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Developments in mid-infrared FT-IR spectroscopy of selected carbohydrates

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Abstract

FT-IR spectroscopy has become a powerful research tool for elucidating the structure, physical properties and interactions of carbohydrates. It provides a new interpretive and experimental framework for the study of complicated systems of natural polymers. This paper gives an overview over new infrared applications in the study of carbohydrates, both small compounds and macromolecules. These include a wide range of studies of carbohydrates in different physical states, from the crystalline solid state to aqueous solution, and special techniques, which expand the experimental framework to the in-muro studies of plant materials, and quantitative determination. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Infrared spectroscopy is one of the most often used spectroscopic tools for the study of polymers. The method is rapid and sensitive, with a great variety of sampling techniques, and yet the instrumentation can still be considered inexpensive. The infrared absorption spectrum of a compound is probably its most unique physical property and the spectrum is often called the fingerprint of a molecule. The method was first of all used as an identification tool for relatively pure compounds. However, new techniques allow more detailed structural analysis of pure macromolecules and their model oligomers, analysis of polymer mixtures and crude samples, and even the investigation of interactions of particular macromolecules.

The pioneering investigations in infrared spectroscopy of carbohydrates date back to the fifties and the whole progress of the technique in the carbohydrate field was summarized by Mathlouthi and Koenig (1986). Updated experimental IR spectroscopy can be found in monographs (i.e. Koenig, 1992; Stuart, 1997; Wilson, 1994).

The mid-infrared region at 4000–400 cm^{-1} was the most widely used range for various applications. Most of the work reviewed in 1986 (Mathlouthi & Koenig, 1986) involved routine applications comprising interpretation of infrared and Raman carbohydrate data. However, they are limited basically to a few monomers and to the polymers cellulose and starch. The current biochemical, biological and food industrial applications, however, cover a wide variety of carbohydrates, including commercial sugars, cellulose, pectins, starch, hemicelluloses, carrageenans, hyaluronates etc., and their low molecular weight models.

In recent years, the use of Fourier-transform infrared (FT-IR) spectroscopy has been revitalized with a new generation of infrared instruments and sampling techniques which benefit structural evaluation (configurational and conformational analysis), systematic fingerprinting of carbohydrates in various physical states, allow special applications such as analysis of crude samples of natural products and FT-IR microspectroscopy with monitoring of structural changes. Chemometric methods are extensively used in quantitative analysis. In the field of physics of synthetic polymers new non-conventional techniques have been recently developed. Although there are some sampling limitations, two-dimensional infrared spectroscopy (2D FT-IR) has also been successfully introduced into the field of carbohydrate research.

2. Conventional IR and FT-IR spectroscopy

Mid-infrared spectroscopy measures the absorption of radiation in the frequency range from about 4000 to 400 cm^{-1}. The absorption involves transitions between vibrational energy states and rotational substates of the

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molecule. A selection rule applies to these transitions, and absorption of infrared light can occur only if the vibration causes a change in the dipole moment of molecule. It is possible to assign absorptions to specific functional groups, making IR spectroscopy very useful in structural elucidation. Because the intensity of the absorption is proportional to the concentration of the absorbing species, quantitative analysis is also possible (Wilson, 1994).

2.1. Monosaccharides and oligosaccharides

The infrared spectra of mono- and oligosaccharides were shown to be important in the structure elucidation of small molecules and also in polymer analysis where they represent structural units. The most frequently used IR spectral range in carbohydrate analysis was the anomeric region at 950–750 cm^{-1} where it is possible to distinguish bands characteristic for α and β conformers or pyranoid and furanoid ring vibrations of mono- and polysaccharides (Mathlouthi & Koenig, 1986). The α and β conformers of glucose, galactose and mannose can be distinguished using the 2a and 2b bands at 870–840 and 890 cm^{-1}, respectively (Mathlouthi & Koenig, 1986).

Xylopyranose, the structural unit of xylans is a pentose differing from glucose by the lack of the C-6 group. For the α-conformer of xylopyranose and derivatives the 2a band is missing due to the absence of an interaction between the anomeric C1-H and a C-6 group, and 2b is characteristic for the β-conformer (Kačuráková, Petráková & Ebringerová, 1990). It has been shown in a series of xylobiosides that the anomeric region was very sensitive to the type and position of glycosidic bond. While the differences were apparent in crystalline form, the freeze-dried amorphous form did not give unambiguous results. Further study of substituted and homo-xylooligosaccharides have been carried out at 1200–800 cm^{-1} (Kačuráková, Ebringerová, Hirsch & Hromádková, 1994). Results showed how the β-(1 → 4)-xylobioside IR spectra were influenced by substituent units such as xylose, glucose and arabinofuranose in different positions. Xylose or arabinose substituents in position O-3 showed significant influence on spectral shape compared to the xylose or glucose unit as substituent on xylobioside in the O-2 position. In conclusion, the type of substituent and the linkage position were found to have a strong effect on the shape of the infrared spectrum.

FT-IR spectra of amylose and four cellulose oligosaccharides between 1500 and 600 cm^{-1} have been recorded in the crystalline state. The two series contain the same monosaccharide building block, glucose, with different configurations of the glycosidic linkages, α- and β-(1 → 4), respectively. Characteristic spectral features associated with the glycosidic linkages and details of the stereochemistry were studied (Sekkal, Dincq, Legrand & Huvenne, 1995).

Monosaccharide (glucose, fructose, galactose, xylose, ribose), and disaccharide maltose complexes of Ni^{2+} and Mn^{2+} have been characterized by diffuse reflectance and aqueous solution absorbance FT-IR (Bandwar & Rao, 1996, 1997).

2.1.1. Hydration of oligosaccharides

Hydration of xylooligomers has been studied in an atmosphere with controlled humidy, ranging from dry to 98% RH (relative humidity). The spectra showed remarkable differences for crystalline and amorphous forms (Kačuráková, Belton, Wilson, Hirsch & Ebringerová, 1998a). The IR spectra of crystalline material gave well distinguished and sharp bands, while amorphous spectra were less resolved. The water deformation band at 1640 cm^{-1} was present in the spectrum of the wet amorphous sample while it was diminished in the spectrum of the dry amorphous form. Most of the oligomers showed one crystalline form in the 56–84% RH range. However, it was also possible to find dry and wet crystalline forms as was the case with xylopyranosyl-β-(1 → 2)-xylopyranoside, where they can be clearly distinguished at 56 and 76% RH, respectively (Fig. 1). Their spectra had slightly different relative intensities and also a band for crystal water (at 76% RH) was found at 1615 cm^{-1}.

The hydration results established the importance of defined conditions for IR measurements and showed that hydration control could be successfully achieved. They suggest that this kind of study can be performed on any kind of saccharide.

