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**INVESTIGATIONS OF A SPRAY-DRYING METHOD FOR
PRODUCING NANOPOROUS/NANO-PARTICULATE
MICROPARTICLES (NPMPs) OF PROTEINS AND PROTEIN-
EXCIPIENT COMPOSITES**

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being a thesis submitted for the degree of Doctor of Philosophy in Pharmaceutics

at the

University of Dublin, Trinity College

under the direction and supervision of

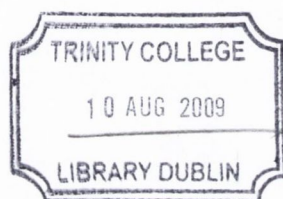
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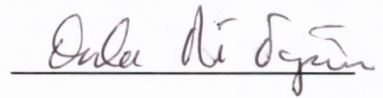
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DECLARATION

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Orla Ní Ógáin

SUMMARY:

The research, presented in this thesis, concerns the application of a spray-drying method to produce nanoporous/nano-particulate microparticles (NPMPs) of proteins, stabilising excipients and composites. The proposed method, involves spray-drying a solution or suspension of the compound(s) plus possibly a process enhancer (for example, ammonium carbonate) from a co-solvent system consisting of at least two volatile solvents. The potential of NPMPs for inclusion in dry powder formulations for pulmonary delivery of proteins or peptides was explored. Particles were visualised by scanning electron microscopy. Process stability was assessed by a selection of thermal analysis, solid-state characterisation (by powder x-ray diffraction), fourier transform infrared spectroscopy, SDS-PAGE and biological activity assays. Storage stability studies were also undertaken under different temperature and relative humidity conditions. Micromeritic characteristics of NPMPs, i.e., specific surface area, particle size, bulk and tapped densities were assessed. In-vitro deposition of powders was evaluated using an Andersen cascade impactor method.

The model protein, lysozyme, was initially investigated and spray-dried as NPMPs from a feed system comprising methanol:water or ethanol:water co-solvent systems with ammonium carbonate or an alternative ammonium salt, as a process enhancer. Phase separation in the feed system, prior to spray-drying, was identified as a required step in the production of NPMPs of lysozyme from these systems. Particle morphology and NPMP production depended on the concentration and type of alcohol and process enhancer. Ethanol and ammonium carbonate were selected for further studies because less process enhancer was required to produce NPMPs with this combination and because ammonium carbonate has a low volatilisation temperature and was thus expected to be volatilised during the spray-drying method. Process stability investigations of NPMPs of lysozyme spray-dried from ethanol:water:ammonium carbonate systems suggested some perturbation in protein folding and secondary structure in the dried state. NPMPs showed excellent recovery of activity and secondary structure, however, on dissolution, suggesting that these were reversible perturbations. A series of mass ratio composite NPMPs of lysozyme:trehalose (9:1 – 1:1) and lysozyme:alternative excipient were spray-dried using an ethanol:water:ammonium carbonate feed system. All lysozyme composites studied also showed excellent retention of biological activity on dissolution.

NPMPs of sugars – trehalose and raffinose were spray-dried alone from methanol:n-butyl acetate:(water) systems using different solvent ratios. The powders were amorphous, as assessed by XRD analysis. These NPMPs potentially represent a carrier system for peptide or protein or other drug compounds. Non-porous spherical particles of lysozyme with a rough surface were spray-dried from methanol:n-butyl acetate systems. Composite NPMPs of lysozyme:trehalose,

lysozyme:raffinose or lysozyme:hydroxypropyl- β -cyclodextrin were spray-dried in mass ratios of 1:4 and 3:7 (protein:excipient) from methanol:n-butyl acetate systems. Lysozyme:excipient composites from these systems showed excellent retention of biological activity on dissolution. NPMPs showed favourable storage stability for twelve weeks at temperatures of 4 °C or 25 °C with desiccant, in terms of physical stability of the particles and biological activity of lysozyme.

NPMPs of trypsin were spray-dried using the ethanol:water:ammonium carbonate feed system, although higher concentration of ethanol and ammonium carbonate were required than for lysozyme, and trypsin suffered a greater loss of activity than lysozyme. Inclusion of trehalose and raffinose with trypsin in composites spray-dried from these systems showed an improvement in biological activity but particle morphology was affected by the inclusion of the sugar, suggesting a less robust system. Composite NPMPs of trypsin:trehalose, trypsin:raffinose, and trypsin:hydroxypropyl- β -cyclodextrin were spray-dried from methanol:n-butyl acetate:water systems in mass ratios of 1:9, 1:4 and 1:1, with excellent retention of biological activity on dissolution. These NPMPs showed favourable storage stability for twelve weeks at temperatures of 4 °C or 25 °C with desiccant, in terms of physical stability of the particles and at 4 °C in terms of biological activity of trypsin.

NPMPs of catalase were not produced on spray-drying from an ethanol:water:ammonium carbonate system and the near complete loss of activity observed from the low concentrations of ethanol and ammonium carbonate used compared to lysozyme or trypsin systems, discouraged further work with this system. NPMPs of catalase:excipient were produced using methanol:n-butyl acetate:water systems but showed almost complete loss of catalase activity, suggesting irreversible denaturation.

A methanol:n-butyl acetate solvent feed system was used to spray-dry NPMPs of salmon calcitonin:trehalose (mass ratio of 1:19), and suggests potential for future work and development of this system, as a therapeutically used peptide drug suitable for pulmonary delivery.

Particle characterisation of all NPMPs produced, showed favourable characteristics for pulmonary delivery including particle size, bulk and tapped density, surface area and in-vitro deposition, compared to spray-dried non-porous particles.

ACKNOWLEDGEMENTS:

Ba mhaith liom buíochas speisialta a ghabháil le mo stiúraitheoirí - an Dochtúir Anne Marie Healy, agus leis an Ollamh Owen Corrigan, as an deis a thabhairt dom céim dochtúireachta a ghlacadh, agus go háirithe as a gcuid cúnamh i rith mo thaighde, agus arís agus me ag scríobh an tráchtas seo. Is mór liom bhur gcomhairle agus bhur dtacaíocht le linn na mbliain.

Tá mé buíoch do Choláiste na Tríonóide as an scoláireacht iarchéime a bhronnadh orm. Tá mo bhuíochas ar fhoirne teicniúla agus rúnaíochta na scoile, ach go háirithe Patrick Quinlan, Brian O'Reilly, Conan Murphy, agus Liesa Eckhardt, agus ar Neal Leddy on lárionad miocrascóipe agus anailíse as a chabhair le miocrascópaíocht. Ba mhaith liom buíochas mór a ghabháil leis na mic linn iarchéime agus leis na taighdeoirí eile, as a gcuid cairdis - Alecia Barry, Ahmad Shawqi Barham, Jennifer Coppins, Deirdre D'Arcy, Cinmoy Dey, Sibylle Endter, Shadeed Gad, Arun Katheri, Jianhe Li, Xue Li, Bo Liu, Fiona Ní Chearúil, Lorraine Nolan, Krystoff Paluch, Katie Ryan, Almath Spooner, Lidia Tajber, Martina White agus Tao Zhang. Do Ahmad, Arun, Shadeed, Tao agus Xue Li – go raibh maith agaibh as na comhráití cultúrtha agus na blaisbíní! Buíochas le Jianhe, Lorraine agus Lidia as buneolas ceirde a thabhairt dom, i leith spré-thriomú agus na modhanna anailíse eile. Buíochas ar leith le Lidia as a tacaíocht agus a comhairle, agus le Lorraine, Alecia, Katie, Martina agus lem dheirfiúr Aisling as a gcúnamh leis na profaí a léamh.

Buíochas ó chroí le hAisling, Niamh, Sadhbh, Ruán, David, agus Aideen as a dtacaíocht agus as an spórt! Dom thuismitheoirí – Dáithí (Ard-Ollamh na cúirte!) agus Caitríona Ó hÓgáin - gan sibh ní fhéadfainn é seo a dhéanamh. Is inspioráid saoil agam sibh. D'fhéadfainn tráchtas eile a scríobh ar ar thug sibh dom - go raibh míle maith agaibh as bhur síorchabhair! Do Ed, go raibh maith agat as gach aon ní i rith na mblianta fada - seo linn ar aghaidh sa saol!

PRESENTATIONS ASSOCIATED WITH THIS THESIS:

POSTER PRESENTATIONS:

- “Spray drying proteins for oral inhalation - the production and characterisation of nanoporous microparticles” All-Ireland Pharmacy Schools Conference, Belfast, April 2006 [Ní Ógáin O, Tajber L, Corrigan OI, Healy AM]
- “Nanoporous Microparticles (NPMs) - a novel drug delivery system for the pulmonary delivery of proteins” American Association of Pharmaceutical Scientists Annual Meeting, San Antonio, November 2006 [Ní Ógáin O, Tajber L, Corrigan OI, Healy AM]
- “Spray dried porous particles of sugars and sugar-protein composites with application for pulmonary delivery” ExcipientFest Europe meeting, Cork, June 2008 [Ní Ógáin O, Shawqi Barham A, Tajber L, Corrigan OI, Healy AM]

ORAL PRESENTATIONS:

- “Characterisation of nanoporous microparticles of lysozyme and stabilising excipient trehalose, produced using a novel spray-drying method” Socrates European Pharmaceutical Students conference, Athens, July 2006 [Ní Ógáin O, Tajber L, Corrigan OI, Healy AM]
- “Investigations of a novel method for producing nanoporous/nano-particulate microparticles (NPMs) of proteins, sugars and protein-sugar composites.” All-Ireland Pharmacy Schools Conference, Dublin, April 2007 [Ní Ógáin O, Tajber L, Corrigan OI, Healy AM]

ABBREVIATIONS AND SYMBOLS:

A 450 (or other)	absorbance at wavelength = 450 (or other) nm
AA	ammonium acetate
AHC	ammonium hydrogen carbonate
AC	ammonium carbonate
ACI	Andersen cascade impactor
AF	ammonium formate
ANOVA	analysis of variance
AUC	area under the curve
BCA	bincinchonic acid
bp	bulk density
BA	n-butyl acetate
b.p.	boiling point
cAMP	cyclic adenosine monophosphate
Cat	catalase
CD	cyclodextrin
CI	Carr's compressibility index
CM	closed mode configuration of spray-dryer
comp	composite
CPS	counts per second
Δ	difference
D	diffusion coefficient
d_a	aerodynamic diameter
d_p	physical diameter
$d(0.1)/(0.5)/(0.9)$	particle diameter corresponding to the 10 %/50 %/90 % point on the cumulative percent undersize curve
DPI	dry powder inhaler
DM- β -CD	dimethyl- β -cyclodextrin
DSC	differential scanning calorimeter
ECD	effective cut-off diameter
Exo	exothermic
F	bioavailability
FD	freeze-dried
FPF	fine particle fraction
FTIR	fourier transform infrared spectroscopy
GSD	geometric standard deviation
H	enthalpy

HP	high performance cyclone
HP- β -CD	2-hydroxypropyl- β -cyclodextrin
HPLC	high performance liquid chromatography
λ	wavelength
LPP	large porous particles
Lys	lysozyme
(p)MDI	(pressurised) metered dose inhaler
MMAD	mass median aerodynamic diameter
MMAD _e	experimental mass median aerodynamic diameter
m.p.	melting point
MW	molecular weight
N	number of replicates
<i>N/d</i>	not determined
NPMPs	nanoporous/nano-particulate microparticles
O.D.	optical density
OM	open mode configuration of spray-dryer
P	porous
Ph. Eur.	European Pharmacopoeia
PH	minus log of hydrogen ion concentration
PI	isoelectric point
PLGA	poly(lactic-co-glycolic) acid polymer
PVP	polyvinylpyrrolidone
Q	flow rate
R	radius
R ²	correlation coefficient
Raff	raffinose
REG/Reg	regular cyclone
RD	recovered dose
R.H.	relative humidity
r.p.m	revolutions per minute
ρ	particle density
ρ_0	unit density
S	shape factor
SD	spray-dried
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sCT	salmon calcitonin
SE	scanning electron

SEM	scanning electron microscopy
stdev	standard deviation
T	time
T	temperature
τ_D	time required for a droplet to dry
Tg	glass transition
TGA	thermogravimetric analysis
Tm	apparent denaturation temperature
tp	tapped density
Treh	trehalose
Tryp	trypsin
t.s.c.	total solid concentration
Twb	wet bulb temperature
U	units
Unp	unprocessed
USP	United States Pharmacopoeia
v/v	volume in volume
w/v	weight in volume
w/w	weight in weight
XRD	X-ray diffraction (powder)

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ORIGIN AND SCOPE

The inhaled route is widely used to deliver low molecular weight drugs to treat lung disease such as asthma, chronic obstructive pulmonary disease and infections. More recently the potential of the pulmonary route for local and systemic delivery of biological macromolecules has been explored, and this is the focus of this thesis. Systemic delivery of proteins or peptides via inhalation may be a more patient-friendly and cost-effective alternative to the parenteral route. Factors determining successful inhalation therapy include physicochemical aspects of the formulation, biological aspects of the active ingredient(s) and the performance of the delivery device (inhaler/nebuliser). This thesis is concerned with formulation aspects, and in particular dry powder formulation. Dry powder inhalers offer advantages over other delivery systems in terms of stability, and delivery efficiency and are the most popular studied method of protein delivery to the lungs (Agu et al., 2001; Cryan, 2005). Significant formulation challenges exist however, in terms of maintaining adequate protein stability during processing and storage, and ensuring efficient and reproducible delivery to the lungs. Efficient delivery of dry powders to the peripheral lung for systemic delivery, requires aerodynamic diameters of $\sim 1\text{-}3\ \mu\text{m}$ (Lee, 2002a). Spray-drying is a widely applied method for producing fine powders of proteins. Non-reducing sugars or other stabilising excipients may be added to the formulation to improve the process and storage stability of proteins (Lee, 2002a). Excipients may also be employed as carriers in DPI systems, to improve lung delivery either in a passive or an active capacity (Cryan, 2005). Porous particles represent a particle engineering solution to improving lung deposition. They may have smaller aerodynamic diameters than represented by their geometric diameters, as a result of low particle densities due to void spaces. Advantages of porous particles for pulmonary delivery have been extensively described in the literature, principally improved dispersibility and reduced inter-particulate interactions compared to non-porous particles (Edwards et al., 1997; Vanbever et al., 1999; Maa et al., 1999; Hirst et al., 2002; Newhouse et al., 2003).

A one-step spray-drying method for producing nano-porous/nano-particulate microparticles (NPMPs) of low molecular weight drugs was initially described by Tajber (2005). Li (2005), McDonald (2005), and Nolan (2008) subsequently continued this work, developing the method for drugs or excipients. The spray-drying method, involved spray-drying a solution of one or more compounds of interest, with in some cases, a volatile process enhancer (ammonium carbonate) from a co-solvent system consisting of at least two (volatile) solvents. The method represented an advantage over other methods for producing porous particles, in that it was a simple one-step method producing substantially pure microparticles (microparticles with no or only trace amounts of any additional processing excipient). The spray-drying method was successfully used for spray-drying NPMPs of low molecular weight drugs including several thiazide, steroid and sulfa-compound drugs (Tajber 2005, McDonald 2005, Nolan 2008), for excipients including the larger cyclodextrins, and polymer polyvinylpyrrolidone (Li 2005). Dependence of NPMP production on

spray-drying conditions (for example the operational mode of the spray-dryer, inlet drying temperature, drying gas) and feed conditions (for example, solvent selection, co-solvent composition, amount of process enhancer, and total solid concentration) were described and optimal conditions were worked out on a case-by-case basis, with some general trends identified. NPMPs produced were characterised in terms of powder density, surface area, particle size, in-vitro deposition, solubility and stability, and showed favourable characteristics making them suitable for inhaled drug delivery.

The scope of this thesis was:

- To assess the feasibility of the proposed spray-drying method to produce NPMPs of proteins and to examine process and storage stability of the spray-dried protein. To investigate different spray-drying and feed conditions. To investigate particle characteristics that may be relevant when considering inhaler delivery (i.e., particle size, bulk and tapped density, surface area and in-vitro deposition).
- To spray-dry NPMPs of hydrophilic excipients (non-reducing sugars and polyols) and to investigate different spray-drying and feed conditions. Rationale for investigation of hydrophilic excipients include: the literature described use as protein stabilisers, potential use as a carrier NPMP system or in co-spray-dried composite systems with proteins or peptide, and to complement existing knowledge regarding the use of the method with low molecular weight hydrophilic compounds. To investigate process stability and particle characteristics of NPMPs.
- To spray-dry composites of protein:excipient or peptide:excipient at different mass ratios, using spray-drying and feed conditions identified from the earlier studies with proteins and excipients alone. To investigate potential production of composite NPMPs, process and storage stability, and particle characterisation.

CHAPTER 1: INTRODUCTION

1.1 PULMONARY DELIVERY OF PROTEINS AND PEPTIDES

Significant developments in the fields of medicinal chemistry and biotechnology in the last ~ 25 years, have resulted in the discovery of many novel protein and peptide drugs, and enabled production of large quantities of high purity compounds (Patton, 1997; Scheuch et al., 2006). According to a survey by the Pharmaceutical Research and Manufacturers of America in 2006 (PhRMA, 2006) there were 418 biotechnology medicines in development and 125 already FDA approved and available to patients. This sector growth has prompted considerable research in the area of drug delivery of macromolecular drugs.

Protein and peptide drugs are generally poorly absorbed across biological membranes. Reasons for this include their large size, ionisation, high surface charge, and enzymatic and chemical instability (Adjei and Gupta, 1997). The majority of proteins and peptides are thus formulated as parenterals (intravenous, intramuscular and subcutaneous). The parenteral route has several disadvantages however, including patient discomfort, potential high cost (reconstitution, equipment and requirement for health professional input) and the risk of needle-stick injuries (Patton, 1997; Shoyele and Cawthorne 2006). The issue of patient discomfort is not trivial as protein drugs are often used in chronic conditions, requiring frequent administration and this may impact on compliance to the dosage regimen. Less invasive and more patient-acceptable routes have been sought, and the oral, pulmonary, nasal, buccal, transdermal and ocular routes have been investigated. Conventional oral delivery of proteins is extremely challenging, because of the harsh acidic and enzymatic conditions encountered in the digestive system and reduced bioavailability due to the first pass effect. Although research is ongoing, including, for examples, the use of penetration enhancers and enzyme inhibitors, liposomal formulations, and chemical modifications, an oral protein formulation is yet to be developed (Goldberg and Gomez-Orellana, 2003; Shoyele and Cawthorne, 2006; des Ricux et al., 2006). The nasal route has shown some success for the delivery of peptides and vaccines, and several nasal peptide products are on the market including oxytocin, vasopressin analogues, salmon calcitonin and luteinising hormone-releasing hormone analogues (Illum and Fisher, 1997). However, most peptide and protein drugs are not significantly absorbed through the nose without the use of absorption enhancers (Illum and Fisher, 1997). The buccal, transdermal and ocular routes have also shown very limited success for systemic delivery of proteins and peptides and may represent less socially acceptable delivery routes (Adjei and Gupta, 1997; Shoyele and Slowey, 2006). The pulmonary route has emerged as a particularly interesting and viable alternative. Advantages of the pulmonary route include a very large absorptive surface area (~80-140 m²), decreased metabolism and efflux transporter activity

compared to the oral route, avoidance of first pass effect and potential for rapid onset of action (Adjei and Gupta, 1997; Scheuch et al., 2006; Patton, 2004).

The inhaled route is commonly used to deliver low molecular weight drugs to treat local lung disease such as asthma, chronic obstructive pulmonary disease and infections. Recombinant human deoxyribonuclease (rhDNase, with molecular weight ~33 kDa) (Pulmozyme[®]), was the first protein drug approved for lung delivery (Okamoto et al., 2002). Pulmozyme[®] is used to reduce lung sputum viscoelasticity in the treatment of cystic fibrosis (CF) patients (by hydrolysing DNA found in very high concentrations in the pulmonary secretions of these patients) (Roche, 2006). The site of action of inhaled rhDNase is thought to be the airways (local action). Neither short-term nor long-term (24 weeks) exposure to rhDNase aerosols were shown to result in significant elevation of serum concentration of DNase in CF patients (Gonda, 1997). The formulation of Pulmozyme[®] is an unbuffered aqueous solution intended for nebulisation, and is packaged in ampoules of 2500 U (2.5 mg) in a 2.5 ml volume (Roche, 2006). Other examples of proteins or peptides investigated for lung delivery to treat local disease are shown (Table 1.1).

Table 1.1 Examples of candidate macromolecules studied for inhalation for local effects adapted from Cryan (2005), with molecular weight where available, ¹www.sigmaldrich.com, ²Hubbard, 1997, ³Carp et al., 1982, ⁴Bingle and Tetley, 1996, ⁵Maquin et al., 1991.

Peptide/Protein	Condition	Molecular weight
Alpha-1-antitrypsin	Emphysema/ Cystic fibrosis	~52 kDa ²
Alpha1 proteinase inhibitor	Emphysema/ Cystic fibrosis	~ 50 kDa ³
Secretory leukoprotease inhibitor	Emphysema/ Cystic fibrosis	~12 kDa ⁴
Cyclosporin A	Prophylaxis or treatment of rejection following lung transplant	1.2 kDa ¹
Interferon-γ	Cancer/Pneumocystis carinii	~17 kDa ⁵
Interleukin-2	Cancer/Pneumocystis carinii	~15.5 kDa ⁵
Interleukin-1R	Asthma	
Anti-IgE Mab	Asthma	
Muramyl dipeptide	Anti-TB vaccine	492 Da ¹
Catalase	Oxidative stress	~250 kDa ¹
Superoxide dismutase	Oxidative stress	~32 kDa ¹

Numerous studies have shown the feasibility of the pulmonary route for the systemic delivery of proteins and peptides. The first protein for systemic delivery via inhalation to be marketed was insulin (Exubera[®]), for the treatment of type 1 and type 2 diabetics. Exubera[®] insulin was launched in January 2006. The formulation is a dry powder of recombinant human insulin (60%) spray-dried with excipients (mannitol, glycine, sodium citrate, sodium hydroxide) to produce a glassy amorphous material (White et al., 2005). Pfizer withdrew Exubera[®] from the market in October 2007 however, due to quoted failure to meet customer needs or financial expectations (www.exubera.com; Mack, 2007). Other proteins/peptides currently in development for pulmonary

delivery include interleukin-1 for asthma, interferons for multiple sclerosis or hepatitis and calcitonin for osteoporosis (Mansell, 2007). A pulmonary form of calcitonin inhaled by mouth has been proposed to have several advantages compared to the commercially available nasal or subcutaneous formulations, including reduction of side-effects with the potential for patients to adjust the delivered dose (Patton, 2000). The lung bioavailability of a particular protein/peptide will impact on its suitability for systemic delivery by the inhaled route. Higher bioavailability may be advantageous in terms of decreasing cost and possibly increasing reproducibility of the dose delivered to the systemic circulation and of the response (Edwards et al., 1998). The absolute bioavailability (F) is defined as the percentage of the administered dose absorbed intact into the systemic circulation. It is expressed relative to the same dose delivered by intravenous bolus injection (100% bioavailability), and calculated using equation 1.1 (Ashford, 2002). In the context of drug delivery by inhalation, differences in interpretation regarding the delivered dose exist, with some researchers defining bioavailability in terms of the actual administered dose (nominal dose), whereas others account for losses of drug in the inhaler, mouth and throat and define bioavailability in terms of the dose delivered to the lungs only (Edwards et al., 1998). Bioavailability may also be quoted as the relative bioavailability, for example relative to the subcutaneous route instead of the intravenous route. Relative bioavailability may represent an overestimation of bioavailability, but may be useful in comparison studies, particularly in the case where a subcutaneous product is available.

$$F = (AUC/AUC_{iv}) \times (dose_{iv}/dose) \times 100 \quad \text{Equation 1.1}$$

(where AUC is the integrated area under the curve of systemic plasma concentration of the drug with time for a particular dose and AUC_{iv} is the comparable area after intravenous injection of the same drug at $dose_{iv}$).

Examples of macromolecules studied for pulmonary delivery to achieve systemic effects are presented in Table 1.2.

Table 1.2 Examples of candidate macromolecules studied for inhalation to achieve systemic effects, adapted from Cryan (2005) and Shoyele & Slowey (2006) with molecular weight where available. ¹Qiu et al., 1997, ²www.sigmaaldrich.com, ³Martindale (2007), ⁴absolute pulmonary bioavailability (from animal studies) adapted from Patton (2000)

Peptide/Protein	Condition	Molecular weight	Tmax ¹ (hr)	Pulmonary bioavailability ⁴
Salmon Calcitonin Human Calcitonin	Paget's disease, Osteoporosis	~3.5 kDa ²	0.25	17%
Parathyroid hormone	Osteoporosis	~4.1 kDa ¹	0.25	> 20%
Human growth hormone	Growth deficiency	~22 kDa ¹	0.75	9%
Interferon-β	Multiple sclerosis			
Insulin	Diabetes mellitus – type 1 and type 2	~5.8 kDa ²	0.25	57%
Amylin	Diabetes mellitus – type 1	~3.9 kDa ²		
Luteinising hormone releasing hormone analogues	Cancer	~1.1 kDa ¹	1.0	95%
Interferon-α	Viral infection, cancer	~ 19 kDa ¹	6.0	> 56%
Ribavirin	Viral infection	244 Da ³		
Granulocyte colony stimulating factor	Neutropenia	~18.6 kDa ¹	1.5	62%
Erythropoietin	Anemia	~34 kDa ²		
Heparin	Anticoagulation			
dDAVP (1-deaminocysteine-8- D-arginine vasopressin	Diabetes insipidus	~1.2 kDa ¹	0.5	

1.1.1 Methods of Pulmonary Delivery

Factors determining successful inhalation therapy and optimal bioavailability using aerosols include physicochemical aspects of the formulation, biological aspects of the active ingredient(s) and the performance of the inhaler or delivery device, as summarised in Figure 1.1. The formulation of dry powders is the focus of this thesis, thus the formulation factors will be briefly discussed.

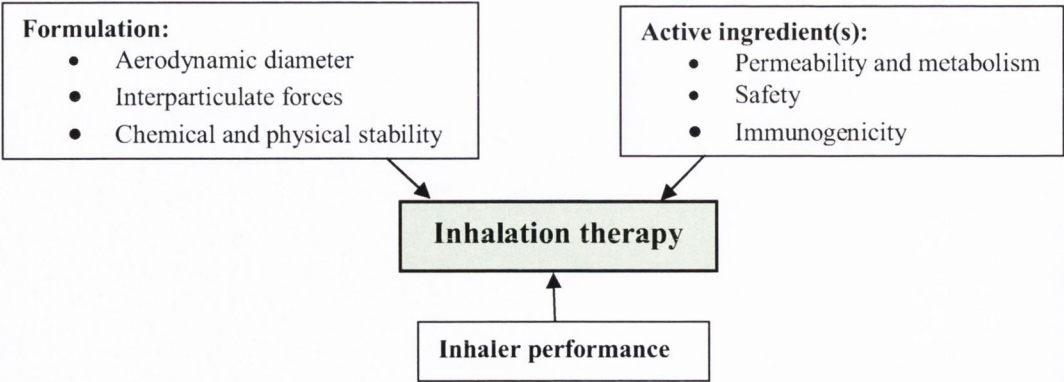


Figure 1.1 Factors determining successful inhalation therapy with dry powders adapted from Okamoto et al., 2002.

Three main delivery systems are used for aerosol delivery - pressurised metered dose inhalers (pMDIs), dry powder inhalers (DPIs) and nebulisers. The devices currently on the market were predominantly developed to achieve local delivery of small molecule drugs to the lungs, and require some modifications so that they can be used for systemic delivery of proteins and peptides. In the case of pMDIs the use of propellants to atomise the drug solution and the large air-liquid interfaces generated during aerosolisation may adversely affect protein stability (Agu et al., 2001). Similarly, the solution state and high shear stress exerted during nebulisation can lead to denaturation of many proteins (Agu et al., 2001). Droplets produced by nebulisation may also have a heterogenous size and resulting poor delivery to the lower respiratory tract (Agu et al., 2001). Modifications of devices including the AERx system (Aradigm) and Respimat (Boehringer), have been developed to minimise these difficulties. Dry powder inhalers offer advantages over pMDI and nebuliser delivery systems in terms of stability (as proteins are generally more stable in the dried state compared to the solution state) and delivery efficiency, and are the most popular studied method of protein delivery to the lungs (Agu et al., 2001; Cryan, 2005). A large selection of passive (breath-driven) and active (power-driven), single or multiple-dose devices are available, to optimise stability and delivery efficiency (Cryan, 2005).

1.2 DRY POWDER FORMULATIONS

1.2.1 Aerodynamic diameter and interparticulate forces

The aerodynamic particle diameter (d_a) is a key determinant of aerosol performance, and unlike the physical particle diameter is influenced by the size, shape and density of the particles. It is defined as the physical diameter of a unit density sphere with the equivalent settling velocity through the air, as the particle, and for roughly spherical particles is described by equation 1.2. (Shoyele and Cawthorne, 2006; Taylor, 2002). This relationship does not hold for non-spherical particles, although it can be modified in some cases by applying a shape factor (s) as in equation 1.3. This shape factor parameter is defined as the ratio of the drag force on a particle to the drag force on the volume equivalent sphere at the same velocity. It has also been found, for elongated or needle-like particles that the aerodynamic diameter is controlled principally by the short axis (Shoyele and Cawthorne, 2006).

$$d_a = d_p \times (\rho/\rho_o)^{0.5} \quad \text{Equation 1.2}$$

$$d_a = d_p \times [(\rho/\rho_o) * 1/s]^{0.5} \quad \text{Equation 1.3}$$

(where d_p is the physical diameter, ρ is the particle density and ρ_o is unit density, s is a shape factor).

Dry powders for inhalation are generally poly-disperse, and particle size distribution may be described by a log normal distribution. The log of the particle diameter is usually plotted against mass (other possibilities are particle number, or surface area) on a linear or probability scale. The experimental mass median aerodynamic diameter (MMAD), which is the particle diameter that has 50% of the aerosol mass above it and 50% below it is read from a cumulative distribution curve. The geometric standard deviation (GSD) describes the size variability and is calculated from the ratio of the particle diameter at the 84.13% point on the cumulative distribution curve to the 15.87% point, as in equation 1.4 (USP, 2008). A mono-dispersed aerosol would have a GSD of 1, and a poly-dispersed aerosol > 1.2 (Labiris and Dolovich, 2003; Codrons et al., 2003).

$$\text{GSD} = [\text{size } 84.13\% / \text{size } 15.87\%]^{0.5} \quad \text{Equation 1.4}$$

1.2.2 Pulmonary deposition

It is generally accepted that particles with an aerodynamic particle size of 1-5 μm are required to avoid deposition in the mouth or throat and reach the lungs. Larger particles (aerodynamic diameters $> \sim 6 \mu\text{m}$) may impact in the mouth or throat and be swallowed, and smaller particles ($< 1 \mu\text{m}$ but greater than a few hundred micrometers) may be exhaled on the next breath unless they are aggregated, or sufficient time is available to allow migration to the lung walls (for example by slow

deep breathing and breath holding) (Lee, 2002a; Edwards et al., 1998; Chow et al. 2007). In order to understand the nature of particle deposition in the lungs, the structure of the upper and lower respiratory tracts components should be considered as their shapes and dimensions affect the deposition profiles of particles (Figure 1.2). The lower respiratory system may involve ~23 levels of branching. These are sometimes classified in terms of generation numbers as in Figure 1.2 (Annapragada et al., 1997). Surface areas increase markedly in the lower alveolar areas compared to the airways. The three main physical mechanisms and contributing forces leading to deposition of aerosol particles in the lungs are inertial impaction, sedimentation by gravity, and brownian diffusion (Labiris and Dolovich, 2003) (Figure 1.2). The best areas to target the lungs for absorption are not completely understood (Patton and Byron, 2007). Small peptides and insulin appear to be best absorbed in the lowest stages (higher generation number) (Patton et al., 1997; Patton and Byron, 2007). Particles with aerodynamic diameters of 1-3 μm may thus be considered optimal, because they are sufficiently small to reach the deep lung and alveoli.

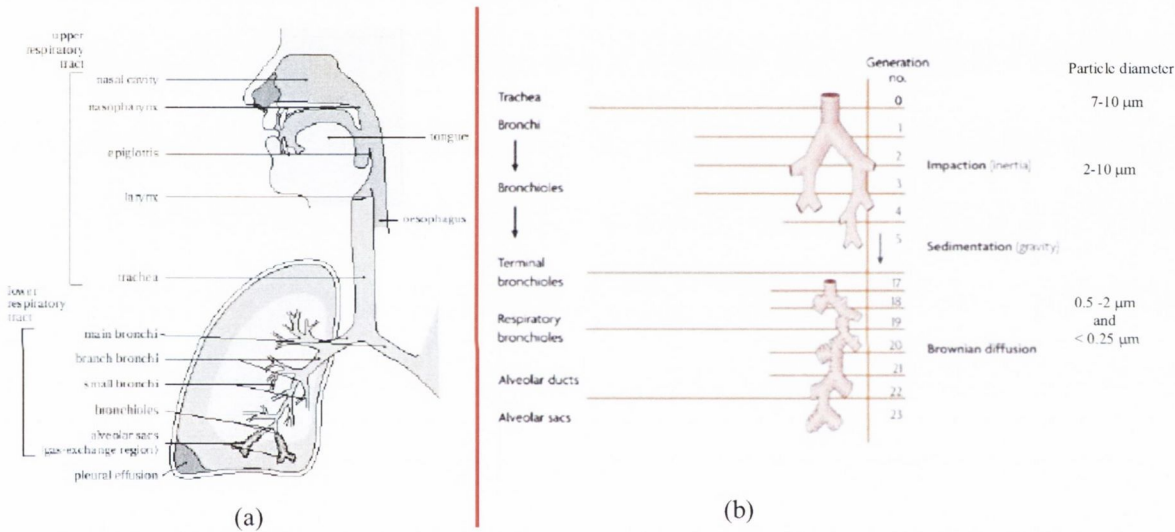


Figure 1.2 Schematic showing (a) structure of pulmonary system, (Hussain et al., 2004) and (b) branching in the lower respiratory tract according to the Weibel model with the primary mechanism of particle deposition occurring (adapted from Annapragada et al., 1997 and Patton and Byron, 2007).

1.2.3 Porous particles

Low aerodynamic diameters can be achieved by the use of physically small particles, porous particles, elongated particles, and particles of rough surface (to increase the drag force on the particles thus reducing settling velocity) (Chan, 2006). This can be seen from examination of equation 1.3 as decreasing d_p , ρ or increasing the shape factor s , will all lead to an increased d_a .

Physically small particles have the disadvantage of strong interparticulate cohesive forces and poor dispersibility, however, and may also exhibit poorer flow properties than larger particles (French et al., 1996; Li et al., 1996; Zeng et al. 2001). Forces involved in interparticulate interactions include van der Waals forces, electrostatic forces, capillary forces and mechanical interlocking (Zeng et al., 2001). The relative and overall magnitude of forces involved for a particular powder varies, depending on many complex factors including the nature of the drug and particle factors including size, shape and surface energetics. Direct experimental determination of aggregate strength is technically difficult. The following general conclusions have been made, however, based on theoretical analyses of particle surface energy of fine powders: aggregate strength decreases with increasing geometric diameter, low powder bulk density gives rise to loose and weak aggregates, decreased particle surface energy promotes dispersion, and particles with an irregular surface have a smaller aggregate strength than a smooth particle of the same geometric diameter, because of reduced contact area and reduced interparticulate forces (Chow et al., 2007).

Porous particles represent a particle engineering solution to improving lung deposition and potentially bioavailability of dry powder drugs. They have low aerodynamic diameters as a result of low particle densities due to void spaces. Advantages of porous particles have been extensively described in the literature, principally improved dispersibility and reduced inter-particulate interactions compared to non-porous particles. A further possible advantage to using large porous particles may be a decreased phagocytic clearance in the lungs and subsequent increased bioavailability (Edwards et al., 1997). Porous particles have been produced by a number of different processes including double solvent emulsion evaporation methods, spray-drying methods including spray-drying emulsions with pore-forming agents, spray-freeze drying and supercritical fluid processing. Proprietary methods include the PulmoSphere™ (Nektar Therapeutics) and Acusphere (Acusphere Inc.), both methods involving spray-drying. A selection of studies showing the advantages of porous particles and these methods will be briefly discussed.

Large porous particles (LPPs) produced by a double emulsification solvent evaporation method were described and characterised by Edwards et al. (1997, 1998). Particles containing insulin, or testosterone were produced by encapsulation in a biodegradable copolymer (poly(lactic-co-glycolic) acid polymer) using a double emulsification solvent evaporation method (to produce large porous particles) or a single emulsification solvent evaporation method (to produce small non-

porous particles), with similar aerodynamic diameters. [The double emulsification solvent evaporation method involved the following – an aqueous solution was emulsified with a 50:50 PLGA solution in methylene chloride on ice. This emulsion was poured into 1% aqueous polyvinyl alcohol solution, homogenised and stirred for 3 hours. Microspheres were collected by centrifugation, washed with water, and freeze-dried into a free-flowing powder (Edwards et al. 1997).]. Large porous particles had low densities ($< 0.4 \text{ g/cm}^3$) and large mean geometric diameters ($> 5 \text{ }\mu\text{m}$) and resulted in higher respirable fraction, than small non-porous particles. These effects were observed despite similarity of aerodynamic diameters, and were attributed to the smaller surface-to-volume ratio of the large porous particles, leading to less aggregation and hence improved dispersibility. Edwards et al. (1997) also showed a prolonged action (~ 96 hours) for large porous particles containing insulin, and postulated that this was due to the larger geometric size of the particles avoiding phagocytic clearance from the lungs.

Vanbever et al. (1999) prepared large porous particles of combinations of excipients which were generally regarded as safe (lactose and dipalmitoylphosphatidylcholine (DPPC)) and drug (insulin, albuterol or estradiol) by a standard spray-drying process. Water soluble excipients and/or drug were dissolved in an aqueous component and water insoluble excipients and/or drug were dissolved in 95% EtOH, and combined to give an overall 87% EtOH feed material with a total solid content of 0.1% w/v. Formulation and spray-drying parameters were varied and found to have an affect on the overall particle morphology. Increasing DPPC concentration resulted in increase in volume diameter and decrease in density of particles, potentially as a result of the surface activity of DPPC and its tendency to adsorb on the droplet surface (Chow et al., 2007). Particles produced had geometric diameters of $3 - 15 \text{ }\mu\text{m}$ and tapped densities of $0.04 - 0.6 \text{ g/cm}^3$. Particles with high porosity, large geometric size and theoretical MMAD values (calculated from the geometric diameter and tapped density) of $1 - 3 \text{ }\mu\text{m}$ showed excellent emitted dose of $\sim 96\%$ when aerosolised from a Spinhaler[®] device, and respirable fractions of $49 - 92\%$ depending on the method of analysis (Andersen cascade impactor or Aerosizer data respectively).

The PulmoSphere[™] process is also currently used to produce porous particles with low density for use in pMDI or DPI. Production involves two steps, firstly preparation of an emulsion of fluorocarbon (for example, perfluorooctyl bromide) in water, stabilised by phospholipid (for example, distearoylphosphatidylcholine), with the drug either dissolved or dispersed in the external aqueous phase which may also contain excipients, followed by spray-drying. The fluorocarbon acts as a pore-forming agent. Porous particles produced are hollow, with geometric diameters $< 5 \text{ }\mu\text{m}$, and low tapped densities ($< 0.1 \text{ g/cm}^3$) (Hirst et al., 2002). Excellent aerosolisation efficiencies have been described in-vitro, with high respirable fractions of $> 60\%$ (Dellamary et al., 2000; Duddu et al., 2002). Promising in-vivo results in healthy humans have also been reported, for example, Newhouse et al. (2003) reported a nine-fold improved lung deposition of tobramycin

PulmoSpheres delivered from a passive DPI compared to the commercial nebulised product over a 15-minute administration period. Duddu et al. (2002) also showed that in-vivo delivery of a budesonide PulmoSphere formulation was more efficient and more reproducible (postulated to be due to lower dependence on patient airflow) compared to standard micronised drug.

Straub et al. (2005) also used a spray-drying method to produce porous PLGA microparticles as a preliminary step in the production of AI-700 (investigated as an intravenously administered ultrasound contrast agent). These studies involved spray-drying an emulsion of PLGA and DPPC in methylene chloride, and ammonium bicarbonate (pore-forming agent) in water. This approach has been used in Acusphere Inc. patented porous microparticle technology, HDDS (hydrophobic drug delivery system) and PDDS (pulmonary drug delivery system).

Maa et al. (1999) produced porous particles by spray-freeze drying and compared their aerodynamic properties (fine particle fraction via multi-stage liquid impinger) to non-porous dimpled spherical particles produced by conventional spray-drying. The authors concluded that the high surface area low density porous particles demonstrated a significantly higher fine particle fraction compared to non-porous particles, and attributed this to better aerodynamic properties.

Non-porous particles with corrugated or rough surfaces have similarly shown improvements over non-porous smooth spherical particles in terms of fine particle fraction and in-vitro deposition. Chew and Chan (2001) investigated this for spray-dried bovine serum albumin. Particles were similar in terms of chemical entity, and non-surface morphology related solid-state characteristics including particle size distribution, water content, and amorphous nature. Significantly higher fine particle fraction was measured for the corrugated particles compared to the smooth spherical particles. Less dependence of fine particle fraction on flow rate was also observed for the corrugated particles. These data imply that the corrugated particles exhibited less cohesion and greater dispersibility, and this was postulated to be due to a lower true surface area of contact between particles.

1.3 CHEMICAL AND PHYSICAL PROPERTIES OF PEPTIDE AND PROTEIN DRUGS

Proteins and peptides are polymers of the twenty common amino acids, or chemical modifications or derivatives of these. Several amino acids have ionisable functional groups in the side chains. The ionisation of these groups as a function of changes in the solution pH is responsible for the overall charge on a given peptide or protein. Most proteins or peptides have a net charge of zero at some pH, called the isoelectric point (Porter, 1997). Proteins are generally larger than peptides. The distinction between them may be based on the fact that physical chemical behaviour (for example, solubility) of peptides is generally predicted from the behaviour of the constituent amino acids, whereas the properties of the larger proteins also depend on three-dimensional conformational effects (Porter, 1997). Four levels of structure may be described for proteins (Stryer, 1995):

- Primary structure refers to the amino acid sequence.
- Secondary structure refers to the spatial arrangement of neighbouring amino acid residues in a sequence, which may constitute a periodic structure for example the α -helix and β -strand.
- Tertiary structure refers to the spatial arrangement of amino acids that are far apart in the linear sequence and to the pattern of disulfide bonds.
- Quaternary structure refers to the spatial arrangement of subunits and the nature of their contacts, found in some proteins.

Protein drugs differ from low molecular weight drugs in that they can undergo various degradation reactions at the level of primary structure (chemical degradation) or higher order structures (physical degradation). Chemical degradation reactions may include deamidation and peptide bond isomerisation, reaction of disulfides and thiols, proteolysis and hydrolysis, and oxidation. Physical degradation may involve partial unfolding, aggregation and precipitation. Complete unfolding is rarely seen, unless, for example all disulfide bonds in a protein are broken. Proteins can be unfolded by environmental changes such as in solution pH, addition of a strong hydrogen bond disrupting (chaotropic) agent, addition of surface active agents or by heating. Unfolding can be reversible or irreversible, resulting in recovery of native state and activity or permanent damage and loss of activity. Reversal of denaturation may be observed for most small proteins, if the environmental changes are done reversibly and the primary structure of the protein is not damaged by the process. Reversal of denaturation is generally not as successful for larger proteins however (Porter, 1997).

1.4 SPRAY-DRYING

Spray-drying has several advantages over other powder processing techniques, particularly for the production of dry powders of proteins and protein-exipient composites. It is a well-established and well-studied method in the pharmaceutical, food, chemical and other industries, with industrial application since the 1920s. Requirements of final product, capacity and safety differ between industries and products, and spray-drying has been extensively adapted to satisfy these requirements (Masters, 1991; Maa and Prestrelski, 2000). Regarding proteins, it is a realistic and commercially used (for example in the case of Exubera[®] insulin) alternative to air-jet milling, which is most commonly used to produce micron sized particles of low molecular weight drugs for pulmonary delivery. Air-jet milling is generally unsuitable for protein processing due to physical instability (Lee, 2002a). Freeze-drying, which is the most established drying process used for proteins, results in poor particle size and shape control and would generally require a further size reduction step to ensure suitable particle characteristics for pulmonary use (Maa and Prestrelski, 2000). Another advantage of spray-drying is that it can be used to form composites of proteins with stabilising excipients or carriers (Lee, 2002a). Other newer techniques such as spray-freeze drying, and supercritical fluid processing have also shown promising results for production of protein particles in suitable size ranges, but these techniques are yet to be established commercially for drying protein pharmaceuticals.

Spray-drying is a one step atomisation/drying technique that involves rapid conversion of a liquid feed to a particulate product. The ability to control particle size by careful selection of spray-drying and feed conditions has been well documented. However, the control of particle morphology (shape, surface and internal structure characteristics) has been studied more recently (Maa and Prestrelski, 2000; Vehring, 2007).

1.4.1 Overview of spray-drying process

The physical flow of a simple spray-drying process is shown in Figure 1.3, and proceeds as follows: the liquid feed (1) is heated (2) and atomised (3) as it passes through a nozzle, and mixes with heated drying gas in the drying chamber (4) and the solvent is evaporated. Powder mixed with gas passes through to the cyclone separator (5), where they are separated. The separated powder is collected in the collecting vessel (6). Gases and very fine powder pass to the filter, where fines are retained (7), and gases are removed to atmosphere through an exhaust (8). This configuration describes an open mode system. In the case where explosive/flammable solvents or materials are used or where an explosive reaction of the dried product on contact with drying medium within the spray-dryer is possible, an inert drying gas is used instead of air and spray-drying is carried out in a closed cycle. In closed mode drying, drying gas passes through a cooler unit, where solvent condenses and is collected, and drying gas is recycled.

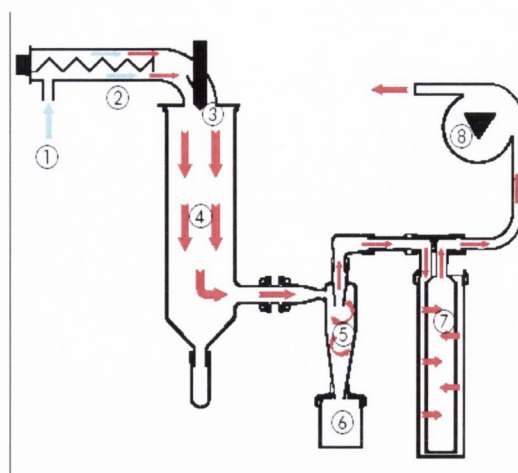


Figure 1.3 The physical flow of a typical co-current laboratory bench-top spray-dryer operating in the open mode (Büchi, 2003).

The complete spray-drying process can be divided into four stages as described by Masters, (1991). These include atomisation of the feed material, spray-gas contact, drying of spray materials and separation of dried product from the drying medium. Each stage of the drying process may influence the final particle and stability characteristics.

1.4.1.1 Atomisation

The atomisation stage creates a spray for optimum evaporation and is achieved by the use of a rotary atomiser, pressure nozzle or two-fluid nozzle, depending on the nature of the feed and final product requirements (Masters, 1991). The spray-dryer utilised in this work is equipped with a two-fluid nozzle, which is most common in small scale and pilot scale spray-dryers. In this type of nozzle the feed and drying gas are passed separately to the nozzle head, where high air velocities break up the feed into a spray of fine droplets. Adjustment of the air flow controls the degree of atomisation at constant feed rate. The viscosity of the feed may affect the particle size of the droplets and subsequent dried particles with medium coarse powders produced from high viscosity feeds, and fine powders (may be agglomerated) produced from low viscosity feeds. The dimensions of the nozzle may also affect the particle size (Elversson et al., 2003).

1.4.1.2 Spray-gas contact

Spray-gas contact occurs in the drying chamber and can be co-current (spray and drying gas are introduced to the drying chamber in the same direction), counter-current (spray and drying gas are introduced from and in opposite directions) or mixed arrangement. For heat sensitive materials such as pharmaceuticals the co-current arrangement is generally employed as it imparts a lower thermal load to the particles (Masters, 1991).

1.4.1.3 Droplet drying

The drying of the droplet involves two phases (1) a constant drying rate period (2) a steadily decreasing drying rate period (Masters, 1991; Lee, 2002a). During the first period, as evaporation takes place, moisture/solvent diffuses from within the droplet and maintains the saturated surface conditions and the temperature of the surface is equivalent to the wet bulb temperature (T_{wb}), which is considerably lower than the inlet or outlet drying temperature. As the drying continues, critical moisture/solvent content is reached and this becomes insufficient to diffuse to the surface and maintain saturated conditions. This may coincide with a shell forming at the droplet surface. Evaporation is controlled by diffusion through this outer shell, which becomes thicker as drying continues (as such the drying rate decreases) and the product experiences an increase in temperature above this T_{wb} . Energy is required for vaporization and so the temperature does not reach the temperature of the surrounding gas until all the free moisture/solvent has been removed (Masters, 1991; Lee, 2002a). Maa et al. (1997) showed that the temperature at 3 different locations in the drying chamber (5, 17, 33 cm below the nozzle) of a Büchi-190 spray-dryer corresponded most closely to the outlet drying temperature, and that this outlet drying temperature (rather than the inlet drying temperature) is the most useful parameter for determining droplet drying speed. Morphology of particles may appear as solid or hollow spheres or irregular in shape including deviations from sphericity for examples dimples, holes, wrinkled or collapsed particles, depending on the nature of the shell formed and the rate of droplet drying. Tsapis et al. (2002) described two characteristic times as critical in the drying process leading to particles different morphology – the time required for a droplet to dry (τ_d), and the time required for a solute or a nano-particle to diffuse from the edge of the droplet to its centre (R^2/D , where R = radius of the droplet and D is the solute or nano-particle diffusion coefficient). A Peclet number (Pe) was defined as the ratio of these characteristic times as $Pe = R^2/(\tau_d D)$.

1.4.1.4 Separation and collection

Separation of dried product from the drying gas occurs at the end of the process, once drying is completed. Primary separation may take place in the drying chamber followed by separation equipment - a cyclone as a dry collector (powder) and a wet scrubber (gas/evaporated moisture/solvent), or alternatively the total separation takes place in the separation equipment. In the benchtop spray-dryer the second system is used. Separation occurs in the cyclone by means of inertial deposition of the particles from the gas stream. The powder and gas enter the cyclone tangentially, producing centrifugal forces which greatly exceed the gravitational force. The particles move towards the wall and the base of the cyclone, driven by the centrifugal force, while the gas spirals upwards to the gas outlet at the top of the cyclone. The dimensions of the cyclone will impact on the efficiency of this separation (Brandenberger, 2003).

1.4.2 Spray-drying of proteins and associated stresses

Selection of spray-drying and feed conditions can affect the residual moisture content of the powders, and the process and storage stabilities of the protein (Lee, 2002a). Residual moisture content of spray-dried powders is determined by the nature of the spray-dried material and the interaction of water with the protein or excipients and by the relative humidity of the drying conditions (as an equilibrium exists between the moisture content of the powders and the environmental humidity) (Maa and Prestrelski 2000). Spray-dried protein powders generally have higher residual moisture contents (~7%) than freeze-dried powders (~3%), owing to the much shorter drying times (Maa et al., 1998). Residual moisture may be decreased by increasing the outlet drying temperature but this may pose an unacceptable thermal stress to the protein. Alternatively secondary vacuum drying can be performed post spray-drying (Maa et al., 1998). Increased residual moisture may negatively influence the storage stability. It has been found in spray-drying experiments of oxyhaemoglobin/sucrose and lactate dehydrogenase/trehalose that high inlet and outlet temperatures can reduce the process stability but increase storage stability of the protein, possibly as a result of decreased residual moisture (Adler and Lee, 1999; Labrude et al., 1989).

Spray-drying of an aqueous solution of protein without excipients can lead to structural instability, aggregation or ultimately inactivation for many proteins. Stresses potentially encountered by proteins during the spray-drying process include shear, thermal, and air-liquid interfacial stresses (Figure 1.4). Significant thermal denaturation of proteins is not generally anticipated during spray-drying because the temperature encountered by the droplet is significantly lower than the inlet drying temperature, and drying contact times are short (Lee, 2002a). Many proteins for example β -galactosidase (Maa et al., 1997), oxyhaemoglobin (Labrude et al., 1989), insulin (Stahl et al., 2002) and lactate dehydrogenase (Adler and Lee, 1999) have shown increased aggregation with increased inlet temperature however, and as such use of a low inlet temperature may be prudent (Maa and Prestrelski, 2000). Air-liquid interfacial stresses may be encountered during the atomisation stage of spray-drying. Proteins are amphiphilic polyelectrolytes and as such may adsorb to surfaces or interfaces. This can lead to unfolding as the protein orients hydrophobic amino acids towards the non-aqueous (air) side (Tzannis and Prestrelski, 1999). Several studies have identified the air-liquid interface as a cause of aggregation, for example human growth hormone, bovine serum albumin, lactate dehydrogenase, β -galactosidase, while other proteins, for example rhDNase, may be less affected by this stress (Maa and Prestrelski, 2000).

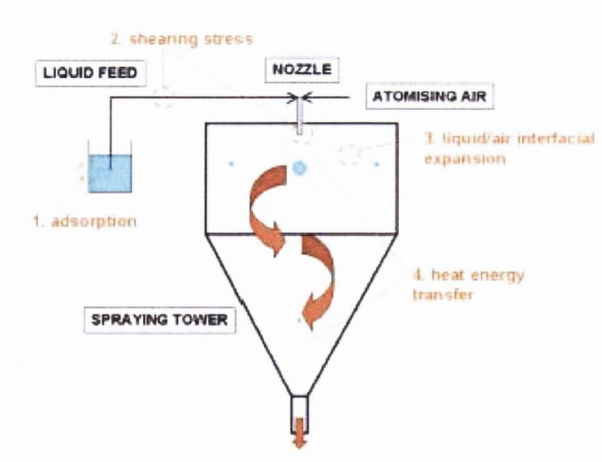


Figure 1.4 A summary schematic of the possible stresses experienced by a protein/peptide during the spray-drying process from Lee (2002b).

1.4.2.1 Use of excipients in protein formulation

The use of excipients to stabilise proteins during drying was initially adopted from freeze-drying data, and has shown considerable success also in spray-drying experiments (Lee, 2002a). Sugars (particularly disaccharides), polyols, amino acids or organic salts have been used to protect native protein structure during drying processes, including spray-drying, and also on storage. Two hypotheses are described for the mechanism of stabilisation – the glassy immobilisation hypothesis and the water replacement hypothesis. The glassy stabilisation hypothesis states that stabilisation is achieved by formation of an amorphous glass during drying. In the glassy state, structural changes are significantly delayed and only minor molecular motions for example rotational motions of side chains and vibrations occur (Lee, 2002a). Glass stabilisation was introduced to spray-dried microparticles commercially by Nektar therapeutics, under the trademark Pulmosol[®], and forms the basis of the Exubera insulin spray-dried product. In the water replacement hypothesis, stabilisation is attributed to the formation of H-bonding between the sensitive component and the excipient, when water is removed, thus maintaining the structural integrity of proteins, and membranes. An amorphous state may be important to allow maximal H-bonding between the excipient and protein (Arakawa et al., 2001).

Lactose, sucrose, trehalose and raffinose have been investigated as examples of stabilising sugars in spray-drying and other process drying experiments and against specific experimentally-induced stresses for example liquid-solid interfacial stress (Lee, 2002a; Liao et al., 2002, Wendorf et al., 2004). Sucrose, trehalose and raffinose are non-reducing sugars and as such have the advantage that they will not undergo the Maillard browning reaction with proteins. These sugars tend to form amorphous glasses on spray-drying, and have been shown to stabilise proteins during processing and also on storage below the T_g of the formulation. Sucrose has a lower glass transition temperature (~ 74 °C), compared to either trehalose or raffinose however (Lee, 2002a). Mannitol is the polyol most commonly studied for stabilisation of proteins in solution and in the dried state

(Singh and Singh, 2003; Lee, 2002a). Its ability to stabilise proteins in the dried state may be inversely related to its tendency to crystallise however (Izutsu et al., 1993, 1994). Fully or partially amorphous forms of mannitol may be present in certain formulations depending on the type and ratio of other excipients and on the method of preparation (Yu et al. 1998, Kim et al. 1998). Poyols may also improve dispersibility of powders due to low hygroscopicity. The large cyclodextrin – hydroxypropyl- β -cyclodextrin (HP- β -CD) has also been investigated as a stabiliser of proteins or peptides in solution and in the dried state, and during spray-drying (Irie and Uekama, 1999, Branchu et al., 1999). The cyclodextrin may interact locally with hydrophobic side-chains of the protein/peptide and inhibit intermolecular association of proteins/peptides. Cyclodextrins are further interesting as excipients because of the reported application of some as transmucosal penetration enhancers (DM- β -CD) (Schipper et al., 1993; Hussain et al., 2003), and release modifiers from PLGA microparticles prepared by spray-drying, due to complexation with proteins (HP- β -CD) (De Rosa et al., 2005). No one excipient represents all desirable attributes and as such an anticipated dry powder protein formulation may include a combination of excipients.

Stabilising effects depend on the selection of an optimal concentration of appropriate excipient(s). Sub-optimal excipient concentration(s) may not adequately stabilise the protein, while excessive excipient can lead to destabilisation (Tzannis and Prestrelski, 1999). Excipients may also be employed as carriers in DPI systems, to improve lung delivery either in a passive or an active capacity (Cryan, 2005). Regulatory approval for all excipients intended for lung delivery is a consideration. Lactose is licensed for pulmonary applications, but it is not intended to enter the lungs, and its size is large enough to be deposited in the oropharyngeal region (Cryan, 2005). Mannitol has also been approved for use in pulmonary formulation, as a component of the Exubera formulation (White, 2005).

1.4.3 Background to the spray-drying method used in this thesis

Initial work on developing the spray-drying method for producing nanoporous/nano-particulate microparticles (NPMPs) was carried out by Tajber (2005). NPMPs of budesonide, bendroflumethazide or sulphadimidine were produced by spray-drying a solution feed of the compound with the process enhancer ammonium carbonate (AC) in ethanol/water co-solvent mixes (Tajber, 2005). Li (2005), Mc Donald (2005), and Nolan (2008) subsequently continued this work, developing the method for drugs or excipients, using different solvent combinations and with or without the process enhancer, and characterising NPMPs produced in terms of powder density, surface area, particle size, in-vitro deposition, solubility and stability. The spray-drying method was successfully used for spray-drying NPMPs of small molecule drugs including several thiazide, steroid and sulfa-compound drugs (Tajber 2005, McDonald 2005, Nolan 2008) for excipients including the larger cyclodextrins, and polymers chitosan, and polyvinylpyrrolidone (PVP) (Li 2005). A dependence of NPMP production on a number of factors including spray-drying

conditions (for examples the operational mode of the spray-dryer, inlet drying temperature, drying gas) and feed conditions (for examples solvent selection, co-solvent composition, amount of process enhancer, and total solid concentration) was noted and optimal conditions were worked out on a case by case basis, with some general trends identified. One such trend is the hydrophilicity/hydrophobicity of a compound, which aids solvent selection. The hydrophobic sulfa and steroid compounds were spray-dried as NPMPs from alcohol/water systems, whereas the hydrophilic HP- β -CD, and PVP were spray-dried as NPMPs from methanol/butyl acetate systems. NPMPs produced were found to have excellent in-vitro deposition compared to non-porous spray-dried samples, high surface areas, and low bulk and tapped densities making them suitable for oral inhalation (Nolan, 2006). Not all drugs and excipients that were studied, have been successfully formulated as NPMPs to date. Where relevant, details of previous research are referenced in the text of this thesis.

Based on results from these previous studies, a hypothesis was proposed for the formation of NPMPs. This hypothesis assumes that the more volatile solvent of the co-solvent mix should be a good solvent for the drug, and the less volatile solvent a poor solvent for the drug (Corrigan, 2005).

The evaporation rate of a solvent will depend on its vapour pressure at the processing temperature, the boiling point, specific heat, enthalpy and heat of vaporisation of the solvent, the rate of heat supply, the degree of association between solvent molecules and between solvent and solute molecules, the surface tension of the liquid, the rate of air movement above the liquid surface, and humidity of air surrounding the liquid surface (Wypych, 2001).

During the atomisation stage of the spray-drying process, droplets are formed containing the drug/excipient in the co-solvent mix, rapid drying of these droplets proceeds on contact with the warm drying gas and the more volatile solvent phase may evaporate to a greater extent, resulting in the droplet becoming richer in the less volatile solvent component. The fall in solubility of the drug/excipient may be dramatic and they may condense out initially as a nano-sized liquid phase within the droplet. The drug becomes in effect the dispersed phase in an external phase. As drying proceeds and further solvent loss occurs, the drug phase droplets become less fluid and come closer together and they will need to fall below the T_g as or before they touch to avoid complete fusion. The hypothesis excludes a role for the process enhancer AC, except that it may cause a salting out effect and as such lower solubility (Corrigan, 2005). Alternatively the salt may have an effect on the relative volatility of solvents in a co-solvent mix. The effect of a salt may be relevant as when the salt is readily soluble in one constituent of a mixture but not in another, the volatility of the constituent in which it is soluble is decreased and the other constituent is unaffected. This issue may be further complicated in the case of the process enhancer ammonium carbonate however, as

the salt is expected to be volatilised (volatilisation temperature 59 °C) during the spray-drying process.

Another issue that may be pertinent when considering this proposed mechanistic hypothesis is where two solvents form an azeotropic mix. Ethanol and water form a well-known example of a positive azeotrope, comprised of 95.6% w/w EtOH, 4.4% w/w water (azeotropic ratio), where ethanol boils at 78.4 °C, water boils at 100 °C, and the azeotrope boils at 78.1 °C. Azeotropes are generally described in terms of distillation experiments. Data from such experiments cannot be directly related to the very fast drying in the droplet seen in the spray-drying process, but would agree with the hypothesis that the droplet becomes richer in the less volatile water component as drying proceeds for ethanol/water ratios at least for all EtOH/H₂O ratios below the azeotropic ratio. Examination of literature data on azeotropes of other solvent combinations, used to produce NPMPs, shows that methanol does not form an azeotrope with water. It does however form a positive azeotrope with methyl acetate and ethyl acetate (www.chemeng.ed.ac.uk). No information was available on formation of azeotropes with butyl acetate in the literature.

Spray-drying of macromolecular biological compounds (proteins/peptides) using the novel method has not been attempted previously. These compounds differ from the compounds previously examined in their large size and higher order structure. They can undergo a variety of degradation reactions at the level of the primary structure and higher order structures. One major consideration is the fact that the proposed spray-drying method involves the use of organic solvents. This raises concerns regarding the solubility and stability of proteins

Considering solubility, hydrophilic proteins generally exhibit very poor solubility in alcohols and most non-aqueous solvents, with the exception of glycerol and DMSO (Stevenson, 2000). Solubility for some proteins (for examples lysozyme, insulin, myoglobin), particularly in MeOH, but also in other organic solvents, can be increased by freeze-drying a protein from aqueous solution at a pH remote from its isoelectric point, or alternatively by complexation with neutral polymers (Bromberg and Klibanov, 1995; Chin et al., 1994).

Regarding stability concerns, alcohols have been used to both denature proteins and increase the structural stability (by stabilisation of α -helical structures). Alcohols may also alter the ratio of secondary structure or induce non-native conformations that may be reversible or irreversible depending on the protein and the conditions. Peptides are usually less adversely affected by non-aqueous conditions than proteins (Stevenson, 2000). It has been found that formulation in neat organic solvents (non-aqueous) conditions may increase the thermo-stability of protein, but even a small amount of water in the system may reverse this effect (Volkin et al., 1991). Bromberg and Klibanov (1995) reported no loss of activity of lysozyme on dissolution in MeOH or EtOH, and

concluded that no irreversible damage was caused to the protein. The use of EtOH/aqueous systems with anti-solvent precipitation have been investigated in supercritical fluid processing of proteins, with good retention of biological activity of several different proteins, and significant loss of activity of others (Jovanovic et al., 2004).

1.5 BACKGROUND TO PROTEINS/PEPTIDE USED

1.5.1 LYSOZYME

Lysozyme is a globular, compact protein composed of 129 amino acids, arranged as a single polypeptide chain, and crosslinked with 4 disulfide bridges. It has a molecular weight of ~14.3 kDa and an isoelectric point (pI) of 11.35 and is active over a broad pH range (6.0 to 9.0). Lysozyme preferentially hydrolyses the β -1, 4 glycosidic linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues (NAM-NAG) in peptidoglycan and between *N*-acetyl-D-glucosamine residues in chitodextrin. Peptidoglycan residues are particularly present in the cell wall of certain micro-organisms, notably gram-positive bacterial cells making them most susceptible to this hydrolysis. This is exploited as the basis of a convenient activity assay, in which lysis of *Micrococcus lysodeikticus* cells by the enzyme, is measured by a turbidimetric rate determination method (www.sigmaaldrich.com, Shugar, 1952). Lysozyme is a commercially valuable protein, used as a food additive in milk products, for extraction of bacterial intracellular products, as a component in artificial saliva products and in a variety of other oral preparations to treat minor painful viral infections e.g. Herpes zoster (Ghosh and Cui, 2000; Martindale, 2007). It is also widely used in research as a model protein for biochemical and biophysical studies (Ghosh and Cui, 2000).

1.5.2 TRYPSIN

Trypsin is comprised of a single chain polypeptide of 223 amino acid residues. It is a member of serine protease S1 family, is activated from trypsinogen (proenzyme form, 229 amino acids) and produced in the pancreas. It has molecular weight of 23.5 kDa and pI 10.1-10.5 and is reversibly denatured at high pH (>11). This native form of trypsin is referred to as β -trypsin. Trypsin can self-digest (autolysis) and β -trypsin is cleaved at Lys131-Ser132 in the bovine sequence and results in α -trypsin – two chains held together by disulfide bridges. Autolysis is slow in solutions of 1 mM HCl (pH 3), stored at -20 °C, and is further retarded by the presence of Ca^{2+} (20 mM) (www.sigmaaldrich.com). Trypsin will cleave peptides at the C-terminal side of lysine and arginine residues and will also hydrolyse ester and amide linkages of synthetic derivatives of amino acids, for example benzoyl L-arginine ethyl ester (BAEE), which is the basis of the enzymatic activity assay. The pH for optimal activity is 7-9.6 (www.sigmaaldrich.com).

1.5.3 CATALASE

Catalase from bovine liver is a large protein with MW ~250 kDa and pI 5.4. It is composed of 4 identical subunits, each containing iron bound to a protoheme IX group. Catalase catalyses the destruction of H_2O_2 , by two reaction pathways: catalytic reaction and peroxidative reaction, which is exploited as a basis for the activity assay. It acts as a natural antioxidant. It is soluble ~1 mg/ml in 50 mM potassium phosphate buffer at pH 7 (www.sigmaaldrich.com).

1.5.4 SALMON CALCITONIN

Calcitonins are peptide hormones involved in the complex system of calcium regulation, found in the thyroid glands of mammals or the ultimobranchial gland in amphibians. Salmon calcitonin (sCT) has a MW of ~3.5 kDa, shares ~ 50% homology with human derived calcitonin (hCT), and has more potent biological activity. In common with the other calcitonins, SCT has 32 amino acids and the following features: - a disulfide bridge between cysteine residues (position 1 and 7), forming a seven amino acid residue ring at the N-terminal, with a free amino group and a proline amide group at the C terminal. These features are considered essential for activity (Azria, 1989). Salmon calcitonin has a higher pI (10.4) than human calcitonin (8.7), which may be responsible for the increased solution stability of sCT around neutral pH compared to hCT (Baudys et al., 1996). Potency, expressed in terms of international units (IU), is defined as the quantity of calcitonin which will produce a fall in blood calcium level after 1 hour when given to young rats compared to an international reference standard. Potency for sCT is ~6000 IU/mg. SCT has very high solubility in water of ~100 mg/ml, and in 70% EtOH ~50 mg/ml (Stevenson and Tan, 2000).

Salmon calcitonin is currently formulated as a sterile solution for intramuscular or subcutaneous injection or as a nasal spray (Miacalcic[®]). The bioavailability of the nasal formulation compared to the parenteral route varies from ~2-15%, and the product is given as a once daily dose of 200 IU (~ 0.167 µg) (Novartis, 2007). Bioavailability of salmon calcitonin via the pulmonary route has been assessed in animal and in human studies. Rat studies have shown an absolute bioavailability of 17% following intratracheal administration (Patton et al., 1994). Bioavailability in humans (n=10) of a dry powder formulation of sCT showed relative bioavailability compared to the intramuscular route of ~ 28%, and a bioactivity of ~ 66% (Deftos et al., 1997).

1.6 BACKGROUND TO MAIN EXCIPIENTS USED

Excipients were selected based on literature use (in spray-drying or other drying processes) as stabilisers of proteins and peptides (Section 1.4.2.1, and Chapters 4, 5). Other desirable aspects of the excipients were, in the case of trehalose and raffinose and hydroxypropyl- β -cyclodextrin - a high T_g (to maximise physical stability on storage) and methanol solubility (to dissolve in the proposed MeOH:BA solvent system of the spray-drying method, for hydrophilic compounds), and in the case of mannitol and myo-inositol a non-hygrosopic character.

1.6.1 TREHALOSE (DIHYDRATE)

Trehalose is a disaccharide comprised of two glucose molecules connected via an α,α -1,1 linkage (Figure 1.5). It is a non-reducing sugar, which can be explained by examination of its structure (the reducing end of one glucosyl residue is connected with the other). It does not therefore react with the amino acids of proteins to cause the Maillard browning reaction. It is widely found in nature, where it is used as a source of energy and as a stabiliser of membranes and other macromolecular assemblies under extreme environmental conditions (for example dryness, freezing and osmopressure). It has a high thermo-stability and wide pH-stability range. No degradation was observed when 4% trehalose solutions at pH 3.5-10 were heated at 100 °C for 24 hours (Higashiyama, 2002). It exists in the crystalline form as a stable dihydrate. Trehalose undergoes phase transitions, e.g. moisture-induced transition from an amorphous to crystalline phase, or from crystalline to amorphous or α -anhydrous crystalline form during fast or slow dehydration, respectively (Kilburn et al., 2006). The melting point of the dihydrate is quoted as 95 – 97 °C (fusion enthalpy 57.8 kJ mol⁻¹), although there is debate regarding its thermal behaviour in the literature, with some evidence of transformation to the anhydrous form which has melting point of ~203/210 °C (fusion enthalpy 53.4 kJ mol⁻¹) (Higashiyama, 2002; Pharmaceutical Excipients, 2006). Dry amorphous trehalose undergoes a glass transition (T_g) at ~118 - 120 °C (Higashiyama, 2002). Trehalose is soluble in water (68.9 g/100ml H₂O at 20 °C), and slightly soluble in 95% EtOH (Higashiyama, 2002; Pharmaceutical Excipients, 2006).

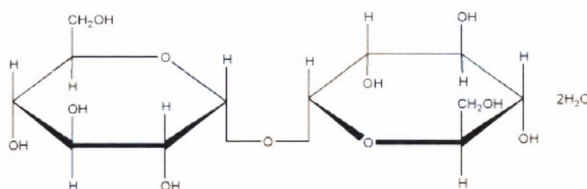


Figure 1.5 Schematic structure of trehalose dihydrate

1.6.2 RAFFINOSE (PENTAHYDRATE)

Raffinose is a non-reducing trisaccharide, comprised of the monosaccharide moieties galactose, glucose and fructose (Figure 1.6). It is found frequently naturally occurring in plants. It commonly exists as a pentahydrate crystal but there is evidence that it may form lower hydrates (tri-, tetra-) as

metastable crystalline forms on dehydration/rehydration of pentahydrate crystalline or amorphous forms respectively. No evidence of existence of an anhydrous crystalline form has been found although an anhydrous crystalline methanol solvate form has been produced by recrystallisation from a saturated methanol solution at 50 °C (Hogan and Buckton, 2001, Saleki-Gerhardt et al., 1995). The glass transition temperature of the dry amorphous form has been determined from DSC experiments to be 109 °C (heating scan rate of 5 °C/min) (Moura Ramos et al., 2005), 116 °C (heating scan rate 2 °C/min) (Miller and Lechuga-Ballesteros, 2006) and 103 °C (heating scan rate 20 °C/min) (Saleki-Gerhardt et al., 1995). The Tg decreases with increasing water content and is reportedly equal to ~25 °C at about 43% RH, and ~11 °C at about 53% RH at which recrystallisation begins to be detected from XRD (Miller and Lechuga-Ballesteros, 2006). Heating of the pentahydrate to ~80 °C leads to dehydration. This has sometimes been reported as the melting temperature of the pentahydrate (Moura Ramos et al., 2005; Saleki-Gerhardt et al., 1995). Solubility of raffinose has been reported as 1 in 10 in MeOH, 1 in 7.1 in water, and slightly soluble in EtOH (95%) (Pharmaceutical Excipients, 2006).

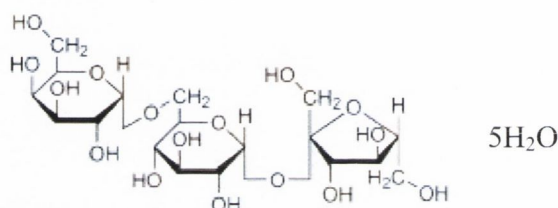


Figure 1.6 Schematic structure of raffinose pentahydrate.

1.6.3 MANNITOL

Mannitol is a polyol (Figure 1.7), used in pharmaceutical formulations, particularly as a diluent in tablet formulations and in lyophilized preparations, and has also been investigated to enhance dispersibility in DPIs (Cryan, 2005). It has an advantage over sugars in that it is non-hygroscopic, but it can have a plasticising effect in a co-spray-dried system due to a low Tg of ~13 °C, and melting temperature of ~167 °C (Pharmaceutical Excipients, 2006). Mannitol has been shown to reduce the glass transition temperature when freeze dried with various disaccharides including trehalose, compared with the disaccharide alone, in a manner well predicted by the Gordon-Taylor equation (Kim et al., 1998). It is chemically stable and does not undergo hydrolysis at either high or low pH, nor does it undergo the Maillard browning reaction with proteins.

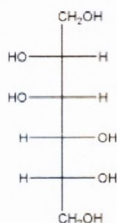


Figure 1.7 Schematic structure of mannitol.

1.6.4 MYO-INOSITOL

Myo-inositol is a low-hygroscopic polyol (Figure 1.8). It has a melting point of 225 °C (Watt et al., 2004). No information on experimentally derived glass transition temperature could be found in the literature. Mosén (2003) spray-dried myo-inositol, and found it to be crystalline. Sievers et al. (2007) investigated myo-inositol as a stabilizer during drying with a CO₂-Assisted nebulisation with a bubble dryer (CAN-BD) process and described myo-inositol based powders as amorphous by XRD, with a residual moisture content of < 0.5%. Other components dried with myo-inositol however included gelatin, arginine-HCl, alanine, histidine, lactalbumin hydrolysate and tricine, which likely influenced the solid state of the powder.

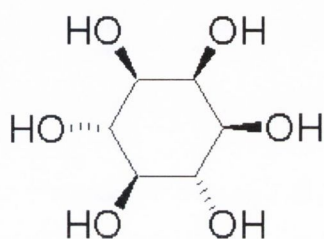


Figure 1.8 Schematic structure of myo-inositol

1.6.5 2-HYDROXYPROPYL- β -CYCLODEXTRIN (HP- β -CD)

Cyclodextrins (CDs) are a family of cyclic oligosaccharides comprising either 6 (α -CD), 7 (β -CD) or 8 (γ -CD) glucopyranose units, linked by α 1-4 glycosidic bonds. Cyclodextrins have been described as nearly perfectly circular in shape, approximating a truncated cylindrical cone, with the primary hydroxyl groups at the C6 positions outlining the narrower rim of the cone, and secondary hydroxyl groups at the C2 and C3 positions outlining the wider rim of the cone. The interior walls are outlined by the hydrophobic hydrogen atoms at the C3 and C5 positions, and thus a relatively hydrophobic micro-environment exists in the inner core (Yong et al., 2008; Davis and Brewster, 2004) (Figure 1.9). Chemical modifications of CDs are by etherification or substitution of other functional groups at the C2-, C3-, and C6- hydroxyl groups of glucose residues. Pharmaceutical uses of CDs include solubility, bioavailability or stability enhancements, odour/taste masking and prevention of admixture incompatibilities (Davis and Brewster, 2004). The mechanism of solubility enhancement of lipophilic drugs may be by inclusion complexation, within the more hydrophobic cavity of the cyclodextrin.

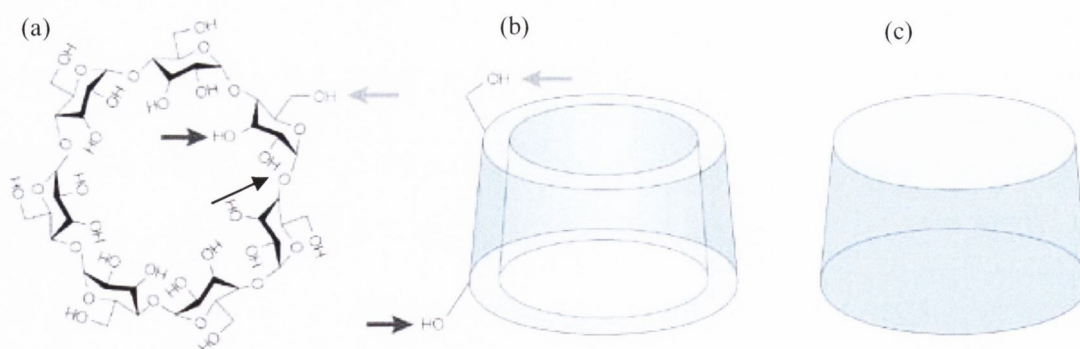


Figure 1.9 Schematic of β -CD showing (a) arrangement of glucopyranose units, with arrows marking hydroxyl groups at the C2-, C3- and C6- positions (b), (c) showing truncated cylindrical cone shape of the molecule (Davis and Brewster, 2004).

2-Hydroxypropyl- β -cyclodextrin (HP- β -CD) is modified from the naturally occurring cyclodextrin β -CD through substitution of hydroxypropyl groups. Modification improves the water solubility and the toxicity profile of the cyclodextrin. It is produced by treating a base-solubilised solution of β -CD with propylene oxide. Different degrees of substitutions are possible, with the pharmaceutical grade normally averaging ~ 0.65 hydroxypropyl groups per glucose residue. (The sample used in this thesis has an average degree of substitution of 0.67). Hydroxypropyl groups may occur at different positions in batches of HP- β -CD with similar degrees of substitution, depending on the reaction conditions. This may impact on the physicochemical properties, including complexation abilities, and contribute to differences seen in HP- β -CD from different suppliers or even between batches from the same supplier (Loftsson and Duchêne, 2007).

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

	Supplier	Product no.	Lot
Acetic acid glacial	Riedel-de-Haën, Germany	27225	-
Ammonium Acetate	Fluka, U.S.A.	17836	1180445
Ammonium Carbonate	Sigma, Ireland	A8045	092K0113
Ammonium Formate	Fisher, U.K.	A15080126	0086558
Ammonium Hydrogen Carbonate	Rectapur – Prolabo, France	21218.298	90164
Ammonium Persulfate	Sigma, Ireland	A3678	98H0750
N _α -Benzoyl-L-arginine ethylester hydrochloride	Fluka, Switzerland	12880	12222932
Brilliant Blue R (Coomassie Blue)	Sigma, Ireland	B7920	073K3789
2-Butanol	Riedel-de-Haën, Germany	33066	S290500
n-Butyl Acetate, extra pure	Merck, Germany	-	-
Calcitonin (Salmon) Ph Eur (as Calcitonin acetate; 5149 IU/mg)	Polypeptide, Denmark	-	204029-01
Catalase	Sigma, Ireland	C1345	035K7037 036K7008
Compressed Air	Haug Kompressoren, Switzerland	-	-
Coumarin-6, laser grade	Polysciences, Inc., U.S.A.	8037L	543680
Ethanol	Solvent stores – Cooley distillery, Ireland	-	-
Glycerol (sterile)	5 Prime, Inc. U.S.A.	-	IJ172A
Glycine	Sigma, Ireland	G8898	055K0189
Hydrochloric Acid 37%	Riedel-de-Haën, Germany	07102	021090
32% extra pure	Riedel-de-Haën, Germany	07155	S070694
Hydrogen Peroxide solution 30%	Merck, Germany	-	K35043897539
Hydroxy-propyl-β-cyclodextrin	Sigma, Ireland	C0926	044K1766
Inulin	Sigma, Ireland	I2255	49F7175
Lysozyme dialysed lyophilised powder, ~50,000 units/mg.	Sigma, Ireland	L7651 salt-free L6876 ~5% salts as sodium acetate, sodium chloride	103K7044 016K1189 094K1454 016K1189
d-Mannitol	Sigma, Ireland	M9546	042K0071

2-Mercaptoethanol	Sigma, Ireland	M7154	45H05081
Methanol	Lab Scan Analytical Sciences, Ireland	-	-
Methanol, HPLC grade	Lab Scan Analytical Sciences, Ireland	-	-
Micro BCA protein assay kit	Pierce Biotechnology Inc., U.S.A.	23235	G198670 HD103662
<i>Micrococcus lysodeikticus</i>	Sigma, Ireland	M3770	51K8613
Myo-inositol	Sigma-Aldrich, Ireland	I5125	106K0019
liquid Nitrogen	Air products, Ireland	-	-
Nitrogen gas (high purity, oxygen free)	BOC Gases, Ireland	-	-
Polyvinylpyrrolidone PVP-k15 – Ave. MW 10,000	Aldrich, U.S.A.	81390	1199370
Polyvinylpyrrolidone PVP-40 – Ave. MW 40,000	Sigma, Ireland	PVP-40	18H0044
Potassium Bromide - Spectranal	Sigma Aldrich, Ireland Riedel de Haën, Germany	22,186-4 34919	S44777-487 30190
Potassium Dihydrogen Phosphate	Merck, Germany	-	A651773524
Potassium Hydroxide pellets	Merck, Germany	-	B545632 432
Prestained protein marker, broad range, premixed for SDS-PAGE	Cell Signaling Technology, U.S.A.	7720S	33-8
Protogel Ultra Pure 30% w/v Acrylamide : 0.8% w/v Bis	National Diagnostics, U.K.	-	-
Sigma 7-9 (Tris[hydroxymethyl]aminomethane)	Sigma, Ireland	T1378	87H5407
Silica gel self-indicating with moisture indicator (blue gel)	Avonchem, U.K. Merck, Germany	A76655-N -	3858-162 K35894825 622
Sodium Bromide	Fluka, U.S.A.	71329	440459/1
Sodium Chloride	Aldrich, Ireland	43,320-9	U01861
Sodium Dihydrogen Phosphate anhydrous	Riedel de Haën, Germany	04270	21760
Sodium Hydroxide pellets	Riedel de Haën, Germany	30620	60500
Sodium Dodecyl Sulphate	Sigma, Ireland	L3771	043K0013 116K0039
Sulforhodamine 101, free acid	Fluka, U.S.A.	86184	1300985
d-Raffinose Pentahydrate	Aldrich, U.S.A.	20,667-9 R0250	LO 02324BI 08K0072
d-(+)-Trehalose Dihydrate	Sigma, Ireland	18,835-2 T9531	00519 Batch1 112K7029 Batch2 076K650 Batch3

		T5251	16H3816 Batch4
N, N, N,'N,' Tetramethylethylenediamine (TEMED)	Sigma, Ireland	T9281	112K0586
Trypsin from Bovine Pancreas lyophilised powder, salt free ~9000 units/mg	Fluka, U.S.A.	93610	S37763-396 S32904-146
Water grade 1, 2	Purite Prestige Analyst	-	-

2.2 METHODS

2.2.1 SPRAY-DRYING

A Büchi mini spray-dryer B-290 (Büchi Labortechnik AG, Switzerland) was used to spray-dry all systems. Spray-drying was carried out using either the open suck mode (negative pressure) or closed blowing mode (positive pressure) configuration. An inert loop B-295 accessory (Büchi Laboratories-Technik AG, Switzerland) was attached and used for systems spray-dried in the closed mode configuration. The drying gas used, was either compressed air (oil-free) supplied by an attached compressor unit (Haug Kompressoren AG, Switzerland) or nitrogen gas (BOC gases, Ireland) supplied by an attached cylinder in the open mode, or exclusively nitrogen gas in the closed mode. Either a regular cyclone separator or a high performance (high efficiency) cyclone separator was used, as indicated in Appendix 1. The regular cyclone may be limited in its capacity to collect particles smaller than 2 μm leading to product losses into the filter. The high performance cyclone has a smaller diameter than the regular cyclone (Figure 2.1), leading to better separation and collection of smaller particles, and thus resulting in a higher collectable yield. Spray-drying with a high performance cyclone may result in a slightly lower outlet temperature and a higher pressure drop through the cyclone, measured during spray-drying (Brandenberger, 2003). A nozzle tip of 0.7 mm and nozzle screw cap of diameter 1.5 mm were used, unless indicated otherwise in the text or in Appendix 1.

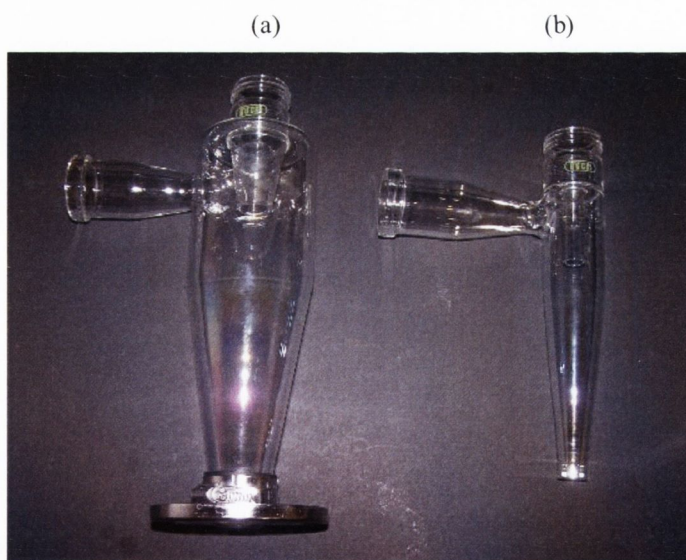


Figure 2.1 Photographs of (a) regular cyclone, (b) high performance cyclone used

All conditions used for spray-drying are listed in Appendix 1. The B-290 spray-dryer allows the inlet drying temperature to be controlled, the outlet drying temperature however is dependant on a combination of settings and is measured. Systems were spray-dried as solutions or suspensions as indicated, from single or mixed solvent systems. Ammonium carbonate (AC), ammonium

hydrogen carbonate (AHC), ammonium formate (AF), or ammonium acetate (AA) were included in some of the systems and the quoted % represents the % of total solid content which was the ammonium salt. Systems which were spray-dried from a mixture of alcohol and de-ionised water are described in terms of the % alcohol content (volume %) e.g., 80% EtOH represents 80% EtOH, 20% H₂O.

Feed solutions/suspension for spray-drying, were prepared using the following order of addition of components, depending on the particular system: In the protein/excipient systems spray-dried from alcohol/water/ammonium salt systems, the protein with or without excipient was first dissolved in the water component with stirring using a magnetic stirrer, followed by addition of the ammonium salt, before addition, with stirring, of the alcohol component. Samples spray-dried from methanol and n-butyl acetate (MeOH:BA) systems involved first dissolving the protein/excipient in the MeOH component with stirring, followed by addition of the BA component. Samples spray-dried from MeOH:BA:H₂O systems involved first dissolving the protein/excipient in the H₂O component, followed by addition of the MeOH component with stirring, before addition of the BA component. Samples which were spray-dried as suspensions (on visual inspection) were occasionally stirred during the spray-drying process, and a nozzle cleaning setting of 2 or 3 (with the higher setting allowing cleaning at shorter intervals) was used, depending on the nature of the precipitate - fine or coarse respectively. Spray-dried product was collected from the collecting vessel of the spray-dryer using a small synthetic bristle brush. A static gun (Zerostat 3; Aldrich) or a static controller (Stat Attack; Static Solutions Ltd., MA, USA) were used as needed, depending on the static nature of the powder. Collected samples were stored in amber or clear glass screw cap jars or clear glass sample tubes in a sealed desiccator containing silica gel, at either 4 °C (sugars) or -20 °C (proteins/protein-excipient composites), until further analysis. The collectable product yield (expressed as%) was calculated as (mass of product collected/mass of product spray-dried – mass of AC/AHC if applicable)*100. Collectable product yield % was not calculated for systems including AF/AA due to the likelihood of residual contamination by the process enhancer because of higher decomposition temperatures, and is expressed as amount of powder collected. Collectable product yield was also not calculated for systems requiring early stoppage of spray-drying due to nozzle blockage or other technical difficulty.

2.2.2 FREEZE-DRYING

Aqueous solutions (10 mg/ml – lysozyme/trypsin; 20 mg/ml myo-inositol) were prepared and adjusted to the desired pH value using 0.1 M NaOH or HCl as required. Solutions were placed in 40 ml or 75 ml flasks (VirTis Ltd, NY, USA) to a maximum half flask capacity as instructed by the manufacturer, and flash frozen by immersion and swirling in liquid nitrogen. Freeze-drying was

carried out in a single step cycle using a VirTis Benchtop 6k freeze-dryer (VirTis Ltd, NY, USA) at an initial temperature of $\sim -80^{\circ}\text{C}$, and vacuum ~ 100 mTorr for a suitable time period (generally 24-48 hours). Freeze drying was deemed to be complete when no moisture was evident on the outside of the flasks and the product appeared to be dry, as per manufacturer's instructions. Freeze-dried product was collected and stored in amber glass screw cap jars in a sealed desiccator, at -20°C (protein) or 4°C (myo-inositol). The collected product yield % was calculated as (mass of product collected/mass of product freeze dried)*100.

2.2.3 SCANNING ELECTRON MICROSCOPY (SEM)

Powder samples were visualised using a Hitachi S-4300 field emission scanning electron microscope (Hitachi Scientific Instruments Ltd., Japan). Samples were initially fixed on aluminium stubs using double-sided adhesive tabs. Samples were then sputter-coated with gold. Samples were visualised at 5 kV. SEM micrographs were taken from at least 2 different areas of sample, and at different magnifications. Mean particle diameter was estimated in some cases from SEM micrographs. Particle diameters were measured manually from micrograph printouts and sized using the provided scale.

2.2.4 THERMAL ANALYSIS

2.2.4.1 Differential scanning calorimetry (DSC)

Differential scanning calorimetry measurements were carried out under nitrogen purge using a Mettler Toledo DSC 821^e (Mettler Toledo Ltd., U.K.) calorimeter. Samples (approximately 4 - 10 mg) were accurately weighed using a Mettler MT50 microbalance into 40 μl aluminium pans, which were hermetically sealed with a lid with three vent-holes. A heating rate of $10^{\circ}\text{C}/\text{min}$ and temperature range of $25 - 200^{\circ}\text{C}$ or $25 - 300^{\circ}\text{C}$, was used for all measurements not requiring a heating cooling step. In some cases a heating/cooling step was carried out either to eliminate overlapping solvent evaporation effects, and involved heating to 100°C followed by cooling to 70°C before heating to $200/300^{\circ}\text{C}$ as previously, or to check the reversibility of a thermal event by heating above, cooling below and reheating through the thermal event, using a heating rate of $10^{\circ}\text{C}/\text{min}$. Mettler Toledo STAR^e software was used for capture and analysis of data.

2.2.4.2 Thermogravimetric analysis (TGA)

Residual moisture/solvent in the powder samples was calculated using TGA, from the measured mass loss as a function of temperature. TGA measurements were carried out on a sample under nitrogen gas purge, using a Mettler TG50 module with attached Mettler MT5 balance (Mettler Toledo Ltd., U.K.). Samples (approximately 4 - 12 mg) were accurately weighed, using a Mettler

MT50 microbalance, into 40 µl aluminium pans or lids. Aluminium pans or lids were left open for the duration of the analysis. A heating rate of 10 °C/min and temperature range of 25-200/300 °C, was used for all TGA experiments. Mettler Toledo STAR^c software was used for capture and analysis of data.

2.2.4.3 Moisture uptake (raffinose)

Powder samples (approximately 4 - 10 mg) were weighed into 40 µl aluminium pans and placed on the upper balance of the Mettler MT50 microbalance of the Mettler TG50 module and the increase in weight measured for 30 minutes. The ambient temperature and relative humidity were measured using a thermohygrometer (CJH clocks, UK). Samples were run at least in duplicate, and followed by TGA analysis from 25-200/300 °C. To investigate the effect of sample size, a larger volume powder (approximately 100 mg) was placed on a plastic dish on the balance and the process was repeated. Following the experiment the powder sample was collected and stored in a desiccator in a cold room (temperature 2-8 °C), prior to XRD and SEM analysis.

2.2.5 POWDER X-RAY DIFFRACTION (XRD)

The crystalline/amorphous nature of the powder was ascertained by powder XRD, using either a Siemens D-500 diffractometer or a Philips XRD. In the case of the Siemens XRD, this diffractometer consisted of a DACO MP wide-range goniometer with a 1.0° dispersion slit, a 1.0° anti-scatter slit and a 0.15° receiving slit. The Cu anode X-ray tube was operated at 40 kV and 30 mA in combination with a Ni filter to give monochromatic Cu K α X-rays. The Philips XRD consisted of a PW1050/80 goniometer with a Cu fine focus tube (1.5 kW) and 1.0° dispersion slit, a 1.0° anti-scatter slit and a 0.2° receiving slit, operated at 40 kV and 20 mA. Powder samples were analysed in a low background small volume sample mounts, of cavities 0.2 mm deep and 0.4 mm diameter. Measurements in all cases were taken from 5° to 40° on a theta 2 scale at a step size of 0.05° per second for qualitative analysis. Data were captured using XRD Commander software (Siemens D-500) or HBX Hiltonbrooks XRD Link (Philips XRD).

2.2.6 PARTICLE SIZE ANALYSIS

Measurements of particle size distributions were undertaken using a laser diffraction particle sizer - Mastersizer 2000 (Malvern Instruments, UK). Dry powders were dispersed using a Scirocco dry powder feeder attachment, using 2 - 3 bar pressure setting and vibration feed rate of 50 - 80% as indicated for each sample, to achieve obscuration within the limits of 0.5% - 6%. Refractive and absorption indices were chosen appropriate for the material measured. These indices were chosen such as to optimise the fit of the model based on the Mie theory, and minimise weighted residuals. These are listed in the table below. Samples were run in triplicate, unless otherwise indicated. Results are expressed as the particle diameter at 10% - d(0.1), 50% - median volume diameter or d(0.5) and 90% - d(0.9) of the cumulative volume distribution. The shape of the distribution was also considered, - being symmetrical, skewed, mono-modal, bimodal etc. The span of the volume distribution, (a measure of the width of the distribution relative to the median diameter), was calculated by the software according to the equation: Span = [d(0.9) - d(0.1)]/d(0.5) (Maa et al., 1997), with a large span indicative of a heterogeneous size distribution.

Table 2.1 Refractive and absorption indices of materials used in particle sizing determinations

Material	Refractive Index	Absorption Index
Trehalose	1.538	0.01
Raffinose	1.538	0.01
Lysozyme	1.5	1
Lysozyme:Trehalose (1:1)	1.5	0.1

2.2.7 BULK AND TAPPED DENSITY MEASUREMENTS

The bulk density (bp) was calculated by determining the weight of powder required to occupy a 1 ml volume in a graduated glass syringe (Lennox Laboratory supplies, Dublin 12, Ireland), by pouring under gravity. The tapped density (tp) of the powders was determined by vertically tapping this sample onto a level bench-top surface from a height of 5 cm x 100 times. The tapped density was calculated as the ratio of the mass to the tapped volume of the sample. Measurements were performed in triplicate unless indicated, and are expressed as an average ± standard deviation. Carr's Index (CI)% values were calculated, according to the equation:

$$\text{Carr's Index (CI)\%} = [(tp - bp)/tp] \times 100$$

This simple index can be used to give an indication of the powder flow (Table 2.2) (de Villiers, 2005).

Table 2.2 CI values indicative of powder flow behaviour (de Villiers, 2005).

Carr's Index (%)	Powder flowability
5-15	excellent
12-16	good
18-21	fair to passable
23-25	poor
33-38	very poor
>40	extremely poor

2.2.8 SURFACE AREA ANALYSIS

Surface area analyses were performed using a Micromeritics Gemini 2370 analyser, with nitrogen gas (BOC) as the analytical (adsorptive) gas and helium (BOC) as the reference gas for free space measurements, as required. Prior to analysis, samples were degassed under nitrogen gas, using a Micromeritics FlowPrep 060 degasser for 24 hours at 25 °C, to remove residual moisture and contaminants. Sample weights measured before and after degassing were recorded. These mild degassing conditions were chosen because of the strong dependence of physical stability of particles containing sugars, on temperature and relative humidity, and were similar to degassing conditions (25 °C for 16 hours) used by Maa et al. (1999). SEM analysis taken after degassing ensured that particle morphology was unaffected by the degassing process. Free space measurements (particularly when the measured surface area was < 25 m²/g) were performed using glass beads of negligible surface area in the reference tube. The number of glass beads used was such that approximate volume balance was achieved, i.e., the measured free space was between -0.5 and + 0.5 cm³. In cases where the measured surface area was ≤ 1.0 m²/g, filler rods with bulb tubes were used to optimise accuracy and BET fit. Saturation pressure was determined periodically between analyses. The evacuation conditions used were as follows: rate of 500 mmHg/min, time 1 minute. Equilibration time for adsorption was 10 s. The amounts of nitrogen gas adsorbed at the relative pressures of 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3, were determined and the BET multipoint surface area was calculated. Where it improved the correlation coefficient for the BET plot, the first or last points were omitted. All analyses were performed in duplicate and are expressed as the average and range, unless indicated otherwise.

2.2.9 AERODYNAMIC ASSESSMENT OF FINE PARTICLES

Aerodynamic assessment of fine particles was carried out using apparatus D (Andersen cascade impactor) of the European Pharmacopoeia 6.0 (Ph. Eur. 6.0). This is an 8-stage device as shown in Figure 2.2 (a). Each stage contains a series of holes, and stages are assembled in order of decreasing diameter holes, with a collection plate or filter arranged under each stage. Cascade impactors classify particles on the basis of aerodynamic diameters. The aerosol passes through each stage, particles having enough inertia will impact on that particular stage collection plate, while smaller particles will follow the stream lines and pass to the next impaction stage (Figure 2.2

(b)) (Copley, 2007; USP, 2008). The effective cut-off diameter of each stage is the minimum size of the particle that will be retained on the collection stage and associated plate. The standard Andersen cascade impactor is designed for use at a flow rate of 28.3 l/min. Modified configurations for operation at higher flow rates are also available. Effective cut-off diameters of stages will depend on the particular flow rate used (Copley, 2007).

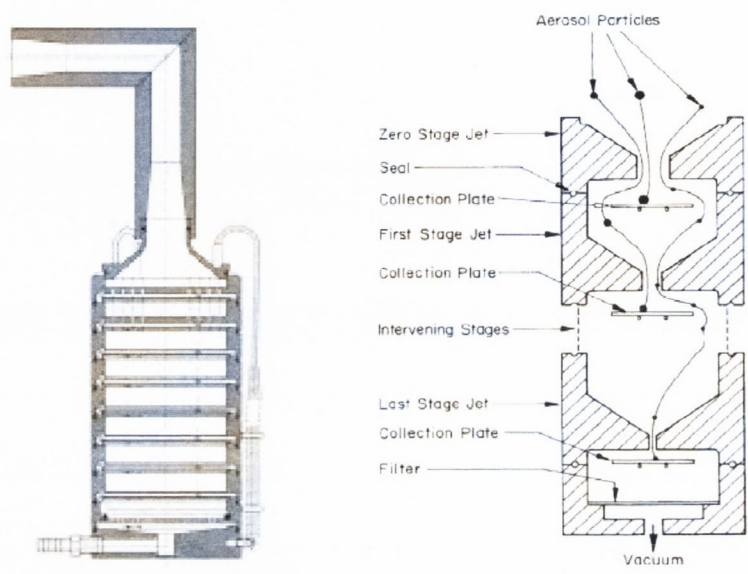


Figure 2.2 (a) Assembly of an Andersen cascade impactor, from Ph. Eur. (6.0), (b) schematic representation of the principle of operation of a cascade impactor, from USP (2008)

A modified 60 l/min Andersen cascade impactor (ACI) apparatus (Copley Scientific Ltd. UK) was used. A size 3 hard gelatin capsule (Farillon Ltd., U.K.) was filled with approximately 25 mg powder, and placed in a Handihaler device (Boehringer Ingelheim, Germany). The pre-separator of the ACI was not used, as the particle size was $< 5 \mu\text{m}$ (as estimated from SEM/particle sizing). The preseparator may be omitted depending on the product characteristics (Ph. Eur. 6.0), and is used to collect large masses of non-inhalable powder (USP, 2008). Collection plates were uncoated. A pressure drop of 4 kPa was established over the Handihaler device using a Critical Flow Controller Model TPK (Copley Scientific Ltd. UK) and sample collection tube. Flow rate stability was ensured by measuring the absolute pressure on either side of the flow control valve (p_2, p_3), with a ratio of $p_3/p_2 \leq 0.5$ indicating sonic flow. The flow rate, Q , required to produce a pressure drop of 4 kPa, was measured by attaching a flow meter model DFM2 (Copley Scientific Ltd. UK) in place of the inhaler. A 4 l inspiration volume was achieved by setting the timer so that $t = [4 \cdot 60 / Q] \text{ s}$. Two actuations were used to empty the capsule from the Handihaler device, as per the inhaler manufacturer's instructions (Boehringer Ingelheim; USP, 2008). Effective cut-off diameters (ECD) of the stages of the ACI, at a given flow rate (Q), were calculated using the equation: $\text{ECD}_Q = \text{ECD}_{Q_{60}} (Q_{60}/Q)^{0.5}$, where: ECD_Q = effective cut-off diameter at flow rate Q , and with standard effective cut-off diameters at $Q = 60 \text{ l/min}$ (Q_{60}) as detailed by Copley (2007). The average measured flow produced by the Handihaler device arrangement used was $\sim 50 \text{ l/min}$. Table

2.3 gives the ECD_{Q60} and ECD_{Q50} (μm) for the stages of the ACI. Minor variations in flow rate may have been the result of small inter-run differences in inhaler performance or equipment set-up including the seal between the inhaler and the mouthpiece of the impactor.

