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Prediction of Relative Tissue Proportions in Wheat Mill Streams by Fourier Transform Mid-infrared Spectroscopy

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Supporting Information

ABSTRACT: Fourier-transform mid-infrared (FTIR) spectroscopy was investigated as a method to quantify the relative wheat grain tissue proportion in milling fractions. Spectra were acquired with a FTIR spectrometer equipped with an attenuated total reflectance device on ground samples, and the relative tissue proportion was determined according to the biochemical marker methodology as the reference method. Partial least-squares models were developed independently to predict the amount of outer pericarp, aleurone layer, starchy endosperm, and an intermediate layer (made up of inner pericarp plus seed coat plus nucellar epidermis). Good quality of prediction was obtained regardless of the target tissue. The standard errors of prediction obtained for the outer pericarp, intermediate layer, aleurone layer, and starchy endosperm quantification were, respectively, 3.4, 1.3, 3.4, and 4.6%.

KEYWORDS: FTIR spectroscopy, chemometrics, wheat, aleurone layer

INTRODUCTION

Wheat bran is a byproduct of the conventional milling industry and commercially available in large quantities. Although it is mainly used as a low-value ingredient in animal feed, its use as a source of dietary fiber or micronutrients is increasing. Indeed, bran has been shown to concentrate most of the minerals, vitamins, and fibers encountered in the wheat grain. However, these nutrients are not evenly distributed within the bran.[1] This technological fraction is constituted of starchy endosperm adhering to the outer layers, which are made up of, from the outer to the inner surface, the pericarp, the seed coat, the nucellar epidermis, and finally the aleurone layer. If minerals and vitamins are concentrated in the aleurone cells, lignans were mainly observed in the seed coat. According to the tissue (aleurone/hyaline or pericarp) the nature of the polysaccharide (the main source of dietary fibers) or phenolic moieties changes.[2] Alternative technologies in dry fractionation refinery have been designed or are under development to better exploit the grain potential found in the bran.[3] Monitoring the tissue proportion in the produced fractions is essential to control the quality of the product and also to adapt the processes. To gain sensitivity, quantitative tools are needed. Among various methodologies, the measurement of the amounts of biochemical markers found specifically in wheat grain tissue has been proved to be an efficient tool for the assessment of the grain tissue (aleurone, outer pericarp, intermediate layer, and germ) proportions in milling fractions exhibiting contrasting compositions, as flours, brans, or aleurone-rich fractions.[4,5] However, this method implies several biochemical analyses, and its application to batches of grain of unknown origin launches a loss of accuracy.[6] Therefore, its application to control quality on a production line is not practically feasible, but it could be used as a reference for the calibration of a method based on physical signal. More rapid methods, such as fluorescence spectroscopy, were evaluated to distinguish pericarp from aleurone.[7,8] Flour contamination after milling was then efficiently determined with commercial equipments based on the specific fluorescence properties of both of these tissues. However, their adaptability to other fractionation diagrams yielding very different tissue proportions of fractions has not yet been shown. Moreover, other tissues, such as the seed coat, cannot be identified, due to the lack of specific fluorescence signature. Mid-infrared spectroscopy was also used to identify different mill streams,[9] and, more recently, specific spectral signatures of each peripheral tissue were identified.[10] On the basis of the polysaccharide spectral signature, multivariate models allowed the accurate prediction, regardless of the cultivar, of the histological origin of hand-isolated tissues (pericarp, nucellar epidermis, aleurone layer, and seed coat but with a lower performance). However, the preservation of the specificity of these FTIR signatures and the quantification ability have not been checked on powdered samples.

The objective of this work was to evaluate the ability of mid-infrared spectroscopy, coupled to multivariate data analysis, to quantify the histological composition of milling fractions. The biochemical marker methodology was used as the reference method to calibrate the predictive models.

