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Investigation by Confocal Raman Microspectroscopy of the Molecular Factors Responsible for Grain Cohesion in the *Triticum aestivum* Bread Wheat. Role of the Cell Walls in the Starchy Endosperm

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ABSTRACT

Confocal Raman microspectroscopy has previously been employed to investigate the protein content and composition of the starchy endosperm of the wheat grain. With the same objective, that is to determine the molecular basis of grain cohesion and more specifically of kernel hardness, the contribution of endosperm cell walls in the kernel structure and cohesion was explored. The technique showed that endosperm cell walls consist not only of arabinoxylan chains with ramifications of ferulic esters, but also of others components such as proteins and lipids that could play some role in the mechanical properties of the endosperm cell walls. A new model of interaction between ferulic ramifications and a phospholipid component was proposed. The investigation of cell wall composition at successive stages of grain development revealed a decrease in the protein to arabinoxylan ratio and simultaneously an increase of the ferulic acid to arabinoxylan ratio that could be associated with a strengthening of the cell wall structure. The study confirms the effectiveness of confocal Raman microspectroscopy to approach the structure of wheat grain at the micrometer scale and to identify specific molecular factors responsible for grain cohesion and involved in the fracture modes generated during the milling process.

Keywords: Raman microspectroscopy, *Triticum aestivum* wheat, hardness, endosperm cell wall.

INTRODUCTION

Processing of wheat grain into flour during milling consists of two distinct cracking operations: (i) separation between outer layers (bran) and starchy part of the kernel, the starchy endosperm (Fig. 1), and (ii) fragmentation of the starchy endosperm into fine particles (flour). The present study is focused on the fragmentation of the starchy endosperm, which is composed of cells or compartments containing starch granules embedded in a protein matrix. The size of the cells and the thickness of their walls depend on their location within the starchy endosperm (central or peripheral).

Hardness is the characteristic of the wheat kernel that affects the fragmentation and milling performance. Hardness primarily depends on a genetic factor which determines the difference between *soft* varieties whose endosperm has a friable behaviour, and *hard* varieties whose endosperm is mechanically resistant. Hardness may be also influenced, to a lesser extent, by environment. According to their level of hardness, wheat

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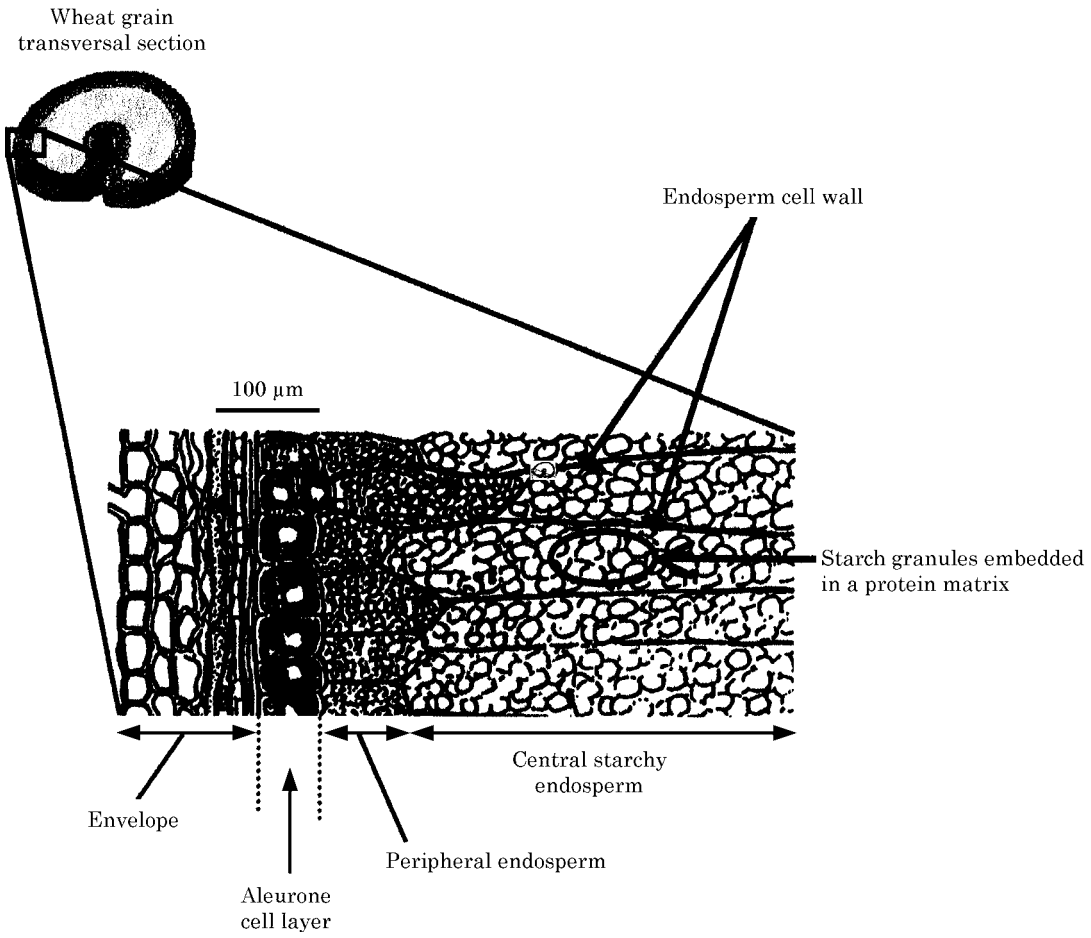

Figure 1 Schematic view of the structure of a wheat grain transverse section.

varieties present very different milling behaviours (e.g. milling energy, flour yield, easiness of flour to flow) and are oriented towards specific uses, as a consequence of the characteristics of their flours (starch damage, particle size and shape, hydration capacity). For instance, *hard* varieties are desirable for bread production, whereas *soft* varieties are generally used for cake or biscuit production.

Several hypotheses were proposed to explain the physico-chemical bases of endosperm hardness, respectively based on the nature and the structure of (i) the protein matrix surrounding starch granules[1,2] and (ii) of the interface between this protein matrix and the starch granules[3,4] (Fig. 1). Accordingly, it is likely that the starch–protein interface is the site of a chemical difference which could form an important basis of endosperm hardness. However, previous studies never totally ruled out a possible contribution of the endosperm cell walls to the mechanical characteristics of the endosperm. It is well known that there is a basic difference between the modes of fracture propagation of *hard* and *soft* wheats: whereas *soft* kernels fracture through the endosperm cells at the starch-protein interface, leaving the starch granules undamaged, *hard* kernels tend to fracture at cell walls (or, when cells are broken, within the starch granules)[5]. Even if the role of cell walls is minor compared to that of the interface between starch granule and protein matrix, it may be sufficient to significantly influence endosperm cohesion and milling performance and, for that reason, it has to be clarified.

So far, however, the implication of the kernel wall in the cereal grain cohesion is not well documented, as cell wall components were essentially studied for their effects on properties of the bread dough, and in particular on the hydration capacity

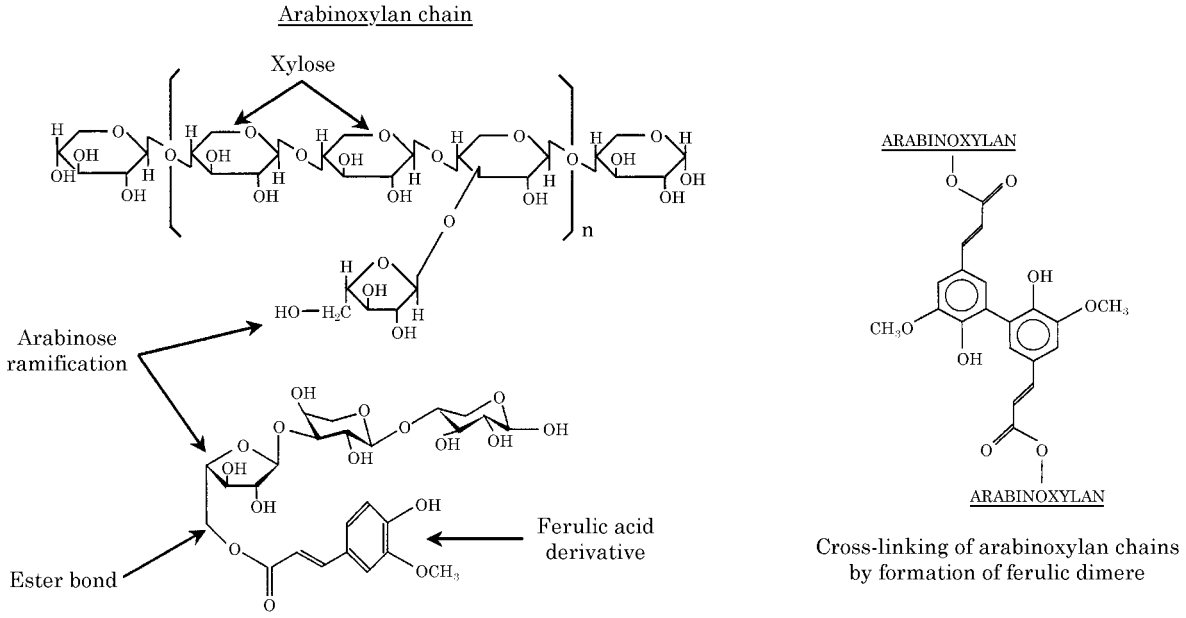

Figure 2 Chemical structure of the cell wall component.