Several saccharides such as sucrose, glucose, and nigerose were hydrated (unpublished results) and showed that they are in the hydrated crystalline state at about 76–84% RH. The film spectra were free of the broad adsorbed water deformation band at 1645 cm^{-1} typical for KBr spectra. In the spectra of nigerose at 1564 cm^{-1} (Kačuráková, unpublished result), α,α-trehalose at 1680 cm^{-1}, and (1 → 3)-β-xylan at 1663 and 1588 cm^{-1} medium intensity absorption bands were found which belong to structural water (Kačuráková, Wellner, Ebringerová, Wilson & Belton, 1999). Because of the random occurrence of the water band there is no interpretation of the type of water so far. However, by observing the temperature dependence of the water bending band of α, α-trehalose dihydrate, it has been found that at 70°C the property of the bound water molecules changes from the ice-like water at 1680 cm^{-1} to liquid-like one. This finding indicated that the trehalose–water complex behaved like a small water cluster (Akao, Okubo, Ikeda, Inoue & Sakurai, 1998).

2.1.2. Aqueous solutions

A structural study of the sugars (glucose, fructose and sucrose) in aqueous solution has been achieved by attenuated total reflection (ATR) FT-IR spectroscopy. A comparison of the mono- and disaccharide spectra provided evidence for the conformational equilibrium, the intra- and intermolecular interactions and the “dimerization

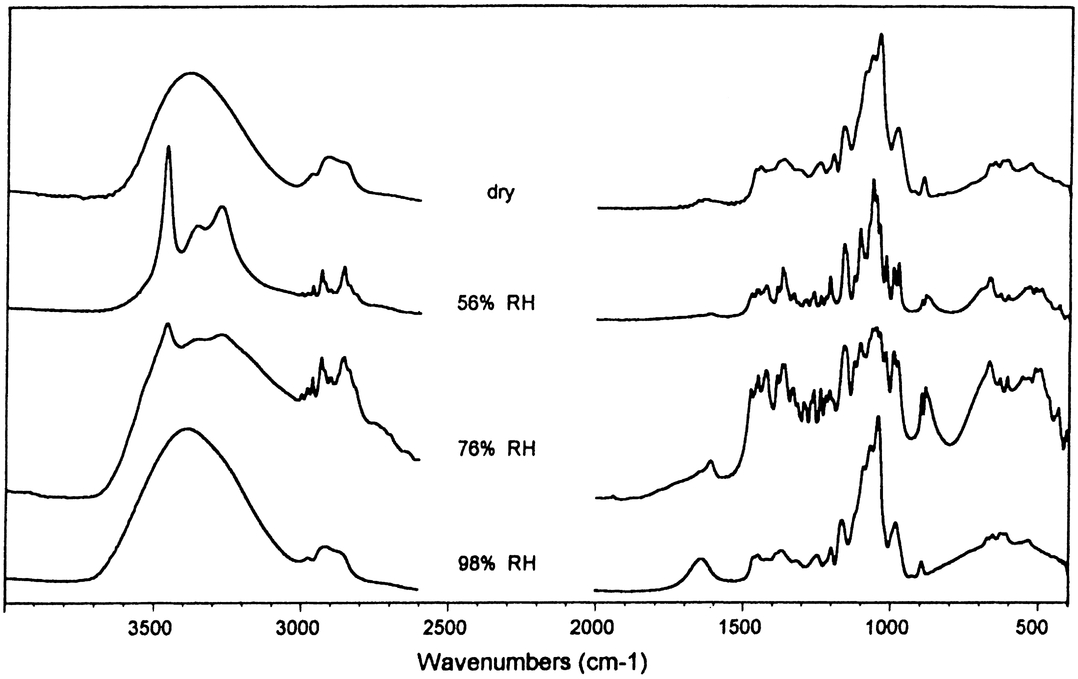

Fig. 1. FT-IR spectra of X*ylp*-β-(1 → 2)-*xylp*-(1-OMe) film on AgCl plate. The sample was conditioned at four relative humidity steps, %RH. Ordinate in arbitrary units. (Adapted from Kačuráková et al., 1998, with permission from John Wiley and Sons, Ltd.)

effect” on monosaccharide structures (Kodad, Mokhlisse, Davin & Mille, 1994).

Changes in conformation, and constraints imposed by hydrogen bonding with the solvent, have been studied in aqueous solution of oligosaccharides such as trehalose, sucrose, maltose, melibiose, lactose, maltotriose, raffinose and stachyose, differing in positions of the glycosidic bond (Kačuráková & Mathlouthi, 1996). The spectral data of the oligosaccharides at concentrations varying from dilute to saturated solution showed that trehalose was particularly stable in water while the sucrose molecule, as concentration increased, performed folding around its glycosidic bond in order to establish intramolecular hydrogen bonds. The ratio of integrated intensities A δ(CH2)/A δ(COH) at (1470–1200) and (1150–950) cm^{-1} ranges have been calculated to check the concentration effect. Almost all oligosaccharides showed a steady increase of the ratio, except for the sucrose, which exhibited abrupt changes in the slope at 27 and 65% (w/w).

The most pronounced spectral features differentiating the oligosaccharides from their hydrolysis products were found in the 1160–1150 and 1000–960 cm^{-1} regions which can also be used to distinguish the non-reducing from reducing sugars (Kačuráková & Mathlouthi, 1996).

2.1.3. Vibrational frequency caculations

The infrared spectra of α- and β-D-galactose were recorded, both in the mid-IR range (4000–500 cm^{-1}) and in the far-IR (500–50 cm^{-1}). These spectra constitute the basis of a crystalline-state force field established for these two molecules through a normal coordinate analysis. A modified Urey–Bradley–Shimanouchi force field was combined with an intermolecular potential energy function which included van der Waals interactions, electrostatic terms and an explicit hydrogen bond function. The computed potential energy distributions have been found to be compatible with previous assignments for glucose, particularly for the modes involving C6 and C–OH groups. For β-D-galactose the same force field was used with modified force constants due to the C1 and C6 groups (Sekkal, Legrand, Vergoten & Dauchez, 1992). The same procedure was applied on trehalose, sophorose and laminaribiose, which have been recorded in the crystalline state in the 4000–100 cm^{-1} region. The force field parameters were adapted from initial works on both anomers of glucose. The vibrational assignments of the observed bands were made on the basis of the potential energy distributions. Although the greatest part of the vibrational modes is very highly coupled, the calculated vibrational frequencies agreed very well with the observed frequencies (Dauchez, Derreumaux, Lagant, Vergoten, Sekkal & Legrand, 1994).

