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# Variability in cell wall preparations: quantification and comparison of common methods

Catherine M.G.C. Renard*

*Unité de Recherches Cidricoles—Biotransformation des Fruits et Légumes, Institut National de la Recherche Agronomique, BP 35327, F-35653 Le Rheu Cédex, France*

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**Abstract**

Cell wall materials were prepared from apple tissue by seven different procedures, some of which are methods in current use in laboratories. The yield, the composition and the fractionation patterns of the pectin content, following sequential extraction in water, chelating agent (CDTA) and a pectin lyase treatment, was compared for each CWM. Variability of the yields and compositions of the CWM were small and few differences were statistically significant. There were differences in the partitioning pattern of the pectin during fractionation but also a high standard deviation of the yields between repetitions.


*Keywords:* Apple; *Malus domestica* Borkh.; Pectin; Chelating agent; Pectin-lyase; Procyanidin

## 1. Introduction

To isolate the cell walls from a given plant tissue, one must remove the cell contents, macromolecular as well as small molecules, and inactivate endogenous enzymes with minimal alteration of the cell wall components (Fry, 1988; Selvendran & O'Neill, 1987). There are many methods used, indeed almost each laboratory will have its own procedure but they belong to three types. In most of the early work on cell wall, alcohol (ethanol or methanol) or acetone extractive-free residues were used, which lead to co-precipitation of intracellular macromolecules. Simple extraction with water or buffer has also been used but may result in marked modifications due to endogenous enzymes. Selvendran and O'Neill advised in 1987 the exclusive use of aqueous solvents, with a preliminary step of milling in detergents such as sodium dodecylsulfate or sodium deoxycholate, followed by phenol:acetic acid:water. This procedure, which was widely adopted, has been shown to alter pectin solubility and calcium content of cell walls (Huber, 1991), and use of buffered phenol was advised. A drawback of the phenol is its toxicity. Mafra et al. (2001) therefore proposed replacing phenol by 1-propanol.

In our laboratory, we commonly test apple varieties for their polyphenol contents and have developed strategies for sampling and conservation, to compare varieties, modes of culture, maturation stages, etc. We wanted to know how to apply or adapt these methods for studies of the cell walls and to have an indication of the inherent robustness of comparisons. We aim to assess in this work the reproducibility and ease of a number of procedures for cell wall preparations, discern characteristic artefacts if any and in particular compare methods starting from fresh and freeze-dried material. We start from ripe apple fruits, a parenchyma with a relatively low protein content but rich in polyphenols, and at a maturity stage such that they are devoid of starch.

## 2. Materials and methods

### 2.1. Apples

Apples of the cultivar 'Golden delicious' were purchased at a local supermarket. They were homogenous qua size and apparent maturity. Ten apples were tested by the visual iodine test (Reid, Padfield, Watkins, & Harman, 1982)

* Tel.: +33 223 485 217; fax: +33 223 485 210.
*E-mail address:* crenard@lerheu.rennes.inra.fr, crenard@rennes.inra.fr

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and found to be devoid of starch. Three triplicates of 10 apples were constituted. Each apple was peeled, cored, and dropped into an antioxidant bath (ascorbic acid, 2 g/L). Apples were then divided as follows: each apple was cut vertically into eight equal portions; each of the eight portions was cut in two vertically, then again in two lengthwise giving 32 pieces per apple. These 32 pieces were divided between eight containers in such a way that each container received two top and two bottom pieces, coming each from one quarter of the apple (see Fig. 1). The eight containers were as follows: one dropped in boiling ethanol; one dropped in cold ethanol; six frozen; out of those six, two were freeze-dried.

### 2.2. Cell wall preparations

*Hot alcohol insoluble solids* (AISh; Renard, Voragen, Thibault, & Pilnik, 1990). The fresh slices were dropped in hot ethanol (three times the weight of slices) and left for 20 min at boiling (temperature: 82–85 °C). They were filtered off in a sieve, ground by six successive bursts of 15 s in a Braun kitchen processor and washed repeatedly with cold 70% ethanol in a G3 sintered glass filter (20 min incubation before filtration each time) until the filtrates were sugar-free (presence of sugars tested by the phenol–sulfuric method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956)). They were then dried by solvent exchange with 96% ethanol (three times) and acetone (three times), then overnight in an oven at 40 °C.

