Characterisation of Spray-Dried Protein/Carbohydrate Formulations using Gravimetric Vapour Sorption/Near Infrared Spectroscopy

Abigail Elizabeth Moran

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University of London School of Pharmacy
29-39 Brunswick Square
London
WC1N 1AX

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Abstract

Protein drug formulations are commonly produced in the solid form due to their degradation in solution. Drying may alter protein conformation and cause loss of activity. Stabilising excipients, particularly disaccharides, are used to protect proteins during drying. The mechanism of this protection remains unclear. The aims of this work were to investigate the use of the combined technique of dynamic vapour sorption and near infrared spectroscopy (DVS/NIRS) for the analysis of spray-dried protein/trehalose formulations and to examine the mechanism of protein stabilisation by trehalose upon drying.

Anhydrous forms of trehalose (a disaccharide) were produced according to methods in the literature and by drying in the DVS analyser. Characterisation was performed using Differential Scanning Calorimetry (DSC), X-Ray Powder Diffraction analysis (XRPD) and Thermogravimetric Analysis (TGA). NIR spectra of samples were recorded. A new anhydrous form of trehalose was produced in the DVS analyser. Assignment of peaks in the NIR spectra for the polymorphic forms of trehalose was achieved.

Spray-dried trehalose was prepared by spray-drying solutions of α,α-trehalose dihydrate in water, of varying concentration, using parameters appropriate for the drying of proteins. Crystallisation was induced at 75 % RH and NIR spectra recorded during the experiments. Spray-dried trehalose was also produced using parameters commonly used for drying sugars. Crystallisation was induced by repetitive exposure to 75 % RH in the DVS analyser and NIR spectra were recorded. Variability was observed in the behaviour of trehalose samples dried from solutions of low trehalose concentration. Small amounts of anhydrous trehalose in otherwise amorphous samples were identified by NIRS. The cause of variability/anhydrous nature was proposed to be the less uniform droplets atomised from solutions of low concentration. DVS/NIR allowed the examination of the crystallisation of amorphous trehalose in real-time, which was shown not to be instantaneous. The presence of anhydrous trehalose in otherwise amorphous samples was proposed to act as a seed for subsequent crystallisation, causing the formation of an unstable dihydrate with a tendency to an anhydrous state.

Co-spray-dried samples of catalase (a protein) and trehalose were prepared and DVS/NIR experiments were performed in the same way as for spray-dried trehalose samples. The activity of the samples was determined before and after the experiments. Mathematically produced theoretical NIR spectra were compared with the spectra of co-spray-dried samples. A lower ratio of catalase: trehalose was required for the effective stabilisation of catalase during drying. Upon exposure to 75 % RH, (mimicking storage), the presence of trehalose in the formulation was detrimental to the stability of the protein. Catalase: trehalose 50:50 was the most effective ratio of components for the overall stabilisation of catalase during/following spray drying. The data supported the water replacement hypothesis of protein stabilisation upon drying because the greatest interaction between components was expected at a 50:50 ratio and evidence of hydrogen bonding between co-spray-dried components was shown in the NIR spectra.

Multiple Linear Regression (MLR) and Partial Least Squares Regression (PLSR) were used to determine the feasibility of NIRS for the quantification of components in co-spray-dried catalase/trehalose formulations. Feasibility was demonstrated and PLSR gave a more specific calibration model.

The simultaneous use of DVS/NIRS was shown to be very useful to analyse sample transitions in real-time. The method allowed conclusions to be drawn that would be difficult, or impossible to arrive at by the use of either method in isolation.
"It must be nearly finished, surely?"
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I'd like to dedicate this thesis to my Mum, my Dad and my sister Gill. It's been a tough few years in more ways than one and not only have they adapted to everything that life has thrown at them, but they've had the reserves to be lovely to me when I've been tired and ratty. I can't begin to express my awe and appreciation. Thank you.

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<td>Alpha anhydrous trehalose</td>
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<td>T&lt;sub&gt;β&lt;/sub&gt;</td>
<td>Beta anhydrous trehalose</td>
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<td>T&lt;sub&gt;β-vac&lt;/sub&gt;</td>
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<td>T&lt;sub&gt;d&lt;/sub&gt;</td>
<td>DVS-anhydrous trehalose</td>
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<td>T&lt;sub&gt;g&lt;/sub&gt;</td>
<td>Glass Transition Temperature (°C)</td>
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<td>α,α-Trehalose dihydrate</td>
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Introduction
1 Introduction

Protein drug formulations are commonly produced in solid form due to the tendency of proteins in solution to degrade. However, the processes involved in the production of solid protein formulations such as spray drying or freeze drying can be detrimental to the protein. A change in protein conformation can lead to a reduction or total loss of the biological activity of the protein (Prestrelski et al., 1993a).

It has been shown that certain organisms are capable of surviving almost complete desiccation (Crowe, 1971). Large concentrations of disaccharides have been found in such organisms and evidence has been provided to suggest that it may be these sugars, in the amorphous (glassy) state that afford protection against protein denaturation upon total removal of water. The mechanism of this preservation is a subject of debate and it is this area of research that is the focus of this thesis.

This chapter serves to give a general overview of the literature surrounding the topic of protein stabilisation to date. This will be followed by four chapters of results opening with more detailed introductions based upon the specific content of each chapter.

1.1 Anhydrobiosis

Anhydrobiosis is the term used to describe the state in which organisms exist with as little as 0.1 % water available in their tissues and remain viable (Crowe et al., 1992). On addition of water, the cells of the anhydrobiotic organisms swell and recommence active life as before their dehydration. Examples of such organisms include the 'resurrection plants', *Craterostigma plantagineum* and *Selaginella lepidophylla* (Roser, 1991). These plants demonstrate the ability to remain viable over tens of years in extremely hot, dry conditions in a 'metabolic stasis' in which metabolic processes are undetectable. Upon exposure to water, these plants are able to resume metabolic activity and become as they were prior to their dehydration (Gil et al., 1996). Other such 'anhydrobiotes' include the cysts of the brine shrimp, *Artemia*, certain nematodes and some fungal spores (Crowe et al., 1992). These organisms have been the fascination of scientists for many years owing to the possibility of harnessing the mechanism of anhydrobiosis to protect organisms or structures without this functional ability to remain viable upon dehydration.
In the investigation of the mechanism of anhydrobiosis, it was discovered that a common feature of anhydrobiotic organisms was an increased intracellular concentration of disaccharides, particularly trehalose (Crowe et al., 1992). It was considered that the presence of trehalose, which became amorphous under conditions of dehydration, was fundamental to the mechanism of anhydrobiosis (Crowe et al., 1992).

It has been known for several years that many sugars, not just trehalose, are useful as excipients in terms of their biostabilisation capabilities. The biostabilisation properties of trehalose in particular have received attention due to its abundance in nature in organisms that are able to withstand environments of extreme dehydration. Alongside research into the effect of various stabilising additives on the maintenance of phospholipid membranes upon dehydration (an in vitro method to mimic the situation in anhydrobiotic organisms, Crowe et al., 1984, Crowe and Crowe, 1988) similar investigations have been carried out to investigate the effect of stabilising additives during the drying of proteins (e.g. Carpenter and Crowe, 1988, 1989). In the pharmaceutical industry, the stabilisation of proteins by excipients in the amorphous state has been of interest in the fields of biotechnology and drug delivery, where the drying of proteins is required for the necessary shelf life of the eventual product. This is discussed in more detail later in this chapter.

1.2 The Crystalline vs. the Amorphous Form

A crystalline material is defined as one exhibiting long-range order and well-defined molecular packing (Yu, 2001). Crystalline solids have defined thermodynamic properties such as melting temperature and solubility and can exist in several polymorphic or solvated forms (Hancock and Zografi, 1997). Such materials are more physically and chemically stable compared with their amorphous counterparts, having lower free energy and hence reduced molecular motion within the crystal lattice (Hancock and Zografi, 1997).

Amorphous materials may exhibit short-range molecular order within their structures but lack long-range order. The amorphous form is less stable both chemically and physically than the crystalline form due to increased internal energy and molecular motion (Yu, 2001). It has better dissolution properties (Shekunov and York, 2000) and may retain some free water within the less ordered structure.
Amorphous materials have been studied in the pharmaceutical industry for many years owing to the storage of dry products in the amorphous state and the desirable formulation properties of such forms, e.g. rapid dissolution. Latterly, materials existing in the amorphous or glassy state have been the subject of much research as a result of their apparent ability to stabilise biotechnology products such as peptides and proteins (Franks et al., 1991).

1.3 Glass Formation

Hancock and Zografi (1997) described four main ways in which an amorphous material can be produced:

1. Condensation from the vapour state.
2. Mechanical activation of crystalline material (through milling or compaction).
3. Precipitation from solution (during spray or freeze drying).
4. Supercooling of a liquid below its melting point.

The processes involved in the formation of partial or full amorphous character may be accidental or deliberate, a reason for the latter taking place being to improve the dissolution characteristics of the solid product. However, the formation of a glass is usually described in the literature by the process of supercooling as described below (Hancock and Zografi, 1997, Craig et al., 1999).

When a liquid is cooled, rather than supercooled, crystallisation occurs when the liquid reaches its melting temperature, \( T_m \) (Figure 1.1). Here, the molecules within the liquid state have adequate time to order themselves into the lattice-like crystalline structure. This exothermic process is accompanied by a decrease in the specific volume (V) of the system and a decrease in enthalpy (H). Conversely, when a liquid is supercooled, i.e. rapid cooling below the melting temperature, molecules within the liquid do not have enough time to orientate themselves into the crystal lattice formation and attain equilibrium. There is no change seen in volume or enthalpy as the liquid passes \( T_m \), and the liquid is termed a ‘supercooled liquid’. This state is also known as the ‘rubbery state’, owing to the pliable characteristics of some polymers under these conditions. The viscosity of supercooled liquids is in the region of \( 10^3 - 10^{12} \) Pa.S although this varies with temperature (Angell, 1995). If the supercooled liquid is cooled further, it will eventually reach a point at which molecular mobility is so low that it may be
termed as 'fixed' in the glassy state. This point is known as the glass transition temperature (T_g). Below T_g, the material exists in the glassy state that is thermodynamically unstable, having a viscosity of greater than 10^{12} Pa.S. The material could theoretically be further cooled below T_g to another temperature point known as the Kauzmann temperature, T_K (Figure 1.1). This temperature is generally ≈ 20K below T_g and is thought to represent the theoretical lower limit of T_g, where the excess entropy of the system reaches zero (Kauzmann, 1948). This temperature has also been deemed the Zero Mobility Temperature, T_0, when molecular motion is brought to a standstill (Hatley, 1997).

![Figure 1.1](image)

**Figure 1.1.** A phase diagram to illustrate the changes in enthalpy or specific volume of a model solid substance upon changes in temperature (adapted from Hancock and Zografi (1997)).

### 1.4 Plasticisation and the amorphous to crystalline transition

As the glass transition of water is very low (-135 °C, Crowe *et al.*, 1996), the presence of water in a given amorphous system will serve as a plasticiser, lowering the overall T_g of the system, as demonstrated by Elamin *et al.*, (1995). If the T_g of an amorphous system is reduced to below the experimental temperature, then it may be expected that the system will be converted from the glassy to the rubbery state. In the rubbery state, molecular mobility is increased to such an extent that crystallisation of the substance is likely to occur, leading to destabilisation of the system. Such crystallisation will begin
with a nucleus (seed) of one crystal within the otherwise amorphous system, triggering the crystallisation of the bulk.

The understanding of the crystallisation kinetics of the system in the presence of excipients and at the proposed water content is advantageous for the prediction of behaviour and long-term stability of drugs formulated in the amorphous state (Shekunov and York, 2000). The existence of a substance in more than one crystal form (polymorphism) is important to consider during drug product formulation. Solid-state recrystallisation must be suppressed in formulations of substances known to have more than one polymorphic form, in order to be able to accurately predict the stability of the formulation.

1.5 The Glassy State and Stability

Amorphous substances are generally thought to be unstable above their Tg owing to increased molecular mobility, and stable below their Tg, when reduced molecular mobility within the system prevents destabilisation of the disordered form due to its higher viscosity. However, this general concept is not clear-cut, as molecular motion still occurs in amorphous products below their glass transition temperatures (Angell, 1995, Streefland et al., 1998). Indefinite stability is therefore not guaranteed for amorphous products stored below their Tg (e.g. Duddu et al., 1997b, Streefland et al., 1998).

Hancock et al. (1995) described the molecular mobility of amorphous solids below their glass transition temperatures. They measured the molecular mobility of three substances commonly used in pharmaceutical formulations (indomethacin, polyvinyl pyrrolidone (PVP)) and sucrose following storage at various temperatures and for various time periods below their known glass transition temperatures. By the use of Differential Scanning Calorimetry for such measurements, these researchers were able to conclude that storage at a temperature at least 50 K below the Tg of each substance was required in order to be certain that the molecular motions detected would be negligible over the shelf life of the products. Similar research has been carried out to investigate the stability of amorphous substances at varying storage temperatures with respect to their glass transition temperatures (e.g. Elamin et al., 1995, Duddu et al., 1997b, Hatley, 1997). Such research is useful to allow the accurate prediction of the
shelf life of an amorphous product at given temperatures/water contents (e.g. Duddu and Dal Monte, 1997a; Hatley, 1997).

The glassy state is particularly important in the pharmaceutical industry, particularly with respect to the bioavailability and stability of drug products. Many drug products are formulated in the amorphous state to improve their dissolution characteristics and hence improve their bioavailability. As the amorphous form has more associated energy due to its more disordered state, it is more hygroscopic and hence more liable to dissolve and degrade faster than the associated crystalline form.

Production techniques such as milling and compression can induce small amounts of amorphous character into a formulation (Saleki-Gerhardt et al., 1994; Buckton et al., 1995b). These amorphous, highly reactive regions tend to be on the surface of the material and are available to interact with other components of the formulation. These regions can have a large and often detrimental impact on the performance and stability of the final product. As changes to the crystallinity of a powder surface can cause batch-to-batch variation, the detection and quantification of such low levels of amorphous content has been a subject of much research in the pharmaceutical industry in the last decade (Briggner et al., 1994; Saleki-Gerhardt et al., 1994; Sebhatu et al., 1994; Buckton and Darcy, 1995a; Buckton et al., 1995b; Hogan and Buckton, 2000; Al-Hadithi et al., 2004). It is thought that the understanding of the properties of amorphous systems is important to be able to predict the behaviour of pharmaceutical products in which process-induced amorphous regions have been produced (Elamin et al., 1995).

Spray drying and freeze drying are methods used in the pharmaceutical industry to produce stable formulations of biotechnological products such as proteins. With the advent of gene therapy it is likely that the demand for stable biological formulations will increase. Both drying techniques may produce amorphous products; therefore issues surrounding the stability of the amorphous state over the time periods required for the storage of medicines have become increasingly important. There are many texts available that discuss the spray drying and freeze drying of biotechnology products (Franks et al., 1991, Fåldt and Bergenståhl, 1994, Skrabanja et al., 1994, Carpenter et al., 1997, Franks, 1998, Maa et al., 1997, Maa et al., 1998), all of which concentrate on the variables most likely to have unfavourable effects on fragile biotechnology
formulations. Much research has been performed to investigate the ideal methods to stabilise biotechnological products such as proteins in the solid state.

Many parenteral products are formulated in the freeze-dried, amorphous state because of their instability in solution. This state is sometimes preferable to the crystalline state because of its more rapid dissolution characteristics, meaning that reconstitution of the product prior to administration to the patient can be performed in a fast and easy fashion. Conversely, these favourable properties can also be a disadvantage in terms of the stability of the product formulation over the shelf life of a medicinal product, because the hygroscopic nature of the material encourages degradation of the product. Craig et al., 1999, presented a review of the relevance of the amorphous state to pharmaceutical systems, including methods for the measurement of the glass transition temperature in such systems and the relation of the glassy state to the chemical and physical stability of amorphous drug formulations.

1.6 Protein Structure and Function
Proteins are the main nitrogenous constituents of living organisms. Amino acids are the so-called 'building blocks' of peptides and proteins, with the general chemical formula R-CH(NH2)COOH. The variation in the nature of the R group in the general formula determines the properties of the individual amino acid. Amino acids link through the formation of peptide bonds (-CO.NH-) to form peptides, containing amino acids linked through peptide bonds (Nelson and Cox, 2005). Peptides containing less than ten amino acid residues are generally named individually, i.e. dipeptide and tripeptide, whereas those consisting of between ten and thirty amino acid residues are termed oligopeptides (Thornton and Barlow, 1991). Peptides formed of more than thirty amino acids are generally termed polypeptides. Peptides and polypeptides constitute the primary structure of proteins.

The secondary structure of a protein describes the spatial arrangement of amino acids in the polypeptide chain and the repetitive conformation of the polypeptide (Nelson and Cox, 2005). Types of secondary structures include the alpha helix, beta conformation or beta turn. The secondary structure of a protein is highly dependent upon the amino acid residues making up the polypeptide chain because hydrogen bonding between peptide CO and NH groups occurs in order to maintain the structure (Thornton and Barlow, 1991; Nelson and Cox, 2005). For example, the helical structure of the alpha
helix is maintained by hydrogen bonding between repetitive amino acids in the polypeptide chain. Thus, if the size or charge of the amino acids in the polypeptide chain differ, the formation of the alpha helix may be prevented, necessitating a different secondary structure such as the beta conformation (Nelson and Cox, 2005).

The tertiary and quaternary structures of a protein describe the overall arrangement of all atoms comprising the protein and the arrangement of the polypeptide chains into three-dimensional complexes, respectively (Nelson and Cox, 2005). Tertiary structure differs from secondary structure in that it describes the long-range arrangement of the amino acids in the polypeptide chains, where amino acids that are far apart in the chain may interact in the folded state by weak bonds such as covalent disulphide cross-links (Nelson and Cox, 2005). Proteins in such folded states are more compact (Thornton and Barlow, 1991) and are generally termed fibrous proteins or globular proteins dependent upon their tertiary structure. The polypeptide chains in fibrous proteins are generally formed into strands or sheets whereas those in globular proteins are folded into globular or spherical shapes. The arrangement is termed the quaternary structure of the protein (Nelson and Cox, 2005).

Water molecules are found to bind strongly to the surface of proteins and essentially surround the three-dimensional structure in a layer of water (hydration layer). The water monolayer of a protein (M₀) is the water content required to exhaust all of the possible water-binding sites on the surface of the protein with additional clustering of water molecules (Hagemann, 1992). Water molecules bound to some proteins are bound so tightly so as to appear part of the overall structure (Nelson and Cox, 2005). The bound water molecules are thought to be fundamental to the maintenance of the native protein structure because they remain even when the protein is in solution.

1.7 Protein Formulation Issues
With the advent of recombinant DNA techniques and the generation of peptides and proteins as drugs, much research has been undertaken to develop protein formulations that remain stable in the time scale required for pharmaceutical products. Proteins have the tendency to degrade in solution due to chemical reactions such as oxidation or deamidation or through aggregation, precipitation or extremes of pH (among many causes). Although stabilizing additives can be used in such formulations to retain the protein in its native form, such as carbohydrates and polyols (Back et al., 1979,
Arakawa and Timasheff, 1982, Costantino et al., 1998d), formulation in the solid state is favoured for greater long-term stability.

The most commonly used method for producing solid protein formulations is freeze drying (lyophilisation), although other methods are utilized. Spray drying is a method that is currently being used in the production of protein powders for inhalation e.g. inhaled insulin. As freeze drying results in a solid 'cake' that is easy to reconstitute, it is a more useful process for the production of formulations for injection. However, the characteristics of freeze-dried material (static, limited flow) prevent its use in the formulation of products for inhalation. Although spray drying is useful in this respect, in that inhalable particles may be produced, care must be taken in the drying of more temperature-labile proteins that may be destroyed by the heat applied during the process.

During the final stages of drying (freeze or spray drying), the hydration shell surrounding the protein is removed, causing denaturation upon loss of the protein's native structure (Prestrelski et al., 1993a and 1993b). The native, three-dimensional structure of a protein is critical to its biological activity and hence therapeutic use (Franks et al., 1991) and so loss of the conformation of proteins upon dehydration can lead to loss of protein activity (Prestrelski et al., 1994, Nelson and Cox, 2005). The stresses involved in the processing of proteins must be overcome to prevent the denaturation and loss of activity of the protein during formulation.

1.8 Process-induced vs. storage-induced stresses
Crowe et al. (1990) highlighted the fact that the stresses imposed upon proteins through different processes such as freezing and dehydration are fundamentally different. A discussion regarding the appropriate stabilising additives for proteins undergoing specific freezing or drying stresses was presented by Carpenter et al. (1994). It is logical to suppose therefore, that stabilising additives for dried protein formulations should be chosen with the potential process-related stresses in mind, to create a balanced, stable formulation. Carpenter et al. (1993) corroborated this by demonstrating that the selection of stabilising additives specific to the stresses (freezing and drying) predicted for the processing of protein formulations could optimise the stability of the protein following subsequent storage and rehydration. These researchers went on to prove this theory further by demonstrating the optimal recovery of lactate
dehydrogenase and phosphofructokinase following freeze drying by using a combination of polyethylene glycol to protect the protein during freezing (cryoprotection) and a carbohydrate such as trehalose, to protect the conformation of the protein during drying (Prestrelski et al., 1993b). It may be supposed that during a spray drying process, the ideal combination of stabilising additives should include an agent to prevent degradation in solution, an agent such as a carbohydrate to protect the conformation of the protein upon drying, and finally an agent to raise the Tg of the formulation to encourage stability over the product's shelf life.

Prestrelski et al. (1995) suggested that stabilizing additives for the long-term storage stability of dried proteins should ideally have high molecular weights and high glass transition temperatures. They stated that the characteristics of stabilisers to prevent drying-associated denaturation alone are different to the characteristics of protectants required to ensure long-term stability, owing to the additional problems of chemical degradation. It was proposed that the relative ability of an additive to effect stabilisation during drying and subsequent storage is dependent upon the degradation pathway specific to the protein (i.e. unfolding, chemical decomposition or aggregation). A review of the deterioration of freeze-dried proteins by Costantino et al. (1998d) describes the deleterious processes that may cause degradation of proteins in the solid state (e.g. aggregation). These authors emphasised the necessity to understand the mechanism of deterioration of individual proteins prior to formulation into the solid state (Costantino et al., 1998d). Liao et al. (2002a and 2002b) supported this proposal following their observation that the mechanism of stabilisation conferred onto proteins by carbohydrates appeared to be different for different types of proteins. It may be concluded that when formulating proteins, additives should be chosen with respect to potential process-related stresses and the potential routes by which the protein may degrade.

1.9 Excipients used as stabilising agents

Many research groups have tried to find the ideal combination of additives to protect proteins from the stresses induced during spray drying or freeze drying, although most have concentrated on the use of disaccharides such as trehalose, owing to their implied role in anhydrobiosis. Examples of other additives that have been investigated for their potential for biostabilisation are given in Sections 1.9.1, 1.9.2 and 1.9.3. Many combinations of excipients have been tested for their potential ability to stabilise
proteins upon drying and storage, often including polymers (e.g. Carpenter et al., 1994; Izutsu et al., 1995; Hinrichs et al., 2001). Two reviews outlining practical advice for the selection of formulations for freeze drying proteins have been published, which give more detailed information regarding the potential of excipients for the stabilisation of proteins during and after lyophilisation (Pikal, 1990b and Carpenter et al., 1997).

1.9.1 Surfactants
Adler and Lee (1999) showed that the addition of polysorbate 80 to solutions of the protein, lactate dehydrogenase (LDH) and trehalose prior to spray drying improved the stability of the protein following spray drying. The surfactant was added in order to reduce the aggregation of the protein at the inlet prior to atomisation into the spray dryer apparatus. As Mumenthaler et al. (1994) demonstrated that denaturation of proteins during spray drying was primarily caused by exposure of the protein at the air/water interface of droplets upon spray drying, the surfactant was thought to act by excluding the protein from the air/water interface of the atomised droplets; thus preventing its degradation (Adler and Lee, 1999).

Adler et al. (2000) followed the study of Alder and Lee (1999) by examining the combinations of trehalose and a surfactant (polysorbate 80 or Sodium Dodecyl Sulphate (SDS)) as additives to potentially stabilise the protein Bovine Serum Albumin (BSA) upon spray drying. These workers used Electron spectroscopy for Chemical Analysis (ESCA) to assess the nature of the elements at the surface of the spray-dried particles; thus allowing quantification of the components of the particle at the surface (Adler et al., 2000). These researchers showed that the addition of a surfactant to the solution to be spray-dried resulted in a concentration-dependent obstruction of protein adsorption at the surface of the spray-dried particles. It was observed that at the molar ratio of protein: surfactant required to fully exclude the protein; the surface was not saturated with the surfactant. Adler et al. (2000) proposed that a complex was formed between the protein and surfactant in the solution to be spray-dried, encouraging the removal of the protein from the air/water interface. This removal was thought to prevent denaturation of the protein during the drying process. Although the addition of the surfactant, polysorbate 80 was shown to stabilise LDH upon spray drying, its presence was shown to be detrimental to the stability of the protein during storage (Adler and Lee, 1999).
Hillgren et al. (2002) investigated the protection mechanism of the non-ionic surfactant, Tween 80 (polyoxyethylene 20 sorbitan monooleate) on the protection of the protein, LDH, during freezing and thawing, freezing being a fundamental part of the freeze drying process. These authors tentatively proposed that Tween 80 might protect LDH from denaturation by preventing its interaction with ice. The concentration of Tween 80 required to effect stabilisation was shown to be dependent upon the freezing rate, thought to be related to the area of the ice crystals formed (Hillgren et al., 2002). The ideal concentration of Tween 80 was observed below the critical micelle concentration, allowing greater interaction with the ice crystals and thus hindering the contact between the protein and the ice. Lower concentrations were shown not to protect the protein during freezing because of the reduced coverage of the surface of the ice crystals, allowing increased protein-ice contact (Hillgren et al., 2002).

1.9.2 Salts
Several studies have been performed to investigate the potential of salts to stabilise proteins during drying (discussed below). The potential of salts for this protection was alluded to in a study of the effect of salts on aqueous sugar systems, in which the inhibition of water crystallisation was observed in the presence of salts (Mazzobre et al., 2001).

In a study by Miller et al. (1998), the effects of trehalose and trehalose/sodium tetraborate mixtures on the recovery of lactate dehydrogenase (LDH) activity following freeze thawing and vacuum drying were investigated. The presence of sodium tetraborate in LDH/trehalose formulations was shown to be detrimental on the activity of LDH after freeze thawing, however it was shown to improve the storage stability of LDH when compared with the use of trehalose as the sole stabiliser (Miller et al., 1998). These results suggest that a balance of stabilising agents is required during the freeze drying process in order to prevent protein degradation during the stresses of freezing, drying and subsequent storage.

Mazzobre and Buera (1999) freeze-dried the enzyme β-galactosidase in the presence of trehalose and different salts (MgCl2, CaCl2, ZnCl2, CsCl, NaCl or KCl) to investigate the relative stability of the protein in the formulations by measurement of the enzyme activity and determination of the formulation glass transition temperatures. This study concentrated on the ability of the cations in the salts to delay trehalose crystallisation at
44% RH and hence enhance the stabilising effect on the protein. The crystallisation of the amorphous trehalose in the formulation was delayed by the incorporation of cations, particularly Mg\(^2+\), which caused an improvement in the stability of the enzyme at 44% RH (Mazzobre and Buera, 1999). As the glass transition temperatures of the formulations containing salts were no different to those of the enzyme formulations containing trehalose as the sole additive, it was hypothesised that the improvement in the stability of the protein following freeze drying was caused by the inhibition of trehalose crystallisation (Mazzobre and Buera, 1999). Further discussion regarding the inhibition of crystallisation of the stabilising additive is given in the introduction to Chapter 5.

1.9.3 High molecular weight carbohydrates

The high molecular weight polymeric carbohydrate, dextran, has been investigated for its potential to stabilise proteins upon freezing and drying (Allison et al., 1998 and 2000). Dextrans of varying molecular weight have particularly high glass transition temperatures and for this reason were thought to be potential stabilising excipients for proteins in the glassy state. Although dextran has a high Tg, it was found that used alone, it was unable to protect the protein, Actin from denaturation during lyophilisation (Allison et al., 1998). However, a combination of dextran and the disaccharide sucrose was shown to be as effective as sucrose alone for the protection of the structure of the protein (Allison et al., 1998). In their study, Allison et al. (2000) were able to show that freeze drying actin with trehalose or sucrose alone prevented the process-induced denaturation of the protein and the protein formulations were active immediately after lyophilisation. However, protein aggregates were formed upon rehydration following storage in those samples freeze-dried with sucrose or trehalose alone. The use of dextran alone to protect the protein during the drying process was shown to be unsuccessful; however the degree of aggregation of the protein upon rehydration was greatly reduced (Allison et al., 2000). A combination of a disaccharide (sucrose or trehalose) and dextran was shown to improve the stabilisation of the Actin through freeze drying and subsequent rehydration. It was proposed that the disaccharide protected the protein from unfolding during the drying process and dextran, by raising the Tg of the amorphous formulation, prevented the aggregation of the protein during storage and rehydration (Allison et al., 2000).
1.10 Disaccharides as excipients for protein stabilisation

It is clear from Section 1.9 that most successful studies into the stabilisation of proteins upon drying and subsequent storage have involved the use of disaccharides such as sucrose and trehalose. Disaccharides have been shown to protect proteins from denaturation in the final stages of drying but the mechanism of this protection remains a matter of contention (Carpenter and Crowe, 1988) and will be discussed in Section 1.11.

1.10.1 The Trehalose Anomaly

The disaccharide, trehalose has received particular attention with respect to its biostabilisation properties because of its abundance in nature in organisms that are able to withstand environments of extreme dehydration (for the structure of trehalose, see p71). Trehalose has been implicated in the lyoprotection of membranes (Crowe et al., 1984), liposomes (Crowe and Crowe, 1988) and proteins (Carpenter and Crowe, 1988) and has been highlighted as having an increased propensity to stabilise proteins upon drying (e.g. Carpenter and Crowe, 1988; Crowe et al., 1990; Duddu and Dal Monte, 1997a; Terebiznik et al., 1998). Sun and Davidson (1998) ascribed the superior qualities of trehalose to the unique properties of trehalose glass (low free volume, restricted molecular mobility and resistance to phase separation).

Roser (1991) also stated that trehalose is unique in its biostabilising capabilities however Levine and Slade (1992) disputed these claims. These researchers claimed that rather than being unique, the properties of trehalose were simply unusual, and that by combining similar low molecular weight mono- and disaccharides (with lower glass transition temperatures than trehalose) with higher molecular weight carbohydrates such as dextrans, comparable biostabilisation results could be achieved.

Although the superiority of trehalose as a biostabilising agent over other sugars remains a subject of debate, it is clear that trehalose is unusual with respect to its high Tg considering its low molecular weight and the fact that it is often the disaccharide observed in high concentration in anhydrobiotic organisms. For these reasons it is worthy of further investigation to determine the mechanism of its biostabilising action and it is the main subject of this thesis. The properties of trehalose are described in greater detail in the introduction to Chapter 3.
1.11 Mechanisms of Protein Stabilisation by Disaccharides

Different mechanisms have been proposed to describe the protection of the native structure and activity of proteins by glassy carbohydrates in solution and upon drying. These mechanisms are described in the following sections. Arakawa et al. (2001) reviewed the factors affecting the short-term and long-term stabilities of proteins. This review provides a useful breakdown of the stresses placed upon proteins during processing and the corresponding hypotheses for the mechanisms of stabilisation of stabilising additives, including carbohydrates. The mechanism proposed for the stabilisation of proteins by glassy disaccharides during freezing (prior to drying in the lyophilisation process) is similar to that described for proteins in solution.

1.11.1 Protein Stabilisation in Solution

Proteins in solution can become denatured by changes in the environment such as extremes of pH or high temperature or by chemical means such as oxidation, hydrolysis and deamidation. A common problem with protein solutions is the tendency of proteins to aggregate and precipitate, causing denaturation and hence loss of activity (Franks et al., 1991). Protein degradation in solution is something that must be considered regardless of the method of drying (freeze or spray drying), because proteins must be dissolved in a solvent prior to the drying process in order to produce an amorphous product.

Timasheff and colleagues proposed and proved a mechanism (the ‘Timasheff Mechanism’) for the stabilization of proteins in solution by various additives (ligands) such as disaccharides (Timasheff, 1992). Thermodynamically, a protein in solution exists in equilibrium between its native and denatured state. Under ‘normal’ conditions at room temperature, the protein will favour its native state because it has lower free energy than the denatured state. If a ligand binds to either the native or the denatured form of the protein, the free energy of that state will be lowered. The lower energy state in any system will always be favoured over the higher energy state. According to thermodynamics, a ligand will only bind to a protein if the ligand-protein complex has a lower free energy value than the protein alone. If the protein alone had a lower free energy value than the ligand-protein complex, binding would not occur because this would not be thermodynamically favourable. In the case of a stabilizing additive (ligand) for proteins, Timasheff and colleagues found the ligand to be ‘preferentially excluded’ from the surface of the protein. This means that the protein is preferentially
hydrated by the solvent (water). The ligand is said to have ‘negative binding’ to the protein, meaning that instead of the free energy of the protein being lowered by binding, the free energy in the preferentially hydrated state is increased. As there is a greater degree of ‘preferential exclusion’ of the ligand from the surface of the denatured state of the protein as opposed to the native state, the native state has lower overall free energy than the denatured state. The lower energy state is the more thermodynamically favourable and so more of the protein will exist in its native form than in the denatured form, resulting in overall stabilisation of the protein in solution in its native, active conformation. A diagram to illustrate the ‘Timasheff Mechanism’ of protein stabilisation in solution is given in Figure 1.2. Binding of a non-stabilising solute to the protein in solution is shown to cause unfolding of the protein and therefore its denaturation (labelled A on Figure 1.2). Preferential exclusion of a stabilising solute from the protein surface promotes maintenance of the native structure of the protein and therefore, stability (labelled B on Figure 1.2). A change to the native conformation of a protein can lead to a reduction or total loss of the biological activity of the protein; therefore maintenance of the protein in its native conformation is fundamental to the successful formulation of proteins into an appropriate dosage form (Prestrelski et al., 1993a).

**Figure 1.2.** Illustration to depict the preferential binding of solute molecules to a denatured protein (A) and the preferential exclusion of solute molecules from a native, folded protein as per the Timasheff Mechanism (B). Adapted from Crowe et al. (1990).

### 1.11.2 Protein Stabilisation upon Drying

During the initial removal of bulk water from the system, proteins are subject to the denaturing stresses of increased solute concentration, contact with the air-water interface and possible intermolecular interaction with protein molecules that may
already have become unfolded (Crowe et al., 1998). The mechanism of protein stabilization by additives during this initial stage of drying is thought to be the ‘Timasheff Mechanism’ as described for proteins in solution (Section 1.11.1).

In the final stages of drying, the hydration layer surrounding the protein molecule is partially or wholly removed and the level of moisture retained within the dry protein is not sufficient to maintain a full hydration layer. When this hydration layer is removed, the protein is no longer able to retain its complicated native structure and so becomes unfolded (Prestrelski et al., 1993a). It has been shown that the ability of the stabilizing additive (e.g. disaccharide) to retain the protein in its native structure upon removal of the hydration layer is directly related to the recovery of protein activity on rehydration of the dried solid (Prestrelski et al., 1993a and 1993b). Most of the solutes that confer stabilization to proteins in solution or during the freeze-thawing process do not seem to offer any protection to proteins during desiccation (Arakawa et al., 2001), rather it seems that the ability to confer stabilisation to proteins upon the complete removal of water is peculiar to disaccharides alone. There are several hypotheses as to the mechanism of stabilisation of proteins exhibited by disaccharides, including ‘The Glassy Matrix Theory’, ‘The Water Replacement Hypothesis’ and theories relating to the desiccation or bonding within the amorphous protein-disaccharide system upon exposure to moisture, or the ability of the disaccharide to take part in the Maillard reaction. These hypotheses will be discussed individually in the following sections.

### 1.11.3 The Glassy Matrix Theory

There appears to be agreement among research groups that the disaccharide must be in a glassy state with the protein in order to confer adequate protection from drying stresses. However the mechanism of this protection remains a matter of debate.

- **Evidence in favour of the ‘Glassy Matrix Theory’**

It was Franks et al. in 1991 who proposed that the proteins are simply ‘fixed’ in the glassy matrix of the disaccharide upon drying, thus preventing molecular mobility, unfolding of the protein or intermolecular interactions.

Green and Angell (1989) observed that the Tg of trehalose in the trehalose-water phase diagram was higher at all water contents than other saccharides (glucose, maltose and sucrose). As trehalose is known for its role in anhydrobiosis, these workers proposed
that the high Tg of trehalose could be related to its mechanism of biostabilisation. This proposal was supported by Crowe et al. (1996), who also proposed that the superior efficacy of trehalose for stabilisation of dry biomaterials is related to the observation that amorphous trehalose has a higher Tg at all water contents when compared with sucrose (Crowe et al., 1996).

A study by Izutsu et al. (1994) involved the manipulation of mannitol content during freeze drying to investigate how its amorphicity affected the denaturation of the protein. When the mannitol remained amorphous in the formulation, protection of the protein, lactate dehydrogenase occurred on drying. However, with an excess of mannitol, its crystallisation was observed and the protein became denatured upon lyophilisation. This data highlighted the importance of the maintenance of the amorphous state of excipients for the protection of proteins during drying.

**Evidence against the 'Glassy Matrix Theory'**

Other researchers have disagreed with the 'Glassy Matrix Theory', because many proteins already exist in the glassy state (Angell, 1995) and do not readily crystallise. If it were simply the glassy state that was necessary to confer stabilisation, amorphous proteins alone would be stable upon drying, which is not the case. The role of 'vitrification' in anhydrobiosis was the subject of an extensive review by Crowe et al. (1998), who concluded that the glassy state of stabilising components in anhydrobiotes is not sufficient to cause the stabilisation exhibited upon extreme dehydration of such organisms. These researchers resolved that it must be a combination of vitrification and interaction (water replacement, as described in Section 1.11.4) that is responsible for the stabilisation of proteins/membranes by glassy carbohydrates observed in anhydrobiotic organisms.

Tanaka and colleagues (1991) investigated the effect of increasing the chain length of saccharides and dextrans in dried protein formulations. One would expect the glassy state to be formed more readily as saccharide chain length increased meaning that the greater the molecular weight of the additive, the better the protein stabiliser. However, these researchers found that the protective effect of saccharides decreased with chain lengths greater than 3 and the percentage denaturation of the protein increased with increasing molecular weight of dextran. These findings corroborate the theory that it is more than the glassy state alone that is responsible for protein stabilization by
Allison et al. (1998) investigated the effects of drying methods and additives on the structure and function of the protein, Actin and reached a similar conclusion. It was found that amorphous dextran alone (known to have a high Tg) failed to protect the structure of air-dried or freeze-dried actin. These researchers confirmed their conclusions in another study, in which the amorphous disaccharides, sucrose and trehalose were shown to protect actin upon drying, whereas dextran, having a higher Tg, did not (Allison et al., 2000).

Davidson and Sun (2001) investigated the potential of raffinose, a non-reducing trisaccharide to stabilise the protein, Glucose-6-phosphate dehydrogenase during lyophilisation. Different ratios of sucrose to raffinose were used as stabilising excipients in the formulations and it was found that despite the higher Tg of amorphous raffinose compared with amorphous sucrose, the formulations containing sucrose alone provided the most effective protection for the protein upon lyophilisation (Davidson and Sun, 2001). According to the glassy matrix theory of protein stabilisation, raffinose should have been the more effective stabilising agent compared with sucrose because of its higher Tg, however this was not the case.

Mazzobre and Buera (1999) were also able to conclude that the protective effects of glassy excipients cannot always be related to increased glass transition temperatures. The enhanced protective effect of disaccharides in the presence of cations compared with disaccharides alone could not be attributed to an increase in Tg.

The results of the studies described above suggest that there is more evidence in opposition of the ‘Glassy Matrix Theory’ of protection of proteins by amorphous disaccharides than there is in support. From this, it may be concluded that the mechanism of protection of proteins by amorphous disaccharides upon drying is more complex than that proposed by this theory.

**1.11.4 The Water Replacement Hypothesis**

A second proposal of how disaccharides confer protection to proteins on drying, based on correlated evidence, assumes that the carbohydrate and protein are in the glassy state and suggests that the carbohydrate binds to the polar residues of the protein in place of water molecules as the hydration shell is removed (Crowe, 1971). This theory has since been termed the ‘Water Replacement Hypothesis’, a description of which was given by
Crowe et al. (1993a and 1993b). Carpenter and Crowe (1989) investigated the feasibility of the water replacement hypothesis in a study of the interactions of carbohydrates with dried proteins through infrared spectroscopy. In this study, the researchers observed that when carbohydrates were dried with proteins, the extent to which the carbohydrate molecules were able to form intermolecular hydrogen bonds was reduced, because of hydrogen bonds formed between the carbohydrate and protein molecules. To a limited extent, carbohydrate molecules appeared to satisfy protein hydrogen-bonding requirements on the removal of the hydration layer. When dried alone, the infrared spectrum for the protein was shown to lack bands in the region corresponding to the hydrogen bonding of water to the carboxylate groups of the protein. When the protein was dried in the presence of a carbohydrate, the band representing hydrogen bonding was present, suggesting a stabilizing interaction between the carbohydrate and protein (Carpenter and Crowe, 1989).

- **Evidence in favour of the 'Water Replacement Hypothesis'**

A study conducted by Tanaka and colleagues in 1991 supported the water replacement hypothesis. In this research, as the molecular weight of the saccharide additive was increased in the protein formulation, its ability to stabilise the protein against the stress of dehydration decreased. It was suggested that this reduction was due to steric hindrance of the larger saccharide molecules prohibiting hydrogen bonding to the polar groups of the protein. The failure of maltodextrins and dextrans to stabilise proteins/cells fully during the drying process has been attributed to their inability to effectively hydrogen bond to the proteins, membranes or polymers by several other research groups (Taylor and Zografi, 1998; Allison et al., 1999; Cerutti et al., 2000). Taylor and Zografi (1998) observed that the tendency of carbohydrates to hydrogen bond with the polymer, PVP, correlated inversely with the Tg of the sugar. Thus, those carbohydrates having high molecular weight such as the maltodextrins, were shown to have a reduced tendency to hydrogen bond with the polymer, compared with those additives of lower molecular weight, such as sucrose or trehalose (Taylor and Zografi, 1998).

Prestrelski et al. (1993a) provided supporting evidence for the water replacement theory of protein stabilization by the investigation of the effects of lyophilisation on the polypeptide, poly-L-lysine. In the aqueous form, poly-L-lysine exists in three different conformational forms dependent on conditions of pH and temperature, the α and β
forms and the disordered structure. Upon drying, all three conformations of the protein revert to the β form. The preferred β conformation of the dried protein allows more intermolecular bonding within the protein structure, thus replacing those hydrogen bonds that are lost through the removal of the hydration layer, suggesting that hydrogen bonding is necessary for stabilisation.

Souillac et al. (2002) used calorimetric techniques to establish the existence of protein/carbohydrate interactions in the amorphous state. These researchers examined the enthalpy of solution of freeze-dried and physical mixes of four proteins with mannitol, sucrose or trehalose. Deviations from a linear relationship between the percentage of protein in the formulation and the enthalpy of solution were deemed indicative of an interaction between the protein and excipient. Physical mixtures of the dried proteins and excipients exhibited linear relationships between protein content and enthalpy of solution whereas freeze-dried formulations exhibited non-linear relationships. From this study and the examination of DSC data for the samples, Souillac et al. (2002) concluded that direct interaction between proteins and sucrose and trehalose existed to a greater extent than with mannitol and the most likely interaction was hydrogen bonding, in line with that proposed for the water replacement hypothesis.

Liao et al. (2004) investigated the stabilisation of freeze-dried and spray-dried lysozyme formulations, using glycerol, trehalose or sucrose as stabilising excipients. Using DSC and FT-IR, these researchers related the stabilising capacity of the excipients to their propensity to form hydrogen bonds with the protein. A relationship between the protein-stabilising capacities of the excipients upon drying and the glass-forming capacity of the excipients was not established, however it was stated that the glassy state could be useful to prevent aggregation and dissociation of the protein (Liao et al., 2004). Two previous studies by these researchers using FT-IR spectroscopy, circular dichroism and DSC were also concluded in support of the water replacement hypothesis of protein stabilisation by carbohydrates such as sucrose and trehalose upon freeze drying and spray drying (Liao et al., 2002a and 2002b).

Many research groups have provided evidence to indicate that sugars included in protein formulations as stabilising agents must be in the glassy state and be able to hydrogen bond with the protein upon drying (Prestrelski et al., 1995; Remmele et al., 1997; Kreilgaard et al., 1998; Allison et al., 1999; Cerutti et al., 2000; Pikal-Cleland and
The water replacement hypothesis has therefore become the most widely accepted hypothesis for the stabilisation of proteins by disaccharides upon drying.

**Evidence against the ‘Water Replacement Hypothesis’**

There is less evidence to discount the water replacement hypothesis of protein stabilisation upon drying than there is to corroborate it. Crowe *et al.* (1984), attempted to correlate structural data for various stabilising additives with their ability to stabilise membranes during extreme dehydration. These researchers examined the stabilising additives for the number and density of –OH groups available to form hydrogen bonds, and the position of these –OH groups (axial/equatorial). It was observed that these parameters had little correlation with the effectiveness of biopreservation. The striking example from their study was the comparison of the effectiveness of trehalose with cellobiose. Trehalose and cellobiose were shown to contain an identical number and density of –OH groups available for intermolecular bonding, with all of the groups in the equatorial position, however trehalose was shown to be more than twice as effective a stabilising agent for the maintenance of the integrity of membranes (Crowe *et al.*, 1984). Crowe *et al.* (1984) speculated that there was a lack of evidence to prove that the stabilising action of carbohydrates was due to water replacement in dry membrane/protein samples. It is important to note that one of the authors of this paper later went on to provide evidence for the water replacement hypothesis (Carpenter and Crowe, 1989) and so this research is somewhat outdated.

Belton and Gil (1994) investigated the interaction of trehalose with lysozyme upon freeze drying using FT-IR spectroscopy. These researchers were unable to concur with the conclusions proposed by Carpenter and Crowe (1989) that the protein and carbohydrate directly interacted in the dried state. Although they observed changes in the FT-IR spectrum of the lysozyme/trehalose sample compared with the spectra of the individual dried components, these researchers could not find evidence of trehalose/protein interactions in the spectra of the freeze-dried trehalose/lysozyme sample. They proposed that the effects observed in the spectrum of trehalose by Carpenter and Crowe (1989) (assigned to hydrogen bonding between the protein and carbohydrate), were not because of an interaction with the protein, but were because of the presence of water in the sample (Belton and Gil, 1994).
Other Theories of Protein Stabilisation by Carbohydrates

- **The Timasheff Mechanism (see Section 1.11.1)**

The 'Timasheff Mechanism' is used to describe the mechanism of stabilisation conferred by stabilising excipients to proteins in the primary stages of drying, when bulk water and the hydration layer of the protein are still present. It is generally not a mechanism thought to be possible in the dried state, because of the theories relating to interaction between proteins and stabilisers (e.g. Carpenter and Crowe, 1989, Arakawa et al., 2001). However, some evidence has been presented to support the 'Timasheff mechanism' for the protection of proteins by carbohydrates in the dried state. Belton and Gil (1994) contradicted the work of others suggesting that an interaction between proteins and carbohydrates exists in the dried state as a mechanism of protein stabilisation. From their analysis of freeze-dried lysozyme/trehalose powders by FT-IR spectroscopy, they proposed that the 'Timasheff mechanism' remained in the dried state. This proposal was based upon the observation that the protein in the freeze-dried lysozyme/trehalose samples more closely resembled the spectra of the protein in the hydrated state. It was thought that the trehalose, by being preferentially excluded from the protein surface, caused the concentration of remaining water in the sample surface of the protein, thus effecting hydration and therefore, stability (Belton and Gil, 1994). Changes in the spectrum of trehalose when co-freeze-dried with lysozyme were assigned to the dehydration of trehalose in the samples.