MATERIALS AND METHODS

Isolation of Pure Tissues. The aleurone layer, the intermediate layer (made of the inner pericarp plus seed coat plus nucellar epidermis), the outer pericarp, and the starchy endosperm were hand isolated as described in ref 4 from various common wheat cultivars (*Triticum aestivum* L.) selected from the 26 lines analyzed in the HEALTHGRAIN diversity screen[11] to describe the overall variability encountered.[6] Common wheat grains from Atlas-66, Campari, Disponent, Estica, Herzog, Isengrain, Rialto, San Pastore, Spartanka, and Valoris cultivars were

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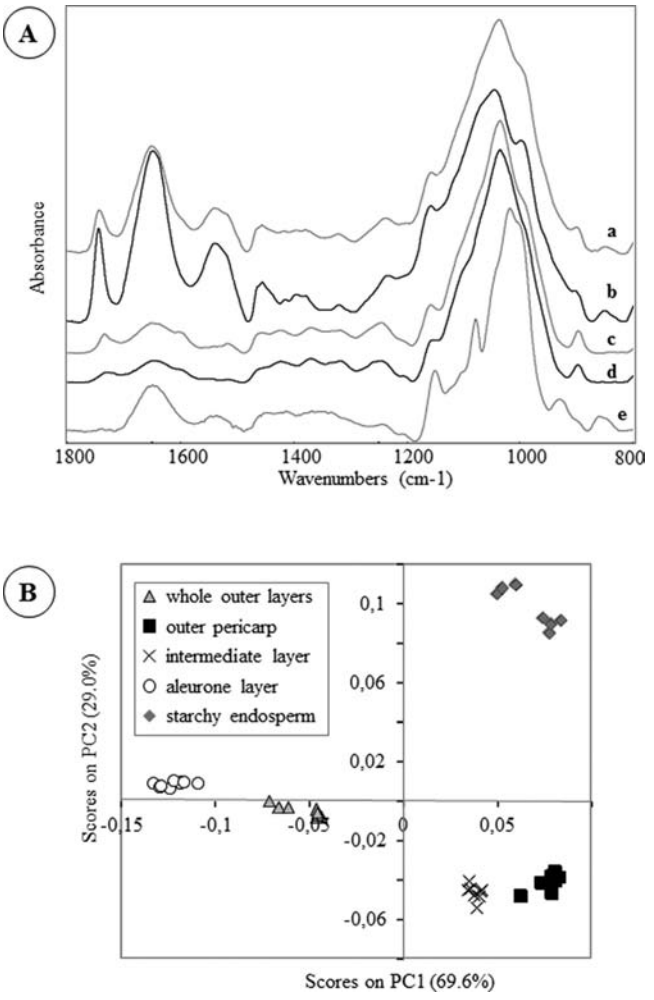

Figure 1. FTIR spectroscopy of pure hand-isolated tissues from two wheat cultivars (Crousty and Tiger): (A) ATR-FTIR spectra of liquid nitrogen ground spectra from (a) the whole outer layers, (b) the aleurone layer, (c) the intermediate layer, (d) the outer pericarp ,and (e) the starchy endosperm; (B) similarity map built from the first and second principal components accounting for 98.6% of the variability. PCA was carried out from baseline corrected and normalized spectra with the 800−1800 cm^{-1} spectral range.

kindly supplied by M. Rakszegi at the Agricultural Research Institute of the Hungarian Academy of Science, Martonvásár, Hungary. Commercial wheat grains were also added: Tiger, Crousty, Monopol, Isengrain (harvest 2006), and Recital as common wheat (*T. aestivum* L.) cultivars and Nefer, Orlu, and Pescadou as durum wheat (*Triticum durum* Desf) cultivars.

Wheat Mill Streams. Wheat mill streams were provided by the Department of Safety and Quality of Cereals, Federal Research Centre for Nutrition and Food (BFEL, Germany), and Bühler A.G. (Uzwil, Switzerland). They were produced from two wheat cultivars (Crousty and Tiger) as described by Hemery et al.[4] To cover a wide range of tissue proportions, samples were chosen in conventional milling diagram, debranning technologies, and also bran fractionation process.

