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1 **Structural and chemical analysis of native and malted barley**
2 **kernels by polarized Raman spectroscopy (PRS)**

3

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13 **Abstract**

14 In this work, barley kernel sections embedded in resin were mapped using polarized
15 Raman spectroscopy (PRS) for evaluation of both chemical and structural changes in
16 endosperm upon malting. A multivariate analysis that includes Band-Target Entropy
17 Minimization (BTEM) was used to resolve the individual spectrum of components
18 present on sections and to reconstruct images based on their relative signal. BTEM-
19 resolved spectra matched with several individual model compound spectra that allowed
20 the identification of protein, starch, non-starch carbohydrates, esterified ferulic acid and
21 embedding media. Raman images also revealed chemical changes in the cell walls within
22 starchy endosperm and inside cells due to enzymatic reactions in malting. The anisotropic
23 response of the C-O-C Raman band associated with the orientation of ordered structures

inside starch granules remains despite a notorious shape distortion of large starch granules.

Keywords: Chemical imaging, Malting, Multivariate analysis, Polarized Raman spectroscopy

Abbreviations

(AFM) Atomic Force Microscopy, (BTEM) Band-Target Entropy Minimization, (CV) Coefficient of Variation, (LLS) Linear Least-Squares fit, (PRS) Polarized Raman Spectroscopy, (SD) Standard Deviation

1. Introduction

The chemical composition and microstructure of cereal kernels determines the outcome of several food processing techniques such as milling, gelatinization and malting (Holopainen et al., 2005). For example, cereal kernels experience a series of chemical and structural changes during malting that lead to both release of fermentable carbohydrates and production of precursors related to desired organoleptic properties for beer production. In this regard, the endosperm microstructure of cereal kernels is also known to play an important role in malting that greatly depends on properties of the protein matrix and packing density of starch in endosperm cells (Chandra et al., 1999). Optical microscopy constitutes the standard technique to obtain information regarding chemical distribution of different compounds inside cereal kernels but requires the implementation of several staining protocols of histological sections. Moreover, the evaluation of microstructure in cereal kernels often requires the use of other complementary microscopic techniques. For instance, confocal scanning laser microscopy (CSLM) and scanning electron microscopy (SEM) have been used to study starch granules of different

botanical sources (Glaring et al., 2006, van de Velde et al., 2002, Nair et al., 2011). Atomic force microscopy (AFM) has also been employed in the study of isolated starch granules, thus enabling the visualization of ultra-structures on native and mutant pea starch (Ridout et al., 2002). In this context, *in situ* scanning techniques that provide both chemical and structural information at micron scale in cereal grains open up the opportunity of increasing the knowledge regarding cereal endosperm transformations during malting.

Briefly, the malting process begins by the imbibition of barley grains in steeping vessels for 1-2 days in a stage called “steeping”, which induces seed germination. Subsequently, germinating grains are incubated for 3 to 4 days in boxes for sprouting. The aim of this phase is the production of cell wall and protein-degrading enzymes. Finally, the seedlings are dried at 50 to 90°C to stop all metabolic processes inside the seeds by water deprivation and heat effects during a stage called “kilning” (Kleinwächter et al., 2014).

Polarized Raman micro-spectroscopy (PRS) constitutes a suitable technique to study cereal kernels during malting since both chemical composition and molecular orientation of materials can be obtained at high spatial resolution (0.6-1µm) without undergoing staining of prepared sections (Jääskeläinen et al., 2013). PRS is based on the analysis of the inelastic scattering of light interacting with molecules, in which the frequency shift between the incident and scattered light is associated with a particular vibration mode of a chemical bond. Several cereal kernels have already been studied by PRS. For instance, wheat kernels were used to investigate the relationship between interstitial protein structure and hardness (Piot et al., 2002) and to characterize arabinoxylans present in the cell wall during the endosperm development (Philippe et al., 2006). Additionally, PRS

revealed starch granule structure in the endosperm of wild and mutant maize kernels (Wellner et al., 2011), and also the compound distribution of aleurone layer in wheat and barley kernels using basis spectral analysis (Jääskeläinen et al., 2013).