Tzannis and Prestrelski (1999a) discounted the proposal that the Timasheff mechanism is a possible mechanism of protein stabilisation upon drying of proteins. These researchers reported that if the protein, trypsinogen was spray-dried from a solution in which there was a high degree of preferential exclusion of the stabilising agent, a reduction in the stability of the protein in the dried state was observed. It was proposed that the cause of this degradation was the formation of sucrose-rich and protein-rich phases in the dried state, hence reducing the likelihood of potentially stabilising protein-sucrose interactions (Tzannis and Prestrelski, 1999a). This hypothesis was supported by a further study by these researchers (Tzannis and Prestrelski, 1999b).

- **Desiccation**

The origin of the seemingly superior ability of trehalose to stabilise dried proteins/liposomes is uncertain. Aldous et al. (1995) proposed several theories as to the stabilising action of trehalose, one of which was the crystallisation of amorphous
trehalose providing desiccation for the remaining amorphous phase. The removal of water from the remaining amorphous phase of the formulation prevents plasticisation of the system; therefore the Tg is maintained at a high enough temperature to ensure storage stability (Aldous et al., 1995). It was proposed that trehalose is able to crystallise and phase-separate in an amorphous product, dependent upon the amount of trehalose present and the water content of the sample. To this effect, it would be predicted that carbohydrates with the ability to form higher hydrates, such as iso-trehalose (synthetic β,β-trehalose, which forms a hydrate with four moles of water per mole of trehalose) or raffinose (a trisaccharide, which forms a pentahydrate upon crystallisation), would be better biostabilisers than those carbohydrates unable to form a hydrate or forming lower hydrates. This, however, was proven not to be the case by Davidson and Sun (2001) who reported that despite the higher Tg of the freeze-dried protein, glucose-6-phosphate dehydrogenase (G6PDH) with a mixture of sucrose/raffinose, the protein in samples containing sucrose alone had enhanced storage stability compared with samples containing a mixture of sucrose and raffinose. Sucrose does not crystallise to a hydrate; therefore it would be unable to remove water from the system through crystallisation in order to desiccate the remaining amorphous phase.

Crowe et al. (1996) supported the view of Aldous et al. (1995), in that they observed that amorphous trehalose in dried liposome formulations was in the form of α,α-trehalose dihydrate as well as the glassy form upon rehydration. They agreed that the sequestration of water molecules by trehalose, to effect its crystallisation upon rehydration of the liposome/trehalose preparation, was critical in maintaining the Tg of the rest of the sample and therefore the stability of the formulation. However, the effect of trehalose crystallisation on the stability of liposomes may not be necessarily mirrored in the stability of proteins, which are vastly more complex structures.

The proposal by Aldous et al. (1995) that the crystallisation of trehalose may be responsible for its stabilising properties was refuted by several reports of the crystallisation of amorphous sugars in dried protein formulations leading to degradation of the protein (e.g. Isutzu et al., 1994; Cardona et al., 1997; Sarciaux and Hageman, 1997; Kreilgaard et al., 1999). However, upon heating, Terebiznik et al. (1997) observed a rapid decrease in the activity of freeze-dried α-Amylase as crystallisation of trehalose in the samples to its dihydrate form occurred. The rate of protein deactivation reached a plateau upon further heating, indicating the stabilisation of the protein. It was
proposed that this stabilisation occurred because of water removal from the amorphous sample, caused by the crystallisation of trehalose to its dihydrate form and raising the Tg of the remaining amorphous phase. Upon melting of the crystallised trehalose after a further period of heating, the rate of protein deactivation increased. It was proposed that this acceleration occurred as water was released during melting of the trehalose dihydrate crystals, thus increasing the mobility of the remaining amorphous phase and consequently causing deactivation of the protein at a faster rate (Terebiznik et al., 1997). It was proposed that this released water could then serve to cause crystallisation of some or all of the remaining amorphous trehalose in the sample, thus again reducing the water content of the remaining amorphous phase, effecting reduced mobility and enhanced protein stabilisation.

Few research papers have specifically investigated the proposed desiccation action of trehalose dihydrate upon the remaining amorphous phase of dried protein/trehalose formulations; therefore the hypothesis of Aldous et al. (1995) remains contentious.

**Chemical reactivity**

Another proposal for the mechanism of protein stabilisation by carbohydrates upon drying was based upon the relative reactivity of carbohydrates in the Maillard reaction (Colaco et al., 1994). The Maillard reaction (non-enzymatic browning) involves the reaction of the carbonyl groups of reducing sugars with free amino groups of amino acids to form brown compounds with distinctive flavour and aroma. The outcome of a Maillard reaction will depend upon the sugars and amino acids available to react and the conditions of the reaction (e.g. temperature, pH).

Colaco et al. (1994) hypothesised that in addition to the more common theories of protein stabilisation in the dried state (i.e. the glassy matrix and water replacement theories), the chemical inertness of the carbohydrate may be important. To this end, they suggested that the superiority of trehalose as a stabilising agent for the long-term storage stability of proteins might be related to its chemical inertness. This proposal was logical with respect to the storage stability of the protein but less applicable to the mechanism of the stabilising action of carbohydrates during the process of dehydration. Another flaw to this hypothesis was that sucrose was stated as being more chemically reactive than trehalose (Colaco et al., 1994), however it has been reported to be nearly as effective, and in some cases, more effective than trehalose for the stabilisation of
proteins (e.g. Liao et al., 2002a). Terebiznik et al. (1998) also contradicted the hypothesis regarding the relationship of the biostabilising properties of carbohydrates with chemical reactivity. These researchers investigated the browning activity of α-amylase when freeze-dried in carbohydrate/PVP matrices. They concluded that the stabilising efficiency of the carbohydrates studied did not correlate with the browning observed in the samples, or the 'browning-inhibiting properties' (chemical reactivity).

1.12 Summary
The 'Water Replacement Hypothesis' (Crowe, 1971) and the 'Glassy Matrix Theory' (Franks et al., 1991) are generally thought to be the two most likely mechanisms through which disaccharides are able to stabilise proteins upon drying. It is however clear that a consensus has not been reached on the issue and there are outstanding questions regarding the relative efficacy of various disaccharides to provide such protection.

1.13 Aims of the Thesis
The overall aims of this thesis are:

- To investigate the use of the combined technique of dynamic vapour sorption and near infrared spectroscopy for the purposes of the solid-state analysis of spray-dried protein/carbohydrate formulations

- To enhance the understanding of the unusual properties of trehalose for the purposes of protein stabilisation in the dried state

- To provide evidence as to the mechanism of stabilisation provided for the model protein catalase in the spray-dried state by trehalose

Specific aims for each individual results chapter will be stated at the start of each chapter.
Materials and Methods
2 Materials and Methods

Materials

Table 2.1. Sources and specific information for materials used in the studies described in Chapters 3-6.

<table>
<thead>
<tr>
<th>Material</th>
<th>Source</th>
<th>Specific Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>α,α-Trehalose dihydrate</td>
<td>Sigma Aldrich, St. Louis, USA</td>
<td>D- (+) trehalose&lt;br&gt;Product code T-5251&lt;br&gt;Lot 111K3797&lt;br&gt;From <em>Saccharomyces cerevisiae</em>&lt;br&gt;C_{12}H_{22}O_{11}.2H_{2}O&lt;br&gt;Mwt: 378.3 g/mol</td>
</tr>
<tr>
<td>Bovine liver catalase</td>
<td>Sigma Aldrich, St. Louis, USA</td>
<td>Catalase from bovine liver&lt;br&gt;Product code C-9322&lt;br&gt;Lot 100K7270&lt;br&gt;EC 1.11.1.6&lt;br&gt;Approx. 1588 units/mg</td>
</tr>
<tr>
<td>Potassium dihydrogen orthophosphate</td>
<td>BDH Laboratory Supplies, Poole, England</td>
<td>KH_{2}PO_{4}&lt;br&gt;Product code 102034B&lt;br&gt;Lot A116725&lt;br&gt;Mwt: 136.09 g/mol</td>
</tr>
<tr>
<td>Di-sodium hydrogen orthophosphate</td>
<td>BDH Laboratory Supplies, Poole, England</td>
<td>Na_{2}HPO_{4}&lt;br&gt;Product code: 102494C&lt;br&gt;Lot F1018981&lt;br&gt;Mwt: 141.96 g/mol</td>
</tr>
<tr>
<td>Hydrogen peroxide 30% solution</td>
<td>Sigma Aldrich, St. Louis, USA</td>
<td>H_{2}O_{2}&lt;br&gt;Product code: H-0904&lt;br&gt;Lot 41K3442&lt;br&gt;31.3 % Assay&lt;br&gt;ACS reagent&lt;br&gt;Mwt: 34.01 g/mol</td>
</tr>
<tr>
<td>Phosphorus pentoxide 98%</td>
<td>Acros Organics, Geel, Belgium</td>
<td>C.A.S. 1314-56-3&lt;br&gt;Mwt: 141.94 g/mol&lt;br&gt;Lot 16215801</td>
</tr>
<tr>
<td>Purified water</td>
<td>Elga Option 4 water purifier</td>
<td>Purified water USF</td>
</tr>
</tbody>
</table>

Methods

2.1 Spray Drying

2.1.1 Introduction

Spray drying is the process of drying a feed solution by spraying it into a hot air stream to produce solid, dry particles. The technique is widely used in the food industry to dry unstable foodstuffs (e.g. milk) and is also used in the pharmaceutical and biochemical industries. The process of spray drying consists of the following stages:
• Atomisation of feed materials through feed nozzle into spray cylinder
• Contact of feed material droplets with hot air stream
• Drying of feed material droplets as the air stream passes through the apparatus and begins to cool
• Collection of dry particulate product

Parameters that can contribute to the characteristics of the final, dry product produced in the spray drying process are outlined in Table 2.2. These have been widely studied by researchers in attempts to optimise products for particular uses (e.g. Maa et al., 1997; Walton and Mumford, 1999; Ståhl et al., 2002).

Spray drying is particularly favoured by the pharmaceutical industry owing to the possibility of optimising product characteristics such as flowability, porosity, bulk density and moisture content (Corrigan, 1995). The technique has been used to dry pharmaceutical products such as vaccines, enzymes and antibiotics (Masters, 1991) and in the development of inhalation products, where optimisation of particle characteristics is especially important for the performance of the product. With the increased use of biotechnology to create novel drug therapies, more protein drugs have entered the pharmaceutical market. As proteins are generally unstable in solution, drying is a method by which these products may be stabilised (Franks et al., 1991).

Spray drying is a known method by which amorphous material may be produced (Hancock and Zografi, 1997). The amorphous state is inherently less stable than the crystalline form, having increased entropy, enthalpy and internal free energy (Duddu and Grant, 1995). However, the higher thermodynamic activity of the amorphous state can give rise to favourable characteristics applicable to the pharmaceutical industry, such as increased apparent solubility (Haleblian, 1975; Corrigan et al., 1984).

2.1.2 Instrumentation
A Buchi 191 mini spray dryer (Buchi, Switzerland) was used to spray dry all amorphous products in this thesis unless otherwise stated.

2.1.3 Experimental
The preparation of spray-dried trehalose formulations for the studies in Chapters 3 and 4 is described in the relevant chapters.
2.1.3.1 Production of Co-Spray-dried Catalase/Trehalose Formulations

Co-spray-dried catalase/trehalose formulations were prepared according to the 7-sample set plan outlined in Table 2.3. The appropriate mass of catalase was first dissolved in water and then the corresponding mass of trehalose added after initially being dissolved in water. Solutions were made to volume in volumetric flasks and stored over ice prior to spray drying.

A major problem encountered was the foaming of solutions on dissolving the catalase. To keep this to a minimum, a swirling technique was adopted to dissolve the catalase and the entire solution stored over ice to disperse the bubbles formed prior to making the solution to volume. The act of producing each formulation solution was undertaken in the same manner and as swiftly as possible to avoid the more rapid denaturation of the protein in solution.

Table 2.2. *Spray drying parameters and the product characteristics they may affect.*

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Possible Product Characteristics Affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inlet temperature (°C)</td>
<td>Particle size</td>
</tr>
<tr>
<td>Outlet temperature (°C)</td>
<td>Moisture content</td>
</tr>
<tr>
<td>Atomising air flow rate (normliter/h)</td>
<td>Particle morphology</td>
</tr>
<tr>
<td>Drying air flow pressure/rate (Bar)</td>
<td>Residual activity (biologicals)</td>
</tr>
<tr>
<td>Liquid feed:</td>
<td>Stability profile</td>
</tr>
<tr>
<td>• Rate (mL/min)</td>
<td></td>
</tr>
<tr>
<td>• Solution or suspension</td>
<td></td>
</tr>
<tr>
<td>• Excipient selection</td>
<td></td>
</tr>
<tr>
<td>• % Component composition</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.3. Formulation plan for co-spray-dried catalase/trehalose formulations.

<table>
<thead>
<tr>
<th>Formulation % Catalase Content</th>
<th>Catalase</th>
<th>Trehalose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>20</td>
<td>20%</td>
<td>80%</td>
</tr>
<tr>
<td>40</td>
<td>40%</td>
<td>60%</td>
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<tr>
<td>50</td>
<td>50%</td>
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<tr>
<td>60</td>
<td>60%</td>
<td>50%</td>
</tr>
<tr>
<td>80</td>
<td>80%</td>
<td>20%</td>
</tr>
<tr>
<td>100</td>
<td>100%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Total solids = 0.5% = 5 mg/mL.

Operating parameters for the co-spray drying of the catalase/trehalose solutions were based upon those used by Tzannis and Prestrelski (1999b) and are outlined in Table 2.4. The newer version of the Büchi 190 Mini Spray dryer, the Büchi 191 Mini Spray Dryer (Büchi, Switzerland) was used to spray dry the formulations. Validation of the feed pump was carried out in order to confirm the feed solution volume per minute corresponding to the setting (%) of the dryer. The volume of water per unit time was measured at a range of feed pump settings and a calibration curve produced.

Table 2.4. Operating parameters for the preparation of co-spray-dried catalase/trehalose powders using the Buchi 191 Mini Spray Dryer.

<table>
<thead>
<tr>
<th>Operating Parameters</th>
<th>Settings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inlet temperature (°C)</td>
<td>128-130</td>
</tr>
<tr>
<td>Outlet temperature (°C)</td>
<td>75-77</td>
</tr>
<tr>
<td>Feed rate (mL/min)</td>
<td>4</td>
</tr>
<tr>
<td>Pressure (bar)</td>
<td>3</td>
</tr>
<tr>
<td>Atomiser flow rate (normliter/h)</td>
<td>600</td>
</tr>
<tr>
<td>Machine settings (%)</td>
<td></td>
</tr>
<tr>
<td>- Aspirator</td>
<td>80</td>
</tr>
<tr>
<td>- Feed rate</td>
<td>18</td>
</tr>
</tbody>
</table>

Dry catalase/trehalose products were confirmed to be amorphous by X-ray powder diffraction (producing a halo pattern).

2.2 Dynamic Vapour Sorption and Near Infrared Spectroscopy

2.2.1 Introduction

Dynamic vapour sorption (DVS) and near infrared spectroscopy (NIRS) comprise a combined technique, currently unique to this research facility. Dynamic vapour
sorption is a gravimetric technique that is used primarily to investigate the water sorption behaviour of samples. The combined technique of DVS/NIRS was described by Lane and Buckton (2000) and has since predominantly been used to investigate crystallisation behaviour and solid-state transitions.

The near infrared (NIR) region of the electromagnetic spectrum is from approximately 750-2500 nm (13300-4000 cm\(^{-1}\)). Absorption bands in this region originate from overtones or combinations of fundamental bond vibrational movement. NIR spectroscopy is based upon the concept of vibrational spectroscopy, in which the atom-to-atom bonds in molecules vibrate with particular absorption frequencies. The frequency of vibration is determined by the properties of the atoms contributing to the bond. Different bond strengths or lengths due to atom characteristics therefore affect the frequency at which the absorption band (also referred to as peak in this thesis) may appear.

Absorption bands in the NIR spectrum originate from fundamental vibrations in the mid infrared region. Bonds between atoms in molecules vibrate with the absorption of light of a particular frequency, with resulting excitation to a higher energy level. Different energy level transitions for vibrations of different bonds give rise to different types of transitions. Absorptions in the NIR spectrum arise from overtones and combinations of fundamental vibrations (Bugay, 2001). Overtone and combination bands are essentially 'not allowed' in quantum mechanics (Ciurczak, 2001). In the classical view of diatomic harmonic bond oscillation, bond excitation of one energy level is allowed. These are described as fundamental vibrations in the mid infrared (MIR) region of the electromagnetic spectrum. In this model of harmonic oscillation, absorption in the near infrared is not possible. The quantum theory of anharmonic oscillation describes a model of bond vibration that is more applicable in practice. This model takes into account the fact that 'ideal' diatomic bonds rarely exist and that in vibration to higher energy states, bonds will not necessarily return to the ground state (lowest energy vibration) and may be disrupted (broken). This model describes the excitation of bonds across more than one energy level. For example, excitation from ground state to vibrational energy level 1 (v=0 to v=1) illustrates a fundamental frequency in the mid IR region whereas excitation from v=0 to v=2 corresponds to the first overtone of the fundamental frequency, shown in the near infrared region (Figure 2.1). Owing to the 'illegal' nature of overtones and combination bands in quantum theory, bands rather
than specific wavelengths are often assigned to particular bond vibrations, as these peaks cannot be defined precisely.

Overtone peaks generally arise from X-H stretching vibrations. This is due to the fact that overtones are generally much weaker than fundamental transitions and thus the weaker bond vibrations, such as bending or rotating, would be in the third or fourth overtone in the NIR spectrum, due to the relative low strength of the original fundamental vibrational band (Ciurczak, 2001). Combination peaks arise from the combination of two or more (additive or subtractive) vibrations to give a single band (Ciurczak, 2001). These are also much weaker than the original fundamental vibration.

\[ v = 0, 1, 2, 3 \]

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{vibrational_energy_levels.png}
\caption{Vibrational energy levels for a diatomic molecule (Reproduced with permission from Jee, 2003).}
\end{figure}

Near infrared spectroscopy is becoming more popular in the pharmaceutical industry owing to the non-destructive nature of data collection and the development of better computer software designed to deal with mathematical manipulation of the data. It is becoming a preferred technique in the area of process control, where the development of NIR probes utilising fibre-optic technology have been developed for on-line process checks.

In the pharmaceutical field, near infrared spectroscopy as an individual technique has been used to determine the degree of crystallinity within physical mixes of crystalline
and amorphous substances (Seyer et al., 2000) and to detect polymorphism (Aldridge et al., 1996). It has also been used to quantify silica content and to discern the difference between silicified microcrystalline cellulose and silica/microcrystalline cellulose physical mixes (Buckton and Yonemochi, 2000) and to study of the differences of celluloses before and after wet granulation (Buckton et al., 1999). The combined technique of DVS/NIRS has been used in several published studies: to quantify low amorphous content in predominantly crystalline lactose (Hogan and Buckton, 2001a), to investigate the unusual crystallisation behaviour of raffinose (Hogan and Buckton, 2001b), to study unusual crystallisation processes and the water sorption behaviour of salbutamol sulphate (Columbano et al., 2002) and to investigate the crystal transitions of theophylline (Vora et al., 2004). As qualitative and quantitative analysis of NIR spectra can be performed, this technique has the potential to be a very useful tool in sample analysis.

2.2.2 Instrumentation

The DVS apparatus (Figure 2.2) consists of a humidity controlled ultra sensitive microbalance housed within an accurately controlled temperature chamber (DVS-1, Surface Measurement Systems, London, UK). The apparatus comprises identical sample and reference pans, made from quartz glass, housed within glassware within which the relative humidity (RH) is accurately controlled. Mass changes were measured to ± 1 x 10^-4 mg. Humidity control was achieved by the regulation of the flow of dry nitrogen gas into the apparatus. The desired relative humidity was attained through the mixing of dry and wet gas into the glass sample compartment of the apparatus. Switching valves were used to alternate the flow of nitrogen either directly to the sample and reference pans (0 % RH) or through a glass humidification vessel, containing a solvent reservoir (water). By mixing the gas from the ‘dry’ or ‘wet’ inlets in the correct proportions, the pre-determined relative humidity was reached. Humidity and temperature probes were positioned close to the sample and reference pans to give an independent reading of the system parameters. The DVS experiment was controlled via a remote computer using the DVS-1 software (Surface measurement systems, London, UK). This software allowed the RH within the DVS apparatus to be controlled between 0-98 % RH. A series of time-controlled RH ramps could be pre-programmed or alternatively, a method could be prepared by which the mass change over time (dm/dt) was required to stabilise before the next RH ramp was initiated.
Adaptations were made to the DVS apparatus to allow the simultaneous collection of near infrared spectra during experiments. A specially adapted optical reflectance near infrared probe (Foss NIRSystems, Maryland, USA) was positioned approximately 4 mm beneath the quartz, flat-bottomed sample pan of the DVS instrument, thus enabling NIR spectra to be collected during the DVS experiment. The Vision® software (version 2.21) was used to allow timed acquisition of NIR spectra throughout the DVS experiment and for data analysis. Spectra were recorded as log 1/R where R is reflectance; each spectrum being a mean of 32 scans recorded over approximately 40 s.

Figure 2.2. Photograph of the Dynamic Vapour Sorption (DVS) apparatus, with the fibre-optic near infrared probe in place [1 - fibre optic cable for the near infrared probe; 2 - entry of the near infrared probe into the DVS apparatus; 3 - dome protecting the ultra sensitive microbalance; 4 - Glass casing held together by a metal clip, enclosing the reference pan (right) and the sample pan (left identical casing); 5 - humidity probe (one on each side); 6 - solvent reservoir humidification vessel; 7 - Outer casing of temperature controlled incubator (door shown as open).]
2.2.3 Experimental
The reference pan remained empty during experiments whilst the sample pan contained sample masses of between 25 – 70 mg. Between experiments the sample pan was rinsed with distilled water followed by absolute alcohol and returned to the DVS apparatus to dry under a 0 % RH atmosphere. After approximately 10 min the DVS balance was tared and the baseline examined for static. If static appeared on the baseline, sample and reference pans were exposed to 95 % RH for approximately 15 min. Over this period at 95 % RH, the balance trace was monitored and when stable, the RH was restored to 0 %. The balance was then once again tared and monitored again for 10 min to ensure a steady baseline (± 0.001 mg).

2.2.4 Calibration
2.2.4.1 Microbalance Calibration
A weight calibration was performed once every month, if the instrument was moved, turned off, if the temperature of the incubator was changed or if the sample or reference pans were replaced. The DVS microbalance with empty sample and reference pans was tared and a 100 mg calibration weight (class M) placed onto the sample side. Any difference between that shown by balance and that expected was registered and taken into account by the DVS software.

2.2.4.2 Relative Humidity
The relative humidity of the DVS apparatus was validated every three months. The validation method relied upon the principle that the vapour pressure of water above a saturated salt solution in equilibrium with its surroundings is constant at a particular temperature. Saturated salt solutions of sodium chloride and lithium chloride were produced according to the method of Nyqvist (1983). An amount of the saturated salt solution with crystals was placed on the sample side of the DVS microbalance. An experiment was set up whereby the RH was ramped by 1-2 % per hour over a 10% RH range around the critical RH quoted for each salt in the literature (Nyqvist 1983). By plotting the change of mass with time, the critical RH of the salt could be calculated, allowing the RH of the system to be calibrated.

This method only accounted for validation of the system RH. A service engineer carried out calibration of the RH probes on a yearly basis because no in-house facility for this existed.
2.2.4.3 Near Infrared Spectroscopy

The NIR optical reflectance probe required calibration every month or when the NIR lamp was turned off. A reference spectrum of a ceramic disc was taken when necessary and this spectrum was used as a background against which experimental data were recorded. Wavelength linearisation and a performance test were performed at intervals using the Vision® software. Any failure of such tests led to the attention of an NIRS service engineer.

2.3 X Ray Powder Diffraction (XRPD)

2.3.1 Introduction

In the production and processing of crystalline material, disruption of crystal structure may occur, leading to disordered, amorphous regions. These small regions of disorder can affect the behaviour of the material as a whole and so it is vital to be able to quantify the extent of disorder within the bulk. X Ray Powder Diffraction (XRPD) is a technique that has been used primarily to assess quantitatively, mixtures of different crystalline forms through a range of approaches (Stephenson et al., 2001). To a lesser degree, XRPD has also been used to quantify the degree of crystallinity of samples (Klug and Alexander, 1974), an example of which was the estimation of the degree of crystallinity in digoxin samples (Black and Lovering, 1977). More accurate methods of quantitatively assessing crystallinity have since been favoured, such as differential scanning calorimetry (Guinot and Leveiller, 1999), isothermal microcalorimetry (Briggner et al., 1994; Sebhatu et al., 1994; Mackin et al., 2002; Darcy and Buckton, 1998), solution calorimetry (Hogan and Buckton, 2000) and water vapour sorption (Saleki-Gerhardt et al., 1994; Mackin et al., 2002). These methods have been preferred due to their specificity for measuring the crystallisation or thermal behaviour of the highly reactive region of disorder of the sample alone, as opposed to XRPD, which measures the amount of disorder through the bulk. The lower the amount of amorphous material within the sample, the less sensitivity XRPD has, meaning that its quantification detection limit is approximately 10 %, compared with other techniques that can quantify disorder to as low as approximately 0.5 %. Although other techniques have overshadowed XRPD in the determination of crystallinity, it is still widely used for this purpose as an additive technique in materials characterisation and remains an important tool in the analysis of crystalline forms.
2.3.2 Instrumentation
A Philips PW3710 X-Ray Powder Diffractometer (Philips, Cambridge, UK) was used in all studies.

2.3.3 Experimental
Sample (< 1 g) was loosely filled into the shallow well of the XRPD sample holder. A small purpose made block of Perspex (made in-house for the purpose) was used to press the sample carefully into the sample holder cavity to create a smooth, level, finished powder surface. Any excess sample surrounding the circular sample holder cavity was removed using a piece of tissue or cotton bud swab. The powder bed achieved by this method measured a depth of approx. 2 mm.

The sample holder was loaded into the diffractometer and scanned between 5-50 °2θ Samples were measured at 45 kV and 30 mA.

2.4 Thermogravimetric Analysis

2.4.1 Introduction
Thermogravimetric analysis (TGA) is a thermal analysis technique that is commonly used in the pharmaceutical industry to assess the water content of, or to analyse phase changes within, samples. TGA measures the magnitude and rate of change of mass of a sample as a function of time and temperature. It can thus be used to monitor the phase changes as a result of dehydration of a sample prior to decomposition and those that may be due to chemical reactions occurring within the sample. Sample mass behaviour can be assessed when subjected to ramped temperature experiments or under isothermal conditions, all carried out in a controlled atmosphere.

2.4.2 Instrumentation
A TGA 2950 Thermogravimetric Analyser (TA Instruments, Surrey, UK) was employed for all studies. The TGA operates on a 'null balance' principle, meaning that the weight signal generated by a change of mass in the sample pan is based upon the production of an unbalanced signal within the balance. The balance arm is held in a horizontal position ('null position') through optical control within the balance mechanism. A flag positioned centrally on the balance arm blocks an equal amount of light (supplied by a current infrared LED) to each of two photodiodes. If mass is lost or gained from the sample pan, causing movement of the balance arm, the light beam
becomes partially blocked, causing an unequal amount of light to strike the photodiodes. This creates an unbalanced signal within the circuitry, which is accounted for and the balance arm returned to its null position. The change in current within the control circuitry to allow return of the balance arm to the null position is directly proportional to the change of mass of the sample and is converted into a mass signal. 100 µl platinum sample holders were used on both the sample and reference sides of the balance and open aluminium sample pans (Perkin Elmer cat. No 0219-0062) were used.

Sample temperature is measured by a thermocouple located above the sample pan. The thermocouple also measured the heating rate within the TGA furnace.

2.4.3 Experimental
TGA was used in all studies primarily to measure the water content of samples and to assess the proportions of free or bound water within the sample structure.

Between 5 – 10 mg of sample was used for each TGA experiment. Experiments were run at a ramp rate of 10 °C/min from room temperature to above the melting point of the sample. Experiments were run in triplicate.

2.4.4 Calibration
Two calibration procedures were carried out on a regular basis: weight and temperature.

2.4.4.1 Weight Calibration
A weight calibration was carried out once a month during continuous use and 100 mg and 1 g calibration weights were used to calibrate the weight signal. Weight calibration was carried out following the specific instructions within the TGA software. A sample pan was tared and according to directions in the software, the 100 mg and 1000 mg calibration weights were loaded. Any difference between the weight shown by the balance and that expected was registered and taken into account by the TGA calibration software.

2.4.4.2 Temperature Calibration
Calibration of the thermocouple was carried out on the same day as the weight calibration where possible. This calibration was repeated monthly, or if the heating rate, purge gas flow rate or thermocouple position was changed. This calibration relied
on the melting point of the high purity metal indium. The observed melting temperature of indium was found by analysis of the derivative of temperature versus time curve of a temperature ramping experiment run at the same scan rate as that to be used in sample runs. The observed and expected melting temperatures of indium were entered into the calibration software. Any difference between the observed and expected values was registered and taken into account.

2.5 Scanning Electron Microscopy

2.5.1 Introduction

Scanning electron microscopy is a technique that enables the visualisation of surface structures down to sizes as small as 1 μm. It can be used to size very small particles although such sizing may be subjective and relies on homogeneity of the sample. Scanning electron micrographs produced by this technique appear almost three dimensional due to the large depth of field this technique allows. This makes it particularly useful in the analysis of particle morphology and crystal form.

2.5.2 Instrumentation/Experimental

Following mounting onto adhesive carbon discs attached to SEM stubbs, samples were coated with gold by sputtering for 4 min at 30 mA (Emitech K550 sputter coater, Emitech, Kent, UK). A Philips L20 SEM (Philips, Eindhoven, Netherlands) was then used to obtain scanning electron micrographs of samples, the voltages and scales of which are shown on individual micrographs.

2.6 Differential Scanning Calorimetry

2.6.1 Introduction

Differential scanning calorimetry (DSC) is a thermal analysis technique in which the difference in the rate of heat flow supplied to the sample and reference is measured whilst they are exposed to temperature change. Two types of DSC are available, heat flux DSC and power compensation DSC. Heat flux DSC consists of a single furnace containing the sample and reference. The temperature difference between sample and reference is measured and a calorimetric calibration is required to assign the actual heat flow rate difference. Power compensation DSC consists of two furnaces, one each for the sample and reference. Each furnace has its own heater and temperature sensor. Control of heat supplied to the furnaces is by a control loop that ensures that the temperature difference between both furnaces remains at its minimum. A residual
temperature difference is always kept between furnaces, which on its own relates to the difference in heating power between the heaters for sample and reference. When a sample undergoes a transition, this produces an endothermic or exothermic response, requiring power compensation from the furnace heaters to ensure that the temperature difference between furnaces remains at its minimum. The compensatory power supplied is directly proportional to the difference in heat flow rates to the furnaces and therefore to the heat flow rate of the transition occurring within the sample. Power compensation DSC was employed in the studies in this thesis.

DSC can be used to measure a range of thermal events that may take place within a sample, such as vaporisation (exothermic), crystallisation (exothermic) or melting (endothermic). Glass transitions, demonstrated by a change in the heat capacity of the sample are observed as a change in the baseline of the temperature/heat flow graph. The sensitivity of DSC to detection of these transitions depends upon several parameters such as the mass of the sample used, the heating scan rate and the thermal history of the sample. The experimental method can be optimised to improve the resolution of transitions. For example, using a high scan rate may improve the sensitivity of the technique to detect glass transition temperatures or for the resolution of the complex transitions of polymorphs (McGregor et al., 2004), whilst a slower scan rate may give higher resolution in terms of higher temperature melting peaks but may not be sensitive enough to detect a glass transition temperature.

2.6.2 Instrumentation

A DSC 7 Differential Scanning Calorimeter (Perkin-Elmer Instruments, Beaconsfield, Bucks, UK) was employed in thermal analysis studies requiring lower temperature scan rates. The Pyris 1 Differential Scanning Calorimeter (Perkin-Elmer Instruments, Beaconsfield, Bucks, UK) was used in studies requiring higher temperature scan rates. Pyris® software (Version 3.8) was used with both instruments.

2.6.3 Experimental

Perkin-Elmer aluminium non-hermetically sealed pans (cat. no. 0219-0062) were used, with sample mass of between 2-5 mg (accurately weighed). Sample preparation was particularly important for ‘ideal’ data to be attained from this technique. The contact surface between the sample and the sample pan was particularly important, with a thin layer of sample loaded into the bottom of sample pans prior to crimping. Crimped pans
were checked to be flat prior to loading into the sample holder to ensure maximum contact between the pan and furnace. An empty crimped sample pan was used as a reference in all experiments. A nitrogen purge (20 mL/min) was used during all experiments.

2.6.4 Calibration

The DSC was calibrated using high purity metals, with known temperature and energy transitions (see Table 2.5). Two metal standards were used for every calibration. Data for the melting transition was determined for each metal by running a scanning experiment at the same heating rate at that to be used in subsequent work. The observed and expected melting transition information (temperature and enthalpy of fusion) was entered into the calibration software. Any difference between the observed and expected values was registered and taken into account. Calibration was carried out whenever the heating rate was changed, the instrument was turned off, or otherwise every two weeks. Calibration checks using indium were carried out regularly during normal use.

Table 2.5. Melting points and enthalpy value(s) for high purity metal standards used in calibration of the DSC.

<table>
<thead>
<tr>
<th>Metal Standard</th>
<th>Onset of Melting Point (°C)</th>
<th>Enthalpy of Fusion (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indium</td>
<td>156.60</td>
<td>28.45</td>
</tr>
<tr>
<td>Zinc</td>
<td>419.47</td>
<td>-</td>
</tr>
<tr>
<td>Lead</td>
<td>327.46</td>
<td>-</td>
</tr>
</tbody>
</table>
Chapter Three
3 The Application of Dynamic Vapour Sorption and Near Infrared Spectroscopy in Combination to Examine the Physical Forms of the Model Carbohydrate, Trehalose

3.1 Introduction

The number of polymorphic forms that a substance can assume can greatly affect its behaviour. This fact has long since been acknowledged in the field of pharmaceutical science and knowledge of the various states in which an excipient can exist is vital for the prediction of product shelf life and performance. This is particularly important where drug formulations are concerned, when a significant change in form can greatly affect the therapeutic efficacy of the product.

As discussed in Chapter 1, disaccharides have been shown to be particularly successful in the protection of proteins from drying stresses, but trehalose in particular seems to have unique properties for this purpose (Roser, 1991). In an overview by Colaco et al. (1994), stability studies of a restriction enzyme, \textit{Pst} I when vacuum-dried with various excipients, showed that \textit{\alpha,\alpha}-trehalose was a better stabilising excipient than the wide range of monosaccharides, disaccharides and polymers tested. The superiority of trehalose as a biostabiliser is thought to be related to its remarkable in vivo action, where it is found in high concentrations in many anhydrobiotic organisms (Crowe, 2002). Upon rehydration, these organisms exhibit the ability to recover activity after experiencing conditions of extreme dehydration. There have been several theories to explain the mechanism behind the unique biostabilising properties of trehalose. One such theory is that it is related to the fact that trehalose is the only disaccharide to have an absence of intramolecular hydrogen bonding within its crystalline architecture (Aldous \textit{et al}., 1995). However, as trehalose in dried organisms is generally found to be in the amorphous state or supersaturated solution, the relevance of the internal bonding of the crystalline form is unclear. As most disaccharides exhibit the ability to bond with proteins, as described in the ‘water replacement hypothesis’ (Chapter 1), it is unlikely that the ability of trehalose to do this contributes to its superior stabilising qualities. A plausible suggestion for the remarkable qualities of trehalose was presented by Aldous \textit{et al}. (1995). These researchers rationalised the stabilising properties of dried amorphous trehalose by its ability to crystallise and phase separate, in real-time, when the dried product is stored above its \textit{Tg}. This, in effect, leaves an amorphous phase of the biological product/trehalose and a separate crystalline phase of trehalose dihydrate.
If this were the case, then trehalose in the dried product mixture would act as a desiccant, by removing water from the amorphous phase in order to effect its own crystallisation, thus increasing the Tg of the remaining amorphous phase. This desiccant action of α,α-trehalose dihydrate would theoretically enable biological products to be stored above their glass transition temperatures for extended periods of time. In their research, Aldous et al. (1995) commented that if their theory proved correct, then other sugars with the ability to crystallise to stoichiometric hydrates from the amorphous state would surely exhibit the same biostabilising properties as α,α-trehalose. This being so, it would be expected that those sugars able to crystallise to higher hydrates from the amorphous state, such as the trisaccharide, raffinose, which can form a pentahydrate (Saleki-Gerhardt et al., 1995), would be superior to α,α-trehalose in its stabilising ability. These sugars would provide an enhanced desiccating effect on the remaining amorphous phase within dried biological products due to their need for more molecules of water in order to effect crystallisation. In a study comparing the relative glassy properties of sucrose and trehalose, Crowe et al. (1996) found that increasing the water content of both amorphous sucrose and trehalose led to a significant reduction in the Tg of sucrose, whereas the Tg of trehalose remained high. They discovered that water added to amorphous trehalose tended to be used to form a trehalose dihydrate phase, thus ‘protecting’ the remaining amorphous phase from plasticisation and supporting the hypothesis of Aldous et al. (1995). Since the work of Aldous et al. (1995) and Crowe et al. (1996), little seems to have been published with regard to the biostabilising properties of those sugars able to form higher hydrates such as β,β-trehalose (4 mol water per mol trehalose) and raffinose, but much has been done to characterise further the polymorphic behaviour of α,α-trehalose.

Trehalose is known to exist in the amorphous and dihydrate forms as well as in three possible anhydrous forms. Perlin and colleagues (Reisener et al., 1962) reported two anhydrous forms produced from the dehydration of trehalose dihydrate (α and β anhydrous forms). These forms were later referred to as alpha and beta anhydrous trehalose (Tα and Tβ respectively, Sussich et al., 1998). Sussich et al. (1998) characterised a further anhydrous form (Tγ) through the thermal dehydration of trehalose dihydrate and went some way to determine the structural transformations of trehalose dihydrate under conditions of dehydration stress (Sussich et al., 2001, Figure
These researchers proposed that it might be the ‘extended capacity’ of trehalose polymorphism that may give it its unique properties of biostabilisation.

3.1.1 Polymorphic Forms of Trehalose

3.1.1.1 α,α-Trehalose Dihydrate (Tₐ)

α, α- Trehalose is a non-reducing disaccharide of glucose (α-D-glucopyranosyl-α-D-glucopyranoside). The structure of the native crystalline form of trehalose, α,α-trehalose dihydrate, is shown in Figure 3.2.

![Figure 3.1. Schematic outlining the transformation pathways of the polymorphic forms of α,α-trehalose. Adapted from Sussich et al. (2001).](image)

![Figure 3.2. Structure of the disaccharide α,α-trehalose (α-D-trehalose).](image)
Uniquely for a disaccharide, α,α-trehalose dihydrate exhibits an absence of intramolecular hydrogen bonding (Aldous et al., 1995). This form of trehalose is found naturally in many plants and insects. Sussich et al. (1998) described the dehydration of T_h using different heating rates in a DSC (Differential Scanning Calorimeter) to effect its dehydration. This produced very different DSC thermograms. A slow heating rate gave an endotherm for the dehydration of the dihydrate at ~100 °C leading to an anhydrous amorphous phase. A cold crystallisation was seen at ~180 °C followed by a melt. Higher scan rates led to the development of a crystallisation exotherm immediately followed by an endotherm, after the initial dehydration endotherm at ~100 °C. The data by Sussich et al. (1998) illustrate the variability of the dehydration of α,α-trehalose dihydrate, hence giving rise to anhydrous forms. It is not yet understood whether this behaviour may be related to the biostabilisation properties of trehalose.

3.1.1.2 Alpha Anhydrous Trehalose (T_a)

Holding a sample of α,α-trehalose dihydrate at 85 °C for 4 h under vacuum produces the alpha anhydrous form of trehalose, T_a (Reisener et al., 1962). T_a has been described as having the same molecular morphology as the dihydrate form of trehalose, due to hydrate water removal occurring slowly enough to prevent relaxation of the structure and hence form change (Sussich et al., 2001). However, this hypothesis was opposed by Nagase et al. (2002), who compared a computer-generated XRPD diffraction pattern for T_h devoid of crystal water molecules, to the XRPD diffraction pattern of T_a as described by Sussich et al. (1998). They concluded that the diffraction pattern of T_a did not match the theoretical pattern for the ‘dehydrated dihydrate’ and thus did not possess the same lattice parameters as T_h. However, it has been shown that T_a can be converted back directly into the dihydrate form by exposure to controlled relative humidity (i.e. 50 % RH for 24 h (Sussich et al., 2001)). DSC thermograms of T_a show an endotherm indicative of a melt of the anhydrous T_a solid at ~125 °C. A recrystallisation exotherm representing the conversion to T_b is observed immediately prior to an endotherm representing the melt of T_b above 200 °C (Sussich and Cesàro, 2000).

3.1.1.3 Beta Anhydrous Trehalose (T_b)

Holding a sample of α,α-trehalose at 130 °C for 4 h produces the crystalline beta anhydrous form of α,α-trehalose, T_b (Reisener et al., 1962). The beta form appears to
be the more stable of the anhydrous forms of α,α-trehalose identified thus far, having a high melting point shown as an endotherm on a DSC thermogram at ~215 °C and being the form to which Tα converts upon heating (Sussich and Cesàro, 2000).

3.1.1.4 Gamma Trehalose (Tγ)

The gamma form of α,α-trehalose was identified by Sussich et al. (1998). Tγ has only been produced in a differential scanning calorimeter through a process of cold crystallisation. Sussich et al. (1998) first described the formation of Tγ as follows:

- Upon heating in the calorimeter using a scan rate of 5-10 °C/min, Th dehydration is initiated
- Assuming that all of the water in the dihydrate is not removed at the same time, it is conceivable that an amorphous solid may be formed, containing the small amount of water remaining from the original dihydrate
- 'Cold crystallisation’ occurs at ~120 °C when the small amount of water that hasn’t already been removed in the heating process plasticises the amorphous solid above the Tg, producing a crystalline material, in this case, Tγ

Sussich et al. (1998, 2000) appear to be the only group to have reported the existence of Tγ thus far and the exact structure of this form has yet to be elucidated. Tγ is often referred to as an anhydrous form but is now thought to consist of a hydrated core of α,α-trehalose dihydrate and an external layer of the anhydrous Tβ form (Sussich and Cesàro, 2000). As such, this form of trehalose is in a metastable state. When subjected to heating in a DSC, Tγ undergoes a solid-solid transition at ~125 °C from Tγ → Tβ and then an endotherm for the melt of Tβ is observed at ~220 °C.

3.1.1.5 ‘Form II’

Belton and Gil introduced the anhydrous form of trehalose, Form II, in 1994. They described this form as a metastable form of trehalose, created by leaving α,α-trehalose dihydrate under vacuum for 48 h at 50 °C. In this paper, these workers described the FT-IR spectra of Form II compared with the spectra of freeze-dried trehalose alone and freeze-dried protein-trehalose formulations. They suggested that in dried protein-trehalose formulations, any remaining water might be concentrated at the protein-trehalose interface, thus causing dehydration of the trehalose bulk and altering its
conformation to an anhydrous crystalline form. Gil et al. further characterised Form II in a later publication (1996) using vibrational and solid-state NMR spectroscopy. The recognition of Form II as a new crystalline form of anhydrous trehalose was disputed by Taylor et al. (1998), who argued that the Raman spectrum of Form II was identical to that obtained through the dehydration of \(\alpha,\alpha\)-trehalose dihydrate particles of smaller particle size (< 45 \(\mu\)m). For this reason, they stated that Form II was not a new crystalline form but rather a disordered product of the dehydrated dihydrate. However, Akao et al. (2002) went on to describe the dehydration of \(\alpha,\alpha\)-trehalose dihydrate to produce Form II by supercritical CO\(_2\) fluid extraction under appropriate temperature and pressure conditions (80 °C and 20 MPa). They were in agreement with Nagase et al. (2002); that Form II was most probably identical to T\(_\alpha\) (see T\(_\kappa\) discussion in Section 3.1.1.6 for details).

### 3.1.1.6 Kappa Anhydrous Trehalose (T\(_\kappa\))

A further anhydrous form of \(\alpha,\alpha\)-trehalose, T\(_\kappa\) was reported by Nagase et al. (2002). This form was prepared from \(\alpha,\alpha\)-trehalose dihydrate by either heating under vacuum or by heating in hot air. T\(_\kappa\) was converted to an amorphous form at 127 °C. Nagase et al. (2002) showed that T\(_\kappa\) was very hygroscopic and was rapidly converted to the dihydrate form at 25 °C and 43 % RH. When T\(_\kappa\) was mixed with an amorphous form of trehalose and exposed to 43 % RH, water adsorption to the amorphous form was ten-fold slower than to the amorphous form alone. From this result, these researchers suggested that T\(_\kappa\) has a role in the bio-protective effect of trehalose in Anhydrobiosis. This hypothesis was based upon that by Aldous et al. (1995), who proposed that trehalose has the ability to crystallise and phase separate above its T\(_g\) in order to effect desiccation of the remaining amorphous phase. Nagase et al. (2002) appeared undecided over whether T\(_\kappa\) is an entirely new polymorphic form of trehalose. They showed that the XRPD pattern of partially hydrated T\(_\kappa\), (i.e. a mixture of T\(_\kappa\) and T\(_\beta\)) was similar to the XRPD pattern of Form II. These researchers also showed that T\(_\kappa\) had similarities to the alpha anhydrous form described (differently) in various papers by Sussich and co-workers (1997, 1998, 2000), thus they tentatively supposed that T\(_\alpha\), Form II and T\(_\kappa\) were identical. It seems that elucidation of the crystal structure of each form is necessary to determine the true similarities of these anhydrous forms.
3.1.1.7 Amorphous Trehalose (\(T_{\text{am}}\))
The properties of the glassy state of trehalose were investigated by Crowe et al. (1996). The amorphous state of trehalose is reported to have the highest glass transition temperature of all amorphous carbohydrates at around 115 °C (Crowe et al. 1996). Sussich et al. (1998) reported a \(T_g\) of amorphous trehalose of 120 °C whereas Roos (1993) reported a \(T_g\) for trehalose of 107 °C (midpoint). The differences are likely to be due to different water contents of the samples tested.

