Highlights

- A larger sample set (61) of starches than has been used in previous studies
- Full structural characterisation using a range of techniques
- A non-linear, hydration dependent response occurs in the infrared spectrum
- Complex changes in the infrared spectra of starch with increasing structural order
Infrared spectroscopy as a tool to characterise starch ordered structure - a joint FTIR-ATR, NMR, XRD and DSC study

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Title Running Head: Infrared spectroscopy to characterise starch ordered structure

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Abstract

Starch has a heterogeneous, semi-crystalline granular structure, and the degree of ordered structure can affect its behaviour in foods and bioplastics. A range of methodologies are employed to study starch structure; differential scanning calorimetry, $^{13}$C nuclear magnetic resonance, X-ray diffraction and Fourier transform infrared spectroscopy (FTIR). Despite the appeal of FTIR as a rapid, non-destructive methodology, there is currently no systematically defined quantitative relationship between FTIR spectral features and other starch structural measures. Here, we subject 61 starch samples to structural analysis, and systematically correlate FTIR spectra with other measures of starch structure. A hydration dependent peak position shift in the FTIR spectra of starch is observed, resulting from increased molecular order, but with complex, non-linear behaviour. We demonstrate that FTIR is a tool that can quantitatively probe short range interactions in starch structure. However, the assumptions of linear relationships between starch ordered structure and peak ratios are overly simplistic.

Graphical abstract
1. Introduction

Starch is one of the most important biopolymers in the world. It is the main source of energy, in the form of exogenous glucose supply, in the human diet, and is a key feedstock for a number of industries such as bioethanol production and biodegradable plastics manufacture (Butterworth, Warren & Ellis, 2011; John, Anisha, Nampoothiri & Pandey, 2011; Shen, Worrell & Patel, 2010). Starch granules are made up of two glucose polymers, amylose (an essentially linear polymer composed of $\alpha$-1$\rightarrow$4 linked anhydro-glucose residues, typically reported to have a molar mass $\sim 10^5$–$10^6$ g mol$^{-1}$) and amylopectin (a highly branched polymer consisting of short chains of $\alpha$-1$\rightarrow$4 linked anhydro-glucose residues, interspersed with 5-6% branch points of $\alpha$-1$\rightarrow$6 linkages, with a molecular weight perhaps as high as $10^7$–$10^9$ g mol$^{-1}$) (Gidley et al., 2010). These two polymers are arranged into a complex semi-crystalline granular arrangement, based on aggregates of double helices formed primarily from adjacent branches of amylopectin molecules.

The ratio of crystalline to amorphous material in the native starch granule varies greatly, from 10% to 50% crystallinity, depending on the botanical origin of the starch (Lopez-Rubio, Flanagan, Gilbert & Gidley, 2008). Further alterations in structure can occur as a result of subsequent processing of the starch. For example, the most common processing step is gelatinisation, where the starch is heated in excess water at a temperature between 50 and 70°C. The starch undergoes a cooperative transition whereby water enters the amorphous regions of granules, causing them to swell and destabilise the crystalline regions. This results in rapid swelling of the granule, melting of the crystallites and a complete loss of ordered structure (Bogracheva, Wang, Wang & Hedley, 2002; Cooke & Gidley, 1992; Perry & Donald, 2002; Waigh, Gidley, Komanshek & Donald, 2000). Other processing steps which
may increase or decrease starch molecular order include annealing (Tester & Debon, 2000), extrusion (Brümmer, Meuser, van Lengerich & Niemann, 2002) and enzymatic digestion (Lopez-Rubio, Flanagan, Shrestha, Gidley & Gilbert, 2008). For all processing applications involving starch, measuring the degree of ordered structure is important, but doing so is far from facile. As different analytical methods measure ordered structure in different ways, and may measure subtly different aspects of starch structure, it is informative to compare and contrast analytical methods. In the present paper, four of the most common analysis methods are used: x-ray diffraction (XRD), differential scanning calorimetry (DSC), \(^{13}\)C cross-polarization magic angle spinning nuclear magnetic resonance spectroscopy (\(^{13}\)C CP-MAS NMR) and Fourier transform infrared spectroscopy with attenuated total internal reflectance (FTIR-ATR).