In this work, we have studied the chemical and structural properties of both native and malted barley grains endosperm by PRS. The computational analysis carried out enabled us to reconstruct individual components using Band-Target Entropy Minimization (BTEM) and to elaborate images on selected regions corresponding to the aleurone layer-starchy endosperm boundary and the centre of the starchy endosperm. To our knowledge, this is the first application of this type of chemometric techniques to the study of cereal kernels. Additionally, we have focused on the analysis of the anisotropic response of the starch Raman band that is related to the orientation of ordered structures inside native and malted barley.

2. Materials and Methods

2.1. Barley sections

Kernels of malting barley (*Hordeum vulgare* L.) cultivar Fairytale from crop 2010 were studied as native and malted. The malting process was carried out in an industrial production system as follows: barley kernels were steeped twice in conical steeping vessels at 15°C during 13 and 3 hours respectively with a 16-hour air-rest between the steps. After steeping, the barley kernels were transferred to germination boxes for 6 days at 16°C. The germination process was terminated during kilning using air at 50°C for 17h and finally at 84°C for 4 hours.

Malted and native barley kernels were then fixed with a solution of 3% paraformaldehyde and 1% glutaraldehyde in 0.10 mol/L sodium potassium phosphate buffer pH 7. Subsequently, kernels were dehydrated in a series of ethanol solutions, embedded in Historesin© (Leica microsystem; Mensheim, Germany) and cut into semi-thin sections (4 µm thick) with a rotary microtome (Microm Labogeräte GmbH, Walldorf, Germany) using a tungsten carbon knife. Longitudinal and cross-sections of native and malted barley kernels were then mounted onto glass slides and dried on a heating plate at 40°C.

2.2. Model compounds

In order to validate the resolved spectra from BTEM, Raman spectra of different model compounds, namely gliadin (Sigma-Aldrich), arabinoxylan from rye (*Secale cereal* L.; Leuven Bioproducts), barley starch (Berner), (1→3,1→4)-β-D glucan (Megazyme) and ferulic acid (Sigma Aldrich), were recorded.

2.3. Atomic force microscopy (AFM)

A single large starch granule embedded in the endosperm of native barley seeds was mapped by atomic force microscopy using an AFM instrument that is coupled to a WITec alpha 300 Raman microscope (alpha 300, WITec, Ulm, Germany). The image was obtained in air using silicon NCR cantilevers (Nanoworld Germany) with a tip radius below 10 nm and a nominal force constant of 42 N.m⁻¹, operating in tapping mode (resonance frequency 285 kHz). Topographic and amplitude mode images were recorded. The image size was 25x25 µm² and the scan frequency 1Hz. No further image processing was performed.

2.4. Raman spectroscopy

For Raman experiments, a continuous excitation laser beam was focused down to a micrometre sized spot on the barley sections through a confocal Raman microscope equipped with a piezo-scanner. A frequency doubled Nd:YAG, 532 nm linear polarized excitation laser (~20 mW) was used in combination with a 100X (Nikon, NA = 0.90) microscope objective. The polarization angle of the laser was rotated using a half-wave plate in the optical pathway. The spectra were acquired using a CCD camera (Andor Newton DU970-BV, Andor Technology plc, Belfast, UK) behind a grating (600 g mm^{-1}) spectrograph with a spectral resolution of $2\text{-}3 \text{ cm}^{-1}$. For mapping, the surface of the barley sections were scanned with steps of $1 \mu\text{m}$, integrating the signal for 0.3 s and using 0° polarization of the exciting laser. Several histological sections of native and different malting time points were available to study under PRS. Optical microscope images were employed to select the more representative of malted kernels. For PRS mapping, ten histological sections (five longitudinal and five cross-sections) of each native and malted kernels were used. For polarization experiments, the starch spectra were collected on different spots of a single large starch granule in native barley, integrating the signal for 1 s at different polarization direction ranging from -90° to 90° with steps of 15° .