3.1.1.8 Dehydration behaviour of \(\alpha,\alpha\)-trehalose dihydrate
The dehydration behaviour of trehalose has been investigated in attempts to elucidate its mechanism of bioprotection. Taylor and York (1998) investigated the phase behaviour of different particle size fractions of trehalose dihydrate upon dehydration. They reported two possible routes through which trehalose anhydrate could be formed upon heating trehalose dihydrate. Small particles were shown to dehydrate and collapse into an amorphous phase that became a highly viscous liquid above its \(T_g\) (< 80 °C), thus allowing crystallisation into an anhydrate form. Larger particles were shown to convert to the anhydrous form through a solid-solid conversion directly from the dihydrate to the anhydrate form on removal of the dihydrate water through heating (> 80 °C). This conversion occurred at a higher temperature than the dehydration and collapse of the smaller particles because the larger particles are able to retain the dihydrate water to higher temperatures. These researchers did not, however, refer to any other \(\alpha\)-trehalose forms other than the dihydrate, ‘anhydrate’ and amorphous forms and dispute the suggestion of Form II being an anhydrous form.

3.2 Aims
As mentioned in Section 2.2, the combination of Dynamic Vapour Sorption analysis and Near Infrared Spectroscopy (DVS-NIRS) has been used to study the crystallisation behaviour of some pharmaceutical actives and excipients (Hogan and Buckton, 2001b, Columbano et al., 2002) and more recently, to examine the polymorphic forms and corresponding transitional states of theophylline (Vora et al., 2004). Data obtained from this combined method was used in the study described in this chapter, with the aim to realise a better understanding of the dehydration of \(\alpha,\alpha\)-trehalose dihydrate. It was hoped that DVS-NIRS would be a suitable method to detect changes in trehalose samples in real-time, as transitions occurred. The purpose of this chapter was to assign
peaks in the NIR spectrum for the various polymorphic forms of α,α-trehalose, with the objective to allow investigation of the behaviour of trehalose in its dehydrated form as an excipient in dried protein formulations. As the polymorphism of trehalose is thought to be central to its biostabilising action, further understanding of its solid state transitions may be important to allow better formulation of protein pharmaceuticals in the future.

3.3 Experimental

3.3.1 Production of Anhydrous Trehalose Forms

Two anhydrous forms of trehalose, alpha anhydrous trehalose (Tα) and beta anhydrous trehalose (Tβ) were produced according to the method described for the production of ‘a’ and ‘b’ anhydrous forms respectively by Reisener et al. (1962). Trehalose dihydrate (Tdh) was held under vacuum for 4 h at 85 °C to produce Tα and held for 4 h at 130 °C at ambient pressure to produce Tβ. Owing to confusion in the description of production of Tβ in the literature, trehalose dihydrate was initially held at 130 °C under vacuum to produce the beta anhydrous form. Although this method was subsequently changed and performed under atmospheric pressure, the vacuum-prepared sample was retained and named beta-vac anhydrous trehalose (Tpvac). ‘Anhydrous’ samples were immediately loaded onto the DVS quartz sample pan to allow collection of NIR spectra via the NIR probe as described in Section 2.2.

A further anhydrous form of trehalose was produced by holding trehalose dihydrate at 0 % RH (under nitrogen purge gas) and 25 °C for 28 h in the Dynamic Vapour Sorption (DVS) analyser. NIR spectra were recorded every 30 min during the dehydration to enable Near Infrared (NIR) peak changes to be tracked and for comparison with the spectra of other polymorphic forms. For ease of discussion this anhydrous form was named TΔ. In order to examine the reversibility of the production of this anhydrate, a sample of α,α-trehalose dihydrate was exposed to 0 % RH for 30 h followed by 75 % RH for 30 h and finally 0 % RH for 30 h.

Samples were checked for crystallinity using X Ray Powder diffraction analysis as described in Section 2.3 and water content was assessed using thermogravimetric analysis, as outlined in Section 2.4. DSC was carried out on samples as described in
Section 2.6 and results were compared with those in the literature. A scan rate of 10 °C or 200 °C per min was used with aluminium non-hermetically sealed pans (Section 2.6).

3.4 Results and Discussion

3.4.1 Production and characterisation of anhydrous forms of trehalose.

Anhydrous forms were produced as described in Section 3.3.1. NIR spectra, DSC and TGA thermograms and X-ray powder diffraction patterns will be presented for each polymorphic form of trehalose followed by NIR peak assignations for each form. A detailed analysis of the anhydrous form produced in the DVS apparatus is presented in Section 3.4.1.1. The preparation of amorphous trehalose was prepared as described in Chapter 4, Section 4.3.2.

3.4.1.1 Production and reversibility of T_d (DVS-anhydrous trehalose)

α,α-Trehalose dihydrate was subjected to a dehydration-rehydration-dehydration cycle in the DVS analyser as described in Section 3.3.1. The water sorption plot obtained for the process is shown in Figure 3.3. The product of each dehydration step was named T_d.

Figure 3.3. DVS plot for α,α-trehalose dihydrate exposed to 0 % RH for 30 h, 75 % RH for 30 h and 0 % RH for 30 h in order to cause dehydration, rehydration and dehydration respectively. Labelled points represent those time points from which NIR spectra discussed were collected.
NIR spectra (1800-2200 nm) from the labelled time-points (A-G) of the DVS plot in Figure 3.3 are displayed in Figure 3.4. These NIR spectra enable the time-points of form change to be more accurately pinpointed. Throughout the discussion, the term ‘peak’, when referring to near infrared spectra, will be used to describe downward pointing troughs corresponding to mathematically treated SNV normalised, second derivative spectra. Spectra from time-points A, D and E all showed a double peak characteristic of α,α-trehalose dihydrate at 1954/1978 nm and spectra were identical throughout the entire wavelength region 1100-2250 nm (data not shown). NIR spectra from time-points B, C, F and G were also similar to each other. NIR spectra between time-point A and B (Figure 3.5) showed gradual changes as water was removed from the sample, until time-point B, when the sample stabilised at a mass corresponding to that of an anhydrate form. Changes in the spectra were not limited to regions of the NIR spectrum related to water within the sample, indicating that the sample was undergoing structural change to compensate for the loss of the hydrate water. On average the mass change between the dihydrate and the anhydrate forms was 9.57 % (± 0.10 %), corresponding to the loss of two molecules of hydrate water from each molecule of trehalose. The second dehydration gave almost identical results to the first in terms of mass change (%) and time-periods corresponding to form changes.

![Figure 3.4. NIR spectra from specified time-points of the DVS plot in Figure 3.3.](image-url)
The water sorption plot of the dehydration-rehydration-dehydration of Th shows that the dehydration of α,α-trehalose dihydrate to an anhydrate form took approximately 25 h to complete (shown between points A and C on Figure 3.3). The rehydration of the anhydrate form, Tₐ to the dihydrate form took less than 0.5 h. The dehydration of the newly formed dihydrate took approximately 25 h to complete, mirroring the initial dehydration step. This behaviour is consistent with the description of the formation and rehydration of an isomorphic desolvate by Stephenson et al. (1998). In this paper, an isomorphic desolvate was described as retaining the crystal lattice structure of the parent solvate form on removal of solvate molecules. Isomorphic desolvates have extreme hygroscopicity and thus rapidly rehydrate in conditions of increased relative humidity to become restored to the original parent solvate form (Stephenson et al., 1998). Desolvate (anhydrate) forms generally have a higher overall free energy compared with parent solvate (hydrate) forms and are therefore less stable (Hancock and Shamblin, 1998). This being so, it would be expected that the rehydration of the desolvate into the solvate form would be a less energetic process than the dehydration of the solvate to the desolvate. The dehydration (desolvation) of Th shown in Figure 3.3 took a longer period of time compared with the rehydration of the anhydrate (desolvate) form, suggesting that either more energy was associated with the dehydration of Th compared
with the rehydration of the anhydrate, or that the kinetics of desolvation process were slower. In a review by Hancock and Shamblin (1998), the dehydration and rehydration of a parent hydrate molecule to an anhydrate retaining the crystalline structure of the parent solvate was discussed. They described the ready addition or removal of hydration water through solvate tunnels in the crystal lattice structure in such isomorphous forms. The dehydration of crystal hydrates requiring hydrate water in order to maintain crystal lattice integrity would be expected to be slower than those hydrates in which solvate tunnels in the crystal structure facilitate easy removal of hydrate water. The dehydration of \( T_h \) to \( T_d \) took much longer than its rehydration, but as the rehydration rate may be dependent on the \% RH the sample was exposed to, this was not a conclusive contradiction of \( T_d \) being an isomorphous desolvate of \( T_h \). Stephenson et al. (1998) however, proposed that the rate of moisture sorption of an isomorphous desolvate is independent of \% RH and is limited principally by the rate of diffusion of water molecules into the solvent tunnels created by removal of the water of hydration.

Changes in the XRPD pattern of a crystalline sample upon desolvation are reported to be small (Stephenson et al., 1998). If \( T_d \) were indeed an isomorphous desolvate of \( T_h \), small but reproducible shifts in reflection to higher \( 2\theta \) values would be expected in the XRPD pattern of \( T_d \) compared with that of \( T_h \) (Stephenson et al., 1998). This would indicate a general trend towards a smaller unit cell volume and stabilisation of the dehydrated lattice. In order to assess whether \( T_d \) was an isomorphous desolvate of \( T_h \), comparison of the XRPD patterns for \( T_d \) and \( T_h \) was undertaken in order to assess similarities. Figures 3.6a and 3.6b show the XRPD patterns of \( T_h \) and \( T_d \). The XRPD pattern of \( T_d \) showed significant noise on the baseline, indicating that amorphous content was produced during the dehydration of \( T_h \) to \( T_d \). The superimposed peaks on the XRPD pattern of \( T_d \) are different to those on the XRPD pattern of \( T_h \) and the pattern is not a shift from that of \( T_h \). If \( T_d \) were an isomorphous desolvate of \( T_h \), the XRPD patterns of both would be very similar. It can therefore be concluded that \( T_d \) was not an isomorphous desolvate of \( T_h \). As the superimposed peaks on the XRPD pattern of \( T_d \) are different to those on the XRPD patterns of the alpha and beta anhydrous forms (Figures 3.7a, 3.7b and 3.8a), it can be proposed that \( T_d \) is a different anhydrous form to \( T_a \) or \( T_b \).
Figures 3.6a and 3.6b. XRPD patterns for samples of α-α-trehalose dihydrate and DVS-anhydrous trehalose respectively.
Figures 3.7a and 3.7b. XRPD patterns for samples of alpha anhydrous trehalose and beta anhydrous trehalose respectively.
Figure 3.8a and 3.8b. XRPD patterns for samples of beta-vac anhydrous trehalose and spray-dried amorphous trehalose (spray-dried from a 10% trehalose feed solution in water) respectively.
3.4.1.2 X-Ray Powder Diffraction

X-ray powder diffraction patterns for all of the polymorphic forms of trehalose prepared are displayed in Figures 3.6a, 3.6b, 3.7a, 3.7b, 3.8a and 3.8b.

\(\alpha,\alpha\)-Trehalose dihydrate (T\(_h\))

The XRPD pattern for the dihydrate crystal form of trehalose showed no halo effect indicating that it was a crystalline sample (Figure 3.6a). Peaks are fairly consistent with those seen in the literature for T\(_h\). However, exact peak positions are not stated in the literature, therefore making precise comparison difficult (Sussich et al., 1997, 1998; Nagase et al., 2002).

DVS-anhydrous trehalose (T\(_d\))

The XRPD pattern of the anhydrous form of trehalose produced in the DVS analyser is shown in Figure 3.6b and was discussed in Section 3.4.1.1.

Alpha Anhydrous Trehalose (T\(_\alpha\))

The XRPD pattern for T\(_\alpha\) showed a distinct ‘halo’ effect, with a rise of the pattern at around 20°2\(\theta\) (Figure 3.7a). The pattern showed superimposed peaks indicating that the sample was partially crystalline. The intensity of crystalline peaks was reduced compared with the peaks for T\(_\beta\) but peak angle assignments were still possible. Visual comparison of the XRPD pattern of T\(_\alpha\) to that in the literature (Sussich et al., 1998) showed peaks at approximately the same positions; however, the literature pattern showed no halo effect. Although partially amorphous, the sample remains useful for NIR spectral comparison with other polymorphic forms because of the superimposed crystalline peaks. As the NIR spectrum for the amorphous form was known, peaks for the amorphous form could be discounted when assigning NIR peaks to the alpha form.

Beta anhydrous trehalose (T\(_\beta\))

The baseline of the XRPD pattern for T\(_\beta\) was of poor quality and a rise in the pattern at ~ 20°2\(\theta\) suggested the presence of amorphous content in the sample (a small halo effect, Figure 3.7b). However, crystalline peaks were superimposed onto the baseline, suggesting that the sample was partially crystalline and partially amorphous. As this was the case for every anhydrous sample made, it may be that the amorphous form of trehalose was simultaneously formed in the dehydration process. The XRPD pattern
obtained did not match that in the literature with respect to the halo effect, but the peaks superimposed onto the baseline appeared to be similar to those reported (Nagase et al., 2002). This suggested that the sample was partially amorphous trehalose and partially the anhydrous form, $T_\beta$. Although this was not ideal, the $T_\beta$ proportion of the sample would generate absorption bands in the NIR region of the electromagnetic spectrum and therefore the sample would remain useful in terms of NIR peak analysis. As the amorphous trehalose NIR spectrum was known, peaks corresponding to the amorphous form could be discounted when assigning peaks to the beta anhydrous form.

**Beta-vac anhydrous trehalose ($T_{\beta\text{vac}}$)**

The XRPD pattern for the beta form of anhydrous trehalose prepared under vacuum rather than at atmospheric pressure showed very similar peaks to the pattern of $T_\beta$ described above. Crystalline peaks were superimposed onto an amorphous halo pattern; however, the halo effect of the pattern for the beta-vac sample was more pronounced than that of the beta form prepared at atmospheric pressure (Figure 3.8a). This observation suggested that the $T_{\beta\text{vac}}$ sample contained more amorphous content than the $T_\beta$ sample. The rate of water removal from the dihydrate crystal upon dehydration could be responsible for this result. A more 'gentle' removal of water from the dihydrate crystal lattice would be expected to allow the structural rearrangement of the lattice to compensate for water removal and thus maintain morphology. A faster rate of water removal, such as that under vacuum, would not allow time for the crystal lattice to compensate for water removal, thus allowing relaxation of the lattice structure into a different form. This suggestion was consistent with the observation of Sussich et al. (2001), who described the gentle removal of water molecules to produce the alpha form of trehalose, so that the lattice structure could not relax into a more compact form.

**Amorphous trehalose**

The XRPD pattern for amorphous trehalose exhibited the characteristic 'halo' effect demonstrated by amorphous material and had no crystalline peaks, as expected (Figure 3.8b).
3.4.1.3 Differential Scanning Calorimetry

A summary of the thermal transitions observed in all of the anhydrous samples is given in Table 3.1, followed by a discussion of the DSC data for each anhydrous form. The temperatures of the transitions were calculated using the Pyris® software (Perkin-Elmer, Version 3.8). The glass transition temperatures stated were calculated from the mid-point of the transitions whereas the temperatures all other transitions represent the peak maxima unless otherwise stated.

Table 3.1. Summary of thermal transitions for the proposed anhydrous forms of trehalose described in Section 3.4.1.3 obtained from DSC data (n=3).

<table>
<thead>
<tr>
<th>Anhydrous Form</th>
<th>Scan rate (°C/min)</th>
<th>Tg(°C)</th>
<th>Tam→Tβ (°C)</th>
<th>Melt (°C)</th>
<th>Tα→Tβ (°C)</th>
<th>Tβ Melt (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha (Tα)</td>
<td>10</td>
<td>?</td>
<td>~122 (Tα)</td>
<td>~170</td>
<td>210</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>?</td>
<td>125-130 (Tα)</td>
<td>~190</td>
<td>210</td>
<td></td>
</tr>
<tr>
<td>Beta (Tβ)</td>
<td>10</td>
<td>114</td>
<td>~170</td>
<td></td>
<td>208</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>126</td>
<td>~190</td>
<td></td>
<td>221</td>
<td></td>
</tr>
<tr>
<td>Beta-vac (Tβvac)</td>
<td>10</td>
<td>~115</td>
<td>~170</td>
<td></td>
<td>207</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>115-120</td>
<td>~190</td>
<td></td>
<td>217</td>
<td></td>
</tr>
<tr>
<td>DVS (Tδ)</td>
<td>10</td>
<td>~82</td>
<td>~125 (Tδ or Tα?)</td>
<td>~195</td>
<td>212</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Alpha anhydrous trehalose (Tα)

Examples of DSC thermograms recorded for Tα with scan rates of 10 °C per min and 200 °C per min are shown in Figures 3.9a and 3.9b. At heating scan rates of 10 °C per min, a reproducible endotherm representing the melt of Tα was shown at 122 °C. A 'cold crystallisation' event was observed with an onset of ~170 °C, representing conversion to the beta anhydrous form. This was followed by a reproducible endotherm with a peak maximum of 210 °C representative of the Tβ melt. These transitions all confirm the presence of Tα in the sample, although at this scan rate, a double peak was seen reproducibly at 95-100 °C that was not shown in DSC thermograms in the literature. This double peak could possibly be the result of the dehydration of remaining...
un-dehydrated T\textsubscript{h} within the sample. It is also possible that the first part of this double peak could relate to a low T\textsubscript{g} of possible amorphous content in the sample. This, however, does not explain the second part of the peak. A faster scan rate of 200 °C per min was used to try to clarify the unknown peaks in the thermogram at the slower scan rate. In the example (Figure 3.9b), the double peak at around 95-100 °C was absent and a broader endotherm was shown for the melting of T\textsubscript{\alpha} at a slightly higher temperature of 125-130 °C compared with that shown at the slower scan rate. A cold crystallisation to the beta anhydrous form was again observed as an exotherm but this time was seen at a higher temperature of ~190 °C. The melt of T\textsubscript{\beta} was noted in exactly the same position as that shown when a slower heating rate was used, with a peak maximum of 210 °C. The absence of the double peak at 95-100 °C could indicate the presence of amorphous content in the sample that did not have time to recrystallise and melt at the faster heating rate. This would be consistent with XRPD patterns.

**Beta Anhydrous Trehalose (T\textsubscript{\beta})**

Examples of DSC thermograms recorded for T\textsubscript{\beta} with scan rates of 10 °C per min and 200 °C per min are shown in Figures 3.10a and 3.10b. An endotherm representing the melt of T\textsubscript{\beta} would be expected at ~215 °C for the pure anhydrous form. In the DSC thermograms produced using a slower scan rate of 10 °C per min, the baseline was distorted due to the decomposition of the sample at higher temperatures. A reproducible T\textsubscript{g} was observed at 114 °C for the amorphous content within the sample. An exotherm for the re-crystallisation of this amorphous content to the beta anhydrous form of trehalose was observed at ~170 °C, prior to an endotherm representative of the melt of the beta anhydrous form, with a reproducible peak maximum of 208 °C. A shoulder was pronounced in the lower temperature region of the melting endotherm for the beta anhydrous form. This has been described in the literature and explained as corresponding to the melting of less perfectly formed anhydrous crystals (Sussich and Cesàro, 2000). As would be expected, this shoulder disappeared when a faster heating rate was used (Figure 3.10b), where a sharper endotherm was observed at 221 °C with a reproducible peak height. The T\textsubscript{g} of amorphous trehalose in the sample was shown at a higher temperature of 126 °C at a scan rate of 200 °C/min, with recrystallisation of the amorphous content at a higher temperature of 190 °C. The DSC data confirmed the presence of T\textsubscript{\beta} with some amorphous content in the T\textsubscript{\beta} sample, which was in agreement with XRPD data.
**Beta-vac anhydrous trehalose (Tβvac)**

Examples of DSC thermograms recorded for Tβvac with scan rates of 10 °C per min and 200 °C per min are shown in Figures 3.11a and 3.11b. The DSC thermograms produced using a slower scan rate of 10 °C per min look nearly identical to those for the Tβ sample, the only difference being the larger recrystallisation exotherm in the thermogram for the Tβvac sample. This would suggest more amorphous content in the Tβvac sample compared with the Tβ sample, a suggestion supported by the XRPD data for both samples. The DSC thermograms produced using a faster heating rate looked similar to those for the Tβ sample, the only differences being the larger size of the recrystallisation exotherm for the Tβvac sample, indicating an increased proportion of amorphous content in the sample and the lower temperatures of both the Tg and the endotherm for the melt of the beta anhydrous form. The Tg featured at 115-120°C for the Tβvac sample compared with 126 °C for the Tβ sample whereas the melt for the beta anhydrous trehalose form was shown at a peak of 217 °C for the Tβvac sample compared with a peak of 221 °C for the Tβ sample.

**DVS-anhydrous trehalose (Td)**

An example of a DSC thermogram recorded for Td with a scan rate of 10 °C per min is shown in Figure 3.11c. Collection of DSC data for the DVS-produced anhydrous trehalose proved particularly difficult as the sample was very hygroscopic and became hydrated very quickly during removal of the sample from the DVS apparatus and transferral to the DSC. This effect could have been reduced by loading the α,α-trehalose dihydrate onto the DVS pan in aluminium DSC sample pans, allowing weighing and crimping immediately after removal from the DVS apparatus under a stream of nitrogen. The DSC thermogram obtained is similar to that for Tα at the higher heating scan rate, however a possible Tg was observed at ~82 °C, indicative of the amorphous content in the sample (consistent with the halo shown in the XRPD pattern). From the DSC data alone, Td appears similar to Tα, however the XRPD (Section 3.4.1.2) and NIR spectra (discussed Section 3.4.1.5) indicate distinct differences in the structure of the two forms. These observations highlight the need for more than one characterisation technique in order to reach conclusions on the identity of unknown substances.
**Figures 3.9a and 3.9b.** DSC thermograms recorded for alpha anhydrous trehalose ($T_0$) at heating scan rates of (a) 10 °C/min and (b) 200 °C/min.
Figures 3.10a and 3.10b. DSC thermograms recorded for beta anhydrous trehalose ($T_p$) at heating scan rates of (a) 10 °C/min and (b) 200 °C/min.
Figures 3.11a, b and c. DSC thermograms recorded for beta-vac anhydrous trehalose ($T_{\text{p,vac}}$)$^{(a+c)}$ and DVS-anhydrous trehalose ($T_{\text{d}}$)$^{(c)}$ at heating scan rates of $(a+c) 10 ^\circ\text{C}/\text{min}$ and $(b) 200 ^\circ\text{C}/\text{min}$. 
3.4.1.4 Thermogravimetric Analysis (TGA)

Data from the thermogravimetric analysis of each polymorphic form of trehalose are presented in Table 3.2. Samples of $\alpha,\alpha$-trehalose dihydrate showed a mass loss of $\sim 9.5\%$ upon heating, corresponding to two molecules of water per molecule of trehalose (dihydrate water). Water content in the anhydrous forms was likely caused by transferral and handling of the samples following production. Generally the water content increased in these samples over the time period required for testing due to brief exposure to atmospheric humidity upon sample transferral. Samples containing suspected higher levels of amorphous content contained more water due to the disordered nature of the amorphous structure allowing increased absorption of water.

Table 3.2. Initial water content of polymorphic forms of trehalose prepared by different methods, calculated from TGA analysis.

<table>
<thead>
<tr>
<th>Polymorphic Form</th>
<th>Water Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha,\alpha$-Trehalose dihydrate ($T_h$)</td>
<td>9.54 (0.04)</td>
</tr>
<tr>
<td>Alpha anhydrous trehalose ($T_\alpha$)</td>
<td>0.68 (0.26)</td>
</tr>
<tr>
<td>Beta anhydrous trehalose ($T_\beta$)</td>
<td>0.45 (0.17)</td>
</tr>
<tr>
<td>Beta-vac anhydrous trehalose ($T_{\beta vac}$)</td>
<td>0.64 (0.09)</td>
</tr>
<tr>
<td>Amorphous trehalose ($T_{am}$)</td>
<td>3.52 (0.40)</td>
</tr>
</tbody>
</table>

3.4.1.5 Near Infrared Spectroscopy

Near infrared spectra were recorded for each polymorphic form produced as described in Section 3.3.1. Figures 3.12, 3.13 and 3.14 show the collated spectra for all of the proposed polymorphic forms of trehalose, separated into sections of the spectrum for clarity.
Figure 3.12 NIR spectra between 1100-1500 nm for the polymorphic forms of trehalose produced as described in Section 3.3.1.

Figure 3.13 NIR spectra between 1500-1850 nm for the polymorphic forms of trehalose produced as described in Section 3.3.1.
Figure 3.14 NIR spectra between 1850-2250 nm for the polymorphic forms of trehalose produced as described in Section 3.3.1.

Table 3.3. shows the assignment of peaks within the NIR region of the electromagnetic spectrum for each polymorphic form of trehalose produced in this study. Emboldened peak positions in the table were chosen as identifying peaks for each form because they discriminated the sample from any other form. Shaded cells refer to those peaks that appear in the NIR spectrum of every sample and therefore must relate to a ‘core’ part of the trehalose structure. Spectra for the $T_p$ and $T_{pvac}$ forms were combined because they were very closely matching or identical. Differences between these two forms seemed to arise from differences in amorphous content because the peaks for $T_{pvac}$, the more amorphous of the two forms (by XRPD and DSC), tended away from those displayed for $T_p$ and towards those characteristic for the amorphous form. It was noted that the spectrum for $T_d$ (the anhydrous form prepared by a single drying step in the DVS analyser) was most similar to that of the crystalline $\alpha,\alpha$-trehalose dihydrate form ($T_h$) than any of the other proposed anhydrate forms, with the obvious absence of the peaks for the dihydrate water (bound water) at 1954 and 1978 nm. As the NIR spectrum for $T_d$ was different to that of any of the other anhydrous forms tested, it was hypothesised that it was identical to Form II, proposed by Gil et al. (1996), an anhydrous form
described as the ‘dehydrated dihydrate’. As the proposal that \(T_d\) was an isomorphous desolvate of \(T_h\) was discounted by its XRPD data, this hypothesis was considered unlikely. Gil et al. (1996) said that Form II retained the crystal lattice structure of the original dihydrate following dehydration, in the absence of the dihydrate water. However, as the XRPD pattern of Form II was not compared with that of \(T_h\), further characterisation is required to determine whether Form II is an isomorphous desolvate of \(T_h\). The NIR spectrum of \(T_d\) is not identical to that of \(T_h\) without peaks for the dihydrate water and so more characterisation of this crystal structure would be required to confirm its actual form. Its NIR spectrum is different to that of any of the other anhydrous forms studied; therefore it may be hypothesised that it is a new polymorphic form of anhydrous trehalose.

**Table 3.3.** Assignment of peaks in the NIR spectrum between 1100-2250 nm for polymorphic forms of trehalose produced as described in Section 3.3.1. Emboldened peaks correspond to those used as identifying peaks for each form.

<table>
<thead>
<tr>
<th>(\alpha,\alpha)-Trehalose Dihydrate ((T_h))</th>
<th>Alpha Anhydrous Trehalose ((T_\alpha))</th>
<th>Beta Anhydrous Trehalose ((T_\beta))</th>
<th>Beta-vac Anhydrous Trehalose ((T_{\beta vac}))</th>
<th>DVS Anhydrous Trehalose ((T_d))</th>
<th>Amorphous Trehalose ((T_{am}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1466</td>
<td>1470</td>
<td>1448</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1498</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1518</td>
<td></td>
<td></td>
<td></td>
<td>1556/1594</td>
<td></td>
</tr>
<tr>
<td>1604</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1688/1722</td>
</tr>
<tr>
<td>1688/1722</td>
<td>1692/1720</td>
<td>1694/1726</td>
<td>1684/1718</td>
<td>1696/1728</td>
<td></td>
</tr>
<tr>
<td>1768</td>
<td></td>
<td></td>
<td>1764</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1788</td>
<td></td>
<td></td>
<td></td>
<td>1804</td>
<td></td>
</tr>
<tr>
<td>1816</td>
<td></td>
<td>1820</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1860/1882</td>
<td></td>
<td></td>
<td>1936</td>
</tr>
<tr>
<td>1954/1978</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2054</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2174</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2168</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2242</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

It was hoped that the assignment of peaks in the NIR spectrum for each polymorphic form of trehalose would help to elucidate any structural transitions of trehalose (form
changes) within the co-spray-dried catalase/trehalose powders described in Chapter 5 of this thesis. The theory by Aldous et al. (1995) that amorphous trehalose within such powders removes water from the system to form the dihydrate, thus ‘protecting’ the remaining amorphous phase of the formulation may be investigated using the peak assignments listed in Table 3.3.

### 3.5 Conclusions: Trehalose Dehydration and Polymorphism

- Two forms of ‘crystalline’ anhydrous trehalose, alpha anhydrous trehalose (Tα) and beta anhydrous trehalose (Tβ) were produced according to methods described in the literature and characterised by DSC.

- A ‘new’ anhydrous form of trehalose, named Td, was produced by the dehydration of α,α-trehalose dihydrate in the DVS analyser by drying at 0 % RH for 28 h. This form was not an isomorphous desolvate of Th (as confirmed by XRPD) and therefore is unlikely to be the same as the anhydrous ‘Form II’ as described in Section 3.1.1.5.

- A form of beta anhydrous trehalose, beta-vac anhydrous trehalose (Tβvac), was produced under vacuum in otherwise identical conditions to that used to produce Tβ. This method produced a more amorphous, less crystalline product than that produced by heating alone, because of the faster removal of water molecules during the dehydration process.

- Assignment of characteristic peaks for the different polymorphic forms of α,α-trehalose in the near infrared spectra of solid samples was shown to be possible.

### 3.6 Trehalose Dehydration and Polymorphism - Application of Results

Conclusions reached in this section could be used to examine the behaviour of trehalose in co-spray-dried protein-trehalose formulations to try to elucidate the mechanism of bioprotection conferred by trehalose in such systems.
Chapter Four
4 Analysis of the Crystallisation of Spray-dried Trehalose

4.1 Introduction

Following the characterisation of the different polymorphic forms of trehalose by the use of XRPD, DSC and DVS/NIRS in Chapter 3 and prior to the characterisation of co-spray-dried protein/trehalose formulations in Chapter 5, it was considered necessary to understand the nature of the crystallisation of trehalose from the amorphous, spray-dried form, using DVS/NIRS. With the knowledge of the assigned peaks in the NIR spectra of the polymorphic forms of trehalose obtained in Chapter 3 and the understanding of the crystallisation of amorphous, spray-dried trehalose, it was hoped that the behaviour of trehalose in co-spray-dried protein/trehalose formulations could be realised.

Two studies were performed to investigate the properties and crystallisation of amorphous trehalose. The first was an investigation into the effect of the concentration of trehalose in the spray dryer feed solution on the form of the solid trehalose produced (Section 4.2). This study was performed prior to the work described in Chapter 5, in which solutions of protein and trehalose of low feed concentration (0.5 % w/v in water) were to be spray-dried. As all previous work had been performed using feed solutions of relatively high concentration for spray drying, the aim of this work was to determine the effect of reducing the feed solution concentration prior to the analysis of co-spray-dried protein/carbohydrate samples. The second study was an analysis of the crystallisation of amorphous trehalose at 75% RH (Section 4.3), in order to assess whether peaks for intermediate forms in the crystallisation process would be visible in the near infrared spectrum (i.e. peaks indicative of the collapsed form of trehalose).

4.2 Study of the variability of spray-dried trehalose as a function of spray dryer feed solution concentration

4.2.1 Aim

The aim of the first part of this chapter was to investigate the effect of varying the concentration of trehalose in the feed solution prepared for spray drying, on the form and water sorption behaviour of the spray-dried trehalose product. It was hoped that this would provide useful insight into the behaviour that might be predicted for trehalose in co-spray-dried catalase (protein)/trehalose formulations in Chapter 5.
4.2.2 Experimental

4.2.2.1 Spray Drying

α,α-Trehalose dihydrate was spray-dried from 0.5, 5 or 10 % solutions in water (250 mL volume) using a Büchi 191 Mini Spray Dryer (Büchi, Switzerland). Parameters used are outlined in Table 4.1 and were the same as those used to co-spray-dry catalase and trehalose solutions in Chapter 5 of this thesis. Spray-dried products were stored in a desiccator over phosphorus pentoxide (0 % RH) until use.

Table 4.1. Operating parameters for the preparation of spray-dried trehalose powders for the feed concentration variability study using the Buchi 191 Mini Spray Dryer.

<table>
<thead>
<tr>
<th>Operating Parameters</th>
<th>Settings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inlet temperature (°C)</td>
<td>128 - 130</td>
</tr>
<tr>
<td>Outlet temperature (°C)</td>
<td>75 - 77</td>
</tr>
<tr>
<td>Feed rate (mL/min)</td>
<td>4</td>
</tr>
<tr>
<td>Pressure (bar)</td>
<td>3</td>
</tr>
<tr>
<td>Atomiser flow rate (normliter/h)</td>
<td>600</td>
</tr>
<tr>
<td>Machine settings (%)</td>
<td></td>
</tr>
<tr>
<td>- Aspirator</td>
<td>80</td>
</tr>
<tr>
<td>- Feed rate</td>
<td>18</td>
</tr>
</tbody>
</table>

4.2.2.2 Scanning Electron Microscopy

Scanning electron micrographs of spray-dried trehalose samples were recorded as soon after spray drying as possible, using the method described in Section 2.5.

4.2.2.3 Gravimetric Vapor Sorption/Near Infrared Spectroscopy

Gravimetric studies of solid trehalose samples spray-dried from various trehalose feed solution concentrations were carried out using a humidity and temperature controlled microbalance Dynamic Vapour Sorption (DVS) apparatus (Surface Measurement Systems, London, UK) as described in Section 2.2. Samples were loaded onto the flat-bottomed quartz glass sample pan of the DVS apparatus, were dried at 0 % RH and subsequently exposed to 75 % RH for 10 h to induce crystallisation of amorphous material before a second drying stage. The NIR spectrometer recorded a mean of 32 scans over the wavelength region 1100 – 2500 nm every 2.5 or every 15 min of the DVS experiment, via a fibre optic probe (Foss NIRSystems, UK) as described in Section 2.2.
Samples were checked for crystallinity using X-ray powder diffraction as described in Section 2.3.

4.2.3 Results and Discussion
4.2.3.1 Scanning Electron Microscopy
Scanning electron micrographs of trehalose samples spray-dried from 0.5, 5.0 and 10.0 % w/v in water solutions are displayed in Figures 4.1, 4.2 and 4.3. The particles of trehalose spray-dried from 0.5 % w/v trehalose solutions in water were smaller than those spray-dried from 5.0 % w/v feed solutions, which were smaller than those spray-dried from 10.0 % w/v feed solutions. All of the particles appeared spherical and smooth, regardless of the concentration of trehalose in the spray dryer feed solution. The largest particles were observed when the trehalose was spray-dried from a 10 % w/v solution, and the largest particle in the SEM for this sample appeared ‘dimpled’. Maa et al. (1997) reported a similar phenomenon in the morphology of co-spray-dried protein/lactose powders, in which such ‘dimpling’ occurred in particles spray-dried from solutions containing higher concentrations of solids. These researchers concluded that the ‘pitting’ or ‘dimpling’ observed in particles formed following spray drying from solutions of higher solids concentration was indicative of the particles of increased density being more resistant to collapse. The particles they produced from solutions of lower concentrations of solids showed deeper holes (reminiscent of donuts), indicating collapse of the particles. This was not observed in the trehalose samples spray-dried from different feed concentrations, because the particles produced from feed solutions of lower concentrations were spherical, with no evidence of pitting or dimpling.

Giunchedi and Conte (1995) described the formation of dimpling on the surface of microparticles produced by spray drying. These researchers attributed the dimpling to insufficient permeability of the saturated crust of the drying droplet, leading to a increase in pressure in the particle and subsequent fracture (observed as holes or dimples on the particle surface). This theory may be applied to the observations noted in the SEMs of the trehalose samples. At the highest feed concentration, the surface of the drying droplet may have been saturated with trehalose, with crust formation. The reduced permeability of this crust may have caused an increase in the pressure within the droplet leading to the dimpling observed. The droplets of the solutions containing lower concentrations of trehalose would be less likely to reach saturation at the droplet surface during drying; therefore smooth, spherical, dry particles would be expected.
Figures 4.1a, b and c. Scanning electron micrographs of trehalose samples spray-dried from 0.5, 5.0 and 10.0 % w/v in water solutions respectively.
It must be emphasised that scanning electron micrographs illustrate the particle morphology of one small part of a larger sample and that they may not always be representative of the whole sample.

4.2.3.2 DVS/NIRS: Trehalose spray-dried from 0.5 % w/v solutions (in water)

- Water sorption data (0.5 % feed solution)

On exposure to 75 % RH in a dynamic vapour sorption analyser, a sample of a dry amorphous sugar would be expected to absorb moisture. A mass increase associated with moisture absorption would be observed, until such point that the mobility of the sample would have increased to a level at which crystallisation could occur. At this point, collapse of the sample with related mass loss indicative of the expulsion of excess water from the system would occur. Rearrangement of molecules to the crystal form would follow. On rearrangement of the system to the crystal state, the sample mass would be expected remain stable at 75 % RH. Subsequent exposure to 0 % RH would cause the sample to dry to an anhydrous state, with the eventual loss of any hydrate water associated with the crystal structure.

Samples 1, 2 and 3 were spray-dried from 0.5 % w/v trehalose solutions in water as described in Section 4.2.2.1 and dynamic vapour sorption-near infrared (DVS-NIR) experiments were carried out on samples as described in Section 4.2.2.3. DVS plots for these experiments are shown in Figure 4.2.

The water sorption behaviour of the three samples (1, 2 and 3) differed greatly. All of the samples had different water contents prior to the DVS experiments (~5.6 %, ~3.3 % and ~2.8 % for Samples 1, 2 and 3 respectively), already suggesting the variability of products produced by spray drying from low concentration feed solutions. The most variable aspect of the water sorption behaviour of the three samples was the mass loss upon crystallisation of the samples. The dotted area highlighted in Figure 4.2 is magnified in Figure 4.3. All three samples demonstrated a crystallisation process indicated by mass loss at higher RH. However, Samples 1 and 3 crystallised at a faster rate than Sample 2, which showed a much broader peak corresponding to water expulsion upon crystallisation, and a greater percentage mass loss at 75 % RH compared with the other two samples.
Figure 4.2. DVS plots for three spray-dried trehalose samples (runs 1-3) exposed to 0 % RH for 6 h, 75 % RH for 10 h and then 0 % RH for a further 6 h (at 25°C). Samples were spray-dried from 0.5 % w/v trehalose in water solutions. The black-dotted box represents the area magnified in Figure 4.3.

Figure 4.3. Magnified region indicated by black-dotted area in Figure 4.2.

The percentage mass change of Sample 2 showed near-stabilisation during the 75 % RH step of the DVS experiment, as would be expected after crystallisation of amorphous
material. Samples 1 and 3 however, showed gradual, consistent mass loss following crystallisation at 75 % RH, suggesting that slower crystallisation processes were occurring. As all three samples showed such variable water sorption, it was hypothesised that they contained regions of crystalline material as well as amorphous content. This theory would explain why some samples crystallised at a faster rate than others, because a crystalline ‘seed’ within an otherwise amorphous sample would encourage crystallisation of amorphous material at a faster rate than a wholly amorphous sample. All samples were confirmed amorphous within the limits of X-ray powder diffraction (~10 %). The three samples gained between 10.25-11.25 % in weight between the end of the first drying stage and the end of the 75 % RH stage of the experiments. The gain of 2 molecules of water per molecule of trehalose to produce the dihydrate crystal form would equate to a mass increase of approximately 10.5 %. It was therefore considered that the samples were at different stages of their crystallisation at the end of the 75 % RH stage and that stabilisation of the form of the samples had not occurred. The continued mass loss at 75 % RH suggested that the samples were tending towards a form other than the dihydrate, such as one of the anhydrous forms discussed in Chapter 3. It was hoped that analysis of the near-infrared spectra collected at 15 min intervals during the DVS experiments would allow elucidation of the form of the samples prior to, during and after their crystallisation.

- **NIR Spectral Analysis (0.5 % feed solution)**
Throughout this chapter, the term ‘peak’, when referring to near infrared spectra, will be used to describe downward-pointing troughs corresponding to mathematically treated SNV normalised, second derivative spectra.

**NIR spectra at the end of the first drying stage of DVS experiments**
SNV, second-derivative near infrared spectra of Samples 1, 2 and 3 from the end of the first drying stage of the DVS experiments shown in Figure 4.2 are displayed in Figures 4.4, 4.5 and 4.6. The three wavelength ranges shown were chosen because spectral differences between the samples were greatest in these regions. The spectra of the three samples at the end of the first drying stage of the DVS experiments were compared with:
- Each other
- The spectra of a typical amorphous form and that of the original crystalline $\alpha,\alpha$-trehalose dihydrate
- The spectra of 'known' crystalline anhydrous forms described in Section 3.4.1.5.

The 'dry' spectra of Samples 1, 2 and 3 were all observed to be different to each other, suggesting variability based upon the spray drying process. All three sample spectra exhibited small similarities to that of the original crystalline $\alpha,\alpha$-trehalose dihydrate. An example of this is shown in the spectrum of Sample 3 in Figure 4.5. The NIR spectrum of Sample 3 showed a shallow peak at 1516 nm, not found in the spectrum of a 'typical' amorphous form. A peak at 1518 nm was present in the spectrum of $\alpha,\alpha$-trehalose dihydrate, suggesting an area of crystallinity within the otherwise amorphous Sample 3.

All three samples exhibited peaks in their spectra that did not correspond to those of an amorphous or crystalline dihydrate form of trehalose. These peaks were examined for similarities to the spectra of anhydrous crystalline forms of trehalose. The majority of these 'unknown' peaks seemed to have similarities to, but were not an exact replica of, peaks of an anhydrous form. Most of these similarities appeared in the wavelength ranges, 1350-1390 or 1860-1890 nm. The first of these ranges is described in the literature as corresponding to the first overtone of C-H combinations in $-CH_2$ moieties (Foss NIRSystems Technical Literature). The latter range is not described as corresponding to the vibrations of any particular chemical group in the literature. Examples of peaks in the 'dry' trehalose spray-dried samples are shown in Figure 4.4 and 4.6. In Figure 4.4, a peak is shown at 1378 nm in the NIR spectrum of Sample 2. This corresponds to the peak at 1380 nm characteristic to the spectrum of $T_\alpha$, the anhydrous form produced by drying for 28 h in the DVS analyser. In Figure 4.6, peaks are shown at 1860 and 1880 nm in the spectrum of Sample 2, corresponding to the characteristic peaks exhibited by alpha anhydrous trehalose, $T_\alpha$. No 'unexplained' peaks (peaks not corresponding to the amorphous or dihydrate trehalose forms) in the sample spectra corresponded to peaks of the beta anhydrous form of trehalose. Unexplained peaks in the sample spectra are listed in Table 4.2, along with any possible peak assignments to anhydrous forms.
Figure 4.4. SNV-2\textsuperscript{nd} derivative NIR spectra (between 1320-1390 nm) of spray-dried trehalose Samples 1, 2 and 3 from the end of the first drying stage of the DVS experiment shown in Figure 4.2.

Figure 4.5. SNV-2\textsuperscript{nd} derivative NIR spectra (between 1440-1620 nm) of spray-dried trehalose samples 1, 2 and 3 from the end of the first drying stage of the DVS experiment shown in Figure 4.2.
Figure 4.6. S/NV-2nd derivative NIR spectra (between 1800-1960 nm) of spray-dried trehalose samples 1, 2 and 3 from the end of the first drying stage of the DVS experiment shown in Figure 4.2.

Table 4.2. Description of peaks in dry spray-dried trehalose sample NIR spectra remaining unexplained after comparison with NIR spectra of the amorphous or dihydrate forms with possible assignment of peaks to those of anhydrous forms described in Section 3.4.1.5.

<table>
<thead>
<tr>
<th>Trehalose Sample</th>
<th>Unexplained peak (nm)</th>
<th>Possible assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>1488</td>
<td>T_d (1480 nm)</td>
</tr>
<tr>
<td>Sample 2</td>
<td>1352/1374 (double)</td>
<td>T_d (1378 nm)</td>
</tr>
<tr>
<td></td>
<td>1860</td>
<td>T_α (1860 nm)</td>
</tr>
<tr>
<td></td>
<td>1886</td>
<td>T_α (1882 nm)</td>
</tr>
<tr>
<td>Sample 3</td>
<td>1380</td>
<td>T_d (1378 nm)</td>
</tr>
<tr>
<td></td>
<td>1474</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>1908/1922/1938 (triple)</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

NIR spectra at the end of the 75 % RH stage of the DVS experiments

The NIR spectra of spray-dried trehalose Samples 1, 2 and 3 from the end of the 75 % RH stage of the DVS experiments showed little difference in peak position, with the
only small difference in peak position between samples being at around 1560 nm. A difference in this region would usually be attributed to the first overtone of bound –OH groups, corresponding to intramolecular hydrogen bonding (Seisler et al., 2002). However, crystalline α,α-trehalose dihydrate is unusual because of the absence of intramolecular hydrogen bonding within the crystal lattice structure (Aldous et al., 1995), therefore such a peak would not be expected to refer to intramolecular hydrogen bonding and thus remains unexplained. Differences in peak intensities were the only other differences between sample spectra at the end of the 75 % RH stage of the DVS experiments.

**NIR spectra at the end of the final drying stage of DVS experiments**

The main differences in the NIR spectra of spray-dried trehalose Samples 1-3 from the end of the DVS experiments shown in Figure 4.2 were in the 1350-1390 nm wavelength range, corresponding to the first overtone of C-H combination vibrations (Foss NIRS Systems Technical Literature). These differences are shown in Figure 4.7.

![SNV-2nd derivative NIR spectra](image)

**Figure 4.7.** SNV-2nd derivative NIR spectra (between 1330-1400 nm) of spray-dried trehalose samples 1, 2 and 3 from the end of the second drying stage of the DVS experiments shown in Figure 4.2.

On comparison of the sample spectra from the end of the second drying stage of the DVS experiments to those of known trehalose polymorphic forms (Figure 4.8), it was
clear that Samples 2 and 3 were tending towards either the alpha anhydrous form or the DVS anhydrous form, owing to the appearance of peaks at around 1375 nm producing double peaks in this region for both samples.

Samples 2 and 3 both showed differences in this wavelength region at the end of the first drying stage of the DVS experiment, prior to crystallisation, whereas Sample 1 did not. This result suggests that some of the trehalose molecules may retain the form in which they existed prior to crystallisation, after the event. This finding could be important with respect to the protective effect of trehalose in anhydrobiosis, in which the many polymorphic forms of trehalose may play a role.

![Diagram](image)

**Figure 4.8.** SNV-2nd derivative NIR spectra of amorphous trehalose, α,α'-trehalose dihydrate and crystalline anhydrous trehalose samples between 1330-1400 nm as described in Section 3.4.1.5.

4.2.3.3 DVS/NIRS: Trehalose spray-dried from 5 % w/v solutions (in water)

- Water sorption data (5 % feed solution)

Samples 4, 5 and 6 were spray-dried from 5 % w/v solutions in water as described in Section 4.2.2.1 and dynamic vapour sorption-near infrared experiments were carried out on samples as described in Section 4.2.2.3. DVS plots for these experiments are shown in Figure 4.9.
The gravimetric data of Samples 5 and 6 appeared almost identical whereas that of Sample 4 was very different, showing an increased mass drop upon crystallisation at higher relative humidity and a faster rate of mass loss across the 75 % RH stage of the experiment. None of the samples showed mass stabilisation at 75 % RH, suggesting that the crystal dihydrate forms produced were unstable, or that crystallisation was not entirely complete. The samples exhibited mass gains of 9.5-10.5 % over their dry weights, suggesting the variability of the form of the trehalose at the end of the 75% RH stage of the DVS experiment. The variability shown between samples from different spray drying runs was evident, though to a lesser extent than that seen when trehalose was spray-dried from 0.5 % w/v feed solutions.