Diffraction based methods, such as XRD, are the only methods available for the assessment and quantification of long-range crystalline order in starch. Crystalline order in starch is conventionally determined using a two-phase model, introduced by Sterling (Sterling, 1960) and Nara (Nara, Mori & Komiya, 1978), where an amorphous halo is subtracted from the ordered parts of the scattering pattern, and the ratio of the two phases is used as an estimate of long range crystalline order. A more nuanced peak fitting approach has recently been suggested (Lopez-Rubio, Flanagan, Gilbert & Gidley, 2008), taking into account incoherent scattering from crystalline regions, but due to its simplicity and robustness, the two-phase model is still commonly employed.

While XRD is the only method that can measure long-range crystalline order, there are a number of methods available to measure short-range double helical order in starch. DSC measures the enthalpy change associated with gelatinisation of starch, as gelatinisation involves the disruption of ordered regions in the granule, and therefore the magnitude of the enthalpy change is proportional to the amount of ordered structure. Early work assumed that
the enthalpy change was predominantly the result of crystallite melting, and was in essence a
direct measure of long-range crystalline order (Biliaderis, Page, Maurice & Juliano, 1986;  
Donovan, 1979; Liu, Lelievre & Ayoung-Chee, 1991). More recent studies, however, have
indicated a more complex picture, in which the contribution from disruption of crystalline
order is only one small component, with contributions to the overall enthalpy change also
coming from swelling, hydration and disruption of short-range double helical structure, and
that gelatinisation is a kinetic event that is dependent on both heating rate and water content
(Bogracheva, Meares & Hedley, 2006; Cooke & Gidley, 1992; Gidley, 1992). The enthalpy
change associated with starch gelatinisation, under standard conditions, can therefore be used
as an indicator of ordered structure, but any further inferences regarding long-range
crystallinity should be drawn with caution.

In the mid-1980’s Gidley and Bociek (Gidley & Bociek, 1985) noted that the $^{13}$C CP-MAS
NMR spectra of starch shows differences in chemical shift displacements due to changes in
the conformation of the glucan chain. These changes were particularly pronounced at the C1
and C4 positions, and were observed between amorphous starch polymers and double helices,
and between A and B type crystalline starch. This observation was subsequently exploited to
allow direct quantification of the proportion of double-helices in starch, giving a direct
measure of short-range order (Cooke & Gidley, 1992). This method has subsequently been
refined through use of peak fitting procedures and, more recently, chemometric approaches to
give a reliable estimate of ordered structure (Flanagan, Gidley & Warren, 2015; Tan,
Flanagan, Halley, Whittaker & Gidley, 2007). It has been consistently noted that the
proportion of double-helices in starch is greater than the amount of crystallinity, suggesting
that there are a significant proportion of double helices not in crystalline register (Cooke &
More recently, FTIR-ATR has been suggested as a method to measure short range order in
starch, and even as an “infrared crystalline phase index” (Capron, Robert, Colonna, Brogly &