2.5. Analysis of Raman images

All spectra were first pre-processed by removing cosmic ray peaks, correcting the baseline drift, restricting the wavenumber range to $320\text{-}1800 \text{ cm}^{-1}$, smoothing and normalizing by the Euclidean norm. Cosmic peaks were removed using an in-house written algorithm based on the comparison of differential spectra among neighbouring

pixels. The baseline was corrected by an iterative polynomial fitting of peak-free points, recognized from the continuous wavelet transform of the signal (Bertinetto & Vuorinen, 2014). The smoothing was done using a Whittaker smoother with 2nd order polynomial and smoothing parameter $\lambda = 5$.

The pre-processed spectra were analysed with BTEM, a technique used for identifying pure components within spectral mixtures (Widjaja et al., 2003). It works by performing a Singular Value Decomposition (SVD) on the data matrix and finding linear combinations of a selected set of singular vectors (commonly denoted as V^T) that satisfy the following criteria: a) minimum entropy; b) non-negativity in spectral intensity; c) presence of a certain peak (named *band-target* in this context). They are expressed by the objective function H :

$$H = -\omega_1 \sum_v h_v \ln h_v + \omega_2 \sum_v \frac{d^n a_v}{dv^n} + \omega_3 \sum_v a_v + P \quad (\text{eq.1})$$

where v are the wavenumbers at which measurements are taken, a_v is the Raman intensity for one particular wavenumbers, h_v is defined as:

$$h_v = \left| \frac{d^m a_v}{dv^m} \right| / \sum_v \left| \frac{d^m a_v}{dv^m} \right| \quad (\text{eq.2})$$

$\omega_1, \omega_2, \omega_3$ were set as 10, 1, 1, respectively, and m, n as 2 and 1, respectively. The first three terms are a generalized entropy function that combines Shannon entropy, sum of spectral derivative and integrated spectral area. The minimum entropy criterion is based

on the principle that a pure component always has lower entropy than a mixture containing it. The fourth term P in eq. 1 is a penalty function that takes very large values in the occurrence of negative spectral intensities. The presence of the band-target in the final reconstructed spectrum is guaranteed by a peak-normalization step before every evaluation of H . The BTEM algorithm is run several times yielding a different solution, and thus a possible pure component, for each band-target. In our implementation, the band-targets were searched automatically (Tan et al., 2009) and the optimum for H was found by means of a Particle Swarm Optimizer (<http://psotoolbox.sourceforge.net/>, 2014).

In parallel with the application of BTEM directly to the whole matrix of pre-processed spectra, BTEM was also applied to two sets of cluster-averaged spectra. Clustering was performed on the pre-processed data compressed by PCA, retaining the principal components explaining 85-90% of the total variance. The first set was obtained with hierarchical clustering, using city-block distance, complete linkage and selecting a partition with a number of clusters approximately equal to 1/100 of the total number of pixels in the image. The second set was obtained with the k -means method, using city-block distance, setting a number of clusters $\sim 1/10$ of the total number of pixels, running the algorithm 1000 times with different random initializations and retaining the partition with the lowest sum of squared errors. Prior to the clustering step, the noisiest spectra were filtered out to avoid the risk of averaging them with good ones and thus severely distorting the signal. The cluster-averaging suppressed some noise that was not eliminated during pre-processing and brought about a more compact SVD, with fewer V^T

needed to describe the data. The number of V^T used for running the BTEM algorithm was chosen by visual inspection; in a few cases, different numbers of V^T were tried.