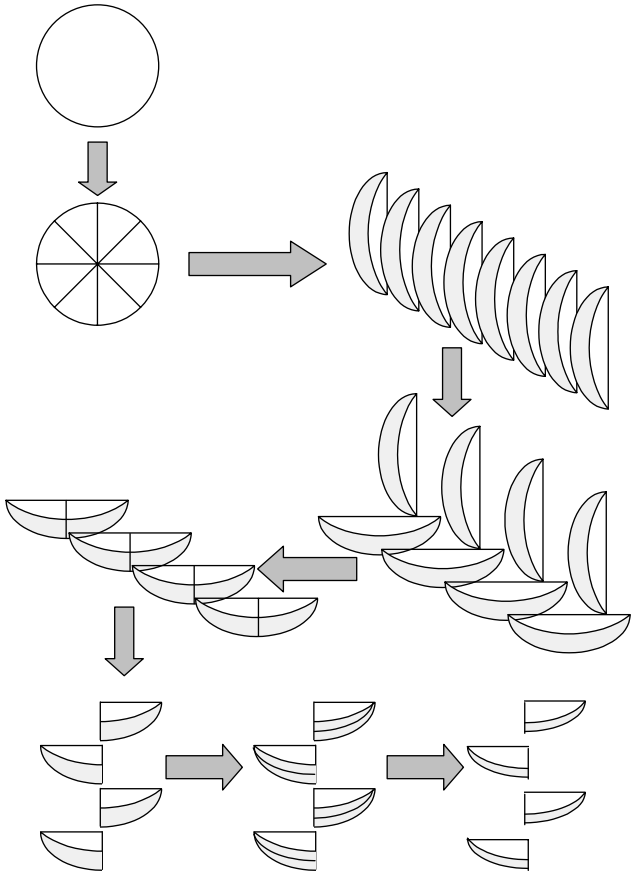

Fig. 1. Scheme for fractionation of apples into eight samples, with a systematic repartition of top and bottom, sunny and shaded faces.

*Cold alcohol insoluble solids* (AISc). The same procedure was used except that the boiling step was omitted and the fresh slices were directly ground in cold ethanol.

*Buffer—phenol method* (BP; Renard & Jarvis, 1999). A buffer simulating the ionic conditions in apple juice (1.2 mM $CaCl_2$, 2.0 mM $MgCl_2$, 0.5 g/L KCl, 60 mg/L ascorbic acid and 4 g/L malic acid, plus sodium disulfite (antioxidant) at 1 g/L, adjusted to pH 3.5 with 5 M NaOH) was used throughout the procedure (this solution will be called 'buffer' in the following paragraphs). Frozen apple slices (∼100 g) were suspended in chilled buffer (500 mL) plus Triton 100 (2 g/L) and octanol (4 mL) and blended for six successive bursts of 15 s in a Braun kitchen blender. The detergent was then washed off with chilled buffer in a cold room (4 °C) on a G3 sintered glass filter until foaming disappeared. The cell walls were then suspended in chilled acetone:water (60:40, v:v) and transfered to a G3 sintered glass filter, still in the cold room. After washing with acetone:water, excess solvent was removed by aspiration under vacuum, the remaining paste was weighed and suspended in four times its weight of phenol for 1 h at room temperature. The saturated phenol solution was removed by extensive washing with buffer on G3 sintered glass (until the phenol smell disappeared). The sample was finally solvent-exchanged in 70% ethanol (three times) then 96% ethanol and acetone and dried as above.

*Water insoluble solids* (WIS). The frozen slices were suspended in chilled buffer and ground by six successive bursts of 15 s in a Braun kitchen blender. They were washed with chilled water on a G3 sintered glass filter (20 min incubation before filtration) in a cold room (4 °C) until the filtrates were sugar-free. The remaining paste was freeze-dried.

*Propanol–acetic acid–water* (PAW; Mafra et al., 2001). The frozen slices were suspended in chilled SDS (sodium dodecyl sulfate) solution (15 g/L) and ground by six successive bursts of 15 s in a Braun kitchen blender. They were filtered off on a G3 sintered glass filter, washed once by SDS at 5 g/L (in the cold room) then extensively by a propanol–acetic acid–water mixture (2:1:1 volumes) until the filtrates were sugar-free (at room temperature). The sample was finally solvent-exchanged in 70% ethanol (three times) then 96% ethanol and acetone and dried as above.