Table 2.3 Effective cut-off diameters (μm) for stages of the ACI modified 60 l/min apparatus operated at a flow rate of 60 l/min (Copley, 2007), and calculated effective cut-off diameters (μm) for stages of the same apparatus operated at a flow rate of 50 L/min

Stage of impactor	ECD_{Q60} (μm)	ECD_{Q50} (μm)
Stage – 1	9.0	9.86
Stage – 0	5.8	6.35
Stage 1	4.7	5.15
Stage 2	3.3	3.61
Stage 3	2.1	2.30
Stage 4	1.1	1.20
Stage 5	0.7	0.77
Stage 6	0.4	0.44
Filter	0.2	0.22

After the experiment, retained/deposited powder was collected from the capsule shell, device, mouthpiece, induction port, and each individual stage plus the impaction plate/filter below it, by rinsing and making up to suitable volume with solvent. Mass deposited was calculated from the measured concentration and a prepared standard curve, using an appropriate method of analysis (Table 2.4).

Table 2.4 Summary of collecting solvents and methods of analysis used for the different spray-dried materials analysed by ACI

Material	Collecting solvent	Method of analysis
Lysozyme	H ₂ O	Pierce micro BCA protein assay kit
Lysozyme/Trypsin:Trehalose	H ₂ O	Pierce micro BCA protein assay kit
Lysozyme/Trypsin:Raffinose		
Lysozyme/Trypsin:HP- β -CD		
Trehalose/Raffinose + coumarin-6	80% EtOH	Fluorescence detection: $\lambda_{\text{ex}} = 485 \text{ nm}$ $\lambda_{\text{em}} = 520 \text{ nm}$
Trehalose/Raffinose + sulforhodamine101	H ₂ O	Fluorescence detection: $\lambda_{\text{ex}} = 584 \text{ nm}$; $\lambda_{\text{em}} = 612 \text{ nm}$

The “recovered dose”, being the total amount of powder collected from the device, capsule and impactor was calculated, and the analysis was accepted if this fell within 75% - 125% of the

nominal loaded dose (USP, 2008). A table of cumulative mass% (as% of the total recovered emitted dose) vs effective cut-off diameter for each stage of the impactor was constructed. Fine particle fraction (fraction of the emitted dose having aerodynamic diameter $< 5 \mu\text{m}$) and particle fraction with aerodynamic diameter $< 3 \mu\text{m}$ were calculated by interpolation from the linear portion of the log normal graph of this data, by plotting in Microsoft Excel the inverse of the standard normal distribution of the cumulative mass% against the natural logarithm of the effective cut-off diameter. Experimental mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were similarly determined by interpolation from the linear portion of the log normal plot of cumulative mass% (as% of the total dose recovered from the impactor stages) versus effective cut-off diameter. MMAD was determined from the intercept of this plot on the x-axis (50% mark) and the GSD was determined from the slope of the line (Ph. Eur., 6.0, USP, 2008). Analyses were run in triplicate and expressed as mean $\pm$ standard deviation, or in duplicate and expressed as mean and range.

2.2.10 FLUORESCENCE DETECTION

For ACI analyses of trehalose and raffinose powders, a fluorescent marker was incorporated at a low load (+ 0.2% by weight of sugar content) during spray-drying, to enable subsequent quantitation of powder. For porous particles, produced from non-aqueous systems, the fluorescent dye coumarin-6 was used. For non-porous particles, produced from aqueous systems, sulforhodamine 101 was used because of increased water solubility of this marker. Fluorescence measurements were carried out using either an RF 1501 spectrofluorometer with attached sipper unit, or a Fluostar optima microplate reader (BMG Labtech, Germany) using black 96 well flat-bottomed plates (Sarstedt, Inc., Germany) and a 200 μl sample volume, with excitation and emission wavelengths as detailed in Table 2.4. All sample and standard measurements were carried out in duplicate. Calibration curves were prepared in triplicate on each apparatus, with the same spray-dried batch of powder and collecting solvent as used in the ACI analysis, and was either freshly prepared or checked simultaneously with each analysis.

2.2.11 PROTEIN QUANTITATION

Protein quantitation was carried out using a Pierce Micro BCA protein assay kit[®] (Pierce, Rockford, IL, USA). Standards were prepared using the same protein/peptide and solvent system as for the test samples and dilutions for at least 2 standard curves were prepared (range 0.04 – 0.005 mg/ml). The assay was performed following the plate assay protocol recommendations, as provided by the manufacturer. Briefly, 150 μl of appropriately diluted solutions of test/standard were transferred into a clear flat-bottom 96 well micro-plate (Sarstedt, Inc) in duplicate. 150 μl of

the working reagent [prepared by mixing 25 parts of Micro BCA reagent A (sodium carbonate, sodium bicarbonate, and sodium tartrate in 0.2 N NaOH), 24 parts of reagent B (4% bicinchoninic acid in water and 1 part of BCA reagent C (4% cupric sulfate pentahydrate in water))] was added to each well. Plates were covered, manually shaken and incubated in an oven (Economy incubator with fan size 1, Gallenkamp) for 2 hours at 37 °C, and allowed to cool to room temperature. The samples were analysed for a colorimetric change measured as the optical density (O.D.) at 562 nm using a UV plate reader (Microplate Autoreader EL311, Bio-Tek Instruments, U.S.A.). RS232 Data Logger software (Eltima[®], Germany) was used to capture the data and all data were processed in Microsoft Excel. The O.D. of each sample was compared to the average standard curve, and the concentration of the protein/peptide was extrapolated from this standard curve.

2.2.12 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

Secondary structural changes in the processed proteins were investigated by fourier transform infrared (FTIR) spectroscopy in the solid or in the solution state. Other methods that may be used to examine the secondary structure of proteins including circular dichroism, (limited to clear protein solutions), raman spectroscopy, nuclear magnetic resonance (limited to proteins of $\leq \sim 200$ amino acids), and X-ray crystallography (Singh, 2000), were not considered in this thesis. Each method has specific advantages and limitations, but FTIR was considered the most suitable because of the potential to examine solid and solution states. Solid state FTIR was also used to compare batches of trehalose dihydrate exhibiting different thermal and solubility properties (Section 4.2.1.4.1).

2.2.12.1 Solid samples

Solid state samples were prepared as KBr disks. Protein, excipient or protein:excipient composite samples were mixed with KBr at a ratio of 1:100 or 1:200 using an agate mortar and pestle. Disks were compressed at 8 tonnes pressure for 2 minutes using a 13 mm KBr die set (Apollo scientific, U.K.). FTIR spectra were obtained using a Nicolet Magna 560 Spectrometer and Omnic software 4.1. 64 scans were run for each sample at resolution of 2 cm⁻¹.

2.2.12.2 Interpretation of protein spectra – solid samples

The spectrum was baseline corrected, and smoothed using automatic functions in Omnic software 4.1. The areas between 1720 – 1600 (amide I), 1600 – 1500 (amide II) and 1350 – 1200 (amide III) were truncated as described by Van De Weert et al. (2001) and exported into Origin software. Baselines of isolated peaks were corrected by subtraction of a straight line or left as exported, as indicated in the text. Areas of comparative peaks were normalised to 100 by integration and simple math functions. The absolute difference spectrum of two area-normalised peaks was calculated and

stored in a new column using the formula [abs (col(x) - col(y))]. Integration of this absolute difference spectrum gave the area of non-overlap between two area-normalised spectra (Griebenow et al., 1999). Area of overlap was then calculated from 100 – area of non-overlap, giving a semi-quantitative measure of the similarity of the shape of the peaks.

2.2.12.3 Liquid samples

Aqueous solutions (50 mg/ml protein) were analysed under continuous nitrogen purge in a demountable liquid FTIR cell (SpectraTech, U.S.A.), with 32 mm CaF₂ windows, separated by 0.006 mm mylar spacers (Specac Ltd., U.K.). 256 scans were run for each sample at a resolution of 4 cm⁻¹. Solvent background was subtracted from each run. The spectrum was baseline-corrected, smoothed and the amide 1 peak was isolated using Omnic Software and spectra were analysed as with the solid samples. The criterion used for accepting that water and vapour were successfully subtracted, was a flat baseline between 2000-1750 cm⁻¹ (Jackson, 1995).

2.2.13 SDS-PAGE

Gel electrophoresis was carried out using an SDS-PAGE chamber (ATTO corp., Tokyo, Japan) where the runs were controlled by a Labnet Powerstation 300 (Labnet International Inc., U.K.). Sample buffer (non-reducing) was prepared by combining the following: 3.55 ml H₂O, 1.25 ml 0.5M Tris-HCl pH 6.8, 2.5 ml glycerol, 2.0 ml 10% w/v SDS, 0.2 ml 0.5% w/v bromophenol blue. Reducing sample buffer was prepared by adding 0.05 ml 2-mercaptoethanol to 0.95 ml of this mixture immediately before use. Powder samples were dissolved in phosphate buffer (0.5 - 1 mg/ml protein in phosphate buffer, pH 6.24 for lysozyme samples, pH 7.6 for trypsin samples or pH 7 for catalase samples) and mixed with prepared sample buffer in volume ratios of 4:1 protein solution:sample buffer. Samples were immediately mixed by inversion and heated to 85 °C for three minutes in water. Protein marker and samples (approximately 10 - 25 µl) were introduced into the sample wells of the gel (12% polyacrylamide + tris/SDS buffer), using a micro-syringe. The samples were then separated according to their molecular weight under reducing conditions (sample buffer including mercaptoethanol) or non-reducing conditions (sample buffer without mercaptoethanol). Output settings of 150 mA and 80 V were set and electrophoresis was allowed to continue until the protein marker had completely resolved on the gel sheet by visual inspection. The gel was removed and stained by immersing in a 0.25% w/v solution of Coomassie blue dye in 50% v/v methanol and 10% v/v acetic acid, and placed in a Precision reciprocal shaking water bath (Precision Scientific, U.S.A.) at 25 °C overnight with gentle agitation (speed 30 r.p.m.). Destaining was carried out similarly with agitation in two steps, firstly immersion in a 50% v/v methanol, 10% v/v acetic acid solution for approximately 2 hours (with one change of solvent), followed by 7% v/v methanol, 10% v/v acetic acid for approximately 3 hours (with one change of solvent). Bands observed for test solutions were compared to the standard protein marker, to estimate molecular

weight. Gels were placed on clear plastic backing support and either scanned or photographed using a Gel Doc gel documentation system (Bio-Rad Laboratories Inc, USA).

2.2.14 LYSOZYME ACTIVITY ASSAY

Activity of lysozyme samples was assessed by a turbidimetric rate determination assay proposed by Shugar (1952), and described by Sigma Aldrich. This method is based on the rate of lysis of a suspension of *Micrococcus lysodeikticus* cells by lysozyme enzyme. Measurements were carried out at 25 °C, pH 6.24, using a helios UV/Vis spectrometer, with attached Thermo Spectronic Single Cell Peltier (Thermo Electron Corp., U.S.A.). Substrate for the assay (0.015% w/v *Micrococcus lysodeikticus* suspension in 66 mM phosphate buffer pH 6.24), was freshly prepared daily, and stored in a water bath at 25 °C until use. Lysozyme enzyme solution (200-400 units; 0.008 mg/ml) in cold 66 mM phosphate buffer pH 6.24, was prepared immediately before use. 2.5 ml substrate was transferred by pipette to a quartz cuvette and allowed to equilibrate to 25 °C in the spectrometer for 3-4 minutes. 0.1 ml of buffer (blank run)/enzyme solution (test run) was pipetted into the cuvette, capped and mixed by inversion. Absorbance at λ = 450 nm (A 450) was recorded every 30 s for 5 min 30 s. The Δ A 450/minute was obtained from the average decrease in A 450 /minute for the linear portion of the plot of A 450 against time. The biological activity (Units/mg solid) of the lysozyme sample was then calculated by the following: [df = dilution factor; 0.001 = change in absorbance as per unit definition; 0.1 = volume (in millilitre) of enzyme solution used]

$$\begin{aligned} \text{Units/ml test solution} &= \frac{(\Delta A\ 450/\text{minute Test} - \Delta A\ 450/\text{minute Blank})(df)}{(0.001)(0.1)} \\ \text{Units/mg solid} &= \frac{\text{units/ml test solution}}{\text{mg solid/ml test solution}} \end{aligned}$$

Protein concentration in the test solution was assessed by BCA protein quantitation using a Pierce Micro BCA protein assay kit (Section 2.2.11). Lysozyme standard samples were prepared and used to create a standard curve. These data were used to calculate specific biological activity (Units/mg protein):

$$\text{Units/mg protein} = \frac{\text{units/ml test solution}}{\text{mg protein/ml test solution}}$$

2.2.15 CATALASE ACTIVITY ASSAY

Enzymatic activity of catalase samples was assessed by a continuous spectrophotometric rate determination assay as described by Sigma. Measurements were carried out at 25 °C, pH 7, using a

helios UV/Vis spectrometer, with attached Thermo Spectronic Single Cell Peltier to maintain the temperature (Thermo Electron Corp., U.S.A.). This method is based on the rate of reduction of hydrogen peroxide by catalase as below:



50 mM phosphate buffer was freshly prepared and pH adjusted to 7 using 1 M KOH and stored at room temperature (buffer A). Substrate for the assay (0.036% w/w H_2O_2 in buffer A) was prepared using 30% w/w H_2O_2 (0.12g of 30% w/w H_2O_2 in 100g total weight solution), and the absorbance $\lambda = 240 \text{ nm}$ (A 240) was checked. This A 240 was adjusted to between 0.520 and 0.550 using either H_2O_2 or phosphate buffer as necessary. Catalase enzyme solution (50-100 units; 0.04 mg/ml) in cold buffer A was prepared immediately before use. 2.9 ml substrate (3 ml for the blank run) was transferred using a p-5000 micro-pipette to a quartz cuvette and allowed to equilibrate to 25 °C in the spectrometer for 3-4 minutes. 0.1 ml of enzyme solution (test run) was pipetted into the cuvette. The contents of the cuvette were immediately mixed by inversion and the time required for A 240 to decrease from 0.450 to 0.400 was recorded. Biological activity (Units/mg solid) was calculated using the formula: [df = dilution factor; 3.45 corresponds to the decomposition of 3.45 micromoles of hydrogen peroxide in a 3.0 ml reaction mixture producing a decrease in the A240 from 0.450 to 0.400 absorbance units; min = time in minutes required for the A240 to decrease from 0.450 to 0.400 absorbance units; 0.1 = volume (in milliliter) of enzyme solution used]

$$\text{Units/ml test solution} = \frac{(3.45)(\text{df})}{(\text{min})(0.1)}$$

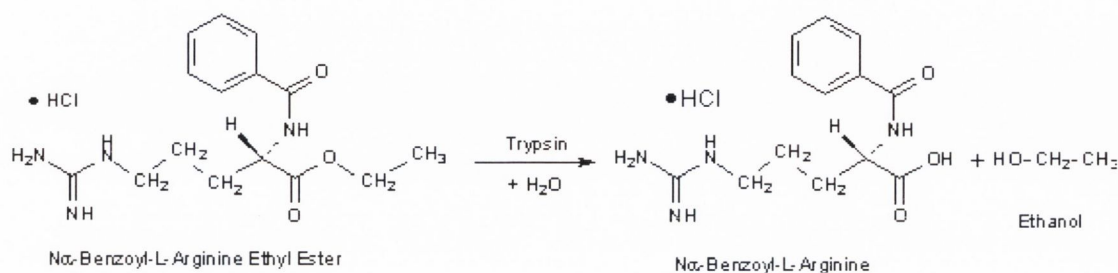
$$\text{Units/mg solid} = \frac{\text{units/ml test solution}}{\text{mg solid/ml test solution}}$$

Protein concentration in the test solution was assessed by BCA protein quantitation using a Pierce Micro BCA protein assay kit (Section 2.2.11). Catalase standard samples were prepared and used as a standard curve. All test samples were diluted 1 in 2 to fall within range of the standard curve. These data were used to calculate the specific biological activity (Units/mg protein), using the equation:

$$\text{Units/mg protein} = \frac{\text{units/ml test solution}}{\text{mg protein/ml test solution}}$$

2.2.16 TRYPSIN ACTIVITY ASSAY

Activity of trypsin samples was assessed by a continuous spectrophotometric rate determination assay as described by USP (2008) for crystallised trypsin and by Sigma. This assay is based on the reaction below. Measurements were carried out at 25 °C, pH 7.6, using a helios UV/Vis spectrometer with attached Thermo Spectronic Single Cell Peltier (Thermo Electron Corp., U.S.A.).



Substrate for the assay (0.25 mM Na-Benzoyl-L-arginine ethyl ester hydrochloride in 67 mM Sodium phosphate buffer pH 7.6) was prepared. 1 mM HCl solution was freshly prepared in grade 1 water, and used within 2 hours of preparation (blank). Trypsin enzyme solution (~500 BAEE units/ml; 0.044 mg/ml) in cold 1 mM HCl solution, was prepared immediately before use. 3.0 ml substrate was transferred by pipette to a quartz cuvette and allowed to equilibrate to 25 °C in the spectrometer for 3-4 minutes, until the absorbance at $\lambda = 253$ nm (A_{253}) was constant. 0.2 ml of 1 mM HCl solution (blank run) or enzyme test solution (test run) was pipetted into the cuvette, which was capped and immediately mixed by inversion. A_{253} was recorded every 30 s for approximately 5 minutes or until A_{253} plateaued. The $\Delta A_{273}/\text{minute}$ was obtained from the average increase in A_{273} /minute for the linear portion of maximal increase in the plot of A_{253} against time. The biological activity (Units/mg solid) of the trypsin sample was then calculated by the following: [df = dilution factor; 0.001 = change in absorbance as per the unit definition; 0.20 = volume (in millilitre) of enzyme solution used]

$$\text{Units/ml test solution} = \frac{(\Delta A_{253}/\text{minute Test} - \Delta A_{253}/\text{minute Blank})(\text{df})}{(0.001)(0.20)}$$

$$\text{Units/mg solid} = \frac{\text{units/ml test solution}}{\text{mg solid/ml test solution}}$$

Protein concentration in the test solution was assessed by BCA protein quantitation using a Pierce Micro BCA protein assay kit (Section 2.2.11). Trypsin standard samples were prepared and used as a standard curve. All test samples were diluted 1 in 2 to fall within range of the standard curve.

These data were used to calculate the specific biological activity (Units/mg protein), using the equation:

$$\text{Units/mg protein} = \frac{\text{units/ml test solution}}{\text{mg protein/ml test solution}}$$

2.2.17 SALMON CALCITONIN ACTIVITY ASSAY (Dr Sinead Ryan, UCD)

The cAMP-secreting activities of sCT and sCT:trehalose composite were assessed using T47D (human breast cancer cells). Bioactivity was quantified by incubating samples with the breast cancer cell line T47D, which over-expresses calcitonin receptors and results in intracellular cAMP release upon activation. Briefly, human breast cancer cells (T47D) were maintained in a RPMI-1640 culture medium (Gibco) containing 1% penicillin–streptomycin (Gibco), 10% foetal bovine serum (Gibco) and insulin (0.2 IU/ml). The cells were plated at an initial density of 1.0×10^6 cells/well and incubated in a 95% air and 5% CO₂ atmosphere at 37 °C for 2 days. After washing with Hank's balanced salt solution (Sigma), the cells were pre-incubated with the same medium supplemented with 0.2 M 3-isobutyl-1-methyl-xanthine (ISBM) and a phosphodiesterase inhibitor at 37 °C for 30 min. The cells were then incubated with the test samples of different concentrations of sCT and sCT:Trehalose at 37 °C for 15 min. After removing the supernatant, the intracellular cAMP was extracted from the cells by cell lysis and measured by ELISA (R&D Systems). Concentration response curves were performed on sCT and sCT:trehalose composite. The concentrations of sCT:trehalose used were based on the concentration of SCt present in the sCT:trehalose sample only so it was comparable with the unprocessed SCt. Bioactivity was plotted against concentration on a logarithmic scale and data were fitted using non-linear regression, and a variable slope according to the SSlogis model (parameters defined in Appendix 7). The parameters of the model are presented, with 95% confidence intervals.

Data were also fitted independently using GraphPad Prism5 software (30 day trial) non-linear regression, dose response curve (variable slope) option (Model is defined in Appendix 7). The parameters of the model are presented, with 95% confidence intervals.

2.2.18 PH MEASUREMENT

pH measurements were carried out using an Orion 420A+ (Thermo Electron Corp., U.S.A.) pH-meter. The pH-meter was calibrated daily using standard buffer solutions (Orion) of pH 4, 7, 10 (± 0.01) sufficient to cover the desired pH range.

2.2.19 STATISTICAL ANALYSIS

Comparison of means was done by one way ANOVA and by two sample t-tests using Minitab v14 software. A difference or effect was considered to be significant at a significance level of $\alpha = 0.05$. The assumption of normality of data was checked by constructing a normal probability plot of the calculated residuals. Residual values versus fitted values were plotted to check that there was no trend in the variability of the data.

2.2.20 STORAGE STABILITY STUDY

Storage stabilities of porous and non-porous spray-dried and unprocessed lysozyme and porous composites of lysozyme:trehalose (weight ratio 1:1) were assessed under temperature and relative humidity storage conditions – long-term and accelerated stability - as defined by the ICH (2003) for a period of 20 weeks. Samples were placed in screw cap jars that were left open during the study and placed in a glass chamber at the required relative humidity, in an incubator oven (Gallenkamp, UK) set at the desired temperature. Conditions studied were: 4 °C/desiccant, 25 °C/60% R.H. (achieved by inclusion of a saturated solution of NaBr in the glass chamber), and 40 °C/75% R.H. (achieved by inclusion of a saturated solution of NaCl in the glass chamber). Lysozyme:trehalose composites were removed from the study conditions of 40 °C/75% R.H. before the week 5 time-point due to collapse of the powder into a sticky mass. Samples were removed at appropriate time intervals and analysed as outlined in Table 2.5 and Table 2.6.

Table 2.5 Layout of storage stability study for lysozyme [P = porous spray-dried lysozyme; NP = non-porous spray-dried lysozyme; Unp = unprocessed lysozyme]

	SEM	TGA	DSC	XRD	FTIR (Solid)	ASSAY	SDS-PAGE
Day 0	P/NP/Unp	P/NP/Unp	P/NP/Unp	P	P/NP/Unp	P/NP/Unp	P/NP/Unp
Day 1	P/NP						
Week 1	P	P			P	P	
Week 2	P/NP	P/NP			P/NP/Unp	P/NP/Unp	
Week 5	P	P			P	P	
Week 9	P/NP	P/NP			P/NP/Unp	P/NP/Unp	
Week 12	P	P			P	P	
Week 16	P/NP	P/NP			P/NP/Unp	P/NP/Unp	
Week 20	P/NP	P/NP	P/NP	P	P/NP/Unp	P/NP/Unp	P/NP/Unp

Table 2.6 Layout of storage stability study for lysozyme:trehalose (1:1) spray-dried composite, produced using different spray-drying conditions

	SEM	TGA	DSC	XRD	FTIR (Solid)	ASSAY	SDS-PAGE
Day 0	√	√	√	√	√	√	
Day 1	√						
Week 1	√	√			√		
Week 2	√	√	√	√	√	√	
Week 5	√	√	√	√	√	√	
Week 9	√	√	√	√	√	√	
Week 12	√	√	√	√	√	√	
Week 16	√	√	√	√	√	√	
Week 20	√	√	√	√	√	√	√

Storage stabilities of porous composites of lysozyme:excipient and trypsin:excipient (weight ratio 1:4) spray-dried from MeOH:BA:(H₂O) systems, were assessed under desiccant conditions and storage temperatures 4 °C and 25 °C for a period of 12 weeks. Samples were placed in screw cap jars that were left open during the study and placed in a glass chamber with desiccant in an incubator oven (Gallenkamp, UK) set at the desired temperature. Samples were removed at appropriate time intervals and analysed as outlined in Table 2.7 below.

Table 2.7 Layout of storage stability study for lysozyme:trehalose (1:1) spray-dried composite, produced using different spray-drying conditions.

	SEM	TGA	DSC	XRD	ASSAY
Day 0	√	√	√	√	√
Day 1	√		√		
Week 1	√	√	√		
Week 4	√	√	√	√	
Week 8	√	√	√	√	
Week 12	√	√	√	√	√

3.1 INTRODUCTION

Lysozyme was chosen as a model protein because it is well-characterised, with information regarding spray-drying and other drying processes available in the literature, and because of the availability of a convenient activity assay, and low cost for initial studies compared to therapeutically used proteins. It is quite a stable protein, and can reportedly withstand the various stresses of spray-drying, with little loss of biological activity on re-hydration. Perturbations in secondary structure have been described in the solid state after spray-drying however and ascribed to dehydration and to processing stresses (Elkordy et al., 2002; Liao et al. 2002, 2005; Bögelein, 2007). Some examples of spray-dried lysozyme found in the literature, include the following: Elkordy et al. (2002) spray-dried lysozyme from aqueous solution on a Büchi B-190 spray-dryer at a total solid concentration of 1% w/v and inlet and outlet temperatures of 120 ± 2 °C and 52 ± 3 °C, respectively. Biological activity measured following spray-drying was reported as $84.9 \pm 4.1\%$ of original activity. Deterioration of secondary structure of spray-dried lysozyme in the solid state was found by solid-state FT Raman but was not reported under re-hydration conditions. Liao et al. (2002, 2005) reported similar biological activity of $87.2 \pm 2.1\%$ and $86.8 \pm 2.45\%$ of original activity when spray-dried from 5 mM phosphate buffer (pH 7.0) also on a Büchi B-190 spray-dryer, at inlet temperatures 95 °C and 120 °C with perturbation in secondary structure in the dried state assessed by FTIR. Bögelein (2007) spray-dried lysozyme from aqueous solution at different inlet temperatures (90 - 220 °C) on a Büchi B-290 spray-dryer equipped with either a regular or a high efficiency specially-constructed cyclone with smaller radius and greater collectable yield compared to the regular cyclone. Biological activity was reported as 94.3 – 91.5% of original activity for samples spray-dried using the regular cyclone, and 87 – 78.3% of original activity for samples spray-dried using the high efficiency cyclone. Morphology of particles was a combination of spherical and donut-shaped particles with smooth surfaces.

The background to the spray-drying method used in this thesis, and difficulties anticipated with its application to spray-drying proteins, have been discussed previously (Chapter 1). This method has not been previously applied to macromolecules, and as such the work in this section is largely empirical in nature. Lysozyme was initially freeze-dried from aqueous solution in which the pH had been altered away from the protein's isoelectric point (11.35), to increase its solubility in methanol, as described by Chin et al. (1994) and by Bromberg and Klivanov (1995). This was undertaken as a prelude for future incorporation into alcohol:water and non-aqueous methanol:n-butyl acetate systems. Preliminary characterisation of the freeze-dried material was undertaken to establish similarity with the unprocessed material. Spray-drying of freeze-dried lysozyme and unprocessed lysozyme from alcohol:H₂O:AC systems was examined, with particular consideration

given to the morphology of spray-dried particles. The influence of solvent ratio and AC content on feed material and spray-dried particle morphology was examined. Different spray-drying parameters were also investigated. Alternative process enhancers being ammonium hydrogen carbonate (AHC), ammonium acetate (AA) and ammonium formate (AF) were considered. Lysozyme was spray-dried from water with/without AC as comparison systems. Finally freeze-dried lysozyme was spray-dried from a non-aqueous (MeOH:BA) system, prior to later formulation of such systems with excipients (Chapter 5). Some spray-dried systems, representative of different morphologies and spray-drying conditions, were selected for further characterisation in terms of process and storage stability. Particle characterisation and in-vitro deposition of different particle morphologies was carried out to assess the potential aerodynamic properties of spray-dried particles and suitability for formulation in a dry powder inhaler.

3.2 FREEZE-DRYING STEP TO INCREASE METHANOL SOLUBILITY

Lysozyme was freeze-dried from aqueous solution at a solid concentration of 1% w/v and pH 6 (pH adjusted using 1 M NaOH; initially pH ~4.6) in a method similar to that described by Chin et al. (1994) and Bromberg and Klibanov (1995). Freeze-dried lysozyme was XRD amorphous, showing a broad diffuse halo pattern with absence of any sharp distinguishing peaks. Unprocessed material was also amorphous by XRD analysis (Figure 3.1). Residual moisture contents of ~ 4-6%, (calculated from mass loss over the temperature range 25-100 °C by thermogravimetric analysis) were determined for freeze-dried and unprocessed lysozyme (Figure 3.2 (a), (b)). DSC showed a similar pattern to unprocessed material with a broad endothermic peak from 25~160 °C, partially comprising moisture loss, and a sharp endothermic peak at ~204 °C indicative of the apparent denaturation temperature (Tm), similar to published data (Elkordy et al., 2004) and slightly higher than unprocessed material at ~ 200 °C (Figure 3.2 (c), (d)). The freeze-drying process did not alter the solid-state characteristics of lysozyme, analysed by these methods.

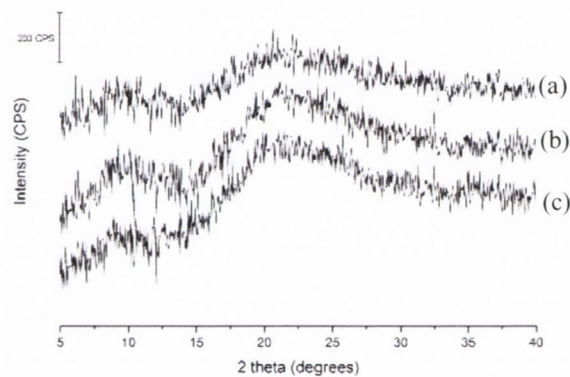


Figure 3.1 XRD trace of (a) freeze-dried (b) unprocessed material (Prod. No. L7651) (c) unprocessed material (Prod. No. L6876).

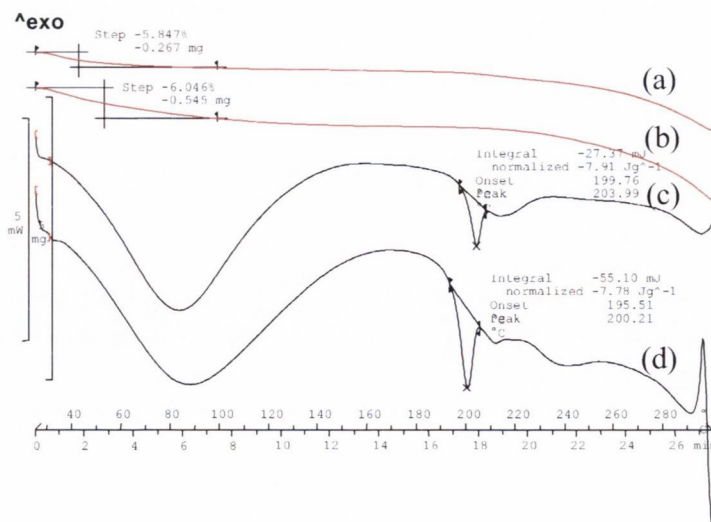


Figure 3.2 TGA (traces shown in red) and DSC traces (shown in black) of (a), (c) freeze-dried lysozyme; (b), (d) unprocessed lysozyme (L7651).

The freeze-dried powder had very low bulk (bp) and tapped densities (tp) of $0.0332 \pm 0.0042 \text{ g/cm}^3$, ($n = 3$) and $0.0586 \pm 0.0019 \text{ g/cm}^3$ ($n = 3$) respectively, with a calculated high Carr's index of 43.3 (indicative of extremely poorly flowing powder (de Villiers, 2005)). The material appeared as thin flakes as visualised by SEM, typical of lyophilised material (Figure 3.3 (a)). These compared to higher measured bulk and tapped density values, and lower calculated Carr's indices for unprocessed material, (2 batches of lysozyme products L7651 and L6876) (Table 3.1). SEM analysis showed large and irregular shaped particles, similar for the two unprocessed products, as shown under two magnifications (Figure 3.3 (b), (c)).

Table 3.1 Bulk and tapped density measurements for different batches of unprocessed lysozyme.

Product No.	bp (g/cm ³)	tp (g/cm ³)	Carr's index
L7651	0.453 ± 0.099	0.504 ± 0.019	10.1
	0.488 ± 0.0046	0.609 ± 0.006	19.9
L6876	0.528 ± 0.006	0.622 ± 0.008	15.1
	0.497 ± 0.0013	0.609 ± 0.020	18.4

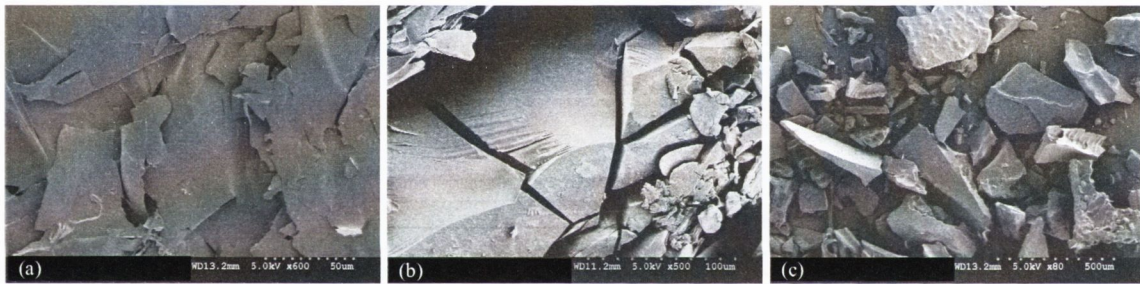


Figure 3.3 SE micrograph of (a) freeze-dried lysozyme (b) unprocessed lysozyme (L7651) high magnification (c) unprocessed lysozyme (L6876) low magnification.

Biological activity of freeze-dried lysozyme was well retained with no statistically significant loss of activity measured by enzymatic activity assay following freeze-drying ($n = 6$), relative to unprocessed material (control) ($n = 3$) (at significance level $\alpha = 0.05$). Results are shown below in table 3.2 and expressed as % of control activity (calculated from the units/mg solid) and % specific activity (calculated from units/mg protein as assessed from micro BCA analysis of test solutions), to account for any difference in protein content of test solutions.

Table 3.2 Summary of biological activity assay results for freeze-dried lysozyme; *± pooled stdev % (in the case of % activity and % specific activity) is calculated from the pooled standard deviation of all samples run for the daily assay as a % of control solution.

	Units/ml test	Units/mg solid	µg protein/test	Units/mg protein	% Activity (*± 4.9%)	% Specific activity (*± 4.5%)
Freeze-dried	326 ± 16.7	40781 ± 2085	8.4 ± 0.26	38937 ± 2026	93.2	99
Control	350 ± 18.5	43750 ± 2312	8.9 ± 0.4	39323 ± 855	100	100

Solubility of lysozyme, (freeze-dried from aqueous solution (pH 6)) in methanol has been reported as > 5 mg/ml (Bromberg and Klibanov, 1995) and > 10 mg/ml (Chin et al., 1994) and for unprocessed lysozyme in methanol ~ 0.011 mg/ml (Bromberg and Klibanov, 1995). MeOH solubility of freeze-dried and unprocessed lysozyme was preliminarily checked by visual inspection, to check similarity with these published reports. 10 mg of freeze-dried lysozyme was dissolved in 2 ml MeOH (5 mg/ml) with manual agitation, yielding a clear solution. 10 mg of unprocessed lysozyme was not observed to dissolve in up to 10 ml MeOH (< 1 mg/ml). These are similar to reported findings.

3.3 SPRAY-DRIED LYSOZYME SYSTEMS

3.3.1 Spray-dried lysozyme from methanol:water systems with ammonium carbonate

Spray-drying lysozyme from a methanol:water plus AC system was first considered based on the work of McDonald (2005) as a starting point for preliminary research with the protein. Initial feed conditions of 75% v/v MeOH, 0.3% w/v total solid concentration (t.s.c.), 16.67% w/w of t.s.c. being AC were selected. The low t.s.c. of 0.3% w/v was chosen based on the low solubility of unprocessed lysozyme in MeOH. The inlet temperature of 90 °C was chosen, based on the work of McDonald (2005). Spray-drying was carried out in the closed mode configuration, with N₂ as drying gas, as dictated by safety considerations when spray-drying with MeOH. Other relevant spray-drying conditions are given in Appendix 1. These resulted in outlet drying temperatures, similar to Elkordy et al. (2002) of approximately 53 °C. Freeze-dried (Section 3.2) or unprocessed lysozyme was initially dissolved in the H₂O component, followed by the addition of AC and finally addition of MeOH, with stirring. In a third system, the pH of the solution of unprocessed lysozyme in water was adjusted to pH 6, similar to the pH from which lysozyme was freeze-dried, and prepared similarly. A fine cloudiness/phase separation was found to occur on addition of MeOH to lysozyme and AC in the H₂O component, regardless of the initial pH of the lysozyme solution or whether a freeze-drying step was used to increase the methanolic solubility of lysozyme. This indicated that the reported improvement in MeOH solubility from the freeze-drying step was not maintained under these feed conditions.

Spray-dried particles consisted in all cases of non-porous spheres, being < 3 µm in diameter from SEM estimations. These spheres had an interesting surface morphology when viewed using a high magnification in SEM, being apparently rough in texture with some tiny pores/gaps in the surface (marked by a red arrow in Figure 3.4). This was more noticeable at a lower magnification in the sample initially freeze-dried at altered pH (Figure 3.4 (a)). Particle morphology was not considered sufficiently different however, to merit the added step of freeze-drying. This step was therefore omitted in all future alcohol:H₂O:AC systems. Collectable powder yields were extremely low (≤ 3%).

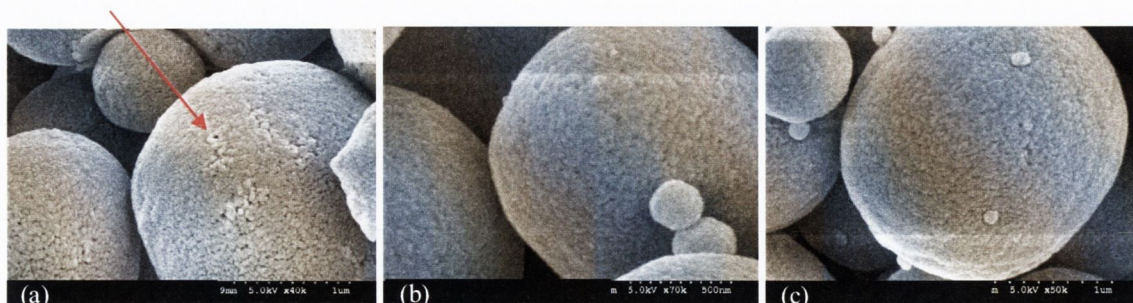


Figure 3.4 SE micrographs of lysozyme (a) initially freeze-dried (from solution pH 6), (b) unprocessed and spray-dried as received (c) unprocessed (pH of water component adjusted to 6), all spray-dried from 75% v/v MeOH, 16.67% of t.s.c. AC, inlet temperature 90 °C (note magnifications differ).

McDonald (2005), Nolan (2008) reported a strong dependence of NPMP production on spray-drying and feed conditions, including inlet drying temperature, mode of spray-dryer (open or closed), and solvent choice or concentration and amount of AC process enhancer. The mode of the spray-dryer and type of drying gas could not be altered due to safety considerations while spray-drying with MeOH in the feed material. The effect of changing inlet drying temperature was therefore initially considered. The inlet temperature was changed to 110 °C and 70 °C, maintaining all other conditions as in initial experiments, resulting in outlet temperatures of 64 °C and 40 °C, respectively. Spray-dried particles were spherical and non-porous. Alteration of inlet drying temperature in this range was not found to markedly impact on particle shape or size, as observed from SE micrographs (Figure 3.5).

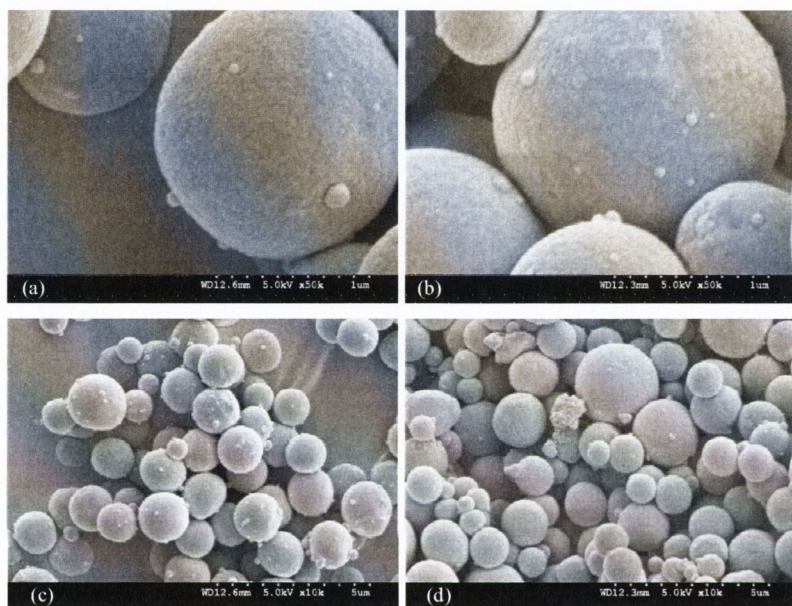


Figure 3.5 SE micrographs of lysozyme spray-dried from 75% v/v MeOH, 16.67% (t.s.c.), with change in inlet temperature (a), (c) 110 °C (b), (d) 70 °C.

Alteration of solvent ratio (55 - 80%) and amount of AC (0 - 40%) were considered and effects on particle morphology and phase separation were simultaneously investigated. All systems in this study were spray-dried at an inlet temperature of 90 °C for uniformity and to ensure adequate drying occurred especially in the higher water content samples, and also to minimise thermal stress to the protein. A t.s.c. of 0.3% was employed in the case of the lower AC content (0%, 16.67%, 33.33%), and a t.s.c. of 0.4% for higher (40%) AC content.

Three distinct particle morphologies were found and a relationship with phase separation of the feed material was noted. Non-porous collapsed/irregular particles were produced from systems spray-dried from 55% or 60% MeOH, 16.67% AC; 75% MeOH, 0% AC and 60% MeOH, 40% AC (Figure 3.6). This particle morphology has been described as “donut” shaped and postulated to occur when the outer crust formed during droplet drying collapses due to vapour pressure within the droplet, and is more common during rapid droplet drying (Maa et al., 1997). These systems,

with the exception of a slight cloudiness in the case of 60% MeOH, 40% of t.s.c. AC, were spray-dried as clear solutions.

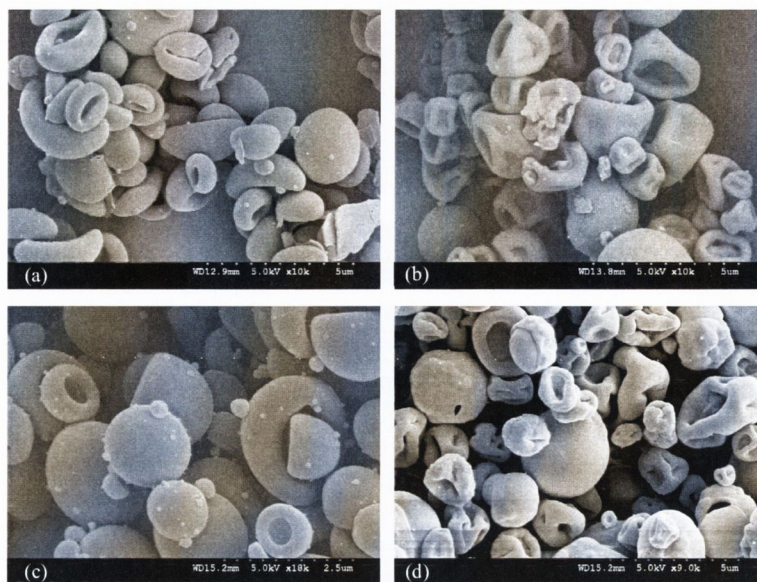


Figure 3.6 SE micrographs showing lysozyme spray-dried from (a) 55% MeOH, 16.67% of t.s.c. AC; (b) 60% MeOH, 16.67% of t.s.c. AC; (d) 75% MeOH; (e) 60% MeOH, 40% of t.s.c. AC.

Non-porous intact spheres, were observed in SE micrographs of lysozyme spray-dried from 65%, 70%, 80% MeOH with 16.67% AC and 80% MeOH with 33.3% AC (Figure 3.7). All these systems were spray-dried as cloudy suspensions.

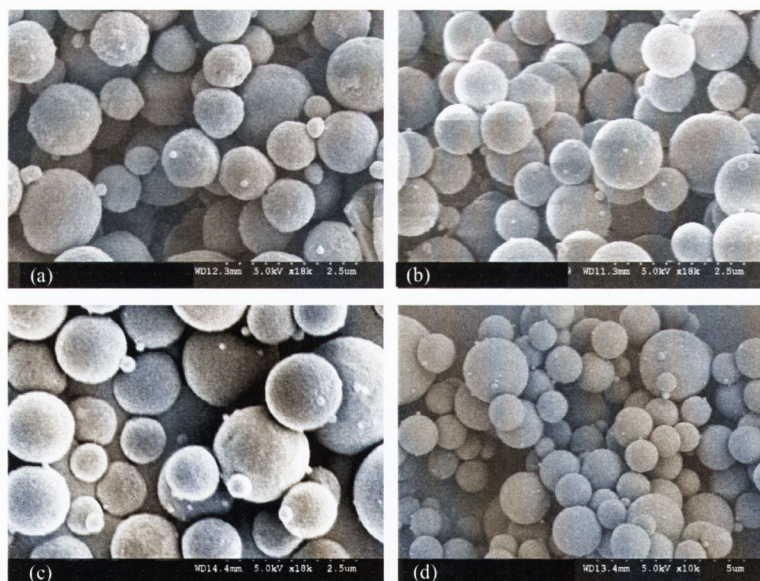


Figure 3.7 SE micrographs showing lysozyme spray-dried from (a) 65% MeOH; 16.67% AC; (b) 70% MeOH, 16.67% AC; (c) 80% MeOH, 16.67% AC; (d) 80% MeOH, 33.3% AC.

Spherical porous particles were produced from systems spray-dried from 65-80% MeOH with 40% of t.s.c. AC, which were similar in size (diameter < 3 μm from SEM estimations) and in morphology, as observed from SEM analysis (Figure 3.8 (a) - (d)). Particle surface resembled

fused irregular-shaped nano-particles or highly folded morphology with some holes in the surface as marked by the red arrow in Figure 3.8 (e). Shattered or broken particles, useful for assessing hollow/solid nature of particles, were difficult to find. One broken particle marked by a red arrow in Figure 3.8 (f), appeared to comprise internal voids or a honeycomb structure rather than being hollow particles with well-defined outer walls. All these systems were spray-dried as cloudy suspensions.

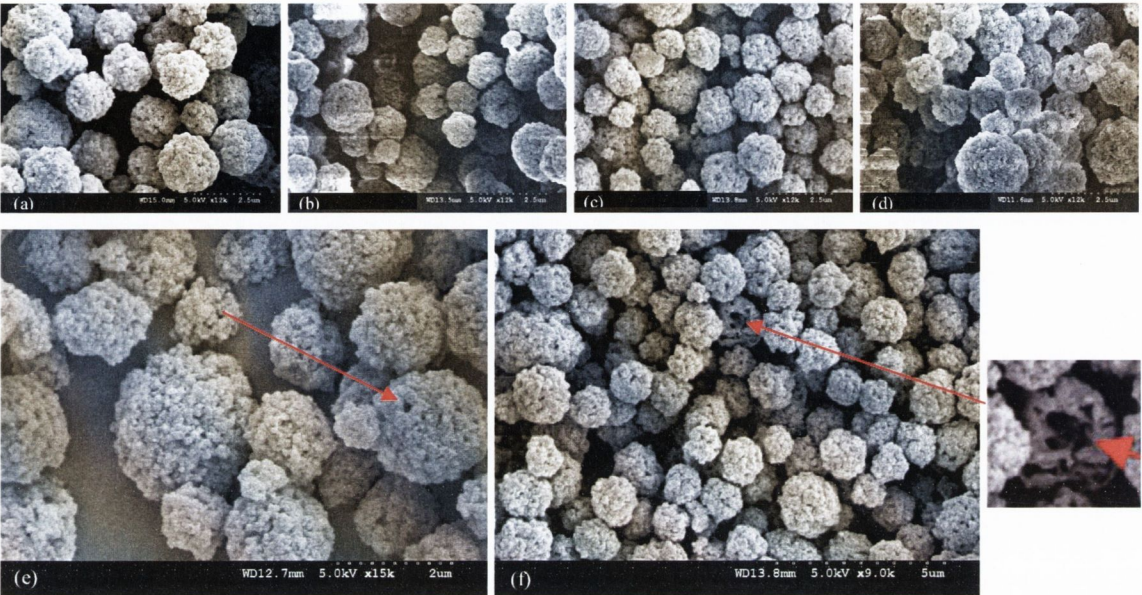


Figure 3.8 SE micrographs showing lysozyme spray-dried from (a) 65% MeOH; 40% of t.s.c. AC, (b) 70% MeOH; 40% of t.s.c. AC, (c) 75% MeOH; 40% of t.s.c. AC, (d) 80% MeOH; 40% of t.s.c. AC, (e) Higher magnification showing holes in particle surface of system in (d), (f) Example of broken particle showing internal voids/honeycomb structure of system in (c).

From these experiments, conditions resulting in cloudiness/phase separation in the feed material were essential to produce spherical particles and ultimately NPMPs, as this was found in all systems producing NPMPs (Figure 3.9).

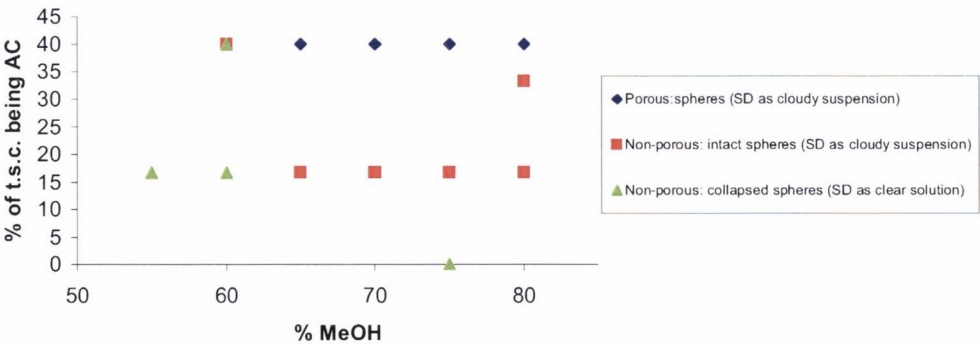


Figure 3.9 Particle morphology and feed material appearance with % MeOH /% of t.s.c. AC.

Collectable product yield varied from < 0.5% - 18.3%, with the higher yields being obtained for the non-porous particles than for the porous particles. A number of factors may have contributed to

this. A dependence of collectable yield on spray-dried volume has been noted elsewhere in this work (Section 4.2.1.3), and porous particles were spray-dried from 50 ml volumes compared to 100 ml volumes for non-porous particles in this study, except in one case as shown (Figure 3.10). Porous particles also comprised a greater % of t.s.c. being AC which is postulated to be volatilised during the spray-drying process (due to the low volatilisation temperature of AC); therefore % of total protein content in the feed material should be considered as a lower total protein content may also affect the yield. [In the case of 40% AC (0.4% t.s.c.), this represented a total protein content of 0.24% compared to 0.25% in the case of 16.67% AC (0.3% t.s.c.) and 0.2% in the case of 33.33% AC (0.3% t.s.c.)]. Porous particles and intact spherical non-porous particles were spray-dried as cloudy suspensions (displaying some degree of phase separation), with some material deposited in the feed container and as such unavailable for spray-drying. This may have further contributed to the lower yields noted in these samples (Figure 3.10).

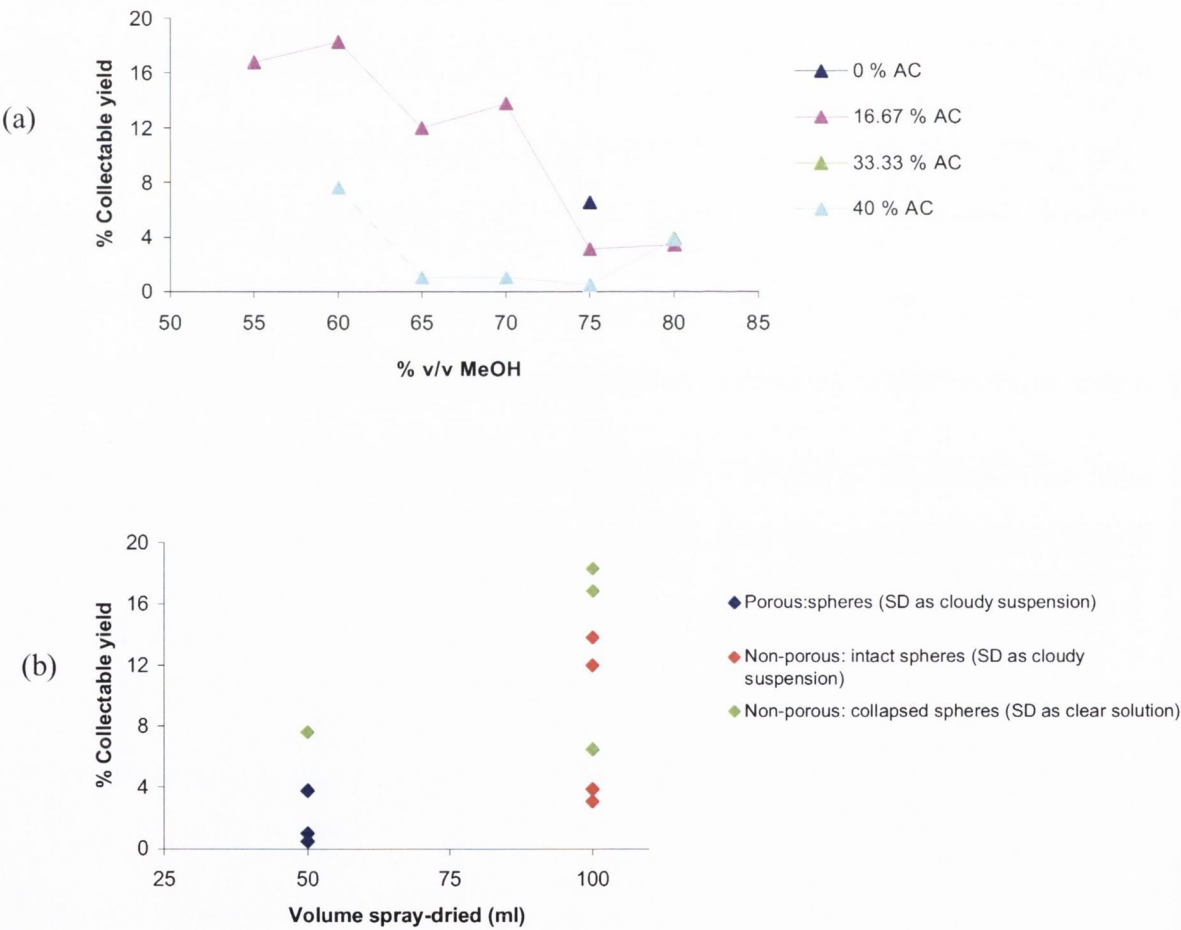


Figure 3.10 Relationship between collectable yield and (a) feed conditions and (b) volume spray-dried and morphology.

3.3.2 Spray-dried lysozyme from ethanol:water systems with ammonium carbonate

It was decided to investigate the use of ethanol as an alternative alcohol. Ethanol is a more pharmaceutically acceptable solvent, classified by the ICH (2005) as a solvent with low toxic potential (class 3 solvent) compared to methanol which should be limited in pharmaceutical products because of inherent toxicity (class 2 solvent). It is also possible to spray-dry ethanol systems in the open mode configuration due to a higher flash point and thus less stringent safety requirements. This may potentially lead to increased product yield, as a contributory to the low yield in the closed mode drying may have been powder loss from glassware connections when the spray-dryer was operated at positive pressure (Section 4.2.1.3). An increase in product yield would allow further characterisation of spray-dried powders. Ethanol may also be a stronger precipitating alcohol due to increased hydrophobicity compared to MeOH (Bromberg and Klivanov, 1995). Phase separation was identified from Section 3.3.1 as necessary for NPMP production, with NPMPs produced from systems spray-dried as cloudy suspensions. Phase separation and NPMP production may potentially be achieved at lower alcohol (EtOH) and/or AC concentrations than for MeOH:H₂O:AC, because of the greater hydrophobicity of EtOH. It was considered desirable to minimise the AC content in order to minimise any residual contamination of the spray-dried product, after volatilisation of the process enhancer.

Feed conditions that did not produce NPMPs from MeOH:H₂O:AC systems were initially attempted with EtOH:H₂O:AC systems. Lysozyme was spray-dried from 75% EtOH without AC, 0.3% t.s.c., inlet temperature 90 °C, in the closed mode similar to a comparable system with MeOH. The feed material was spray-dried as a clear solution and spray-dried particles were comprised of collapsed spheres and donut-shaped particles (Figure 3.11 (a)), similar to those produced with MeOH. Lysozyme was then spray-dried from 60% EtOH with 16.67% of t.s.c. being AC, 0.3% t.s.c., inlet temperature 90 °C in the open mode with N₂ as drying gas. This system was spray-dried as a cloudy suspension, unlike the equivalent MeOH system, and spray-dried particles had a wrinkled, raisin-like morphology (Figure 3.11 (b)).

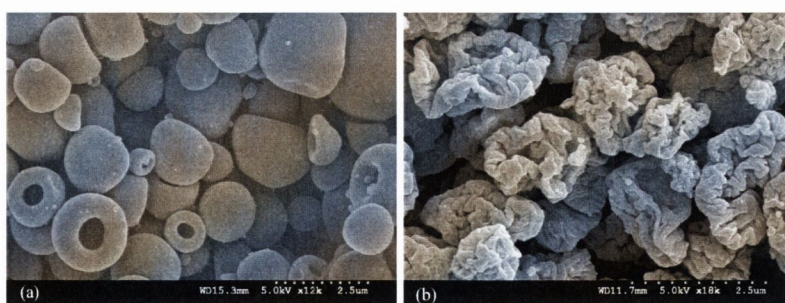


Figure 3.11 SE micrographs showing lysozyme spray-dried from (a) 75% EtOH, no AC; (b) 60% EtOH, 16.67% of t.s.c. AC.

Further alteration of EtOH:H₂O:AC was considered to produce NPMPs and lysozyme was spray-dried from 75% and 80% EtOH; 10% of t.s.c. AC; 0.3% w/v t.s.c. in the open mode. The % EtOH and % of t.s.c. AC were chosen based on previous studies using these systems for low molecular weight drugs by Tajber (2005) and Nolan (2008). These systems were spray-dried as cloudy suspensions. The inlet drying temperature used was 78 °C. This lower inlet temperature compared to the standard 90 °C used in MeOH:H₂O:AC systems, was selected based on experience by Tajber (2005) with spray-drying various EtOH:H₂O solvent systems in the open mode, and also to minimise thermal stresses to the protein. NPMPs were produced under these conditions (Figure 3.12). Collectable product yields were low however at 6.0% (from 75% EtOH) and 4.9%, 7.7% (from 80% EtOH, results from two different runs), with 50 ml volumes spray-dried.

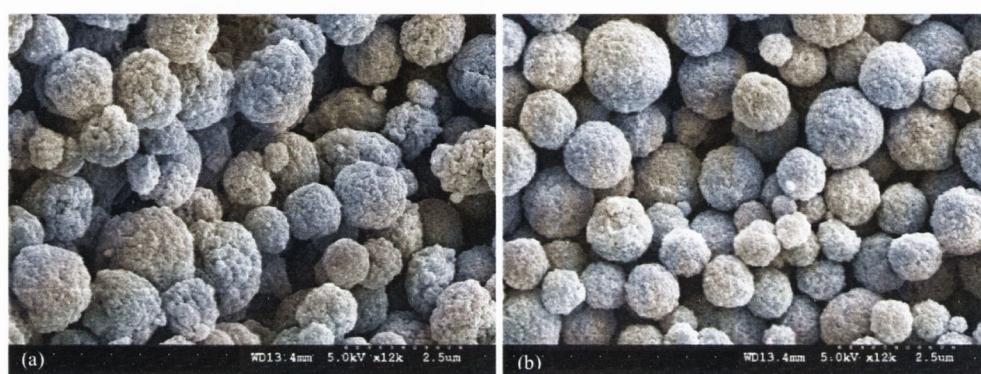


Figure 3.12 SE micrographs showing lysozyme spray-dried from (a) 75% and (b) 80% EtOH with 10% of t.s.c. being AC.

Strategies considered for increasing the collectable yield included increasing the t.s.c. and increasing the volume spray-dried. Selecting the system spray-dried from 80% EtOH, 10% of t.s.c. AC, as an example, t.s.c. was increased to 0.4% w/v, 0.5% w/v, 1% w/v and 2% w/v. Volumes spray-dried were maintained at 50 ml. Increasing t.s.c. resulted in an increase in collectable product yield to 24.6% (0.4% w/v t.s.c.); 32.8%, 20.2% (0.5% w/v t.s.c, results from two different runs); 35.8% (1% w/v t.s.c.); 53.4% (2% w/v t.s.c.). Particle morphology was similar where t.s.c. was increased up to 1% w/v (Figure 3.13). Further increase of the t.s.c. to 2% w/v resulted in more non-porous particles produced (one example marked by red arrow in Figure 3.13 (d)), than were seen for the lower t.s.c. suggesting a concentration related effect on morphology. Higher t.s.c. of 1% w/v and 2% w/v also resulted in a more extensive phase separation, necessitating a higher nozzle cleaning setting (setting 3) and intermittent stirring of the feed suspension, during spray-drying. The t.s.c. of 0.5% w/v was thus selected for future experiments, as this resulted in an acceptable yield without these additional requirements. Increasing the volume spray-dried (t.s.c. 0.5% w/v) increased the collectable yield to 35.4% (250 ml), and 38.9%, 52.3%, 58.8% (500 ml, results from three different runs).

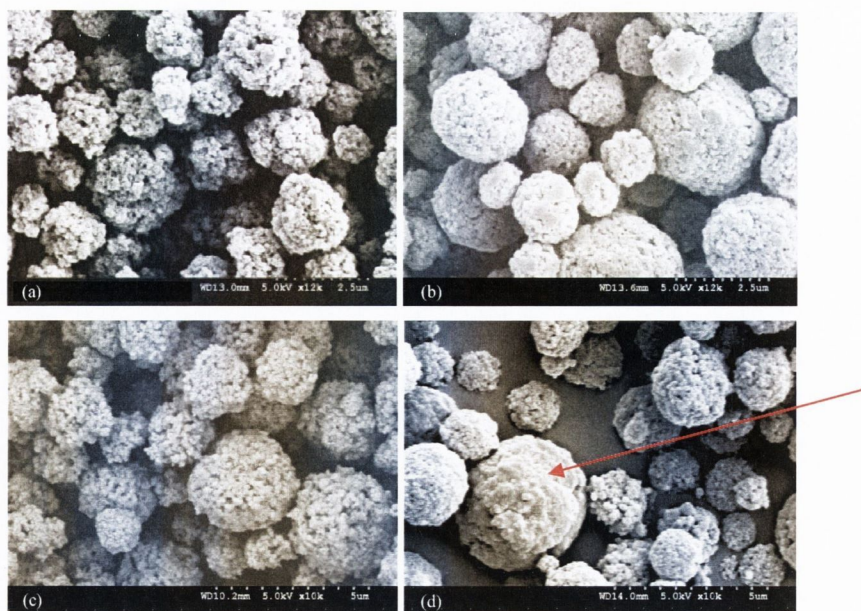


Figure 3.13 SE micrographs showing lysozyme spray-dried from 80% EtOH, 10% of t.s.c. AC, inlet temperature 78 °C, (a) 0.4% t.s.c. (b) 0.5% t.s.c. (c) 1% t.s.c. (d) 2% t.s.c.

From initial experiments the concentrations of EtOH and AC in the feed material were observed to have a major effect on particle morphology with NPMPs produced using 75% and 80% EtOH; 10% of t.s.c. AC. In order to examine this in more detail, further concentrations of EtOH (50, 60, 65, 70%) were considered with a standard AC content of 10% of t.s.c.. Lysozyme was also spray-dried from 80% EtOH, with a reduced AC content of 5% and 0% of t.s.c.. It was found that in systems including 10% of t.s.c. AC, EtOH concentration of 70% v/v produced NPMPs. This system involved phase separation in the feed material prior to spray-drying and spray-drying of a cloudy suspension feed material. Lower EtOH concentrations of 50%, 60%, 65% with 10% of t.s.c. AC were spray-dried as clear solutions and spray-dried product resembled collapsed non-porous particles (Figure 3.14). Spray-drying from 80% EtOH, 5% of t.s.c. AC (cloudy suspension) resulted in spherical non-porous particles, which had a roughened surface (Figure 3.14). Spray-drying from 80% EtOH, no AC (clear solution) resulted in collapsed, non-porous particles (Figure 3.14). These results are summarised in the graph in Figure 3.15.



(50% EtOH; 10% of t.s.c. AC)

(60% EtOH; 10% of t.s.c. AC)

(65% EtOH; 10% of t.s.c. AC)

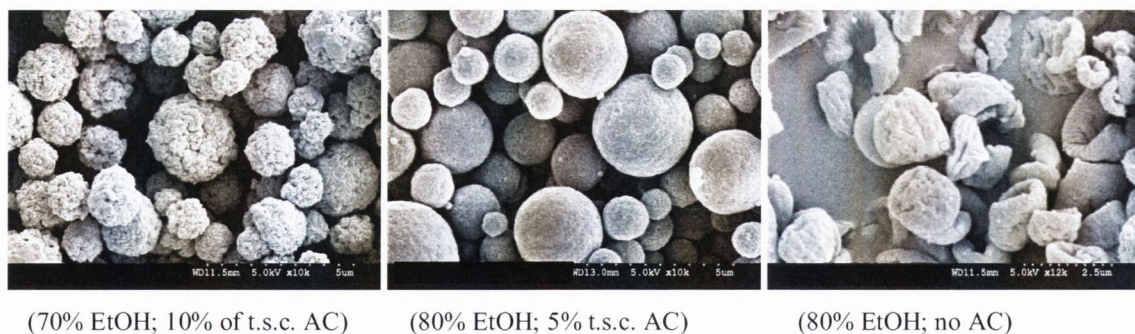
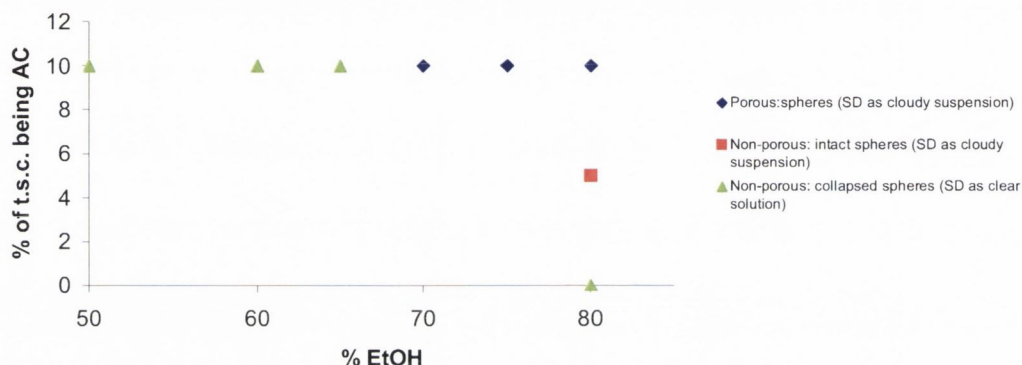


Figure 3.14 SE micrographs showing lysozyme spray-dried at different % EtOH; % AC as shown; inlet temperature 78 °C.

Figure 3.15 Particle morphology and feed material appearance with % EtOH and % t.s.c. AC.



Higher magnification SE micrographs of NPMPs spray-dried from 70% and 80% EtOH, 10% AC, are shown (Figure 3.16). Evidence that NPMPs spray-dried from 70% EtOH may be hollow was suggested from the broken particle, marked by the red arrow in Figure 3.16 (a). Surface morphology was seen to extend through the particle wall, with the inside of the particle similar to the external surface morphology. Surface morphology appeared to consist of fused irregular shaped nano-particles and was similar for the two EtOH feed concentrations.

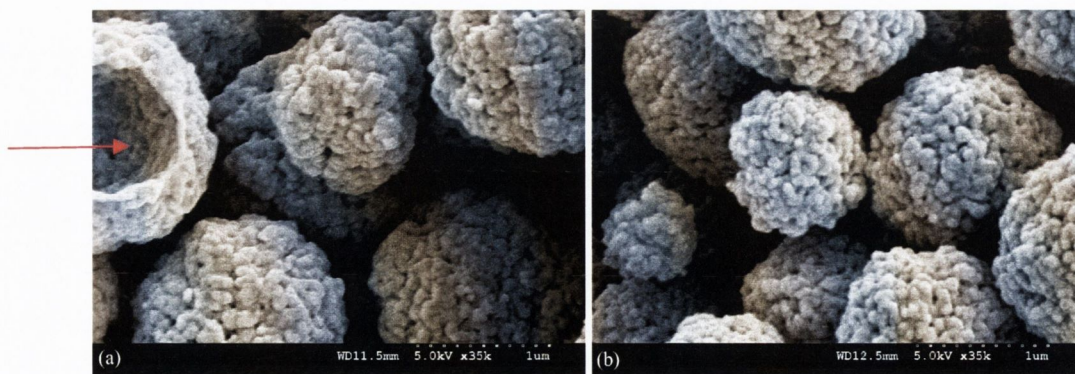


Figure 3.16 SE micrographs showing spray-dried porous lysozyme from (a) 70% and (b) 80% EtOH, 10% of t.s.c. AC.

An effect due to increasing inlet temperature was not anticipated considering previous experience with spray-drying from MeOH:H₂O:AC systems but was investigated by increasing the inlet temperature of the system spray dried from 80% EtOH with AC to 85 °C and the system spray-dried from 70% EtOH with AC to 90 °C. This latter system was interesting for future experiments spray-drying lysozyme in a composite with trehalose, as these conditions were found to be favourable for producing discrete amorphous particles of the sugar (Section 4.2.1.2). No distinct effect on particle morphology was observed on increasing inlet drying temperature, although a higher prevalence of non-porous particles were observed in the system spray-dried from 80% EtOH with AC spray-dried at 85 °C (Figure 3.17).

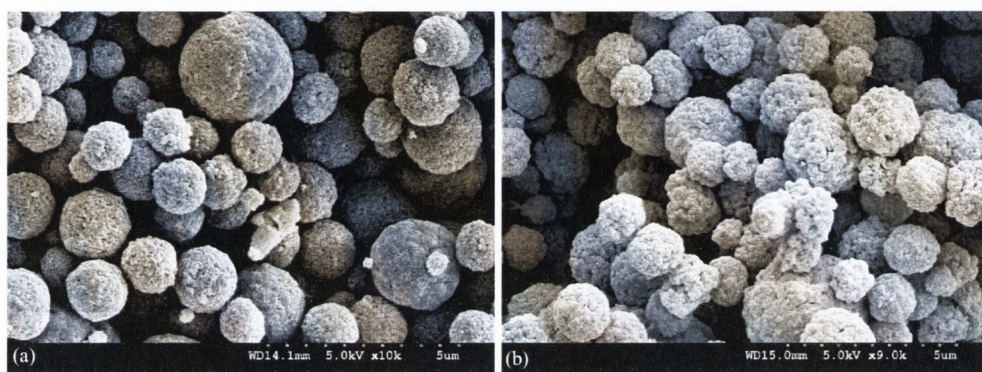


Figure 3.17 SEM micrographs showing effect of changing inlet temperature on particle morphology (a) 80% EtOH, 10% of t.s.c. AC, inlet temperature 85 °C (b) 70% EtOH, 10% of t.s.c. AC, inlet temperature 90 °C.

3.3.3 Investigation of phase separation conditions

Precipitated material was collected from the feed system - 80% EtOH, 10% of t.s.c. AC, 90% of t.s.c. lysozyme, 0.5% t.s.c., on a 0.2 μm filter by filtering the suspension under vacuum, and dried at 40 °C with desiccant overnight. SE micrograph of the collected material showed large irregular shaped particles morphologically similar to unprocessed material (Figure 3.18).

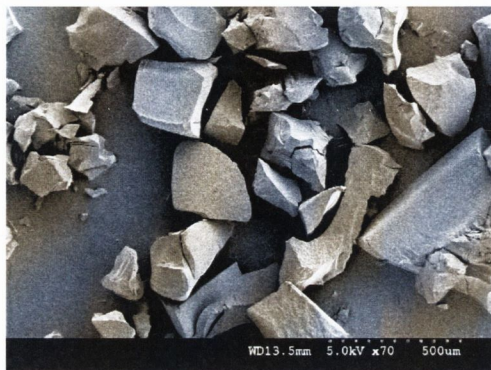


Figure 3.18 SE micrograph of collected and dried precipitate from 80% EtOH, 10% of t.s.c. AC, 90% of t.s.c. lysozyme, 0.5% t.s.c.

Phase separation of a protein in solution (precipitation), can be described as a reduction in solubility such that most of the molecules can no longer stay in solution (Maa and Prestrelski, 2000), and is classified as reversible (in which the protein structure is recovered on dissolution) or irreversible (in which the protein structure is damaged and not recoverable on dissolution). In their native (solution) state, globular proteins have non-polar amino acid side chains oriented towards the interior of the protein and polar side chains oriented outwards. The stability of this conformation is influenced by hydrophobic interactions within the interior of the molecule, the electrostatic interactions and hydrogen bond interactions at the protein-water interface. Disruption of these interactions may alter the balance between the intra- and intermolecular interactions. Protein-protein contact increases and this can lead to aggregation and ultimately precipitation or, depending on the conditions, crystallisation (Pingoud et al., 2002). Phase separation can be induced by adjusting the pH of a solution towards the protein's isoelectric point, by change in temperature, by addition of an anti-solvent, or by addition of a salt.

Isoelectric precipitation is based on the fact that proteins exhibit minimal solubility at their isoelectric point. This is not a strong precipitation technique alone, because of the limited pH range at which proteins may be stable and also because many proteins may still show solubility even at the pI (Maa and Prestrelski, 2000).

Considering the case of salt-induced precipitation, this may depend on the type of protein, type of salt and concentration of salt. Different anions or cations are found to have different effectiveness

for “salting out” proteins and these have been arranged in a series, referred to as the Hofmeister series (Figure 3.19) (Zhang and Cremer, 2006). From this series the ions contributed by AC would be expected to have a strong protein precipitating power.

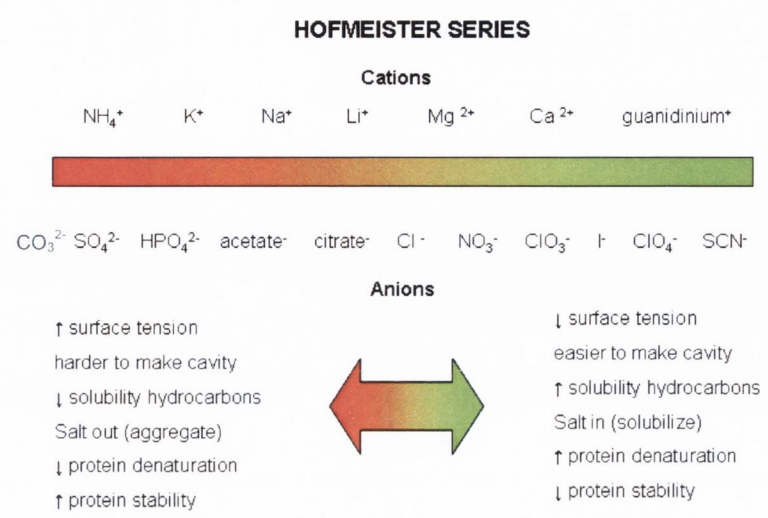


Figure 3.19 Summary of Hofmeister series – relative effectiveness of cations or anions to precipitate proteins, adapted from Zhang and Cremer (2006), www.tinyurl.com/ed5gj.

Addition of miscible organic solvent – primarily methanol, diethyl ether, acetone and ethanol are also used to precipitate protein from solution. The organic cosolvent decreases the solvation power of water, and also decreases the dielectric constant (Porter, 1997; Maa and Prestrelski, 2000).

Phase separation of lysozyme from the alcohol:H₂O:AC systems has been observed to depend on the concentration of alcohol and AC in this work, and was thus postulated to be achieved by a combination of effects being addition of salt, and addition of anti-solvent (alcohol). The effect of change in solution pH by the addition of AC may also be important however and it was decided to look in more detail at these effects.

The effect on the pH of aqueous lysozyme solution by the addition of AC was investigated by measuring solution pH at different concentrations of lysozyme and AC (Figure 3.20). Addition of AC to lysozyme solution (pH ~ 4) resulted in a pH of ~ 8 - 8.5 (towards the isoelectric point of ~11.35) in the aqueous component, similarly for all concentrations of AC and lysozyme (product no.: L6876, L7651) investigated. [The pH of the aqueous solution component only was considered as pH relates to purely aqueous conditions with considerable difficulty associated with interpretation and theoretical meaning of pH in non-aqueous or co-solvent solution (Stevenson, 2000).]

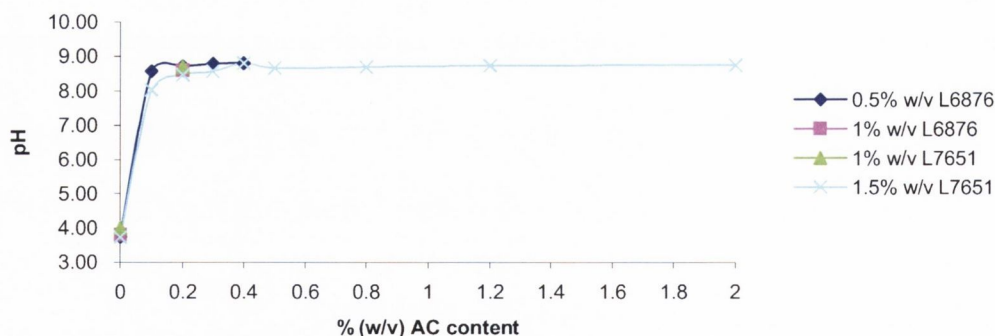


Figure 3.20 pH of solutions containing different concentrations of lysozyme and AC.

The effect of pH change, without the addition of the AC salt, on phase separation and spray-dried morphology from 80% EtOH was investigated, by adjusting the pH of lysozyme dissolved in H₂O component to ~8 using 1M KOH, prior to the addition of EtOH (to 80% v/v) and spray-drying (inlet temperature 78 °C). No phase separation was found in this system and spray-drying resulted in non-porous spherical or partially collapsed spheres (Figure 3.21), similar to particles produced from these feed conditions without pH adjustment (Section 3.3.2, Figure 3.14). This suggested that a pH effect similar to the addition of AC and anti-solvent effect by addition of EtOH (80%) were not sufficient to produce a phase separation, without the salt.



Figure 3.21 SE micrograph of lysozyme spray-dried from 80% EtOH, inlet temperature 78 °C, pH of water component adjusted to pH 8 using 1M KOH.

In order to further probe the effect of the salt and pH effects, other ammonium salt preparations - ammonium hydrogen carbonate (AHC), ammonium formate (AF), ammonium acetate (AA), were investigated in place of AC, as all these preparations result in lower solution pH, have different physicochemical properties, contain different anion species and contain different proportions of ammonia. AHC is most closely related to AC in that AC is a mix of variable proportions of AHC and ammonium carbamate, comprising at least 30% by weight NH₃ (www.sigmaaldrich.com). AHC alone comprises ~ 22% by weight NH₃. It is a slightly weaker base than the mix ammonium carbonate, with a pH of 0.1N aqueous solution at 25 °C of 7.8 (www.sigmaaldrich.com). It is

similarly volatile in solution and releases ammonia and CO₂ on decomposition, and is similar also in that it is insoluble in EtOH. AHC was considered as an alternative process enhancer to investigate whether this small difference in pH and ammonium content in a very similar preparation impacted on NPMP production. Lysozyme was spray-dried from 80% EtOH with 5% and 10% of t.s.c. AHC, inlet drying temperature 78 °C, as comparison to similar systems using AC as the process enhancer. These particles had a similar morphology, assessed by SEM analysis (Figure 3.22), to comparative particles spray-dried using AC (Figures 3.13 (b), 3.14) in that 10% of t.s.c of process enhancer resulted in NPMPs and 5% resulted in spherical particles with a rough non-porous surface. In the example of 10% of t.s.c. AHC, there appeared to be slightly more non-porous large (> 5 µm) particles present than for AC. Measured pH of lysozyme with 10% AHC in water was 7.4, and with 5% AHC was 7.2.

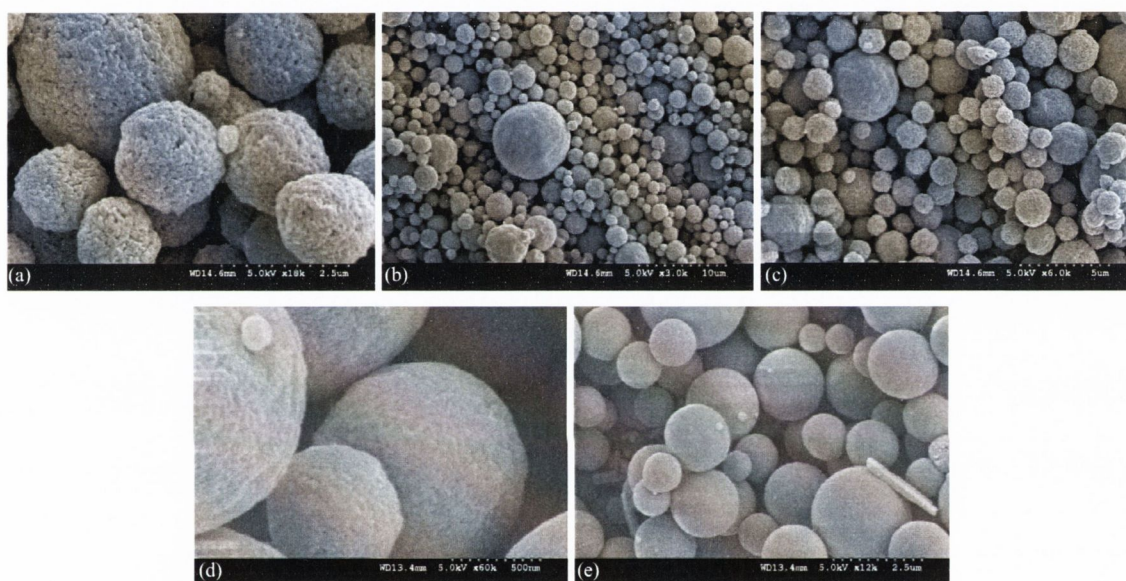


Figure 3.22 SE micrographs of lysozyme spray-dried from 80% EtOH, 0.5% t.s.c, inlet temperature 78 °C with (a), (b), (c) 10% of t.s.c. AHC, taken at different magnifications; (d), (e) 5% of t.s.c. AHC, taken at different magnifications.

Ammonium formate and ammonium acetate are more acidic than AC with reported pH (1M in H₂O at 25 °C) of 5.5 - 7.5 and 6.2 - 7.5, respectively (www.sigmaaldrich.com). They also have higher melting points of 119 - 121 °C and 110 - 112 °C, respectively, and are more soluble in alcohol (www.sigmaaldrich.com). AF comprises ~ 27%, and AA comprises ~ 18% by weight ammonia. They may not be completely volatilised during the spray-drying process with inlet drying temperatures of 78-90 °C and there is a potential for residual contamination of formic or acetic acid in the spray-dried product. They were thus not considered as desirable process enhancers but were examined to give some information on the conditions of phase separation and NPMP formation. Inlet drying temperatures were increased to 90 °C and 80% EtOH solvent system was chosen. A higher quantity of AF or AA were predicted to be required to produce phase separation and

NPMPs, based on their lower solution pH and lower precipitating power of the contributed anions from the Hofmeister series (Zhang and Cremer, 2006; www.tinyurl.com/ed5gj). Lysozyme was initially spray-dried with 27.3% (pH of lysozyme and AF in H₂O component 5.45), 40%, 50% and 60% w/w of t.s.c ammonium formate. All systems were spray-dried as cloudy suspensions, indicating phase separation. Non-porous spherical particles were obtained with the lowest AF content in feed material. These particles appeared to be smaller than any of the other systems spray-dried (Figure 3.23 (a)). Spray-dried systems including 40% AF content resulted in spherical particles with a more brain-like or highly folded surface morphology (Figure 3.23 (b)) rather than the fused irregular shaped nano-particulate surface seen with 80% EtOH/10% AC systems. Spray-dried systems including 50% and 60% AF displayed similar morphology with some holes identified in the surface (Figure 3.23 (c), (d)).

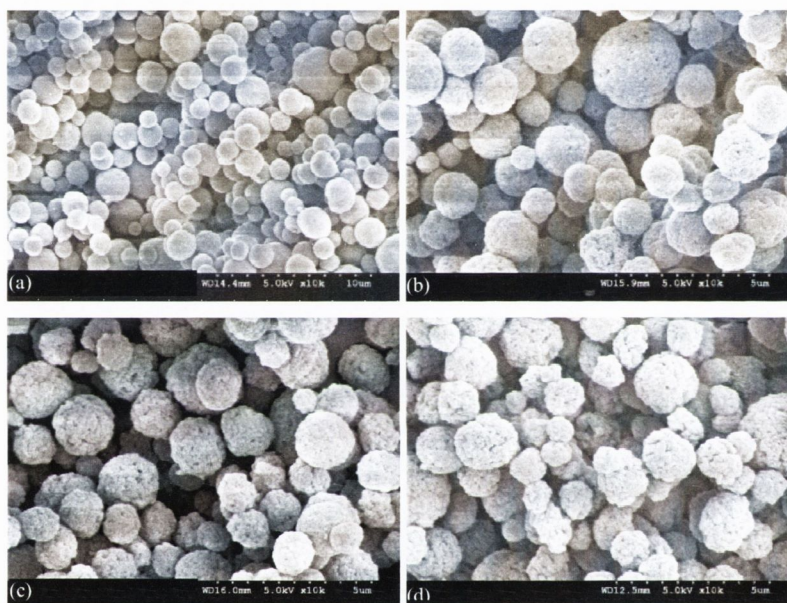


Figure 3.23 Lysozyme spray-dried with AF from 80% EtOH, inlet temperature 90 °C (a) 27.3% of t.s.c. AF (b) 40% of t.s.c. AF (c) 50% of t.s.c. AF (d) 60% of t.s.c. AF.

Based on the results with AF, lysozyme was spray-dried with 40% (pH of AA and lysozyme in H₂O component 6.21), 50% (pH of AA and lysozyme in H₂O component 6.6), and 60% w/w of t.s.c. ammonium acetate (pH of AA and lysozyme in H₂O component 6.76) from 80% EtOH. All feed material showed phase separation prior to spray-drying. Non-porous spherical particles with a rough surface were obtained with the lowest AA content in feed material (Figure 3.24 (a)). Spray-dried systems including 50% AA and 60% AA content resulted in spherical particles with a brain-like or highly folded surface morphology similar to 40% AF (Figure 3.24 (b), (c)).

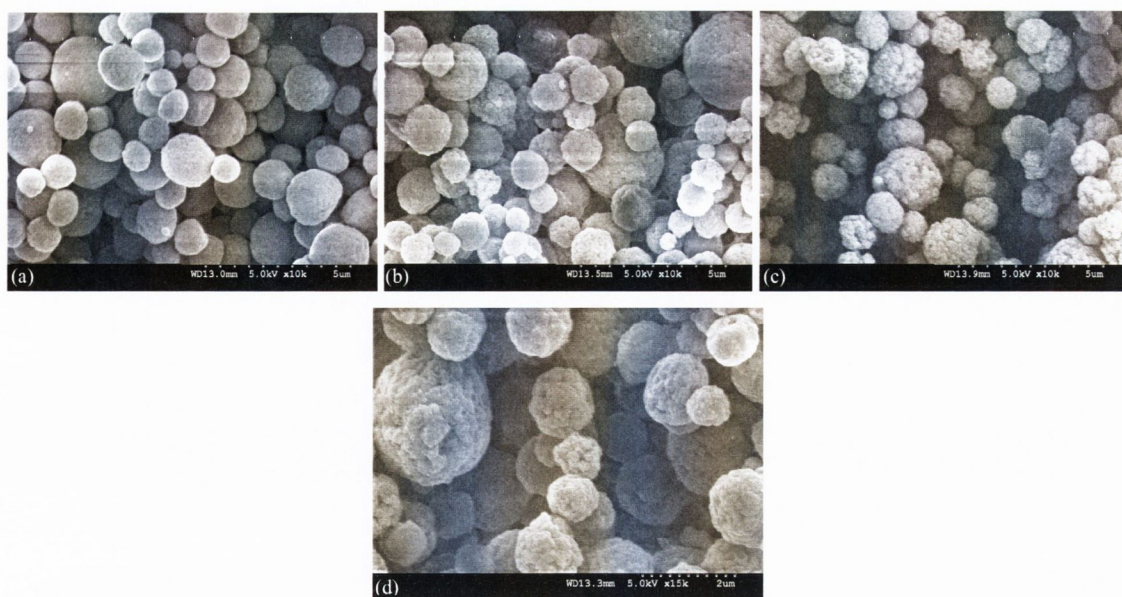


Figure 3.24 SE micrographs showing lysozyme spray-dried with AA from 80% EtOH, inlet temperature 90 °C (a) 40% of t.s.c. AA (b) 50% of t.s.c. AA (c) 60% of t.s.c. AA (d) Higher magnification of system spray-dried with 50% of t.s.c. AA.

3.3.4 Spray-dried lysozyme from water

Lysozyme was spray-dried from H₂O with or without AC, as comparison spray-dried systems for stability and particle characterisation, and to investigate the effect of AC on the protein spray-dried from simple aqueous systems. Spray-drying was carried out in the open mode configuration with air as drying gas. An intermediate inlet drying temperature of 120 °C was chosen, to ensure adequate drying of the powder under these conditions and to minimise thermal stresses to the protein. Lysozyme was initially spray-dried from aqueous solution at a t.s.c. of 0.3% and 16.67% of t.s.c. being AC, similar to initial studies involving spray-drying from MeOH:H₂O:AC systems. This was then increased to a t.s.c. of 0.5% and either 20% AC or no AC in subsequent spray-drying experiments to increase collectable product yield, and for the purpose of further characterisation. All systems were spray-dried as clear solutions.

Inclusion of AC had an effect on the morphology of the particles as noted from SEM analysis. Inclusion of AC, particularly at the higher 20% of t.s.c. level resulted in a greater amount of collapsed particles and indentations (marked by red arrows) in particle surface compared to the relatively uniform spherical shape of lysozyme spray-dried without AC under similar conditions (Figure 3.25). Particles appeared to be hollow as noted from these collapsed particles. All particles spray-dried from aqueous solution had a smooth non-porous surface.

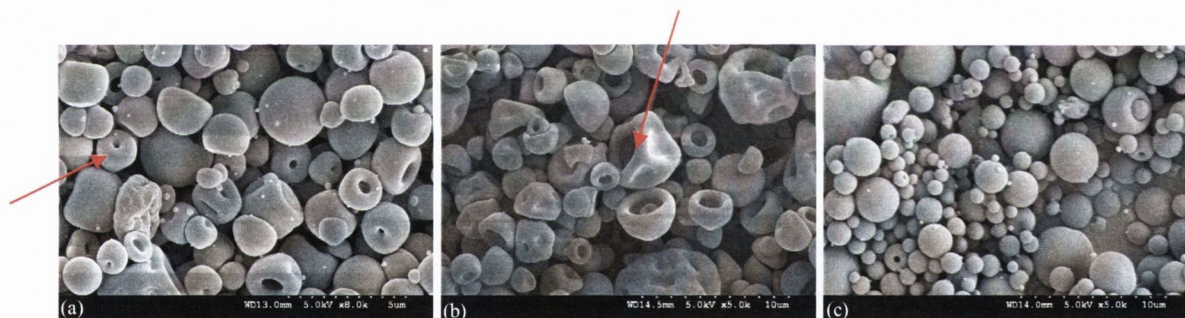


Figure 3.25 SE micrograph of lysozyme spray-dried from aqueous solution at inlet drying temperature 120 °C (a) 0.3% t.s.c., 16.67% of t.s.c. AC (b) 0.5% t.s.c., 20% of t.s.c. AC (c) 0.5% t.s.c. no AC.

3.3.5 Spray-dried lysozyme from methanol:n-butyl acetate (non-aqueous)

Spray-drying lysozyme from MeOH:BA non-aqueous systems was considered based on results by Li (2005) with the water soluble excipients polyvinylpyrrolidone (PVP) and hydroxypropyl- β -cyclodextrin, and in this present work with sugar excipients – trehalose and raffinose (Chapter 4). Lysozyme was initially freeze-dried from aqueous solution (pH ~6) to increase solubility in methanol (Section 3.2). Reported solubilities for similarly prepared freeze-dried lysozyme are > 10 mg/ml in MeOH or > 5 mg/ml, and < 0.05 mg/ml in n-butyl acetate (Chin et al., 1994; Bromberg and Klibanov, 1995). Unlike the alcohol:water:AC systems this solvent system more accurately satisfies the hypothesis of the mechanism underlying the method for producing NPMPs (based on observations for low molecular weight compounds), in that the drug should be soluble in the more volatile solvent (methanol – b.p. 65 °C) and less soluble in the less volatile component (n-butyl acetate – b.p. 126 °C) (Corrigan, 2005).

Freeze-dried lysozyme was dissolved in the MeOH component with stirring, before the BA component was added. Solvent ratios of 80:20 and 1:1 were selected based on previous studies with non-protein excipients (Li, 2005; Chapter 4). Low total solid concentration of 0.2% and 0.15% w/v were selected because of the very low solubility of lysozyme in BA. The solvent ratio system of 80:20 was spray-dried as a solution. Some phase separation was noted on addition of the BA solvent in the solvent ratio of 1:1 and was spray-dried as a fine suspension. Spray-drying was carried out in the closed mode configuration (conditions detailed in Appendix 1), with an inlet drying temperature of 100 °C. Porous particles were not produced using either solvent ratio, but particles were mainly spherical and had a rough surface morphology (Figure 3.26).

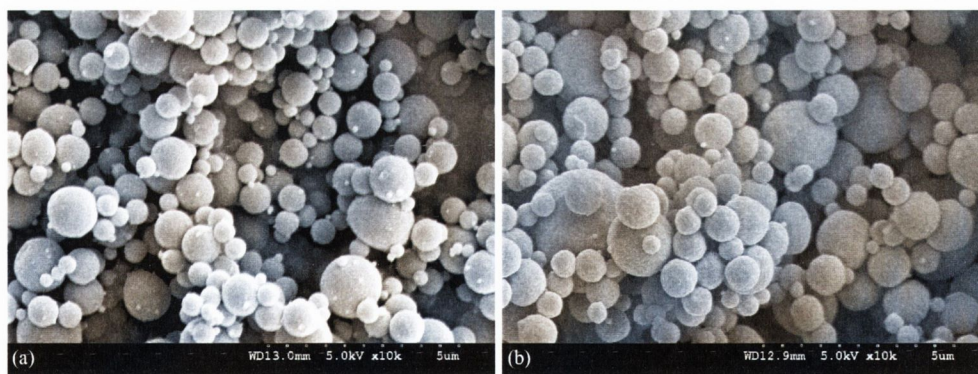


Figure 3.26 SE micrographs showing lysozyme spray-dried from (a) 80:20 MeOH:BA, (b) 1:1 MeOH:BA; inlet temperature 100 °C.

3.4 PROCESS STABILITY

3.4.1 Solid-state characterisation and thermal analysis

Spray-dried porous particles (from 80% EtOH, 0.5% t.s.c., 10% of t.s.c. AC, inlet temperature 78 °C, Figure 3.13 (b)) and non-porous particles (from H₂O, 0.5% t.s.c., inlet temperature 120 °C with 20% of t.s.c. AC or without AC Figure 3.25 (b), (c)) of lysozyme were compared in terms of XRD, DSC and TGA. All spray-dried powders were amorphous by XRD, with an absence of characteristic sharp peaks and a halo pattern, as was found for freeze-dried and unprocessed lysozyme (Section 3.2). The shapes of the XRD traces were similar for all spray-dried particle morphologies (Figure 3.27).

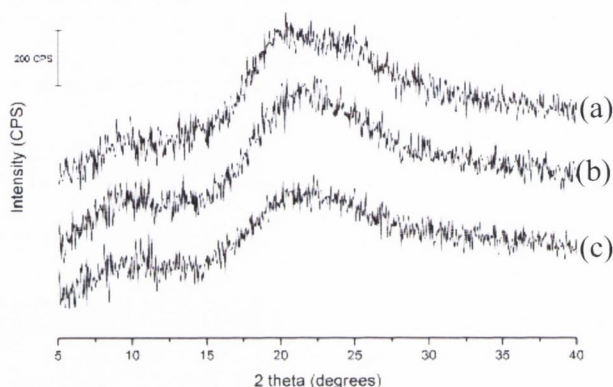


Figure 3.27 XRD of (a) Porous lysozyme (b) Non-porous lysozyme spray-dried without AC (c) Non-porous lysozyme spray-dried with AC.

DSC traces (Figure 3.28) showed a slight difference for the porous protein particles compared to the non-porous spray-dried and unprocessed material. All spray-dried samples showed broad endotherms from 25-160 °C and apparent denaturation endotherms at ~ 200 °C. This was smaller and less sharp in the porous sample compared to the other samples however. All spray-dried samples showed a subsequent broad endotherm with a peak ~ 220-225 °C, which was slightly later than an equivalent peak seen in the unprocessed material (~210 °C). This endotherm appeared to be most pronounced in the porous sample. DSC analyses of proteins are quite complicated and generally require modulated DSC to separate overlapping thermal events. Apparent thermal degradation temperature (T_m) can be identified from conventional DSC however and may give information on protein structure, as a change in or lack of a T_m peak has been associated with changes in native protein structure or denaturation (Hill et al., 2005; Elkordy et al., 2002, 2004). The change in shape of the T_m peak and in the area of decomposition in the porous sample may thus suggest some change in the protein structure.

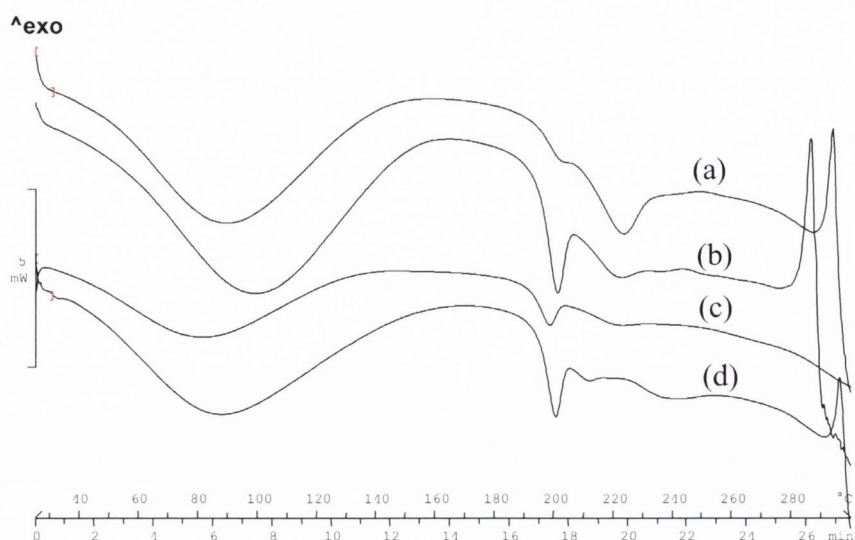


Figure 3.28 DSC of (a) Porous lysozyme (b) Non-porous lysozyme spray-dried without AC (c) Non-porous lysozyme spray-dried with AC (d) Unprocessed lysozyme.

