acid from apple pectin: acid or enzymatic or combined chemical and enzymatic hydrolysis.

In the first part of this work, the hydrolysis conditions were investigated. We studied the kinetic of liberation and degradation of galacturonic acid chains as a function of chemical hydrolysis parameters such as acid concentration, type and temperature. Finally, enzymatic pectin hydrolysis and combined chemical and enzymatic hydrolysis were also studied. In the second part of this work, we compared two analytical techniques: the colorimetric *m*-hydroxydiphenyl method and the HPAEC-PAD.

2. Materials and methods

Chemicals: Glucuronic acid, D-galacturonic acid, sodium acetate trihydrate, *m*-hydroxydiphenyl, sodium tetraborate and apple pectin are purchased from Fluka chemical Company (Buchs, Germany); sodium hydroxide natronlauge 50% from JT Baker (Devenier, Holland); sodium hydroxide pellets from Merck (Darmstadt, Germany).

Pectins: High methoxyl (HM) apple pectin (DM = 75%); Low methoxyl (LM) citrus pectin (DM = 9%). Sugar beet pectin is extracted in our laboratory at pH 1.5 at 85 °C for 4 h. It is characterized by 58.7% of DM, 28.9% of DA and 6.0% (w/w) of neutral sugars. Chicory pectin is extracted in our laboratory at pH 1.5 at 85 °C for 30 min. It is characterized by 48.7% of DE and 9.1% (w/w) of neutral sugars.

Standard solutions: Standard solution containing galacturonic acid and glucuronic acid with concentration 1 mM are prepared from a 10 mM stock solution to determinate the relative response factors.

Enzymes: The enzyme used, VL9, is a commercial liquid preparation (Viscozyme L9) obtained from Novo Nordisk (Copenhagen, Denmark). This enzyme preparation is produced from a selected strain of *Aspergillus aculeatus*.

2.1. Chemical hydrolysis

All experiments are duplicated.

H_2SO_4 hydrolysis: 10 mg of apple pectins are hydrolyzed (for different times) with 0.2, 1 and 2 M of H_2SO_4 (2.5 ml) at 80 and 100 °C, respectively. The reaction medium is neutralized with NH_4OH (14 M); 2 ml glucuronic acid (10 mM) is added as internal standard. This solution is adjusted pH 7 and diluted to 25 ml. Aliquot of hydrolysate are taken and filtered through a 0.45 μm filter membrane before injection in HPAEC-PAD.

TFA and HCl hydrolysis: 10 mg of pectins are hydrolyzed, respectively, with (2.5 ml) 1 M TFA and HCl at 100 °C. Then, the hydrolysate is treated as in the case of H_2SO_4 hydrolysis.

2.2. Enzymatic hydrolysis

The hydrolysis is optimized with a mixture of apple pectin (0.02% w/v), 0.5 mM glucuronic acid as internal standard and VL9 diluted 500-fold in 20 mM pH 5 sodium acetate buffer. The mixture is incubated at 50 °C. During the hydrolysis (0 h up to 72 h), aliquots of hydrolysate are taken and heated at 100 °C for 3 min to inactivate the enzymes and filtered through a 0.45 μm filter membrane before injection in HPAEC-PAD.

2.3. Combined chemical and enzymatic hydrolysis

10 mg of apple pectin are subjected to treatment with 5 ml of 0.2 M TFA for 72 h at 80 °C. The final pH is adjusted at pH 5 and the hydrolysate is diluted to 25 ml. 20 ml of this solution are mixed with 20 ml of VL9 diluted 500-fold in 20 mM pH 5 sodium acetate buffer and contained glucuronic acid (2 mM) as internal standard. The mixture is incubated at 50 °C for 24 h and treated as described in the precedent procedure.