Figure 4.9. DVS plots for three spray-dried trehalose samples (runs 4-6) exposed to 0 % RH for 6 h, 75 % RH for 10 h and then 0 % RH for a further 6 h (at 25 °C). Samples were spray-dried from 5.0 % w/v trehalose in water solutions.

- NIR Spectral Analysis (5 % feed solution)

It was proposed that the NIR spectrum of Sample 4 would be significantly different to those of Samples 5 and 6, and that the spectra of the latter two samples would be similar owing to their near-identical DVS plots. Upon examination of the spectra shown in Figure 4.10, taken at 15 min intervals during the DVS experiments shown in Figure 4.9, the spectra of Sample 6 appeared to be different to that of Samples 4 and 5, which appeared almost identical. This was unexpected, because the water sorption data would
suggest otherwise. This finding gives further credibility to the combined DVS/NIR technique, because water sorption data alone would point towards a different conclusion to that of the combined data.

The differences in the NIR spectra of Sample 6 compared with Samples 4 and 5 were examined in order to determine their importance. Prior to the feed variability study, it was hypothesised that differences in the physical form of trehalose samples would be responsible for any differences in their water sorption behaviour and NIR spectra. To investigate this, the NIR spectrum of Sample 6 from the end of the first dry stage of the DVS experiment was compared with the spectra of the polymorphic forms of trehalose as described in Section 3.4.1.5 (Figure 4.11).

![NIR spectra comparison](image)

**Figure 4.10.** SNV-2nd derivative NIR spectra of spray-dried trehalose Samples 4, 5 and 6 from the end of the first drying stage of the DVS experiment shown in Figure 4.9.
Figure 4.11. Comparison of the SNV-2nd derivative NIR spectra of spray-dried trehalose Sample 6 (prior to exposure to 75 % RH and crystallisation) with the spectra of the polymorphic forms as described in Section 3.4.1.5.

On examination of the spectra of the three spray-dried trehalose samples, the greatest differences between the samples before and after crystallisation were shown in the wavelength range of 1340-1390 nm. Differences between the spectra of samples were shown in one other wavelength region only (1820-1880 nm). However, as these differences were shown prior to, but not after crystallisation and thus bore no relevance to the form of the crystallisation product, it was decided to concentrate on the difference shown in the region of shorter wavelength.

The spectral comparison shown in Figure 4.11 suggests that the characteristic shoulder and peak at 1358/1372 nm in the spectrum of Sample 6 at the end of the first drying stage of the DVS experiment was most similar to the pattern shown at 1356/1374 nm by the alpha anhydrous form of trehalose. It was confirmed that this pattern was not present in the NIR spectrum of the ‘typical’ amorphous trehalose sample as described in Section 3.4.1.5 and that it had been present in the spectra of Sample 6 since the start of the DVS experiment and was therefore not an artefact of the initial drying stage.
Tracking of the NIR spectra of Samples 4, 5 and 6 through the crystallisation process at 75 % RH showed that the difference at 1358/1372 nm in the NIR spectrum of sample 6 in its dry state had no effect on the sample’s crystallisation to the dihydrate form. All three spray-dried trehalose samples crystallised to yield crystals displaying NIR spectra identical to that of α,α-trehalose dihydrate.

The NIR spectra of Samples 4, 5 and 6, between 1340-1380 nm at the end of the final drying stage of the DVS experiment, are shown in Figure 4.12. At this stage of the DVS experiment, all three trehalose samples displayed gradual mass loss, indicating the removal of hydrate water molecules to produce anhydrous form(s). The shallow double peak in the NIR spectrum of Sample 6 at 1358/1372 nm reappeared during the final drying stage of the DVS experiment and there was evidence of the double peak in the spectrum of Sample 5. As the double peak was more developed in the NIR spectrum of Sample 6, it was proposed that as this sample displayed these peaks in its spectrum prior to crystallisation, this region of anhydrous nature within the otherwise amorphous trehalose sample acted as a seed for nucleation and conversion to the particular anhydrous form upon drying, subsequent to crystallisation of the sample.

![Figure 4.12. SNV-2nd derivative NIR spectra of spray-dried trehalose Samples 4, 5 and 6 from the end of the second drying stage of the DVS experiment shown in Figure 4.9.](image)

The double peak at 1358/1372 nm shown in the spectrum of Sample 6 during the final stage of the DVS experiment is not present in the spectrum of α,α-trehalose dihydrate
but is present in that of alpha anhydrous trehalose ($T_a$). Figure 4.13 shows the comparison of the spectra of $T_a$ with that of the anhydrous form produced through the drying of $\alpha,\alpha$-trehalose dihydrate in the DVS apparatus ($T_d$). As there was a double peak in the 1340-1380 nm wavelength ranges of both anhydrous forms, the full wavelength range (1100-2250 nm) of both spectra was examined to determine similarities to Samples 4, 5 and 6 at the end of the DVS experiments shown in Figure 4.9. As more similarities existed between the spectra of $T_d$ and Samples 4, 5 and 6 than between $T_a$ and the sample spectra, it is hypothesised that all three spray-dried trehalose samples were tending towards the $T_d$ anhydrous form at the end of the DVS experiments.

![Figure 4.13. Comparison of the SNV-2nd derivative NIR spectra of alpha anhydrous trehalose ($T_a$) and DVS-anhydrous trehalose ($T_d$) between 1340-1390 nm.](image)

**Figure 4.13.** Comparison of the SNV-2nd derivative NIR spectra of alpha anhydrous trehalose ($T_a$) and DVS-anhydrous trehalose ($T_d$) between 1340-1390 nm.

### 4.2.3.4 DVS/NIRS: Trehalose spray-dried from 10 % w/v solutions (in water)

- **Water sorption data (10% feed solution)**

Samples 7, 8 and 9 were spray-dried from 10 % w/v solutions in water as described in Section 4.2.2.1 and dynamic vapour sorption-near infrared experiments were carried out on samples as described in Section 4.2.2.3. DVS plots for these experiments are shown in Figure 4.14. The gravimetric data of all three spray-dried trehalose samples appear almost identical, suggesting that less variability is induced upon spray drying from solutions of a higher solute concentration compared with spray drying from solutions of
low solute concentration. All three samples showed a mass increase of ~11 % over the dry mass at the end of the 75 % RH stage (compared with the expected mass gain of 10.5 % in order to produce the dihydrate), suggesting that crystallisation of the samples had not been completed (also evident from the continued mass loss at 75% RH).

- **NIR Spectral Analysis (10 % feed solution)**

There are few differences between the NIR spectra of Samples 7-9 at the end of the first drying stage of the DVS experiments compared with the differences observed between Samples 1-3 and 4-6. Only a slight difference is noted in the 1330-1390 nm wavelength range, with broader peaks noticed for 2 samples, however the positions of the peaks were the same. All three samples crystallised to the form of α,α-trehalose dihydrate, exhibiting almost identical spectra (data not shown), which all matched the spectrum of α,α-trehalose dihydrate as discussed in Section 3.4.1.5. At the end of the second drying stage, the NIR spectra of Samples 7-9 appeared identical, indicative of the lack of variability between batches of spray-dried trehalose spray-dried from solutions of higher solute concentration (data not shown).

![Figure 4.14](image.png)

**Figure 4.14.** DVS plots for three spray-dried trehalose samples (Samples 7-9) exposed to 0 % RH for 8 h, 75 % RH for 10 h and then 0 % RH for a further 6 h (at 25 °C). Samples were spray-dried from 10.0 % w/v trehalose in water solutions.
4.2.4 Conclusions: Study of the variability of spray-dried trehalose as a function of spray dryer feed solution concentration

a) Variability upon spray drying
Variability between spray-dried samples dried under identical conditions was greater in samples spray-dried from feed solutions of low solute concentration compared with the samples spray-dried from feed solutions of high solute concentration. This was not wholly unexpected, because in very dilute solutions, the molecules of trehalose would be further apart and therefore the amount of trehalose in the droplets to be spray-dried would be less likely to be uniform. The amount of trehalose in droplets from solutions of high solute concentration would be more likely to be uniform, thus reducing variability in the spray-dried form.

b) Anhydrous crystal formation
By the use of NIR spectroscopy it was possible to identify the form of the trehalose in spray-dried formulations. Spray drying trehalose from solutions of low solute concentration led to areas of anhydrous trehalose in the otherwise amorphous samples, which may have acted as a ‘seed’ for the form of trehalose produced through the subsequent crystallisation of the sample. This conclusion was prompted by continual mass loss shown by the samples following the initial crystallisation event, suggesting the tendency of the samples towards the anhydrous form following crystallisation to the dihydrate form. This phenomenon was more obvious in those trehalose samples spray-dried from solutions of low solute concentration.

c) Tendency towards the form of DVS-anhydrous trehalose ($T_d$)
During the final drying stage of the DVS experiments, spray-dried (and subsequently crystallised) samples were shown to tend towards the DVS-anhydrous form of trehalose, described in Chapter 3.

These results have shown that the combination technique of DVS/NIRS is useful for the examination of sample transitions in real-time. The use of the combination technique allowed conclusions to be drawn that would be difficult, or impossible to arrive at by the use of either method in isolation. This work provided a valuable insight into the behaviour of spray-dried trehalose formulations that may be useful for the determination of spray drying parameters for pharmaceutical or biopharmaceutical formulations. Such knowledge would help to prevent batch-to-batch variability and hence enhance stability. Possible further work will be discussed in Section 4.6.
4.3 Analysis of the Crystallisation Event of Amorphous Trehalose

4.3.1 Aims
The aim of part two of this chapter was to investigate the crystallisation of amorphous trehalose at 75 % RH in order to simplify the analysis of co-spray-dried protein/trehalose formulations under the same conditions in Chapter 5.

4.3.2 Experimental
4.3.2.1 Production of spray-dried trehalose
Trehalose α- (D) dihydrate was spray-dried from a 10 % solution in water (250 mL volume) using a Büchi 191 Mini Spray Dryer (Büchi, Switzerland, see Chapter 2, Section 2.1). Parameters used are outlined in Table 4.3. The parameters were different to those described in Table 4.1 because the study was carried out prior to the feed concentration variability study described in Section 4.2, in which parameters reflected those used to create spray-dried protein/carbohydrate formulations described in Chapter 5 of this thesis.

Spray-dried products were stored in a desiccator over phosphorus pentoxide (0 % RH) until use. Samples were confirmed as amorphous using X-ray powder diffraction as described in Section 2.3.

Table 4.3. Operating parameters for the preparation of co-spray-dried trehalose powders using the Büchi 191 Mini Spray Dryer.

<table>
<thead>
<tr>
<th>Operating Parameters</th>
<th>Settings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inlet temperature (°C)</td>
<td>140-150</td>
</tr>
<tr>
<td>Outlet temperature (°C)</td>
<td>70-80</td>
</tr>
<tr>
<td>Feed rate (mL/min)</td>
<td>2</td>
</tr>
<tr>
<td>Pressure (bar)</td>
<td>3</td>
</tr>
<tr>
<td>Atomiser flow rate (normliter/h)</td>
<td>600</td>
</tr>
<tr>
<td>Machine settings (%)</td>
<td></td>
</tr>
<tr>
<td>- Aspirator</td>
<td>60</td>
</tr>
<tr>
<td>- Feed rate</td>
<td>16-20</td>
</tr>
</tbody>
</table>

4.3.2.2 Crystallisation of amorphous spray-dried trehalose via single exposure to a 0% RH→75% RH→0% RH DVS cycle
Samples of amorphous spray-dried trehalose prepared as described in Section 4.3.2.1 (approximately 30 mg) and crystalline α,α-trehalose dihydrate were subjected to a 0%
4.3.2.3 Crystallisation of amorphous spray-dried trehalose via repeated exposure to a 0% RH→75% RH→0% RH DVS cycle

In addition to exposing the amorphous trehalose to a single DVS cycle as described in Section 4.3.2.2, an equivalent quantity of the spray-dried trehalose produced as described in Section 4.3.2.1 was exposed to a repeated cycling programme of 0 % RH and 75 % RH. Hour-long periods at 0 % RH were divided by increasing periods of time at 75 % RH. Amorphous spray-dried trehalose was exposed to 0 % RH for 6 hours (initial drying phase), 75 % RH for 5 min, 0 % RH for 1 h, 75 % RH for 10 min, 0 % RH for 1 h, 75 % RH for 15 min, 0 % RH for 1 h, 75 % for 30 min, 0 % RH for 1 h, 75 % RH for 35 min, 0 % RH for 1 h, 75 % RH for 40 min and finally 0 % RH for 1 h. NIR spectra were recorded every 150 s throughout the DVS experiment. Cycling of relative humidity was carried out in order to examine the reversibility of structural changes in the original single RH ramp DVS experiment (Section 4.3.2.2) and to analyse structural changes occurring during crystallisation by near spectroscopy.

4.3.3 Results and discussion

4.3.3.1 Crystallisation of amorphous spray-dried trehalose via single exposure to a 0% RH→75% RH→0% RH DVS cycle

- Water sorption data (Single RH cycle)

DVS water sorption isotherms for spray-dried samples of amorphous trehalose are shown in Figure 4.15, labelled Sample 1, 2 and 3. Sample mass values were determined at positions A – H of the DVS runs for each spray-dried trehalose sample. These values are displayed in Table 4.4. Samples showed an initial mass loss at 0 % RH as drying occurred (from an initial mass = 100 % to ca 97 %) and then a rapid mass gain when moisture was absorbed by the sample on exposure to 75 % RH, equating to ca. 14.1 % mass gain over the dry weight. This was followed after an average of 35 min by a rapid mass loss as moisture was expelled from the sample due to crystallisation of the amorphous trehalose. It was expected that samples would stabilise at 75 % RH following crystallisation to the dihydrate crystal form of trehalose (containing ~ 9.2 %
water). Samples labelled 1 and 3 in Figure 4.15 showed stabilisation at 75 % RH whereas Sample 2 continued to lose mass over the entire 75 % RH stage, showing a move away from the dihydrate water content. This continued loss of water indicated an unexpected trend to lose available water suggesting that Sample 2 may have formed a less stable dihydrate than the other two samples and was tending towards another, possibly anhydrous, state.

On returning the three recently crystallised trehalose samples to 0% RH (at 16 h after the start of the DVS experiment), all three samples showed gradual mass loss at a faster rate than that shown by Sample 2 during the 75% RH step, indicating diffusion of water from the samples, presumably due to loss of the dihydrate water. The sample that was in the crystalline form (trehalose dihydrate) at the start of the experiment lost mass during the final 0 % RH stage of the experiment, at an equal rate to those samples that were originally in the amorphous state. This suggested that all four trehalose samples were likely to be tending towards an anhydrous form during the final drying stage.

![Figure 4.15](image)

**Figure 4.15.** DVS plots for three spray-dried trehalose samples (1, 2 and 3) and one α,α-trehalose dihydrate sample exposed to 0 % RH for 6 h, 75 % RH for 10 h and then 0 % RH for a further 6 h (at 25 °C).
Table 4.4. Mass changes (%) between different time points of the DVS plots for each spray-dried trehalose sample as described in the discussion of Figure 4.15.

<table>
<thead>
<tr>
<th></th>
<th>B - A (%)</th>
<th>D - B (%)</th>
<th>F - D (%)</th>
<th>G - B (%)</th>
<th>H - B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>-2.30</td>
<td>14.08</td>
<td>-4.58</td>
<td>9.11</td>
<td>6.46</td>
</tr>
<tr>
<td>Sample 2</td>
<td>-2.58</td>
<td>14.10</td>
<td>-3.59</td>
<td>8.80</td>
<td>6.60</td>
</tr>
<tr>
<td>Sample 3</td>
<td>-2.91</td>
<td>14.20</td>
<td>-3.96</td>
<td>9.46</td>
<td>6.75</td>
</tr>
<tr>
<td>Average</td>
<td>-2.60</td>
<td>14.13</td>
<td>-4.04</td>
<td>9.12</td>
<td>6.60</td>
</tr>
</tbody>
</table>

- NIR spectral analysis of spray-dried trehalose Sample 3

From the water sorption data alone, it was impossible to tell exactly why the crystal structure of Sample 2 showed such instability as all the amorphous trehalose samples crystallised at 75% RH. The combination of DVS with near infrared spectroscopy (NIRS) allowed the crystallisation process to be examined on a more structural level. During the DVS experiment described in Figure 4.15 for spray-dried trehalose Sample 3, NIR spectra were recorded every 2.5 min as opposed to every 15 min, in order to follow the crystallisation process in more detail.

In Figure 4.16, NIR spectra between wavelengths of 1300-1500 nm are presented for spray-dried trehalose Sample 3 as it absorbed and expelled moisture at 75% RH as crystallisation occurred. Labels on the Figure refer to the time points of the DVS experiment shown on Figure 4.15, at which the spectra were recorded.

The downward-pointing peak at 1432 nm shown in Figure 4.16 was reduced as moisture was absorbed by the sample between points B to D and a shoulder at ~ 1468 nm began to form. Peaks for water in the NIR region are well characterised (Shenk et al., 1992; Seisler et al., 2002; Kamat et al., 1989). Peaks between 1435 nm and 1480 nm are referred to as first overtone bound –OH alcohol in tables of chemical group frequencies in the NIR region and refer to intermolecular hydrogen bonding (Seisler et al., 2002). It was therefore likely that the peaks shown in Figure 4.16 corresponded to changes in the bonding of water within the trehalose molecular structure as water was introduced into the system. It was proposed that the water introduced to the sample was converted from being free (within the trehalose structure) to becoming bound (-OH). This proposal was consistent with the onset of crystallisation.
Figure 4.16. SNV-2nd derivative NIR spectra of spray-dried trehalose Sample 3 between 1300 – 1500 nm from different time points through the DVS experiment described in Figure 4.15.

Figure 4.17. SNV-2nd derivative NIR spectra of spray-dried trehalose Sample 3 between 1860 – 2000 nm from different time points through the DVS experiment described in Figure 4.15 compared with the original crystalline trehalose dihydrate prior to spray drying.
In Figure 4.17, NIR spectra between wavelengths of 1860 nm – 2000 nm are presented for the same time points as shown in Figure 4.16. The peaks in Figure 4.17 are easier to explain in terms of the sorption and desorption of water into and out of the sample. The peak at 1932 nm refers to free water in the sample (Seisler et al., 2002). This peak increased in intensity as the sample was held at 75% RH and water adsorbed onto and diffused into, the trehalose structure. This peak began to reduce in intensity as the sample approached its threshold for moisture uptake prior to crystallisation. At this point the peak reduced in both intensity and shape, whilst a shoulder was formed at ~ 1952 nm. This observation suggested that water was initially sorbed and then gradually rearranged within the sample to form the dihydrate. It does not appear that this was an instantaneous process; a smaller peak at 1932 nm with a shoulder at 1952 nm was seen at the point of maximum mass of the sample (time point D). The next spectrum, recorded 2.5 min later, showed a large, sharp peak at 1954 nm and a smaller peak at 1978 nm. The peak at 1932 nm and shoulder at 1952 nm were absent, signifying that crystallisation of the sample had occurred. On examination of the spectra of the dry crystalline α,α- trehalose dihydrate (prior to dissolution and subsequent spray drying), it can be concluded that the peaks at 1954 nm and 1978 nm were indicative of the dihydrate crystalline form of trehalose because they were present in the NIR spectrum of the original α,α-trehalose dihydrate. This information points towards the spectrum at point D being that of the collapsed form of amorphous trehalose, prior to crystallisation, because the peaks corresponding to the dihydrate form, shown at point H, were not yet present.

Between points D and E on Figure 4.15, Samples 1 and 3 both stabilised eventually at a mass close to that expected if trehalose dihydrate had formed. The stabilisation of the mass of Sample 1 occurred faster than that of Sample 3. Sample 2 continued to lose mass gradually over the entire 75% RH period, suggesting that this sample had not formed a stable dihydrate. Work outlined in the feed variability study in Section 4.2 showed that spray drying trehalose produced variable results in terms of water sorption behaviour, seemingly dependent on the concentration of the trehalose solution to be spray-dried. It was found that the lower the concentration of trehalose in the solution to be spray-dried, the more variable the final spray-dried product. As all three samples shown in Figure 4.5 were spray-dried from 10% w/v solutions, it would be expected that the variability would be fairly low between these samples. If Sample 2 were forming the dihydrate, it would be expected that the mass of the sample would stabilise...
at 75 % RH, much like Samples 1 and 3. It seems therefore that Sample 2 was tending towards a form other than the dihydrate, perhaps an anhydrous form. The NIR spectrum of Sample 2 from the end of the 75 % RH stage (time-point G) was compared with those of Samples 1 and 3. The only major difference between the spectra is shown in Figure 4.18.

![Figure 4.18](image)

**Figure 4.18. Comparison of SNV-2nd derivative NIR spectra of spray-dried trehalose Samples 1, 2 and 3 and α,α-trehalose dihydrate (Sigma) from time-point G on Figure 4.15.**

Both Samples 1 and 3 showed a single peak in the region at 1360 and 1366 nm respectively whereas Sample 2 exhibited a double peak with minima at 1358 nm and 1376 nm. The spectrum of the original crystalline trehalose sample, prior to spray drying, showed a single peak in this region at 1360 nm. Interestingly, in the spectra from time-point B in Figure 4.15, Samples 1 and 3 showed a single peak at 1362 nm whereas Sample 2 again showed a double peak at 1358/1376 nm (Figure 4.19). As these peaks present in the amorphous form were present in the supposed ‘crystalline’ spectrum at point G for Sample 2, it suggests that the sample had not entirely crystallised to the dihydrate form. Although the ‘crystalline’ spectra of Samples 1 and 3 were not identical to that of the α,α-trehalose dihydrate, they displayed similar peak morphology in the same wavelength regions and had water sorption behaviour suggestive of crystallisation to the dihydrate form. Figure 4.20 shows the spectrum of Sample 2 showing the double peak at 1358/1376 nm prior to crystallisation of the
sample, compared with spectra of the polymorphic forms of trehalose as described in Section 3.4.1.5. The spectrum of Sample 2 is most similar to that of alpha anhydrous trehalose ($T_\alpha$), although shows similarities to that of $T_d$ and $T_\beta$. Comparing the spectra across the entire wavelength region, 1100-2250 nm, the spectrum of Sample 2 is again most similar to that of $T_\alpha$.

![Figure 4.19. Comparison of SNV-2nd derivative NIR spectra of spray-dried trehalose Samples 1, 2 and 3 from time-point B on Figure 4.15.](image)

![Figure 4.20. SNV-2nd derivative NIR spectra of Sample 2 compared with spectra of the polymorphic forms described in Section 3.4.1.5 in the region 1340-1390 nm.](image)
As amorphous forms are inherently disordered, it is understandable that the SNV-2\textsuperscript{nd} derivative NIR spectra of such forms would differ due to structural dissimilarities. However, crystalline forms of any substance, exhibiting long-range order and well-defined molecular packing would be expected to have near-identical NIR spectra (Yu, 2001). The behaviour and spectra of spray-dried trehalose Sample 2 suggest that the crystallisation of amorphous forms cannot be relied upon to be reproducible. The feed concentration variability study showed that samples spray-dried from higher feed concentrations gave greater reproducibility in terms of water sorption behaviour and NIR spectra. However, this study, using samples produced from the highest feed concentration used and spray drying parameters that produced samples that appeared to give more classical gravimetric water vapour sorption plots for amorphous forms, showed that even at higher feed concentration, reproducibility of form may not be relied upon.

- **Water sorption behaviour of crystalline $\alpha,\alpha$-trehalose dihydrate**

The water sorption isotherm for the original crystalline $\alpha$, $\alpha$- trehalose dihydrate is shown alongside those of the spray-dried samples in Figure 4.15. The crystalline sample lost mass rapidly during the initial drying stage and then a mass increase of $\sim$ 3 % was observed on exposure to 75 % RH as water sorption took place. As expected, mass loss from this sample was not shown at 75 % RH to indicate crystallisation because the sample was already crystalline.

**4.3.3.2 Crystallisation of amorphous spray-dried trehalose via repeated exposure to a 0% RH$\rightarrow$75% RH$\rightarrow$0% RH DVS cycle**

- **Water sorption data (Repeated RH cycle)**

The DVS plot of the crystallisation of amorphous spray-dried trehalose via repeated exposure to a 0 % RH$\rightarrow$75 % RH$\rightarrow$0 % RH DVS cycle is shown in Figure 4.21. The DVS plot in Figure 4.20 shows the repeated exposure of a spray-dried trehalose sample to 75 % RH as described in Section 4.3.2.3. Increasing periods of time under conditions of 75 % RH (to gradually induce crystallisation) were separated by drying under conditions of 0 % RH, in order to examine the water sorption processes that occur during crystallisation and to examine the reversibility of the crystallisation process. Water uptake by the sample was shown during each 75 % RH period, followed by mass loss during the drying periods. This was consistent until time-point 13 (indicated on Figure 4.21) at which crystallisation was assumed to have taken place, indicated by
mass loss at 75 % RH. From the DVS data it was assumed that crystallisation took place at time-point 13, however the combined use of DVS with NIRS allowed a more detailed examination of the crystallisation process.

![DVS plot of the crystallisation of amorphous spray-dried trehalose via repeated exposure to a 0 % RH → 75 % RH → 0 % RH DVS cycle. Time points from which NIR spectra were collected are numbered for ease of discussion.](image)

**Figure 4.21.** DVS plot of the crystallisation of amorphous spray-dried trehalose via repeated exposure to a 0 % RH → 75 % RH → 0 % RH DVS cycle. Time points from which NIR spectra were collected are numbered for ease of discussion.

- **NIR data analysis (Repeated RH cycle)**

During the analysis of the NIR spectra recorded during the crystallisation of spray-dried trehalose via repeated exposure to 75% RH shown in Figure 4.21, it was expected that as sorption and desorption occurred, there would be some peaks that would reversibly change with water going into and out of the system and some peaks that would irreversibly change to signify structural changes within the sample. Two investigations ensued. The aim of the initial analysis was to assign peaks within the spectra to the amorphous, crystalline dihydrate or ‘collapsed’ forms, by examination of those peaks remaining unchanged subsequent to changes in RH (thus discounting reversible peaks due to water sorption/desorption). A second investigation was carried out to examine the peaks corresponding to water moving into and out of the system corresponding to changes in RH, and analysis of the mechanism and kinetics of crystallisation.
DVS Time-points 2 → 6: Reversible NIR peaks due to water sorption/desorption

Between time-points 2 and 6 of the DVS experiment shown in Figure 4.21, the DVS plot showed repeated water sorption followed by water desorption, indicated by mass gain and loss respectively. The mass after each desorption returned to approximately the same as it was prior to the previous stage of water sorption, suggesting that water was absorbed and desorbed equally over these time periods. This being so, it was considered likely that any major changes within the NIR spectra between time-points 2 and 6 would be due to this reversible water movement. Figure 4.22 shows the spectra between 1850-2000 nm recorded at time-points 2-6 of the DVS plot shown in Figure 4.21.

![SNV-2nd derivative NIR spectra recorded at numbered time-points during the DVS experiment shown in Figure 4.21. The arrow indicates the reversible nature of the peak at 1932 nm.](image)

As indicated by the arrow in Figure 4.22, the peak at 1932 nm increased in intensity as water sorption took place and reduced in intensity with water desorption. A peak at this wavelength is likely to have arisen from the combination of O-H stretch and O-H bend vibrations in free water in the sample, corroborating the theory that this peak movement was due to free water sorption/desorption. Other such reversible peaks are described in Table 4.5 along with other peak changes within the sample over this period.
Table 4.5. Table of peak changes observed between time-points 2-6 of the DVS plot shown in Figure 4.21. (Seisler et al., 2002; Foss NIRSystems Technical Literature; Shenk et al., 1992).

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Description of observation</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1374</td>
<td>Reversible Intensity ↓ with H2O</td>
<td>O-H anti-symm. Stretch and O-H symm. Stretch – Combination band (H2O)</td>
</tr>
<tr>
<td>1432</td>
<td>Reversible Intensity ↓ with H2O</td>
<td>O-H first overtone (H2O)</td>
</tr>
<tr>
<td>1884</td>
<td>Reversible Intensity ↓ with H2O + gradual shift to 1882</td>
<td>No known absorption band for water or O-H groups in this wavelength region</td>
</tr>
<tr>
<td>1932</td>
<td>Reversible Intensity ↑ with H2O</td>
<td>O-H stretch and O-H bend – Combination band (H2O)</td>
</tr>
<tr>
<td>2072</td>
<td>Reversible Intensity ↓ with H2O + Shoulder development at 2096</td>
<td>Free –OH alcohol band frequency (O-H stretch and O-H bend) – Combination band</td>
</tr>
</tbody>
</table>

The description of the development of a shoulder at 2096 nm could mean a change in the hydrogen bonding within the sample. Peak broadening or shifting to a longer wavelength can be indicative of more hydrogen-bonded species within a sample (Shenk et al., 1992); however as there are no peaks stated in the literature in this region of the spectrum for bound –OH groups, this is less likely. As band assignments differ depending on the chemical and physical environment of the sample (Zhou et al., 2003), the possibility of hydrogen bonding cannot be discounted.

DVS Time-points 6 → 10: Changes in NIR spectra upon diffusion of water into the sample

Between time-points 6-10 of the DVS experiment shown in Figure 4.21, the DVS plot showed repeated water sorption followed by water desorption, similar to that shown between time-points 2-6, except that the mass did not return to ‘baseline’ upon desorption. If the water were merely absorbing and desorbing from the sample, then the % mass loss would be expected to return to ‘baseline’ upon drying. The drying stages between time-points 6-10 were not long enough to allow water to fully desorb from the sample as the periods of time at 75 % RH increased, suggesting that water had penetrated further into the sample structure with the possibility of binding. Water
desorption from within the sample structure would be slower than that from the sample surface, resulting in mass gain because water was unable to ‘escape’ from the sample structure during the short period of drying at 0 % RH. Differences between the NIR spectra of the sample at each ‘dry’ time-point (6, 8, 10) would be expected upon mass gain of the sample because of water absorption between these points. However, no obvious trend was observed in the changes in intensity of the peaks corresponding to water in the literature between time-points 6-10 (Figures 4.23a and 4.23b). This indicated that the process of water diffusion into the disorganised amorphous trehalose structure was variable. No peak shifts were observed during this time period.

![Figure 4.23a](image1.png)  
![Figure 4.23b](image2.png)

*Figures 4.23a and 4.23b.* SNV-2nd derivative NIR spectra from wavelength regions corresponding to water between 1410-1460 nm (a) and 1900-1960 nm (b) from time-points 6, 8 and 10 of the DVS experiment shown in Figure 4.21.
DVS Time-points 11 → 13: Crystallisation of amorphous trehalose

SNV-2\textsuperscript{nd} derivative NIR Spectra from time-points during the DVS plot shown in Figure 4.21 are shown in Figure 4.24. Crystallisation of the amorphous trehalose was evident in the spectrum recorded at time-point 12. This was determined by tracking the peak for O-H first overtone absorption (for free water) at 1432 nm. This peak decreased in intensity from time-point 11 of the DVS experiment onwards, shifting to become a peak at 1468 nm, corresponding to the first overtone vibrations of bound –OH alcohol groups. This indicated an increase in the intermolecular hydrogen bonding in the sample and hence the onset of crystallisation (Seisler \textit{et al}., 2002).

\begin{figure}[h]
\centering
\includegraphics[width=0.7\textwidth]{figure.png}
\caption{SNV-2\textsuperscript{nd} derivative NIR spectra between 1350-1500 nm, recorded at numbered time points over the trehalose crystallisation event during the DVS experiment shown in Figure 4.21.}
\end{figure}

The period between time-points 11 and 12 was next examined for evidence of crystallisation, to investigate the speed of the crystallisation process. Zhou \textit{et al}.
(2003) described the analysis of combination bands of water at ~1920 nm to determine bound and free water within pharmaceutical samples. Figure 4.25 shows this region of the NIR spectrum for the spray-dried trehalose sample throughout the entire crystallisation event shown in Figure 4.21.
Between time-points 11 and 12, a drying stage at 0 % RH meant that no extra water was absorbed by the sample and mass loss occurred during this period. The spectra in Figure 4.24 showed a decrease in the intensity of the peak at 1932 nm (relating to free water in this sample according to previous assignment of peaks). This peak appeared to shift and transform into the peaks for bound water at 1954 nm and 1978 nm between time-points 11-13 rather than simply reducing in intensity as would be expected if only water desorption were taking place. This observation would suggest that the sample had absorbed enough water at time-point 11 to form the dihydrate but had yet to rearrange fully to the crystal form. The sample went on to take up further water (shown by mass gain on the DVS plot) prior to time-point 13, when crystallisation was shown to occur, indicated by mass loss on the DVS plot at 75 % RH. The peak at 1932 nm for free water in the sample did not increase in intensity between time-points 12 and 13, when the sample was being held at 75 % RH. This observation signified that all of the water absorbed by the sample during the 75 % RH stage between time-point 12 and 13 was immediately utilised by the sample to form the dihydrate.

Figure 4.25. NIR spectra between 1850-2000 nm, recorded at numbered time points over the trehalose crystallisation event during the DVS experiment shown in Figure 4.21.
Peaks corresponding to the crystalline $\alpha,\alpha$-trehalose dihydrate form (assigned in Section 3.4.1.5) developed and increased in intensity after time-point 13 on the DVS plot, indicating that the structure was reordering following crystallisation from the amorphous form. During each period of drying at 0% RH after time-point 13, the sample lost mass at a slower rate than that shown in the drying stages prior to crystallisation (Figure 4.26). This was thought to be indicative of the start of the dehydration of the dihydrate form, a process shown to take ~25 h in Section 3.4.1.1. On examination of the peaks at the start and end of the drying stage immediately following time-point 13, it was observed that the peaks indicative of the dihydrate water at 1954/1978 nm increased in intensity (Figure 4.27). This suggests that at point A on Figure 4.26, the sample had not completed reordering to the dihydrate form following collapse in the crystallisation process. This is also shown by the fact that the rate of water loss was faster during the first part of the section A-B, indicating the continued expulsion of free water from the sample, shown by mass loss. The sample did not stabilise between points A and B, suggesting that dehydration of the sample had begun following crystallisation and subsequent exposure to 0% RH.

The spectrum at point B in Figure 4.26, shown in Figure 4.27, is very similar to that at time-point 14, indicating that the crystallisation of amorphous trehalose to the dihydrate form had been completed by point B. The water sorption behaviour of the sample when exposed to 75% RH subsequent to the crystallisation event was similar to that expected for a sample of $\alpha,\alpha$-trehalose dihydrate, in that the sample adsorbed a small amount of water, indicated by the slight increase in sample mass at the start of the 75% RH stages. The sample then showed mass loss at 75% RH, suggesting the instability of the dihydrate form and a tendency towards an anhydrous state. If the crystal form were stable, it would be expected that mass stabilisation would be achieved during the 75% RH stages, which wasn't observed. The phenomenon of mass loss at 75% RH was reported earlier in this chapter for those samples of 'amorphous' trehalose spray-dried from trehalose solutions of lower concentrations and in a paper by Al-Hadithi et al. (2004), in which the crystallisation behaviour of trehalose at 75% RH was shown to be different to that at 53% RH. These researchers proposed that the continual mass loss observed at 75% RH following the initial mass loss indicative of crystallisation was because of the rapid crystallisation of the surface of particles, forming a 'shield' of crystalline material; thus preventing the access of water to the centre of the particles to effect crystallisation. It was proposed that the slow diffusion of water from the centre of
particles through the crystalline material on the surface, produced the continued mass loss shown at 75% RH. This left an open question with regard to the state of trehalose in the centre of the particles (Al-Hadithi et al., 2004).

From the studies described in this chapter, the continual mass loss shown by trehalose samples at 75 % RH was thought to be indicative of a continuing, slow crystallisation process, or the formation of an unstable dihydrate form. From the data given in this section, it seems most likely that following crystallisation, the trehalose samples were unstable and therefore tending towards an anhydrous state, because there was no evidence of any other form of trehalose (the peaks for which were identified in Section 3.4.1.5) in any region of the NIR spectrum of the crystallised sample at time-point 13 in Figure 4.26. The proposal that the particles of trehalose may be coated with molecules of trehalose dihydrate and that the centre of the particles remained anhydrous or amorphous could not be addressed in this study.

![Figure 4.26](image)

**Figure 4.26.** Magnified part of Figure 4.21 (DVS plot of the crystallisation of amorphous spray-dried trehalose via repeated exposure to a 0 % RH → 75 % RH → 0 % RH DVS cycle) between time-points 13-14.
4.3.4 Conclusions: Analysis of the Crystallisation of Amorphous Trehalose

a) Variability

Spray drying from solutions of high solute concentration and the use of spray drying parameters that are more common for the drying of sugars such as trehalose, did not entirely prevent variability between spray-dried samples.

b) Water sorption behaviour and anhydrous crystal formation

Similar water sorption behaviour to that shown by samples in Section 4.2 (the feed-variability study) was observed for spray-dried trehalose samples in this study. Analysis of the near infrared spectra of one of the spray-dried trehalose samples indicated the presence of crystalline anhydrous material in the otherwise amorphous material. This anhydrous nature was observed in the spectrum of the same sample following crystallisation to the dihydrate form, suggesting that the anhydrous material acted as a seed for crystallisation and subsequent dehydration.

c) The crystallisation process

The combination technique of DVS/NIRS enabled the crystallisation of amorphous trehalose to be examined in real-time. Crystallisation was not shown to be an instantaneous process; peaks/shoulders suggestive of the formation of trehalose...
dihydrate were observed in the NIR spectra immediately prior to the mass loss indicative of crystallisation shown in the DVS data. These changes in the NIR spectra were indicative of the increased mobility of the amorphous trehalose with increased water content. Following crystallisation, the continued gradual loss of mass of the samples observed at 75 % RH was proposed to be indicative of the instability of the dihydrate form and of the tendency towards an anhydrous state. The continued mass loss was not thought to be indicative of a continuing crystallisation process.

4.4 Summary of the Conclusions of Chapter Four

One of the main observations from the work presented in Chapter 4 was the inherent variability observed in spray-dried trehalose samples. Anhydrous crystals were identified in some of the spray-dried samples using near infrared spectroscopy and the knowledge of trehalose polymorphism gained in Chapter 3. Following crystallisation of the majority of spray-dried trehalose samples, gradual mass loss continued at 75 % RH at variable rates dependent upon the sample. This was attributed to the instability of the dihydrate crystals formed and the tendency of samples towards an anhydrous state. It is possible that these attributes of spray-dried trehalose samples may be involved in the mechanism by which trehalose is able to confer stability to proteins upon drying.

The use of the combined technique of DVS/NIRS was shown to be successful for the analysis of crystallisation processes in real-time and showed potential for the future analysis of more complex systems.

4.5 Further Work

Optimisation of the spray drying parameters in order to produce fully amorphous samples and reduce the variability observed would improve the stability of spray-dried formulations.

Quantification of the amorphous content in samples of trehalose spray-dried from solutions of different feed solutions would be useful in order to be able to predict the form of the product obtained through the spray drying process. Some methods that could be used for such quantification studies are batch isothermal microcalorimetry, solution calorimetry or differential scanning calorimetry. Differential scanning calorimetry could also be used to determine the glass transition temperatures of spray-
dried trehalose formulations, the knowledge of which may be useful for the determination of the potential stability of such formulations upon storage.

Differential scanning calorimetry could also be useful to test the hypothesis that particles of amorphous trehalose, under conditions of 75 % RH, form particles of anhydrous or amorphous material, coated with crystalline trehalose dihydrate molecules (Al-Hadithi et al., 2004). If this were the case, then the behaviour of the 'shielded particles' would be different to those of fully crystalline particles of trehalose dihydrate.

The combination of dynamic vapour sorption analysis and near infrared spectroscopy would be useful for the analysis of co-spray-dried samples, for which the water vapour sorption data alone is complex. Following analysis of the individual components to be co-spray-dried, it may be possible to use DVS/NIRS to determine the state or nature of interaction of the individual components in the co-spray-dried samples. This would be particularly useful for the analysis of co-spray-dried protein/sugar formulations, for which the mechanism of stabilisation conferred by the sugar, is unclear (see Chapter 5).
Chapter Five
5 Characterisation of co-spray-dried catalase/trehalose formulations using Dynamic Vapour Sorption Analysis and Near Infrared Spectroscopy

5.1 Introduction

5.1.1 Catalase

Catalase is a primarily intracellular enzyme, found in all but a few aerobic organisms (Deisseroth and Dounce, 1970). This enzyme is usually isolated from cells in which it is contained in high concentrations, such as mammalian hepatic cells or erythrocytes, or from a bacterial source, such as Micrococcus lysodeikticus, which can be easily manipulated to produce a high yield of the enzyme. In hepatic or kidney cells, catalase is mainly found in the organelles located in the cytoplasm of the cells, for example, the mitochondria and in particular, the peroxisomes (Aebi, 1983). Here, catalase is thought to act with other enzymes to regulate the level of hydrogen peroxide in the cells. In red blood cells, catalase acts to prevent the accumulation of methaemoglobin: the product of the oxidation of the iron in haemoglobin. Methaemoglobin is unable to combine reversibly with oxygen; therefore an increase in the levels of this product leads to a reduction in the oxygen-releasing capacity of the blood to the body tissues. An increase of methaemoglobin in red blood cells leads to cyanosis.

Catalase is a hydrogen peroxidase enzyme, meaning that it causes the breakdown of two moles of hydrogen peroxide to produce two moles of water and one mole of oxygen. However, catalase is an unusual enzyme in that it not only exerts this ‘catalatic’ activity to breakdown hydrogen peroxide, but also employs ‘peroxidatic’ activity, in which it catalyses the oxidation of hydrogen donors (e.g. ethanol) with the consumption of one mole of a peroxide (Aebi, 1983). The predominant type of reaction will depend on the concentration of hydrogen donor and hydrogen peroxide in the system.

Structurally, catalase is a tetrameric haemoprotein, meaning that it consists of four subunits, each containing one haem prosthetic group, with a total molecular mass of approximately 240 000 g/mol (Aebi, 1983). The structure of native catalase is thought to consist of 50 % helix, the percentage of which is reduced upon denaturation, when the molecule is converted to a random coil (Yang and Samejima, 1963). It is often used as a model protein because of its high concentration in the liver of large mammals such as cows, meaning that it is economical to use.
The catalase used in the studies described in this chapter was obtained from Sigma Aldrich in a freeze-dried form. Freeze drying itself is known cause dissociation of the native catalase tetramer into conformationally altered monomer and tetramer units, the proportions of which are thought to be dependent on the starting material (Sichak and Dounce, 1987). It was observed that lyophilisation of a Sigma Company source of catalase produced both a catalase tetramer and a monomer, with the tetramer retaining more of the native catalase conformation than the monomer and thus having more enzymatic activity (Sichak and Dounce, 1987). Some lyophilised forms of catalase have also been observed to retain up to 35 % of the native, undissociated enzyme, retaining more intrinsic activity than any of the subunits (Tanford and Lovrien, 1962). It must therefore be concluded that the final form of catalase in a lyophilised product is dependent on the starting material and the conditions of freeze drying.

A freeze-dried form of catalase was used in a study to investigate the physical stability of spray-dried protein/sugar powders using water vapor sorption (Forbes et al., 1998) and in a study of pressure-induced activity loss in solid-state catalase (Wurster and Ternik, 1995). Other model proteins such as insulin, lysozyme, β-lactoglobulin and pepsin have also been used in their freeze-dried forms for investigations of protein stabilisation or hydration (Forbes et al., 1998, Liao et al., 2002b and Vandermeulen and Ressler, 1980a). As catalase in its freeze-dried form retains a significant proportion of its original activity, it is a suitable alternative to the crystalline form of the enzyme for the purposes of investigating its stability. Catalase in its pure, crystalline form can only be obtained in smaller quantities and owing to its purity, is vastly more expensive than the freeze-dried alternative. As spray drying was to be used for the studies in this chapter, which is a process leading to often very low yields of product, the use of the freeze-dried form of catalase was considered justified.

5.1.2 Water vapour sorption studies with proteins

The study of the water vapour sorption behaviour of proteins to investigate stability is very useful, given that water is often the cause of instability. Water vapour sorption studies on proteins have generally been aimed at modelling the water sorption behaviour of proteins and peptides by examining water sorption isotherms of such substances in comparison to isotherms calculated by mathematical models. Shamblin et al. (1998), presented a review of the water sorption behaviour of proteins, with discussion on the influence of formulation additives. These authors described the
complexities of the water sorption behaviour of multicomponent formulations of proteins and additives as being dependent upon the water vapour sorption behaviour of the individual components as well as upon any interactions between the components. As sugars are often added to protein formulations prior to drying in order to confer some protection to the complex native protein structure upon the removal of water from the system, it is presumed that the sugars interact in some manner with the protein (Shamblin et al., 1998). Whether sugars with a high glass transition temperature simply provide an amorphous matrix to prevent increased molecular mobility in the protein or whether they interact with the protein to replace the water lost upon dehydration, it would be expected that exposure to high relative humidity would plasticise the amorphous nature of the dried product, thus increasing molecular mobility and decreasing the stability of the protein. Indeed, if the carbohydrate additive were conferring stability to the protein by its amorphous nature, exposure to high relative humidity would likely cause phase separation of the amorphous structure and re-crystallisation of the amorphous carbohydrate.

The water monolayer of a protein ($M_0$) has been described as the water content required to exhaust all of the possible water-binding sites on the surface of the protein with additional clustering of water molecules (Hagemann, 1992). The water monolayer of a protein is exceeded when further additional water is shown to display 'bulk' properties (Hagemann, 1992). Costantino et al. (1998a) studied the water sorption behaviour of three model proteins when co-lyophilised with mannitol, sucrose or trehalose. These workers calculated the water monolayer for the individual components and compared the predicted and actual calculations for the water monolayer of co-lyophilised samples. Their study was based on the theory that proteins dried to water contents above or below that of the water monolayer content exhibited instability compared with those proteins dried to a water content equivalent to the water monolayer (Costantino et al., 1994, Hagemann, 1992, Hsu et al., 1991). It was found that the water monolayer for co-lyophilised protein-disaccharide samples was reduced in comparison with that expected from the contributions of each of the components (Costantino et al., 1998a). These researchers supposed that the disaccharides interacted with the proteins in the dried state, masking sites for the water monolayer on both components. The interaction between the proteins and carbohydrates in the dried form was not characterised.
In addition to the proposed interaction of proteins with carbohydrates on co-
lyophilisation, Costantino *et al.* (1998a, b and c) also observed that co-lyophilisation of 
carbohydrates with proteins inhibited the crystallisation of the amorphous 
carbohydrates. This observation, reported in several previous papers (e.g. te Booy *et 
Tzannis and Prestrelski, 1999a and 1999b, Zeng *et al.*, 2001) was also supported by 
Forbes *et al.* (1998), who investigated the water vapour sorption behaviour of three 
proteins, including catalase, when co-spray-dried with lactose or mannitol. It was 
observed that catalase inhibited the crystallisation of amorphous lactose at 75% relative 
humidity (RH) to a greater extent than the other proteins studied (insulin and 
ribonuclease A), however this observation was reported to be a kinetic feature because 
the same behaviour was not observed following a longer period of exposure to 75% RH 
(Forbes *et al.*, 1998).