2.2. Polysaccharides

2.2.1. Cellulose

The most studied polysaccharide using infrared spectroscopy is cellulose, a β-(1 → 4)-linked glucan, which is universal in plants and occurs in algae and bacteria (Aspinall, 1983). The FT-IR studies cover identification of cellulose types (Fengel & Ludwig, 1991; Langkilde & Svantesson, 1995), determination of the degree of crystallinity (Hulleman, van Hazendonk & van Dam, 1994;

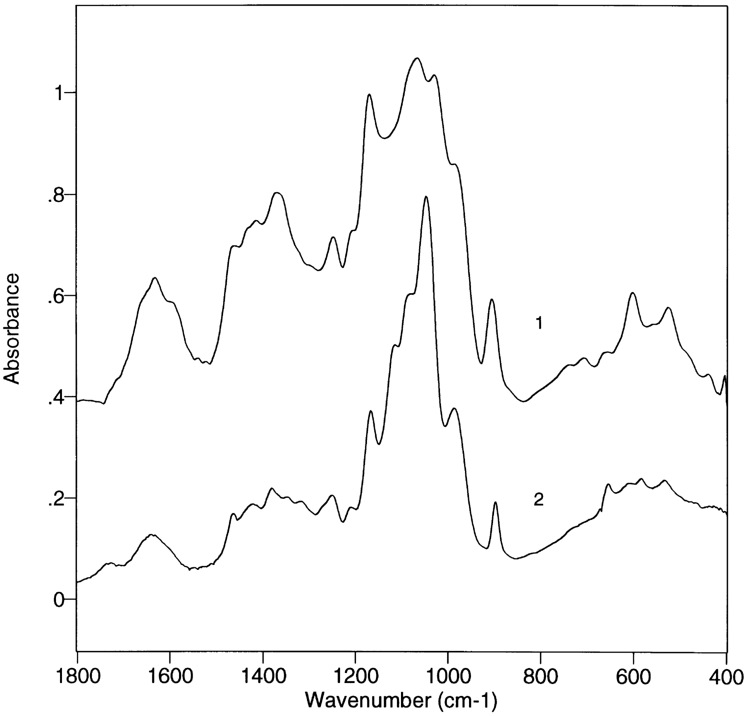

Fig. 2. FT-IR spectra of β-(1 → 3)-D-*xylan* (1) and β-(1 → 4)-D-xylan (2) measured in KBr pellets. (Adapted from Kačuráková et al., 1999, with permission from Elsevier Science.)

Kataoka, Hishikawa & Kondo, 1996; Rowe, McKillop & Bray, 1994) and the effect of batch and source variation on the crystallinity of microcrystalline cellulose (Rowe et al., 1994). Some differences in FT-IR spectra between cotton, linen and regenerated fibers were observed. Cotton linters and bacterial cellulose are restricted in the intensity of various bands whereas the positions of IR bands are the same (Fengel & Ludwig, 1991). FT-IR spectra supported the hypothesis that celluloses can be divided into algal–bacterial and cotton–ramie–wood types according to the spectra which are sensitive to the change from cellulose I → II (Michell, 1990).

FT-IR spectroscopy is a particularly useful tool for orientation studies of functional groups attached to the polymer chain and in orientational measurements the dichroic ratio is an important parameter. The spectrum of an oriented crystalline sample of native cellulose recorded in polarized radiation provided information about the direction of transition moments of various functional groups with respect to the fiber axis (Cael, Gardner, Koenig & Blackwell, 1975; Marchessault & Sundararajan, 1983; Mathlouthi & Koenig, 1986). Cellulose and glucose are some of the few carbohydrates on which normal coordinate analysis has been performed (Cael et al., 1975; Marchessault & Sundararajan, 1983; Mathlouthi & Koenig, 1986; Zhbankov, 1992).

2.2.2. Xylan

Xylan structure varies with the nature and degree of branching of the xylopyranan main chains which characteristically carry side chains of 4-O-methyl-D-glucuronic acid and L-arabinose (Aspinall, 1983). The β-(1 → 4)-xylans occur in higher plants and β-(1 → 3)-xylans in algae. Xylans with β-(1 → 4)- and β-(1 → 3)-linked xylose units as backbone showed marked differences in spectral shape (Fig. 2) due to different conformation and water content (Kačuráková et al., 1999). The spectra of substituted (1 → 4)-xylans depended on the degree of substitution and position of the substituent. In the case of arabinoxylan highly substituted with α-L-arabinofuranose at the C3 position the glycosidic (C–O–C) band intensity at 1150 cm^{-1} was diminished. This is due to conformation caused by significant steric interaction between the arabinose side chain, the backbone (Yui, Imada, Shibuya & Ogawa, 1995) and resulting water linkage. Similarly to the xylooligosaccharides the arabinofuranose or glucuronic substituents in position C-2 did not significantly affect the spectra (Kačuráková et al., 1998a; Kačuráková et al., 1999).

Stereochemical features of β-(1 → 4)-xylan and arabinoxylan have been studied by X-ray fiber diffraction, FT-IR spectroscopy, and conformational analysis (Kačuráková et al., 1994; Lelliott, Atkins, Juritz & Stephen, 1978; Yui et al., 1995). The water sorption of 4-O-methyl-glucuronoxylan in the (OH) stretching and 1200–1000 cm^{-1} region was reported earlier (Kalutskaya, 1988). It has been shown that absorbed water influences the molecular orientation and consequently the degree of order. These results motivated us to examine the xylan spectra as a function of relative humidity. The samples were conditioned in the atmosphere of controlled humidity and then measured as thin film layers on the surface of AgCl or BaF_2 windows. The xylans did not show any significant changes in the 1800–900 cm^{-1} region. The cause may be the structural water which is kept in cavities of the polymer network and which is not possible to remove even after drying above P_2O_5.

Infrared spectroscopy was utilized in many studies of xylan-type polysaccharides looking at solubility, fragmentation products after gamma-radiation, and xylan derivatives (quarternized, carboxybenzyl bromide) (Ebringerová, Kačuráková, Hromádková & Pružinec, 1989; Ebringerová, Hromádková, Kačuráková & Antal, 1994; Ebringerová, Novotná, Kačuráková & Machová, 1996; Ebringerová, Sroková, Talába, Kačuráková & Hromádková, 1998). With xylans usually are found lignins and other phenolics. Lignin, ferulic acid and proteins can be detected in the 1700–1500 cm^{-1} region, however, they occur in combination and so these are not easy to distinguish or determine quantitatively. For that reason FT-Raman spectroscopy was helpful (Kačuráková et al., 1999). Wheat straw (Stewart & Morrison, 1992; Sun, Lawther & Banks, 1996; Sun, Fang, Rowlands & Bolton, 1998a), fiber (Sun & Hughes, 1998), and sugar beet (Sun & Hughes, 1998, 1999) hemicelluloses have been fractionally and structurally characterized by FT-IR spectroscopy.