All the BTEM runs mentioned above (i.e. on whole image, on two sets of cluster-averaged spectra and on occasional alternate V^T sets) yielded a superset of solutions, on which a post-screening was carried out to identify the pure components in the sample. The best solutions were selected by removing near-identical ones and by comparing with the spectra of model compounds. Images of the relative concentration of each identified compound were generated by a linear least-squares (LLS) fit of the selected BTEM solution (basis set) on the Raman spectra. In a few cases, different basis sets were tried and the one with the best fit and no negative concentrations (with some tolerance for noisy points) were retained.

2.6 Analysis of anisotropic response in starch spectra

Analysis of the signal variation at different polarization directions of the laser was initially carried out to evaluate the anisotropic behaviour of spectral peaks within a starch granule. Firstly, the spectra were pre-processed by removing cosmic ray peaks, correcting the baseline (using the same algorithms as above), restricting the wavenumber range to 280-1650 cm^{-1} , smoothing and dividing every spectrum by the sum of the Euclidean norms of the spectra belonging to the same pixel. This last operation homogenized the total intensity at each pixel, which was affected by non-uniform sample depth, without suppressing the variability related to the polarization angle. Secondly, a spectrum of the Standard Deviation (SD) of the Raman intensities among thirteen polarization angles was calculated for every pixel of the image. These SD spectra were then processed by

hierarchical clustering (using Euclidean distance and Ward linkage) to identify the region in the image showing the most pronounced Raman anisotropic behaviour. For this region, a spectrum of the Coefficient of Variation (CV) was computed by dividing the SD spectrum by the average (pixel-normalized) Raman spectrum. The anisotropy of each peak was then evaluated by the average value of the CV spectrum within the peak range for all pixels in the selected region. All the calculations mentioned above were performed using MATLAB® version 8.2 R2013b (The Mathworks, USA).

The amplitude of the anisotropic variation in different locations of the starch granule was quantified by the Raman response of the 865 cm^{-1} band (the most anisotropic band) normalized by the one at 1343 cm^{-1} (the least anisotropic). The band intensities were evaluated by curve fitting as follows: in the interval 815-896 cm^{-1} with one Gaussian function at $\sim 865 \text{ cm}^{-1}$ and in the interval 1180-1595 cm^{-1} with four Gaussians functions located at, $\sim 1263 \text{ cm}^{-1}$, $\sim 1343 \text{ cm}^{-1}$, $\sim 1386 \text{ cm}^{-1}$ and 1460 cm^{-1} using the WITec Project Plus module (WITec GmbH, Germany). These values were calculated on raw spectra pre-processed only by Savitzky-Golay smoothing (using a fourth degree polynomial and 7-points window) and background subtraction using a straight line in the range of 815-1525 cm^{-1} .

3. Results and discussion

In the present work, the endosperm from both native and malted barley seeds was studied using Raman micro-spectroscopy. Prior to Raman mapping and image reconstruction of kernel sections, individual spectra of the main components including the embedding media were recorded (**Fig. 1**). Cells in the starchy endosperm of barley contain starch

granules embedded in a protein matrix and cell walls composed of arabinoxylan and (1→3, 1→4)-β-D glucans. Other minor components of the cell walls are cellulose, phenolic acids and heteromannans (Fincher and Stone, 1986, Bacic and Stone, 1981).

The polysaccharides spectra represented by (1→3,1→4)-β-D glucans, starch and arabinoxylan (**Fig. 1A**) exhibit bands in the three major regions: (1000-1200 cm⁻¹) that are the typical fingerprint of carbohydrates assigned to C-O-C, C-O and C-C stretching; (1200-1500 cm⁻¹) associated to CH/CH₂ deformation and C-O-H bending modes, and below 800 cm⁻¹ that is attributed to the skeletal and torsional vibration modes (Barron et al., 2006,Philippe et al., 2006). It is in the last region that starch displays a strong band at 485 cm⁻¹ that is assigned to the skeletal vibration of glucopyranose unit (Liu et al., 2004). The starch spectrum also exhibits a band located at 865 cm⁻¹ assigned to stretching C-O-C / ring-breathing that could indicate sensitivity to molecular orientation of ordered structures in starch grains (Wellner et al., 2011).

