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Fourier-Transform Raman and Fourier-Transform Infrared Spectroscopy[1]

An Investigation of Five Higher Plant Cell Walls and Their Components

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Infrared and Raman spectra of sequentially extracted primary cell walls and their pectic polymers were obtained from five angiosperm plants. Fourier-transform Raman spectrometry was shown to be a powerful tool for the investigation of primary cell-wall architecture at a molecular level, providing complementary information to that obtained by Fourier-transform infrared microspectroscopy. The use of an extraction procedure using imidazole instead of cyclohexane *trans*-1,2-*N*,*N*,*N*′,*N*′-diaminotetraacetate allows the extension of the infrared spectral window for data interpretation from 1300 to 800 cm^{-1}, to 2000 to 800 cm^{-1}, and allows us to obtain Raman spectra from extracted cell-wall material. Wall constituents such as pectins, proteins, aromatic phenolics, cellulose, and hemicellulose have characteristic spectral features that can be used to identify and/or fingerprint these polymers without, in most cases, the need for any physical separation. The Gramineae (rice [*Oryza sativa*], polypogon [*Polypogon fugax steud*], and sweet corn [*Zea mays*]) are spectroscopically very different from the nongraminaceous monocotyledon (onion [*Allium cepa*]) and the dicotyledon (carrot [*Daucus carota*]); this reflects differences in chemical composition and cross-linking of the walls. The possibility of a taxonomic classification of plant cell walls based on infrared and Raman spectroscopies and the use of spectral fingerprinting for authentication and detection of adulteration of products rich in cell-wall materials are discussed.

The macroscopic properties of fruits, vegetables, cereal crops, and plant-derived materials of industrial importance such as wood, paper, and cotton derive from the properties of plant cells and their surrounding walls. Cell walls form a major component of the bulk of plant material, and much research is now being channeled into characterizing the mechanical and rheological properties of cell walls and the biochemical basis that underpins these properties. Much cell-wall analysis has relied on chemical or enzymic cleavage of wall polymers with subsequent sugar and methylation analysis (Carpita and Gibeaut, 1993). We are now searching for methods that give information about the wall in situ, and vibrational spectroscopies such as FT-IR and FT-Raman offer this opportunity.

In dicotyledonous and nongraminaceous monocotyledonous plants, cellulose accounts for roughly 30% of the dry weight of the wall. The principal hemicellulose is xyloglucan, thought to interlock the cellulosic microfibrils to form a network in the wall (McCann et al., 1990; Carpita and Gibeaut, 1993). Other noncellulosic polysaccharides such as glucomannans, galactomannans, galactoglucomannans, (1–3)-β-D-glucans, and glucuronoarabinoxylans are found in much lower amounts. Three major pectic polymers have been characterized: homogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II (McNeil et al., 1980; Stevenson et al., 1988), although rhamnogalacturonan II is too scarce to be a major structural component. Rhamnogalacturonan I may have side chains of arabinan or arabinogalactan attached to the O-4 of the rhamnosyl residues, and one-third of the galacturonic acid residues are acetylated at the O-3 position (Komalavilas and Mort, 1989). The degree of esterification of pectins varies widely among tissues and among species. Pectins may form a network through Ca^{2+} cross-linking and ester linkages (Jarvis, 1984). Ester linkages with dihydroxycinnamic acids such as diferulic acid (Fry, 1986) between pectins and other pectins or noncellulosic polysaccharides have been proposed. Large multigene families of proteins that can account for 1 to 20% of the dry mass of the wall are being characterized (Cassab and Varner, 1988; Keller, 1993). In addition, the presence of many enzymes in the wall allows potential modifications to wall polymers in situ.

The walls of grasses contain very different biopolymeric constituents (Carpita and Gibeaut, 1993). Cellulose is also a major structural component of the walls of the Gramineae, but the principal hemicellulose is glucuronoarabinoxylan with only small amounts of xyloglucan. Glucuronoarabinoxylans vary widely in their degree of substitution, from those

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Abbreviations: CDTA, cyclohexane *trans*-1,2-*N*,*N*,*N*′,*N*′-diaminotetraacetate; CWM, cell wall material; FT-IR, Fourier transform infrared; FT-Raman, Fourier transform Raman.

with nearly all xylosyl units branched to those with only 10% or less of the xylosyl units bearing side groups. Graminaceous walls are enriched in ferulate and *p*-coumarate esters (Kato and Nevin, 1985) and glucuronoarabinoxylans may be cross-linked by both esterified and etherified hydroxycinnamates and other phenolic substances (Scalbert et al., 1985). The Gramineae are notably poor in pectin, but antibodies to methyl-esterified or unesterified pectins do recognize particular tissue patterns within these walls (Knox et al., 1989). Chemically these pectins are homogalacturonans and rhamnogalacturonan I, but highly substituted glucuronoarabinoxylan is closely associated with these pectins (Carpita, 1989). Mixed-linkage β-(1–3),(1–4)-D-glucan is synthesized during cell expansion in the Gramineae (Gibeaut and Carpita, 1993).

However, models of plant cell-wall architecture are still at a very early stage of development. To reveal the chemical composition and structural details of the wall requires the application of new analytical methods that can suitably probe the wall in a native state. IR microspectroscopy is a noninvasive technique that is suitable for analysis at the single cell-wall level (Griffiths and de Haseth, 1986; McCann et al., 1992). Polarized IR spectroscopy has been used to give information about polymer orientations in situ of matrix polysaccharides of pea cell walls (Morikawa et al., 1978; Hayashi et al., 1980) and carrot cell walls (McCann et al., 1993). Molecular orientation of lignin (Atalla and Agarwal, 1984) and cellulose (Atalla et al., 1980) in plant cell walls and their macromolecular organization in secondary walls of woody tissues (Agarwal and Atalla, 1986) were investigated by conventional Raman microspectroscopy (Atalla and Agarwal, 1986). In this FT-IR and FT-Raman preliminary study, we have chosen five plants (three Gramineae, one nongraminaceous monocotyledon, and one dicotyledon) to assess the potential of vibrational spectroscopies for qualitative and quantitative analysis of cell walls.

