Spatial Distribution of Phenolic Materials in Durum Wheat Grain as Probed by Confocal Fluorescence Spectral Imaging

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ABSTRACT

Microspectrofluorometry has been employed to study the spatial distribution of phenolic material in cereal grain. Transverse sections of the grain were used for the spectral characterisation of different molecular species present in *Triticum durum* grains. Auto-fluorescence emission spectra were recorded on micro regions of each section. The analysis of the whole set of spectra permitted the characterisation of three principal spectral features; indicators of phenolics in specific regions of wheat sections. The comparison with model reference spectra showed that spectral components could be attributed to ferulic and p-coumaric acids. Using these spectral components, spectral fluorescence imaging was performed allowing the relative fluorescence intensity of each phenolic feature to be mapped. Images generated were used for the generation of the 3D organisation of auto-fluorescent phenolic materials within the grain. This new and rapid method was further used for the spectral characterisation of the various aleurone cell walls with high sensitivity. Analysis of the data showed that outer and inner aleurone cell walls exhibited similar fluorescence profiles but with significantly different intensities.
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INTRODUCTION

Microspectrofluorometry, which is a proven technique in the study of the behaviour of drugs in single living cells[1-3] can be useful to map phenolic material in wheat grain. The technique allows *in situ* characterisation of phenolic components on the basis of fluorescence emission spectra using transverse and longitudinal sections of the grain. Phenolic compounds are the most auto-fluorescent materials in cereal grains[4] and have already been detected as esterified forms in cereal walls[5-7]. In the wheat grain the distribution of phenolics has been previously studied by different microscopic methods such as fluorescence[4] or ultra-violet absorption and microspectrophotometry[8], and it has been shown that the exterior layers of the grain are strongly auto-fluorescent compared to the starchy endosperm.

The feruloyl function of ferulic acid (4-hydroxy-3-methoxycinnamic acid) was found to be responsible for the intense auto-fluorescence of the cell wall of the aleurone layer[5], where ferulic acid is highly concentrated. The occurrence of ferulic acid as component of the wheat kernel has been established[9], and quantified in wheat milling fractions by high-performance chromatography[10,11]. Most of the ferulic acid in wheat grains is esterified to cell wall constitutents such as arabinoxylans[12].

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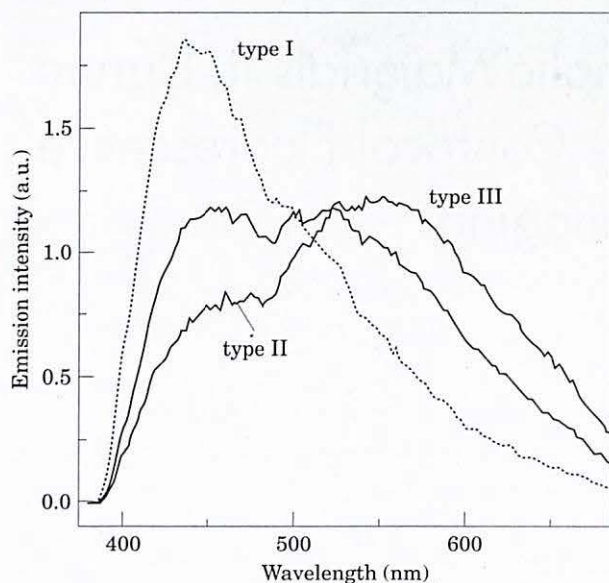
Figure 1 The three principal types of emission spectra recorded on the transverse section surface of durum wheat.

Several compounds in wheat can be used as precise indicators of botanical constituents[11,13,14]. The auto-fluorescence of phenolics in wheat flour can be used as a specific probe to characterise and estimate aleurone contamination in mill-streams[11,14,15]. In previous studies[11,13,14], individual constituents (pericarp, aleurone, and endosperm), used for the fluorescence characterisation, are generally separated by manual dissection or milling.

Microspectrofluorometry is a rapid method, which is ideal for analysing cereal grain section without preliminary separation. Characteristic emission spectra of specific components or constituents can be directly obtained from sections. This method offers the possibility of obtaining a highly specific spectrum which is characteristic of each tissue.