López-Díez and Bone (2004) used gravimetric water sorption experiments to investigate 
the mechanism of protein (trypsin) preservation following freeze drying with trehalose. 
These researchers examined hydration isotherms for freeze-dried trypsin/trehalose and 
trypsin/sucrose formulations. A reduction in hydration was determined to indicate 
interaction between the trypsin and carbohydrates and it was concluded that trehalose 
was the more effective preservative than sucrose because of its increased level of 
interaction with the protein (reduced hydration).

In summary, several research groups have used water vapour sorption analysis in 
attempts to probe the relationship between proteins and carbohydrates in the dried state. 
It has been shown that the hydration of amorphous components in spray-dried systems 
is likely to be reduced by an interaction between the components (Sarcaiaux and 
separation/crystallisation of stabilising amorphous additives in dried protein-additive 
systems has been shown to destabilise the system, causing loss of protein activity 
(Tzannis and Prestrelski, 1999a and 1999b). By increasing the proportion of protein in 
dried protein/disaccharide formulations, inhibition of crystallisation of amorphous 
disaccharides has been observed (French *et al.*, 1995, Sarcaiaux and Hageman, 1997, 
Costantino *et al.*, 1998b and Forbes *et al.*, 1998), however, the ideal proportion of 
disaccharide to protein in the formulations to result in optimal protein stabilisation is 
unclear. Some researchers have proposed that over a disaccharide: protein ratio of 1:1, a
reduction in the extent of biostabilisation will be observed (Tzannis and Prestrelski, 1999a and 1999b). This proposal was contradicted by López-Díez and Bone (2004), who observed an increased retention of protein activity at higher disaccharide: protein ratios. It is clear that the physical form of the excipient intended to confer stabilisation to the protein in the dried state is important, however there appears to be no consensus as to the mechanism through which biostabilisation is achieved by amorphous disaccharides such as sucrose or trehalose. Given that the partition of water between the components of the amorphous protein/carbohydrate matrix upon drying is likely to be fundamental to the stability of the protein in the formulation, gravimetric vapour sorption analysis is considered a useful tool for further examination of the mechanism of biostabilisation. In combination with near infrared spectroscopy, such analysis may reveal the form of the disaccharide in dried protein/carbohydrate formulations and aid in the elucidation of the mechanism of biostabilisation conferred by disaccharides to proteins in the dried state.

5.1.3 Near infrared spectroscopy and proteins

As proteins are notoriously complicated, heterogeneous structures, very little has been published regarding spectra/structure correlations in the near infrared region for these molecules. One of the main reasons for this is that extensive work has been carried out using infrared and Raman spectroscopy and therefore more evidence exists to justify the use of these types of spectroscopy as investigative techniques. As water significantly affects spectra in the near infrared region, this is another reason why other forms of spectroscopy other than NIR have been preferred to examine the structure of proteins in the past. A summary of the relevant literature regarding the spectra/structure correlations for proteins in the near infrared region of the electromagnetic spectrum is given below, followed by a table outlining the main points for the NIR analysis of proteins (Table 5.1).

Sadler et al. (1984) examined the NIR spectra of proteins and assigned peaks for these in the solution and solid states using photoacoustic NIR spectroscopy. Importantly, these workers found that the NIR spectra of denatured proteins were very similar to those of their native forms, with the only small differences between the native and denatured protein spectra being alteration in the peak at 1350 nm and small changes in the 1700-1800 nm and 1400-1500 nm regions. Many of these small changes appeared to be specific to individual proteins and the spectra appeared to be insensitive to the
proteins' secondary structures. Sadler et al. (1984) concluded that it was difficult to assign peaks in the NIR region for proteins with confidence, because of the complexity of bond vibrations in the macromolecular structures.

Liu et al. (1994) undertook a study to compare the FT-IR and FT-NIR spectra of proteins and polypeptides with the aim to find a 'marker band' for the protein/polypeptide structure. These workers focussed on the more isolated combination band for amide A/II at approximately 4850 cm⁻¹ (2062 nm), the assignment for which was rationalised by Wang et al. (1994). From their studies of several polypeptides in the solid state, such as poly-L-leucine and poly-L-alanine, Liu et al. concluded that the frequency of the amide A/II band is sensitive to the strength of the hydrogen bonding of the amide groups of the polypeptides. These workers also proposed that this band could be used to indicate the strength of hydrogen bonding, if the secondary structure of the polypeptides was the same. Comparison of the FT-IR and FT-NIR spectra of proteins such as lysozyme and chymotrypsinogen revealed that all proteins except some types of collagen revealed the amide A/II band between 2053-2057 nm indicating the insensitivity of this band to the secondary structure of the proteins (Liu et al., 1994). By examination of pepsin that had been denatured to different extents, Liu et al. (1994) also proposed that the position of the amide A/II combination band in the NIR region was sensitive to the degree of denaturation of the protein studied. A shift to higher frequency (shorter wavelength) was proposed to be indicative of destruction of the hydrogen bonds in the amide groups of the protein studied (pepsin) and therefore of the denaturation of the protein.

Miyazama and Sonoyama (1998) measured the FT-NIR spectra of seven globular proteins including lysozyme and myoglobin and also found a common absorption band for Amide A/II at 2055 nm, regardless of the secondary structure of the proteins studied. These workers were able to assign an absorption band at 2210 nm to sheet structures within the globular proteins studied and observed bands at 2141 nm and 2169 nm in all proteins, which although unassigned, were proposed to provide information on the secondary structure of proteins.

Robert et al. (1999) used a generalised canonical correlation analysis to take into account data from the mid-IR and NIR analysis of twelve proteins as well as reference data in order to determine whether the secondary structure of proteins could be
monitored using near infrared spectroscopy. The potential of NIRS for this purpose was confirmed by these workers, with the successful assignment of bands representative of the α helix (2172 and 2289 nm), β sheet (2205, 2264 and 2313 nm) and unordered structures (2265 nm) for the proteins studied.

In a review of NIR interpretive spectroscopy by Workman (1996), several band positions were assigned to the functional groups of proteins. These are included in Table 5.1.

**Table 5.1.** Features of protein NIR spectra from the literature, highlighted for their expected value in the analysis of the spectra of co-spray-dried protein/carbohydrate formulations.

<table>
<thead>
<tr>
<th>Observation*</th>
<th>Significance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Altered peak at 1350 nm</td>
<td>Indicative of denaturation of the protein</td>
<td>Sadler <em>et al.</em> (1984)</td>
</tr>
<tr>
<td>Protein band assignments 1500-1530 nm</td>
<td>N-H stretch 1st overtone</td>
<td>Workman, (1996)</td>
</tr>
<tr>
<td></td>
<td>N-H stretching vibrations</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Primary amide carbonyl stretch (+ maillard-bound protein)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N-H bend/C=O stretch and N-H bend/C-N stretch combination bands</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amide A/II band ~2053-2057 nm</td>
<td>Insensitive to protein 2° structure</td>
</tr>
<tr>
<td></td>
<td>If 2° structure is the same, this is indicative of the strength of H-bonding of amide groups</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Shifts to shorter wavelength (higher frequency) with increased denaturation of the protein (H-bond breakage in the amide groups)</td>
<td></td>
</tr>
<tr>
<td>Band at 2172 nm</td>
<td>Indicative of α helix secondary structure</td>
<td>Robert <em>et al.</em> (1999)</td>
</tr>
<tr>
<td>Band at 2205 nm</td>
<td>Indicative of β sheet secondary structure</td>
<td>Robert <em>et al.</em> (1999)</td>
</tr>
<tr>
<td>Band at 2210 nm</td>
<td>Assigned to sheet structures in globular proteins</td>
<td>Miyazama and Sonoyama (1998)</td>
</tr>
</tbody>
</table>

*Observations above 2250 nm in the literature were omitted because of interference above this wavelength when using the fibre optic probe for NIR spectral collection.*
5.1.4 Water and hydrogen bonding in the near infrared region

Bound water can be distinguished from free water by NIRS because of the different types and strengths of bonds that water can form (Subramanian and Fisher, 1972). Free water is the product of three separate molecular species, with one, two or three hydrogen bonds (Workman, 1996). These different bonds vibrate in different ways and therefore cause the evolution of separate peaks in the NIR region. The largest observable changes in the NIR region are due to changes in the hydrogen bonded species in the sample being studied; changes to the hydrogen bonding in a sample can cause peak shifts or broadening/sharpening in the spectra. The formation of hydrogen bonds results in peak shifts in the near infrared region to longer wavelengths, whereas bond breakage leads to peak shifts to shorter wavelengths (Shenk et al., 1992). Generally, the more hydrogen bonds in a sample, the more intense the X-H peaks in the NIR spectrum for that sample (Workman, 1996).

There have been several publications related to the absorption bands of water in the NIR region and also to the investigation of the hydration of macromolecules, including proteins. A summary of the relevant literature is given below, followed by a table outlining the main points for the NIR analysis of hydration related to proteins that may be useful for the analysis of the NIR spectra in this chapter (Table 5.2).

Subramanian and Fisher (1972) examined the hydration of polypeptides by NIRS, using 'hydration spectra', based on the work of McCabe and Fisher (1970). This technique of comparing the spectrum of an aqueous solution of the solute to the spectrum of water enabled the spectra corresponding to bulk, bound or excluded water to be separated. From studies of the hydrated spectra of coiled and uncoiled polypeptides, Subramanian and Fisher were able to tentatively assign a peak at 1405 nm ± 3 nm to a combination band representative of water molecules hydrogen-bonded through their lone pairs to the –NH$_2$ groups of the polypeptides. These workers also assigned a peak at 1530 nm to the absorption related to free –NH groups and a shoulder at 1540 nm to hydrogen-bonded –NH groups in the polypeptides studied.

Bonner and Choi (1974) identified the 1900 nm peak in pure water to correspond to those water molecules with free –OH groups. They reported that more extensively bound water molecules caused this peak to shift to higher wavelengths. McCabe et al. (1970) indicated that the peaks between 1350-1400 nm could be assigned to free –OH
groups in water molecules, whereas a peak nearer 1500 nm could be assigned to that of completely bound water. Vandermeulen and Ressler (1980a) were able to record the near infrared spectra of intact proteins in solution, by compensating for the excluded volume of water around the proteins in the NIR spectra. These workers found that the water of hydration for the proteins studied (pepsin and bovine serum albumin) gave peaks near 1500 nm and 1950 nm, whereas the peaks for pure water were found at 1450 nm and 1928 nm.

Vandermeulen and Ressler (1980b) investigated the effect of the interactions with sodium dodecyl sulphate (SDS) on the NIR spectra of three proteins (bovine albumin, ovalbumin and β-lactoglobulin) in aqueous solution. It was observed that the absorption band for water bound to proteins at 1490 nm shifted to 1430 nm in the presence of SDS, consistent with decreased hydration of the protein surface and the hydrophobic environment induced by the SDS alkyl chains. As Kuntz and Kauzmann (1974) proposed that the proportion of water molecules binding to proteins was greatest for charged groups of proteins, Vandermeulen and Ressler (1980b) claimed that as the SDS interacted with these charged protein groups, that water was displaced; therefore the hydration of the protein reduced.

In a study of water sorption behaviour of amorphous raffinose (Hogan and Buckton, 2001b), the authors were able to identify the different hydrate forms of raffinose using the combined technique of gravimetric vapour sorption and near infrared spectroscopy, concentrating on the peak at 1440 nm in the NIR spectrum of raffinose, assigned to –OH interactions. Zhou et al. (2003) carried out an NIR study to determine the water content of a drug substance using Principal Component Analysis (PCA). They researchers were also able to identify the different hydrate forms of a drug substance using this technique and attributed a peak at 1904 nm to surface water and a peak at 1936 nm to bound water. In this way, Zhou and colleagues were able to distinguish between samples containing both surface and bound water.
Table 5.2. A summary of the features of water/hydration in the NIR region from the literature, highlighted for application to the analysis of the spectra of co-spray-dried protein/carbohydrate formulations.

<table>
<thead>
<tr>
<th>Observation</th>
<th>Significance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak at 1405 nm ± 3 nm</td>
<td>Water molecules hydrogen-bonded through their lone pairs to –NH₂ groups of polypeptides</td>
<td>Subramanian and Fisher (1972)</td>
</tr>
<tr>
<td>Peak at 1440 nm</td>
<td>Hydrate water of raffinose</td>
<td>Hogan and Buckton, (2001b)</td>
</tr>
<tr>
<td>1490 nm peak shift to shorter wavelength (1430 nm)</td>
<td>Decreased hydration of protein due to interaction of charged protein groups with SDS</td>
<td>Vandermeulen and Ressler (1980b)</td>
</tr>
<tr>
<td>Peak at 1530 nm + shoulder at 1540 nm</td>
<td>Free –NH groups and hydrogen-bonded –NH groups respectively</td>
<td>Subramanian and Fisher (1972)</td>
</tr>
<tr>
<td>Peak at 1900 nm</td>
<td>Water molecules with free –OH groups</td>
<td>Bonner and Choi (1974)</td>
</tr>
<tr>
<td>1904 nm</td>
<td>Surface (free) water</td>
<td>Zhou et al. (2003)</td>
</tr>
<tr>
<td>Peaks at 1450 nm and 1928 nm</td>
<td>Pure bulk water (in aqueous solutions of protein)</td>
<td>Vandermeulen and Ressler (1980a)</td>
</tr>
<tr>
<td>Peaks near 1500 nm and 1950 nm</td>
<td>Water of hydration for proteins in aqueous solution</td>
<td>Vandermeulen and Ressler (1980a)</td>
</tr>
</tbody>
</table>

The above reviews of studies of water/hydration and proteins using near infrared spectroscopy highlighted the variability in absorption peak/band positions in the NIR region specific to particular samples. Given the complicated structure of some samples, particularly macromolecules, it is unsurprising that the NIR spectra are complicated, with overlapping bands due to the many different types of bonds in their structures. The band positions shown in Tables 5.1 and 5.2 are those specific to the research from which they were taken and it must be assumed that there may be variation of the position of the assigned bands, dependent upon the sample being examined. Although these difficulties in the use of near infrared spectroscopy have been highlighted, its use has many benefits over other forms of spectroscopy. The use of a near-infrared probe such as that used in the studies in this thesis allows the fast, non-destructive collection of spectral data for samples in real-time. No sample preparation is required, which is particularly useful for samples of limited mass or for samples that may degrade on exposure to the atmosphere, for which spectra may be measured through the packaging, if appropriate.
5.1.5 Rationale and Aims of Chapter Five

Gravimetric vapour sorption analysis was used in Chapters Three and Four of this thesis to examine the polymorphism and crystallisation behaviour of spray-dried trehalose samples. A simple three stage dynamic vapour sorption experiment was devised, in which the form of trehalose in the dried state before and after exposure to 75% RH could be examined. Following the conclusion of this work it was decided that the same experimental design could be used to examine the behaviour of trehalose in co-spray-dried protein/carbohydrate formulations, in which the water vapour sorption behaviour would be much more complex. The aim of this work was to determine the form of trehalose in the co-spray-dried formulations and to examine the gravimetric water vapour sorption behaviour for evidence of phase separation of the components at increased relative humidity. If the carbohydrate were in fact protecting the protein simply by the maintenance of an amorphous matrix alone ('Glassy Matrix Theory' of biostabilisation), then phase separation of the carbohydrate at 75% RH would be expected, in line with that expected of the carbohydrate component in isolation. If the carbohydrate were interacting with the protein (i.e. the 'Water Replacement Hypothesis'), then the gravimetric water vapour sorption behaviour of the sample would be more complex at 75% RH, with either a lack of phase separation or phase separation of the free fraction of the carbohydrate component with the remaining carbohydrate being bound to the protein.

Analysis of the gravimetric vapour sorption data alone was expected to be difficult given the complexity of the co-spray-dried systems. From the assignment of peaks in the NIR spectra of different polymorphic forms of trehalose in Chapter Three and the knowledge gleaned from the review of relevant literature on the analysis of proteins, hydration and hydrogen bonding by NIRS given above, it was hoped that near infrared spectra, collected at intervals during gravimetric vapour sorption experiments, would provide some evidence as to the form of the components in the co-spray-dried protein/carbohydrate formulations.
5.2 Experimental

5.2.1 Production of spray-dried catalase samples
Co-spray-dried samples were produced according to the formulation plan outlined in Section 2.1. The ratio of catalase and trehalose in the co-spray-dried formulations in each sample set is given in Table 5.3. All feed solutions consisted of 0.5 % w/v total solids in water and spray drying was performed using a Büchi 191 Mini Spray Dryer (Büchi, Switzerland). Spray-dried samples were stored in desiccators over phosphorus pentoxide (0 % RH) until analysis.

Samples were checked for crystallinity using X Ray Powder diffraction as described in Section 2.3 and water content was assessed using thermogravimetric analysis, as outlined in Section 2.4. All samples were imaged before and after the DVS experiments, using scanning electron microscopy as described in Section 2.5.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>% Catalase</th>
<th>% Trehalose</th>
</tr>
</thead>
<tbody>
<tr>
<td>ct 0100 x</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>ct 2080 x</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>ct 4060 x</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>ct 5050 x</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>ct 6040 x</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>ct 8020 x</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>ct 1000 x</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

5.2.2 Gravimetric Vapor Sorption/Near Infrared Spectroscopy
Gravimetric studies of co-spray-dried catalase/trehalose samples were carried out using a humidity and temperature controlled microbalance Dynamic Vapor Sorption (DVS) apparatus (Surface Measurement Systems, London, UK) as described in Section 2.2. Samples of approx. 30 mg were loaded onto the flat-bottomed quartz glass sample pan of the DVS apparatus, dried at 0 % RH for 8 h and subsequently exposed to 75 % RH for 10 h to induce re-crystallisation of free amorphous trehalose in the samples, before a second drying stage for 6 h. The NIR spectrometer recorded a mean of 32 scans over the wavelength region 1100 – 2500 nm every 15 min of the DVS experiment, via a fibre optic probe (Foss NIRSSystems, UK) as described in Section 2.2. Throughout this chapter, the term ‘peak’, when referring to near infrared spectra, will be used to describe
downward-pointing troughs corresponding to mathematically treated SNV normalised, second derivative spectra.

5.2.3 Determination of Sample Catalase Content

The haem content of the catalase enzyme structure means that the enzyme displays a characteristic absorption at 405 nm (Aebi, 1983; Tanaka et al., 1991). This absorption could therefore be used to establish the concentration of catalase in samples.

Preparation of 50 mM Phosphate Buffer Solution

6.81 g of KH$_2$PO$_4$ was dissolved in purified water and made up to 1000 mL, to produce buffer solution A. 7.10 g of Na$_2$HPO$_4$ was dissolved in purified water and made up to 1000 mL to produce buffer solution B. Solutions A and B were mixed in the proportion 1:1.5 (v/v) to produce 50 mM phosphate buffer. The pH of the solution was determined as pH 7.0.

Scanning UV spectroscopy was used to ensure that the phosphate buffer used in the assay or the trehalose in the samples did not interfere with the characteristic absorption of catalase at 405 nm. No interference was observed. A sample of the catalase product obtained from Sigma dissolved in phosphate buffer was scanned against a phosphate buffer reference in order to check the presence of the absorption band at 405 nm.

Preparation of Calibration and Sample Solutions

Standard solutions of 0, 1, 10, 100, 250, 500 and 750 μg/mL were produced through dilution of a 1000 μg/mL solution prepared using the original catalase product used to produce solutions for spray drying. Approximately 25 mg of each co-spray-dried catalase/trehalose sample was accurately weighed, dissolved in phosphate buffer pH 7.0 and made up to a volume of 50 mL. The absorbance of each calibration and test sample was measured at 405 nm in a UV spectrophotometer (Cary 3E, Varian, Australia), in triplicate. The results of the calibration samples were plotted to produce a calibration curve of catalase concentration (μg/mL) against absorbance (405 nm), the equation for which was applied to the results of the test samples to allow the concentration of catalase in the samples to be calculated.

Particular difficulties were experienced in achieving full dissolution of the co-spray-dried samples in phosphate buffer pH 7. In order to bring about dissolution of the
samples, particularly those containing a higher proportion of catalase, vigorous shaking of the solution was required, leading to frothing. This frothing in turn led to difficulties achieving accurate sample volumes prior to analysis. Samples were made up to volume as accurately as possible, however this was a substantial source of error in the assay method. Other sources of error are outlined in (Table 5.4). However, good calibration curves were produced using the original catalase as supplied and the results for the samples were around those expected. The calculated catalase percentage of the 100% spray-dried catalase product in each sample set was designated as 100% and percentages adjusted in line with this.

**Table 5.4.** Possible sources of error and/or variability in the method for the determination of catalase content described in Section 5.2.3. *Denotes additional sources of error in the catalase activity assay described in Section 5.2.4.

<table>
<thead>
<tr>
<th>Possible Sources of Error/ Variability in the Catalase Activity Assay Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>▪ Weighing of samples</td>
</tr>
<tr>
<td>▪ Intrinsic pipette error</td>
</tr>
<tr>
<td>▪ Different batches of</td>
</tr>
<tr>
<td>- Phosphate buffer</td>
</tr>
<tr>
<td>- Hydrogen peroxide solution*</td>
</tr>
<tr>
<td>▪ Incomplete dissolution of samples</td>
</tr>
<tr>
<td>▪ Sample frothing – leading to inaccurate solution volumes</td>
</tr>
<tr>
<td>▪ Loss of activity of samples in solution prior to measurement*</td>
</tr>
<tr>
<td>▪ Intrinsic error of the UV spectrophotometer</td>
</tr>
<tr>
<td>▪ Human error</td>
</tr>
</tbody>
</table>

**Calculation of catalase content**

An example calibration curve of catalase concentration (µg/mL) against absorbance (405 nm) is shown in Figure 5.1.

**Example Calculation** (assuming 100% catalase in 100% spray-dried catalase sample):

\[
\text{Abs}_{405} \text{ct8020a (}= 80\% \text{ catalase content}) = 0.076
\]

Equation from calibration curve:

\[
y = 0.0002x + 0.0007
\]

Catalase concentration (µg/mL) of sample = \[
0.076 - 0.0007 \\
0.0002
\] = 376.5 µg/mL

Calculated Catalase in 100mL = 37.65 mg/100mL
Actual weight of sample in 100 mL = 49.20 mg
Expected % Catalase in sample = 80%
Actual % Catalase in sample = 76.52%

Figure 5.1. An example of a calibration curve of catalase concentration (µg/mL) against absorbance (405 nm) used in the calculation of catalase content in the co-spray-dried catalase/trehalose samples.

5.2.4 Determination of Catalase Activity

\[
2\text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} \text{O}_2 + 2\text{H}_2\text{O}
\]

Catalase activity is generally measured in two ways: either by following the decomposition of hydrogen peroxide to produce oxygen and water (as shown in the reaction above), or by measurement of the volume of oxygen produced by the decomposition (Aebi, 1983). The most preferred assay method because of its sensitivity and ease of use is the UV method based upon that developed by Chance and Herbert (1950) and Maehly and Chance (1954). This method is based upon the fact that the decomposition of hydrogen peroxide by catalase can be measured directly, by the decrease in absorbance at 240 nm (Aebi, 1983).
As no assay method was provided specific to the catalase product used in this study, a UV method documented in the product literature for a similar catalase product produced by Kikkoman was used, based upon the UV method published by Aebi (1983).

**Preparation of Hydrogen Peroxide Solution**

0.75 mL of 30% H₂O₂ solution was diluted with phosphate buffer (preparation as described in Section 4.1.2.3) to 100 mL to produce substrate solution A. The absorbance at 240nm (1 cm light path) of 2.0 mL of the resultant solution with 1.0 mL phosphate buffer was checked against phosphate buffer in the reference cell with a target A₂₄₀nm of 0.85 ± 0.02. Substrate solution A was subsequently diluted with an appropriate volume of phosphate buffer to produce the desired absorbance reading and the absorbance re-checked at 240 nm in the same manner (substrate solution B). The final solution was stored over ice, protected from light and a small volume allowed to equilibrate to room temperature prior to each sample measurement.

**Preparation of Sample Solutions**

A mass of each co-spray-dried catalase/trehalose sample was accurately weighed to ensure an approximate proportional mass of 6mg catalase in each sample test solution. Each sample was dissolved in phosphate buffer and made up to 50mL to produce an initial sample solution. A 1 mL aliquot of each initial sample solution was removed and diluted to a volume of 50 mL with phosphate buffer to produce the final sample solution for measurement. Sample solutions were produced individually, immediately prior to measurement, to reduce the degradation of the protein in solution. Samples were stored over ice between triplicate measurements and allowed to equilibrate to room temperature prior to measurement.

**Sample Measurement**

The decomposition of hydrogen peroxide by catalase to produce water and oxygen is a first order reaction, meaning that the rate of hydrogen peroxide decomposition is directly proportional to the initial concentration of both the substrate and the enzyme. Assuming that hydrogen peroxide concentrations at the start and end of the assay measurement period are known, the activity of the known amount of enzyme may be determined.
The rate of hydrogen peroxide was followed by measuring the decrease in absorbance at 240 nm of each sample solution over 5 min. 2.0 mL of each final sample solution was pipetted into a 3 mL quartz, far-uv cuvette (1 cm light path) and 1.0 mL of the prepared hydrogen peroxide sample added. A blank solution, consisting of 2 mL phosphate buffer in place of the sample solution was also tested. The decrease in absorbance of each sample was measured over 5 min against phosphate buffer in the reference cell, using a Cary 3E UV-Visible spectrophotometer (Varian, Australia). A timed measurement programme was used in order that absorbance readings could be measured every 10 s of the 5 min measurement period. Each sample was run in triplicate and the results were transferred into the Excel software package for data manipulation.

**Calculation of Catalase Activity**

Graphs of time (min) against absorbance were plotted for each sample tested (e.g. Figure 5.2). The activity of catalase was calculated according to the following formulae (Kikkoman product literature):

\[
\text{Volume Activity (U/mL)} = \frac{(\Delta A_S - \Delta A_0) \times 3.0 \text{ (mL)} \times \text{df}}{0.0436 \times 2.0 \text{ (mL)}}
\]

\[
= \Delta A \times 34.4 \times \text{df}
\]

\[
\text{Weight Activity (U/mg)} = (\text{U/mL}) \times \frac{1}{C}
\]

Where:

\( \Delta A_S \) = The change in absorbance over time for the sample, using the linear proportion of the curve

\( \Delta A_0 \) = The change in absorbance over time for the blank solution, using the linear proportion of the curve

0.0436 = Millimolar extinction coefficient of \( \text{H}_2\text{O}_2 \) at 240 nm (\( \text{cm}^2/\mu\text{mol} \))

\( \text{df} \) = Dilution factor of catalase sample

\( C \) = Content of catalase preparation in sample (mg/mL)

\( U \) = Units of activity of catalase, where one unit is defined as the amount of catalase which decomposes 1 \( \mu\text{mol} \) of \( \text{H}_2\text{O}_2 \) per minute under the conditions of the assay method.
The volume activity referred to the activity of the catalase in each mL of the initial sample solution; therefore a dilution factor was included in the equation to take into account the dilution of the initial sample solutions during the assay method. The dilution factor used in this study was 50; denoting the dilution of 1 mL of each the initial sample solution to 50 mL in phosphate buffer. $\Delta A_S$ and $\Delta A_0$ were determined from the linear part of the time (min) against absorbance curves produced for each test sample. For the samples, this was determined to be between an absorbance of 0.6 and 0.7.

An example of the calculation performed to determine the activity of the catalase in each sample, using a sample of the original catalase as supplied, is shown below.

**Sample Information**

- **Sample name:** Original Catalase as supplied 1
- **Weight of sample:** 9.22 mg
- **Concentration of catalase in initial solution:** $0.1844 \text{ mg/mL}$
- **Expected Activity (as stated by the product manufacturer):** $1588 \text{ U/mg}$

![Graph of time (min) against absorbance (only linear proportion of curve shown) for the decomposition of $H_2O_2$ by sample 1 of the original catalase as supplied.](image)

**Figure 5.2.** Graph of time (min) against absorbance (only linear proportion of curve shown) for the decomposition of $H_2O_2$ by sample 1 of the original catalase as supplied.

**Activity Calculation**

$\Delta A_0 = 0.0011$

$C = 0.1844 \text{ mg/mL}$
Volume Activity (U/mL) = \frac{(\Delta A_5 - \Delta A_0) \times 3.0 \text{ (mL)} \times df}{0.0436 \times 2.0 \text{ (mL)}}

= \frac{(0.1766 - 0.0011) \times 3.0 \text{ (mL)} \times 50}{0.0436 \times 2.0 \text{ (mL)}}

= 301.89 \text{ U/mL}

Weight Activity (U/mg) = (U/mL) \times \frac{1}{C}

= 301.89 \times 5.42

= 1637.16 \text{ U/mg}

Validity of the Activity Calculation
The value of 1637.16 U/mg for the weight activity of the original catalase sample as supplied was just 3.08 % higher than that specified by the product manufacturer (1588 U/mg). Given the assay limit range that the manufacturer will have placed on the catalase product for the activity stated on the products of at the very least, ± 5 %, the calculated value was considered the same as that stated by the manufacturer and indicated the validity of the assay method. The mean weight activity of three samples from the same initial sample solution of the original catalase as supplied was calculated to be 1678.52 U/mg with a standard deviation of ± 49.42 U/mg. This was equivalent to a standard deviation of 2.94 %, which indicated the repeatability of the assay method. The standard deviation of the mean weight activity values of triplicate testing of three initial sample solutions of the original catalase product as supplied was calculated to be ± 63.03 U/mg, equivalent to ± 3.64 %. This suggests the very low inter-batch and inter-day variability of the assay, which is noteworthy considering the errors related to the assay as shown in Table 5.4 (Section 5.2.3). The activity assay method could therefore be considered valid for the purposes of this study.

5.3 Results and Discussion
5.3.1 Determination of sample catalase content
Catalase content in the co-spray-dried samples was determined as described in Section 5.2.3. The averages of the mean results of triplicate testing of the samples on three different days are presented in Table 5.5.
Table 5.5. Results of the catalase concentration assay for co-spray-dried catalase/trehalose samples.

<table>
<thead>
<tr>
<th>Sample Expected % Catalase Content</th>
<th>Average % Catalase Content</th>
<th>Standard deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>19.15</td>
<td>2.09</td>
</tr>
<tr>
<td>40</td>
<td>39.14</td>
<td>3.50</td>
</tr>
<tr>
<td>50</td>
<td>46.64</td>
<td>2.08</td>
</tr>
<tr>
<td>60</td>
<td>62.17</td>
<td>3.79</td>
</tr>
<tr>
<td>80</td>
<td>82.06</td>
<td>3.22</td>
</tr>
</tbody>
</table>

5.3.2 Determination of catalase activity

A summary of the activity data for the co-spray-dried catalase/trehalose samples is given in Table 5.6. It was observed that the activity of the original catalase as supplied reduced over time (from 1801 U/mg to 1678 U/mg over a period of six months). However, the determination of the activity of catalase in co-spray-dried samples was generally carried out within a week of spray drying, meaning that the activity of the catalase prior to spray drying was effectively the same as that tested in the activity assay for the spray-dried sample set.

From the data it was observed that the catalase denatured upon spray drying, with the 100% spray-dried catalase sample retaining just 46.9% of its original activity. This phenomenon was clear with all 100% spray-dried catalase samples, however the extent of denaturation differed between batches, with recovered percentage activity values ranging between 46.9 and 71.7%. A higher retention of enzyme activity was observed when catalase was co-spray-dried with trehalose, with a maximum retention of activity of 89.8% shown by the catalase: trehalose 20:80 (% w/w) sample in the batch data shown in Table 5.6. In all batches of co-spray-dried samples, the highest retention of activity of catalase was observed by those samples containing the lowest proportion of catalase to trehalose, however as can be observed in the batch data, no trend was observed in the retention of the activity of catalase across the range of catalase/trehalose contents.
Table 5.6. Summary of activity data for co-spray-dried catalase/trehalose samples before and after the DVS experiments described in Section 5.2.2.

<table>
<thead>
<tr>
<th>Sample % Catalase Content</th>
<th>Wt. Activity (U/mg) Pre-DVS (± SD %, n=3)</th>
<th>Average % Recovery of Activity Pre-DVS (Original catalase* = 100 %)</th>
<th>Wt. Activity (U/mg) Post-DVS (± SD %, n=3)</th>
<th>Average % Recovery of Activity Post-DVS (Compared with activity pre-DVS)</th>
<th>Average Overall % Recovery of Activity post-DVS compared with original</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>1507.57 (5.29)</td>
<td>89.8</td>
<td>794.41 (6.26)</td>
<td>52.7</td>
<td>47.3</td>
</tr>
<tr>
<td>40</td>
<td>919.40 (1.98)</td>
<td>54.8</td>
<td>461.63 (0.87)</td>
<td>50.2</td>
<td>27.5</td>
</tr>
<tr>
<td>50</td>
<td>1418.32 (0.82)</td>
<td>84.5</td>
<td>897.01 (1.51)</td>
<td>63.2</td>
<td>53.4</td>
</tr>
<tr>
<td>60</td>
<td>1010.14 (0.74)</td>
<td>60.2</td>
<td>575.80 (5.95)</td>
<td>57.0</td>
<td>34.3</td>
</tr>
<tr>
<td>80</td>
<td>1020.88 (2.05)</td>
<td>60.8</td>
<td>807.06 (5.57)</td>
<td>79.1</td>
<td>48.1</td>
</tr>
<tr>
<td>100</td>
<td>787.19 (2.94)</td>
<td>46.9</td>
<td>658.67 (7.13)</td>
<td>83.7</td>
<td>39.2</td>
</tr>
</tbody>
</table>

*Weight activity of original catalase as supplied = 1678.52 U/mg (± 2.94 %).

Following exposure to 75% RH and a further drying stage during the DVS experiments, the activity of the catalase in the formulations was compared with those activities recorded prior to the DVS experiments (Column 5 in Table 5.6). A further reduction in the activity of the catalase in all of the formulations was observed, with no trend observed across the range of catalase/trehalose contents. The 100% spray-dried catalase sample retained the highest activity following the DVS experiment compared with its activity pre-DVS, and the second highest retained activity following exposure to 75% RH was observed for the catalase in the co-spray-dried 80:20 catalase: trehalose formulation. The lowest retained activities were observed in the two samples containing the lowest catalase: trehalose ratio. This suggested that although the co-spray-dried sample containing the highest proportion of trehalose protected the protein from denaturation during spray drying, this same stabilisation was not observed during the exposure of the co-spray-dried sample to conditions of high relative humidity.

These findings suggest that separate mechanisms are responsible for the protective effect of trehalose during drying and shelf life. It has been previously reported that different stabilisation mechanisms and hence different stabilising additives are required for stabilisation of proteins during freezing and subsequent drying (e.g. Crowe et al., 158.
1990, Prestrelski et al., 1993b); therefore it is feasible that an additive that stabilises a protein during spray drying may not protect the protein from degradation during its shelf life. The recovery of protein activity upon reconstitution from the dried state following freeze drying is known to correlate directly with the maintenance of the protein native structure in the dried state (Carpenter et al., 1994); therefore it is vital to consider the processing and storage of the protein when considering the choice of appropriate stabilising additive(s).

The data in column 6 of Table 5.6 show the activities of the co-spray-dried catalase/trehalose formulations after the DVS experiments, as a percentage of the initial activities, calculated following spray drying (pre-DVS). These data took into account the denaturation of the protein following spray drying and subsequent exposure to high relative humidity (mimicking accelerated stability trials); therefore the formulation with the highest retention of activity would be favourable for a spray-dried catalase/trehalose formulation that would require storage following the drying process and before ‘use’. The formulation with the highest overall retained activity was the co-spray-dried catalase: trehalose 50:50 formulation, a result that was consistent with the findings of Tzannis and Prestrelski (1999a and 1999b), who found a higher retention of activity of spray-dried trypsinogen when it was formulated in a 1:1 ratio with sucrose.

5.3.3 X-ray powder diffraction
All co-spray-dried catalase/trehalose samples were confirmed to be amorphous by X-ray powder diffraction. The X-ray powder diffraction patterns of all samples exhibited the characteristic ‘halo’ effect demonstrated by amorphous material and had no peaks consistent with crystalline character (data not shown).

5.3.4 Thermogravimetric analysis
Water content of the samples was determined to be 3.3 – 5.2 % w/w by thermogravimetric analysis (see Table 5.7). There was no apparent trend in water content that could be correlated to the ratio of catalase and trehalose in the co-spray-dried samples.
Table 5.7. The water content of co-spray-dried catalase/trehalose as determined by thermogravimetric analysis.

<table>
<thead>
<tr>
<th>Sample % Catalase Content</th>
<th>Water Content (% ± SD, n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.4 (0.4)</td>
</tr>
<tr>
<td>20</td>
<td>4.1 (0.7)</td>
</tr>
<tr>
<td>40</td>
<td>5.3 (0.4)</td>
</tr>
<tr>
<td>50</td>
<td>4.3 (0.1)</td>
</tr>
<tr>
<td>60</td>
<td>4.0 (0.3)</td>
</tr>
<tr>
<td>80</td>
<td>3.4 (0.7)</td>
</tr>
<tr>
<td>100</td>
<td>3.5 (0.3)</td>
</tr>
</tbody>
</table>

5.3.5 Scanning electron microscopy

Scanning electron micrographs of the co-spray-dried catalase/trehalose samples are shown in Appendix 1. The co-spray-dried catalase: trehalose particles appeared ‘fluffy’ to the naked eye, consistent with the appearance of an amorphous material. The electron micrograph of spray-dried trehalose alone showed small, smooth, spherical particles, consistent with those discussed for the spray-dried trehalose samples described in Chapter Four.

No obvious differences were observed in the micrographs between samples containing different proportions of catalase and trehalose, or between the same samples before and after the DVS experiments described in Section 5.2.2. The micrographs of all of the samples containing a proportion of catalase appeared similar, with a shrivelled appearance (see Appendix 1). This observation was consistent with other reports of the appearance of co-spray-dried protein/carbohydrate formulations in the literature. In one such report, the addition of a small quantity of a protein to a solution of lactose caused a dramatic drop in surface tension, indicating the higher surface activity of the protein compared with the carbohydrate alone (Fäldt and Bergenståhl, 1994). Following subsequent spray drying of the solution, the increased surface activity of the protein was reflected in the appearance of the particles, with ‘dents’ appearing in the particles, indicating the presence of the protein at the particle surface (Fäldt and Bergenståhl, 1994). In another paper, the morphology of spray-dried protein particles were reported to be different specific to the individual protein, with recombinant human deoxyribonuclease exhibiting spherical particles, regardless of the addition of lactose to the solution to be spray-dried, and spray-dried particles of bovine serum albumin exhibiting raisin-like morphology, similar to the morphology of the co-spray-dried
catalase: trehalose particles described above (Maa et al., 1997). Since no obvious
difference was noted in the appearance of the co-spray-dried catalase: trehalose samples
before and after the DVS experiments, this suggested that any trehalose that had
crystallised during exposure to 75% RH was contained within the particles, with the
protein dominating the particle surface, causing the morphology of all particles to
appear similar regardless of the composition.

5.3.6 Comparison of original catalase samples (as supplied) with 100 % spray-
dried catalase samples using DVS/NIRS

5.3.6.1 Water Vapour Sorption Behaviour

Batches A, B and C were spray-dried from 0.5 % w/v catalase in water solutions as
described in Section 5.2.1 and dynamic vapour sorption-near infrared experiments were
carried out on samples as described in Section 5.2.2. Identical DVS/NIR experiments
were carried out on the catalase as supplied. Results are shown in Figure 5.3.

![Figure 5.3. DVS plots for three batches of spray-dried catalase and two samples of
the original catalase as supplied, exposed to 0 % RH for 8 h, 75 % RH for 10 h and
then 0 % RH for a further 6 h (at 25 °C). Spray-dried samples were dried from 0.5%
solutions in water.](image-url)
As activity data showed that catalase lost activity upon spray drying (see Section 5.2.5.2.), it was expected that this loss of activity would be reflected in the water sorption behaviour and NIR spectra of the spray-dried samples when compared with the original catalase as supplied. Contrary to this hypothesis, the DVS plots shown in Figure 5.3 showed very little variation between the spray-dried batches of catalase and the original catalase as supplied. Samples lost a mean of 3.3 % mass during the initial drying stage, followed by an mean mass increase of 17.6 % upon exposure of the samples to 75% RH. The sample masses then reached a plateau at 75% RH. Fluctuations shown in the mass of the samples at 75% RH were most likely caused by the intrinsic variability of the sample RH produced in the DVS analyser (± 0.5 % RH), because the change in sample masses over this period do not exceed ± 0.5 %. Upon exposure to 0% RH during the final drying stage of the DVS experiments, all sample masses returned to approximately the same as shown at the end of the first drying stage of the experiments. From these results, the DVS plots of the spray-dried catalase samples may be said to be essentially similar to those of the original catalase as supplied. Given that the calculated activities of the individual 100% catalase original and spray-dried samples were different, it can be concluded that the water vapour sorption behaviour of the samples was not related to activity or stability.

5.3.6.2 Near infrared spectroscopy

Although the water sorption behaviour of the spray-dried catalase samples was essentially similar to that of the original catalase as supplied, the NIR spectra collected during the DVS experiments suggested differences between all of the samples tested. NIR spectra of the samples from the end of the first drying stage of the DVS plots shown in Figure 5.3 were compared. The spectra of the catalase as supplied and those of the spray-dried samples gave peaks at similar wavelengths across the majority of the spectrum, an example of which is shown between 2000-2225 nm in Figure 5.4. The amide A/II peak was shown at 2054 nm for all samples and the peaks at 1976, 2168 and 2208 nm were observed in all samples, with the intensity of the peaks being the only obvious difference between sample spectra. The small peak at 2104 nm varied between samples, however the differences did not correlate with the activity of the samples and there was no reference to a stability indicating peak at this wavelength in the literature.
Figure 5.4. SNV 2nd-derivative NIR spectra between 1950-2225 nm of four batches of spray-dried catalase samples and two samples of the original catalase as supplied. Spectra were recorded at the end of the first drying stage of the DVS plots shown in Figure 5.3.

Although the spectra of the spray-dried samples and the spectra of the samples of the original catalase as supplied were similar at most wavelengths, there were two regions of the spectrum (1320-1400 nm and 1820-1940 nm) in which the samples showed distinct differences, as shown in Figures 5.5 and 5.6.

In Figure 5.5, differences between the spray-dried batches of catalase and the original catalase as supplied are shown between 1320–1400 nm. Peaks in this region are generally indicative of the first overtone of C-H combination bands (-CH3 group C-H stretch and bend, 1355-1365 nm) (Siesler et al., 2002). Alternatively, peaks in this region may be representative of H2O combination vibrations (1375-1385 nm, OH symmetrical and anti-symmetrical stretch). All of the samples exhibited two negative peaks between 1320–1400 nm, the maximum of the first peak ranging between 1346–1360 nm (C-H combination band) and the second peak maximum ranging between 1376–1382 nm (H2O combination band). The samples’ peak maxima are outlined in Table 5.8. No trend was observed as to the positioning of the two peak maxima between the spray-dried samples and the catalase as supplied.
Figure 5.5 (top) and 5.6 (bottom). SNV 2nd-derivative NIR spectra between 1320-1400 nm (top) and 1820-1940 nm (bottom) of four batches of spray-dried catalase samples and two samples of the original catalase as supplied. Spectra were recorded at the end of the first drying stage of the DVS plots shown in Figure 5.3.
Table 5.8. Peak maxima in the first region of variation in the NIR spectra of the 100% catalase samples, between 1320 – 1400 nm.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Negative peak maxima between 1320 – 1400 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st Peak</td>
</tr>
<tr>
<td>Batch A</td>
<td>1350</td>
</tr>
<tr>
<td>Batch B</td>
<td>1358</td>
</tr>
<tr>
<td>Batch C</td>
<td>1360</td>
</tr>
<tr>
<td>Catalase as supplied 1</td>
<td>1346</td>
</tr>
<tr>
<td>Catalase as supplied 2</td>
<td>1354</td>
</tr>
</tbody>
</table>

Differences between 1820–1940 nm in the spectra of spray-dried batches of catalase and the original catalase as supplied are shown in Figure 5.6. Very few absorption peaks are described in the literature for peaks within the 1820-1900 nm range; the only absorptions reported are those indicative of phosphorous group moieties (P-H, 1890 – 1900 nm (Siesler et al., 2002)) and free –OH groups in water molecules at 1890 nm (Vandermeulen and Ressler, 1980a). In the 1900–1940 nm region, there are several reported absorption bands, indicative of the absorption of H₂O (combination OH stretch and bend band, 1930–1940 nm), the second overtone of secondary amide groups (-CONH-) and the first overtone of phosphorous groups (P-OH, 1900–1910 nm). In the 1820–1940 nm region, positions of peaks in the spectrum of spray-dried catalase batch A were similar to those in the spectra of both samples of the ‘catalase as supplied’, whereas the spectra of spray-dried catalase batches B and C were almost identical to each other but different to the other samples. No peak assignments have been made in the literature for peaks between 1820–1890 nm, however it was likely that the peaks around 1910 nm were due to the 2nd overtone of secondary amide groups. The peak at approximately 1934 nm was indicative of the combination absorptions of OH stretch and bend of free water in the samples, not removed during the first drying stage of the DVS experiments owing to entrapment within the convoluted protein structure.

The regions of variation in the 100% catalase samples (1320-1400 nm and 1820-1940 nm) did not differentiate the spray-dried catalase samples from the original catalase samples as supplied. Indeed, the differences in the spectra in these regions denoted intra-batch variability in the original catalase as supplied as well as inter-batch variability in the spray-dried batches of catalase. Although the results of the activity assays described in Section 5.3.2 showed a reduction of activity of the enzyme upon spray drying, no trends were observed in the spectra to indicate peaks corresponding to
the denaturation of the protein in the spray-dried samples when compared with those of
the original catalase as supplied. In a study of the applicability of photoacoustic near-
infrared spectroscopy to examine the conformational changes in the solid state by Sadler
et al. (1984), conformational changes of 20 proteins and polypeptides (not including
catalase) were induced by heating them in solutions of low pH prior to lyophilisation.
These researchers observed that the denatured proteins gave spectra remarkably similar
to those of the proteins in their native form, reporting a small peak at 1350 nm in the
denatured proteins compared with the spectra of the native forms. In the study of the
catalase samples in this chapter, no obvious trend was observed in the position of the
peaks at around 1350 nm in the spectra of the spray-dried catalase and the catalase as
supplied (Table 5.8) nor any correlation in the activity data. This suggested that the
peak at 1350 nm reported in the literature for denatured proteins could not be applied to
denaturation in catalase samples. Further work would be required to confirm this
statement.

The variability and lack of trends in the NIR spectra of the catalase samples was
unsurprising because of the complex nature of the protein molecule. Proteins are
notoriously heterogeneous in their structural character, all the more so in the disordered,
amorphous state, in which both the original catalase as supplied and the spray-dried
catalase were proved to be by X-ray powder diffraction. Although specific peaks in the
near infrared spectra of the samples could not be assigned to active or denatured
elements of catalase, this was not surprising given the evidence described for similar
studies in the literature. The combined technique of dynamic vapour sorption and near
infrared spectroscopy was able to illustrate differences between the spectra that the
water vapour sorption experiments in isolation, could not, indicating the potential of this
technique for future studies in this area of research.

5.3.7 Comparison of co-spray-dried catalase/trehalose samples using DVS-NIRS
Co-spray-dried catalase/trehalose samples were spray-dried from 0.5 % w/v total solids
in water solutions as described in Section 5.2.1 and dynamic vapour sorption-near
infrared experiments were carried out on samples as described in Section 5.2.2.