IR and Raman spectroscopies are based on two different physical processes: an absorption process and a scattering effect. In IR spectroscopy the ratio of transmitted to incident radiation is detected, whereas in Raman spectroscopy the loss of energy in a light beam scattered by the sample is recorded. In both cases the absorbed energy is removed from the incident radiation and excites vibrations within the molecule. However, to absorb IR radiation a molecule must undergo a net change in dipole moment as a consequence of its vibrational motion. In contrast, for Raman activity a change in polarizability during the vibration is required. Consequently, only strongly polarized asymmetric chemical bonds give rise to intense IR bands, whereas strong Raman scattering may be observed from nonpolar but polarizable bonds. Thus, the two techniques are complementary, and the fact that many vibrations are seen in both spectra serves as valuable confirmatory evidence in the case of complex samples.

The shapes of the IR and Raman spectral bands and their positions on the energy scale are characteristic of particular functional groups within the molecule. In some cases the bands in a particular spectral region cannot be assigned to a specific functional group, but they are characteristic of the molecule as a whole and constitute a fingerprint for the molecule.

MATERIALS AND METHODS

Biological Material

Primary cell walls from onion (*Allium cepa*), carrot (*Daucus carota*), rice (*Oryza sativa*), black Mexican sweet corn (*Zea mays*), and polypogon (*Polypogon fugax steud*) were prepared as described by McCann et al. (1990).

The CWM of onion and carrot were isolated from parenchyma tissues. Sweet corn suspension cells were grown in Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 2% Suc, 2 mg/L 2,4-D, pH 5.7. Rice and polypogon suspension cells were grown in Murashige and Skoog medium supplemented with 3% Suc, 2 mg/L 2,4-D, 0.5 g/L casein hydrolysate, pH 5.7. The suspension culture cells were harvested after 7 d.

Extraction Sequence

Two different extraction procedures were used to remove pectic polymers from isolated CWM.

(a) CWM was extracted according to the method of Redgwell and Selvendran (1986) with 0.1 M CDTA (Na salt, pH 6.5) at 20°C overnight and then with fresh CDTA for an additional 2 h; and with a solution of 0.1 M Na_2CO_3 and 20 mM $NaBH_4$ at 1°C overnight and then with fresh solution for 3 h at 20°C.

(b) CWM was extracted with 2 M imidazole, pH 7.0, at 20°C for 24 h and then for 15 h and with the Na_2CO_3 solution as described above. To extract an equivalent amount of pectin, the extraction procedure proposed by Mort et al. (1991) was modified by increasing the concentration of imidazole and the duration of the extraction as indicated above.

Purification of Polymers

After each extraction step, the CWM was collected by centrifugation and washed with distilled water. The supernatant and washings containing extracted polymers were neutralized to pH 7.0 with HCl and then dialyzed against distilled water. The samples were freeze-dried and stored at 4°C.

Vibrational Spectroscopy

IR transmission spectra were recorded on a Bio-Rad FTS-40 FT spectrometer interfaced to a Spectra-Tech IR-Plan microscope. An area of the sample of approximately 100 × 100 μm was selected as described by Hammouri (1991). All spectra were obtained at a resolution of 8 cm^{-1} and 256 interferograms were co-added.

About 0.5 mL of water was added to roughly 10 mg of the freeze-dried CWM or extracted polymers, and a drop of the suspension or solution was applied to a BaF_2 microscope window (13 mm diameter by 2 mm thick) and dried down in a 30°C oven for 1 h. An area of 100 × 100 μm was selected from areas of single cell-wall fragments of 0.1 to 0.2 μm thickness. A single-beam background spectrum of the clear BaF_2 window was recorded first. The single-beam spectrum of the sample was ratioed to the background before conversion to an absorbance spectrum. Sample homogeneity was

assessed by obtaining spectra from different areas on the BaF_2 windows and from different preparations of wall material and polymers.

Raman spectra were measured with a Bruker IFS66 spectrometer (with FRA 106 module) using an unpolarized CVI c/w neodymium:YAG laser emitting in the near-IR at 1.064 nm. The spectra of polymers were obtained at a resolution of 16 cm^{-1}, and between 2,000 and 6,000 interferograms were co-added. The spectra of the CWM were recorded at a resolution of 8 cm^{-1}, and between 5,000 and 15,000 interferograms were co-added. Spectral baseline correction of IR and Raman spectra was performed when appropriate. Raman and IR spectra of all extractants were also obtained. Unlike the IR spectrometer, the Raman spectrometer was not equipped with a microscope attachment: the Raman spectra were recorded from bulk samples of approximately 1 mg that were homogeneous by FT-IR microspectroscopy. For Raman spectroscopy, about 1 mg of freeze-dried sample was tamped into an open-ended aluminum cavity, and a Raman spectrum was directly recorded without any further sample preparation.

RESULTS AND DISCUSSION

Imidazole Is a Better Chelating Agent than CDTA for Vibrational Spectroscopy

Pectins can be extracted from cell walls by extractants that chelate calcium such as EDTA or CDTA (Redgwell and Selvendran, 1986) or, more recently, imidazole (Mort et al., 1991). It is clear from both IR and Raman spectra that CDTA remains associated with pectins and extracted CWM even after extensive dialysis. However, no contribution to IR and Raman spectra from imidazole can be detected after dialysis, eliminating the need for the digital subtraction of the chelating agent, a process that can lead to the production of spectral artifacts due to variations in the conformation of the chelating agent arising from the complexation states of CDTA in the CWM. CDTA has strong absorbances around 1600 and 1406 cm^{-1} that severely limit the use of this region of the IR domain (Fig. 1). Furthermore, CDTA is a strong Raman scatterer and gives rise to intense bands over the 2000 to 200 cm^{-1} range, frequently masking Raman spectral features from the sample.

To evaluate the equivalence of the two extraction methods, IR data for polymers and cell walls extracted with imidazole and CDTA have been compared over the 1300 to 800 cm^{-1} range, where there is no interference from CDTA. It is clear that the two sets of spectra are very similar (Fig. 2). This result has also been found by sugar analysis (Mort et al., 1991). X-ray fluorescence energy-dispersive scanning EM of cell-wall fragments shows that calcium is entirely removed by dialysis after the imidazole treatment (data not shown). In conclusion, imidazole is a more suitable chelating agent that extends the useful IR range and allows Raman spectra to be obtained from extracted CWM.