of the flour. Consequently, the isolation protocols and the characterisation of their macromolecular properties were largely investigated[6–8].

Cell wall composition

In the physiological context of the wheat kernel, cell wall composition has to be appropriate to allow both resistance to the turgor pressure, and wall expansion during cell growth. The walls that surround the cells in the starchy endosperm consist predominantly of arabinoxylans[9] (60–70%), i.e. linear chains of α 1–4 xylose with branchings of arabinose (generally as monomers, but with a small percentage of oligomers) to which ferulic acid can be covalently linked via ester bonds (Fig. 2). The esterified ferulic units can provide cross-linking between arabinoxylan chains by formation of diferulic covalent bonds and, more rarely, formation of truxillic acid by photodimerisation[10,11]. The degree of branching, the spatial arrangement of arabinosyl substituents along xylan backbone, and the ferulic content are the main factors that determine the physico-chemical properties of the arabinoxylan chains, such as their mechanical resistance or their elasticity. These properties are also likely to contribute in the mode of fracture propagation at endosperm cell walls and consequently to the easiness of endosperm fragmentation.

In addition to arabinoxylans, other constituents are likely to influence the kernel structure. Proteins are known to contribute in the cohesion of plant cell walls as, for instance, primary cell walls contain a typical protein named extensin possessing structural properties[11,12]. Extensin forms a network by an intramolecular cross-linking resulting from the formation of an isodityrosine bond between two tyrosine residues. In cereal cell walls, although few studies were carried out on the possible role of protein networks, proteins covalently linked to arabinoxylan via ferulic units esterified to arabinose branches might be involved in maintenance of the structure. Various speculations were made regarding covalent cross-links between ferulic acid esterified to arabinoxylan and Tyr or Cys residues[10], even though no direct evidence of their occurrence has been brought so far. Alternatively, there might be formation of a C-S thiol bond between the sulphur atom of cysteine and one carbon atom of the C=C double bond of the propenoic chain of ferulic unit. Also, there might be formation of a diphenyl bond between the respective phenolic rings of tyrosine and of ferulic acid. Another possible bond involves the formation

of an ether linkage between the hydroxyl group of tyrosine and the ferulic ring, in the same way as for intramolecular cross-linking of extensin involving isodityrosine formation[11].

Lipids are also likely to play a role in the cell wall structure. In wood structure, lipid polymers called suberin and cutin are clearly involved[13]. These aliphatic polymers are linked to ferulic or para-coumaric acids, by covalent bonds, either by ester bonds on the acid group, or by an ether bond to the hydroxyl group of the phenol ring, or alternatively to the unsaturated carbon atom of the propenoic group of the phenolic acids. In contrast, no implication of lipids has been so far reported in cereal cell walls. This is probably due to its poor content, which makes it undetectable even by modern analytical techniques.

The present study aims at investigating the composition of endosperm cell walls by Confocal Raman microspectroscopy in order to understand how the composition of cell walls could influence their mechanical resistance and endosperm hardness. On a longer term perspective, the purpose of this research is to identify the molecular bases of the wheat grain texture and mechanical properties in order to (i) better control the operation of fragmentation during the grinding steps of conventional milling and ultra-fine milling, and (ii) to provide direction for breeding of wheat genotypes better suited to the fragmentation process.

Confocal Raman spectroscopy

The lack of data on the role of kernel cell walls in the grain cohesion certainly reflects the absence of appropriate analytical tools. The state of art indicates that the techniques so far employed are destructive and that the results concern destructured materials after extraction and/or purification steps. The advantage of our methodology[14–16] is the ability to obtain molecular information from a non-destructive analysis performed *in situ*, directly on the grain. The high spatial resolution also represents an asset to this investigation at the microscopic scale.

In cereals, confocal Raman spectroscopy and microspectroscopy have only been employed to study the microstructure of the wheat kernel[17] and the secondary conformation of specific wheat proteins[18]. In these previous studies, Raman microspectroscopy appeared to be very efficient for characterising the various types of molecules present in the wheat kernel. Regarding polysaccharides, Raman vibration bands appeared to be sensitive to the degree of branching and to the conformation of glycosidic bonds[19,20]. In the protein characterisation, the technique permitted not only to determine the composition in certain amino acid residues but also to analyse the secondary structure (α-helix, β-sheet, β-turn, random coil. . .) especially through the vibration of the amide I band[19,21]. Concerning lipids, it was possible to determine the degree of insaturation of the aliphatic chain and to detect the presence of phospholipids through specific vibrations of the PO_2^- head. The phenolic components such as ferulic and paracoumaric acids were also easily detected by a doublet of vibrations corresponding to the stretching of the conjugated C=C double bonds of the phenyl ring and of the propenoic chain[17,19].

In the present paper, we will show how Raman microspectroscopy allows to approach the composition of the endosperm cell walls. We will also demonstrate that Raman data help to characterise the bonds between molecules. In addition, the composition of endosperm cell walls at different stages of the grain development will be followed in order to identify molecular factors responsible for the cell wall structure and mechanical resistance.

MATERIALS AND METHODS

Plant material

Experiments were carried out on wheat (*Triticum aestivum*) samples supplied by INRA (Montpellier, France) and by Champagne Céréales (Reims, France).

Wheat grains of different varieties were supplied by Champagnes Céréales in order to investigate differences in microscopic structure between *soft* and *hard* endosperms. The varieties were classified by Champagne Céréales according to their degree of hardness (from *soft* to *hard* types).

Wheat grains (from *soft* to *hard* types) were also collected at successive stages of development in the experimental field of Champagne Céréales, at 3-day intervals during the last phase (pasty phase) of grain development (starting 1 month before the harvest). Because of the large variability of the material, special care was taken for sampling. Wheat grains were always collected at the same location in the field, at the same position on the