*Alcohol insoluble solids from freeze-dried material* (AISfd). The apple slices were freeze-dried and ball-milled with 10 steel balls (∅ 0.7 cm) in an hermetically closed plastic container under fast planetary agitation at room temperature for 2 h. The powder was suspended in 70% ethanol, washed with 70% ethanol and dried as described for AISh (all at room temperature).

*Buffer—phenol from freeze-dried material* (PBfd). The freeze-dried apple slices were ground as above and

suspended in a water:phenol (1:4 weight) mixture (100 mL for 10 g of dried powder, i.e. presence of salts and electrolytes (from the apple) at concentrations close to those used in the buffer solution) and incubated for 1 h at room temperature, after which the phenol was filtered off on a G3 sintered glass filter, and the suspensions washed with buffer solution until the phenol smell disappeared. The cell wall material was then washed twice with acetone:water (60:40) and dried by increasing concentrations of acetone (80:20, three times, pure acetone three times) then overnight in an oven at 40 °C.

### 2.3. Sequential pectin extraction

Each cell wall preparation (i.e. 21 samples) was extracted sequentially by water (three times 2 h at room temperature), CDTA (0.05 M, pH 5.8–6, overnight then 8 h) and pectin-lyase (PL) (Megazyme, Bray, Ireland; 11 U/g of cell wall, overnight). For each extraction approximately 500 mg of cell wall preparation and 30 mL of extracting medium were incubated at room temperature in an 35 mL empty Sep-pack prep column (Interchim™) equipped with a sinter of porosity 20 μm under slow planetary agitation at 25 °C. After incubation, the extract and the cell wall were separated by filtration under vacuum. The three water extracts were pooled and dialysed against purified water (three times) prior to freeze-drying. The two CDTA extracts were pooled and dialysed first against 0.1 M NaCl then against purified water prior to freeze-drying. The PL extracts, likely to contain oligomers, were freeze-dried without dialysis.

### 2.4. Analytical

The individual neutral sugars were analysed by gas chromatography (capillary column of 30 m×0.25 mm i.d. coated with DB225, 0.15 μm film thickness, J & W Scientific, Folsom, USA) at 215 °C, using hydrogen as carrier gas, after sulfuric acid hydrolysis (1 M, 3 h, 100 °C) and derivation to alditol acetates (Englyst & Cummings, 1984). Cell wall preparations were submitted to prehydrolysis in 13 M sulfuric acid (1 h, room temperature; Saeman, Moore, Mitchell, & Millet, 1954). Myo-inositol was used as internal standard. To determine the degree of methylation, samples were saponified with 0.2 M KOH, 2 h, at room temperature. Methanol was determined in the supernatants according to Klavons and Bennet (1986). Galacturonic acid was determined by the automated *m*-hydroxydiphenyl (Thibault, 1979) assay on an Alliance instruments (Méry/Oise, France) autoanalyzer after acid hydrolysis (cell walls) or saponification (extracts). The degree of methylation (DM) was calculated as molar ratio of methanol to galacturonic acid. Nitrogen was analysed by the Kjeldahl method. Proteins were calculated as N×6.25. Polyphenols were measured by HPLC-DAD on a Purospher RP-18 endcapped (60 Å, 5 μm, 250×4 mm) column (Merck) after thioacidolysis (Guyot, Marnet, & Drilleau, 2001).

The molecular weight distribution of polysaccharides was determined using a HPLC system involving a laboratory data control (LDC) programmable pump equipped with four Bio-Gel TSK columns (300×7.8 mm each) in series (50, 40, 30 and 25 PWXL; Tosohaas, Stuttgart, Germany), in combination with a TSK XL guard column (40×6 mm) at 35 °C. Solutions (200 μL) of the extracts (2 mg/mL) were injected. They were eluted with 0.4 M sodium acetate buffer pH 3.6 at 0.8 mL/min. The eluate was continuously monitored using the automated *m*-hydroxydiphenyl (Thibault, 1979) and orcinol (Tollier & Robin, 1979) assays on an Alliance instruments (Méry/Oise, France) autoanalyzer.