The IR and Raman Spectra Contain Characteristic Bands for the Main Biopolymers of the Cell Wall

The main IR spectral features of CWM have been previously assigned (McCann et al., 1992, 1993). Partly due to their comparatively sharper bands, the Raman spectra appear more complex than their IR counterparts. The Raman spectra are complementary to the IR spectra: Raman spectra can be used to differentiate biopolymers that are indistinguishable in the IR and vice versa. Generally speaking, when relatively pure polymers are extracted from the cell walls they can more unambiguously be assigned in the IR, since, in the absence of spectral interferences from cellulose, etc., they often give a characteristic profile. This rule applies to a lesser extent to the Raman spectra, but polymers in a mixture can more easily be assigned in Raman. For the purposes of investigating compositional differences, the Raman spectra are diagnostic primarily for phenolic materials and, to a lesser degree, for pectins, whereas the IR spectra are primarily diagnostic for pectin and protein. The assignment of the main bands in the IR and Raman spectra is summarized in Table I.

In the IR region 1200 to 900 cm^{-1}, pectins have a profile similar to that of polygalacturonic acid, and can easily be distinguished from nonpectic polysaccharides. Pectins give rise to several Raman bands in the region 900 to 200 cm^{-1}, and the sharp peak at about 854 cm^{-1} appears to be a useful

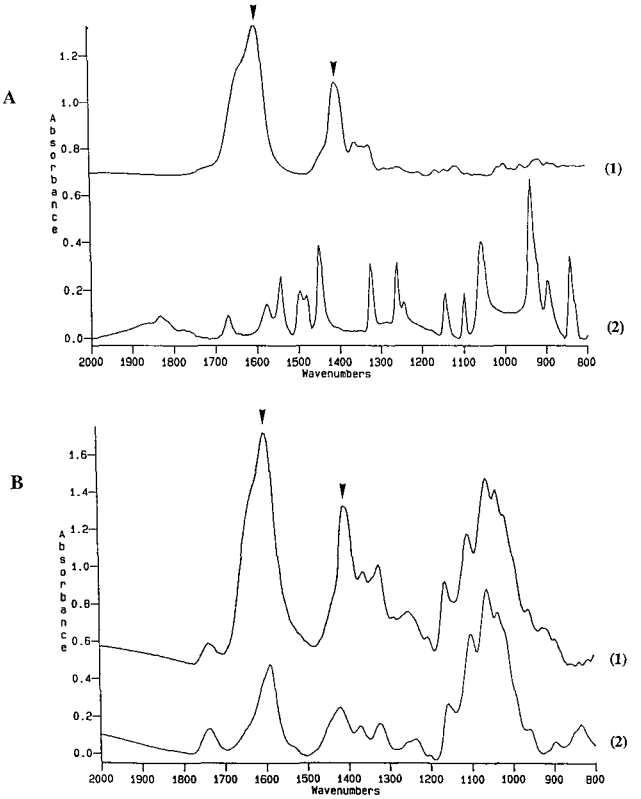

Figure 1. FT-IR spectra of pure CDTA (A, 1), pure imidazole (A, 2), and rice cell walls after extraction with CDTA (B, 1) and imidazole (B, 2). CDTA has strong absorbances around 1600 and 1406 cm^{-1} and these bands can be seen in the spectrum of CDTA-extracted CWM. The spectrum of imidazole-extracted CWM is free from any interferences from imidazole. The use of CDTA as a chelating agent for the extraction of the wall limits the spectral region to 1300 to 800 cm^{-1}, whereas with imidazole the entire mid-IR region can be used for spectral interpretation.

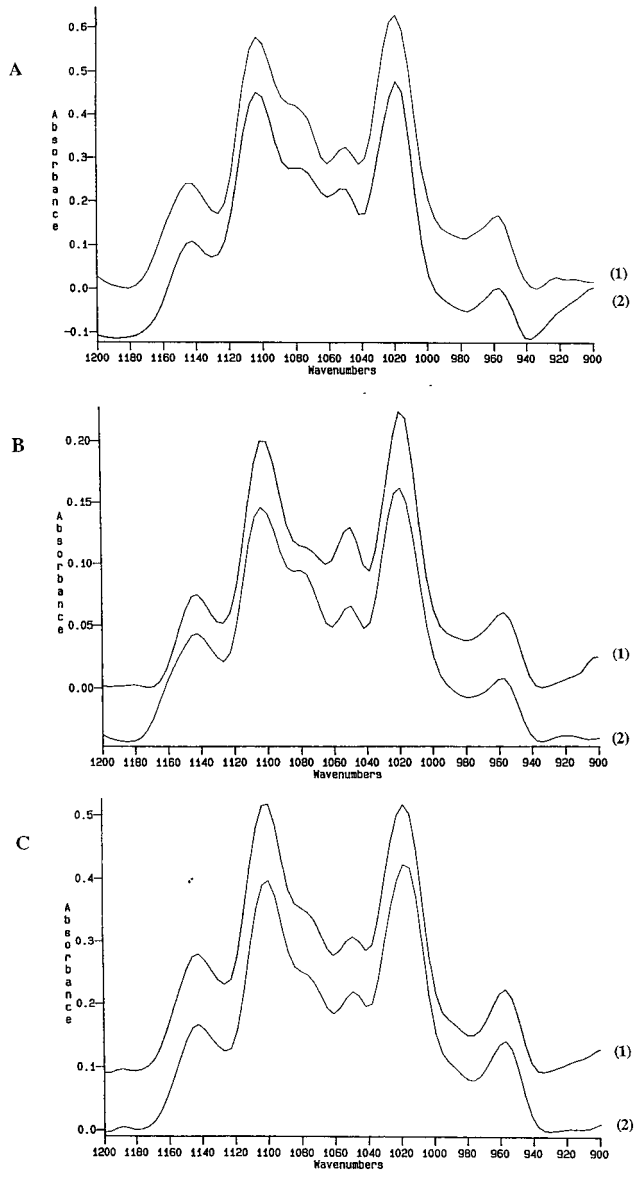

Figure 2. FT-IR spectra of pectic polysaccharides extracted from onion CWM with CDTA or imidazole, and sodium carbonate. A, First fraction extracted with CDTA (1) and imidazole (2). B, Second fraction extracted with CDTA (1) and imidazole (2). C, First sodium carbonate-extractable fraction after the extraction with CDTA (1) and imidazole (2). The IR spectra corresponding to the two extractions are very similar, indicating that the CDTA and imidazole extraction methods are equivalent.

analytical spectral band for acidic pectins. When the pectins are treated with base, the ester linkage will be destroyed, and in such cases two absorptions may be seen at about 1600 and 1420 cm^{-1} (antisymmetric and symmetric COO^- stretches): these two peaks are then diagnostic for pectin in salt form. The most diagnostic peak in the IR spectra of CWM is the peak centered at about 1745 cm^{-1}, arising from the ester carbonyl stretching associated with pectin. Its exact frequency and bandshape reveal the type (saturated-alkyl or aryl esters) and/or environment of the ester groups.