Microspectrofluorometry is described along with its application to the study of the auto-fluorescence in durum wheat samples. Data obtained by the technique are compared to those which have been reported by other methods.

EXPERIMENTAL

Microspectrofluorometer

Fluorescence emission spectra from section of wheat kernels were recorded using a confocal laser microspectrofluorometer (Dilor, Lille, France) equipped with an optical microscope (Olympus BH2) with 4× and 100× objectives. A UV argon ion laser (2065A, Spectra Physics; 365 nm excitation) was coupled to the microspectro-fluorometer to acquire X–Y fluorescence emission spectra from a confocal section. The sample was positioned under an objective lens adapted for the total transmission of UV radiation. The position of the sample was obtained by a computer-controlled motorised stage (Märzhäuser, model MCL-2). The stage allowed the Y-scanning to be done with an
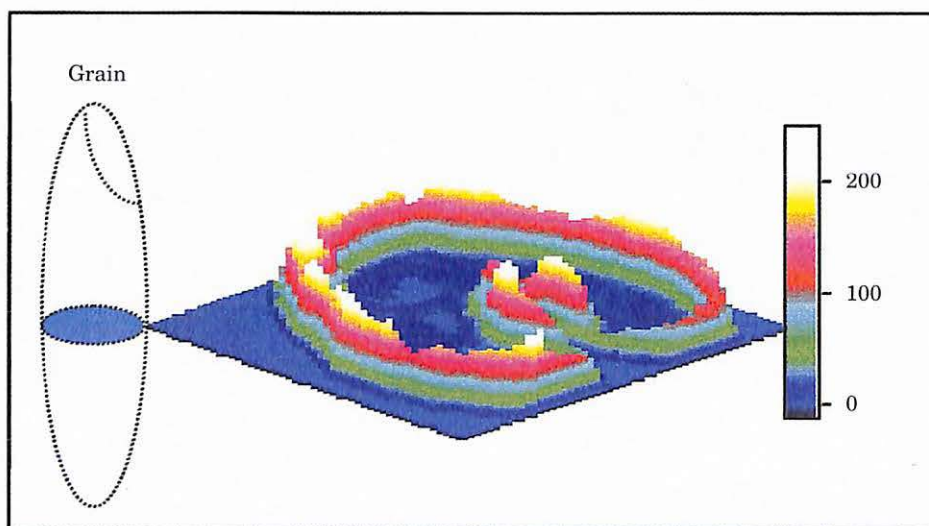

Figure 2 Distribution map of phenolics auto-fluorescence in a middle transverse section of the durum wheat grain, outer layers are highly fluorescent. The spectral image was created on the basis of the intensities of the spectra in the selected frequency interval between 380 and 680 nm; (excitation at 365 nm).
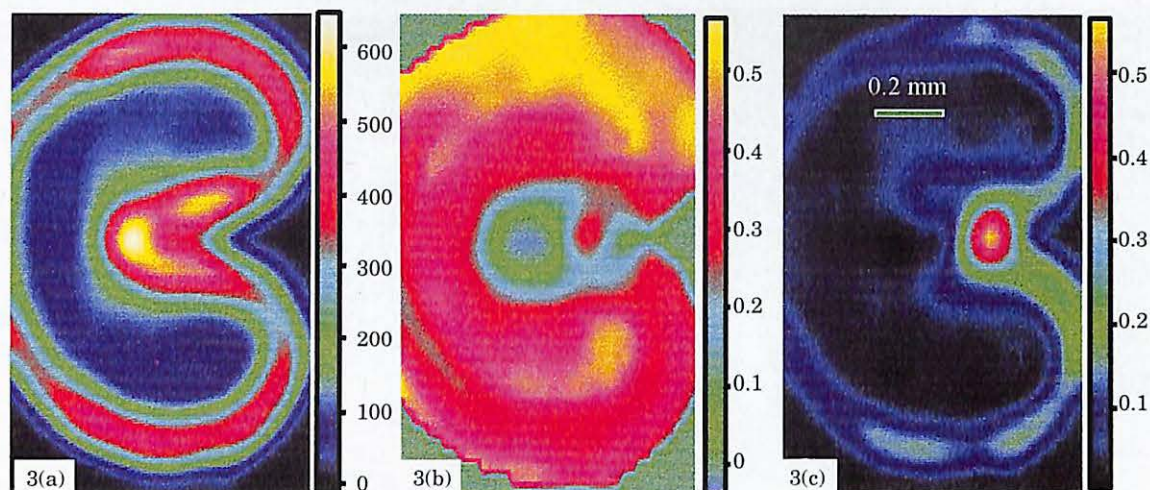
Figure 3 Distribution of the three principal spectra on reconstituted surface of a transverse section of durum wheat kernel. Spectral contribution of: type I (a), type II (b) and type III (c) spectra in the spectral image of wheat cross section. The colour scale is an artificial colour palette, which express the variation in intensity of a particular spectral component. Warm colours such as white and yellow represent maximum intensities whereas cold colours like blue and black are representative of low or no intensities.

