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Research Paper

Detection of Low Levels of Amorphous Lactose using H/D Exchange and FT-Raman Spectroscopy

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Purpose. To demonstrate the potential of monitoring H/D exchange by FT-Raman spectroscopy as a tool for the detection and quantification of low levels of amorphous lactose in formulations.
Methods. Samples containing different proportions of amorphous and crystalline lactose were prepared. H/D exchange was carried out by exposing the samples to a flow of D_2O vapour. A calibration curve was constructed from the FT-Raman spectra of the deuterated samples by integrating the ν(OD) band and normalizing to an internal standard. This method was benchmarked against a conventional approach using Raman spectroscopy where the ratio of Raman bands associated with crystalline and amorphous lactose is used to estimate the amorphous content.
Results. The H/D exchange method revealed a linear response over the entire composite range with an excellent correlation coefficient (R^2=0.999). The sensitivity of this approach in detecting the amount of amorphous lactose present in a blend is significantly greater than that offered by conventional FT-Raman in the 0–10% level of amorphous material.
Conclusions. A non-destructive method that is capable of providing reproducible measurements of low levels of amorphous material in lactose has been demonstrated and this method has enhanced sensitivity relative to approaches using Raman spectroscopy without deuteration.

KEY WORDS: amorphous; deuteration; lactose; quantification; Raman.

INTRODUCTION

In drug development there is significant emphasis placed on the importance of achieving reproducibility in terms of the biological, chemical and physical characteristics of the product (1). However, in practice this can be difficult since processing steps such as size reduction (2–4), spray drying (5), freeze drying (6), granulation (7) and compression (3,8) can induce phase change during the production of pharmaceutical materials. The presence of the amorphous phase can have a profound effect on the physical and chemical properties of both drug and excipient samples. Even very low levels (a few %) of amorphous material can have a dramatic effect on both the physical and chemical properties of pharmaceuticals and techniques capable of reliably quantifying such amorphous content to low levels are not routinely available (9–11). The characterisation methods commonly used for determining amorphous content in pharmaceutical materials include powder X-ray diffraction (PXRD) (5), calorimetric methods such as differential scanning calorimetry (DSC) (12–14), isothermal microcalorimetry (IMC) (15,16) and solution calorimetry (17), and spectroscopic methods such as solid-state NMR (18,19), near-infrared (12) and Raman (20,21). In general, the limit of detection and limit of quantification of the PXRD and DSC are >5%, and the determination of experimental parameters for calorimetric methods can also be time consuming (9). Solid state ^{13}C NMR, whilst promising, has difficulties associated with differences in relaxation time between carbon in different environments and this can result in the analysis being problematic (18,19).

The development of new techniques to probe the level of amorphous material is beneficial to the pharmaceutical industry. In this paper we describe a modified spectroscopic method, using deuterium substitution of exchangeable protons, to enhance the detection level of Raman spectroscopy to the amorphous phase in the model compound, α-lactose monohydrate. The application of deuterium exchange allows selection between exchangeable and non-exchangeable protons (i.e. amorphous and crystalline phases) and the exchange process can be monitored using Raman spectroscopy. Once exchanged, the ν(OH) band (3,500–3,100 cm^{-1}) is replaced in the Raman spectrum by a ν(OD) band (2,700–2,300 cm^{-1}) as the deuterium signal is shifted by approximately $1/\sqrt{2}$ to lower frequency. Furthermore, this spectral region is largely free from any interfering peaks.

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Of particular relevance to this paper are the investigations using deuterium exchange to probe water diffusion in sugars (22) and water dynamics in channel hydrates (23). To the best of our knowledge there have been no other examples where this approach has been applied to pharmaceutically relevant compounds. The quantification of the amorphous phase using isotopic substitution has been confined to polymers (24,25). In this paper we report a method to probe low levels of the amorphous phase using FT-Raman spectroscopy to monitor H/D exchange. α-lactose monohydrate is an ideal model compound since it contains eight hydroxyl functional groups and is an important pharmaceutical excipient used as a bulking agent in tableting and as a carrier in dry powder inhalation formulations. In these applications the presence of the amorphous phase, even at low levels, can have a profound impact on the performance of the drug product (11).