2.2.3. Pectin

Pectins are a family of polysaccharides made up of a backbone of linear (1 → 4)-linked α-D-galacturonan. The main pectin chain bears side chains of α-D-galactopyranose, α-L-arabinofuranose and α-(1 → 2)-linked rhamnopyranosyl residues which form a large portion of the cell wall of higher plants. With the investigation of the carboxyl group state, infrared spectroscopy offers the possibility of determining some functional groups of pectic derivatives in the 1900–1500 cm^{-1} region (Engelsen & Norgaard, 1996; Filippov, 1992). Determination of the esterification degree of carbonyl groups of pectin by means of infrared spectroscopy was carried out at 1745 and 1605–1630 cm^{-1} for ester vs. carboxylate regions, respectively (Chatjigakis, Pappas, Proxenia, Kalantzi, Rodis & Polissiou, 1998; Filippov & Kohn, 1974).

The interaction of divalent cations with potassium pectate and three potassium pectinate samples with degree of esterification, E, of 23, 59 and 93% have been studied by FT-IR (Wellner, Kačuráková, Maloviková, Wilson & Belton, 1998). Characteristic band shifts occured in (C–O) groups and ring vibrations in the 1200–900 cm^{-1} region as well as in the ν_{as} and ν_s carboxylate bands at around 1617 and 1420 cm^{-1}, indicating a metal coordination by the pectate chains in accordance with the egg-box hypothesis. The FT-IR spectra showed some interaction between pectate and K^+ and Mg^{2+} even when no gels were formed. Ca^{2+} and Sr^{2+} interacted strongly with pectate and low methoxyl pectinates. Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+} also showed complex formation with pectinates at 59%, and Pb^{2+} and Cu^{2+} to some extent even with very highly esterified pectinate (93%) (Wellner et al., 1998).

2.2.4. Starch

Widespread in plants, starch occurs mainly as α-(1 → 4)-linked glucan. When heated in water above a critical temperature, gels are produced on cooling. The process of gelation and the return of the system to the ordered state are important factors in food processing. The gelation and retrogradation of the two main constituents of starch, amylose and amylopectin, have been studied (Wilson & Belton, 1988; Wilson, Kalichevsky, Ring & Belton, 1987). Characteristic IR bands for starch gels have been found at 1046 and 1019 cm^{-1}. The infrared changes observed were related to changes in molecular conformation and explained in terms of models of random coil, helix forms (fast) and helix aggregates (slow). IR ratio measurements were used to follow the gelation and retrogradation with time. Retrogradation studies of waxy maize starch and wheat starch have shown that spectra are sensitive to the conformation and degree of hydration of constituent biopolymers (Goodfellow & Wilson, 1990). Such studies of starch components and carrageenans indicated that FT-IR is a powerful technique for studying conformational changes in biopolymer systems (Belton, Goodfellow & Wilson, 1989) even if it is not possible to determine the absolute conformation of a biopolymer.

Retrogradation and physical ageing of model starch systems with respect to their glass transition temperatures have been investigated by diffuse reflectance (DRIFT) Fourier transform infrared spectroscopy which detected the onset of retrogradation of starch materials by changes in peak line shapes and intensities in the characteristic area between 995 and 1020 cm^{-1} (Smits, Ruhnau, Vliegenthart & vanSoest, 1998). Self-supporting, smooth and translucent starch films were prepared by drying starch gels at various temperatures and air humidities. Light microscopy showed different structures among the films dried at elevated temperatures compared with those dried at room temperature. Attenuated total reflectance infrared spectroscopy (ATR) revealed an increasing absorbance ratio of the peaks at 1047 and 1032 cm^{-1}, which correlates with an increasing crystallinity in the films (Rindlav, Hulleman & Gatenholm, 1997). Retrogradation kinetics for a potato starch gel was monitored by FT-IR spectroscopy and compared with waxy maize starch. The spectra showed the C–C and C–O stretching region 1300–800 cm^{-1} to be sensitive to the retrogradation process. Different retrogradation rates were found for amylopectin and amylose; whereas amylose crystallization occured within a few hours, amylopectin crystallization was slow and took a few weeks (Vansoest, Dewit, Tournois & Vliegenthart, 1994).

2.2.5. Other polysaccharides

FT-IR spectroscopy was suggested as a possible method for the determination of the relative ratio of α- and β-glycosidic linkages in glucans which is important for the estimation of purity of glucan preparations (Šandula, Kogan, Kačuráková & Machová, 1999). The carboxylate anion’s asymmetric and symmetric stretching vibrations are characteristically at 1600 and 1421 cm^{-1} in carboxymethyl–glucan (Muzzarelli, Illari & Petrarulo, 1994a; Šandula et al., 1999).

FT-IR spectroscopy is suitable for analysis of the amino groups of aminosugars such as chitin and chitosans (Ritthidej, Chomoto, Pummangura & Menasveta, 1994) and has been applied to chitin polymorphs (Focher, Naggi, Torri, Cosani & Trebojevich, 1992), crystallinity of krill chitin derivatives (Wlochowicz, Przygocki & Polowinski, 1987), chitosan acylation (Xu, McCarthy, Gross & Kaplan, 1996; Aiba, 1994; Muzzarelli, Illari & Tomasetti, 1994b) and chitin–glucan (Šandula et al., 1999) complex characterization. A complex application of FT-IR analysis of polysaccharides using low molecular weight models involved sodium hyaluronate in amorphous state and aqueous solution (Gilli, Kačuráková, Mathlouthi Navarini & Paoletti, 1994).

Infrared study have been reported on treatment of solutions of propylene glycol alginate (PGA) with morpholine, which resulted in the formation of a stable gel which occurred with a lower PGA concentration then it was required for gelation by the high pH mechanism (Gray & Philp, 1991). The gelation was the result of replacement of the propylene glycol groups to give morpholine amide groups on the alginate chains.

A review of literature data on vibrational spectroscopy of sulfated polysaccharides and new results have been presented (Matsuhiro, 1996). Information from classical infrared spectroscopy studies has been of significance for characterizing seaweed galactans. Agar-type polymers showed two diagnostic second derivative bands in the region 800–700 cm^{-1}. Carrageenans exhibited a number of bands in the region 1600–1000 cm^{-1}. The second-derivative mode of the FT-IR spectra can be applied to distinguish agarophyte and carrageenophyte seaweed samples, which showed the same bands as the corresponding polysaccharides (Matsuhiro, 1996; Matsuhiro & Rivas, 1993). Variable temperature ATR technique was applied to starch and carrageenans in the 1400–1000 cm^{-1} region, where K^{+}, Rb^{+} and Cs^{+} sulfate groups were assigned. In the 1100–800 cm^{-1} region conformational changes were assigned (Wilson, Goodfellow & Belton, 1988). The infrared microspectrometry identified agars without any extraction directly in various seaweeds. The main bands of agars are all observed, especially the intense ones between 1000 and 1100 cm^{-1} and below 1000 cm^{-1} (Sekkal, et al., 1993a).