The shapes of TGA plots were similar for all spray-dried samples. Mass loss over 25-100 °C calculated from TGA was similar for all spray-dried lysozyme samples, showing an initial one-step mass loss of ~ 4-5%, indicative of residual moisture loss from amorphous spray-dried material (Figure 3.29). Further mass loss occurred at a slower rate until ~ 200 °C, when mass loss increased from 200-300 °C (~ 17%), corresponding to decomposition in the DSC scan.

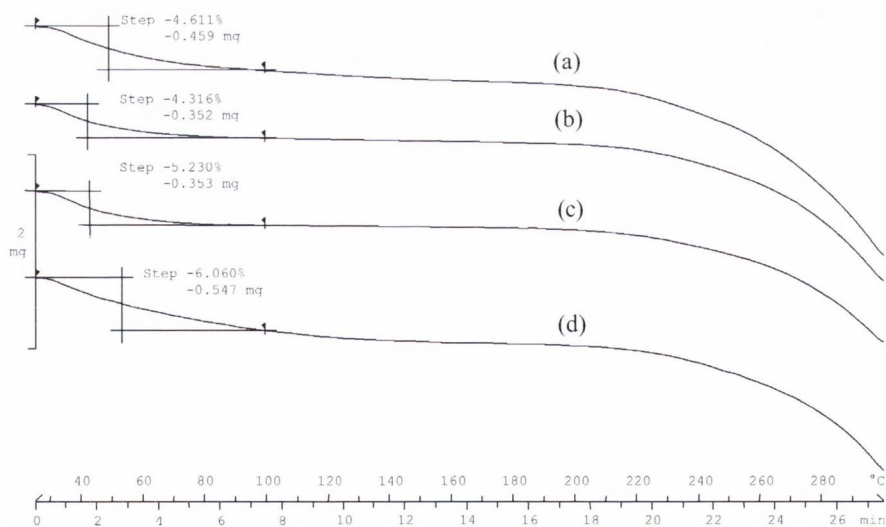


Figure 3.29 TGA of (a) Porous lysozyme (b) Non-porous lysozyme spray-dried without AC (c) Non-porous lysozyme spray-dried with AC (d) Unprocessed lysozyme.

DSC and TGA of the collected precipitate (Section 3.3.3) and of lysozyme spray-dried from different EtOH:H₂O:AC systems were further investigated. The DSC and TGA of the collected

precipitate was unlike the model porous lysozyme system (Figures 3.28 (a), 3.29 (a)), particularly in the temperature area of decomposition (Figure 3.30). DSC showed a broad endotherm between 25-140 °C followed by a second endotherm beginning at ~ 206 °C, similar but broader than the corresponding T_m peak seen in unprocessed lysozyme, and followed by an exothermic (~240 °C) and disordered pattern. The TGA plot also showed a difference in shape compared to spray-dried or unprocessed material. A continuous mass loss was observed from 25 - ~200 °C, until a sharp step decrease in mass, at the same temperature as the beginning of this endothermic decomposition peak. This data suggested that the collected precipitate also showed some change in the folding of the protein, but not directly comparable to the model porous lysozyme system examined. Changes in the DSC and TGA of the precipitate may have been influenced by the feed conditions and also by exposure to vacuum during collection or temperature during drying, whereas changes in the DSC of the model porous system may have been influenced by the feed conditions and by spray-drying stresses.

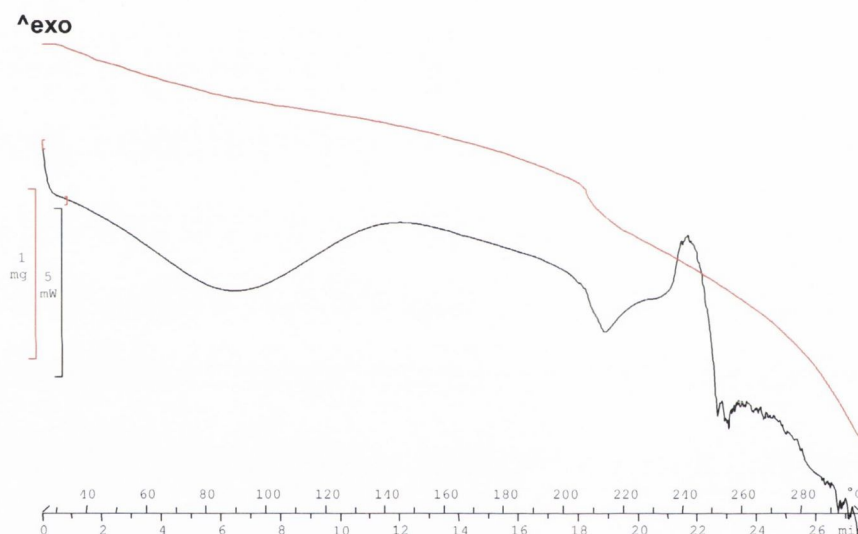


Figure 3.30 TGA (in red) and DSC (in black) traces of the collected precipitate (Section 3.3.3).

Analysis of the DSC of spray-dried lysozyme from different EtOH:H₂O:AC systems (Figure 3.31) showed that lysozyme spray-dried from 50% EtOH; 10% of t.s.c. AC, inlet temperature 78 °C showed a large distinct endotherm at ~ 200 °C. All the other systems investigated showed smaller peaks in the same area or evidence of an endothermic peak super-imposed on a following larger broad endotherm, similar to the model porous system investigated. Changes in the T_m and patterns of decomposition seen in DSC were found to depend on feed conditions, although a direct relationship with phase separation in the feed or production of NPMPs was not found. TGA data (Figure 3.32) were similar to spray-dried porous and non-porous lysozyme (Figure 3.29) showing an initial area of mass loss from 25-100 °C followed by a slower mass loss, and finally a more rapid mass loss due to decomposition from 200 – 300 °C.

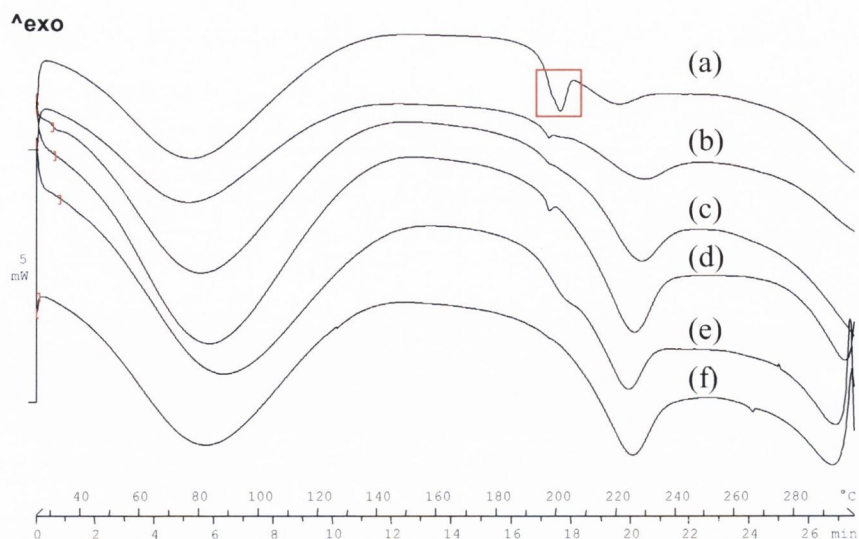


Figure 3.31 DSC of lysozyme spray-dried from (a) 50% EtOH; 10% of t.s.c. AC (b) 60% EtOH; 10% of t.s.c. AC (c) 65% EtOH; 10% of t.s.c. AC (d) 70% EtOH; 10% of t.s.c. AC (e) 75% EtOH; 10% of t.s.c. AC, at 78 °C and from (f) 80% EtOH 5% t.s.c. AC at 85 °C.

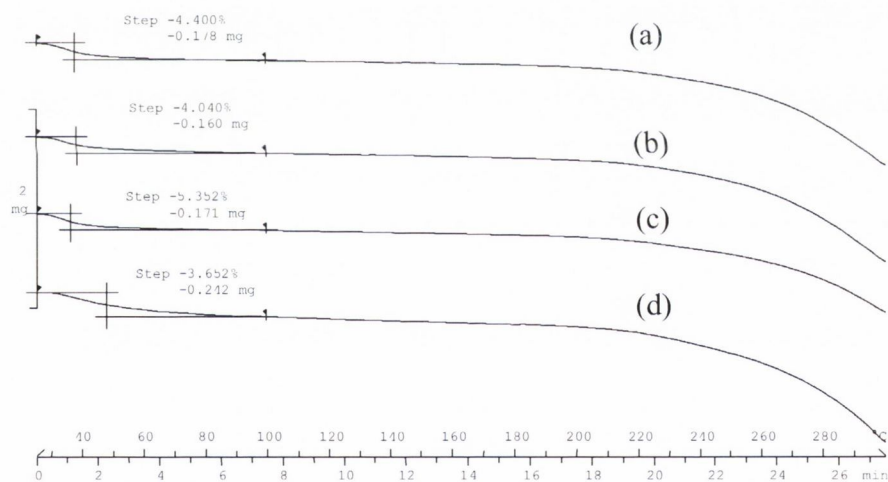


Figure 3.32 TGA of lysozyme spray-dried from (a) 50% EtOH; 10% of t.s.c. AC (b) 60% EtOH; 10% of t.s.c. AC (c) 70% EtOH; 10% of t.s.c. AC, at 78 °C and from (d) 80% EtOH 5% t.s.c. AC at 85 °C.

DSC and TGA data of lysozyme spray-dried with the alternative process enhancers AF (60%), AA (60%) were examined to ascertain whether any contamination by these process enhancers could be identified from the shapes of these scans (Figure 3.33). DSC scans showed initial broad endotherms from 25 - ~120 °C, followed by a second broad endotherm from 120 - ~160 °C (which was not found in other spray-dried lysozyme samples without AF or AA). TGA of the samples, showed an initial mass loss from 25 - ~80 °C, followed by a second step of mass loss to ~180 °C, before decomposition above 200 °C. The magnitude of these mass losses were not equivalent to

the ammonium salt content (60%) in the total solid content of the feed system prior to spray-drying however, indicating that some of the process enhancer was removed during spray-drying or that contamination in the sample was not removed over the temperature range studied in DSC and TGA experiments (25 – 300 °C).

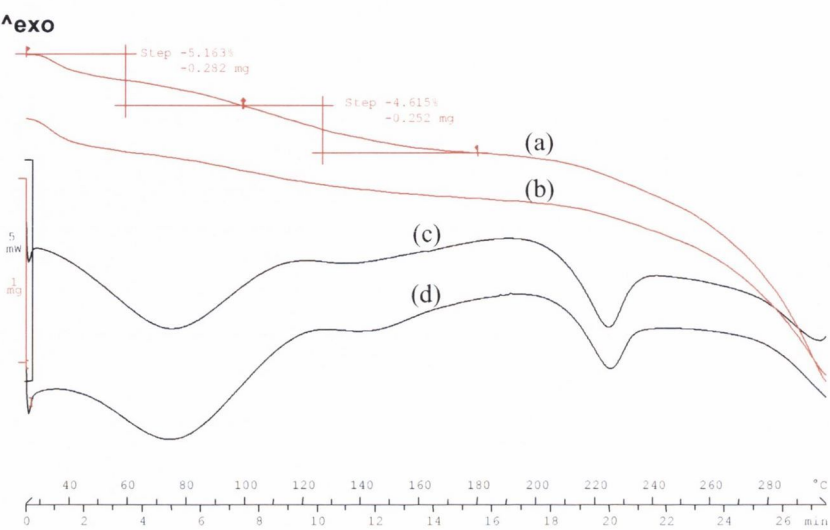


Figure 3.33 DSC (in black) and TGA (in red) of (a), (c) lysozyme spray-dried with 60% AA, and (b), (d) lysozyme spray-dried with 60% AF.

DSC and TGA of lysozyme spray-dried from non-aqueous MeOH:BA solvent systems (Figure 3.34) were examined. DSC showed initial broad endotherms from 25-140 °C followed by a second broad endotherm beginning at ~ 200 °C with a peak at ~ 217-220 °C. No sharp endothermic peak at Tm could be identified due to this overlapping peak. TGA data showed an initial mass loss, followed by larger more gradual mass loss in the second stage of mass loss than seen for other spray-dried samples before a rapid mass loss due to decomposition. Calculated residual solvent or moisture losses were 2.8, 5.1% over the temperature range of 25 – 100 °C, respectively for lysozyme spray-dried from the solvent ratios of 80:20 and 1:1 MeOH:BA.

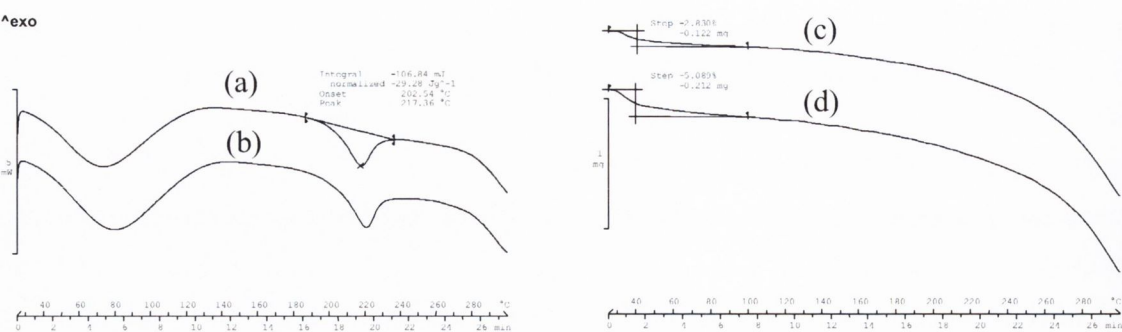


Figure 3.34 DSC and TGA of lysozyme spray-dried from (a), (c) 80:20 MeOH:BA, and (b), (d) 1:1 MeOH:BA; inlet temperature 100°C.

3.4.2 Secondary structure

Solid state FTIR was used to assess changes in protein secondary structure. Spectra were collected as described (Section 2.2.12). Comparison of spectra was carried out in order to investigate changes in the spray-dried versus the native state. Visual comparison may suffice to detect deviations, although to give a semi-quantitative measure of this, the area overlap method or the correlation coefficient method have been proposed (Van de Weert et al., 2005). These can be calculated using raw spectra or band-narrowed spectra (for example by applying second derivation or fourier self-deconvolution functions). In this work, raw spectra were used as they essentially contain the same structural information and are less affected by operator bias, signal/noise ratio and/or water vapour contributions (Van de Weert et al., 2001).

Inter-run repeatability was checked by replicate FTIR measurements of unprocessed material, showing good repeatability with area of overlap of amide 1, 2, 3 peaks $\sim 95 - 99.7\%$ although one run in the amide 3 peak area showed an apparently different shape that was not reflected in the high % area of overlap (95%) (Figure 3.35). Inter-batch reproducibility for the amide 1 band was checked by analysis of 3 batches of porous spray-dried lysozyme compared to unprocessed material (Figure 3.36). Results showed a high level of reproducibility with a standard deviation in area of overlap compared to unprocessed material of 0.72%.

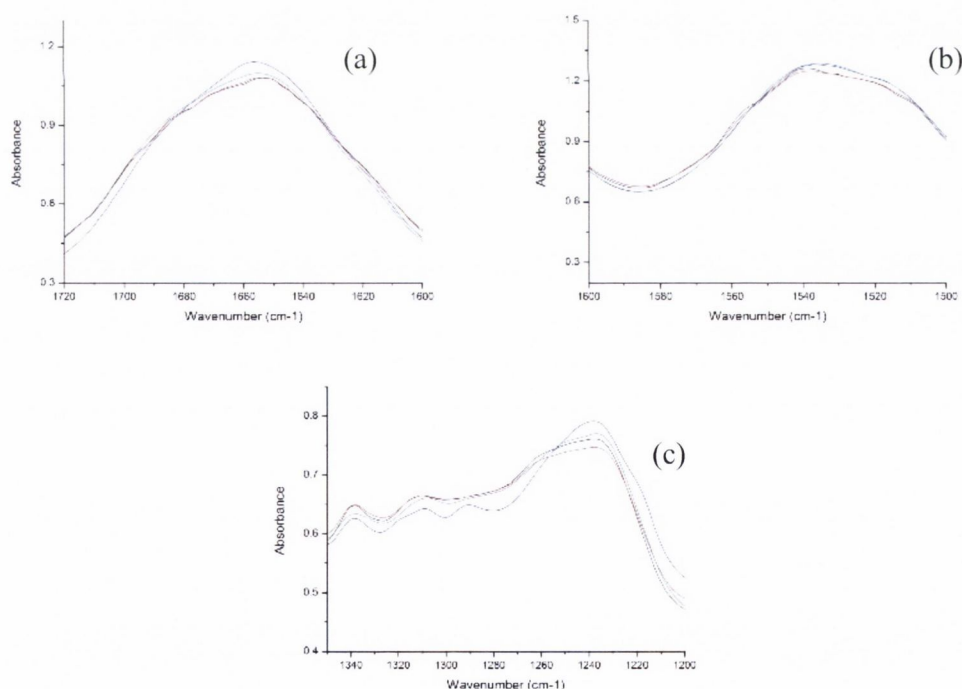


Figure 3.35 Inter-run repeatability for 4 runs of unprocessed lysozyme (L7651), each run represented by a different colour (a) amide 1, (b) amide 2, (c) amide 3 areas using spectra processing/calculation method 1.

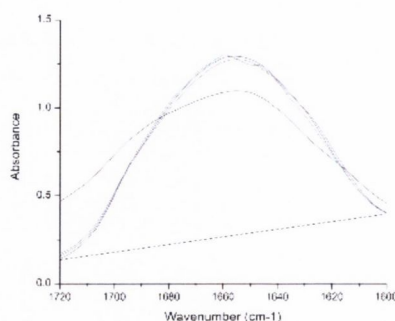


Figure 3.36 Inter-batch reproducibility for 3 batches of porous spray-dried lysozyme (coloured lines) vs unprocessed material (black line) using spectra processing/calculation method 1.

The % area of overlap between the area-normalised amide 1 peak of spray-dried porous or non-porous and unprocessed lysozyme were calculated (Table 3.3). All spray-dried samples showed a change in the shape of the amide 1 peak, as reflected in the area of overlap calculations.

On analysis of the data, the difference in the baseline of the isolated amide peak between samples processed differently in this work is likely to have contributed to the measurement of area of non-overlap. The data were thus recalculated with initial manual correction of the individual peak baseline by subtraction of a straight line in Origin software across the base of the peak (dotted line shown in Figure 3.36) prior to area normalisation, and calculation of the absolute difference spectrum and area of non-overlap as before. This calculation method is similar to that described by van de Weert (2001) in that baseline correction of the individual isolated peaks was performed.

Using this calculation method (calculation method 2), inter-run repeatability calculated for unprocessed material for the amide 1 peak showed good repeatability in the amide 1 area (area of overlap 98-99%) and in the amide 3 peak area for 3 of the runs (~99%) (Figure 3.37). The amide 3 area of a fourth run showed a difference in peak shape, similar to that obtained by the previous calculation method. In this method of calculation the difference was more apparent in the % area overlap however with overlap with the other runs of ~ 82%. This suggested different sensitivities of the two calculation methods. Because of the high inter-run repeatability of the amide 1 region and the preponderance of analyses in the literature using this peak, this area was chosen for characterisation of spray-dried samples.

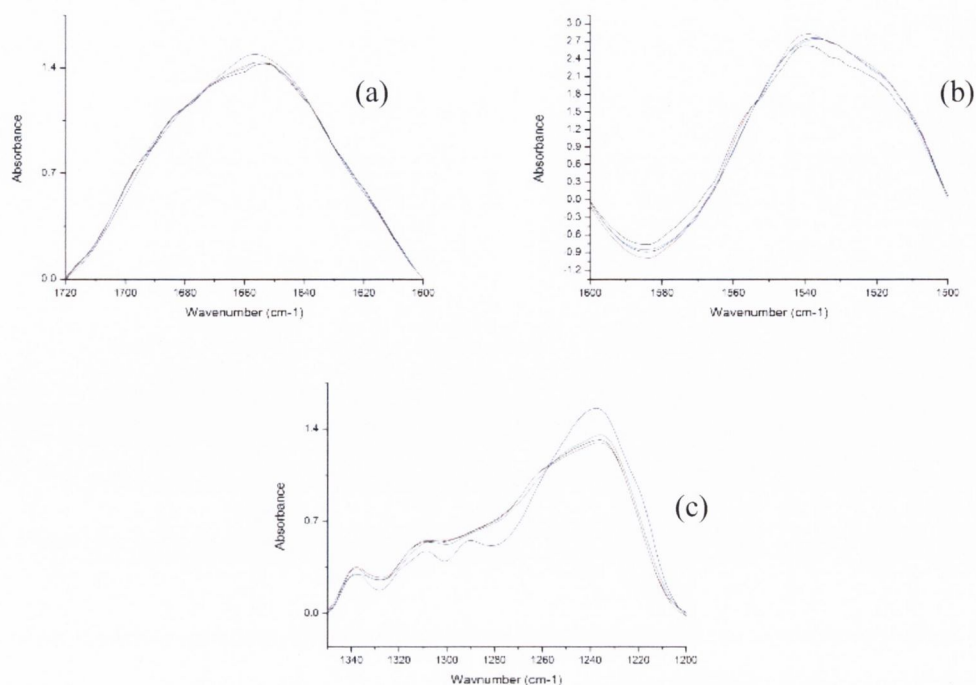
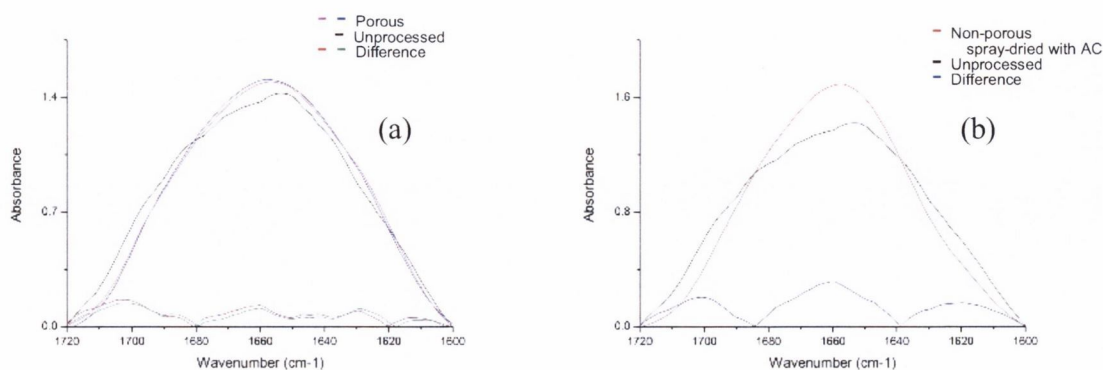


Figure 3.37 Inter-run repeatability for 4 runs of unprocessed material (a) amide 1, (b) amide 2, (c) amide 3 peak areas, using spectra processing/calculation method 2.

Average area of overlap between porous particles and unprocessed material in the amide 1 region was $91.5 \pm 0.52\%$ ($n=3$) (Figure 3.38 (a)). Visual inspection of normalised peaks confirmed a difference between the shapes of the spectra. Results are presented for porous and non-porous lysozyme spray-dried with/without AC vs unprocessed material in the amide 1 region (Figure 3.38, Table 3.3). This calculation method resulted in higher % area overlap for spray-dried vs unprocessed material, compared to the previously used automatic baseline correction of the entire spectrum, but similarly showed change in the shape of the amide 1 peak area, suggesting some change in the secondary structure. Data could also be more intuitively visually compared by this calculation method.



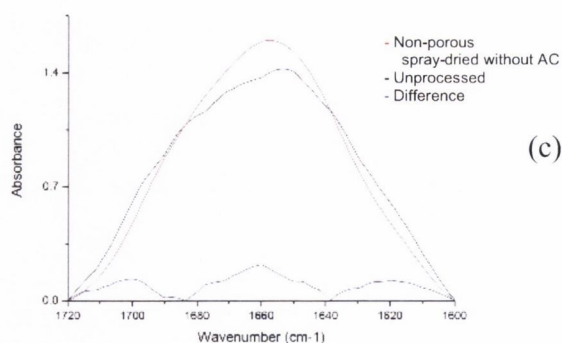


Figure 3.38 Area-normalised, baseline-corrected amide 1 peaks of spray-dried vs unprocessed material and absolute difference peaks (a) porous lysozyme (b) non-porous lysozyme spray-dried with AC (c) non-porous lysozyme spray-dried without AC.

Table 3.3% Area overlap of area-normalised amide 1 peaks of spray-dried with unprocessed lysozyme (at different spray-drying conditions), using calculation method 1 (whole spectrum baseline correction) or calculation method 2 (individual peak baseline correction).

Lysozyme system	Calculation method 1	Calculation method 2
	% Area overlap of Amide 1 peak	% Area overlap of Amide 1 peak
Porous	82.7 ± 0.72%	91.3 ± 0.52%
Non-porous with AC	77.4%	83.6%
Non-porous without AC	84.1%	88.7%

Van de Weert et al. (2001) cautioned against the interpretation of spectra of materials with different levels of hydration i.e. very dry lyophilised protein spectra vs unprocessed material spectra as different levels of hydration may affect spectra. Spray-dried samples did not show higher dehydration compared to unprocessed material from TGA data however. As such dehydration effects were not considered the only contributor to change in spectra, seen in this work. The amide 1 peaks of porous and non-porous lysozyme spray-dried without AC were compared to each other to examine spray-dried samples with similar levels of hydration and different methods of preparation. A difference between these spray-dried samples was observed visually (Figure 3.39 (a)), with a calculated area of overlap of 93.6%. Analysis of the collected precipitate, dried overnight at 40 °C (Section 3.3.3) showed deviation from unprocessed material and an area of overlap of 87.8%. This difference, as well as the difference between spray-dried porous/non-porous samples, suggests a role in feed conditions in the change seen.

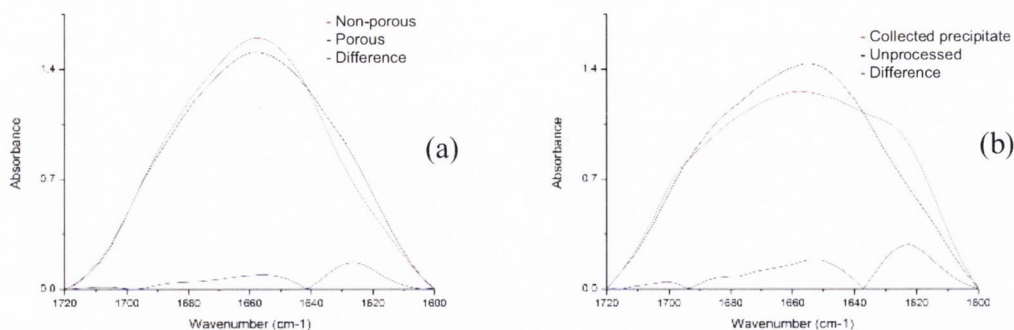


Figure 3.39 Area-normalised, baseline-corrected amide I peaks and absolute difference spectra (a) porous vs non-porous spray-dried lysozyme (b) collected precipitate vs unprocessed material.

Liquid FTIR analysis was carried out on porous lysozyme to compare the amide I peak shape to unprocessed material in solution. Peak shape has been observed to be different in the solution and solid state (Van de Weert et al., 2001). 50 mg/ml protein solution was prepared and analysed in a liquid cell using a path-length of 0.006 mm. Unprocessed protein, was used as a standard and heat denatured protein (prepared by exposing protein solution to 40 °C temperature overnight in an oven) was used to check the ability of the method to detect change in structure in a heat-denatured protein. Porous lysozyme showed a visually similar peak to unprocessed protein and an area overlap of 94.2%, suggesting similarity of secondary structure in solution. Heat denatured protein showed a shift in the amide I peak (Figure 3.40) and % area of overlap of 57% with unprocessed protein.

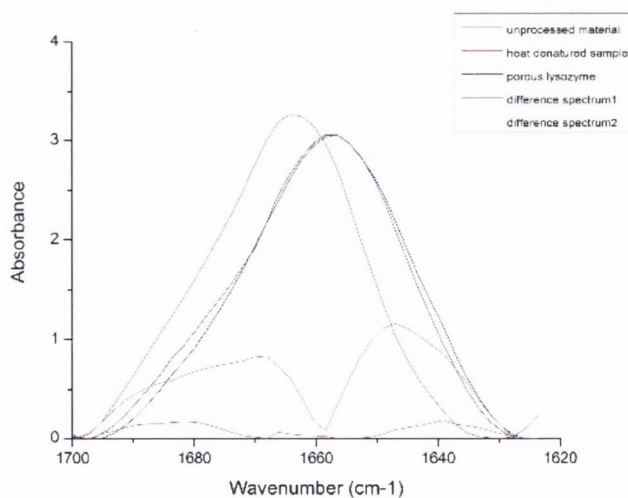


Figure 3.40 Area-normalised amide I peaks showing unprocessed protein, heat denatured protein, porous lysozyme and absolute difference spectra – 1 (unprocessed-heat denatured sample). – 2 (unprocessed-porous).

3.4.3 Biological activity assay

Spray-dried porous particles (from 80% EtOH, 0.5% t.s.c., 10% of t.s.c. AC, inlet temperature 78 °C) and non-porous particles (from H₂O, 0.5% t.s.c., inlet temperature 120 °C with 20% of t.s.c. AC or without AC) of lysozyme were compared in terms of biological activity using a turbidimetric rate determination assay based on the lysis of a *Micrococcus lysodeikticus* suspension. Micro BCA protein analysis was carried out on test solutions to correct for any difference in protein content, as a result of solution preparation, residual moisture, solvent or AC contamination in the powders. Unprocessed lysozyme (control) was assayed with the spray-dried samples. ANOVA was carried out on assay results to assess differences between spray-dried samples and control. Activity is expressed as % of the activity of the control and % of the specific activity of control (samples corrected for protein content of test solution), considering the pooled standard deviation from the data. Excellent retention of activity was found for these spray-dried powders produced, with no statistically significant differences in either activity/specific activity compared to the control solution. Micro BCA analysis of the amounts of protein in test solutions also showed no statistically significant difference between batches. Similarity between test solution protein concentrations suggests protein content in the spray-dried powders were similar. The results from one batch of each spray-dried material are presented below (Table 3.4). Inter-batch variability was found to be good, with results from other batch assays giving similar specific activity of 102.8, 100.8 ± 6.97% and activity of 95.6, 102.9 ± 21.9% for porous and non-porous lysozyme spray-dried with AC, respectively. The standard deviation for activity (calculated from units/mg solid) was particularly high in this assay. Residual analysis was carried out to further investigate this. Residuals were found to be similarly distributed around means, and no outlier was identified from the normal probability plot of residuals (p = 0.136). Micro BCA analysis of the amount of protein in each test solution was examined. Although a large variation was noted, no statistically significant difference was found between batches (p = 0.903), suggesting that protein content was similar in all cases and that experimental conditions in the preparation of solutions were likely to have influenced the variability seen.

Table 3.4 Biological activity assay results for porous, non-porous particles spray-dried with/without AC; (n=3, *± pooled standard deviation).

	Units/ml test	Units/mg solid	µg protein/test	Units/mg protein	% Activity* (± 11.1%)	% Specific activity * (± 4.6%)
Porous	301 ± 26.9	37656 ± 3362	7.6 ± 0.66	39633 ± 328	101.3	97.1
Non-porous (SD without AC)	323 ± 23.1	40313 ± 2894	8.5 ± 1.07	37981 ± 2579	108.4	93.1
Non-porous (SD with AC)	334 ± 28.2	41719 ± 3532	8.1 ± 0.70	41401 ± 1794	112.2	101.5
Control	298 ± 48.3	37188 ± 6035	7.3 ± 0.85	40796 ± 2095	100	100

[As a check on ability to detect loss of activity, lysozyme solution was exposed to boiling conditions (98 °C) for 20 minutes, < 50% biological activity was retained; preliminary experiments showed that the filter collected precipitate and oven-dried (Section 3.3.3) retained 96% activity compared to control solution (n=1)].

Examination of all the data from process stability studies, suggested that some perturbation was apparent in the solid state for the spray-dried porous lysozyme (DSC, solid state FTIR), which was not observed to the same degree in the solution state (biological activity assay, solution state FTIR). Liao et al. (2002) reported similar findings for spray-dried lysozyme from aqueous solution in that perturbation of secondary structure of lysozyme was found, assessed by examination of the intensity of the α -helix band in solid state FTIR. This was only equivalent to 60% of unprocessed or native state lysozyme for the spray-dried lysozyme. Similar loss in activity was not observed. Dehydration stress was concluded to be the main factor responsible for this perturbation. Elkordy et al. (2002), also postulated that the lower water content in the spray-dried protein produced changes in the solid but not in the (re-hydrated) solution state, and concluded that this anomaly represented a limitation to the use of lysozyme as a model protein in that it is a stable protein and able to revert to its active state, and that the solution studies may not reflect the conformational damage that may have occurred during solid processing.

Differences between porous and non-porous spray-dried lysozyme found in this work, and unique patterns seen in the DSC and FTIR of the collected precipitate, suggested that different stresses may contribute to changes seen in the porous lysozyme. These stresses may stem from the precipitation step and from exposure to organic solvents. Examination of studies in the literature involving exposure of lysozyme to solvents and solvent interfaces include the following: Van de Weert et al. (2000) studied the effect of a water/methylene chloride interface during an emulsification process, and found some precipitation at the solvent interface. Recovery and lyophilisation of this precipitate was analysed for any difference compared to unprocessed material. FTIR was carried out in the dried state and a decreased α -helical content and evidence of intermolecular β -sheet formation was reported by analysis of the inverted second derivative of the amide I region. A decreased α -helical content was also found in a lyophilised control lysozyme sample but this was to a lesser extent. Re-dissolved sample in 1M guanidine HCl, showed no change in secondary structure by circular dichroism, in specific activity or in SDS-PAGE or size exclusion chromatography. The authors concluded that lysozyme precipitate consisted of non-covalent aggregates that refolded into their native form on dissolution. Goda et al. (2000) examined amyloid protofilament formation of hen egg lysozyme in highly concentrated ethanol solution. In this study the α -helical content of lysozyme was found to increase with ethanol concentration up to 80%, but decreased at 90% and content of β -structure increased, as assessed by circular dichroism. Addition of 10 mM NaCl to a solution of lysozyme in 90% EtOH resulted in

precipitation, which had the characteristics of amyloid protofilament from staining with Congo red, electron microscopy and XRD analysis. Collection and re-dissolution of this precipitate was carried out and circular dichroism showed native secondary structure was recovered. It was concluded that the lysozyme precipitates could recover the native conformation and enzymatic activity by removal of ethanol. A similar mechanism may explain the results in the present study, showing changes in the DSC and solid state FTIR of spray-dried lysozyme from EtOH:H₂O:AC systems but less change from liquid FTIR and biological activity assay.

3.5 STORAGE STABILITY

The storage stabilities of porous (spray-dried from 80% EtOH, 0.5% t.s.c. including 10% AC, inlet temperature 78 °C, Figure 3.13 (b)) and non-porous lysozyme particles (spray-dried from H₂O, 0.5% t.s.c. inlet temperature 120 °C, Figure 3.25 (c)) were assessed under different temperature and relative humidity conditions during a 20-week storage period. Particles were analysed by SEM, TGA, FTIR and biological activity assay at regular time points during the study (Section 2.2.20). DSC was carried out on porous/non-porous particles and XRD was also carried out on porous particles at the end of the study period. SDS-PAGE analysis was carried out on all protein powders at the end of the study period and compared to unprocessed lysozyme and spray-dried powders stored in a desiccator at –20 °C, and is considered separately (Section 3.5.4). Unprocessed material was similarly investigated on stability in terms of biological activity, FTIR and SDS-PAGE only.

3.5.1 Storage at 4 °C / Desiccant

Particle morphology of porous/non-porous particles was unaffected by storage under these conditions as can be seen from SEM photo-micrographs taken after 24 hours and 20 weeks storage under 4 °C / desiccant conditions (Figure 3.41).

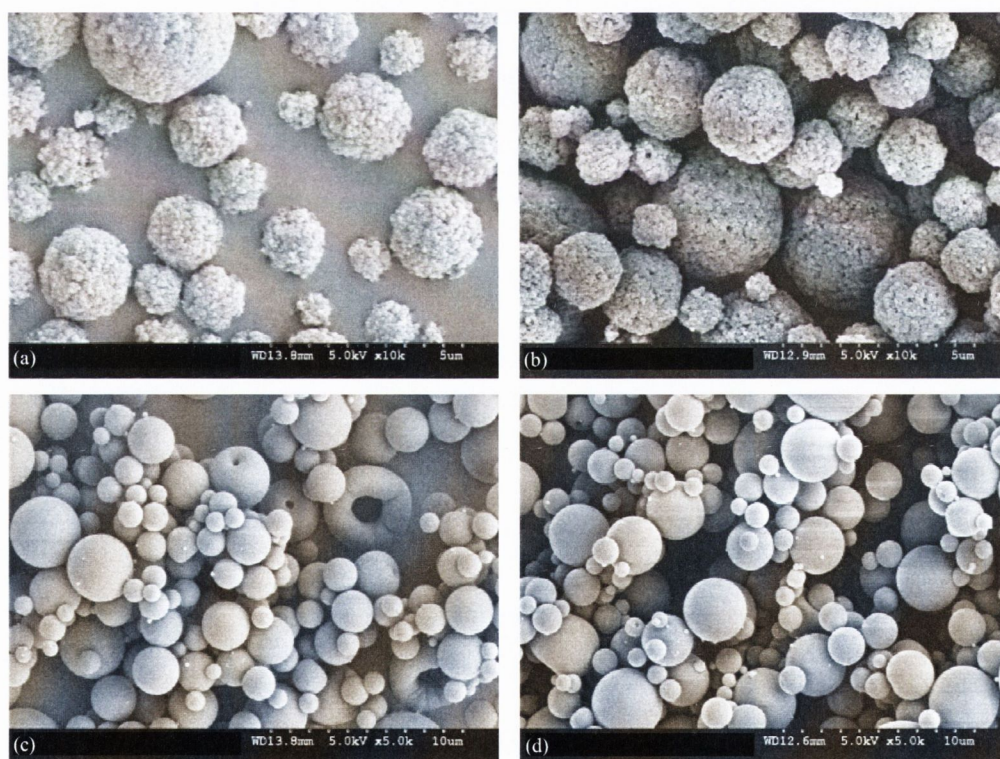


Figure 3.41 SE micrographs showing porous/non-porous lysozyme (a)/(c) after 24 hours (b)/(d) after 20 weeks storage at 4 °C/desiccant.

Mass loss from 25 - 100 °C calculated from TGA, showed that the moisture content of powders was not markedly changed during storage. Porous lysozyme showed a starting residual moisture loss of 4.6%, a moisture loss of 5.1% at week 20 and a range of 2.8 – 5.1% over all the study

measurements. Non-porous lysozyme showed a comparatively similar starting residual moisture loss of 4.3%, a moisture loss of 2.9% at week 20 and a range of 2.9 – 4.9% over all measurements (Figure 3.42). XRD (Figure 3.43) and DSC (Figure 3.44 (a)) of porous microparticles showed no change compared to starting porous material following storage for 20 weeks. DSC of non-porous lysozyme also showed no change in the DSC trace compared to starting non-porous spray-dried material (Figure 3.44 (b)).

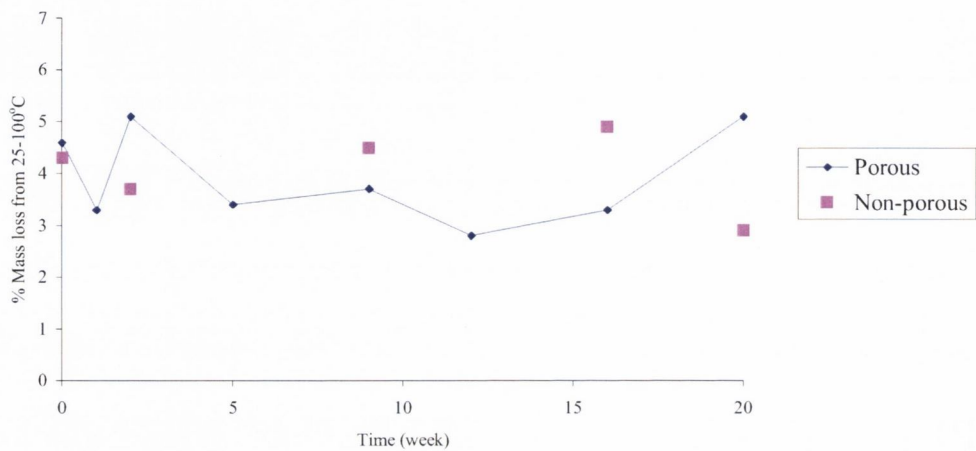


Figure 3.42 Moisture content (calculated from mass loss 25-100 °C from TGA) per week, for porous and non-porous spray-dried lysozyme, during 20 weeks storage at 4 °C/desiccant conditions, n=1.

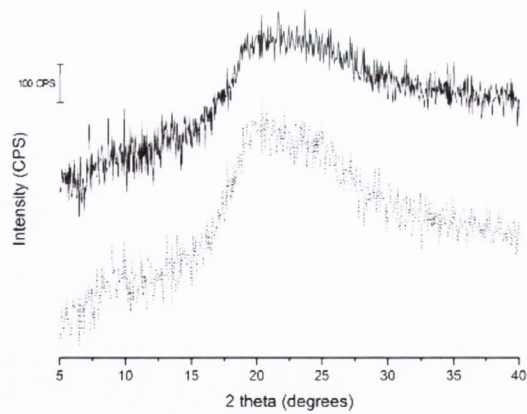


Figure 3.43 XRD of porous lysozyme stored at 4 °C/ desiccant for 20 weeks (solid line), freshly prepared porous lysozyme stored in a desiccator at -20 °C (broken line).

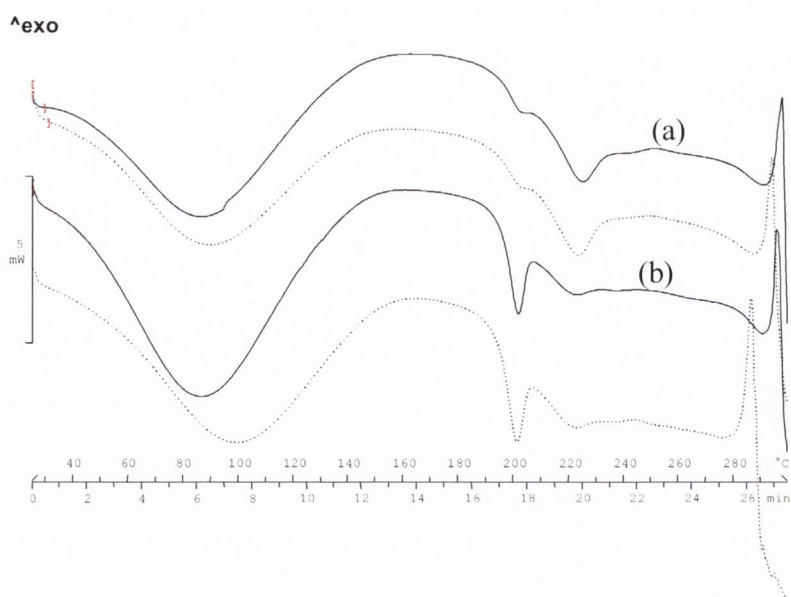


Figure 3.44 DSC of (a) porous, (b) non-porous spray-dried lysozyme following 20 weeks storage at 4 °C/desiccant conditions (solid line), broken line shows freshly prepared material.

Solid state FTIR showed no change in the shape of the amide 1 peak compared to the respective starting material following 20 weeks storage (i.e. porous vs porous; non-porous vs non-porous; unprocessed vs unprocessed material), suggesting that secondary structure did not change further on storage following change as a result of processing (Figure 3.57).

There was no statistically significant change (determined by ANOVA analysis; significance level $\alpha = 0.05$) in biological activity or specific biological activity seen for spray-dried porous/non-porous or unprocessed lysozyme after 20 weeks storage at 4°C/desiccant compared to unprocessed control (stored at -20°C/desiccant). Table 3.5 shows results of the assay carried out at week 20. Figures 3.45, 3.46 show biological activity and specific biological activity with time.

Table 3.5 Biological activity assay results for porous, non-porous, unprocessed lysozyme after 20 weeks storage at 4 °C/desiccant conditions (n=2, *± pooled standard deviation).

	Units/ml test	Units/mg solid	µg protein/test	Units/mg protein	% Activity* (± 6.4%)	% Specific activity * (± 7.0%)
Porous	311 ± 35.4	38863 ± 4358	8.55 ± 1.2	36436 ± 1045	96.8	100.2
Non-porous	354 ± 9.9	44250 ± 1237	9.75 ± 0.07	36312 ± 1278	110.3	99.9
Unprocessed	354 ± 2.8	44222 ± 314	9.2 ± 0.14	38461 ± 865	110.2	105.8
Control	321 ± 18.4	40111 ± 2318	8.85 ± 0.92	36346 ± 1680	100	100

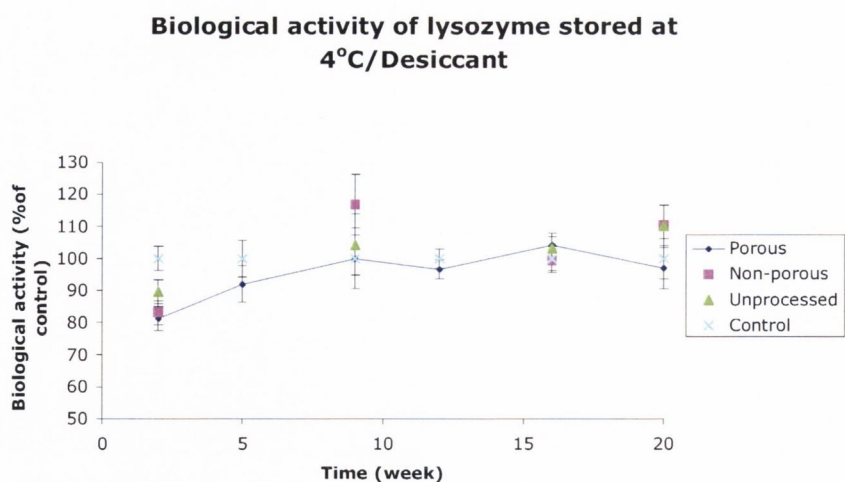


Figure 3.45 Biological activity of spray-dried porous/non-porous and unprocessed lysozyme as % of corresponding control solution activity. Error bars shown represent $\pm$ pooled standard deviation for the samples.

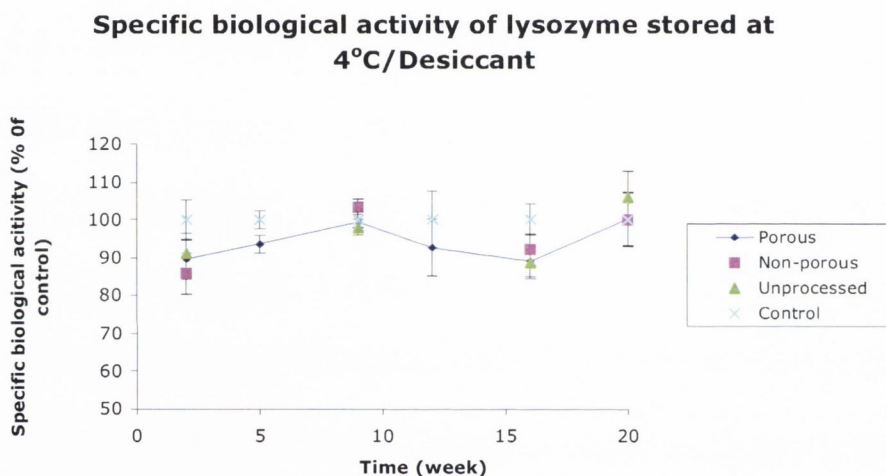


Figure 3.46 Specific biological activity of spray-dried porous/non-porous and unprocessed lysozyme as % of corresponding control solution activity. Error bars shown represent $\pm$ pooled standard deviation for the samples.

3.5.2 Storage at 25 °C / 60% R.H.

Particle morphology of porous/non-porous particles was unchanged by storage under these conditions over 20 weeks, as can be seen from SE photo-micrographs (Figure 3.47).

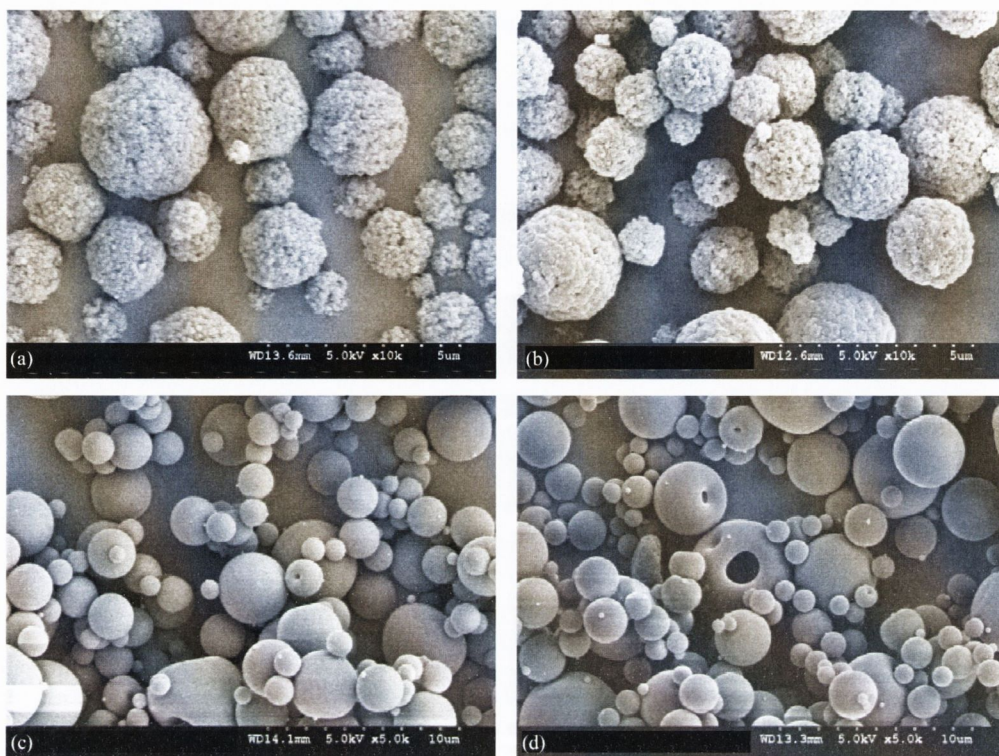


Figure 3.47 SE micrographs showing porous/non-porous lysozyme (a), (c) after 24 hours (b), (d) after 20 weeks storage at 25 °C/60% R.H..

Mass loss from 25 - 100 °C calculated from TGA analysis, showed that the moisture content of powders was increased slightly during storage (Figure 3.48). Variability was increased compared to storage at 4 °C/desiccant conditions, as seen from the increase in the range from all measurements taken. A moisture loss of 5.0% was calculated for porous lysozyme stored for 20 weeks, and a range of 3.3 – 7.3% was calculated over all time-point measurements. A moisture loss of 7.3% was calculated for non-porous lysozyme stored for 20 weeks, and a range of 3.5 – 7.3% was calculated over all time-point measurements. XRD (Figure 3.49) and DSC (Figure 3.50 (a)) of porous microparticles showed no change compared to starting porous material following storage for 20 weeks. DSC of non-porous lysozyme also showed no change in the DSC trace compared to starting non-porous spray-dried material (Figure 3.50 (b)).

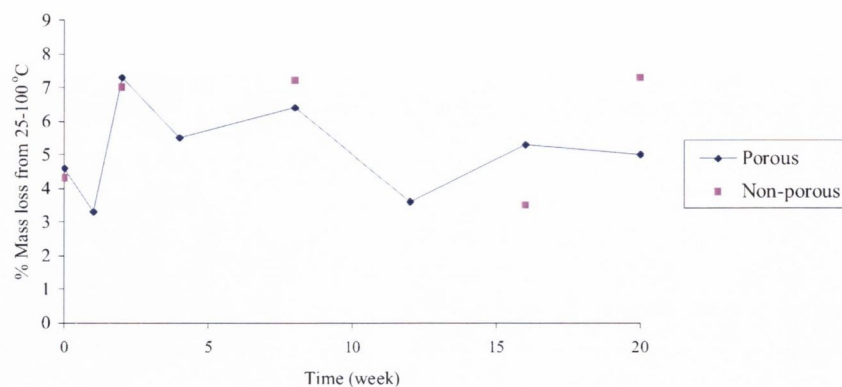


Figure 3.48 Moisture content (calculated from mass loss 25-100 °C from TGA) per week, for porous and non-porous spray-dried lysozyme, following 20 weeks storage at 25 °C/60% R.H., n=1.

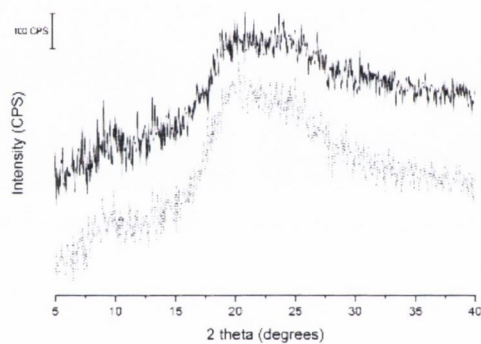


Figure 3.49 XRD of porous lysozyme stored at 25 °C/ 60% R.H. for 20 weeks (solid line), freshly prepared porous lysozyme stored in a desiccator at -20 °C (broken line).

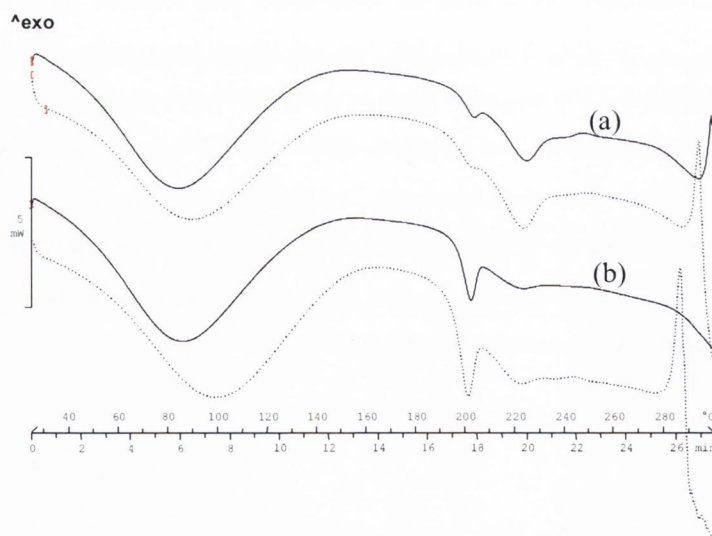


Figure 3.50 DSC of (a) porous, (b) non-porous spray-dried lysozyme following 20 weeks storage at 25 °C/60% R.H. (solid line), broken line shows freshly prepared material.

Solid state FTIR showed no change in the shape of the amide 1 peak compared to the respective starting material following 20 weeks storage (i.e. porous vs porous; non-porous vs non-porous; unprocessed vs unprocessed material), suggesting that secondary structure did not change further on storage following change as a result of processing (Figure 3.57).

There was no statistically significant change (determined by ANOVA analysis; $\alpha = 0.05$) in biological activity seen for spray-dried porous/non-porous or unprocessed lysozyme compared to control after 20 weeks storage. Non-porous spray-dried lysozyme showed a statistically significant higher specific activity from ANOVA analysis but this was likely to be an artifact of one low BCA result, as no such trend was noted in other weeks. This evidence suggests that the biological activity of spray-dried porous, non-porous or unprocessed lysozyme was not adversely affected by storage under these conditions for 20 weeks. Table 3.6 shows results of the assay carried out at week 20. Figures 3.51 and 3.52 show biological activity and specific biological activity with time.

Table 3.6 Biological activity assay results for porous, non-porous, unprocessed lysozyme after 20 weeks storage at 25 °C/60% R.H. (n=2, *± pooled standard deviation).

	Units/ml test	Units/mg solid	µg protein/test	Units/mg protein	% Activity* (± 5.2%)	% Specific activity * (± 4.0%)
Porous	342 ± 12.7	42787 ± 1583	9.1 ± 0.28	37611 ± 223	106.7	103.5
Non-porous	344 ± 3.5	42925 ± 380	7.9 ± 0.14	43472 ± 393	107.0	119.6
Unprocessed	327 ± 24	40903 ± 3045	9.6 ± 1.34	34426 ± 2293	102.0	94.7
Control	321 ± 18.4	40111 ± 2318	8.85 ± 0.92	36346 ± 1680	100	100

Biological activity of lysozyme stored at 25°C/60% RH

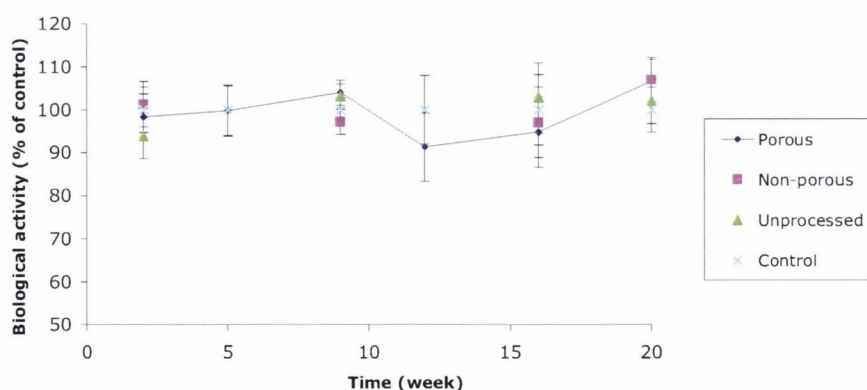


Figure 3.51 Biological activity of spray-dried porous/non-porous and unprocessed lysozyme as % of corresponding control solution activity. Error bars shown represent ± pooled standard deviation for the samples.

Specific biological activity of lysozyme stored at 25°C/60% RH

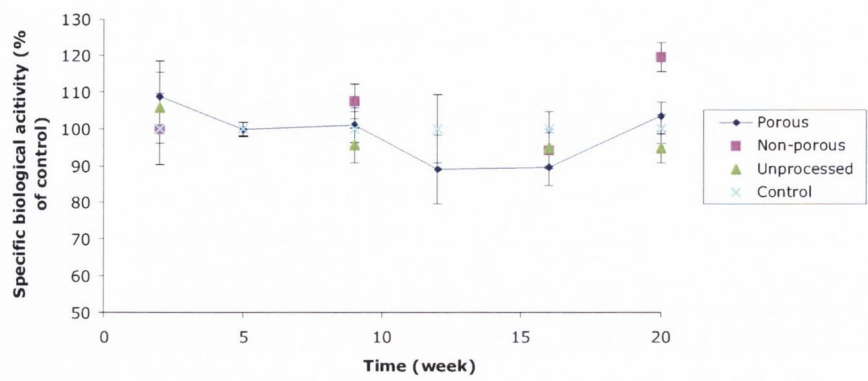


Figure 3.52 Specific biological activity of spray-dried porous/non-porous and unprocessed lysozyme as % of corresponding control solution activity. Error bars shown represent $\pm$ pooled standard deviation for the samples.

3.5.3 Storage at 40 °C / 75% R.H.

Particle morphology was considerably affected by these storage conditions. Loss of porous surface morphology was noted after 24 hours and particle fusion was considerable from 1 week onwards for porous lysozyme particles. Non-porous particles also showed a change in surface morphology after 24 hours with some particle fusion and indents to the surface. Storage for 20 weeks resulted in less fusion for non-porous than for porous particles however. SE micrographs (Figure 3.53) show particle morphology following 24 hours, 2 weeks and 20 weeks storage.

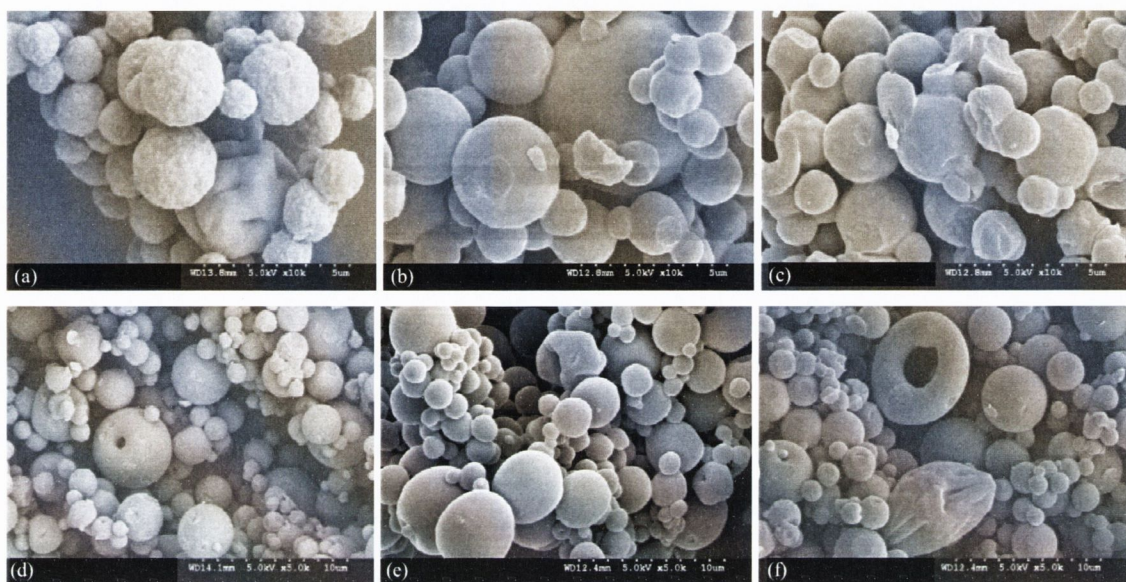


Figure 3.53 SE micrographs showing porous (top), non-porous (bottom) lysozyme (a), (d) after 24 hours (b), (e) after 2 weeks (c), (f) after 20 weeks storage at 40 °C/75% R.H..

Mass loss from 25 - 100 °C calculated from TGA analysis, showed that the moisture content of powders was increased during storage (Figure 3.54). Moisture content showed a maximum after storage for 2 weeks, with mass losses of 11.0 and 11.5%, respectively for porous and non-porous particles at this time point. Variability was increased compared to storage at 4 °C / desiccant and 25 °C / 60% R.H. conditions, as seen from the range of all measurements of 4.6 - 11% (mass loss of 7.9% calculated after 20 weeks storage) for porous lysozyme and 4.3 - 11.5% (mass loss of 9.6% calculated after 20 weeks storage) for non-porous lysozyme. XRD of porous microparticles remained amorphous after 20 weeks storage (Figure 3.55). DSC of porous lysozyme showed a change in the area of decomposition and T_m peak following storage under these conditions (Figure 3.56 (a)). The T_m peak was larger than in the freshly prepared porous sample and more similar to the unprocessed sample. This suggests some change in folding compared to the freshly prepared spray-dried samples. DSC of non-porous lysozyme was not changed compared to the freshly prepared sample (Figure 3.56 (b)).

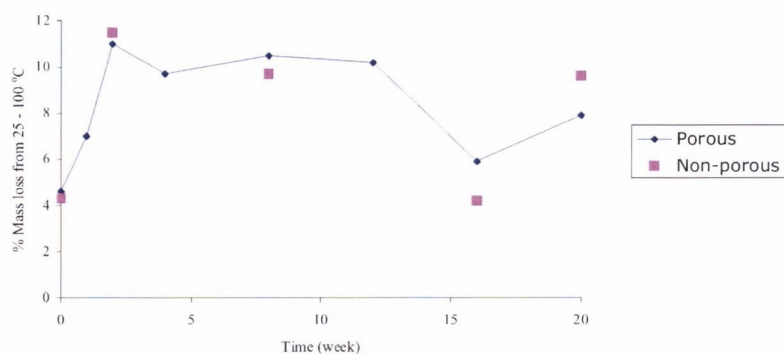


Figure 3.54 Moisture content (calculated from mass loss 25-100 °C from TGA) per week, for porous and non-porous spray-dried lysozyme, following 20 weeks storage at 40 °C/75% R.H., n=1.

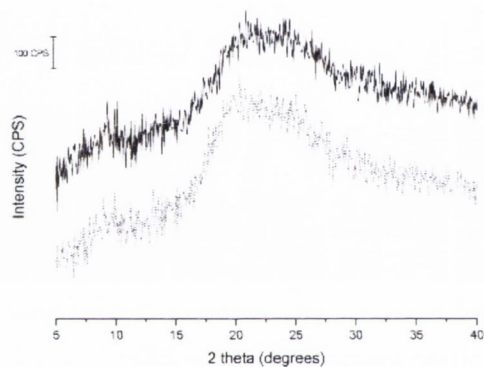


Figure 3.55 XRD of porous lysozyme stored at 40 °C/ 75% R.H. for 20 weeks (solid line), freshly prepared porous lysozyme stored in a desiccator at -20 °C (broken line).

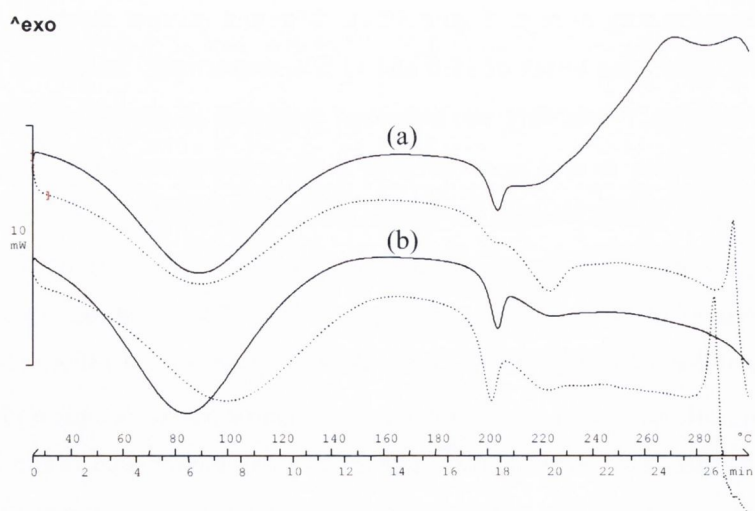


Figure 3.56 DSC of (a) porous, (b) non-porous spray-dried lysozyme following 20 weeks storage at 40 °C/75% R.H. (solid line), broken line shows freshly prepared material.

Solid state FTIR showed no change in the shape of the amide 1 peak compared to the respective starting material for spray-dried porous lysozyme, following 20 weeks storage. Interestingly non-porous and unprocessed material did show a change in the shape of the amide 1 areas compared to respective starting materials (Figure 3.57). Calculation of area of overlap between this peak and the respective starting peaks showed an area of overlap of 92.1% (unprocessed material: starting vs stability), and 94.8% (non-porous material: starting vs stability).

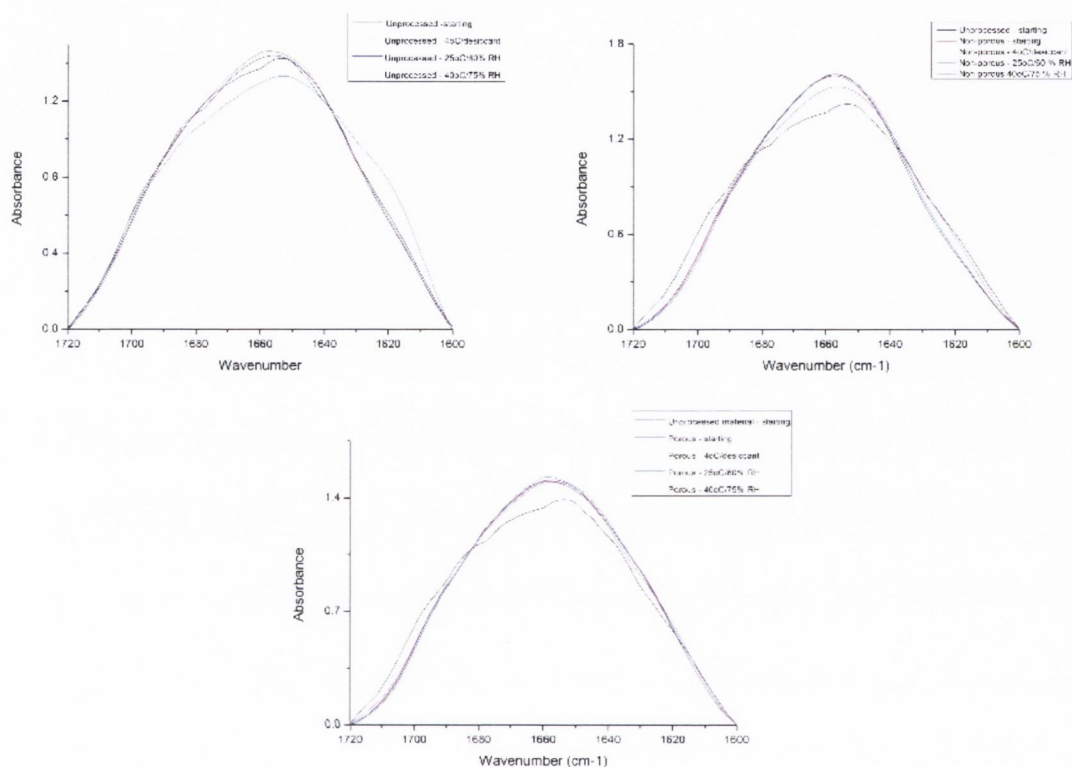


Figure 3.57 Area-normalised, baseline-corrected amide 1 peak for unprocessed (black trace)/non-porous/porous lysozyme (coloured traces) stored under stability conditions as indicated in the legend for each of the graphs (20 weeks).

Biological activity of spray-dried samples was found to decrease on storage with a statistically significant decrease compared to control. This was significantly greater in the case of porous spray-dried lysozyme. Decrease in biological activity was associated with a decrease in solubility of the protein, as observed from the decrease in μg protein/test from micro BCA analysis (Table 3.7). This suggests that the decrease in activity was related to formation of insoluble aggregates. No decrease in specific activity was found because this measurement involved correction for protein content. Unprocessed material also showed a decrease in % activity, although there was a large variability observed at week 20, resulting in a high standard deviation. The trend observed over 20 weeks resembled that for the porous spray-dried sample (Figures 3.58, 3.59).

Table 3.7 Biological activity assay results for porous, non-porous, unprocessed lysozyme after 20 weeks storage at 40 °C/75% R.H. (n=2, *± pooled standard deviation).

	Units/ml test	Units/mg solid	µg protein/test	Units/mg protein	% Activity* (¹ ± 3.9; ² ± 28.0)	% Specific activity * (¹ ± 3.6 ² ± 2.5)
Porous	206 ± 7.1	25757 ± 874	5.35 ± 0.21	38520 ± 220	64.2 ¹	106 ¹
Non-porous	270 ± 8.5	33750 ± 1105	6.3 ± 0.42	42907 ± 220	84.1 ¹	118.1 ¹
Control 1	321 ±18.4	40111 ± 2318	8.85 ± 0.92	36346 ±1680	100 ¹	100 ¹
Unprocessed	211 ± 119.5	26328 ± 14916	4.5 ± 2.5	46485 ± 528	67.4 ²	119.0 ²
Control 2	313 ± 33.2	39063 ± 4198	8.0 ± 0.57	39061 ± 1295	100 ²	100 ²

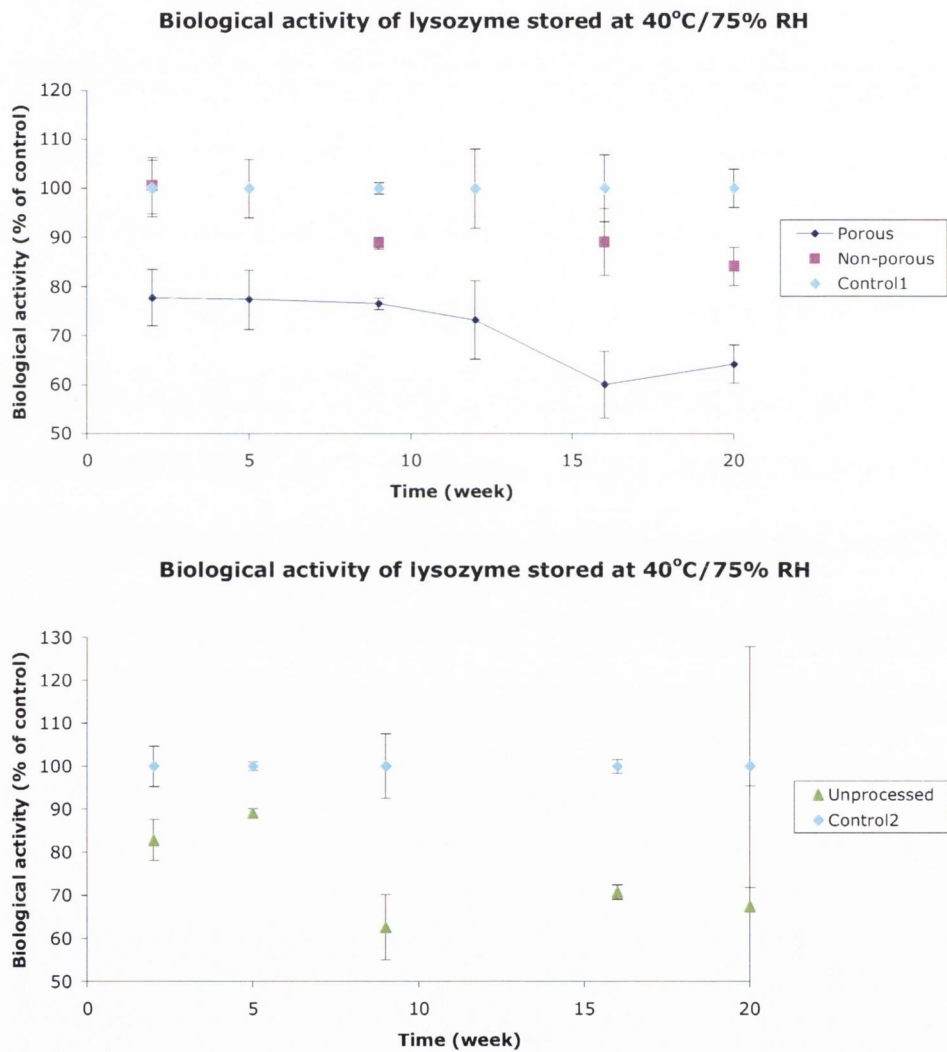


Figure 3.58 Biological activity of spray-dried porous/non-porous and unprocessed lysozyme as % of corresponding control solution activity. Error bars shown represent ± pooled standard deviation for the samples.

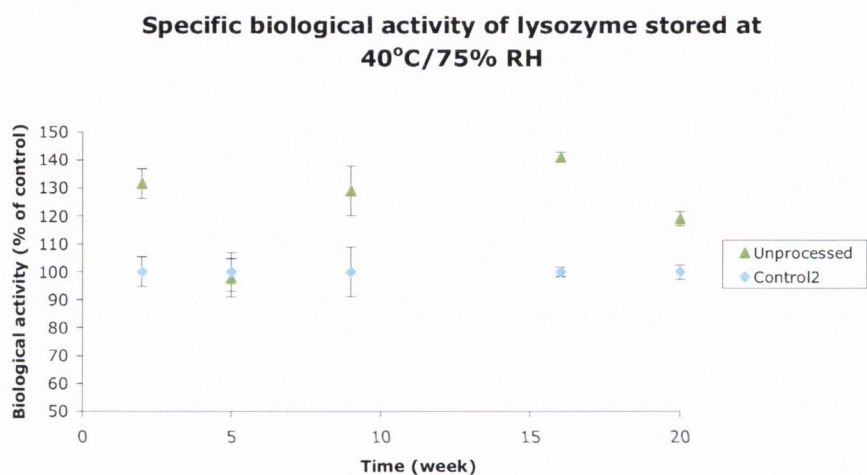
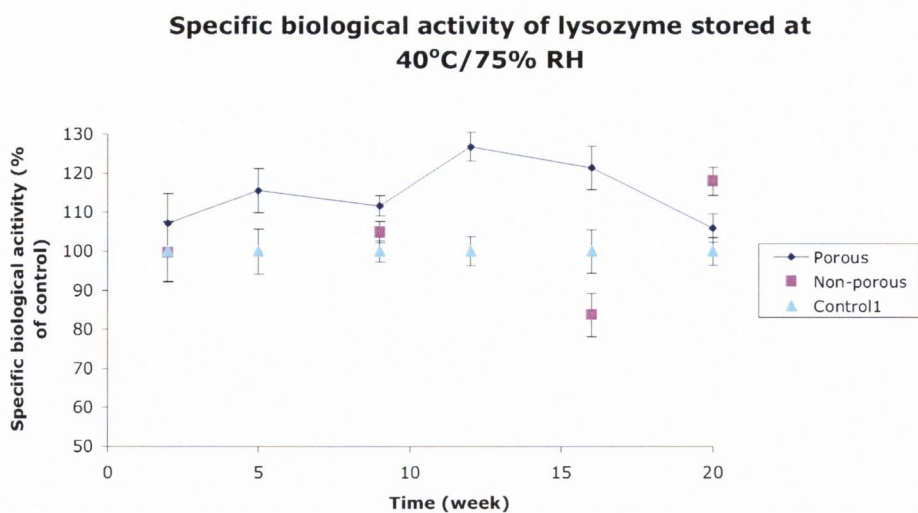


Figure 3.59 Specific biological activity of spray-dried porous/non-porous and unprocessed lysozyme as % of corresponding control solution activity. Error bars shown represent $\pm$ pooled standard deviation for the samples.

3.5.4 SDS-PAGE

Reducing SDS-PAGE was carried out on spray-dried porous, non-porous (without AC) stored at -20 °C, and stored under 4 °C/desiccant, 25 °C/60% R.H., 40 °C/75% R.H. stability conditions for 20 weeks and unprocessed lysozyme stored at -20 °C, and stored under 4 °C/desiccant, 25 °C/60% R.H., stability conditions for 20 weeks (Figure 3.60). Gel sheet analysis showed that no appreciable soluble covalent protein aggregation took place following spray-drying and storage at -20 °C in a desiccator. However, a weak higher molecular weight band (~32 kDa by comparison to the standard), indicating the presence of amounts of aggregated protein (dimer), was identified for spray-dried (porous and non-porous) stored at 4 °C/25 °C and 40 °C under stability study conditions and unprocessed samples stored at 25 °C under stability study conditions.

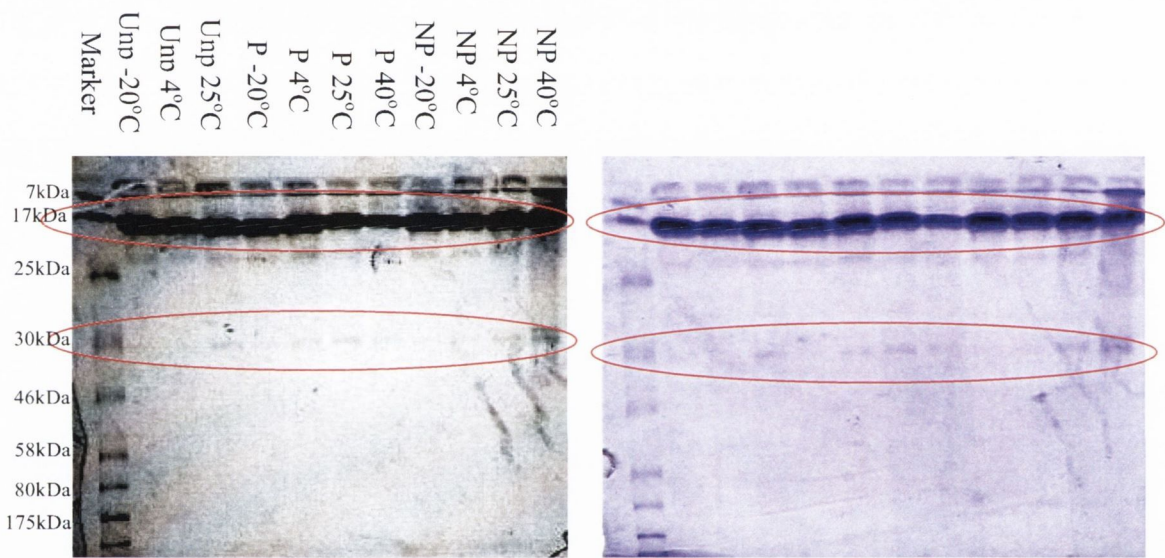


Figure 3.60 SDS-PAGE carried out under reducing conditions: picture taken using gel documentation system under UV light (left) and scanned image (right). [Unp = unprocessed; P = porous; NP = non-porous, temperature labels correspond to stability conditions -20 °C/desiccant; 4 °C/desiccant; 25 °C/60% R.H.; 40 °C/75% R.H.]

To further investigate this, SDS-PAGE was carried out under non-reducing conditions on the spray-dried porous lysozyme samples (Figure 3.61). A small amount of aggregation was again evident from a higher molecular weight band, particularly for samples stored under the more challenging conditions of 25 °C/60% R.H. and 40 °C/75% R.H..

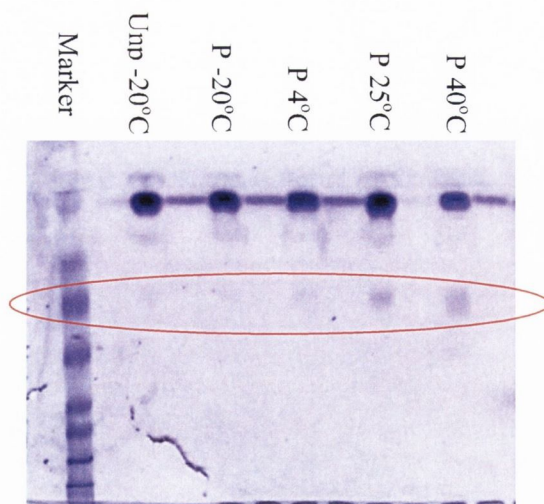


Figure 3.61 SDS-PAGE carried out under non-reducing conditions: scanned image. [Unp = unprocessed; P = porous; temperature labels correspond to stability conditions -20°C /desiccant; 4°C /desiccant; 25°C /60% R.H.; 40°C /75% R.H.]

Stability, as assessed by SEM, TGA, DSC, FTIR and biological activity, were similar for porous and non-porous material, stored at 4°C /desiccant and 25°C /60% R.H.. The stability of non-porous lysozyme was better than porous lysozyme stability, stored at 40°C /75% R.H. as noted from a greater biological activity of non-porous particles stored under these conditions for similar lengths of time. Evidence of aggregation from SDS-PAGE for porous and non-porous and unprocessed lysozyme and changes in DSC traces of porous lysozyme (but not non-porous lysozyme) stored under these more challenging conditions also suggests loss of stability.

3.6 PARTICLE CHARACTERISATION OF SPRAY-DRIED LYSOZYME

Observation of the surface morphology of the porous spray-dried particles suggested a high surface area, because of the fused nano-particular appearance. Particle diameter appeared to be favourable for pulmonary delivery from SEM analysis (less than $\sim 3 \mu\text{m}$). In order to examine these and to assess aerodynamic properties, surface area, bulk and tapped densities, particle size distribution and in-vitro drug deposition using an Andersen cascade impactor were undertaken. The model porous system lysozyme spray-dried from 80% EtOH with 10% of t.s.c. (0.5% w/v) AC, inlet temperature 78°C was chosen (Figure 3.13 (b)). Lysozyme spray-dried from water without any AC and with 20% of t.s.c. AC (t.s.c. 0.5%, inlet temperature 120°C) were chosen as model non-porous systems with different morphology (Figure 3.25).

Average particle diameter was initially estimated from SE micrographs (Table 3.8). The sizes of three batches of porous lysozyme were estimated before further analysis and to examine inter-batch variation. All spray-dried samples (porous/non-porous) appeared to have similar particle size. One batch of spray-dried porous lysozyme – batch 3 had the smallest estimated particle size and standard deviation. Statistical significance was not established due to the crude nature of these estimates, however variation of this batch from other batches was suspected as a result of small changes in spray-dryer performance noted by the research group and necessitating nozzle replacement, shortly after this batch was spray-dried. Particle sizing using laser diffraction and in-vitro drug deposition was carried out on this batch – referred to as ‘porous lysozyme – small particle size batch’ as a comparison but the suspected atypical nature of this batch should be noted. Results of ‘porous lysozyme – regular batch’ represent spray-dried lysozyme particles that were spray-dried well before or after nozzle replacement was required.

Table 3.8 Average particle diameter and standard deviation estimated from SEM analysis.

	Average particle diameter $\pm$ standard deviation (μm) [number of particles considered]
Porous lysozyme – batch 1	2.16 ± 0.87 [150]
Porous lysozyme – batch 2	1.65 ± 0.73 [162]
Porous lysozyme – batch 3	1.54 ± 0.45 [351]
Non-porous lysozyme SD with AC	1.92 ± 0.82 [73]
Non-porous lysozyme SD without AC	1.70 ± 1.07 [123]

Particle size distribution was estimated using laser diffraction particle sizing at the standard operational settings of 2 bar air pressure and 50% vibration feed rate for porous particles. A higher vibration feed rate of 70 – 80% was required to adequately disperse non-porous particles. This did not influence the measured particle size distribution of these particles, as observed from replicate runs at 50%, 70%, 80% vibration feed rates. Porous particles and non-porous particles spray-dried

with AC showed a mono-modal, highly reproducible intra-batch size distribution. Non-porous particles spray-dried without AC, showed a skewed distribution (on the log scale) with some tailing in the particle size distribution below 1 μm diameter. The narrowest size distribution was found for the porous lysozyme – small particle size batch, with a span averaging 1.018 compared to 1.523 for the regular batch of porous lysozyme, 1.605 for non-porous lysozyme spray-dried with AC and 1.847 for non-porous lysozyme spray-dried without AC. The two spray-dried batches of porous particles showed smaller median volume diameters than non-porous particles with the smallest $d(0.5)$ measured for the porous lysozyme – small particle size batch (Table 3.9). The percentage of particles in the nanometer size range was similar for porous particles and non-porous particles spray-dried without AC, but significantly less for the non-porous particles spray-dried with AC.

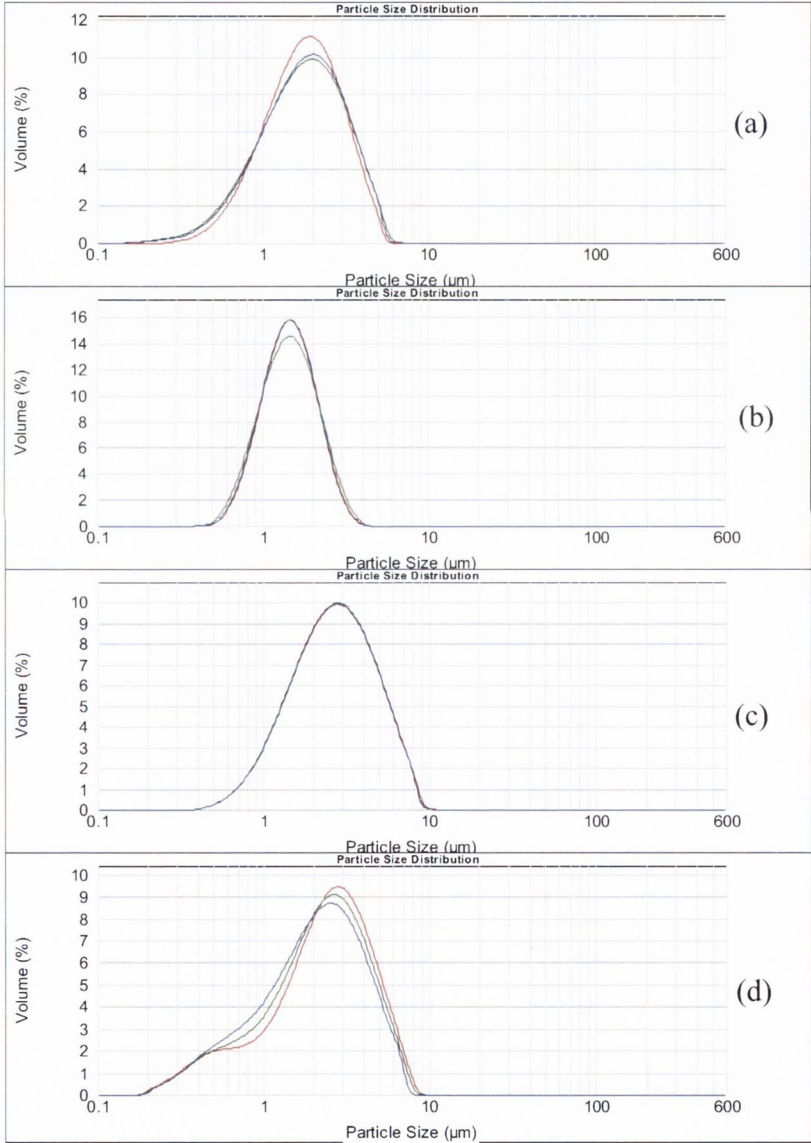


Figure 3.62 Particle size distributions of (a) spray-dried porous lysozyme, (b) spray-dried porous lysozyme – small particle size batch, (c) spray-dried non-porous lysozyme (spray-dried with AC), (d) spray-dried non-porous lysozyme (spray-dried without AC), n = 3 (different colour traces represent different runs).

Bulk and tapped densities were similar for the porous lysozyme and non-porous lysozyme particles spray-dried without AC (Table 3.9). These were higher than expected for the porous lysozyme, compared to bulk and tapped densities of other porous particles produced by the method (Section 4.2.3, Mc Donald (2005), Nolan (2008)). Bulk and tapped densities were lower for the non-porous lysozyme spray-dried with AC (Table 3.9). Non-porous lysozyme particles spray-dried with AC were hollow, with evidence of collapsed particles from SEM analysis. The calculated Carr's index for porous particles was 35.7% which is indicative of a very poor flowing powder. Values for non-porous particles spray-dried without AC and with AC were 53.1% and 22.2% indicative of extremely poor and poor flowing powders, respectively. The extremely poor flow of non-porous particles spray-dried without AC may have contributed to the low dispersibility of the powder during particle sizing measurements.

Surface area measurements of porous particles were approximately twice those of non-porous spray-dried particles spray-dried with/without AC (Table 3.9). The increase in surface area was smaller than expected, particularly considering the approximately 40-fold increase in surface area of porous trehalose and raffinose particles compared to non-porous particles (Section 4.2.3).

Table 3.9 Particle sizing (n = 3; mean ± standard deviation), surface area (n = 2 mean (range); *n = 1;) and bulk and tapped densities (n = 3 mean ± standard deviation; *n = 1;) for porous lysozyme, porous lysozyme (small particle size batch), non-porous lysozyme (spray-dried without AC), non-porous lysozyme (spray-dried with AC).

	Porous Lysozyme – regular batch	Porous Lysozyme – small particle size batch	Non-porous Lysozyme (SD without AC)	Non-porous Lysozyme (SD with AC)
Particle sizing				
d(0.1) (µm)	0.786 ± 0.052	0.872 ± 0.023	0.632 ± 0.013	1.187 ± 0.004
d(0.5) (µm)	1.785 ± 0.011	1.442 ± 0.010	2.221 ± 0.162	2.660 ± 0.013
d(0.9) (µm)	3.505 ± 0.112	2.340 ± 0.048	4.725 ± 0.219	5.457 ± 0.035
% < 1 µm	17.93 ± 1.83	17.80 ± 1.40	18.62 ± 1.84	5.95 ± 0.052
Surface area (m²/g)	6.7867 [6.5457, 7.0277]	<i>N/d</i>	2.7223 [3.6781, 1.7665]	*3.3270
Bulk density (g/cm³)	0.27 ± 0.011	<i>N/d</i>	0.23 ± 0.014	*0.14
Tapped density (g/cm³)	0.42 ± 0.017	<i>N/d</i>	0.49 ± 0.003	*0.18

Andersen cascade impactor (ACI) data showed that porous lysozyme particles had a higher fine particle fraction (< 5 µm) and particle fraction < 3 µm than non-porous particles. Due to the large variability however, there was not a statistically significant difference between this batch and non-porous particles spray-dried with AC (< 5 µm p = 0.061; < 3 µm p = 0.119), but there was statistically significant difference between this batch and non-porous particles spray-dried without AC (< 5 µm p = 0.014; < 3 µm p = 0.016). The batch of porous lysozyme with a small particle size, gave the highest fine particle fraction and particle fraction < 3 µm. Mass median aerodynamic

diameters for porous and non-porous particles spray-dried with AC were almost identical. A larger MMAD was found for non-porous particles spray-dried without AC. Edwards et al. (1997) described a case involving large porous and non-porous PLGA particles, where differences were noted in respirable fraction but not in MMAD. They attributed the advantage of porous particles, to decreased aggregation on leaving the inhaler and decreased impaction in the non-respirable stages of the ACI. In this study porous particles also showed less deposition in the mouthpiece adaptor and induction port than non-porous particles with AC (Figure 3.63). The average emitted dose (expressed as % of nominal dose emitted from the inhaler) was similar for all systems with no statistically significant difference between particle morphologies.

Table 3.10 Summary of results from Andersen cascade impactor analysis; mean $\pm$ s.d.; n = 3.

	% < 5 μm	% < 3 μm	MMAD (μm)	GSD	% emitted (of nominal dose)
Porous lysozyme	57.98 $\pm$ 4.48%	32.55 $\pm$ 3.72%	2.83 $\pm$ 0.23	2.01 $\pm$ 0.48	88.4 $\pm$ 11.4
Porous lysozyme -small particle size batch	72.30 $\pm$ 5.05%	48.08 $\pm$ 4.93%	2.05 $\pm$ 0.04	1.91 $\pm$ 0.07	73.1 $\pm$ 9.9
Non-porous lysozyme -spray-dried with AC	46.31 $\pm$ 5.25%	25.38 $\pm$ 4.38%	2.83 $\pm$ 0.21	2.08 $\pm$ 0.2	71.3 $\pm$ 13.0
Non-porous lysozyme -spray-dried without AC	37.60 $\pm$ 5.13%	19.50 $\pm$ 2.73%	4.79 $\pm$ 0.75	2.46 $\pm$ 0.44	75.4 $\pm$ 7.0

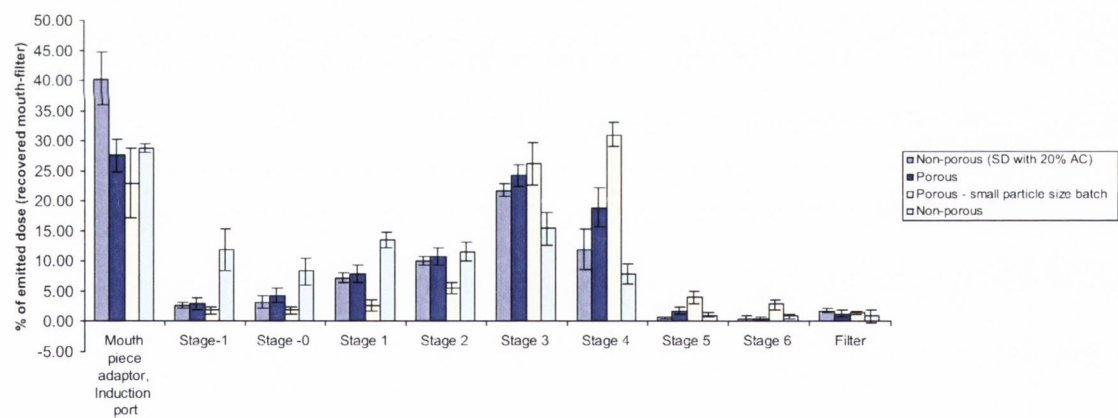


Figure 3.63 Deposition patterns on the stages of the ACI apparatus showing differences between product morphologies. Calculated as % of recovered emitted dose (mouth – filter), used to calculate fine particle fraction.

3.7 CONCLUSIONS

Porous particles of lysozyme were produced from a range of alcohol:H₂O:AC systems. The importance of the concentration of alcohol and AC and of phase separation of the feed prior to spray-drying was observed. The fact that inlet temperature did not affect surface morphology was noted on two occasions. The deviation of the solvent system from the hypothesis proposed for NPMP production by the method involving solvent selection was also noted. These may indicate that a slightly different mechanism of NPMP production is involved in these systems than in the systems spray-dried by other researchers in the group using the method, and than seen for hydrophilic systems spray-dried from the non-aqueous systems in this work. Alternative process enhancers ammonium formate and ammonium acetate were considered and required higher concentrations of salt to be used to produce porous particles from an 80% EtOH solvent system. Their lower efficacy by weight of salt, compared to AC and AHC were proposed to be due to a lower precipitating power for the protein.

Process stability determinations suggested that porous lysozyme produced from an EtOH:H₂O:AC system experienced some perturbations in folding/secondary structure in the dried state, with recovery of activity on re-hydration. Comparison with non-porous lysozyme spray-dried from water and collected precipitate (not spray-dried), suggested different stresses imposed by the method. Storage stability determinations also suggested a slightly different behaviour for spray-dried porous versus non-porous particles with greater loss of activity seen for the porous system after 20 weeks storage at 40 °C/75% R.H..

Porous lysozyme showed favourable aerodynamic characteristics compared to non-porous spray-dried lysozyme. Specific surface area and bulk and tapped density measurements deviated from expected outcomes as compared to porous particles of other materials produced by the method.

4.1 INTRODUCTION

This section involves spray-drying excipients, to investigate whether NPMPs can be produced. These studies are conducted as a prelude to spray-drying excipients with a protein or peptide in composite systems. The rationale for using excipients in dry powder protein formulations, has been introduced in Chapter 1. The non-reducing sugars trehalose and raffinose, and polyols mannitol and myo-inositol are investigated. A significant amount of work was previously carried out by Li (2005) in producing NPMPs of HP- β -CD, so it is not considered further in this section, with regard to preliminary research. It is however considered as a model cyclodextrin component in composite systems with proteins in Chapters 5 and 6, and in one composite system in this section with trehalose. Table 4.1 provides a summary, from the literature, of some of the physicochemical and solid-state characteristics following spray-drying, of the excipients used in this chapter.

Previous research in developing the spray-drying method, particularly for the water soluble saccharides, include lactose spray-dried from water with AC as process enhancer by McDonald (2005), and HP- β -CD spray-dried from mixed organic solvents by Li (2005). McDonald (2005) spray-dried lactose:AC in different mass ratios, from water. These experiments suggested an interesting morphology for the particles spray-dried in a mass ratio of 75:25 lactose:AC. Surface morphology of spray-dried particles was dissimilar to other NPMPs produced using the spray-drying method (for example the fused nano-particulate appearance of NPMPs of lysozyme from EtOH:H₂O:AC (Chapter 3), and some thiazide and steroid compounds produced by Tajber (2005), McDonald (2005), Nolan (2008)) and consisted of hollow thin-walled particles with pores or blow holes through the surface. These particles had high surface area and low bulk and tapped densities. They were described as porous particles of “poor quality” (Corrigan, 2005). Li (2005) spray-dried HP- β -CD from a selection of different solvent systems and conditions. NPMPs were not produced from any of the alcohol:H₂O:AC systems. NPMPs were however produced from mixed organic solvent systems for example MeOH:BA (1:1), MeOH:IPA (1:1), MeOH:BA:H₂O (15:15:1 and 6:6:1) (inlet temperatures 100-120 °C) using the Büchi B-290 spray-dryer in the closed mode configuration (Li, 2005). These previous studies are considered when spray-drying excipients in this chapter.

Particle properties, including particle size, shape, surface morphology, residual moisture/solvent content, solid-state characteristics and thermal behaviour are the main criteria for formulation evaluation. Applicability of the spray-drying conditions for future incorporation of proteins is also assessed. Each excipient is considered separately regarding spray-drying and thermal analysis and solid-state characteristics. Particle characterisation including micromeritics and in-vitro deposition

is considered together for NPMPs of trehalose and raffinose, because of similarities between the NPMPs produced, in terms of morphology and feed conditions. Preliminary examples of composites of excipients are also considered at the end of the chapter.

Table 4.1 (^aElversson and Millqvist-Fureby, 2005; ^bBouchard, 2007b; ^cLechuga-Ballesteros et al., 2003, ^dYu et al., 1998; ^eHigashiyama, 2002, ^fPharmaceutical excipients, 2006; ^gSurana, 2004; ^hMosén, 2003; ⁱMiller and Lechuga-Ballesteros, 2006; ^jLi, 2005).