METHODS AND MATERIALS

Materials

Crystalline α-lactose monohydrate (Respitose™, SV003) was obtained from (DMV International, Veghel, The Netherlands) and was used as received. Amorphous lactose was generated by spray drying a 10% w/v solution using a Buchi mini spray drier 190, inlet temperature was 166°C; outlet temperature was 97°C with a flow rate of 13 ml/min. This material was stored under a phosphorous pentoxide desiccant prior to deuteration and analysis. For calibration, sample blends with 0%, 2%, 4%, 6%, 8%, 10%, 25%, 50%, 75% and 100% (*w*/*w*) amorphous content were prepared by combining 100% amorphous and 100% crystalline components, which were then mixed using a vortex mixer. The particle size of both crystalline and amorphous components was determined using a scanning electron microscope (LEO 1430 VP). The calibration was validated using blends at 3%, 5% and 7% (*w*/*w*) amorphous content. Further testing was also performed in which a 5% drug loaded model formulation was prepared containing 5% amorphous lactose, 5% carbamazepine III (Sigma) and 90% crystalline α monohydrate lactose.

Dynamic H/D Exchange Process

Deuterium oxide vapour (25% relative humidity) was generated and controlled using a Triton Humidity Generator (Triton Technology, UK). The vapour was flowed at a rate of approximately 200 ml/min over the samples, which were prepared in 2 ml clear glass vials, and inert, oxygen-free nitrogen was used as the carrier gas. Having been exposed to the deuterium oxide vapour (for up to 16 hours) the system was "flushed" using dry nitrogen gas for a further two hours in order to remove residual D_2O from the samples.

FT-Raman Spectroscopy

Spectra were recorded using a Bruker RFS 100/S FT-Raman spectrometer. Analysis was carried out at room temperature with the Nd:YAG laser set at 1,064 nm and 450 mW, with a spot size of 150 μm in diameter. For each sample a total of ten FT-Raman spectra were collected both before and after exposure to D_2O vapour (8 cm^{-1} resolution, 5 min/spectrum), the samples were mixed between each measurement and these spectra were then averaged to minimize the effects of possible inhomogeneity. This process was then repeated for at least three independently prepared samples at each amorphous composition. The Raman spectra were not corrected.

RESULTS

FT-Raman spectra representing samples of amorphous lactose and crystalline α-lactose monohydrate are shown in Fig. 1. It is evident from these spectra that increasing amorphicity results in peak broadening. Furthermore, it is clear from Fig. 1a that the ν(OH) stretch has significantly more structure in the crystalline material relative to the broad and featureless band in the amorphous material. This observation has been discussed in detail with respect to cellulose by Maréchal and Chanzy (26).

Raman spectroscopy has been used previously to estimate the amount of amorphous lactose content by using the ratio between bands assigned to amorphous and crystalline lactose (21,27,28). We have used this approach to benchmark our new methodology in which a combination of deuteration and Raman spectroscopy is used to determine the amount of amorphous material present in particular blends. This conventional approach analyses the Raman spectra recorded of sample blends without deuteration using two bands, centred at 440 and 470 cm^{-1} (Fig. 1b), representing the amorphous and crystalline phases respectively. The area under these bands was integrated between 490–420 and 490–450 cm^{-1} and the ratio of these regions were determined. The result for each sample was plotted as a function of amorphous content, Fig. 2a. These data have been fit using a second order polynomial with a reasonable correlation coefficient (R^2= 0.996). However, closer scrutiny of the low amorphous content region (<10%; Fig. 2b) shows that the correlation is poor below the 10% limit, Fig. 2b.