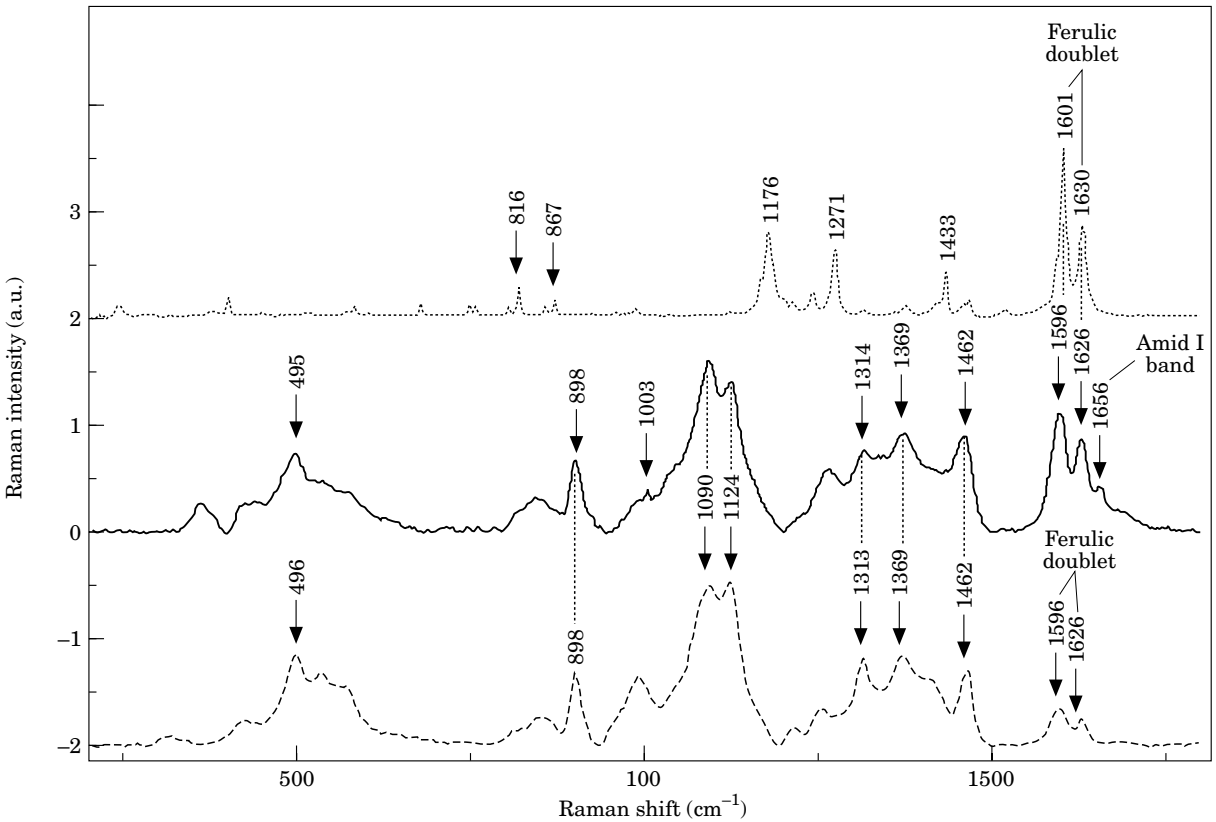

Figure 3 Raman spectra of endosperm cell wall recorded *in situ* on a grain section (spectrum 3a: ——) and of reference products, pure ferulic acid (reference 1 = spectrum 3b:. . . . . .) and reference water-extractable arabinoxylan (reference 2 = spectrum 3c:– – –).

ear (between one-third and one-half of the ear's height) and in the same atmospheric conditions (no sampling under rainy or wet weather conditions). Grains were stored at controlled room temperature (20 °C) and hygrometry (30%).

In order to calibrate the Raman microspectroscopy, various reference products such as ferulic acid, water extractable arabinoxylan, and phospholipid were used. In the paper, these reference products will be named as follows: reference 1: pure ferulic acid (Sigma); reference 2: water extractable arabinoxylan extracted of wheat flour and purified according to Figueroa-Espinoza and Rouau[7]; reference 3: pure phospholipid (Sigma) whose chemical structure is close to that of wheat phospholipids.

Sample preparation

For Raman experiments carried out on grains, spectra were recorded on 50-µm sections. Sectioning of grains was done transversally in ice using a cryomicrotome. In this study, only transverse sections at half-height of the kernels were considered. Samples were examined, at controlled room temperature and hygrometry (30%), immediately after sectioning.

Spectroscopic technique

Confocal Raman microscopy[15–17] was conducted on kernel sections using a Labram microspectrometer (Horiba, Jobin Yvon, France) that results from the coupling between a Raman spectrometer and an optical microscope. The microspectrometer was equipped with a He/Ne laser delivering 8 mW of red light, an optical unit permitting the illumination of the sample by the laser light, an optical microscope (equipped with a high stability BX 40 Olympus, 100× (NA 0,9) objective), a collection optical unit, which takes into account the confocal hole, the dispersive system

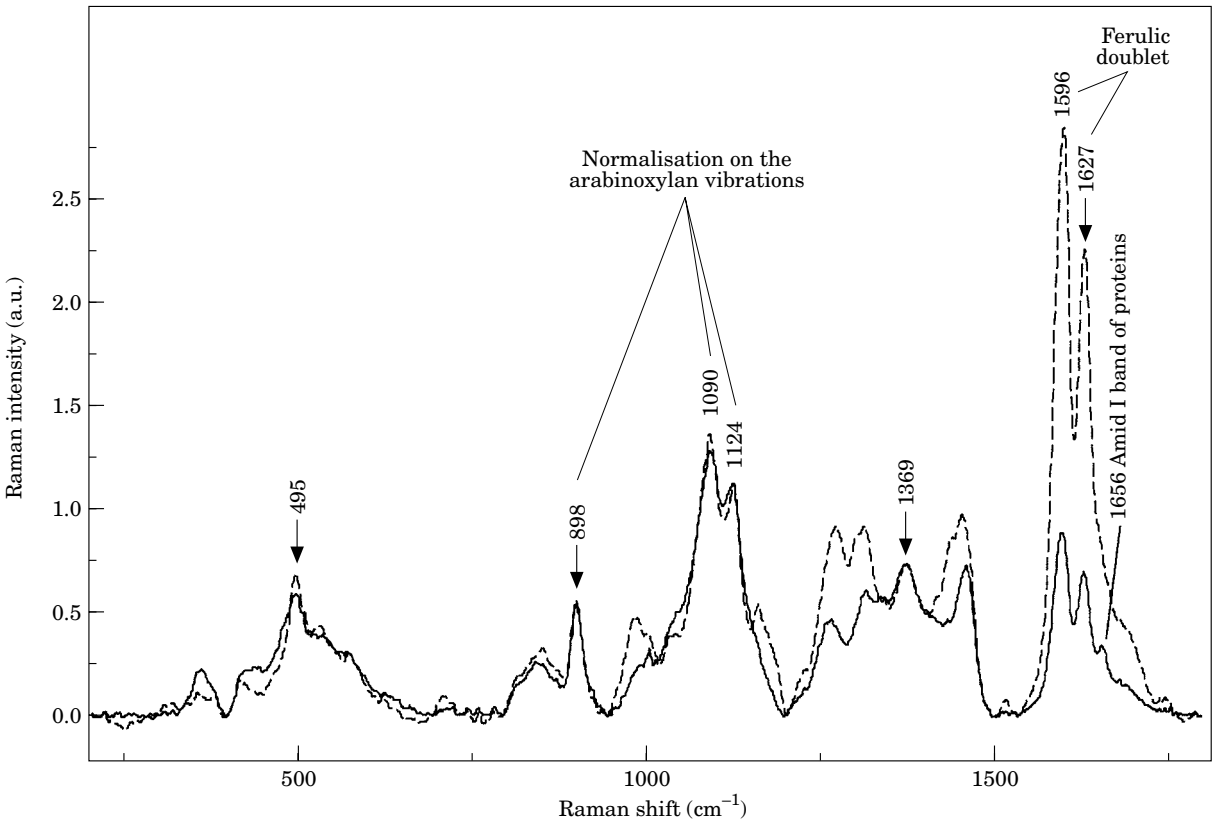

Figure 4 Comparison between Raman spectra of endosperm cell wall (——) and of aleurone cell wall (– – –).

(1800 grooves/mm holographic grating) and the multichannel CCD detector of 1024 × 256 pixels. The spectral resolution depends on the slit size located on the input of the grating. In the typical conditions of our experiments, the resolution was 2 cm^{-1}. This equipment permits a spatial resolution of about 1 μm both in X and Y dimensions. In comparison, the width of the endosperm cell[22] wall was examined at about 3 μm. Considering the opacity of our sample, the analysed depth was not taken into account. The acquisition time was of the order of 5 min per spectrum.

The confocality was assured by a diaphragm located in the image focal plane of the sample. The advantage of confocal sampling is the considerable reduction of the depth of focus and thus an increased discrimination in the z-direction. For a wheat grain section, it can be useful to reduce the confocal hole to be sure of recording spectra of very specific parts of the kernel. The reduction of the confocal pinhole permits improvements of both the lateral and axial spatial resolution. For the *in situ* experiment, the confocal aperture was set to 200 μm, which ensures recording of signal arising only from the cell wall without any contribution of the adjacent tissue.

Repeatability test has been performed by recording a total of 100 Raman spectra at different points of the endosperm cell wall, on sections (half height of the grain) of 10 different grains of a same batch (same variety and same maturation stage). It appears, in the particular conditions of sampling, that the variability does not exceed 5% in intensity of the same vibration.