The main protein components in barley kernels are hordeins and glutelins. The protein spectrum in this set of model compounds was represented by wheat gliadin, as shown in **Fig. 1B**. Gliadins are evolutionarily related to hordein and have similar Raman spectrum to glutelin (Jääskeläinen et al., 2013). This spectrum shows the characteristic signature of proteins given by the amide I band that is assigned to the vibration of the trans-peptide group CONH (1600-1700 cm⁻¹), the amide III band (1230-1340 cm⁻¹) that arises from the combination of N-H bending and C-N stretching of the peptide group and also the sharp band at 1003 cm⁻¹ assigned to the phenylalanine ring vibration. Phenolic compounds are represented by ferulic acid, whose spectrum is displayed in **Fig. 1B**. It exhibits sharp

bands at 1604 and 1631 cm^{-1} that are assigned to the aromatic ring vibration and to C=C side-chain stretching, respectively. The same doublets on the region 1590-1630 cm^{-1} are observed in the arabinoxylan spectrum due to the presence of covalently bonded ferulic acid to its structure (Kačuráková et al., 1999). Due to band overlapping among the Raman spectra of the carbohydrates, protein and the embedding media, a multivariate analysis that includes BTEM was performed in order to reconstruct the Raman spectrum of the main components present in barley sections. The selected areas mapped by PRS for native and malted barley grains correspond to the boundary starchy endosperm-aleurone layer and the centre of the starchy endosperm. An example of the BTEM-reconstructed spectra of the components present in boundary starchy endosperm-aleurone layer is shown in **Fig. 2**. Reconstructed spectra match well with the authentic spectra corresponding to protein, starch, non-starch carbohydrates and the embedding media. The spectrum D in **Fig. 2** shows the typical doublet band of ferulic acid but with a slight frequency shift of the band located at 1604 cm^{-1} in pure ferulic acid to 1599 cm^{-1} in the reconstructed spectra. This shifting has been attributed to the influence of the environment on the esterified form of ferulic acid present in the aleurone cell wall (Piot et al., 2000). Another interesting aspect of this reconstructed spectrum is the presence of a small band at ~1660-1725 cm^{-1} that is associated with the carbonyl group present in esters (Movasaghi et al., 2007, Calheiros et al., 2008). Moreover, peaks in the range ~1100-1500 cm^{-1} are less sharp than in the model compound, which is probably due to the pure crystalline state of the latter as opposed to the complex mixture of substances present in the barley kernel.

The reconstructed spectrum E related to proteins clearly shows the characteristic amide I (1600-1700 cm^{-1}), amide III (1230-1340 cm^{-1}) and phenylalanine ring ($\sim 1003 \text{ cm}^{-1}$) vibrations. The spectrum F did not match any of the model compounds but appears as a result of the contribution of anisotropic starch bands during BTEM analysis, especially the one at 865 cm^{-1} that is sensitive to the orientation of molecules within starch granules (see discussion below). It is worth noting such detailed information is often lost during the pre-processing phase, therefore the presence of this spectrum among the BTEM solutions denotes a very effective chemometric analysis. On the other hand, it was not possible to obtain clearly distinct reconstructions for the spectra of arabinoxylan and (1-3) (1-4) β -D-glucans, probably because of their highly similar spectral features or insufficient signal-to-noise ratio in the relevant areas of the image. In this sense, it is better to account for this spectrum as ‘non-starch cell wall carbohydrate’ (spectrum A) that according to its location in barley seeds might represent arabinoxylan (71%) in the endosperm aleurone layer or (1-3) (1-4) β -D-glucans (75%) in the starchy endosperm (Bacic and Stone, 1981).

