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Quantification of amorphous content in mixed systems: amorphous trehalose with lactose

Dima Al-Hadithi, Graham Buckton*, Stephen Brocchini

Department of Pharmaceutics, School of Pharmacy, University of London, 29-39 Brunswick Square, London WC1N 1AX, UK

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Abstract

There has been a lot of interest in quantification of the amorphous content of materials, especially when the amorphous content is a small percentage of the total mass. Whilst there has been success in studies on single materials, there has been little work showing how quantification of the amorphous content of one material can be undertaken in the presence of another. In this work isothermal microcalorimetry was used to measure the content of amorphous trehalose following mixing with crystalline lactose. Gravimetric water sorption studies revealed that trehalose did not form a complete dihydrate when exposed to 75% RH, presumably due to the rapid crystallisation of the outer regions of the particles. At 53% RH, the gravimetric studies showed dihydrate formation. The calorimetry data revealed that the crystallisation response was directly related to the mass of amorphous material in the mixture and was not affected by the mass of non-crystallising sample. It was shown that as long as there was a minimum mass of amorphous material (in this case 4 mg), it was possible to measure a crystallisation response with sufficient accuracy to allow quantification. Lower masses of amorphous content allowed detection, but less accurate quantification, as the response was superimposed on the initial calorimetric heat flow response. It was also found that the response at 53% RH in the TAM was less accurate due to the low peak height and long duration (compared to that seen at 75% RH). It can be concluded that the TAM method is well suited to both detection and quantification of amorphous content when there is one amorphous sample mixed with another (and thus presumably more than one) non-crystallising material.
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1. Introduction

The desire to measure and quantify the physical form present in materials stems from the fact that changes in physical form can have major effects on the properties of formulations, in terms of the way the formulation behaves during processing and in subsequent use. The amorphous form is known to have different physico-chemical properties from crystalline forms. On occasions it may be desirable to have an amorphous material, for example when trying to stabilise formulations of proteins in the solid state. Trehalose is a sugar that is used to stabilise protein formulations. On other occasions, it may be desirable to remove all traces of amorphous content from a product, for example, to ensure that the product behaves in a consistent way during its shelf life and that no changes in performance occur as a consequence of a physical form transition.

Recently, there has been a lot of interest in quantification of amorphous material in samples. Whilst it is reasonably easy to detect crystal forms, to detect and quantify the presence of amorphous materials is considerably more difficult. Techniques that have been used to study samples of single pure powders include water sorption [1,2], isothermal microcalorimetry [3,4] Raman spectroscopy [5], solid state NMR [6], near infra-red spectroscopy [7,8] and solution calorimetry [9–11]. The ability to quantify amorphous content, even when it exists as a low percentage of the total material mass, is therefore now established. The next challenge is to develop methods that allow quantification in mixtures of powders and eventually complete formulations. It is clear that the methods used for single powders will be much harder to apply to mixtures. For example, solution calorimetry, which is possibly one of the best methods for studying single powders, is difficult to use for mixtures as

* Corresponding author. Tel.: +44-207-753-5858; fax: +44-207-753-5858.
E-mail address: graham.buckton@ulsop.ac.uk (G. Buckton).

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the dissolution of multiple components results in complex data. Equally, spectroscopic methods, which are very useful for pure powders, may prove harder to use for mixtures due to interfering peaks.

Isothermal microcalorimetry was one of the first methods to be explored as a tool for studying the amorphous content of single powders [4] although it was subsequently found to be subject to greater variability than techniques such as solution calorimetry [11,12]. However, mixtures of materials have been investigated using calorimetric methods: for example, Buckton and Darcy [13] studied the crystallisation of amorphous lactose in the presence of powders such as microcrystalline cellulose and magnesium stearate. It is obvious from previous work that additional components will affect the crystallisation response for an amorphous sample, but also that the crystallisation should be measurable. The aim of this study is therefore to see if it is possible to obtain a calibration curve for amorphous content in mixed powder systems and if so to see what detection sensitivity may be possible.