Name (Number of saccharide units)	Hydrate (s)	MW ^f	Tg (°C)	MP (°C)	Solid state after spray- drying	Solubility ^f
Trehalose (2)	Dihydrate	378.33 (dihydr) 342.31 (anhyd)	117 ^b	95 – 97 °C ^e (Dihydrate - dehydration) 203 – 210 °C ^e (Anhydride)	Amorphous ^g	68.9g/100ml in H ₂ O (20 °C) Slightly soluble in 95% EtOH
Raffinose (3)	Pentahydrate, Meta-stable tetra- and tri- hydrates	594.52 (penta-) 504.44 (anhyd)	118 ^b 113 ^c	80 °C (Pentahydrate - dehydration)	Amorphous ⁱ	0.14g/ml in H ₂ O 0.10g/ml in MeOH Slightly soluble in 95% EtOH
Cyclodextrin; HP-β-CD (7)	-	1380	250 ^b		Amorphous ^j	H ₂ O > 1 in 2 (20 °C)
Mannitol	-	182.17	12.6 ^d	167-170 °C ^f	Crystalline (89-100%) ^a	1 in 5.5 H ₂ O 1 in 83 EtOH
Myo-inositol	-	180.16		222-227 °C ^f	Crystalline ^h	50 mg/mL in H ₂ O

4.2 NON-REDUCING SUGARS

4.2.1 TREHALOSE

Maa et al. (1997), described trehalose as having a “sticky” nature and spray-dried composite particles from aqueous solution, with a high ratio of trehalose:protein (3:2, 7:3) as highly agglomerated. They concluded that concentrations of trehalose greater than 50% w/w were not appropriate for aerosol applications. SEM pictures presented, showed fused spherical particles. Spray-drying in this study was carried out using a Büchi B-190 spray-dryer, equipped with a two-fluid nozzle (0.5 mm). The following conditions were employed: inlet temperature 90 °C, drying air flow rate 0.6 m³/min, atomizing air flow rate 0.9 m³/hr, liquid feed rate 5 ml/min. These conditions resulted in an outlet drying temperature of approximately 53 °C. Spray-drying conditions may not have been optimal however, and this may have contributed to the poor quality of the particles obtained, rather than stickiness on spray-drying being an inherent property of the spray-dried sugar. The inlet temperature of 90 °C and resulting outlet temperature appear quite low for spray-drying from aqueous solution, and may have resulted in slow or incomplete particle drying. It may have been chosen however, to minimise degradation of the protein in the study.

Subsequent to this work, Adler and Lee (1999) investigated the effect of increasing inlet drying temperature on spray-dried trehalose. Spray-drying in this study was carried out from aqueous solution, also on a Büchi B-190 equipped with a pneumatic nozzle (0.7 mm) driven at 6 bar air pressure. Spray-drying conditions included: drying inlet temperatures of 110, 130 and 150 °C, atomizing air flow rate 0.7 m³/hr, and liquid feed rate 4 ml/min. No evidence of agglomeration was found in the SEMs presented, although the use of the lower inlet temperature of 110 °C may have resulted in agglomerated particles which, while not collected, were deposited on the walls of the drying chamber or cyclone separator, resulting in the lower powder yield obtained (Adler and Lee, 1999).

4.2.1.1 SPRAY-DRIED FROM WATER

The influence of inlet temperature on spray-dried trehalose from water (2% t.s.c., 75:25 trehalose:AC), using the Büchi B-290 spray-dryer in the open mode configuration, was investigated. The feed conditions were chosen based on the results of McDonald (2005), for spray-drying lactose from water. This spray-dryer was equipped with a two-fluid nozzle of diameter 0.7 mm, with nozzle screw cap 1.5 mm, and with air as drying gas (gas flow setting 40 mm, equivalent to 473 Normlitre/hour). Liquid feed rate of 9 ml/min was used. Different inlet temperatures of 100, 110, 130 and 150 °C were investigated, resulting in outlet temperatures of 56-51, 64-61, 69 and 80 °C, respectively.

Product recovered from spray-drying at the lower inlet temperatures of 100 °C and 110 °C had a “sticky” consistency on collection. Collectable yields were low at 20.4% (25 ml spray-dried) and 8.5% (50 ml spray-dried), respectively, with visible deposition of the material on the walls of the drying chamber and cyclone separator. Particles were fused when viewed under SEM, with particles spray-dried at 100 °C most affected (Figure 4.1 (a), (b)). The lower inlet temperatures of 100 °C and 110 °C appeared to be unsuitable for spray-drying trehalose from water. Inlet temperatures of 130 °C and 150 °C were more successful, however. Collectable powder yields were higher at 36.2% (50 ml spray-dried) and 41.3% (50 ml spray-dried), respectively, with more of the powder deposited in the collecting vessel, and visibly less deposition on the walls of the drying chamber and cyclone separator. Discrete particles were collected as seen from SEM analysis (Figure 4.1 (c), (d)). Particles were spherical, and appeared to be hollow, with thin walls, as noted from some shattered particles and blow holes through the surface of the particles (marked by red arrows in Figure 4.1). They did not display the porous morphology of lactose particles spray-dried by McDonald (2005) however.

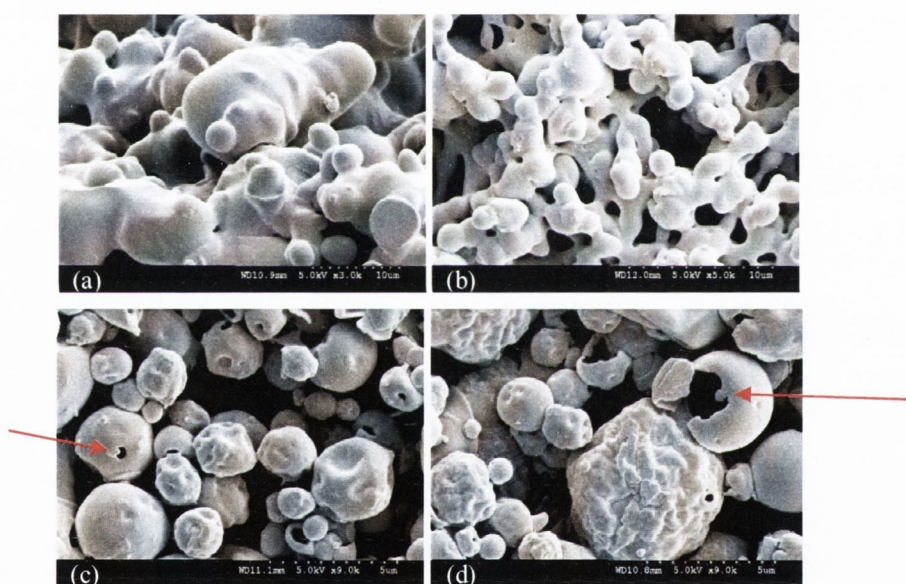


Figure 4.1 SE micrographs showing trehalose spray-dried from water, 2% w/v t.s.c., 25% of t.s.c. AC, inlet temperature (a) 100 °C, (b) 110 °C, (c) 130 °C, (d) 150 °C.

McDonald (2005) observed an increase in particle fusion under SEM and rapid visible deliquescence of spray-dried lactose powders, with increasing AC content. To investigate this effect regarding spray-dried trehalose powders, spray-drying was carried out with no AC and with increased AC (40% of t.s.c.), at an inlet temperature of 130 °C. Spray-drying without AC resulted in spherical particles with no evidence, observed from SEM, of shattered or fractured particles (Figure 4.2 (a)). Spray-drying with 40% of t.s.c. AC showed shattered particles in SE micrographs (Figure 4.2 (b)). No evidence of increased hygroscopic behaviour during preparation for SEM was found with trehalose samples on increasing AC content.

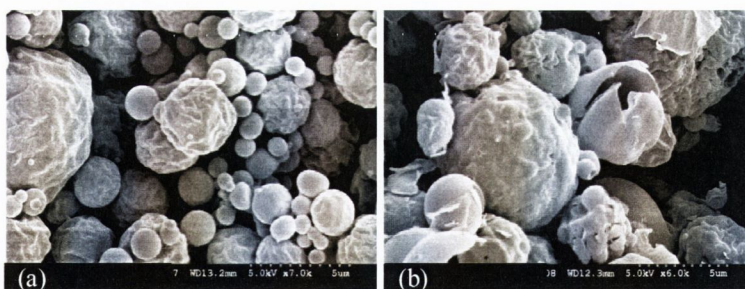


Figure 4.2 SE micrographs, showing trehalose, spray-dried from water, inlet temperature 130 °C with (a) no AC, (b) 40% of t.s.c. AC.

4.2.1.2 SPRAY-DRIED FROM ETHANOL:WATER SOLUTIONS

Trehalose:AC were initially spray-dried from 80% and 90% (v/v) ethanol at an inlet temperature of 78 °C and from 80% EtOH at a higher inlet temperature of 85 °C. These conditions were chosen considering the EtOH:H₂O:AC systems used to produce NPMPs of lysozyme (Chapter 3). It was not expected that these systems would produce NPMPs of trehalose alone, considering the hydrophilic nature of the sugar, and the hypothesis that production of NPMPs depended on solvent selection, as introduced in Chapter 1. It was considered desirable, however, that discrete amorphous particles could be produced using these spray-drying conditions, for incorporation into composites with protein NPMPs (Chapters 5, 6).

Collected products from these systems had a ‘sticky’ consistency, making further characterisation difficult. SE micrographs showed fused particles, an example of which is shown below (Figure 4.3). The sticky nature of the product on collection appeared unsuitable for formulation with a protein, and so these samples were not characterised further.

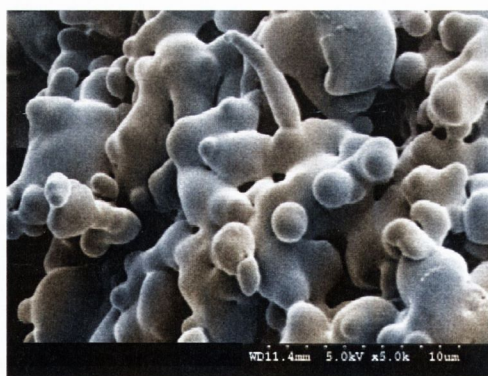


Figure 4.3 SE micrograph showing fused particles spray-dried from 80% EtOH, 10% of t.s.c. AC; inlet temperature 85 °C.

Strategies considered to overcome this problem were alteration of EtOH concentration, and the use of higher inlet temperatures as this had shown success with spray-drying trehalose from water. Total solid concentration was increased to 1% w/v to increase the product yield. AC was not expected to affect the production of fused particles based on results from spray-drying trehalose

from water, but was altered from 10-25% of t.s.c. to examine any effect on particle morphology. (10% was chosen because this had been used to produce NPMPs of lysozyme from an EtOH:H₂O:AC system, 25% was chosen to compare to the results from the aqueous systems.) EtOH concentrations of 60-80%, AC content (10-25%) and inlet temperatures (90-95 °C) were varied.

Spray-dried powders, produced using the higher inlet temperatures of 90-95 °C, were not noticeably “sticky” on collection and comprised non-porous spheres and shattered particles similar to those spray-dried from water, when viewed under SEM (Figure 4.4). Fusion between particles was reduced compared to the lower inlet temperatures of 78 and 85 °C used. Shattering and fracture of particles was more evident from SE micrographs of trehalose spray-dried with the higher AC content of 25% of t.s.c.. Particles appeared to be hollow and consisted of thin walls, as observed from these shattered particles.

The EtOH:H₂O:AC system did not produce NPMPs of the hydrophilic sugar alone. Composite lysozyme:trehalose NPMPs may be possible, however, considering the optimal spray-drying conditions to minimise fusion of the sugar, and to produce NPMPs of lysozyme alone.

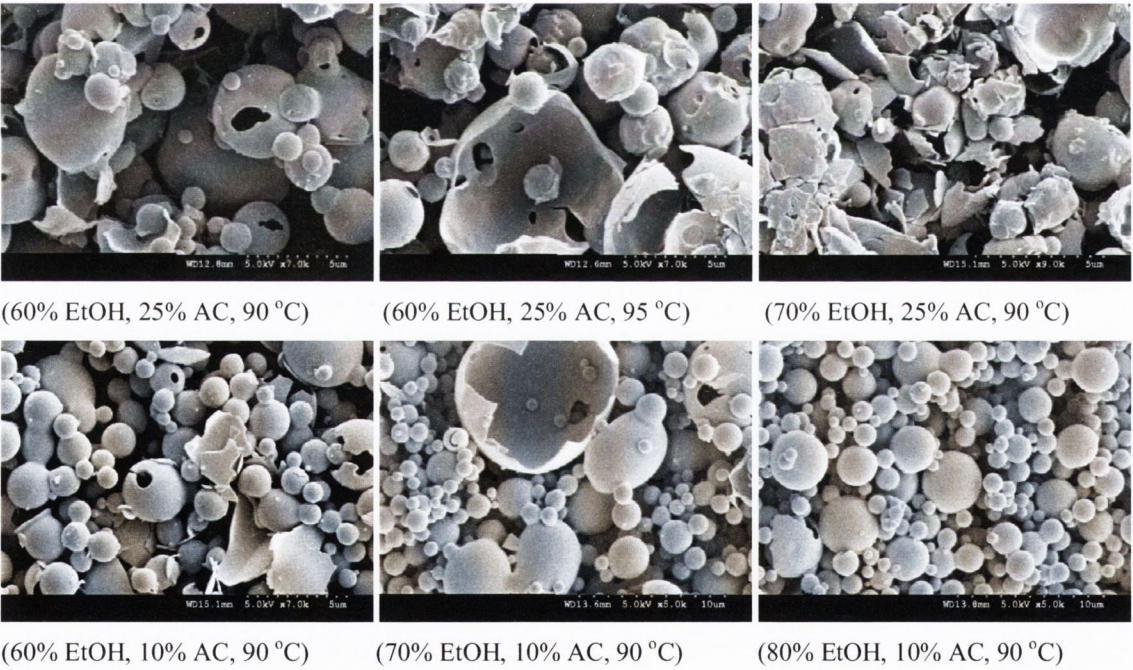


Figure 4.4 SE micrographs of trehalose spray-dried from EtOH:H₂O:AC systems, all 1% t.s.c., % EtOH, % of t.s.c. AC, inlet drying temperature as shown.

4.2.1.3 SPRAY-DRIED FROM METHANOL:N-BUTYL ACETATE

The use of a non-aqueous methanol:n-butyl acetate (MeOH:BA) solvent system, as had been successfully used by Li (2005) to produce NPMPs of HP- β -CD, was considered for trehalose because of the hydrophilic nature of the sugar similar to HP- β -CD.

Trehalose was initially spray-dried from MeOH:BA in solvent ratios of 50:50 and 80:20 and a t.s.c. of 0.5%. Spray-drying was carried out in the closed blowing mode configuration, using N₂ as drying gas, due to safety requirements while spray-drying with these organic solvents. The solvent ratio of 50:50 was chosen similar to Li (2005), and the solvent ratio of 80:20 was chosen because of the increased solubility of the sugar in the MeOH component compared to the BA component. An inlet temperature of 100 °C was chosen. Spray-drying resulted in NPMPs as shown below (Figure 4.5). NPMPs spray-dried from both solvent ratio systems showed a similar morphology, which resembled fused spherical nano-particles. There was a problem with an extremely low collectable product yield (< 2%) limiting characterisation of particles to SEM analysis. Contributories to this low yield may have included the small volume spray-dried (50 ml), low total solid concentration (limited by the solubility in MeOH) and inefficient trapping or escape of smaller particles to the filter and from the connections of glassware of the spray-dryer in the blowing mode. (Evidence for this included the greater than usual visible presence of powder on the filter after spray-drying and particles seen to escape in a white cloud between the cyclone separator and the collecting vessel during drying). This suggested the need for process optimisation, in order to have a usable system, and for operator safety. Variable factors, being the total solid concentration (t.s.c), volume spray-dried and the type of cyclone separator used were considered. Preliminary experiments showed that increasing these factors and use of a high performance (HP) cyclone, increased the collectable yield.

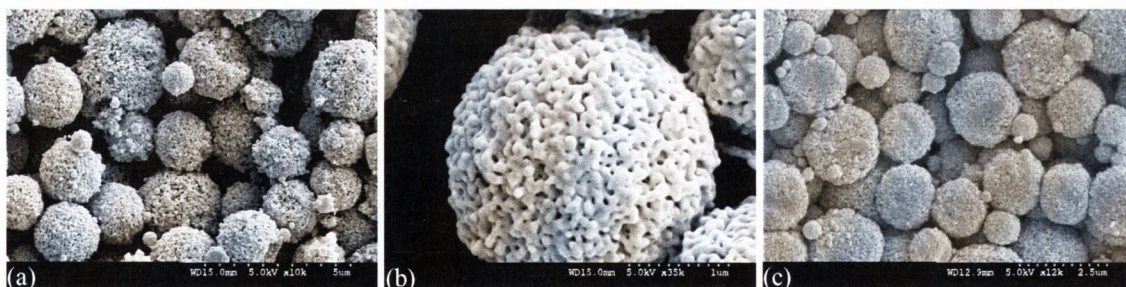


Figure 4.5 SE micrograph showing trehalose spray-dried from MeOH:BA from a solvent ratio of (a), (b) 80:20; (c) 50:50; 0.5% t.s.c., inlet temperature 100 °C.

The t.s.c. was maintained at 0.5%, because of limited solubility of trehalose in the non-aqueous system, and the volume spray-dried was increased. A high performance (HP) cyclone was used in place of the regular (REG) cyclone. Use of the HP cyclone was found to result in spray-drying being carried out at a lower pressure (~20 mbar compared to ~30 mbar) and a slightly lower outlet temperature. NPMPs were produced using the two cyclone separator types, irrespective of these

differences (Figure 4.6). The particles produced were similar, although the spherical shape appeared to be slightly looser in the case where the HP cyclone was used. Yield was increased to ~10% for the regular cyclone (200 ml spray-dried) and ~50% (200 ml spray-dried) for the HP cyclone, enabling further characterisation of the powder. On collection, the product spray-dried using the HP cyclone, appeared more static, which may have been due to the lack of grounding of the collecting vessel of the HP cyclone. Because of the increased yield, the HP cyclone was generally used for future spray-drying of NPMPs of trehalose, unless indicated.

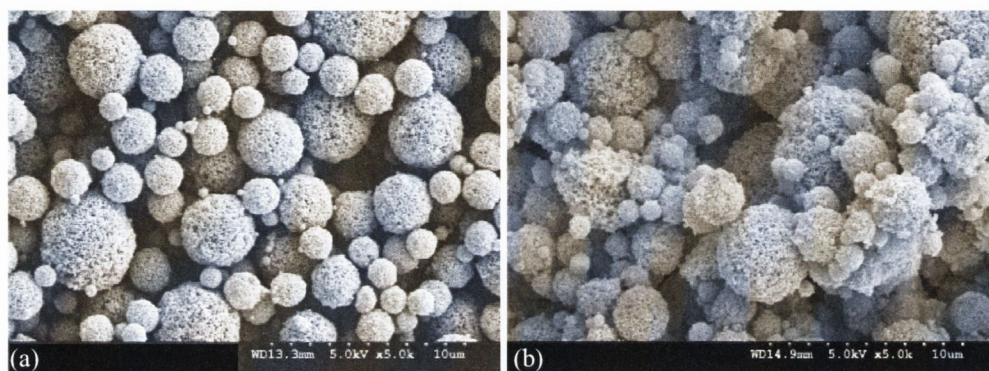


Figure 4.6 SE micrograph showing trehalose spray-dried from MeOH:BA (80:20 solvent ratio) using the (a) REG cyclone or (b) HP cyclone.

The effect of changing the solvent ratio on particle morphology was investigated by spray-drying from 90:10, 70:30, 60:40 (MeOH:BA) ratios (see Appendix 1 for details). It was found that spray-drying from a 90:10 solvent ratio gave porous particles which were irregular in shape, while spray-drying from 70:30, 60:40 ratios produced porous spheres (Figure 4.7). There was no trend seen in the collectable product yield, with yields of 32.9% (50 ml spray-dried), 26.7% (50 ml spray-dried) and 35.9% (50 ml spray-dried) obtained, respectively, for one batch each of trehalose spray-dried from MeOH:BA ratios 90:10, 70:30 and 60:40.



Figure 4.7 SE micrograph showing trehalose spray-dried from MeOH:BA in solvent ratios of (a) 90:10, (b) 70:30, (c) 60:40.

The effect of introducing a small amount of water into the MeOH:BA solvent system was investigated by spray-drying trehalose from 80:20:10, 49:49:2 and 15:15:1 MeOH:BA:H₂O solvent ratio systems (see Appendix 1 for details). This was desirable, when considering possible future composite systems with proteins, to aid dissolution of a water soluble protein or peptide

component. Li (2005) had successfully spray-dried NPMPs of HP- β -CD from the 15:15:1 solvent ratio system and from the higher water content solvent ratio of 6:6:1 (MeOH:BA:H₂O). Non-porous slightly fused particles were spray-dried from the 80:20:10 ratio using both the REG and the HP cyclone. Fusion between particles was more extensive where the HP cyclone was used, and particle size was observed to be smaller from SEM analysis (Figure 4.8 (a), (b)). Particles appeared to be hollow, as noted from several shattered particles and holes in particle walls (one example is indicated by a red arrow in Figure 4.8 (b) below). A phase separation/precipitation was found on addition of BA for the 49:49:2 and 15:15:1 MeOH:BA:H₂O solvent ratio systems. This appeared to be more extensive and coarser in the higher water content of the 15:15:1 ratio, necessitating early stoppage of spray-drying due to nozzle blockage. This precipitation may have contributed to the mixed particle morphology of NPMPs and large rods seen in SEM analysis for this system (Figure 4.8 (c)). NPMPs were produced from the 49:49:2 solvent ratio system, using both the REG and the HP cyclones (Figure 4.8 (d), (e)). The particles produced using the REG cyclone appeared to have a larger particle size, as observed from SEM analysis, which may be a reflection of the greater trapping efficiency for small particles of the HP cyclone. A lower t.s.c. of 0.3% was tried in the production of NPMPs using the HP cyclone (Figure 4.8 (f)), in order to minimise precipitation in the feed prior to spray-drying. Morphology of the particles was very similar to the higher 0.6% t.s.c., although the collectable product yield was lower at only ~10% (30 ml spray-dried) compared to ~24% (50 ml spray-dried) for the higher 0.6% t.s.c..

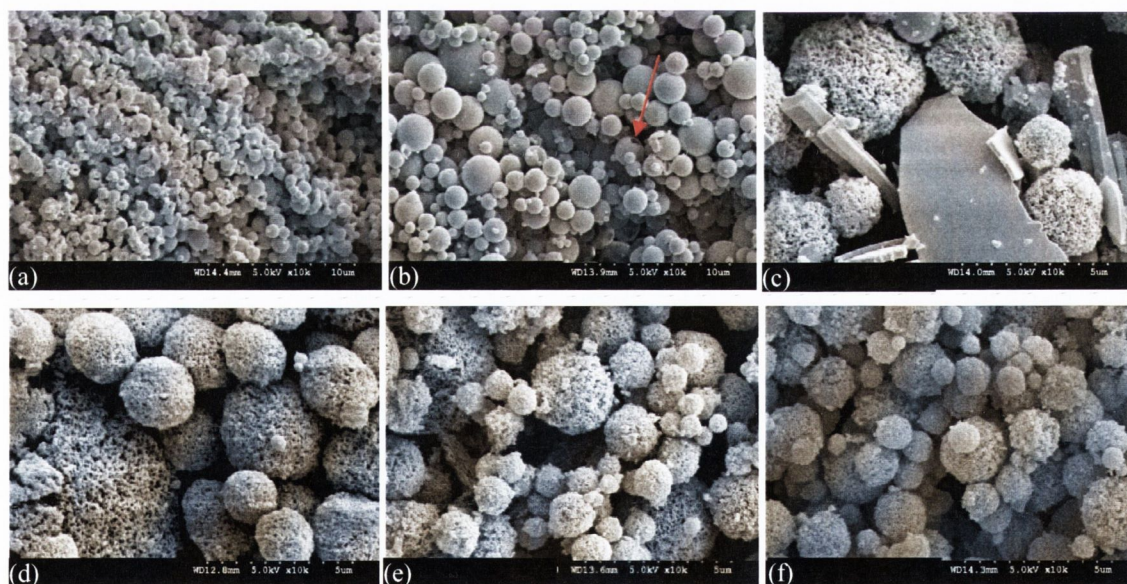


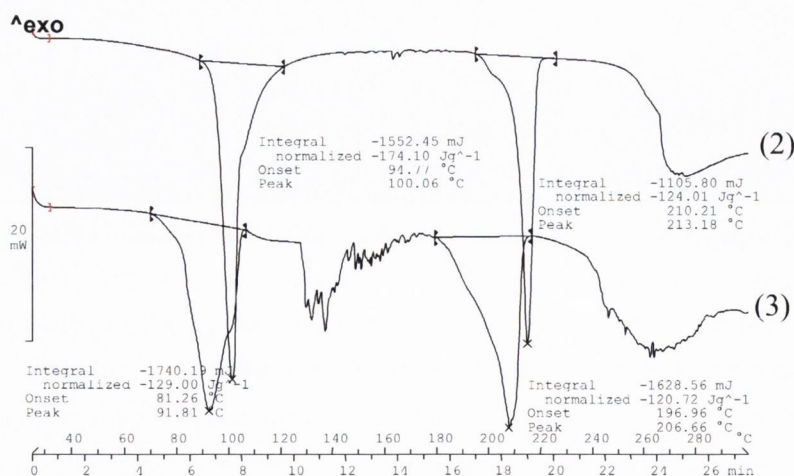
Figure 4.8 SE micrographs showing trehalose spray-dried using the indicated cyclone, solvent ratios (MeOH:BA:H₂O) and t.s.c., inlet drying temperature 100 °C (a) HP cyclone, 80:20:10, 0.45% t.s.c. (b) REG cyclone, 80:20:10, 0.45% t.s.c. (c) REG cyclone, 15:15:1, 0.48% t.s.c. (d) REG cyclone, (49:49:2), 0.6% t.s.c. (e) HP cyclone, (49:49:2), 0.6% t.s.c. (f) HP cyclone (49:49:2), 0.3% t.s.c..

4.2.1.4 SOLID-STATE CHARACTERISATION AND THERMAL ANALYSIS

4.2.1.4.1 Unprocessed Material: Trehalose dihydrate

Four different batches of α,α -trehalose dihydrate were used in the course of this thesis. (These are labelled batch 1-4 here for simplicity, see Materials (Chapter 2) for details of the batch and product numbers.) Batch 3 was found to behave differently to the other batches in spray-drying experiments. It was not possible to dissolve this batch of trehalose dihydrate in the MeOH component, and subsequently spray-dry it as a solution from non-aqueous systems, even at a reduced total solid content of 0.3% w/v and despite sonication. Spray-drying as a suspension produced a crystalline material as indicated by XRD analysis. Spray-drying this batch of trehalose dihydrate from water was possible and resulted in non-porous particles, similar to those produced using other batches of trehalose dihydrate. The powder was found to be extremely static on collection however. Differences between spray-drying and dissolution behaviour of batches were surprising, particularly between batches 2 and 3, since these batches had the same product number and thus preparation method and origin. Batch 3 was not used further for spray-drying experiments because of these problems, but solid-state, thermal, FTIR, SEM and laser diffraction particle size analysis were carried out to investigate possible reasons for these differences in behaviour, with particular attention to any differences between batches 2 and 3.

DSC scans (Figure 4.9) showed endothermic peaks - the first at ~ 91 - 97 °C and the second at ~ 206 - 211 °C. The first endotherm, at ~ 91 - 97 °C, can be attributed to loss of the dihydrate, the second endotherm at 206 - 211 °C to melting of the anhydrous β crystal form (Ohashi 2007, Taylor and York, 1998). Batch 4 differed from the other three batches in that a second peak was found immediately after the first endotherm. A non-specific endothermic area occurring after ~ 130 °C was noted in some batches. This may correspond to a melting transition of the α crystal form (Jones et al., 2006). Comparison of batches 2 and 3 showed that this area was greater with batch 3, in duration and also in amplitude relative to the other two peaks. Resolution between peaks was also poorer with batch 3.



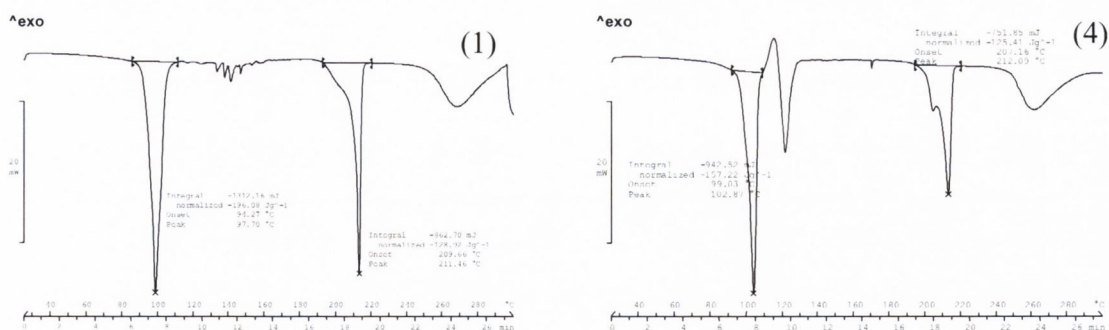


Figure 4.9 DSC scans of trehalose dihydrate batches 1-4 as numbered.

Mass losses of ~9.5% were calculated from TGA, occurring in one stage for batches 1, 2, 4 and in two stages for batch 3 (Figure 4.10). This level of mass loss was close to the theoretical value of 9.5% for the dihydrate sugar in all batches.

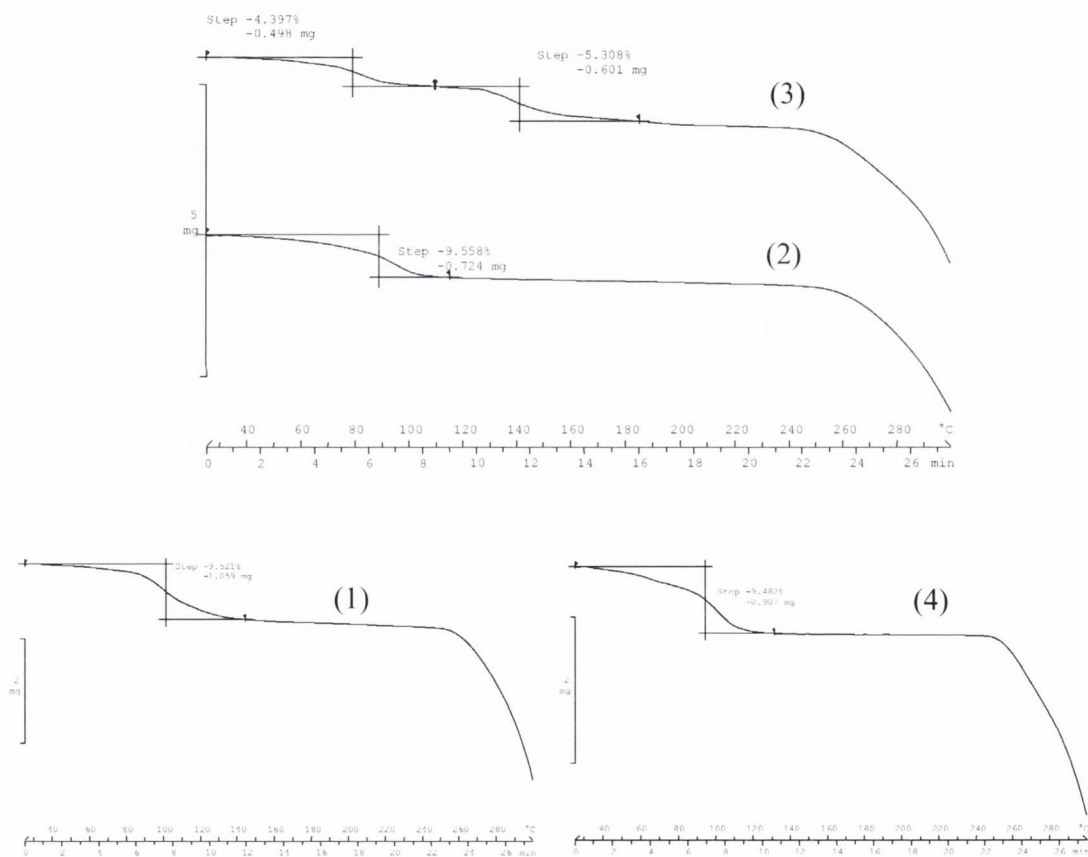


Figure 4.10 TGA scans of trehalose dihydrate, batches 1-4 as numbered.

FTIR spectra were similar for all the batches (Figure 4.11). XRD analysis showed that all batches had peaks in similar positions, and characteristic peak positions (~9°, 24°) were consistent with published peak positions for the dihydrate crystal form (8.7°, 23.8°) (Kilburn et al. 2006, Furuki et al., 2005, Ohashi et al., 2007) (Figure 4.12). Relative peak heights differed between batches. These results supported the dihydrate nature of all trehalose batches.

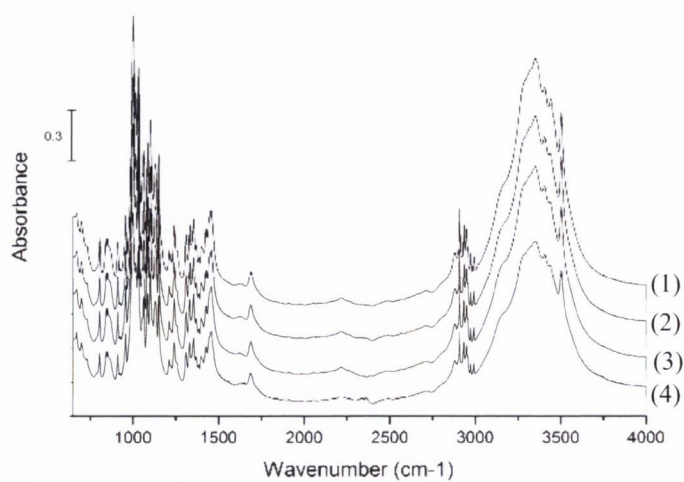


Figure 4.11 FTIR of trehalose dihydrate batches 1-4 as numbered.

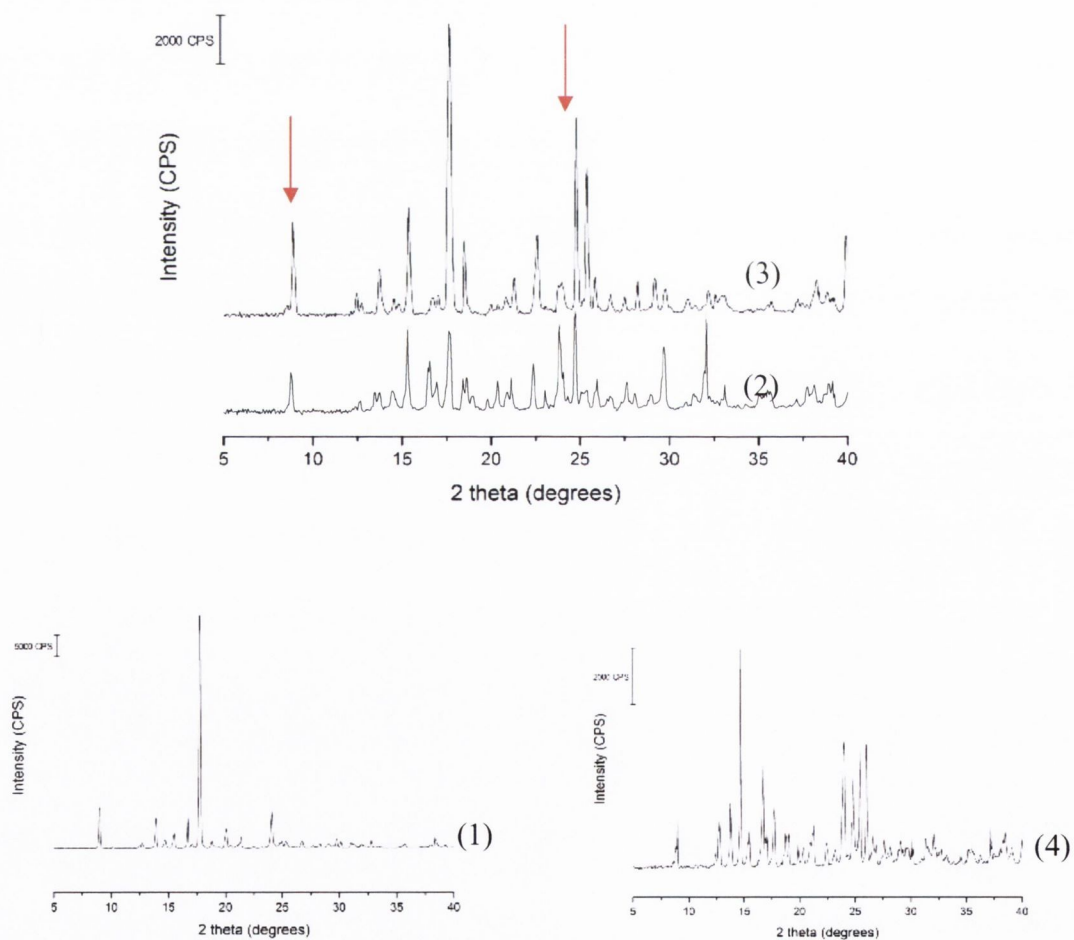


Figure 4.12 XRD traces of trehalose dihydrate batches 1-4 as numbered.

Despite the fact that FTIR and XRD confirmed the dihydrate nature of all batches, there were differences in thermal behaviour of batches as observed in DSC and TGA scans – particularly in the area of dehydration. Similar differences were noted by Taylor and York (1998) for different particle size fractions of trehalose dihydrate. The authors compared samples using hot stage microscopy, DSC, TGA, and XRD. DSC studies showed that the onset and completion of dehydration occurred at lower temperatures, with greater peak resolution, for the smaller compared to the larger particle size fractions. The TGA of the dehydration transition also occurred earlier and in one step for the smaller particle sizes and at a later onset and in two steps for the larger particle sizes. They concluded that the larger surface area to volume ratio and potentially increased crystal lattice defects of the smaller particles may have facilitated water removal and impacted on dehydration kinetics. Armstrong et al. (1996) also reported differences in thermal behaviour studied by DSC for three alpha, alpha-trehalose dihydrate batches, which persisted despite milling and concluded that sample origin and crystallisation history could affect crystal texture and thermal behaviour.

SEM analysis was carried out on the four batches and photo-micrographs are shown below (Figure 4.13). Batch 3 appeared to have the largest particle size. Particles from this batch also had a rough surface compared to the smooth surface of the crystals from the other batches. The size and shape of particles (for particles in the micrometre range) can affect solubility (Martin, 1993). From SEM analysis batch 3 did appear to be composed of larger crystals than the other batches, although all particles were relatively large and not in the fine particle micrometre range.

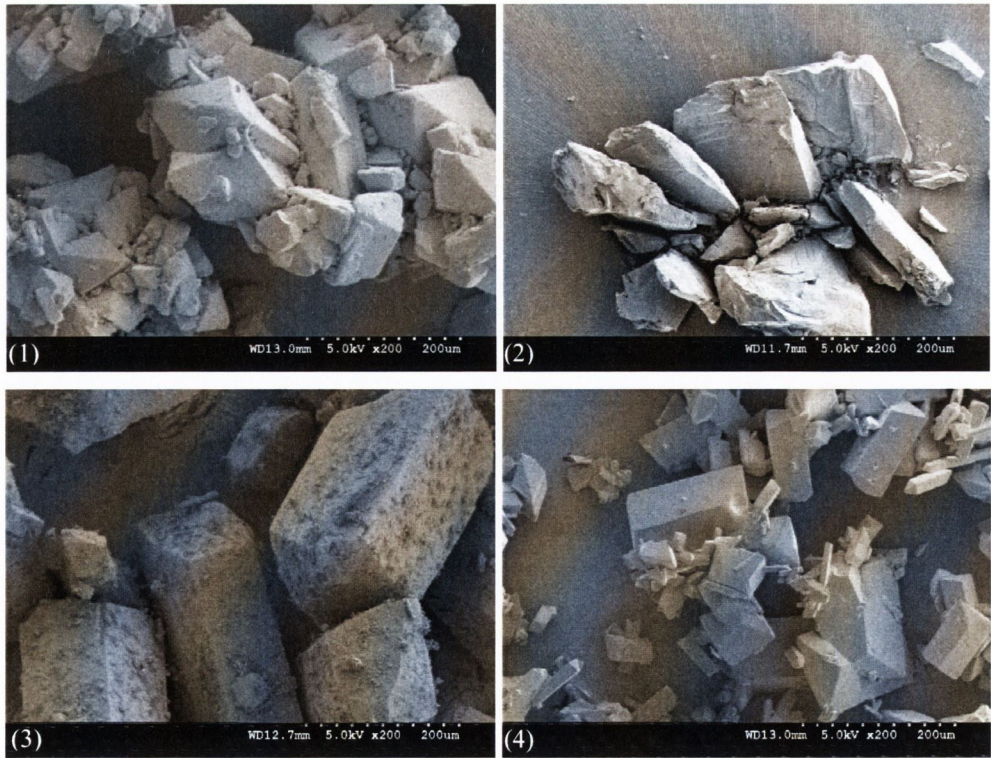


Figure 4.13 SE micrographs showing trehalose dihydrate batches 1-4 as numbered.

The particle size distributions of batches 2, 3 were investigated. Particle sizing was carried out at 2 bar pressure, 50% vibration rate and 2.5 bar pressure, 60% vibration rate. One analysis only was carried out for each sample due to scarcity of material. Particle size $d(0.1)$, $d(0.5)$, $d(0.9)$ are shown in Table 4.2. The particle size distributions are also shown (Figure 4.14). Both batches had a bimodal size distribution. The results showed that at the lower pressure and vibration settings, batch 3 appeared to have a slightly larger particle size, which agreed with observations from SEM analysis. This difference was not apparent in the higher pressure and vibration setting however with both batches displaying a very similar particle size distribution. These higher settings may have created a milling effect however, and resulted in fracture of larger crystals.

Table 4.2 Particle sizes of trehalose dihydrate batches 2, 3

	d(0.1)	d(0.5)	d(0.9)
Conditions: 2 bar/50%			
Batch 2	39.676	299.263	896.451
Batch 3	59.228	445.555	1080.593
Conditions: 2.5 bar/60%			
Batch 2	32.205	276.916	917.269
Batch 3	33.709	278.871	873.452

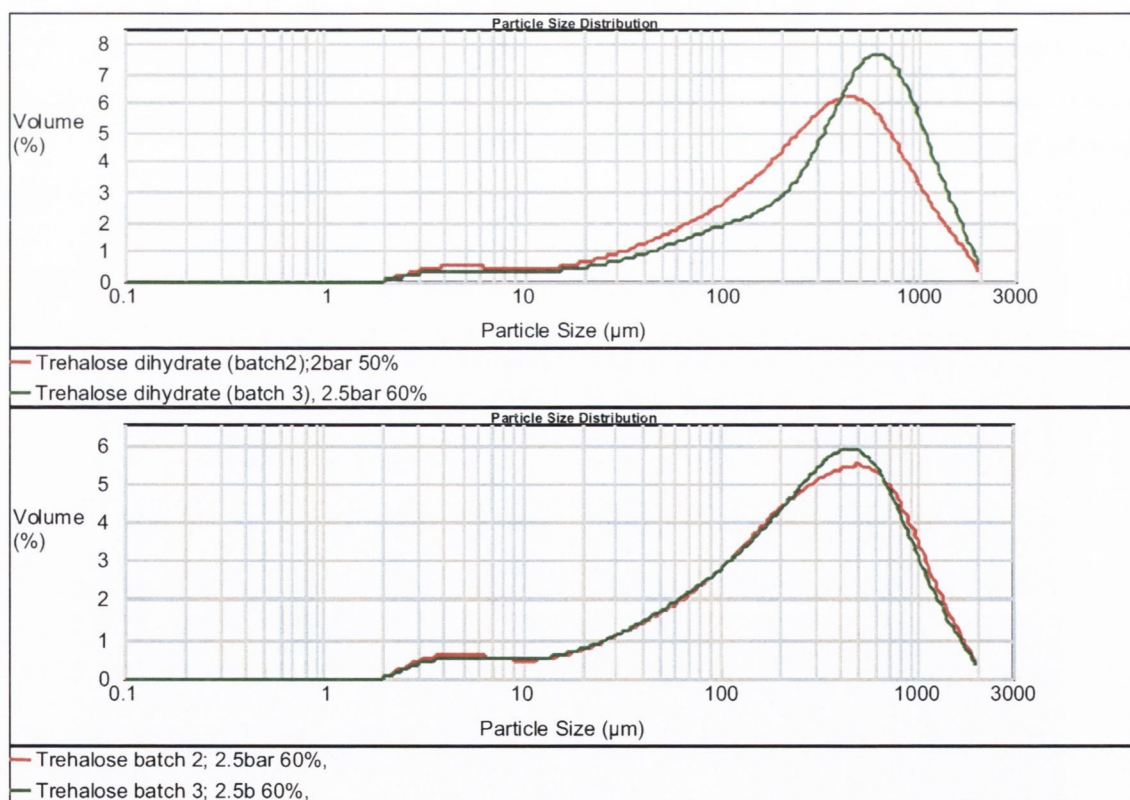


Figure 4.14 Particle size distributions of batches 2, 3.

These results suggest that a higher particle size and difference in crystal texture may have influenced dehydration behaviour and solubility of batch 3, although an in-depth study of these phenomena was outside the scope of this thesis.

4.2.1.4.2 Spray-dried trehalose from water

The “sticky” consistency of powder samples spray-dried from inlet temperatures 100 and 110 °C did not enable sample preparation for XRD analysis, and crystallinity could not therefore be established. The DSC and TGA scans of the sample spray-dried at inlet temperature 100 °C were examined for information about the solid state of the sugar however (Figure 4.15). The initial DSC scan, taken immediately after sample collection (Figure 4.15 (a)) showed an exotherm at onset ~52 °C (peak ~67 °C), followed by two endothermic peaks at ~94 °C, and at ~211 °C. The second endotherm was preceded by a disordered pattern on the DSC. The initial exotherm may correspond to a recrystallisation transition, based on the presence of the two subsequent endotherms. The first endotherm may be indicative of loss of the dihydrate, and the second endotherm may correspond to melting of the anhydrous trehalose, on comparison to similar peaks in trehalose dihydrate. The DSC scan was repeated on another sample of the same spray-dried batch, after the first DSC scan had finished (~45 minutes). The repeat scan (Figure 4.15 (b)) showed similar but smaller endotherms, and no evidence of an initial exotherm or disordered area before the second endotherm. TGA showed a one-step mass loss of ~10.4% which is slightly higher than the unprocessed material (~9.5%), consistent with mass loss of 2 moles of dihydrate water (Taylor and York, 1998). These data, particularly the difference between the two runs on the DSC, suggest that trehalose was in a metastable state and in the process of crystallisation.

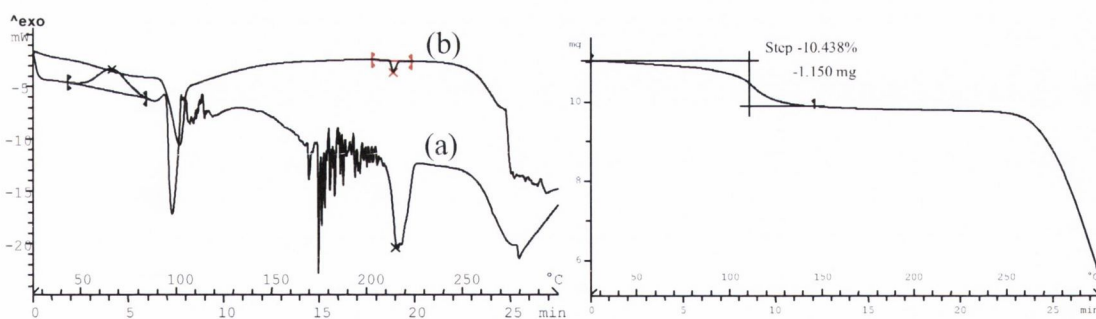


Figure 4.15 DSC initial (a), repeat (b) scans and TGA scan of trehalose spray-dried from aqueous solution at inlet temperature 100 °C.

All powders spray-dried from higher inlet temperatures of 130 or 150 °C were amorphous, as assessed by XRD, which were characterised by the absence of any sharp distinguishing peaks (Figure 4.16). DSC and TGA data were similar for all these systems (Figure 4.17). A broad endotherm was seen from 25~100 °C in DSC scans, indicative of moisture loss. A transition ascribed to the T_g was identified at ~120 °C on the DSC scans. Two of the samples (spray-dried at 25% AC, inlet 130 °C or 150 °C additionally showed an earlier transition at ~100 °C, which may

correspond to a small amount of dihydrate material. A recrystallisation peak was not identified in any case. The melting endotherm of anhydrous trehalose at ~210 °C was either not identified or was considerably smaller than the unprocessed material. Mass loss over the temperature range 25-100 °C from TGA (~3-5%), for these systems, was consistent with residual moisture or solvent loss.

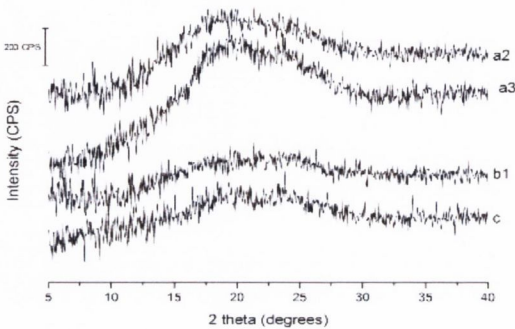


Figure 4.16 XRD scans of trehalose spray-dried from aqueous solution (a2) inlet temperature 130 °C, 25% t.s.c. AC, (a3) inlet temperature 150 °C, 25% t.s.c. AC, (b) inlet temperature 130, °C 0% t.s.c. AC, (c) inlet temperature 130 °C, 40% t.s.c. AC.

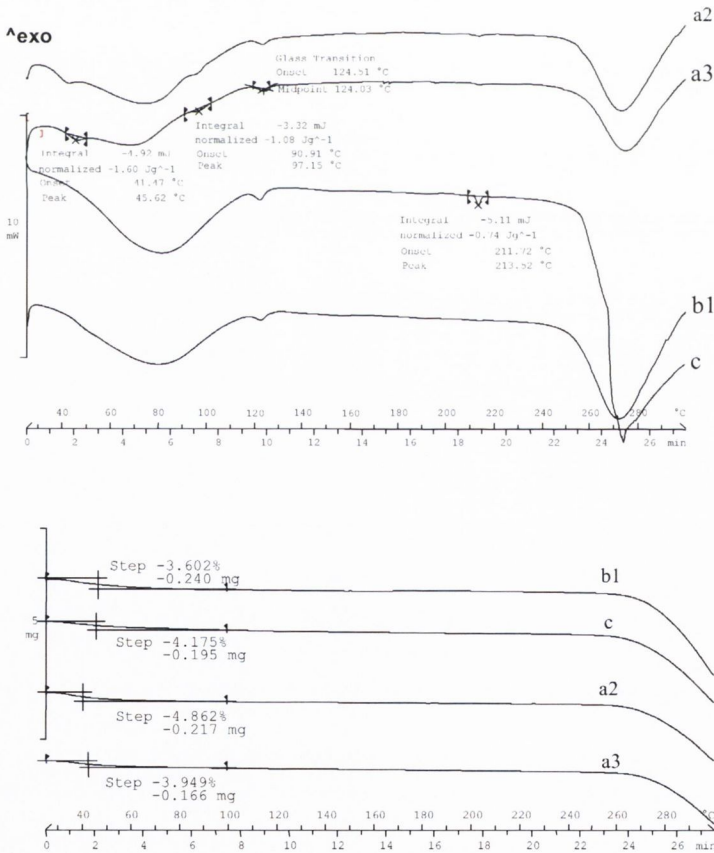


Figure 4.17 DSC and TGA scans of trehalose spray-dried from aqueous solution (a2) inlet temperature 130 °C, 25% t.s.c. AC, (a3) inlet temperature 150 °C, 25% t.s.c. AC, (b) inlet temperature 130, °C 0% t.s.c. AC, (c) inlet temperature 130 °C, 40% t.s.c. AC.

4.2.1.4.3 Spray-dried trehalose from ethanol:water solutions

Fused particles spray-dried from the lower inlet temperatures of 78 or 85 °C were not examined due to the sticky nature of the powders. The results presented relate to trehalose samples spray-dried from EtOH:H₂O solutions at the higher inlet temperatures of 90-95 °C. Powders were mainly amorphous as confirmed by XRD, although one sample (Figure 4.18 (b)) showed a small peak at ~24 °, indicative of the dihydrate form of trehalose. This sample had been spray-dried from 60% EtOH, with 25% of t.s.c. AC and at the higher inlet temperature (95 °C). DSC traces (Figure 4.19) were similar for all systems, except for the samples spray-dried from 80% EtOH, inlet temperature 90 °C. Glass transitions were identified at ~120 °C, no recrystallisation exotherms and small or absent melting endotherms were noted. Trehalose spray-dried from 60% EtOH with 25% of t.s.c. AC (inlet temperature 95 °C), additionally showed an earlier small endotherm in the DSC superimposed on the broad endotherm at ~88 °C. This small depression and the evidence from XRD analysis suggest an amount of dihydrate material present in the mainly amorphous sample (for which a T_g was detectable). This may have occurred as a result of processing or following conversion on exposure to ambient temperature and humidity conditions during sample preparation. The sample spray-dried from 80% EtOH, inlet temperature 90 °C, showed a disordered area between 120-140 °C and a considerably larger melting endotherm at ~210 °C in the DSC scan.

Residual moisture or solvent contents of 5-8% were calculated from TGA data (Figure 4.19). Samples, spray-dried from 70-80% EtOH, 10% of t.s.c. AC, inlet temperature 90 °C, showed a further small mass loss from 100 – 125 °C.

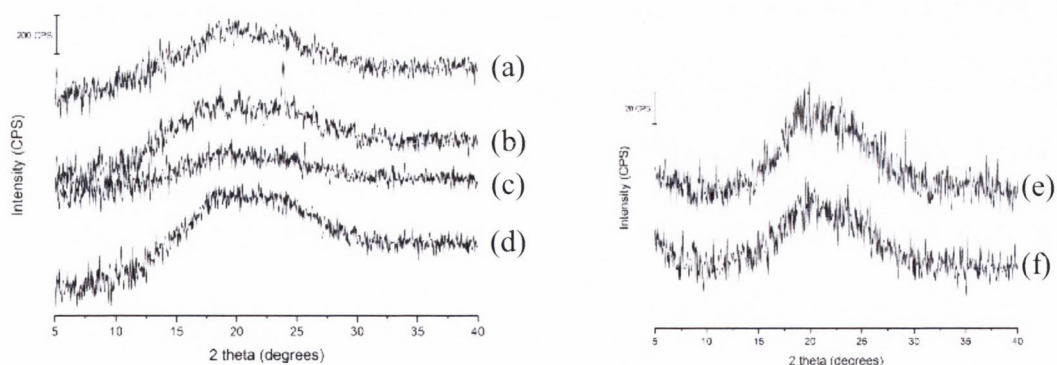


Figure 4.18 XRD scans of trehalose spray-dried from following conditions % EtOH, % AC of t.s.c., inlet temperature (a) 60%, 25% AC, 90 °C (b) 60%, 25% AC, 95 °C (c) 70%, 25% AC, 90 °C (d) 60%, 10% AC, 90 °C (e) 80%, 10% AC, 90 °C (f) 70%, 10% AC, 90 °C. Note (a)-(d) scans taken using Siemens XRD; and (e), (f) scans taken using Philips XRD.

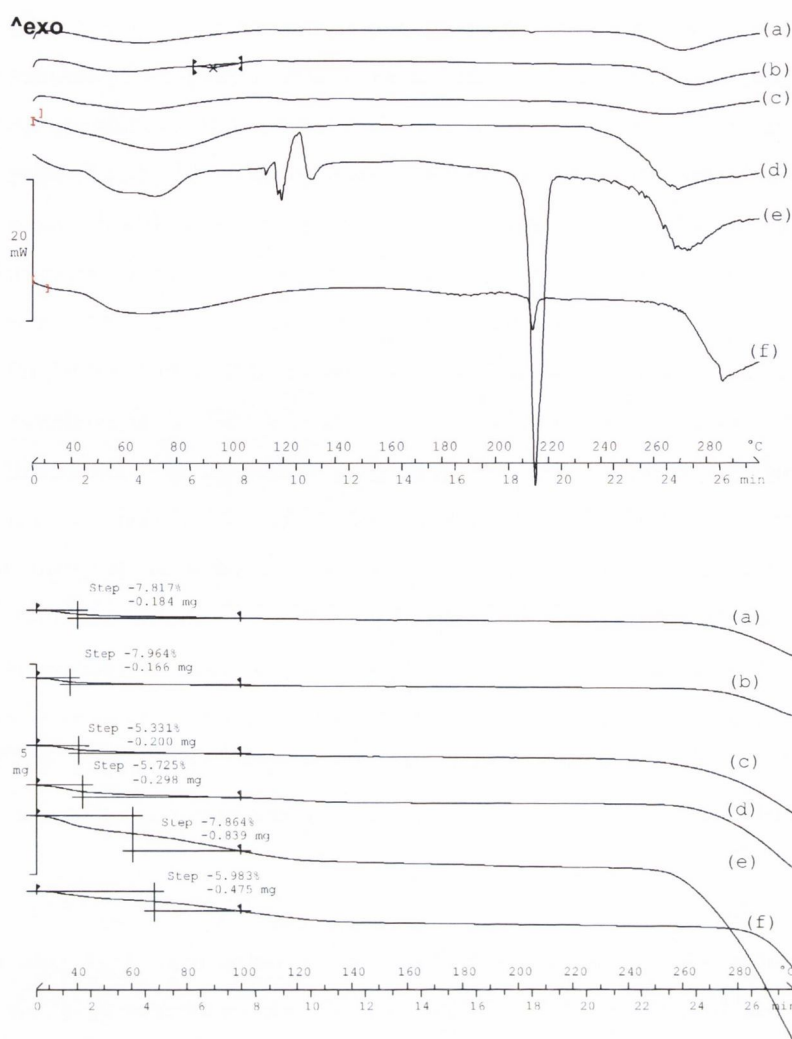


Figure 4.19 DSC (top), TGA (bottom) scans of trehalose spray-dried from following conditions % EtOH, % of t.s.c. AC, inlet temperature (a) 60%, 25% AC, 90 °C (b) 60%, 25% AC, 95 °C (c) 70%, 25% AC, 90 °C (d) 60%, 10% AC, 90 °C (e) 80%, 10% AC, 90 °C (f) 70%, 10% AC, 90 °C.

4.2.1.4.4 Spray-dried trehalose from methanol:n-butyl acetate systems

XRD analysis showed that NPMPs of trehalose were amorphous (Figure 4.20). DSC and TGA data were similar for trehalose spray-dried from 80:20 MeOH:BA solvent ratio, using either the HP or the REG cyclone (sample batches shown in Figure 4.21). A broad endotherm was observed from 25~120 °C, indicative of solvent or moisture loss. Tg could be determined from DSC data at ~120 °C in most cases but in some batches was obscured due to the broad endotherm. A recrystallisation exothermic peak occurred slightly later in the sample that was spray-dried using the regular cyclone in the sample batches shown. However, this could not be attributed to the choice of cyclone, because of a difference between onset of recrystallisation between batches (from ~144-180 °C) (see Appendix 2). Melting endotherms consistent with anhydrous trehalose at ~218 °C were identified. Residual solvent or moisture contents of ~3-4% were calculated from TGA.

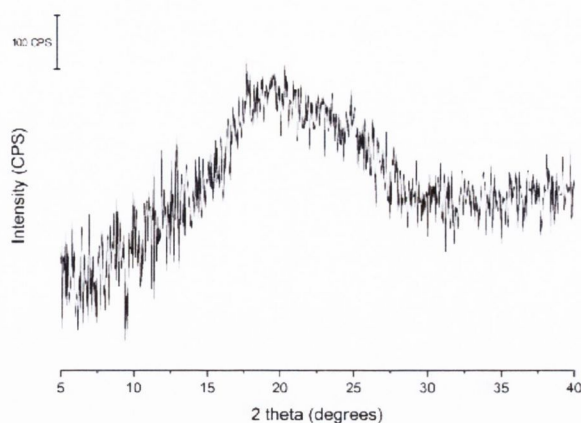


Figure 4.20 XRD scan of trehalose NPMPs spray-dried from 80:20 MeOH:BA.

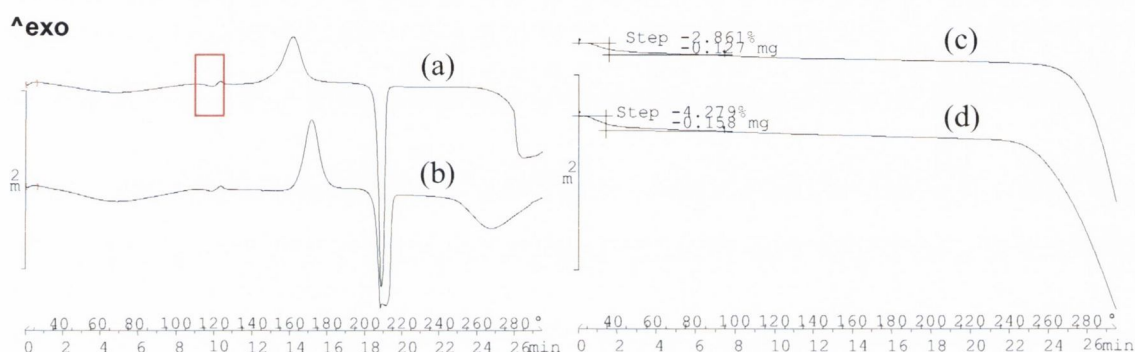


Figure 4.21 DSC and TGA scans of trehalose spray-dried from 80:20 MeOH:BA using (a), (c) HP cyclone (b), (d) REG cyclone.

An interesting behaviour was noted in the DSC scans of the NPMPs spray-dried from 49:49:2 MeOH:BA:H₂O using the REG cyclone (t.s.c. 0.6%), or HP cyclone (t.s.c. 0.6% or 0.3%) (Figure 4.22). No recrystallisation exotherm or melting endotherm was found in the system that had been spray-dried using the REG cyclone, but these were apparent in both cases where the HP cyclone was used to spray-dry trehalose from this system. Trehalose spray-dried from the lower t.s.c. of 0.3% using the HP cyclone, showed a later recrystallisation exotherm, immediately before melting. Similar residual moisture/solvent contents were calculated for all three samples spray-dried from 49:49:2 MeOH:BA:H₂O (Figure 4.22) and as such the difference in DSC could not be attributed to a difference in residual moisture or solvent.

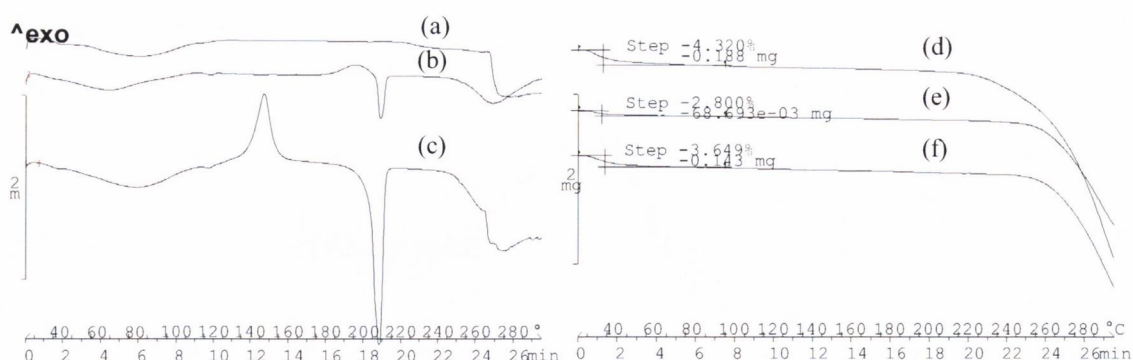


Figure 4.22 DSC and TGA scans of trehalose spray-dried from 49:49:2 MeOH:BA:H₂O using (a), (d) REG cyclone, 0.6% t.s.c.; (b), (e) HP cyclone, 0.3% t.s.c.; (c), (f) HP cyclone, 0.6% t.s.c..

The differences between thermal behaviour of batches and more significantly spray-drying conditions, as evident from DSC data, were interesting, considering the similarity between XRD and TGA analyses. Examination of the literature also showed some examples of amorphous trehalose with different crystallisation or thermal behaviour, as assessed by DSC. Surana et al. (2004) showed differences in the DSC scan for amorphous trehalose produced by different methods. A similar thermal behaviour for spray-dried trehalose, described by DSC analysis, was found in this paper as in the present work, in that freshly prepared spray-dried trehalose (from water) did not crystallise readily and no recrystallisation peak or melting endotherm of the anhydrous form was found. This was unlike amorphous trehalose produced by other methods, for example freeze dried, dehydrated, or melt-quenched (Surana et al. 2004), which resembled the trehalose samples spray-dried from MeOH:BA in this work, with recrystallisation and melting endotherms observed in the DSC scans.

The effect that the feed concentration in water had on the crystallisation tendency of spray-dried amorphous trehalose, was investigated by Moran and Buckton (2007). DSC studies were not presented in this publication and crystallisation was instead studied by a combination of dynamic vapour sorption and near infrared analysis. The samples spray-dried from the highest feed concentration were found to reproducibly and readily recrystallise when exposed to water vapour, whilst the samples spray-dried from the lower feed concentrations exhibited greater variability between batches and required a larger amount of water to be absorbed before crystallisation occurred. Differences in the drying rates of trehalose, spray-dried from different feed concentrations, was suggested by Moran and Buckton (2007) as influencing packing in the amorphous trehalose and as such differences in local or short-range order, despite the lack of long-range order. A similar explanation may be involved in the differences in thermal behaviour and recrystallisation tendencies of amorphous trehalose spray-dried from different spray-drying conditions, in the current work.

Tajber (2005) reported no recrystallisation of amorphous trehalose spray-dried from water (2.5% t.s.c., inlet temperature 110 °C) on a Büchi 190 spray-dryer. Bögelein (2007) reported DSC scans showing a lower T_g at ~80 °C, a recrystallisation exotherm at ~140 °C and decomposition above 175 °C, following spray-drying from a concentrated aqueous solution (35% w/w) on a Büchi 190, and from a 10% w/w solution from a Büchi 290 spray-dryer (inlet temperatures of 130, 138 °C, respectively). The lower T_g measured compared to published values in his work was attributed to the water content of the powders (~3%), and correlated with the predicted value using the Gordon Taylor equation Bögelein (2007). This decrease in T_g was not observed in this work, despite similarity in residual moisture/solvent calculated from TGA. This discrepancy between the values of T_g, and the decomposition or melting peak may be explained by different DSC sampling techniques, however. In the current work, DSC studies were carried out in pans with three pinholes in the lid, thus allowing residual moisture or solvent to escape, whereas in Bögelein's work completely closed DSC pans were used with a heating cooling reheating scan to eliminate interference with enthalpic relaxation.

4.2.2 RAFFINOSE

4.2.2.1 SPRAY-DRIED FROM METHANOL:N-BUTYL ACETATE

Given the positive results with trehalose, it was decided to investigate spray-drying of the trisaccharide - raffinose from similar non-aqueous MeOH:BA solvent systems. Raffinose was observed to be more soluble in methanol than trehalose, and as such a higher t.s.c. of 1% w/v was selected for spray-drying experiments, initially from a 80:20 MeOH:BA solvent ratio system. Increasing the t.s.c. was desirable because this was found to increase the collectable product yield, from preliminary experiments with trehalose. The regular cyclone was used, unless indicated, as this had the advantage of producing a less static powder and easier collection. (See Appendix 1 for full spray-drying conditions). A variable collectable yield was found with spray-drying different batches of raffinose from this solvent system and conditions, ranging from 2.5-27% (50 ml volumes spray-dried) and 44.12% (500 ml volume spray-dried).

NPMPs of raffinose were produced from the 80:20 MeOH:BA solvent ratio system, with similar morphology as trehalose particles. Microparticles were composed of a collection of spherical nano-particles as can be seen clearly from the SE micrograph taken at high magnification (Figure 4.23 (a)). The question as to whether particles were hollow was examined by considering all SE micrographs. Evidence of shattered or broken particles was scarce, and particles were generally observed to be intact. One example of a broken particle is shown in Figure 4.23 (b), marked by a red arrow. From this evidence particles did not appear to be hollow, but were comprised of fused spherical nanoparticles throughout the microparticle.

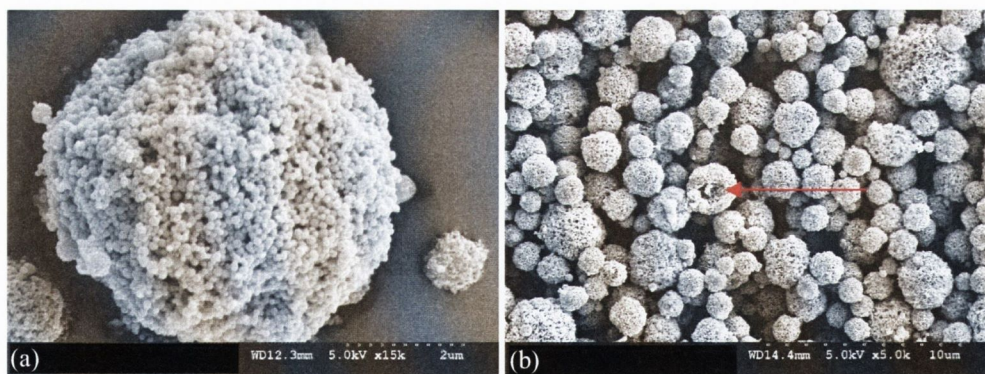


Figure 4.23 SE micrographs of raffinose spray-dried from 80:20 MeOH:BA; inlet temperature 100°C; 1% t.s.c..

The effect of changing solvent ratio on the morphology of the particles was examined, and solvent ratios of 90:10, 70:30, 60:40, 50:50 MeOH:BA were investigated (see Appendix 1 for conditions). Addition of BA to raffinose in the MeOH component in the systems of solvent ratios 60:40 and 50:50 (MeOH:BA) resulted in a phase separation/precipitation of raffinose, and were spray-dried as suspensions with nozzle cleaning set at level 2. The other solvent ratio systems (90:10, 70:30)

were spray-dried as solutions. As was found with similar experiments involving spray-drying trehalose, irregular-shaped NPMPs were produced from the 90:10 solvent ratio systems (Figure 4.24 (a)), and spherical NPMPs were produced from the 70:30, and 60:40 solvent ratio systems (Figure 4.24, (b), (c) respectively). The presence of some fused and non-porous microparticles amongst NPMPs was noted in the case of raffinose spray-dried from the 60:40 solvent ratio system. This was found to a much greater degree in the 50:50 solvent ratio system, as shown in Figure 4.24 (d). The fusion between particles could not be attributed to temperature and R.H. conditions during preparation for SEM coating (Section 4.2.5), as a sample spray-dried from the 80:20 MeOH:BA solvent ratio system prepared similarly did not show any particle fusion.

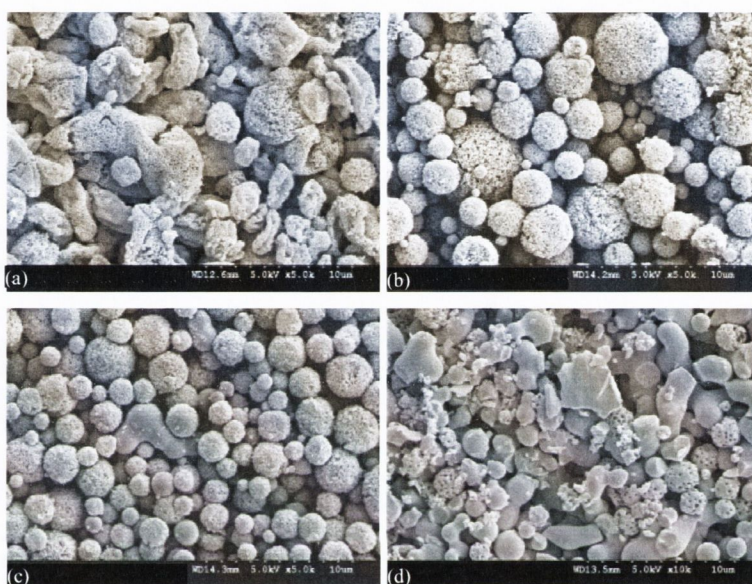


Figure 4.24 SE micrographs showing raffinose spray-dried from MeOH:BA solvent ratios (a) 90:10, (b) 70:30, (c) 60:40, (d) 50:50; all 1% t.s.c., inlet drying temperature 100 °C.

The effect of using a smaller nozzle screw cap of 1.4 mm (compared to 1.5 mm routinely used) on NPMP production was investigated. The lower nozzle screw cap size is recommended to minimise gas consumption and associated operating costs (Büchi, 2003), and resulted in a lower gas flow setting of ~20 mm (246 Normlitre/hour) compared to the usual 40 mm (473 Normlitre/hour), with all other conditions being similar. Manual reduction of the gas flow setting to 20 mm using the larger 1.5 mm nozzle screw cap was also tried as a comparison system. Problems with high O₂ during drying were encountered with this set-up however, necessitating early stoppage of the spray-drying experiment. The problem of high O₂ did not occur with the smaller nozzle diameter and lower gas flow setting of 1.4 mm/20 mm used. NPMPs were produced in all cases and shown in Figure 4.25. Particle size appeared to be largest in the case of raffinose spray-dried using the nozzle screw cap diameter of 1.5 mm and gas flow setting of 20 mm, and smallest for raffinose spray-dried using the nozzle screw cap diameter of 1.4 mm and gas flow setting of 20 mm, from SEM analysis. These results agree with data reported by Elversson and Millqvist-Fureby (2005), in

that particle size of spray-dried carbohydrate solutions was increased following reduction of gas flow and increase in nozzle orifice diameter.

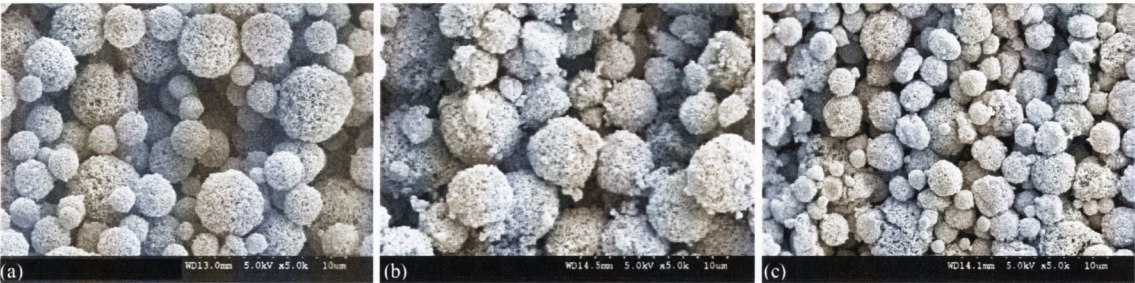


Figure 4.25 SE micrographs showing NPMPs of raffinose spray-dried from 80:20 MeOH:BA solvent system, inlet drying temperature 100 °C, 1% w/v t.s.c., nozzle screw cap diameter/gas flow setting as indicated (a) 1.5 mm/40 mm; (b) 1.5 mm/20 mm; (c) 1.4 mm/20 mm.

A small amount of water was introduced into the 80:20 MeOH:BA system, giving a 79:19:2 MeOH:BA:H₂O solvent ratio, to facilitate inclusion of a water soluble peptide or protein in future composite systems. Particles had a nano-particulate appearance and were slightly irregular in shape. Some evidence of fusion between particles was also noted from SEM analysis (Figure 4.26).



Figure 4.26 SE micrograph showing raffinose spray-dried from 79:19:2 MeOH:BA:H₂O, inlet temperature 100 °C, 1% t.s.c..

4.2.2.2 SPRAY-DRIED FROM WATER

Raffinose was spray-dried from H₂O (t.s.c. of 2.5%) at different inlet temperatures (110, 120, 130 °C) to produce non-porous comparative particles and to investigate the influence of drying temperature on this sugar (see Appendix 1 for conditions). Morphologies of the spray-dried particles were similar from SEM analysis (Figure 4.27), and were independent of inlet temperature. Particles were spherical and had a smooth non-porous surface. Unlike trehalose, no fusion of particles was noted from SEM, even at the lowest inlet temperature. The lowest inlet temperature of 110 °C resulted in deposition, visible as a sticky material, on the walls of the drying chamber and

cyclone separator however. This was also reflected in the collectable yield, with collectable yield increasing with inlet temperature (Appendix 1).



Figure 4.27 SE micrographs showing raffinose spray-dried from H₂O, t.s.c. 2.5% and inlet temperatures (a) 110 °C (b) 120 °C (c) 130 °C.

4.2.2.3 SOLID STATE CHARACTERISATION AND THERMAL ANALYSIS

4.2.2.3.1 Unprocessed material: Raffinose pentahydrate

XRD analysis confirmed the crystalline pentahydrate nature of the unprocessed material on comparison of peaks to published XRD scans (distinct peaks were identified at 10.75, 13.65 and 21.1 °, shown marked by red arrows, in Figure 4.28) (Bates et al., 2007; Leinen and Labuza, 2006).

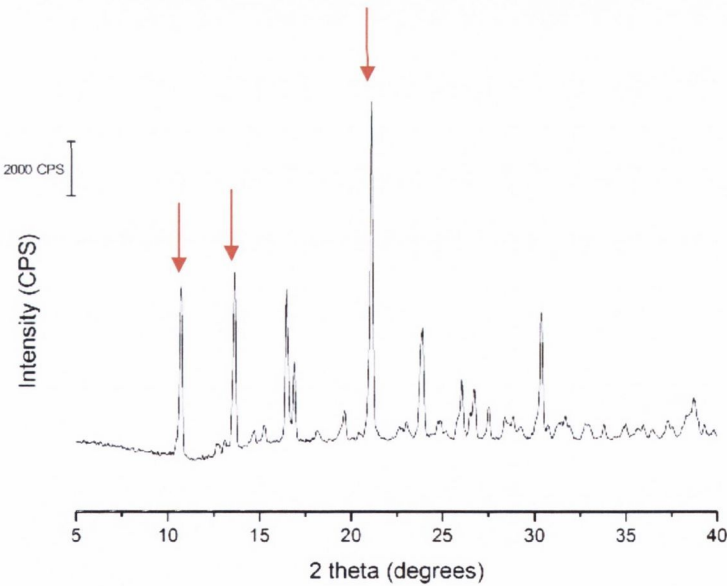


Figure 4.28 XRD scan of raffinose pentahydrate.

TGA showed a large step decrease in mass over the temperature range 25- ~130 °C of ~14% (Figure 4.29). (Mass loss considering the temperature range of 25-100 °C was ~ 12%.) The theoretical stoichiometric mass loss from dehydration of the pentahydrate form is 15%. A sharp endotherm was found in DSC analysis at ~84 °C corresponding to dehydration. This was followed

by an area of disordered pattern, starting at ~ 110 °C, suggestive of structural collapse or caramelisation of the sugar. No clear endothermic melting peak was observed from DSC (Figure 4.29). Moura Ramos et al. (2005), reported a similar pattern in the DSC of crystalline raffinose pentahydrate, and explained that raffinose pentahydrate dehydrated at ~ 80 °C, causing collapse into an amorphous form, and thus no endothermic melting peak was observed. They also found that no crystallisation was found on cooling raffinose in the metastable liquid form.

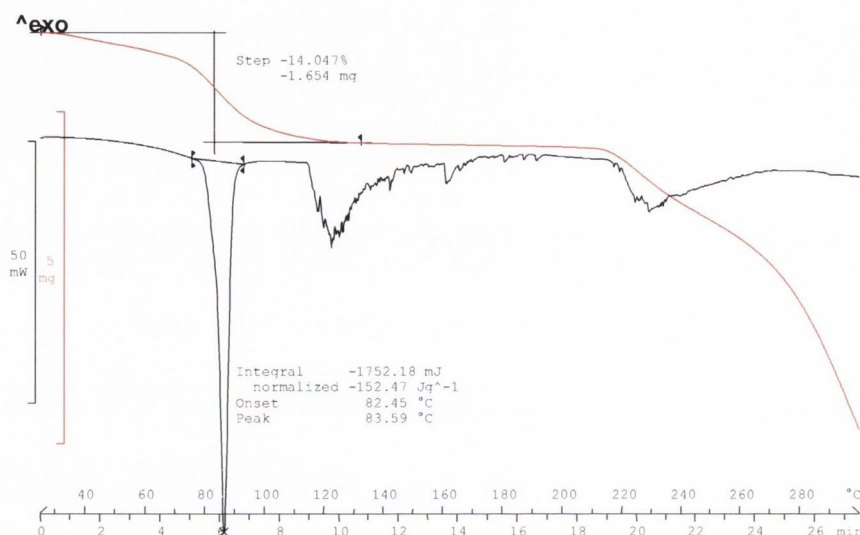


Figure 4.29 TGA (in red) scan and DSC scan (in black) of raffinose pentahydrate.

4.2.2.3.2 Spray-dried raffinose from methanol:n-butyl acetate systems

NPMPs of raffinose, spray-dried from the 80:20 MeOH:BA solvent system were amorphous by XRD, as demonstrated by the absence of any distinguishing peaks and a broad diffuse halo pattern. Fused particles produced from the 50:50 MeOH:BA solvent ratio system were also amorphous, showing a very similar pattern (Figure 4.30). DSC showed similar patterns for both systems with an initial broad endothermic peak and glass transition at ~ 120 °C (Figure 4.31). A small exotherm was also noted immediately after the glass transition, particularly evident in the DSC scan in Figure 4.31 (b). This can be attributed to enthalpy relaxation, on storage below the T_g. TGA showed mass losses of $\sim 4\%$, consistent with residual moisture/solvent loss, with an onset occurring earlier than for hydrate water loss ($> \sim 83$ °C) on comparison to unprocessed material.

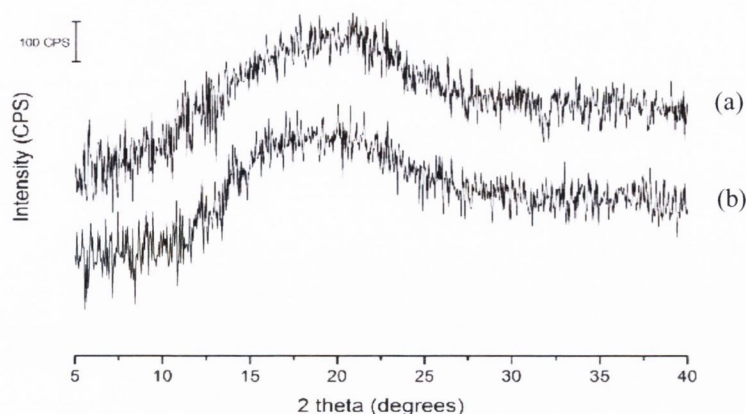


Figure 4.30 XRD scan of (a) raffinose spray-dried from 80:20 MeOH:BA, (b) raffinose spray-dried from 50:50 MeOH:BA.

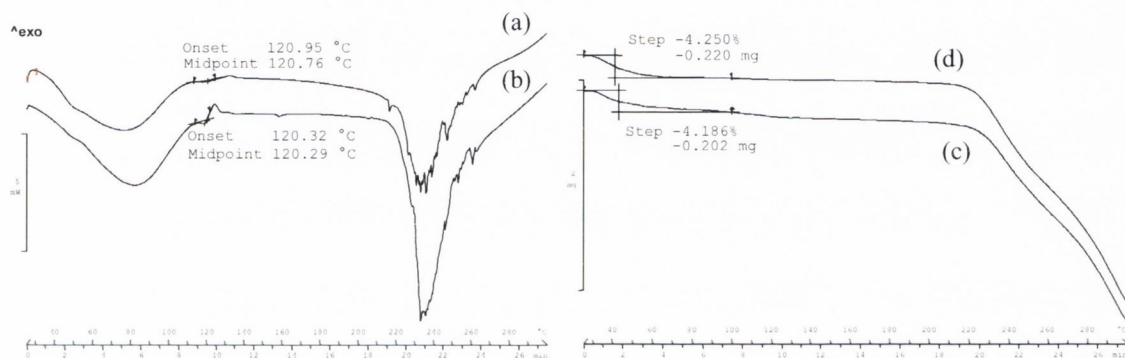


Figure 4.31 DSC and TGA scans (a), (c) raffinose spray-dried from 50:50 MeOH:BA, (b), (d) raffinose spray-dried from 80:20 MeOH:BA.

4.2.2.3.3 Spray-dried raffinose from water

XRD analysis demonstrated that raffinose, spray-dried from water (inlet temperature of 130 °C), was amorphous. DSC analysis (heating scan 25-300 °C) showed a broad endothermic peak from 25~130 °C followed by decomposition at ~220 °C (Figure 4.32 (b)). The broad endotherm obscured the possible identification of a glass transition temperature expected at ~ 115-120 °C. Heating to 100 °C (~15 °C below the glass transition temperature, to try to remove some of the residual moisture) followed by cooling to 70 °C and reheating to 300 °C, showed a glass transition at ~115 °C (Figure 4.32 (a)). Residual moisture was similar for this sample and those spray-dried from inlet temperatures 110 °C and 120 °C with 3.7 - 4.2% mass loss over the temperature range 25-100 °C (Figure 4.33).

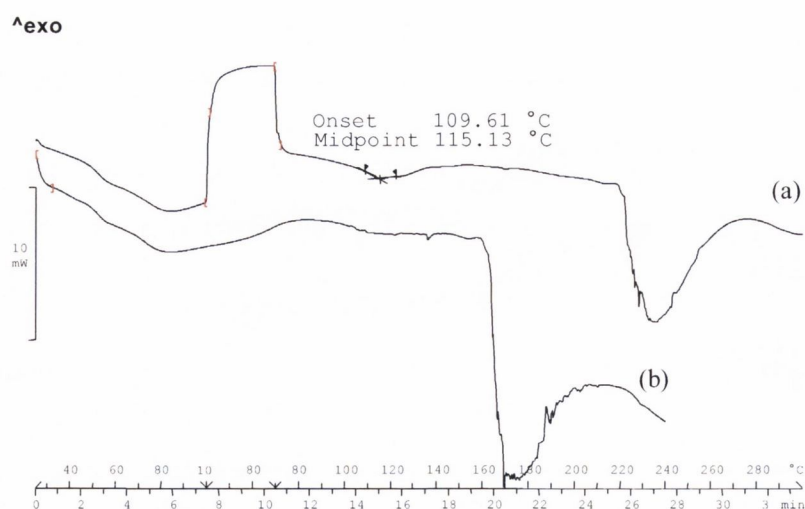


Figure 4.32 DSC of raffinose spray-dried from water, inlet temperature 130 °C, (a) 25-100-70-300 °C at 10 °C/min, (b) 25-300 °C at 10 °C/min.

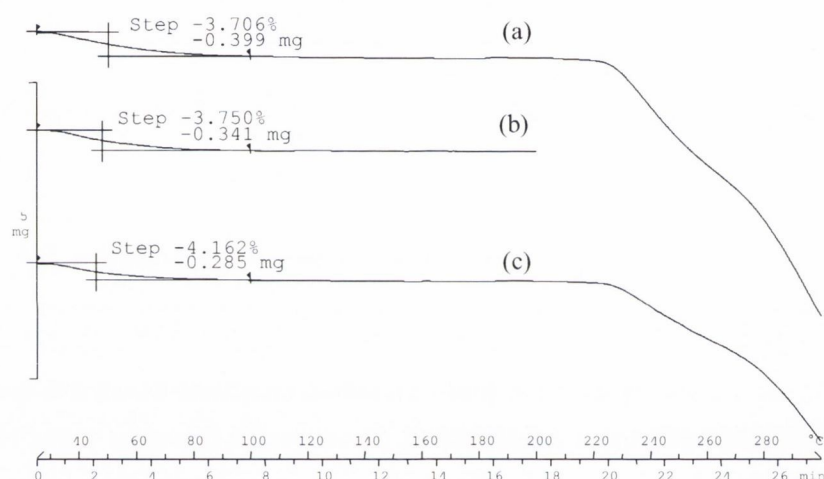


Figure 4.33 TGA of raffinose spray-dried from water, inlet temperatures (a) 130 °C, (b) 120 °C, (c) 110 °C.

Differences in thermal behaviour, from DSC analyses, were not observed between batches of raffinose spray-dried from similar or different solvent systems. This is in contrast with differences seen in the thermal behaviour of spray-dried trehalose in this work. Moura Ramos et al. (2005) concluded in a study of molecular mobility in amorphous raffinose produced by different methods (freeze-drying, annealing at 90 °C under vacuum for 1 hour, and heating above the T_g to 140 °C under inert atmosphere) that different amorphisation procedures did not generate glasses with different properties.

4.2.3 PARTICLE CHARACTERISATION OF SPRAY-DRIED SUGARS

NPMPs of the sugars, spray-dried from MeOH:BA systems, were expected to show favourable aerodynamic and micromeritic properties for pulmonary delivery, and to be superior to non-porous particles, spray-dried from water, from experiments with other NPMPs and non-porous materials (McDonald, 2005; Nolan, 2008). NPMPs (spray-dried from 80:20 MeOH:BA) and non-porous particles (spray-dried from H₂O) of trehalose and raffinose were thus compared in terms of particle size distribution via laser diffraction particle sizing, surface area, and bulk and tapped density and in-vitro deposition using the Andersen cascade impactor apparatus.

Particle size distribution, estimated using laser diffraction particle sizing at the standard operational settings of 2 bar air pressure and 50% vibration feed rate, suggested that NPMPs were slightly smaller than non-porous particles. The significantly smallest particle size was found for the batch of trehalose NPMPs studied (significance level $\alpha=0.05$). This difference in particle size may have been influenced by the choice of cyclone used, as this sample was spray-dried using the HP cyclone, which may have a higher trapping efficiency for smaller particles, whereas all the other batches were spray-dried using the regular cyclone. D(0.5) value are shown in Table 4.3, expressed as mean $\pm$ standard deviation. D(0.1), and d(0.9) values are also included. The particle size distributions are shown in Figure 4.34. All spray-dried sugar sample batches showed a mono-modal, highly reproducible intra-batch size distribution. Inter-batch variation was greater as demonstrated by the replicate single run of another batch each of NPMPs and non-porous trehalose, shown italicised in red in Table 4.3.

NPMPs of the two sugars had similar and very low bulk and tapped densities, which were significantly lower than the respective non-porous particles, by a factor of at least 2. Specific surface area measurements of the two sugars were also significantly higher than the non-porous spray-dried sugar particles, by ~ 40 times. The low bulk and tapped density and high specific surface area values of NPMPs were anticipated from examination of the particle morphology, comprised of fused nano-particles with voids between these nano-particles. Specific surface area values measured for NPMPs were approximately half of those reported by Nolan (2008) for NPMPs of sodium cromoglicate. This differences may be somewhat explained by the reported hollow nature of sodium cromoglicate NPMPs, and the fact that no evidence of hollow particles was found for the sugar NPMPs.

Table 4.3 Particle sizing (n = 3; mean ± standard deviation), values in red italic represent one analysis of a different spray-dried batch, surface area (n = 2; mean (range)) and bulk and tapped densities (n = 3; mean ± standard deviation) for NPMPs and non-porous trehalose and raffinose.

	NPMPs of trehalose	Non-porous trehalose	NPMPs of raffinose	Non-porous raffinose
Particle sizing				
d(0.1) (μm)	1.21± 0.00 [0.988]	1.35± 0.00 [1.534]	1.46 ± 0.03	1.53 ± 0.11
d(0.5) (μm)	2.48 ± 0.04 [2.078]	3.17 ± 0.03 [2.870]	2.96 ± 0.053	3.32 ± 0.23
d(0.9) (μm)	5.28 ± 0.17 [5.071]	7.07 ± 0.09 [5.188]	5.85 ± 0.27	6.77 ± 0.47
Surface area (m ² /g)	44.4854 (44.8366, 44.1342)	1.0963 (1.1551, 1.0374)	44.3023 (43.1865, 45.4180)	1.0681 (0.9312, 1.2049)
Bulk density (g/cm ³)	0.075 ± 0.001	0.225 ± 0.012	0.080 ± 0.003	0.342 ± 0.007
Tap density (g/cm ³)	0.124 ± 0.001	0.445 ± 0.021	0.122 ± 0.004	0.587 ± 0.024

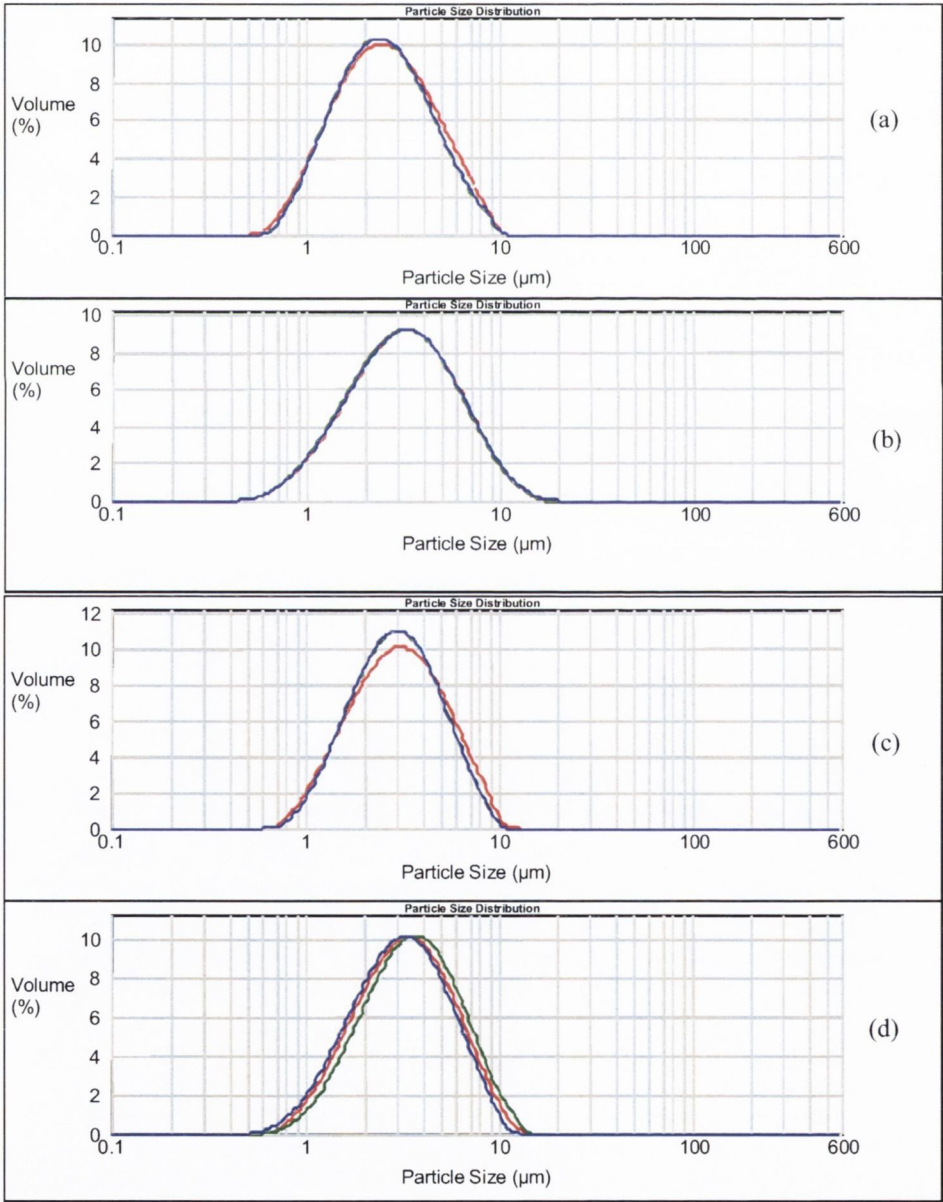


Figure 4.34 Particle size distributions of (a), (b) NPMPs of trehalose and non-porous particles of trehalose, (c), (d) NPMPs of raffinose and non-porous particles of raffinose.

4.2.4 IN-VITRO DEPOSITION VIA ANDERSEN CASCADE IMPACTOR ANALYSIS

Sugar moieties pose difficulties in quantification, as they lack suitable chromophoric groups. Methods used to quantify sugars traditionally involve HPLC analysis on an amino column with refractive index detection or low UV detection. These methods lack sensitivity however - with published concentrations measured using refractive index detection, in the literature ranging from 0.11-71 g/L for the major sugars (Montero et al., 2004). The amino column may also lack ruggedness and be degraded by the use of aqueous mobile phases required for sugar analyses. Alternative methods of saccharide quantification include the megazyme enzyme assay kits (Megazyme International Ireland Ltd.). This company provides specific trehalose and raffinose assay kits having linear test ranges of 20-400 µg/ml and 60-1250 µg/ml respectively. These sensitivities may suffice for ACI analysis although the lower limits of these ranges (particularly for the raffinose kit) may be high, leading to problems particularly in quantifying the amount of sugar deposited on the lowest stage 6 and filter of the impactor (judging by ACI results on protein NPMPs). Non-specific methods of powder quantification in ACI analysis were therefore considered. Two simple methods identified in the literature were measuring the increase in mass of a glass filter placed on each stage (Bosquillon et al., 2004) and inclusion of a small amount of fluorescent material (~0.2% by weight) with the spray-dried particles followed by fluorescence detection and quantification by comparison to a prepared calibration curve (Bosquillon et al., 2001 a, b; Codrons et al., 2003).

Initial attempts found the filter weighing method to be unreliable using the analytical laboratory balance. Inaccuracies were suspected with extremely low recovery of powder following analysis, despite emptying of the capsule and visible powder collected on filters, in some cases a lower post-analysis vs pre-analysis mass of the filter was recorded. A more sensitive gravimetric balance may have enabled increased accuracy with this analysis. The second method as described by Bosquillon et al. (2001 a, b) was thus considered. NPMPs of trehalose and raffinose were spray-dried with the fluorescent dye coumarin-6, by incorporating a low dye load (0.2% w/w of sugar content) with the sugar in the 80:20 MeOH:BA feed solution, followed by spray-drying. Coumarin-6 is a lipophilic molecule, with negligible water solubility, and as such could not be dissolved in the aqueous solution used for production of non-porous particles of trehalose or raffinose. An alternative feed system was initially considered to allow the use of the coumarin-6 dye and spray-dry non-porous sugar particles. Coumarin-6 was thus incorporated with raffinose in a feed solution of 60% EtOH and spray-dried. Resultant particles had a fused morphology and appeared to have a mixed particle size from SEM (Figure 4.35). These particles were unlike standard spray-dried sugar particles and as such were considered unsuitable as comparators to the NPMPs. Sulforhodamine 101 fluorescent dye is a hydrophilic dye (Codrons et al., 2003) and was used subsequently to incorporate with non-porous particles, and spray-dried from water.



Figure 4.35 SE micrograph showing raffinose spray-dried with coumarin-6 from 60% EtOH.

Similarity of particles spray-dried with or without fluorescent markers was ascertained by SEM analysis and by laser diffraction particle sizing. SE micrographs of particles including the fluorescent marker, showed them to be very similar to NPMPs or non-porous particles spray-dried without the fluorescent marker (Figure 4.36). Particle sizing by laser diffraction was carried out on one replicate of the NPMPs or non-porous particles containing the fluorescent marker. Although measured particle sizes were statistically significantly different to the measured batch in triplicate, a similar difference was noted between batches and the difference was likely similar to inter-batch variation.

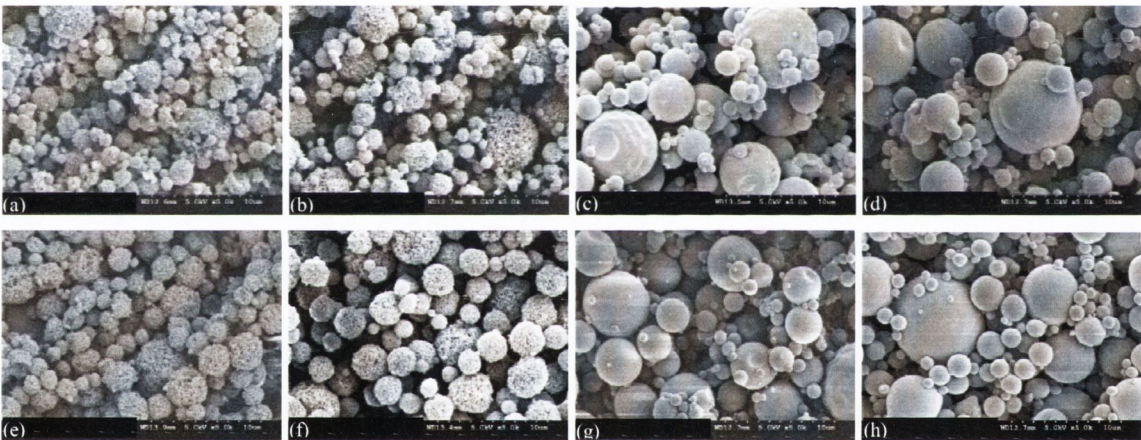


Figure 4.36 SE micrographs showing spray-dried (a) trehalose NPMPs with coumarin-6, (b) trehalose NPMPs without coumarin-6, (c) non-porous trehalose with sulforhodamine 101, (d) non-porous trehalose without sulforhodamine 101; (e) raffinose NPMPs with coumarin-6 (f) raffinose NPMPs without coumarin-6, (g) non-porous raffinose with sulforhodamine 101, (h) non-porous raffinose without sulforhodamine 101.