### 2.5. Statistical analysis

Methods were compared using the two-way ANOVA and Bonferoni *t*-test of the Sigmastat software (Jandel Scientific). Standard deviations were calculated for each series of replicated measurement using the sum of individual variances pondered by the individual degrees of freedom.

## 3. Results

### 3.1. Yields and compositions of the cell walls

Table 1 summarises the results of the cell wall preparation. For all samples, the yields and compositions

Table 1
Yields from fresh weight (mg/g fresh weight) and compositions (mg/g dry matter) of the different cell wall preparations

| | Yields | Rha | Fuc | Ara | Xyl | Man | Gal | Glc | GalA | MeOH | Procyanidins | Nitrogen |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AISc | 18.7a | 11 | 9 | 68 | 61 | 14 | 58 | 263a | 252 | 32 | 1.1 | 9.6b |
| AISfd | 17.7$a | 11 | 9 | 68 | 62 | 14 | 56 | 255a | 254 | 32 | 1.7 | 12.1a |
| AISh | 18.2a | 11 | 9 | 66 | 60 | 14 | 57 | 259a | 262 | 30 | 1.3 | 10.1b |
| PAW | 17.1ab | 12 | 10 | 71 | 62 | 15 | 57 | 284b | 254 | 30 | 1.0 | 5.7c |
| PB | 17.0ab | 11 | 9 | 67 | 61 | 16 | 57 | 284b | 256 | 30 | 0.2 | 6.3c |
| PBfd | 16.3b | 9 | 9 | 71 | 63 | 15 | 59 | 286b | 259 | 30 | 0.6 | 5.4c |
| WIS | 16.8ab | 10 | 10 | 73 | 68 | 15 | 56 | 284b | 257 | 31 | 1.2 | 4.8c |
| SD | 0.8 | 0.9 | 0.8 | 5.1 | 4.1 | 0.9 | 3.8 | 5.9 | 8.5 | 2.2 | 0.5 | 0.6 |

SD, Standard deviation of the mean (degrees of freedom: 13). Different letters denote values which are significantly different. $, Two repetitions only.

were typical of apple cell wall and well within the ranges of the literature values (Aspinall & Fanous, 1984; Ben-Shalom, Conway, Gross, Sams, & Pinto, 1997; Ella Missang, Massiot, Baron, & Drilleau, 1993; Fischer & Amado, 1994; Le Bourvellec, Guyot, & Renard, 2004; Massiot, Baron, & Drilleau, 1994; Percy, Melton, & Jameson, 1997; Renard, Baron, Guyot, & Drilleau, 2001; Renard & Thibault, 1991; Renard et al., 1990; Stevens & Selvendran, 1984; Voragen, Heutink, & Pilnik, 1980; Yoshioka, Kashimura, & Kaneko, 1994). The differences in yields were very limited (between 1.63 and 1.87%), except for an aberrant value for AISfd caused by accidental loss of material. A trend to higher yield was observed for all three AIS procedures versus the other four methods, but it was statistically significant only between the three AIS procedures and PBfd. From a practical point of view, the ball-milling of freeze-dried apple material as carried out for AISfd and PBfd resulted in very fine powders, difficult to recover from sinters and very susceptible to dispersion by drafts (or coughs, etc.). There was a trend for slightly lower yields starting from freeze-dried apple powders rather than the corresponding method starting from fresh or frozen apple slices, but it was not statistically significant.

There were few statistically significant differences between methods for the composition of the cell walls. The main sugars were glucose and galacturonic acid, each close to a quarter of the wall weight. Arabinose, galactose and xylose represented each about 5–8% of the wall, while rhamnose, mannose and fucose were present in low amounts ($<2\%$). Glucose content was significantly lower in the AIS procedures than in the WIS, PB and PAW. Glucose, arising mainly from cellulose, i.e. an insoluble material, should be the least extractable and therefore variable of all the cell wall components. That its concentration was lowered when extraction yields were higher indicated that other components were partially extracted in the other procedures. There were no significant differences in content of the other sugars, including galacturonic acid, or for methanol content and degree of methylation. N content was significantly higher in the AIS procedures (close to 10 mg/g, i.e. protein contents close to 6%) than in other methods (approximately 5 mg/g, i.e. protein contents close to 3%), and among these it was significantly higher in AISfd. The PAW treatment seemed quite good at removing proteins from the walls, probably due to the preliminary detergent step. Procyanidins constituted a minor part of the cell wall preparations, lower than 2 mg/g. Due to the low concentrations, their determination was difficult and high standard deviations inhibited detection of significant differences. However, there was a clear trend for higher procyanidins, i.e. higher polyphenols, in the AIS (and PAW) procedures.