accuracy of 0·1 μm. The X-axis scanning was achieved by a scanner that produced a periodic angular deflection of the UV laser beam. The 365 nm laser beam was deflected along a line on the sample where fluorescence emission was collected and focused onto a confocal square hole. A second scanner oscillating in phase with the first one deflected the emission signal onto the entrance slit of the spectrograph where the emission signal was projected onto a CCD detector (300×1200

pixels). The recorded spectra were then computed to generate a spectral image[1].

Microspectrofluorometric analysis

Sample

Samples of durum wheat grains were chosen from a series of *Triticum durum* used for evaluating milling efficiency at INRA Montpellier, France. Transverse cryo-sections (60 μm thick) were obtained by soaking the grains for approx. 4 h in distilled water, freezing at $-20\,°C$ and sectioning at $-20\,°C$ using a cryostat (Jung 2800). In order to analyse the pure aleurone tissue, thin longitudinal sections (10 μm) were cut, as explained above, at the periphery of the dorsal side (the same side as the germ). Sections thus obtained were placed on quartz slides for drying at room temperature. The objective lens was positioned over a selected field. Sections from three samples were studied and data obtained were analysed.

Spectra from crystals of pure ferulic acid (4-hydroxy-3-methoxycinnamic acid) and p-coumaric acid (4-hydroxycinnamic acid) (Sigma, France) were used as references. A 10% ferulic acid solution prepared in methanol was subsequently absorbed at $3·33\ 10^{-11}\ mg/μm^2$ onto chromatography paper (Whatmann no. 3 chromatography; Polylabo, France) to simulate cellulosic sur-
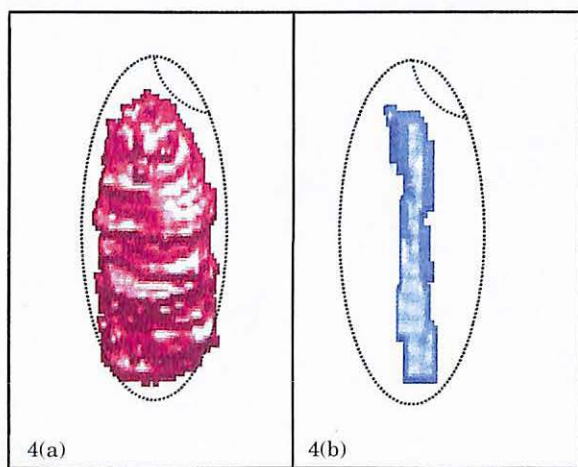