FTIR spectra in the 1600–100 cm^{-1} range have been employed in a structural analysis of biopolymers of the polygalactane type. In spite of the complexity of the spectra in this region, precise assignments have been made, first on the basis of previously calculated frequencies of the basic unit (D-galactose) and secondly by comparing the spectra of kappa-, iota-, and lambda-carrageenans, as well as agar and appropriate disaccharides. The analysis provided a basis for studies of the conformational changes accompanying gelation, a process which is different from one polygalactan to another (Sekkal, Legrand, Huvenne & Verdus, 1993b). The effect of the anomeric structure of D-galactose-3-sulfate (potassium salt) on its spectral characteristics was studied by FT-IR spectroscopy under a variety of conditions. The C–O–S vibrational bands in the region 900–800 cm^{-1} depended on the anomeric structure and on hydration. In the dry state the α-anomer had a band at 868 cm^{-1} while the β-anomer had bands at 882 and 831 cm^{-1}. These differences in frequency can be useful as a diagnostic tool in determining the configuration at C-1 of galactose-3-sulfate units present in many molecules of biological importance (Koshy & Boggs, 1997). Equatorial sulfate ester substitution at O-6 of galactose residues have been identified at 820 cm^{-1} in red alga polysaccharides (Liao, Chiovitti, Munro, Craik, Kraft & Bacic, 1996).

3. Quantitative FT-IR spectroscopy

3.1. Sample identification and classification

The authentication of food is a very important issue both for the consumers and for the food industry with respect to all levels of the food chain from raw materials to finished products. Data from instrumental techniques such as mid-infrared spectroscopy are increasingly being employed for sample identification and classification using chemometric methods based on principal component analysis (PCA), least squares (PLS), artificial neural network (ANN), discriminant analysis (DA), and principal variables (PV).

Quantitative FT-IR analysis of sugar mixtures is rapid and has the potential for on-line monitoring in industry. A matrix method has been applied for soft drinks containing sucrose, glucose and fructose (Kemsley, Zhuo, Hammouri & Wilson, 1992). Jam type classification and fruit content has been performed using PCA, PLS and DA methods (Defernez & Wilson, 1995; Wilson, Slack, Appleton, Sun & Belton, 1993; Defernez, Kemsley & Wilson, 1995). An investigation of the relative effects of sources of instrumental instability, using a model developed for fruit puree classification was carried out on single-beam spectra, which are potentially useful for on-line industrial analysis. Suggestions are given to reduce the instrumental and experimental interferences on chemometric analyses, both when recording spectra and for managing spectral databases (Defernez & Wilson, 1997). It has become apparent that mid-IR can be used to address a wide range of issues and provide solutions for rapid analysis and on-line control with new applications to food, which include new qualitative and quantitative applications and discriminant (classification) methods. The largest number of new applications and technical developments have used attenuated total reflectance (ATR). Novel ATR cells have been designed for high-temperature, high-pressure and a range of on-line applications. The analysis of sugars in various systems has been particularly well studied. The authors (Wilson & Tapp, 1999) predict that the use of mid-IR spectroscopy is likely to continue to increase and develop in the near future. ATR sampling has been used to detect adulteration of raspberry purees with a database collected of over 800 adulterated fruit purees. PLS regression of the spectra gave good classification success (Kemsley, Holland, Defernez & Wilson, 1996). The speed of FT-IR spectroscopy makes this technique a rapid method for screening raspberry purees for adulterants. A comparative study has been carried out between a horizontal ATR cell and a Cylindrical Internal Reflection cell (CIRCLE). Both cells were employed for the determination of glucose, fructose, sucrose and total sugar in soft drinks and fruit juices using absorbance measurements at two resolution values (4 and 8 cm^{-1}). Data were processed by PLS regression. Both cells provided appropriate figures of merit, but the analytical sensitivity obtained using the horizontal ATR cell was three times higher than that obtained using the CIRCLE cell (Garrigues, Rambla & de la Guardia, 1998). Opaque raw sugar cane juices representative of a sugar cane harvest have been analyzed by ATR in the 1250–800 cm^{-1} region and the spectral data have been processed by PCA and PCR (Cadet & Offmann, 1997). The use of ATR in the quantification of individual sugar concentrations (glucose, maltose, maltotriose, and maltodextrines) in real mixtures extracted during starch hydrolysis was performed. Glucose and maltose were detected with the required precision, but not maltotriose or maltodextrines (Bellonmaurel, Vallat & Goffinet, 1995). A method based on diffuse reflectance FT-IR (DRIFT) using PLS analysis has been developed for rapid determination of carbohydrates (arabinose, galactose, glucose, mannose and xylose) in Kraft pulp (Backa & Brolin, 1991).

In the 1200–800 cm^{-1} region multivariate analysis was performed to characterize the pectic polysaccharides from olive and orange. The FTIR spectra were shown to be a good source for quick evaluation and polysaccharide composition of samples of pectic origin (Coimbra, Barros, Barros, Rutledge & Delgadillo, 1998). The possibility of creating a fast method for determining quality parameters in aminated pectins was shown using IR spectroscopy and chemometrics. The models built with the PV methods were found to be superior in performance compared to models with knowledge based selection method and PLS models (Engelsen & Norgaard, 1996).

FT-IR spectroscopy was used for sulfate analysis of bacterial polysaccharides. The data indicated that this technique could be used to determine the chemical composition of the polymers along with a semiquantitative estimation of the sulfate content. A good correlation has been found between FT-IR and other analytical techniques for sulfate concentrations ranging from 2.4 to 20% (Lijour, Gentric, Deslandes & Guezennec, 1994).

3.2. Structural applications of chemometrics

FT-IR and X-ray analyses were employed to determine the relative ratio of cellulose I_α and I_β crystalline phases present in each developmental stage of coniferous tracheid cell wall formation. The IR spectra showed that initially the I_α phase occupies 50% of the crystalline regions in the primary cell wall cellulose and this value drops 20% after ceasing of the cell enlarging growth for the formation of the secondary wall cellulose (the remaining regions are composed of the I_β phase) (Kataoka & Kondo, 1999). Cellulosic fabrics have been studied by the DRIFT technique. The spectra were qualitatively and quantitatively differentiated with the aid of chemometrics techniques, namely principal component analysis and soft independent modelling of class analogies and showed that discrimination on the basis of fabric dye, fabric type and textile processing can be achieved (Gilbert & Kokot, 1995). Hydroxypropyl distarch phosphate, acetylated distarch adipate, acid treated and pregelatinized modifications of the corn starch and waxy starch have been classified and the recognition of their modifications by artificial neural network (ANN) processing of ATR/FT-IR spectra. Natural groupings of similarly modified samples have been obtained using the self-organizing artificial neural network of the Kohonen type (Dolmatova, Ruckebusch, Dupuy, Huvenne & Legrand, 1998). The novel approach of the classification of modified starches with regard to their origin and the recognition of their properties and modifications by chemometric treatments of infrared spectra have been studied using ATR accessory, statistical (PCA, PLS), and neuronal (Kohonen and feed forward networks) methods (Vandeerstraeten, Wojciechowski, Dupuy & Huvenne, 1998).