Table 4.4 Particle size of NPMPs and non-porous particles of trehalose and raffinose including fluorescent marker (single run).

	d(0.1) μm	d(0.5) μm	d(0.9) μm
Trehalose NPMPs + c6	1.069	2.198	4.917
Non-porous trehalose + sulf101	1.640	3.473	6.830
Raffinose NPMPs + c6	1.386	2.636	4.983
Non-porous raffinose + sulf101	1.607	3.613	7.435

Andersen cascade impactor analysis was carried out using a Handihaler® as a delivery device, and a flow rate of ~50 l/min, dictated by the requirements of a pressure drop of 4 kPa over the inhaler device. The two sugars exhibited a similar pattern, with NPMPs having statistically higher particle fraction < 5 µm and < 3 µm (calculated as % of the emitted dose accounted for), and lower MMAD and GSD values, compared to non-porous particles. The difference in MMAD values between NPMPs and non-porous particles did not agree with the much smaller difference between median diameter by volume distribution, and may also be affected by the density, shape and dispersibility and aggregation of the powder. NPMPs and non-porous particles both showed a higher MMAD than measured d(0.5), but NPMPs showed a smaller difference. This may suggest decreased inter-particle interactions and aggregation between particles for NPMPs compared to non-porous particles. The GSD value gives an indication of the variability of the aerodynamic diameter of particles. From this data, NPMPs were less variable than non-porous particles. Emitted dose (expressed as % of nominal dose from the inhaler), was slightly higher for NPMPs but this was not statistically significant, due to the large standard deviations seen. Analysis of deposition patterns showed that all particles deposited primarily in the mouthpiece adaptor and induction port. After this, NPMPs showed higher deposition on the lower stages 3, 4 and non-porous particles showed higher deposition on the upper stages -1, 0, 1.

Table 4.5 Summary of results of ACI analysis; expressed as mean ± standard deviation; for NPMPs (n = 4)/non-porous particles (n = 4) of trehalose and NPMPs (n = 6) and non-porous particles (n = 3) of raffinose.

	% < 5 µm	% < 3 µm	MMAD (µm)	GSD	% emitted (of nominal dose)
Trehalose NPMPs	40.17 ± 6.76	24.17 ± 4.86	3.144 ± 0.616	2.358 ± 0.32	80.6 ± 16.1
Trehalose Non-porous	16.24 ± 2.7	7.97 ± 1.88	12.43 ± 2.3	3.453 ± 0.398	66.5 ± 32.7
Raffinose NPMPs	54.8 ± 11.5	33.56 ± 8.82	2.676± 0.800	2.118 ± 0.171	81.2 ± 11.0
Raffinose Non-porous	17.66 ± 1.9	9.51 ± 1.91	9.154 ± 0.806	3.555 ± 0.352	76.23 ± 7.51

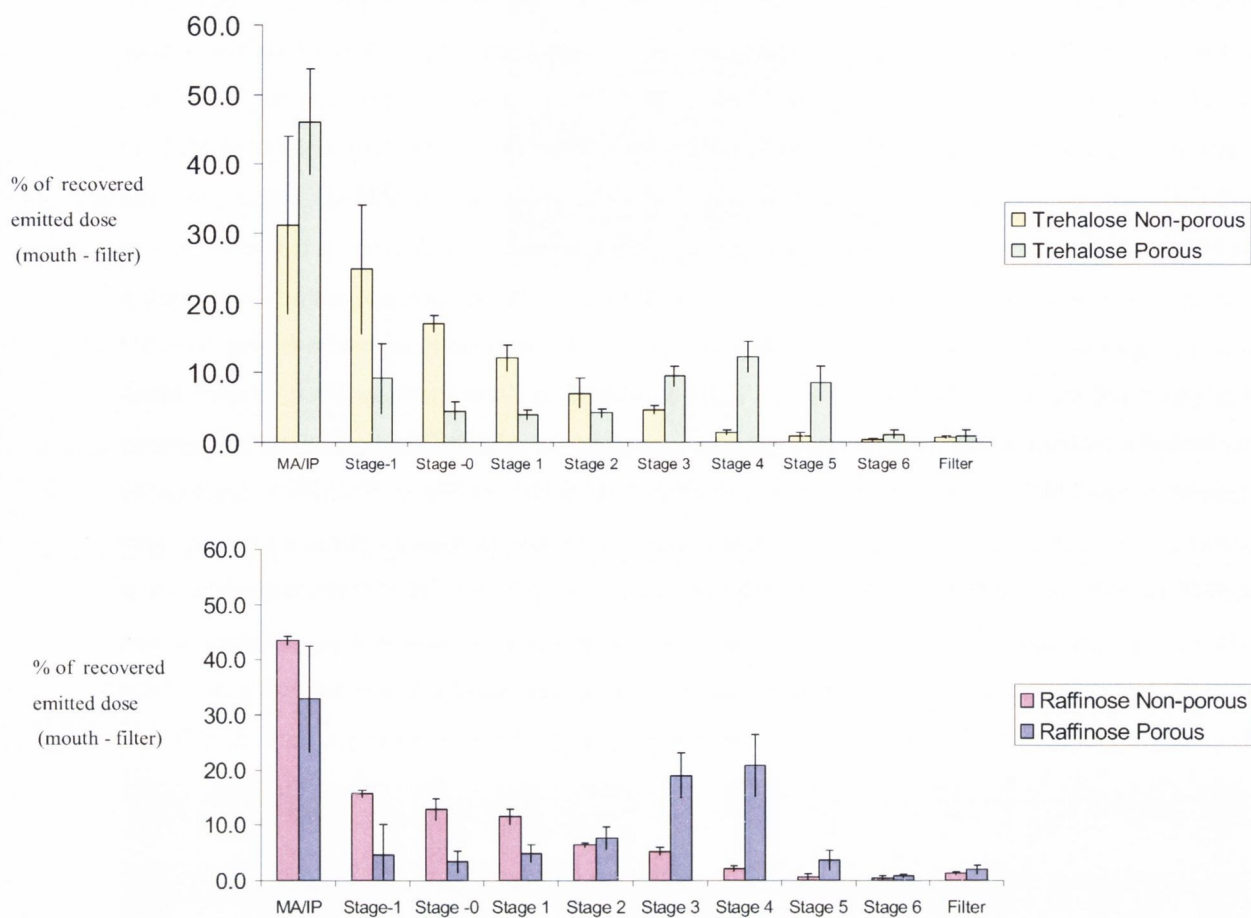


Figure 4.37 Deposition patterns on the stages of the ACI apparatus showing differences between product morphologies. Calculated as % of emitted recovered dose (mouth – filter), used to calculate fine particle fraction. MA – mouthpiece adaptor, IP – induction port.

4.2.5 HYGROSCOPICITY OF SPRAY-DRIED SUGARS

A problem was encountered with spray-dried powders of trehalose and raffinose, which was apparent on high humidity (> 60%) and high temperature days (> 25 °C) as seen during the summer months. Particles were found to visibly absorb moisture when mounted on aluminium stubs prior to sputter coating in SEM, changing from a powder surface to a translucent mass. This was not found during the rest of the year (lower ambient humidity and temperature). Subsequent SE micrographs showed complete particle collapse, with a fused mass visible as shown in Figure 4.38, which is characteristic of deliquescence. This behaviour was noted for NPMPs of the two sugars regardless of batch of sugar or solvent used, drying conditions (inlet temperature 100 °C vs 120 °C, nozzle screw cap diameter, total solid content 0.3% vs 0. 6%, type of cyclone), and storage time prior to SEM analysis. This was partly, overcome by a faster preparation (~20 mins) of stubs for SEM analysis, and minimisation of time between preparation and coating. A similar behaviour was described and photographed by Van Den Berg et al. (1993), in the case of freeze-dried sucrose, where ~20 min exposure to ambient conditions of 50% R.H. and 20 °C immediately prior to sputter coating on EM stubs, resulting in structural collapse.



Figure 4.38 SE micrograph of spray-dried trehalose particles showing deliquescence.

Evidence that the loss of particle stability occurred during preparation of samples for SEM rather than on storage at 4 °C in a desiccator, was obtained from comparing SEM of the same sample taken on two different days, where deliquescence was seen in the first SEM but not on the second. Evidence that trehalose was affected more than raffinose was obtained where NPMPs of raffinose and trehalose prepared similarly and SEM taken on the same day resulted in less visible deliquescence of raffinose compared to trehalose. The chemistry of the sugar (hydrophilicity, and molecular weight), the strength of interactions between the sugar and water and the greater ability of raffinose over trehalose to absorb moisture before crystallisation (5-hydrate vs 2-hydrate) are also likely to affect hygroscopic behaviour. Although trehalose and raffinose are considered to have low hygroscopicity, amorphous sugars usually have a much stronger tendency to take up water vapour (Hancock and Shamblin, 1998).

It was decided to examine in more detail the hygroscopicity of spray-dried raffinose NPMPs. Water uptake of raffinose NPMPs under lower ambient temperature and humidity conditions of $\sim 20.8\text{ }^{\circ}\text{C}$, and R.H. of 37% (measured ambient lab conditions using a hygrometer) was carried out by placing a small amount ($\sim 8\text{ mg}$) of raffinose powder on a gravimetric balance and monitoring the increase in mass over 30 minutes, followed by TGA. This short time frame was selected based on a similar time scale for SEM sample preparation, to compare these milder ambient conditions with those producing deliquescence during SEM sample preparation. Good repeatability was found for the 2 runs with a very small increase in mass ($< 1\%$) after 30 minutes exposure to these ambient conditions. Mass losses over the temperature range $25\text{--}100\text{ }^{\circ}\text{C}$ of $\sim 4.5\%$ were determined from TGA, following this exposure, which are similar to losses determined for freshly spray-dried sample (Figure 4.39). A larger powder sample ($\sim 100\text{ mg}$) was similarly examined and found to demonstrate a similar small increase in mass of $\sim 0.72\%$ after 30 minutes. SEM, XRD and DSC were also carried out on the larger volume sample and no changes were noted compared to freshly spray-dried material, with retention of amorphous character and NPMP morphology.

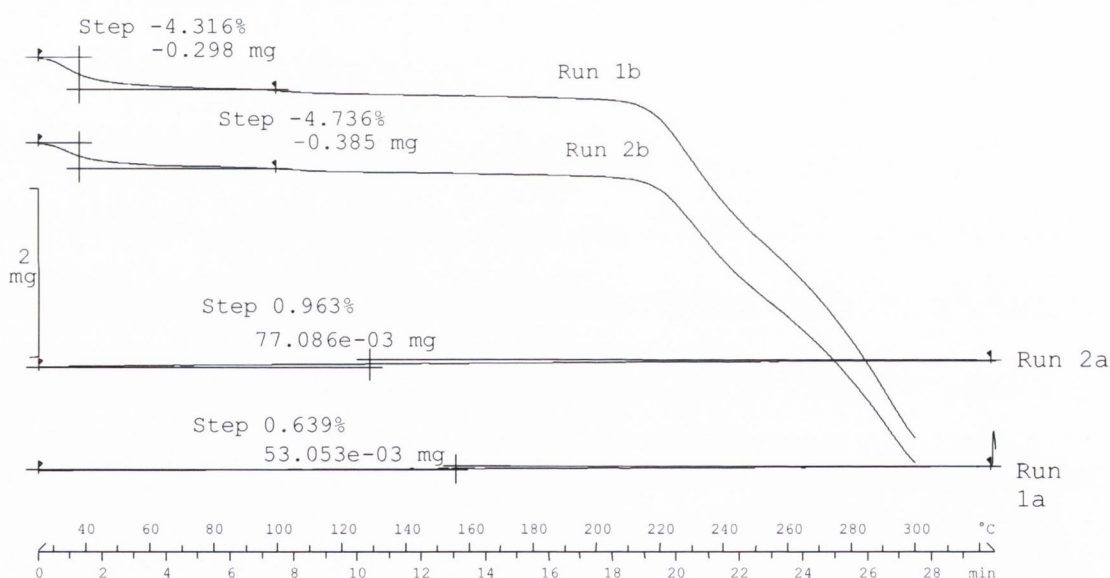


Figure 4.39 Water uptake of raffinose NPMPs (1a, 2a) (ambient conditions of $20.8\text{ }^{\circ}\text{C}$, 37% R.H.), followed by TGA (1b, 2b), 2 runs shown.

Literature studies involving amorphous spray-dried raffinose have examined the influence of temperature and relative humidity on stability. Miller and Lechuga-Ballesteros (2006) found that spray-dried raffinose remained as discrete spherical particles following storage at $25\text{ }^{\circ}\text{C}$ and R.H. up to 38% for 8 weeks. Storage at higher R.H. of 43.2% resulted in liquid bridging of particles and at a high R.H. of $\sim 69\%$ resulted in recrystallisation with SE micrographs showing angular crystalline material. Saleki-Gerhardt et al. (1995), also showed that amorphous raffinose prepared

by two different methods exhibited structural collapse and crystallisation at temperature 30 °C and R.H. > 45%.

To ascertain if a difference was apparent between NPMPs and non-porous spray-dried samples, powder samples of raffinose were placed at 25 °C, 60% R.H. for 24 hours, followed by visual inspection and SEM analysis. Significant moisture uptake and collapse of structure were observed similarly in both cases. Visual inspection of samples revealed that samples had the appearance of a “paste” and a fused mass as seen from SEM analysis (Figure 4.40). These results do not show a difference between NPMPs and non-porous spray-dried particles but the conditions and the time frame studied may have been unsuitable to allow differentiation between the two.

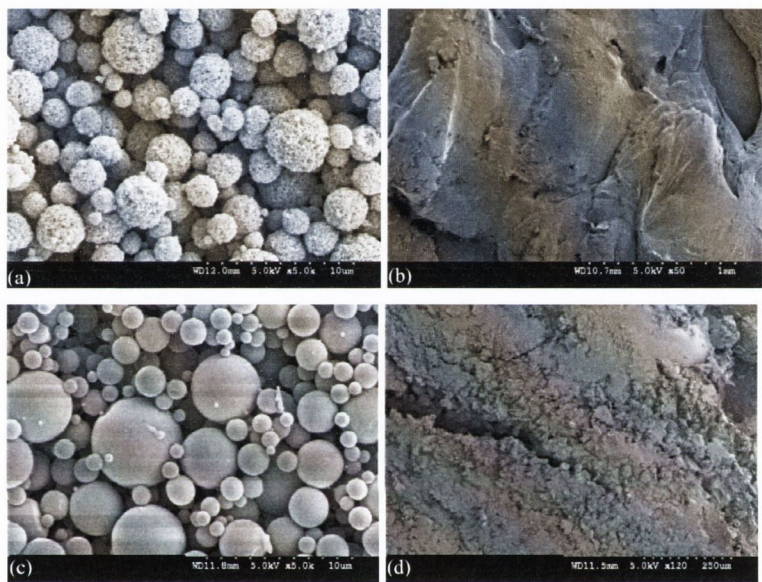


Figure 4.40 SE micrographs showing raffinose spray-dried as (a) NPMPs, (c) non-porous, and following storage at 25 °C / 60% R.H. for 24 hrs (b) NPMPs, (d) non-porous.

4.3 POLYOLS

4.3.1 MANNITOL

Mannitol is generally crystalline on spray-drying, although amorphous material may be found on spray-drying in a composite system for example in mass ratio of up to 30% w/w with protein (rhuMabE25) depending on drying conditions (Maa et al., 1997).

Mannitol was spray-dried from a MeOH:BA:H₂O (7:2:1) system, because of solubility problems in MeOH:BA system. The addition of BA to mannitol dissolved in the MeOH:H₂O component however resulted in some precipitation of the polyol. Further precipitation was seen on spray-drying, with blockage of the nozzle requiring early discontinuation of drying. Recovered product was a mix of spherical, rough or porous particles and rods (Figure 4.41 (a)). This mixed morphology may be due to some mannitol being in solution and some in suspension. The system was repeated with filtering through a 0.45 µm filter prior to spray-drying. Precipitation in feed tubes and around the nozzle was again noted, and spray-drying had to be prematurely stopped and the spray-dryer cleaned. Recovered product comprised of aggregates and larger rods (Figure 4.41 (b)).

This MeOH:BA:H₂O system may be unsuitable for spray-drying mannitol alone, and was not considered further, except for thermal and XRD analysis.

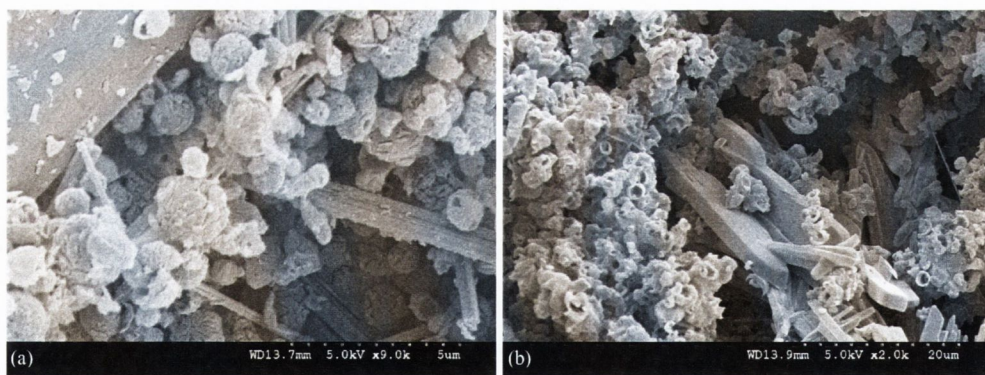


Figure 4.41 SE micrographs showing mannitol spray-dried from MeOH:BA:H₂O (7:2:1), (a) unfiltered, (b) filtered prior to spray-drying.

4.3.1.2 SOLID-STATE CHARACTERISATION AND THERMAL ANALYSIS

XRD data showed that spray-dried mannitol samples (Figure 4.41 (a), (b)) were crystalline but not identical to each other or to mannitol unprocessed material. XRD of unprocessed material compared to the XRD data of Burger et al. (2000), suggests that it is in the mod 1, (also known as the β-), polymorphic form. The XRD of spray-dried samples showed some discrepancies with this unprocessed material XRD trace however, in terms of peak positions and also relative peak

intensities, and could not be specifically assigned to either the mod II (α -) or mod III (δ -) polymorphic forms (Figure 4.42). It is likely that impure mixes of crystalline (polymorphic forms) and amorphous materials are present in these samples. A mixed population may be expected from the feed conditions resulting in precipitation and from the mixed morphology seen in SEM. No difference was noted in the DSC data, which showed melting peaks at 165-167 °C, similar to the unprocessed material and literature value. Mass losses over the temperature range 25-100 °C were low at ~0.5-0.7% from TGA, similar to unprocessed material (Figure 4.43).

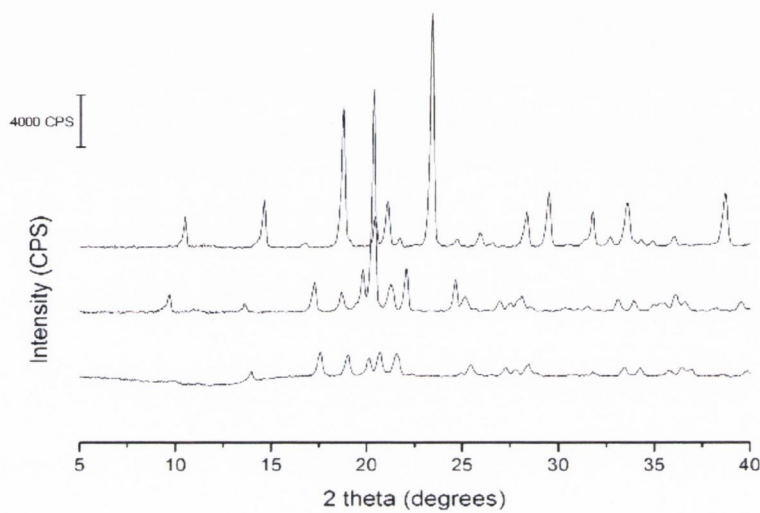


Figure 4.42 XRD scans of (a) unprocessed mannitol, (b) mannitol spray-dried from MeOH:BA:H₂O (7:2:1), unfiltered, (c) filtered prior to spray-drying.

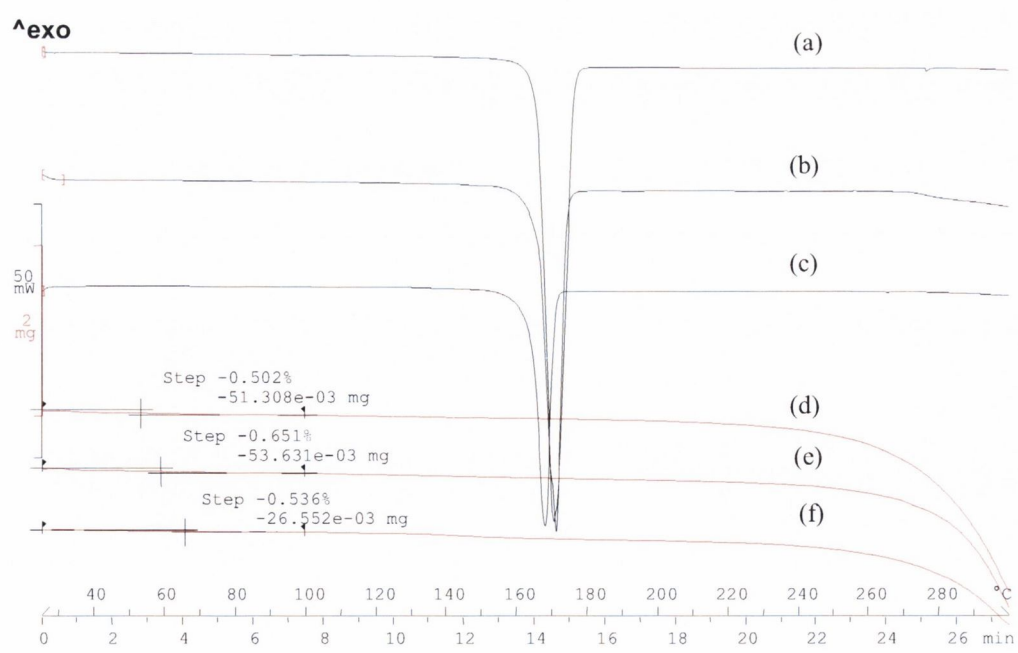


Figure 4.43 DSC (in black) and TGA (in red) scans of (a), (d) unprocessed mannitol, (b), (e) mannitol spray-dried from MeOH:BA:H₂O (7:2:1) - unfiltered, (c), (f) filtered prior to spray-drying.

4.3.2 MYO-INOSITOL

There is a limited amount of information available on spray-drying myo-inositol alone in the literature. It has been spray-dried by Mosén (2003), and found to be crystalline on processing. Regarding other processing techniques, it has been formulated with other compounds in CO₂-assisted nebulisation with a bubble dryer (CAN-BD) drying producing an amorphous non-hygroscopic composite (Sievers, 2007). Preliminary work in this thesis therefore involved spray-drying myo-inositol from H₂O and from EtOH:H₂O systems with/without the polymer PVP to assess its particle morphology and solid-state characteristics and thus potential use in a composite with a model protein. Insolubility in the MeOH:BA system (similar to mannitol) did not enable examination of this solvent combination with regard to spray-drying myo-inositol alone. The MeOH:BA:H₂O system was also not successfully used for spray-drying mannitol alone, and also caused problems with nozzle blockage and as such was not attempted for myo-inositol.

4.3.2.1 SPRAY-DRIED FROM WATER

Myo-inositol was initially spray-dried from water using different inlet temperatures of 130 °C and 150 °C, and a t.s.c. of 2% w/v (See Appendix 1 for details). Spray-dried particles had a very similar morphology from SEM analysis, consisting of fused spheres, with a non-porous rough surface. Collectable product yield was increased with the higher inlet temperature of 150 °C, with a yield of 58.3% (100 ml spray-dried) compared to 31.4% (100 ml spray-dried) with an inlet temperature of 130 °C. Incorporating small amounts of PVP-k15 and the higher molecular weight PVP-40, as used by Tajber (2005), were considered to investigate whether amorphous material and discrete particles could be produced. PVP-k15 was initially included in a mass ratio with myo-inositol of 1:9 and spray-dried from H₂O at an inlet temperature 150 °C. The product was found to have a pliable “sticky” consistency that rapidly solidified forming hard “rock-like” aggregates during collection and short-term exposure to ambient conditions. Inlet drying temperature was decreased to 130 °C and PVP-40 was used in place of PVP-k15 at the same mass ratio of 1:9, and in the third sample the amount of PVP-k15 was increased to a mass ratio of 1:4. Some fusion was noted in all systems. The mass ratio of 1:9 PVP-k15:myo-inositol spray-dried at the higher temperature of 150 °C appeared to be most affected however. SE micrographs of particles are shown in Figure 4.44.

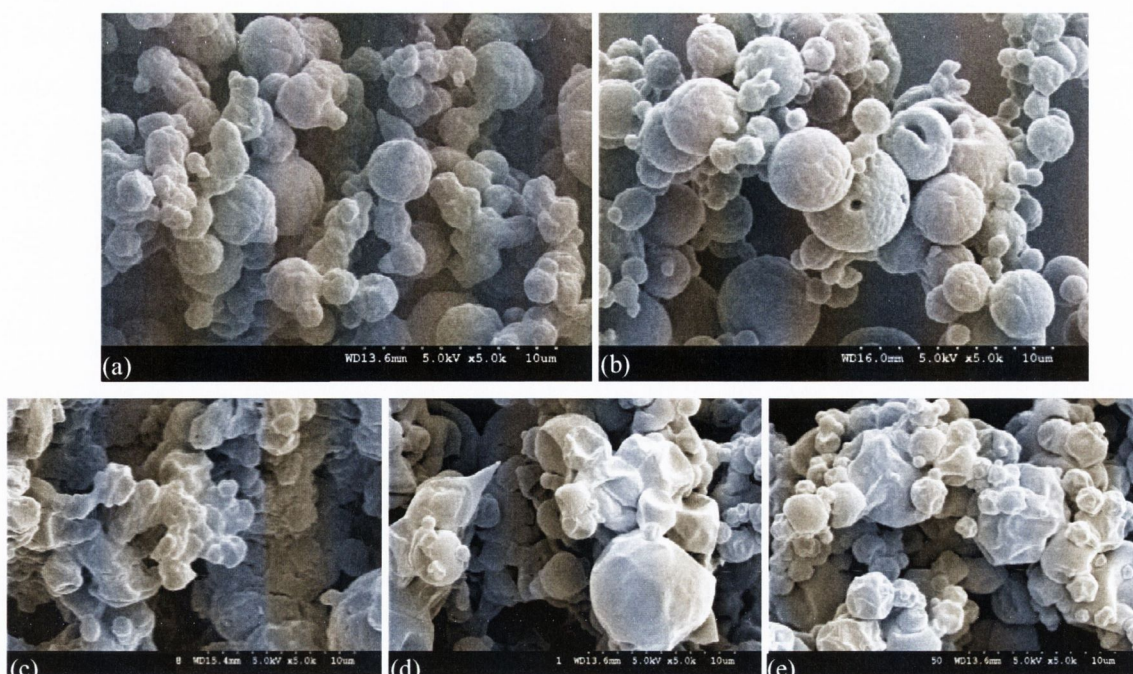


Figure 4.44 SE micrographs of spray-dried myo-inositol (alone/with PVP-k15/with PVP-40) from H₂O, inlet temperature as detailed (a) alone; 130 °C (b) alone; 150 °C, (c) myo-inositol:PVP-k15 [9:1]; 150 °C (d) myo-inositol:PVP-k15 [4:1]; 130 °C (e) myo-inositol:PVP-40 [9:1]; 130 °C.

4.3.2.2 SPRAY-DRIED FROM ETHANOL:WATER SOLUTIONS

Myo-inositol was spray-dried from similar EtOH:H₂O:AC systems, as were used with lysozyme:excipient composites (Chapter 5), to investigate its use as an alternative carrier. It was not expected that porous particles would be produced from these systems, due to the hydrophilic nature of myo-inositol, but discrete particles and either a reduction in crystallinity or amorphous material were considered desirable characteristics. The feed conditions - 70% EtOH, 10% of t.s.c. AC and inlet temperature 90 °C were initially chosen, based on results from spray-drying trehalose, and lysozyme:excipient composites (Chapter 5). SE micrographs (Figure 4.45) showed fused spheres, with a rough surface in the case of the system incorporating AC, and a smoother surface where no AC was used. Particles showed a high degree of fusion. The presence of AC in the feed material, may have contributed to a slightly different surface morphology.

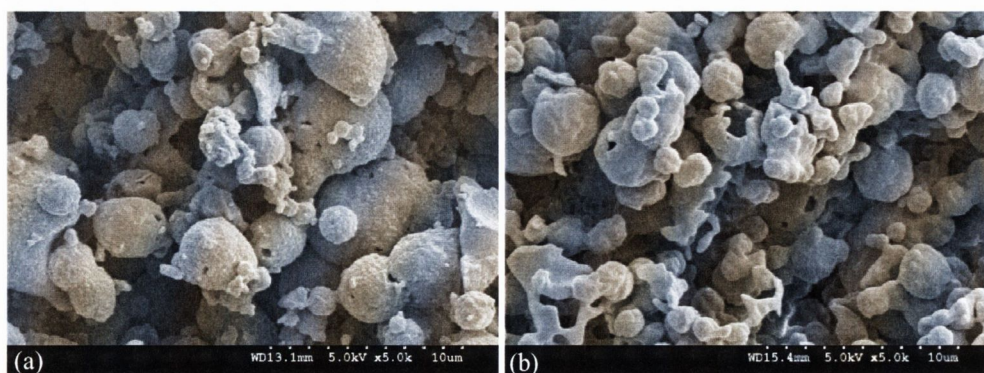


Figure 4.45 SE micrographs of myo-inositol spray-dried from 70% EtOH; inlet temperature 90 °C (a) 10% of t.s.c.AC, (b) no AC.

The use of PVP, having been included to investigate production of amorphous product spray-dried from water, was considered for EtOH:H₂O systems. PVP-k15 was successfully incorporated with lysozyme spray-dried from 80% EtOH at inlet temperature 78 °C, retaining the porous morphology of the spray-dried protein (Chapter 5), and as such a composite system involving lysozyme:myo-inositol:PVP was envisaged, spray-dried under similar conditions. As preliminary studies, myo-inositol:PVP-k15 were spray-dried, in proportions of 4:1 and 3:2 both with 10% AC from 80% EtOH, inlet temperature 78 °C. Morphology of the powders viewed under SEM showed a high degree of fusion, particularly with the lower PVP-k15 content.

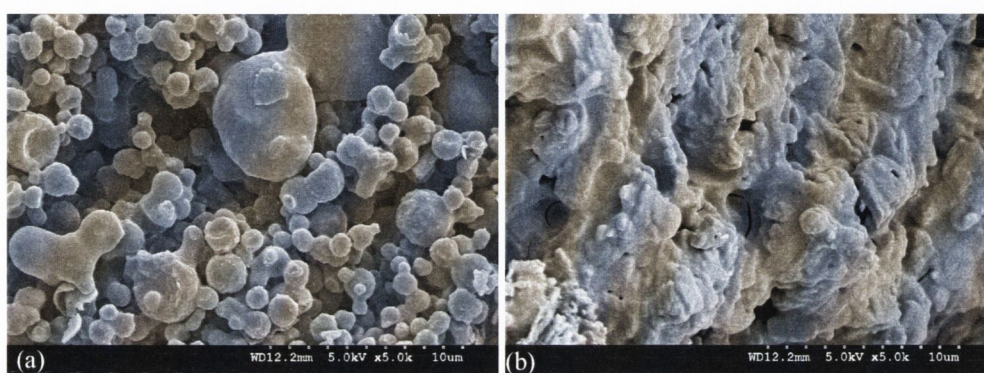


Figure 4.46 SE micrographs of myo-inositol spray-dried with PVP from 80% EtOH; inlet temperature 78 °C (a) myo-inositol:PVP-k15 (3:2), (b) myo-inositol:PVP-k15 (4:1).

4.3.2.3 SOLID-STATE CHARACTERISATION AND THERMAL ANALYSIS

4.3.2.3.1 Unprocessed material: Myo-inositol

XRD data showed that the material was crystalline (Figure 4.47). The DSC scan showed a sharp melting endotherm at ~223 °C consisted with literature published melting point of ~225 °C, with no other thermal events detectable (Figure 4.48 black trace). Mass loss from 25-100 °C, calculated from TGA (Figure 4.48 red trace), was low at ~0.5%, which is also indicative of a crystalline, anhydrous non-hygroscopic material.

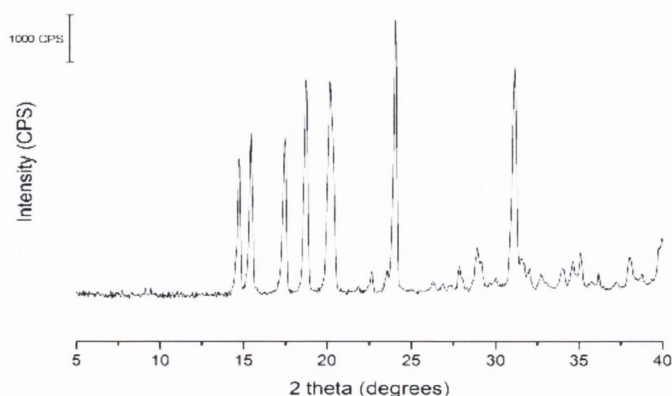


Figure 4.47 XRD of myo-inositol unprocessed material.

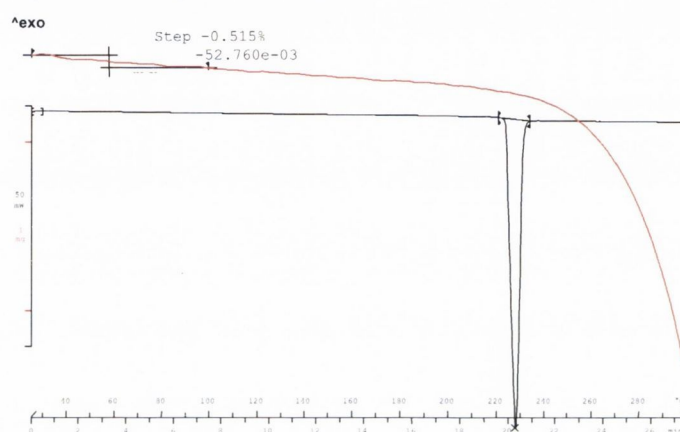


Figure 4.48 DSC (black trace) and TGA (red trace) of myo-inositol unprocessed material.

4.3.2.3.2 Spray-dried and freeze-dried myo-inositol from water

Spray-dried samples of myo-inositol alone from H₂O, were crystalline by XRD showing a very similar pattern as unprocessed material (Figure 4.49). DSC showed one sharp endothermic peak at the melting temperature of ~224 °C, and TGA showed low residual moisture content, as for unprocessed material. In order to investigate a different processing method on solid state characteristics and because of the paucity of literature data, myo-inositol was freeze-dried from water at a solid concentration of 2% w/v. The rationale for this was to compare solid state characteristics of myo-inositol prepared by two different methods, used to produce amorphous material (freeze-drying or spray-drying). Crystalline material was again produced, which was noted from XRD and DSC. Although the amplitudes of the peaks for freeze-dried material from XRD were lower, this may have been due to a lower density of material resulting in less material in the cavity mount, rather than a reduction in crystallinity.

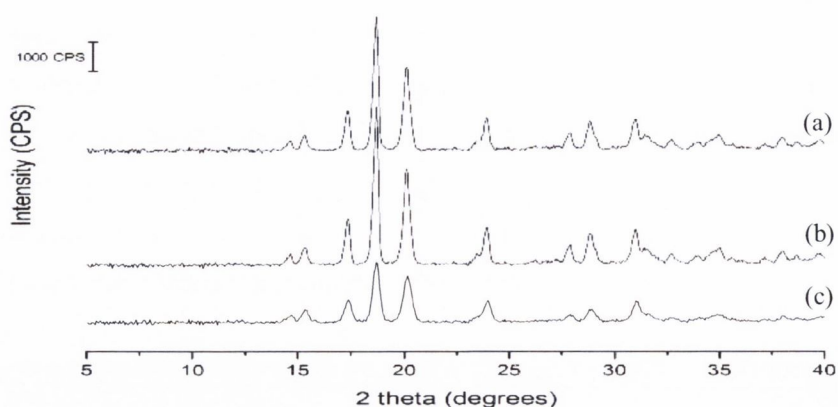


Figure 4.49 XRD of myo-inositol spray-dried from water at inlet temperature (a) 130 °C, (b) 150 °C and (c) freeze-dried myo-inositol.

Myo-inositol:PVP-k15 (9:1) spray-dried from H₂O at 150 °C resulted in crystalline material by XRD analysis (Figure 4.50 (a)), although the relative peak heights were different to the unprocessed material. The material may have been initially amorphous and rapidly recrystallised, on exposure to room temperature and humidity. Evidence for this was the visual observation of powder change on collection. Myo-inositol:PVP-k15 (4:1) spray-dried from H₂O at 130 °C, resulted in primarily amorphous material by XRD analysis (Figure 4.50 (b)). The DSC scan showed an initial broad endotherm followed by two sharp exothermic peaks, followed by a sharp melting endotherm, and was indicative of an amorphous material (Figure 4.51 (a)). Sharp symmetrical exothermic peaks may represent recrystallisation exotherms. The presence of two such peaks is unusual however and was investigated by using heating/cooling/re-heating cycles. DSC scans of 25-160-120-300 °C (initial heating beyond the first exotherm (~146 °C) but before the second exotherm (~176 °C), and reheating over the range of the two exotherms) and 25-190-120-300 °C (initial heating beyond the second exotherm, and reheating over the range of the two exotherms) were used. These data showed that the exotherms were irreversible, as both exotherms disappeared on reheating, following initial heating to 190 °C. The thermal events were exclusive of each other however, and the second exotherm was found on reheating in the 25-160-120-300 °C DSC scan. Myo-inositol:PVP-40 (9:1) spray-dried from H₂O at 130 °C, also resulted in primarily amorphous material by XRD although a small amount of crystallinity may be apparent from small peaks at ~19.7 and 22 ° (Figure 4.50 (c)). The DSC scan was similar to the system in which 20% PVP-k15 was used, showing two exothermic peaks although these peaks were shifted to higher temperatures (Figure 4.51 (b)).

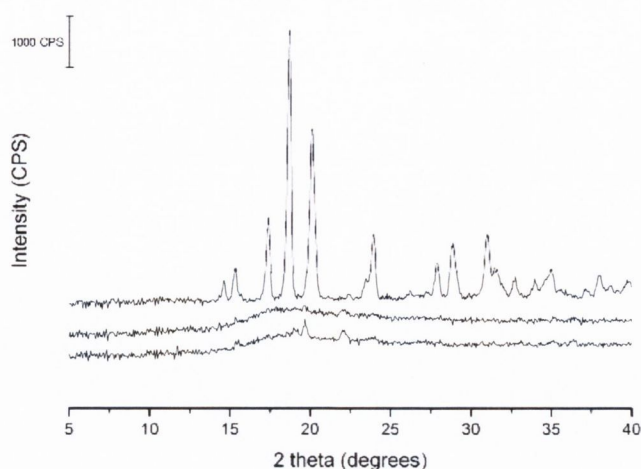


Figure 4.50 XRD analysis of spray-dried (a) myo-inositol:PVP-k15 9:1, (b) myo-inositol:PVP-k15 4:1, (c) myo-inositol:PVP-40 9:1.

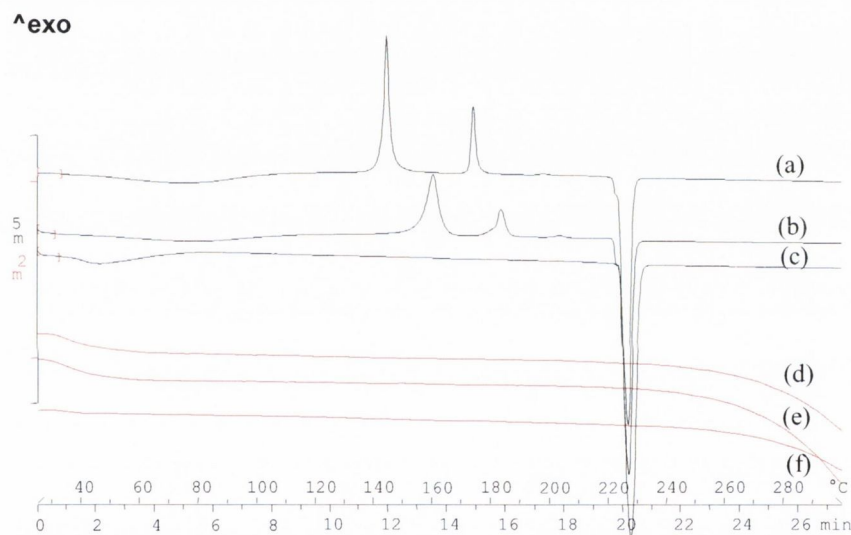


Figure 4.51 DSC (in black) and TGA (in red) of (a), (d) myo-inositol:PVP-k15 4:1, (b), (e) myo-inositol:PVP-40 9:1 (c), (f) myo-inositol:PVP-k15 9:1.

4.3.2.3.3 Spray-dried myo-inositol from ethanol:water solutions

All myo-inositol samples spray-dried from EtOH:H₂O systems were crystalline by XRD analysis (Figure 4.52). DSC data for myo-inositol spray-dried alone also supported the assertion of crystallinity from XRD, with the presence of a single sharp melting endotherm at ~224 °C. TGA showed small mass losses over the temperature range of 25-100 °C, analogous to unprocessed material. The use of PVP-k15 and altered drying conditions, did not enable production of amorphous product by XRD analysis, despite the high proportion of PVP used (ratio 3:2). TGA showed higher mass losses in samples containing PVP however (Figure 4.53 (a), (b)) and in the high PVP content sample (3:2 myo-inositol:PVP-k15) an early recrystallisation peak was noted at ~40 °C from DSC analysis (Figure 4.53 (d)), suggesting the presence of some amorphous material.

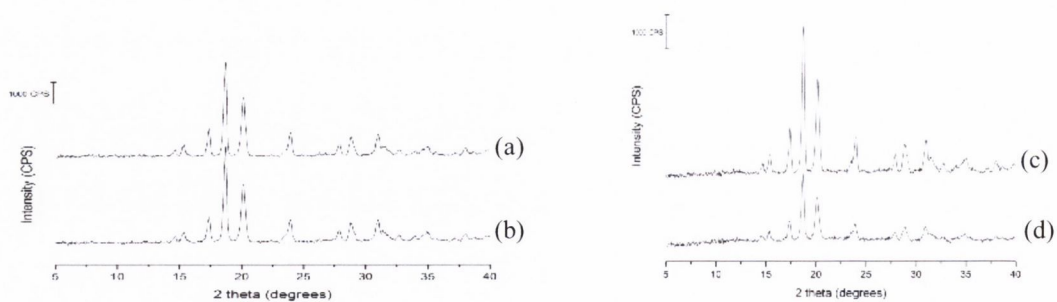


Figure 4.52 XRD of myo-inositol spray-dried (a) including 10% AC of t.s.c.; 70% EtOH, inlet temperature 90 °C, (b) no AC; 70% EtOH, inlet temperature 90 °C, (c) myo-inositol:PVP-k15 4:1; including 10% AC of t.s.c.; 80% EtOH, inlet temperature 78 °C, (d) myo-inositol:PVP-k15 3:2; including 10% AC of t.s.c.; 80% EtOH, inlet temperature 78 °C.

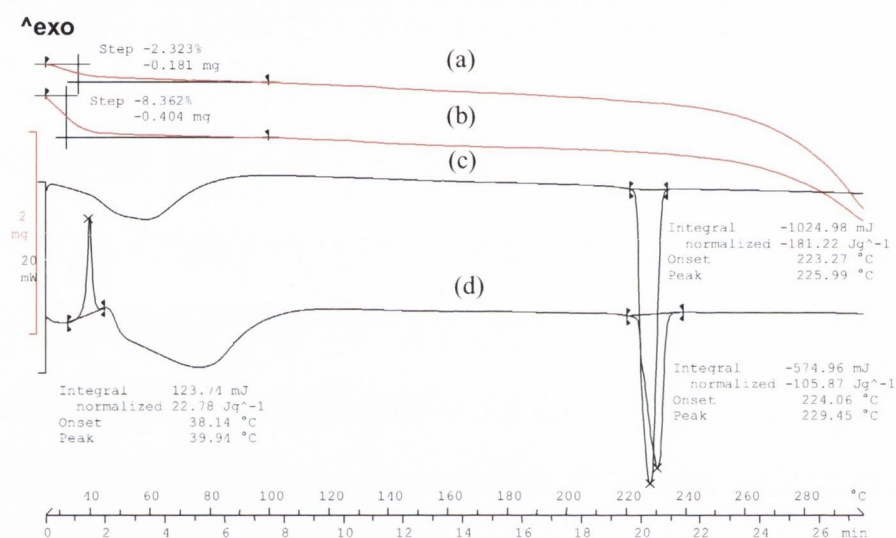


Figure 4.53 TGA (in red) and DSC (in black) of spray-dried (a)/(c) myo-inositol:PVP-k15 4:1; including 10% AC of t.s.c.; 80% EtOH, inlet temperature 78 °C, (b)/(d) myo-inositol:PVP-k15 3:2; including 10% AC of t.s.c.; 80% EtOH, inlet temperature 78 °C.

4.4 SPRAY-DRIED EXCIPIENT COMPOSITES

Trehalose was spray-dried with mannitol and with HP- β -CD in composite systems (mass ratio 9:1 trehalose:mannitol, and 1:1 trehalose:HP- β -CD), from the 80:20 MeOH:BA system that had produced NPMPs of trehalose alone. Composite systems may be desirable to ensure optimal particle and solid-state properties, to combine the advantages and minimise the disadvantages of the individual components.

Mannitol can reduce the water content of a composite particle because of its non-hygroscopic character. It may plasticise owing to its low Tg however. It has been shown to reduce the Tg when freeze-dried with various disaccharides including trehalose, compared with the disaccharide alone, with the Tg of amorphous trehalose:mannitol (9:1 mass ratio) measured at ~ 81.8 °C and calculated by the Gordon-Taylor equation at 77.1 °C (Kim et al., 1998). Trehalose has a high Tg and good protein stabilising potential but is a soft hygroscopic material in the amorphous, spray-dried state. Bögelein (2007) found that spray-drying trehalose:dextran:mannitol in increasing mannitol ratios up to 3:4:3 resulted in an amorphous material, and a reduction in water content of the powders with increasing mannitol content up to 22% w/w. Reduction in the Tg of the composite powders was also found as expected with an increase in mannitol content.

The effect of adding a small amount of mannitol to trehalose in NPMP composite particles was thus considered. The trehalose:mannitol composite powder had a sticky consistency and deliquescence on collection under ambient relative humidity (visual observation). SEM analysis showed a fused mass, compared to NPMPs of trehalose spray-dried alone and prepared similarly (Figure 4.54). The small amount of mannitol (10%) was likely to have exerted a considerable plasticising effect on trehalose during spray-drying and during handling. The composite was amorphous by XRD (Figure 4.55). A Tg was not identified by DSC, but the DSC scan showed an early recrystallisation exotherm at ~ 70 °C followed immediately by a small endotherm which may correspond to dehydration. A small endotherm between 120 - 140 °C may represent melting of the anhydrous crystalline α -form of trehalose (Jones et al., 2006). Two sharp endotherms at 159 °C and at 202 °C were also noted (Figure 4.56). These are slightly lower but likely due to melting peaks of mannitol and anhydrous trehalose. Inclusion of mannitol with trehalose, without another high Tg adjuvant, in a ratio of 1:9 was found to be disadvantageous to particle stability.

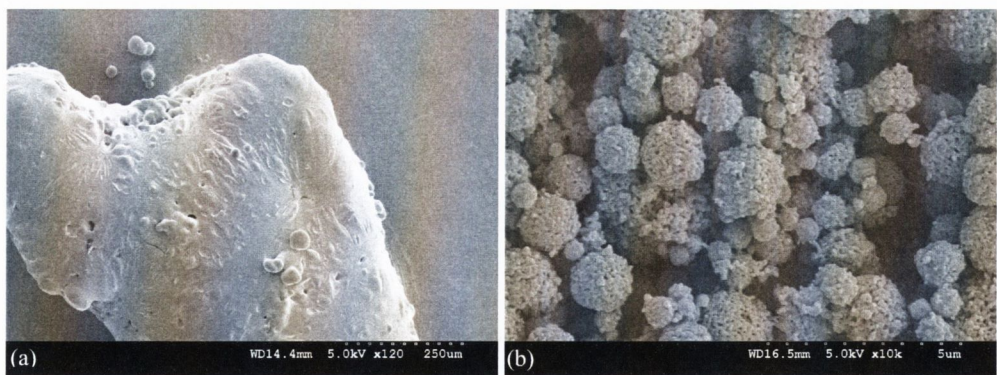


Figure 4.54 SE micrographs showing (a) trehalose:mannitol (9:1) and (b) trehalose alone, spray-dried from 80:20 MeOH:BA, inlet temperature 100 °C.

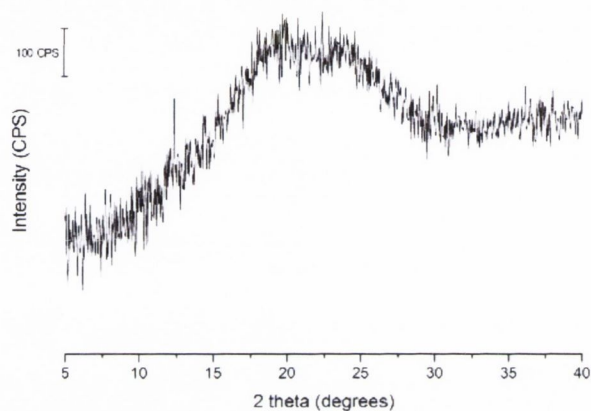


Figure 4.55 XRD of trehalose:mannitol (9:1) spray-dried from 80:20 MeOH:BA, inlet temperature 100 °C.

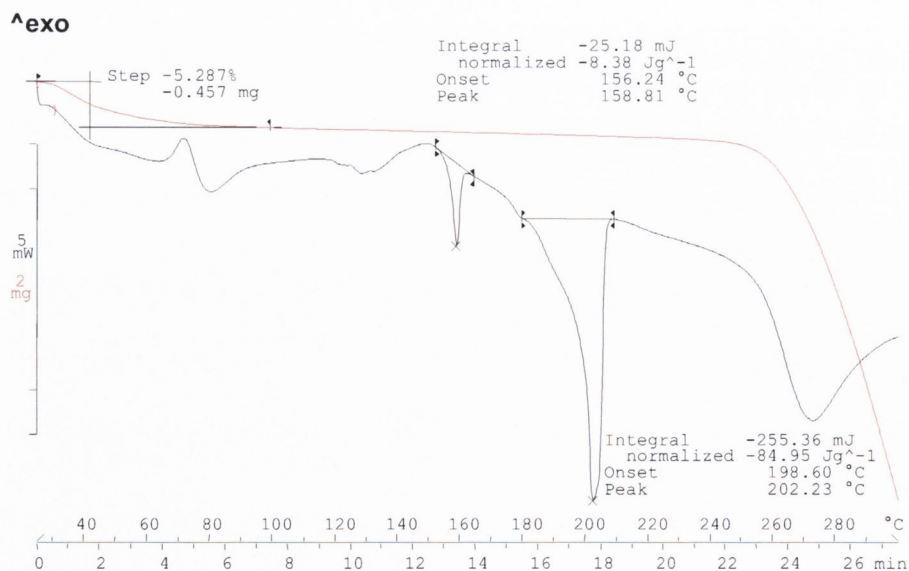


Figure 4.56 DSC (in black) and TGA (in red) scans of trehalose:mannitol (9:1) spray-dried from 80:20 MeOH:BA, inlet temperature 100 °C.

HP- β -CD has a high Tg of $\sim 250^\circ\text{C}$, and has shown protein stabilising effects on spray-drying distinct from those shown by the disaccharide sucrose (Branchu et al., 1999). NPMP composites of protein and HP- β -CD were found to be less hygroscopic than NPMP composites comprising protein and trehalose or raffinose (Chapters 5, 6). A composite of trehalose and HP- β -CD was thus considered.

SEM analysis showed that trehalose:HP- β -CD composite powders were NPMPs, similar to trehalose spray-dried alone from this system (Figure 4.57). DSC showed a broad endotherm indicative of moisture loss, a possible Tg at $\sim 130^\circ\text{C}$ and no recrystallisation or melting peaks (Figure 4.58), similar to trehalose spray-dried alone from water or using the regular cyclone from a MeOH:BA:H₂O system. The absence of a recrystallisation peak and melting of anhydrous recrystallised trehalose may indicate increased thermal stability of the composite, compared to NPMPs of trehalose spray-dried under similar conditions alone, and as such may offer a more physically stable formulation. Improvement in stability and hygroscopic behaviour of this composite may warrant further investigation.



Figure 4.57 SE micrograph of Trehalose:HP- β -CD (ratio 1:1) spray-dried from MeOH:BA 80:20, inlet temperature 100°C .

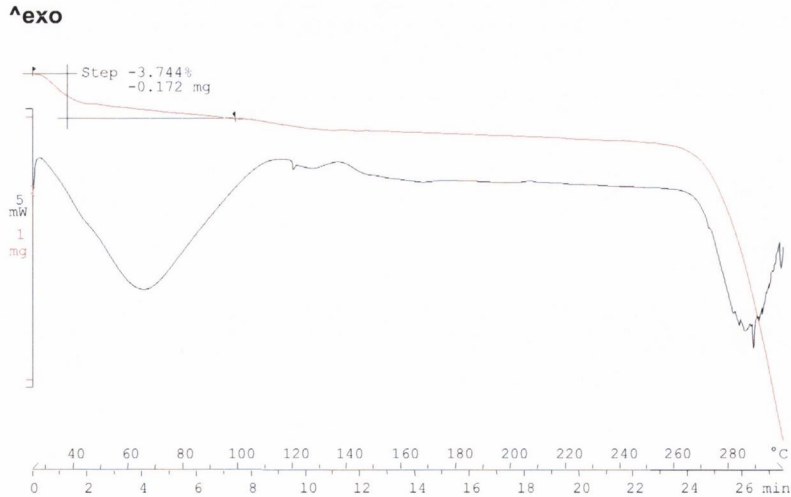


Figure 4.58 DSC (in black) and TGA (in red) of trehalose:HP- β -CD (ratio 1:1) spray-dried from MeOH:BA 80:20, inlet temperature 100°C .

4.5 CONCLUSIONS

NPMPs of trehalose and raffinose were spray-dried from MeOH:BA and from MeOH:BA:H₂O solvent systems. Particles had a surface morphology resembling fused spherical nanoparticles, and did not appear to be hollow, as seen from SEM analysis. NPMPs were amorphous as assessed by XRD. Thermal properties of trehalose, assessed by DSC, were found to depend on spray-drying conditions. This was not found with raffinose, with similar DSC patterns for raffinose spray-dried from different conditions. NPMPs (spray-dried from 80:20 MeOH:BA solvent system) displayed favourable aerodynamic and micromeritic characteristics compared to non-porous particles (spray-dried from H₂O), suggesting potential suitability for pulmonary delivery. Hygroscopicity was noted for spray-dried powders of the two sugars on high temperature and high humidity days. This was more pronounced in the case of trehalose and may represent a disadvantage for formulation in a dry powder for inhalation, potentially requiring further formulation in a composite with non-hygroscopic excipients or inclusion of a desiccant in the delivery device.

A composite particle combining the advantageous properties of the different components may offer the best alternative for protein stabilisation. Mannitol is a non-hygroscopic component. It was not successfully spray-dried as NPMPs from a MeOH:BA:H₂O solvent system alone. Spray-drying with trehalose in a composite particle resulted in increased hygroscopicity and lower thermal stability by DSC, probably due to a plasticising effect of mannitol. Spray-drying trehalose with HP- β -CD appeared more promising and may represent a more stable formulation.

Myo-inositol was spray-dried alone and with PVP from EtOH:H₂O solutions for formulation in a composite particle with lysozyme NPMPs. Spray-dried particles did not appear promising in terms of particle morphology, assessed by SEM analysis, or in terms of solid-state characteristics by XRD and DSC. Formulation with protein (strong glass former) in a composite may further influence these characteristics however (Chapter 5).

5.1 INTRODUCTION

As previously discussed (Chapters 1, 4), proteins are generally formulated with one or more stabilising excipient(s) in the solid state. There are numerous reports in the literature regarding the use of stabilisers with various proteins in spray-drying and other drying processes. Reports specifically concerning spray-drying lysozyme with excipients include articles by Liao et al. (2002, 2003), and Suihko et al. (2005). In the studies by Liao et al. (2002, 2003) lysozyme was spray-dried with sucrose, trehalose and mixtures of these two with dextran, resulting in improved process stability compared to the protein spray-dried alone. A dependence on spray-dried mass ratio was reported with different excipients conferring different advantages. Suihko et al. (2005) examined molecular mobility and phase separation in co-spray-dried lysozyme:trehalose and lysozyme:sucrose composites of different mass ratios on storage under 0% and 75% relative humidity for 5-6 days, and described phase separation and crystallisation (considered undesirable for protein stability) of high sugar content composites stored under high relative humidity. This present work involves using processing methods that have not been previously applied to composite protein:excipient materials. As such, particle morphology and the ability to produce porous particles are of interest as well as any effects on process/storage stability and particle characteristics of composites. These data may be useful when adapting the method for other protein/peptide composites (Chapter 6).

The stabilising excipients investigated in this chapter include sugars (spray-dried alone Section 4.2), polyols (spray-dried alone Section 4.3), and the cyclodextrin HP- β -CD (spray-dried alone, Li (2005)). The hydrophilic polymer PVP is also considered for incorporation in a ternary system with myo-inositol and lysozyme. Two general feed systems of the spray-drying method are considered separately – the EtOH:H₂O:AC system which was previously used to produce NPMPs of lysozyme alone and the non-aqueous MeOH:BA system, which was previously used to produce NPMPs of sugars alone.

The EtOH:H₂O:AC system was used to produce NPMPs of lysozyme (Section 3.3.2) and to produce non-porous particles of trehalose (Section 4.2.1.2). These studies suggested that the EtOH and AC concentrations were important factors in the production of NPMPs of lysozyme alone. Low inlet drying temperatures of 78 and 85 °C, although suitable for spray-drying the protein alone, resulted in sub-optimal drying of trehalose alone and a sticky mass on collection. Considering these experiences, lysozyme:trehalose composite systems were initially spray-dried at a mass ratio of 1:1, from 70-80% EtOH, 10-25% of t.s.c. AC and inlet temperatures 78 and 90 °C. Different mass ratios were further investigated with effects on surface morphology and process

stability examined. Storage stability and particle characterisation of composites of mass ratio 1:1 were investigated for comparison to lysozyme spray-dried alone. Spray-dried lysozyme:raffinose and lysozyme:inulin were preliminarily investigated as alternative saccharide excipients at a mass ratio of 1:1. The non-hygroscopic polyol excipient myo-inositol was considered and lysozyme was spray-dried with myo-inositol, with PVP and with a mixture of myo-inositol:PVP to investigate the possible production of an amorphous composite including myo-inositol.

The non-aqueous MeOH:BA system was used to produce NPMPs of trehalose (Section 4.2.1.3), raffinose (Section 4.2.2.1) and HP- β -CD (Li, 2005) and to produce non-porous spherical particles of lysozyme (Section 3.3.5). Following from these studies, and as presented in this chapter, composites of freeze-dried lysozyme (Section 3.2) and excipients trehalose, raffinose and HP- β -CD were investigated at low protein concentration (mass ratio 1:4 lysozyme:excipient, and further mass ratios 3:7 and 1:1 lysozyme:raffinose). Particle morphology and process stability of composites were investigated. Preliminary particle characterisation and storage stability of mass ratio 1:4 composites at two different temperatures (desiccant conditions) were investigated.

5.2 ETHANOL:WATER:AMMONIUM CARBONATE FEED SYSTEMS

5.2.1 SPRAY-DRIED LYSOZYME:TREHALOSE COMPOSITES

The spray-drying conditions 80% EtOH, 10% of t.s.c. AC, inlet temperature 78 °C (hereafter referred to as conditions 1); 70% EtOH, 10% of t.s.c. AC, inlet temperature 78 °C; 70% EtOH, 10% of t.s.c. AC, inlet temperature 90 °C (hereafter referred to as conditions 2); and 70% EtOH, 25% of t.s.c. AC, inlet temperature 90 °C, were selected for spray-drying lysozyme:trehalose composites at a mass ratio of 1:1. A total solid concentration of 0.5% w/v was used in all cases as for lysozyme systems spray-dried alone, except in the higher AC content (25% of t.s.c.) sample in which a t.s.c. of 1% w/v was used. All other spray-drying conditions and % yields are presented in Appendix 1. All systems showed a phase separation in the feed material on addition of the EtOH component prior to spray-drying, resulting in increased turbidity of the feed solution, and were spray-dried as suspensions. Some evidence of fusion of particles was found for the system spray-dried from conditions 1 (Figure 5.1 (a)). Particle morphology was otherwise similar to lysozyme spray-dried alone from these conditions (Section 3.3.2 Figure 3.13, 3.16). Composite particles spray-dried from 70% EtOH, 10% of t.s.c. AC, inlet temperatures 78 °C and 90 °C did not show fusion of particles on SEM analysis. Surface morphology of particles was highly folded (Figure 5.1 (b), (c)) rather than the nano-particulate appearance of porous particles of lysozyme spray-dried alone. Particles were hollow with thin particle walls as observed from some shattered particles and blow holes through the particle surface (Figure 5.1 (d)). Composite particles spray-dried from 70% EtOH, 25% of t.s.c. AC, inlet temperature 90 °C, showed a mixed morphology of spherical nano-particulate/porous particles and collapsed folded particles (Figure 5.1 (e)).



Figure 5.1 SE micrographs showing lysozyme:trehalose composite particles, spray-dried at a mass ratio of 1:1, % EtOH, % of t.s.c., inlet drying temperature as (a) 80%, 10% AC, 78 °C, (b) 70%, 10% AC, 78°C (c), (d) 70%, 10%, 90 °C (e) 70%, 25%, 90 °C; Note magnifications differ.

Spray-drying conditions 2 were chosen for investigation of different mass ratio composites. Lysozyme was spray-dried with trehalose, in respective mass ratios of 9:1, 4:1, 7:3, and 1:4. Composite particles spray-dried from mass ratios 9:1, 4:1, 7:3 were found to have a folded/porous surface morphology with some shattering of particles (Figure 5.2 (a), (b), (c)). Spray-dried particles having a mass ratio of 1:4 lysozyme:trehalose, consisted of mainly collapsed “raisin-like” particles with some folded/porous particles also present (Figure 5.2 (d)).

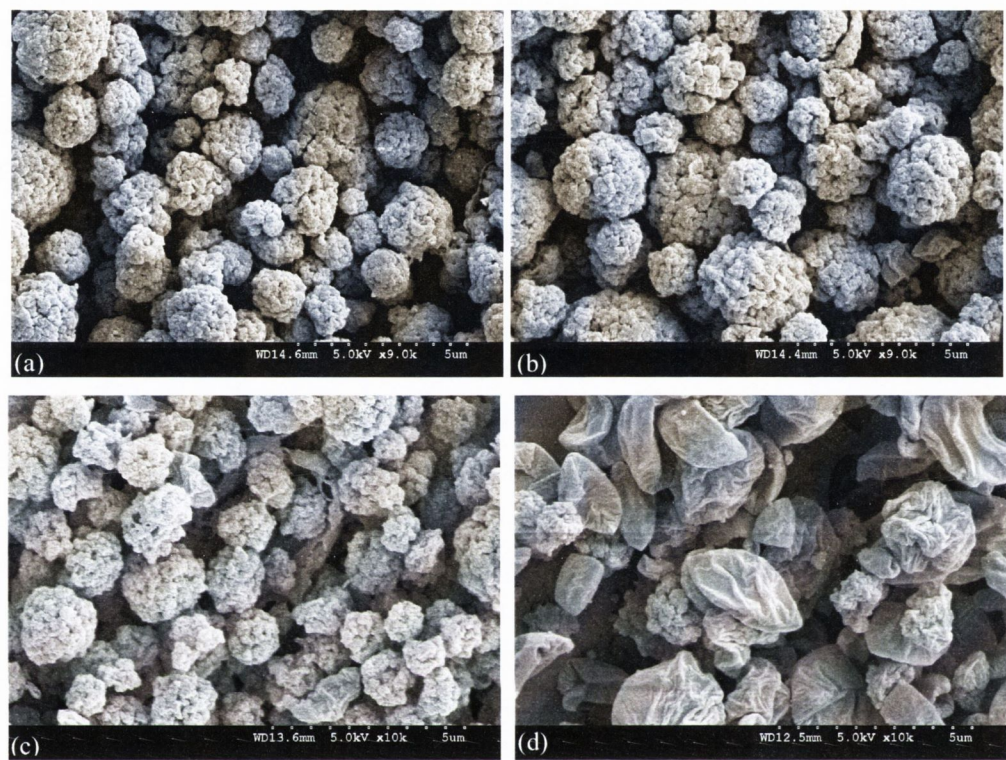


Figure 5.2 SE micrographs showing lysozyme:trehalose spray-dried in mass ratios of (a) 9:1, (b) 4:1, (c) 7:3, (d) 1:4, from 70% EtOH, 10% AC, 90 °C; Note: magnifications x 9 k, x 10 k are presented.

5.2.1.1 PROCESS STABILITY

5.2.1.1.1 Solid-state characterisation and thermal analysis

Lysozyme:trehalose composites (mass ratios 9:1 – 1:4) spray-dried from conditions 2 were amorphous by XRD showing the characteristic halo pattern (mass ratio 9:1 – 1:1 shown Figure 5.3 (a), (c), (d)). The lysozyme:trehalose composite spray-dried from conditions 1, was also XRD amorphous (Figure 5.3 (b)), despite the fusion apparent in SEM analysis, suggesting sub-optimal drying of the sugar.

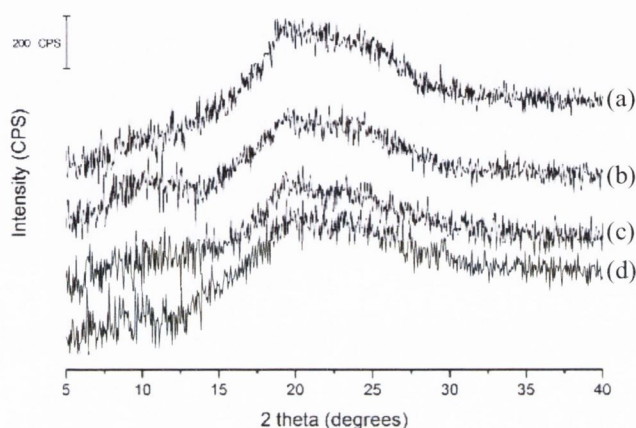


Figure 5.3 XRD of lysozyme:trehalose spray-dried in mass ratios of (a) 1:1, (c) 4:1 (d) 9:1 from conditions 2, and (b) 1:1 from conditions 1.

DSC of composites showed initial broad endothermic peaks from 25 – 120/140 °C, indicative of residual moisture/solvent loss and were similar to lysozyme spray-dried alone under these conditions. No sharp endotherms at ~ 200 °C corresponding to the apparent denaturation temperature of lysozyme (Section 3.4.1) were identified in any of the samples, even in the high lysozyme content sample, spray-dried as mass ratio 9:1. Similar difficulties in identifying this peak were encountered for lysozyme spray-dried alone from 70% - 80% EtOH/10% of t.s.c. AC (Section 3.4.1), and may be due to the processing conditions employed and folding of the protein. A broad endotherm was observed at ~220 °C in the sample spray-dried at a mass ratio of 9:1 lysozyme:trehalose, similar to lysozyme spray-dried alone and attributed to decomposition. This peak was shifted downwards on increasing trehalose content and was not found in the highest trehalose content of spray-dried mass ratio 1:4 (lysozyme:trehalose). There was also no evidence of recrystallisation or the sharp melting endothermic peak (~212 °C) of trehalose. No distinct glass transitions could be identified in the samples of lower sugar content (mass ratios 9:1 – 1:1) due to obscuration by the initial broad endotherm. A transition was identified at ~155 °C in composites spray-dried as mass ratio 1:1 and 1:4 lysozyme:trehalose, and another at ~130 °C for the 1:4 ratio composites (Figure 5.4 – marked by red boxes). The higher transition at ~155 °C may represent a

melting transition of the α -crystal form of trehalose (Jones et al., 2006), and the transition identified at ~ 130 °C may represent a glass transition, from data with spray-drying trehalose alone (Section 4.2.1.4). A heating/cooling/reheating DSC cycle (25-130-70-200 °C) was attempted for the 1:1 mass ratio composite to try to differentiate between overlapping events, due to the reversible nature of the glass transition temperature, and to identify a glass transition in this sample. Transitions at ~ 118 °C (similar to a transition for trehalose spray-dried alone) and also at ~ 155 °C were identified (Figure 5.5) on the second heating run. The identification of a glass transition in pure proteins or of sugar:protein mixes of low sugar ratio is usually not possible or is difficult using conventional DSC (Hill et al., 2005; Katayama et al. 2008). Katayama et al. (2008) examined the glass transition of pure lysozyme using high ramp DSC (HRR-DSC) (heating scan of 100 °C/min) and by extrapolation of the Tg from measured lyophilised mixtures of lysozyme:sucrose in varying mass ratios (heating scans of 20 °C/min and 100 °C/min). These studies identified a glass transition at ~ 199 °C for the pure protein by HRR-DSC and ~ 168 °C by extrapolation from lysozyme:sucrose composites, with a clear anti-plasticising effect on sucrose and increasing Tg with increasing lysozyme content. Liao et al. (2003) recorded glass transition temperatures for spray-dried composites of lysozyme:trehalose and lysozyme:sucrose at different mass ratios. The lysozyme:trehalose mass ratio was not found to have a statistically significant effect on the Tg, but in contrast to Katayama's work a statistically significant decrease in the Tg for spray-dried sucrose-containing composites was reported with increasing lysozyme content. Liao et al. (2003) commented that this trend may be partly or wholly attributed to the interplay between the anti-plasticising effect of lysozyme and the plasticising effect of water, as the moisture content increases with increasing lysozyme in the lysozyme:sugar composites. DSC conditions were not reported in this paper.

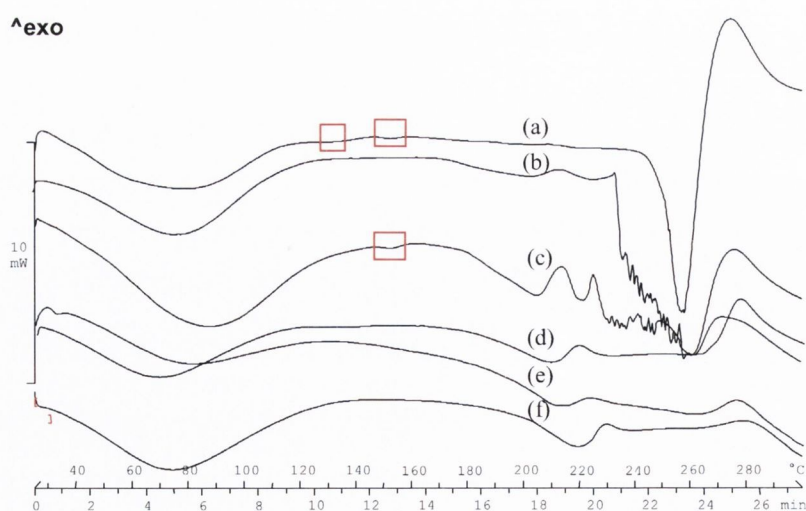


Figure 5.4 DSC of lysozyme:trehalose spray-dried in mass ratios of (a) 1:4, (c) 1:1 (d) 7:3 (e) 4:1 (f) 9:1 from conditions 2, and (b) 1:1 from conditions 1.

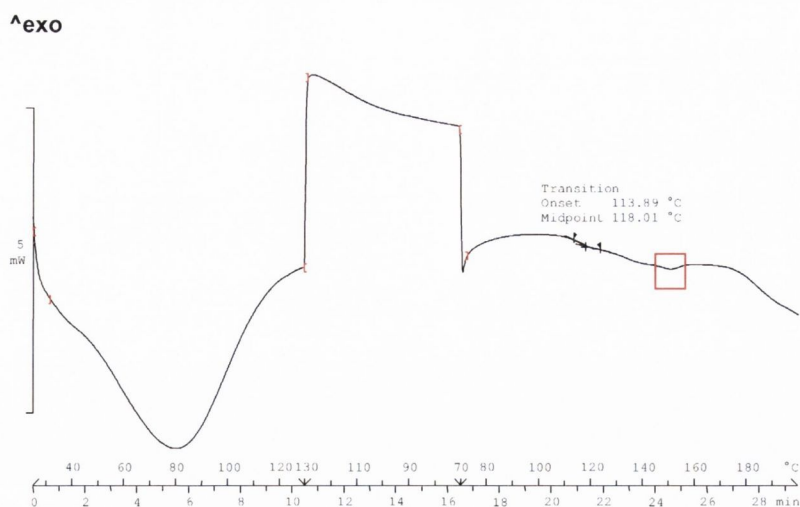


Figure 5.5 DSC of lysozyme:trehalose spray-dried in mass ratios of 1:1 (conditions 2) showing a possible glass transition at 118 °C and a further transition at ~155 °C.

A residual moisture/solvent loss was calculated for all composite samples of 3.38 – 4.80% over the temperature range 25 – 100 °C from TGA. Higher contents were calculated for the higher lysozyme content samples, although the difference between samples was small. Suihko et al. (2005) reported higher moisture content with increased lysozyme content in spray-dried composites stored at ~ 0% R.H., and attributed this to a higher affinity of water to lysozyme than to the amorphous sugar. This was also observed by Liao et al. (2002) although only small decreases in moisture content was found with further increases in sugar concentration above a mass ratio of 1:1. A further smaller step of mass loss (100-140 °C) was observed for samples having high trehalose content (marked with a red box in Figure 5.6). These marked mass losses were > 2% w/w (for spray-dried lysozyme:trehalose mass ratio 1:1 as 2.0% for the composite spray-dried from conditions 1, and 2.4% for the composite spray-dried from conditions 2 and spray-dried lysozyme:trehalose mass ratio 1:4 calculated mass loss of 2.9%). Calculated mass losses over this temperature range for the lysozyme:trehalose 7:3, 4:1, 9:1 mass ratio composites were lower, respectively 0.95%, 0.87% and 0.94%, and a distinct step was not observed from the TGA scan. A mass loss step was observed in this temperature range for trehalose powders spray-dried alone from 70 and 80% EtOH, 10% AC, 90 °C (Section 4.2.1.4.3), with a determined mass loss of 2.58 and 3.40% respectively but was not observed for lysozyme spray-dried alone (Section 3.29) with a determined mass loss of 0.59% for lysozyme spray-dried from conditions 1 and 0.72% for lysozyme spray-dried from conditions 2.

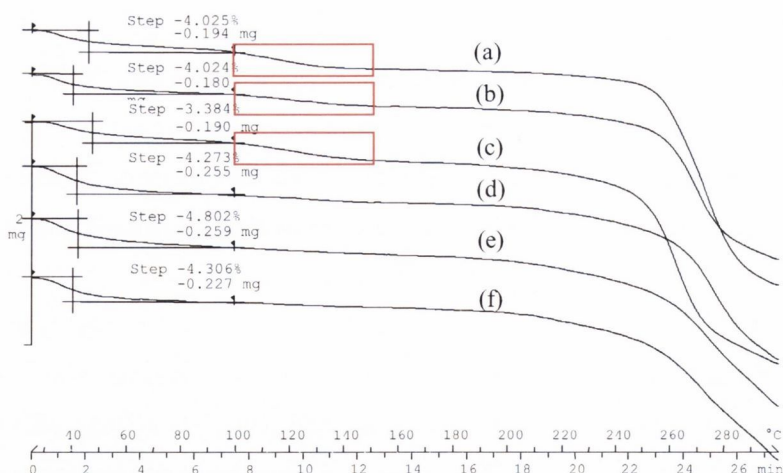


Figure 5.6 TGA of lysozyme:trehalose spray-dried in mass ratios of (a) 1:4, (c) 1:1 (d) 7:3 (e) 4:1 (f) 9:1 from conditions 2, and (b) 1:1 from conditions 1.

5.2.1.1.2 Secondary structure and biological activity assay

Solid state FTIR studies were carried out for different mass ratios of lysozyme:trehalose at a protein:KBr ratio of 1:200. A slightly lower % area of overlap with unprocessed protein was calculated for the composites, compared to lysozyme spray-dried alone from these conditions (Table 5.1). No protection against perturbation in secondary structure, with increasing trehalose content was thus observed. Liao et al. (2002) observed that trehalose partially stabilised the native α -helical structure (assessed by FTIR) of spray-dried lysozyme in the range of mass ratio (lysozyme:trehalose) of 10:1 – 1:1, but was less effective compared to sucrose, and further increase in trehalose content did not enhance preservation of lysozyme structure. Different processing stresses involved in the current method may explain this difference in stabilisation of secondary structure by trehalose.

Table 5.1 Lysozyme:trehalose spray-dried systems (from conditions 2) with % area overlap with unprocessed lysozyme of area normalised amide 1 peaks either using whole spectrum baseline-correction or isolated peak baseline correction. [^{*1} HP cyclone used instead of regular cyclone; ^{*2} spray-dried from conditions 1]

Lysozyme:trehalose	Area overlap amide 1 peak (whole spectrum baseline-corrected)	Area overlap amide 1 peak (peak baseline-corrected)
10:0	83.9%	92.9%
9:1	81.3%	91.1%
4:1	80.7%	89.6%
7:3	81.0% ^{*1} ; 80.3%	89.5% ^{*1} ; 89.2%
1:1	81.9%	90.5%; 91.8% ^{*2}
1:4	80.3%	85.8%

Biological activity assays were carried out for lysozyme:trehalose composites of mass ratios 9:1, 4:1, 7:3, 1:1, 1:4 and presented below with controls run on the same day of analysis as samples. Lysozyme spray-dried alone from similar conditions (conditions 2) is also presented for comparison (Table 5.2). Test samples were prepared containing 0.008 mg/ml solid for spray-dried composites of mass ratios up to 7:3. For higher mass ratios of 1:1 and 1:4, the solid content in test solutions was increased such that protein content was ~ 0.008 mg/ml. All showed good retention of biological activity with no statistically significant difference compared to control in specific activity, (test solutions were corrected for protein content using a micro BCA assay) (significance level $\alpha = 0.05$). The % activity of the solid was similar to the spray-dried ratio of protein:sugar also suggesting good retention of activity and that the mass ratio in the spray-dried composites was similar to the initial mass ratio in solution prior to spray-drying.

Table 5.2 Summary of biological activity assay results for lysozyme:trehalose composites of different mass ratios spray-dried from conditions 2; *all 0.008 mg/ml solid, ** adjusted solid concentration based on spray-dried ratio to give ~0.008 mg/ml protein, n = 3 for each batch; pooled stdev % (in the case of % activity and % specific activity) is calculated from the pooled standard deviation of all samples run for the daily assay as a % of units of control solution.

	Units/ml test	Units/mg solid	µg protein/ml test	Units/mg protein	% Activity (± pooled stdev %)	% Specific activity (± pooled stdev %)
*10:0	300.7 ± 18.7	37597 ± 2308	8.43 ± 0.25	35644 ± 1164	100.4 ± 4.2	97.0 ± 7.5
*9:1	259.3 ± 11.6	32418 ± 1462	7.23 ± 0.12	35868 ± 1926	85.6 ± 4.2	97.6 ± 7.5
*4:1	238.3 ± 7.2	29799 ± 951	6.3 ± 0.53	38046 ± 3873	79.6 ± 4.2	103.6 ± 7.5
*7:3	212.7 ± 15.0	26570 ± 1851	5.93 ± 0.49	36031 ± 4409	70.9 ± 4.2	98.1 ± 7.5
*7:3 (HP)	204.3 ± 2.1	25528 ± 239	5.87 ± 0.06	34815 ± 629	68.2 ± 4.2	94.8 ± 7.5
*Control	300.0 ± 13.2	37305 ± 1756	8.17 ± 0.29	36733 ± 2377	100 ± 4.2	100 ± 7.5
**1:1	360.3 ± 9.5	22521 ± 613	8.43 ± 0.75	42884 ± 2696	51.4 ± 1.5	97.8 ± 5.0
**1:4	342.3 ± 13.6	8562 ± 329	7.87 ± 0.06	43545 ± 2002	19.5 ± 1.5	99.3 ± 5.0
**Control	350.5 ± 9.2	43843 ± 1132	8.00 ± 0.00	43843 ± 1132	100 ± 1.5	100 ± 5.0

5.2.1.2 STORAGE STABILITY

The storage stabilities of lysozyme:trehalose composites, spray-dried as mass ratios of 1:1 from conditions 1, and from conditions 2 were assessed under different temperature and relative humidity conditions during a 20-week storage period, in a comparative study to lysozyme spray-dried alone from conditions 1 (Section 3.5).

Storage at 4 °C / Desiccant

Particle morphology, (from SEM), of composite particles were not affected by storage at 4 °C under desiccant for 20 weeks (Figure 5.7 (b), (d)), compared to freshly prepared material (Figure 5.1 (a), (c), (d)). Powders remained amorphous for the duration of the 20-week study as determined by XRD analysis (Appendix 2). There was also no change in DSC or in the shape of TGA traces during storage (Appendix 2). Mass loss from 25 – 100 °C calculated from TGA ranged from 2.88 – 4.71% (week 20 – 2.96%) for the sample spray-dried from conditions 1 and from 2.78 – 5.01% (week 20 – 4.27%) for the sample spray-dried from conditions 2 (Figure 5.8).

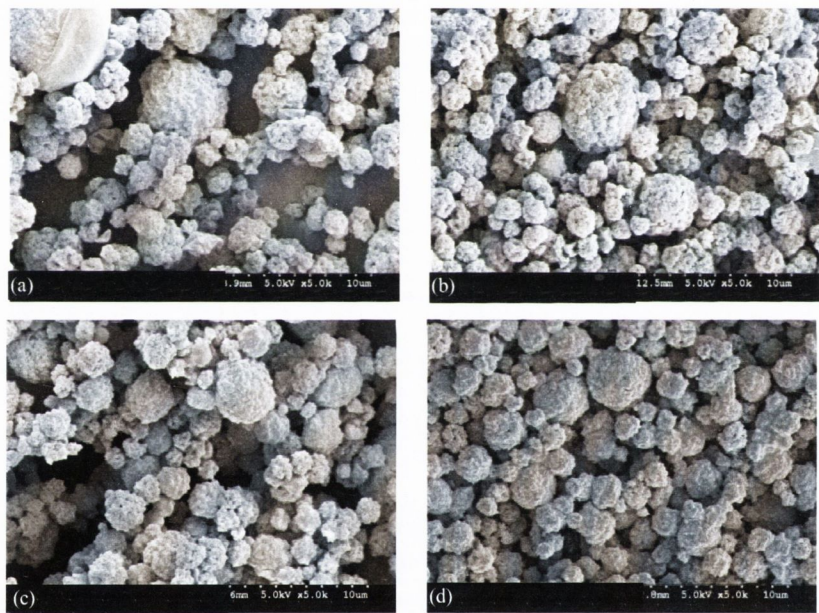


Figure 5.7 SE micrographs showing lysozyme:trehalose 1:1 composite spray-dried from conditions 1, (a) after 24 hours (b) after 20 weeks, and from conditions 2 (c) after 24 hours (d) after 20 weeks.

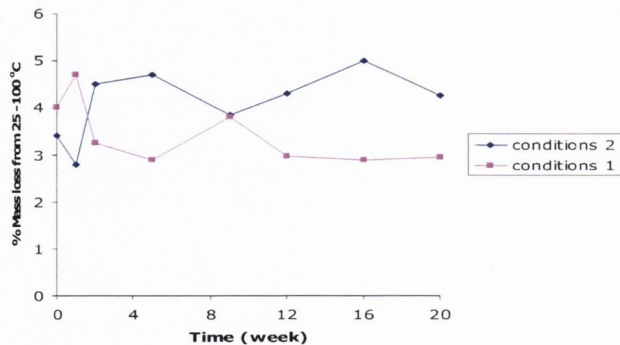


Figure 5.8 Moisture content (calculated from mass loss over temperature range 25-100 °C from TGA, n=1) for lysozyme:trehalose (1:1) spray-dried from conditions 1, 2, during 20 weeks storage at 4 °C/desiccant.

There was no change in the shape of the amide 1 peak on storage at 4 °C/desiccant for 20 weeks, with an area of overlap of area-normalised individually baseline-corrected peaks (calculated using method 2 and freshly prepared composite) of 97.8% (conditions 1) and 97.9% (conditions 2), which is similar to the intra-run repeatability values for the same batch (98 – 99%) (Section 3.4.2). There was also no statistically significant change in the biological activity (units/mg solid) or specific activity (units/mg protein, corrected for protein content using micro BCA protein assay) of the composites on storage under these conditions for 20 weeks (Table 5.3, Figure 5.9). There was no observable decrease in solubility of samples when preparing test solutions.

Table 5.3 Summary of biological activity assay results for spray-dried composites of lysozyme:trehalose (spray-dried mass ratio 1:1) after 20 weeks storage at 4 °C/desiccant. (n=2, *± pooled standard deviation).

	Units/ml test	Units/mg solid	µg protein/ml test	Units/mg protein	% Activity* (± 6.4)	% Specific activity * (± 2.7)
Conditions 1	364.5 ± 7.8	22774 ± 497	9.40 ± 0.14	38784 ± 1267	58.3	99.3
Conditions 2	356.0 ± 17.0	22227 ± 1050	8.85 ± 0.35	40205 ± 354	56.9	102.9
Control	312.5 ± 33.2	39063 ± 4198	8.00 ± 0.57	39061 ± 1295	100	100

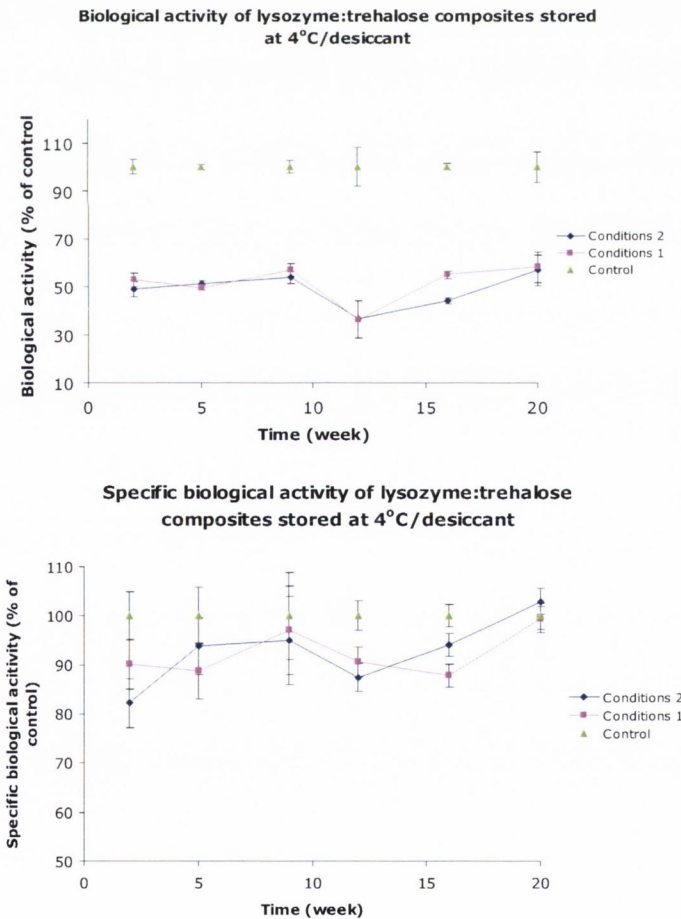


Figure 5.9 Biological activity and specific biological activity of spray-dried composites of lysozyme:trehalose (spray-dried mass ratio 1:1) as % of corresponding control solution activity. Error bars shown represent ± pooled standard deviation for the samples.

Storage at 25 °C / 60% R.H.

Particle morphology of composite particles was affected by storage under these conditions as can be seen from SE photo-micrographs (Figure 5.10). Fusion between particles was increased, and phase separation of a component of the composite was observed (marked by red arrows). Powders spray-dried from conditions 1 and 2, displayed crystallinity by XRD by the first measurement time-point in the study (week 1) (Figure 5.11). Peaks at 2 theta equal to $\sim 23.9^\circ$ and 8.7° are characteristic of the trehalose dihydrate. This peak at position 8.7° is relatively small compared to the peak at $\sim 23.9^\circ$ in the fully crystalline raw trehalose depending on the batch (Section 4.2.1.4.1) and may not be detectable in the diluted composite sample. Crystallisation was also suggested from DSC analyses, with a sharp endothermic peak observed to develop, super-imposed on the initial broad endotherm at $\sim 80^\circ\text{C}$. At the week 2 time-point, in the sample spray-dried from conditions 1, this peak was initially observed at the higher temperature of $\sim 100^\circ\text{C}$ but shifted to $\sim 80^\circ\text{C}$ in later weeks. No melting endotherm of anhydrous trehalose was observed (Figure 5.12). The mass loss over the temperature range $25 - 100^\circ\text{C}$ from TGA was increased on storage compared to freshly spray-dried material (Figure 5.13, Figure 5.14). This increase in water content with crystallisation was expected because trehalose absorbs water and recrystallises as a dihydrate which contains $\sim 9.5\%$ water (Suihko et al., 2005). The second mass loss step from $100 - 140^\circ\text{C}$ identified for the freshly-prepared 1:1 composites was not identified in the TGA scans taken during the stability study (Figure 5.13).

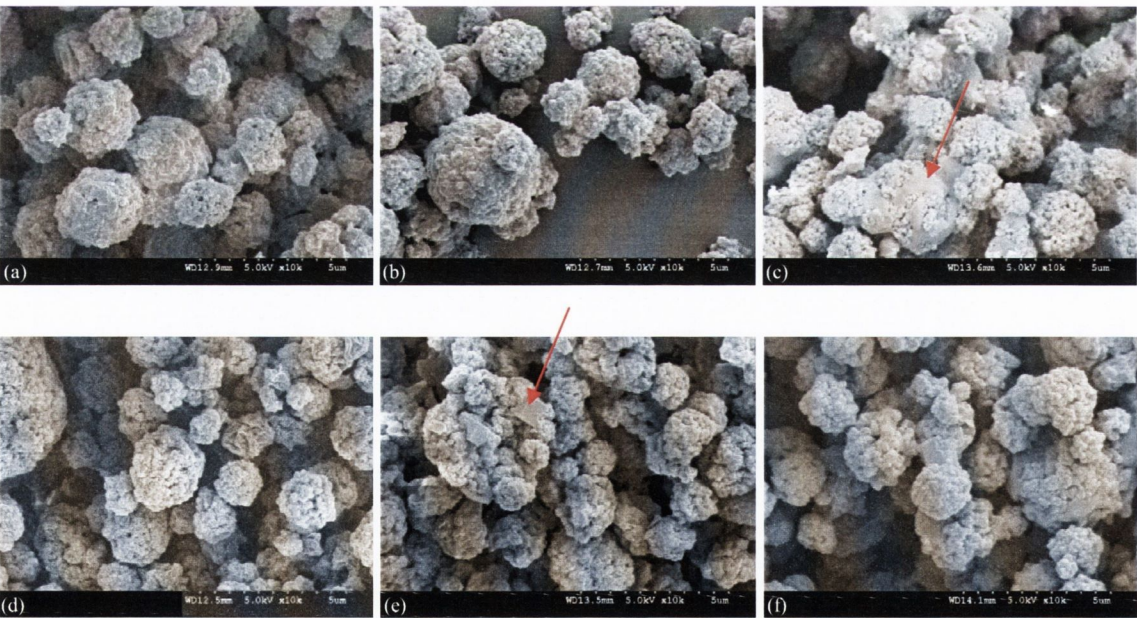


Figure 5.10 SE micrographs showing lysozyme:trehalose composites spray-dried: from conditions 1 after (a) 24 hours (b) 5 weeks (c) 20 weeks, and from conditions 2 after (d) 24 hours (e) 5 weeks (f) 20 weeks storage at $25^\circ\text{C}/60\% \text{ R.H.}$.

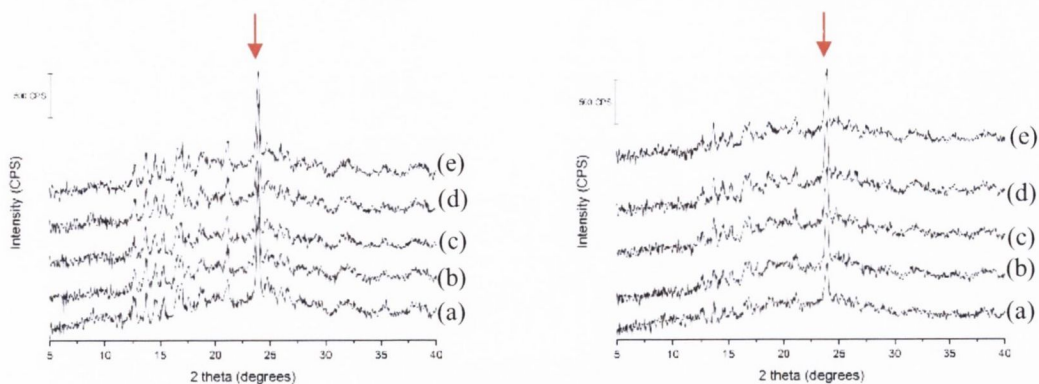


Figure 5.11 XRD of lysozyme:trehalose (1:1) spray-dried from conditions 1 (left) and spray-dried from conditions 2 (right) after (a) week 1 (b) week 2 (c) week 9 (d) week 16 (e) week 20, storage at 25 °C/60% R.H..

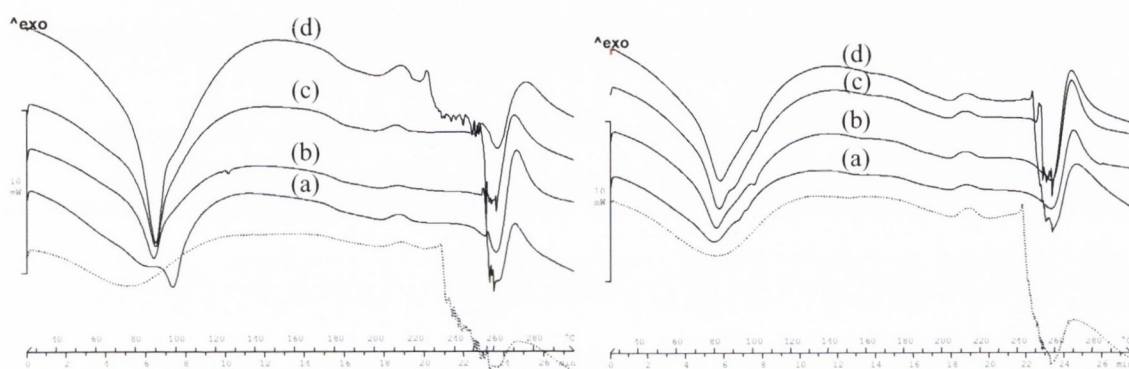


Figure 5.12 DSC of lysozyme:trehalose (1:1) spray-dried from conditions 1 (left) and spray-dried from conditions 2 (right), broken line – freshly prepared material, solid line after (a) 2 weeks (b) 9 weeks (c) 16 weeks and (d) 20 weeks, storage at 25 °C/60% R.H..

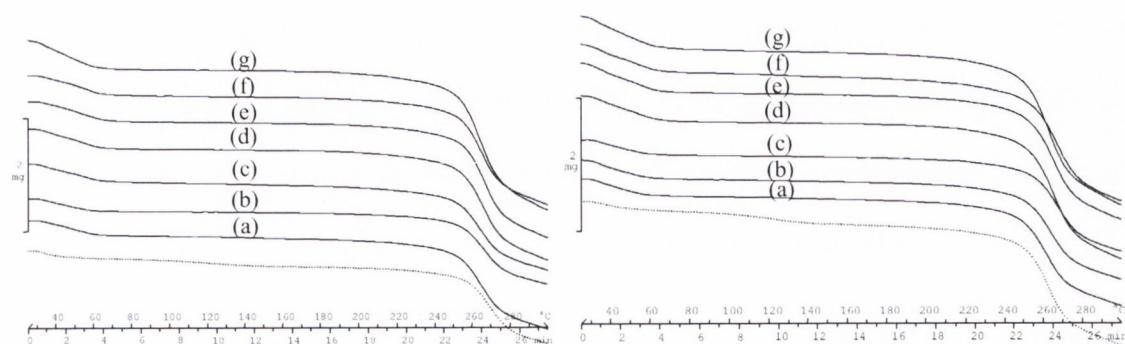


Figure 5.13 TGA of lysozyme:trehalose (1:1) spray-dried from conditions 1 (left) and spray-dried from conditions 2 (right), broken line – freshly prepared material, solid line after (a) 2 weeks (b) 9 weeks (c) 16 weeks and (d) 20 weeks, storage at 25 °C/60% R.H..

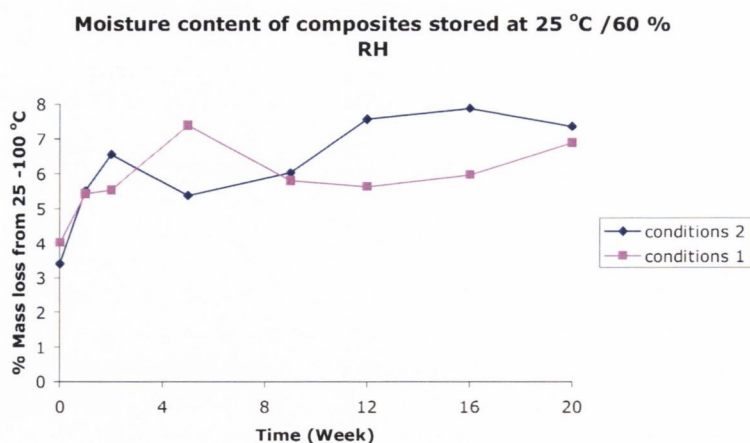


Figure 5.14 Moisture content (calculated from mass loss over the temperature range 25-100 °C from TGA, n=1), lysozyme:trehalose (1:1) spray-dried from conditions 1, 2, during 20 weeks storage at 25 °C/60% R.H..

There was no change in the shape of the amide 1 peak on storage at 25 °C/60% R.H., with an area of overlap of area-normalised individually baseline-corrected peaks (calculated using method 2) with freshly prepared composite of 98.0% (conditions 2) and 98.4% (conditions 1), which is similar to the intra-run repeatability calculated values of 98 - 99% for the same batch (Section 3.4.2).

Reducing SDS-PAGE was carried out on spray-dried composites, from conditions 1 and 2, stored under 4 °C/desiccant, or 25 °C/60% R.H. stability conditions for 20 weeks. Gel sheet analysis showed that no appreciable soluble covalent protein aggregation took place for any of the composite samples, with no evidence of high molecular weight bands identified for any of the samples.

There was also no statistically significant change in the biological activity or the specific activity and no decrease in solubility observed for the composites on storage at these conditions for 20 weeks (Table 5.4, Figure 5.15).

Table 5.4 Summary of biological activity assay results for spray-dried composites of lysozyme:trehalose (spray-dried mass ratio 1:1) after 20 weeks storage at 25 °C/60% R.H. (n=2, *± pooled standard deviation).

	Units/ml test	Units/mg solid	µg protein/ml test	Units/mg protein	% Activity* (± 8.1%)	% Specific activity * (± 5.4%)
conditions 1	336.0 4.2	20977 276	8.25 0.50	40825 1739	53.7	104.5
conditions 2	323.5 55.9	20195 3480	7.85 1.91	41663 2931	51.7	106.7
Control	312.5 33.2	39063 4198	8.00 0.57	39061 1295	100	100

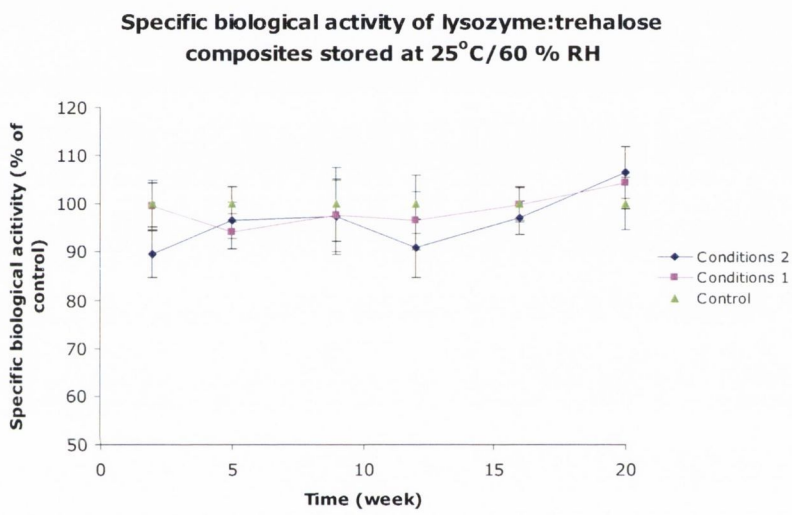
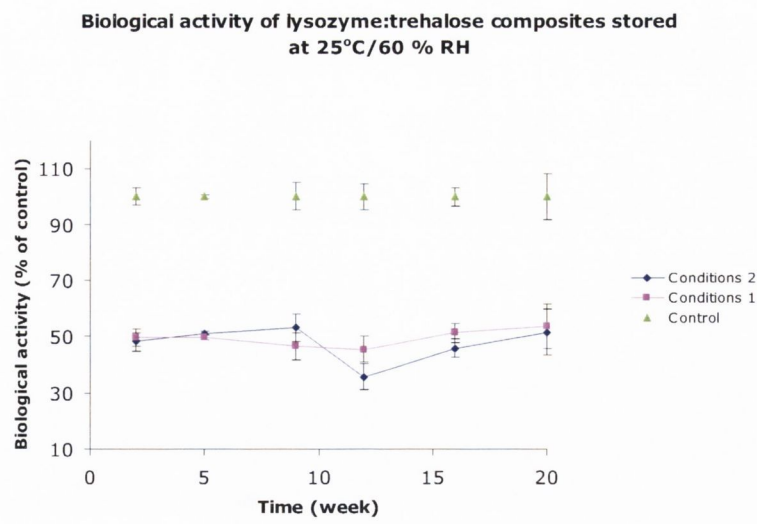


Figure 5.15 Biological activity and specific biological activity of spray-dried composites of lysozyme:trehalose (spray-dried mass ratio 1:1) as % of corresponding control solution activity. Error bars shown represent $\pm$ pooled standard deviation for the samples.