Spectral treatment

All the different mathematical operations (baseline correction, normalisation, subtraction, second derivative) were performed using the Labspec software. Such operations were often useful in order to extract information on particular molecular species, from a multicomponent sample analysed *in situ*.

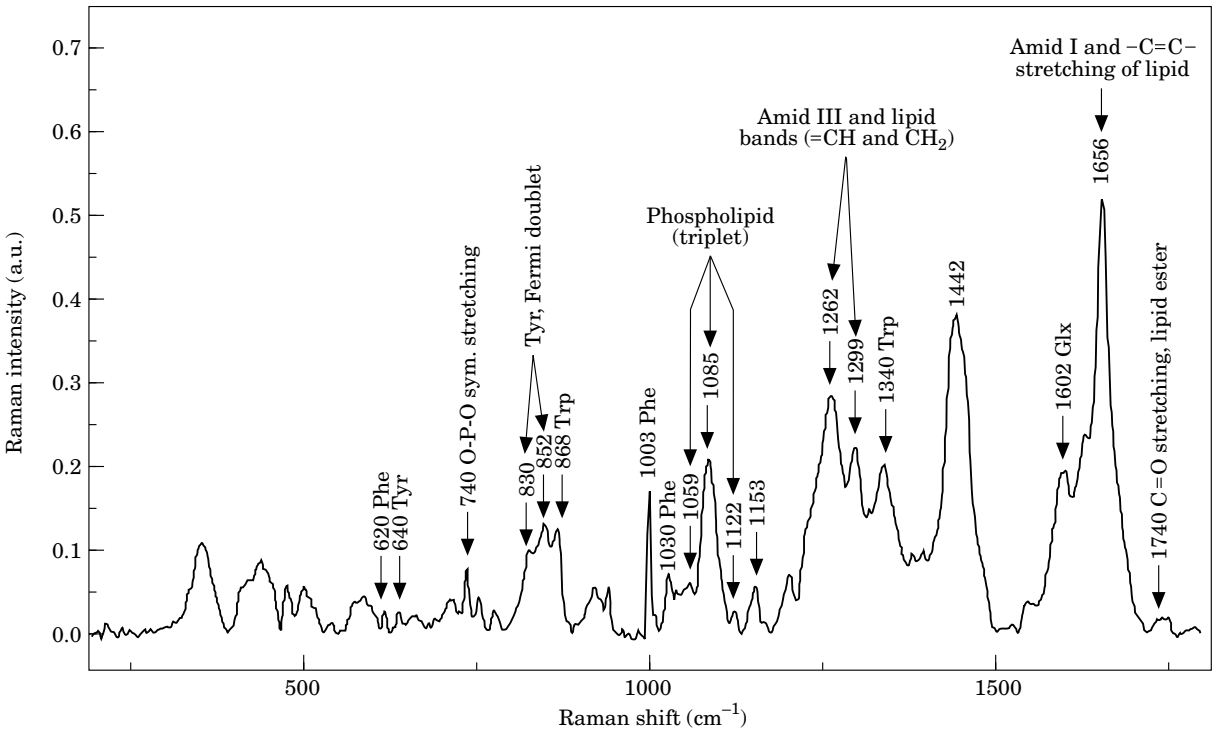

Figure 5 Difference spectrum of the endosperm cell wall obtained by subtracting the water-extractable arabinoxylan spectrum from the *in situ* endosperm cell wall spectrum (reference 2).

RESULTS

Micro-Raman spectra of endosperm cell walls recorded on wheat grain sections

In Fig. 3, the *in situ* spectrum [Fig. (3a)] recorded on endosperm cell wall, was compared with spectra of reference products such as pure ferulic acid (reference 1, spectrum 3b) and water-extractable arabinoxylans sample (reference 2, spectrum 3c). The arabinoxylans, whose molecular structure is presented in Fig. 2, are constituted of a linear backbone of xylose with arabinose branchings on which ferulic acid molecules can be esterified. The ferulic esterified units, contained in the arabinoxylans sample, are easily detectable by the vibration doublet (1596–1626 cm^{-1}) specific to phenolic acids. The comparison of these different spectra reveals that the endosperm cell walls are not only composed of arabinoxylan chains with ferulic units but also of proteins. The protein content is detectable by vibrations at 1003 cm^{-1} and by the amide I band centred at 1656 cm^{-1}.

Concerning the ferulic content, a frequency shift of the vibration doublet can be noticed between the spectrum of pure ferulic acid (vibrations at 1603 and 1630 cm^{-1}) and the spectrum of the endosperm cell walls (vibrations at 1596 and 1626 cm^{-1}). This spectroscopic characteristic is likely to be associated with the modification of the chemical environment of ferulic acid in the pure and esterified form, present in the cell walls. Nevertheless, additional model compounds should allow to elucidate the type of molecular linkages.

In Fig. 4, the spectrum of an endosperm cell wall is compared with that of a lateral (aleurone–aleurone) cell wall. Spectra were normalised on the arabinoxylan vibrations (1089–1128 cm^{-1}), meaning that the content in arabinoxylans was fixed to be equal in both kinds of cell walls. We can then notice that the ferulic content is much more important in the aleurone cell wall than in the endosperm cell wall. If considering that the ferulic acid:arabinoxylan ratio is an indicator of the cell wall resistance[23], particularly because ferulic units can form dimers through a biphenyl bridge (Fig. 2), and consequently strengthen the wall by cross-linking two arabinoxylan chains, this result

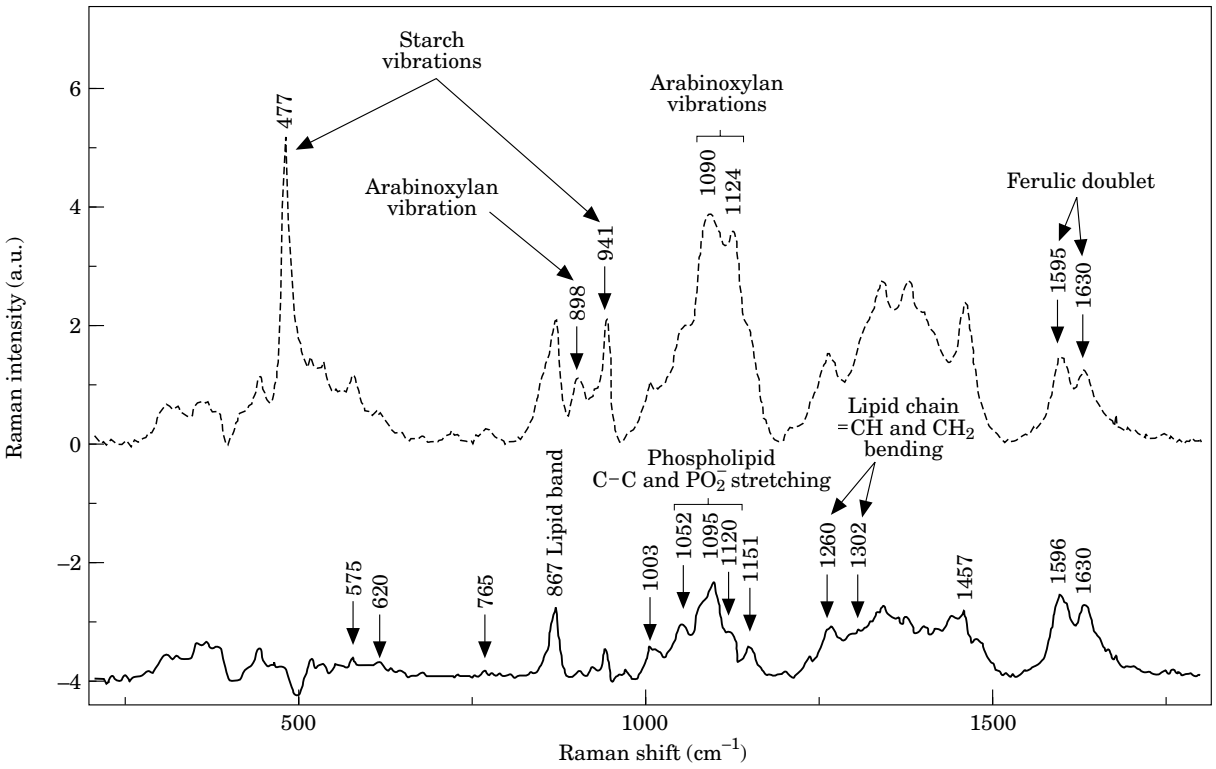

Figure 6 Raman spectrum of total arabinoxylan sample (– –.) and corresponding spectrum (——) after eliminating starch and arabinoxylan chain contributions.

suggests that endosperm cell walls would be mechanically weaker than the aleurone cell walls.