2. Materials and methods

Trehalose was obtained from Sigma. Amorphous trehalose was produced by spray drying in a mini spray drier (Buchi model 191) from a 10% (w/v) aqueous solution of trehalose dihydrate. The temperatures used were 140–150 and 70–80 °C for the inlet and outlet, respectively, with an atomiser airflow rate of 500–600 norml/h. The spray dried sample was tested using X-ray powder diffraction (Philips PW 3710 X-ray Powder Diffractometer, UK) and found to be amorphous. All samples were stored in desiccators containing self-indicating silica gel at 25 °C.

Isothermal microcalorimetry (Thermal Activity Monitor, Thermometric, Sweden) was carried out using 3 ml glass ampoules, each containing the powder sample and a tube of saturated salt solution. The instrument was calibrated electrically to 3000 μW. Previous studies [4,12] have shown that the crystallisation of amorphous lactose was affected by the surface area of the salt solution, the RH and the temperature. In this study, the salt solution surface was held constant at 12.56 mm^2, the temperature was 25 °C and the RH was either 75% (saturated sodium chloride solution) or 53% RH (saturated magnesium nitrate solution). All experiments in the region 0–20% (w/w) amorphous content were $n = 3$, those in the range 20–100% (w/w) (which is a region of less interest as changes in this range are readily detected by many methods) were $n = 1$.

The crystallisation of amorphous trehalose was also studied gravimetrically (Dynamic Vapour Sorption, Surface Measurement Systems Ltd). The instrument was calibrated for mass and RH measurements. Samples of 30–70 mg were loaded onto the instrument and held at 0% RH for 6–10 h to dry the sample. The RH was then changed to either 75 or 53% for 10 and 48 h, respectively, followed by a further 6–10 h at 0% RH. All experiments were at 25 °C.

Thermogravimetric analysis was carried out using a TGA 2950 (TA Instruments, UK), with 10 mg samples and a heating rate of 10 °C/min up to 250 °C. Mass and temperature calibrations were carried out on the instrument before use.

Mixtures were prepared by mixing (Turbula Mixer, 15 min, 42 rpm) known quantities of spray dried amorphous trehalose with crystalline lactose alpha monohydrate (DMV International, The Netherlands). The lactose was tested in the TAM at 75 and 53% RH and did not result in a crystallisation response.

3. Results and discussion

In order to understand the behaviour of a two component system it is necessary to understand the behaviour of the individual components. Consequently, the crystallisation of amorphous trehalose on its own is important. Indeed consideration of this crystallisation is essential in understanding many aspects of the properties of trehalose and how this material may be of value in protecting proteins.

3.1. Gravimetric sorption of amorphous trehalose

Typical gravimetric sorption data are presented in Figs. 1 and 2. It can be seen that at both RH values the sample absorbs excess water and then loses mass as the material crystallises.

At 75% RH, the water content peaks at 14.52% gain. The sorption kinetics are interesting in that the sorption has not come close to equilibrium prior to the onset of crystallisation (the mass gain is still in a rapid rise when crystallisation, and hence mass loss, begins). This would indicate that the entire sample has not been saturated with absorbed water and that crystallisation must have started in the outer regions of the particles (which can be expected to saturate with water first). The mass loss is then seen to go through complex kinetics, firstly a rapid loss for a period of 35 min, followed by a much slower rate of change. The first stage of the mass loss is essentially linear (an apparent zero-order kinetic process), whereas the second stage of the mass loss has a linear fit when mass change is plotted as a function of square root of time (figure not shown), indicating that a slow diffusion process is taking place in this region. The two kinetic regions could relate to water loss from the particle surface initially, followed by slower diffusion of water trapped within, or between, the newly formed crystalline particles.