FTIR-ATR has some notable advantages over other commonly used methods for measuring
starch structure- the equipment is relatively cheap, simple to use and maintain, and offers
highly flexible sample presentation. The ATR accessory gives the flexibility to analyse starch
samples in a range of physical forms, particularly in the hydrated state, making it possible to
observe spectral differences due to the level of hydration. Using microspectroscopy, these
advantages can also be applied to microscopically-localised regions within samples, in
contrast to the other methods which give bulk average data. The FTIR spectra of starch
typically shows bands at 2900-3000 cm\(^{-1}\) (C-H stretching), 1100-1150 cm\(^{-1}\) (C-O, C-C and C-
O-H stretching) and 1100-900 cm\(^{-1}\) (C-O-H bending). It is not possible to assign bands in the
spectra of starch unambiguously, as the absorbance bands are overlapping and poorly
resolved (Van Soest & Vliegenthart, 1997). The bands in the region 1100-900 cm\(^{-1}\) have been
shown to be sensitive to changes in starch structure, in particular bands at 1000, 1022 and
1047 cm\(^{-1}\) have been widely studied, using a range of samples including retrograded starches
(Wilson, Kalichevsky, Ring & Belton, 1987), mixtures of starch and amorphous maltodextrin
(Van Soest & Vliegenthart, 1997), enzyme hydrolysed starches (Sevenou, Hill, Farhat &
Mitchell, 2002) and acid hydrolysis residues (‘lintners’) (Capron, Robert, Colonna, Brogly &
Planchot, 2007). From these studies, the band at 1022 cm\(^{-1}\) seems to increase in more
amorphous samples, while the bands at 1000 and 1047 cm\(^{-1}\) become more defined in more
crystalline samples. This has led to the adoption of the band ratios at 1022:1000 cm\(^{-1}\) and
1047:1022 cm\(^{-1}\) as measures of short-range ordered molecular structure in a range of different
studies (Bello-Pérez, Ottenhof, Agama-Acevedo & Farhat, 2005; Brümmer, Meuser, van
Lengerich & Niemann, 2002; Iizuka & Aishima, 1999; Rubens, Snauwaert, Heremans &
Using chemometric approaches, this procedure has been taken further, demonstrating the hydration sensitivity of the 1022:1000 cm\(^{-1}\) intensity ratio and the possible relationship between crystallinity measured by XRD and FTIR band ratio (Capron, Robert, Colonna, Brogly & Planchot, 2007). Capron and co-workers (Capron, Robert, Colonna, Brogly & Planchot, 2007) found the absorbance at 1047 cm\(^{-1}\) to be essentially independent of the degree of structure in the starch. They linked the hydration sensitivity of the 1022:1000 cm\(^{-1}\) band ratio to the model liquid-crystalline polymeric model of starch structure proposed by Waigh and co-workers (Waigh, Kato, Donald, Gidley, Clarke & Riekel, 2000; Waigh, Perry, Riekel, Gidley & Donald, 1998). This led to the suggestion that the change in the 1022:1000 cm\(^{-1}\) band ratio upon hydration of crystalline (but not amorphous) starches is the result of a nematic-smectic transition. Warren and co-workers (Warren, Perston, Royall, Butterworth & Ellis, 2013) recently investigated the spectral changes that occur during in situ hydrothermal gelatinisation of starch. Their findings showed that changes in the spectra during gelatinisation can accurately determine the gelatinisation temperature reflecting the loss of ordered structure. The intensity ratio of the bands at 1022:1000 cm\(^{-1}\) proved a useful tool to monitor loss of structure, but a detailed appraisal of the spectra suggested greater complexity, with an apparent peak position shift during gelatinisation.

In the present study, we examine a wider range of starches than has previously been used in any single structural study of starch, including native wild type, mutant, amorphous, and extruded starches, both in the dry and hydrated states. Using this extensive sample set, we aim to develop a detailed understanding of the changes that occur in the FTIR spectra of starches with different levels of molecular structure using chemometric analysis tools in addition to peak ratio analysis, to improve the use of FTIR-ATR as a tool to understand
starch ordered structure. The starch samples are also analysed using $^{13}$C CP/MAS NMR, XRD and DSC to explore short-range and long-range molecular order in starch.

2. Materials and Methods

2.1. Materials

Commercially available starches: regular maize, gelose 80 (high amylose) maize and mazaca waxy maize were purchased from National Starch (Australia), waxy maize was bought from Penford (Australia) and potato starch was purchased from National Starch and Chemicals (UK). All commercially obtained starches were used without further treatment. All other starches were gifts from collaborators and were from a range of botanical sources, including native barley and barley mutants (Higgins et al., 2013; Regina et al., 2012) and wild rice and rice mutants (Butardo et al., 2011). Due to the limited amount of mutant rice samples, whole flours were used rather than isolated starches. Additional starch samples were produced under different drying conditions (Zhang et al., 2014), and using extrusion to add variation to the sample set (Zhang, Dhital, Flanagan, Luckman, Halley & Gidley, 2015). Wheat starch (Cerestar, cv. GL04) and pea starches (WT, r and lam (Wang, Bogracheva & Hedley, 1998)) were gifts from Prof. T. Bogracheva and Prof. C. Hedley (formerly of the John Innes Centre, Norwich, UK), waxy rice starch (cv. Remyrise) was a gift from Dr. P. Rayment (Unilever, UK); these starches are described in detail elsewhere (Warren, Royall, Gaisford, Butterworth & Ellis, 2011). Native starch powders from tapioca (Penford, Australia), regular maize (Penford, NZ), waxy maize (Tate and Lyle, Decatur, IL), high amylose maize (HylonVII, Penford, Australia), potato, and wheat (both commercial material, supermarket, Sydney, Australia) were gifts from Dr. E.P. Gilbert (ANSTO, Australia) (Doutch & Gilbert, 2013).
Amorphous starches were prepared following the method of Gidley and Bociek (Gidley & Bociek, 1985) with minor adjustments. Briefly, starches were boiled in water (1% w/v) for 30 minutes and precipitated with ethanol or rapidly frozen in liquid nitrogen and lyophilised.