2.4. Citrus, beet sugar and chicory pectin hydrolysis

2.4.1. Enzymatic hydrolysis

2 ml of pectin solution (1 g/l) was hydrolyzed with 2 ml of VL9 diluted 500-fold in 20 mM pH 5 sodium acetate buffer and contained glucuronic acid (2 mM). The hydrolysate is treated as in the case of enzymatic hydrolysis.

2.4.2. Combined chemical and enzymatic hydrolysis

2 ml of pectin solution (1 g/l) was hydrolyzed with 0.4 M TFA (2 ml) at 80 °C for 72 h. The hydrolysate is adjusted pH 5 with NH_4OH (14 M) and diluted to 25 ml. 2 ml of this solution is treated as previously.

2.5. Analytical methods

Spectrophotometric procedure: 2.4 ml of sulfuric acid/sodium tetraborate solution (476 mg sodium tetraborate in 100 ml 36 N sulfuric acid) were added to 0.4 ml of sample (1 g/l) in test tubes and cooled in an ice bath. Samples were vortexed and heated at 80 °C for different times (0, 1, 2, 3, 4, 5, 6, 8 and 10 h). After cooling in an ice bath, 0.04 ml of *m*-hydroxydiphenol reagent (150 mg *m*-hydroxydiphenol in 100 ml 0.125 N NaOH) were added to each tube. For each sample, absorbance at 520 nm was read at different times (0 min, 30 min and 1 h 30 min) on a Ultrospec 4000 UV/Visible Spectrophotometer (Pharmacia, Cambridge, England). A blank sample in which the hydroxydiphenol reagent was replaced by 0.04 ml 0.125 N NaOH was also prepared.

HPAEC-PAD: The separation of galacturonic acid is done using a Dionex DX-500 Bio-LC system, with a CarboPAc PA10 column (250 * 4 mm) in combination with a CarboPac guard column (25 * 4 mm), Dionex corp., Sunyvale, CA. All determinations are carried out at a temperature of 30 °C and at a flow rate of 1 ml/min. The neutral monosaccharides are coeluted isocratically using 100 mM NaOH for 5 min. Uronic acids are eluted using a gradient reaching 170 mM CH_3COONa ($3H_2O$) and 100 mM NaOH for 13 min. The column is washed with 100 mM NaOH for 10 min before the next injection. 25 μl of sample is injected. Detection is realized using a pulsed amperometric detector with post-injection of 200 μl/min 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 are applied for duration times $T_1 = 0$ 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.

2.6. Statistical analysis

Results of galacturonic acid of citrus, sugar beet and chicory pectin obtained by enzymatic and chemical and enzymatic hydrolysis were evaluated by a standard analysis of variance (Single factor ANOVA) followed by Fisher's protected LSD post-hoc test at 0.05 significance level, whenever applicable.

3. Results and discussion

3.1. HPAEC-PAD assays

We used HPAEC-PAD as an accuracy technique for the quantification of galacturonic acid and pectin sugars. A complete separation of uronic acids has been obtained when we used carboPac PA10 (Fig. 1).

3.2. Chemical hydrolysis of galacturonic acid chains of pectin

The different results obtained by chemical hydrolysis of galacturonic acid chains of pectin with different acids, concentrations at temperatures 80 and 100 °C are shown as follows:

3.2.1. Influence of molarity acid

The results (Fig. 2) show that recoveries of free galacturonic acid after treatment with 0.2, 1 and 2 M sulfuric acid at 100 °C are the highest after 18, 6 and 3 h, respectively. For longer hydrolysis time, the destruction rate of free galacturonic acid is greater than the release rate from the polymer. The release of galacturonic acids was considerably accelerated with higher concentrations of sulfuric acid. Under these conditions of hydrolysis, maximum 30% (w/w) of the total galacturonic acid is quantified. This shows clearly that this procedure alone is not efficient for glycosidic linkage cleavage (Leitão et al., 1995; Quigley & Englyst, 1994). The reason for that is that once released from the pectin, the galacturonic acid is subject to degradation, forming lactones in irreproducible amounts (Blake & Richards, 1968). Garna, Mabon, Wathelet, and Paquot (2004) showed similar effect on the liberation of pectin neutral sugars under drastic hydrolysis conditions. A different optimum was obtained for each carbohydrate. So, these conditions are not ideal to quantify, with precision, the galacturonic acid and pectin neutral sugars.