### 3.2. Yields of the fractional extractions

The yields in total matter and in galacturonic acid are given in Table 2. Water extracted between 2 and 11% of the galacturonic acid, the chelating agent (CDTA) between 14 and 20% and the pectin-lyase gave the highest yields with $>22\%$. Approximately half of the initial uronic acids were recovered in the extracts (between 39% for WIS and 56% for PAW). The yields from the water extraction between the three AIS procedure plus the PAW procedure were much higher (three to four times higher) than those of the PB and WIS procedures, both in total matter and in galacturonic acid. For the CDTA extracts the variations in ponderal yields were much higher than in uronic acid, probably linked to known difficulties (Mort, Moerschbacher, Pierce, & Maness, 1991) in removing CDTA. This was comparable e.g. to the results reported by Fischer, Arrigoni, and Amado (1994), whose CDTA yields varied between 12.7 and 23.1% of AIS of apples at different ripening stages, with total sugar contents of the extracts between 319 and 516 mg/g. There was a tendency to higher yields of CDTA extracts from the cell wall preparations after freeze-drying, but it was not statistically significant.

Table 2
Yields of the sequential pectin extractions: in percent of dry matter and in percent of initial galacturonic acid

| | Water | | CDTA | | Pectin-lyase | |
|---|---|---|---|---|---|---|
| | Total | GalA | Total | GalA | Total | GalA |
| AISc | 4.9a | 8.8a | 16.6 | 18.8 | 25.4 | 22.0a |
| AISfd | 5.9a | 9.5a | 23.7 | 15.6 | 22.8 | 27.1ab |
| AISh | 6.1a | 11.3a | 15.3 | 13.5 | 26.0 | 22.2a |
| PAW | 5.3a | 8.7a | 14.1 | 20.5 | 30.1 | 26.7ab |
| PB | 1.2b | 1.7b | 13.4 | 16.0 | 27.0 | 23.9a |
| PBfd | 1.4b | 2.1b | 19.9 | 17.0 | 21.8 | 34.9b |
| WIS | 1.9b | 2.3b | 14.1 | 15.4 | 18.6 | 21.7a |
| SD | 0.6 | 1.1 | 3.8 | 2.9 | 4.9 | 3.3 |

SD, Standard deviation of the mean (degrees of freedom: 14). Different letters denote values which are significantly different.

The yields of the pectin-lyase extracts were between 22 and 30% for the dry matter and 22–34% for the galacturonic acid. There were no significant differences for global PL yields but the galacturonic acid yields for PBfd were significantly higher than for AISc, AISh, PB and WIS; this extract also had higher neutral sugar contents and therefore higher yields overall.

### 3.3. Compositions of the extracts

The neutral sugar compositions of the water extracts were not analysed due to the low amounts available for some procedures. Galacturonic acid represented between 80 and 87 mol% of the CDTA extracts. There were no significant differences between methods, though the extracts from AISc, AISfd, AISC and PAW had lower contents ($<85$ mol%) than those from PB, PBfd and WIS ($>85$ mol%). In the PL extracts, galacturonic acid only accounted for 64–68 mol% of the extracts, again without significant differences.

The neutral sugar compositions of the CDTA and the PL extracts are given in Table 3. All had arabinose as main