Moisture contents were measured by weighing approximately 200mg of sample into a crucible, drying the sample for 18 hours in an oven at 105°C. The resultant weight of the sample was recorded and the difference in weight is taken as the moisture content.

The amylose contents of the samples were measured according to the iodine binding method of Knutson (Knutson 1986; Knutson and Grove 1994; Knutson 2000). In this analysis starch samples were dissolved overnight by shaking in a 6 mM iodine solution which was made by first dissolving iodine in 9 parts DMSO then adding 1 part water. Once the starch had dissolved, the solution was diluted in 8 parts H$_2$O. After 30 min the iodine-amylose complex formed a stable colour and the absorbance of the starch samples and amylose standards was read spectrophotometrically at 600 nm against the reagent blank.

2.2. Fourier transform infrared spectroscopy

All spectra were collected using a PerkinElmer Spectrum One FTIR spectrometer fitted with a PerkinElmer UATR single bounce ATR accessory with a diamond crystal. Data were collected by an attached computer running PerkinElmer Spectrum 6 software. Dry starches were equilibrated at laboratory humidity (50 % RH) and clamped directly onto the crystal for analysis. Hydrated starches were made into a slurry by mixing with deionised water (50% w/w), and added to the top of the crystal. Spectra of hydrated starches were obtained without applying pressure. 32 spectra were obtained and co-added for each sample at a resolution of 4 cm$^{-1}$. A background spectrum was obtained by collecting 32 co-added scans following cleaning of the crystal with a mixture of ethanol and water. The spectra were vector
normalized by dividing each spectrum by the standard deviation of its absorbance values
(with mean subtraction), prior to subsequent analysis (SNV normalisation).

2.3. Wide Angle X-Ray Diffractometry

X-ray diffraction measurements were performed with an X’Pert Pro X-ray diffractometer
(XRD) (PANalytical, Almelo, the Netherlands) operating at 40 kV and 40 mA with Cu Kα
radiation (λ) at 0.15405 nm. The scanning region was set from 2θ = 3° to 2θ = 40° with a step
interval of 0.02° and a scan rate of 0.5°/min. The crystalline peak area and amorphous area
were separated by PeakFit software (Version 4.12, Systat Software Inc., San Jose, CA, USA)
following the method of Lopez-Rubio, Flanagan, Shrestha, Gidley and Gilbert (2008)
Relative crystallinity was calculated as the ratio of the crystalline peak area to the total
diffraction area.

2.4. $^{13}$C CP/MAS Nuclear Magnetic Resonance Spectroscopy

All starches were analysed by $^{13}$C cross-polarized magic angle spinning (CP/MAS) nuclear
magnetic resonance (NMR) spectroscopy, using a Bruker MSL-300 spectrometer (Bruker,
Billerica, MA, USA) at a frequency of 75.46 MHz. Depending on availability, up to 200 mg
starch was packed in a 4-mm diameter, cylindrical, PSZ (partially stabilized zirconium oxide)
rotor with a Kel-F end cap. When as little as 50 mg of starch was available, the rotor was
packed with Teflon tape above and below the sample to maximize signal and ensure correct
spinning. The rotor was spun at 5 kHz at the magic angle (54.7°). The 90° pulse width of 5 μs
and a contact time of 1 ms were used for all starches with a recycle delay of 3 s. The spectral
width was 38 kHz, the acquisition time was 50 ms, 2 k points were recorded, and zero-filled
to 4 k before transformation. Exponential adopization resulting in 20 Hz of line broadening
was used. At least 1000 scans and up to 2000 scans were accumulated for each spectrum.
Spectral acquisition and interpretation methodology as described by Tan, Flanagan, Halley,
Whittaker and Gidley (2007) and Flanagan, Gidley and Warren (2015) were used to quantify the double helices, single helices, and amorphous conformational features.