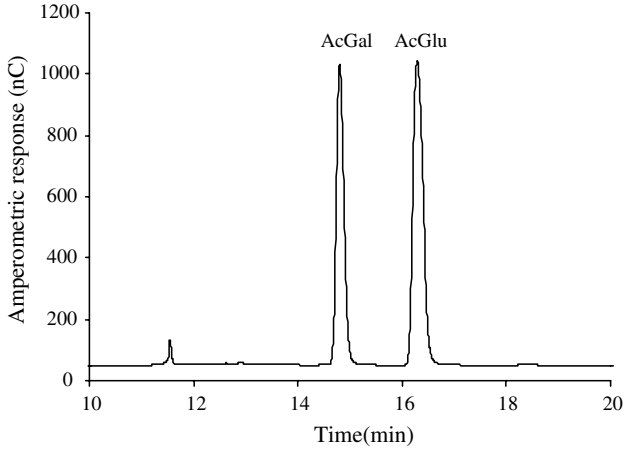

Fig. 1. HPAEC-PAD chromatogram of a standard solution containing uronic acids (1 mM). Operating conditions for HPAEC-PAD: flow rate, 1.0 ml/min; injected sample: 25 μl. Peak identity: AcGal, galacturonic acid; AcGlu, glucuronic acid.

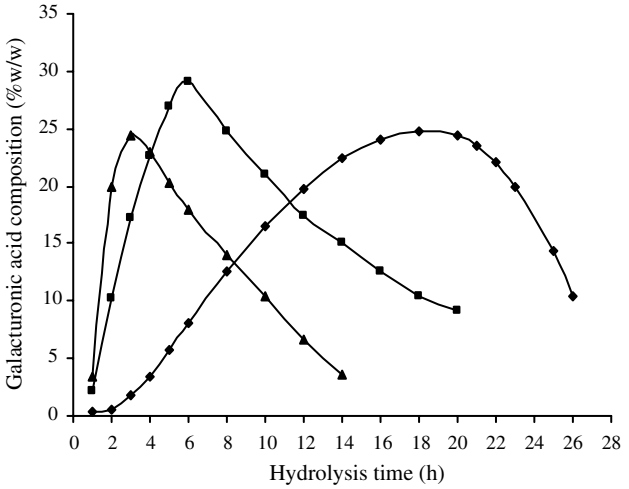

Fig. 2. Recovery of galacturonic acid (g per 100 g dry sample) from apple pectin subjected to treatment with 0.2, 1 and 2 M H_2SO_4 at 100 °C versus different hydrolysis times : 0.2 M H_2SO_4 (♦), 1 M H_2SO_4 (■), 2 M H_2SO_4 (▲).

3.2.2. Influence of acid type

Similar results are obtained, when apple pectin is hydrolyzed with hydrochloric acid (1 M) or trifluoroacetic acid (1 M) at 100 °C (Fig. 3). 40% (w/w) and 30% (w/w) of the total galacturonic acid is quantified, respectively, after 5 h hydrolysis with 1 M HCl and after 6 h hydrolysis with 1 M TFA or 1 M H2SO4. However, we observed that trifluoroacetic acid causes less damages to galacturonic acid than H_2SO_4 and HCl.