Figure 4 Three-dimensional reconstruction of the spectral contributions of types I (a) and type III spectra (b) within the durum wheat grain. The germ is not included.
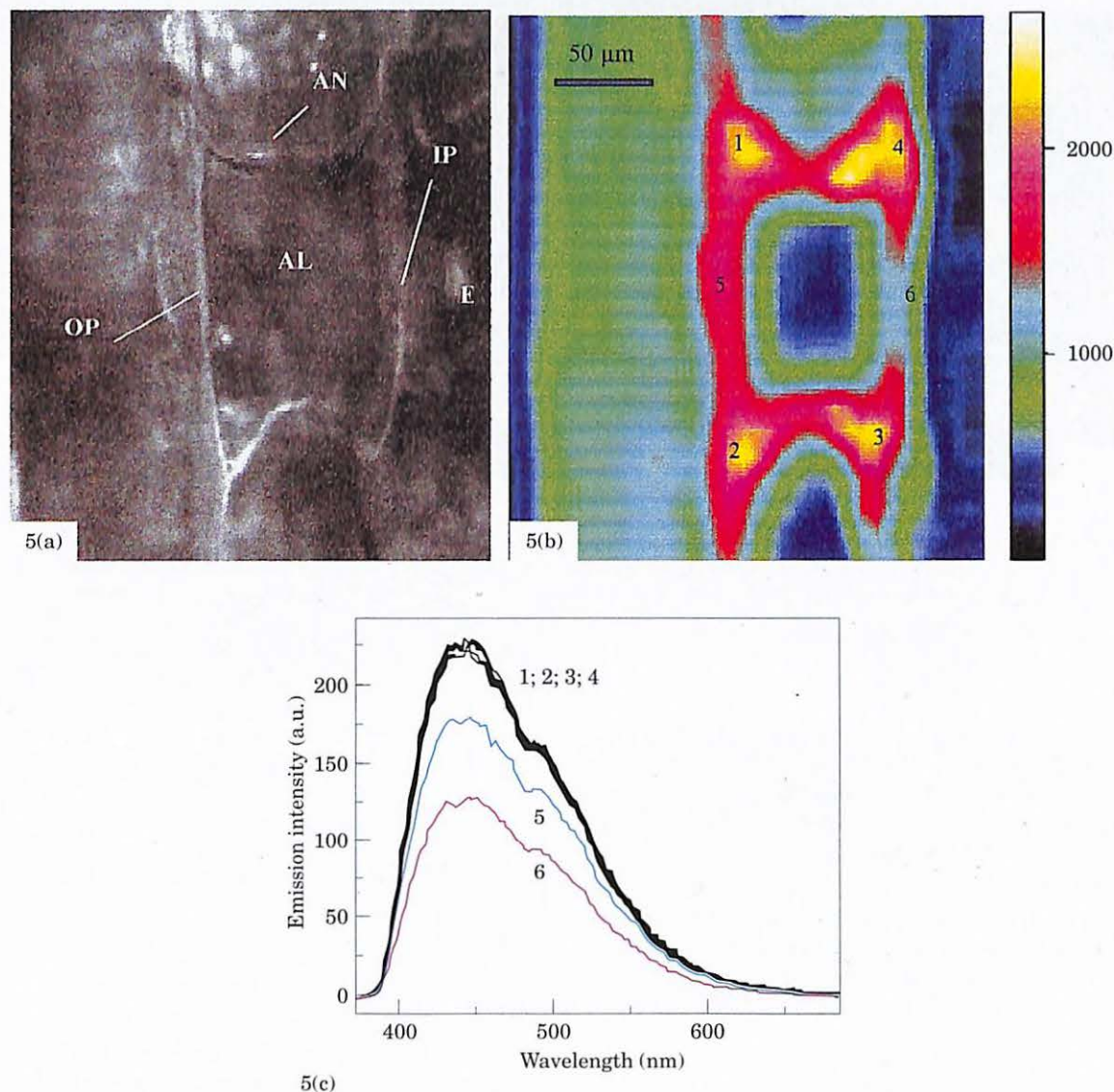
5(a)

5(b)

5(c)

Figure 5   Conventional (a) and spectral (b) images of the outer tissues of wheat transverse section showing spectral differences within aleurone cell walls. Cell wall sites studied are: (AL) aleurone layer; (OP) outer periclinal aleurone wall; (IP) inner periclinal aleurone wall; (AN) anticlinal aleurone wall; (E) endosperm. (c) Comparison of fluorescence intensities of the aleurone cell walls; spectra (c; 1,2,3,4,5,6) were extracted from the spectral image and correspond to the points marked by the same numbers on the spectral image.

roundings. Emission spectra of the ferulic acid simulated cellulosic sample were then collected.

Spectral imaging
The confocal system allows the recording of X–Y spectra from selected areas of sections. Spectra were collected from sections and subsequently decomposed into principal spectral components. When principal component analysis is applied, spectra with maximal distance from each other are first found; these are then corrected to avoid

meaningless negative values of the contribution coefficients. The corrected spectra are then used as model components, which can be approximated to the real phenolic materials of the wheat grain. This procedure allows the extraction of the main components, which best explains the variations observed in the wheat sections.