### 2.5. Differential Scanning Calorimetry

All DSC data were obtained using a TA Instruments Q2000 instrument, using Tzero hermetically sealed aluminium pans. The sample chamber was purged with nitrogen gas at a rate of 40 mL/min. The instrument was calibrated for temperature using indium and tin standards. Samples were prepared in triplicate by accurately weighing approximately 5 mg of starch with 40 mg of deionised water into a pan, mixing, sealing and leaving overnight to equilibrate. Samples were heated from 10 to 95°C at a rate of 5°C/min. Subsequent thermograms were analysed using TA Instruments Universal Analysis software to obtain gelatinisation enthalpies, as described elsewhere (Bogracheva, Wang, Wang & Hedley, 2002).

### 2.6. Data analysis

Preparation of figures, statistical analyses and linear regression fitting was carried out using Sigma Plot 13 (Systat, USA). Principal component analysis (PCA) was carried out using Unscrambler X 10.3 (Camo, Norway).

### 3. Results

#### 3.1. Analytical data

DSC, XRD and $^{13}$C CP/MAS NMR data were obtained for a total of 61 starch samples. The mean DSC enthalpy of the sample set was 9.0 J/g with a range from 0 to 17.6 J/g. $^{13}$C CP/MAS NMR spectra were analysed to calculate total helical order for each of the samples. The mean helical order in the sample set was 30.0 % with range from 4 to 49 % total helical order. XRD data were analysed to obtain relative crystallinity, and the sample set had a mean
of 25.8 % with a range from 0 to 51 % relative crystallinity (see Supporting Information Table S1 for full data sets).

3.2. Fourier transform infrared spectra

Spectra were collected for the starch sample set used in the present study, and are shown in Figures 1 A and B for the dry and hydrated samples, respectively. The hydrated samples showed much more pronounced peaks at 3000-3700 cm\(^{-1}\), 2000-2250 cm\(^{-1}\) and 1500-1750 cm\(^{-1}\), as well as a pronounced baseline slope below 900 cm\(^{-1}\) as a result of adsorption by excess water. The dry samples showed a large number of adsorption bands in the region 1700-1200 cm\(^{-1}\) presumably arising from minor components (protein, lipid) in the starch, as well as the CH- stretch peaks at approximately 2900 cm\(^{-1}\), which were masked by the large water adsorption bands in the hydrated samples. The amide I and amide II peaks at 1640 and 1530 cm\(^{-1}\) are particularly prominent in the rice flour samples, which have a lower degree of purity, and reflect their slightly elevated protein content. The major adsorption bands arising from starch can be observed in the region 1200-1000 cm\(^{-1}\), arising from C-O, C-C and C-O-H stretching and C-O-H bending, as discussed in the introduction. A visual inspection of the spectra indicates that there is significant variation between the different spectra in the region 1200-1000 cm\(^{-1}\), and that the hydrated starches showed significantly more variation in this region than the dry samples. Both the hydrated and dry sample sets were subjected to PCA and the loadings for the first and second principal components (PC) are shown in Figure 2. For the hydrated starches, the loadings for the first and second PC’s (Figure 2A and B) show that the majority of the variance in the spectra occurs in the region 1000-1030 cm\(^{-1}\). The same can be observed for dry samples (Figure 2C and D), although the loadings for PC 2 (Figure 2D) also show a number of peaks in the region 1700-1200 cm\(^{-1}\). This confirms the findings of Capron et al. and Sevenou et al., (Capron, Robert, Colonna, Brogly & Planchot, 2007; Sevenou, Hill, Farhat & Mitchell, 2002) using an extended data set, that the 1000-1022 cm\(^{-1}\)
region is the main source of variation in the spectra of starch, and that there is significant
water sensitivity in the spectra.
Figure 2A
3.3. Relationship between peak height ratios and ordered structure from other analytical methods

As previously discussed, a number of authors have suggested that the peak ratios 995:1022 and 1045:1022 cm\(^{-1}\) may be useful indicators of ordered structure in starch. In the present study, these two peak ratios have been systematically investigated for correlation against data from DSC, XRD and \(^{13}\)C CP/MAS NMR, to investigate if they can be used as predictors of ordered molecular structure in starch (Table 1 and, Supplementary Information Table S1 and Figure S1). In the dry state, there are correlations between both peak ratios and all three analytical methods, although these are all weak correlations. The root mean square error of calibration (RMSEC) (Table 1 and 2) for all of these models are large, representing errors of greater than 25\% for the average sample in the calibration set.