Using computationally reconstructed spectra as opposed to spectra of model compounds to resolve Raman images has two main advantages. The first one, which was already previously mentioned, is that reconstructed spectra are probably more similar to the actual spectrum of the component inside the sample. The second is that this computational approach provides an objective and reliable way to identify which components should form the basis set. The choice of the basis set has indeed a great influence on the subsequent fit; however, when using only a priori chemical knowledge,

it is relatively easy to miss a component or include one in excess and still get results that make physical sense.

Among other existing multivariate curve resolution/unmixing techniques, BTEM is the most appropriate for these data considering the available a priori information and the assumptions that can be made. The “pure wavenumber” requirement of SIMPLISMA (meaning that, for each component, there is at least one wavenumber containing a signal from that component only) cannot be satisfied with such chemically complex samples (Windig et al., 2005). MCR-ALS needs additional information to define constraints on spectral or concentration profiles that enable well defined solutions (Jaumot et al., 2005). For images, the only applicable constraint is defining the number of components in some areas of the image. However, we could not obtain this information with sufficient reliability, not even using local rank methods. Minimum simplex volume methods could in principle be an option, but previous calculations employing this approach (not reported here) were able to satisfactorily reconstruct only 2-3 components; the reason for this outcome is not clear.

The optical microscope images and the resulting Raman images from the BTEM analysis on areas located at the starchy endosperm-aleurone layer boundary in native and malted barley are shown in **Fig. 3**. Colour bars indicate the coefficient of the LLS fit (with the sum of coefficients normalized to 1 for each pixel), which is proportional to the relative concentration of the component. Starch images reveal small rounded and large ellipsoidal starch granules ranging from 2 to 20µm inside the starchy endosperm cells. In native barley, proteins appear distributed in the cells of the aleurone layer and the starchy

endosperm. Malted barley endosperm exhibits a reduction in protein matrix concentration inside the starchy endosperm cells caused by the enzymatic action during malting (Palmer, 1993). Esterified ferulic acid in both native and malted barley is disseminated throughout cell walls of the aleurone layer. Non-starch carbohydrates (arabinoxylan and (1→3, 1→4)-β-D glucans) are distributed throughout the cell wall of the aleurone layer and the endosperm in the native barley but appear completely degraded in the starchy endosperm of malted barley.

Fig.4 shows Raman images corresponding to the centre of starchy endosperm in both native and malted barley containing large starch granules (10-20μm) and also small rounded granules (2-5 μm) embedded in the cells. The small starch granules in the malted barley appeared partially degraded due to the hydrolytic action of amylases. Changes in the shape of the large starch granules were observed in the malted grains. In this case, starch granules appear slightly distorted or swollen compared to native kernel, an effect that might be caused by the thermal treatment during kilning. The central endosperm in native barley show smaller protein concentration compared to the native endosperm areas close to the aleurone layer (**Fig. 3**), but after malting almost no protein matrix was detected (Wijngaard et al., 2007). Similarly, the spectrum of ferulic acid was not detected under the experimental conditions in the cell walls of the central endosperm in native barley, even though the presence of phenolic acids in the central starchy endosperm of barley kernel is reported but only in low concentration (0.1% or less) that might fall below the detection limit of the technique (Nordkvist et al., 1984). Non-starch carbohydrates (mainly (1→3, 1→4)-β-D glucans) are distributed within the cell wall of

native barley but not in the malted barley, thus indicating cell walls hydrolysis during malting.

In order to gain a better understanding of ordered structures within starch granules, the anisotropic Raman scattering of starch at different polarization angles of the laser was analysed in a single ellipsoidal granule in the endosperm of native barley. It was suggested that the 865 cm^{-1} band assigned to stretching C-O-C/ring breathing is sensitive to the molecular orientation of the amylopectin in the semi-crystalline regions and Raman maps based on this band can detect changes in the assembly of crystalline structures within the granule (Wellner et al., 2011).