Principal components, chosen as containing the features characteristic of certain sample components, were used for the construction of a specific spatial distribution map of these components on
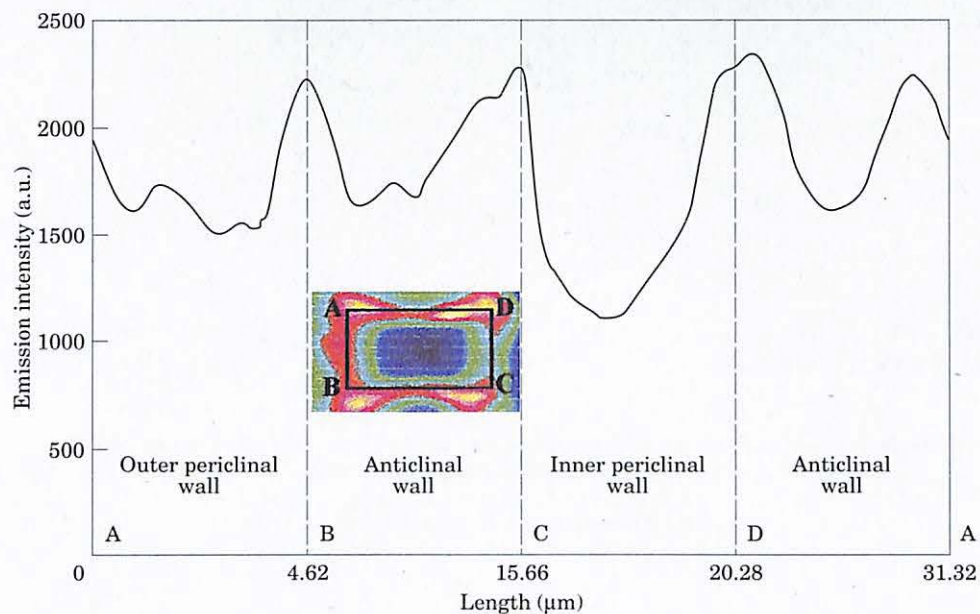
Figure 6 Intensity profile at an emission near 445 nm along the aleurone cell perimeter ABCD indicated on the same spectral image in Figure 5.
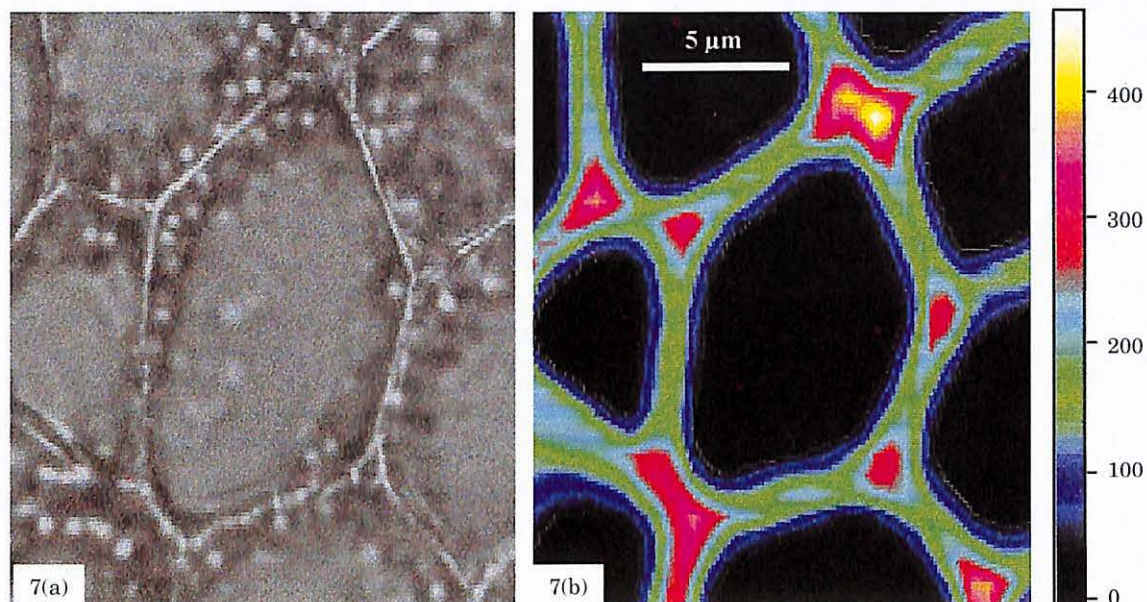