The Raman spectra of amorphous lactose and crystalline α-lactose monohydrate recorded before and after exposure to a flow of deuterium oxide vapour are shown in Fig. 3. It is clear that there is complete loss of intensity of the ν(OH)

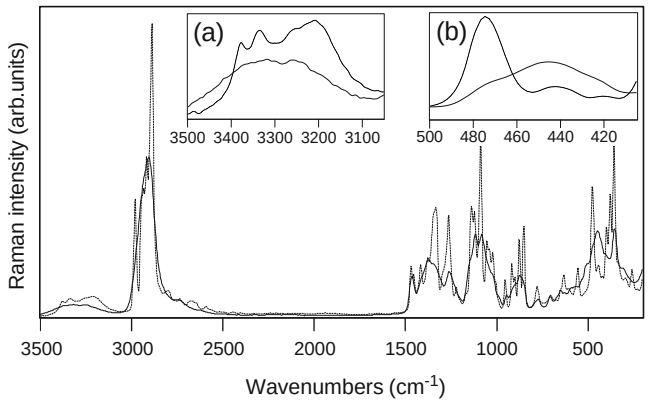

Fig. 1. FT-Raman spectra of amorphous (*solid*) and crystalline (*dashed*) lactose. *Inset* **a** highlights the differences in the ν(OH) band in the crystalline and amorphous components (see text). *Inset* **b** shows bands typically used in Raman analysis of amorphous lactose.

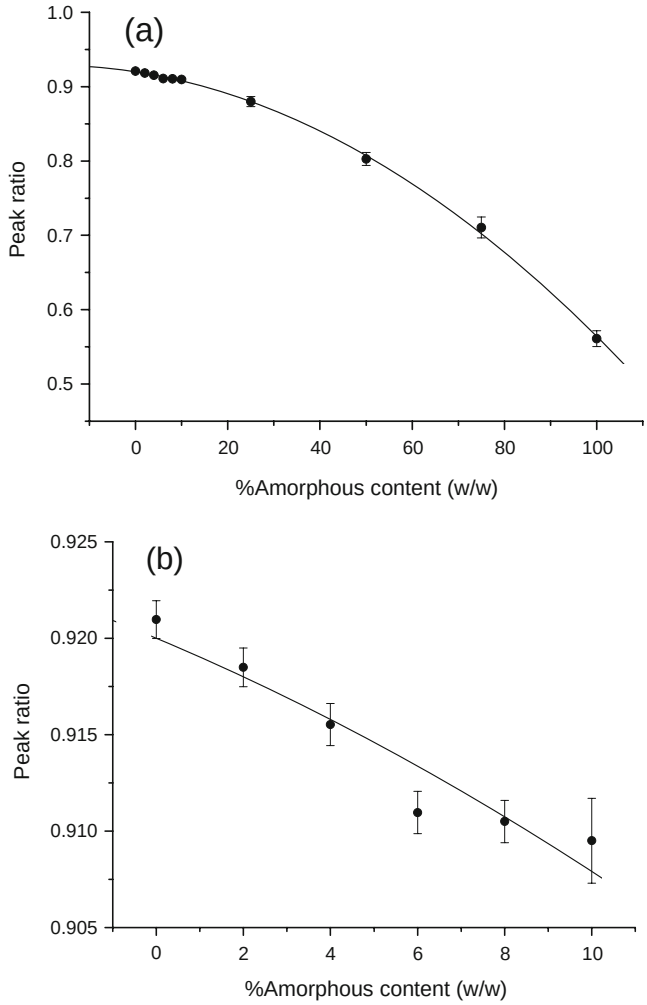

Fig. 2. Calibration plots constructed following analysis of undeuterated blends using a ratio of the peaks highlighted in Fig. 1; **a** 0–100% amorphous content range and **b** 0–10% amorphous content range. The *error bars* represent ± 1SD from the mean, based on a minimum of three measurements.