Relative Tissue Proportions from Biochemical Markers Methodology. The proportions of the different grain tissues were determined by the biochemical marker methodology as the reference method.[4] Therefore, four assays were carried out to measure starch, phytic acid, alkylresorcinols, and phenolic acid amounts on samples and pure tissues isolated from the same wheat cultivars.

FTIR Spectroscopy. Infrared spectra were collected using a Nicolet Nexus 6700 (ThermoScientific, Courtaboeuf, France) spectrometer equipped with an attenuated total reflectance (ATR) Smart DuraSampleIR accessory (ThermoScientific, U.K.) and a Mercury−Cadmium-Telluride-High D detector. Interferograms (128) were collected at 4 cm^{-1} resolution and co-added before Fourier transformation. Spectra were recorded between 800 and 4000 cm^{-1}. Powders were pressed onto the diamond ATR surface (∅ = 2 mm). For each sample three spectra were recorded. A background scan was obtained every three spectra. The ATR diamond surface and pressure rod were cleaned with water and ethanol before each spectral recording. Considering the refractive index of samples and ATR crystal, the depth of penetration is estimated as <5 μm at 1000 cm^{-1}. Depending on the frequency range (4000−800 cm^{-1}), the approximate volume sampled is <3.14 $mm^2 \times 6\,\mu$m. Therefore, before spectral analysis, all of the samples (reference tissues and mill streams) were ground in liquid nitrogen with a Spex CertiPrep 6750 laboratory impact grinder to drastically decrease the particule size (D50 < 50 μm).

Spectral Data Treatment. Prior to statistical analysis (PCA or PLS regression), spectra were baseline corrected (piecewise linear correction) and normalized according to the total intensity. Principal component analysis (PCA) and unsupervised hierarchical cluster analyses were carried out on pure ground tissue spectra based on the 810−1800 cm^{-1} spectral range using the PLS-Toolbox v.3.5 (Eigenvector Research, Inc.) for MATLAB (v7.0.4, Mathworks).

PLS regression was used to quantify the relative proportion in the aleurone layer, intermediate layer, outer pericarp, and starchy endosperm based on the 810−1800 cm^{-1} spectral range using the PLS-Toolbox v.3.5 (Eigenvector Research, Inc.) for MATLAB (v7.0.4, Mathworks). The following pretreatments were used: second-derivative filter (Savitsky and Golay, data points, 11; polynomial order, 2), orthogonal signal correction, and mean-centering. Calibration performance was first calculated as the multiple coefficient of determination (R^2) and root-mean-square error of cross-validation using venetian blind mode (RMSECV). The model was then tested on an independent validation data set ($n = 62$), and performance was reported as the coefficient of determination (r^2), the bias (which corresponds to the average difference between the values calculated by the model and those measured experimentally with the reference method), the slope, and the standard error of prediction (SEP). The ratio of standard error of prediction to the standard deviation of the reference data (RPD) was also calculated.[12] An RPD > 3 could be considered as a good indicator for prediction purposes. The following equations were used:

$$\text{bias} = \frac{\sum_{i=1}^{n} (\hat{Y}_i - Y_i)}{n}$$

$$\text{RMSE (CV or } P) = \sqrt{\frac{\sum_{i=1}^{n} (\hat{Y}_i - Y_i)^2}{n-1}}$$

$$\text{RMSEP}^2 = \text{SEP}^2 + \text{bias}^2$$

$$\text{RPD} = \frac{\text{SD}_{\text{cal}}}{\text{SEP}}$$

Y_i is the tissue proportion obtained with the reference method for the ith sample, $\hat{Y}_i$ the tissue proportion predicted by the model from the FTIR spectrum for the same sample, n the number of samples used in each data set, and SD_{cal} the standard deviation for the tissue proportion in the calibration data set.