In order to analyse more precisely the other components detectable in the spectrum of endosperm cell walls, we have eliminated the arabinoxylan contribution by subtracting the reference spectrum of a water-extractable arabinoxylan from the *in situ* spectrum. The spectroscopic analysis then reveals that the difference spectrum obtained (Fig. 5) mainly corresponds to proteins, but also contains lipid vibrations.

The protein component can be characterised by its composition in phenylalanine (bands at 620 cm^{-1}, 1001 cm^{-1} corresponding to vibration of phenyl ring breathing, and 1030 cm^{-1}), in tyrosine (band at 640, and doublet at 830–852 cm^{-1}), or in tryptophan (bands at 1340 cm^{-1} and at 868 cm^{-1}, sensitive to the hydrogen bond involving the NH group of the indole ring)[18,19,21].

The presence of lipids is suggested by the vibration at 740 cm^{-1} and by the triplet at 1059–1085–1122 cm^{-1}, corresponding to phospholipids[19,24,25]. Two of the triplet bands (1059 and 1122 cm^{-1}) would be attributed to vibrations of C-C stretching of aliphatic chain while central band at 1085 cm^{-1} could be assigned to PO_2^- symmetric stretching vibration. In order to further investigate lipids, and in particular to characterise the bond between the lipid and the arabinoxylan chain (the main component of the cell wall), we recorded the spectrum of a total arabinoxylan (water extractable and insoluble with water) sample extracted from flour according to Rouau and Surget[8]. Indeed, the question stands whether lipids are bound to proteins themselves linked to arabinoxylan via esterified ferulic ramifications or if lipids are directly bound to arabinoxylan. The spectrum of total arabinoxylan is presented in Fig. 6. Besides specific vibrations of arabinoxylans at 898, 1090 and 1124 cm^{-1}, starch vibrations[19,20] at 477 and 941 cm^{-1} were visible, indicating that purification of arabinoxylans from flour is not complete. Starch and arabinoxylans contributions were then respectively eliminated by subtracting spectra of starch and reference water extractable arabinoxylans sample (reference 2). The final spectrum is also presented in Fig. 6. Except the doublet

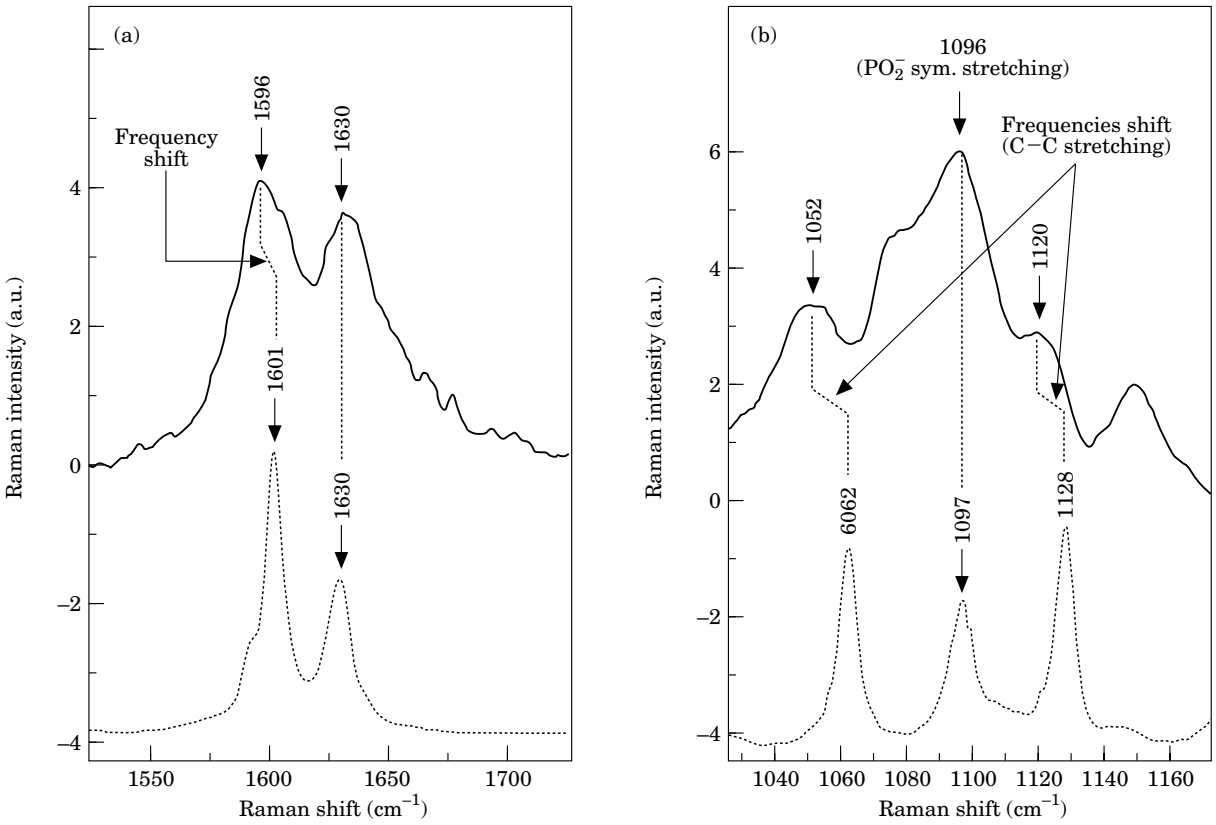

Figure 7 Ferulic doublet (a) (1500–1700 cm^{-1}) and phospholipid triplet (b) (1040–1140 cm^{-1}) spectral regions of the total arabinoxylan (——) respectively compared with the same region for pure ferulic acid (reference 1 ..... in (a)) and pure phospholipid (reference 3 ..... in (b)).

at 1596 and 1630 cm^{-1}, attributed to the ferulic acid component, the main bands may be assigned to lipids vibrations. For instance, the band at 867 cm^{-1} is attributed to the lipid content[19], the triplet at 1052, 1095 and 1120 cm^{-1} originates from phospholipid vibrations, and the doublet at 1260 and 1302 cm^{-1} is known to result from vibrations of C-H bonds in the $=CH$ and $-CH_2$ moiety of the lipid chain. The ratio of intensities of the 1260 and 1302 cm^{-1} bands is classically used to determine the degree of insaturation of the lipids[19].

It can be observed that, in the analysed sample, the ferulic doublet is located at 1596–1630 cm^{-1}, while in a pure ferulic acid it is located at 1601–1630 cm^{-1}. The shift of the lowest frequency of the ferulic doublet vibrations, visible on Figure 7(a), would suggest that the environment of the ferulic acid could be affected by the presence of lipids in the insoluble arabinoxylan sample. However, as reported above (see Fig. 3), when a ferulic acid is esterified to an arabinose branching of the arabinoxylan chain, a shift of both vibration frequencies at 1596–1626 cm^{-1} is usually observed. Moreover, by comparing Raman spectra of trans-cinnamic acid (no substitution of the phenyl ring except the propenoic chain), of paracoumaric acid (one substitution of the phenyl ring by OH group) and of ferulic acid (two substitutions of the phenyl ring by OH and OCH_3 groups), it is possible to show that substitution on the phenyl ring influences the frequency of the first vibration of the doublet (lowest frequency) without shifting the frequency of the second band (highest frequency), which was assigned to a $C=C$ stretching vibration of the propenoic chain (spectral data not shown). The shift of only one vibration band may suggest that the ferulic units play a role in the interaction between arabinoxylans and lipids. In addition, it can be assumed that the phenolic ring of the ferulic units, which is the more accessible part of the molecule, would be specifically involved in the bond between the ferulic acid and lipid components.