Storage at 40 °C / 75% R.H.

Particle morphology of composite particles, spray-dried from conditions 1 and 2, was severely affected by storage under these conditions as can be seen from SE photo-micrographs (Figure 5.16) taken after 24 hours. Collapse of particle structure was evident. Composite powders, spray-dried from the two conditions, showed similar crystallinity by XRD taken at the first measurement time-point (week 1) (Figure 5.17). TGA data were also similar for the two composites at the week 1 time-point with calculated mass losses from 25 - 100 °C of 8.9% and 9.0% (Figure 5.18 (a)). A difference was observed in moisture content and TGA data between the composites at the week 2 time-point however (Figure 5.18 (b)). A larger mass loss on heating was calculated for the composite spray-dried from conditions 2, of 12.4% from 25 – 100 °C and a further 10.5% from 100 – 160 °C compared to a smaller mass loss of 6.3% (25 – 100 °C) for the composite spray-dried from conditions 1. DSC taken at the same time-point (week 2) also showed differences between the samples (Figure 5.19). DSC showed a sharp endotherm super-imposed on the broader endotherm for the sample spray-dried from conditions 1. This was not apparent for the sample spray-dried from conditions 2. Both samples showed a later sharp endotherm at ~ 170-176 °C, possibly a melting endotherm of the sugar. The reason for the difference between the composites at the week 2 time-point was not identified but may be related to a difference in the propensity of the composites to crystallise forming a dihydrate.

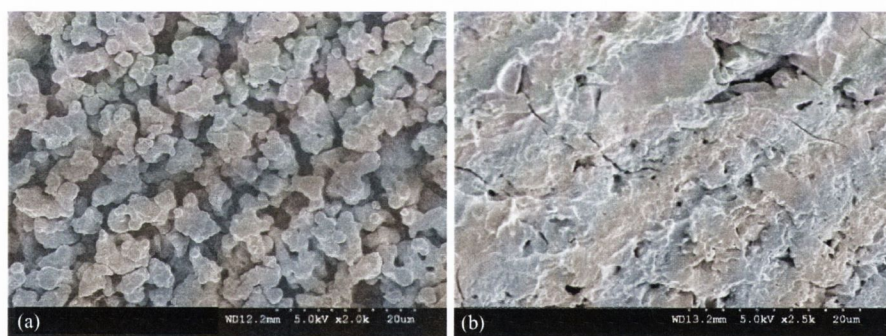


Figure 5.16 SE micrographs showing lysozyme:trehalose 1:1 composite spray-dried from (a) conditions 1 and from (b) conditions 2, after 24 hours storage at 40 °C/75% R.H..

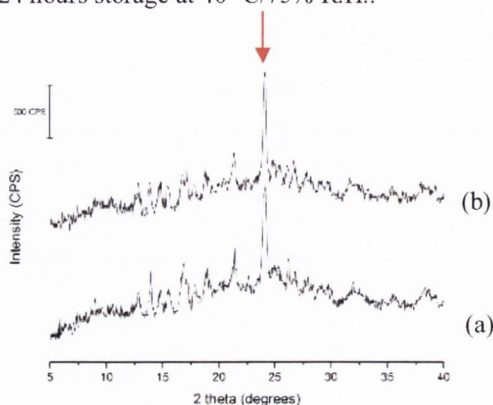


Figure 5.17 XRD of lysozyme:trehalose 1:1 composite spray-dried (a) from conditions 1 and (b) from conditions 2, after 1 week storage at 40 °C/75% R.H..

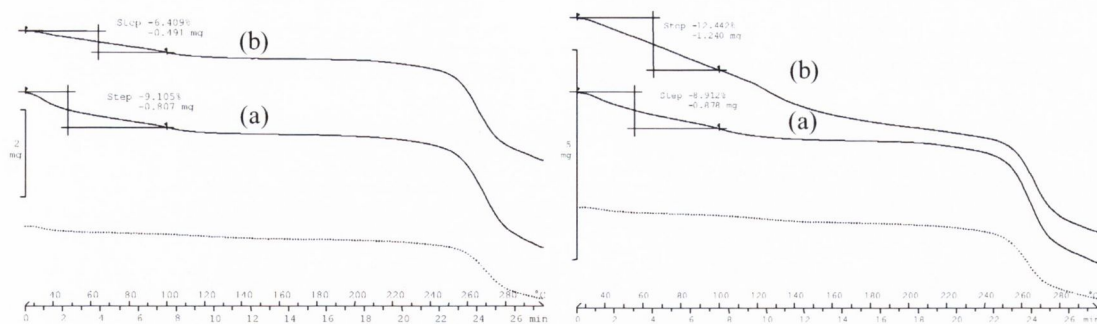


Figure 5.18 TGA of lysozyme:trehalose (1:1 spray-dried from conditions 1 (left) and spray-dried from conditions 2 (right) (a) week 1 (b) week 2 storage at 40 °C/75% R.H., broken line freshly prepared material.

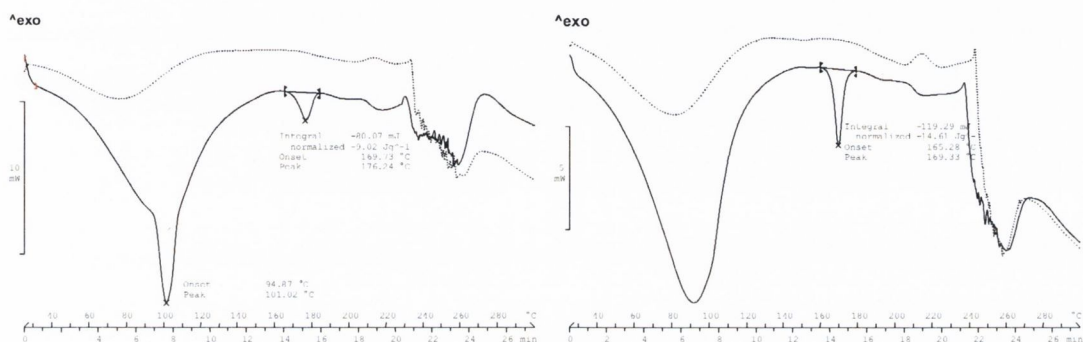


Figure 5.19 DSC of lysozyme:trehalose (1:1) spray-dried from conditions 1 (left) spray-dried from conditions 2 (right) – solid line week 2 storage at 40 °C/75% R.H., broken line freshly prepared material.

There was a change in the shape of the amide 1 peak on storage, under these conditions, with an area of overlap of area-normalised individually baseline-corrected peaks (calculated using method 2) with freshly prepared composite of 91.2% (week 1) and 94.3% (week 2) for the composite spray-dried from conditions 2 and 90.1% (week 1) and 88.9% (week 2) for the composite spray-dried from conditions 1.

There was a decrease in the biological activity and specific activity of the composites spray-dried from conditions 2 but not from conditions 1 on storage under these conditions for 2 weeks (Table 5.5).

Table 5.5 Summary of biological activity assay results for spray-dried composites of lysozyme:trehalose (spray-dried mass ratio 1:1) after 2 weeks storage at 40 °C/75% R.H. (n=2, *± pooled standard deviation).

	Units/ml test	Units/mg solid	µg protein/ml test	Units/mg protein	% Activity* (± 4.4)	% Specific activity * (± 4.3)
conditions 1	357 ± 32.5	22305 ± 2044	10.05 ± 0.92	35510 ± 6	53.4	93.7
conditions 2	265 ± 21.2	16563 ± 1326	8.70 ± 0.14	30444 ± 1943	39.6	80.3
Control	334.5 ± 16.3	41797 ± 2099	8,85 ± 0.92	37888 ± 2038	100	100

The powders were observed to collapse into a sticky mass on storage, and were removed from the study before the week 5 time-point and thus no further analysis was undertaken.

Comparison of the results of the full stability study (4 °C/desiccant; 25 °C/60% R.H. and 40 °C/75% R.H.) to a similar study for lysozyme spray-dried alone from conditions 1 (Section 3.5), suggests a similar FTIR and biological activity stability stored at 4 °C/desiccant and 25 °C/60% R.H.. Inclusion of trehalose resulted in crystallisation and change in particle morphology stored at 25 °C/60% R.H. and also collapse of powders to a sticky mass at 40 °C/75% R.H. however, suggesting a disadvantage in the physical stability of these formulations, compared to lysozyme alone. Interestingly an increased stability of biological activity of spray-dried lysozyme:trehalose spray-dried from conditions 1 (but not conditions 2) compared to lysozyme spray-dried alone stored at 40 °C/75% R.H. after 2 weeks was noted. This was despite crystallisation of the sugar and changes in appearance of the material.

5.2.1.3 PARTICLE CHARACTERISATION

Lysozyme:trehalose spray-dried at a mass ratio of 1:1 from conditions 2, was chosen for further characterisation in terms of surface area, Malvern particle sizing, bulk and tapped density and in-vitro deposition via Andersen cascade impactor analysis. These particular spray-drying conditions were chosen, because the particles produced had a different morphology – i.e. a more corrugated or folded surface (Figure 5.1 (c), (d)) compared to the fused nano-particulate surface appearance of lysozyme particles spray-dried alone and lysozyme:trehalose composites spray-dried from conditions 1 (Figure 5.1 (a)).

Particle size distribution was determined using laser diffraction particle sizing at the standard operational settings of 2 bar air pressure and 50% vibration feed rate (Figure 5.20). Particles had an average d(0.5) of $2.278 \pm 0.052 \mu\text{m}$, average d(0.9) of $4.496 \pm 0.098 \mu\text{m}$, and average d(0.1) of $1.131 \pm 0.027 \mu\text{m}$, which was slightly larger than the porous particles of lysozyme spray-dried alone and similar to non-porous spray-dried particles of lysozyme alone. The particles had a narrow size distribution with an average span of 1.476 ± 0.009 .

Particles were found to have a surface area between that of NPMPs of lysozyme and non-porous lysozyme particles spray-dried alone (Section 3.6 Table 3.9) with an average value of $4.756 \text{ m}^2/\text{g}$. Bulk and tapped density were lower than NPMPs of lysozyme alone at $0.211 \pm 0.011 \text{ g/cm}^3$, and $0.30 \pm 0.015 \text{ g/cm}^3$ respectively, which may be influenced by the hollow nature and thin particle walls, as noted from SEM analysis. Carr’s index, calculated from density data, gave a value of 29.7, indicative of a poorly flowing powder (de Villiers, 2005).

Table 5.6 Particle sizing (n = 3; mean ± standard deviation), surface area (n = 2; mean (range)) and bulk and tapped densities (n = 3, mean ± standard deviation) for lysozyme:trehalose spray-dried as a mass ratio of 1:1 from conditions 2.

	LYSOZYME:TREHALOSE (1:1)
Particle Sizing	
d(0.1) (μm)	1.131 ± 0.027
d(0.5) (μm)	2.278 ± 0.052
d(0.9) (μm)	4.496 ± 0.098
% < 1 μm	6.27± 0.62
Surface Area (m ² /g)	4.756 (4.7575, 4.7542)
Bulk Density (g/cm ³)	0.211 ± 0.011
Tapped Density (g/cm ³)	0.300 ± 0.015

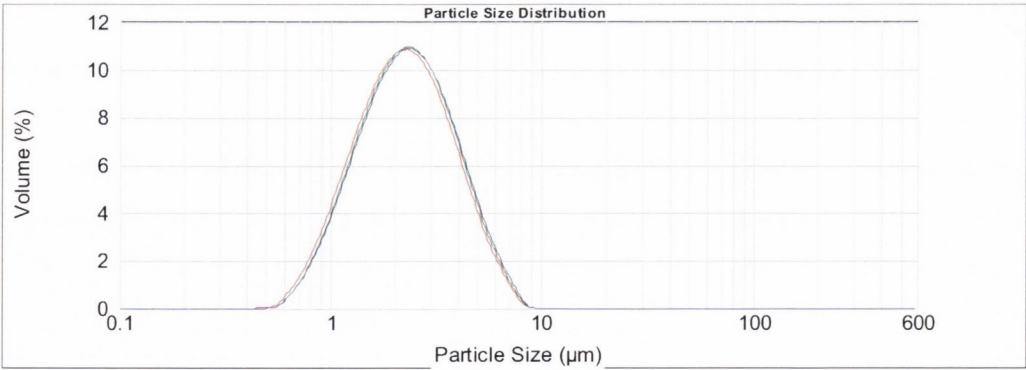


Figure 5.20 Particle size distribution for lysozyme:trehalose spray-dried as a 1:1 mass ratio composite, from conditions 2.

Fine particle fraction ($< 5 \mu\text{m}$) and particle fraction $< 3 \mu\text{m}$ estimated from Andersen cascade impactor analysis were favourable, compared to NPMPs of lysozyme spray-dried alone (Section 3.6). The in-vitro deposition profile also showed deposition mainly in the mid and lower stages (stages 3, 4), and less deposition in the mouthpiece adaptor/induction port as compared to either the NPMPs or the non-porous lysozyme (Figure 5.21). The MMAD of $2.72 \pm 0.05 \mu\text{m}$ is within the desirable range for systemic delivery. A GSD of 1.99 ± 0.09 , indicative of the spread of the data and distribution of aerodynamic particle size was calculated, similar to porous lysozyme spray-dried alone.

Table 5.7 Summary of results from Andersen cascade impactor analysis lysozyme:trehalose (1:1 mass ratio composite, spray-dried from conditions 2); mean $\pm$ stdev; n = 3.

	% < 5 μm	% < 3 μm	MMAD (μm)	GSD	% emitted (of nominal dose)
Lys:trch (1:1)	63.98 ± 2.75	39.15 ± 1.99	2.72 ± 0.05	1.99 ± 0.09	83.8 ± 6.0

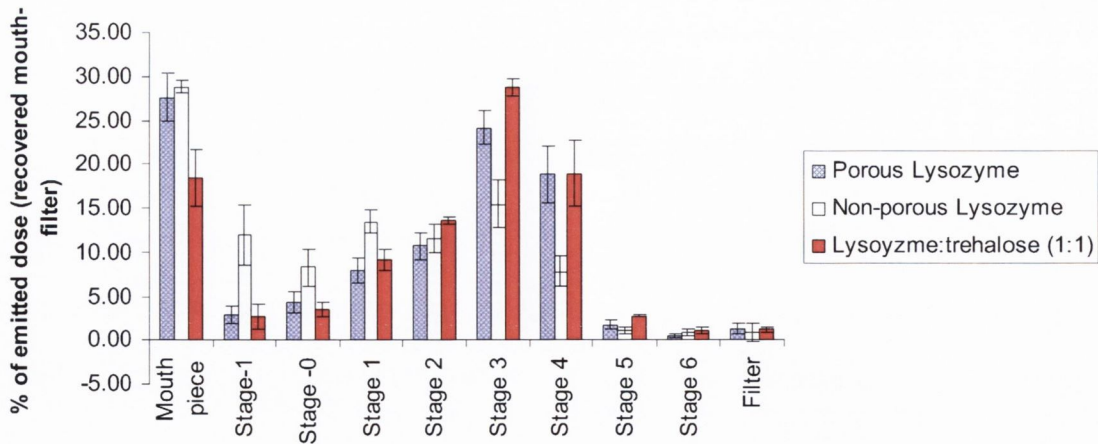


Figure 5.21 Deposition patterns on the stages of the ACI apparatus for lysozyme:trehalose (1:1 mass ratio composite) spray-dried from conditions 2, shown comparative to NPMPs of lysozyme and non-porous lysozyme (Chapter 3). Calculated as % of recovered emitted dose (mouth – filter), used to calculate fine particle fraction.

5.2.2 SPRAY-DRIED LYSOZYME:RAFFINOSE, LYSOZYME:INULIN COMPOSITES

Lysozyme was spray-dried with raffinose as an alternative sugar to trehalose, at mass ratio 1:1 from conditions 1 and from conditions 2. Particles spray-dried from conditions 1 were observed to be discrete, and spherical, with a non-porous rough surface morphology as determined by SEM analysis (Figure 5.22 (a)). Particles spray-dried from conditions 2 were observed to be discrete, and spherical with a folded to porous surface morphology as was evident from SEM analysis (Figure 5.22 (b)). Lysozyme:inulin (mass ratio 1:1) was spray-dried from conditions 2. Particles were observed to be collapsed with a minority of spherical particles and had a folded to porous surface morphology from SEM analysis (Figure 5.22 (c)).

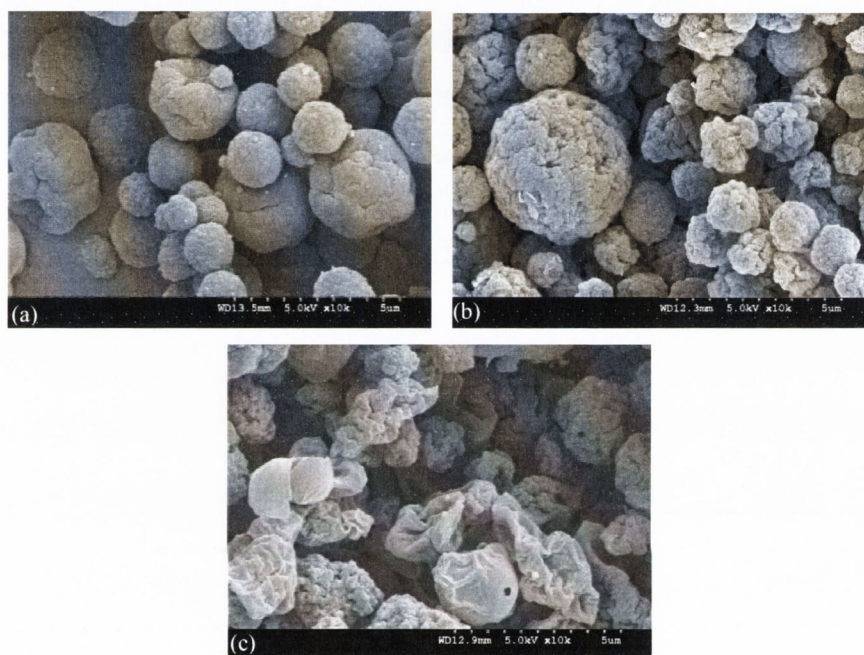


Figure 5.22 SE micrographs showing lysozyme:raffinose spray-dried in a mass ratio of 1:1 from (a) conditions 1 (b) conditions 2; and (c) lysozyme:inulin spray-dried in mass ratio 1:1 from conditions 2.

Lysozyme:raffinose and lysozyme:inulin composites, spray-dried from conditions 1 and 2, were amorphous by XRD. A similar residual moisture or solvent loss was calculated for lysozyme:raffinose composite samples (3.8 – 4.0%) over the temperature range 25 –100 °C from TGA data with a further smaller step of mass loss observed over the temperature range 100-140 °C (Figure 5.23). A higher residual moisture or solvent loss over the temperature range 25 –100 °C was determined from TGA data for the inulin composite ~ 6.7% (Figure 5.24). DSC data (Figure 5.23, 5.24) showed an initial broad endotherm for all composites and a second broad endotherm corresponding to protein decomposition at > ~ 200 °C. No glass transitions were identified in any of the composites. The inulin composite additionally showed a sharp endotherm at ~173 °C, attributed to the melting peak of inulin (151.4 – 186.5 °C depending on the polymerisation degree of the inulin sample) (Blecker et al., 2003). This endotherm was also seen in the DSC scan of unprocessed inulin.

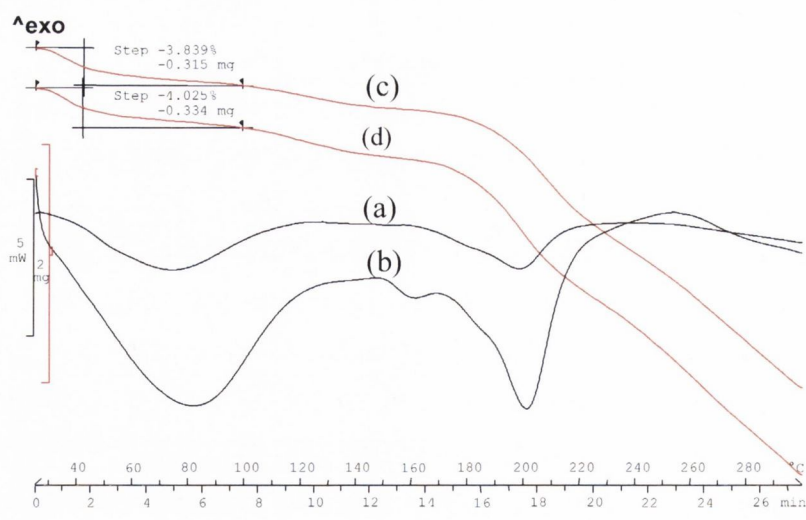


Figure 5.23 DSC (shown in black) and TGA (shown in red) of (a), (c) lysozyme:raffinose (1:1) spray-dried from conditions 2, (b), (d) lysozyme:raffinose (1:1) spray-dried from conditions 1.

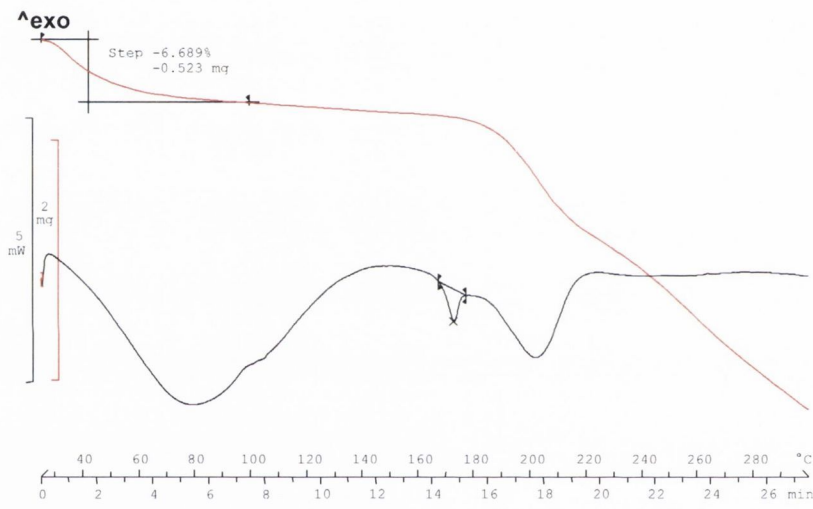


Figure 5.24 DSC (shown in black) and TGA (shown in red) of lysozyme:inulin (1:1) spray-dried from conditions 2.

Biological activity assay was carried out on lysozyme:raffinose composite spray-dried from conditions 2 (Figure 5.22 (a)), and showed no statistically significant decrease in specific activity, compared to unprocessed material (Table 5.8).

Table 5.8 Summary of results from biological activity assay of lysozyme:raffinose spray-dried in a mass ratio of 1:1 from conditions 2 (n=3).

	Units/ml test	Units/mg solid	µg protein/ml test	Units/mg protein	% Activity (± 2.6%)	% Specific activity (± 4.9%)
Lys:raff (1:1)	321.0 ± 18.3	20056 ± 1151	7.17 ± 0.80	44961 ± 2524	45.7	102.6
Control	350.5 ± 9.2	43843 ± 1132	8.0 ± 0.0	43843 ± 1132	100	100

5.2.3 SPRAY-DRIED LYSOZYME:MYO-INOSITOL(:PVP) COMPOSITES

Lysozyme:myo-inositol (mass ratio 1:1) was spray-dried from conditions 2 at a t.s.c. of 0.5% w/v. Resultant particles were spherical and had a folded morphology. Particles appeared to be hollow with thin particle walls (Figure 5.25). XRD analysis showed crystallinity of the composite, with peak positions similar to unprocessed myo-inositol (Figure 5.26). Analysis of DSC data identified the melting endotherm of myo-inositol at 224 °C (Figure 5.27), as compared to ~ 223 °C unprocessed myo-inositol alone (Section 4.3.2.3). A broad endotherm was also detected from 25-120 °C. A residual moisture or solvent content of ~ 4.3% was calculated from 25 – 100 °C from TGA (Figure 5.27). Spray-drying myo-inositol with lysozyme in this mass ratio and from these conditions did not appear promising, due to the solid-state characteristics of the composite. Further characterisation of this system was thus not undertaken.

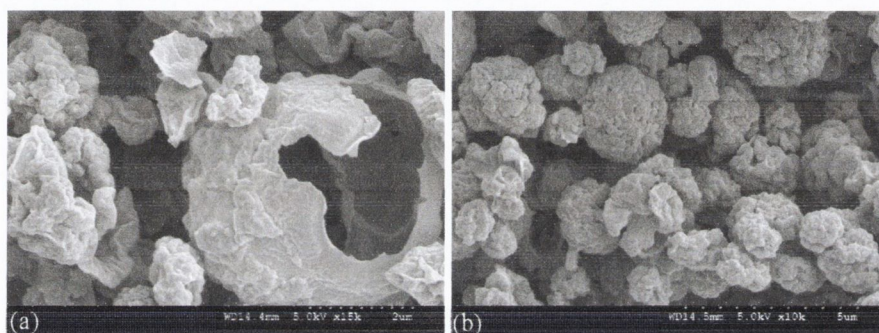


Figure 5.25 SE micrographs showing lysozyme:myo-inositol spray-dried in mass ratio 1:1, 70% EtOH, 10% of t.s.c, AC, inlet temperature 90 °C.

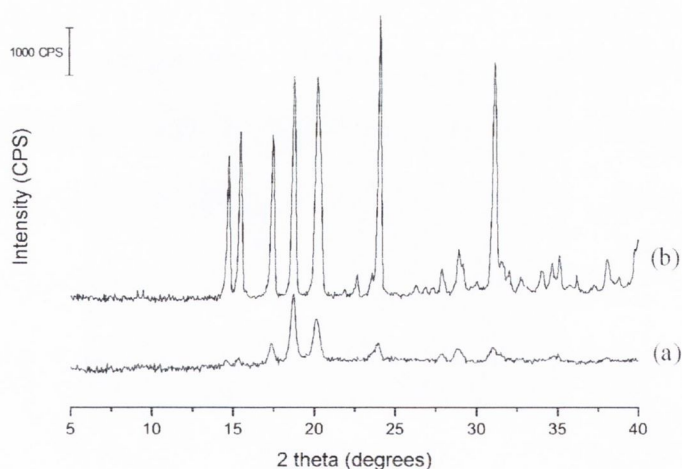


Figure 5.26 XRD of (a) lysozyme:myo-inositol composite (spray-dried mass ratio 1:1), (b) myo-inositol (unprocessed material).

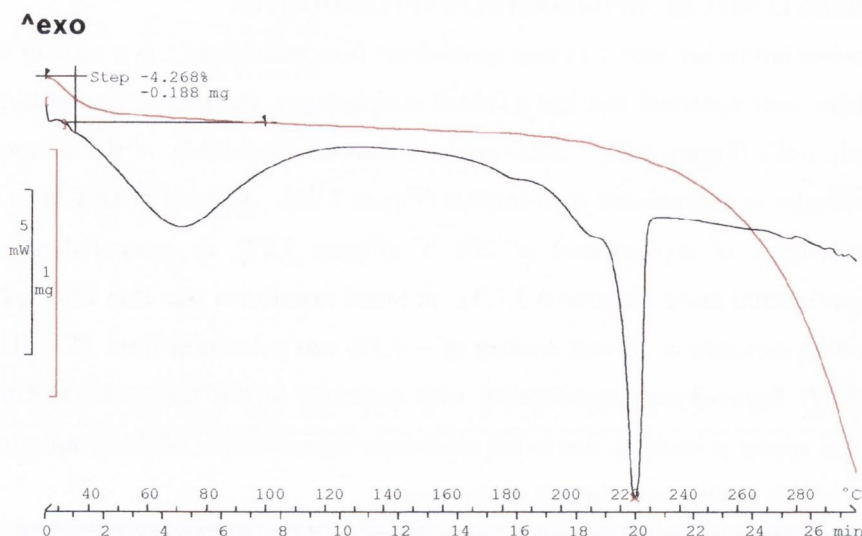


Figure 5.27 DSC (shown in black) and TGA (shown in red) of lysozyme:myo-inositol spray-dried in mass ratio 1:1, 70% EtOH, 10% of t.s.c, AC, inlet temperature 90 °C.

PVP has been previously used to inhibit crystallisation and promote production of an amorphous composite by spray-drying (Tajber, 2005). Lysozyme was initially spray-dried with PVP-k15 (MW 10,000) in a ratio of 9:1 from conditions 1 (used to produce NPMPs of lysozyme alone – chapter 3) to investigate any adverse effects on the morphology of the powder, prior to spray-drying in a ternary system with myo-inositol. Particle morphology was observed to be spherical and porous/nano-particulate from SEM analysis (Figure 5.28).

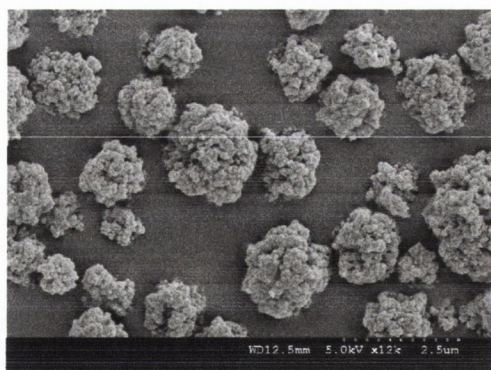


Figure 5.28 SE micrographs showing lysozyme:PVP k15 spray-dried in mass ratio 9:1, from conditions 1.

PVP-k15 or PVP-40 were included with lysozyme:myo-inositol and spray-dried from conditions 1, at mass ratios of 5:3:2 (k-15), and 5:4:1 (k-15,40) lysozyme:myo-inositol:PVP. Spray-dried particles consisted of collapsed spheres and spherical particles with a rough surface, as evident from SEM analysis (Figure 5.29).

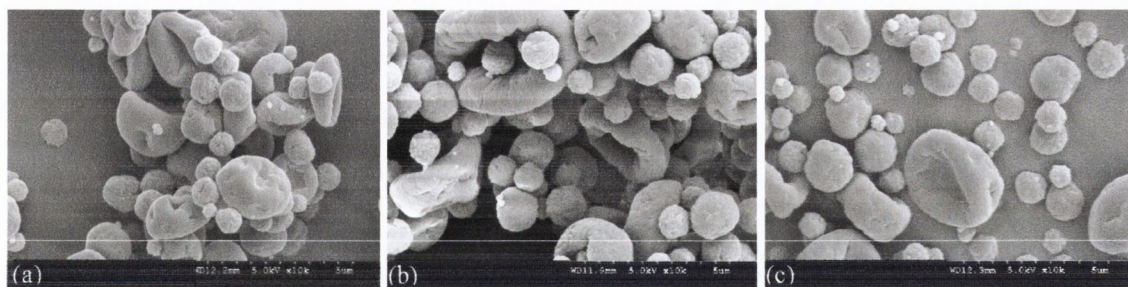


Figure 5.29 SE micrographs showing (a) lysozyme:myo-inositol:PVP-k15 spray-dried in mass ratio 5:4:1, (b) lysozyme:myo-inositol:PVP-40 spray-dried in mass ratio 5:4:1, and (c) lysozyme:myo-inositol:PVP-k15 spray-dried in mass ratio 5:3:2, all from conditions 1.

Lysozyme:myo-inositol:PVP samples were amorphous by XRD (Figure 5.30). DSC scans displayed the following: (1) broad endotherms from 25 - ~120 °C in DSC scans attributed to moisture loss, (2) exotherms starting at ~ 155 - 160 °C corresponding to recrystallisation and (3) sharp endothermic peaks at ~ 224 °C corresponding to melting of myo-inositol (Figure 5.31). Residual moisture/solvent contents of all lysozyme:myo-inositol:PVP composites, calculated from TGA, were 4 – 4.2% (Figure 5.32). Presence of both protein and PVP enabled an amorphous myo-inositol composite to be produced.

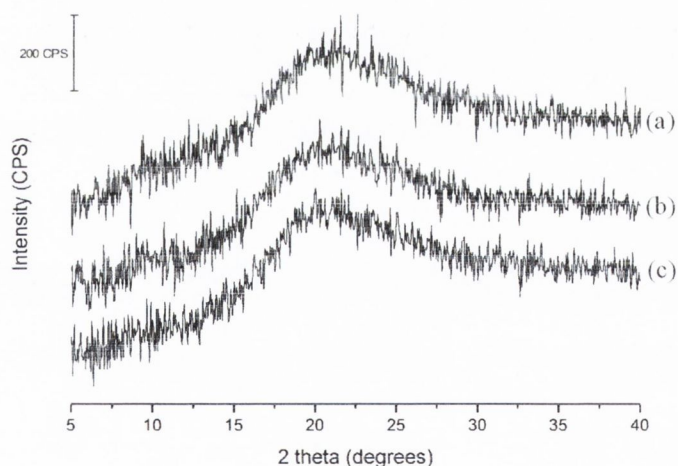


Figure 5.30 XRD of (a) lysozyme:myo-inositol:PVP-k15 spray-dried in mass ratio 5:3:2, (b) lysozyme:myo-inositol:PVP40 spray-dried in mass ratio 5:4:1, (c) lysozyme:myo-inositol:PVP-k15 spray-dried in mass ratio 5:4:1; all spray-dried from conditions 1.

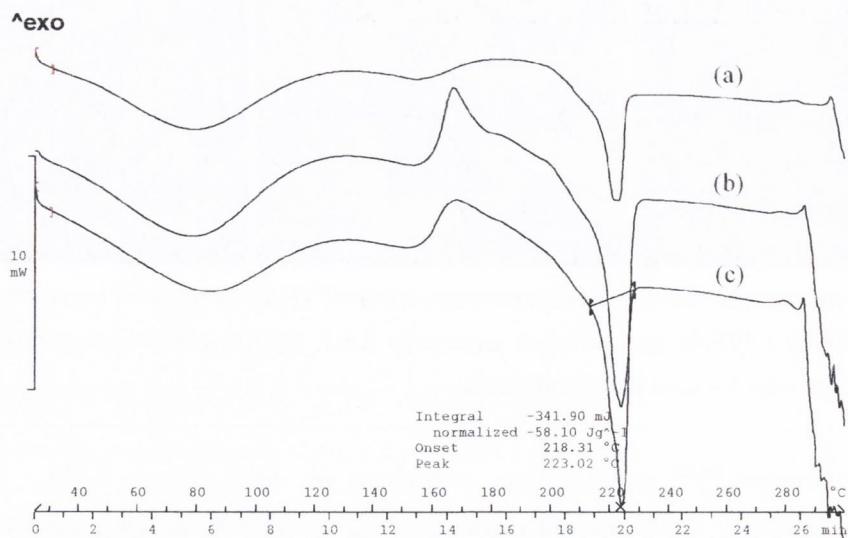


Figure 5.31 DSC of (a) lysozyme:myo-inositol:PVP-k15 spray-dried in mass ratio 5:3:2, (b) lysozyme:myo-inositol:PVP-40 spray-dried in mass ratio 5:4:1, (c) lysozyme:myo-inositol:PVP-k15 spray-dried in mass ratio 5:4:1; all spray-dried from conditions 1.

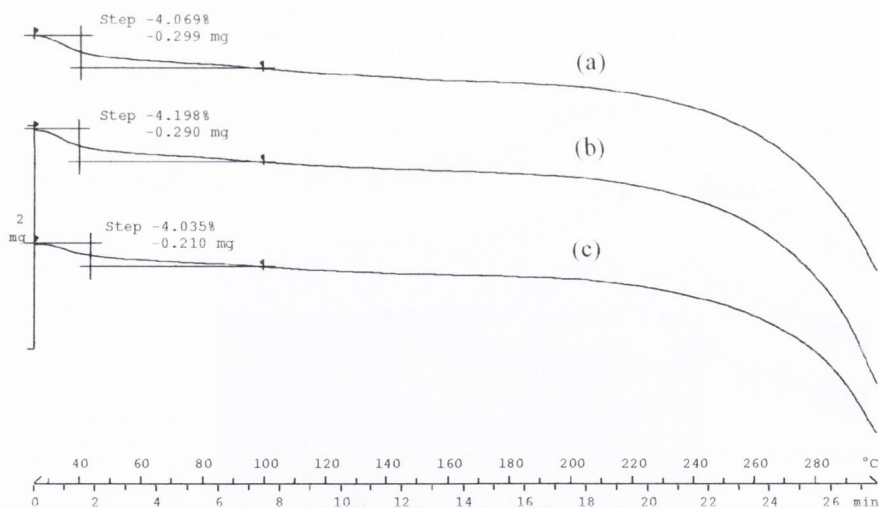


Figure 5.32 TGA of (a) lysozyme:myo-inositol:PVP-k15 spray-dried in mass ratio 5:3:2, (b) lysozyme:myo-inositol:PVP-40 spray-dried in mass ratio 5:4:1, (c) lysozyme:myo-inositol:PVP-k15 spray-dried in mass ratio 5:4:1; all spray-dried from conditions 1.

5.3 METHANOL:N-BUTYL ACETATE SOLVENT SYSTEMS

5.3.1 SPRAY-DRIED LYSOZYME:HP- β -CD COMPOSITES

Lysozyme was freeze-dried to increase its solubility in methanol (Section 3.2), and spray-dried with HP- β -CD from a non-aqueous MeOH:BA system (solvent ratio 1:1, inlet temperature 100 °C). Spray-drying was carried out in the closed mode configuration, using the regular cyclone arrangement. These conditions had been successfully used by Li (2005) to produce NPMPs of HP- β -CD alone. Feed solutions were prepared by first dissolving lysozyme in the MeOH component, followed by addition of HP- β -CD, and finally the BA component. Lysozyme:HP- β -CD mass ratios of 4.8:95.2 and 1:4 were prepared. A total solid concentration of 2.1% w/v was used for the 4.8:95.2 mass ratio similar to that used by Li (2005) (2% w/v). A lower t.s.c. of 1% w/v was used for the 1:4 mass ratio in order to avoid problems with the solubility of freeze-dried lysozyme in the MeOH component (1% w/v t.s.c. = 0.4% w/v lysozyme in the MeOH component) (from Section 3.2 - 0.5% w/v lysozyme should be soluble in the MeOH component).

Some phase separation was noted in the feed material on addition of BA, with a small amount of deposition of material on the walls of the conical flask and the feed material was spray-dried as a suspension. Spray-dried particles were spherical and porous with absence of broken or shattered particles, as observed from SEM analysis (Figure 5.33). Collectable product yields were ~ 40% (mass ratio 4.8:95.2, volume spray-dried 50 ml) and ~ 26% (mass ratio 1:4, volume spray-dried 40 ml).

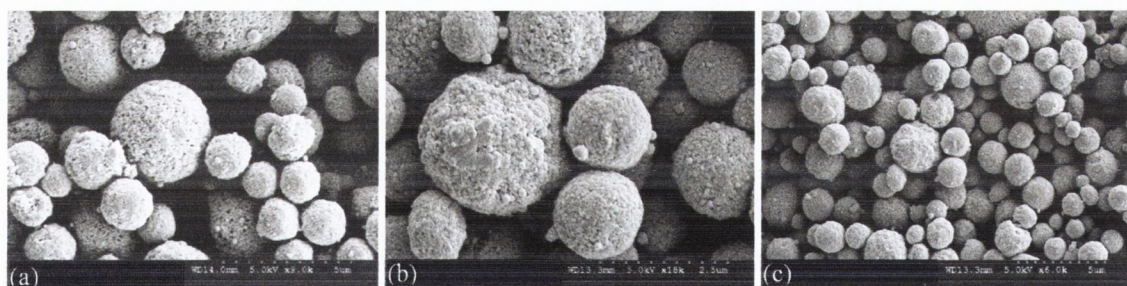


Figure 5.33 SE micrographs showing lysozyme:HP- β -CD spray-dried in mass ratios (a) 4.8:95.2, (b)/(c) 1:4 from a 1:1 MeOH:BA solvent system, inlet drying temperature 100 °C, under different magnifications.

The composite of spray-dried mass ratio of 1:4 lysozyme:HP- β -CD was chosen for further investigation because of a favourable particle morphology and higher lysozyme content. The regular cyclone was replaced with a high performance cyclone and spray-drying was repeated. Spray-dried particles had similar morphology as evident from SEM analysis (Figure 5.34 (a), (b)), being spherical and porous. Collectable product yield was high with a yield of ~ 69% (volume spray-dried 75 ml). HP- β -CD (originally spray-dried from a MeOH:BA system by Li (2005)), was spray-dried using similar conditions as for the lysozyme:HP- β -CD 1:4 mass ratio composite (see

Appendix 1) and the HP cyclone. No phase separation was found in the feed material of HP- β -CD spray-dried alone and the feed material was spray-dried as a clear solution. Collectable product yield was $\sim 63\%$ (volume spray-dried 75 ml). Particle morphology was spherical and nano-porous, as observed from SEM analysis (Figure 5.34 (c), (d)). A large variation in particle size was also evident from SE micrographs particularly for the HP- β -CD spray-dried alone.

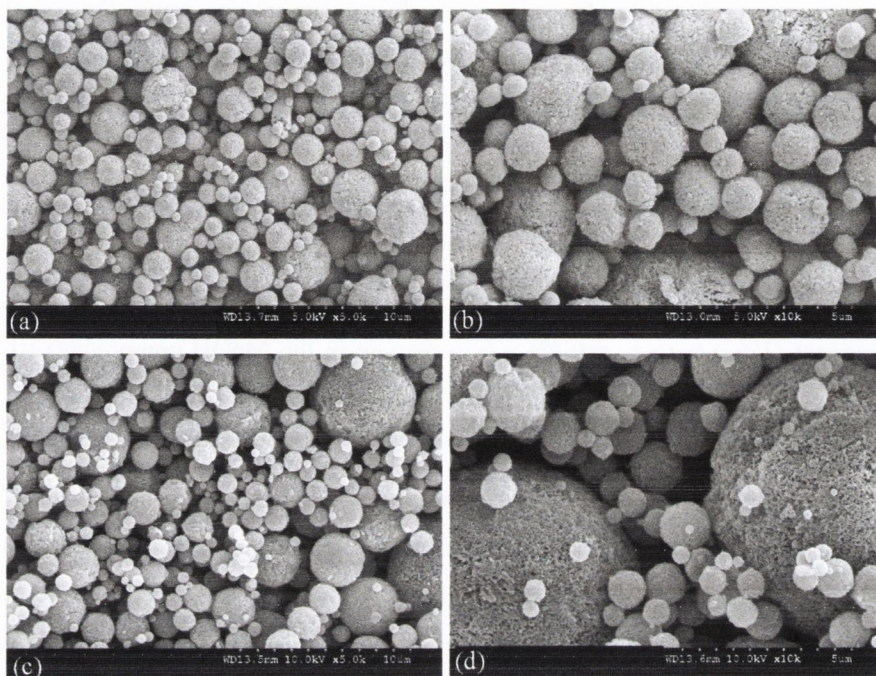


Figure 5.34 SE micrographs showing (a)/(b) lysozyme:HP- β -CD spray-dried in mass ratios of 1:4 and (c)/(d) HP- β -CD spray-dried alone from a 1:1 MeOH:BA solvent system, inlet drying temperature 100 °C, using a HP cyclone.

5.3.2 SPRAY-DRIED LYSOZYME: TREHALOSE COMPOSITES

Freeze-dried lysozyme (Section 3.2) was spray-dried with trehalose dihydrate at a mass ratio of 1:4 (lysozyme:trehalose dihydrate), from an 80:20 MeOH:BA solvent system, as was used for producing NPMPs of trehalose alone (Section 4.2.1.3, Figure 4.6) and a t.s.c. of 0.5%. (See Appendix 1 for details.) A HP cyclone was used. Phase separation/precipitation was not observed to the same extent on addition of BA, as in the lysozyme:HP- β -CD system described above and the appearance of the feed was a clear solution to slightly turbid suspension depending on the batch. Contributory factors to this difference in solubility relative to the HP- β -CD composite system may have included a lower total solid concentration and thus lower total protein concentration, and a lower concentration of the more hydrophobic BA used in this feed system. Spray-dried material consisted of porous spheres as seen from SEM micrographs (Figure 5.35). The pores were smaller (approximately < 90 nm) compared to trehalose spray-dried alone which had a looser nanoparticulate appearance, with surface gaps/pores between nano-particles approximately < 300 nm (Section 4.2.1.3 Figure 4.6). Collectable product yields were high at ~ 54 and $\sim 64\%$ (volume spray-dried 150 ml) for two replicate batches spray-dried.

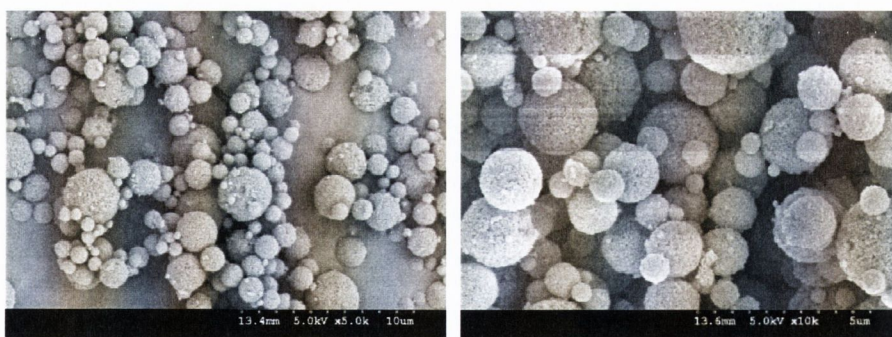


Figure 5.35 SE micrographs showing lysozyme:trehalose spray-dried at a mass ratio of 1:4, from MeOH:BA (80:20 solvent ratio), inlet temperature 100 °C.

5.3.3 SPRAY-DRIED LYSOZYME:RAFFINOSE COMPOSITES

Freeze-dried lysozyme (Section 3.2) was spray-dried with raffinose pentahydrate at a mass ratio of 1:4, in a similar system as produced porous particles of raffinose alone (Section 4.2.2.1). The total solid concentration used was 0.5% compared to 1% for the sugar spray-dried alone, to try to minimise precipitation, as for the trehalose composite system (Section 5.3.2). A regular cyclone was used to allow comparison with the sugar spray-dried alone. Spray-dried particles were spherical and porous (Figure 5.36 (a), (b), (c)). As was observed for the lysozyme:trehalose composite systems, surface gaps/pores were smaller, than for the sugar spray-dried alone (Section 4.2.2.1 Figure 4.23). Collectable yields were ~ 28% and ~ 26% for two different batches spray-dried (volumes spray-dried: 150 ml and 200 ml respectively). Replacing the regular cyclone with a high performance cyclone did not significantly affect the particle morphology as observed in SEM analysis (Figure 5.36 (d), (e)). Collectable yield was approximately doubled to ~ 54% (volume spray-dried 150 ml).

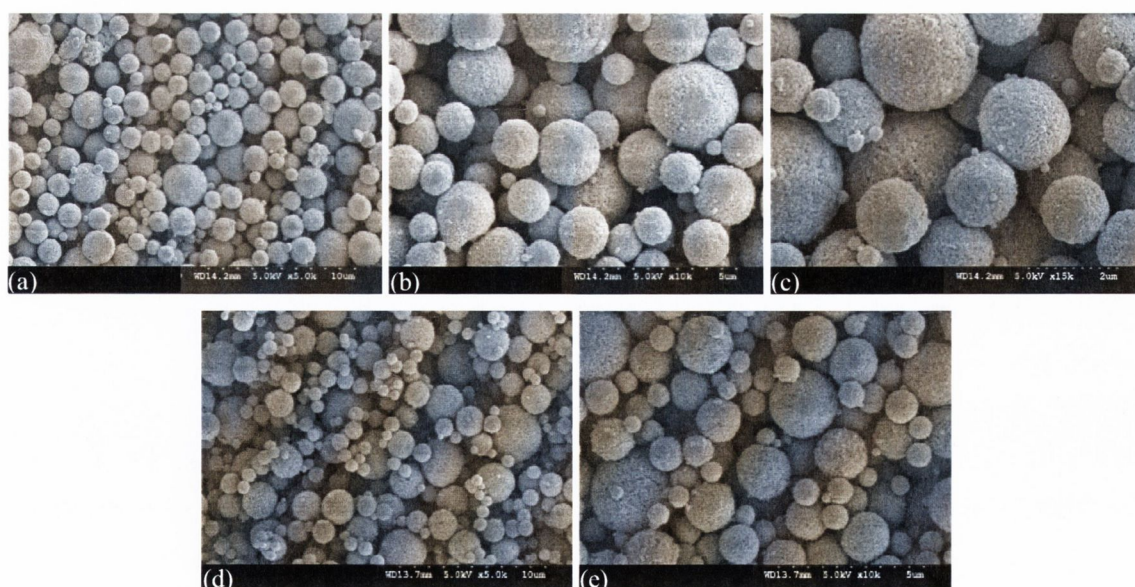


Figure 5.36 SE micrographs showing lysozyme:raffinose pentahydrate spray-dried at a mass ratio of 1:4 from MeOH:BA (80:20 solvent ratio), inlet temperature 100 °C (a), (b), (c) using regular cyclone, and (d), (e) using HP cyclone under different magnifications.

In order to examine different mass ratios of lysozyme:raffinose, ratios of 3:7 and 1:1 were prepared. SEM analysis of particles spray-dried from a 3:7 mass ratio showed some pores on the surface although these appeared more fused than with the lower mass ratio of 1:4 (Figure 5.36 (a)). Particles spray-dried from a mass ratio of 1:1 lysozyme:raffinose had a fused roughened non-porous surface (Figure 5.37 (b), (c)).



Figure 5.37 SE micrographs showing lysozyme:raffinose spray-dried at a mass ratio of 3:7 (a) using a regular cyclone (b) using a HP cyclone, and at a mass ratio of 1:1 (c) using a HP cyclone.

5.3.4 PROCESS STABILITY

5.3.4.1 Solid-state characterisation and thermal analysis

HP- β -CD composites

Spray-dried composites of mass ratio 1:4 lysozyme:HP- β -CD were chosen for further characterisation, due to favourable particle morphology and higher lysozyme content. The composite material was amorphous by XRD, similar to HP- β -CD spray-dried alone (Figure 5.38). DSC and TGA of HP- β -CD spray-dried alone were compared to two batches of composite (Figures 5.39, 5.40). A broad endothermic peak was observed for all samples from 25-120 °C by DSC. A glass transition was detected at 226.5 °C for HP- β -CD spray-dried alone. A small endothermic peak at ~220 °C for the composites may be due to a decomposition peak of lysozyme, which was to be expected in this temperature region from DSC studies on lysozyme spray-dried alone from MeOH:BA (Figure 3.32, Section 3.4.1). Thermal decomposition was observed in the composites at ~ 280 °C, accompanied by mass loss in the TGA scan. This decomposition was not observed in DSC or TGA scans of HP- β -CD spray-dried alone in the temperature range studied. Mass loss over the temperature range of 25-100 °C was 4.8% and 5.6% for the composite samples and slightly higher for HP- β -CD spray-dried alone at 6.3%.

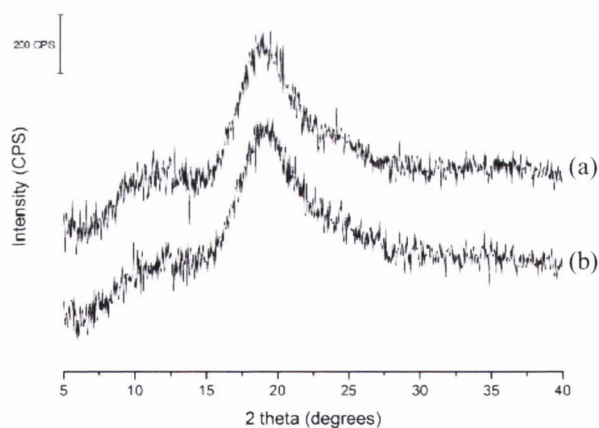


Figure 5.38 XRD of (a) HP- β -CD spray-dried alone (b) lysozyme:HP- β -CD composite dried at mass ratio 1:4.

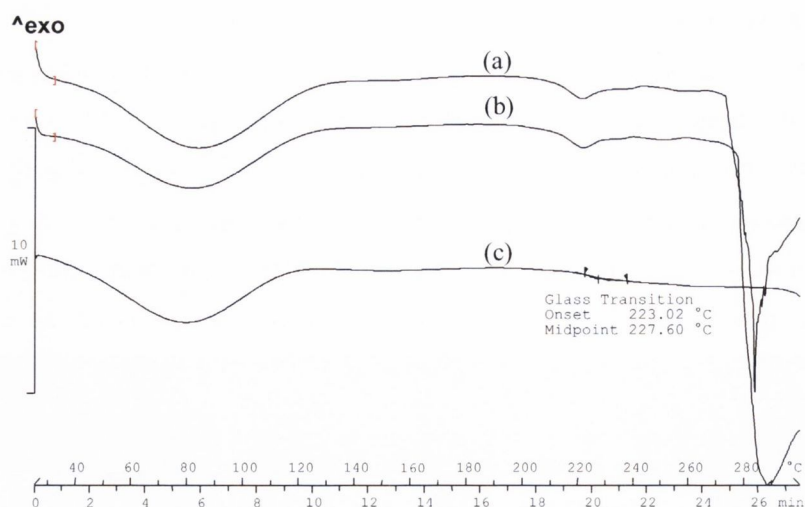


Figure 5.39 DSC scans of (a), (b) 2 batches of lysozyme:HP-β-CD composite dried at mass ratio 1:4 (c) HP-β-CD spray-dried alone.

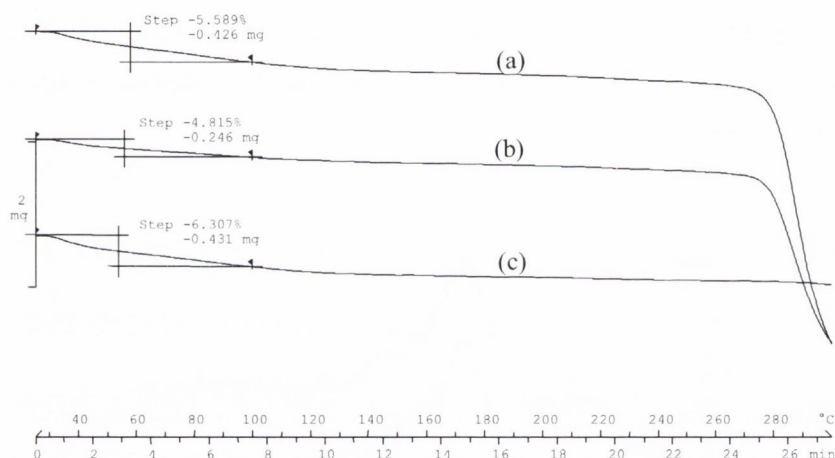


Figure 5.40 TGA scans of (a), (b) 2 batches of lysozyme:HP-β-CD composite dried at mass ratio 1:4 (c) HP-β-CD spray-dried alone.

Trehalose composites

Spray-dried composite (mass ratio 1:4) was amorphous by XRD (Figure 5.41). DSC and TGA data of two batches of composite are shown, compared to trehalose spray-dried under similar conditions alone (Figure 5.42, 5.43). A glass transition at ~120 °C was identified for one batch of the composite lysozyme:trehalose material, similar to trehalose spray-dried alone. A recrystallisation exotherm and subsequent melting of the crystalline sugar (at ~213 °C) observed in the DSC scan of trehalose spray-dried alone was not observed in the DSC scan of this batch of composite however. An endothermic decomposition of the protein at ~220 °C identified for the protein spray-dried alone from similar conditions (Figure 3.32 Section 3.4.1) was also not identified in the DSC of this composite batch, although a later endotherm at ~265 °C was identified. A second batch showed a

high temperature recrystallisation exotherm immediately prior to an endothermic peak similar to the melting peak of anhydrous trehalose at 213 °C, and also showed a later endotherm at ~265 °C. This is a similar situation to trehalose spray-dried alone, in which recrystallisation was identified in the DSC scans of some batches, occurring at different temperatures, or in other cases was absent depending on spray-drying conditions and within a set of conditions depending on the batch (Section 4.2.1.4.4, Appendix 2). The second endotherm identified at ~265 °C showed a corresponding mass loss in TGA suggesting decomposition of the protein. Residual moisture or solvent contents of 4.6% and 4.2% were calculated for the two batches of lysozyme:trehalose composite.

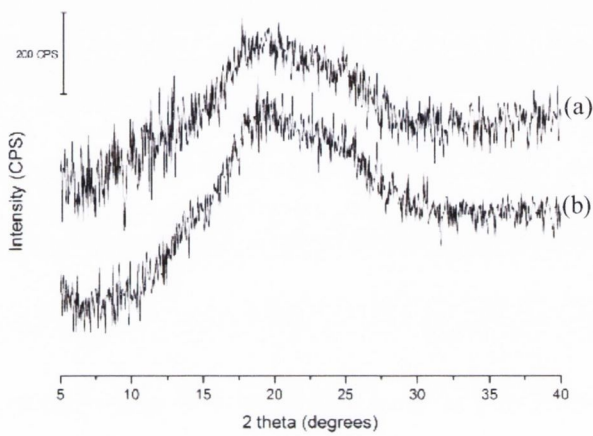


Figure 5.41 XRD of (a) trehalose spray-dried alone (b) lysozyme:trehalose (1:4) composite.

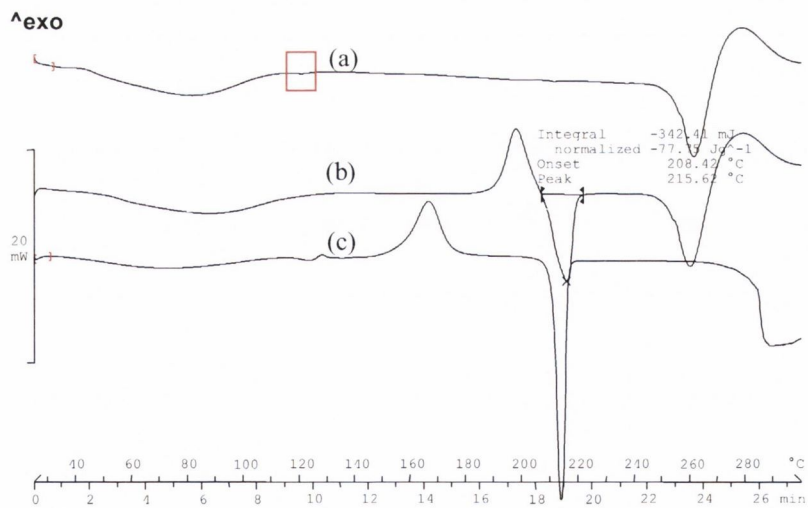


Figure 5.42 DSC scans of (a) (b) 2 batches of lysozyme:trehalose composite dried at mass ratio 1:4 (c) trehalose spray-dried alone.

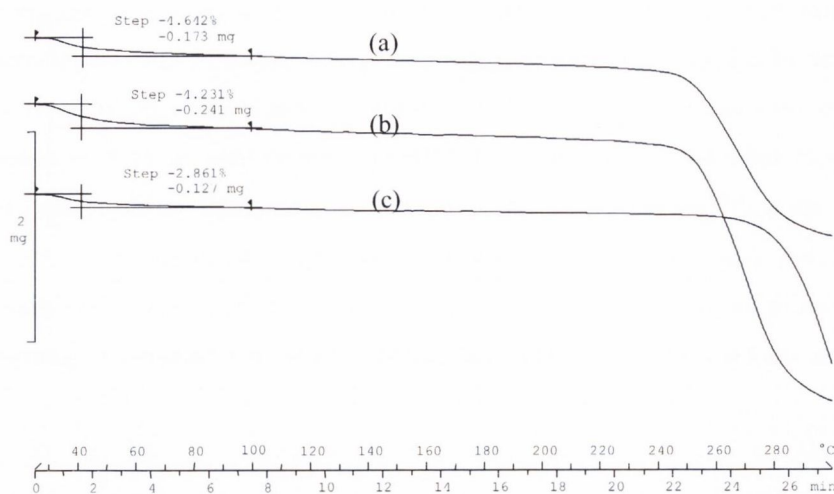


Figure 5.43 TGA scans of (a) (b) 2 batches of lysozyme:trehalose composite dried at mass ratio 1:4 (c) trehalose spray-dried alone.

Raffinose composites

The composites (mass ratios 1:4, 3:7, 1:1 lysozyme:raffinose) were amorphous by XRD (Figure 5.44). DSC (Figure 5.45) and TGA (Figure 5.46) scans of two batches of composite spray-dried in a mass ratio of 1:4 are presented, together with raffinose spray-dried alone. Glass transitions were noted in the two systems at $\sim 120^\circ\text{C}$. An endothermic decomposition peak at $\sim 215^\circ\text{C}$ was observed in the DSC scans of the composites, with corresponding mass losses at similar temperatures in the TGA scans. A later endothermic decomposition ($\sim 230^\circ\text{C}$) was observed in the raffinose sample spray-dried alone. Residual moisture or solvent was calculated from TGA as 4.7% and 4.1% for two batches of composite material.

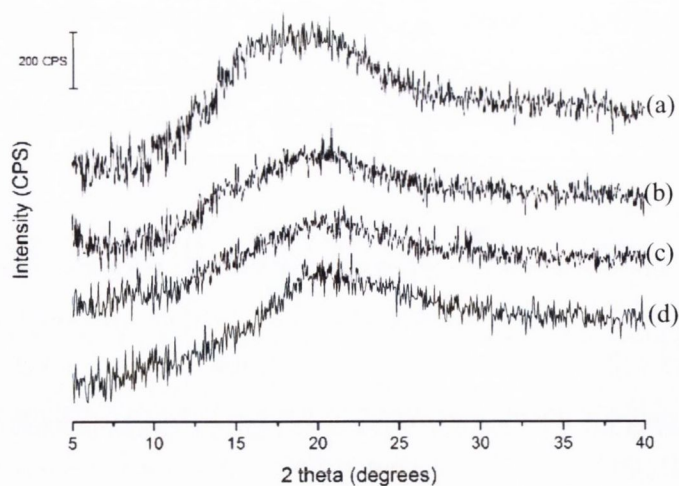


Figure 5.44 XRD of (a) raffinose spray-dried alone (b) lysozyme:raffinose 1:4 (c) lysozyme:raffinose 3:7 (d) lysozyme:raffinose 1:1.

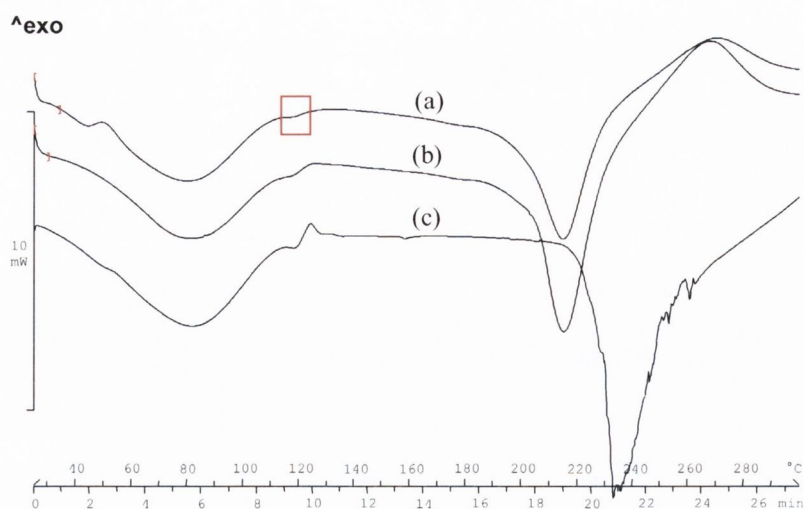


Figure 5.45 DSC scans of (a) (b) 2 batches of lysozyme:raffinose composite dried at mass ratio 1:4 (c) raffinose spray-dried alone.

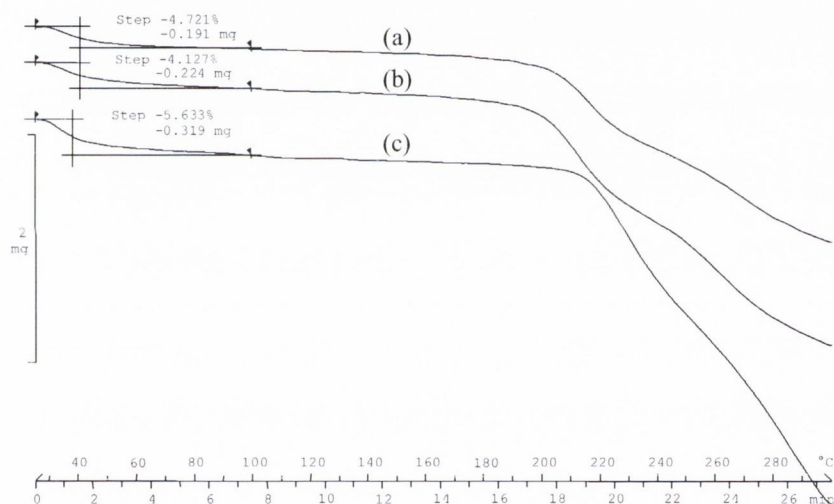


Figure 5.46 TGA scans of (a) (b) 2 batches of lysozyme:raffinose composite dried at mass ratio 1:4 (c) raffinose spray-dried alone.

5.3.4.2 Secondary structure and biological activity assay

No protection of secondary structure was observed with the inclusion of any of the excipients compared to freeze-dried lysozyme spray-dried alone from MeOH:BA. The area of overlap with unprocessed lysozyme was 85.0% for the lysozyme:HP- β -CD (1:4) composite, 86.4% for the lysozyme:trehalose (1:4) composite and 84.4% for the lysozyme:raffinose (1:4) composite. These compared to 89.0% for lysozyme spray-dried alone from the 80:20 MeOH:BA solvent ratio system and 89.9% from the 1:1 MeOH:BA solvent ratio system.

Biological activity assay carried out on composite particles showed excellent retention of activity, with specific activity of the composite particle similar to the original starting material. Table 5.9 shows the results of one batch each of spray-dried composites. The specific activity of all composites was similar and not statistically significantly different from the control (at a significance level of $\alpha=0.05$). Micro BCA analysis showed that the lysozyme:raffinose batch had a slightly higher than expected protein content in the test sample, than would have been predicted from the ratio of components spray-dried in the composite particle (i.e. assuming a 20% lysozyme loading in the composite particle). This was also reflected in a higher calculated activity for units/ml of test sample and for units/mg solid compared to the other composites. This slight discrepancy may be influenced by small errors in weighing/preparation but also may be theoretically influenced by the loss of 5-hydrate water from the starting crystalline raffinose in the feed compared to the spray-dried amorphous anhydrous raffinose in the composite. Even taking into account a potential increased mass from unbound water with the more hygroscopic amorphous raffinose, this could lead to an estimated ~10% loss in mass for the raffinose component which may alter the lysozyme:raffinose mass ratio in the final spray-dried composite material from that added to the initial feed (1:4). (From TGA analysis a mass loss of 4-5% was calculated for amorphous raffinose vs 15% mass loss for crystalline raffinose over temperature range 25-100 °C). Loss of 2-hydrate water from trehalose composite would be expected to influence the ratio to a lesser extent, when taking into account the amount of unbound water in the amorphous trehalose (from TGA analysis a mass loss of 4-5% is calculated for amorphous trehalose vs 9.5% mass loss for crystalline trehalose over temperature range 25-100 °C). HP- β -CD also does not lose bound hydrate water on spray-drying (water content in the unprocessed batch used was 3.4%, assessed by Karl Fischer www.sigmaaldrich.com). Activity assay, and micro BCA analysis of test solutions of replicate batches (Table 5.10) did not show an increased protein content in the raffinose composite however suggesting difficulty in predicting deviations from the mass ratio spray-dried in the final composite.

Table 5.9 Summary of results from biological activity assay of lysozyme:excipient composites spray-dried at a mass ratio of 1:4 (n=3, *pooled standard deviation shown).

	Units/ml test	Units/mg solid	μ g protein/ml test	Units/mg protein	% Activity *($\pm$ 3.7%)	% Specific activity *($\pm$ 4.4%)
Lys:HP-β-CD	337.9 $\pm$ 0.72	8448 $\pm$ 18	8.8 $\pm$ 0.3	38429 $\pm$ 1310	19.3	97.7
Lys:trehalose	352.9 $\pm$ 11.6	8823 $\pm$ 290	8.63 $\pm$ 0.23	40874 $\pm$ 282	20.2	103.9
Lys:raffinose	376.7 $\pm$ 25.3	9417 $\pm$ 631	10.3 $\pm$ 1.25	36763 $\pm$ 2624	21.5	93.5
Control	350.0 $\pm$ 18.5	43750 $\pm$ 2312	8.9 $\pm$ 0.4	39323 $\pm$ 855	100	100

5.3.4.3 SDS-PAGE

SDS-PAGE analysis (under reducing and non-reducing conditions) showed no increase in covalent aggregation for the composite materials or freeze-dried lysozyme compared to the starting material with similar appearance of the stained gel and no strong higher molecular weight bands visible on the gel sheets (Figure 5.47).

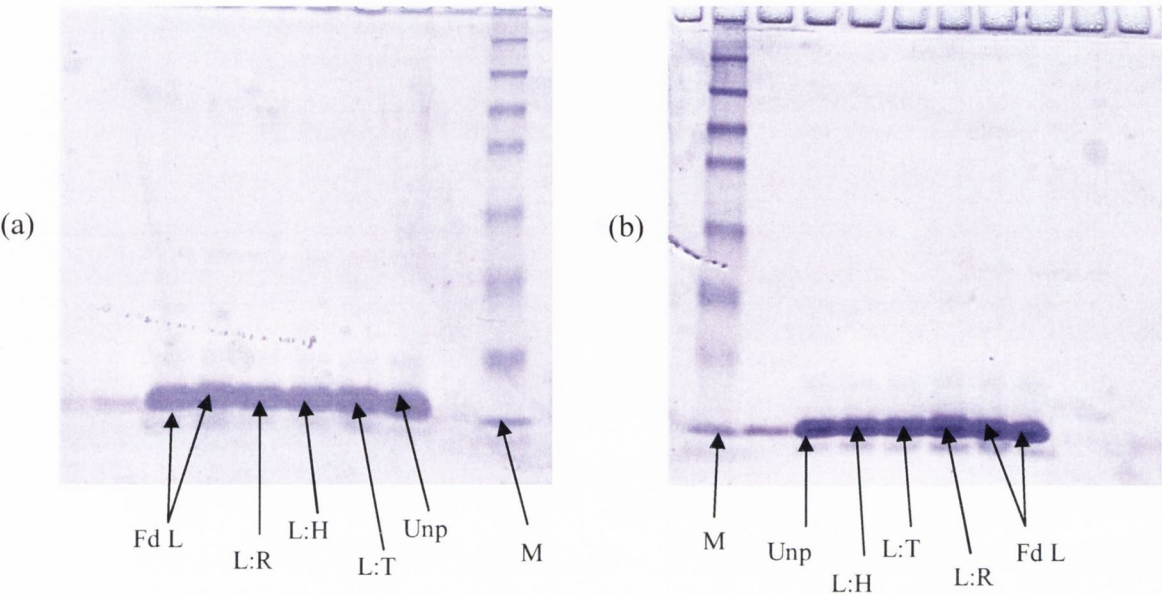


Figure 5.47 (a) Reducing and (b) non-reducing SDS-PAGE of freeze-dried lysozyme (Fd L), lysozyme:raffinose (L:R), lysozyme:HP- β -CD (L:H), lysozyme:trehalose (L:T) composites all spray-dried at a mass ratio of 1:4 from MeOH:BA systems, unprocessed lysozyme (Unp), and standard marker (M).

5.3.5 STORAGE STABILITY

In order to assess the physical stability of the lysozyme:excipient composites (spray-dried mass ratio 1:4) from non-aqueous systems a stability study was carried out. The high efficiency cyclone was used to spray-dry all samples. From previous work with spray-dried raffinose, the conditions 25 °C/60% R.H. (Section 4.2.5), were found to be unsuitable for stability studies, as particles absorbed moisture and dissolved into a sticky substance with complete particle collapse (viewed under SEM) after 24 hours. A small amount of the composite materials was stored under these conditions, for 24 hours whereupon SEM and DSC analysis were performed. The conditions 25°C/desiccant; 4°C/desiccant were selected for a 12-week stability study.

Storage under desiccant conditions at 4 °C and at 25 °C

Particle morphology of composite particles was not affected by storage for 12 weeks under desiccant conditions at 4 °C and at 25 °C, as can be seen by comparison of SE micrographs taken at week 12 (Figure 5.48), with those of the freshly spray-dried material (Figures 5.34, 5.35, 5.36 (d), (e)). All composites remained amorphous during the 12-week stability study at these conditions (Appendix 2). DSC and TGA (Appendix 2) showed no changes, indicating no crystallisation or increased moisture uptake for any the composites stored under desiccant conditions at 4 °C, or lysozyme:HP- β -CD stored under desiccant conditions at 25 °C for 12 weeks. Composites of lysozyme:trehalose and lysozyme:raffinose stored under desiccant conditions at 25 °C for 12 weeks displayed some small changes in the DSC with small endotherms (~ 70 °C) superimposed on the initial broad endotherms in later weeks. Moisture content, calculated from TGA, remained at ~4-5% during 12 weeks of the study (Figure 5.49).

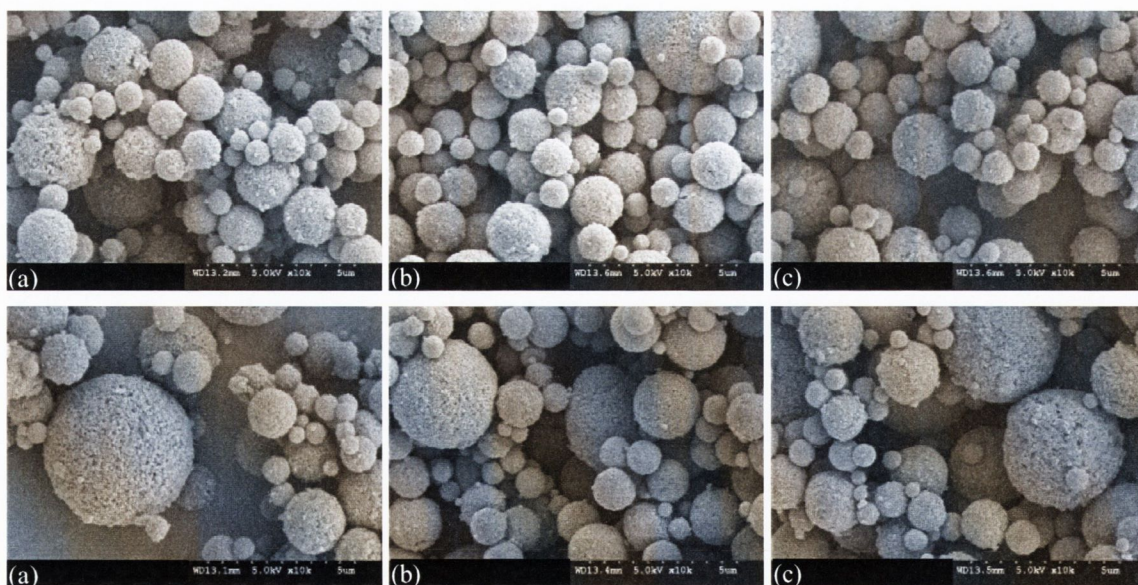


Figure 5.48 SE micrographs of composite particles stored at 4 °C/desiccant (top), 25 °C/desiccant (bottom) after 12 weeks (a) lysozyme:trehalose (1:4) (b) lysozyme:raffinose (1:4) (c) lysozyme:HP- β -CD (1:4).

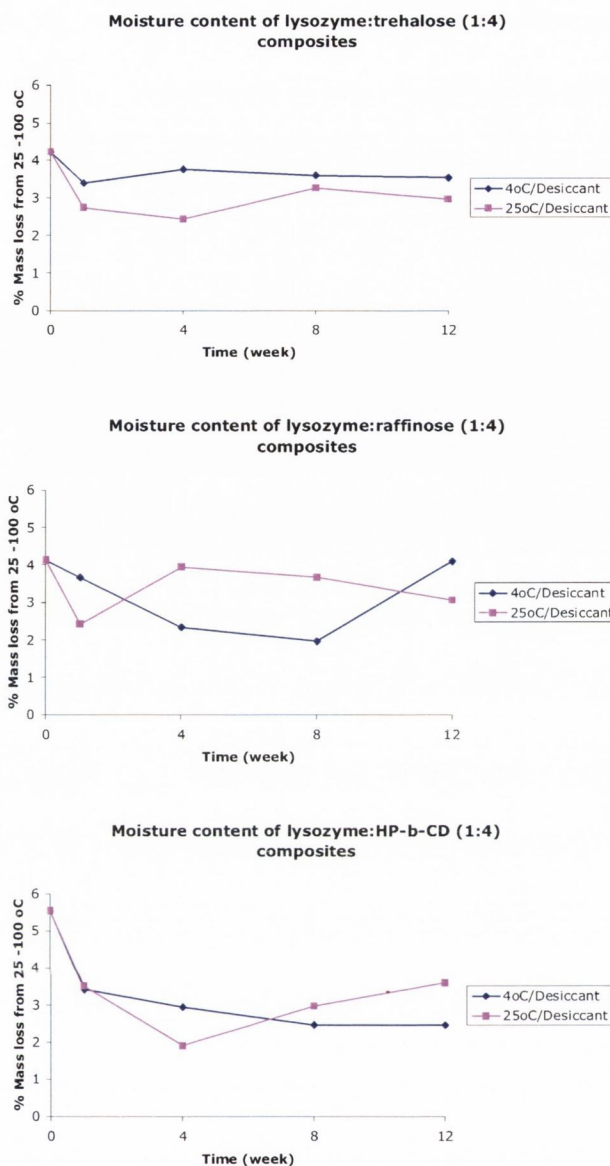


Figure 5.49 Moisture content (calculated from mass loss 25-100 °C from TGA, n=1) for spray-dried lysozyme:excipient composites (spray-dried mass ratio 1:4), during 12 weeks storage at 4 °C/desiccant and 25 °C/desiccant.

Biological activity assay showed good retention of specific activity on storage at 4 °C/desiccant for 12 weeks, with no statistically significant change compared to freshly spray-dried material. Storage at 25 °C for 12 weeks showed that activity was well retained for trehalose and raffinose composites but less well retained for the HP- β -CD composite, although again this was not statistically significant due to a large pooled standard deviation.

Table 5.10 Summary of biological activity assay results for spray-dried composites of lysozyme:excipient, starting and after 12 weeks storage at 4 °C/desiccant and 25 °C/desiccant (* pooled standard deviation, n=2 for each batch).

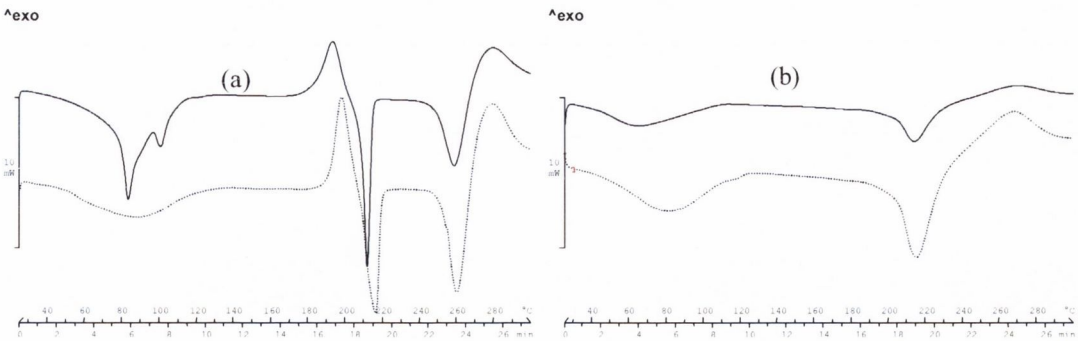
	Units/ml test	Units/mg solid	µg protein/ml test	Units/mg protein	% Activity	% Specific activity
Starting						
Lys:trehalose	287.5 ± 47.4	7188 ± 1193	6.25 ± 0.495	45653 ± 3799	16.5 ± 5.8*	105.4 ± 5.2*
Lys:raffinose	361.5 ± 67.2	9032 ± 1679	8.05 ± 1.768	44906 ± 1292	20.7 ± 5.8*	103.7 ± 5.2*
Lys:HP-β-CD	445 ± 65.1	11125 ± 1635	10.00 ± 1.414	44482 ± 406	25.5 ± 5.8*	102.7 ± 5.2*
Control	349.5 ± 34.7	43672 ± 4309	8.05 ± 0.354	43304 ± 2063	100 ± 5.8*	100 ± 5.2*
4 °C/des. W12						
Lys:trehalose	257 ± 5.7	6422 ± 154	6.55 ± 0.071	39112 ± 281	17.1 ± 1.9*	98.2 ± 7.1*
Lys:raffinose	319.5 ± 5.0	7985 ± 111	8.1 ±0.000	39471 ± 779	21.3 ± 1.9*	99.1 ± 7.1*
Lys:HP-β-CD	313 ± 14.1	7813 ± 309	8.6 ± 0.000	36445 ± 1515	20.8 ± 1.9*	91.5 ± 7.1*
Control	300 ± 14.1	37500 ± 1768	7.5 ± 0.283	39825 ± 453	100 ± 1.9*	100 ± 7.1*
25 °C/des. W12						
Lys:trehalose	251 ± 14.1	6281 ± 154	8.0 ± 1.414	36846 ± 3086	16.7 ± 1.9*	92.5 ± 7.1*
Lys:raffinose	302.5 ± 10.6	7563 ± 265	8.35 ± 0.495	36160 ± 924	20.2 ± 1.9*	90.8 ± 7.1*
Lys:HP-β-CD	307.5 ± 12.0	7688 ± 309	9.6 ± 1.556	32437 ± 6565	20.5 ± 1.9*	81.4 ± 7.1*
Control	300 ± 14.1	37500 ± 1768	7.5 ± 0.283	39825 ± 453	100 ± 1.9*	100 ± 7.1*

Storage at 25 °C / 60% R.H.

Storage at 25 °C / 60% R.H. for 24 hours for lysozyme:raffinose (1:4) and lysozyme:trehalose (1:4) composites resulted in particle collapse when viewed under SEM (Figure 5.50 (a), (b)). This was not observed with composite particles of lysozyme:HP- β -CD (1:4), which retained their porous morphology after 24 hours (Figure 5.50 (c)) as evident from SEM analysis. DSC analysis suggested presence of a hydrate for the trehalose composite stored under these conditions for 24 hours, with a sharp endothermic peaks at 80 and 100 °C (Figure 5.51 (a)). A recrystallisation exotherm occurred, as in the freshly prepared material, immediately before the endothermic melting peak of anhydrous trehalose at $\sim$ 213 °C. This peak suggests the presence of some amorphous sugar and agrees with an observation by Suihko et al. (2005), that crystallisation and phase separation occurred on exposure of high trehalose content (lysozyme:trehalose) composite to high humidity (75% R.H.) but part of the sugar in the phase-separated formulations remained amorphous and in close association with lysozyme. Composite particles of protein:raffinose and protein:HP- β -CD (Figure 5.51 (b), (c)) did not exhibit changes in DSC scans compared to freshly prepared material.



Figure 5.50 SE micrographs of composite particles taken after 24 hour storage at 25 °C/60% R.H. of (a) lysozyme:trehalose (1:4) (b) lysozyme:raffinose (1:4) (c) lysozyme:HP- β -CD (1:4).



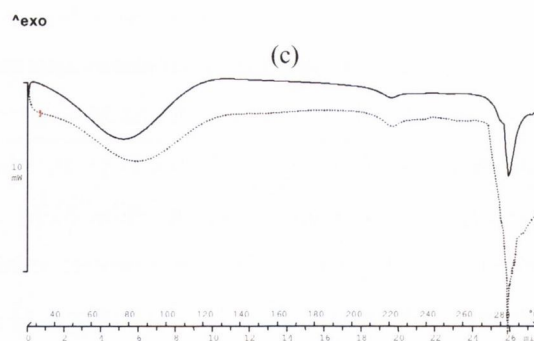


Figure 5.51 DSC scans of composite particles (a) lysozyme:trehalose (1:4) (b) lysozyme:raffinose (1:4) (c) lysozyme:HP- β -CD (1:4) taken after 24 hour storage at 25 °C/60% R.H.; broken line shows freshly spray-dried material.

5.3.6 PARTICLE CHARACTERISATION

Bulk and tapped density measurements showed that the composite NPMPs had low densities but these were higher than the excipient NPMPs spray-dried alone (values shown italicised in red for comparison). The highest density values were calculated for the HP-β-CD system, this was true both for the composite system and for the excipient spray-dried alone. Calculated Carr’s index, indicate that all were very/extremely poor flowing powders. Specific surface area values were high although they were lower than for the excipients spray-dried alone for the lysozyme:HP-β-CD and lysozyme:raffinose composite. Similar surface area results were measured for the lysozyme:trehalose composite as for trehalose NPMPs spray-dried alone. The highest surface area values were measured for the lysozyme:HP-β-CD composite system, which were two-fold higher than the lowest, namely the lysozyme:raffinose composite system.

Table 5.11 Summary of density and specific surface area measurements for lysozyme:excipient composites of mass ratio 1:4 [¹*n=3; average ± standard deviation ²*n=2; average (range) ³*calculated from average bp and tp]. Values shown, italicized in red represent the results for the NPMPs of excipient spray-dried alone (see also Section 4.2.3).

	¹ *Bulk Density (g/cm ³)	¹ *Tapped Density (g/cm ³)	³ *Carr’s Index	² *Surface Area (m ² /g)
Lys:HP-β-CD	0.2374 ± 0.0124	0.3560 ± 0.0035	33.3	54.5729 (54.2213, 54.9245)
[HP- β-CD]	[0.1928 ± 0.0064]	[0.2892 ± 0.0031]	33.3	[75.2253 (74.8092, 75.6413)]
Lys:Treh	0.1428 ± 0.0081	0.2447 ± 0.0018	41.6	43.7426 (45.9236, 41.5615)
[Trehalose]	[0.075 ± 0.001]	[0.124 ± 0.001]	39.5	[44.4854 (44.1342, 44.8366)]
Lys:Raff	0.1575 ± 0.0101	0.2779 ± 0.0064	43.3	25.4586 (25.3166, 25.6006)
[Raffinose]	[0.08 ± 0.0025]	[0.122 ± 0.004]	34.4	[44.3023 (43.1865, 45.4180)]

Aerodynamic assessment of fine particle showed that the composite NPMPs had good *in-vitro* deposition properties (Table 5.12, Figure 5.52). Deposition within the impactor was mainly in the mid and lower stages (stage 3 and 4). High deposition was also observed in the mouthpiece adaptor/induction port. The lysozyme:raffinose composite had a lower fine particle fraction than the other composites (but this was not statistically significant) and a statistically significantly lower particle fraction < 3 μm. This may have been influenced by the spray-drying conditions and in particular the choice of cyclone used for the studied batch (regular cyclone vs HP cyclone for the others), which may have affected the particle size of the particles collected. GSD values, indicative of the spread of the aerodynamic diameter, were similar for all three NPMP composites. Estimation of particle size from SE micrographs was carried out for the three composites by measuring 150 particles (Table 5.13). The results of these estimates support a possible particle size effect on the lower particle fraction < 3 μm calculated for the lysozyme:raffinose composite. The

lowest average % emission was observed for the lysozyme:trehalose composite. This was statistically significant (at level $\alpha = 0.05$).

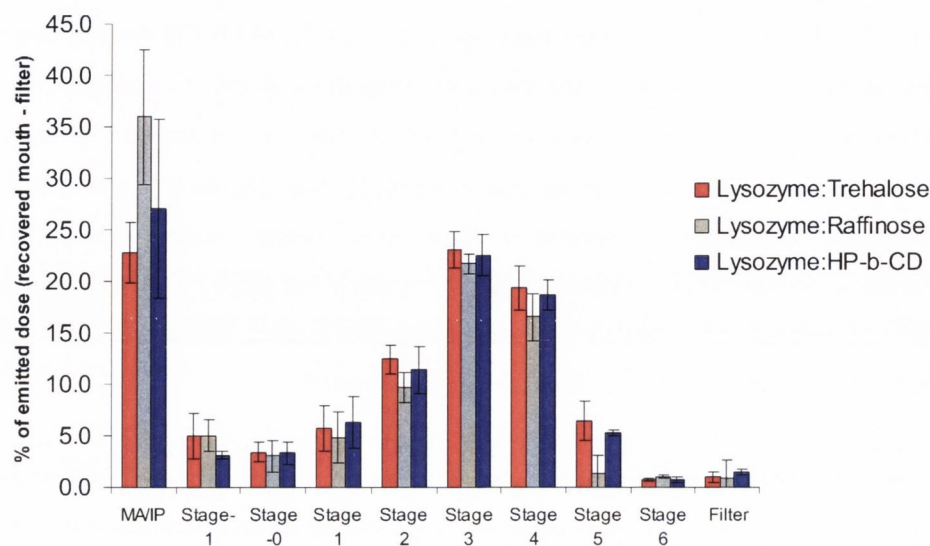


Figure 5.52 Deposition patterns on the stages of the ACI apparatus. Calculated as % of recovered emitted dose (mouth – filter), used to calculate fine particle fraction.

Table 5.12 Summary of results from Andersen cascade impactor analysis; mean ± stdev; n = 3.

Composite (mass ratio 1:4)	% < 5 µm	% < 3 µm	MMAD (µm)	GSD	% emitted (of nominal dose)
Lys:HP-β-CD	59.81 ± 2.16	35.98 ± 1.42	2.47 ± 0.42	2.01 ± 0.28	87.3 ± 2.0
Lys:Treh	62.34 ± 3.91	37.20 ± 3.51	2.77 ± 0.35	2.03 ± 0.06	79.2 ± 0.8
Lys:Raff	50.05 ± 5.21	27.47 ± 2.82	2.92 ± 0.15	2.04 ± 0.02	84.2 ± 0.5

Table 5.13 Particle size estimates from SEM analysis – measurement of 150 particles.

Composite (mass ratio 1:4)	Particle size (µm)
Lys:HP-β-CD	1.02 ± 0.58
Lys:Treh	0.98 ± 0.64
Lys:Raff	1.58 ± 0.63

5.4 CONCLUSIONS

Composite NPMPs of lysozyme:sugar excipient were produced from the two solvent feed systems at varying mass ratios. Lysozyme:trehalose composites spray-dried using the EtOH:H₂O:AC solvent system (conditions 2), were hollow and had a spherical porous or highly folded appearance for mass ratios 9:1 – 1:1, similar to lysozyme spray-dried alone from these conditions. A higher trehalose content composite (mass ratio 1:4) produced mainly collapsed particles. Lysozyme was also spray-dried with raffinose and with inulin from a mass ratio of 1:1. Particles had a highly folded surface morphology. Lysozyme:myo-inositol were spray-dried as NPMP composites, however powders were crystalline from XRD analysis. Inclusion of PVP with lysozyme and myo-inositol resulted in amorphous material although the conditions used did not result in NPMPs. This solvent system may be suitable for spray-drying lysozyme:sugar composites in lower sugar content composites. Lysozyme:trehalose, lysozyme:raffinose and lysozyme:HP- β -CD spray-dried using the MeOH:BA solvent system in mass ratios of 1:4, resulted in porous particles with a fused nanoparticulate appearance similar to the excipient spray-dried alone. Lysozyme:raffinose composites spray-dried from a mass ratio of 3:7 were porous but from a mass ratio of 1:1 had a fused surface morphology. This solvent system may be more suitable for high sugar/excipient content composites. These data suggest different usefulness depending on the choice of excipient and on whether a high or a low excipient content in the composite was required. The morphology of the particles appeared to be influenced by the majority component of the composite, and was similar to the morphology of this component spray-dried alone using the same spray-drying conditions.

All lysozyme composites studied showed good retention of biological activity, although as observed in Chapter 3, biological activity assay of lysozyme spray-dried alone also showed good retention of activity. Bögelein (2007) commented that lysozyme was unsuitable for studying the effects of stabilising agents due to only marginal inactivation assessed by biological activity assay, during spray-drying and subsequent storage (4 °C/25 °C, ~0% R.H.). Application to other less robust proteins or peptides may further test the stabilising effect of the excipients with regard to spray-drying using the described methods.

Sample composites chosen for particle characterisation – lysozyme:trehalose mass ratio 1:1 (from EtOH:H₂O:AC) and lysozyme:excipient (trehalose, raffinose or HP- β -CD) mass ratio 1:4 (from MeOH:BA) had favourable particle characteristics for pulmonary drug delivery with a high fine particle fraction and particle fraction < 3 μ m. Storage stability of sugar containing composites were dramatically affected by relative humidity conditions however.

6.1 INTRODUCTION

Processing methods for proteins and peptides are generally developed on an individual case basis, depending on their suitability to the process and the need for stabilising or other excipient(s). The methods hitherto described in this thesis are based on experiments with lysozyme, excipients and lysozyme:excipient composites, and involve spray-drying using either an alcohol:H₂O:ammonium salt (namely EtOH:H₂O:AC) feed system or a MeOH:BA:(H₂O) solvent feed system. The applicability of these methods to other proteins or peptide and composites of these with excipients is the focus of the current chapter. Each protein or peptide is discussed separately under the headings of the solvent system used.

In the first method, involving spray-drying from EtOH:H₂O:AC feed system, protein precipitation (observed as a phase separation) is postulated to be important in the process, with NPMPs of lysozyme being prepared following phase separation in the feed material and spray-drying (Section 3.3). Factors hypothesised to be important in the case of lysozyme, include the protein's isoelectric point (11.35) and the pH of the solvent system (~ 9), concentrations of EtOH (organic solvent) and the salt ions contributed to by AC (salting-out effect). Two other proteins are considered with this processing method in the present work. Trypsin has a similar size (23.5 kDa) and isoelectric point (10.3-10.5) to lysozyme and similar reported activity following spray-drying (Forbes et al., 2007). Catalase is a much larger protein (~250 kDa) with an isoelectric point of 5.4 and lower reported activity following spray-drying (Liao et al., 2005). The use of stabilising sugars – trehalose or raffinose to protect the proteins against process stresses is also investigated.

The second method, involving spray-drying from a MeOH:BA solvent feed system, has been used in formulation of lysozyme with excipient, to produce composite NPMPs (Section 5.3). It is postulated (from experiments with lysozyme) to be suitable for low protein:high excipient composites, with the morphology of the composite particles similar to the excipient spray-dried alone. A requirement of this system may be that the protein or peptide should be soluble in the MeOH component, as for lysozyme composites. The use of a small amount of water in the feed system to aid dissolution is also considered in this chapter as an alternative MeOH:BA:H₂O. Composites of trypsin:excipient (trehalose, raffinose or HP-β-CD) are investigated from MeOH:BA:(H₂O) systems. The MeOH:BA:H₂O system is developed for different mass ratios of trypsin:excipient, and process and temperature-dependant storage stability studies and preliminary particle characterisation are undertaken for spray-dried trypsin:excipient (1:4 mass ratio)

composites. Preliminary studies on the use of these methods with catalase and with the therapeutic peptide salmon calcitonin (sCT) are introduced.

Literature data concerning spray-drying trypsin, catalase and salmon calcitonin give an indication of their lability to spray-drying stresses (from aqueous or aqueous buffer solution), but without the additional stresses of the proposed methods (i.e., organic solvent and salt-induced precipitation).

Trypsin has been spray-dried from aqueous solution at an inlet temperature 127 ± 4 °C resulting in activity $\sim 89.7 \pm 4.1\%$ of original unprocessed trypsin (Forbes et al., 2007). This is similar to reported activity of lysozyme after spray-drying (Elkordy et al., 2002; Liao et al., 2002, 2005). Trypsin was also spray-dried with various carbohydrates at a low protein load (0.2:99.8, 1:99, 5:95) from aqueous solution (pH 7) at inlet temperature 180 °C (Millqvist-Fureby et al., 1999). Residual activity was in all cases $\geq 82\%$ of original unprocessed trypsin, which was significantly better than the residual activity for the similar ratio of freeze-dried trypsin:carbohydrate.

Catalase appears to be more susceptible to degradation during the spray-drying process. Liao et al. (2005) reported a residual activity of $54.4 \pm 4.1\%$ following spray-drying (from 5 mM phosphate buffer, pH 7 at inlet temperature 95 °C). Bögelein (2007) reported higher residual activities for spray-dried catalase samples from aqueous solution, and a dependancy of biological activity on the inlet temperature, for example residual activities (compared to original unprocessed catalase) of 88.8% (inlet temperature 137 °C), 57% (inlet temperature 190 °C) and 32.8% (inlet temperature 220 °C).

Information regarding stability of salmon calcitonin on spray drying includes a study by Chan et al. (2004) showing no aggregation on spray-drying either alone or in the presence of mannitol from aqueous solution (pH 7), with inlet and outlet temperatures of 125 °C and 70 °C, respectively.

6.2 SPRAY-DRIED TRYPSIN AND TRYPSIN COMPOSITES

6.2.1 ETHANOL:WATER:AMMONIUM CARBONATE FEED SYSTEMS

6.2.1.1 Spray-dried trypsin alone

Trypsin was initially spray-dried using the following conditions - 80% EtOH, 10% of t.s.c. AC, 0.5% t.s.c., inlet temperature 78 °C. These conditions produced NPMPs of lysozyme (Section 3.3.2). Phase separation occurred on addition of EtOH to the feed material with an increase in turbidity of the feed material, which was subsequently spray-dried with intermittent stirring to avoid settling of the suspension. Spray-drying was carried out in the open mode using the regular cyclone arrangement (see Appendix 1 for conditions). Spray-dried particles were non-porous and consisted of collapsed spheres, as was evident from SEM analysis (Figure 6.1 (a)). Collectable yield was low at ~12% (25 ml spray-dried). Figure 6.1 (b) shows unprocessed trypsin for comparison.

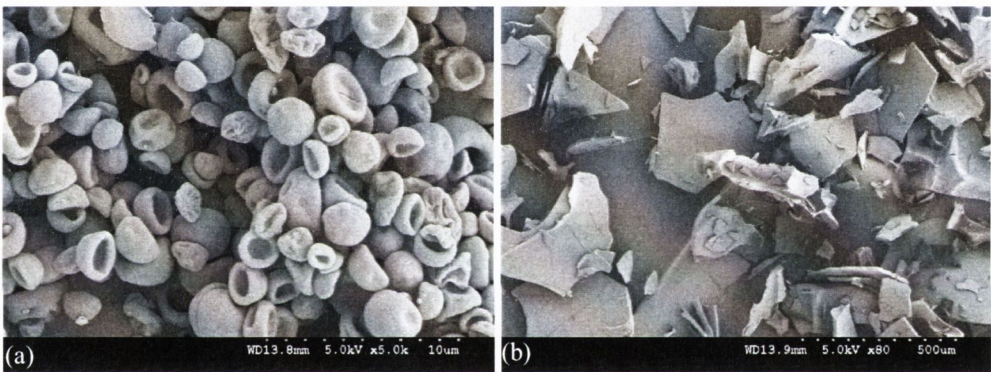


Figure 6.1 SE micrographs of (a) trypsin spray-dried from 80% EtOH, 10% of t.s.c. AC, 0.5% t.s.c., 78 °C (b) unprocessed trypsin. Note different magnifications.