Proteins give rise to two strong bands in the IR arising from the amide linkage. They are seen at about 1650 cm^{-1} (amide I) and 1550 cm^{-1} (amide II), often with an intensity ratio of about 2:1. Their corresponding bands in Raman are weak but serve as confirmatory evidence. Pure protein spectra have relatively little intensity between 1200 and 800 cm^{-1} in IR and below 950 cm^{-1} in Raman (Parker, 1983).

The important phenolic peaks are seen at about 1430 cm^{-1} and as a doublet at 1605 to 1635 cm^{-1} (bands C and D). Only the D band is due to an aromatic stretch; the C band appears to be associated with the α-β double bond on the propanoid side group in lignin-like structures. Atalla and Agarwal (1984) have reported it at about 1630 cm^{-1} in the spectra of ferulic acid. Fortunately, these absorptions are very weak in the IR and do not interfere with the IR assignment of acidic pectins in salt form. However, there is some evidence that the 1420 cm^{-1} IR band of the pectins in salt form has a significant Raman intensity. Therefore, only the 1605 to 1635 cm^{-1} doublet can be used to confirm the presence of phenolic material. The intensity and shape of this band is such that it is unlikely to be confused with the weaker amide I.

It is beyond the scope of this work to complete a full assignment of the spectra acquired, because a considerable number of model compounds must be studied. However, these key assignments are sufficient for the investigation of the differences in extractability, and hence composition, of the cell walls. Implications resulting from the subtle spectral details will be discussed.

Different Types of Ester Are Present in Cell Walls

In dicotyledons and nongraminaceous monocotyledons, galacturonic acid residues of acidic pectins are available for esterification. In grasses, the hemicellulosic glucuronoarabinoxylan and the galactosyluronic acid-rich pectins are the two major polysaccharides containing uronic acids (Carpita and Gibeaut, 1993): however, the glucuronic acid residues in glucuronoarabinoxylans are not esterified (Kim and Carpita, 1992).

CWM of all five plants contain esters (Figs. 3 and 4A), but complexity in the ester band suggests that more than one type of ester may be present. In cell walls and isolated polymers, the maximum of the ester bands (band A) lies between 1741 and 1730 cm^{-1}, indicating that the majority of the wall esters are saturated alkyl esters (Parker, 1983; Colthup et al., 1990), and the presence of methyl-esterified forms of pectin in cell walls is well documented.

A close inspection of the apparently broad ester absorption reveals the presence of shoulders and/or asymmetry in the band shape, indicating that there may actually be a number of different ester types or that the ester groups exist in different local environments. The presence of nonmethyl esters in cell walls of elongating maize coleoptiles (Kim and Carpita, 1992) and tobacco suspension cells (McCann et al., 1994) has been reported. The heterogeneity in the nature of the ester function appears most clearly in the case of cell walls of rice and sweet corn (Fig. 3): the IR spectra of their unextracted cell walls exhibit a shoulder at 1720 cm^{-1} in the ester band A. This frequency is characteristic of aromatic esters. The Raman spectra of CWM of the Gramineae also

Table I. *Assignment of the main bands in IR and Raman spectra (Parker, 1983; Colthup et al., 1990)*
IR, IR active; R, Raman active. Weak activity is indicated with ( ).

Band	Activity		Assignment	Frequency Range	Comments
A	IR	R	C = O stretching of alkyl ester	1745 cm^{-1}	The exact frequency is dependent on attached groups
B	IR	(R)	Amide I; C = O stretching + contribution from C-N stretching	1650 cm^{-1}	The secondary structure affects the frequency
C		R	C = C stretching	≈1635 cm^{-1}	Due to the α-β double bond on the propanoid side chain of hydrocinnamic acids or lignins
D	(IR)	R	Aromatic C = C skeletal stretching	1600 cm^{-1}	Due to the aromatic ring of phenolic
E	IR		COO^- antisymmetric stretching	≈1600 cm^{-1}	Due to the acidic groups of polygalacturonic acids
F	IR	(R)	Amide II: N-H deformation + contribution from C-N stretching	1550 cm^{-1}	The secondary structure affects the frequency
G	IR			1520 cm^{-1}	
H		R	C-H bend	≈1456 cm^{-1}	
I	IR		COO^- symmetric stretching	1420 cm^{-1}	Due to the acidic group of polygalacturonic acids
J	(IR)	R	C-H bend	1370 cm^{-1}	
K	IR		C-O-H deformation and C-O stretching of phenolics + asymmetric C-C-O stretching of ester	≈1245 cm^{-1} (1260–1180 cm^{-1} for phenolics) (1280–1185 cm^{-1} for ester groups)	The exact frequency of this band depends on the attached group
L	IR	R	Amide III: interaction between C-N stretching and C-N-H in-plane bending of protein	≈1230 cm^{-1}	The secondary structure affects the frequency
M	IR	R	Nonlocalized, highly coupled vibrational modes of polysaccharide backbones	1160–970 cm^{-1}	The spectral envelope of the band depends on the crystallinity and conformation of the polysaccharide; fingerprint region for polysaccharides
N	IR		CH_2 and CH_3 rocking	960 cm^{-1}	
O		R	C-O-C antisymmetric stretching of the glycoside linkage	≈ 854 cm^{-1}	Intense in acidic pectins; highly reproducible
P		R	C-O-C symmetric stretching of the glycoside linkage	≈ 684 cm^{-1}	Intense in acidic pectins
Q		R	C-O-C deformation of the glycoside linkage	≈ 441 cm^{-1}	Intense in acidic pectins
R		R	C-O-C deformation of the glycoside linkage	≈ 336 cm^{-1}	Intense in acidic pectins

exhibit spectral features characteristic of phenolics (bands C and D) (Fig. 4B). Along with the presence of bands characteristic of phenolics in the spectra of the pectin-rich fraction of the Gramineae, this may result from esterified hydroxycinnamic acid derivatives such as diferulate ester (Fry, 1989) in the cell wall of grasses.

The comparative degree of esterification of the wall can be crudely assessed by the ratio of the areas of the ester band A to the pectin band M. Figure 5 shows that the walls of onion and carrot have the same degree of esterification, which is higher than in the walls of the Gramineae.