Table 3
Neutral sugar compositions (mol%) of the CDTA and pectin-lyase extracts

| | CTDA extract | | | | | | Pectin-lyase extracts | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Rha | Fuc | Ara | Xyl | Gal | Glc | Rha | Fuc | Ara | Xyl | Gal | Glc |
| AISc | 9.2a | 2.1 | 39.9a | 12.6ab | 24.6a | 11.6a | 8.9ab | 0.8 | 55.4ab | 10.5 | 21.4 | 3.1 |
| AISfd | 10.1ab | 1.8 | 41.7a | 12.5ab | 25.0b | 9.0bc | 7.8a | 0.5 | 58.8a | 9.6 | 20.8 | 2.5 |
| AISh | 9.4a | 1.9 | 42.5ab | 10.2a | 27.8a | 8.2c | 9.4b | 0.8 | 54.0b | 10.4 | 21.7 | 3.7 |
| PAW | 10.1ab | 1.3 | 48.4ab | 11.8ab | 21.7c | 6.7cd | 8.3ab | 0.7 | 54.0b | 12.5 | 21.3 | 3.3 |
| PB | 10.7ab | 1.6 | 51.0b | 11.1ab | 18.3d | 7.3cd | 9.5b | 0.7 | 57.4ab | 10.1 | 19.2 | 3.0 |
| PBfd | 11.8b | 1.4 | 49.3b | 13.1ab | 18.5d | 5.8cd | 7.7a | 0.5 | 57.7ab | 10.0 | 22.0 | 2.1 |
| WIS | 9.0a | 2.0 | 45.5ab | 14.3b | 18.2d | 10.9abc | 8.9ab | 0.7 | 57.3ab | 10.2 | 20.1 | 2.8 |
| SD | 0.8 | 0.5 | 2.2 | 1.1 | 1.2 | 0.9 | 0.4 | 0.1 | 1.5 | 1.2 | 0.9 | 0.6 |

SD, Standard deviation of the mean (degrees of freedom: 14). Different letters denote values which are significantly different.

neutral sugar, the PL extracts being richer in arabinose (>50 mol%), followed by galactose at circa 20%. Next came rhamnose and xylose, each close to 10%, followed by low amounts of glucose and traces of fucose. Again these values are typical for similar extracts from apple cell walls. Though the CDTA and PL-extracted pectins clearly differed in their proportions of neutral sugars to galacturonic acid, globally their neutral sugar profiles were close.

The galactose mole percent of CDTA extracts from AIShc, AISc and AISfd were significantly higher than for PAW, and they both were significantly higher than PB, WIS and PBfd. The mole percent of arabinose were higher in PB and PBfd and lower in AISc and AISfd, but the differences were not as clear as for galactose. This might be interpreted as presence of more galactose and less arabinose in the CDTA extract when there was more water-extractable pectin, i.e. by difference one might infer a higher proportion of arabinose to be present in the water-soluble pectins. Significant differences between methods were also observed for rhamnose, xylose and glucose. The rhamnose content of the CDTA extract from PBfd was high, in contrast to WIS. For xylose, the highest concentrations were obtained in WIS and lowest in AISh, while for glucose the highest concentrations were in the CDTA extracts of WIS and AISc, contrasting with WIS, PB and PBfd.

In the PL extracts, the predominance of arabinose and galactose was even higher than in the CDTA extracts, the fucose and glucose content being noticeably lower. The rhamnose content was statistically significantly lower in the two preparations from freeze-dried material.

### 3.4. Size-exclusion chromatography

The size-exclusion chromatography patterns of the CDTA and the PL extracts are given in Figs. 2 and 3. In both figures, the chromatograms of the three repetitions are given for each method and they are always in the same order. Thus all lower traces are from the same group of apples, etc.

The CDTA extracts had mostly homogeneous distributions of the total and acidic sugars. The HPSEC patterns of AISh and AISc were close. Systematic presence of a peak at the void volume of the column in these samples might be linked to ethanol-induced aggregation of the pectins, though this peak was absent in AISfd. Both AISfd and PBfd showed the same graduation between samples, as did PAW and PB. The profiles for the three repetitions of WIS procedure were particularly variable.

The PL extracts usually showed two peaks of distinctive characteristics, a high molecular peak poor in uronic acids and an uronic acid-rich peak at high $K_{av}$ values, arising from the oligomers generated by the enzyme. Again these