### Table 1. Fitting parameters for correlations between selected FTIR peak ratios and measures of starch ordered structure for hydrated starches

<table>
<thead>
<tr>
<th>Analytical method</th>
<th>Ratio</th>
<th>(r^2) (Calibration)</th>
<th>RMSEC</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSC</td>
<td>995:1022</td>
<td>0.68</td>
<td>2.83 J/g</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NMR</td>
<td>995:1022</td>
<td>0.48</td>
<td>9.53 %</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>XRD</td>
<td>995:1022</td>
<td>0.48</td>
<td>8.61 %</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DSC</td>
<td>1045:1022</td>
<td>0.001</td>
<td>5.02 J/g</td>
<td>0.81</td>
</tr>
<tr>
<td>NMR</td>
<td>1045:1022</td>
<td>0.006</td>
<td>13.25 %</td>
<td>0.57</td>
</tr>
<tr>
<td>XRD</td>
<td>1045:1022</td>
<td>0.006</td>
<td>11.92 %</td>
<td>0.57</td>
</tr>
</tbody>
</table>

### Table 2. Fitting parameters for correlations between selected FTIR peak ratios and measures of starch ordered structure for dry starches

<table>
<thead>
<tr>
<th>Analytical method</th>
<th>Ratio</th>
<th>(r^2) (Calibration)</th>
<th>RMSEC</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSC</td>
<td>995:1022</td>
<td>0.42</td>
<td>3.64 J/g</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NMR</td>
<td>995:1022</td>
<td>0.31</td>
<td>11.34 %</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>XRD</td>
<td>995:1022</td>
<td>0.39</td>
<td>9.50 %</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DSC</td>
<td>1045:1022</td>
<td>0.44</td>
<td>3.17 J/g</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NMR</td>
<td>1045:1022</td>
<td>0.59</td>
<td>8.32 %</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>XRD</td>
<td>1045:1022</td>
<td>0.48</td>
<td>8.53 %</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
For the hydrated starch samples, the peak ratio 1045:1022 cm\(^{-1}\) showed no correlation with any of the analytical methods employed in this study. The 1022:995 cm\(^{-1}\) peak ratio showed a correlation with all three analytical methods, and the best correlation (\(r^2 0.69\)) for any of the peak ratios in either the hydrated or dry states was observed for the relationship between the hydrated starch 1022:995 cm\(^{-1}\) ratio and DSC enthalpy for the same starch samples. To further investigate the relationship between these peak ratios and measures of ordered structure, heat map plots were generated (Figure 3) in which the spectra have been ranked according to DSC enthalpy from high to low, and then plotted in series, with high absorbance values shown as “hot” colours (reds and yellows) and low absorbance values shown as “cold” colours (greens and blues).
The hydrated starch spectra (Figure 3a) show a pronounced peak position shift, with the most intense peak moving from around 1000 cm\(^{-1}\) in starches with a high order (above 14 J/g) to 1020 cm\(^{-1}\) in starches with low order (less than 10 J/g). The bi-plot from the PCA analysis of the hydrated starches (Figure 4) confirms this, showing that the high and low ordered starches divide into two groups. The dry starches show a similar, but much less pronounced, peak position shift.
4. Discussion

In the present study we have subjected a very wide range of starch samples to a range of commonly used analytical methods (XRD, NMR, DSC and FTIR-ATR). This was carried out with the aim of exploring the use of FTIR-ATR as a general method for the analysis of starch ordered structure, and to build calibrations allowing direct quantification of starch ordered structure.

The speed and flexibility in sample presentation of FTIR-ATR make it an attractive alternative to conventional analysis methods, and it has received wide use, but few authors have attempted to validate FTIR-ATR as a quantitative method (Capron, Robert, Colonna, Brogly & Planchot, 2007). The starch samples used in the present study cover the full range of levels of ordered structure that would be commonly encountered in the study of starch structure, from fully amorphous hydrothermally treated or extruded starches through to native tuber starches with levels of crystallinity measured by XRD to be as high as 51%. The samples come from a wide range of botanical origins, including genetic mutants, allowing a thorough assessment of the expected variation in FTIR-ATR spectra.