Three types of esters may be present in the wall: (a) free methyl esters of the acid groups of pectin, (b) other saturated-alkyl esters that may cross-link pectin molecules, and (c) phenolic esters. Differences in the extractability of pectins by de-esterifying agents may be explained by the differences in the relative proportion of these esters. Both non-Gramineae, onion and carrot, show a small decrease in the degree of esterification of their walls during the extraction with chelating agents. Calcium cross-linked pectin chains, which contain very few methyl esters, are solubilized. During the extraction with sodium carbonate, the saturated-alkyl ester cross-links are broken and the pectins, which may also be methyl esterified, are completely removed from the wall.

In contrast, the de-esterification of the Gramineae walls is more gradual. The removal of very small amounts of pectins (approximately 0.05% of the dry weight of the cell wall) cannot entirely account for the sharp decrease in the degree of esterification of the wall. In fact, it is mainly due to the concurrent release of esterified phenolics. These phenolics

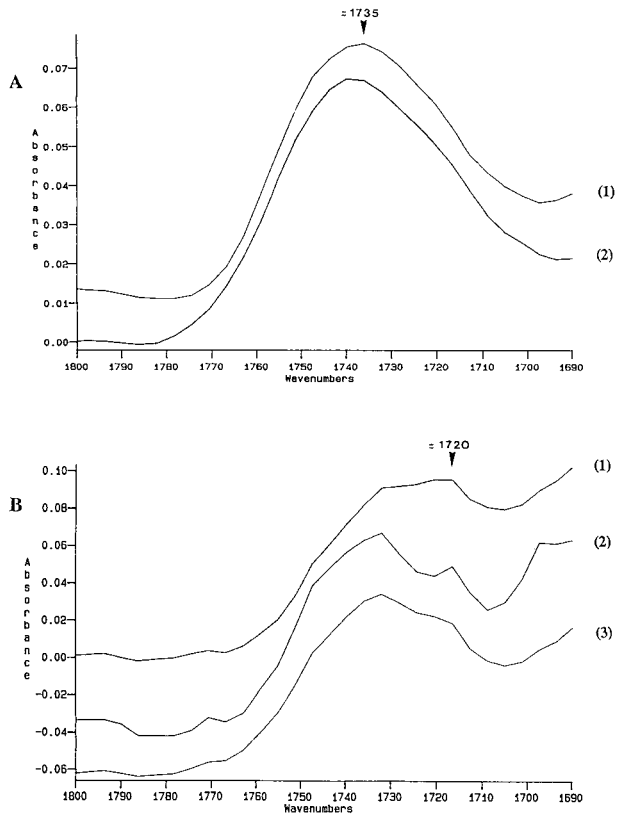

Figure 3. IR ester bands of CWM. The maximum of the ester band of carrot (A, 1) and onion (A, 2) is about 1735 cm^{-1}. This corresponds to the methyl esters of the D-galacturonic acid residues of pectins, but the broadness of the band may indicate the presence of other types of saturated alkyl-esters. The walls of the Gramineae also contain saturated alkyl-esters, but the ester bands of sweet corn (B, 1), rice (B, 2), and polypogon (B, 3) exhibit a shoulder at about 1720 cm^{-1}, which is diagnostic for phenolic esters.

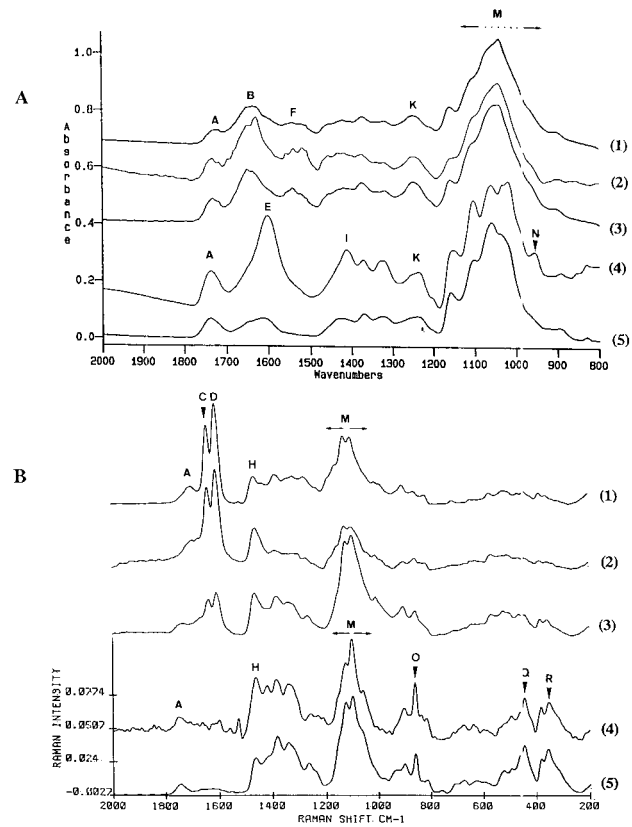

Figure 4. IR (A) and Raman (B) spectra of unextracted CWM of onion (1), carrot (2), polypogon (3), rice (4), and sweet corn (5). It can be seen that all cell walls contain esters (band A, 1745 cm^{-1}). The doublet C-D (1635 and 1600 cm^{-1}) in the Raman spectra shows the presence of phenolics in the unextracted CWM of the Gramineae, whereas a very large amount of pectin is detected in the wall of onion and carrot by Raman (bands O, P, Q, and R). Each cell wall gives highly characteristic vibrational spectra that can be used as a fingerprint for a given cell wall. A distinction between graminaceous and nongraminaceous plant cell walls is possible by examining their vibrational spectra, particularly the Raman.

are clearly seen in the Raman spectra of the imidazole-extractable polymers (Fig. 6D). The walls of the Gramineae are totally de-esterified by sodium carbonate, which brings about the release of most of the pectins in these walls (Fig. 6C). Some esterified phenolics are also extracted from the wall at this stage, as shown by the presence of bands C and D in the Raman spectra of the sodium carbonate-extractable polymers. The low degree of esterification of the wall of polypogon is mirrored by the presence of the relatively weak phenolic doublet in the Raman. There are clear differences in the kinds of ester present in the walls of the Gramineae and non-Gramineae.