RESULTS AND DISCUSSION

Spectral Characteristics of Ground Isolated Tissues. At first, spectra of pure hand-isolated tissues were recorded to

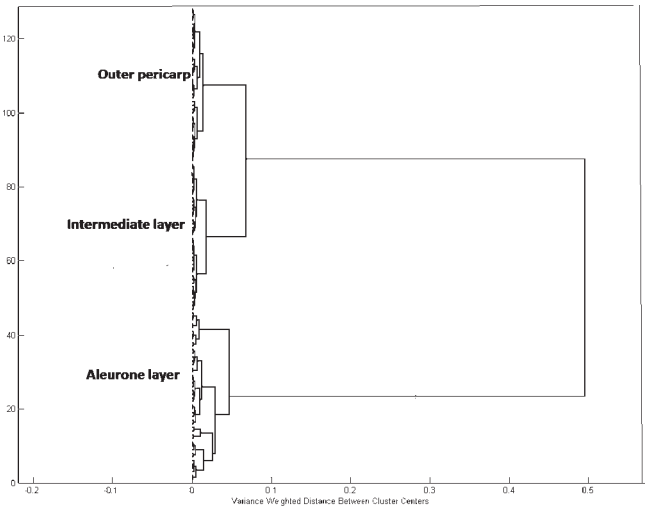

Figure 2. Dendrogram obtained from ATR-FTIR spectra from each pure outer tissue isolated from various wheat cultivars (16 common wheat, 3 durum wheat) based on Ward's clustering method (from baseline corrected and normalized spectra, using the 810−1800 cm^{-1} region).

evaluate the ability to identify a specific spectral signature from ground tissues. FTIR spectra could be related to the main constituents of each tissues (Figure1A). Indeed, specific absorbance at 1648 and 1540 cm^{-1} observed in the starchy endosperm and aleurone layer spectra could be related to their protein content. All of the tissues showed broad absorbance bands for polysaccharides between 1200 and 800 cm^{-1}. In the starchy endosperm spectrum the main band was centered at 1017 cm^{-1}, with shoulders at 930, 1078, and 1151 cm^{-1}, and was assigned to starch.[13,14] In all of the outer layers, the main band was centered at 1044 cm^{-1} with shoulders at 1158 and 997 cm^{-1} that could be related to cell wall polymers (arabinoxylans, β-glucans, cellulose).[14−17] Lipids could be detected by absorbance bands at 3009, 2924, and 2854 cm^{-1} (data not shown) and at 1740 cm^{-1} in the aleurone layer and intermediate layer spectra.[10] Compared to the aleurone layer strip, liquid nitrogen ground aleurone layer showed higher absorbance at 1740, 1648, and 1540 cm^{-1}. Considering the depth of penetration of the ATR signal, this could be explained by the aleurone cell opening during grinding, leading to the analysis of cellular content, which is known to be rich in protein and lipids.[1] As shown on the spectral similarity map built from PCA carried out from 39 spectra (Figure 1B), starchy endosperm was easily discriminated from peripheral tissues. On the contrary, the outer pericarp and intermediate layer showed close coordinates in principal components 1 and 2 that account for 97% of the variability. This could be explained both by (i) the presence of inner pericarp, which could not be distinguished from outer pericarp by FTIR, in the intermediate layer, due to limits in hand dissection possibility, and also by (ii) the slight spectral differences between the seed coat and the outer pericarp.[10] The whole outer layers have intermediate scores between aleurone layer and pericarp, in agreement with their tissue composition (around 50% of aleurone layer, 25% of intermediate layer, and 25% of outer pericarp). (See also the Supporting Information for FTIR spectra of pure hand isolated tissues from two wheat cultivars.)