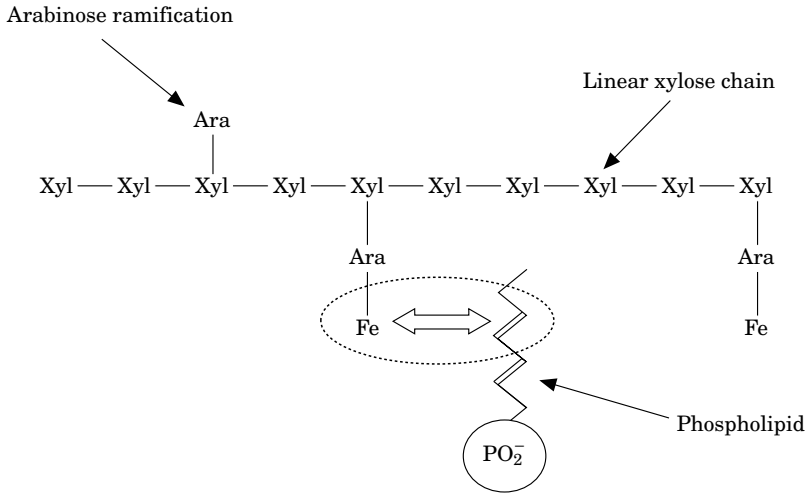

Figure 8 Simplified view of proposed interaction model between arabinoxylan chain and phospholipid, within endosperm cell wall.

Similarly, we have searched for a possible change in the lipid vibrations. In Fig. 7(b), we have zoomed the spectral shift of the phospholipid triplet region (1050–1130 cm^{-1}). By comparing the triplet vibration of the insoluble arabinoxylan sample with that of a pure phospholipid (reference product 3), we noticed that the frequency of the PO_2^- symmetric stretching vibration was not shifted while frequencies of the C-C stretching of the aliphatic chain were shifted toward lower frequencies. The frequency shifts of the ferulic doublet on one hand, and of the triplet phospholipid on the other hand, could be evidence of an interaction between these two molecular entities. Furthermore, this interaction would be likely to involve the aliphatic chain of the phospholipid rather than its phosphate head, as schematically shown in Fig. 8, because PO_2^- symmetric stretching vibration keeps the same vibration frequency. Nevertheless, in order to better characterise this interaction as well as the type of bonding between these molecular entities, further investigations have to be performed on more complex model compounds using complementary analytical techniques[26,27].

In this first part, we have seen from *in situ* experiments that central endosperm cell walls are composed not only of arabinoxylan chains with ferulic units esterified to arabinose ramifications, but also of proteins and lipids in low quantities. Moreover, it has been shown that esterified ferulic acid could be involved in bonds between arabinoxylan chains and protein (more particularly Cys or Tyr residues) or lipids (phospholipid via their aliphatic chain). Such results would suggest that new molecular factors could be involved in the solidity of endosperm cell walls and consequently would be likely to play a role in kernel cohesion.

Raman spectra of endosperm cell walls during grain development

In order to better understand the role of the endosperm cell walls in kernel cohesion, we have followed the evolution of the cell wall composition at various stages of grain development, during the month preceding maturity. This studied period corresponds to the ripening phase of the grain, which involves hardening and dehydration of the kernel. Experiments were carried out on several varieties of different levels of hardness (*soft* and *hard*), but no significant differences are visible between spectra of these varieties. Figure 9 represents a set of Raman spectra of endosperm cell walls recorded at different maturation stages. In order to compare the protein and the ferulic contents of the cell walls during the grain ripening, spectra were normalised on the arabinoxylans (AX) vibrations (898, 1090 and 1124 cm^{-1}). The ferulic acid and the protein components were therefore easily detectable respectively by the vibrations doublet at 1596–1626 cm^{-1}, and by the amide I band centred at 1656 cm^{-1} (alternatively, the ring breathing band of the phenylalanine residue at 1003 cm^{-1} could be used). This experiment clearly shows that the ferulic acid:arabinoxylan ratio is

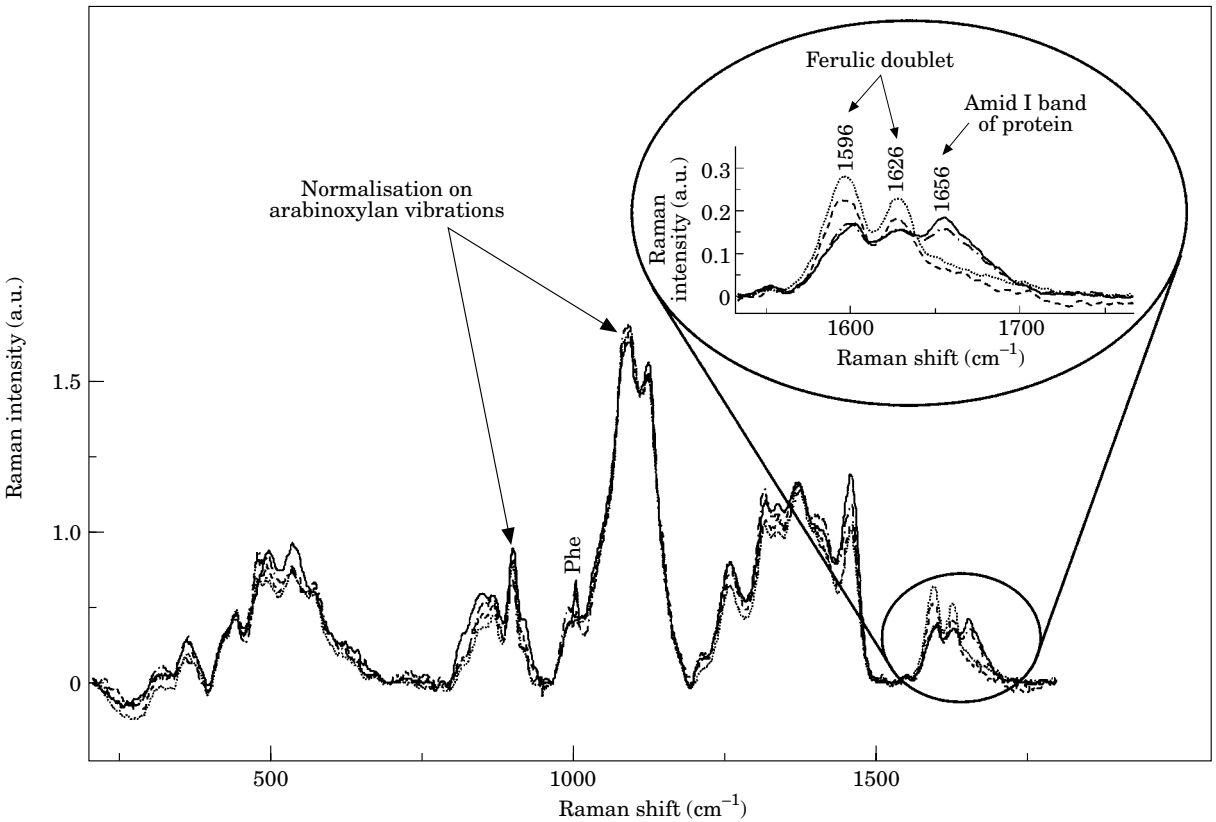

Figure 9 Set of Raman spectra of endosperm cell walls at different stages of grain development for *soft* and *hard* varieties. 7 July: ——; 16 July: —.—.—.; 21 July: ————; 27 July: . . . . . .

increasing when grain maturation proceeds, whereas the protein:arabinoxylan ratio is decreasing. Such a result could indicate that the change in the composition of the cell wall leads to its strengthening, following on from the formation of ferulic acid dimers. Indeed, the observed evolution of the ferulic acid:arabinoxylan and protein:arabinoxylan ratios could express the disappearance of the ferulic–protein bonds for the benefit of the formation of ferulic–ferulic bonds. It could also indicate that ferulic units are synthesised from proteins, and particularly from phenylalanine residues as in the case of lignin synthesis in wood[13]. The evolution of the lipid content at the different maturation stages could not be correctly studied due to the fact that lipid vibrations were not distinctly visible on the spectra, being masked by the arabinoxylan vibrations that are used for the normalisation. In further investigations, the secondary structure of cell wall proteins should be analysed, as it was done in previous studies for protein of the starchy endosperm[17], in order to better understand the mechanism of cell wall strengthening during grain maturation.