3.2.3. Influence of temperature

The chemical hydrolysis of pectin with 0.2, 1 and 2 M H_2SO_4 at 80 °C is characterized by the liberation of galacturonic acid without any degradation for the first 48 h (Fig. 4) and even 72 h of hydrolysis (results not

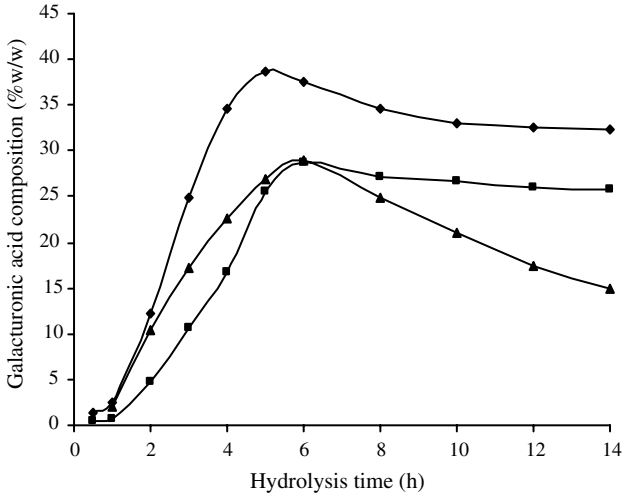

Fig. 3. Recovery of galacturonic acid (g per 100 g dry sample) from apple pectin subjected to treatment with 1 M TFA, HCl and H_2SO_4 at 100 °C versus different hydrolysis times : 1 M TFA (■), 1 M HCl (◆), 1 M H_2SO_4 (▲).

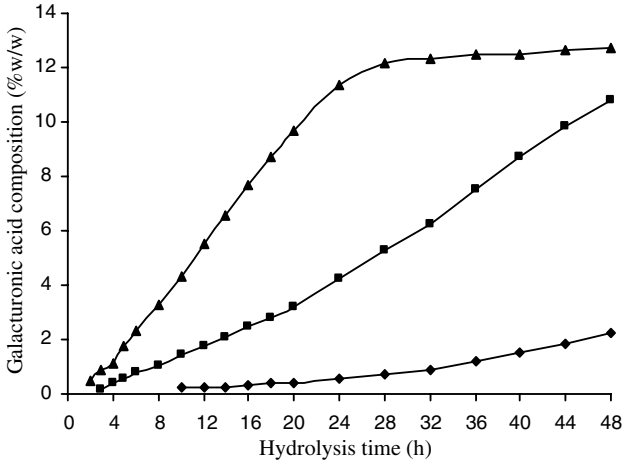

Fig. 4. Recovery of galacturonic acid (g per 100 g dry sample) from apple pectin subjected to treatment with 0.2, 1 and 2 M H_2SO_4 at 80 °C versus different hydrolysis times : 0.2 M H_2SO_4 (◆), 1 M H_2SO_4 (■), 2 M H_2SO_4 (▲).

shown). The galacturonic acid recoveries of apple pectin within 48 h of hydrolysis is of 2%, 10% and 12% with 0.2, 1 and 2 M H_2SO_4, respectively. Compared with hydrolysis in drastic conditions at 100 °C, lower recoveries are obtained, which is due to the higher resistance of the galacturonic linkages under milder hydrolysis conditions (Biermann, 1988; De Ruiter et al., 1992).

Under these conditions, Garna et al. (2004) showed that pectin neutral sugar chains hydrolysis with 1 M H_2SO_4 at 80 °C, contrary to 0.2 M H_2SO_4 for 72 h at 80 °C, is characterized by a slower liberation and degradation of pectin sugars side chains. Thibault et al. (1993) used these milder hydrolysis conditions (0.1 M HCl at 80 °C) for apple pectin. They highlight three fractions, which can each be related to one of the main structural features of pectins. The neutral side chains composed mostly by arabinans and arabinogalactans are 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.