5.3.7.1 General Water Sorption Behaviour Observations
Gravimetric water vapour sorption results for a batch of seven samples containing
different proportions of catalase to trehalose are shown in Figure 5.7. It was noted that
all of the co-spray-dried catalase/trehalose samples increased in mass during the 75 % RH stage of the experiments to a greater extent than that of the catalase or trehalose spray-dried alone. As the 100 % spray-dried trehalose sample absorbed the lowest amount of moisture at 75 % RH, it would be expected that the proportion of trehalose in the co-spray-dried catalase/trehalose sample would absorb less moisture than an equivalent mass of catalase. However, this was not the case, as shown by the mass of the co-spray-dried 50:50 catalase: trehalose sample, which exhibited the greatest mass increase of approximately 22 % at 75 % RH as opposed to the 18.2 % increase shown by the 100% catalase sample under the same conditions. This unexpected result was considered in terms of the proportions of components in the samples and other published work looking at the water vapour sorption behaviour of dried protein/carbohydrate formulations.

Figure 5.7. DVS plots for a batch of co-spray-dried catalase/trehalose samples exposed to 0 % RH for 8 h, 75 % RH for 10 h and then 0 % RH for a further 6 h (at 25 °C). The black-dashed box represents the area magnified in Figure 5.8, discussed later in this Section.

Tzannis and Prestrelski (1999b) used water sorption analysis to investigate their previous observation that high concentrations of sucrose led to a near-complete
reduction in the stabilising effects conferred by the disaccharide onto a co-spray-dried protein (Tzannis and Prestrelski, 1999a). These workers observed that an increase in the concentration of sucrose in the co-spray-dried carbohydrate/protein formulation led to a reduction in the critical percentage RH required to facilitate crystallisation of the amorphous sucrose in the formulations. It was hypothesised that this was because of the increased concentration of sucrose in the formulations facilitating its own crystallisation in the presence of moisture, or because the addition of protein to the co-spray-dried formulation inhibited the crystallisation of the amorphous sugar (Tzannis and Prestrelski, 1999b).

Inhibition of the crystallisation of trehalose in the co-spray-dried catalase/trehalose formulations was observed in the DVS plots shown in Figure 5.7, in which an absence of mass loss at 75% RH in those formulations containing greater than 50% catalase was shown. The absence of mass loss indicated that crystallisation of the trehalose in the sample did not occur. Further evidence for this, combining water vapour sorption and near infrared spectral analysis is described in the individual sample discussions later in this chapter.

Tzannis and Prestrelski (1999b) observed that at lower than 1:1 mass ratios of sucrose to trypsinogen (a model protein) in co-spray-dried samples, the sorptive capacity of the formulations for water was reduced compared with those formulations containing a higher than 1:1 proportion of sucrose. A similar result was observed for the co-spray-dried catalase/trehalose samples (Figure 5.7), however the extent of absorption was also reduced in those formulations containing a lower than 1:1 proportion of trehalose. Tzannis and Prestrelski (1999b) proposed that the amorphous sucrose in their formulations blocked water-binding sites on the protein, providing evidence of the potential for hydrogen bonding between the carbohydrate and protein. They suggested that at greater than 1:1 sucrose: trypsinogen mass ratios, the carbohydrate preferentially interacted with other sucrose molecules, producing a form of phase separation in the formulations (protein–rich and carbohydrate–rich regions). In proposing this theory, Tzannis and Prestrelski (1999b) predicted that the same mechanism of exclusion of the carbohydrate from the surface of the protein in solution was carried through to the solid state upon spray drying, making interaction between the protein and carbohydrate less likely when the carbohydrate was present at higher than 1:1 mass ratios with the protein. As sucrose was shown to crystallise above sucrose mass ratios of 1:1, and protein had
been shown to inhibit its crystallisation, it was hypothesised that the sucrose in these formulations had phase-separated, producing sucrose-rich regions and protein-rich regions, thus reducing the protection of the protein by the carbohydrate (Tzannis and Prestrelski, 1999b). This theory did not hold in the water vapour sorption data of co-spray-dried catalase/trehalose formulations described in this chapter, because although the greatest absorption was observed at a catalase: trehalose ratio of 50:50 (1:1), all of the other formulations containing higher or lower ratios of trehalose to catalase exhibited reduced absorption at 75% RH.

The activity data for the co-spray-dried catalase: trehalose samples in this study (Section 5.3.2) indicated that the highest ratio of trehalose to catalase inferred the greatest protection to the catalase during the spray drying process. Following exposure to 75% RH (causing plasticisation of the amorphous formulations), the highest retention of the original activity prior to exposure was observed in those samples containing the lowest ratio of trehalose to catalase, suggesting that during the shelf life of the co-spray-dried samples, a different mechanism of preservation was required compared with that needed to protect the protein during the drying process. Discussion of the different stresses induced upon proteins with respect to the mechanisms of biopreservation required has been discussed in the literature (e.g. Crowe et al., 1990, Prestrelski et al., 1993b); the majority of such papers referring to the different stresses involved in the freezing and subsequent drying and reconstitution of lyophilised protein products, as discussed in Section 5.3.2. These references, combined with the study described in this chapter, suggest that the mechanism of protection of proteins during processing is complex, and all of the different stresses involved in the processing should be taken into account when choosing the intended preservative agent.

Both of the 100% spray-dried catalase and trehalose formulations exhibited lower levels of moisture absorption under conditions of 75% RH compared with the co-spray-dried samples. This behaviour was unexpected, giving the possibility of an interaction between the catalase and trehalose in the co-spray-dried samples. Reports in the literature described earlier in this section proposed that an increase in interaction between the protein and carbohydrate in dried samples causes a reduction in the hydration of the samples. An opposing view may be expressed based upon the data in this chapter, in which the interaction of proteins with carbohydrates causes an increase in the hydration of co-spray-dried samples. Inhibition of the crystallisation of the
amorphous trehalose in the sample by the protein would allow more moisture to be absorbed by the sample at 75% RH prior to crystallisation. This effect was observed up to and including a 50:50 ratio of catalase to trehalose in co-spray-dried samples. At catalase: trehalose ratios above 50:50, the moisture uptake at 75% RH was reduced, to the same extent as those samples containing less than a 50:50 ratio of catalase: trehalose. Theoretically, the maximum interaction between the catalase and trehalose in the co-spray-dried samples would be expected at a 50:50 catalase: trehalose mass ratio, with a linear reduction expected at higher and lower ratios of the two components. This was observed in the co-spray-dried catalase: trehalose samples, suggesting that an interaction between the catalase and trehalose occurred during spray drying, in line with the water-replacement hypothesis of protein stabilisation proposed by Carpenter and Crowe (1989).

López-Diez and Bone (2004) observed that freeze-dried formulations of trypsin with the highest concentrations of added trehalose exhibited the highest enzyme activities at all time-points during incubation at 77°C for 20 days. It was noted that the initial rapid loss of activity of the enzyme was reduced with increased proportions of the stabilising excipient, regardless of whether it was trehalose or sucrose added. A reduction in hydration in the hydration isotherms for the formulations was determined to indicate interaction between the trypsin and carbohydrate and it was concluded that the greater effectiveness of trehalose as a preservative (compared with sucrose) was because of its increased level of interaction with the protein (López-Diez and Bone, 2004). These researchers hypothesised that the increased level of protein-carbohydrate bonding in trypsin/trehalose formulations compared with trypsin/sucrose formulations may be because of the inability of trehalose to form strong hydrogen bonds with other trehalose molecules in the anhydrous crystal state induced upon dehydration (proposed to be the ‘Form II’ dehydrated dihydrate structure of trehalose, described by Taylor et al. (1998)). This would result in trehalose-protein bonding providing a lower, and therefore more favourable energy state than that provided through trehalose-trehalose bonding, hence causing preservation of the protein (López-Diez and Bone, 2004). This theory may be flawed, because although it has been reported that trehalose is unique in its inability to form intramolecular hydrogen bonds (Aldous et al., 1995) the inability of trehalose molecules to form intermolecular bonds has not been proven. This theory of trehalose forming hydrogen bonds with the protein in the dried state is investigated in this chapter because if this were true, the absorption corresponding to the trehalose-catalase bond
would be evident in the sample NIR spectra, with the intensity increasing up until saturation of the binding sites available on the surface of the protein. Evidence of bonding between the co-spray-dried components in this study is discussed under the results sections for each formulation and in the final summary discussion.

5.3.7.2 Spray-Dried 100 % Trehalose Sample

- Water Vapour Sorption Behaviour
As expected, the water sorption behaviour of the 100 % spray-dried trehalose sample (Figure 5.7) was typical of that shown by the trehalose samples spray-dried from feed solutions of low trehalose concentration described in Chapter 4, Section 4.2. The sample mass decreased as it dried at 0 % RH and then gained 13.4 % as it absorbed moisture upon exposure to 75 % RH. Following this gain, mass loss indicative of crystallisation of the amorphous material within the sample was observed at 75% RH. The sample went on to gradually lose mass at 75 % RH, continuing this loss at the same rate on return to 0 % RH. This water sorption behaviour is consistent with the type of behaviour shown by the trehalose samples spray-dried from 0.5 % feed solutions in Section 4.2, where it was hypothesised that although predominantly amorphous (as shown by the typical halo X-ray powder diffraction pattern), the sample contained areas of anhydrous crystalline material (less than 10 % by XRPD) at the end of the first drying stage of the DVS experiment.

- Near Infrared Spectra
The near infrared spectra of the 100 % spray-dried trehalose sample collected at 15 min intervals throughout the DVS experiment were similar to those discussed in Section 4.2 of this thesis, in which the partly amorphous/partly anhydrous nature of the trehalose samples spray-dried from 0.5 % was discussed.

5.3.7.3 Spray-Dried 100 % Catalase Sample

- Water Vapour Sorption Behaviour
All of the spray-dried samples that contained catalase showed a greater increase in mass at 75 % RH than the sample of 100 % spray-dried trehalose. This was unsurprising because proteins are notoriously difficult to crystallise, usually requiring purification and dialysis. Thus, unlike the amorphous trehalose sample, mass loss indicative of crystallisation was not expected at 75 % RH. The spray-dried protein samples were
expected to absorb moisture under conditions of high relative humidity, until reaching saturation. This was observed with the 100 % spray-dried catalase formulation, which showed an increase in mass of ~ 18.2% before mass stabilisation upon saturation at 75 % RH. The sample then lost mass during the second drying stage of the DVS experiment, to return to its original mass.

- **Near Infrared Spectra**

  The NIR spectra of co-spray-dried 100% catalase samples, collected at 15 min intervals throughout the DVS experiment are described in Section 5.3.6.2.

5.3.7.4 Co-spray-dried Catalase: Trehalose 20:80 Formulation

- **Water Vapour Sorption Behaviour**

  Figure 5.8 shows the magnified region of the DVS plots highlighted in Figure 5.7. After 8 h drying at 0% RH, the co-spray-dried 20:80 catalase: trehalose sample increased in mass by approximately 21.5 % over a period of 55 minutes at 75% RH before exhibiting mass loss (Figure 5.8). The sample lost mass over 40 minutes before mass stabilisation was reached at approximately 12% above the sample's original dry mass. The sample mass remained constant until the end of the 75% RH stage of the DVS experiment, at which point the relative humidity returned to 0% RH. At this point, the sample mass dropped rapidly for approximately 5 minutes and then began to lose mass at a slower rate. This rate of mass loss continued for 100 minutes after which the rate again changed to a faster rate of mass loss, similar to that shown by the co-spray-dried catalase: trehalose 40:60 and 50:50 samples at the same time point. This rate of mass loss continued until the end of the DVS experiment (Figure 5.9).
Figure 5.8. Magnified black-dashed region highlighted in Figure 5.7 (DVS plots for a batch of co-spray-dried catalase/trehalose samples exposed to 0 % RH for 8 h, 75 % RH for 10 h and then 0 % RH for a further 6 h (at 25 °C)).

The DVS plot for the co-spray-dried catalase: trehalose 20:80 sample indicated that recrystallisation of trehalose in the sample occurred at 75% RH. This was shown by mass loss followed by stabilisation at 75% RH. The sample exhibited behaviour typical of a crystallisation response of a wholly amorphous sample of trehalose i.e. after mass loss at high relative humidity, the sample mass stabilised rather than showing gradual mass loss as shown by the 100% spray-dried trehalose sample in which areas of anhydrous material existed. Given that the sample had the lowest proportion of catalase to trehalose, it was not surprising that this sample exhibited the greatest mass loss at 75 % RH out of all of the co-spray-dried samples, indicative of the larger proportion of trehalose in the sample. The mass of the sample stabilised at approximately 12 % above its dry mass, indicating that the sample had absorbed a greater proportion of moisture than that necessary to crystallise the trehalose in the sample. Assuming perfect proportions of 20 % catalase to 80 % trehalose in the sample, if all of the trehalose in the sample had crystallised to the dihydrate form, the mass increase after crystallisation could be approximated at ~7.6 % plus surface water. It was therefore likely that the protein absorbed the moisture unaccounted for in the calculation of the dihydrate water. During the final 0% RH drying stage of the DVS experiment, three consecutive rates of mass loss were observed and the mass did not stabilise during this period (Figure 5.9).
This observation was indicative of the instability of the sample structure at the end of the 75\% RH stage of the experiment, however from the gravimetric vapour sorption data alone it was not clear what changes occurred in the sample structure as it dried at 0\% RH. The NIR spectra of the sample, taken at 15 minute intervals during the DVS experiment, were examined for evidence of the form of the trehalose in the sample at different time points during the DVS experiment and to investigate the behaviour of the sample during the second drying stage at 0\% RH.

**Figure 5.9.**  *DVS plot for the co-spray-dried 20:80 catalase: trehalose sample.*

**NIR Spectra**

NIR spectra of the co-spray-dried 20:80 catalase: trehalose sample from different stages of the DVS experiment are presented in Figure 5.10. The spectra of the 100\% spray-dried catalase sample at the end of the 75\% RH stage and the second drying stage are also presented, in addition to the spectrum of a sample of crystalline \(\alpha,\alpha\)-trehalose dihydrate.

The spectra of the co-spray-dried 20:80 catalase: trehalose sample in Figure 5.10 illustrate the formation of trehalose dihydrate in the sample during the 75\% RH stage of the experiment. This was shown by the absence of the distinctive trehalose dihydrate peak at 1954 nm in the spectrum taken at the end of the first dry stage of the experiment and the subsequent appearance of this peak when a spectrum was collected at 660
minutes into the DVS experiment, after the mass loss at 75% RH indicative of the crystallisation of amorphous trehalose in the sample.

![SNV 2nd-derivative NIR spectra between 1850-2000 nm of co-spray-dried 20:80 catalase: trehalose at various time-points from the DVS plot shown in Figures 5.9, compared with the spectra of α,α-trehalose dihydrate and the 100% catalase and 100% trehalose samples recorded at the end of the 75% RH stage of the DVS experiments.](image)

**Figure 5.10.** SNV 2nd-derivative NIR spectra between 1850-2000 nm of co-spray-dried 20:80 catalase: trehalose at various time-points from the DVS plot shown in Figures 5.9, compared with the spectra of α,α-trehalose dihydrate and the 100% catalase and 100% trehalose samples recorded at the end of the 75% RH stage of the DVS experiments.

From the NIR spectra in Figure 5.10, it was observed that the peak for bulk water shown at 1928 nm in the spectrum of the 100% spray-dried catalase sample at the end of the 75% RH stage was absent from the spectrum of the co-spray-dried 20:80 catalase: trehalose sample taken at the same point. This suggests that all of the free water in the sample was either used to effect the crystallisation of amorphous trehalose in the sample to the dihydrate form, or was bound to the protein. The spectrum of the co-spray-dried sample at the end of the 75% RH stage exhibited a more pronounced peak at 1908 nm compared with that in the spectrum of trehalose dihydrate at 1906 nm. In the dry spectrum of the 100% spray-dried catalase sample, a shoulder was seen at 1914 nm, but a peak at around 1906 nm was absent. Peaks close to 1900 nm are generally considered to be indicative of free –OH groups in water molecules or surface water (Bonner and Choi, 1974 and Zhou *et al.*, 2003, respectively). The assignment of the peak at 1908 nm to free water in the sample at the end of the 75% RH stage of the DVS experiment was
confirmed in the co-spray-dried catalase: trehalose sample because the peak diminished rapidly upon drying at 0% RH.

As the gravimetric vapour sorption behaviour of the co-spray-dried 20:80 catalase: trehalose sample exhibited three different rates of mass loss during the final drying stage of the DVS experiment, the spectrum at the end of the second drying stage was compared with the spectra of the different polymorphic forms of trehalose identified in Chapter 3. This comparison is shown in Figures 5.11 and 5.12.

![Graph showing the comparison of spectra](image)

**Figure 5.11.** A comparison of the SNV 2nd-derivative NIR spectra (between 1300-1600 nm) of the co-spray-dried 20:80 catalase: trehalose sample and the 100% catalase sample from the end of the second dry stage of the DVS plot shown in Figure 5.9, with the spectra of the polymorphic forms of trehalose identified in Chapter Three.

In Figures 5.11 and 5.12 it was clear that at the end of the second drying stage of the DVS experiment, there were at least two forms of trehalose present in the co-spray-dried 20:80 catalase: trehalose sample. In Figure 5.11, peaks corresponding to the ‘DVS-anhydrous’ form of trehalose (identified in Chapter Three) were shown in the spectrum of the co-spray-dried sample at the end of the second dry stage of the DVS experiment. In Figure 5.12, peaks corresponding to α,α-trehalose dihydrate were shown in the spectrum of the co-spray-dried sample at the end of the second dry stage, with the distinctive peak for the dihydrate still present at 1954 nm.
Figure 5.12. A comparison of the SNV 2nd-derivative NIR spectra (between 1850-2000 nm) of the co-spray-dried 20:80 catalase: trehalose sample and the 100% catalase sample from the end of the second dry stage of the DVS plot shown in Figure 5.9, with the spectra of the polymorphic forms of trehalose identified in Chapter Three.

As two forms of trehalose were identified in the spectrum of the co-spray-dried 20:80 catalase: trehalose sample at the end of the second dry stage of the DVS experiment whereas at the end of the 75% RH stage only those peaks corresponding to the dihydrate form were present, it was decided to investigate the development of peaks in the co-spray-dried NIR spectra during the second drying stage. NIR spectra of the co-spray-dried sample taken at intervals during the second drying stage of the DVS experiment are presented in Figures 5.13 and 5.14.

The development of peaks for the DVS-anhydrous form of trehalose is illustrated in Figure 5.13. From the spectra it was shown that the peaks for the DVS-anhydrous form of trehalose had begun to form within 15 minutes after the start of the second dry stage. This was shown by the increase in intensity of the peak at 1384 nm, indicative of the anhydrous form. The double peak with maxima at 1452 and 1476 nm, assigned to the DVS-anhydrous form, did not start to form until 4 hours after the start of the second 0% RH stage of the experiment, at which point a shoulder was evident on the large peak at 1468 nm. The double peak became evident at approximately 45 minutes before the end of the DVS experiment (not shown).
Figures 5.13 (top) and 5.14 (bottom). SNV-2nd Derivative NIR spectra of the co-spray-dried 20:80 catalase: trehalose sample taken at intervals during the second drying stage of the DVS experiment shown in Figure 5.9.

Figure 5.14 shows the development of peaks in the NIR spectra of the co-spray-dried 20:80 catalase: trehalose sample during the final drying stage of the DVS experiment between 1800-2000 nm. The peak indicative of α,α-trehalose dihydrate reduced in intensity over the course of the drying stage at 0% RH, except for during the first 105 minutes, during which the dihydrate peak at 1954 nm did not change in shape or size. These spectra between 1800-2000 nm explain the different rates of mass loss shown in the DVS plot for the sample during the second 0% RH stage of the experiment (see Figure 5.7). The first rate of mass loss was rapid and lasted for approximately 5
minutes. The spectrum taken at the end of the 75% RH stage was compared with the spectra taken 15 minutes later. The only difference between 1800-2000 nm was the diminished peak at 1908 nm. The dihydrate peak at 1954 nm was unchanged at this time point; suggesting that the dihydrate form of trehalose had not yet undergone structural change. It was therefore concluded that the peak at 1908 nm in the spectrum of the co-spray-dried sample at the end of the 75% RH stage was surface water, which was removed in the first 5 minutes of the second drying stage. It would be expected that free water molecules, requiring the least energy to remove from the system would be removed first upon drying; therefore this peak assignment is plausible.

It was hypothesised that if the components of the co-spray-dried samples interacted during the spray drying process, the spectrum of the co-spray-dried sample at the end of the first drying stage would be different to that of a physical mix of the spray-dried components at the same time-point. To investigate this theory, a ‘theoretical physical mix’ of the two 100% spray-dried components was produced, by mathematically summing the appropriate proportions of the SNV-2\textsuperscript{-1}-derivative absorption values of the spectra of the two 100% spray-dried components. This calculation was performed to produce absorption values for the entire range of wavelengths (1100-2250 nm), resulting in a theoretical spectrum for the physical mix of the spray-dried components. By using the SNV-2\textsuperscript{-1}-derivative absorption values of two 100% component spectra from different time-points during the DVS experiments for these samples, theoretical spectra were produced for the different time-points of interest in the DVS experiment. This was preferred rather than producing an actual physical mix of the components because of the known difficulties associated with the mixing of two or more amorphous components to produce a homogeneous mixture. It was decided that a more accurate representation of the ‘mixed’ (non-interacting) components would be achieved through the mathematical calculation of the theoretical spectra from the 100% spray-dried trehalose and catalase samples produced on the same day as the co-spray-dried samples, which would reduce the effect of the variability exhibited between different batches of spray-dried samples described in Chapter Four.

Comparison of the spectrum of the co-spray-dried 20:80 catalase: trehalose sample with the equivalent theoretical spectrum of a physical mix of the two components was performed in order to determine whether the components interacted during the spray drying process and also to determine any behaviour specific to the co-spray-dried
sample that would not have been expected based upon the theoretical spectra. Comparisons of the co-spray-dried 20:80 catalase: trehalose spectra collected at various time-points of the DVS experiment, with the respective ‘theoretical mix’ spectra are shown in Figures 5.15 and 5.16.

*Figures 5.15 (top) and 5.16 (bottom).* A comparison of the co-spray-dried 20:80 catalase: trehalose SNV-2\(^{nd}\) derivative NIR spectra from various time-points of the DVS experiment with the respective ‘theoretical mix’ spectra (SD = spray-dried, TM = theoretical mix).
Differences in the positions of the peaks shown in the spectra of the co-spray-dried sample and the theoretical spectra from the end of the first dry stage of the DVS experiment were determined in order to assess whether there was any interaction between the co-spray-dried components. Two differences in peak position were observed between the two spectra. The first difference was a peak at 1482 nm in the theoretical spectrum, which was observed at 1476 nm in the co-spray-dried spectrum. In a paper by Vandermeulen and Ressler (1980b), a shift of a peak at 1490 nm to a shorter wavelength was found to be indicative of decreased hydration of proteins due to interaction of charged protein groups with the additive, Sodium Dodecyl Sulphate (SDS). The peak shift in that paper was found to be 60 nm, whereas the similar shift noted for the co-spray-dried 20:80 catalase: trehalose sample was just 6 nm. The shift of 60 nm was likely to have been observed in the spectra of the proteins in the presence of SDS because of the nature of the additive. SDS is a highly charged molecule and would be likely to bind with the charged groups of the proteins to a greater extent than lesser-charged trehalose molecules. Vandermeulen and Ressler (1980b) commented that the alkyl chains of the SDS molecules would create a hydrophobic environment upon addition to a solution of protein. Upon binding to the charged groups of the protein, SDS would effect reduced hydration of the protein owing to its hydrophobicity and therefore a large shift in the NIR peak for protein hydration. As trehalose is not a highly charged molecule and is hydrophilic, it would be expected that the shift in the peak for protein hydration upon binding of trehalose molecules would be much smaller than that reported for SDS; therefore the observed peak difference of 6 nm between the co-spray-dried and theoretical spectra may be indicative of an interaction between the protein and carbohydrate.

From the comparison of the co-spray-dried 20:80 catalase: trehalose spectrum at the end of the second dry stage of the DVS experiment with the theoretical spectrum at the same time-point (Figures 5.15 and 5.16), it was observed that the peaks indicative of the DVS-anhydrous form in the spectrum of the co-spray-dried sample were absent from the theoretical spectrum. This suggests that some or all of the trehalose in the co-spray-dried sample formed a less stable dihydrate by the end of the 75% RH stage of the DVS experiment, indicated by the relative ease of loss of the dihydrate to form the anhydrous form at 0% RH. In Chapter 3, the production of the DVS-anhydrous trehalose form from a sample of α,α-trehalose dihydrate took approximately 25 h to complete. It was observed that after 6 h at 0% RH during the production of the DVS-anhydrous form, the
double peak indicative of the DVS-anhydrous form at 1448 and 1480 nm had not yet formed. As this double peak was clear in the spectrum of the co-spray-dried 20:80 catalase: trehalose sample at the end of the second drying stage of the DVS experiment (after 6 h at 0 % RH), this suggests that the dihydrate form shown at the end of the 75% RH stage was unstable. As the peak indicative of α,α-trehalose dihydrate at 1954 nm was also present at the end of the second drying stage, it is clear that dehydration of the trehalose dihydrate formed at the end of the 75% RH stage of the experiment was not complete by the end of the DVS experiment.

From the above analysis, it was hypothesised that the trehalose in the co-spray-dried 20:80 catalase: trehalose sample was partially bound to the protein. However, at 75% RH, trehalose dihydrate was formed and was partially converted to the DVS-anhydrous form during the final 0% RH stage of the experiment. This conversion was more rapid than that shown by a sample of α,α-trehalose dihydrate dried under the same conditions, which indicated the possible instability of the trehalose dihydrate in the co-spray-dried sample at the end of the 75% RH stage.

5.3.7.5 Co-spray-dried Catalase: Trehalose 40:60 Formulation

- Water Vapour Sorption Data

A DVS plot for the co-spray-dried 40:60 catalase: trehalose sample is shown in Figure 5.17. The plot is similar to that of the co-spray-dried 20:80 catalase: trehalose sample except that following crystallisation at 75 % RH, the sample stabilised at a greater mass: ~ 13.5 % over its dry weight compared with approximately ~ 12 % increase in the dry weight of the sample containing 20 % catalase.

The co-spray-dried 40:60 catalase: trehalose sample increased in mass by approximately 21.75 % over a period of 85 minutes at 75 % RH before exhibiting mass loss. The sample lost mass over approximately 95 minutes before the mass began to stabilise at approximately 13 % above the sample’s original dry mass. The sample mass remained constant until the end of the 75 % RH stage of the DVS experiment, at which point the relative humidity was returned to 0 % RH. During the final drying stage of the DVS experiment, the sample exhibited a variable rate of mass loss, similar to that described and discussed for the co-spray-dried 20:80 catalase: trehalose sample.
Figure 5.17. DVS plot for the co-spray-dried 40:60 catalase: trehalose sample.

- NIR Data

NIR spectra of the co-spray-dried 40:60 catalase: trehalose sample from different stages of the DVS experiment are presented in Figure 5.18. The spectra of the 100% spray-dried catalase sample at the end of the 75% RH stage and the second drying stage are also presented, in addition to the spectrum of a sample of crystalline α,α-trehalose dihydrate.

The peak at 1954 nm (assigned to α,α-trehalose dihydrate in Chapter 3) in the spectrum of the sample at the end of the 75% RH stage of the experiment was indicative of the crystallisation of the trehalose in the sample to the dihydrate form. This observation was in line with that observed in the spectrum of the co-spray-dried 20:80 catalase: trehalose sample at the same time point. However, the NIR spectra of the two samples are different due to the fact that the peak indicative of trehalose dihydrate was present at the end of the second dry stage in the spectrum of the co-spray-dried 20:80 catalase: trehalose sample but was absent from the spectrum of the co-spray-dried 40:60 catalase: trehalose sample at the same time-point. The spectrum of the co-spray-dried 40:60 catalase: trehalose sample at the end of the second dry stage of the DVS experiment, compared with the spectra for the various polymorphic forms of trehalose characterised in Chapter 3 is shown in Figure 5.19.
Figure 5.18. SNV-2nd derivative NIR spectra between 1875-2000 nm of co-spray-dried 40:60 catalase: trehalose at various time-points from the DVS plot shown in Figure 5.17, compared with the spectra of α,α-trehalose dihydrate and the 100% catalase and 100% trehalose samples taken at the end of the 75% RH stage of the DVS experiments.

Figure 5.19. A comparison of the SNV-2nd derivative NIR spectra of the co-spray-dried 40:60 catalase: trehalose sample and the 100% catalase sample from the end of the second dry stage of the DVS plot shown in Figure 5.17, with the spectra of the polymorphic forms of trehalose identified in Chapter Three.
From Figures 5.18 and 5.19, it is clear that the trehalose dihydrate present at the end of the 75% RH stage of the DVS experiment had entirely converted to the DVS-anhydrous form by the end of the second drying stage. Comparisons of the co-spray-dried 40:60 catalase: trehalose spectra collected at various time-points of the DVS experiment, with the respective ‘theoretical mix’ spectra are shown in Figures 5.20 and 5.21.

Figures 5.20 and 5.21. A comparison of the SNV-$2^{nd}$ derivative spectra of a co-spray-dried 40:60 catalase: trehalose sample from various time-points of the DVS experiment in Figure 5.17 with the respective ‘theoretical mix’ spectra.
There were no peaks distinctive of the DVS-anhydrous form in the equivalent theoretical spectrum of the co-spray-dried 40:60 catalase: trehalose sample at the end of the second dry stage of the DVS experiment. Peaks corresponding to α,α-trehalose dihydrate were however present in the theoretical spectrum at this time-point. As 6 h at 0% RH was shown not to be sufficient to cause the total dehydration of trehalose dihydrate to the DVS-anhydrous form in Chapter 3, the total dehydration of all of the trehalose dihydrate in the co-spray-dried 40:60 catalase: trehalose sample was unexpected. It was proposed that the trehalose in the co-spray-dried sample had formed an unstable form of the dihydrate at the end of the 75% RH stage of the experiment, similar to the behaviour shown in the co-spray-dried 20:80 catalase: trehalose formulation.

Differences in the positions of the peaks shown in the spectra of the co-spray-dried sample and the theoretical spectra from the end of the first dry stage of the DVS experiment were determined in order to assess whether there was any interaction between the co-spray-dried components. Only one difference in peak position was observed in the spectrum of the co-spray-dried sample compared with the theoretical spectrum. A peak in the theoretical spectrum at 1488 nm appeared at 1482 nm in the spectrum of the co-spray-dried sample. This was similar to the observation noted in the spectrum of the co-spray-dried 20:80 catalase: trehalose sample, which showed a peak at 1476 nm, observed at 1482 nm in the theoretical spectrum. This was again suggestive of an interaction between the protein and carbohydrate because a peak at around 1490 nm is likely to be indicative of the hydration of the protein. A shift in the position of this peak to a shorter wavelength was proposed to be indicative of a reduction in protein hydration due to interaction with an additive (Vandermeulen and Ressler, 1980b).

5.3.7.6 Co-spray-dried Catalase: Trehalose 50:50 Formulation

- Water Vapour Sorption Behaviour

If there were no interaction between the catalase and trehalose during the drying process, then it would be anticipated that the water sorption behaviour of the 50:50 co-spray-dried catalase: trehalose sample would be reflective of the relative amounts of both sample components. Thus, at 75 % RH, an increase in mass would be expected, to a greater extent than that shown by the 100 % spray-dried trehalose sample alone,
owing to the proportion of catalase in the sample, which would absorb a greater quantity of water than the trehalose. Upon saturation of the protein fraction of the sample, it would be expected that the trehalose proportion of the sample would then reach its threshold of moisture uptake prior to recrystallisation of the amorphous carbohydrate to the crystalline, dihydrate form. Mass loss would be expected upon crystallisation of the amorphous trehalose in the sample, followed by mass stabilisation.

In reality the co-spray-dried 50:50 catalase:trehalose sample exhibited what can be described as ‘delayed recrystallisation’ at 75 % RH. The dashed area in Figure 5.7 is magnified in Figure 5.22 to highlight the behaviour of the samples during the 75 % RH stage of the DVS experiments. The DVS plot of the co-spray-dried 50:50 catalase:trehalose sample showed the greatest uptake in mass at 75 % RH than any of the other samples and is shown in full in Figure 5.23. Rather than observing mass uptake followed by a rapid mass loss indicative of crystallisation, a gradual mass uptake was observed, followed by a very gradual loss of mass and eventual mass stabilisation, after approximately 470 minutes at 75 % RH.

![Figure 5.22. Magnified black-dashed region highlighted in Figure 5.7 (DVS plots for a batch of co-spray-dried catalase/trehalose samples exposed to 0 % RH for 8 h, 75 % RH for 10 h and then 0 % RH for a further 6 h (at 25 °C)).](image)
Figure 5.23. DVS plot for the co-spray-dried 50:50 catalase: trehalose sample.

The different rates of mass loss observed during the second dry stage of the DVS experiment that were observed with the co-spray-dried 20:80 and 40:60 catalase: trehalose samples were also observed in the DVS plot of the co-spray-dried 50:50 catalase: trehalose sample. The initial rapid drop in mass during this stage was proven to correspond to free water in the analysis of the co-spray-dried 20:80 catalase: trehalose sample. As the initial mass drop for the 50:50 co-spray-dried sample was larger than that for the other samples, this indicated that the sample contained a higher percentage of free water than the other samples. As the sample showed the greatest uptake in mass at 75 % RH, this was not unexpected.

- Near Infrared Spectra

NIR spectra of the co-spray-dried 50:50 catalase: trehalose sample from different stages of the DVS experiment in Figure 5.23 are presented in Figure 5.24. The spectra of the 100% spray-dried catalase sample at the end of the 75% RH stage and the second drying stage are also presented, in addition to the spectrum of a sample of crystalline α,α-trehalose dihydrate.
Figure 5.24. SNV-2\textsuperscript{nd} derivative NIR spectra between 1875-2000 nm of co-spray-dried 50:50 catalase: trehalose at various time-points from the DVS plot shown in Figure 5.23, compared with the spectra of \(\alpha,\alpha\)-trehalose dihydrate and the 100\% catalase and 100\% trehalose samples taken at the end of the 75\% RH stage of the DVS experiments.

From the spectra of the co-spray-dried 50:50 catalase: trehalose sample, it was clear that amorphous trehalose within the sample underwent recrystallisation during the 75\% RH stage of the DVS experiment, shown by the presence of the characteristic peaks of trehalose dihydrate (assigned in Section 3.4.1.5) at 1952 nm and 1978 nm. These peaks were less defined than those shown in the spectrum of the crystalline 100\% trehalose sample from the end of the 75\% RH stage, and a third peak with a maximum at 1914 nm was observed, with similar intensity to the peak at 1952 nm. A broad peak was shown by the 100\% catalase sample with negative maxima at ~1912 nm and 1932 nm at the end of the 75\% RH stage; therefore it was likely that the third peak at 1914 nm in the co-spray-dried sample corresponded to the proportion of catalase in the sample. The broad peak in the spectrum of the 100\% catalase sample at 1932 nm was attributable to the free water in the sample under the high relative humidity conditions. This peak was absent from the spectrum of the 50:50 catalase: trehalose co-spray-dried sample at the end of the 75\% RH stage of the experiment; therefore it can be hypothesised that the trehalose in the sample utilised free water in the sample in order to effect recrystallisation to the dihydrate form.
There were no detectable peaks characteristic of the amorphous trehalose form in the spectra of the 50:50 catalase: trehalose co-spray-dried sample at the end of the 75% RH stage of the experiment (i.e. absence of the peak for water in amorphous trehalose at 1936 nm as assigned in Chapter 3), suggesting that all of the trehalose in the sample had recrystallised to the dihydrate form or was present in a different form (i.e. bound to the protein).

It was observed that the spectrum of the co-spray-dried 50:50 catalase: trehalose sample at the end of the second drying stage of the DVS experiment was essentially the same as that from the end of the first drying stage of the experiment. The peaks corresponding to the trehalose dihydrate at 1954 and 1978 nm were absent in the final spectrum of the experiment, meaning that the crystalline trehalose dihydrate formed during the 75% RH stage of the DVS experiment had converted to another form. To investigate whether the trehalose in the sample had converted to an anhydrous form of trehalose, the spectrum of the co-spray-dried 50:50 catalase: trehalose sample from the end of the second dry stage of the DVS experiment was compared with the spectra of the polymorphic forms characterised in Chapter Three of this thesis (Figures 5.25 and 5.26).

Figure 5.25. A comparison of the SNV-2nd derivative NIR spectra between 1875-2000 nm, of the co-spray-dried 50:50 catalase: trehalose sample and the 100% catalase sample from the end of the second dry stage of the DVS plot shown in Figure 5.23, with the spectra of the polymorphic forms of trehalose identified in Chapter Three.
Trehalose dihydrate
Alpha anhydrous trehalose
Beta anhydrous trehalose
Amorphous trehalose
ct5050s - end of 2nd drying stage
ctl(HM)x - end of 2nd drying stage
DVS-anhydrous trehalose

From analysis of Figure 5.25, it was unclear whether the trehalose in the co-spray-dried sample had converted to any particular anhydrous form after the final drying stage of the DVS experiment. It was clear however, that the peak at ~ 1978 nm in the co-spray-dried sample was likely to be representative of the catalase in the sample. The same NIR spectra were examined in the region 1300-1600 nm (Figure 5.26), in which it was clear that the trehalose in the co-spray-dried sample had converted to the ‘DVS-anhydrous’ form by the end of the second drying stage of the DVS experiment. The peaks for this form of trehalose appeared to dominate the co-spray-dried sample’s spectrum (owing to its crystalline nature), which could be seen by comparing its spectrum to that of the 100% spray-dried catalase sample from the same time-point. To investigate whether this observation would be the case in a physical mix of the two spray-dried components, the spectrum for the ‘theoretical mix’ of the two spray-dried components, as described under the discussion of the co-spray-dried 20:80 catalase:trehalose sample NIR spectra was compared with that of the co-spray-dried sample. Comparisons of the co-spray-dried 50:50 catalase:trehalose spectra collected at various
time-points of the DVS experiment, with the respective ‘theoretical mix’ spectra are shown in Figures 5.27 and 5.28.

Figures 5.27 and 5.28 highlight the similarities as well as the differences between the spray-dried and theoretical spectra. The co-spray-dried spectra collected at the end of the first dry stage and the end of the 75% RH stage of the DVS experiment were remarkably similar to the theoretical spectra calculated for the 100% spray-dried component spectra at the same time-points. This was evidence that the proportion of the components in the co-spray-dried products remained the same as that in the feed solutions prior to spray drying.

The co-spray-dried spectrum collected at the end of the second dry stage was significantly different to the corresponding theoretical spectrum. The theoretical spectrum indicated the presence of trehalose dihydrate, with dihydrate peaks present at 1954 and 1466 nm (assigned to the dihydrate form in Section 3.4.1.5). There was no evidence of the presence of trehalose dihydrate in the spectrum of the co-spray-dried sample, however the peaks indicative of the DVS-anhydrous form of trehalose were present at 1378, 1480 and 1804 nm within 4.5 h of drying at 0 % RH. This result was unexpected, as the conversion of a sample of 100% α,α-trehalose dihydrate to the DVS-anhydrous form was shown to take approximately 25 h in Chapter Three.
Figures 5.27 and 5.28. A comparison of the SNV-2nd derivative NIR spectra of co-spray-dried 50:50 catalase: trehalose from various time-points of the DVS experiment with the respective 'theoretical mix' spectra.

The full conversion of trehalose dihydrate to the DVS-anhydrous form in the co-spray-dried 50:50 catalase: trehalose sample during the final drying stage of the DVS experiment suggested that the trehalose dihydrate form in the co-spray-dried sample at the end of the 75% RH stage of the experiment was unstable. This observation was in line with the theory of Aldous et al. (1995), who proposed that the special properties of
trehalose for the purposes of biostabilisation were due to its ability to crystallise and phase separate above the glass transition temperature (Tg/°C) of the dried amorphous sample, acting as a desiccant for the rest of the amorphous sample by removing the excess moisture by the formation of the dihydrate. From the results, it was hypothesised that during the 75% RH stage of the DVS experiment, the amorphous co-spray-dried catalase/trehalose sample was plasticised by water vapour, thus greatly reducing its Tg below the experimental temperature. The subsequent crystallisation of the amorphous trehalose in the sample to the dihydrate form protected the protein from free water in the sample, which could otherwise have led to its instability and denaturation. This proposal was strengthened by the observation that the peak for free water at 1932 nm was absent from the spectrum of the co-spray-dried 50:50 catalase: trehalose sample at the end of the 75% RH stage of the DVS experiment. This indicated that free water in the sample was utilised by the amorphous trehalose in the sample to effect crystallisation to the dihydrate form. This hypothesis was applied to the results of the other co-spray-dried catalase/trehalose samples, and will be discussed in the summary discussion for this chapter.

The co-spray-dried and theoretical 50:50 catalase: trehalose spectra at the end of the first drying stage of the DVS experiment were observed to be very similar. The regions of the NIR spectrum in which the peaks assigned to hydrogen bonds are contained are shown in Figures 5.27 and 5.28. A difference in peak position from the ‘expected’ 1490 nm in the theoretical spectrum to 1486 nm in the co-spray-dried spectrum at the end of the first dry stage of the DVS experiment was evidence of the possible interaction of trehalose with catalase in the co-spray-dried sample as remarked upon in the previous discussions of the co-spray-dried 20:80 and 40:60 catalase: trehalose sample data.

5.3.7.7 Co-spray-dried Catalase: Trehalose 60:40 Formulation

- Water Vapour Sorption Data

A gravimetric vapour sorption plot for the co-spray-dried 60:40 catalase: trehalose sample is shown in Figure 5.29. The shape of the plot was similar to that of the 100% spray-dried catalase and that of the co-spray-dried 80:20 catalase: trehalose sample, however the sample absorbed a greater mass of water at 75% RH. The more detailed discussion in Section 5.3.7.8 for the co-spray-dried 80:20 catalase: trehalose formulation also relates to the co-spray-dried 60:40 catalase: trehalose formulation.

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Following mass loss during the first dry stage of the experiment at 0 % RH, the sample mass increased by approximately 21 % before stabilisation at 75 % RH, compared with ~19 % and ~18 % for the co-spray-dried 80:20 catalase: trehalose sample and the 100 % spray-dried catalase sample respectively. Mass loss indicative of the crystallisation of trehalose in the co-spray-dried sample was not observed, suggesting that the trehalose in the sample either remained amorphous or was bound to the protein and therefore unable to crystallise under conditions of 75% RH. Upon exposure to 0% RH during the second drying stage of the DVS experiment, the mass of the sample dropped rapidly as desorption occurred, returning to its original mass by the end of the experiment.

![DVS plot](image)

**Figure 5.29.** DVS plot for the co-spray-dried 60:40 catalase: trehalose sample.

**NIR Data**

NIR spectra of the co-spray-dried 60:40 catalase: trehalose sample from different stages of the DVS experiment are presented in Figures 5.30 and 5.31. The spectra of the 100% spray-dried catalase sample at the end of the 75% RH stage and the second drying stage are also presented, in addition to the spectrum of a sample of crystalline α,α-trehalose dihydrate.

From the examination of the spectra in Figure 5.30 it was concluded that the trehalose in the co-spray-dried sample did not crystallise to the dihydrate form at 75 % RH. The peak assigned to α,α-trehalose dihydrate at 1954 nm in Chapter 3 was absent from the spectrum of the co-spray-dried sample at the end of the 75 % RH stage of the
experiment. The peak at 1470 nm in the spectrum of the co-spray-dried sample at the end of the 75% RH stage was more consistent with the peak at approximately 1472-1474 nm in the spectrum of amorphous trehalose than with the peak at 1466 nm in the spectrum of α,α-trehalose dihydrate (Figure 5.31).

\[ \text{Figure 5.30. SNV-2}^{\text{nd}} \text{ derivative NIR spectra between 1875-2000 nm of co-spray-dried 60:40 catalase: trehalose at various time-points from the DVS plot shown in Figure 5.29, compared with the spectra of α,α-trehalose dihydrate and the 100% catalase spectrum taken at the end of the 75% RH stage of the DVS experiments.} \]

From Figure 5.31 it was concluded that the trehalose in the co-spray-dried 60:40 catalase: trehalose sample was in the same form at the end of the second dry stage as it was at the end of the first dry stage of the DVS experiment because the spectra at both time-points were the same. To investigate whether there was an interaction between the two components of the co-spray-dried sample, the co-spray-dried spectrum at the end of the first dry stage of the DVS experiment was compared with that of the relevant theoretical spectrum. The results of this analysis were the same as those described for the co-spray-dried 80:20 catalase: trehalose, with no evidence of interaction between the catalase and trehalose observed (see discussion under co-spray-dried 20:80 catalase: trehalose section). Although there were differences in peak intensities between the co-spray-dried and theoretical spectra, no differences in peak positions were observed, which would have suggested a possible interaction between the components.
Figure 5.31. SNV-2\textsuperscript{nd} derivative NIR spectra between 1300-1600 nm of co-spray-dried 60:40 catalase: trehalose at various time-points from the DVS plot shown in Figure 5.29, compared with the spectra of α,α-trehalose dihydrate and the 100\% catalase spectrum taken at the end of the 75\% RH stage of the DVS experiments.

Figure 5.32. Comparison of the co-spray-dried 60:40 catalase: trehalose SNV 2nd-derivative NIR spectra (1300-1600 nm) from various time-points of the DVS experiment in Figure 5.29 with the respective ‘theoretical mix’ spectra.
5.3.7.8 Co-spray-dried Catalase: Trehalose 80:20 Formulation

- Water Vapour Sorption Behaviour

The water sorption behaviour of the co-spray-dried 80:20 catalase: trehalose sample was similar to that of the 100 % spray-dried catalase sample, in that the sample absorbed moisture during the 75 % RH stage of the experiment, and then returned to its original mass by the end of the second drying stage. The sample did however absorb more moisture (a greater mass) than the 100 % catalase sample at 75 % RH, showing an increase in mass of ~ 19.0 % (compared with 18.2 % for the 100 % catalase sample) before mass stabilisation upon saturation. This observation, noted for all co-spray-dried samples, was discussed earlier in this Section (‘General Observations’) and was proposed to be indicative of interaction between the co-spray-dried components.

The DVS plot of the co-spray-dried 80:20 catalase: trehalose sample shown in Figure 5.34 indicates that recrystallisation of the amorphous trehalose in the sample did not take place, shown by the absence of mass loss during the 75 % RH stage of the experiment and the return of the sample to its original mass during the second drying stage. There are several possible hypotheses for the absence of recrystallisation implied by the water vapour sorption behaviour of the sample:
a) There was interaction between the protein and carbohydrate upon spray drying, for example, hydrogen bonding of trehalose molecules to catalase in place of the water removed during the drying process. In this case, the trehalose molecules would not be free to recrystallise to the dihydrate form. This theory is consistent with theory of biostabilisation inferred upon dried proteins by disaccharides first introduced by Crowe (1971), later termed the ‘Water replacement Hypothesis’.

b) Smaller trehalose molecules (20% w/w) were diluted by the larger catalase molecules (80% w/w) and were in effect separated by the catalase, preventing extensive lattice formation of the trehalose in its crystalline form. If this were the case, then peaks for the anhydrous or amorphous forms of trehalose would be present in the sample spectrum, unless masked by peaks corresponding to the catalase.

c) The level of moisture in the co-spray-dried sample at 75% RH did not reach a critical level to threaten the stability of the catalase in the sample. If the threshold of water content had been reached, the amorphous trehalose within the sample would have crystallised, thus removing excess water from the system to form the dihydrate.