Just prior to reversion to 0% RH the mass loss had not ended and there was still diffusion of water from the crystallised sample into the 75% RH environment. However, the residual mass gain at the end of 75% RH stage was 6.59 mg, which was equal to 9.05%. The water content expected for the formation of a dihydrate would be 10.5%. Consequently, the sample exposed to 75% RH absorbed more water than

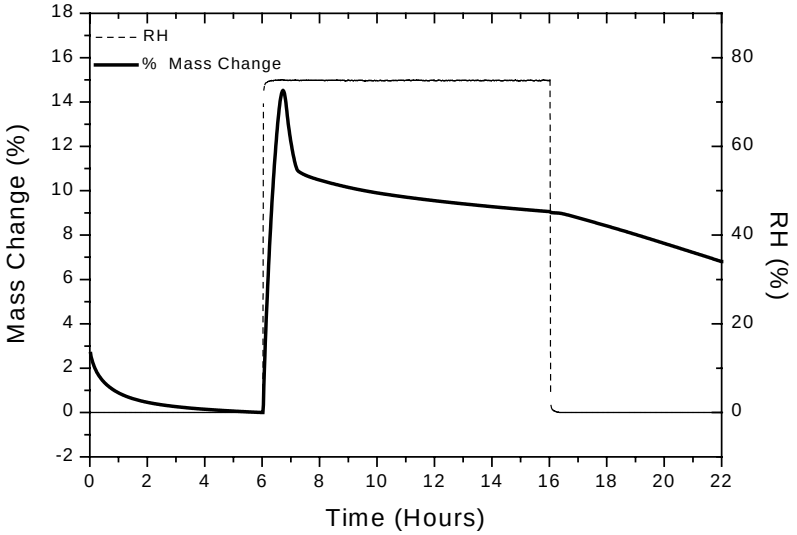

Fig. 1. DVS trace for amorphous trehalose being exposed to 75% RH.

was required to form a dihydrate, then rapidly lost water as crystallisation started and then showed a further slow desorption of water away from the sample, despite the fact that the material had not formed a complete dihydrate at this RH. The dihydrate was then slowly lost at 0% RH.

Exposure of amorphous trehalose to 53% RH in the DVS resulted in a plateau mass gain, indicative of saturation of the sample with absorbed water (plateau water content was a 13.60% increase). This was followed, after a lag period, by a very slow mass loss, again almost certainly consisting of two kinetic processes and resulting in a sigmoidal trace. The residual mass at the end of this phase was that of the dihydrate crystal form. A drop to 0% RH was then seen to indicate the loss of the dihydrate, again showing its instability at low RH. The data indicate that trehalose is either crystallising slowly, or that the water diffusion from the crystallised sample is slow. It follows that the behaviour of the sample at 53% RH is much more as expected and that at 75% RH is idiosyncratic. It can be concluded that at 75% RH in the DVS the rate of supply of water was so fast that parts of the sample were able to crystallise to the dihydrate, but other regions did not have sufficient access to water to allow this to happen. This was possibly due to collapse of amorphous material post-plasticisation. As water continued to diffuse away (rather than form more dihydrate), it can be concluded that the outside of the particles have formed dihydrate and that in doing so they have shielded interior regions from any further water access. On the basis of this hypothesis the slow diffusion of water away from this material must be from voids between newly formed crystal surfaces. The data leave an open question about whether the central regions of the particles are anhydrous crystals or amorphous material.

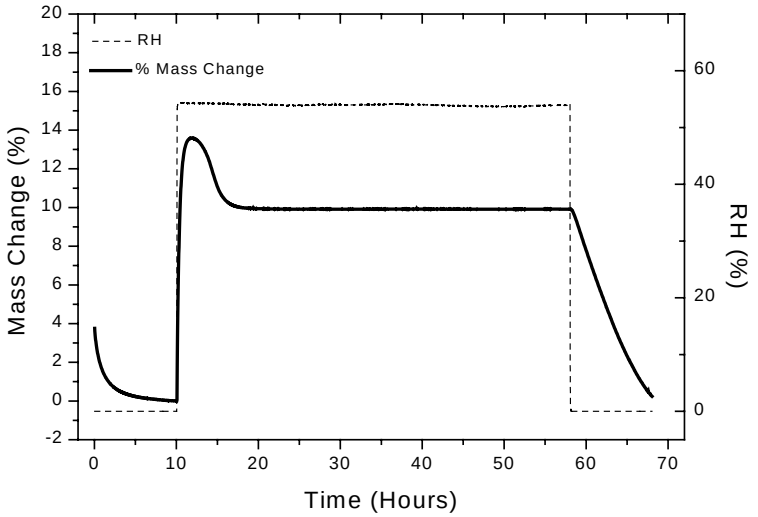

Fig. 2. DVS trace for amorphous trehalose being exposed to 53% RH.