DISCUSSION

Botanical aspect of the cell wall composition

Most of the studies on molecular bases of wheat grain hardness relate to the nature or structure of the protein matrix and of its interface with starch granules[1–4]. In the present study, we attempted to investigate the role of the endosperm cell wall in providing the strength for maintenance of the kernel structure. For this purpose, a new investigative approach for the composition of cell walls was developed based on confocal Raman microspectroscopy. It was observed that endosperm cell walls were comprised not only of arabinoxylan chains with esterified ferulic units, but also of other kinds of components in smaller quantities such as proteins or, more surprisingly,

lipids (particularly phospholipids). The presence of lipids in the endosperm cell walls had never been reported in cereals, but had been found in the bond system that maintains the cell wall structure of wood, in association with phenolic acids[13]. For example, suberin is a mixed polymer of aromatic and aliphatic monomers. The spectroscopic analysis of the shift in specific vibrations frequencies led us to propose a model of interaction between phospholipids and ferulic units esterified to the arabinoxylan chains, within the endosperm cell wall. In this proposed model, aliphatic chains of phospholipids would interact with phenyl rings of ferulic branchings of the arabinoxylan.

Concerning the presence of protein within cell walls, it has been reported that certain proteins classes intervene in the cell wall structure and possess particular specific physico-chemical properties to allow cell wall expansion during the growth of plant tissues[11,12]. For example, extensin forms a network cross-linked by isodityrosine ether linkage that permits the strengthening of the structure of plant cell walls during their development.

The results described here can be, to some extent, useful in the *in situ* characterisation of the bonding between protein and other cell wall components, in view of obtaining some basic information on their toughness. For instance, proteins could be covalently linked to ferulic units through either the cysteine residue or the tyrosine residue[10]. However, in the spectrum shown in Fig. 5, contrary to tyrosine vibrations, no band assigned to cysteine was visible, suggesting that the bond between the cysteine residue and the ferulic unit was absent in the wheat endosperm cell wall. On the other hand, it can be speculated that a biphenyl bridge is formed between the phenolic rings of the tyrosine and of the ferulic unit. Alternatively, the hydroxyl group of tyrosine could form an ether linkage with the ferulic ring, similarly as the isodityrosine formation permitting an intramolecular cross-linking in the extensin[11]. Since the ratio of vibration intensities in the Fermi doublet (I_{852}/I_{830}) is greater than one (Fig. 5), it can be suggested that the tyrosine residue would be exposed towards the protein[19] and would be therefore likely to form intermolecular bond with neighbouring molecules such as ferulic units.

To understand more precisely the respective role of the various molecular components in the mechanical resistance of the endosperm cell wall, it should be interesting to study their orientation by using polarised excitation and by detecting the polarisation of the diffused Raman signal, similarly as polarised FTIR microspectroscopy used to determine the orientation of particular functional groups with respect to the direction of cell elongation in carrot suspension cells[12,28]. Moreover, the orientation of molecules could be characterised when the cell wall undergoes a particular deformation (elongation, stretching) under mechanical constraints that are likely to take place in the endosperm cell wall during the growing phase of the kernel.

Surprisingly, no significant difference in the cell wall composition was detected between varieties of different hardness levels, suggesting that hardness depends more on characteristics of the protein matrix and of its interface with starch granules[1–4] than on the composition of the endosperm cell walls. It is however possible that compositional differences in minor components of cell walls, such as proteins or lipids, are sufficient to make fracture propagation along cell walls more or less easier during the milling process, without necessarily influencing the hardness value. Consequently, when aiming at assessing the whole milling behaviour of wheat grain samples, we suggest that the cell wall composition has to be taken into account, in addition to hardness. This would also explain why varieties with similar hardness levels present sometimes significantly different milling behaviours.

Furthermore, we have shown that wheat grain maturation is associated with changes in molecular composition of kernel cell wall. The evolution of the ferulic content:AX ratio, considered as an indicator of the wall resistance[23], would reveal a reinforcement of the endosperm cell wall structure during grain development, because the increase in ferulic content could be the evidence of the formation of diferulic cross-linking between two arabinoxylan chains (in parallel, the protein:AX ratio is decreasing). This ferulic dimer should present specific vibrations, such as biphenyl bridge stretching, that could be useful to distinguish the ferulic monomer from the dimeric form. Nevertheless, on our spectra, such distinction were not visible, either because the diferulic vibrations were masked by arabinoxylan vibrations, which is particularly true in the 1050–1200 cm^{-1} spectral region in which the biphenyl vibration frequency is located, or simply because the symmetry of the vibration modes does not generate diffused Raman scattering. In the latter case, infrared spectroscopy should be more efficient because it gives

information complementary to that of Raman spectroscopy, although the poor spectral resolution of the infrared microscopy limits its use for our investigation goals.

Changes in endosperm cell wall composition during maturation is certainly associated with molecular rearrangement which occurs under effect of cell wall growth (elongation) and assures a reinforcement.

The work should be pursued on a larger number of varieties in order to be able to draw conclusions on a possible correlation between cell wall structure and milling characteristics (fracture propagation). Concerning the comparison between endosperm cell walls and aleurone cell walls, it appeared that the latter presented a ferulic acid: AX ratio much more important, which could be a basis of the high strength of the aleurone/endosperm interface, and explain the difficulty encountered by the miller to separate the starchy endosperm and the outer envelopes.

Interest and limits of the Raman microspectroscopy technique

All spectroscopic techniques possess the advantage to be non destructive, contrary to most common analytical tools, such as HPLC, that require solubilisation and purification steps prior to analysis.

Akin *et al.*[29] used UV absorption microspectrophotometry in order to investigate aromatic constituents in walls of aleurone and endosperm cells of wheat grains. While information was obtained on the composition of the aleurone cell walls, the technique failed in measuring the aromatic content of endosperm cell walls, because the amount of aromatic components was too low with regard to the sensitivity of the technique. Saadi *et al.*[30] confirmed these results by investigating phenolic constituents through confocal microspectrofluorometry, a technique that measured the signal corresponding to the transitions between electronic states of the molecular system. Raman microspectroscopy, whose diffused signal characterises the vibration states of the molecular system, allows collection of information on aromatics composition of the aleurone cell walls (ester-linked ferulic acid, higher ferulic content in anticlinal aleurone walls than in the outer and inner periclinal walls[17]) similar as that of spectroscopic techniques employed by Akin *et al.*[29] or Saadi *et al.*[30]. It also permits assessment of the ferulic content as well as the arabinoxylan content of the endosperm cell walls, which allows determination of certain features responsible for kernel cohesion.

Compared with FTIR microspectroscopy[31], Raman microspectroscopy presents a much higher spatial resolution because the infrared radiation is limited by the optical diffraction that precludes a $<10\ \mu m$ resolution, unless a special synchrotron source[32] (high brilliance, powerful) is used. Compared with fluorescence, Raman microspectroscopy gives more complete information on chemical structure and bond conformation of molecules, while having a similar sensitivity. Another significant advantage of Raman microspectrometry is the ease with which new equipment can be attached, such as a near infrared laser diode as an excitation source, or optical fibre to perform experiments on distant samples.

The approach of the characterisation of molecular composition of the endosperm cell walls reported in this study clearly demonstrates the efficiency of the confocal Raman microspectroscopy in the *in situ* and non-destructive characterisation of the wheat grain structure, with a spatial resolution better than 1 μm, and in the exploration of new domains in the cereal field.

The potential of the spectral treatment techniques for bringing out information must also be underlined. While spectra recorded *in situ* on wheat grain samples are often relative to a mixture of molecular species, reflecting the complexity of biological systems, the mathematical operations of normalisation and subtraction of spectra are essential for extracting relevant information on each specific component, that could not be identified or quantified separately using any other method of characterisation.

CONCLUSION

New approaches in the understanding of *Triticum aestivum* wheat grain cohesion and hardness have been investigated by confocal Raman microspectroscopy. The results presented in this study mainly concern the composition of endosperm cell walls and to some extent, the type of molecular bonding between their components. Spectroscopic data suggest that cell walls consist not only of arabinoxylan chains with ramifications of ferulic esters, but of other components such as protein and lipids, which could also be involved in the resistance of endosperm cell walls. On the other

hand, the investigation of cell wall composition at successive stages of grain development revealed a decrease in the protein to arabinoxylan ratio and simultaneously an increase of the ferulic acid to arabinoxylan ratio which could be associated with the strengthening of the cell wall structure. Furthermore, this study confirms the usefulness and effectiveness of confocal Raman microspectroscopy to: (i) approach the structure of wheat grain at the micrometer scale and (ii) identify specific molecular factors possibly involved in grain cohesion, propagation of fracture generated during grain milling, and consequently in the determination of flour characteristics obtained from grains of different degrees of hardness.