Extractability of Pectins and Proteins from Cell Walls Shows Differences in Wall Architecture of the Gramineae and Non-Gramineae

Throughout the extraction, few changes in the IR and Raman bands, which arise largely from the contribution of cellulose and hemicellulose (particularly band M), are observed (Fig. 7). Spectrum 3 in Figure 7B possesses most of the characteristics of a spectrum of cellulose but not in the

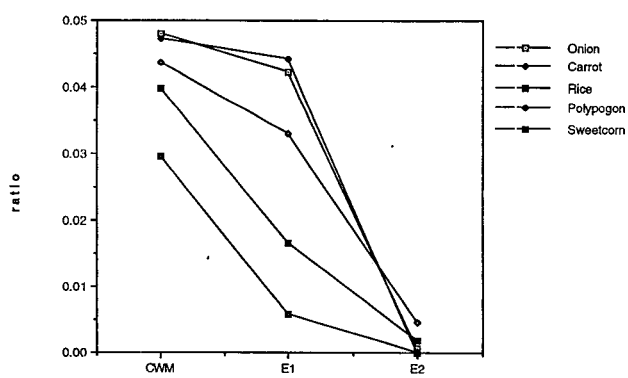

Figure 5. Degree of esterification of the wall versus extraction step. The comparative degree of esterification of the wall can be assessed by calculating the ratio of the area of the ester band A to the area of the pectin band M. Extraction steps: CWM, unextracted cell-wall material; E1, imidazole-extracted cell-wall material; E2, sodium carbonate-extracted cell-wall material.

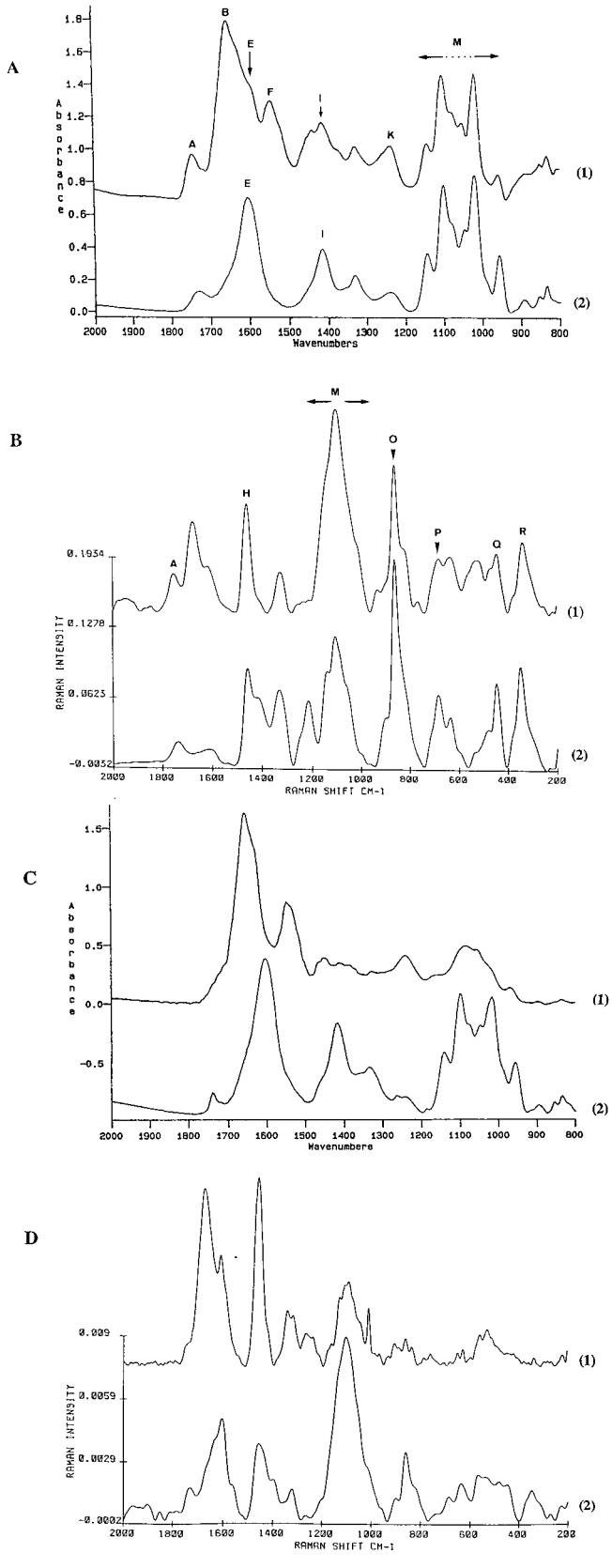

Figure 6. IR and Raman spectra of the polymers extracted from onion (A and B) and polypogon (C and D), obtained during extraction with imidazole (1) and extraction with sodium carbonate (2). As judged by the intensity of the IR bands B and F, a significant amount of protein is present in the imidazole-extractable polymers of onion, with a smaller amount in the sodium carbonate-extractable fraction. They are both rich in pectins as shown by the Raman bands O, P, Q, and R and the IR "fingerprint" region between 1200 and 900 cm^{-1}. In contrast, the imidazole-extractable fraction of polypogon contains large amounts of proteins (bands B and F) and some nonpectic polysaccharides (IR band M), whereas the features in the vibrational spectra show that the sodium carbonate-extractable fraction is primarily acidic pectin (IR band M). A significant amount of aromatic phenolics is also present in the polypogon fractions as shown by the presence of the Raman peaks C and D.

cellulose I form. Because the sample has not been exposed to strong alkali that may alter the structure to cellulose II, this may indicate the presence of cellulose IV in this sample. The presence of cellulose IV in primary walls has been suggested on the basis of electron diffraction studies on cell walls from rosewood cell-suspension cultures (Chanzy et al., 1979). However, the solubilization of pectins and some phenolics from the wall is accompanied by changes in the 1200 to 1500 cm^{-1} region and diminution in the intensity of some bands (bands A and K in the IR and C, O, P, Q, and R in Raman).

According to their vibrational spectra, the imidazole-extractable fractions of the Gramineae consist primarily of protein, whereas the sodium carbonate-extractable fractions consist primarily of acidic pectin with a small amount of protein (Fig. 6C). However, a significant amount of aromatic phenolics is also present in all these fractions, as shown by the presence of the Raman peaks C and D (Fig. 6D). As judged by the intensity of the amide I band in IR and Raman and the amide II band in IR, a significant amount of protein is also present in the imidazole-extractable polymers of onion and carrot, with a smaller amount in their sodium carbonate-extractable fractions (Fig. 6, A and B). The cell walls of the five plants still contain some proteins after the sodium carbonate extraction (although, in the case of onion, protein constitutes less than 1% of the dry weight of the wall).