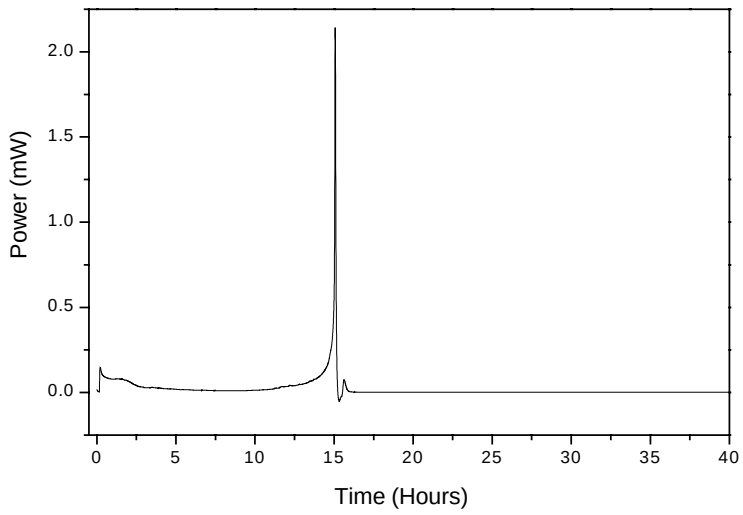

Fig. 3. Typical isothermal microcalorimetry trace for a 30 mg sample of amorphous trehalose exposed to 53% RH.

3.2. Isothermal microcalorimetry (TAM) of amorphous trehalose

The isothermal microcalorimetry experiments are very different from those in the DVS, principally because the rate of supply of water vapour in the TAM is by slow diffusion from a small tube of saturated salt solution whereas in the DVS the supply is a very rapid flow of humidified nitrogen. This will result in the rate-determining step being the supply of water vapour in the TAM (at both 75 and 53% RH), whereas the rate-determining step is the response of the sample in the DVS. This concept of rate of supply of water vapour in the TAM has been discussed previously for crystallisation of lactose [12].

The isothermal microcalorimetry data for amorphous trehalose are shown in Figs. 3 and 4 (53 and 75% RH, respectively). Fig. 3 shows a very long lag time prior to crystallisation. The reason for this is that crystallisation requires the supply of some 2 mg of water to form a dihydrate in the 20 mg of powder that was used in the test. This is a substantial mass of water to evaporate from a small tube of saturated salt solution. Indeed, it was our expectation that the sample may crystallise partially to a dihydrate due to the long time needed to get sufficient water to allow the entire sample to crystallise, especially as anhydrous crystal forms of trehalose have been shown to exist [14–16]. To check for this, samples were removed from the calorimeter after the crystallisation response and tested using thermogravimetric analysis. They were found to contain the water content of the full dihydrate. It follows that the sample requires 15 h in order to obtain sufficient water to cause crystallisation into the dihydrate form. In this case, the supply of water vapour is rate limiting and slower than distribution of water within the powder, hence the surface of the particles never