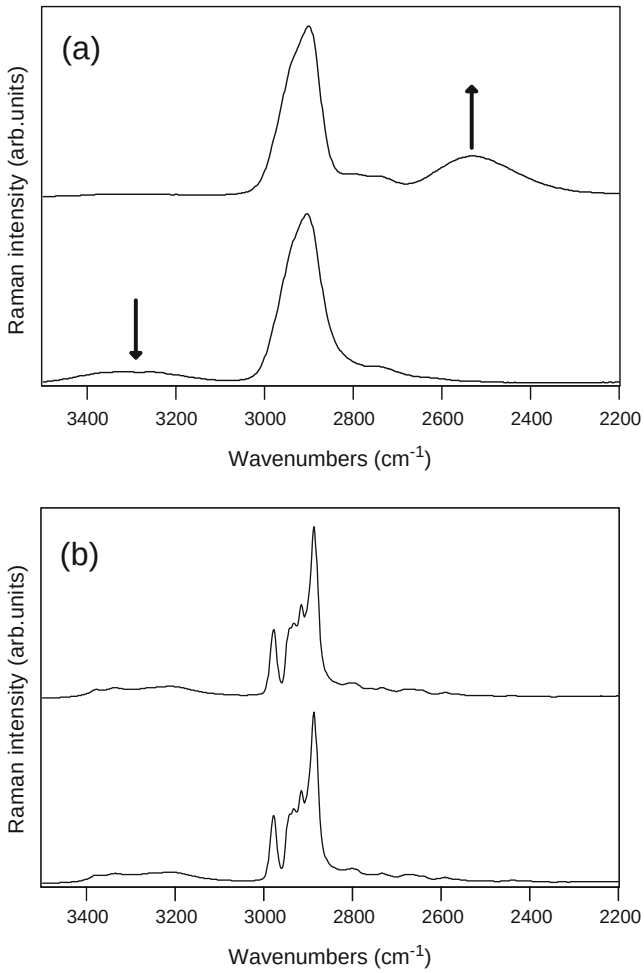

Fig. 3. Averaged FT-Raman spectra of **a** amorphous lactose and **b** crystalline lactose; before (*lower spectrum*) and after (*upper spectrum*) exposure to deuterium oxide vapour. The *arrows* illustrate the loss of intensity in the ν(OH) band and the corresponding intensity gain in the intensity of the ν(OD) band.

band in the region between 3,500 and 3,100 cm^{-1}, and the simultaneous appearance of a ν(OD) band in the region 2,650–2,350 cm^{-1}, Fig. 3a. This indicates that the amorphous lactose sample undergoes complete deuteration of the hydroxyl group hydrogens. In contrast, no such spectral changes were observed following exposure of crystalline lactose to D_2O, Fig. 3b.

Figure 4 shows the Raman spectra following deuteration of four different samples, each containing different quantities of amorphous material. It can be seen from the Figure that the intensity changes in the ν(OH) and ν(OD) band vary with the amount of amorphous material present in each blend. The ν(OD) profile is also consistent with the broad, featureless OH stretch, free from any of the pronounced ν(OH) peaks attributable to intermolecular and intramolecular hydrogen bonding, Fig. 1a (26).

It is important to standardize the Raman spectra in order to allow quantification of the results of the H/D exchange experiments. The results were initially analysed by integrating the regions 2,650–2,350 cm^{-1} and 3,050–2,800 cm^{-1} in the Raman spectra before deuteration. The C–H functional groups are not susceptible to exchange and therefore the ν(CH) stretch can be used as an internal standard to normalize the data (22). Taking a ratio of these two integration values provides a normalized background for the

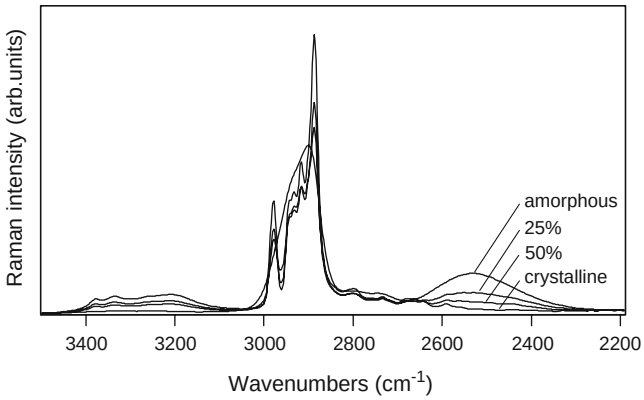

Fig. 4. Averaged FT-Raman spectra of different lactose blends after exposure to deuterium oxide vapour. The spectra show the effect of deuteration on samples with different amorphous contents.