Complex-formation between carbohydrates and cations could have important biological implications. The phenomena of split and shift of two peaks centered at 1053 and 991 cm^{-1} obtained by PCA have been investigated and explained in terms of interaction, involving potassium ions and sucrose molecules which would be responsible for the storage of this cation with essential role in plant metabolism (Cadet & Offmann, 1996).

4. Plant cell wall

The cell wall represents polymer systems in complex mixtures of both variable structure and composition. The spectroscopic probe must permit selective monitoring of structural features to determine not only the type of polymer but also how they are ordered and connected together. 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. It is not possible to assign each IR band in the cell wall spectra because of their complexity. The variation in intensity or absence of some bands in the spectra, however, reflects compositional differences between cell walls. Subtle variation in intensity and position

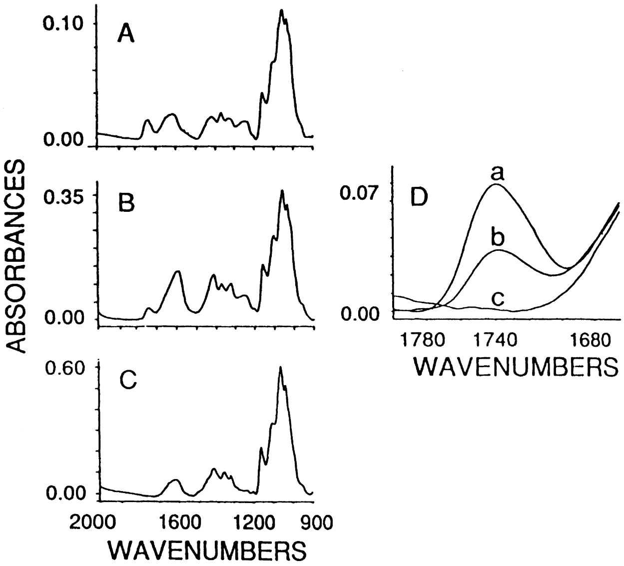

Fig. 3. FT-IR spectra of onion cell wall material. (A) Before extraction; (B) after extraction with cyclohexanediamine-tetraacetic acid; (C) a further extraction with Na_2CO_3; and (D) expanded 1780–1680 cm^{-1} shows the diminishing (a–c) of the carboxylic ester band at 1740 cm^{-1} with these extractions. (Adapted from McCann et al., 1992, with permission from The American Society of Plant Physiology.)

of individual bands can also arise from differences in conformation of the wall polymers and interactions between individual macromolecules. This is useful in both comparative studies and also industrial applications.

FT-IR spectroscopy was shown to be a powerful tool for investigating cellulose crystalline structure (Kataoka & Kondo, 1998) and primary cell wall architecture (McCann, Hammouri, Wilson, Belton & Roberts, 1992) at a molecular level. It provided complementary information to that obtained by FT-Raman spectrometry to aid data interpretation in the 1800–800 cm^{-1} range (Séné, McCann, Wilson & Grinter, 1994). Spectra of extracted onion epidermis are shown in Fig. 3 (McCann et al., 1992). Cell wall composition of dried sections of *Solieria chordalis* have been studied using FT-IR microspectrometry. The results suggested that it is an accurate method for the assessment of cell wall differentiation and also variations in sulfate substitutions on the cell wall polysaccharides in situ. Cortex, sub-cortex and medulla were compared for their iota-carrageenan content through analysis of ratios between peak heights (Fournet, Gall, Deslandeds, Huvenne, Sombert & Floch, 1997). FT-IR microspectrometry has been carried out in order to identify agars without any extraction directly in various seaweeds (Sekkal & Legrand, 1993).

5. Non-conventional FT-IR spectroscopy

5.1. FT-IR microspectroscopy

IR spectroscopy usually measures all polymers in the sample regardless of their spatial distribution. However, small-scale structural changes can produce large effects on the physical and mechanical properties of biopolymer samples. Therefore FT-IR microspectroscopy was applied in the investigation of plant materials, which allows measurements of structural features and monitoring of small changes in a $10 \times 10\ \mu m$ area (Chen, Wilson & McCann, 1997a).

In order to determine the relative ratio of cellulose I_α and I_β cystalline phases in development stages of wood cell wall formation, continuous FT-IR microscopy monitoring was used. The results led to a novel concept of on–off stressed crystallization, which may be induced by cellular enlarging growth for primary wall formation (Kataoka & Kondo, 1999).

FT-IR microspectroscopy was applied to three distinct types of plant tissue (Stewart, 1996). Reflectance microspectroscopy of nutshells highlighted the differences between the chemistries of the inner and outer surfaces and the tissue as a whole. The outer surfaces were suberized, while the inner surfaces contained absorbances indicative of lignin or tannins or both. Transmission microspectroscopy was used to follow the changes in cell wall structure and composition of flax epidermal cells during development (Stewart, McDougall & Batty, 1995; Himmelsbach, Khalili & Akin, 1998). Different chemical components (pectin, protein, lignin, cellulose) and their location in strawbery achene, vascular bundles and cortical cell walls were studied by means of bright-field microscopy and FT-IR microscopy. Both methods showed that lignin was an important component of achene and vascular bundles, whereas the cortical cell walls contained mainly pectin and cellulose in the middle lamella, and protein as deposits in the outer layer (Suutarinen, Anakainen & Autio, 1998).

FT-IR microspectrometry method has been applied on sections of different red algae, whose cell wall is mainly constituted of polygalactanes such as agar and carrageenans. The comparison of the obtained spectra with those previously reported for the extracts from the same seaweeds allowed the confirmation of the nature of each carrageenan (Sekkal et al., 1993b).