Figure 7 Conventional (a) and spectral (b) images of a section of the pure aleurone tissues showing the auto-fluorescence in the aleurone cell walls and high auto-fluorescence of the junction of the cell walls.

the transverse section studied. Images, superimposable to their conventional images, were generated on the basis of the relative contribution (score) of each spectral component (Labspec image treatment software from Dilor S.A., France). Analysis of the images obtained by the mapping procedure allowed not only the generation of component distribution but also the determination of either their presence or absence in certain histological sections.
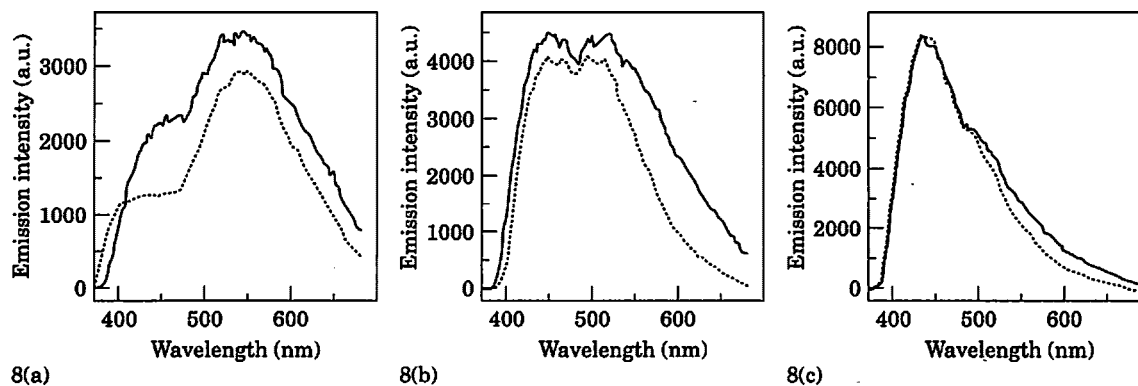
Figure 8 Microspectrofluorimetric comparison of emission spectra profile for phenolics features in the wheat grain and pure phenolic acids. (a) (—) the type III spectrum; (...) pure p-coumaric acid crystals spectrum. (b) (—) the type II spectrum recorded in the starchy endosperm; (...) pure ferulic acid crystals spectrum. (c) (—) the type I spectrum specific to the aleurone tissue; (...) spectrum of chromatography paper (Whatman) including ferulic acid.

RESULTS AND DISCUSSION

Microspectrofluorometric scanning was used to record X–Y emission spectra of points on transverse sections of durum wheat grains. Recorded spectra were composed of several types of spectra, which were represented by three principal spectral components: type I (λ_{max}: 431 nm); type II (λ_{max}: 450, 515 nm) and type III (λ_{max}: 455, 544 nm), as depicted in Figure 1. The three types of spectra were considered to be the principal phenolic features in the durum wheat grain.

Relative contribution of these spectral components was used for the reconstitution of a fluorescence image equivalent to that expected with a conventional fluorescence microscope. Such an image of total auto-fluorescence emitted in the range of 380–680 nm is presented in Figure 2. The total auto-fluorescence emission map indicates that a higher fluorescence intensity was emitted at the exterior regions of the section corresponding to the pericarp and aleurone layers, a region which is high in phenolic materials. Contributions of the three spectral components recorded from the durum wheat sections were mapped by the Labspec image treatment software. The distributions of individual spectral components in a single kernel are shown in Figure 3. The colour scale is an artificial colour palette, which express the variation in intensity of a particular spectral component. Warm colours such as white and yellow represent maximum intensities whereas cold colours like blue and black are representative of low or no intensities.