O–D stretching region. The same regions of the Raman spectra were then integrated in the corresponding spectra following deuteration, and the ratios were used to provide normalized values for the ν(OD) band intensity and quantification of H/D exchange. The change in the intensity of the ν(OD) band was calculated by subtracting the background value, and the resulting data were plotted as a function of amorphous content. Figure 5a shows that there is a linear relationship between the amount of amorphous material present and ν(OD) band intensity. These data can be fit using a linear model giving a correlation coefficient of R^2=0.999. Furthermore, this excellent correlation holds even at low levels of amorphicity (0–10%), Fig. 5b.

For method validation samples were prepared in with 3%, 5% and 7% amorphous content (*w*/*w*) under the same conditions as the calibration set (i.e. using the same batches of amorphous and crystalline lactose and applying the same method of mixing). This process was then repeated twice providing data in triplicate from sample blends prepared on three separate days. An average was taken for each

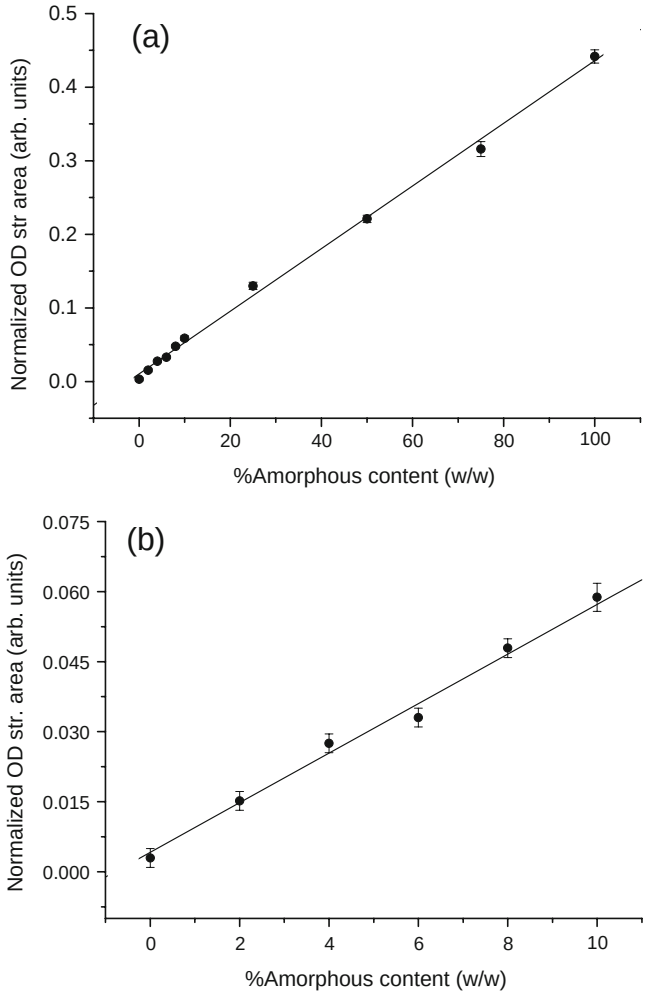

Fig. 5. Calibration plots constructed following analysis of deuterated blends using the integrated area of the ν (OD) vibration; **a** 0–100% amorphous content range and **b** 0–10% amorphous content range. The *error bars* displayed represent±1SD from the mean, based on analysis of data from a minimum of three independently prepared samples.