3.3. Enzymatic hydrolysis

In contrast to the acid hydrolysis, enzymatic preparation led to higher yields of galacturonic acid (66%w/w), which indicates a more complete depolymerization of polygalacturonic chain from apple pectin (Fig. 5). Massiot, Thibault, and Rouau (1989) and Quemener and Thibault (1990) reveal that the enzymatic preparation used (VL9) contain multiple activities mainly polygalacturonase, pectin esterase and a range of hemicellulolytic activities. The latter are especially appropriate for efficient degradation of pectin. VL9 is poor in pectin lyase and devoid of pectate lyase.

Enzymatic approaches to estimate total content of galacturonic acid have previously been used and advocated (Leitão et al., 1995; Matsuhashi et al., 1992; Rumpunen et al., 2002; Quemener & Thibault, 1990). The enzymes from different origin such as SP 249, Rapidase Press, Driselase, Rohament P, petinex 3XL and pectinex ultra SPL were used for pectin hydrolysis. All these enzymes except the Rohament, give a high yield of galacturonic acid. The reason is that they contain various kinds of enzymes able to degrade plant polysaccharides. However, there are some studies that compare

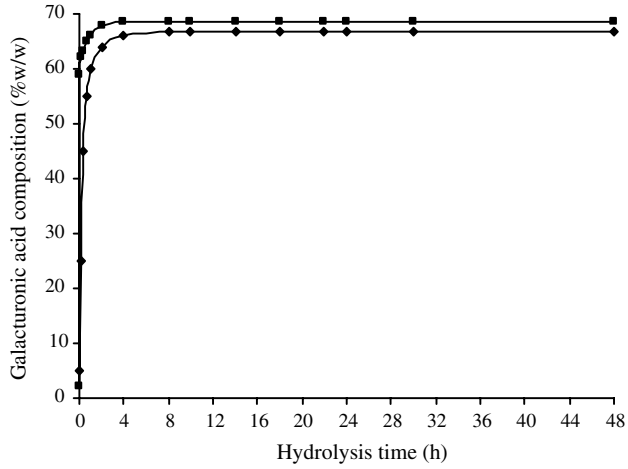

Fig. 5. Release of galacturonic acid from apple pectin subjected to treatment with Viscozyme L9 (VL9) at pH 5 and 50 °C or with combined chemical realized with 0.2 M TFA for 72 h at 80 °C, followed by enzymatic hydrolysis at pH and temperature described previously: VL9 (◆), 0.2 M TFA + VL9 (■).

these enzymes on the basis of galacturonic acid quantification.

3.4. Combined chemical and enzymatic hydrolysis

The content of galacturonic acid obtained in our experiments with combined chemical and enzymatic hydrolysis method (Garna et al., 2004) is close (68% w/w) to those obtained by enzymatic hydrolysis (Fig. 5), but is very different to those obtained by the mild chemical hydrolysis. According to these results, enzymatic hydrolysis with VL9 for 2 h at 50 °C can be used to quantify the galacturonic acid of pectin without degradation. However, if all the glycidic fraction must be analyzed, we showed in our previous work (Garna et al., 2004) that a purification step of the enzymes should be carried out to eliminate contaminants, specially the low molecular weight carbohydrates present in solution. Moreover, this procedure must be combined with chemical hydrolysis realized with diluted acid (0.2 M TFA) for 72 h at low temperature (80 °C) on the one hand and enzymatic hydrolysis for 24 h at 50 °C on the other hand in order to complete pectin depolymerization into monomer components.

Following Garna et al. (2004), the combined chemical and enzymatic hydrolysis gives a high recovery of different pectin neutral sugars, especially for galactose, xylose and glucose, compared to chemical or enzymatic hydrolysis. The presence of xylose and glucose could be explained, in part, by the xyloglucans which have been shown to be associated with pectic material (Selvendran & O'Neill, 1987) or by xylogalacturonan (Kikuchi, Edashige, Ishii, & Satoh, 1996; Schols, Bakx, Schipper, & Voragen, 1995) or eventually by co-products extracted during the industries production of pectins.