Pure peripheral tissues were isolated from various wheat cultivars (n = 18) and their spectra acquired to check if it is still possible to identify the peripheral tissue regardless of the genetic

Table 1. Relative Tissue Proportions in the Calibration and Validation Data Set[a]

	calibration				validation			
	n	mean	range	SD	n	mean	range	SD
outer pericarp (%)	73	17.5	1−57	13.5	62	22.9	1−56	16.1
intermediate layer (%)	73	12.1	1−31	7.4	62	6.1	1−10	2.3
aleurone layer (%)	73	37.6	3−89	19.2	62	32.6	2−71	18.9
starchy endosperm (%)	73	27.4	2−85	21.7	62	34.8	4−84	23.3

[a] Data measured with the biochemical markers methodology.

Table 2. Statistics for FTIR Prediction

	calibration				validation				
	LV[a]	RMSECV	RPD	R^2	bias	slope	RMSEP	r^2	RPD
outer pericarp	5	3.3	4.1	0.974	0.18	0.95	3.4	0.956	4.0
intermediate layer	9	1.8	4.1	0.980	0.02	0.91	1.3	0.72	5.5
aleurone layer	8	4.5	4.3	0.982	−0.04	0.97	3.4	0.968	5.7
starchy endosperm	7	4.5	4.8	0.988	0.28	0.98	4.6	0.962	4.7

[a] Number of latent variates used in the model.

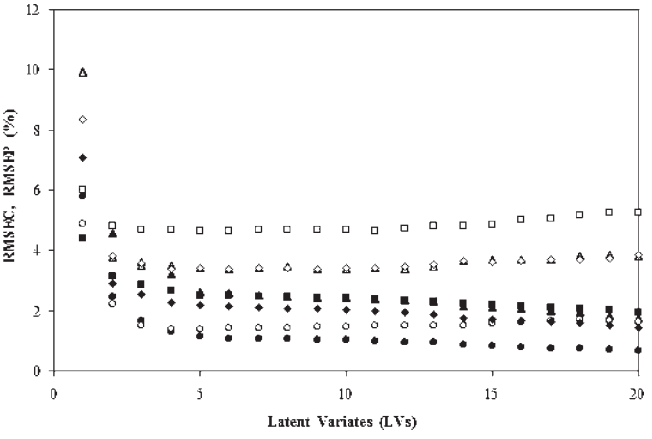

Figure 3. RMSEC (black symbols) and RMSEP (white symbols) obtained according to the number of latent variates used in the PLS models developed to quantify tissue proportions: (□) starchy endosperm; (△) aleurone layer; (○) intermediate layer; (◇) outer pericarp.

variability. Unsupervised hierarchical cluster analysis (such as Ward's method) was carried out on mean-centered spectra using the 1800−810 cm^{-1} spectral range. Regardless of the cultivar, three groups could be easily separated in the dendrogram (Figure 2). This method allowed spectra from the aleurone layer, the intermediate layer, and the outer pericarp to be distinguished. The distance between the aleurone layer spectra group and the intermediate layer or outer pericarp spectra was greater than the distance observed between the intermediate and outer pericarp spectral groups. This proximity was also confirmed using a PCA similarity map (data not shown), drawn from PC scores 1 and 2, respectively accounting for 84.1 and 6.8% of the variability. Regardless of the pigmentation intensity of the seed coat (red vs white cultivars), spectra collected from the intermediate layer were clustered in one group. As the seed coat was not isolated on its own, its color could not be assessed.[10] In summary, the variability