A more detailed analysis of the anisotropic response of different starch bands is shown in **Fig. 5A**, which depicts starch spectra in a large granule collected on a single spot (blue cross on the granule sketch) at 0 and 90° polarization angle of the laser, respectively. **Fig. 5B** shows the mean pixel-normalized spectrum taken at thirteen different polarization angles from the blue region on the granule sketch (see Materials and Methods section), as well as the CV of several bands represented by yellow rectangles. It is possible to observe that most Raman bands in starch granules exhibit anisotropic behavior, although the highest corresponds to the band located at $\sim 865\text{ cm}^{-1}$ that is assigned to the ν_s C-O-C/breathing ring and has the greatest coefficient of variation, while the lowest is found for the $\sim 1120\text{ cm}^{-1}$ band assigned to the glycosidic link C-O-C (Cael et al., 1973). The band at 1343 cm^{-1} assigned to C-O-H bending which has also lower average CV (~ 0.12) is used as normalization band (by ratio of the band intensity) for further analysis.

354 The ultrastructure of starch granules consists of concentric shells of alternating semi-
 355 crystalline and amorphous layers or growth rings. These layers are composed by “parcels
 356 of crystals” or blocklets with amylopectin as the main component. Within the amorphous
 357 layers, the inner structure of the blocklets in particular is defected by non-branching and
 358 lower branching molecules (such as amylose) (Pérez and Bertoft, 2010). As an example
 359 of the anisotropic variation in different locations of a single starch granule, the Raman
 360 response of the 865 cm^{-1} band normalized by the one at 1343 cm^{-1} on confocal spots
 361 located on areas marked [1], [2] and [3] is shown on **Fig. 5C and 5D**. The response of
 362 these spots was fitted to the sinusoidal equation $I = a(1 + b(\cos 2(x - c)))$ by using a non-
 363 linear regression, where I corresponds to the intensity ratio ($865\text{cm}^{-1}/1343\text{cm}^{-1}$), a is the
 364 mean value of the intensity ratio, b the amplitude of the fitting curve, x the polarization
 365 angle of the laser and c the phase shift. This strong spatial variation of the band at 865
 366 cm^{-1} has been attributed to orientation of the crystalline amylopectin elements within the
 367 plane of observation (Wellner et al., 2011). It is also important to notice that due to the
 368 resolution of the technique ($0.6\text{-}1\mu\text{m}$), the anisotropic response observed on a single
 369 confocal spot is the result of the interaction of the laser beam with several amorphous and
 370 semi-crystalline layers. On the other hand, double-helix structures like A-type crystals
 371 prepared from short amylose chains show the C-O-C groups roughly aligned along the
 372 double helix (Popov et al., 2009). In this case, it is possible that the global Raman tensor
 373 of this band in helical structures inside the large starch granules have the largest
 374 polarisability along the double-helix axis and as consequence the maximal Raman
 375 response of the C-O-C band at $\sim 865\text{ cm}^{-1}$ for molecules located “in plane” is obtained
 376 when the polarization of the exciting laser is parallel to double helix axis. The observed

spatial changes in the anisotropic response of 865 cm^{-1} band appear to coincide with the traditional model of radial orientation of the crystalline domain in starch granules. For example, the areas located on [1] in the starch granule might contain crystalline domains more oriented to 90° while in areas [3] these are close to 0° .

In the context of orientation of ordered structures in starch granules of native barley, **Fig. 6A** shows a Raman image of the $\sim 865\text{ cm}^{-1}$ band normalized to the one at 1343 cm^{-1} . The images were taken at 0° polarization of the laser. The higher values observed are related to the orientation of semi-crystalline structures with respect to the polarization angle of the laser. Malted barley endosperm showed a more heterogeneous image due to the degradation of the small starch granules in the endosperm cells but the anisotropic response of the $\sim 865\text{ cm}^{-1}$ band remains inside many of the large starch granules. Similar results are observed in the Raman images of **Figure 6B** for the same set of samples based on the BTEM-reconstructed spectrum F (Fig. 2), which supports the interpretation that reconstructed spectrum F is mainly due to the presence of anisotropic Raman bands in the spectrum of starch present mainly in large starch granules. This result shows that BTEM can provide information on the presence of ordered elements inside the sample, as well as on the abundance of different chemical components. The uses of this algorithm can thus extend beyond its original purpose of unmixing, provided that a careful screening and assignment of BTEM solutions is carried out.