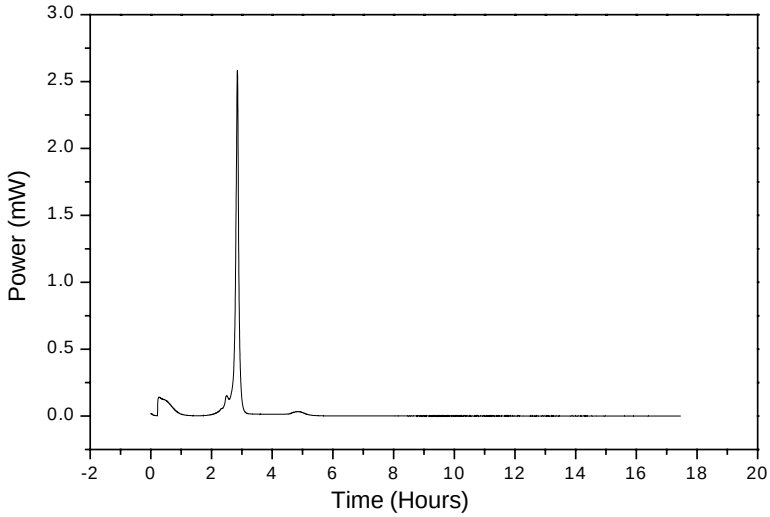

Fig. 4. Typical isothermal microcalorimetry trace for a 20 mg sample of amorphous trehalose exposed to 75% RH.

contains a significantly higher water mass than the bulk (unlike the DVS experiment at 75% RH). The shape of the calorimetric trace (Fig. 3) shows an onset of the response from around 12 h rising to the major peak at 15 h, with a small endotherm after this and a final exotherm. The initial part of the response will relate to the onset of mobility as the water content lowers the glass transition temperature (T_g) to or below 25 °C, the major peak is the rapid propagation of the crystallisation event, with the endotherm being a point where water desorption (endotherm) is momentarily greater than crystallisation (exotherm).

The calorimetric response at 75% RH (Fig. 4) occurs after a much shorter lag time than at 53% RH (Fig. 3). Here, a shoulder is seen at around 3 h, again probably the time when mobility starts as T_g drops below 25 °C, followed by the crystallisation event. Subsequently, the trace does not return to baseline until around 7 h. This result is analogous to the DVS data, where the mass loss was seen to be slow and protracted. It is fair to assume that the water uptake in the calorimetric experiment will be lower than that achieved in the DVS, as the rate of supply of vapour in the DVS is much greater than the rate of evaporation from a small tube of salt solution in the calorimeter. Therefore, the sample in the calorimeter will almost certainly distribute water throughout its mass faster than water is supplied from the salt solution, hence the sample will crystallise when the water content is enough to allow sufficient molecular mobility. The apparent enthalpy of crystallisation for the total peak in Fig. 3 (53% RH) was 58.6 J/g and for Fig. 4 (75% RH) was 61.5 J/g.

3.3. Crystallisation of powder mixtures

Mixtures were exposed to 75% RH in the calorimeter, initially using a total sample mass of ca. 100 mg (Figs. 5 and 6). High amorphous content samples (above 10%, w/w) were very easy to detect and yielded very clear single peaks displaced from the initial heat flow response (the initial response is a consequence of lowering the cell into the calorimeter and equilibration of the air space and the powder with water vapour). Fig. 5 shows an example of such a response for a sample that contained 14% (w/w) amorphous content. It can be seen that this sample crystallised with an onset at 2 h. The reason for this onset time being different from that in Fig. 4 is the total mass of amorphous material, with the 100% (w/w) sample there was 20.9 mg (Fig. 4) and with the 14% (w/w) amorphous sample there was 15.6 mg (14% (w/w) of the 112 mg sample) (Fig. 5), consequently needing less time to saturate. This relationship is shown in Fig. 7 and clearly the single factor that determines lag time is the mass of amorphous trehalose in the ampoule.

Difficulties were encountered when the amorphous content of trehalose fell to 4% (w/w) and below (a total amorphous load of ca. 4 mg for the approximately 100 mg sample load) (Fig. 6). For these samples the crystallisation response started to run into the peak that was seen for the initial equilibration. It follows that to be able to quantify the amorphous content with any confidence the mass of amorphous material must be greater than 4 mg at 75% RH. This means that to quantify samples with a low percentage of amorphous content a large sample mass is required in the calorimeter cell. For example, to have 4 mg of amorphous material, the total mass required for a 0.5% (w/w) amorphous sample would be 800 mg. Consequently to measure reproducible areas for samples with less than 4% (w/w) amorphous content using 75% RH, it was necessary to increase the total sample mass. For samples with 2 and 1% (w/w) amorphous content 300 mg was loaded into the calorimeter. These mixtures gave identical lag times to 100 mg samples that contained the same mass of trehalose. For example, the 2% (w/w)