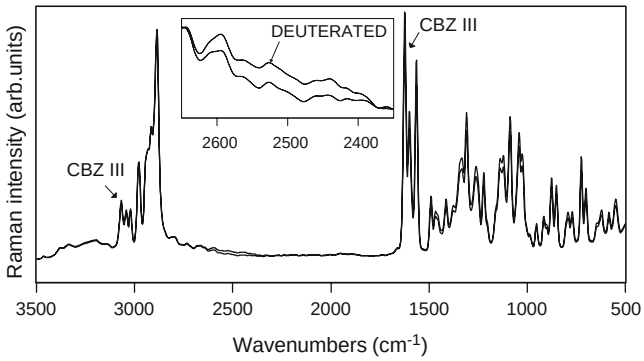

Fig. 6. Averaged FT-Raman spectra of a three component sample blend of CBZ III (5%), amorphous lactose (5%) and α-lactose monohydrate (90%) acquired before and after exposure to deuterium oxide vapour. The *inset* shows an expansion of the ν(OD) band between 2,650 and 2,350 cm^{-1}.

composition and, using the calibration, values of 2.6%, 5.4% and 6.3% respectively were calculated for the amorphous content, which provide a value of 0.6% for the root mean square error of prediction (RMSEP).

Limits of detection (LOD) and quantification (LOQ) have been estimated in accordance with ICH guidelines Q2 (R1).

$$LOD = \frac{3.3\sigma}{S} \quad (1)$$

$$LOQ = \frac{10\sigma}{S} \quad (2)$$

Where σ is the standard deviation and S is the slope of the calibration curve. Using these equations 1.7% and 5.1% are the values calculated for the LOD and LOQ respectively.

Inspection of the averaged FT-Raman spectra acquired before and after deuteration of samples containing 2% amorphous lactose reveals that, for these samples and measurement conditions, the signal is three times the noise. This result supports the statistical estimation we quote for the LOD and, by association, the LOQ. These limits could be improved by refining the experimental protocol, by increasing the spectral acquisition time. For example, by increasing the scan time from 1 hour per sample to 24 hours the signal/noise is reduced approximately sixfold, which would reduce the LOD and LOQ to 0.3 and 1% respectively. In addition, sensitivity to the spectral changes observed upon deuteration could be enhanced by using an excitation source at shorter wavelength.

The application of the deuteration approach was also tested in the presence of a hydrophobic drug. A sample was prepared to be representative of a real formulation containing (by weight) 5% cabamazepine, 5% amorphous lactose and 90% crystalline α-lactose monohydrate. Averaged FT-Raman spectra acquired before and after deuteration are shown in Fig. 6, and the inset clearly demonstrates the appearance of a ν(OD) band in the region 2,650–2,350 cm^{-1} following exposure to deuterium oxide vapour. Some vibrational bands representative of carbamazepine have been highlighted to illustrate that the presence of this

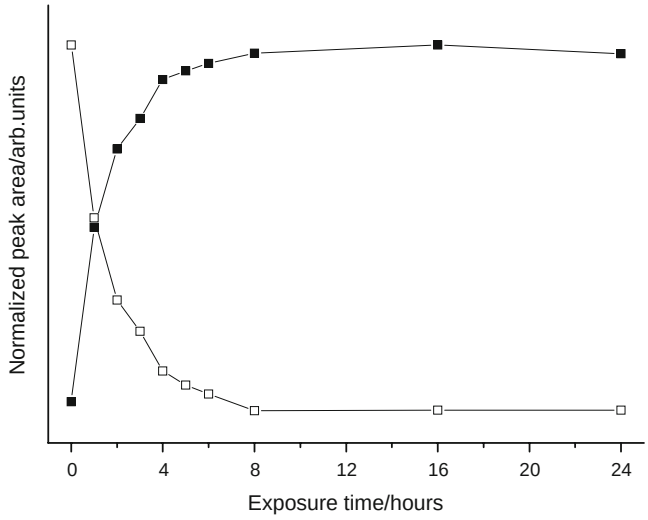

Fig. 7. Normalized peak areas for the ν(OH) (*solid*) and ν(OD) (*open*) bands plotted as a function of exposure time to deuterium oxide vapour.

material does not show interference with quantitative analysis. This will be the case in general, since there are there are few functional groups that are strong Raman scatterers in this spectral region. Using the calibration plot an estimated value of 6% was determined for the amorphous content of the sample.