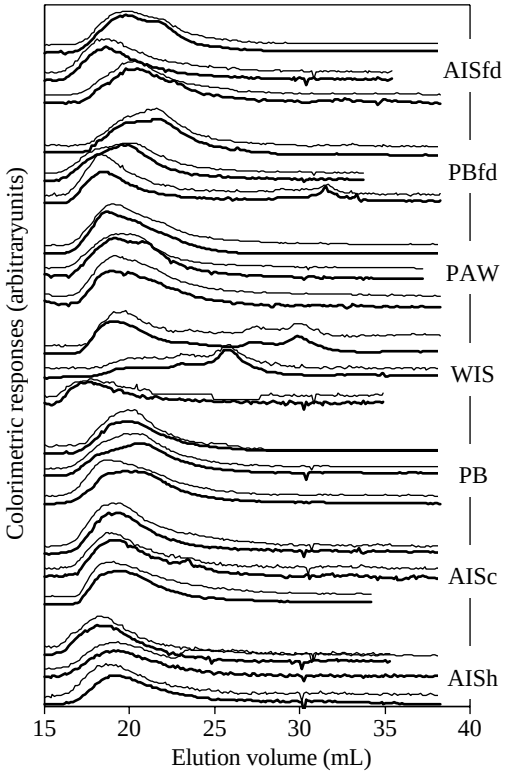


Fig. 2. HPSEC patterns of the CDTA extracts of the different apple cell wall preparations on combined Bio-Gel TSK 50, 40, 30 and 25 PWXL columns (elution: sodium acetate 0.4 M, pH 3.6). The chromatograms of the three repetitions are given for each method and they are always in the same (low, intermediary or high) position. Bold curve, absorbance at 520 nm (uronic acids); thin curve, absorbance at 430 nm (total sugars).

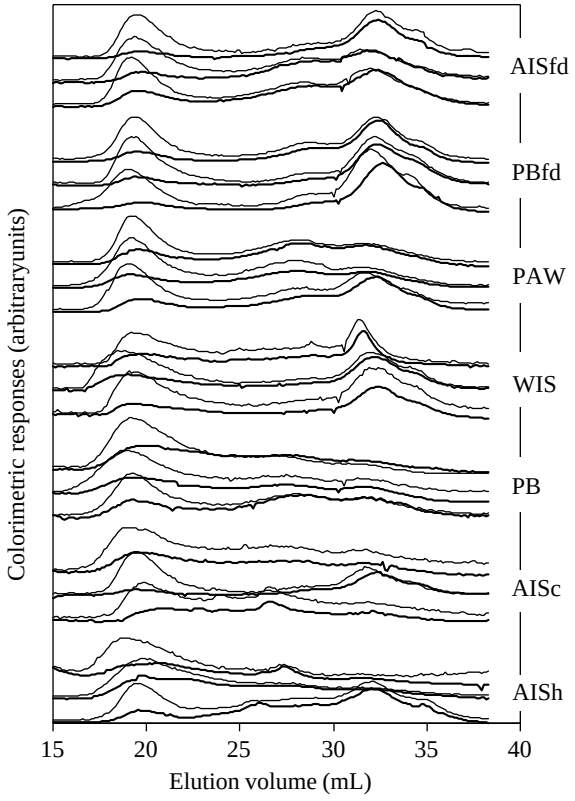


Fig. 3. HPSEC patterns of the pectin-lyase extracts of the different apple cell wall preparations on combined Bio-Gel TSK 50, 40, 30 and 25 PWXL columns (elution: sodium acetate 0.4 M, pH 3.6). The chromatograms of the three repetitions are given for each method and they are always in the same (low, intermediary or high) position. Bold curve, absorbance at 520 nm (uronic acids); thin curve, absorbance at 430 nm (total sugars).

are features reported previously (Fischer et al., 1994; Massiot et al., 1994; Renard et al., 1990; Renard, Voragen, Thibault, & Pilnik, 1991). However, the elution time of the uronic acid peak was very variable: in some samples it distinctly appeared as oligomers, in others a wide range of molecular sizes was covered (AISc, AISh, PAW and PB). However, this did not always occur with the same triplicate of 10 apples and therefore could not be ascribed to differences in e.g. maturity between the repetitions.

## 4. Discussion and conclusions

Good repeatabilities were obtained for the cell wall preparations themselves, with a standard deviation for the yields of 0.8 mg/g fresh weight overall. Qua repeatability of the individual procedures, the best results were obtained with AISc and AISh (approximately 0.2 mg/g for the yields from fresh weight), and the worst for WIS (1 mg/g).