A technique has been developed to produce compositional maps of phase-separated protein/polysaccharide mixed gels using FT-IR microspectroscopy and PLS method. The maps plot out the composition of either the protein, the polysaccharide or the water as a function of position in the sample and are presented in the form of 2D contour plots (Durrani & Donald, 1995). Linear discriminant analysis of FT-IR spectra is a robust method to identify a broad range of structural and architectural alterations in cell walls, appearing as a consequence of regulation, environmental adaptation or genetic modification (Chen, Carpita, Reiter, Wilson, Jeffries & McCann, 1998). A rapid method to screen large numbers of mutant plants for a broad range of cell wall phenotypes using FT-IR microspectroscopy of leaves has been developed. The Fig. 4 shows spectra of *Arabidopsis* plants, one of which had been grown in light

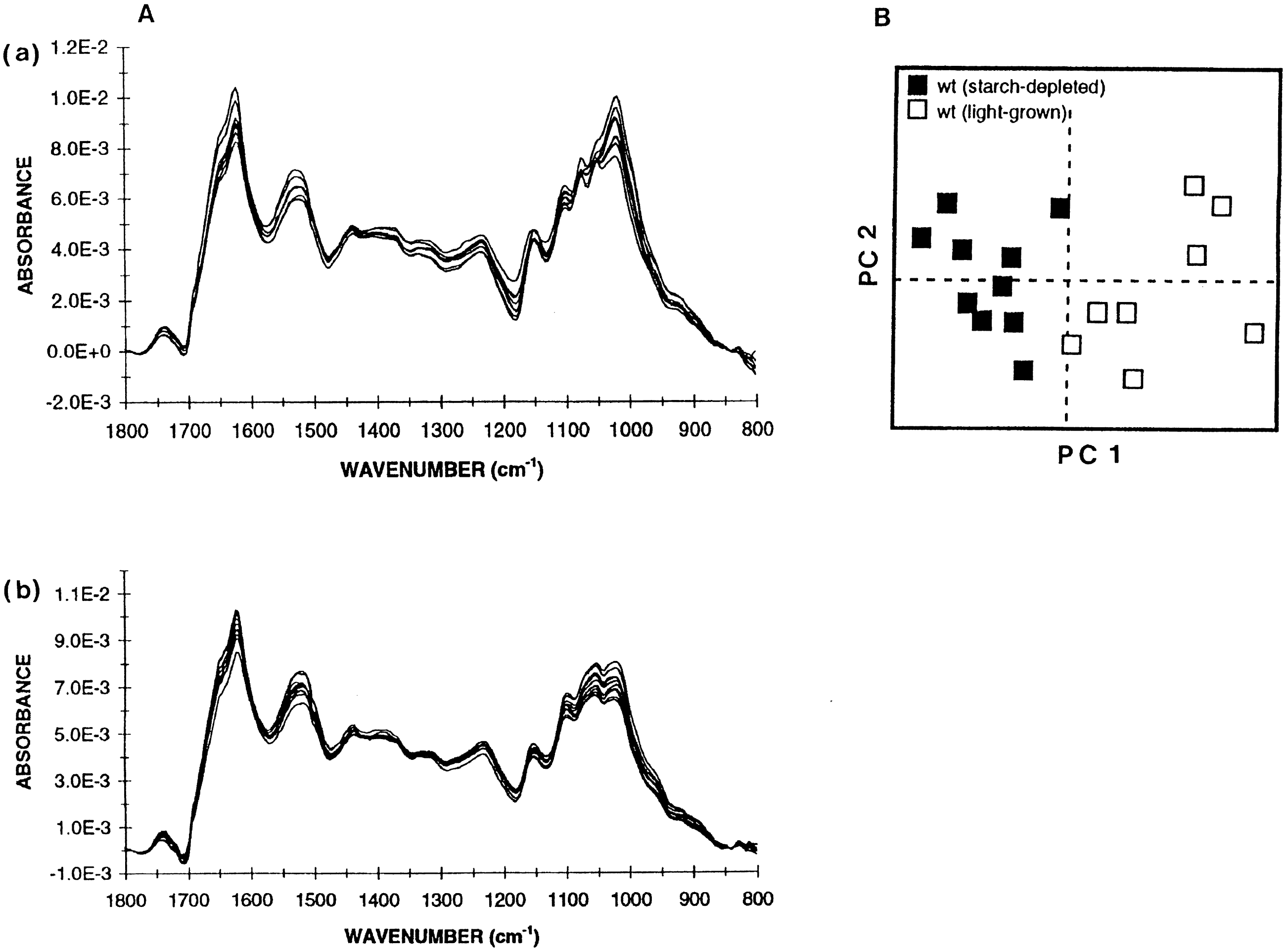

Fig. 4. (A) Area normalized and base-line corrected FT-IR spectra in the range 1800–850 cm^{-1} obtained from leaves of light-grown Arabidopsis (a) and from leaves grown in darkness 2 days (b). (B) A plot of the two principal components (PC) shows groups of light-grown and starch depleted Arabidopsis plants can be separated by the first PC score. Dashed lines indicate the mean PC scores for the entire population. (Adapted from Chen et al., 1998, with permission from Blackwell Science Ltd.)

(Aa), and another which had been starch-depleted in darkness (Ab). Exploratory principal component analysis indicated that mutants deficient in different cell-wall sugars can be distinguished from each other. A single PC score that accounted for over 90% of the total spectral variation could separate the two groups (B).

FT-IR microspectroscopy was used to study glasses of pure carbohydrates and in the cytoplasm of desiccation tolerant plant organs. The position of the OH stretching vibration band (νOH) shifted with temperature. Wavenumber–temperature coefficient (νOH vs. temperature) was determined for dry glucose, sucrose, maltose, trehalose and raffinose. The data suggested that the carbohydrates that are present in the cytoplasm are primary factors contributing to the glassy state (Wolkers, Oldenhof, Alberda & Hoekstra, 1998).

5.2. Molecular orientation

Molecular orientation was shown to be of fundamental importance in studies of plant cell wall polysaccharides (McCann, Chen).

Polyethylene is the most studied example using orientation and it is a good standard to start analyzing any other kind of polymer (Rao, 1963). Biopolymers are much more complicated systems, however, but polarized spectra can be acquired. The measured absorption bands are generally classified as parallel ($\|$) or perpendicular ($\perp$). Absorbance spectra of polymers taken in parallel and perpendicular polarized light with respect to a fixed axis of the sample give information about the orientation of macromolecules. Difference bands (($\|$ minus ($\perp$)) in the positive direction represent frequencies of dipole moments oriented parallel, and those which are negative, perpendicular to the fiber axis. Such difference only occurs when systems have preferential orientation and cancel out with random orientation of macromolecules.

FT-IR provides a powerful and rapid essay for wall components studies by identifying polymers and functional groups non-destructively in-muro (McCann et al., 1992).