From studies involving spray-drying lysozyme (Section 3.3), an association between alcohol and AC concentrations in the feed material, phase separation and spray-dried particle morphology was observed. EtOH and AC content were thus increased to 90% EtOH, 20% of t.s.c. AC; 98% EtOH, 40% of t.s.c. AC; and 95% EtOH, 40% of t.s.c. AC; 50% of t.s.c. AC; and 60% of t.s.c. AC. No significant effect was expected from changing inlet drying temperature (on the basis of experiments with spray-drying lysozyme), and as such this was maintained at 78 °C. T.s.c. was maintained at 0.5% for consistency with previous experiments, and spray-drying was carried out in the open mode using the regular cyclone arrangement (see Appendix 1 for conditions). Samples are further referred to by the% EtOH/% of t.s.c. AC used in the feed material. All feed systems showed phase separation on addition of EtOH to the trypsin and AC solution with an increase in turbidity and deposition of material on the walls of the feed vessel, and were spray-dried as suspensions with intermittent stirring. Some porous particles amongst predominantly non-porous particles were observed in SE micrographs of trypsin spray-dried from 90% EtOH, 20% of t.s.c. AC (Figure 6.2

(a)). Large ($\geq 10\ \mu\text{m}$) and irregular-shaped porous aggregates were observed in SE micrographs of trypsin spray-dried from 98% EtOH and 40% AC (Figure 6.2 (b)). Trypsin spray-dried from 95% EtOH, 40% of t.s.c. AC content resulted in a mixture of non-porous collapsed spherical particles and some porous spherical particles as was evident from SEM analysis (Figure 6.2 (c)). A mixture of large irregular-shaped and spherical porous particles were observed from SEM analysis of trypsin spray-dried from 95% EtOH, 50% and 60% of t.s.c. AC (Figure 6.2 (d), (e)). The higher concentrations of EtOH and AC required to produce porous particles, suggested that this method was less efficient for producing NPMPs of trypsin compared to lysozyme. The porous particles produced were less uniform in shape and size compared to lysozyme NPMPs. Collectable yields (all volumes spray-dried 50 ml) were low (Table 6.1), particularly for trypsin spray-dried from the higher EtOH and AC contents. The increased turbidity indicating phase separation and deposition of material onto the feed vessel walls prior to spray-drying of these systems may have contributed to the lower yields.

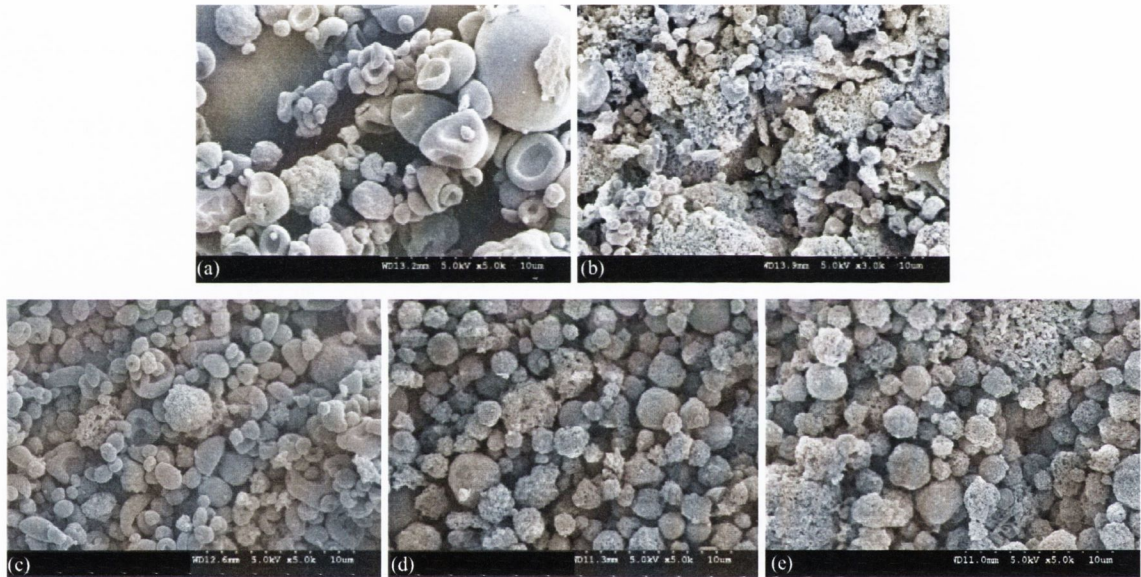


Figure 6.2 SE micrographs of trypsin spray-dried from% EtOH/% AC systems (a) 90%/20% (b) 98%/40% (c) 95%/40% (d) 95%/50% (e) 95%/60%, all t.s.c. 0.5%.

Table 6.1 Collectable yield for trypsin spray-dried from% EtOH/% of t.s.c. AC as shown.

% EtOH/% of t.s.c. AC	% Collectable yield
(a) 90% / 20%	24.9 (50 ml)
(b) 98% / 40%	18.4 (50 ml)
(c) 95% / 40%	22.9 (50 ml)
(d) 95% / 50%	13.6 (50 ml)
(e) 95% / 60%	6.0 (50 ml)

6.2.1.1.1 Process stability

Thermal characterisation

Broad endotherms were observed in DSC scans of spray-dried trypsin (25 – 120~150 °C) and unprocessed trypsin (25 - ~170 °C). A decrease in T_m (apparent denaturation temperature) was noted in the DSC of spray-dried trypsin samples (~ 214 – 218 °C) compared to unprocessed trypsin samples (~227 °C) (Figure 6.3), suggesting a decreased thermal stability. Residual moisture or solvent losses were calculated from TGA, and were between 2.8 - 6.6% for spray-dried trypsin samples, and 4.3% for unprocessed trypsin (Figure 6.4). No trend in increasing mass loss (over the temperature range 25 – 100 °C) with increasing AC in feed material (10 – 50% of t.s.c.), and no significant further mass loss steps before decomposition above 200 °C, were observed from TGA, suggesting no significant residual contamination by AC (volatilisation temperature 59 °C).

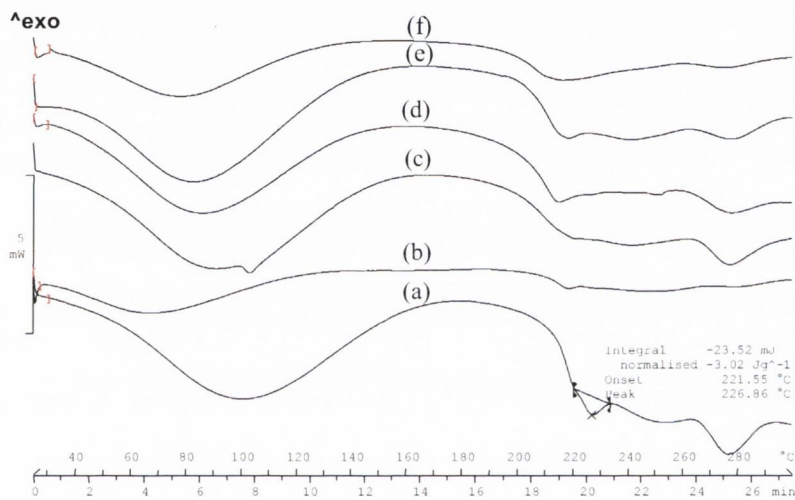


Figure 6.3 DSC scans of unprocessed trypsin (a), and spray-dried trypsin from% EtOH/% AC conditions (b) 80%/10%, (c) 90%/20%, (d) 98%/40%, (e) 95%/40%, (f) 95%/50%.

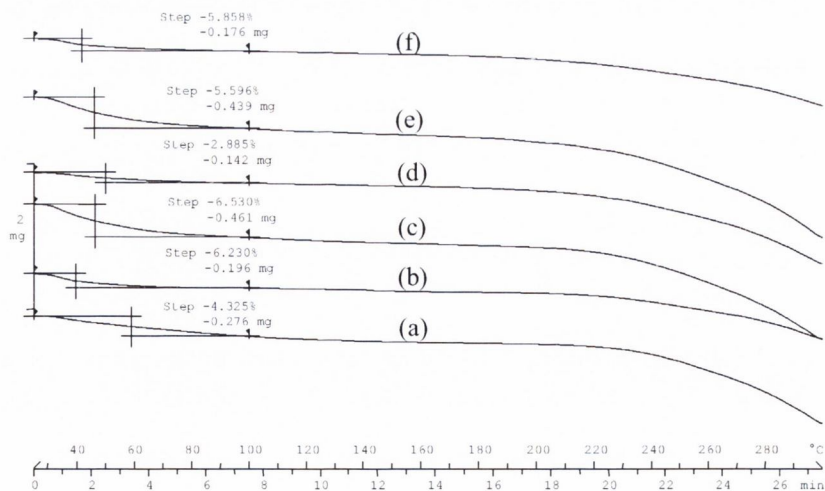


Figure 6.4 TGA scans of (a) unprocessed trypsin, and spray-dried trypsin from% EtOH/% AC conditions (b) 80%/10%, (c) 90%/20%, (d) 98%/40%, (e) 95%/40%, (f) 95%/50%.

Secondary structure and biological activity assay

Solid state FTIR studies suggested perturbations in the secondary structure of spray-dried trypsin compared to unprocessed material, with % area of overlap values in the normalised amide 1 region ranging from 78.6 – 81.9% (calculation method 1), or 81.7 – 84.7% (calculation method 2). % area of overlap values were similar for trypsin spray-dried from all conditions, considering the inter-batch variation - with an area of overlap of two batches of unprocessed material of 97% (calculation method 1), or 97.5% (calculation method 2). % area of overlap values, were lower than values for NPMPs of lysozyme vs unprocessed material (82.7% calculation method 1; and 91.3% calculation method 2).

Table 6.2 % area overlap of area normalised amide 1 peaks of spray-dried trypsin and unprocessed trypsin either using whole spectrum baseline-correction (Calculation method 1) or isolated peak baseline correction (Calculation method 2). Inter-batch value represents the calculated % area of overlap between two different batches of unprocessed trypsin.

% EtOH/% AC	% area overlap amide 1 peak (whole spectrum baseline-corrected) Calculation method 1	% area overlap amide 1 peak (peak baseline-corrected) Calculation method 2
80% / 10%	78.6	84.0
90% / 20%	79.1	83.3
98% / 40%	78.9	81.7
95% / 40%	80.3	84.7
95% / 50%	81.9	83.4
95% / 60%	80.2	83.5
Inter-batch	97.0	97.5

Biological activity assays of spray-dried samples were carried out and compared to unprocessed trypsin (all test or control solutions prepared to concentration ~ 0.044 mg/ml). Activity of spray-dried powders (units/mg solid) and specific activity (units/mg protein) were calculated. Results are expressed in terms of units and % of activity of control solution (Table 6.3). Samples were compared using ANOVA and statistical significance was assessed at a significance level of $\alpha = 0.05$. Trypsin spray-dried from 80% EtOH, 10% of t.s.c. AC; 90% EtOH, 20% of t.s.c. AC and 95% EtOH, 40% of t.s.c. AC, had statistically significantly lower activities calculated from units/mg solid than the control solution. A statistically significant difference was also found in the amount of protein in the test solution (μg protein/ml test) for these samples compared to control, calculated from micro BCA analysis, which may have been due to preparation, or difference in the amount of soluble protein in the test solutions. Residual amounts of the process enhancer (AC) were not indicated from TGA analysis. Specific biological activity was calculated, correcting for protein content in the test solutions, and although a similar reduction in activity was seen for all spray-dried samples this was statistically significant only for trypsin spray-dried from 90% EtOH,

20% of t.s.c. AC. Assay of trypsin spray-dried from 98% EtOH, 40% of t.s.c. AC gave similar results with a statistically significant decrease in biological activity and a decrease in specific activity compared to control solution, which was not statistically significant because of a large pooled standard deviation. Assay of trypsin spray-dried from 95% EtOH, 50% of t.s.c. AC showed the greatest decrease in biological activity and specific biological activity compared to control, which were statistically significant. Biological activity and specific biological activity for this sample were approximately halved compared to unprocessed trypsin. Biological activity assay was not carried out on trypsin spray-dried from 95% EtOH, 60% of t.s.c. AC because of scarcity of material and the poor activity measured for trypsin spray-dried from 95% EtOH, 50% of t.s.c. AC.

Table 6.3 Summary of biological activity assay for trypsin spray-dried from % EtOH/% of t.s.c. AC as shown; n=2; results expressed as average (range); *pooled stdev % (in the case of % activity and % specific activity) is calculated from the pooled standard deviation of all samples run for the daily assay as a % of units of control solution.

	Units/ml test	Units/mg solid	µg protein/ml test	Units/mg protein	% Activity	% Specific activity
80%/10%	363 (366, 359)	8239 (8324, 8153)	38.0 (37.8, 38.2)	9540 (9391, 9689)	79.1 ± 3.4*	98.7 ± 3.2*
90%/ 20%	324 (314, 334)	7358 (7585, 7130)	39.0 (39.1, 38.9)	8302 (8580, 8024)	70.7 ± 3.4*	85.9 ± 3.2*
95%/ 40%	346 (328, 364)	7855 (7443, 8267)	39.8 (38.7, 40.9)	8679 (8894, 8463)	75.4 ± 3.4*	89.8 ± 3.2*
Control	458 (465, 451)	10412 (10568, 10255)	47.5 (49.2, 45.7)	9663 (9874, 9451)	100 ± 3.4*	100 ± 3.2*
98%/40%	318 (328, 308)	7216 (7443, 6989)	43.4 (42.9, 43.9)	7320 (7634, 7005)	70.6 ± 2.4*	77.2 ± 5.7*
Control	450 (445, 454)	10223 (10133, 10313)	47.5 (49.2, 45.7)	9487 (9045, 9929)	100 ± 2.4*	100 ± 5.7*
95%/ 50%	188 (195, 180)	4262 (4091, 4432)	38.4 (40.5, 36.3)	4887 (4851, 4959)	43.5 ± 1.7*	53.7 ± 7.7*
Control	431 (428, 435)	9801 (8689, 9886)	47.5 (49.2, 45.7)	9104 (8689, 9519)	100 ± 1.7*	100 ± 7.7*

Reducing SDS-PAGE was carried out on spray-dried and unprocessed trypsin. A strong band was observed at ~25 kDa compared to the marker standard (expected molecular weight of trypsin 23.5 kDa) – (Figure 6.5 marked by red ring). Low molecular weight bands were also present – (Figure 6.5 marked by black ring). These were also present in the SDS-PAGE patterns of purified trypsin presented in papers by Peng Khng et al. (1998), and Winters et al. (1999). As no differences were noted between the unprocessed and the spray-dried samples and no higher molecular weight bands were detected, no covalent aggregation could be detected.

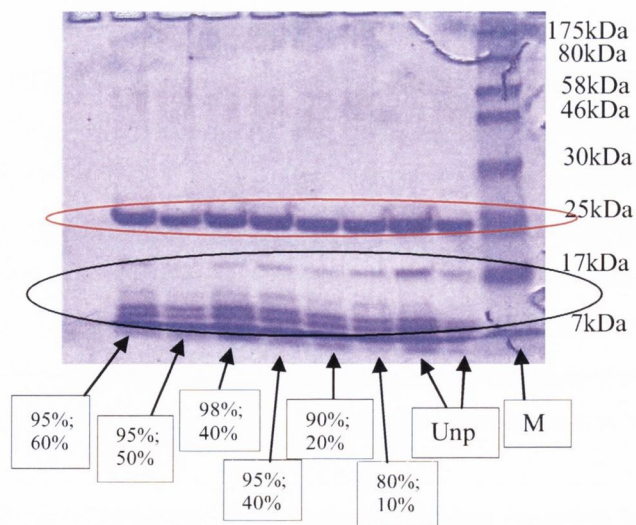


Figure 6.5 Reducing SDS-PAGE, for trypsin spray-dried from % EtOH, % of t.s.c. AC as shown, Unp = unprocessed trypsin, M = standard marker.

6.2.1.2 Spray-dried trypsin composites

Trehalose was investigated as a possible stabiliser of trypsin. Two feed conditions were chosen for preliminary investigation. These were 98% EtOH, 40% of t.s.c. AC and 95% EtOH, 50% of t.s.c. AC. These conditions were used to produce non-spherical porous aggregates for trypsin spray-dried alone (Figure 6.2 (b)) with some loss of activity (Table 6.3), and porous particles of trypsin alone (Figure 6.2 (d)), with significant loss of trypsin activity (Table 6.3).

Trypsin:trehalose were initially spray-dried in a mass ratio of 1:1 from 98% EtOH, 40% of t.s.c. AC, t.s.c. 0.5%, and inlet temperature 78 °C (hereafter referred to as “comp1”). See Appendix 1 for conditions. The spray-dried composite material had a mixed morphology as was evident from SEM analysis (Figure 6.6), including spherical non-porous particles, smaller aggregated particles or porous aggregates and a large fused mass (all marked with red arrows). Fusion was noted between the particles, suggesting non-optimal drying conditions. The powder had a slightly sticky consistency on collection. Collectable yield was similar to trypsin spray-dried alone at 17.2% (50 ml spray-dried).



Figure 6.6 SE micrograph of trypsin:trehalose spray-dried in a mass ratio of 1:1 from 98% EtOH, 40% of t.s.c. AC, inlet temperature 78 °C – comp1.

Trypsin:trehalose were spray-dried from a mass ratio of 4:1 from 95% EtOH, 50% of t.s.c. AC (0.5% t.s.c.), and inlet temperature 78 °C (hereafter referred to as “comp2”). The inlet temperature of 78 °C was chosen to minimise thermal stresses because of the lability of trypsin spray-dried alone. Particle morphology was mainly collapsed non-porous spheres with a small minority of porous particles as observed from SEM analysis (Figure 6.7 (a)). No evidence of fusion was noted in this system from SEM analysis. Trypsin:trehalose were also spray-dried in a mass ratio of 9:1 at inlet temperature 85 °C (“comp3”). Morphology of particles (Figure 6.7 (b)) was very similar to the comp2 with a minority of porous particles as was evident from SEM analysis. Collectable yields (Table 6.4) were slightly higher than trypsin spray-dried alone from 95% EtOH, 50% of t.s.c. AC (Table 6.1)

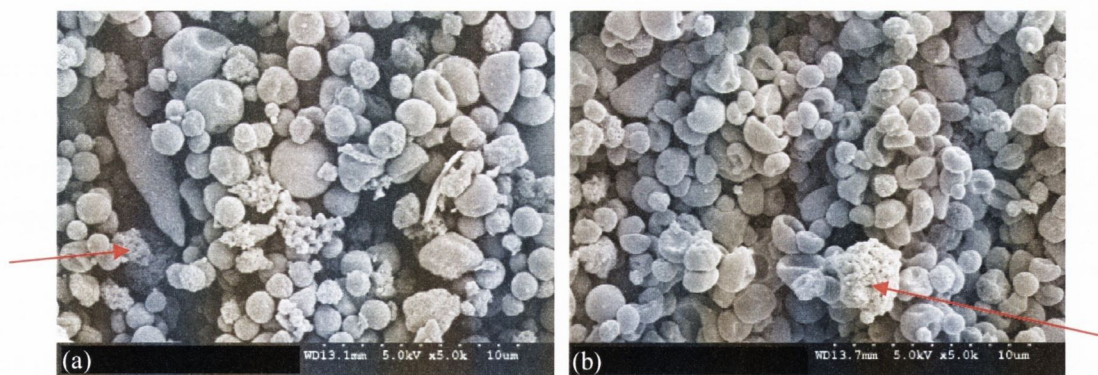


Figure 6.7 SE micrographs of trypsin:trehalose spray-dried from 95% EtOH, 50% of t.s.c. AC (0.5% t.s.c.), in mass ratios (a) 4:1; inlet temperature 78 °C – comp2 (b) 9:1; inlet temperature 85 °C – comp3.

Addition of trehalose to trypsin was thus found to have a marked effect on the morphology of the spray-dried particles even at low trehalose contents. This effect was not observed with lysozyme:trehalose composites spray-dried from EtOH:H₂O:AC systems, with retention of porous or folded morphology up to a lysozyme:trehalose mass ratio of 1:1 (Section 5.2). The EtOH:H₂O:AC system for spray-drying NPMPs of trypsin appears to be less robust, with regard to particle morphology.

Comp3 (9:1 trypsin:trehalose mass ratio composite, spray-dried from 95% EtOH, 50% of t.s.c. AC at 85 °C) was repeated at a higher t.s.c. of 1% w/v in the feed solution (protein content in the feed solution 0.9% w/v) (hereafter referred to as “comp4”), to investigate if this had an effect on the morphology of the spray-dried composite. Spray-dried particles were a mixed population of porous spherical or irregular-shaped and non-porous particles as observed from SEM analysis (Figure 6.8 (a)). Trypsin was similarly spray-dried with raffinose instead of trehalose (“comp5”). SEM analysis showed a similar mix of particles with some porous spherical and irregular-shaped and some non-porous particles (Figure 6.8 (b)). Collectable yields were higher than for comp2, or comp3 (Table 6.4). This trend in increasing collectable yield with t.s.c. was previously seen for spray-drying lysozyme (Section 3.3.2).

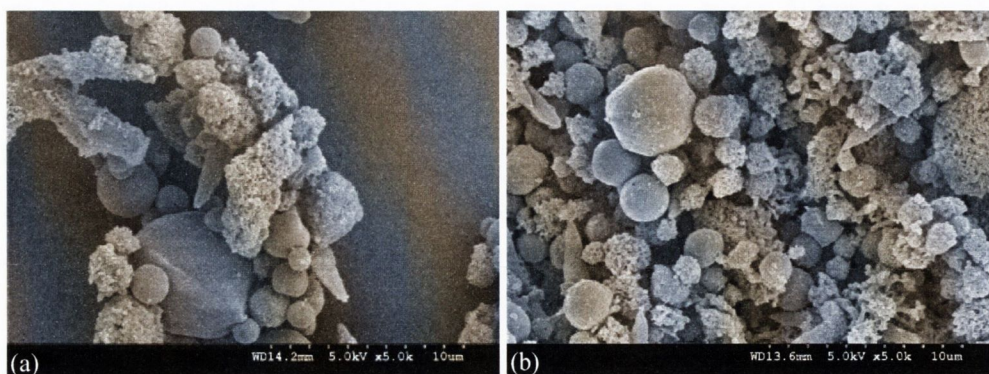


Figure 6.8 SE micrographs of (a) trypsin:trehalose – comp4 (b) trypsin:raffinose – comp5 spray-dried in mass ratios 9:1 from 95% EtOH, 50% of t.s.c. AC, inlet temperature 85 °C (1% t.s.c.).

Table 6.4 Collectable yield for trypsin composites as shown (volume spray-dried ml).

Composite system	% Collectable yield
Comp2	17.3% (50 ml)
Comp3	22.8% (50 ml)
Comp4	43.7% (50 ml)
Comp5	29.6% (50 ml)

6.2.1.2.1 Process stability

Thermal characterisation

Broad endotherms from 25 - ~100 or 140 °C were identified from DSC scans of all trypsin:sugar composites (Figures 6.9, 6.11). Distinct endothermic peaks attributed to the T_m of trypsin were difficult to determine. Where they were determined (marked by a red box) these were lower than for unprocessed trypsin and, similarly to spray-dried trypsin alone, suggested a reduced thermal stability compared to the unprocessed trypsin. No glass transitions, distinct sugar recrystallisation exotherms or melting endotherm corresponding to anhydrous trehalose or raffinose were identified. This was similarly found for lysozyme:sugar composites at spray-dried mass ratios up to 1:1 (Section 5.2.1.1.1). Mass losses of ~ 5.3% - 7.9% were calculated over the temperature range 25 - 100 °C from TGA scans (Figures 6.10, 6.11). Further mass loss steps (marked by red boxes) were also observed from 100 - ~140 °C for comp1 (determined mass loss 2.8%) and comp3 (determined mass loss 3.3%). These were not observed for trypsin spray-dried alone, but were observed for lysozyme:trehalose composites (Section 5.2.1.1.1) and for trehalose spray-dried alone from EtOH:H₂O:AC systems (Section 4.2.1.4.3).

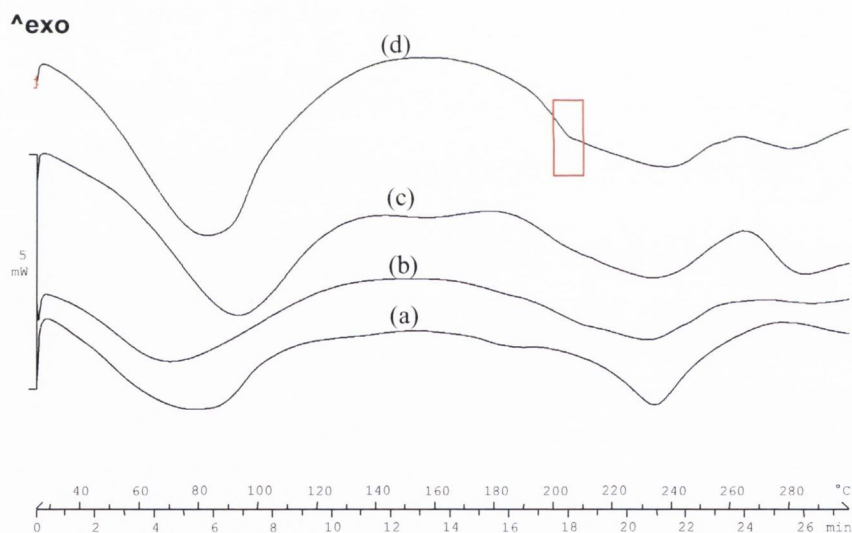


Figure 6.9 DSC scans of trypsin:trehalose spray-dried from mass ratio, conditions: (a) 1:1, 98% EtOH, 40% AC, 78 °C, t.s.c. 0.5% - comp1 (b) 8:2, 95% EtOH, 50% AC, 78 °C, t.s.c. 0.5% - comp2 (c) 9:1, 95% EtOH, 50% AC, 85 °C, t.s.c. 0.5% - comp3 (d) 9:1, 95% EtOH, 50% AC, 85 °C, t.s.c. 1% - comp4.

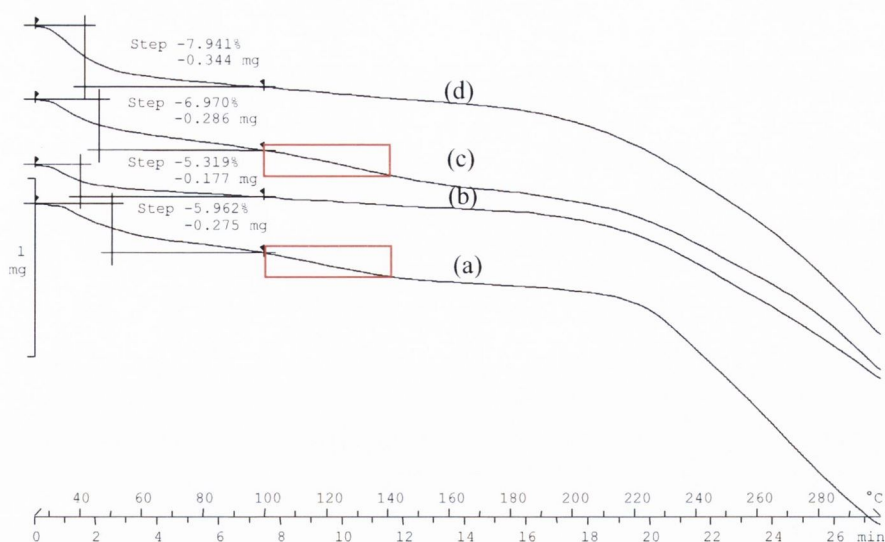


Figure 6.10 TGA scans of trypsin:trehalose spray-dried from mass ratio, conditions: (a) 1:1, 98% EtOH, 40% AC, 78 °C, t.s.c. 0.5% - comp1 (b) 8:2, 95% EtOH, 50% AC, 78 °C, t.s.c. 0.5% - comp2 (c) 9:1, 95% EtOH, 50% AC, 85 °C, t.s.c. 0.5% - comp3 (d) 9:1, 95% EtOH, 50% AC, 85 °C, t.s.c. 1% - comp4.

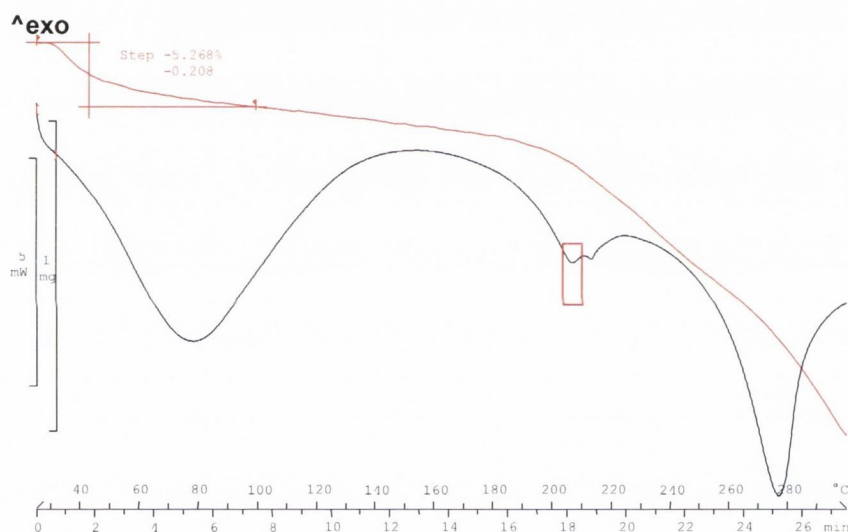


Figure 6.11 DSC (in black) and TGA (in red) scans of trypsin:raffinose spray-dried from mass ratio 9:1 from 95% EtOH, 50% AC, inlet temperature 85 °C, t.s.c. 1% - comp5.

Secondary structure and biological activity analysis

No increase in % area of overlap values in the normalised amide 1 region with unprocessed trypsin were found for trypsin:trehalose or trypsin:raffinose composite (Table 6.5) compared to trypsin spray-dried alone (Table 6.2), suggesting no improvement in secondary structure preservation. This was similarly noted for lysozyme composites spray-dried from EtOH:H₂O:AC systems. The lowest% area of overlap value was calculated for comp1 (trypsin:trehalose mass ratio 1:1, spray-dried from 98% EtOH, 40% of t.s.c. AC). This was similar to the trend seen for trypsin spray-dried alone from different% EtOH/% AC, with the lowest% area of overlap value with unprocessed trypsin calculated for trypsin spray-dried alone from the conditions 98%/ 40%.

Table 6.5% area overlap of area normalised amide 1 peaks of spray-dried trypsin and unprocessed trypsin using isolated peak baseline correction (Calculation method 2).

Composite	% area overlap amide 1 peak (peak baseline-corrected) Calculation method 2
Comp1	80.2
Comp2	82.4
Comp3	82.6
Comp5	83.2

Biological activity assays of spray-dried trypsin:excipient composites were carried out and compared to unprocessed trypsin (all test solutions were prepared to contain a protein concentration of ~ 0.044 mg/ml, based on the mass ratio added to the initial feed system prior to spray-drying). Activity of spray-dried powders (units/mg solid) and specific activity (units/mg protein) were calculated. Results are expressed in terms of units and% of activity of control solution (Table 6.6). Statistical significance was assessed at a significance level of $\alpha = 0.05$, and samples were compared to control solution, using ANOVA, as for trypsin spray-dried alone. The amounts of protein in the test solutions (μg protein/ml test), calculated using micro BCA protein analysis, were lower than expected from the trypsin:excipient mass ratios in the composites (based on the mass ratio in the initial feed prior to spray-drying) for all composites except comp4. A deviation in the ratio of trypsin:excipient in the spray-dried composites compared to that expected from the mass ratio in the initial feed, may be due to loss of protein material as a result of phase separation and deposition of material on the walls of the feed vessel. Lower than expected amounts of protein in test solutions were also seen from micro BCA analysis of test solutions of trypsin spray-dried alone from EtOH:H₂O:AC. % activity of spray-dried test solutions (calculated from the Units/mg solid compared to control solution) were lower than the spray-dried ratio as a result of the lower amount of protein in test solution and also as a result of loss of activity as seen from the results for specific activity (corrected for protein content). The highest % specific activity was calculated for comp1, with almost complete retention of activity compared to unprocessed control solution. This represents an improvement in specific biological activity compared to trypsin spray-dried alone from these conditions (98% EtOH, 40% of t.s.c. AC) (Table 6.3). Considering the composite systems spray-dried from 95% EtOH, 50% of t.s.c. AC – comp2, 3, 4, 5, a statistically significant decrease in % specific activity compared to control was not calculated for comp2, although a similar average decrease was calculated for comp5 (which was statistically significant decrease due to a smaller pooled standard deviation). Larger (statistically significant) decreases in specific activity were calculated for comp3 and comp4 compared to control. The % specific activity for all composites was better than for trypsin spray-dried alone from these conditions (Table 6.3). Comp2, comp3 and comp4 were spray-dried composites of trypsin:trehalose in mass ratios 8:2, 9:1 (0.5% t.s.c.), 9:1 (1% t.s.c.), respectively. Comparison between the % specific activity of these composites suggests a sugar concentration effect on the retention of activity. Comp5 was a spray-

dried composite of trypsin:raffinose in a mass ratio of 9:1 similar to comp4 (trypsin:trehalose). Comparison between comp4 and comp5 thus may indicate a greater stabilising effect by weight of raffinose compared to trehalose for spray-dried trypsin from this system.

Table 6.6 Summary of biological activity assay for trypsin:excipient composites (see Section 6.2.1.2 for descriptions; n=2, results expressed as average (range); n=3, results expressed as average ± standard deviation; *pooled stdev % (in the case of % activity and % specific activity) is calculated from the pooled standard deviation of all samples run for the daily assay as a % of units of control solution.

	Units/ml test	Units/mg solid	µg protein/ml test	Units/mg protein	% Activity	% Specific activity
Comp1	295 (301, 289)	3352 (3423, 3281)	33.8 (31.6, 35.9)	8788 (9533, 8043)	34.2 ± 1.7*	96.5 ± 7.7*
Control	431 (428, 435)	9801 (8689, 9886)	47.5 (49.2, 45.7)	9104 (8689, 9519)	100 ± 1.7*	100 ± 7.7*
Comp2	304 (299, 309)	6904 (6790, 7017)	38.3 (37.7, 38.8)	7941 (7924, 7957)	65.6 ± 1.7*	81.5 ± 5.0*
Control	464 (458, 470)	10540 (10398, 10682)	47.5 (49.2, 45.7)	9742 (9299, 10284)	100 ± 1.7*	100 ± 5.0*
Comp3	284 (285, 283)	5815 (5840, 5789)	40.4 (40.7, 40.1)	7024 (7002, 7045)	58.3 ± 2.0*	70.1 ± 3.2*
Control	439 ± 10.9	9972 ± 248	43.8 ± 0.3	10026 ± 322	100 ± 2.0*	100 ± 3.2*
Comp4	251 ± 3.8	5140 ± 74	56.7 ± 5.1	5026 ± 173	59.0 ± 4.1*	74.2 ± 3.7*
Control	383 ± 26.9	8703 ± 607	49.9 ± 2.3	6770 ± 387	100 ± 4.1*	100 ± 3.7*
Comp5	326 ± 23.3	6902 ± 198	39.4 ± 1.5	8273 ± 390	69.2 ± 2.0*	82.5 ± 3.2*
Control	439 ± 10.9	9972 ± 248	43.8 ± 0.3	10026 ± 322	100 ± 2.0*	100 ± 3.2*

Reducing SDS-PAGE of spray-dried trypsin composites showed a similar pattern to trypsin spray-dried alone and unprocessed trypsin with strong bands at ~25 kDa compared to the marker standard (expected molecular weight of 24 kDa) and presence of low molecular weight bands (Figure 6.12). Soluble covalent aggregation was thus not detected in the composite samples.

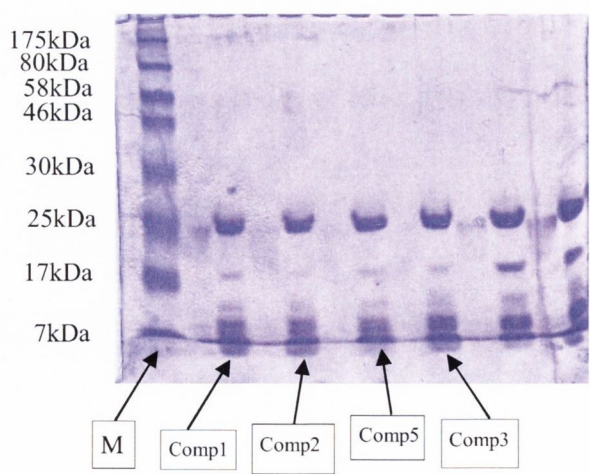


Figure 6.12 Reducing SDS-PAGE, for trypsin composites as shown, M = standard marker.

6.2.2 METHANOL:N-BUTYL ACETATE:(WATER) SYSTEMS

6.2.2.1 Spray-dried trypsin:raffinose from methanol:n-butyl acetate

No published data on freeze-drying trypsin at altered pH to increase methanolic solubility could be found. A similar method as was previously used for lysozyme (Section 3.2) and as also reported by Chin et al. (1994), Bromberg & Klibanov (1995)) was thus attempted, and trypsin was freeze-dried from aqueous solution at pH 3. This was a lower solution pH than was used for freeze-drying lysozyme (pH 6) and was chosen because of the lower pI of trypsin (10.1-10.5) compared to lysozyme (11.35). Freeze-drying resulted in a recovered yield of 93.9%.

Trypsin (initially freeze-dried from aqueous solution at pH3) was spray-dried with raffinose at a mass ratio of 1:4, from an 80:20 MeOH:BA solvent system, inlet temperature 100 °C, and t.s.c. of 0.5% w/v. Spray-drying was carried out in the closed mode using the HP cyclone. See Appendix 1 for details. These conditions were previously used to spray-dry composite NPMPs of lysozyme:raffinose (Section 5.3.3). Freeze-dried trypsin could not be dissolved with manual agitation or with stirring in the initial MeOH component (0.125% w/v). The feed mixture was spray-dried as a suspension. Resultant spray-dried material comprised of a mixed morphology of flakes and small fused spheres when viewed under SEM (marked by red arrows, Figure 6.13 (a)). A lower trypsin:raffinose mass ratio of 2:98 was attempted. Freeze-dried trypsin could not be dissolved in the MeOH component (0.0125% w/v) however. The morphology of the spray-dried material (Figure 6.13 (b)) was similar to the 1:4 mass ratio system, as was evident from SEM analysis. This approach did not appear promising for spray-drying trypsin:excipient composites even at a low protein load. Difficulty with the solubility of trypsin in the non-aqueous system was postulated to be a factor.

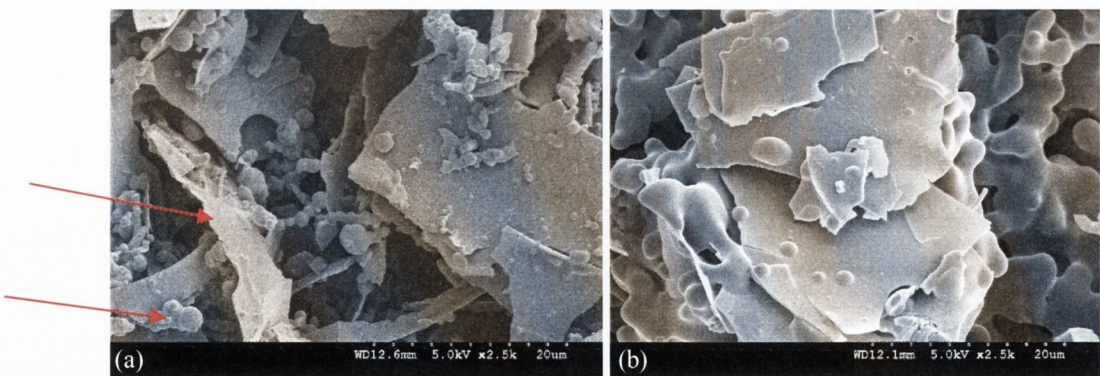


Figure 6.13 SE micrographs showing trypsin:raffinose spray-dried from 80:20 MeOH:BA solvent system in a mass ratio of (a) 1:4 (b) 2:98.

6.2.2.1.1 Process Stability

Preliminary process stability characterisation was carried out on trypsin:raffinose spray-dried from MeOH:BA, despite the unfavourable morphology from SEM analysis. The stability of trypsin spray-dried from the non-aqueous system was of interest because of the lack of published data on spray-drying proteins from organic solvents.

Thermal characterisation

DSC of the spray-dried trypsin:raffinose samples (Figure 6.14 (b), (c)) showed initial broad endotherms from 25 – 120/140 °C, obscuring the identification of a glass transition temperature. Decomposition endotherms starting at ~ 180 °C (mass ratio 1:4) and 200 °C (mass ratio 2:98) were observed in the DSC of spray-dried trypsin:raffinose. Corresponding mass loss steps beginning at these temperatures were observed in TGA scans (Figure 6.15). This decomposition occurred at lower temperatures for the composites, than for raffinose spray-dried alone (~ 220 °C) or freeze-dried trypsin (T_m identified at ~ 225 °C). Mass losses over the temperature range 25 - 100 °C determined from TGA data were similar for raffinose spray-dried alone and for the spray-dried trypsin:raffinose sample mass ratio 2:98 (5.4 - 5.6%), slightly lower for the mass ratio 1:4 (4.3%), and higher for the freeze-dried trypsin, (9%). No further pronounced mass loss steps were observed until decomposition (> 180 °C depending on sample).

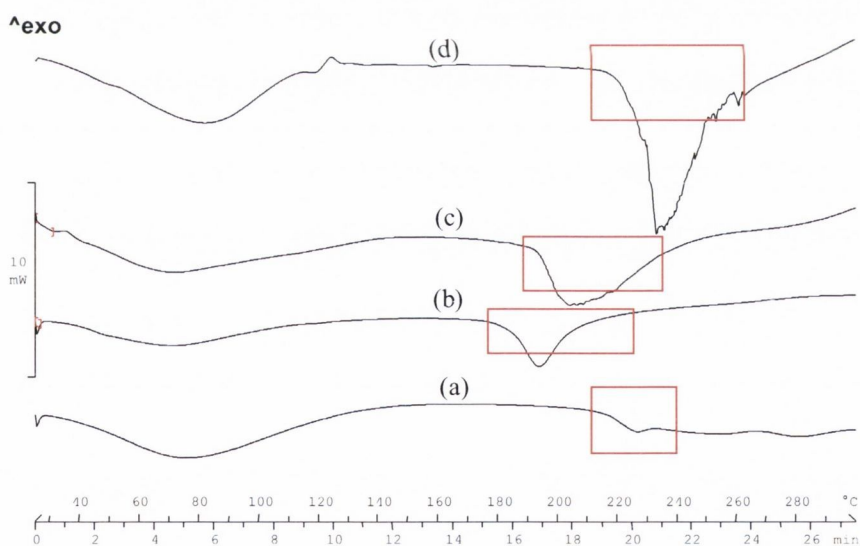


Figure 6.14 DSC of (a) freeze-dried trypsin, (b) spray-dried trypsin:raffinose mass ratio 1:4, (c) spray-dried trypsin:raffinose mass ratio 2:98, (d) spray-dried raffinose alone.

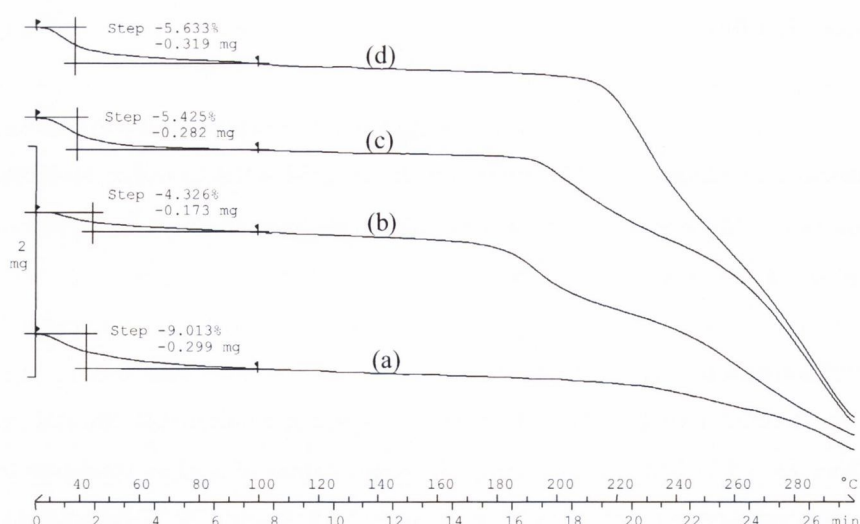


Figure 6.15 TGA (a) freeze-dried trypsin, (b) spray-dried trypsin:raffinose mass ratio 1:4, (c) spray-dried trypsin:raffinose mass ratio 2:98, (d) spray-dried raffinose alone.

Secondary structure and biological activity

The % area of overlap of the normalised amide 1 region of trypsin:raffinose (spray-dried in mass ratio 1:4) with unprocessed trypsin, was calculated using calculation method 2 as 85.2%.

Biological activity assay of freeze-dried trypsin, trypsin:raffinose spray-dried in a mass ratio of 1:4 ($n = 3$) and preliminary investigation of trypsin:raffinose spray-dried in a mass ratio of 2:98 ($n = 1$) were undertaken, and compared to control solution (unprocessed trypsin). Test solutions were prepared such as to contain a protein concentration of ~ 0.044 mg/ml, based on the mass ratio added to the initial feed system prior to spray-drying. Micro BCA protein quantitation of test solutions showed considerably (~ 2 -fold) higher than expected protein content (for the spray-dried mass ratio of 1:4 - 0.0813 ± 0.0029 mg/ml and for the spray-dried mass ratio of 2:98 - 0.0841 mg/ml). This is unlike the case with trypsin:excipient composites or trypsin spray-dried alone from EtOH:H₂O:AC systems in which a lower than expected trypsin content was calculated for the test solutions. This suggests that the protein and sugar were not present in the dried product in the same ratio as added to the formulation, maybe as a result of differing cyclone trapping efficiency of the different morphologies - large flake particles and spherical particles. These test solutions were diluted 1 in 2 prior to activity assay to ensure that the samples were within the measurable linear region of the reaction (~ 500 Units/ml). Comparison of % specific activity showed excellent retention of activity of freeze-dried trypsin and spray-dried trypsin from a mass ratio of 1:4 (trypsin:raffinose) in the initial feed material (Table 6.7). Similar retention of specific activity was seen from the preliminary assay of spray-dried trypsin from a mass ratio of 2:98 (trypsin:raffinose) in the initial feed material ($n = 1$), with a % specific activity of 102.0% of control solution.

Table 6.7 Summary of biological activity assay for trypsin:excipient composites (1:4 mass ratio = trypsin:raffinose spray-dried in a mass ratio of 1:4 from MeOH:BA, FD trypsin = freeze-dried trypsin); n=3; results expressed as average ± standard deviation **micro BCA result for the prepared sample ; *pooled stdev % (in the case of % activity and % specific activity) is calculated from the pooled standard deviation of all samples run for the daily assay as a % of units of control solution.

	Units/ml test	Units/mg solid	µg protein/ml test	Units/mg protein	% Activity (*± 4.2%)	% Specific activity (*± 5.5%)
1:4 mass ratio	401 ± 23.1	3664 ± 209	**81.3 ± 2.9	9856 ± 211	34.6	105.9
FD Trypsin	433 ± 12.8	9839 ± 292	46.0 ± 1.0	9443 ± 102	92.8	101.4
Control	467 ± 30.0	10603 ± 681	50.2 ± 2.3	9309 ± 857	100	100

6.2.2.2 Spray-dried trypsin:excipient from methanol:n-butyl acetate:water

A small amount of water was added to the MeOH:BA solvent system to enable protein dissolution in this component, prior to addition of the organic solvents. Solvent ratios for the MeOH:BA:H₂O system varied depending on whether trehalose, raffinose, or HP- β -CD were used as excipients and was based on previous experiments with spray-drying the excipients alone from a MeOH:BA:H₂O system.

6.2.2.2.1 Spray-dried trypsin:trehalose from methanol:n-butyl acetate:water

A solvent ratio of 49:49:2 (MeOH:BA:H₂O) and a total solid concentration of 0.3% w/v was selected for spray-drying trypsin:trehalose composites, based on results with spray-drying trehalose alone (Section 4.2.1.3). All systems were spray-dried using the HP cyclone in the closed mode (see Appendix 1 for details). Trypsin:trehalose were spray-dried at mass ratios of 1:9, 1:4, 1:1 and are hereafter referred to by this mass ratio. Trypsin and trehalose were first dissolved in the H₂O component, followed by addition of MeOH and subsequently BA, resulting in a precipitation in the feed for all systems. Spray-drying was carried out with stirring of the feed material to avoid settling of the suspension. Particle morphology for the 1:9 and 1:4 mass ratio composites as seen from SEM analysis was spherical nano-particulate/nanoporous particles (Figure 6.16 (a), (b)). Particle morphology for the mass ratio 1:1 composite was a mixture of spherical nano-particulate/nanoporous particles and non-spherical nano-particulate aggregates (Figure 6.16 (c)). There was no trend in the collectable yield with changing mass ratio of the composite, with collectable yields: 28.7% (100 ml spray-dried) for the 1:9 composite, 42.4% (100 ml spray-dried) for the 1:4 composite and 36.9% (100 ml spray-dried) for the 1:1 composite.

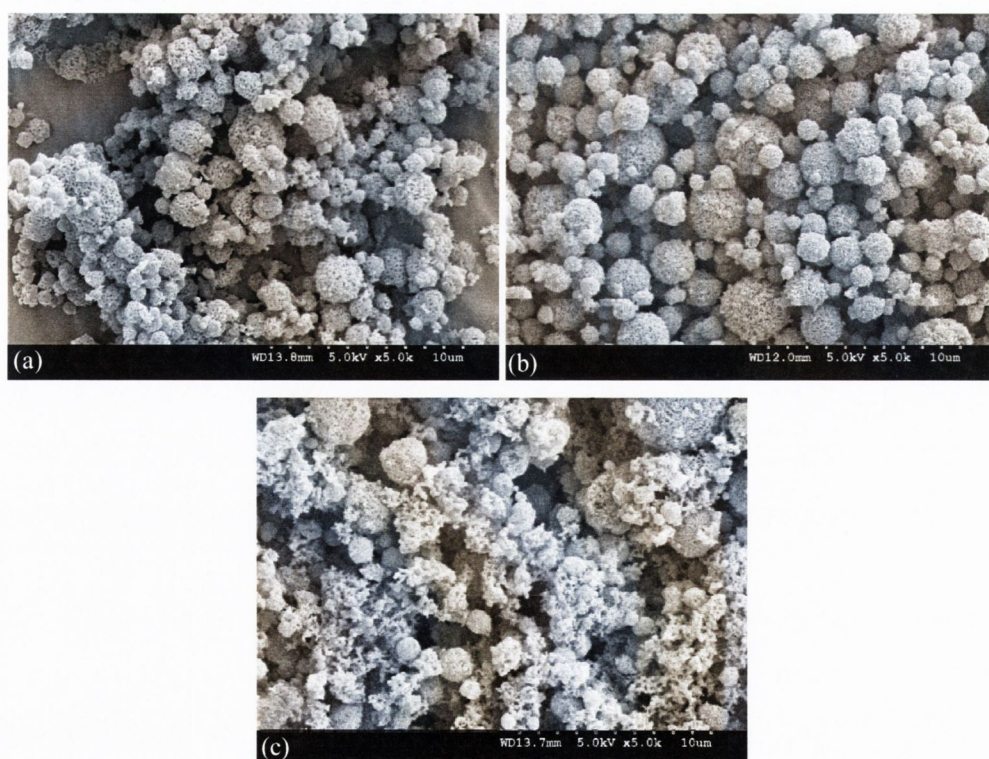


Figure 6.16 SE micrographs of trypsin:trehalose spray-dried in mass ratios (a) 1:9 (b) 1:4 (c) 1:1.

6.2.2.2.2 Spray-dried trypsin:raffinose from methanol:n-butyl acetate:water

A solvent ratio of 79:19:2 (MeOH:BA:H₂O) was selected for spray-drying trypsin:raffinose composites, based on results with spray-drying raffinose alone (Section 4.2.2.1). A slightly lower t.s.c. of 0.5% was chosen to enable dissolution of trypsin and raffinose in the small volume water component. All systems were spray-dried using the HP cyclone in the closed mode (see Appendix 1 for details). Trypsin:raffinose were spray-dried in mass ratios of 1:9, 1:4, 1:1 and are hereafter referred to by this mass ratio. Trypsin:raffinose were first dissolved in the H₂O component, followed by addition of MeOH and subsequently BA resulting in a precipitation in the feed for all systems, with a marked observable increase in turbidity of the feed. The 1:1 mass ratio exhibited the greatest precipitation, resulting in a coarse suspension. Spray-drying was carried out with stirring of the feed material to avoid settling of the suspension. Particle morphology for the composites of mass ratios 1:9 and 1:4 and 1:1 were similar, as seen from SEM analysis (Figure 6.17) being a mix of spherical to slightly irregular nano-particulate/nanoporous particles and non-spherical nano-particulate aggregates. Collectable yields were similar to the trypsin:trehalose composite for the 1:9 mass ratio, with a collectable yield of 31.3% (100 ml spray-dried), a lower yield of 24.9% (100 ml spray-dried) for the 1:4 composite and 20.7% (50 ml spray-dried) for the 1:1 composite, although the lower volume spray-dried is likely to have had an effect on this as previously described.

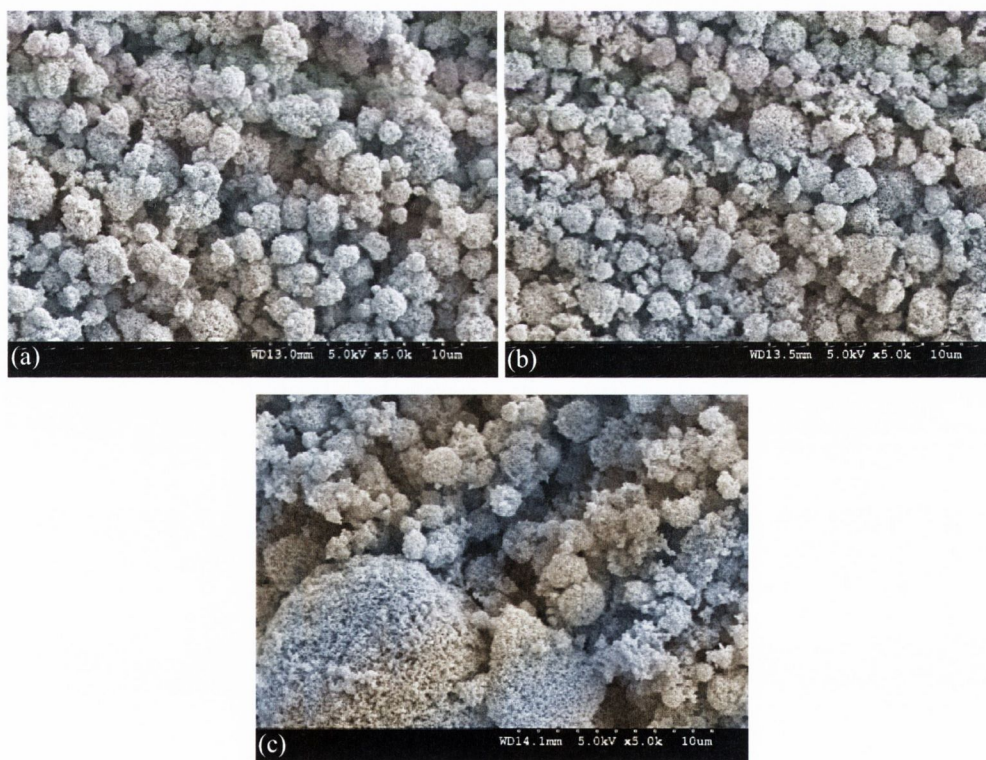


Figure 6.17 SE micrographs of trypsin:raffinose spray-dried in mass ratios (a) 1:9 (b) 1:4 (c) 1:1.

6.2.2.2.3 Spray-dried trypsin:HP-β-CD from methanol:n-butyl acetate:water

A solvent ratio of 15:15:1 (MeOH:BA:H₂O) was selected for spray-drying trypsin:HP-β-CD composites, as this was used to produce porous HP-β-CD particles by Li (2005). A total solid concentration of 1.61% w/v was selected for the 1:9 and 1:4 mass ratio samples (based on a total solid content of 500 mg in 31 ml solvent mix), which was slightly lower than that used by Li (2005) of 1.9% w/v. This total solid concentration was further decreased to 0.81% w/v for the 1:1 mass ratio to try to minimise precipitation and loss of protein material of the walls of the feed material as was considerable with the higher concentrations. All systems were spray-dried using the HP cyclone in the closed mode (see Appendix 1 for details). Trypsin and HP-β-CD were first dissolved in the H₂O component, addition of MeOH and subsequently BA resulting in a precipitation in the feed for all systems, resulting in a coarse suspension. Spray-drying was carried out with stirring of the feed material to avoid settling of the suspension. Particle morphology was similar for the three mass ratio composites as evident from SEM analysis (Figure 6.18). Spherical nano-particulate/nanoporous particles and non-spherical nano-particulate aggregates were observed. Some fusion/contact between smaller particles was observed. High collectable yields were obtained with these systems (~ 2-fold higher than for the trypsin:trehalose or trypsin:raffinose composites) of: 58.6% (31 ml spray-dried) for the 1:9 composite, 63.1% (31 ml spray-dried) for the 1:4 composite, and 43.6% (31 ml spray-dried) for the 1:1 composite. The higher t.s.c. used in these systems, despite the lower volume spray-dried is likely to have contributed to this increased yield.

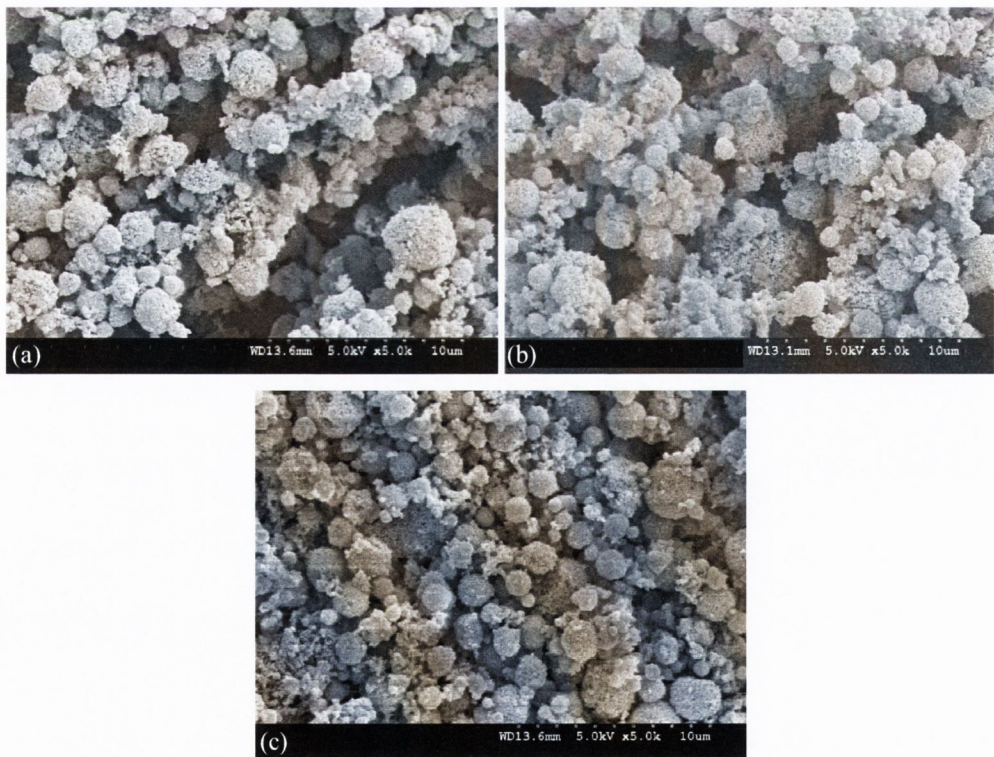


Figure 6.18 SE micrographs of trypsin:HP-β-CD spray-dried in mass ratios (a) 1:9 (b) 1:4 (c) 1:1.

6.2.2.2.4 PROCESS STABILITY

Solid-state characterisation and thermal analysis

Trypsin:trehalose composites

The spray-dried composite (mass ratio 1:4) was amorphous by XRD (Figure 6.19). DSC and TGA scans of composites of mass ratio 1:9, 1:4, 1:1 are presented (Figures 6.20, 6.21). Broad endothermic peaks were observed from 25 - ~120 °C in DSC scans. Glass transitions were identified for mass ratio composites 1:4, and 1:1 at ~125 °C. This area was obscured in the 1:9 mass ratio composite by the initial endothermic peak. Two exotherms were identified in the DSC of the sample of mass ratio 1:4 (similarly identified in a replicate batch used for stability study – see Section 6.2.2.2.5) and were also identified, though smaller, in the 1:9 mass ratio composite, before a sharp endothermic peak (~ 213 °C) attributed to melting of anhydrous trehalose. These exotherms may correspond to recrystallisation of the sugar. The presence of two exotherms is unusual, but may represent mixing irregularities within the composite resulting in two onsets of recrystallisation, or may be contributed to by decomposition of trypsin and may warrant further investigation. The issue of amorphous/amorphous phase separation of sugars and globular proteins and experimental limitations to its detection have been discussed by Hill et al. (2005). The authors argue that observations including those from lyophilisation of polymer:sugar systems may give some insight into these phenomena and that such studies suggest that there may be a greater tendency towards micro-phase separation of trehalose or raffinose:protein than sucrose:protein system, and that this may contribute to the apparent advantage of sucrose rather than trehalose in stabilising the native structure of lysozyme during spray-drying in a study by Liao et al. (2002). Exothermic recrystallisation peak(s) or a melting peak of anhydrous trehalose were not identified in the DSC scan of the composite of mass ratio 1:1. Decomposition was further noted in DSC scans as endothermic areas starting at ~ 225 – 240 °C. Initial mass losses over the temperature range 25-100 °C of 4.5 – 6.7% (repeat of a replicate batch mass ratio 1:4 used for stability study was 2.3% - see section 6.2.2.2.5) were calculated and further mass losses corresponding to decomposition starting at ~ 220 °C were observed from TGA.

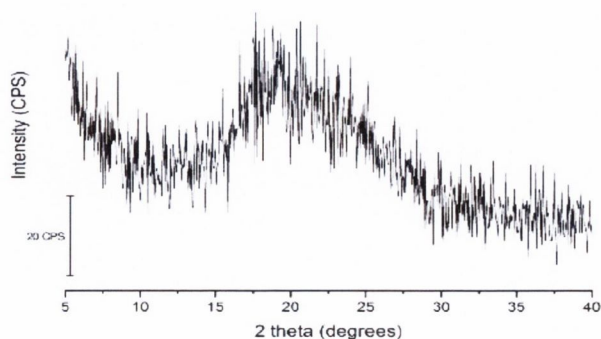


Figure 6.19 XRD scan of trypsin:trehalose mass ratio 1:4 (taken using Philips XRD).

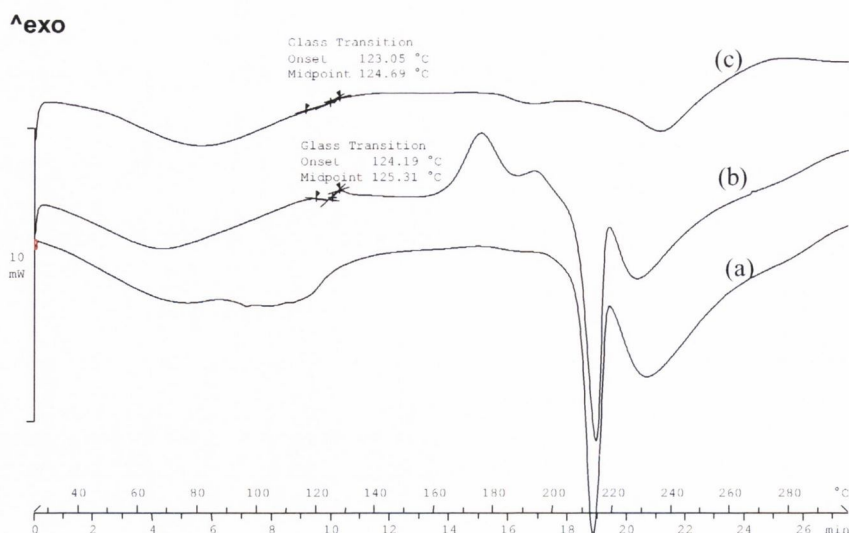


Figure 6.20 DSC scans of trypsin:trehalose spray-dried in mass ratios of (a) 1:9 (b) 1:4 (c) 1:1.

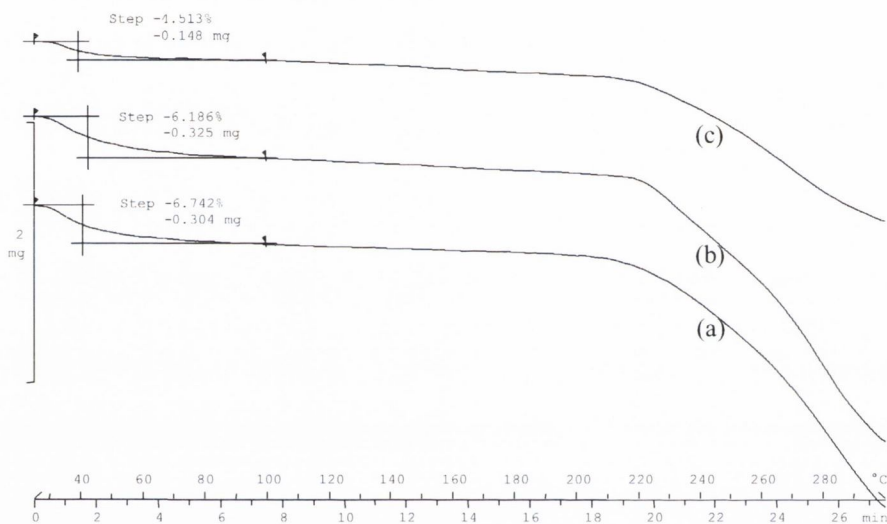


Figure 6.21 TGA scans of trypsin:trehalose spray-dried in mass ratios of (a) 1:9 (b) 1:4 (c) 1:1.

Trypsin:raffinose composites

The spray-dried composite (mass ratio 1:4) was amorphous by XRD (Figure 6.22). DSC and TGA scans of composites of mass ratio 1:9, 1:4, 1:1 are presented (Figures 6.23, 6.24). Glass transitions were detected in the DSC scans of mass ratio composites 1:9 and 1:4 at ~ 120 °C. No recrystallisation peaks were identified, similar to raffinose spray-dried alone (Section 4.2.2.3). An endothermic peak was identified in all samples at ~ 190 °C, attributed to decomposition, which is slightly lower than for raffinose spray-dried alone (~ 220 °C Section 4.2.2.3). Initial mass losses over the temperature range 25 - 100 °C of $\sim 4.2 - 4.6\%$ (repeat of a replicate batch mass ratio 1:4 used for stability study was 3.9% - see section 6.2.2.2.5) were calculated. The shape of these mass loss steps were similar to spray-dried raffinose alone (amorphous material), rather than the later step seen for raffinose pentahydrate (Section 4.2.2.3). Further mass losses corresponding to decomposition starting at $\sim > 180$ °C were observed from TGA.

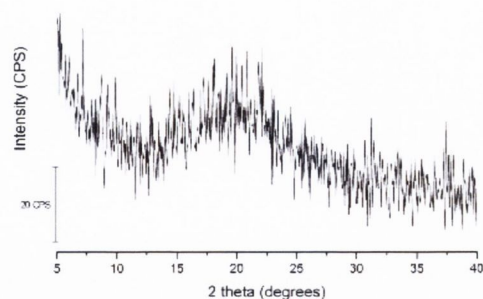


Figure 6.22 XRD scan of trypsin:raffinose spray-dried in mass ratio of 1:4 (Philips XRD).

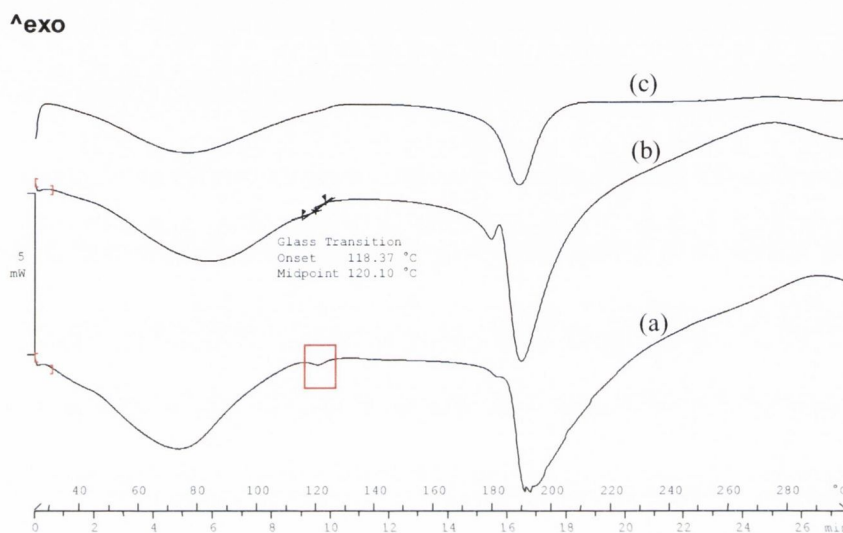


Figure 6.23 DSC scans of trypsin:raffinose spray-dried in mass ratios of (a) 1:9 (b) 1:4 (c) 1:1.

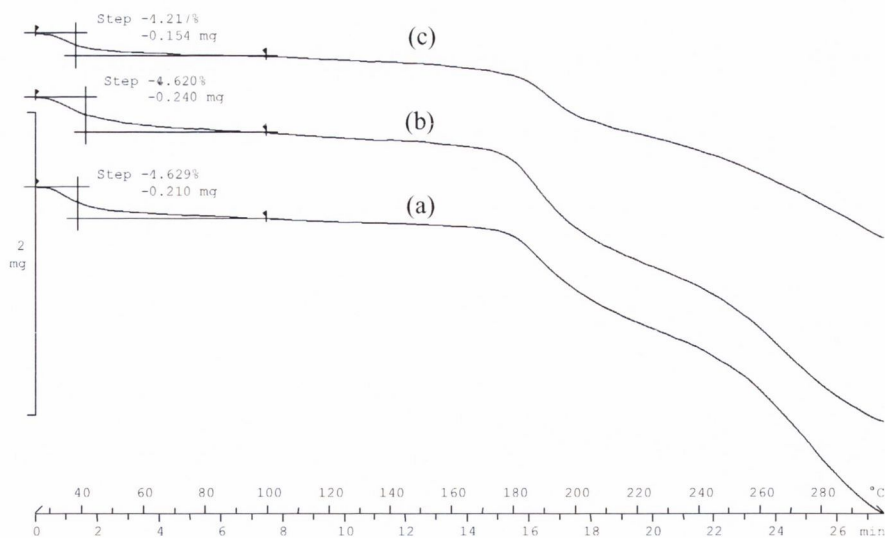


Figure 6.24 TGA scans of trypsin:raffinose spray-dried in mass ratios of (a) 1:9 (b) 1:4 (c) 1:1.

Trypsin:HP-β-CD composites

The spray-dried composite (mass ratio 1:4) was amorphous by XRD (Figure 6.25). DSC and TGA scans of composites of mass ratio 1:9, 1:4, 1:1 are presented (Figures 6.26, 6,27). Broad endothermic peaks were observed from 25 - ~120 °C in DSC scans, followed by small endotherms at ~ 220 °C, which may correspond to the Tm of the trypsin component. This peak enthalpy (J/g) increased with increasing trypsin content. Decomposition was further noted in DSC scans as endothermic areas starting at ~ 255 – 260 °C. Initial mass losses over the temperature range 25 - 100 °C of 5 – 6.6% (repeat of a replicate batch mass ratio 1:4 used for stability study was 4.4% - see section 6.2.2.2.5) were calculated, with further mass losses corresponding to decomposition starting at ~ 250 - 260 °C observed from TGA.

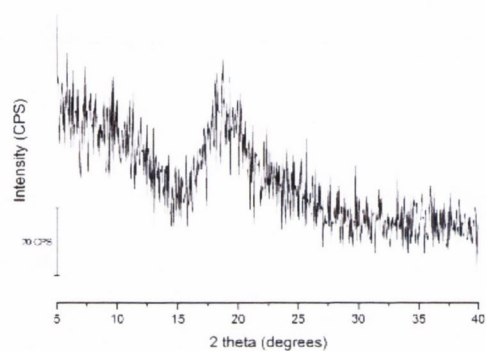


Figure 6.25 XRD scan of trypsin:HP-β-CD spray-dried in a mass ratio of 1:4 (Philips XRD).

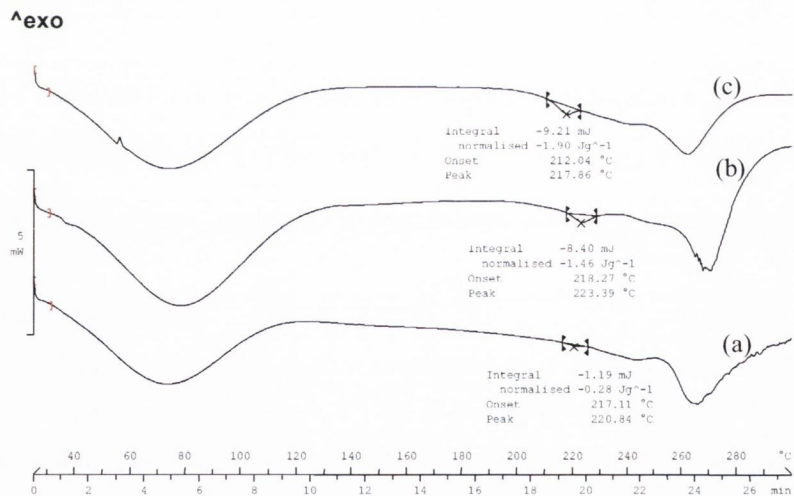


Figure 6.26 DSC scans of trypsin:HP-β-CD spray-dried in mass ratios of (a) 1:9 (b) 1:4 (c) 1:1.

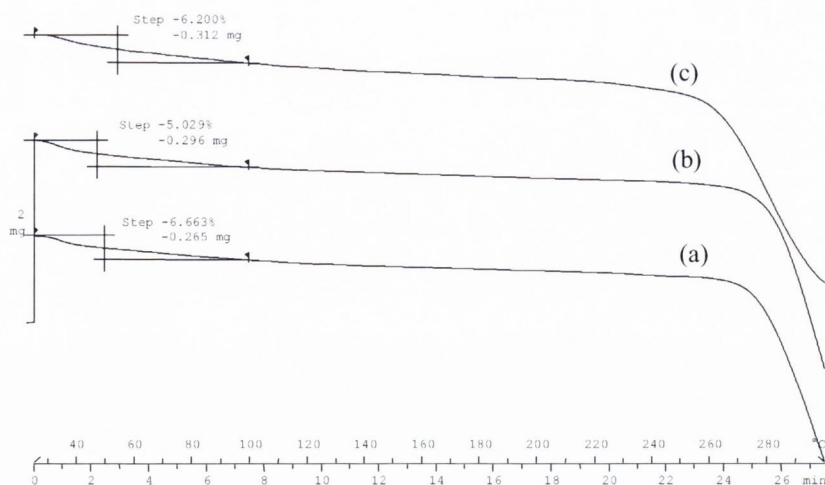


Figure 6.27 TGA scans of trypsin:HP-β-CD spray-dried in mass ratios of (a) 1:9 (b) 1:4 (c) 1:1.

Secondary structure and biological activity

% area of overlap of the trypsin:trehalose mass ratio composite 1:4 with unprocessed trypsin using calculation method 2 was 81.9%, and 80.2% for the trypsin:HP-β-CD 1:4 mass ratio composite and 85.1% for the trypsin:raffinose 1:4 mass ratio composite. This value obtained for the trypsin:raffinose composite was very similar to that obtained for trypsin:raffinose spray-dried from the non-aqueous MeOH:BA system (Section 6.2.2.1.1).

Biological activity assays were carried out on the 1:9, 1:4 and 1:1 trypsin:excipient composites, and compared to unprocessed trypsin. Activity of spray-dried powders (units/mg solid) and specific activity (units/mg protein) were calculated. Results are expressed in terms of units and % of activity of control solution (Tables 6.8). Statistical significance was assessed at a significance level of $\alpha = 0.05$, and samples were compared to control solution, using ANOVA. Test solutions were prepared such as to contain a protein concentration of ~ 0.044 mg/ml. The amount of protein in the test solutions were quantified by micro BCA protein analysis, and were similar to the expected concentrations. There was no statistically significant decrease in specific activity compared to control for any of the composites, except for a slight decrease for the trypsin:raffinose 1:9 mass ratio composite (92.0%).

Table 6.8 Summary of biological activity assay for trypsin:excipient composites (mass ratios as shown, spray-dried from MeOH:BA:H₂O); n=3; results expressed as average ± standard deviation; *pooled stdev % (in the case of % activity and % specific activity) is calculated from the pooled standard deviation of all samples run for the daily assay as a % of units of control solution.

1:9 mass ratio	Units/ml test	Units/mg solid	µg protein/ml test	Units/mg protein	% Activity	% Specific activity
Tryp:trehalose	374 ± 9.6	851 ± 22	40.6 ± 2.2	9239 ± 333	8.5 ± 2.1*	95.4 ± 5.9*
Control	441 ± 15.8	10025 ± 358	45.7 ± 3.6	9682 ± 525	100 ± 2.1*	100 ± 5.9*
Tryp:raffinose	480 ± 25.2	1091 ± 57	54.3 ± 1.9	8841 ± 247	11.2 ± 1.7*	92.0 ± 2.9*
Control	428 ± 14.0	9738 ± 319	44.6 ± 0.9	9606 ± 356	100 ± 1.7*	100 ± 2.9*
Tryp:HP-β-CD	559 ± 12.8	1270 ± 29	59.0 ± 4.1	9499 ± 763	12.7 ± 2.1*	98.1 ± 5.9*
Control	441 ± 15.8	10025 ± 358	45.7 ± 3.6	9682 ± 525	100 ± 2.1*	100 ± 5.9*

1:4 mass ratio	Units/ml test	Units/mg solid	µg protein/ml test	Units/mg protein	% Activity	% Specific activity
Tryp:trehalose	447 ± 14.0	2030 ± 65	49.0 ± 2.7	9120 ± 260	20.8 ± 1.7*	94.9 ± 2.9*
Control	428 ± 14.0	9738 ± 319	44.6 ± 0.9	9606 ± 356	100	100
Tryp:raffinose	373 ± 10.7	1696 ± 48	52.0 ± 0.7	7181 ± 121	22.5 ± 1.0*	100.8 ± 1.5*
Control	332 ± 4.4	7546 ± 99	46.6 ± 0.9	7122 ± 41	100	100
Tryp:HP-β-CD	429 ± 13.6	1950 ± 62	46.1 ± 0.4	9305 ± 218	20.0 ± 1.7*	96.9 ± 2.9*
Control	428 ± 14.0	9738 ± 319	44.6 ± 0.9	9606 ± 356	100	100

1:1 mass ratio	Units/ml test	Units/mg solid	µg protein/ml test	Units/mg protein	% Activity	% Specific activity
Tryp:trehalose	350 ± 7.4	3972 ± 84	48.0 ± 0.2	7287 ± 170	52.6 ± 1.0*	102.3 ± 1.5*
Tryp:raffinose	368 ± 4.1	4180 ± 46	50.8 ± 0.9	7238 ± 57	55.4 ± 1.0*	101.6 ± 1.5*
Control	332 ± 4.4	7546 ± 99	46.6 ± 0.9	7122 ± 41	100	100
Tryp:HP-β-CD	277 ± 1.7	3147 ± 22	43.3 ± 0.3	6379 ± 88	36.2 ± 4.1*	94.2 ± 3.7*
Control	383 ± 26.9	8703 ± 607	49.9 ± 2.3	6770 ± 387	100	100

6.2.2.2.5 Storage Stability

Trypsin:excipient NPMP composites (spray-dried mass ratio of 1:4 from MeOH:BA:H₂O – section 6.2.2.2) were selected for investigation of temperature-dependant storage stability. The conditions 4 °C/desiccant and 25 °C/desiccant were selected for a 12-week stability study. A small amount of the composite materials was also stored at 25 °C/60% R.H., with SEM and DSC analysis after storage for 24 hours. This was a similar study as was carried out previously for lysozyme:excipient composites spray-dried from a MeOH:BA system (Section 5.3.5).

Storage under desiccant conditions at 4 °C and at 25 °C

Particle morphology of trypsin:trehalose, trypsin:raffinose and trypsin:HP-β-CD particles were unaffected by storage for 12 weeks under desiccant conditions at 4 °C and at 25 °C, as can be seen from SE micrographs taken at week 12 (Figure 6.28) and compared to SE micrographs of freshly spray-dried composites (Figures 6.16 (b), 6.17 (b), 6.18 (b)).

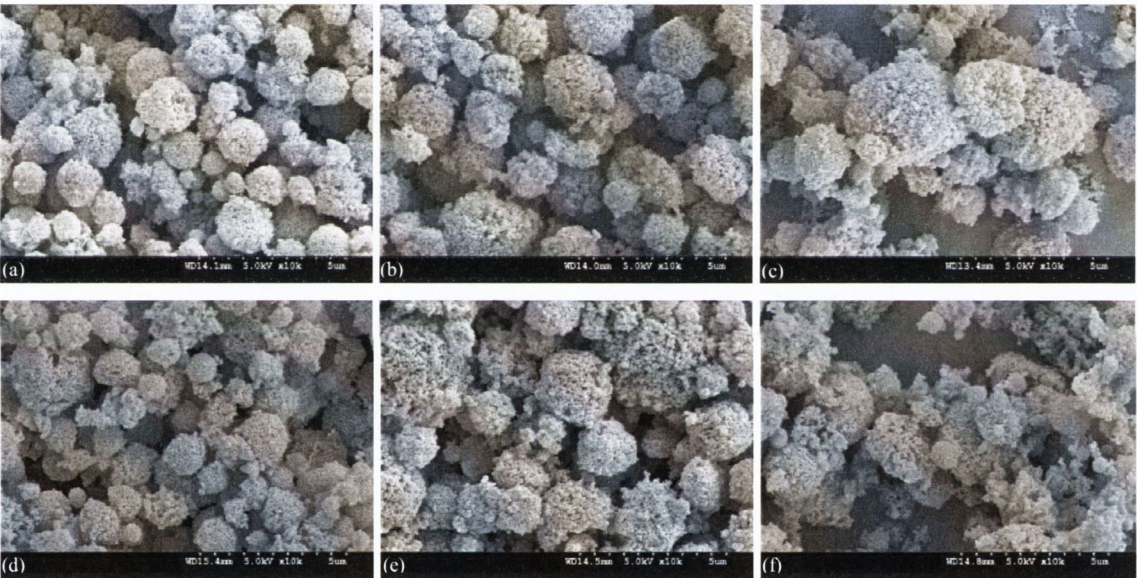


Figure 6.28 SE micrographs composite particles (spray-dried ratio 1:4) after 12 weeks storage at 4 °C/desiccant, at 25 °C/desiccant (a), (d) trypsin:trehalose; (b), (c) trypsin:raffinose; (e), (f) trypsin:HP-β-CD

Trypsin:trehalose, trypsin:raffinose, and trypsin:HP-β-CD composites were predominantly XRD amorphous after 12 weeks storage at 4 °C/desiccant and at 25 °C/desiccant (Appendix 2). Trypsin:raffinose composite stored at 4 °C/desiccant showed one small sharp peak at 16.6°. A peak was identified in crystalline raffinose pentahydrate at 16.5° (Section 4.2.2.3.1). Distinctive peaks attributed to crystalline raffinose pentahydrate at 10.75°, 13.65° and 21.1° (Section 4.2.2.3.1) were not identified for this composite sample stored at 4 °C on stability for 12 weeks however. No changes indicating crystallisation or increased moisture uptake were observed from DSC or TGA (Appendix 3, Figure 6.29) for any the composites stored under desiccant conditions at 4 °C, or at 25 °C for 12 weeks.

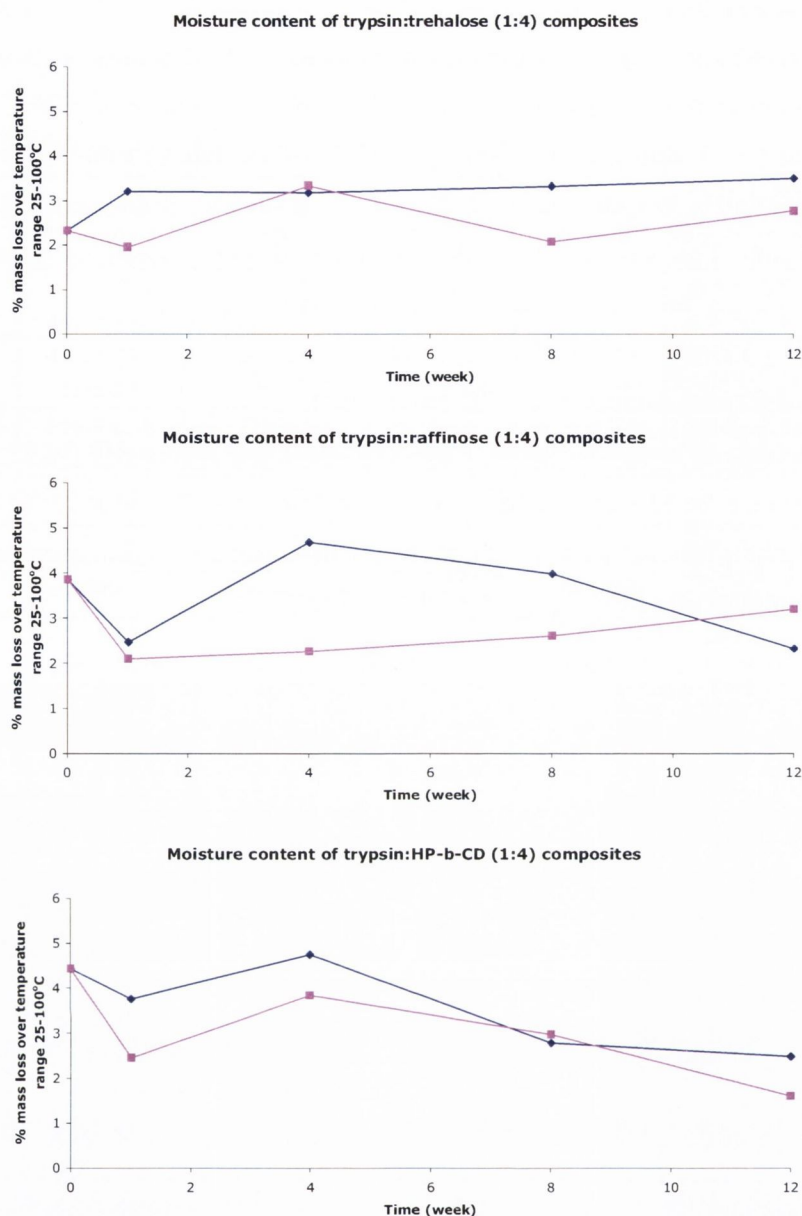


Figure 6.29 Moisture content calculated from TGA for spray-dried trypsin:excipient composites (1:4) during 12 weeks storage at 4 °C/desiccant (blue diamond) and 25 °C/desiccant (pink square).

No decrease in % specific activity was observed for trypsin:excipient composites (1:4) stored at 4 °C/desiccant for 12 weeks compared to control, (similar to the freshly spray-dried starting material batches) (Table 6.9). A decrease in % specific activity was observed for all composites stored at 25 °C/desiccant for 12 weeks compared to control however, and this was statistically significant ($p = 0.002$), with no excipient statistically significantly better than another. No associated decrease in solubility was observed in preparation of these samples, and this was reflected by the relative amounts of protein in test solutions compared to control ($\mu\text{g protein/ml}$ test), from micro BCA analysis. This suggests that loss of activity was not as result of decrease in solubility.

Table 6.9 Summary of biological activity assay for trypsin:excipient composites – freshly prepared, stored at 4 °C/desiccant or 25 °C/desiccant for 12 weeks (1:4 mass ratio, spray-dried from MeOH:BA:H₂O); n=2; results expressed as average (range); *pooled stdev % (in the case of % activity and % specific activity) is calculated from the pooled standard deviation of all samples run for the daily assay as a % of units of control solution.

	Units/ml test	Units/mg solid	µg protein/ml test	Units/mg protein	% Activity	% Specific activity
<u>Starting</u>						
Tryp:trehalose	341 (360, 322)	1550 (1636, 1464)	42.6 (43.1, 42.0)	8012 (8353, 7671)	19.1 ± 2.1	102.8 ±3.3
Tryp:raffinose	391 (399, 382)	1775 (1813, 1736)	49.1 (50.3, 47.9)	7949 (7922, 7976)	21.9 ± 2.1	102.0 ±3.3
Tryp:HP-β-CD	339 (328, 350)	1541 (1491, 1591)	44.3 (42.9, 45.7)	7650 (7646, 7653)	19.0± 2.1	98.2 ±3.3
Control	357 (366, 347)	8102 (8318, 7886)	45.8 (46.2, 45.3)	7790 (7919, 7661)	100± 2.1	100 ±3.3
<u>4 °C/des. W12</u>						
Tryp:trehalose	341 (359, 323)	1549 (1631, 1466)	46.5 (46.4, 46.5)	7339 (7740, 6938)	18.1 ± 1.9	97.1 ± 4.4
Tryp:raffinose	406 (403, 409)	1844 (1830, 1858)	54.3 (52.6, 56.0)	7480 (7657, 7302)	21.6 ± 1.9	99.0 ± 4.4
Tryp:HP-β-CD	342 (345, 339)	1554 (1568, 1540)	46.3 (46.0, 46.5)	7398 (7503, 7292)	18.2 ± 1.9	97.9 ± 4.4
Control	376 (385, 366)	8537 (8750, 8324)	49.7 (50.1, 49.3)	7557 (7684, 7430)	100 ± 1.9	100 ± 4.4
<u>25 °C/des. W12</u>						
Tryp:trehalose	314 (308, 320)	1427 (1398, 1455)	48.3 (47.4, 49.2)	6499 (6488, 6510)	16.7 ± 1.8	86.0 ± 1.8
Tryp:raffinose	328 (331, 325)	1492 (1506, 1477)	52.8 (52.4, 53.1)	6221 (6318, 6123)	17.5 ± 1.8	82.3 ± 1.8
Tryp:HP-β-CD	300 (311, 289)	1364 (1415, 1313)	48.1 (49.1, 47.0)	6243 (6345, 6140)	16.0 ± 1.8	82.6 ± 1.8
Control	376 (385, 366)	8537 (8750, 8324)	49.7 (50.1, 49.3)	7557 (7684, 7430)	100 ± 1.8	100 ± 1.8

Storage at 25 °C / 60% R.H.

Storage of trypsin:trehalose and trypsin:raffinose (1:4) composites at 25 °C / 60% R.H. for 24 hours resulted in particle collapse – loss of particle structure and surface morphology when viewed under SEM (Figure 6.30 (a), (b)). Storage of trypsin:HP- β -CD (1:4) composite at 25 °C / 60% R.H. for 24 hours did not exhibit this, with retention of particle structure and shape and porous surface morphology when viewed under SEM (Figure 6.30 (c))

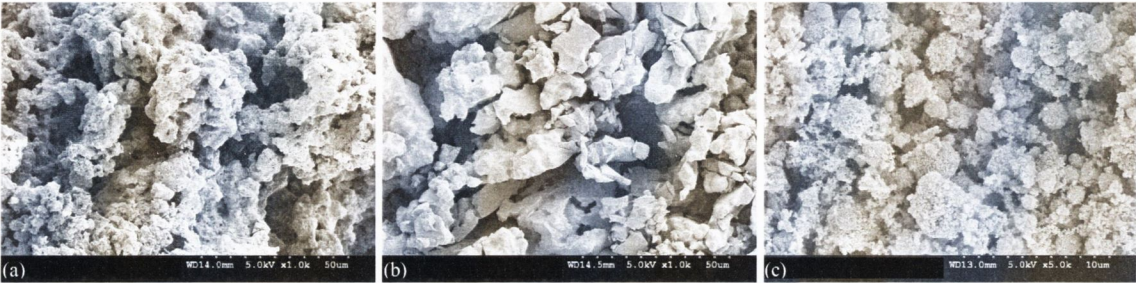


Figure 6.30 SE micrographs composite particles after 24 hour storage at 25 °C/60% R.H. (spray-dried ratio 1:4) (a) trypsin:trehalose (b) trypsin:raffinose (c) trypsin:HP- β -CD,

Crystallisation of trypsin:trehalose and trypsin:raffinose (1:4) composites stored at 25 °C / 60% R.H. for 24 hours was indicated by sharp endothermic peaks observed in the temperature range 80 – 100 °C, suggesting loss of hydrate water, in DSC scans (Figure 6.31 – marked by red boxes). No change was observed in the DSC scan of trypsin:HP- β -CD (1:4) composite stored at 25 °C / 60% R.H. for 24 hours, compared to starting material.

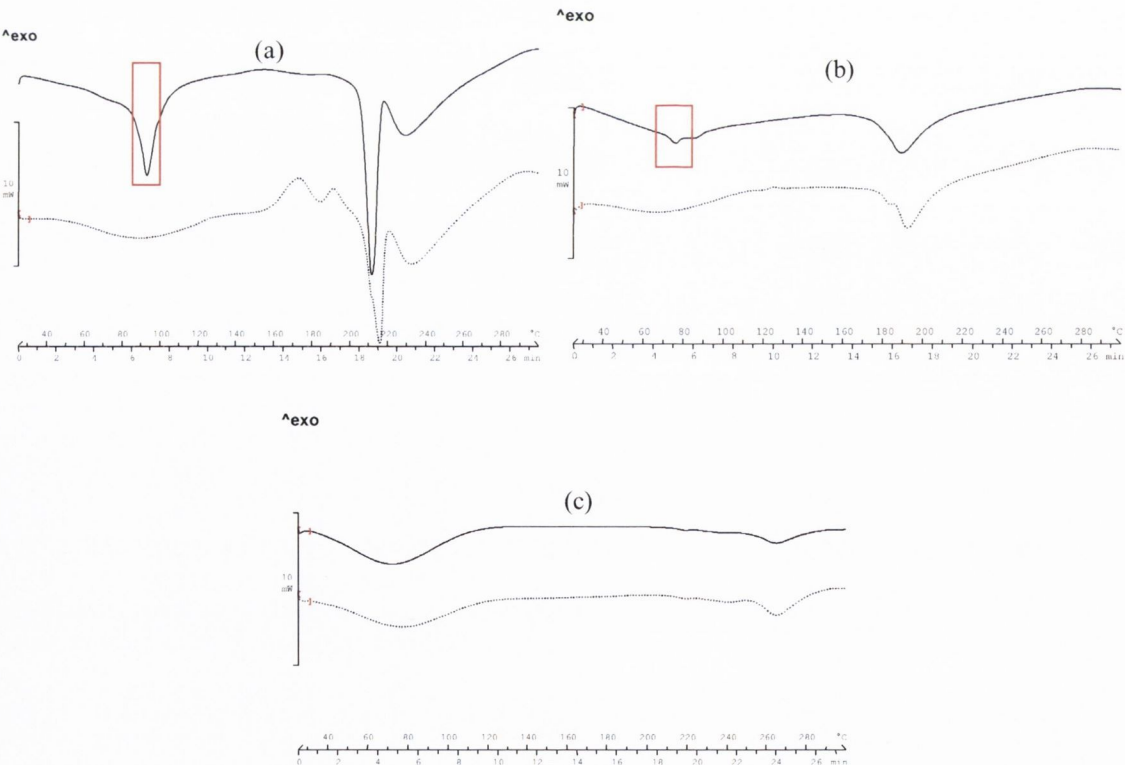


Figure 6.31 DSC scans of composite particles (mass ratio 1:4) (a) trypsin:trehalose (b) trypsin:raffinose (c) trypsin:HP- β -CD taken after 24 hours storage at 25 °C/ 60% R.H (solid line), freshly prepared (broken line).

6.2.2.2.6 PARTICLE CHARACTERISATION

Trypsin:excipient NPMP composites (spray-dried mass ratio 1:4) were selected for preliminary particle characterisation, in terms of bulk and tapped density and in-vitro deposition via Andersen cascade impactor analysis.

Composite powders had low measured bulk and tap densities as shown in Table 6.10. The highest bulk density value was calculated for the trypsin:HP-β-CD composite and the lowest bulk density value calculated for the trypsin:raffinose composite. Analyses were limited to one run for trypsin:trehalose, and trypsin:raffinose composites due to powder quantity, but the standard deviation calculated from 3 replicate measurements of the trypsin:HP-β-CD composite, indicative of inter-batch sample variation, was low. Calculated Carr’s indices were indicative of extremely poorly flowing powders (de Villiers, 2005).

Table 6.10 Bulk and tapped densities for composites as shown (spray-dried mass ratio 1:4); *n=3; average ± standard deviation, **n=1; result only

Composite (mass ratio 1:4)	Bulk Density (g/cm ³)	Tapped Density (g/cm ³)	Carr’s Index (%)
trypsin:trehalose	**0.0878	**0.1596	45.0
trypsin:raffinose	** 0.0649	**0.118	45.0
trypsin:HP-β-CD	*0.0946 ± 0.0015	*0.1523 ± 0.0063	37.9

In-vitro deposition experiments were conducted with two replicates on one batch each of trypsin:trehalose, trypsin:raffinose and trypsin:HP-β-CD. Results were compared using ANOVA to determine statistical significance (significance level α = 0.05). No statistically significant difference was determined between the trypsin:excipient composites for any of the calculated parameters (Table 6.11). Average fine particle fractions (< 5 μm) of 41.55 – 44.82% and average particle fractions < 3 μm of 26.21 – 29.60% were calculated from ACI analysis (Table 6.11). Breakdown of deposition by stage of the apparatus showed high deposition in the mouthpiece adapter and induction port, and within the stages of the impactor, deposition in the mid and lower stages – particularly stages 3, 4, and 5 (Figure 6.32). Average MMAD values of 2.76 - 3.18 μm were calculated for the composites. High average GSD vales of 2.29 – 2.65 were calculated, suggesting variation in aerodynamic diameter.

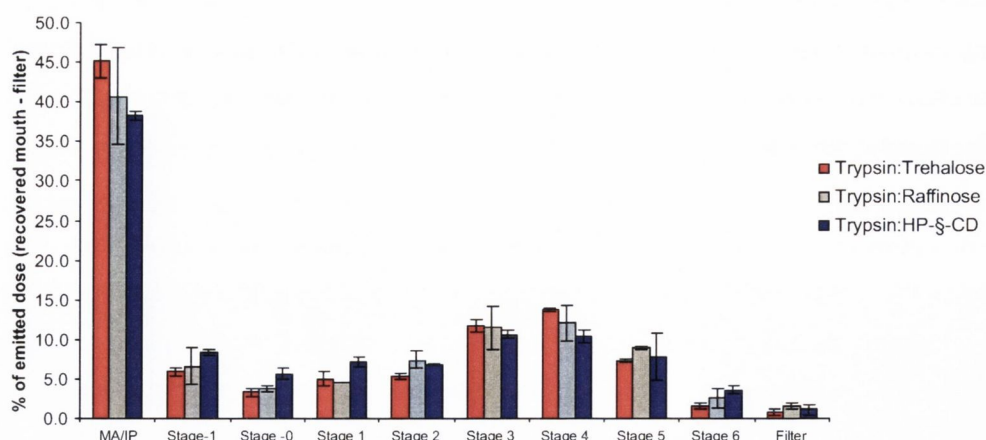


Figure 6.32 Deposition patterns on the stages of the ACI apparatus for the composites as indicated (spray-dried mass ratio 1:4). Calculated as % of recovered emitted dose (mouth – filter), used to calculate fine particle fraction.

Table 6.11 Summary of results from Andersen cascade impactor analysis for trypsin:trehalose, trypsin:raffinose and trypsin:HP-β-CD NPMP composites spray-dried in a mass ratio of 1:4; n = 2, mean (range).

Composite (mass ratio 1:4)	% < 5 μm	% < 3 μm	MMAD (μm)	GSD	% emitted (of nominal dose)
trypsin:trehalose	42.71 (41.57, 43.86)	26.21 (24.80, 27.62)	2.83 (2.92, 2.74)	2.29 (2.23, 2.34)	77.6 (77.7, 77.4)
trypsin:raffinose	44.82 (39.96, 49.68)	29.60 (25.53, 33.68)	2.76 (2.99, 2.52)	2.51 (2.56, 2.46)	81.7 (85.0, 78.4)
trypsin:HP-β-CD	41.55 (43.08, 40.02)	27.82 (29.22, 26.42)	3.18 (3.329, 3.031)	2.65 (2.55, 2.76)	80.3 (79.8, 85.0)

Estimation of particle size from SEM images (150 particles) showed a similar trend as observed in ACI results with the largest average particle size and standard deviation calculated for the trypsin:HP-β-CD composite (Table 6.12). The lower average particle size estimated from SEM analysis compared to MMAD suggests particle aggregation. Fusion was also observed in SEM analysis, between particles, which may also have contributed to the high deposition in the mouthpiece adaptor/induction port.

Table 6.12 Particle size estimates from SEM analysis – measurement of 150 particles.

Composite (mass ratio 1:4)	Particle size (μm)
trypsin:trehalose	1.51 ± 0.74
trypsin:raffinose	1.79 ± 0.81
trypsin:HP-β-CD	1.84 ± 1.17

6.3 SPRAY-DRIED CATALASE AND CATALASE COMPOSITES

6.3.1 AQUEOUS AND ETHANOL:WATER:AMMONIUM CARBONATE SYSTEM

6.3.1.1 Spray-dried catalase alone

Catalase is poorly soluble in unbuffered aqueous solution, due to its pI of 5.4, and in organic solvents. As such preliminary experiments with spray-drying catalase from buffered and then aqueous solutions with AC or AHC were attempted prior to a mixed ethanol:H₂O:AC system.