![DVS plot for the co-spray-dried 80:20 catalase: trehalose sample.](image)
The plausibility of these hypotheses was investigated during the examination of the near infrared spectra collected at 15 minute intervals during the DVS experiment.

- **NIR Spectra**

As the water sorption data alone were insufficient to prove any of the hypotheses described in the previous section, the NIR spectra collected during the experiment were examined for evidence of the form of the trehalose in the sample (anhydrous, dihydrate or amorphous) at different points throughout the DVS experiment. Standard normalised second derivative NIR spectra of the co-spray-dried 80:20 catalase: trehalose sample taken at various points during the DVS experiment shown in Figure 5.34, compared with the spectra of the 100% catalase and crystalline trehalose dihydrate samples are shown in Figure 5.35.

![NIR Spectra](image)

**Figure 5.35.** SNV 2nd-derivative NIR spectra of co-spray-dried 80:20 catalase: trehalose at various points from the DVS plot shown in Figure 5.34, compared with the spectra of the 100% catalase spectrum recorded at the end of the second drying stage of the DVS experiment and a sample of crystalline α,α- trehalose dihydrate as supplied.

All peaks specific to the α,α-dihydrate form of trehalose (as assigned in Chapter 3 of this thesis) were absent in the spectra of the co-spray-dried catalase: trehalose 80:20 sample at all time points of the DVS experiment (Figure 5.35). The peak at 1974 nm consistent with the presence of α,α-trehalose dihydrate appeared to be present at the end
of the 75% RH stage, however the other ‘dihydrate peaks’ at 1954 nm and 2174 nm were absent from the spectrum. As the 100% spray-dried catalase sample showed a peak at 1974 nm, this peak in the co-spray-dried catalase/trehalose spectra could not be used to discriminate between the 100% catalase and 100% trehalose formulations and thus was of little use in analysis of the co-spray-dried formulations. As no other peaks for the dihydrate form of trehalose were present, these data suggest that the trehalose in the sample did not crystallise to the crystalline dihydrate form under conditions of 75% RH, which is in agreement with absence of mass loss at 75% RH shown in the water vapour sorption data.

It was observed in Figure 5.35 that the spectra collected at the end of the first and second 0% RH stages were the same. At 75% RH, a peak for bulk water was observed at 1922 nm, which then diminished during the second drying stage of the DVS experiment. Peaks in this region are generally due to water and hydrogen bonding; therefore this large, broad peak was likely to encompass the peaks of more than one hydrogen-bonded species. Shoulders at 1908 and 1938 nm were evident on this large peak and so it was proposed that the peaks at 1904, 1920 and 1938 nm in the spectra of the sample at the end of the first and second dry stages were in effect superimposed onto the large peak corresponding to bulk water in the sample at the end of the 75% RH stage of the DVS experiment. This observation confirmed that the three peaks in the ‘dry’ spectra were likely to be indicative of hydrogen-bonded species in the sample.

In order to investigate the form of the trehalose in the co-spray-dried 80:20 catalase:trehalose sample at the end of the second dry stage of the DVS experiment, the NIR spectrum of the sample at this time point was compared with those of the polymorphic forms of trehalose identified in Chapter Three. This comparison is shown in Figure 5.36, between 1300-1600 nm, which is the region of the NIR spectrum in which the differences between the polymorphic forms are most obvious.
Figure 5.36. A comparison of the SNV 2nd-derivative NIR spectra between 1300-1600 nm, of the co-spray-dried 80:20 catalase: trehalose sample and the 100% catalase sample from the end of the second dry stage of the DVS plot shown in Figure 5.34, with the spectra of the polymorphic forms of trehalose identified in Chapter Three.

In Figure 5.36, the spectrum of the co-spray-dried 80:20 catalase: trehalose sample at the end of the second dry stage of the DVS experiment was most similar to the 100% spray-dried catalase sample and the spectrum of amorphous trehalose at the same time point. This suggested that the trehalose in the sample remained amorphous throughout the DVS experiment. No obvious similarities are observed between the co-spray-dried spectrum and the spectra of the other polymorphic forms of trehalose, again indicating the stability of the amorphous form of the sample to exposure to 75% RH. This observation was reflected in the superior stability of this formulation to exposure to 75 % RH described in the activity data in Section 5.3.2.

If the trehalose molecules were not bound to the catalase in the co-spray-dried 80:20 catalase: trehalose sample, it would be expected that the sample would be plasticised at 75% RH and that the Tg of the amorphous trehalose in the sample would be lowered below the experimental temperature, effecting crystallisation. As crystallisation was not observed, three hypotheses were proposed. Firstly, the greater proportion of larger catalase molecules in the sample ‘protected’ the smaller trehalose molecules from
crystallisation owing to the preferentially binding of water to the protein rather than the carbohydrate at 75 % RH. Secondly, as the larger catalase molecules were in excess compared with the proportion of smaller trehalose molecules, it was hypothesised that the trehalose molecules were separated by the catalase and therefore prevented from forming a crystalline lattice exhibiting the long-range order necessary for crystalline character to be observed. Finally, it was proposed that the trehalose was bound to the catalase in the co-spray-dried formulation, in agreement with the 'Water Replacement Hypothesis' of biostabilisation by disaccharides (see Chapter 1). If the trehalose were bound to the catalase, crystallisation to the dihydrate form would be prevented. In order to find evidence for these three hypotheses, spectra of the co-spray-dried 80:20 catalase:trehalose formulation at the end of the three RH stages of the DVS experiment were compared with those of the mathematically prepared 'theoretical spectra' prepared in the same manner as described under the discussion for the co-spray-dried 20:80 catalase:trehalose sample (Figures 5.37 and 5.38).

Figures 5.37 and 5.38 show that the first of the proposed hypotheses, that the protein absorbed most of the moisture at 75 % RH, can neither be proved nor disproved by the data because it is impossible to separate the components of the co-spray-dried sample to determine which component absorbed the most moisture at 75 % RH. However, as the co-spray-dried spectra are different to the theoretical spectra, it is likely that the two components do not exist as a simple 'mix' in the co-spray-dried sample, otherwise the co-spray-dried spectra would be very similar to the theoretical spectra.

The second hypothesis, that the trehalose was diluted in a larger 'catalase matrix', thus preventing its recrystallisation, can also neither be proved nor disproved by the data. However, this is a likely hypothesis, as a single catalase molecule is vastly larger than a single trehalose molecule (molecular weights 378.3 g/mol for α,α-trehalose dihydrate compared with 240 000 g/mol for catalase); therefore it is feasible that trehalose molecules were separated in the amorphous matrix produced by spray drying. This is a similar, yet inverse, hypothesis to the theory of biostabilisation proposed by Franks et al. (1991), who suggested that proteins are essentially 'fixed' into a glassy matrix produced by disaccharides upon drying.
Figures 5.37 (top) and 5.38 (bottom). Comparisons of the co-spray-dried 80:20 catalase: trehalose SNV 2nd-derivative NIR spectra from various time-points of the DVS experiment in Figure 5.34 with the respective 'theoretical mix' spectra.

Figures 5.37 and 5.38 were next examined for evidence for the third hypothesis as to the form of trehalose in the co-spray-dried 80:20 catalase: trehalose sample, the 'Water Replacement Hypothesis'. If there were no interaction between the protein and carbohydrate during spray drying, the co-spray-dried spectra at the end of the first dry stage would be expected to be the same as the theoretical spectra i.e. representative of a physical mix of the components of the sample. As this was not the case, the difference between the co-spray-dried and theoretical spectra at the end of the first dry stage of the
DVS experiment were compared for evidence of interaction between the components of the co-spray-dried sample. Although there were several obvious differences in the intensity of peaks between the co-spray-dried and theoretical spectra, an indication of interaction in the form of hydrogen bonding between the sample components would be expected to be shown by peak shifts. The only differences in peak position shown between the co-spray-dried and theoretical spectra were in the 1900-1950 region, in which a triple broad peak is observed with maxima at 1904, 1920 and 1938 nm in the co-spray-dried spectrum whereas a less defined and broader doublet is observed in the theoretical spectrum, with maxima at approximately 1906 and 1922 nm. These observations are not conclusive of an interaction between the catalase and trehalose in the co-spray-dried sample because the peak differences shown are not in positions consistent with the peaks known for the water of hydration of proteins. However, the differences are in the region applicable to water in the NIR spectrum and are likely to reflect differences in bound and free water.

5.3.7.9 Overall Summary Discussion
The water vapour sorption behaviour and near infrared spectra of a batch of seven samples of the co-spray-dried formulations composed of different proportions of catalase and trehalose was discussed in Section 5.3.6.2, with respect to individual sample data. In this summary discussion, the results will be discussed as a whole data set, in relation to the activity data for the batch and with respect to proposed theories of protein-disaccharide biostabilisation in the literature.

a) Water vapour sorption behaviour
The water vapour sorption behaviour of the co-spray-dried catalase/trehalose samples is shown in Figure 5.39.
Figure 5.39. DVS plots for a batch of co-spray-dried catalase/trehalose samples exposed to 0 % RH for 8 h, 75 % RH for 10 h and then 0 % RH for a further 6 h (at 25 °C).

The method of the DVS experiment was such that the spectra of the co-spray-dried samples could be examined at the end of the first dry stage in the dry state, so that the water content of the samples would be similar and the differences in free water would not interfere with the NIR spectra. The stage at 75% RH was designed to cause plasticisation of the samples, with an aim to increase molecular mobility, attempting to mimic the effects of moisture on the formulations during their shelf lives. It was intended that the final drying stage of the experiment would identify which samples had altered over the course of the DVS experiments and to determine the stability of the samples to drying after exposure to 75% RH.

The water vapour sorption behaviour of the co-spray-dried samples shown in Figure 5.39 indicated that at proportions of trehalose in the sample up to 40% w/w, the trehalose in the formulation remained in the amorphous state throughout the experiments, with the samples returning to their original mass during the final drying stage. At proportions of trehalose of 50% w/w and above, the trehalose in the formulations was shown to crystallise at 75% RH, indicated by mass loss at 75% RH. These results suggest that the proportion of catalase in the formulations inhibited the crystallisation of trehalose at 75% RH, similar to other such observations in the
literature. This inhibition may have been simply a bulk effect due to the relative size of the larger catalase molecules compared with the smaller trehalose molecules causing dilution of the trehalose, or the inhibition may be more complex, with trehalose molecules bound to the catalase and thus prevented from crystallising. In this second theory, only the free trehalose in the sample would be able to crystallise at 75 % RH; therefore in those samples with a higher proportion of trehalose, the mass loss shown at 75% RH would be indicative of the free, unbound trehalose in the sample.

The theory as to the interaction of the catalase with the trehalose in the sample was considered plausible given that greatest interaction between the components would be expected at a mass ratio of 50:50 catalase: trehalose. The moisture uptake at 75 % RH was greatest for the co-spray-dried 50:50 catalase: trehalose compared with all of the other formulations. If the interaction between the protein and carbohydrate inhibited the crystallisation of trehalose in the sample, this would enable more water to be absorbed at 75 % RH prior to crystallisation, and so the greatest mass uptake would be expected by the co-spray-dried 50:50 catalase: trehalose formulation, as was shown. Moisture uptake at 75 % RH was decreased upon increasing or decreasing the mass ratios of catalase: trehalose in the samples, which was expected in line with an interaction occurring between the catalase and trehalose causing the inhibition of trehalose crystallisation. This theory was thought more plausible with respect to the data from this study, when compared with the theories of Tzannis and Prestrelski (1999b) and López-Díez and Bone (2004) as described in Section 5.3.7.1.

b) Differences in peak positions between co-spray-dried and theoretical spectra of dry samples following spray drying

A summary of the differences in peak positions of the peak corresponding to protein hydration described by Vandermeulen and Ressler (1980b) between the co-spray-dried spectrum and the theoretical spectrum of each formulation at the end of the first drying stage of the DVS experiment is given in Table 5.9.
Table 5.9. Comparison of the position of the reported peak for protein hydration in the SNV 2nd-derivative NIR spectra of co-spray-dried samples at the end of the first dry stage of the DVS experiments and in the theoretical spectra at the same time-point.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Catalase</th>
<th>% Trehalose</th>
<th>Peak position in theoretical spectrum</th>
<th>Peak position in cospray-dried spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>80</td>
<td>1482 nm</td>
<td>1476 nm</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>60</td>
<td>1488 nm</td>
<td>1482 nm</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>1490 nm</td>
<td>1486 nm</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>40</td>
<td>1490 nm</td>
<td>1490 nm</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>20</td>
<td>1490 nm</td>
<td>1490 nm</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>1492 nm</td>
<td>1492 nm</td>
<td></td>
</tr>
</tbody>
</table>

There are no peaks described in the table for the 100% trehalose sample because the peak for protein hydration at 1490 nm was not present in the trehalose spectrum. The closest peak was a shoulder at 1974 nm. The spectra relating to the data in Table 5.9 are shown in Figures 5.40 and 5.41.

The shift in the position of the peak at 1492 nm in the 100% spray-dried catalase sample to shorter wavelengths in the spectra of the co-spray-dried samples was due to a dilution effect of the increasing proportions of trehalose in the formulations. The difference in the peak position in the co-spray-dried spectra when compared with the appropriate theoretical spectra was the important feature. As no other significant changes occurred across the rest of the wavelength range between the co-spray-dried and theoretical dry spectra, it may be considered that the difference in the position of the peak at ~1490 nm may be due to a change in the structure of the co-spray-dried sample. Vandermeulen and Ressler (1980b) indicated that a shift in this peak to a shorter wavelength was indicative of reduced protein hydration due to the binding of an additive to the water-binding sites on the protein. From the data given for the above batch of co-spray-dried samples, it was proposed that an interaction existed between the catalase and trehalose in those samples with a trehalose concentration of 50% or more. As the catalase in the co-spray-dried catalase: trehalose 50:50 and 20:80 formulations retained the highest weight activity following spray drying (Section 5.3.2), it was proposed that an interaction of the trehalose with the protein in these formulations (via hydrogen bonding) was partially responsible for this higher retention of activity. However, as this theory did not hold with respect to the lower retention of activity after spray drying shown by the co-spray-dried 40:60 catalase: trehalose formulation, it could not be
validated. Further work is required to further investigate the possibility of an interaction between the protein and carbohydrate upon drying.

**Figures 5.40 and 5.41.** SNV 2nd-derivative NIR spectra to highlight the positions of the reported protein hydration peak in the co-spray-dried (bottom) and theoretical (top) spectra.
c) Trehalose dihydrate and DVS-anhydrous trehalose peaks in the spectra of the co-spray-dried samples during the DVS experiments

A summary of the form(s) of the trehalose in the co-spray-dried formulations at the end of the 75% RH stage and the second 0% RH stage of the DVS experiments is given in Table 5.10. The form of the trehalose in the sample was identified by the use of the NIR peak assignments for the polymorphic forms of trehalose given in Chapter 3.

Table 5.10. A summary of the forms of the trehalose identified in the spectra of the co-spray-dried formulations at the end of the 75% RH stage and the final dry stage of the DVS experiments.

<table>
<thead>
<tr>
<th>Co-spray-dried sample</th>
<th>Peaks at end 75% RH stage</th>
<th>Peaks at end of 2&lt;sup&gt;nd&lt;/sup&gt; dry stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Catalase</td>
<td>% Trehalose</td>
<td>α,α-Trehalose dihydrate</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>Yes</td>
</tr>
<tr>
<td>20</td>
<td>80</td>
<td>Yes</td>
</tr>
<tr>
<td>40</td>
<td>60</td>
<td>Yes</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>Yes</td>
</tr>
<tr>
<td>60</td>
<td>40</td>
<td>No</td>
</tr>
<tr>
<td>80</td>
<td>20</td>
<td>No</td>
</tr>
</tbody>
</table>

The NIR spectra of the co-spray-dried formulations containing 60% w/w catalase or more were shown to be the same at the end of the first and second 0% RH stages of the DVS experiments. It was hypothesised that the trehalose in these formulations was either bound to the protein or simply dispersed between the larger catalase molecules in the amorphous matrix of the sample. No peaks for any form of trehalose were identified in the spectra at these time points, with the spectra appearing similar to the 100% catalase spectrum from the same time-points. As crystalline peaks tend to dominate the NIR spectrum owing to their homogeneous, ordered character, it was assumed that the trehalose in the formulations remained in the amorphous form. It was therefore proposed that the peaks for the larger proportion of catalase in the formulation masked those peaks corresponding to amorphous trehalose in the formulations.

The NIR spectra of the co-spray-dried formulations containing 50% w/w catalase or less were shown to be different at the end of the first and second 0% RH stages of the DVS experiments. This was indicative of structural change in the formulations during the 75% RH stage of the experiments. With the lowest ratio of catalase to trehalose in
the samples, a stable form of trehalose dihydrate was formed at the end of the 75% RH stage of the DVS experiments, which was retained at the end of the final drying stage. In those samples containing a higher ratio of catalase to trehalose (> 50:50), crystallisation of the trehalose in the samples was inhibited. In the co-spray-dried 40:60 and 50:50 catalase:trehalose samples, an unstable dihydrate was formed at end of 75% RH stage of the DVS experiments, which had converted to another form by the end of the final drying stage. This phenomenon could be simply due to the quantity of the trehalose in the sample crystallising. In the samples in which there was a greater proportion of trehalose to crystallise, the dihydrate peaks in the NIR spectra remained at the end of the second drying stage of the DVS experiments. This could be a bulk effect, meaning that the hydrate water could not be removed in the 6 h of drying time. In those samples containing less trehalose, the quantity of the dihydrate formed at the end of the 75% RH stage of the DVS experiments may have been reduced to such an extent that all of the hydrate water could be removed within 6 h at 0% RH. This theory could be investigated by performing the same experiments on physical mixes of the amorphous components if homogeneous mixtures could be produced (without adverse effects on the components).

Another theory as to the formation of the 'unstable dihydrate' could be that the prior binding characteristics of the trehalose in the sample may have affected the stability of the dihydrate formed at the end of the 75% RH stage of the DVS experiments. In this hypothesis, the trehalose that had been 'free' in the co-spray-dried samples would have been more likely to form a stable dihydrate at the end of the 75% RH stage and the trehalose that had been bound to the protein in the samples would have formed unstable dihydrate molecules. The basis for this hypothesis is unclear and further work is required to investigate this and the previous theory regarding the mass of trehalose available to crystallise in the sample.

d) Free water at 75% RH
In order to determine the distribution of water in the samples at 75% RH, the sample spectra were examined in the 1900-2000 nm region, in which the absorption of water and hydrogen bonds appears most strongly (Figure 5.42). This analysis was undertaken in order to attempt to answer the following questions about the co-spray-dried catalase/trehalose formulations:
• Does the protein preferentially bind free water at 75% RH as per the Timasheff mechanism of protein stabilisation in solution?
• Does the water content reach a threshold above which the hydration layer of the protein becomes saturated and therefore additional water would serve to destabilise the protein?
• Does the trehalose in the sample utilise excess water (above that required for hydration of either component) to crystallise to the dihydrate, effectively protecting the catalase by acting as a desiccant?

Figure 5.42. SNV 2nd-derivative NIR spectra for co-spray-dried catalase trehalose formulations of varying catalase content recorded at the end of the 75% RH of the DVS experiments shown in Figure 5.39.

• Does the protein preferentially bind free water at 75% RH as per the Timasheff mechanism of protein stabilisation in solution?

The spectra in Figure 5.42 clearly show the presence of trehalose dihydrate in the formulations with up to and including a 50:50 ratio of catalase: trehalose. This was determined by the presence of the characteristic peaks for trehalose dihydrate at 1954 and 1978 nm. These peaks were absent in those samples containing a ratio of greater than 50:50 catalase: trehalose, indicating that the crystallisation of amorphous trehalose in these formulations was inhibited. The peaks in the spectra in the 1900-2000 nm region of the co-spray-dried 60:40 and 80:20 catalase: trehalose samples were most similar to the spectrum of the 100% spray-dried catalase at the same time-point.
However, where in the 100% spray-dried catalase sample a peak maximum was observed at 1930 nm, with a shoulder visible at 1914 nm, in the spectra of the 60:40 and 80:20 catalase: trehalose samples, a peak maximum was observed at 1922 nm, with shoulders at 1908 and 1934 nm. These peaks were shown in the spectrum of the 100% catalase sample at the end of the first dry stage (peak maximum at 1924 nm, a small superimposed peak at 1906 nm and a shoulder at 1936 nm) and in the spectrum of the 100% spray-dried trehalose sample at the same time point. This observation suggests that in the co-spray-dried catalase: trehalose samples at ratios above 50:50, the water in the sample interacted with the sample in a different way to that shown in the spectrum of the 100% spray-dried catalase. At the end of the first dry stage, the only water remaining in the 100% spray-dried catalase or trehalose samples would be expected to be strongly bound water of hydration or water trapped within the amorphous matrix. As these peaks were also present (at greater intensity) at the end of the 75% RH stage of the DVS experiments for the co-spray-dried catalase: trehalose 60:40 and 80:20 formulations, it may be hypothesised that the presence of trehalose in the formulation increased the level of bonding of water introduced into the system, because the peak for free water at 1930 nm in the 100 % spray-dried catalase sample under conditions of 75% RH was not evident in the spectra of the co-spray-dried samples. It may therefore be assumed that under conditions of 75 % RH, the water introduced into the co-spray-dried 60:40 and 80:20 catalase: trehalose formulations was either trapped within the amorphous matrix of the sample or bound to the protein. The reduction of free water in the sample appeared to increase the stability of the protein upon storage, given that the recovery of the activity of the protein following the DVS experiments was similar to that of the 100% catalase sample in these samples. However, as the greatest recovery of activity after the DVS experiments was observed with the 100 % spray-dried catalase formulation compared with the activity observed prior to the experiments, this suggests that once in the dried state, the stability of catalase was hindered by the presence of trehalose in the formulation. This was the opposite of that shown upon drying, in which the activity of the 100 % catalase sample was reduced to a greater extent than any of the other formulations, with the formulation containing the greatest proportion of trehalose exhibiting the highest recovery of catalase activity.

From the previous discussion, it may be hypothesised that the free water introduced into co-spray-dried catalase: trehalose systems at 75 % RH is indeed bound to the protein or trapped within the amorphous matrix retained throughout the period of high relative
humidity. As the peaks for the protein appeared to dominate the spectra of those samples containing a higher proportion of the catalase to trehalose, and both catalase and trehalose in the dried state exhibited similar peaks in the 1900-2000 nm region, it was difficult to conclusively determine the distribution of the water that was introduced to the sample. It would be logical to predict that any water introduced to the system would be preferentially bound to the protein, as this would create a lower energy state according to the ‘Timasheff mechanism’ described in Chapter 1.

- **Does the water content reach a threshold above which the hydration layer of the protein becomes saturated and therefore additional water would serve to destabilise the protein?**

From the data analysed it was not possible to attempt to answer this question. This analysis may be possible in future work if the proportion of catalase in the formulations was kept the same with only the proportion of trehalose varying. This would allow the effect of water and the proportion of trehalose in the formulation to be assessed in a more quantitative manner, whereas with the results from the data collected, this was difficult.

- **Does the trehalose in the sample utilise excess water (above that required for hydration of either component) to crystallise to the dihydrate at 75% RH, effectively protecting the catalase by acting as a desiccant?**

It was unlikely that the trehalose protected the catalase by acting as a desiccant following spray drying, because the formulations containing a lower proportion of catalase to trehalose, in which the trehalose crystallised to the dihydrate form at 75 % RH, exhibited the lowest retention of activity following the DVS experiments. This suggested that conversion of the trehalose to the crystalline form destabilised the amorphous system, leading to denaturation of the protein.

Figure 5.43 shows the NIR spectra of a sample of co-spray-dried 20:80 catalase:trehalose during the 75% RH stage of the DVS experiment shown in Figure 5.39. From the spectra it was clear that trehalose in the formulation had crystallised to the dihydrate form within 75 minutes under conditions of 75% RH, shown by the formation of the dihydrate peaks at 1954 and 1978 nm. Crystallisation of the trehalose began within an hour of exposure to 75% RH, shown by the suggestion of the dihydrate peaks at 1954 and 1978 nm in the spectrum at 540 min.
Figure 5.43. SNV 2nd-derivative NIR spectra of a sample of co-spray-dried 20:80 catalase: trehalose taken at time-points during the 75% RH stage of the DVS experiment shown in Figure 5.39.

It is clear from the spectra shown in Figure 5.43 that the formation of the dihydrate removed free water from the system, shown by the gradual disappearance of the peak at around 1930 nm shown in the spectrum at 495 min. The peaks for the crystalline α,α-trehalose dihydrate dominate the spectra as soon as they form, owing to the more ordered state of the crystal compared with the disorder of the previously amorphous state. This meant that the peaks for trehalose dihydrate in the formulation masked the peaks for the protein, preventing assessment of water bound to the catalase. This also prevented the analysis of whether all or just a proportion of the trehalose in the formulation had crystallised to the dihydrate form, because any peaks for amorphous trehalose or trehalose that may have remained in the amorphous state due to interaction with the protein were masked by the dominance of the crystalline peaks in the spectra.

It may be hypothesised that crystallisation of trehalose in the co-spray-dried formulations caused destabilisation of the remaining amorphous matrix, thus causing denaturation of catalase in the sample. Removal of water from the system to effect crystallisation of the trehalose to the dihydrate form may have induced too strong a desiccant action on the system, removing all excess water from the protein, thus affecting its hydration layer and structural integrity.
e) Evidence of protein denaturation in NIR spectra in relation to activity data

Liu et al., (1994) reported that the Amide A/II band at approximately 2053-2057 nm was shown to shift to shorter wavelength (higher frequency) with increased denaturation of the protein (H-bond breakage in the amide groups). A comparison of the position of this peak at the end of the first dry stage of the DVS experiments, with that at the end of the second drying stage is presented in Table 5.11.

Table 5.11. A comparison of position of the Amide A/II peak in the spectra of the co-spray-dried formulations at the end of the first and second dry stage of the DVS experiments (numbers in brackets refer to the % recovery of activity of the formulations at the start of the DVS experiments, prior to the first drying stage).

<table>
<thead>
<tr>
<th>Sample % Catalase Content</th>
<th>Peak position at end of first dry stage of the DVS experiments</th>
<th>Peak position at end of second dry stage of the DVS experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>2058 (89.8)</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>2056 (54.8)</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>2056 (84.5)</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>2056 (60.2)</td>
<td>2056</td>
</tr>
<tr>
<td>80</td>
<td>2056 (60.8)</td>
<td>2056</td>
</tr>
<tr>
<td>100</td>
<td>2054 (46.9)</td>
<td>2054</td>
</tr>
</tbody>
</table>

From the data in Table 5.11, it may be proposed that at the end of the first drying stage of the DVS experiments, the position of the Amide A/II peak at approximately 2055 nm was indicative of the relative activity of the catalase in the formulations (see Section 5.3.2), with peak in the spectrum of the sample exhibiting the lowest activity being in the lowest position, indicative of increased denaturation. However, this hypothesis may be discounted, considering that the position of the Amide A/II peak in the spectra of samples of the original catalase as supplied (designated as possessing 100% activity) was in the same position as that in the spectrum of the sample exhibiting the lowest activity, at 2054 nm.

Owing to the greater intensity of peaks in crystalline samples because of the more ordered state, the peaks for trehalose dihydrate, if present, dominated the spectra, preventing examination of the Amide A/II peak at ~ 2055 nm and therefore the position of the peak following exposure to 75 % RH in some of the samples.
It was concluded that the Amide A/II absorption band in the co-spray-dried catalase/trehalose samples was not indicative of the denaturation of the protein in the samples.

5.4 Conclusions
Conclusions and possible hypotheses that may be drawn from this chapter are:

a) Crystallisation events were not observed in the water sorption plots or NIR spectra of the 100% catalase samples. Spray-dried samples and the original catalase as obtained showed intra-batch and inter-batch variability in their NIR spectra, indicating the heterogeneous nature of the amorphous protein. No peaks could be assigned to the denaturation of the protein in the NIR spectra.

b) A lower ratio of catalase: trehalose was required for the most effective stabilisation of catalase during the spray drying process. The most effective stabilisation of catalase was observed with the co-spray-dried 20:80 catalase: trehalose formulation (the lowest ratio of catalase to trehalose).

c) A higher ratio of catalase: trehalose was required for the most effective stabilisation of catalase upon exposure of co-spray-dried formulations to 75% RH. The most effective stabilisation of catalase was observed with the 100% spray-dried catalase formulation, in which no trehalose was present.

d) A ratio of 50:50 catalase: trehalose was required for the most effective overall stabilisation of catalase following spray drying and exposure to 75% RH. As this was the ratio in which the greatest interaction between the protein and carbohydrate was expected, this adds to the evidence to support the ‘Water Replacement Hypothesis’.

e) From the observations noted in (b) and (c), it may be hypothesised that the mechanism of stabilisation of catalase was dependent upon the specific stress applied to the protein. The mechanism of stabilisation upon drying appeared to be different to that during shelf life (accelerated by exposure to 75% RH in this study). This conclusion is consistent with the stress-specific protection of proteins (lactate
dehydrogenase and phosphofructokinase) reported in the literature during freezing and drying (Prestrelski et al., 1993b).

f) Based on differences of peaks between co-spray-dried and theoretical spectra assigned to the hydration of the protein in this study and the water vapour sorption behaviour of the samples of different catalase: trehalose mass ratios, it was hypothesised that an interaction existed between catalase and trehalose in the co-spray-dried formulations. The most likely interaction was hydrogen bonding, for which some evidence is provided in the NIR spectra of the samples.

g) The formation of an ‘unstable dihydrate’ at the end of the 75 % RH stage of the DVS experiments was proposed for those samples in which there was greatest interaction between the components. This phenomenon may have been an effect of the different kinetics of dehydration in samples of different catalase: trehalose mass ratios.

5.5 Further Work

Following analysis of the data for this chapter, many different avenues of further work were revealed. These are:

- A comparison of co-spray-dried samples of catalase: trehalose with the concentration of catalase remaining the same between samples, in order to assess the behaviour of the trehalose in the sample in a more quantitative manner.

- Production of ‘actual physical mixes’ of 100% spray-dried catalase and trehalose in the proportions discussed, to assess the influence of mass (bulk) effects of the components on the crystallisation kinetics of the trehalose. This would be problematic to perform accurately, because the homogeneous mixing of two amorphous materials is difficult and could lead to process-related changes in the samples prior to analysis.

- Further examination of the stress-specific stabilisation of proteins upon spray drying and subsequent storage. Reference to the stresses invoked on protein/carbohydrate formulations in the literature has focussed mainly upon the effects of freezing and subsequent drying, rather than spray drying and subsequent storage.
Given the variation of spray-dried samples of trehalose alone observed in Chapter 4, repetition of this work with more sample batches should be performed in order to confirm the findings with greater confidence.
Chapter Six
6 Quantification of Formulation Components by Near Infrared Spectroscopy

6.1 Introduction

6.1.1 Current methods to determine protein concentration in formulations

There are many methods used to quantify the concentration of protein in a given sample, the simplest of which is the measurement of a protein's intrinsic UV absorbance. More commonly, one of the colorimetric (spectrophotometric) methods is used, such as the Bradford Dye assay (Bradford, 1976), the Lowry assay (Lowry et al., 1951), or the Bicinchoninic Acid assay (BCA, based on the assay developed by Smith et al. (1985)). The first of these methods, the Bradford assay, is based upon a dye-binding method of protein determination whereas the others rely upon the biuret reaction. In this reaction, the peptide bond in biuret reacts with aqueous sodium hydroxide and copper II sulphate to produce a purple complex. Peptide bonds in proteins can undergo the biuret reaction, thus forming the basis of a colorimetric assay for protein determination. Spectrophotometric assay methods are generally preferred for their simplicity and low cost and are based upon the detection of either the whole protein or parts of the protein such as the peptide bonds. Each method has its own merits (such as low detection limit or shorter assay duration) and drawbacks (such as poor sensitivity, complicated/time-consuming methods or interfering substances). Examples of some of the more popular spectrophotometric methods used to determine protein concentration with their merits and drawbacks are outlined in Table 6.1. It must be noted that this is not an exhaustive summary of the methods available for protein determination. Other spectrophotometric methods are available, for example, the Kjeldahl method (e.g. BP 2003), based upon determination of total protein nitrogen in the sample or the Ninhydrin reaction, based upon the reaction of amino acids with ninhydrin to form a purple complex (e.g. Duggan, 1966). These methods are useful and in the case of the Kjeldahl Method, very accurate. However, they are complicated, long procedures that are not conducive to the low cost, simple and fast determination of protein concentration. The BCA assay is often the preferred method because of its greater sensitivity compared with the Bradford or Lowry assays, however the choice of assay is highly dependent upon the nature of the protein formulation to be tested.
Table 6.1. Description of some common spectrophotometric methods used to quantify protein concentration (for references, see text).

<table>
<thead>
<tr>
<th>Assay</th>
<th>Theory of method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| Bradford| • Dye binds to whole protein  
• Uses coomassie brilliant blue dye  
• Protein-dye complex with absorbance at 595 nm | • Rapid  
• Reproducible  
• Not affected by reducing sugars | • Interference by detergents  
• Relies on whole protein |
| Biuret  | • Peptide bonds in protein undergo biuret reaction  
• Protein (biuret) + copper solution = purple/blue complex | • Specific to peptide bond | • Relies on polypeptide character  
• Insensitive (1-20 mg range of detection) |
| Lowry   | • Modified biuret method  
• Two stage assay – 1st stage = biuret reaction, 2nd stage = reduction of Folin reagent by copper-protein complex and other specific amino acids  
• Result of 2nd stage is a blue molybden/tungsten product with absorbance at 750 nm | • More sensitive than Biuret method alone (1-500 µg/mL) | • 2 step process  
• Unstable product in alkaline conditions  
• Many interfering substances  
• Protein to protein variation |
| BCA     | • Modified Lowry assay  
• Bicinchoninic acid (BCA) replaces Folin reagent  
• Protein + Cu^{2+} → Cu^{1+} (under alkaline conditions)  
• 1Cu^{1+} + 2BCA → BCA-Cu^{1+} complex (purple)  
• BCA-Cu^{1+} complex (purple) has absorbance at 570 nm | • More sensitive than Lowry method due to 2:1 reaction of BCA with Cu^{+}  
• Various protocols available (standard, room temperature, enhanced) with varying sensitivity due to type of reaction to induce Cu^{2+} → Cu^{1+} | • Many interfering substances |
All of the protein concentration assays mentioned above require the protein sample to be in solution. This in itself is a major shortcoming of all of the spectrophotometric methods, because proteins are inherently less stable in solution. The protein may denature or precipitate during sample preparation or during the assay procedure. In methods relying on a whole or part of the protein structure to be intact, instability of the protein in solution may give rise to inaccurate protein concentration data. Development of a method to determine protein concentration in the solid state would avoid problems due to instability of the sample in solution and prevent sample destruction.

6.1.2 The use of near infrared spectroscopy for the purpose of quantification

A search of the literature for published studies in the quantitative use of near infrared spectroscopy is summarised in Table 6.2. Little appears to have been published regarding the quantification of proteins by the use of near infrared spectroscopy. In the research by Olesberg et al. (2000) an experimental method to monitor protein crystal growth from solution was described, and further published work from the same laboratory outlined a quantitative method to determine protein concentration in solution using FT-NIR (Hu and Arnold, 2000). However, no evidence of the use of NIRS to determine protein concentration in the solid state was uncovered in the literature search. It was noted that most literature regarding quantification using NIRS was published in the last decade, coinciding with the increased use of chemometrics to perform complicated mathematical analyses. The success of NIRS in solid-state quantification studies outlined in Table 6.2 suggests that it may be possible to quantify proteins in the solid-state using similar techniques. Advantages of such quantification by NIRS are outlined below:

- Faster, non-destructive technique compared with current protein quantification methods
- Useful for samples of limited mass
- Several analyses may be replaced by one (e.g. protein and water content)
- Useful for measurement through packaging e.g. quality control of hygroscopic/sterile product
Table 6.2. Summary of the literature search on the use of NIRS as a quantitative analytical method.

<table>
<thead>
<tr>
<th>Authors, Year</th>
<th>Analyte of concern</th>
<th>Reflectance or transmittance</th>
<th>Number of samples</th>
<th>Mathematical method for model preparation</th>
<th>Liquid or solid state</th>
<th>Reference procedure</th>
<th>Successful?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shaw et al., 1996</td>
<td>Protein, creatinine and urea in urine</td>
<td>Transmittance</td>
<td>173</td>
<td>MLR, PLS</td>
<td>Liquid</td>
<td>Chemical assay methods</td>
<td>Yes (useful for screening but not as accurate as ref. method)</td>
</tr>
<tr>
<td>Cinier and Guilment, 1996</td>
<td>Resorcinol in water</td>
<td>FT-NIR probe Transflectance</td>
<td>21</td>
<td>PLS</td>
<td>Liquid</td>
<td>UV-Vis</td>
<td>Yes</td>
</tr>
<tr>
<td>Harthun et al., 1997</td>
<td>Recombinant protein in animal cell culture supernatant</td>
<td>Transmittance</td>
<td>100</td>
<td>PLS</td>
<td>Liquid</td>
<td>Immunologic assay</td>
<td>Yes (Faster method but lower sensitivity)</td>
</tr>
<tr>
<td>Smola and Urleb, 2000</td>
<td>Oxytetracycline</td>
<td>Reflectance</td>
<td>Unclear</td>
<td>PLS, PCA, PCR</td>
<td>Solid</td>
<td>Colorimetric assay, KF titration, FT-IR</td>
<td>Yes</td>
</tr>
<tr>
<td>Patel et al., 2000</td>
<td>Polymorphs in binary/multi-component mixtures</td>
<td>Reflectance</td>
<td>Unclear</td>
<td>Univariate regression analysis, PLS, MLR</td>
<td>Solid</td>
<td>XRPD, thermal analysis</td>
<td>Yes</td>
</tr>
<tr>
<td>Olesberg et al., 2000</td>
<td>Glucose-isomerase and lysozyme during crystal growth</td>
<td>Single-beam spectra (Transmittance)</td>
<td>60</td>
<td>Experimental approach based upon PCA to identify non-analyte components</td>
<td>Liquid</td>
<td>UV</td>
<td>Yes (Comparable to PLS method for monitoring protein crystal growth)</td>
</tr>
<tr>
<td>Authors, Year</td>
<td>Analyte of concern</td>
<td>Reflectance or transmittance</td>
<td>Number of samples</td>
<td>Mathematical method for model preparation</td>
<td>Solution or solid state</td>
<td>Reference procedure</td>
<td>Successful?</td>
</tr>
<tr>
<td>---------------</td>
<td>--------------------</td>
<td>-----------------------------</td>
<td>-------------------</td>
<td>------------------------------------------</td>
<td>-------------------------</td>
<td>---------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Hu and Arnold, 2000</td>
<td>Lysozyme concentration during crystallisation</td>
<td>Digital Fourier filtering of FT-NIR spectra (Transmittance?)</td>
<td>17</td>
<td>Univariate calibration, PLS</td>
<td>Liquid</td>
<td>UV-Vis</td>
<td>Yes (PLS versus univariate analysis comparison)</td>
</tr>
<tr>
<td>Buckton and Yonemochi, 2000</td>
<td>Silica content in silicified microcrystalline cellulose</td>
<td>Reflectance</td>
<td>7</td>
<td>Non-chemometric peak analysis</td>
<td>Solid</td>
<td>None – physical preparation</td>
<td>Yes</td>
</tr>
<tr>
<td>Moffat et al., 2000</td>
<td>Paracetamol in intact tablets</td>
<td>Reflectance</td>
<td>45 tablet batches</td>
<td>MLR</td>
<td>Solid</td>
<td>UV</td>
<td>Yes. Validated to demonstrate ICH guidelines.</td>
</tr>
<tr>
<td>Morris Jr. and Forbes, 2001</td>
<td>Potency of narasin extracts</td>
<td>Transmittance</td>
<td>41</td>
<td>MLR</td>
<td>Liquid</td>
<td>HPLC</td>
<td>Yes</td>
</tr>
<tr>
<td>Hogan and Buckton, 2001a</td>
<td>Low amorphous content of crystalline lactose</td>
<td>Reflectance</td>
<td>27</td>
<td>MLR</td>
<td>Solid</td>
<td>None – physical preparation</td>
<td>Yes</td>
</tr>
<tr>
<td>Forbes et al., 2001</td>
<td>Potency and lipids in monensin broth</td>
<td>Transmittance</td>
<td>100</td>
<td>Several MLR analyses</td>
<td>Viscous liquid</td>
<td>HPLC (potency) Chloroform extraction</td>
<td>Yes Validated</td>
</tr>
<tr>
<td>Wilson et al., 2002</td>
<td>Citral in lemongrass and lemon oils</td>
<td>Transflectance</td>
<td>61</td>
<td>MLR</td>
<td>Liquid</td>
<td>BP titration assay</td>
<td>Yes</td>
</tr>
<tr>
<td>Authors, Year</td>
<td>Analyte of concern</td>
<td>Reflectance or transmittance</td>
<td>Number of samples</td>
<td>Mathematical method for model preparation</td>
<td>Liquid or solid state</td>
<td>Reference analytical method</td>
<td>Successful?</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------------------------------------------------------</td>
<td>------------------------------</td>
<td>-------------------</td>
<td>------------------------------------------</td>
<td>----------------------</td>
<td>--------------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Rager et al., 2002</td>
<td>Constituents in St John’s Wort</td>
<td>Reflectance</td>
<td>35</td>
<td>MLR</td>
<td>Solid</td>
<td>Rev-phase HPLC</td>
<td>Yes</td>
</tr>
<tr>
<td>Stokvold et al., 2002</td>
<td>Moisture in freeze-dried drug product</td>
<td>FT-reflectance (transformed)</td>
<td>80</td>
<td>PLS</td>
<td>Solid</td>
<td>Karl-Fischer titration</td>
<td>Yes</td>
</tr>
<tr>
<td>Gombás et al., 2003</td>
<td>Crystallinity of alpha-lactose monohydrate</td>
<td>Diffuse reflectance</td>
<td>13</td>
<td>MLR</td>
<td>Solid</td>
<td>XRPD</td>
<td>Yes</td>
</tr>
<tr>
<td>Gislum et al., 2004</td>
<td>Nitrogen in ryegrass end red fescue</td>
<td>Reflectance</td>
<td>837</td>
<td>PLS</td>
<td>Solid</td>
<td>Dumas method</td>
<td>Yes</td>
</tr>
<tr>
<td>Laasonen et al., 2004</td>
<td>Thickness of plastic in blister packaging</td>
<td>Diffuse reflectance</td>
<td>193</td>
<td>PLS</td>
<td>Solid</td>
<td>Digital micrometer</td>
<td>Yes</td>
</tr>
<tr>
<td>Harbeck et al., 2004</td>
<td>Betaine following molasses desugarisation</td>
<td>Transmittance?</td>
<td>Unclear</td>
<td>Direct peak analyses</td>
<td>Solution</td>
<td>Liquid chromatography</td>
<td>Yes</td>
</tr>
<tr>
<td>Fertig et al., 2004</td>
<td>Amylose in starch</td>
<td>Reflectance</td>
<td>28</td>
<td>MLR</td>
<td>Solid</td>
<td>None – physical preparation</td>
<td>Yes</td>
</tr>
</tbody>
</table>
6.1.3 Spectral Data Analysis for Quantification by Near Infrared Spectroscopy

Unless otherwise specified, information for this Section was referenced from The Vision® Spectral Analysis Software for Windows User Manual, Version 2.11 (Foss NIRSystems), a review of NIR spectroscopy calibration basics by Workman (1992) and the PhD thesis of Nicola Wilson (2002).

The Vision® software (Foss NIRSystems, Silver Spring, U.S.A.) offers two mathematical methods for the quantitative analysis of sample spectra. The first of these is multiple linear regression analysis (MLR) and the second is partial least squares regression analysis (PLS). Both methods have advantages and disadvantages as outlined in Sections 6.1.4 and 6.1.5 respectively.

One of the main problems with quantitative NIR analysis is that the technique relies upon external calibration (reference) data in order to impart meaning to the sample data. Such reference methods generally measure chemical or physical properties of samples whereas the vibrational characteristics measured by NIR spectral analysis take into account both physical and chemical properties (Shenk et al., 1992). Such reference methods may therefore not be entirely applicable to NIR spectral analysis. NIRS has become a ‘hot topic’ in the pharmaceutical industry, owing to the rapid, non-destructive collection of spectra and the relatively simple quantification methods following development and validation of a calibration model. A guideline for the use of near infrared spectroscopy in the pharmaceutical industry was developed by the Committee for Proprietary Medicinal Products (CPMP) and the Committee for Veterinary Medicinal Products (CVMP) and came into operation in August 2003 (CPMP/QWP/3309/01 or EMEA/CVMP/961/01). This note for guidance is intended for applicants wishing to use near infrared spectroscopy as an analytical method to produce supporting data for a marketing authorisation application in one of the EU member states. The development of such a guideline indicates the growing popularity and importance of NIRS as an analytical method in the Pharmaceutical Industry.

6.1.4 Multiple Linear Regression Analysis (MLR)

Linear regression analysis is a mathematical method used in the quantitative analysis of NIR spectra, in which the absorbance (A) of samples at one or more wavelengths is correlated to reference values. Reference values are obtained externally to the NIR
method and will vary dependent upon the analyte to be quantified. When a good correlation is obtained between reference values and a single wavelength in the sample spectra, a univariate calibration model may be applied to the data, using simple linear regression. In such a model, the assumption is made that analyte concentration values are a linear function of the absorbance (A) at a single wavelength. The correlation coefficient, r^2 describes how well the data at the particular wavelength are represented by the calibration equation, with r^2 of −1 or 1 indicative of a perfect negative or positive correlation respectively. However, when a good correlation cannot be obtained between spectra and reference values at a single wavelength, correlation at more than one wavelength may be obtained, leading to the development of a calibration model through multiple linear regression (MLR).

Multiple linear regression is generally used for quantitative NIR analysis when the system to be analysed is a simple one. For example, in a two-component formulation where the components do not interact, MLR would be the preferred chemometric technique for quantitative analysis. However, in systems where there are many components, where interaction may occur, or where there is overlapping of spectra, partial least squares regression analysis is a more suitable regression method for quantification of the specific components.

The Vision® software performs a ‘forward-search’ MLR analysis on the spectral data set supplied. A single wavelength is chosen at a point where the highest linear correlation between spectral and reference data is observed. A second wavelength is then selected by the software, not because it has the second highest correlation to reference data, but because it shows the highest correlation to reference data in combination with the first wavelength selected. A second wavelength may be chosen either by summation or division. Summation describes the process whereby a second wavelength is added to the model so that the combination of the first wavelength added to the second produces a higher correlation with reference data. Alternatively, the combination of the first wavelength divided by the second may be chosen because a higher correlation may be produced (division).
6.1.4.1 Colinearity and Overfitting of Data

Two major problems with MLR are colinearity and overfitting of data. Colinearity occurs when two or more wavelengths, which are related in some way, are chosen to construct the calibration model. For example, peaks at the first wavelength may indicate the presence of the analyte to be measured, whereas peaks at the second wavelength may indicate its absence. Colinearity may cause the predictive ability (the robustness) of the calibration model to lower, with the calibration being unrepresentative of samples to be analysed in the future. When mathematical pretreatments have been applied to the data, such as normalisation or derivatisation, it must be checked that peaks in the spectra at the wavelengths chosen for the calibration model are pointing in the same direction as the analyte of interest. For example, when standard normal variate and second derivative mathematical treatments are applied to spectra, peaks due to the presence of the analyte point in a negative direction rather than in the positive direction shown in the raw spectra.