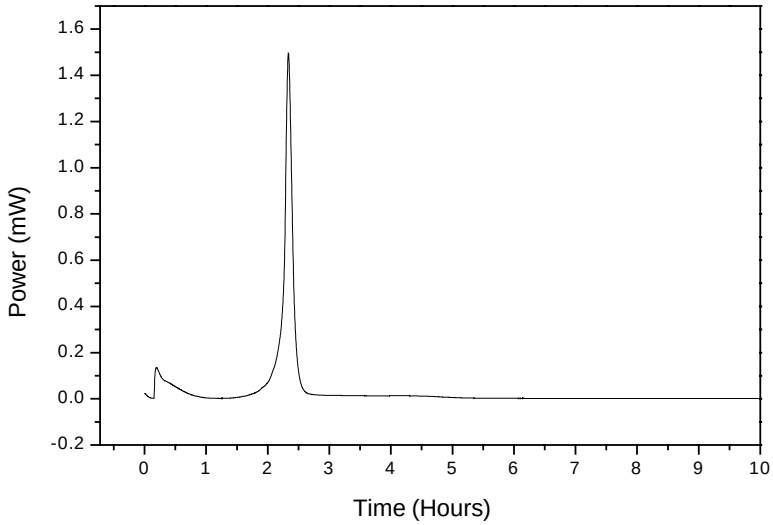

Fig. 5. Typical isothermal microcalorimetry trace for a 112 mg sample containing 14% (w/w) amorphous trehalose and 86% (w/w) crystalline lactose monohydrate exposed to 75% RH.

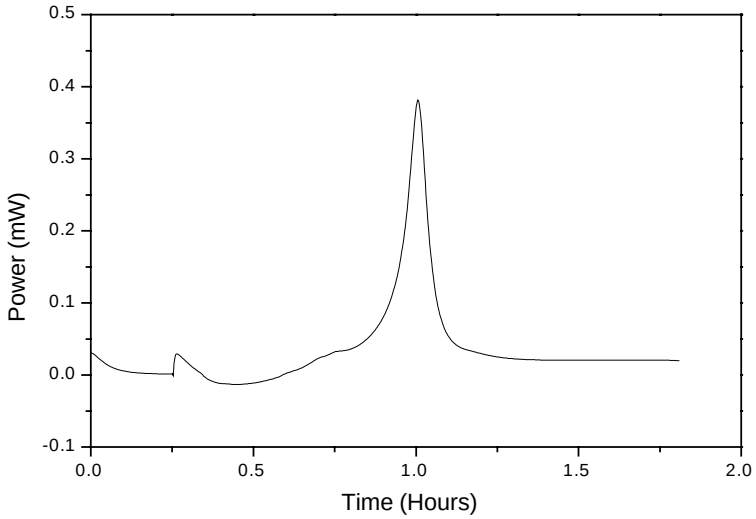

Fig. 6. Typical isothermal microcalorimetry trace for a 100 mg sample containing 4% (w/w) amorphous trehalose and 96% (w/w) crystalline lactose monohydrate exposed to 75% RH.

trehalose 300 mg sample contains 6 mg of amorphous trehalose, as does the 100 mg 6% (w/w) trehalose sample, and both crystallised after a lag time of 1.3 h.

Using 300 mg for the 1 and 2% (w/w) and 100 mg for 4–14% (w/w) (and lower sample masses at very high amorphous contents to keep the response on scale in the TAM), the total areas under the crystallisation response (normalised for sample mass) were plotted as a function of amorphous content (Fig. 8). It can be seen that a good linear correlation was observed for the region 2–100% (w/w) amorphous trehalose content ($r = 0.999$) and also for the region of interest 2–14% (w/w) ($r = 0.995$). The region 0–10% (w/w) was our major area of interest as this is the region where it is very difficult even to detect, let alone quantify, the amorphous content by many standard techniques. In this region, it is clearly possible both to detect and quantify the amorphous content in the mixed powder system. Given the small error bars on Fig. 8, quantification is estimated to be good to 1% (w/w) or better.