It is clear that large variability exists across FTIR-ATR spectra of different starches, and Figure 3 highlights this variation. A key observation is that in the hydrated state there is a pronounced peak position shift from around 1022 cm\(^{-1}\) to around 1000 cm\(^{-1}\) between the low and high ordered starches, and that this change is far less pronounced for starch in the dry state, where all but the most amorphous samples have a mean absorbance peak at around 1000 cm\(^{-1}\). Thus, for starch in the hydrated state, it is possible to use the 1000:1022 cm\(^{-1}\) peak ratio to indicate (but not to fully quantify) whether a starch has high or low levels of ordered structure. The peak position shift from 1000 to 1022 cm\(^{-1}\) is the most dramatic change in the spectra as a result of changes in ordered structure, far more so than those at 1045 and 1080
cm⁻¹, which have been suggested by some authors to be alternative indices of ordered
structure (Rubens, Snauwaert, Heremans & Stute, 1999). This is observation in good
agreement with previous work (Warren, Perston, Royall, Butterworth & Ellis, 2013)
demonstrating that when native (and therefore ordered) starch in excess water undergoes
hydrothermal treatment to an amorphous form there is a distinct peak position change from
1000 to 1022 cm⁻¹.

It is possible to speculate on the reasons for this peak shift occurring, and as to why it may be
more pronounced in hydrated samples as opposed to dry samples. It is well established that
hydration of the starch granule leads to significant structural rearrangements (Waigh, Gidley,
Komanshek & Donald, 2000; Waigh, Kato, Donald, Gidley, Clarke & Rickel, 2000) at longer
length scales (10-100 nm) than are directly probed by infrared spectroscopy. From the data in
the present paper, and from previous work (Capron, Robert, Colonna, Brogly & Planchot,
2007), these starch structural rearrangements have a significant impact at the scale of
individual chemical bonds probed by infrared spectroscopy. It is interesting to note that for
FTIR spectra collected from hydrated samples, a considerably better correlation was seen
between the infrared spectra and DSC enthalpy, than NMR and XRD data (Table 1 and 3).
The DSC enthalpy change associated with starch gelatinisation is dependent, in a complex
manner, on a range of short range molecular interactions which are altered upon heating in a
hydrated environment (Cooke & Gidley, 1992). It may be speculated that the FTIR spectra of
hydrated samples reflects these short range interactions, rather than the longer range
associations which result in the formation of helices (detected by NMR), and the arrangement
of these helices into crystalline arrays (detected by XRD), although clearly structures
detected at all these length scales contribute to the overall structure of the starch granule.

5. Conclusions
The present study uses a wider range of starch samples than has been available in previous studies to compare different measures of starch ordered structure, with the aim of probing the relationship between FTIR spectra of starch in the dry and hydrated states, and other measures of starch ordered structure. It has been confirmed that in the spectra of hydrated starch, the main difference between starches with high and low degrees of order is a peak position shift from 1000 to 1022 cm\(^{-1}\), but using the wide range of starches available in this study it has been shown that there is a more complex relationship between infrared spectra of starches with different degrees of order than has previously been appreciated. There is a clear and distinct shift in the spectra, in particular of hydrated starches, as starch molecular order increases, however this is a non-linear transition. While this complexity means that FTIR spectra cannot be used directly in place of alternative quantitative measures of starch structure, we demonstrate the feasibility of using FTIR to characterise short range molecular interactions in starch, so long as care is taken in the analysis of the spectra.

6. Acknowledgements

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gelatinization using differential scanning calorimetry, X-ray, and birefringence measurements.

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**Figure legends**

**Figure 1.** Overlay SNV normalised FTIR-ATR spectra for all the starch samples analysed in dry (A) and hydrated (B) states. The spectra have been zoomed to show the fingerprint region (full spectra inset).

**Figure 2.** Principal component loadings for the PCA analysis of complete sample sets for dry and hydrated starch FTIR-ATR spectra. A and B hydrated starch, PC1 and PC2 respectively; C and D dry starch, PC1 and PC2 respectively.

**Figure 3.** Heat map plots of the SNV normalised FTIR-ATR spectra for the starch samples used in this study. The spectra have been plotted relative to DSC enthalpy (y-axis) and absorbance intensity is shown in colours, with “hot” colours (reds and yellows) indicating high absorbance and “cool” colours (blues and greens) indicating low absorbance. A; hydrated starch samples, B; dry starch samples.

**Figure 4.** Bi-plot showing the first two PC’s for a PLS model of the hydrated FTIR-ATR spectra. Samples with a DSC enthalpy below 10 J/g have been marked with circles.