Two distinct groups could be observed for the cell wall preparations: the AIS procedures resulted in higher yields, higher protein contents and lower glucose content than either phenol (PB and PBfd), WIS or PAW. The difference seemed to be linked to protein, pectin and procyanidin precipitation by ethanol in AIS methods, all other methods having similar efficiencies in terms of bulk protein removal, while some extraction of the other sugars (from soluble pectins) occurred during the washing steps with water or buffer included in PB, PBfd, WIS and PAW methods. Indeed when the galacturonic acid contents were calculated back to the initial apple fresh weight, there appeared a trend for lower recovery in PB, PBfd, WIS and PAW (4.4, 4.2, 4.3 and 4.4 mg/g fresh apple, respectively) than in AIS samples (4.7, 4.5 and 4.8 mg/g fresh apple for AISh, AISfd and AISc, respectively), but this was not statistically significant.

The results for the pectin extraction procedures were more disturbing as high standard deviations were obtained. For example here CDTA yields (average of triplicates) of 15 and 20% of the galacturonic acid were not significantly different. Here again two groups were obtained, AISc, AISfd, AISh and PAW having high yields in the water extraction and the other three low yields. It would be attractive to attribute the difference solely to a precipitation of the water-soluble pectins by ethanol in the initial stages of the cell wall preparation. However, the PAW did include as a first step an aqueous treatment and was among the samples with relatively low galacturonic acid recoveries. Therefore, in this sample the presence of water-extractable pectin cannot be simply traced back to an early precipitation versus an early extraction during extensive washing steps. It is commonly assumed that CDTA-extractable pectins are a specific fraction (at least in a constant ionic environment, which we have taken care to have except for PAW) and should therefore have the same composition and yields relative to initial fresh weight of the fruit. This fraction represented 0.63–0.78 mg of galacturonic acid per gram fresh apple for most methods, with no discernible trend, but 0.93 for AISc and 1.00 for PAW. In AISc this might be ascribed to some apple PME action, though that would have been expected to occur rather for the WIS. The highest yields during the PL extraction were obtained for the freeze-dried, ball-milled samples. The fine grinding of these samples might have changed accessibility to the PL or even broken covalent bonds (Bock et al., 1977), allowing diffusion of more pectic neutral side chains once the homogalacturonans were degraded.

In PAW something different clearly occurred: high water-soluble pectin yields in spite of the initial SCS washes, the highest CDTA-extractabilities of all samples and high PL yields. A possible cause could be modification of the ionic state of pectins by acetic acid. However, our results are in contradiction both with expectable effects of acidification and literature. Conversion of pectins to the protonated form should decrease both water solubility and CDTA effect as it removes calcium cross-links and decreases intrinsic solubility of this polyelectrolyte. Huber (1991) had noted a decreased distilled water solubility (but increased buffer solubility) of pectin from phenol–acetic acid–water isolated walls compared to AIS, and that CDTA did not enhance extraction of pectin from his phenol:acetic acid:water residue compared to a buffer treatment.

The behaviour of the cell walls in this new procedure thus needs more documentation.

The striking fact about the SEC chromatograms was their variability, which contrasted with the good agreement of the compositional data between repetitions. Different features were observed both per method and per repetition, and neither could be traced back to systematic differences between methods or repetitions. This means that in further work, any interpretation of such data in terms of comparison between samples will have to be very conservative, and can also explain the difficulties encountered earlier, e.g. by Fischer et al. (1994). Curiously similar variations of the patterns between the samples (and within the methods) were observed for similar pre-cell wall isolation histories (freezing, freeze-drying). This might be due to differences encountered by the samples in their kinetics of freezing and drying, i.e. to differences in cell damage and disruption of the cell wall matrix during freezing? The differences observed here between methods seem to be related to the early stages of sample preparation and should caution us to have a very good control of these steps.

In summary, qua ease of the various procedures, AIS methods were by far the easiest, least time-consuming and least in terms of toxic risk for the person conducting the experiment. They also gave low standard deviations and rather comparable SEC patterns. They are to be preferred for experiments involving comparison of large numbers of samples. However, no conclusions on cell wall proteins can be made after such a preparation, and some phenols co-precipitate. If structural work is the aim of an experiment, clearly the phenol procedures are to be preferred, provided the ionic conditions are chosen with care. Freezing and even freeze-drying can be used to differ cell wall preparation, but some care should be taken to control this step. The milling after freeze-drying did not cause major differences in pectin extractability, though very fine particles were obtained. Last, the yields of pectin extraction and SEC chromatograms had high variabilities, which means that it should not be possible to interpret limited variations in extraction patterns in terms of cell wall structure.

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