Overfitting of data can occur when too many wavelengths are used to produce the calibration model, making the model too specific. Although this will increase the accuracy of the calibration equation, the predictive ability (robustness) of the model for future data will be reduced, with the calibration being solely representative of the data used to create it. As a general rule, one wavelength may be added to the calibration model for every ten samples in the calibration data set.

6.1.4.2 Multiple Linear Regression Statistics

- **Multiple Correlation Coefficient (MCC, \(R^2\))**

The multiple correlation coefficient describes how well data at the particular wavelengths used to produce the calibration model are represented by the calibration equation. This number is indicative of the accuracy, but not the predictive ability of the calibration model. Unlike simple linear regression, in MLR, the value of \(R^2\) has a range of 0 to 1, with 1 indicating a perfect relationship, and 0 indicating a total lack of relationship between the sample and reference data when the calibration equation is applied.

Vision® presents supplementary statistics to assess the quality of the calibration equation. Brief descriptions of these are given below.
• **Standard Error of Calibration (SEC)**

When the calibration equation is applied to the calibration set, the SEC is calculated from the residual of the predicted and reference values at each data point. The SEC indicates the upper limit of accuracy of future predictions using the calibration equation.

\[
SEC = \sqrt{\frac{\sum f_i^2}{N - K - 1}}
\]

Where:
- \(N\) = number of samples
- \(K\) = number of wavelengths or factors
- \(F\) = residuals

• **Standard Error of Prediction (SEP)**

The SEP is the same as the SEC except that the calibration equation is applied to the validation data set rather than the calibration set. This gives a better assessment of the future predictive ability of the calibration model.

• **The F Value**

The F value is used by Vision® as an indication of the goodness of fit between the spectral and reference data, taking into account the number of samples, the number of wavelengths used in the correlation and the MCC. On addition of another wavelength to the calibration model, the F value will rise if the addition is warranted and will drop if the number of samples does not warrant the addition, indicating overfitting of the data. It is useful to look at the F value in conjunction with the SEP to assess the predictive ability of the calibration model. The equation for the calculation of the F value is given below:

\[
F = \frac{R^2 (N - W - 1)}{W (1 - R^2)}
\]

Where:
- \(N\) = Number of samples
- \(W\) = Number of wavelengths used in the calibration equation
- \(R^2\) = Multiple correlation coefficient
Relative Error (%)
The % Relative Error may be calculated for each individual sample of the data set. This value gives the % error of the predicted values (calculated from the calibration model) relative to the values calculated by the reference method. This value can therefore indicate the reliability and accuracy of the calibration model and may give more meaningful results than the SEC or SEP.

\[
\text{% Relative Error} = \frac{\text{Predicted value} - \text{Reference value}}{\text{Reference value}} \times 100
\]

6.1.4.3 The Use of Statistics
None of the statistics described should be used in isolation. For example, the SEC may be very small, suggesting that the calibration equation accurately represents the data in the calibration set. However, if the SEP is large, then the fact that the SEC is small is meaningless, as the future predictive ability of the calibration equation will not be accurate. If the multiple regression correlation coefficient (MCC) is very close to 1, this suggests a good correlation of the calibration equation with the calibration data set. However, the MCC does not take into account the number of wavelengths used in the calibration model, so the F value should also be examined. A large F value suggests a good fit between the spectral and reference data whilst taking into account the number of wavelengths used.

6.1.5 Partial Least Squares Regression Analysis (PLS)
PLS is another mathematical method used by the Vision® software for the purpose of quantification. This method is generally used when the system to be analysed is more complex, for example when there may be interaction between components of the sample or when the component spectra overlap to a great extent. PLS uses large parts or even the whole NIR spectrum in compiling the calibration model rather than the use of few wavelengths as with MLR, thus avoiding the problem of colinearity.

PLS is a regression method that takes into account variation between test spectra across the entire NIR spectrum whilst comparing this variation to values obtained from the reference method. PLS uses a series of factors (parts of the spectral data) along with the corresponding reference data to explain variation in the spectra, the first factor
describing the largest variance in the data, with the explained amount of variance reducing with increasing factor number. Factors are similar to the Principle Components used in Principle Component Analysis (PCA). In PLS regression analysis, a ‘mini’ calibration is performed to model the variance explained for each separate factor. Each individual calibration is applied to the validation set of data, and the Prediction Residual Error – Sum of Squares (PRESS) value is calculated for each factor. The ‘predicted’ value for the sample calculated using the calibration equation for the individual factor is subtracted from the value calculated for the same sample by the reference method, and the resultant difference (residual) is squared. The resultant residual values of all of the unknown samples are squared and summed to create the PRESS value for each factor. All of the individual factor calibrations are taken into account when constructing the full calibration model. The danger exists of using too few or too many factors to create the final calibration equation. The use of too few factors will produce a calibration model that will not take into account enough variance in the data. The use of too many factors will lead to a weak model that overfits the data. To prevent this, the factor number having the lowest PRESS value (and therefore closer predicted versus reference values) is the number of factors recommended for use in the final calibration model.

When using PLS, the entire sample set may be used to examine the predictive ability of the calibration model rather than splitting the sample set into calibration and validation subsets. This is made possible by cross-validation of the calibration model. The data set is split into individual samples or groups of samples, which are removed individually from the rest of the samples and tested as unknowns against a calibration model constructed using the rest of the samples. The Standard Error of Cross-Validation (SECV) is an indicator of the validity of the calibration model and is calculated in a similar manner to the SEP described in Section 6.1.4.2.

6.2 Aim

The aim of this study was to carry out a feasibility study to quantify the amount of catalase in solid co-spray-dried catalase/trehalose formulations using near infrared spectroscopy. The objectives of this study were to assess the most applicable regression methods to perform the quantification study and to determine whether full method validation studies would be warranted. If the quantification of protein concentration in
solid-state formulations were shown to be possible, this could lead to the possibility of developing a fully validated method for this purpose.

6.3 Experimental
6.3.1 Preparation of co-spray-dried catalase/trehalose formulations
Co-spray-dried catalase/trehalose formulations were prepared as described in Section 2.1 of this thesis. The formulations used for this study are outlined in Table 6.3. Sample spectra for each formulation were taken from the end of the first dry stage (following 8 h at 0 % RH) of the dynamic vapour sorption experiments carried out as described in Chapter 5, Section 5.2.2, so that all of the samples would be dry and unaffected by varying levels of moisture content. This study could also have been carried out using a Rapid Content Analyser (RCA - NIRS analysis in isolation), however it was considered sensible to use the NIR probe in the combined DVS/NIRS apparatus in order to ensure drying of the sample to constant mass prior to analysis.

Table 6.3. Table outlining the physical composition of co-spray-dried catalase/trehalose formulations used in the quantification studies.

<table>
<thead>
<tr>
<th>Formulation Name</th>
<th>% Catalase</th>
<th>% Trehalose</th>
</tr>
</thead>
<tbody>
<tr>
<td>ct0100d</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>ct0100x</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>ct0100j</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>ct2080d</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>ct2080x</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>ct2575j</td>
<td>25</td>
<td>75</td>
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<tr>
<td>ct4060d</td>
<td>40</td>
<td>60</td>
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<td>40</td>
<td>60</td>
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<tr>
<td>ct5050d</td>
<td>50</td>
<td>50</td>
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<tr>
<td>ct5050j</td>
<td>50</td>
<td>50</td>
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<tr>
<td>ct5050x</td>
<td>50</td>
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<td>ct8020d</td>
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<td>20</td>
</tr>
<tr>
<td>ct8020x</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>ct1000d</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>ct1000j</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>ct1000m</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>ct1000x</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>
6.4 Multiple Linear Regression (MLR) Analysis of dry co-spray-dried catalase/trehalose formulations

6.4.1 MLR Methods

A forward-search MLR method using the Vision® software was performed in the wavelength range of 1100-2200 nm. The region 2200-2500 nm was excluded in all quantification studies owing to spectral noise in this region when a fibre optic probe is used for spectra collection. It was decided to attempt the separate quantification of catalase and trehalose in co-spray-dried samples. Constituent values for both % catalase and % trehalose were assigned to the sample spectra and mathematical pre-treatments to convert the spectra into SNV 2nd Derivative spectra (analyte peaks pointing in the negative direction) were applied. These mathematical pre-treatments were performed by the Vision® software in order to improve the resolution of peaks in the spectra and to remove the effects of particle size. Two MLR methods for production of the calibration model were applied to the sample data, each applied once for the quantification of catalase and once for the quantification of trehalose:

**MLR Method One**
- All 22 samples assigned to the calibration set (no validation set)
- 2 Wavelengths used in each regression analysis by summation

**MLR Method Two**
- Samples assigned either to the calibration or the validation set (60% cal, 40 % val)
- 2 Wavelengths used in each regression analysis by summation

The advantage of Method One was that all samples were assigned to the calibration set, leading to the construction of a more accurate MLR calibration model. The calibration equation created through this method would be expected to be more successful in describing the sample data owing to the greater number of samples used to create the calibration equation. However, as no samples were assigned to a validation set, the predictive ability of the model could not be tested. This means that the calibration equation produced may be specific to the samples used to construct the calibration equation and may not have accuracy for future predictions.
Method Two had the advantage of allowing predictive ability of the calibration model to be tested on the samples in the validation set. However, fewer samples were used in the construction of the calibration equation, meaning that this calibration model may be less accurate than that constructed using all of the available samples.

Reference values for the analysis were the formulation component percentages used to produce the spray drying feed solutions.

6.4.2 Results: Multiple Linear Regression

The results of the MLR calibrations for the quantification of catalase and trehalose using both Method One and Method Two are shown in Table 6.4.

6.4.2.1 Results: MLR Method One

(All samples assigned to the calibration set)

The wavelengths chosen and the statistical data calculated using Method One were the same regardless of whether catalase or trehalose was the constituent of interest. As the proportion of catalase varied in relation to the proportion of trehalose and vice versa, and the samples used for both the catalase model and the trehalose model were the same, this result is not surprising. The wavelengths (1178 nm and 1990 nm) used in the calibration models are highlighted in Figures 6.1 and 6.2. Both the spectra for 100% spray-dried trehalose and 100% spray-dried catalase showed peaks at these wavelengths and an obvious trend was observed at each wavelength upon variation of the ratio of catalase to trehalose. Neither peak was specific to the single analyte of interest (i.e. catalase or trehalose), indicating a lack of specificity of the calibration models due to overlapping peaks.

The graph of reference % catalase values against the values calculated from the calibration models is shown in Figure 6.3. The points on the graph for catalase were identical but inverse in the graph for the trehalose calibration (graph not shown), because the same points of reference in the spectra were used by the software to create the separate calibration models. The calibration model for catalase and that for trehalose are in effect, the same, owing to the use of the same samples for both models and overlapping spectra.
Two wavelengths were used in both calibration models using MLR Method One. The use of the second wavelength to the model was justified because the F value increased and the SEC decreased upon its addition. The calibration equations for both catalase and trehalose adequately represented the sample data in each calibration set, with a multiple correlation coefficient ($R^2$) of 0.992 in both cases. Although this was the case, the predictive ability of the calibration models could not be tested as all of the available samples were assigned to the calibration set only, and the specificity of the models for a specific analyte could not be relied upon due to the overlapping of peaks.
Table 6.4. Summary of multiple linear regression results using all 22 samples of the full data set.

<table>
<thead>
<tr>
<th></th>
<th>Catalase</th>
<th></th>
<th>Trehalose</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calibration only (MLR Method One)</td>
<td>Calibration set (~ 60 %) &amp; Validation set (~ 40 %) (MLR Method Two)</td>
<td>Calibration only (MLR Method One)</td>
<td>Calibration set (~ 60 %) &amp; Validation set (~ 40 %) (MLR Method Two)</td>
</tr>
<tr>
<td>Number of samples in set</td>
<td>22</td>
<td>13 (calibration set) 9 (validation set)</td>
<td>22</td>
<td>13 (calibration set) 9 (validation set)</td>
</tr>
<tr>
<td>Wavelengths (nm)</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; 1990 1178</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; 1988 1230</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; 1990 1178</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; 1990 1296</td>
</tr>
<tr>
<td>R²</td>
<td>0.982 0.992</td>
<td>0.990 0.995</td>
<td>0.982 0.992</td>
<td>0.990 0.996</td>
</tr>
<tr>
<td>F-value</td>
<td>1087 1128</td>
<td>1044 1050</td>
<td>1087 1128</td>
<td>1094 1309</td>
</tr>
<tr>
<td>Standard Error of Prediction (SEP)</td>
<td>- - -</td>
<td>7.067</td>
<td>- - -</td>
<td>5.660</td>
</tr>
</tbody>
</table>
Figures 6.1 (top) and 6.2 (bottom). The SNV 2nd-derivative NIR spectra of a range of co-spray-dried catalase/trehalose formulations indicating those wavelengths used in the MLR Method One calibration models.
6.4.2.2 Results: MLR Method Two

(Samples assigned to the calibration or validation sets in a 60:40 ratio)

By splitting the samples into calibration and validation sets (MLR Method Two), the accuracy of the calibration models was likely to decrease but the predictive ability of the models could be assessed. Samples were randomly assigned to either the calibration or validation sets and checks were made to ensure that samples from across the entire concentration range were included in the both sets (especially the calibration set).

Two wavelengths were again used in both the catalase calibration model and the trehalose calibration model. This was justified by an increase in the F value and a decrease in the SEC in both cases, even though the sample number for both calibration sets was below the recommended 20 samples prior to the addition of another wavelength. However, unlike the calibration models containing all the samples in the calibration set, assigning the samples to the calibration or validation sets in a 60:40 ratio caused the models for the different analytes to be different. Wavelengths of 1988 nm and 1230 nm were chosen by the software for the Calibration model for the quantification of catalase whereas 1990 nm and 1296 nm were selected for the trehalose...
The wavelengths of 1988 nm and 1990 nm are similar to those chosen for the MLR Method One calibration models. The wavelengths of 1230 nm and 1296 nm are shown in Figure 6.4.

**Figure 6.4.** SNV 2\textsuperscript{nd}-derivative NIR spectra of a range of co-spray-dried catalase/trehalose formulations indicating the wavelengths selected for use in the MLR Method Two calibration models.

Neither second wavelength chosen in either the catalase or trehalose MLR Method 2 calibration models appeared to have been chosen by the software at a wavelength where there were large peaks for either constituent. In fact, the peaks for the appropriate analyte (catalase or trehalose) at either wavelength showed little magnitude and were therefore unlikely to be robust peaks to use for the construction of calibration models. A calibration model may achieve greater sensitivity if wavelengths at which larger peaks appear are used in its construction due to the reduced likelihood of interference from other sample component peaks. This is one of the disadvantages of MLR for the construction of calibration models when the sample spectra are more complex. MLR does not take into account the dependent variables (i.e. the largest peaks for the constituents of interest), rather the correlation of the data at particular wavelengths.

The calibration equations for both catalase and trehalose adequately represented the sample data in each calibration set, with a multiple correlation coefficient ($R^2$) of 0.995
for the catalase model and 0.996 for the trehalose model, higher than the value of 0.992 achieved by the calibration models in which all samples were assigned to the calibration set (MLR Method One). The linear fit (linear correlation coefficient, $r^2$) of reference values with calculated values (calculated from the calibration sets) are shown in Figure 6.5 (catalase, $r^2 = 0.998$) and Figure 6.6 (trehalose, $r^2 = 0.998$). The predictive ability of the calibration models was less accurate (catalase, $r^2 = 0.983$, trehalose, $r^2 = 0.984$), shown by the linear fit of samples in the validation sets (which weren't assigned to the sample set used to produce the calibration model).

Calculation of the standard error of prediction for the validation sets indicated that the predictive ability of the calibration models produced for the quantification of both catalase and trehalose was fairly poor, with an SEP of 7.067 for the catalase model and 5.660 for the trehalose model. To examine the performance of the calibration model for catalase more closely, the % relative error for each individual sample was calculated and plotted against the reference % catalase content (Figure 6.7). This was intended to give a clearer view of the accuracy and reliability of the calibration models produced using MLR Method One and MLR Method Two.

![Figure 6.5. Reference % catalase content against % catalase content predicted from the calibration model constructed using MLR Method Two, for both the calibration and validation sets.](image)

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Figure 6.6. Reference % trehalose content against % trehalose content predicted from the calibration model constructed using MLR Method Two, for both the calibration and validation sets.

Figure 6.7. Graph depicting the relative error (%) for individual samples in the full data set (n = 22) used for the multiple linear regression analysis of catalase content.
Figure 6.7 clearly shows one of the samples to be an outlier, giving relative error values of up to 55% compared with the rest of the population generally showing relative errors below 10%. As this sample behaved in the same manner when analysed as part of the calibration set (MLR Method One) or as part of the validation set (MLR Method Two), it was decided to remove the sample and construct calibration models for the quantification of both catalase and trehalose based upon the remaining samples. It was expected that the re-calculated calibration models would give a more accurate indication of the applicability of MLR analysis for the co-spray-dried samples. The results of the MLR analysis using the reduced sample set of 21 samples are shown in Table 6.5.

The results in Table 6.5 show that the calibration models for the quantification of catalase and trehalose were improved following the removal of the outlier from the sample set. The SEP for the catalase model was reduced from 7.067 \((n=22)\) to 3.086 \((n=21)\) and the SEP for the trehalose model was reduced from 5.660 to 5.012. This indicates that the predictive ability of the trehalose calibration equation is less accurate than that of the catalase calibration equation. The wavelengths chosen for the calibration models by the software were not specific the presence or absence of either component; therefore it is possible that the models could be improved by the manual selection of the wavelengths according to knowledge of the absorbance of the components.

Figure 6.8 shows the calculated relative error for each individual sample plotted against the reference % catalase content (looking at the quantification of catalase only). The majority of samples show a % relative error of 5 % or less, suggesting the increased overall accuracy and reliability of the regression method using the reduced 21-sample set.
Table 6.5. Summary of multiple linear regression results after removal of outlier from the data set (n = 21).

<table>
<thead>
<tr>
<th></th>
<th>Catalase</th>
<th></th>
<th>Trehalose</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calibration only (MLR Method One)</td>
<td>Calibration set (~ 60 %) Validation set (~ 40 %) (MLR Method Two)</td>
<td>Calibration only (MLR Method One)</td>
<td>Calibration set (~ 60 %) Validation set (~ 40 %) (MLR Method Two)</td>
</tr>
<tr>
<td>Number of samples in set</td>
<td>21</td>
<td>13 (calibration set) 8 (validation set)</td>
<td>21</td>
<td>13 (calibration set) 8 (validation set)</td>
</tr>
<tr>
<td>Wavelengths (nm)</td>
<td>1st 1990 2nd 1178</td>
<td>1st 1990 2nd 1178</td>
<td>1st 1990</td>
<td>2nd 2118</td>
</tr>
<tr>
<td>R²</td>
<td>0.991 0.996</td>
<td>0.989 0.997</td>
<td>0.991 0.996</td>
<td>0.986 0.994</td>
</tr>
<tr>
<td>F-value</td>
<td>2167 2368</td>
<td>963 1834</td>
<td>2167 2367</td>
<td>769 791</td>
</tr>
<tr>
<td>Standard Error of Calibration (SEC)</td>
<td>3.251 2.205</td>
<td>3.530 1.817</td>
<td>3.251 2.205</td>
<td>3.513 2.460</td>
</tr>
<tr>
<td>Standard Error of Prediction (SEP)</td>
<td>- -</td>
<td>3.086</td>
<td>- -</td>
<td>5.012</td>
</tr>
</tbody>
</table>
6.4.3 Conclusions: Multiple Linear Regression Analysis

- The assignment of all samples to the calibration set for both the catalase quantification model and the trehalose quantification model gave good results for the calibration equation in terms of multiple correlation coefficient and SEC (MLR Method One). However, the wavelengths selected by the software were identical for both the catalase calibration and the trehalose calibration when all samples were assigned to the calibration set. This suggests colinearity within the spectra, meaning that the behaviour at the selected wavelengths was related rather than independent. On examination it was clear that this was the case, with overlapping of component peaks within the spectra adding to the lack of specificity of the calibration models.

- The random assignment of samples to the calibration or validation sets (MLR Method Two) produced good calibration results for both the catalase and trehalose calibration models. However, the standard errors calculated for both the catalase and trehalose validation sets were high, suggesting the low predictive ability of the calibration models. Plotting of catalase content against relative error indicated an outlier that was removed from all data sets. Repetition of both MLR Methods

Figure 6.8. Graph depicting the relative error (%) for individual samples in the reduced data set (n=21) used for multiple linear regression analysis of catalase content, the results of which are described in Table 6.5.
following removal of the outlier gave better results. The predictive ability of the catalase content calibration model was better than that for the trehalose model.

- The low number of samples used in this feasibility study means that no firm conclusions can be made as to the applicability of the multiple linear regression technique for the quantification of catalase or trehalose in the solid state. However, results from the study are promising and a larger validation study is warranted.

### 6.4.4 Further Work: Multiple Linear Regression Analysis

- The major drawback with the MLR technique for the quantification of components of co-spray-dried catalase/trehalose solid samples was colinearity and lack of specificity of the calibration models. This problem may be addressed through the use of an alternative regression technique, such as Partial Least Squares Regression Analysis (see Section 6.5). Alternatively, manual selection of the wavelengths to use in the calibration model could be tested. Wavelengths could be selected according to knowledge of where the absorbance of the component to be quantified is high and where the absorbance of the other component is zero.

- To investigate the specificity and robustness of the MLR model for quantification, the calibration model could be tested on samples of catalase and another additive (e.g. lactose or sucrose). If the calibration model were specific and robust, it should be able to quantify the amount of catalase in a co-spray-dried sample, regardless of the excipient.

### 6.5 Partial Least Squares Regression Analysis of co-spray-dried catalase/trehalose formulations

#### 6.5.1 Method

A fully cross-validated PLS regression analysis for the quantification of catalase and trehalose in the co-spray-dried catalase/trehalose formulations (described in Section 6.3.1) was performed by the Vision® software (Foss NIRSystems), in the wavelength range of 1100-2200 nm. One sample was removed from the sample set at a time to perform the cross-validation. A reduced data set of 21 co-spray-dried catalase/trehalose formulations was used; omitting the outlier identified during the MLR analyses. Reference values for the analysis were the formulation component percentages used to
produce the spray drying feed solutions. For explanation of PLS regression analysis, see Section 6.1.5.

6.5.2 Results: Partial Least Squares Regression Analysis

A fully cross-validated PLS regression analysis of the co-spray-dried catalase/trehalose formulations was performed as described in Section 6.5.1.

The PRESS value calculated by the Vision® software (for definition see Section 6.1.5) indicated the use of four factors for the final calibration as can be seen by the initial minimum point shown in Figure 6.9.

A summary of the statistics for the first four factors is shown in Table 6.6. As factor number increases, the F Value increases, indicating a better goodness of fit between spectral and reference data. The standard error of calibration (SEC) reduces as the factor number increases, indicating the increasing accuracy of the calibration model. The standard error of cross validation (SECV, equivalent to the SEP) also drops, indicating the increased predictive accuracy of the calibration model as more factors are taken into account. Although the F value was higher when factor 7 was used, and the SEC and SECV also reduce, the use of seven factors in the construction of the final calibration model would risk overfitting of the data. As can be seen in Figure 6.9, the
PRESS value reaches a plateau at factor 4 and thus the use of further factors past this point would cause insignificant variation in the spectral data to be taken into account, leading to overfitting of the data and thus reducing the predictive ability of the calibration model.

**Table 6.6. Summary of the first 7 factors proposed for the Partial Least Squares Regression analysis of the dry NIR spectra of co-spray-dried catalase/trehalose formulations (based on % catalase content). Only the first 4 factors were used in the final calibration, according to the PRESS value (see Figure 6.9).**

<table>
<thead>
<tr>
<th>Factor</th>
<th>R Squared</th>
<th>Standard Error of Calibration</th>
<th>PRESS</th>
<th>F Value</th>
<th>Standard Error of Cross Validation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor 1</td>
<td>0.959</td>
<td>7.019</td>
<td>1178</td>
<td>450</td>
<td>7.490</td>
</tr>
<tr>
<td>Factor 2</td>
<td>0.977</td>
<td>5.421</td>
<td>1086</td>
<td>384</td>
<td>7.192</td>
</tr>
<tr>
<td>Factor 3</td>
<td>0.980</td>
<td>5.215</td>
<td>729</td>
<td>278</td>
<td>5.893</td>
</tr>
<tr>
<td><strong>Factor 4</strong></td>
<td><strong>0.994</strong></td>
<td><strong>2.915</strong></td>
<td><strong>372</strong></td>
<td><strong>676</strong></td>
<td><strong>4.211</strong></td>
</tr>
<tr>
<td>Factor 5</td>
<td>0.997</td>
<td>2.328</td>
<td>402</td>
<td>850</td>
<td>4.375</td>
</tr>
<tr>
<td>Factor 6</td>
<td>0.998</td>
<td>2.020</td>
<td>357</td>
<td>941</td>
<td>4.120</td>
</tr>
<tr>
<td>Factor 7</td>
<td>0.998</td>
<td>1.813</td>
<td>390</td>
<td>1002</td>
<td>4.310</td>
</tr>
</tbody>
</table>

Figure 6.10 shows the reference % catalase content plotted against the % catalase content predicted using the calibration equation based upon the use of four factors. There is good correlation to the data, suggesting the feasibility of this method for the quantification of catalase. The PLSR method was also applied to the quantification of trehalose in the co-spray-dried catalase/trehalose formulations. As with the calibration set-only MLR Method One, the results were identical to those shown above for the quantification of catalase. Again, this was expected, because the same samples and method were used for both analyses, with the catalase content varying in line with the trehalose content.

Wavelength loadings are plots produced by the Vision® software, which describe the variation being modelled by a particular factor. The first factor will generally describe the largest variation in the data, with the second factor describing the second largest variation, and so on. For this reason, factors one and two may often be attributed to the effects of particle size or water on the spectrum. On examination of the wavelength loadings for the Partial Least Squares Regression (PLSR) analyses for the catalase and trehalose components (Figures 6.11 and 6.13), it was evident that although the statistics
were the same for both the catalase model and the trehalose model, the variance explained by each factor of the two regression models was different.

![Graph](attachment:image.png)

**Figure 6.10.** Reference % catalase content against % catalase content predicted from the calibration model constructed using PLSR analysis.

Figures 6.11 and 6.13 show the wavelength loadings of factors 1, 2, 3 and 4 for the catalase calibration model and the trehalose calibration model, respectively. Wavelength loadings indicate the wavelengths at which the spectral variance, modelled by the specific factor, occurs. In effect, the wavelength loading plots indicate the parts of the spectrum that are responsible for the differences between samples explained by a particular factor. The variation modelled by the factors for catalase or trehalose are shown as mirror images of each other in the loadings, meaning that the overall variation modelled by the four factors in each model is likely to be the same. On comparison of the wavelength loadings of factor 1 from the catalase calibration model and factor 1 from the trehalose calibration model with the *actual* spectra of 100% spray-dried catalase and 100% spray-dried trehalose (Figures 6.12 and 6.14), the similarities are evident. This is apparent particularly between factor 1 of the catalase calibration and the spectrum of 100% spray-dried catalase. This finding demonstrates the ability of the PLSR method to take into account variation across the entire spectrum in the construction of a calibration model, therefore enabling it to produce calibration equations more applicable to the spectral data in question. It is clear that although both
the catalase and trehalose calibration models gave rise to the same results (as expected), the PLSR method modelled the variance due to the individual constituents more closely.

Figure 6.11. Wavelength loadings of factors 1, 2, 3 and 4 from the Partial Least Squares regression analysis of catalase content in dry co-spray-dried catalase/trehalose formulations. Key: Black = factor 1, red = factor 2, blue = factor 3, green = factor 4).

Figure 6.12. SNV 2nd-derivative NIR spectra of 100 % spray-dried catalase and 100 % spray-dried trehalose after drying for 8 h at 0 % RH in the DVS apparatus.
Figure 6.13. Wavelength loadings of factors 1, 2, 3 and 4 from the Partial Least Squares regression analysis of trehalose content in dry co-spray-dried catalase/trehalose formulations. Key: Black = factor 1, red = factor 2, blue = factor 3, green = factor 4).

Figure 6.14. SNV 2nd-derivative NIR spectra of 100 % spray-dried catalase and 100 % spray-dried trehalose after drying for 8 h at 0 % RH in the DVS apparatus.
The wavelength loadings are indicative of the chemistry of the variation modelled by the factors; hence the similarity of the wavelength loading plots to the spectra of the individual components. The variation modelled by factors 1 and 2 in the PLSR analysis for both models (catalase and trehalose) was similar to the catalase and trehalose spectra (inverted in the opposing constituent models). The main areas of variation modelled by factors 3 and 4 were those regions of the spectrum corresponding to water (around 1400 and 1900 nm), essentially the third component of the co-spray-dried samples, albeit at low levels. These data suggest the specificity of the PLSR method for separating the components of multi-component samples, something that was not evident in the MLR analyses.

6.5.3 Conclusions: Partial Least Squares Regression Analysis

- The fully cross-validated PLS calibration model for the quantification of catalase gave good results with the use of four factors, with an SEC of 2.9 % and an SECV of 4.2 %. This suggested the feasibility of this regression method with NIRS to quantify proteins in the solid state.

- Although the calibration models for catalase and trehalose were identical using the PLSR technique, the wavelength loadings for each factor of the models suggested the greater specificity of these models compared with the calibration models constructed using MLR. It was evident that the two greatest sources of variance accounted for by each calibration model were due to the proportions of catalase or trehalose in the samples. This was unlike MLR, where the construction of the calibration models was constrained to using 2 wavelengths in the entire NIR spectrum.

- To use PLS regression analysis as an accurate tool, sample numbers must be high. It is important to have at least 5 or 6 samples per factor used in the regression analysis (Hu and Arnold, 2000). In this study, 21 samples were used and 4 factors, which was just adequate to create an accurate calibration model. Although the study demonstrated the feasibility of PLSR to quantify catalase or trehalose in the solid state, the addition of more samples to the data set to allow the formation of calibration and validation data subsets would be necessary to produce a fully validated calibration model.
6.6 Overall Conclusion (MLR and PLSR Analyses)

From the data presented in this chapter, the potential of near infrared analysis for the quantification of components of co-spray-dried formulations, including proteins, was demonstrated. It was proposed that the PLSR analysis method might be more robust for the quantification of components in such complex systems because it took into account the variation of the samples across the entire wavelength range.

It must be stressed that these studies were feasibility studies and were not designed to form part of rigorous method development. In order for these methods to be assessed more fully and to be sure of their specificity and robustness, many more samples would be required. The data shown will have been prone to some degree of overfitting because the sample numbers were the minimum for that required for such analyses.
Conclusions and Further Work
7. Conclusions

Protein drug formulations are commonly produced in solid form owing to the tendency of proteins in solution to degrade. The processes involved in the production of solid protein formulations such as spray drying or freeze drying may alter the conformation of proteins, which may in turn lead to loss of biological activity (Prestrelski et al., 1993a). Stabilising excipients, in particular disaccharides, are used to protect proteins from the effects of process-induced stresses. The mechanism of the preservation of proteins upon drying by the addition of disaccharides such as trehalose remains a subject of debate and was the focus of this thesis. Many research groups have highlighted the disaccharide α-trehalose for its superior role in the protection of proteins and membranes during drying processes; therefore this disaccharide was chosen as the model carbohydrate in this work. The aims of this thesis were:

- To investigate the use of the combined technique of dynamic vapour sorption and near infrared spectroscopy for the purposes of the solid-state analysis of spray-dried protein/carbohydrate formulations
- To enhance the understanding of the unusual properties of trehalose for the purposes of protein stabilisation in the dried state
- To provide evidence as to the mechanism of stabilisation provided for the model protein catalase in the spray-dried state by trehalose

As the polymorphism of trehalose is thought by some to be central to its superior biostabilising action (Aldous et al., 1995 and Liao et al., 2004), further understanding of its solid state transitions was thought to be important to try to elucidate the mechanism of protein stabilisation by trehalose upon drying. The purpose of Chapter Three was to realise a better understanding of the dehydration of α,α-trehalose dihydrate and to assign peaks in the NIR spectra of the various polymorphic forms of α,α-trehalose, with the objective to allow investigation of the behaviour of trehalose in its dehydrated form as an excipient in dried protein formulations.

The alpha and beta forms of trehalose (Ta and Tp) were prepared according to methods described in the literature and characterised by Differential Scanning Calorimetry (DSC), Thermogravimetric Analysis (TGA) and X-Ray Powder Diffraction analysis.
A ‘new’ anhydrous form of trehalose, named Tₐ, was produced by the dehydration of α,α-trehalose dihydrate in a DVS analyser by drying at 0 % RH for approximately 25 h. The combined method of Dynamic Vapour Sorption and Near Infrared Spectroscopy (DVS/NIRS) allowed the detection of changes in α,α-trehalose dihydrate as dehydration occurred in real-time. From analysis of XRPD data it was concluded that the anhydrous form of trehalose produced in the DVS analyser was different from the other anhydrous forms of trehalose prepared and different to ‘Form II’ described in the literature (Belton and Gil, 1994). Faster removal of water from trehalose dihydrate under vacuum was shown to produce a more amorphous product, owing to insufficient time being available for the sample to completely reorder to the crystalline anhydrous state.

Assignment of characteristic peaks in the SNV second-derivative NIR spectra of the different polymorphic forms of α,α-trehalose in the near infrared spectra of solid samples was shown to be possible. These results, supported by the results of the other characterisation studies (DSC, TGA, XRPD) were intended to be used to examine the behaviour of trehalose in co-spray-dried protein-trehalose formulations and to try to elucidate the mechanism of bioprotection conferred by trehalose in such systems. Further work with the aim to produce ‘cleaner’ samples of the anhydrous forms could be performed to substantiate work in this initial chapter, with the aim to reduce exposure to the atmosphere during product handling and optimise the dehydration process to produce wholly crystalline forms.

Prior to the examination of two-component co-spray-dried protein/trehalose formulations using DVS/NIRS, it was necessary to understand the data for the individual components. The work in Chapter Four aimed to examine the water vapour sorption behaviour of spray-dried trehalose alone, and to identify transitions occurring in such samples throughout the water vapour sorption experiments by the simultaneous collection of near infrared spectra.

Firstly, the variability of spray-dried trehalose samples spray-dried from solutions of different trehalose concentration (in water) was examined. Variability in the water vapour sorption behaviour of spray-dried trehalose samples dried under identical conditions was observed in samples spray-dried from feed solutions of low solute concentration. Such variability was reduced, but not eliminated with increasing solute
concentration of the feed solution to be spray-dried. Particles spray-dried from solutions of low solute concentration were shown to be smaller than those from high concentration solutions. The variability in spray-dried trehalose samples dried from solutions of low solute concentration was proposed to be because of the less uniform nature of the atomised droplets, prior to drying.

By the use of NIR spectroscopy it was possible to identify the form of the trehalose in spray-dried formulations. The NIR peak assignments for the polymorphic forms of trehalose from Chapter Three were used to identify the form of the trehalose in spray-dried samples. NIR spectra of spray-dried samples indicated that spray drying trehalose from solutions of low solute concentration led to areas of anhydrous trehalose in the otherwise amorphous samples. This anhydrous nature was observed in the NIR spectra of samples following crystallisation to the dihydrate form, suggesting that the anhydrous material acted as a seed for crystallisation and subsequent dehydration. This observation was reduced, but not eliminated, in samples spray-dried from solutions of high trehalose concentration. Continual mass loss at 75 % RH shown by the spray-dried samples following the initial crystallisation event to the dihydrate form, particularly by those samples spray-dried from solutions of low solute concentration, indicated the tendency of the samples towards the anhydrous form following crystallisation. Samples of spray-dried trehalose that had crystallised during exposure to 75 % RH were shown to tend towards the DVS-anhydrous form of trehalose ($T_d$) during the final drying stage of the DVS/NIRS experiments.

The combined application of DVS/NIRS enabled the crystallisation of amorphous trehalose to be examined in real-time. Crystallisation was shown not to be an instantaneous process. Peaks/shoulders suggestive of the formation of trehalose dihydrate were observed in the NIR spectra of samples prior to crystallisation, indicating the increased mobility of the amorphous trehalose with increasing water content. It was proposed that the continued gradual mass loss from samples at 75 % RH following crystallisation was indicative of the instability of the dihydrate form and of the tendency towards an anhydrous state, rather than a continuing crystallisation process.

The work in Chapter Four provided a valuable insight into the behaviour of spray-dried trehalose formulations, which was considered useful for the analysis of co-spray-dried
protein/trehalose formulations, in which the DVS/NIRS results would be much more complex. The use of the combined technique of DVS/NIRS was shown to be successful for the analysis of crystallisation processes in real-time and showed potential for the future analysis of more complex systems. Results of the analysis of the variability of spray-dried products dependent upon the composition of the original solution to be spray-dried would be useful for the future selection of spray drying parameters for pharmaceutical or biopharmaceutical formulations appropriate to prevent batch-to-batch variability and enhance product stability.

Future work to build on the work in Chapter Four could be based upon the quantification of the amorphous content in samples of trehalose spray-dried from solutions of different feed solutions. DSC or isothermal calorimetry could be used for such studies, the results of which would be useful to substantiate the observations of peaks for an anhydrous form of trehalose in the spectra of spray-dried trehalose samples. Quantification of amorphous content could be coupled with studies to optimise spray drying parameters in order to produce trehalose samples with predictable behaviour, thus reducing the variability observed in such spray-dried products. This would be of value in the field of biopharmaceutics, in which trehalose is often used as a protectant for proteins, liposomes or nanoparticles during drying processes.

Further work to substantiate the data from Chapter Four could focus on the use of differential scanning calorimetry to determine differences in the glass transition temperature of trehalose spray-dried using different spray drying parameters. Knowledge of this kind would be useful for the determination of the potential stability of dried formulations upon storage. DSC could also be useful to test the hypothesis that particles of amorphous trehalose, under conditions of 75 % RH, form particles of anhydrous or amorphous material, coated with crystalline trehalose dihydrate molecules (Al-Hadithi et al., 2004). If this were the case, then the behaviour of the ‘shielded particles’ would be expected to be different to that of fully crystalline particles of trehalose dihydrate.

Initial studies in Chapter Five aimed to characterise the water vapour sorption behaviour of samples of spray-dried catalase alone, to couple with the knowledge gleaned from Chapters Three and Four in order to allow easier analysis of co-spray-dried catalase/trehalose formulations. Crystallisation was not observed in the 100 % spray-
dried catalase samples at 75 % RH, nor was there any evidence of crystallisation in the NIR spectra of the samples. Both spray-dried catalase samples and the original catalase as obtained showed intra-batch and inter-batch variability in their NIR spectra, indicating the heterogeneous nature of the amorphous protein.

From studies of the activity of co-spray-dried catalase/trehalose formulations, it was concluded that a lower ratio of catalase: trehalose was required for the most effective stabilisation of catalase during the spray drying process. However, upon exposure of the co-spray-dried catalase/trehalose samples to 75 % RH, mimicking the effects of storage, a higher ratio of catalase: trehalose was required for the most effective stabilisation of catalase. In fact, the most effective stabilisation of catalase at 75 % was observed for the 100 % spray-dried catalase sample, indicating that the behaviour of trehalose under conditions of high relative humidity caused disruption to the catalase in co-spray-dried formulations. It was concluded that the mechanism of stabilisation upon drying was different to that during shelf life, consistent with the stress-specific protection of proteins reported in the literature. Analysis of the effects of both spray drying and subsequent exposure to 75 % RH, indicated that a ratio of 50:50 catalase: trehalose was the most effective ratio of components for the overall stabilisation of catalase during and following spray drying in this study. As this was the ratio at which the greatest interaction between the protein and carbohydrate was expected, this result suggested that the ‘Water Replacement Hypothesis’ was the most likely mechanism of stabilisation of catalase by trehalose in the co-spray-dried samples.

From the analysis of the differences in peaks assigned to the hydration of the proteins in the NIR spectra of the co-spray-dried catalase/trehalose samples and mathematically produced theoretical spectra, it was hypothesised that an interaction existed between catalase and trehalose in the co-spray-dried formulations. Given the comparison of the spectral observations with those of other protein systems in the literature, the most likely interaction was hydrogen bonding; however further work would be necessary to substantiate these observations.

From the examination of the NIR spectra of the co-spray-dried samples and the theoretical spectra during the water vapour sorption experiments, the formation of an ‘unstable dihydrate’ at 75% RH stage was proposed for those samples in which there was greatest interaction between the co-spray-dried components. This phenomenon may
be a simple effect of the mass of trehalose available to crystallise in the sample meaning that the kinetics of dehydration were altered between samples of different catalase: trehalose mass ratios. In order to conclude this work with greater confidence, further work to produce ‘actual physical mixes’ of 100% spray-dried catalase and trehalose in the proportions discussed in this study would be useful as an indicator of the mass effects of the components on the crystallisation kinetics of the trehalose in the samples. This may be problematic to perform accurately, because the homogeneous mixing of two amorphous materials is difficult and can lead to changes in the sample prior to analysis. Additionally, a comparison of co-spray-dried samples of catalase: trehalose in which the concentration of catalase is fixed would be useful to assess the effect of varying the ratio of trehalose in the sample in a more quantitative manner. In such a study, the effect of changing the concentration of solids in the solution to be spray-dried would have to be taken into account when analysing the results.

A separate study of interest and relevance to the work described in this thesis would be the further examination of the stress-specific stabilisation of proteins. Given that reference to the stresses invoked on protein/carbohydrate formulations in the literature has focussed mainly upon the effects of freezing and subsequent drying rather than spray drying and subsequent storage, an analysis of the stresses of spray drying and subsequent storage would be interesting.

Chapter Six described a separate study to the other work in the thesis, with the aim to assess the applicability of near infrared spectroscopy for the quantification of components of co-spray-dried protein/carbohydrate samples as an alternative to other quantification methods.

The first method tested for the quantification of the components of co-spray-dried catalase/trehalose samples was Multiple Linear Regression analysis (MLR). The use of 21 samples in the MLR Analysis produced a good calibration model for the quantification of catalase or trehalose in co-spray-dried samples with the use of two wavelengths in the calibration model. However, the wavelengths selected by the software were identical for both the catalase calibration and the trehalose calibration when all samples were assigned to the calibration set, suggesting colinearity within the spectra. This meant that the behaviour of the components at the selected wavelengths was related rather than independent. The random assignment of samples to the
calibration or validation sets using MLR analysis produced good calibration results for both the catalase and trehalose calibration models and the predictive ability of the models was considered good, with standard errors of prediction of 5% or less. The predictive ability of the catalase content calibration model was better than that for the trehalose model.

Results from the MLR study were promising and a larger validation study is warranted. In future studies it may be interesting to manually select wavelengths to use in the calibration model, selected according to knowledge of where the absorbance of the component to be quantified is high and where the absorbance of the other component is minimal/zero. To investigate the specificity and robustness of the MLR model for quantification, the calibration model could be tested on samples of catalase and another additive (e.g. lactose or sucrose). If the calibration model were specific and robust, it should be able to quantify the amount of catalase in a co-spray-dried sample, regardless of the excipient.

The second method tested for the quantification of the components of co-spray-dried catalase/trehalose samples was a fully cross-validated Partial Least Squares (PLS) regression analysis. The PLS calibration model for the quantification of catalase using 21 co-spray-dried catalase/trehalose samples gave good results with the use of four factors, with a standard error of cross-validation (similar to the standard error of prediction in MLR analysis) of 4.2%. This suggested the feasibility of this regression method with NIRS to quantify proteins in the solid state. The calibration model for the quantification of trehalose was identical to that for catalase except for the wavelength loadings for each factor of the calibration models. The two sources of greatest variance accounted for by each calibration model were due to the proportions of catalase and trehalose (inverted in the opposing constituent models), suggesting the specificity of the models for the components to be analysed. This was unlike MLR, where the construction of the calibration models was constrained to using two wavelengths from the entire NIR spectrum.

From the data presented in Chapter Six, the potential of near infrared analysis for the quantification of components of co-spray-dried formulations, including proteins, was demonstrated. It was proposed that the PLSR analysis method might be more robust for the quantification of components in such complex systems, as it can take into account
the variation of the samples across the entire wavelength range. These studies were feasibility studies and were not designed to form part of rigorous method development. In order to assess the applicability of near infrared spectroscopy for the quantification of co-spray-dried components, including proteins, the addition of more samples to the data set to allow the formation of larger calibration and validation data subsets would be necessary to produce fully validated calibration models. The data shown will have been prone to some degree of overfitting because the sample numbers were the minimum for that required for such analyses. Tests for the robustness and specificity of the models would be required in order to complete the assessment of the calibration techniques.

To conclude, the work outlined in this thesis has shown that the simultaneous use of dynamic vapour sorption analysis and near infrared spectroscopy is very useful for the examination of sample transitions in real-time. The use of the combination technique of DVS/NIRS allowed conclusions to be drawn that would be difficult, or impossible to arrive at by the use of either method in isolation. A 'new' anhydrous form of trehalose was produced in the DVS analyser, for which NIR spectral changes corresponding to its formation were observed in real-time. Coupled with other characterisation techniques such as DSC and XRPD, specific peaks in NIR spectra were assigned to polymorphic forms of trehalose, enhancing the possibility of future analysis of the sugar in other, more complex systems. The variability of spray-dried trehalose samples could be assessed using DVS/NIRS, as well as the tracking of the crystallisation of amorphous trehalose in real-time, a task difficult to perform by any other method. Following the systematic analysis of the components of the co-spray-dried catalase/trehalose system, an examination of co-spray-dried catalase/trehalose samples was performed using the same methods, the results of which supported the water replacement hypothesis of protein stabilisation upon drying. Near infrared spectroscopy was shown to be a promising technique for the quantification of components in co-spray-dried catalase/trehalose systems, warranting larger validation studies in the future. Avenues of further work have been highlighted, with the aim to substantiate the work in this thesis and perform those studies that were not possible to carry out in the time-scale of the PhD.
Appendix One
Appendix 1.

Scanning electron micrographs of co-spray-dried catalase/trehalose samples as described in Chapter 5, Section 5.3.5:

Figure A.1. 100% Spray-dried trehalose (pre-DVS).

Figure A.2. Co-spray-dried 20:80 catalase: trehalose (pre-DVS).
Figure A.3. Co-spray-dried 20:80 catalase: trehalose (post-DVS).

Figure A.4. Co-spray-dried 40:60 catalase: trehalose (pre-DVS).

Figure A.5. Co-spray-dried 40:60 catalase: trehalose (post-DVS).
Figure A.6. Co-spray-dried 50:50 catalase: trehalose (pre-DVS).

Figure A.7. Co-spray-dried 50:50 catalase: trehalose (post-DVS).

Figure A.8. Co-spray-dried 60:40 catalase: trehalose (pre-DVS).
Figure A.9. Co-spray-dried 60:40 catalase: trehalose (post-DVS).

Figure A.10. Co-spray-dried 80:20 catalase: trehalose (pre-DVS).

Figure A.11. Co-spray-dried 80:20 catalase: trehalose (post-DVS).
Figure A.12. 100% Spray-dried catalase (pre-DVS).

Figure A.13. 100% Spray-dried catalase (post-DVS).
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