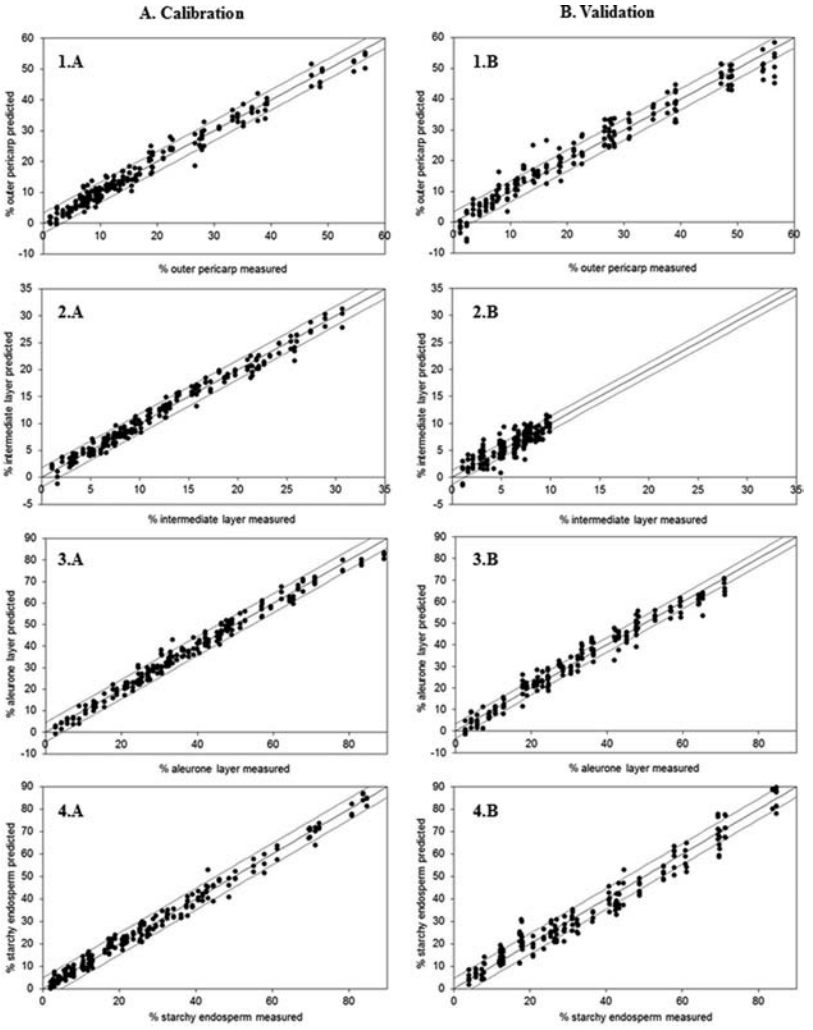

Figure 4. Relative proportion of wheat grain tissues deduced from FTIR spectra compared to reference data obtained by biochemistry: calibration (A) and validation (B) data sets. Dotted lines represent the target, more or less, RMSECV and RMSEP, respectively, in the calibration and validation data sets.

encountered between tissues was higher than the variability encountered for each tissue according to cultivar variability, leading to the potential application of this technique to unknown wheat cultivar batches.

Reference Method Determination of Tissue Proportions. The sample set was constituted of fractions obtained from several milling diagrams (conventional milling, friction/abrasion debranning, bran fractionation).[4] The reference values of each tissue proportion were obtained by the biochemical markers methodology. As some tissues (e.g., germ) were not identified and considering also the error inherent to this method, the sum of each tissue proportion was not always found equal to 100%. The average proportion of undetermined tissue was about 6%, but the largest was 32.7%. No correlation between each tissue proportion was observed. To well cover the whole range in tissue proportion and avoid any correlation between the data, mixtures of different liquid nitrogen ground milling fractions were carried out. In that case, the reference values were calculated, considering the values of the initial samples and the proportion coefficient.

The calibration data set accounts for 73 samples (Table 1). The relative proportions of outer pericarp, intermediate layer, aleurone layer, and starchy endosperm were respectively contained between 1 and 57%, between 1 and 31%, between 3 and 89%, and between 2 and 85% (Table 1). A wide range was then covered, especially for the aleurone layer and starchy endosperm proportions. As the objective of this work was not to detect any flour contamination, the highest proportion of starchy endosperm was 85% and did not cover pure white flour (e.g., 76% extraction rate). No correlation between the variables was observed. The validation data set accounts for 62 samples. A similar range was covered, except for the intermediate layer percentage, which was lower (1−10%) (Table 1).