DISCUSSION

The techniques currently used for detecting and quantifying the amorphous phase have been comprehensively reviewed in the literature (9,10,28). These methods measure various properties of the crystalline or amorphous states. For example, in PXRD the disorder in the crystalline lattice is measured to provide a lower detection limit of 5–10%, whereas IMC depends upon the higher energy state associated with the amorphous phase and limits of detection as low as 1% have been quoted (9).

Previous studies have demonstrated that Raman spectroscopy is also capable of detecting amorphous material at low levels (20,21,27), and likewise near-infrared (12,29,30). Raman spectroscopy has the advantage of being a non-destructive technique requiring no sample preparation. Consequently, partially amorphous systems can be studied without any change of phase occurring either through processing or measurement of the sample. In recently reported Raman spectroscopic studies, the amorphous content of a sample is determined by taking a ratio of two regions without deuteration (21,27,28). The first encompasses a relatively intense feature attributable to the crystalline component and the second comprises both the crystalline component and a broad hump representing the amorphous phase. This method intrinsically leads to a non-linear relationship between Raman intensity and amorphous content. The determination of amorphous content requires the accurate measurement of a broad, low intensity feature in a predominantly crystalline region of the spectrum and as a result this approach is not as sensitive to amorphous material at low levels.

In this work we have demonstrated that the sensitivity of Raman spectroscopy in detecting the amorphous fraction can be enhanced by exploiting the fact that hydroxyl group hydrogens are exchangeable in the amorphous phase but not in the crystalline. As a result of the change in the reduced mass brought about by deuteration of the amorphous hydroxyl functional groups, intensity is shifted from the OH stretch to lower frequency by a factor of approximately $1/\sqrt{2}$. In the Raman spectra this represents a significant shift from 3,500–3,100 to 2,700–2,300 cm^{-1}, resulting in the complete deconvolution of the amorphous and crystalline components. In addition, because the ν(OD) band does not suffer interference from other vibrational bands, analysis of the data is greatly simplified. A further advantage of this method is that, unlike conventional Raman methods, the data reveal a linear correlation over the full amorphous content range (0–100%) making quantification straightforward.

To achieve accurate quantification it is essential that the deuteration of amorphous material is complete. Figure 7 reveals that no further deuteration occurs in a sample of amorphous lactose after about 16 h exposure time. The OH stretch was shown to have reached baseline in the FT-Raman spectrum acquired of amorphous lactose after deuteration (Fig. 3a) by integrating the region between 3,500 and 3,100 cm^{-1}. It is worth noting that during the study it was observed that rates of deuteration appeared to be dependent upon the amount of amorphous material present in the blend, with blends of <10% amorphous material requiring less than four hours for complete deuteration to occur.

The major source of error within the data is attributed to difficulties associated with mixing the amorphous and crystalline components. The importance of homogeneous mixing has been discussed in an earlier study (20) where it was concluded that recording spectra from different regions of the sample and taking an average would reduce the variance in the data and lead to a lower limit of detection. Consequently, in this work error caused by inhomogeneous mixing has been minimized by acquiring single spectra from ten different regions within each sample blend, the average of which has then been calculated and used in subsequent analysis.

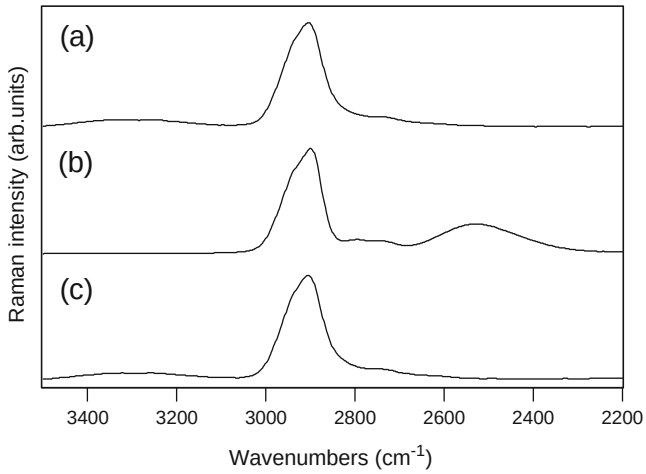

Fig. 8. FT-Raman spectra of amorphous lactose acquired **a** before deuteration, **b** after deuteration and **c** after re-protonation.