3.5. Validation of the analysis method

To validate our analysis method for galacturonic acid quantification, we studied its application on others sources of pectins such as citrus, sugar beet and chicory. These polysaccharides are hydrolyzed with VL9 and with combined chemical and enzymatic hydrolysis. The results of this study are presented in Tables 1 and 2. We observed in the case of citrus and chicory pectin

Table 1
Content of galacturonic acid (% w/w) in citrus, sugar beet and chicory pectin determined by enzymatic hydrolysis with Viscozyme L9 (VL9) for 2 h at pH 5 and 50 °C and chemical hydrolysis with 0.2 M TFA for 72 h at 80 °C, followed by enzymatic hydrolysis as before (values in parenthesis are relative standard deviation in %; $n = 3$)

	VL9	0.2 M TFA + VL9
Citrus	77.4 (0.4)	78.2 (2.1)
Sugar beet	52.9 (1.0)	56.7 (1.8)
Chicory	74.0 (1.1)	76.0 (1.9)

Table 2
Analysis of variance (Single factor ANOVA) evaluating the enzymatic hydrolysis and the combined chemical and enzymatic hydrolysis for estimation of content of galacturonic acid in citrus, sugar beet and chicory pectin

	SS	*df*	MS	*F*	*P*-Value
Citrus	1.5	1	1.5	0.87	0.4
Sugar beet	25.63	1	25.63	12.74	0.02
Chicory	6.615	1	6.615	3.95	0.12

that there is no significant difference between method (citrus pectin: $P = 0.4$; chicory pectin: $P = 0.12$, Table 2). Contrary, the sugar beet pectin we have obtained a significant difference between method ($0.01 < P (0.02) < 0.05$). This is probably due to the high content of acetic acid in sugar beet pectin which can limit the enzyme action to depolymerize completely pectin. So, we can recommend using combined chemical and enzymatic hydrolysis for the estimation of the content of galacturonic acid in sugar beet pectin.

3.6. Spectrophotometric method and HPAEC-PAD assays

Nowadays, colorimetric *m*-hydroxydiphenyl method is commonly used to quantify galacturonic acid in pectin. Many authors have studied interferences of neutral sugars and proteins that influence the uronic acid quantification. Therefore, the sample preparation differs from one author to another (especially in thermal treatment of tubes). In the procedure of Blumenkrantz and Asboe-Hansen (1973), tubes were heated in a water bath at 100 °C for precisely 5 min, compared to 4 min at 80 °C in Thibault method (1979). Fig. 6 represents the coloration kinetic of the pure galacturonic acid with

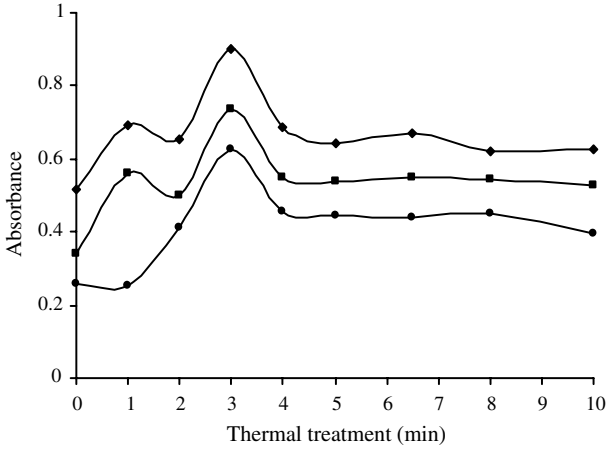

Fig. 6. Kinetic of the galacturonic acid (0.1 g/l) reaction in the *m*-hydroxydiphenyl method (galacturonic acid solution is treated with 36 N H_2SO_4 at 80 °C for 0 min up to 10 min). Absorbance measurement at 520 nm at 0 min (◆), 30 min (■) and 1 h 30 min (▲) after addition of *m*-hydroxydiphenyl.

m-hydroxydiphenyl at 80 °C and the absorbance measurements at 0 min, 30 min and 1 h 30 min after addition of *m*-hydroxydiphenyl. We can see that the maximum of absorbance is obtained after 3 min, and then decreases to become stable after 1 h 30 min. Kinter and Van Buren (1982) attributed these variations to the formation of gas bubbles and recommended that absorbance measurements be taken within 1 h, compared to 5 min for Blumenkrantz and Asboe-Hansen (1973).