Results of mapping showed that the three selected spectral parameters are distributed through the cross-section surface in a well-organised manner. Type I spectrum was primarily located in the outer layers of the grain which coincided with the aleurone layer and it was more intense in the centre of the transverse section, i.e. in the zone which surrounds the pigment strand [Fig. 3(a)]. The high intensity values suggested that the type I phenolic compound can be used as an indicator of the non-endosperm tissues of the grain in order to characterise and estimate aleurone contamination in millstreams. Spectral contribution of the type II was distributed over much of a section as shown in Figure 3(b). The type II spectrum was observed with much lower intensities in the inner starchy endosperm than at its periphery or in the aleurone layer. The type III spectral component was highest in the centre of the crease zone (pigment strand) as seen in Figure 3(c).

Total phenolic material present in the kernel was mapped by doing 3D reconstruction of spectral maps acquired from serial sections of an entire kernel. The 3D images of the spatial distribution of the two phenolic features (Fig. 4) allowed visualisation of individual phenolic components within the durum wheat grain. The type I feature surrounds the grain forming the bran of the 3D model [Fig. 4(a)], whereas the type III feature ran nearly along the entire length of the grain at the crease [Fig. 4(b)].

Aleurone cells are the outermost layer of the endosperm and represent the only living en-

dosperm tissue at maturity. Recently, Akin[8] showed that significant spectral differences occurred within the aleurone layer, between the anticlinal walls (side region between aleurone cells) and the inner and outer periclinal walls of common wheat. All of the aleurone cell walls exhibit only the type I spectrum. A close study, more particularly in the proximity of the aleurone cells allowed to analyse these surfaces on the transverse sections of the grain. Fluorescence images of aleurone cell walls were generated on the basis of the relative contribution of the type I spectrum. Results showed that the fluorescence intensities were significantly different as can be seen by inspecting the fluorescence image in Figure 5(a,b). The fluorescence intensity in the anticlinal walls (AN) is higher than that in the outer (OP) and inner (IP) periclinal walls. The inner periclinal walls exhibit the lowest fluorescence intensity while the highest intensity was recorded at the junction of anticlinal and periclinal cell walls. Relative fluorescence intensities of the various aleurone walls as determined at different points in the same section are displayed in Figure 5(c). The intensity profile emitted near 445 nm was followed and recorded along the aleurone cell perimeter (ABCDA in Fig. 6).

The analysis of pure aleurone tissue obtained by cutting longitudinally along the dorsal side of the grain shows that the junction of aleurone walls exhibits the most intense fluorescence, as shown in Figure 7. This result shows that phenolic material is concentrated in the anticlinal walls and at their intersections with the outer walls of aleurone cells.

The first objective of this study was to characterise the spectral features of the durum wheat kernel with regards to phenolics. Then we attributed spectral features to specific phenolics by comparing the resulting spectra with those of the pure phenolic acids, ferulic acid and p-coumaric acid. Both phenolics have been described as the most abundant simple phenolics in cereals[16]. The spectra of ferulic and p-coumaric acid were used as references showing that the type III and the pure p-coumaric acid solid crystals spectra have identical profiles [Fig. 8(a)] and the type II spectrum has approximately the same profile as that of pure ferulic acid [Fig. 8(b)]. It seems that p-coumaric acid could contribute principally to the fluorescence emission at the centre of the crease zone and that ferulic acid could be at the basis of the weak fluorescence emission spectrum observed in the endosperm.

The true origin of the emission spectrum arising from the wheat section is not easily determined because the model reference spectra are not taken under the same conditions as in the grain section; i.e. the presence of other compounds in the kernel complicates the spectral output.

Ferulic acid was absorbed onto chromatography paper to simulate the grain environment. The absorbed material produced a fluorescence emission spectrum comparable to that which was observed in the outer cell walls [Fig. 8(c)]. We suggest that the type I spectrum could be attributed to the presence of ferulic acid in a particular form, either bound to carbohydrates or influenced by specific interactions such as in cellulosic surroundings in the aleurone cell walls.

These results demonstrate the possibility of analysing the distribution of phenolic material within the durum wheat grain, and characterising its various principal tissues. When applied to other varieties of wheat this specific and sensitive method should enable the determination of the phenolic distribution in whole kernels and should serve as a quality measure to determine the milling efficiency.

Acknowledgements

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