FTIR Models for Quantitative Measurement of the Relative Tissue Proportions. Statistics for the optimized FTIR predictions of the relative tissue proportions in mill streams are shown in Table 2. The spectral range varied from 810 to 1800 cm^{-1}, and no improvement of models was noted by limiting the spectral range to the polysaccharide fingerprint region. The model performance was evaluated by cross-validation and also by an external data set (62 samples). The optimal number of latent variates (LVs) was first evaluated by the evolution of the RMSECV and RMSEP (Figure 3). Both errors decreased as the number of LVs increased. No increase of RMSEP for the highest LV was observed, suggesting no overfitting. The optimal number of LVs varied according to the target tissue from 5 to 9. Regardless of the models, bias is negligible (from −0.04 to 0.28), indicating no major systematic errors in the validation results.

Therefore, SEP could be well evaluated by the RMSEP. RPDs calculated from the validation data set are higher than 4 or even 5, indicating a fair/good ability for quantification of each tissue.[12] The best results were obtained for the aleurone layer and starchy endosperm quantification.

The regression coefficients for the developed PLS models were analyzed to estimate which wavenumbers were contributing to each tissue quantification in regard to pure tissue spectra (Figure 5). The regression coefficient obtained for the starchy endosperm quantification gave opposite peaks to those obtained to quantify the outer tissues. This could be related to the strong differences observed between the starchy endosperm group and peripheral tissues in the PCA similarity map built from pure tissue spectra (Figure 1). This regression coefficient vector showed strong similarities with the second-derivative spectrum of pure starchy endosperm, such as negative peaks at 1151, 1078, 1020, and 994 cm^{-1} assigned to starch content. Moreover, negative peaks were also observed at 1654, 1631, and 1543 cm^{-1} that could be related to amide I and amide II absorption bands. This spectral region is also useful to quantify the aleurone layer and could be related to the amount of proteins in the aleurone layer. The total amount of protein in grains from which the technological fractions were produced were quite similar (about 13.7 and 12.8%, respectively, for Tiger and Crousty cultivars). The influence of variation in protein amount was not introduced in this sample set and could affect the robustness of this model. Moreover, the regression coefficients obtained in models developed to quantify peripheral tissues showed peaks not only in the polysaccharide fingerprint regions but also in the 1700−1745 cm^{-1} region in relation with the presence of lipidic constituents. Slight variations in these two spectral regions were used to distinguish the aleurone layer from the most peripheral tissues. The similarity between the regression coefficient vector and pure tissue spectra was not straightforward for these peripheral tissues.

In the 1−56% range, the outer pericarp proportion could be obtained with RMSECV and RMSEP respectively equal to 3.3 and 3.4%. Linear regression of reference data against FTIR-predicted data gave intercepts and slopes respectively equal to 1.26 (±0.42) and 0.95 (±0.01) (Figure 4A,B). Residues were randomly distributed according to the outer pericarp proportion range. Quantification performance was fair (RPD around 4 in both the calibration and validation data sets, R^2 and r^2 values respectively equal to 0.974 and 0.956), leading to potential application in screening.

Nine LVs were necessary to obtain the proportion of the intermediate layer with RMSECV and RMSEP respectively equal to 1.8 and 1.3%. The quality of prediction could be evaluated both by the RPD and by the coefficient of determination (r^2): if the RPD value was high (5.7), the r^2 was low (0.72). Considering the limited range of the validation data set (1−10% compared to 1−31% in the calibration data set), more data are needed to fully address the effective quantification of such tissue. Nevertheless, promising results were obtained, opening the potential applications of such method.