In conclusion, there seem to be three sets of differentially extractable protein in the wall: one associated with the calcium cross-linked pectins, one with the ester cross-linked pectins, and one with the hemicellulosic-cellulosic network. From these data alone, it is not possible to determine whether the proteins of the wall are selectively solubilized because (a) their association with the matrix polysaccharides is disrupted by the extractant, or (b) they are bound to the extracted polysaccharides, or (c) the accessibility of the protein is increased by previous extraction steps. Preliminary results suggest that proteins are not bound to the polysaccharides in rice, polypogon, and sweet corn (data not shown).

Graminaceous walls are poor in pectin: a ratio of 10:1 for the quantitative amount of pectins extracted from non-Gramineae and Gramineae has been observed. As shown by the intensity of band O in the Raman and band M in the IR, the pectins of the Gramineae are almost exclusively extracted with sodium carbonate compared to 40% found in the imidazole-extractable fractions of onion and carrot. A very small amount of acidic pectin is detected in the imidazole-extract-

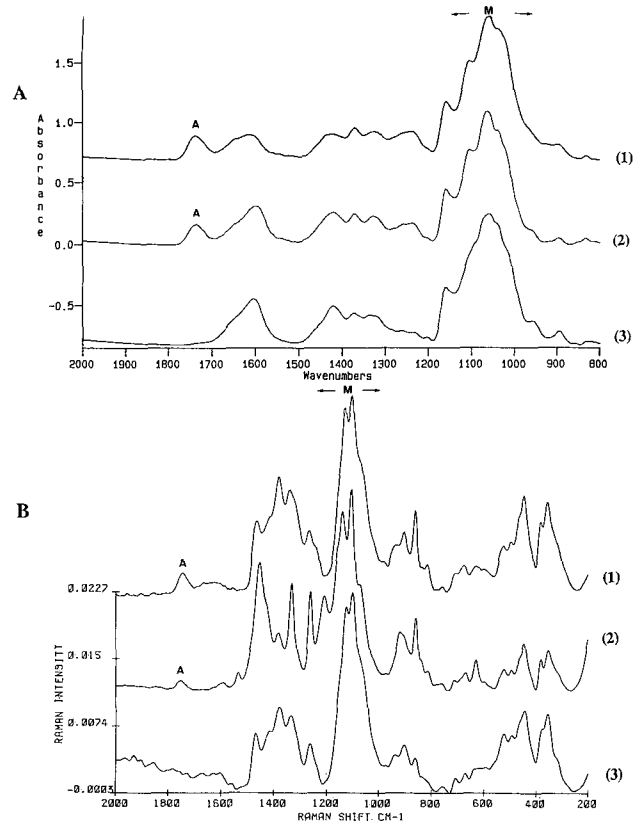

Figure 7. IR (A) and Raman (B) spectra of the unextracted cell wall of onion (1), and their cell-wall residues after the imidazole extraction (2) and after the sodium carbonate extraction (3). Throughout the extraction, few changes in the IR bands that arise largely from the contribution of cellulose and hemicellulose (particularly band M) are observed. However, the solubilization of pectin, esters, and some proteins from the wall is accompanied by changes in the 1200 to 1800 cm^{-1} region. These changes are more marked in the Raman spectra.

able fractions of the grasses by Raman spectroscopy. The arrangement of pectins in the wall architecture is clearly different in the Gramineae and non-Gramineae, with very little calcium-bound pectin present in the Gramineae.

Vibrational Spectroscopy Is a New Way of Fingerprinting the Plant Cell Wall

Plant cell walls contain a great variety of polymers of different types (proteins, aromatics, polysaccharides), each having a particular molecular conformation and interacting with neighboring molecules in a specific way. This complexity appears in the vibrational spectra of the cell walls and it is not possible to assign each spectral band to a particular polymer. However, this complexity can be used to fingerprint a particular cell wall or wall polymer, and this is illustrated in Figure 4.

The variation in intensity or absence of some bands in the spectra reflect compositional differences between cell walls. Subtle variation in intensity and position of individual bands can also arise from differences in conformation of the wall polymers and interactions between individual macromolecules. This is useful in comparative studies like ours, but applications in the food industry also exist. Thus, the characteristic nature of the spectra of CWM from different plants opens up the possibility of using vibrational spectroscopy for the verification of product authenticity and the detection of adulteration in food samples that are rich in CWM, such as jams and fruit purees (Wilson et al., 1993). The sensitivity of vibrational spectra to molecular conformation and crystallinity means that care must be taken to adopt standardized preparative procedures for such comparisons, and that the spectral range below 900 cm^{-1}, which is particularly sensitive to conformation, should not be used.

For example, the primary walls of rice and sweet corn are rich in proteins and phenolics but poor in pectins. Despite these compositional similarities, they can be differentiated by their vibrational spectra. For example, in the spectra of rice and sweet corn, peaks E and M in the IR and peaks A and H in the Raman have different line shapes and intensity. These variations are sufficient for spectral discrimination (Kemsley et al., 1994). Using multivariate analysis on the spectra should enable the determination of the nature of the adulteration such as a change in the content in monomeric sugars.

Figure 4 shows that it is possible to differentiate between Gramineae and non-Gramineae. Bands E, O, Q, and R arise in the spectra of the CWM of the non-Gramineae, whereas C, B, D, and F are characteristic of the Gramineae. Consequently, a distinction between graminaceous and nongraminaceous plant cell walls is possible by examining their vibrational spectra. However, the spectra of cell walls belonging to the different species are not totally identical, and subtle differences can be seen in, for example, rice and sweet corn (Fig. 4).

Taxonomy of plants relies on the study of ecological, morphological, cytological, and more recently genetical characteristics of the plants. Chemical data have also been used in the past (Stace, 1980). It is assumed that a classification will be more predictive the more characteristics on which it is based, and a multivariate approach has been introduced to increase the level of predictivity of a natural classification of plants. Because the vibrational spectra not only contain qualitative and quantitative information on the chemical structure, but also information on molecular conformation and interaction between neighboring molecules, vibrational spectroscopic data may be appropriate as a new variate for numerical taxonomy, although chemometric approaches such as principal components regression and discriminant analysis (Sharaf et al., 1986) may be required to extract the data.