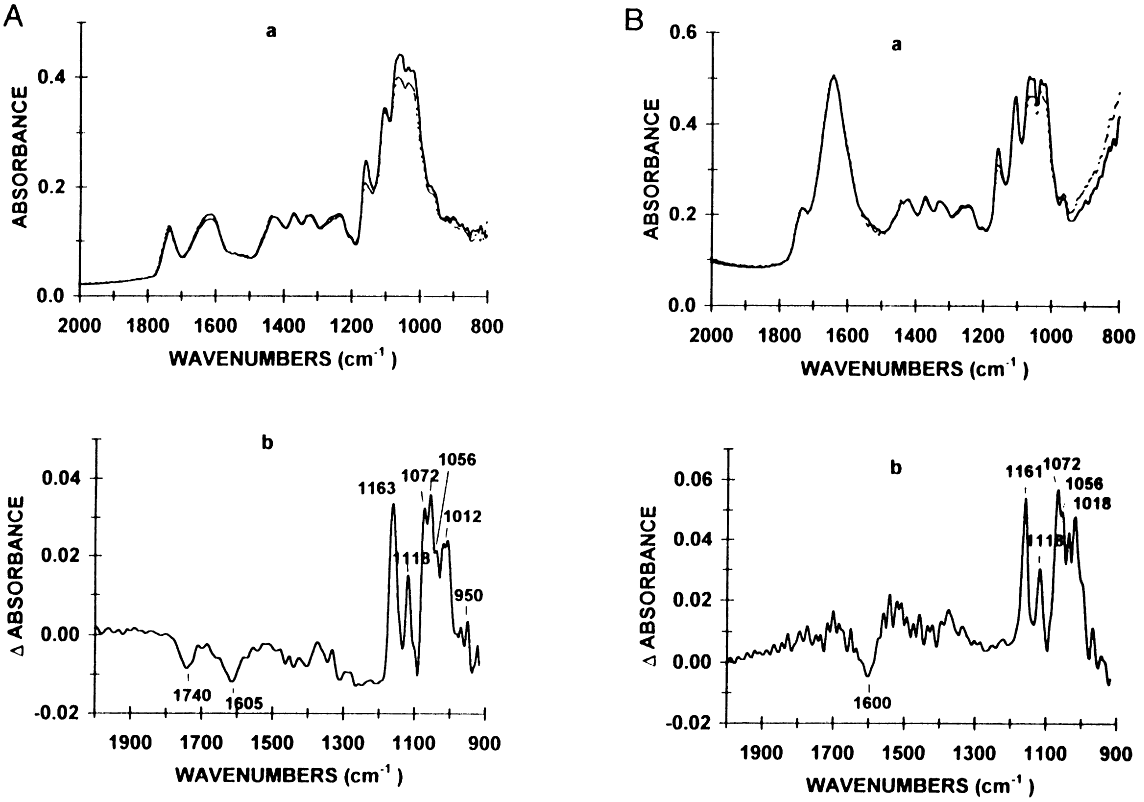

Fig. 5. (A) FT-IR microscope spectra of dry onion epidermal cell walls taken with (a) parallel (solid line) and perpendicular polarization (dotted line) with respect to the long axis of the cell, and (b) the difference spectrum between the parallel and perpendicular polarization directions. (B) FT-IR microscope spectra of hydrated onion epidermal cell walls taken with (a) parallel (solid line) and perpendicular polarization (dotted line) with respect to the long axis of the cell, and (b) the difference spectrum between the parallel and perpendicular polarization directions. (Adapted from Chen et al., 1997a, with permission from Blackwell Science Ltd.)

The direct visualization with polarized FT-IR microspectroscopy permits determination of the orientation of particular functional groups with respect of the cell elongation. The results showed that the single cell wall polymers, such as pectin and protein, not previously thought to be ordered are strictly ordered in some cell wall types (McCann, Stacey, Wilson & Roberts, 1993). FT-IR spectroscopy was used to map changes in wall composition during elongation and between tissue types (McCann & Roberts, 1994). FT-IR microspectroscopy was also used to study the effects of applied mechanical stress. The cell wall biopolymers under stress align in the direction of applied stress (Wilson, Smith, Kačuráková, Saunders, Wellner & Waldron, 2000).

Very little was known about the effects of hydration on polymer conformations in the wall. Therefore a hydration cell has been constructed that allows the study of samples with controlled moisture content in-situ and to analyze composition and architecture of complete cell walls using FT-IR microspectroscopy. The technique can detect large conformational changes in pectic polymers on removal from the cell wall and on drying (Chen et al., 1997a; Chen, Wilson & McCann, 1997b; Chen et al., 1998). A pair of polarized spectra of the dry sample of onion epidermal cell walls is shown on Fig. 5Aa. There are clearly differences in some band intensities between parallel and perpendicular polarization directions, indicating that there are molecular alignments within the walls. The difference spectrum ((||) minus (⊥)) showed that the IR bands at 1163–950 cm^{-1} are more intense with parallel orientation and can be correlated with the polysaccharides backbone (Fig. 5Ab). This suggest that the cellulose with bands at 1162 and 1056 cm^{-1} is arranged predominantly parallel to the long axis of the epidermal cell. The IR bands at 1740 and 1605 cm^{-1} represent the vibrations from pectin ester and carboxylate groups, respectively, which are stronger in perpendicular orientation. The spectra of hydrated onion epidermal cell walls are shown in Fig. 5Ba and b, where the changes are basically the same showing an IR band at 1018 characteristic for pectin gels (Chen et al., 1998; Wellner et al., 1998). The results showed also that the molecular orientation is intrinsic and does not result from drying. The rigorous models of cell wall architecture and roles of individual molecules in such structures can only be constructed with hydrated samples, particularly if a full structural analysis including molecular conformation and crystallinity is required (Chen et al., 1997a,b).

5.3. Dynamic 2D-FTIR spectroscopy

The complex structure of plant cell walls is an important factor in determining the biophysical properties of plant tissue but the macromolecular interactions, which control the mechanical properties of the cell wall, are poorly understood. Numerous applications of IR spectroscopy are nowadays used in polymer physics and can be used to study the effects of applied mechanical stress on highly oriented samples (Noda, Dowrey & Marcot, 1988, Noda, 1989, 1990, 1993; Budevska, Manning & Griffiths, 1994).

FT-IR microscopy showed that stretching induces molecular orientation (Kačuráková, Smith, Waldron & Wilson, 1998b) and because of this induced dichroism of the polysaccharides, IR spectroscopy can be used to study the effects of mechanical stress. It was shown that dynamic 2D FT-IR can provide information about the interactions of biopolymers in the cell walls non-invasively. Infrared spectra of onion epidermis, one of the most thoroughly investigated system for cell wall studies, reveal the prevalence of cellulose and pectin, while minor constituents such as protein, ferulic acid, lignin, and hemicelluloses can also be detected. The time-dependent response of the infrared spectra to an applied mechanical perturbance was studied. The results provided evidence that the major components (cellulose and pectin) exhibited different reorientation rates in the wet onion cell walls (Wilson et al., 2000).

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