In the 3−89% range, the aleurone layer proportion could be obtained with RMSECV and RMSEP respectively equal to 4.5 and 3.4%. Linear regression of reference data against FTIR-predicted data gave intercepts and slopes respectively equal to 0.86 (±0.48) and 0.97 (±0.01). The aleurone proportion seemed to be slightly underestimated for the highest values (>60%), but more data are needed in the highest range (70−90%) to confirm this observation. The highest quality of prediction was obtained for this model (RPD = 5.7, r^2 = 0.968). Good quantification could be then obtained for this tissue with an error similar to that of the reference method.[4]

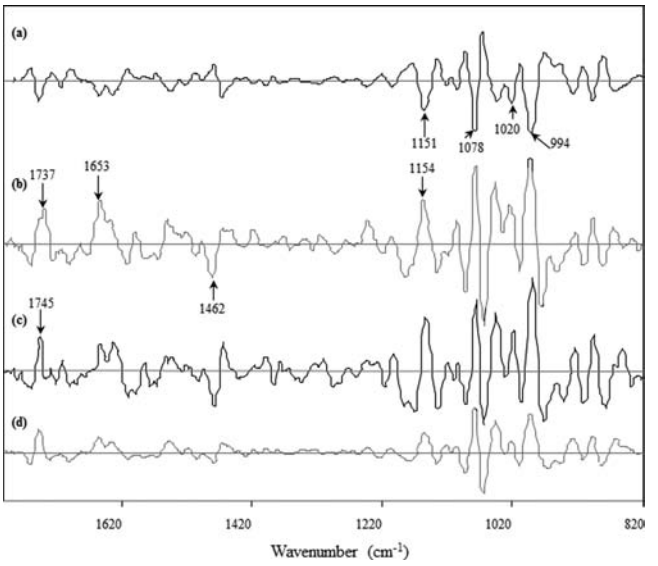

Figure 5. Regression coefficients obtained in PLS model for the quantification of (a) the starchy endosperm, (b) the aleurone layer, (c) the intermediate layer, and (d) the outer pericarp.

The starchy endosperm proportion in these milling fractions could be obtained with RMSECV and RMSEP respectively equal to 4.5 and 4.6%. Linear regression of reference data against FTIR-predicted data gave intercepts and slopes respectively equal to 0.35 (±0.59) and 0.98 (±0.01). Residues were randomly distributed according to the starchy endosperm proportion range. As observed for the aleurone layer, a good quality of prediction was achieved with such a model.

In summary, regardless of wheat species or cultivars, ground wheat grain tissues showed specific ATR-FTIR signatures, allowing tissue identification in wheat powders. Moreover, the potential of ATR-FTIR spectroscopy for a rapid prediction of tissue proportions within mill streams without the need for exhaustive sample preparation has been demonstrated. Models developed to calculate the proportions of starchy endosperm and aleurone layer gave the best quality of prediction, and these tissues could be predicted with absolute errors respectively equal to 4.6 and 3.4%. FTIR spectroscopy has the potential to dramatically reduce the analytical time required to monitor fractionation processes. Further studies are needed to improve the calibration specificity, accuracy, and robustness that will be dependent on sought objectives (quality control in terms of deviation to a standard, focus on a specific tissue and/or proportion ranges). In particular, this must be extended with mill streams obtained from a higher number of cultivars and/or different harvest years.

■ ASSOCIATED CONTENT

Supporting Information. Figure 6: PCA loadings from FTIR spectra of pure hand-isolated tissues from two wheat cultivars (Crousty and Tiger). PCA was carried out from baseline corrected and normalized spectra with the 800−1800 cm^{-1} spectral range (complementary data to Figure 1B). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

ATR, attenuated total reflectance; FTIR, Fourier transform infrared; LV, latent variate; PCA, principal component analysis; PLS, partial least-squares; RPD, residual predictive deviation; RMSECV, root-mean-square error of cross-validation; RMSEP, root-mean-square error of prediction; r^2, coefficient of determination (validation data set); R^2, multiple coefficient of determination (calibration data set); SEP, standard error of prediction.

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