With the aim to link together spectroscopic data to mechanical data, it would now be essential to approach the mechanical properties of the cell walls using micro rheological methods and to simulate their cohesive strengths by molecular modelling. To go further in the interpretation of the spectral information, it would also be valuable to acquire data from specific model compounds as references and to associate different spectroscopic techniques such as Raman scattering and fluorescence emission, or to use techniques of correlation[33] between Raman spectra recorded under various excitation frequencies to find relevant Raman vibration bands of particular components such as diferulic units.

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REFERENCES

1. Barlow, K., Buttrose, M.S., Simmonds, D.H. and Vesk, M. The nature of the starch-protein interface in wheat endosperm. *Cereal Chemistry* **50** (1973) 443–454.
2. Stenvert, N.L. and Kingswood, K. The influence of the physical structure of the protein matrix on wheat hardness. *Journal of Science, Food and Agriculture* **28** (1977) 11–19.
3. Greenwell, P. and Schofield, J.D. A starch granule protein associated with endosperm softness in wheat. *Cereal Chemistry* **38** (1986) 379–380.
4. Greenblatt, G.A., Bettge, A.D. and Morris, C.F. Relationship between endosperm texture and the occurrence of friabilin and bound polar lipids on wheat starch. *Cereal Chemistry* **72** (1995) 172–176.
5. Abecassis, J., Mabille, F., Haddad, Y., Autran, J.-C. and Benet, J.-C. La dureté des blés: états des connaissances actuelles. *Industries des Céréales* **101** (1997) 11–18.
6. Dervilly, G., Saulnier, L., Roger, P. and Thibault, J.-F. Isolation of the homogenous fractions from wheat water-soluble arabinoxylans. Influence of the structure on their molecular characteristics. *Journal of Agricultural and Food Chemistry* **48** (2000) 270–278.
7. Figueroa-Espinoza, M.-C. and Rouau, X. Oxidative cross-linking of pentosans by a fungal laccase and horseradish peroxidase: mechanism of linkage between feruloylated arabinoxylans. *Cereal Chemistry* **75** (1998) 259–265.
8. Rouau, X. and Surget, A. A rapid semi-automated method for the determination of total and water-extractable pentosans in wheat flours. *Carbohydrate Polymers* **24** (1994) 123–132.
9. Izydorczyk Marta, S. and Biliaderis Costas, G. Cereal arabinoxylans: advances in structure and physicochemical properties. *Carbohydrate Polymers* **28** (1995) 33–48.
10. Iiyama, K., Bach-Tuyet Lam, T. and Stone, B.A. Covalent cross-links in the cell wall. *Plant Physiology* **104** (1984) 315–320.
11. McNeil, M., Darvill, A.G., Fry, S.C. and Albersheim, P. Structure and function of the primary cell walls of plants. *Annual Review of Biochemistry* **53** (1984) 625–663.
12. Carpita, N. and Gibeaut, D. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal* **3** (1993) 1–30.
13. Kolattukudy, P.E. Lipid polymers and associated phenols, their chemistry biosynthesis, and role in phytogenesis. In 'The structure, Biosynthesis and Degradation of Wood' (F.A. Loewus and V.C. Runeckles, eds), Recent advances in Phytochemistry, Volume 11, Plenum Press, New York and London (1977) pp 185–246.
14. Sharonov, S., Chourpa, I., Valisa, P. and Manfait, M. Confocal Raman microscopic imaging of biological samples. In 'Advances in Fluorescence Sensing Technology III' (R.B. Thompson, ed.), Proceedings of SPIE—The International Society for Optical Engineering, Bellingham (1997) pp 210–216.
15. Sockalingum, G.D., Chourpa, I., Millot, J.M., Nabiev, I., Sharonov, S. and Manfait, M. Optical microspectroscopy and imaging analysis: probing drugs and metal ions in living cells. In 'Advances in Spectroscopy', (R.J.H. Clarck and R.E. Hester, eds), John Wiley & Sons, Chichester (1996) Volume 25 Biomedical Applications of Spectroscopy, Chapter 2 pp 49–87.
16. Sharonov, S., Nabiev, I., Chourpa, I., Feofanov, A., Valisa, P. and Manfait, M. Confocal three-dimensional scanning laser Raman/SERS/fluorescence microprobe. Spectral imaging and high-resolution applications. *Journal of Raman Spectroscopy* **25** (1994) 699–707.
17. Piot, O., Autran, J.-C. and Manfait, M. Spatial distribution of protein and phenolic constituents in wheat grain as probed by confocal Raman spectral imaging. *Journal of Cereal Science* **30** (2000) 57–71.
18. Le Bihan, T., Blochet, J.E., Désormeaux, A., Marion, D. and Pézolet, M. Determination of the secondary structure and conformation of puroindolines by infrared

and Raman spectroscopy. *Biochemistry* **35** (1996) 12712–12722.
19. Parker, F. *Applications of Infrared, Raman and Raman Resonance Spectroscopy in Biochemistry*. Plenum Press, New York (1983) Chapter 3 pp. 83–154 and Chapter 11 pp. 421–480.
20. Barron, L.D., Gargaro, A.R. and Wen, Z.Q. Vibrational Raman optical activity of carbohydrates. *Carbohydrate Research* **210** (1991) 39–49.
21. Ma, C.Y., Rout, M.K., Chan, W.M. and Phillips, D.L. Raman microspectroscopic study of oat globulin conformation. *Journal of Agricultural and Food Chemistry* **48** (2000) 1542–1547.
22. Larkin, R.A., MacMasters, M.M. and Rist, C.E. Relation of endosperm cell wall thickness to the milling quality of seven pacific northwest wheats. *Cereal Chemistry* **29** (1952) 407–413.
23. Lempereur, I., Surget, A. and Rouau, X. Variability in dehydrodiferulic acid composition of durum wheat (*Triticum durum* Desf.) and distribution in milling fractions. *Journal of Cereal Science* **28** (1998) 251–258.
24. Grdadolnik, J., Hadzi, D. FT infrared and Raman investigation of saccharide-phosphatidylcholine interactions using novel structure probes. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* **54A** (1998) 1989–2000.
25. Borchman, D., Lamba, O.P., Ozaki, Y. and Czarnecki, M. Raman structural characterization of clear human lens lipid membranes. *Current Eye Research* **12** (1993) 279–84.
26. Nabet, A., Auger, M. and Pezolet, M. Investigation of the temperature behaviour of the bands due to the methylene stretching vibrations of phospholipid acyl chains by two-dimensional infrared correlation spectroscopy. *Applied Spectroscopy* **54** (2000) 948–955.
27. Picard, F., Pezolet, M., Bougis, P.E. and Auger, M. Hydrophobic and electrostatic cardiatoxin-phospholipid interactions as seen by solid-state P-31 NMR spectroscopy. *Canadian Journal of Analytical Sciences and Spectroscopy* **45** (2000) 72–83.
28. McCann, M.C. and Roberts, K. Changes in cell wall architecture during cell elongation. *Journal of Experimental Botany* **45** (1994) 1683–1691.
29. Akin, D.E. Microspectrophotometric characterisation of aromatics constituents in cell walls of *hard* and *soft* wheats. *Journal of Science, Food and Agriculture* **68** (1995) 207–214.
30. Saadi, A., Lempereur, I., Sharonov, S., Autran, J.-C. and Manfait, M. Spatial distribution of phenolic materials in durum wheat (*Triticum durum* Desf.) grain as probed by confocal fluorescence spectral imaging. *Journal of Cereal Science* **28** (1998) 107–104.
31. Wetzel, D.L. and Reffner, J.A. Using spatially resolved Fourier transform infrared microbeam spectroscopy to examine the microstructure of wheat kernels. *Cereal Foods World* **38** (1993) 9–20.
32. Wetzel, D.L., Eilert, A.J., Pietrzak, L., Miller, S. and Sweat, J. Ultraspatially-resolved synchrotron infrared microspectroscopy of plant tissue in situ. *Cellular and Molecular Biology* **44** (1998) 145–167.
33. Ozaki, Y. and Wang, Y. Two-dimensional near infrared correlation spectroscopy: principle and its application. *Journal of Near Infrared Spectroscopy* **6** (1998) 19–31.