These results show the influence of the moment chosen to measure the absorbance and the fluctuations of this method when analyzing a pure fraction. Divergent results observed in the literature could be explained by the choice of this method (Matsuhashi et al., 1992; Rumpunen et al., 2002; Scott, 1979).

These results show that sample preparation can greatly influence the analysis of galacturonic acid in pectin. For this reason we recommend the use of HPAEC-PAD for the galacturonic acid analysis. With this method galacturonic acid can be analyzed accurately with high selectivity and sensitivity without any derivatization.

4. Conclusions

We showed that acid hydrolysis of pectin into galacturonic acids with strong acid (0.2, 1 or 2 M) at high temperature (100 °C) seems to be far from ideal to cleave the linkages of galacturonic chains and to quantify with accuracy the content of this compound. This type of hydrolysis seems to combine two simultaneous phenomena: firstly, the release of galacturonic acid as a product of hydrolysis of the pectin and secondly, its degradation under the action of the acid and the heat.

The use of trifluoroacetic acid for hydrolysis of pectin galacturonic acid chains causes less damages to this compound compared to H_2SO_4 and HCl.

A lower recovery of galacturonic acids is obtained by a chemical pectin hydrolysis with 0.2 and 1 M H_2SO_4 at 80 °C. This result shows that these hydrolysis conditions do not completely liberate galacturonic acid from pectin chains. So, galacturonic acid linkages are more stable and resistant to mild chemical hydrolysis.

Enzymatic and combined chemical and enzymatic hydrolysis of pectin, give high recoveries of galacturonic acid. Compared to enzymatic hydrolysis, combined hydrolysis takes more time and needs a purification of the enzymatic preparation VL9 to eliminate contaminants such as glucose, galactose and mannose. However, this method is very useful for the total liberation of pectin neutral sugar chains and thus for simultaneous analysis of galacturonic acid and monosaccharides.

The spectrophotometric method using *m*-hydroxydiphenyl is the most common method to quantify free acids, lactones and uronic acids in soluble polymers. With this method, values for individual uronic acids are not obtained. The handling of hot, concentrated sulfuric acid still needs special precautions with respect to personal safety and laboratory equipment. Hence, this assay is difficult to apply routinely and not easy to use for large numbers of samples. An additional complication is that the presence of galacturonates in the free and conjugated forms, or a high concentration of neutral sugars or proteins may cause erratic results. Our method, enzymatic hydrolysis, does not show all these inconveniences.

The titrimetric method was also used to determine the galacturonic acid composition of pectin. However, the presence of impurities such as salts (cation) that were attached to the galacturonic acid in the pectin samples can lead to a lower quantification of this component.

High-performance anion-exchange chromatography (HPAEC) using pellicular resin and pulsed-amperometric detection (PAD) is a selective and sensitive method for uronic acids analysis. They are analyzed accurately without any derivatization.

In conclusion, the enzymatic hydrolysis and HPAEC-PAD method for galacturonic acid analysis presents three major advantages. Firstly, galacturonic acid is liberated without any degradation thanks to the hydrolysis method. Secondly, more accuracy and repeatability is provided by the HPAEC-PAD. Thirdly, the reagents are more in use.

Acknowledgments

This research has been supported by the ''Ministère de la Région Wallonne, Direction Générale de l'Agriculture''. The authors are grateful to Mme C. Robert, Mr. R. Novakov, Mr. Y. Muhovski and Mr. H. Legros for their assistance.

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