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LITERATURE CITED

Agarwal UP, Atalla RH (1986) In-situ Raman microprobe studies of plant cell walls: macromolecular organization and compositional variability in the secondary wall of *Picea mariana* (Mill.) B.S.P. Planta **169:** 325–332

Atalla RH, Agarwal UP (1984) Raman microprobe evidence for lignin orientation in the cell walls of native woody tissue. Science **227:** 636–638

Atalla RH, Agarwal UP (1986) Recording Raman spectra from plant cell walls. Journal of Raman Spectroscopy **17:** 229–231

Atalla RH, Whitemore RE, Heimbach CJ (1980) Raman spectral evidence for molecular orientation in native cellulosic fibers. Macromolecules **13:** 1717–1719

Carpita NC (1989) Pectin polysaccharides of maize coleoptiles and proso millet cells in liquid culture. Phytochemistry **28:** 121–125

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J **3:** 1–30

Cassab GI, Varner JE (1988) Cell wall proteins. Annu Rev Plant Physiol Plant Mól Biol **39:** 321–353

Chanzy H, Imada K, Mollard A, Vuong R, Barnoud F (1979) Crystallographic aspects of sub-elementary cellulose fibrils occurring in the wall of rose cells cultured *in vivo*. Protoplasma **100:** 303–316

Colthup NB, Daly LH, Wiberley SE (1990) Introduction to Infrared and Raman Spectroscopy, Ed 3. Academic Press, New York, pp 301–305

Fry SC (1986) Cross-linking of matrix polymers in the growing cell walls of angiosperms. Annu Rev Plant Physiol **37:** 165–186

Fry SC (1989) Analysis of cross-links in the growing cell walls of higher plants. *In* HS Linsten, JS Jackson, eds, Modern Methods in Plant Analysis, New Series, Vol 10. Springer-Verlag, Berlin, pp 1-40

Gibeaut DM, Carpita NC (1993) Synthesis of (1–3),(1–4)-b-D-glucan in the Golgi apparatus of maize coleoptiles. Proc Natl Acad Sci USA **90:** 3850–3854

Griffiths PR, de Haseth JA (1986) *In* Fourier Transform Infrared Spectrometry. Wiley-Interscience, New York, pp 426–433

Hammouri M (1991) The investigation of plant cells using vibrational micro-spectroscopy. PhD thesis. University of East Anglia, Norwich, UK

Hayashi R, Morikawa H, Nakajima N, Ichikawa Y, Senda M (1980) Oriented structure of pectic polysaccharides in pea epidermal cell walls. Plant Cell Physiol **21:** 999–1005

Jarvis MC (1984) Structure and properties of pectin gels in plant cell walls. Plant Cell Environ **7:** 153–164

Kato Y, Nevin DJ (1985) Isolation and identification of O-(5-O-feruloyl-α-L-arabinofuranosyl)-(1–3)-O-β-D-xylopyranosyl-(1–4)-D-xylopyranose as a component of *Zea* shoot cell-walls. Carbohydr Res **137:** 139–150

Keller B (1993) Structural cell wall proteins. Plant Physiol **101:** 1127–1130

Kemsley EK, Belton PS, McCann MC, Ttofis S, Wilson RH, Delgadillo I (1994) Spectroscopic method for the authentication of vegetable matter. Food Control (in press)

Kim JB, Carpita NC (1992) Changes in esterification of uronic acid groups of cell wall polysaccharides during elongation of maize coleoptiles. Plant Physiol **98:** 646–653

Knox JP, Linstead PJ, King J, Cooper C, Roberts K (1989) Pectin esterification is spatially regulated both within cell walls and between developing tissues of root apices. Planta **181:** 512–521

Komalavilas P, Mort AJ (1989) The acetylation at O-3 of galacturonic acid in the rhamnose-rich portion of pectins. Carbohydr Res **189:** 261–272

McCann MC, Hammouri M, Wilson R, Belton P, Roberts K (1992) Fourier transform infrared microspectroscopy is a new way to look at plant cell walls. Plant Physiol **100:** 1940–1947

McCann MC, Shi J, Roberts K, Carpita NC (1994) Changes in pectin structure and localisation during the growth of unadapted and NaCl-adapted tobacco cells. Plant J **5:** 773–785

McCann MC, Stacey NJ, Wilson R, Roberts K (1993) Orientation of macromolecules in the walls of elongating carrot cells. J Cell Sci **106:** 1347–1356

McCann MC, Wells B, Roberts K (1990) Direct visualisation of cross-links in the primary plant cell wall. J Cell Sci **96:** 323–334

McNeil M, Darvill AG, Albersheim P (1980) Structure of plant cell walls. X. Rhamnogalacturonan-I, a structurally complex pectic polysaccharide in the walls of suspension-cultured sycamore cells. Plant Physiol **66:** 1128–1134

Morikawa H, Hayashi R, Senda M (1978) Infrared analysis of pea stem cell walls and oriented structure of matrix polysaccharides in them. Plant Cell Physiol **19:** 1151–1159

Mort AJ, Moerschbacher BM, Pierce ML, Maness NO (1991) Problems encountered during the extraction, purification and chromatography of pectic fragments, and some solutions to them. Carbohydr Res **215:** 219–227

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 473–497

Parker FS (1983) Applications of Infrared, Raman, and Resonance Raman Spectroscopy in Biochemistry. Plenum Press, New York

Redgwell RJ, Selvendran RR (1986) Structural features of cell wall polysaccharides on onion *Allium cepa*. Carbohydr Res **157:** 183–199

Scalbert A, Monties B, Lallemand JY, Guittet E, Rolando C (1985) Ether linkage between phenolic acids and lignin fractions from wheat straw. Phytochemistry **24:** 1359–1362

Sharaf MA, Illman DL, Kowalski BR (1986) Chemometrics. Vol 82 of Chemical Analysis. Wiley-Interscience, New York

Stace CA (1980) Plant Taxonomy and Biosystematics: Contemporary Biology. Edward Arnold Ltd, London

Stevenson TT, Darvill AG, Albersheim P (1988) Structural features of the plant cell-wall polysaccharide rhamnogalacturonan-II. Carbohydr Res **182:** 207–226

Wilson RH, Slack PT, Appleton GP, Li Sun, Belton PS (1993) Determination of the fruit content of jam using Fourier transform infrared spectroscopy. Food Chem **47:** 303–308