Clearly, the TAM response at 75% RH shows great promise as a means of detecting and quantifying amorphous content in mixtures, however there is a limitation in the mass of amorphous material that is needed to ensure the crystallisation response is displaced from the initial response for the instrument. It could be expected that the sample mass required would be lower at 53% RH as the lag time is much longer at lower RH values (Fig. 2). In order to check this, mixtures were prepared with low trehalose amorphous contents (not shown). It was found that the crystallisation peak yielded a low intensity prolonged flat peak which did not separate well from the initial response and for which it was very difficult to measure a reproducible area under the curve. Consequently, the response at 53%

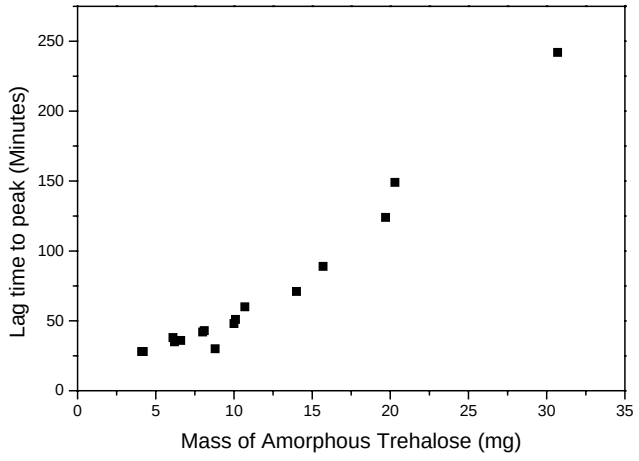

Fig. 7. The relationship between lag time to peak crystallisation and the absolute sample mass of amorphous trehalose exposed to 75% RH.

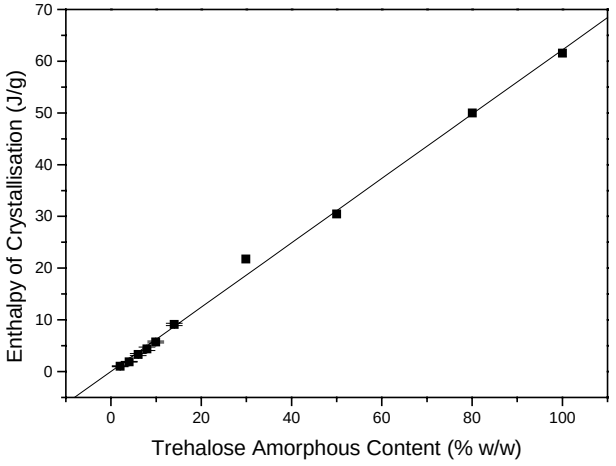

Fig. 8. The relationship between apparent enthalpy of crystallisation and the amorphous content for mixtures of amorphous trehalose and crystalline lactose at 75% RH.

RH was much less suitable for quantification than the much sharper peak that was observed at 75% RH.

4. Conclusion

It has been shown that amorphous trehalose does not crystallise to a full dihydrate when water vapour is supplied very rapidly to the sample in a DVS system (75% RH). When water is supplied at 53% in the DVS the dihydrate is formed. This observation on differences in crystallisation of amorphous trehalose could be of significance for studies on the stability of protein systems stabilised with amorphous trehalose, and requires further study.

The TAM method has been shown to be very suitable both to detect and quantify the amorphous trehalose when it is mixed with crystalline lactose. There is no reason to believe that the presence of other non-crystallising components (other than, or as well as, lactose) would prevent the detection of the amorphous content, however there could be difficulties in cases where more than one material is present in the amorphous state.

The only limitation of the TAM method was the need to have sufficient mass of amorphous content to separate the crystallisation peak from the initial response. It is only necessary to separate these two events if quantification is needed as for simple detection of amorphous content accurate measurements of the area under the crystallisation peak would not be required.

Acknowledgements

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