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

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ABSTRACT

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



## 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 3–4 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 50–90 °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 (0.6–1 μm) without

*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.

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undergoing staining of prepared sections (Jääskeläinen et al., 2013). PRS is based on 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 the 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 technique 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 h respectively with a 16-hour air-rest between the steeps. 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 17 h and finally at 84 °C for 4 h.

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 25 × 25 μm$^2$ and the scan frequency 1 Hz. 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 100× (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 2–3 cm$^{-1}$. For mapping, the surface of the barley sections were scanned with steps of 1 μm, 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 directions 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 320–1800 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 and Vuorinen, 2014). The smoothing was done using a Whittaker smoother with 2nd order polynomial and smoothing parameter λ = 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 $H$:

$$H = -\omega_1 \sum_{\nu} h_\nu \ln h_\nu + \omega_2 \sum_{\nu} \frac{\mathrm{d}^n a_\nu}{\mathrm{d}\nu^n} + \omega_3 \sum_{\nu} a_\nu + P \qquad (1)$$

where $\nu$ are the wavenumbers at which measurements are taken, $a_\nu$ is the Raman intensity for one particular wavenumbers, $h_\nu$ is defined as:

$$h_\nu = \left|\frac{\mathrm{d}^m a_\nu}{\mathrm{d}\nu^m}\right| \Big/ \sum_{\nu} \left|\frac{\mathrm{d}^m a_\nu}{\mathrm{d}\nu^m}\right| \qquad (2)$$