The application of Raman spectroscopy to the quantitative analysis of solids can be affected by the particle size of the analyte. First, Raman scattering intensities are known to be dependent upon particle size with a disparity of the order of 100 μm between two components having been reported as leading to observable differences (31). For this reason, a fine grade of crystalline lactose was used in order to minimize the discrepancy in particle size between the amorphous (<10 μm) and crystalline (approximately 40 μm) fractions. Secondly, if the spot size of the Raman laser is of the same order of magnitude as the particles in the sample blend then the measurement may not be representative of the bulk sample and spectra recorded from different areas of a sample blend can demonstrate significant variance. The spectrometer used in this study is equipped with a laser that has a 150 μm diameter beam and provides an analysis volume of about 0.02 mm^3. Within this volume the disparity between the sizes of the amorphous and crystalline fractions can be considered to be insignificant. It is worth noting that, since the batches of material and the sample preparation have remained constant throughout both calibration and validation, this method has not been tested against variations in particle size or method preparation. It is our aim to address this by refining our validation method to consider amorphous lactose prepared by alternative routes e.g. freeze drying or quench cooling of the melt, and to test the effect of particle size issues by introducing different grades of crystalline material. In addition, the method could be further tested by replacing the α monohydrate lactose component with the anomeric β anhydrous form.

A key concern in our experiments was that exposing samples to D_2O may result in recrystallization of the amorphous component, which would affect the accuracy of the calibration. It has been reported that a morphological change attributable to a moisture induced glass transition occurs around 30% relative humidity and 25°C (32,33), and that below this relative humidity no morphological changes were observed (34). Furthermore, these conditions have been defined as a threshold for molecular mobility, therefore by working below this threshold effective H/D exchange was achieved without inducing any phase transition.

The integrity of the amorphous phase following deuteration was further demonstrated by re-protonating a deuterated amorphous sample. This reverse exchange took place to completeness, Fig. 8, thus demonstrating that there was no hysteresis. In addition, it is worth noting that the laser may result in heating of the sample, which in turn may induce recrystallization. Using the intensity ratio of Stokes and anti-Stokes peaks in an FT-Raman spectrum of α-lactose monohydrate an approximate value of 320 K has been calculated for the temperature of the sample under measurement. This is well below the T_g of anhydrous amorphous lactose and we can conclude that the measurement process does not induce crystallization.

In this work the potential for using H/D exchange to probe a material with exchangeable hydroxyl hydrogens has been demonstrated. It is conceivable that this approach may also be of use when applied to similar functional groups such as −NH and −SH, broadening the range of materials suitable for study.

CONCLUSION

A non-destructive method has been developed for the detection and quantification of amorphous material in binary mixtures of amorphous and crystalline lactose. Using this method, a linear correlation curve has been constructed over the full composite range. It has been demonstrated that H/D exchange can be employed to enhance the sensitivity of FT-Raman spectroscopy in the analysis of the amorphous phase in lactose, and that values of 1.7% and 5.1% for the LOD and LOQ are achievable. In addition, we have demonstrated that deuterium exchange can be applied even to samples which contain a hydrophobic compound.

Significant experimental challenges have been highlighted, notably the risk of recrystallization upon exposure to D_2O and heating caused by the Raman laser. In addition, relevant issues raised in previously reported work have been discussed, in particular the effect of particle size on Raman signal intensity and the importance of homogeneous mixing. Finally, the potential for applying this method more broadly, to study compounds containing functional groups such as −NH and −SH has been suggested.

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