4. Conclusions

In this work, PRS was used to study the chemical and structural changes of barley endosperm upon malting. Multivariate analysis of the endosperm spectra using BTEM appeared suitable to reconstruct individual spectra of the main components and elaborate distribution maps over the barley sections. The BTEM-reconstructed spectra may not only correspond to different compounds present in the sample, but also to the Raman anisotropic response of certain substances, as it was observed for starch.

PRS was able to detect chemical changes in malting, whose major impact is observed not only through the degradation of small starch granules inside the endosperm cells, but also in the shape distortion of large granules and the hydrolysis of cell walls in starchy endosperm. It was also shown that the Raman anisotropic response of the C-O-C band at $\sim 865\text{ cm}^{-1}$ was preserved in many of the big starch granules after malting. Finally, it is possible that a clear spatial description of the anisotropic response of the $\sim 865\text{ cm}^{-1}$ band in starch helical structures will enable the elaboration of *in-situ* orientation maps of amylopectin in starch granules that can be used for the study of different cereals kernels.

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Figure captions

Figure 1: **A)** Raman spectra of authentic carbohydrates, (1→3, 1→4)-β-D-glucan, starch and arabinoxylan present in barley kernels. **B)** Raman spectra of other model compounds used in this study: embedding media, ferulic acid and gliadin.

Figure 2: Reconstructed spectra (A-F) from BTEM analysis performed on native barley kernel at the boundary of the aleurone layer-starchy endosperm. The reconstructed spectrum F appears as a result of anisotropic Raman bands in starch spectrum.

Figure 3: Raman images based on the BTEM-reconstructed spectra of compounds present in the boundary aleurone layer-starchy endosperm in native and malted barley. They correspond to: starch, protein, ferulic acid and non-starch carbohydrates. Color bars indicate the fraction of the LLS fit coefficient, which is proportional to the relative concentration of the component.

Figure 4: Raman images based on the BTEM-reconstructed spectra of starch, protein and non-starch carbohydrates present in the centre of starchy endosperm of native and malted barley. Reconstructed spectra of non-starch carbohydrates were not resolved for malted barley. Color bars indicate the fraction of the LLS fit coefficient as in Fig. 3.

Figure 5: A) Raman spectra of a native starch granule on a single spot (blue cross on the starch granule sketch). Spectra were recorded at 0° and 90° polarization of the laser. The higher anisotropic response is observed in the Raman band located at 865 cm^{-1} assigned to ν_s C-O-C/ring breathing. **B)** Mean Raman spectrum (blue line) obtained at thirteen different polarization angles of the laser from the blue region in the granule sketch (with high anisotropic behavior). Yellow rectangles: the height represents the average coefficient of variation (CV) of the Raman intensity (standard deviation/mean intensity) in the selected band ranges, indicated by the width. **C)** AFM image in topography mode of a sectioned starch granule with ellipsoidal shape found in native barley endosperm. **D)** Experimental and fitted anisotropic response of the band ratio $865\text{cm}^{-1}/1343\text{cm}^{-1}$ on the areas marked as [1], [2] and [3] in C.

Figure 6: A) Raman images of the band ratio $865\text{cm}^{-1}/1343\text{cm}^{-1}$ taken at 0° polarization angle of the laser on a region located on the center of the endosperm for native (top) and malted (bottom) of barley kernel. Higher ratio values on the images ratio are due to the anisotropic response of the band at 865 cm^{-1} , which is sensitive to the molecular orientation of order structures inside the starch granule. **B)** Raman Images for the same samples based on the BTEM-reconstructed spectrum F (Fig. 2).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6