Catalase was initially spray-dried from 50 mM phosphate buffer pH 7, at its quoted solubility of 1 mg/ml (0.1% w/v) (www.sigmaaldrich.com), yielding a spray-dried product containing ~ 87.2% buffer salts (hereafter referred to as “catalase1”). This is a higher salt concentration in the final product than spray-dried by either Liao et al. (2005), or Forbes et al. (1998). An inlet drying temperature of 120 °C was chosen to ensure adequate drying of the aqueous system, and also to minimise thermal stress. Spray-drying was carried out in the open mode using the regular cyclone arrangement (see Appendix 1 for details). Particle morphology was irregular shaped particles and aggregates (Figure 6.36 (a)), and was likely to be influenced by the high salt content of the formulation. Catalase was thus spray-dried from a lower salt content buffer - 5 mM phosphate buffer pH 7, at a total protein concentration of 0.5% w/w in order to reduce the salt content of the spray-dried product (spray-dried product theoretically containing ~ 12% w/w buffer salts) (hereafter referred to as “catalase2”). Other spray-drying conditions were similar to those used for the catalase1 sample. Particle morphology of this system was collapsed particles with a smooth surface as evident from SEM analysis (Figure 6.33 (b)). SEM analysis of unprocessed material is presented for comparison (Figure 6.33 (c)).

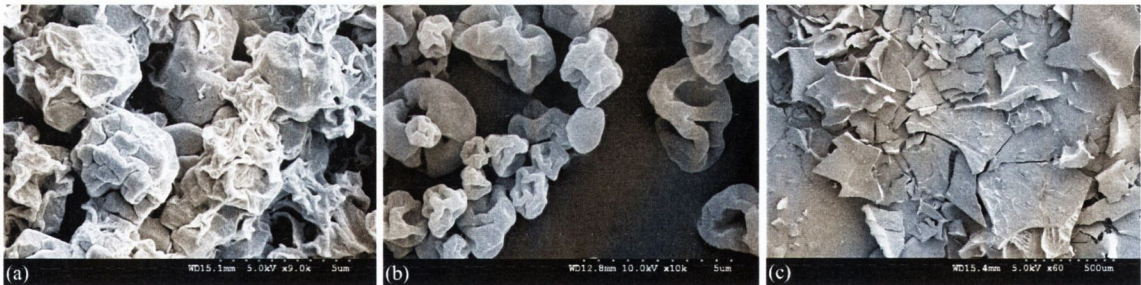


Figure 6.33 SE micrograph of catalase spray-dried from (a) 50 mM phosphate buffer pH 7; 0.1% w/v catalase; 120 °C; - catalase1 (b) 5 mM phosphate buffer pH 7; 0.5% w/v catalase ; 120 °C; - catalase2 and (c) unprocessed catalase, note difference in magnification

AC and AHC increase the pH of aqueous solution (Section 3.3.3) and as such were postulated to enable dissolution of catalase, without additional buffer salts or pH adjustment. Catalase was thus dissolved in aqueous non-buffered solutions with either 10% of t.s.c. AC (hereafter referred to as “catalase3”) or AHC (hereafter referred to as “catalase4”) at a t.s.c. of 0.25% and spray-dried in the open mode with inlet temperature 120 °C (see Appendix 1 for conditions). Spray-dried product

morphology was collapsed raisin-like particles with a smooth non-porous surface as evident from SEM analysis (Figure 6.34).

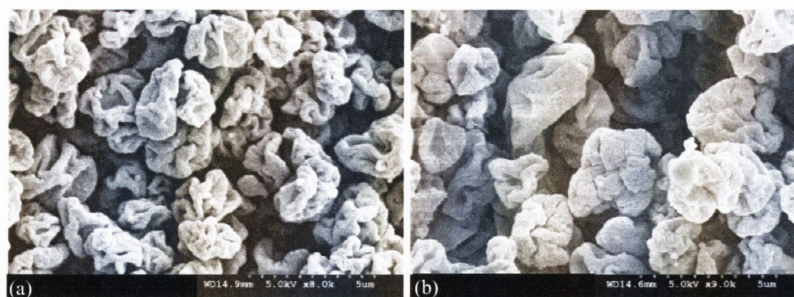


Figure 6.34 SE micrographs of catalase spray-dried from aqueous solutions, (a) 10% AC, 120 °C, 0.25% t.s.c., - catalase3 (b) 10% AHC, 120 °C, 0.25% t.s.c. – catalase4

Addition of EtOH to an aqueous solution of catalase and AC was attempted, and a system containing 50% v/v EtOH, 10% of t.s.c. AC, 0.125% t.s.c. was prepared (hereafter referred to as “catalase5”). Large fibrous aggregates were formed, visible on addition of EtOH and stirring of the solution. This was unlike the appearance of phase separation observed to occur for trypsin and lysozyme systems in EtOH:H₂O:AC systems, which were characterised by sudden marked increases in turbidity. Spray-drying was carried out in the open mode at an inlet temperature of 78 °C (see Appendix 1). The spray-dried product consisted of a mixture of collapsed or folded particles and spherical particles with thin particle walls as evident from SEM analysis (Figure 6.35).

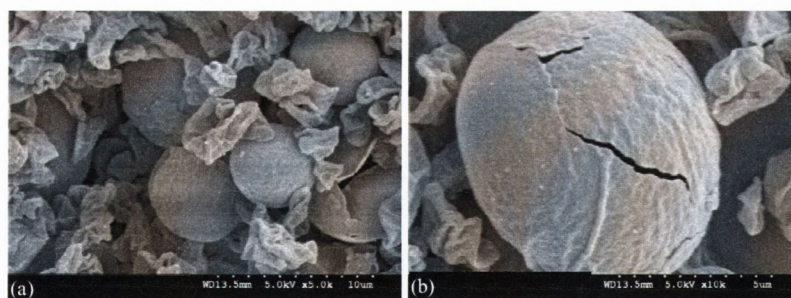


Figure 6.35 SE micrographs of catalase spray-dried from 50% EtOH, 10% of t.s.c. AC, 0.125% t.s.c., inlet temperature 78 °C – catalase5.

6.3.1.2 Spray-dried catalase composite

Catalase was spray-dried with trehalose at a mass ratio of 1:1 to investigate stabilisation of the protein by the sugar and any effect on morphology. Similar spray-drying conditions as catalase3 were used – with 10% of t.s.c. AC from water at inlet temperature 120 °C. A higher t.s.c. of 0.5% w/v was chosen due to the trehalose content. Morphology of the composite was collapsed particles with some fusion or bridging between particles (Figure 6.36).

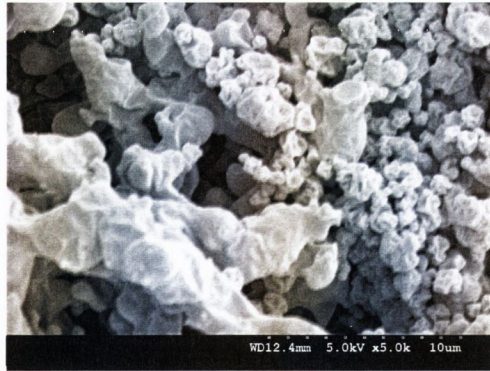


Figure 6.36 SE micrographs of catalase:trehalose 1:1 spray-dried from aqueous solutions, 10% of t.s.c. AC, 120 °C, 0.5% t.s.c.

6.3.1.3 Process stability

Solid-state and thermal characterisation

XRD analysis was carried out for the spray-dried samples - catalase1 and catalase2 to determine the crystallinity of the catalase:buffer salt mix. Catalase1 (Figure 6.37 (c)) was XRD crystalline with some peak overlap with the buffer salt - potassium dihydrogen phosphate. Catalase2 (Figure 6.37 (b)) was mainly amorphous or any crystallinity was below the level of detection of XRD analysis. The amorphous nature of unprocessed catalase (Figure 6.37 (a)) was confirmed by XRD.

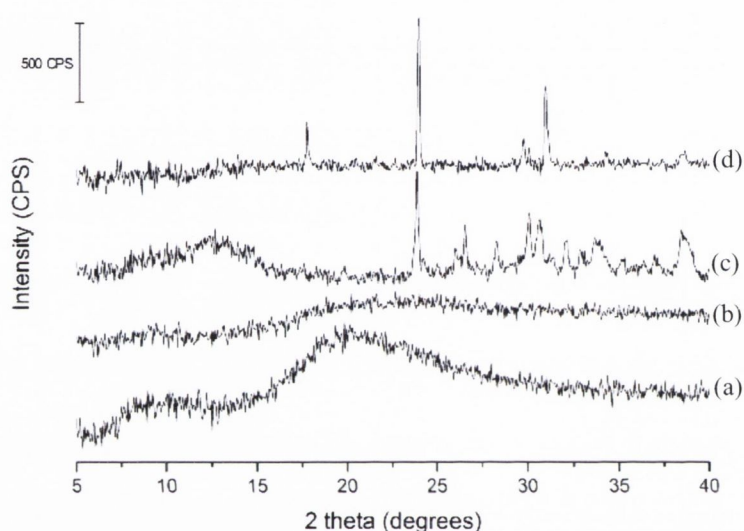


Figure 6.37 XRD of (a) unprocessed catalase (b) catalase2 (c) catalase1 (d) potassium dihydrogen phosphate

DSC analyses were carried out on unprocessed catalase and spray-dried catalase samples - catalase2-5 and the catalase:trehalose composite spray-dried in a mass ratio of 1:1 (Figures 6.38, 6.40). Unprocessed catalase showed a very broad initial endotherm from 25 – 170 °C, with a small sharp endotherm at 125 °C (T_m). A similar pattern in the DSC scan was observed for catalase2. No sharp peaks were identified in DSC scans of spray-dried samples – catalase3-5 or catalase:trehalose composite. These DSC scans were also quite flat in appearance compared to unprocessed catalase or spray-dried catalase2, with no significant thermal events. This may imply a change in folding or unfolding of catalase in these samples. Mass losses calculated from TGA were similar for spray-dried and unprocessed catalase as shown (Figure 6.39). Mass loss occurred in at least three steps and the first two 25 - 100 °C and 100 - 160 °C are quantified.

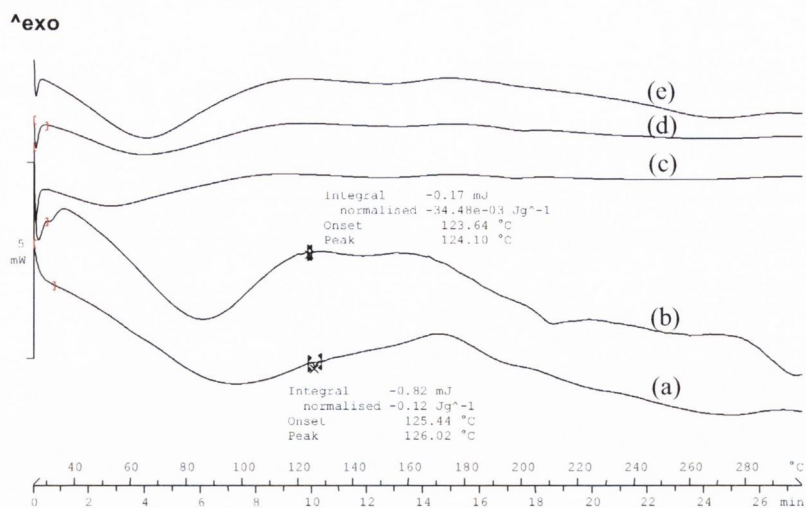


Figure 6.38 DSC scans of (a) unprocessed catalase and spray-dried (b) catalase2 (c) catalase3 (d) catalase4 (e)catalase5

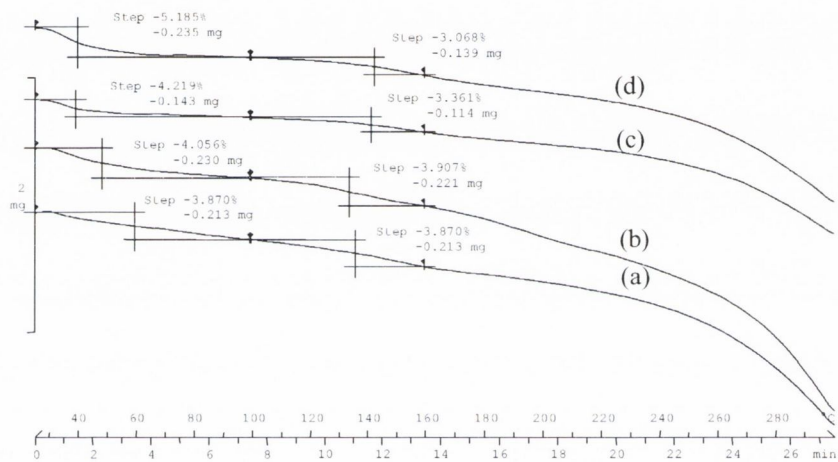


Figure 6.39 TGA scans of (a) unprocessed catalase and spray-dried (b) catalase2 (c) catalase3 (d) catalase4

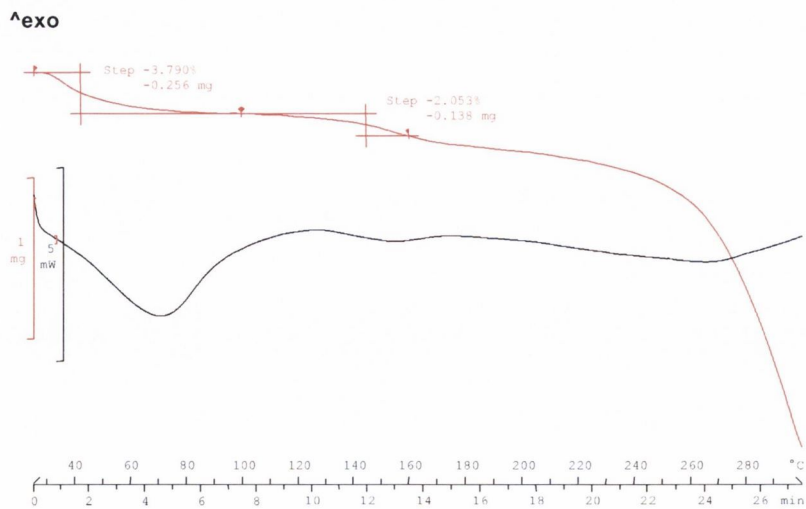


Figure 6.40 DSC (shown in black) and TGA (shown in red) of spray-dried catalase:trehalose mass ratio 1:1

Biological activity

Biological activity assays (with micro BCA protein quantitation) were carried out on catalase samples and used to calculate activity of spray-dried powders (units/mg solid) and specific activity of the protein (units/mg protein) in the powders. Results are expressed as units of activity and % of original specific activity of unprocessed catalase and are summarised in Table 6.13.

Catalase1 (spray-dried with a high quantity of buffer salts) showed a large (statistically significant) loss in % specific activity compared to control solution of ~ 35%. Specific activity was not calculated for catalase2 (spray-dried from lower quantity buffer salts), but % activity (per mg solid) suggested an improvement at 71.8% of control solution. Catalase3 and catalase4 (spray-dried from unbuffered solution with, respectively, 10% AC or AHC) resulted in some loss of % specific activity, which were statistically significant compared to control solution. Inclusion of stabilising sugar trehalose in the mass ratio of 1:1 in the system using AC resulted in protection of this loss with full retention of specific activity (at 105%). Attempts to assay spray-dried catalase5 (spray-dried from 50% EtOH, 10% of t.s.c. AC) were unsuccessful as the solubility of the protein was significantly reduced. Although the A 240 of the H₂O₂ substrate solution decreased very slowly on addition of catalase5 test solution, it did not reach the assay starting absorbance of 0.45 after 15 minutes and so was not completed. This result suggests near complete loss of activity of the protein and suggests that the process carried out under these conditions is highly unsuitable for spray-drying catalase.

Table 6.13 Summary of biological activity assay for spray-dried catalase and catalase:trehalose composite; n=3; results expressed as average $\pm$ standard deviation; *pooled stdev % (in the case of % activity and % specific activity) is calculated from the pooled standard deviation of all samples run for the daily assay as a % of units of control solution.

	Units/ml test	Units/mg solid	μ g Protein/ml test	Units/mg protein	% Activity ($\pm$ 5.8%*)	% Specific activity ($\pm$ 5.3%*)
Catalase1	38.8 $\pm$ 0.9	125 $\pm$ 2.7	29.2 $\pm$ 0.9	1333 $\pm$ 56	5.5	63.6
Catalase2	73.0 $\pm$ 9.2	1621 $\pm$ 203	N/d	N/d	71.8	N/d
Catalase3	69.2 $\pm$ 4.6	1730 $\pm$ 116	38.1 $\pm$ 0.8	1819 $\pm$ 119	76.6	86.7
Catalase4	63.6 $\pm$ 5.0	1591 $\pm$ 125	46.9 $\pm$ 4.8	1590 $\pm$ 125	70.4	75.8
Catalase5	N/d	N/d	22.2 $\pm$ 11.1	N/d	N/d	N/d
Cat:treh	91.4 $\pm$ 2.4	1142.0 $\pm$ 29	41.6 $\pm$ 2.0	2201 $\pm$ 164	50.5	105.0
Control	90.4 $\pm$ 7.1	2259 $\pm$ 178	43.2 $\pm$ 4.1	2097 $\pm$ 35	100	100.0

6.3.2 SPRAY-DRIED CATALASE COMPOSITES FROM METHANOL:N-BUTYL ACETATE:WATER

Catalase was spray-dried with trehalose, raffinose or HP-β-CD from MeOH:BA:H₂O solvent systems similar to those used for trypsin:excipient composites, as preliminary experiments. (See Appendix 1 for details). A protein:excipient mass ratio of 1:9 was selected. Catalase was dissolved in the water component with the aid of 0.1 M NaOH (pH of water component altered to ~ 10) prior to addition of organic solvents. Phase separation occurred on addition of organic solvents with increased turbidity of the feed material and also the appearance of large fibrous aggregates. SEM analysis showed porous particles, and also some larger agglomerates – one example marked with a red arrow (Figure 6.41).

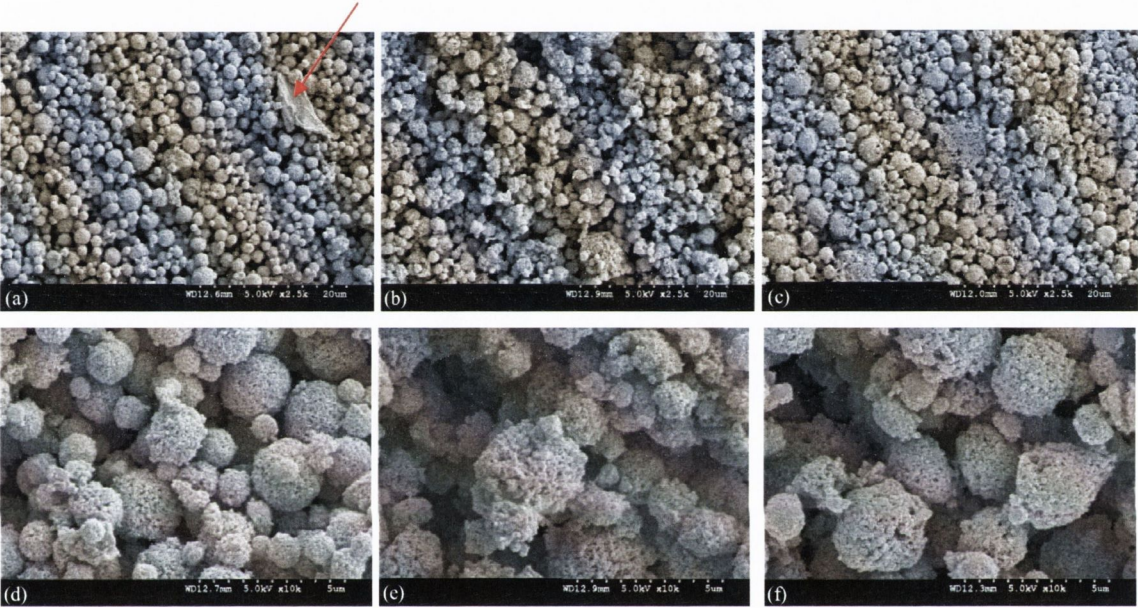


Figure 6.41 SE micrograph of (a), (d) catalase:trehalose, (b), (e) catalase:raffinose, (c), (f) catalase:HP-β-CD, all spray-dried in a mass ratio of 1:9

No difference in DSC or TGA scans were observed for the catalase:excipient composites compared to excipient spray-dried alone. No T_m peaks or decomposition events of catalase were identified. This was not unexpected because of the low protein load and the lack of distinct events seen with the unprocessed catalase, but the lack of any characteristic thermal events including decomposition events may also signify unfolding of the protein.

Biological activity assays on the catalase:excipient samples spray-dried from MeOH:BA:H₂O systems showed almost complete loss of activity of the samples (< 10% for all samples). Loss of protein solubility was also observed, with the presence of insoluble aggregates on preparation of test solutions and difference in protein content of filtered and unfiltered test solutions (protein contents in filtered test solutions were ~ half those in unfiltered solutions) by micro BCA analysis. The loss of activity was likely contributed to but could not be explained solely by the formation of

insoluble aggregates. These data suggest irreversible unfolding and denaturation of the protein. The system used was thus unsuitable for spray-drying catalase, even at a low load (10%).

The solution pH (~10) of catalase in the H₂O component may have been unsuitable however and contributed to this loss of activity. On consideration of the experimental conditions a high pH, although chosen to enable dissolution of catalase, may have been too high and adversely affected the stability of catalase by itself and also the stability of the protein towards thermal and organic solvent stresses. Prajapati et al. (1998) described alkaline unfolding of bovine liver catalase, incubated at high pH (~2 hours). Enzymatic activity was found to reduce rapidly for catalase samples incubated at a pH between pH 9.5 – 11 with a complete loss of activity for catalase samples incubated at pH 11 and above.

6.4 SPRAY-DRIED SALMON CALCITONIN COMPOSITE

6.4.1 Salmon calcitonin:trehalose composite spray-dried from methanol:n-butyl acetate

Salmon calcitonin (sCT) was spray-dried with trehalose in a mass ratio of 5:95 (salmon calcitonin:trehalose dihydrate) from an 80:20 MeOH:BA solvent system, at a t.s.c of 0.5% (see Appendix 1 for details). The use of the MeOH:BA system was possible due to the observed solubility of sCT in MeOH. This was preferable to the MeOH:BA:H₂O system due to the favourable in-vitro deposition characteristics found with the lysozyme:excipient composites spray-dried from a MeOH:BA system compared to trypsin:excipient composites spray-dried from the MeOH:BA:H₂O system. A low load of peptide (5% of total solids) was selected based on the peptide loading used by other researchers in spray-drying or other process production of microspheres containing salmon calcitonin (Yang et al., 2007; Clark et al., 2008; Dani and DeLuca, 2001), and consideration of pulmonary bioavailability (Deftos et al., 1997; Patton, 1994; Komada et al., 1994) and comparison with the intra-nasal dose (Novartis, 2007).

Morphology of spray-dried particles were spherical porous and similar to trehalose spray-dried alone as seen in SE micrographs. Surface morphology consisted of fused nanoparticles (Figure 6.42). Particle size was $1.36 \pm 0.69 \mu\text{m}$ estimated from measurement of 150 particles in SE micrographs.

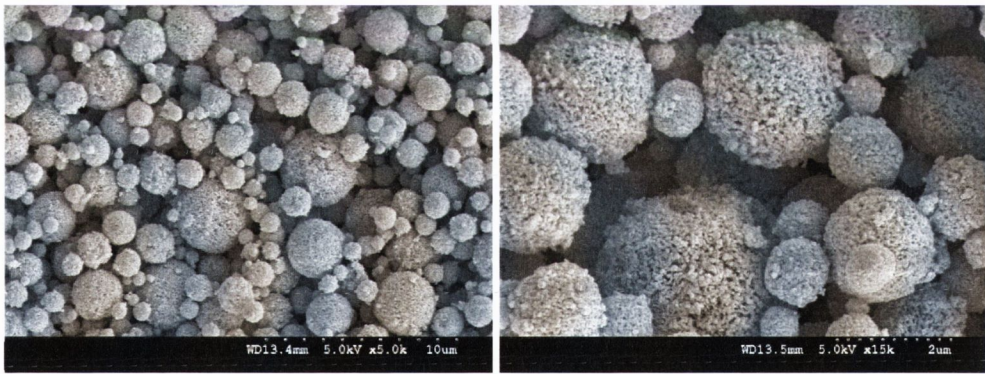


Figure 6.42 SE micrographs of sCT:trehalose composites (spray-dried mass ratio 5:95) shown at different magnifications.

The DSC scan of the sCT:trehalose composite was similar to trehalose spray-dried alone with a glass transition detected at $\sim 123^\circ\text{C}$. Similar to lysozyme and trypsin:trehalose composites, a recrystallisation exothermic peak occurred at a high onset temperature ($\sim 190^\circ\text{C}$ onset). The presence of the glass transition temperature and this recrystallisation peak suggest an amorphous form. A sharp symmetrical endotherm at a temperature of onset of $\sim 212^\circ\text{C}$ (with an enthalpy of fusion of $\sim 37.7 \text{ J/g}$) was attributed to the melting endotherm of anhydrous trehalose. A broad endothermic decomposition peak at onset $\sim 255^\circ\text{C}$, and a corresponding mass loss in the TGA was

earlier than that in the DSC of trehalose spray-dried alone (~280 °C) and may include decomposition of calcitonin. Mass loss between 25 - 100 °C was determined at ~ 4.9% consistent with residual solvent or moisture loss, rather than stoichiometric loss of hydrate or bound water. The shape of this mass loss step in the TGA was also consistent with loosely bound water, as opposed to mass loss seen for trehalose dihydrate (later, more pronounced step).

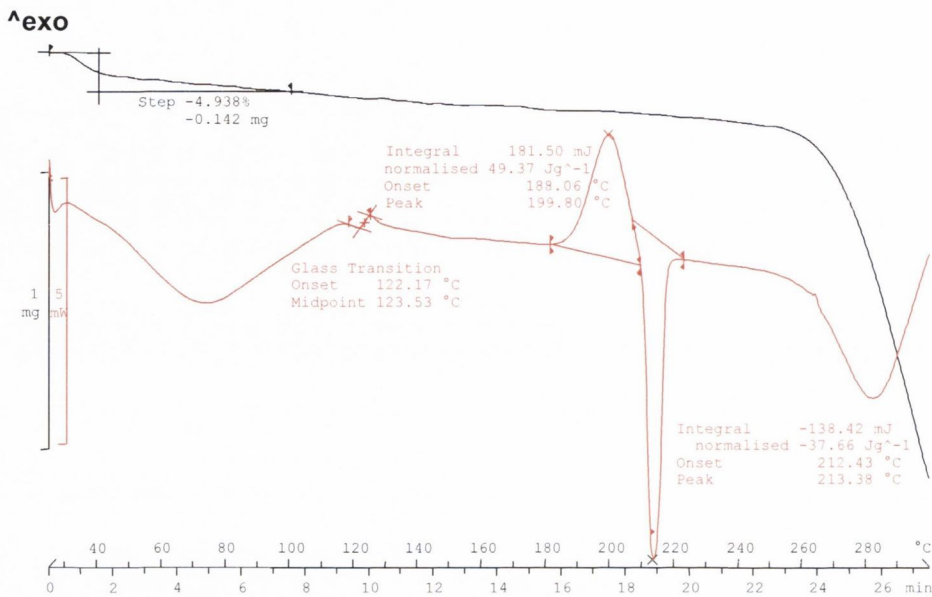


Figure 6.43 DSC (shown in red) and TGA (shown in black) scans of SCT:trehalose composites (spray-dried in a mass ratio of 5:95).

Micro BCA analysis of solutions of composite samples (0.2 mg/0.5 ml buffer $\approx$ 20 μ g sCT /ml) were carried out to check that the ratio of sCT:trehalose in the final composite was similar to the mass ratio added to the feed material prior to spray-drying. An average peptide content of 20.45 ± 4.7 μ g/ml (range 17.1-23.8 μ g/ml) was calculated on comparison to a standard curve of sCT (5-40 μ g/ml, $R^2 = 0.9998$). This suggested that sCT in the final composite particle was similar to the spray-dried concentration of 5% w/w of total solid content.

Activity of sCT in the composite was compared by means of a concentration response curve to unprocessed sCT, assuming a calcitonin load of 5% in spray-dried material, as described in Chapter 2. The assay was undertaken by Dr Sinead Ryan, of Prof. David Braydon's group, UCD. It was concluded that the sCT:trehalose composite was bioactive and quite comparable to unprocessed sCT (Figure 6.44). Data for the concentration response curve were fitted using a SSlogis model (model and parameters in Appendix 7). These parameters are presented in Table 6.14. EC_{50} values were slightly higher for the sCT composite than for unprocessed sCT and this was statistically significant (at a significance level of $\alpha = 0.05$). The EC_{50} value of the sCT:trehalose composite was expressed as a percentage of the EC_{50} of unprocessed sCT (as by Youn et al., (2006) as a measure of biological activity) giving a biological activity of 54.1%. The scale factor (measure of

the “steepness” of the curve) was also statistically significantly lower for the composite, indicative of a steeper dose response curve for the sCT:trehalose composite, which is also relevant when considering the biological activity. Explanation for this difference could not be found from these preliminary experiments.

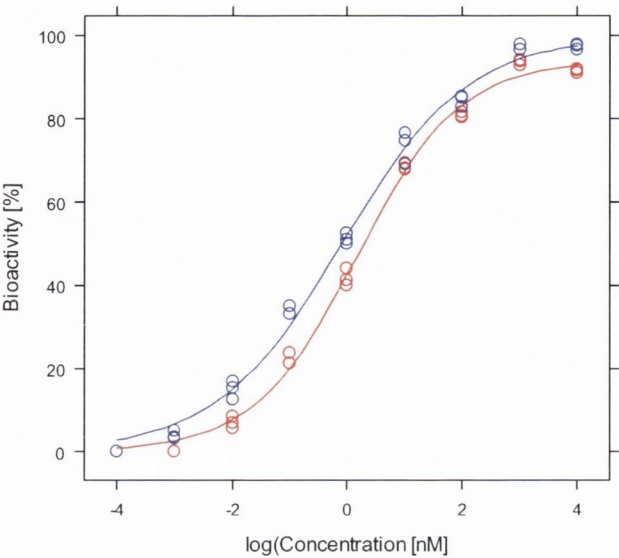


Figure 6.44 Concentration response curves of the sCT (o) and the sCT:trehalose (o) formulations.

Table 6.14 Parameters of model expressed as average (95% confidence interval).

	Efficacy (%)	EC ₅₀ (nM)	Scale Factor
sCT:trehalose	93.99 (91.47, 96.51)	1.4699 (1.1322, 1.8075)	0.8934 (0.8130, 0.9738)
sCT	99.83 (97.03, 102.63)	0.7962 (0.5837, 1.0087)	1.0970 (1.0067, 1.1873)

The data was replotted in GraphPad Prism5 software using the dose-response curve (variable slope) option (Figure 6.45). This model uses the parameters “Top”, “Bottom” similar to “Efficacy” in the SSlogis model, and “Hill slope” as a measure of the steepness of the curve (See Appendix 7 for model and parameters). A higher EC₅₀ was calculated for the sCT:trehalose, composite compared to the unprocessed sCT (giving a biological activity expressed as a %, of 61.2%) but this was not statistically significant at a significance level of $\alpha = 0.05$ (Table 6.15, Appendix 7). The average Hill slope was higher for the sCT:trehalose composite compared to the unprocessed sCT, (which would be indicative of a steeper dose-response curve), but this was also not statistically significant.

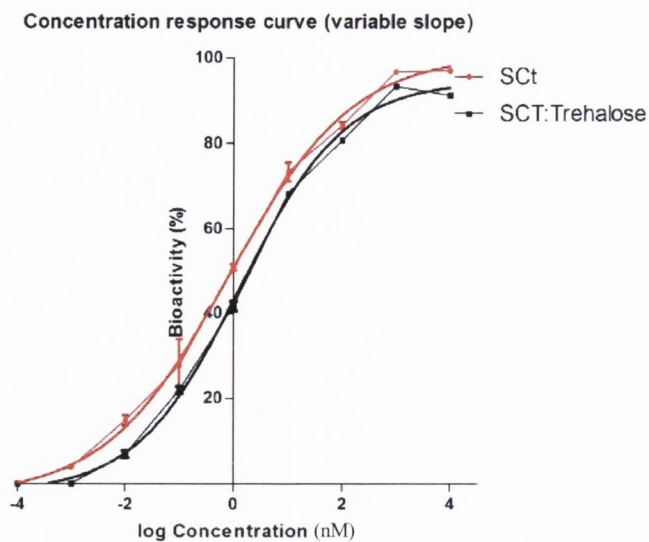


Figure 6.45 Concentration response curves of unprocessed sCT (shown in red) and sCT:trehalose (shown in black) formulations.

Table 6.15 Parameters of model, expressed as average (95% confidence interval).

	Top (%)	Bottom (%)	EC ₅₀ (nM)	Hill slope
sCT:trehalose	94.76 (92.34, 97.18)	-2.215 (-4.458, 0.02764)	1.336 (1.069, 1.671)	0.4556 (0.4096, 0.5016)
sCT	100.9 (95.63, 106.2)	-3.085 (-8.709, 2.539)	0.8181 (0.5027, 1.332)	0.3816 (0.3058, 0.4574)

6.5 CONCLUSIONS

Porous particles of trypsin were spray-dried using the EtOH:H₂O:AC feed system. Higher concentration of EtOH and AC were required, however, than were required for spray-drying NPMPs of lysozyme. Porous trypsin particles produced were also less uniform in size, shape and surface morphology than NPMPs of lysozyme, as observed from SEM analysis. NPMPs of trypsin suffered a greater loss of activity. The use of stabilising sugars was considered. Trehalose and raffinose were included with trypsin and spray-dried from EtOH:H₂O:AC systems. Biological activity was somewhat improved compared to trypsin spray-dried alone. Particle morphology was affected by the inclusion of the sugar, however, even at low sugar contents (4:1, 9:1 trypsin:sugar). The use of MeOH:BA:(H₂O) systems were investigated for spray-drying composites of trypsin:excipients. The MeOH:BA system was unsuccessful for spray-drying NPMP composites and resulted in a mixed morphology of flake-like and spherical particles. The insolubility of freeze-dried trypsin in the MeOH component was postulated to be problematic in this system. The MeOH:BA:H₂O system was used to produce porous particles of trypsin:excipient (trehalose, raffinose and HP- β -CD) with excellent retention of biological activity in mass ratios of 1:9, 1:4 and 1:1. Particle characterisation of the 1:4 mass ratio trypsin:excipient systems showed favourable bulk and tapped density and particle size estimated from SEM analysis. In-vitro deposition experiments showed that the 1:4 mass ratio composites had good in-vitro deposition properties. This method of spray-drying protein with excipient from a MeOH:BA:H₂O system may represent an alternative method for proteins/peptides which are insoluble in MeOH.

An attempt to produce NPMPs of catalase from an EtOH:H₂O:AC system was unsuccessful. The near complete loss of activity observed from the comparatively low concentrations of EtOH and AC discouraged further work with this system. Spray-drying from aqueous solutions with AC and AHC resulted in collapsed particles with some loss of activity. Catalase spray-dried with trehalose from an aqueous solution with AC resulted in protection of this activity. The use of MeOH:BA:H₂O systems were preliminarily investigated for spray-drying composites of catalase, and excipients trehalose, raffinose or HP- β -CD at a mass ratio of 1:9. On consideration of these experiments, the pH of the water component may have been high, and contributed to the loss of stability of the catalase composites. Investigation of the effects on process stability of spray-drying catalase:excipient from MeOH:BA:H₂O systems prepared using different pH of the water component may be an interesting case for future research.

Salmon calcitonin (as a therapeutically used peptide) was preliminarily spray-dried, from a MeOH:BA (80:20) system in a mass ratio of 5:95. NPMPs were produced. SCT in these composites lost some biological activity as assessed by Dr Sinead Ryan, with a statistically significant increase in EC₅₀.

7.1 INTRODUCTION

The research presented in this thesis, concerns the application of a spray-drying method, involving the use of a mixed solvent feed system and potentially a process enhancer (for example ammonium carbonate), to produce nanoporous/nano-particulate microparticles (NPMPs) of proteins, (hydrophilic) stabilising excipients and composites of these. This method, recently proposed and developed for low molecular weight drugs and excipients has advantages over current technologies in that it involves a simple one-step method and produces substantially pure microparticles (microparticles with no or only trace amounts of any additional processing excipient). The background to the spray-drying method proposed has been described in Chapter 1. Spray-drying of proteins or peptides using the method had not been attempted and investigations were thus initially empirical. The model protein lysozyme was used for first studies (Chapter 3). Difficulties anticipated with the proposed method were poor solubility and stability of proteins in co-solvent systems including organic solvents. These difficulties were investigated by attempting to increase the methanolic solubility of lysozyme by a freeze-drying method (Bromberg and Klibanov, 1995) and by assessment of process stability using FTIR, biological activity assay, SDS-PAGE and thermal analysis, following processing. Alternative proteins – trypsin and catalase were subsequently investigated (Chapter 6). Hydrophilic stabilising excipients, were investigated for two reasons - firstly because of their potential application in dry powder formulations of macromolecules as stabilisers or carriers, and secondly to augment existing knowledge regarding the use of the method with low molecular weight hydrophilic compounds. Prior to this research, the method was primarily developed for hydrophobic molecules including steroids, thiazides and sulfa-drugs (Tajber, 2005; McDonald, 2005; Nolan, 2008), with limited studies on hydrophilic excipients – cyclodextrins and PVP (Li, 2005). In the current project stabilising excipients - trehalose, raffinose, mannitol, and myo-inositol were spray-dried using a range of conditions (Chapter 4). Composite systems involving spray-drying lysozyme or other protein with excipient were also investigated (Chapters 5 and 6). Selected NPMPs and non-porous particles of equivalent material were characterised in terms of process stability, storage stability and particle characteristics, to determine advantages of NPMPs for inhalation delivery.

7.2 PRODUCTION OF NPMPs

Two solvent/feed systems, namely alcohol:H₂O:ammonium salt and MeOH:BA:(H₂O), were successfully used in this thesis to produce NPMPs of proteins and excipients alone or composites as outlined in Table 7.1. Attempts to use the method to spray-dry the large protein catalase were unsuccessful in terms of obtaining NPMPs (using the alcohol:H₂O:ammonium salt system) and in terms of process stability with the protein exhibiting catastrophic loss of activity (using both solvent systems).

Table 7.1 Compounds spray-dried as NPMPs from different solvent feed systems in the present work, and mass ratio of NPMP composites.

Compound	Feed system	Mass ratio (composites)
Lysozyme	MeOH:H ₂ O:AC EtOH:H ₂ O:AC EtOH:H ₂ O:AHC EtOH:H ₂ O:AF EtOH:H ₂ O:AA	
Trypsin	EtOH:H ₂ O:AC	
Trehalose	MeOH:BA MeOH:BA:H ₂ O	
Raffinose	MeOH:BA MeOH:BA:H ₂ O	
Trehalose:HP-β-CD	MeOH:BA	
Lysozyme:trehalose	EtOH:H ₂ O:AC MeOH:BA	9:1, 4:1, 7:3, 1:1 1:4
Lysozyme:raffinose	EtOH:H ₂ O:AC MeOH:BA	1:1 1:4, 3:7
Lysozyme:HP-β-CD	MeOH:BA	1:4
Lysozyme:inulin	EtOH:H ₂ O:AC	1:1
Lysozyme:myo-inositol	EtOH:H ₂ O:AC	1:1
Lysozyme:PVP	EtOH:H ₂ O:AC	9:1
Trypsin:trehalose	MeOH:BA:H ₂ O	1:9, 1:4, 1:1
Trypsin:raffinose	MeOH:BA:H ₂ O	1:9, 1:4, 1:1
Trypsin:HP-β-CD	MeOH:BA:H ₂ O	1:9, 1:4, 1:1
Salmon calcitonin:trehalose	MeOH:BA	5:95 (1:19)

7.2.1 NPMPs of lysozyme, trypsin from alcohol:H₂O:ammonium salt

The success in producing NPMPs of the (hydrophilic) proteins from these systems, does not concur with the hypothesis for NPMP production using the proposed spray-drying method (based on experiments with low molecular weight compounds), introduced in Chapter 1 and also described in section 7.2.2, in that the more volatile solvent (for example EtOH or MeOH) would be a poor solvent for the protein and the less volatile solvent (H₂O) would be a good solvent. A different hypothesis for NPMP production is thus proposed for these systems, at the end of this section. The EtOH:H₂O:AC was the predominantly studied system, and as such is primarily discussed.

Addition of EtOH (in concentrations of 70, 75, 80% v/v) and AC (in concentrations of 5 and 10% of t.s.c.) to lysozyme in the aqueous component of the feed, resulted in a phase separation in the feed (Chapter 3). This was observed as a transition from a clear solution to a fine white cloudy

suspension appearance, which occurred almost immediately following addition of EtOH and AC (Appendix 8). The suspension was stable, and did not settle out under gravity for the duration of the spray-drying experiments (from ~5 - ~30 minutes, depending on the volume spray-dried). Phase separation in trypsin feed systems, also occurred quickly on addition of EtOH (in concentrations of 80, 90, 95, 98% v/v) and AC (in concentrations of 10, 20, 50, 60% of t.s.c.). The suspension had a similar white cloudy appearance, although some larger and coarser particles were observed and deposited on the walls of the feed vessel (Appendix 8).

Three factors of the feed system contributing to the phase separation of lysozyme from the EtOH:H₂O:AC system, were identified in Chapter 3 (Section 3.3.3), being the effect of altering the pH of the solution towards the isoelectric point (isoelectric precipitation/phase separation), a salting-out effect as a result of cations and anions (NH₄⁺ and CO₃²⁻ contributed by ammonium carbonate), and the addition of anti-solvent (EtOH). All of these factors may also be responsible for the phase separation seen in trypsin feed systems, and in the case of alternative ammonium salts, and alcohol used for lysozyme systems.

Phase separation of the protein from the alcohol:H₂O:ammonium salt system was required to produce NPMPs on spray-drying, as NPMPs were not produced from any feed system which did not show this. The observation of phase separation in the feed material, did not guarantee NPMP production on spray-drying however, which depended on the concentration and type of alcohol and ammonium salt, and on the particular protein (Table 7.2). The following general observations were made, based on spray-drying experiments:

- A higher % of AC was required when MeOH was used in place of EtOH, to spray-dry NPMPs of lysozyme. This suggests that EtOH, (which is a stronger precipitating alcohol, as it is more hydrophobic) may be a better choice, as less process enhancer is required. Another advantage of EtOH is that it is more pharmaceutically acceptable due to a better safety profile.
- Ammonium carbonate was identified from studies with lysozyme as the best ammonium salt to use, because it was effective for producing NPMPs at lower concentrations than the other salts (ammonium formate and ammonium acetate), and also due to the fact that it is vaporised at a lower temperature and thus removable during the spray-drying process.
- Higher concentrations of EtOH and AC were required to produce NPMPs of trypsin compared to lysozyme.
- Lysozyme and trypsin have molecular weights of respectively 14.3 kDa, 23.5 kDa, isoelectric points of respectively 11.35, 10.1-10.5, and tend to be monomeric, suggesting suitability of the method for proteins with similar physicochemical characteristics.
- Spray-drying conditions for example inlet drying temperature, type of drying gas, type of cyclone were found to have less impact, than feed conditions, on the production of NPMPs.

Table 7.2 Selected feed conditions (alcohol:H₂O:ammonium salt) studied for lysozyme and trypsin producing NPMPs.

Protein	Alcohol	Ammonium salt
Lysozyme	EtOH – 70, 75, 80%	AC – 10%
	EtOH – 80%	AA – 50%, 60%
	EtOH – 80%	AF – 40%, 50%, 60%
	MeOH – 65%, 70%, 75%, 80%	AC – 40%
Trypsin	EtOH – 95%, 98%	AC – 40%, 50%, 60%

Parallels between spray-drying of lysozyme or trypsin from EtOH:H₂O:AC feed systems and drying of proteins by some supercritical fluid (SCF) processing methods for example as described by Thiering et al. (2000), and Bouchard et al. (2007a), can be drawn based on the fact that phase separation/precipitation occurs in the two methods as a result of ethanol (organic solvent), and process enhancer (for the spray-drying method ammonium carbonate and for the SCF methods supercritical CO₂). [Supercritical fluids are materials whose temperatures and pressures are higher than the corresponding critical points (highest temperature and pressure at which a liquid phase may co-exist in equilibrium with a separate vapour phase)]. Thiering et al. (2000) described the production of dry lysozyme powders from 95% EtOH:5% H₂O solution, using CO₂ as antisolvent. The morphology of protein particles was insensitive to changes in operating conditions such as temperature, and solute concentration, but were significantly affected by the solvent system. The predominant effect of feed conditions on particle morphology, compared to very minor effects of temperature change or cyclone choice was noted when spray-drying lysozyme in the current work (Chapter 3). This was in contrast to marked effects described by Nolan (2008) and Mc Donald (2005) when spray-drying low molecular weight hydrophobic compound using the EtOH:H₂O:AC system.

Bouchard et al. (2007a) described lysozyme particle formation during supercritical fluid drying, from aqueous solution using ethanol (as co-solvent for CO₂) and CO₂ as supercritical fluid (as anti-solvent to precipitate the protein). It was found that under fast protein precipitation conditions (high EtOH flow rates or high EtOH:CO₂ ratio) agglomerated nanoparticles were produced. Microspheres, or microparticles (with nanoparticles in the shell or with a spongy core) were obtained using different conditions. The microparticles with nanoparticles described in this paper, also resembled the fused nano-particulate outer appearance with hollow core of lysozyme particles produced from 80% EtOH, 10% AC (Chapter 3).

The following hypothesis for formation of NPMPs of lysozyme and trypsin from EtOH:H₂O:AC systems is proposed, based on the results of the present work, and examination of surface morphology from SEM analysis (appearance of fused irregular shaped nanoparticles), and is shown schematically in Figure 7.1. A similar mechanism may be involved in the case of other alcohol or ammonium salt systems, except that for other ammonium salts investigated (AA, and AF), the salt would not be expected to be completely volatilised during the spray-drying process and there is potential for residual contamination of formic or acetic acid respectively in the dried particle.

- The protein separates in the feed solution, as a result of AC, isoelectric phase separation/precipitation and alcohol. This can occur either as a solid-liquid phase separation with formation of amorphous primary particulates or a liquid-liquid phase separation resulting in a protein-rich phase and a protein poor phase.
- The feed system is atomised producing high surface area droplets. In the case of solid-liquid phase separation, the droplet would contain primary particulates as suspended particles, and in the case of a liquid-liquid phase separation the droplet would contain areas of protein-rich phase and protein poor phase.
- Droplet drying continues and ethanol and water evaporate, and AC is vaporised. In the case that a solid-liquid separation was initially involved, the particulates are already suspended in the droplet; in the case that a liquid-liquid phase separation was involved, the protein will condense out from the protein-rich phase forming suspended material, also resulting in suspended primary particles.
- Small amounts of protein remaining dissolved in the first case, or in the protein poor phase in the second case, will also precipitate out as drying continues and may form a fragile network, keeping the primary particles agglomerated to each other either at the particle surface or throughout the particle, and resulting in the fused nano-particulate appearance of the spray-dried particles.
- The evidence from spray-drying experiments with lysozyme suggests that NPMPs were hollow or with internal voids as noted from SEM analysis of broken lysozyme particles (Chapter 3).

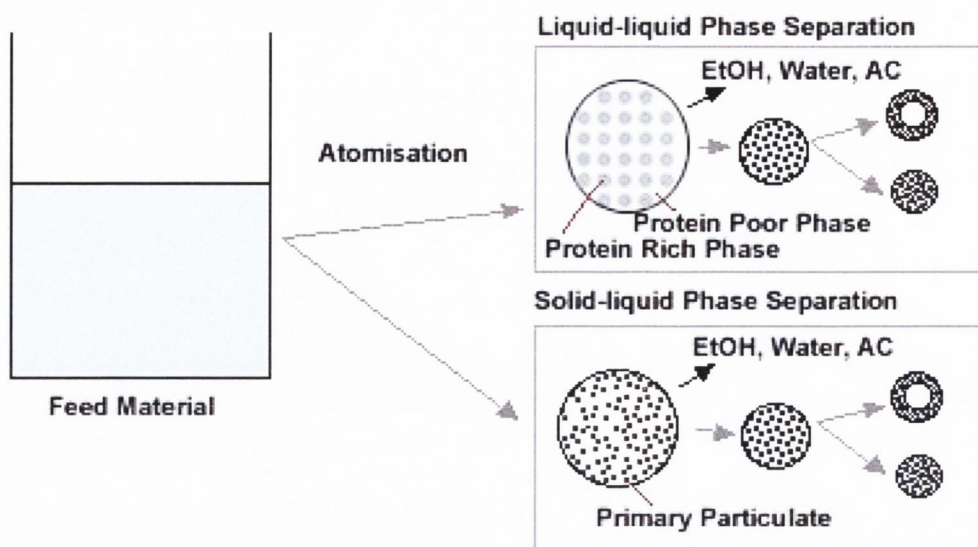


Figure 7.1 Schematic of porous particle formation in the case of lysozyme or trypsin, spray-dried from EtOH:H₂O:AC systems.

Attempts to estimate the particle size in the feed material using Malvern particle sizing (using the liquid cell) were inconclusive, because of difficulties in replicating the conditions in the feed material in the liquid cell, and may warrant further investigation to further explore this mechanism.

The formation of crystalline structure requires specific solvent conditions and long periods of time for the globular proteins to orient themselves in a crystalline structure (Thiering et al., 2000). A rapid non-specific aggregation process will conversely tend to form amorphous and irregular to spherical shaped particulates (Bouchard et al., (2007a); Krebs et al., (2007)). The rapid phase separation encountered in this research, would be expected to produce amorphous and irregular or spherical particulates. The amorphous nature of spray-dried lysozyme and trypsin material (as for unprocessed lysozyme and trypsin) was confirmed by XRD studies.

7.2.2 NPMPs of trehalose, raffinose from MeOH:BA:(H₂O)

Spray-drying of sugars is predominantly reported from aqueous solutions in the literature. Amorphous forms of trehalose and raffinose following spray-drying from aqueous solutions have been reported (Moran and Buckton, 2007; Maury et al., 2005; Surana et al., 2004, and Lechuga-Ballesteros et al., 2003). The solubility of sugars in aqueous solution is generally very high (e.g. trehalose 68.9g/100ml, raffinose 14g/100ml (Pharmaceutical excipients, 2006), resulting in late precipitation from the droplet in the drying process. The metastable zone (the solute concentration above the solubility but below unstable supersturation conditions where spontaneous crystallisation is probable) of sugars is also normally wide and high levels of supersaturation may be reached before precipitation occurs (Bouchard et al., 2007b). Trehalose and raffinose are expected to have lower solubility in MeOH:BA systems than aqueous solutions, and also to undergo faster precipitation as a result of evaporation of MeOH and drop in solubility in BA (according to the hypothesis for NPMP production – see below) and thus the time required to achieve saturation will be reduced. Differences between drying times, and time to saturation in the droplet depending on feed conditions, are postulated to contribute to the differences in morphology of the sugars spray-dried from different conditions, and the thermal behaviour of spray-dried trehalose samples (Chapter 4).

Physicochemical properties of the sugar, such as solubility, T_g, nucleation, crystallisation and viscosity (of the solution) are expected to influence behaviour during processing (Bouchard et al., 2007b) and thus spray-dried product characteristics, in terms of morphology and solid-state characteristics. Trehalose and raffinose are crystalline in the unprocessed form and exist as stable hydrates (trehalose as a dihydrate and raffinose as a pentahydrate), although lower hydrates or anhydrous metastable crystalline forms have been described in the literature (Kilburn et al., 2006; Hogan and Buckton, 2001; Saleki-Gerhardt et al., 1995). DSC analysis of both unprocessed sugars, at a heating rate of 10 °C/min, showed dehydration, at a slightly lower temperature for raffinose pentahydrate (~84 °C) than for trehalose dihydrate (~91-97 °C, depending on the batch and with a possible particle size effect). Spray-dried sugars as NPMPs or as non-porous discrete particles spray-dried from aqueous solution were amorphous by XRD. T_g of spray-dried amorphous forms of the two sugars were high and similar to each other – from the work in this thesis these were ~124 °C (trehalose) and ~120 °C (raffinose). The two sugars were also soluble in methanol (higher for raffinose, allowing a higher t.s.c. to be used in spray-drying experiments), and could as such be dissolved in the MeOH component of the MeOH:BA system. Similarity between the chemical structures of the sugars are also apparent (Section 1.6) as trehalose is comprised of two glucose molecules, and raffinose is comprised of galactose, glucose and fructose. The following features may be important in the production of high T_g amorphous NPMPs of the sugars

– solubility in MeOH or MeOH:H₂O to allow dissolution in the feed system prior to the addition of BA, and poor solubility in BA.

The MeOH:BA solvent system or the MeOH:BA:H₂O solvent system, was used to produce NPMPs of trehalose and raffinose alone. The feed and spray-drying conditions required for production of NPMPs of the two sugars were similar and found to depend on solvent composition. Non-spherical, oblong or torroidal porous particles of the two sugars were obtained on spray-drying from 90:10 MeOH:BA, and spherical porous particles were obtained on spray-drying from 80:20, 70:30 and 60:40 MeOH:BA. Spherical porous particles of trehalose were spray-dried from 50:50 MeOH:BA, whereas a mixture of spherical porous particles and fused particles of raffinose were spray-dried from this solvent ratio. Different solvent conditions were employed for the two sugars spray-dried from MeOH:BA:H₂O (49:49:2 was used to produce NPMPs of trehalose, and 79:19:2 was used to produce NPMPs of raffinose). Spherical NPMPs of trehalose were spray-dried, and slightly irregular shaped to spherical porous particles of raffinose were spray-dried. [A mix of some porous particles and some large non-porous rods of mannitol were also spray-dried from MeOH:BA:(H₂O) systems. Filtering the feed material to attempt to remove some of the suspended material was unsuccessful. This system may require further modification to check whether NPMPs could be reproducibly spray-dried without the problems of mixed morphology, either by using feed conditions or by formulation with other excipients compounds in a composite.]

Phase separation was not generally observed in MeOH:BA feed systems producing NPMPs of sugars alone (except in one example of raffinose spray-dried from MeOH:BA solvent ratio 60:40 and to a greater extent from MeOH:BA solvent ratio 50:50, resulting in particles of mixed morphology including some porous and some non-porous fused particles, following spray-drying), and was seen in varying degrees (from no phase separation and spray-drying of clear solution to phase separation and spray-drying of a slightly turbid or cloudy suspension) in some of the systems or batches producing lysozyme:excipient NPMP composites. Phase separation was observed in some systems or batches using the MeOH:BA:H₂O systems to produce NPMPs of the sugars and trypsin:excipient composites, and was also observed (to a greater extent) in systems (e.g., 15:15:1 used for spray-drying trehalose) resulting in a mixed morphology of porous particles and non-porous material. Phase separation was thus not directly related to the formation of NPMPs from these systems.

The following hypothesis for formation of NPMPs of trehalose and raffinose from MeOH:BA:(H₂O), based on the solubility of the compound(s) and volatility of solvents, and initially proposed by Corrigan (2005), Nolan (2008) based on experiments with low molecular weight drugs (see Chapter 1), is shown schematically in Figure 7.2. The more volatile solvents

(MeOH and H₂O) are better solvents for the sugar and the least volatile solvent (BA) is a poor solvent.

- During the atomisation stage of the spray-drying process, droplets are formed containing the sugar in the co-solvent mix.
- Rapid drying of these droplets proceeds on contact with the warm drying gas and the more volatile solvent phase (MeOH with a b.p. of 65 °C, H₂O with a b.p. of 100 °C) in which the sugar is more soluble, may evaporate to a greater extent, resulting in the droplet becoming richer in the less volatile solvent component (BA with a b.p. of 126 °C), in which the sugar is less soluble. The fall in the solubility of the sugar may be dramatic and it may condense out initially as a nano-sized liquid phase within the droplet. The sugar becomes in effect the dispersed phase in an external phase.
- As drying proceeds and further solvent loss occurs, the sugar phase droplets become less fluid and come closer together, and the sugar may precipitate out as primary nanoparticles and agglomerate together either at the particle surface or within the particle, leading to nano-particulate microparticle formation.
- The evidence from spray-drying trehalose and raffinose in this thesis (Chapter 4) suggests that particles were not hollow, and comprised of fused spherical nanoparticles, throughout the particle.

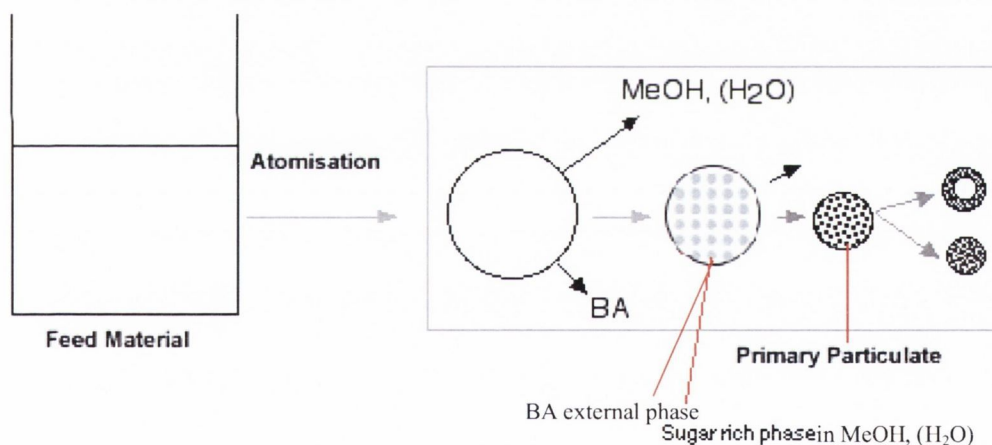


Figure 7.2 Schematic of porous particle formation in the case of trehalose or raffinose spray-dried from MeOH:BA:(H₂O).

A mechanistic difference between production of NPMPs from the two solvent systems (i.e., trehalose or raffinose from MeOH:BA:(H₂O) and lysozyme or trypsin from alcohol:H₂O:ammonium salt, may be in terms of timing of phase separation during the drying process, either occurring before atomisation in lysozyme or trypsin based systems spray-dried from alcohol:H₂O:ammonium salt systems or following atomisation and during droplet drying for the sugar or cyclodextrin based systems spray-dried from MeOH:BA:(H₂O) systems. There may be

some cross-over between the mechanism of NPMPs using the two solvent feed systems, as was evident from the phase separation seen in some of the systems spray-dried from MeOH:BA:(H₂O).

7.2.3 NPMP composites or co-spray-dried systems

Co-spray-dried NPMP systems may involve a more complicated production mechanism than single component systems. The concept of the Peclet number was introduced in Chapter 1, and describes the ratio of characteristic times involved in the drying of a compound from a droplet. This is important when considering co-spray-dried systems because two components may have different Peclet numbers, resulting in different diffusional velocities within an evaporating droplet, which may ultimately lead to separation of components within a microparticle (Vehring, 2007). For compounds with high Peclet numbers (> 1), τ_d (the time required for a droplet to dry) is small and the surface moves faster than the dissolved or suspended components (larger R^2/D - the time required for a solute or a nano-particle to diffuse from the edge of the droplet to its centre) and as such the surface becomes enriched with the component associated with the high Peclet number. The resulting surface accumulation and formation of a thin shell are intuitive in this case (Tsapis et al., 2002; Vehring, 2007). For compounds with low Peclet numbers (for example saccharides dried from aqueous solutions at moderate to low drying gas temperatures), surface enrichment is small and the characteristic time for the solutes to reach saturation at the surface is close to the droplet lifetime, resulting in solid particles (Vehring, 2007). The Peclet number associated with a component, may change during drying, however, in particular in co-solvent systems where one solvent evaporates faster than the other (Vehring, 2007). Surface activity (for example in the case of a surface active protein) of one component may also result in surface enrichment with that component. Millqvist-Fureby et al. (1999) spray-dried trypsin with various excipients, and observed that trypsin was over-represented at the particle surface. Another separation mechanism may be the sequential precipitation of components based on different characteristic times to saturation (Vehring, 2007), which may be particularly pertinent in protein:sugar systems spray-dried in this thesis.

The lysozyme:trehalose, lysozyme:raffinose and trypsin:trehalose composites are used as examples, in discussing production of co-spray-dried composites from the two solvent feed systems. A similar discussion may also describe production of other protein:excipient composites using the spray-drying method.

Considering the lysozyme or trypsin:trehalose composites spray-dried from the EtOH:H₂O:AC (Chapters 5 and 6), the protein would be expected to precipitate in the feed system (from experiments with spray-drying the protein alone). The hydrophilic sugar would not be expected to precipitate in the feed system (from experiments with spray-drying trehalose alone) and would also

not be expected to precipitate rapidly during droplet drying (on consideration of the hydrophilic nature of the sugar and the volatility of the solvents). The sugar would be expected to have a lower Peclet number than the phase-separated protein component, although it may also have a high Peclet number as was evident from the hollow thin walled particles of trehalose spray-dried from EtOH:H₂O:AC. The protein component of the co-spray-dried system would thus be expected to be the main shell former, depending on the mass ratio of protein:excipient. Surface morphology in the case of lysozyme:trehalose co-spray-dried systems up to a ratio of 1:1 was similar to lysozyme NPMPs spray-dried alone. The morphology of trypsin NPMPs was much more strongly affected by the inclusion of sugar, with loss of porous morphology with the inclusion of low mass ratio of trehalose however. The distribution of the components in a co-spray-dried composite material may warrant further investigation, for example by electron spectroscopy for chemical analysis (to probe the elemental composition of the powder surface), as described by Millqvist-Fureby et al. (1999). In this paper the authors calculated the surface coverage of trypsin in trypsin:sugar composites, from comparison of the nitrogen content of pure trypsin as measured by ESCA and the nitrogen content of the spray-dried composite samples.

In the cases of lysozyme:trehalose or lysozyme:raffinose systems spray-dried from MeOH:BA, both the protein and the excipient would be expected to show rapid precipitation either before atomisation due to the addition of hydrophobic solvent (BA) or during droplet drying, as a result of a drop in solubility in the BA component following evaporation of MeOH, and as such a less pronounced difference in distribution of the sugar and protein in the particle, compared to the EtOH:H₂O:AC system, may be expected. Examination of particle morphology showed that the composite particle had a similar fused nanoparticulate surface appearance as sugars spray-dried alone, up to a mass ratio of 3:7 (lysozyme:raffinose), although surface morphology appeared more compact (with smaller gaps/pores). This suggests that the saccharide and protein influenced the surface morphology in the higher saccharide content composites. SE micrographs of mass ratio composite of 1:1 resembled the spherical non-porous particles, with a fused rough surface morphology similar to lysozyme spray-dried alone from MeOH:BA solvent system. For trypsin:trehalose composites spray-dried from MeOH:BA:H₂O, NPMPs were produced to a higher ratio of 1:1 than for the lysozyme:excipient composites spray-dried from MeOH:BA. NPMPs also had a looser appearance. A greater phase separation in these feed systems compared to lysozyme:excipient systems, particularly in the highest protein content composites (1:1) may have influenced the morphology of the particles. A more uniform distribution of sugar and protein throughout the composite particle is hypothesised, compared to the EtOH:H₂O:AC system.

7.3 SUITABILITY OF NPMPs PRODUCED AS A POTENTIAL DRUG DELIVERY SYSTEM

Suitability of NPMPs produced, as potential novel dry powder protein delivery systems for inhalation, involves consideration of the process stability, storage stability and particle characteristics of investigated proteins, excipients and co-spray-dried composite systems.

7.3.1 Process stability and storage stability of NPMPs

The amorphous or crystalline nature, the thermal behaviour and residual moisture or solvent content of NPMP powders may affect the physical stability of particles and also the chemical stability of proteins or protein:excipient composites, and have been described in relevant sections of this thesis. The glassy stabilisation and the water replacement hypothesis for the stabilisation of proteins by excipients favours amorphous character of stabilising excipients in a composite powder (Lee, 2002a; Arakawa et al., 2001). All protein and composite NPMPs, produced in this thesis, were amorphous following spray-drying except for lysozyme:myo-inositol spray-dried from EtOH:H₂O:AC.

Hygroscopicity of sugar NPMPs was observed under high humidity conditions, alone (Chapter 4) and in composite systems (Chapters 5, 6). This may represent a limitation to the use of trehalose or raffinose as sole excipient, in terms of long-term storage, without desiccation. Separation and crystallisation of trehalose in the lysozyme:trehalose (1:1) composites was observed in stability studies at 25 °C/60% R.H. and collapse of particle structure was observed at 40 °C/75% R.H.. A rapid collapse of particle structure was observed for lysozyme:trehalose (1:4), lysozyme:raffinose (1:4), trypsin:trehalose (1:4) and trypsin:raffinose (1:4) NPMPs stored at 25 °C/60% R.H. overnight, but not for lysozyme:HP- β -CD (1:4) or trypsin:HP- β -CD (1:4). No crystallisation or loss of particle structure and morphology were identified in lysozyme:excipient or trypsin:excipient (1:4) NPMP composites stored at 4 °C or 25 °C with desiccant for 12 weeks. Maintaining physical storage stability of composite NPMPs including trehalose or raffinose may be possible, if a desiccant is included in the delivery device.

Process stability of proteins was primarily assessed by biological activity assay, and also by FTIR, SDS-PAGE and thermal analysis. Excellent process stability, from investigation of biological activity, was observed for lysozyme spray-dried alone or as composite with excipients using the two solvent systems of the spray-drying method. Evidence that reversible structural changes occurred included evidence of changes in solid state FTIR and DSC analysis of lysozyme spray-dried alone, and no change in biological activity and solution state FTIR.

Trypsin NPMPs spray-dried from EtOH:H₂O:AC, showed poorer process stability from activity assay, and FTIR, which may have been influenced by differences in the higher EtOH and AC

concentration required to produce NPMPs of trypsin, as well as the lability of the proteins to the stresses involved. Comparison of biological activity of trypsin spray-dried alone or as composites from EtOH:H₂O:AC, to composites of higher excipient content from MeOH:BA:H₂O, showed a higher retention of activity for the latter systems. Reasons may be due to different stresses posed by the feed systems. An increased thermostability of proteins dissolved or suspended in very low or non-aqueous systems has been described in the literature (Walsh, 2002). This may have contributed to the increased stability of trypsin spray-dried from the MeOH:BA:H₂O. The higher stabilising excipient content used in these systems may also contribute to the increased stability. The high process stability of both lysozyme and trypsin in composite particles spray-dried from MeOH:BA:(H₂O) suggests potential wider applicability of this method, than for the EtOH:H₂O:AC method.

The process stability of NPMPs of sCT:trehalose was only preliminarily assessed, and as described in Chapter 6 showed some decrease in activity compared to unprocessed sCT, as was evident from a statistically significant reduction in the EC₅₀, using the SSlogis model to fit the data. Re-plotting the data in Prism software using the dose-response curve (variable slope) option also showed a similarly higher EC₅₀ for the sCT:trehalose, but this was not statistically significant at a significance level of $\alpha = 0.05$. A higher Hill slope of the sCT:trehalose composite was calculated, this may also be relevant when comparing biological activity. Literature data suggests that sCT can adopt different conformations in a range of co-solvents and in particular α -helical structures with an N-terminal loop was observed in MeOH (Meadows et al., 1991; Arvinte and Drake, 1993). Arvinte and Drake (1993) also assessed the reversibility of conformation and found that fluorescence and circular dichroism changes found in sCT and human calcitonin (hCT) in MeOH were reversible. In-vivo biological activity following hCT exposure to 99% MeOH showed no loss of activity (Arvinte and Drake, 1993). Investigation of structural changes as well as biological activity assay, corrected for sCT content in test solutions, may allow further elucidation of the suitability of formulation of this peptide in NPMP composites. The use of alternative feed conditions (such as different concentrations of MeOH or BA or different solvents for example EtOH or propyl acetate) and stabilising excipient combinations (for example trehalose and HP- β -CD, or raffinose and HP- β -CD) should also be considered.

7.3.2 Particle characteristics and suitability for pulmonary delivery

NPMPs are proposed to be particularly suitable for pulmonary delivery and in particular dry powder inhaler formulation (Tajber, 2005; Nolan, 2008). The importance of the aerodynamic behaviour of dry powder and the influence of physical size, density, shape and surface properties of particles and resulting interparticulate forces, on efficient inhaler delivery has been discussed in Chapter 1. Particle characteristics – size, surface area, bulk and tapped density, and in-vitro deposition behaviour (fine particle fraction, particle fraction $< 3\ \mu\text{m}$, MMAD and GSD) of a selection of NPMPs produced were investigated, and in the case of single component systems compared to examples of non-porous spray-dried particles of similar material (Chapters 3, 4). In-vitro deposition data showed the advantage of NPMPs produced over non-porous comparison systems in terms of fine particle fraction and particle fraction $< 3\ \mu\text{m}$. Higher specific surface area values (which may be indicative of increased surface asperities (Zeng et al., 2001)) were also obtained for porous compared to non-porous particles of the same material, although these differences varied depending on the material (for example lysozyme NPMPs showed an increase $\sim 2\text{-}3$ fold compared to non-porous lysozyme, whereas trehalose or raffinose NPMPs showed an increase of ~ 40 fold compared to non-porous material). Bulk and tapped densities were lower for porous compared to non-porous sugars, but were higher for porous lysozyme compared to non-porous lysozyme spray-dried with AC. Particle characteristics and in-vitro deposition data of all NPMPs produced are compared (Table 7.3), to ascertain whether any general trends could be observed in these data.

A strong linear correlation was observed between fine particle fraction ($< 5\ \mu\text{m}$) and particle fraction $< 3\ \mu\text{m}$ ($R^2 = 92.6\%$, statistically significant). Otherwise, only weak or no correlations were observed when plotting fine particle fraction against MMAD ($R^2 = 52.2\%$, statistically significant), bulk density ($R^2 = 25\%$, not statistically significant), tapped density ($R^2 = 18.6\%$, not statistically significant) or surface area ($R^2 = 0\%$, not statistically significant). Insufficient data was obtained to investigate any possible correlation with particle sizing as determined by laser diffraction (only 3 NPMP systems). The lack of correlation in the case of surface area and fine particle fraction is in contrast to a strong positive correlation described by Nolan (2008), with NPMPs of sulfa compounds, sodium cromoglicate and sulfa or sodium cromoglicate:PVP composites.

The lowest surface area measurements for NPMPs were obtained for lysozyme and lysozyme:trehalose (mass ratio 1:1) spray-dried from EtOH:H₂O:AC. These were significantly lower than NPMPs of trehalose or raffinose or HP- β -CD alone or lysozyme:excipient (mass ratio 1:4) composites, spray-dried from MeOH:BA. Inclusion of lysozyme in composites with raffinose and HP- β -CD (but not trehalose) also reduced the surface area compared to the excipient spray-

dried alone, which may have been as a result of the more compact surface appearance as described in Chapter 5. Inclusion of lysozyme in these systems also resulted in increased bulk and tapped density compared to the excipients spray-dried alone.

Table 7.3 Particle characteristics of NPMPs produced; * HP- β -CD spray-dried as comparison system, originally spray-dried as NPMPs by Li (2005).

	Particle size μm d(0.5)-Malvern <i>Average-SEM</i>	Surface area m^2/g	Bulk density g/cm^3	Tapped density g/cm^3	MMAD μm (<i>GSD</i>)	Fine particle fraction %	Particle fraction < 3 μm %
EtOH:H₂O:AC							
Lysozyme	1.785 ± 0.011 2.16 ± 0.87	6.7867 [6.5457, 7.0277]	0.270 ± 0.011	0.420 ± 0.017	2.83 ± 0.23 (2.01 ± 0.48)	57.98 ± 4.48	32.55 ± 3.72
Lysozyme:trehalose 1:1	2.278 ± 0.052 -	4.756 [4.7575, 4.7542]	0.211 ± 0.011	0.300 ± 0.015	2.72 ± 0.05 (1.99 ± 0.09)	63.98 ± 2.75	39.15 ± 1.99
MeOH:BA							
Trehalose	2.48 ± 0.04 1.29 ± 0.77	44.4854 [44.1342, 44.8366]	0.075 ± 0.001	0.124 ± 0.001	3.144 ± 0.616 (2.358 ± 0.32)	40.17 ± 6.76	24.17 ± 4.86
Raffinose	2.96 ± 0.053 -	44.3023 [43.1865, 45.4180]	0.080 ± 0.003	0.122 ± 0.004	2.676 ± 0.800 (2.118 ± 0.171)	54.8 ± 11.5	33.56 ± 8.82
*HP- β -CD	-	75.2253 [74.8092, 75.6413]	0.193 ± 0.006	0.289 ± 0.003	-	-	-
Lysozyme:trehalose 1:4	- 0.98 ± 0.64	43.7426 [45.9236, 41.5615]	0.143 ± 0.008	0.245 ± 0.002	2.77 ± 0.35 (2.03 ± 0.06)	62.34 ± 3.91	37.20 ± 3.51
Lysozyme:raffinose 1:4	- 1.58 ± 0.63	25.4586 [25.3166, 25.6006]	0.158 ± 0.010	0.278 ± 0.006	2.92 ± 0.15 (2.04 ± 0.02)	50.05 ± 5.21	27.47 ± 2.82
Lysozyme:HP- β -CD 1:4	- 1.02 ± 0.58	54.5729 [54.2213, 54.9245]	0.237 ± 0.012	0.356 ± 0.004	2.47 ± 0.42 (2.01 ± 0.28)	59.81 ± 2.16	35.98 ± 1.42
MeOH:BA:H₂O							
Trypsin:trehalose 1:4	- 1.51 ± 0.74	-	0.088	0.160	2.83 [2.92, 2.74] (2.29 [2.23, 2.34])	42.71 (41.57, 43.86)	26.21 (24.80, 27.62)
Trypsin:raffinose 1:4	- 1.79 ± 0.81	-	0.065	0.118	2.76 [2.99, 2.52] (2.51 [2.56, 2.46])	44.82 (39.96, 49.68)	29.60 (25.53, 33.68)
Trypsin:HP- β -CD 1:4	- 1.84 ± 1.17	-	0.095 ± 0.002	0.152 ± 0.006	3.18 [3.329, 3.031] (2.65 [2.55, 2.76])	41.55 (43.08, 40.02)	27.82 (29.22, 26.42)

Fine particle fractions (FPF) of NPMPs produced were compared by ANOVA (Figure 7.3). The NPMPs with the highest FPF were lysozyme:trehalose (1:4), lysozyme:HP-β-CD (1:4) and raffinose spray-dried from MeOH:BA and lysozyme and lysozyme:trehalose (1:1) spray-dried from EtOH:H₂O:AC. Lower FPFs were obtained for lysozyme:raffinose (1:4) and trehalose spray-dried alone from MeOH:BA and for trypsin:excipient (1:4) spray-dried from MeOH:BA:H₂O. The influence of the cyclone (regular cyclone) and effect on collected particle size of lysozyme:raffinose (1:4) composite compared to lysozyme:trehalose (1:4) or lysozyme:HP-β-CD (1:4) spray-dried with the HP cyclone, has been previously noted (Chapter 5).

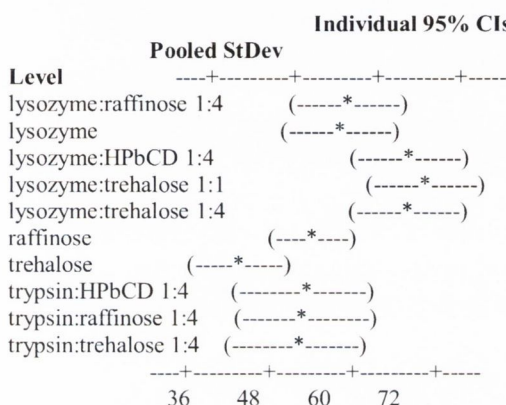


Figure 7.3 ANOVA analysis of fine particle fraction of NPMPs produced.

ANOVA uses a pooled standard deviation, and assumes a similar variability for all data sets. ACI experiments of NPMPs involved two different methods of analysis – the micro BCA assay for all systems including a protein, and fluorescence detection for sugars (including a fluorescent marker). In the event that these methods of analyses contributed to different variability, all ACI of NPMPs using micro BCA assay (i.e., protein or protein:excipient NPMPs) were compared (Figure 7.4). To take account of any differences in variability of particles as a result of method of production, all NPMPs produced from MeOH:BA systems (i.e., trehalose, raffinose and lysozyme:excipient composites) were also compared separately using ANOVA (Figure 7.5). These data suggest that the lysozyme composite NPMPs spray-dried from MeOH:BA systems with the HP cyclone resulted in statistically significantly higher FPF than trypsin composite NPMPs spray-dried from MeOH:BA:H₂O systems also using the HP cyclone. Lysozyme and lysozyme:trehalose spray-dried from EtOH:H₂O:AC also showed statistically significantly higher FPF than composite trypsin NPMPs spray-dried from MeOH:BA:H₂O. The looser surface morphology and SEM evidence of deviation from sphericity and particle-particle contact of trypsin composite systems may have influenced the lower FPF calculated.

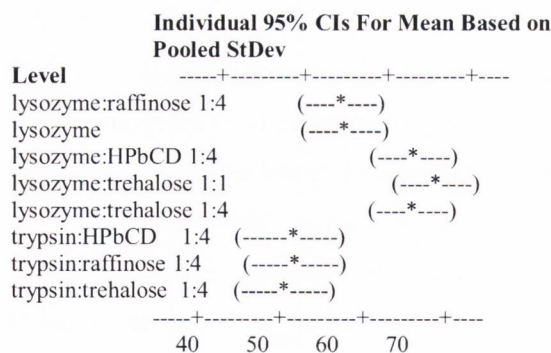


Figure 7.4 ANOVA analysis of fine particle fraction of NPMPs including protein and analysed by micro BCA analysis.

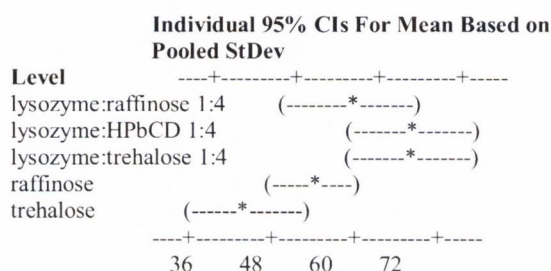


Figure 7.5 ANOVA analysis of fine particle fraction of NPMPs produced from MeOH:BA system.

The issue of hygroscopicity of sugars may be relevant in the discussion of pulmonary delivery of composites of high sugar (trehalose and raffinose) content. Edwards (1997) compared two polymer formulations of large porous particles in terms of respirable fraction determined in a cascade impactor – one type exhibiting hygroscopicity (consisting of poly(lactic acid-co-lysine-graft-lysine), and the other type no hygroscopicity (poly(lactic-co-glycolic acid)). The relative humidity was not specified in this paper. No statistically significant difference was observed and the absolute particle mass density rather than particle chemistry or hygroscopicity was considered the primary determinant of the relatively high respirable fractions observed. The lungs have a very high relative humidity (~ 99.5% at 37 °C) (Telko and Hickey, 2005) however, which is considerably higher than for example the ambient relative humidity in the laboratory at which ACI measurements were carried out (generally ~ 40% R.H., higher in summer months). Hygroscopic particles may be subject to particle growth and loss of particle structure following inhalation, which may in turn affect lung deposition (Telko and Hickey, 2005). Formulation of several excipients together for example trehalose with HP-β-CD as proposed in Chapter 4 may take advantage of the qualities conferred by different excipients, and result in decreased hygroscopicity, and may warrant further investigation.

7.4 MAIN FINDINGS OF THE THESIS

- NPMPs of lysozyme and trypsin alone and composites of lysozyme with stabilising excipients were spray-dried from EtOH:H₂O:AC systems. Substantially pure porous particles of proteins had not been previously described in the literature by a spray-drying method. Susceptibility to stresses of the method depended on the protein, with trypsin showing lower process stability than lysozyme. This solvent feed system may be particularly suitable for proteins with lower molecular weights (~ 20 kDa) and high pI (~10–11), similar to lysozyme and trypsin model proteins, considering the process stability.
- NPMPs of trehalose and raffinose were spray-dried from MeOH:BA and MeOH:BA:H₂O systems, potentially representing a carrier system for peptide or protein or other drug compounds. NPMPs of pure, low molecular weight hydrophilic sugars were not previously described in the literature. Data obtained may also be useful when adapting the spray-drying method to other low molecular weight hydrophilic compounds.
- Hygroscopicity of spray-dried sugars was identified as a potential formulation difficulty. Composites of trehalose:HP- β -CD were spray-dried and may represent a more stable system.
- NPMP composites of lysozyme or trypsin with trehalose, raffinose or HP- β -CD were spray-dried from MeOH:BA:(H₂O). Lysozyme and trypsin in composite systems showed excellent process stability. Storage stability studies of composite systems (1:4 mass ratios) showed retention of particle morphology, amorphous character and thermal behaviour, stored at 4 °C and 25 °C with desiccant for 12 weeks. Biological activity of the two proteins in these composites was unchanged following storage at 4 °C with desiccant. A small decrease in the biological activity of trypsin, and no statistically significant loss of biological activity of lysozyme in these composites were observed, following storage under 25 °C with desiccant. Adequate storage stability of composite NPMPs including trehalose or raffinose may require a desiccant to be included in a potential delivery device.
- A NPMP composite of trehalose with the therapeutically used peptide salmon calcitonin was produced using a MeOH:BA solvent feed system, suggesting potential for further development of this system.
- All NPMPs produced displayed favourable particle characteristics and in-vitro deposition indicating good potential for pulmonary delivery.

7.5 PROPOSED FUTURE WORK

- Investigation of hygroscopic behaviour of sugar NPMPs using dynamic vapour sorption measurements, to classify them in terms of hygroscopicity and to investigate whether they exhibit increased hygroscopicity compared to non-porous spray-dried particles, or depending on spray-drying and feed conditions. Investigation of improvement of hygroscopicity and stability of a composites of excipients for example trehalose:HP- β -CD.
- Development of the spray-dried catalase systems, and investigation of causes of failure to produce NPMPs and loss of stability.
- Application of the method (for example the MeOH:BA:H₂O system) to other candidate proteins or peptides and to potential therapeutic polysaccharides for example heparin or low molecular weight fragments.
- Further development of the salmon calcitonin:excipient system, including thorough analysis of process stability, and optimisation of excipient carrier system and feed conditions.
- Investigation of distribution of components in a co-spray-dried composite system.

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APPENDIX 1: DETAILS OF SPRAY-DRYING AND FEED CONDITIONS

LYSOZYME

Lysozyme – MeOH:H₂O:(AC)

Title [lab book entry] <i>Notes</i>	Solvent	Process enhancer (% of t.s.c.)	T.s.c. (% w/v)	Mode (drying gas)/Cyclone	Inlet/Outlet temp (°C/°C)	Aspirator (%) /Gas flow (cm)	Pump setting (%)	Appearance of feed	Yield (volume spray-dried)
S2 [1_7] *L6876	75% MeOH	16.67 AC	0.3	CM (N ₂)/Reg	90/53	100/4	30	cloudy	1.53% (60ml)
S3 [1_8] L6876	75% MeOH	16.67 AC	0.3	CM (N ₂)/Reg	90/53	100/4	30	cloudy	3.12% (100ml)
S17 [1_40] L7651	75% MeOH	16.67 AC	0.3	CM (N ₂)/Reg	90/55	100/4	30	cloudy	6.56% (100ml)
S1 [1_6] **L6876	75% MeOH	16.67 AC	0.3	CM (N ₂)/Reg	90/53-56	100/4	30	cloudy	N/d
S9 [1_21] L6876	75% MeOH	16.67 AC	0.3	CM (N ₂)/Reg	110/64	100/4	30	cloudy	1.76% (100ml)
S10 [1_23] L6876	75% MeOH	16.67 AC	0.3	CM (N ₂)/Reg	70/40	100/4	30	cloudy	N/d
S16 [1_36] L6876	55% MeOH	16.67 AC	0.3	CM (N ₂)/Reg	90/52	100/4	30	clear	16.76% (100ml)
S12 [1_30] L6876	60% MeOH	16.67 AC	0.3	CM (N ₂)/Reg	90/52	100/4	30	clear	18.32% (100ml)
S4 [1_11] L6876	75% MeOH	-	0.25	CM (N ₂)/Reg	90/56	100/4	30	clear	6.48% (100ml)
S46 [2_11] L7651	60% MeOH	40 AC	0.4	CM (N ₂)/Reg	90/50	100/4	30	cloudy	7.58% (50ml)
S13 [1_31] L6876	65% MeOH	16.67 AC	0.3	CM (N ₂)/Reg	90/57	100/4	30	cloudy	12.04% (100ml)
S14 [1_32] L6876	70% MeOH	16.67 AC	0.3	CM (N ₂)/Reg	90/58	100/4	30	cloudy	13.8% (100ml)
S15 [1_35] L6876	80% MeOH	16.67 AC	0.3	CM (N ₂)/Reg	90/53	100/4	30	cloudy	3.36% (100ml)
S8 [1_20] L6876	80% MeOH	33.33 AC	0.3	CM (N ₂)/Reg	90/57	100/4	30	cloudy	3.88% (100ml)

Chapter 1. The History of the United States

The United States is a country with a rich and diverse history. It is a country that has been shaped by the dreams and aspirations of its people. The history of the United States is a story of exploration, discovery, and the pursuit of the American dream.

The first chapter of this book will explore the early history of the United States, from the first settlers to the founding of the nation. It will look at the challenges and triumphs of the early years, and the role of the government in shaping the country.

The second chapter will focus on the Civil War, one of the most significant events in American history. It will examine the causes of the war, the battles, and the impact it had on the nation.

The third chapter will discuss the Reconstruction era, the period after the Civil War when the country was rebuilding itself. It will look at the challenges of Reconstruction and the role of the federal government.

The fourth chapter will explore the Progressive Era, a time of social and political reform. It will look at the efforts to improve the lives of the poor and the working class, and the role of the government in social reform.

The fifth chapter will focus on the Great Depression, a time of economic hardship and social unrest. It will look at the causes of the Depression and the role of the government in responding to the crisis.

The sixth chapter will discuss the World War II era, a time of global conflict and the rise of the United States as a superpower. It will look at the challenges of the war and the role of the government.

The seventh chapter will explore the Cold War era, a time of tension between the United States and the Soviet Union. It will look at the arms race and the role of the government in foreign policy.

The eighth chapter will focus on the Vietnam War, a conflict that had a profound impact on the United States. It will look at the causes of the war and the role of the government.

The final chapter will discuss the present day, a time of rapid change and technological advancement. It will look at the challenges of the future and the role of the government.

S45 [2_10] <i>L7651</i>	65% MeOH	40 AC	0.4	CM (N ₂)/Reg	90/52	100/4	30	cloudy	1% (50ml)
S44 [2_9] <i>L7651</i>	70% MeOH	40 AC	0.4	CM (N ₂)/Reg	90/53-56	100/4	30	cloudy	1% (50ml)
S43 [2_8] <i>L7651</i>	75% MeOH	40 AC	0.4	CM (N ₂)/Reg	90/55-58	100/4	30	cloudy	0.5% (50ml)
S34 [1_84] <i>L7651</i>	80% MeOH	40 AC	0.4	CM (N ₂)/Reg	90/56	100/4	30	cloudy	3.75% (50ml)

* L6876 first lyophilised from solution at pH ~6 (adjusted using NaOH); ** pH of L6876 in water component of spray-drying feed system adjusted to pH6 (using NaOH)

Lysozyme – EtOH:H₂O:(AC or alternative ammonium salt)

Title [lab book entry] <i>Notes</i>	Solvent	Process enhancer (% of t.s.c.)	T.s.c. (% w/v)	Mode (drying gas)/Cyclone	Inlet/Outlet temp (°C/°C)	Aspirator (%)/Gas flow (cm)	Pump setting (%)	Appearance of feed	Yield (volume spray-dried)
S5 [1_12] <i>L6876</i>	75% EtOH	-	0.3	CM (N ₂)/Reg	90/54	100/4	30	clear	7.24% (100ml)
S7 [1_16] <i>L6876</i>	60% EtOH	16.67 AC	0.3	OM (N ₂)/Reg	90/53	100/4	30	cloudy	4.88% (100ml)
S23 [1_56] S27 [1_64] <i>L7651</i>	80% EtOH	10 AC	0.3	OM (N ₂)/Reg	78/46 78/49	100/4	30	cloudy	4.89% (50ml) 7.7% (50ml)
S29 [1_66] <i>L7651</i>	75% EtOH	10 AC	0.3	OM (N ₂)/Reg	78/49	100/4	30	cloudy	6% (50ml)
S30 [1_72] <i>L7651</i>	80% EtOH	10 AC	0.4	OM (N ₂)/Reg	78/48	100/4	30	cloudy	24.61% (50ml)
S31 [1_73] S40 [1_144] S54 [2_48] S54b [2_116] S54c [3_65] S54d [3_89] <i>L7651</i>	80% EtOH	10 AC	0.5	OM (N ₂)/Reg	78/49 78/42 78/46-47 78/48 78/49-45 78/46-47	100/4	30	cloudy	32.8% (50ml) 20.22% (50ml) 35.4% (250ml) 38.9% (500ml) 52.3% (500ml) 58.8% (500ml)
S117 [3_103] <i>L7651</i>	80% EtOH	10 AC	1	OM (Air)/Reg	78/47	100/4	30%	cloudy	35.8% (50ml)
S118 [3_104] <i>L7651</i>	80% EtOH	10 AC	2	OM (Air)/Reg	78/44-42	100/4	30%	cloudy	53.4% (50ml)

S39 [1_143] <i>L7651</i>	50% EtOH	10 AC	0.5	OM (N ₂)/Reg	78/40-39	100/4	30	clear	7.51% (50ml)
S38 [1_142] <i>L7651</i>	60% EtOH	10 AC	0.5	OM (N ₂)/Reg	78/42	100/4	30	clear	10.76% (50ml)
S42 [1_147] <i>L7651</i>	65% EtOH	10 AC	0.5	OM (N ₂)/Reg	78/42	100/4	30	clear	14.89% (50ml)
S37 [1_141] <i>L7651</i>	70% EtOH	10 AC	0.5	OM (N ₂)/Reg	78/40	100/4	30	cloudy	16.76% (50ml)
S33 [1_76] <i>L7651</i>	80% EtOH	5 AC	0.5	OM (N ₂)/Reg	78/49	100/4	30	cloudy	26.02% (50ml)
S24 [1_57] S28 [1_65] <i>L7651</i>	80% EtOH	-	0.3	OM (N ₂)/Reg	78/48 78/49	100/4	30	clear	1.13% (50ml) 21.33% (50ml)
S32 [1_75] <i>L7651</i>	80% EtOH	10 AC	0.5	OM (N ₂)/Reg	85/53-55	100/4	30	cloudy	24.8% (50ml)
S47 [2_23] <i>L7651</i>	70% EtOH	10 AC	0.5	OM (N ₂)/Reg	90/50	100/4	30	cloudy	9.96% (50ml)
S41 [1_146] <i>L7651</i>	75% EtOH	10 AC	0.5	OM (N ₂)/Reg	78/42	100/4	30	cloudy	13.24% (50ml)
S63 [2_98] <i>L7651</i> <i>pH in H₂O 8.00</i> <i>using KOH</i>	80% EtOH		0.5	OM (Air)/Reg	78/50-48	100/4	30	clear	28.96% (50ml)
S62 [2_97] <i>L7651</i>	80% EtOH	10 AHC	0.5	OM (Air)/Reg	78/47	100/4	30	cloudy	36.57% (100ml)
S65 [2_105] <i>L7651</i>	80% EtOH	5 AHC	0.5	OM (Air)/Reg	78/41	100/4	20	cloudy	22.27% (50ml)
S69 [2_114] <i>L6876</i>	80% EtOH	27.3 AF	0.55	OM (Air)/Reg	78/	100/4	20	cloudy	0.0387g (50ml)
S68 [2_108] <i>L6876</i>	80% EtOH	27.3 AF	0.55	OM (Air)/Reg	90/59-57	100/4	20	cloudy	0.0226g (50ml)
S74 [2_123] <i>L6876</i>	80% EtOH	40 AF	0.5	OM (Air)/Reg	90/67-65	100/4	35	cloudy	0.0067g (50ml)
S75 [2_124] <i>L6876</i>	80% EtOH	50 AF	0.5	OM (Air)/Reg	90/61-63	100/4	20	cloudy	0.0245g (100ml)
S76 [2_125] <i>L6876</i>	80% EtOH	60 AF	0.5	OM (Air)/Reg	90/57	95/4	5	cloudy	0.0331g (100ml)

S87 [2_157] <i>L6876</i>	80% EtOH	40 AA	0.5	OM (Air)/Reg	90/50-53	100/4	5	cloudy	0.0223g (100ml)
S93 [2_164] <i>L6876</i>	80% EtOH	50 AA	0.5	OM (Air)/Reg	90/55	100/4	5	cloudy	0.0705g (100ml)
S94 [2_165] <i>L6876</i>	80% EtOH	60 AA	0.5	OM (Air)/Reg	90/57	100/4	5	cloudy	0.027g (100ml)

Lysozyme – H₂O

Title [lab book entry] <i>Notes</i>	Solvent	Process enhancer (% of t.s.c.)	T.s.c. (% w/v)	Mode (drying gas)/Cyclone	Inlet/Outlet temp (°C/°C)	Aspirator (%)/Gas flow (cm)	Pump setting (%)	Appearance of feed	Yield (volume spray-dried)
S6 [1_15] <i>L6876</i>	H ₂ O	16.67 AC	0.3	OM (Air)/Reg	120/52	100/4	30	clear	17.8% (100ml)
S55 [2_52] S55b [3_77] S55c [3_127] <i>L7651</i>	H ₂ O	20 AC	0.5	OM (Air)/Reg	120/56-55 120/53 120/59-55	100/~3 100/4 100/4	30	clear clear clear	36% (200ml) 8.3% (100ml) 34.5% (400ml)
S58 [2_70] S58b [3_67] S58c [4_112] <i>L7651</i>	H ₂ O	-	0.5	OM (Air)/Reg	120/56-58 120/45 120/60	100/4	30	clear	41.4% (200ml) 50.1% (200ml) 48.7% (200ml)

Lysozyme – MeOH:BA

Title [lab book entry] <i>Notes</i>	Solvent	T.s.c. (% w/v)	Mode (drying gas)/Cyclone	Inlet/Outlet temp (°C/°C)	Aspirator (%)/Gas flow (cm)	Pump setting (%)	Appearance of feed	Yield (volume spray-dried)
S96 [2_170] <i>*L7651</i>	80:20 MeOH:BA	0.2	CM (N ₂)/HP	100/54	100/4	30	clear	18.4% (50ml)
S97 [2_171] <i>*L7651</i> <i>Nozzle 1.4mm</i>	1:1 MeOH:BA	0.15	CM (N ₂)/HP	100/61	100/2	30	~cloudy	24.9% (50ml)

* L7651 first lyophilised from solution at pH ~6 (adjusted using NaOH)

EXCIPIENTS

TREHALOSE

Title [lab book entry] <i>Notes (mass ratio)</i>	Solvent	Process enhancer (% of t.s.c.)	T.s.c. (% w/v)	Mode (drying gas)/Cyclone	Inlet/Outlet temp (°C/°C)	Aspirator (%) /Gas flow (cm)	Pump setting (%)	Appearance of feed	Yield (volume spray-dried)
A1 [2_1]	H ₂ O	25 AC	2	OM (Air)/Reg	100/56-51	100/4	30	clear	8.5% (50ml)
S36 [1_121]	H ₂ O	25 AC	2	OM (Air)/Reg	110/64-61	100/4	30	clear	20.4% (25ml)
A2 [2_1]	H ₂ O	25 AC	2	OM (Air)/Reg	130/69	100/4	30	clear	36.2% (50ml)
A3 [2_2]	H ₂ O	25 AC	2	OM (Air)/Reg	150/80	100/4	30	clear	41.3% (50ml)
S127 [3_176]	H ₂ O	-	2	OM (Air)/Reg	150/82-80	100/4	30	clear	9.4% (100ml)
B1 [2_13] B2 [2_13] B3 [4_95]	H ₂ O	-	2	OM (Air)/Reg	130/66 130/67 130/67-65	100/4	30	clear	55% N/d 24.7% (150ml)
S150 (SS1) [4_94] +0.2% <i>Sulforhodamine 101</i>	H ₂ O	-	2	OM (Air)/Reg	130/ 64	100/4	30	clear (purple)	46.4% (100ml)
C [2_13]	H ₂ O	40 AC	2	OM (Air)/Reg	130/66	100/4	30	clear	40.1% (50ml)
S19 [1_50]	80% EtOH	10 AC	0.5	OM(N ₂) / Reg	78/ 49	100/4	30	clear	20.17% (40ml)
S20 [1_51]	90% EtOH	10 AC	0.5	OM (N ₂) / Reg	78/48	100/4	30	clear	24.4% (40ml)
S21 [1_53]	80% EtOH	10 AC	0.5	OM (N ₂) / Reg	85/ 51	100/4	30	clear	28.39% (40ml)
S22 [1_54]	80% EtOH	15 AC	0.5	OM (N ₂) / Reg	78/47	100/4	30	clear	29.24% (40ml)
D [2_14]	60% EtOH	25 AC	1	OM (N ₂) / Reg	90/ 49	100/4	30	clear	26.6% (50ml)
E [2_14]	60% EtOH	25 AC	1	OM (N ₂) / Reg	95/54	100/4	30	clear	25.6% (50ml)
F [2_15]	70% EtOH	25 AC	1	OM (N ₂) / Reg	90 /51-52	100/4	30	clear	28.1% (50ml)
G [2_15]	60% EtOH	10 AC	1	OM (N ₂) / Reg	90/	100/4	30	clear	30.3% (50ml)
S175 [4_182]	70% EtOH	10 AC	1	OM (Air)/Reg	90/ 56	100/4	30	clear	23.7% (25ml)
S174 [4_181]	80% EtOH	10 AC	1	OM (Air)/Reg	90/ 60-58	100/4	30	clear	36.3% (25ml)
S50 [2_26] S50c [2_168] S121 [3_117]	80:20 MeOH:BA	-	0.5	CM (N ₂)/ Reg	100/ 65 100/ 61 100/ 65	100/4	30 35 30	clear	1.8% (50ml) 1.5% (50ml) 10.6% (200ml)
S51[2_29][2_59] S51r [3_142]	50:50 MeOH:BA	-	0.5	CM (N ₂)/ Reg	100/64 100/64	100/4 100/4	30	clear	N/d 2.9% (50ml)

S67 [2_107] S67r1 [2_180] S67r2 [3_13] S67r3 [3_21] Nz 1.4mm S67r4 [3_23] S67r6 [3_43] S67r7 [3_41] S67r8 [3_45] S67r9 [3_107] S67r10	80:20 MeOH:BA	-	0.5	CM (N ₂)/HP	100 /63 100/64-60 100/61-59 100/62 100/64-62 100/63 100/61-57 100/61 100/58-56 100/58	100/2.5 100/4 100/4 100/2 100/2 100/4 100/4 100/4 100/4 100/4	30 30 30 30 30 30 25 25 30 30	clear	20.94% (100ml) 43.3% (100ml) 8.08% (50ml) 17.44% (50ml) 21.4% (50ml) 30.64% (50ml) N/d N/d 51.3% (200ml) 39.9% (200ml)
SS2 [4_118] + 0.2% coumarin6	80:20 MeOH:BA	-	0.501	CM (N ₂)/HP	100/ 60	100/4	30	clear (yellow)	42.4% (200ml)
S82 [2_135] S82r [2_135]	60:40 MeOH:BA	-	0.6	CM (N ₂)/HP	100/58-57 100/59-56	100/4	30	clear	23.4% (50ml) 35.9% (50ml)
S81 [2_134] S81r [2_134]	70:30 MeOH:BA	-	0.6	CM (N ₂)/HP	100/62-58 100/59-57	100/4	30	clear	25.1% (50ml) 26.73% (50ml)
S80 [2_133] S80r [2_133]	90:10 MeOH:BA	-	0.6	CM (N ₂)/HP	100/61-58 100/58-54	100/4	30	clear	24.8% (50ml) 32.83% (50ml)
S72 [2_120]	80:20:10 MeOH:BA:H ₂ O	-	0.45	CM (N ₂)/HP	100/55	100/4	30	clear	51.3% (55ml)
S73 [2_121]	80:20:10 MeOH:BA:H ₂ O	-	0.45	CM (N ₂)/Reg	100/62	100/4	30	clear	29.8% (55ml)
S122 [3_119]	15:15:1 MeOH:BA:H ₂ O	-	0.48	CM (N ₂)/HP	100/64	100/4	30	coarse suspension	N/d
S83b [3_106; 2_136]	49:49:2 MeOH:BA:H ₂ O	-	0.6	CM (N ₂)/ Reg	100/61	100/4	30	suspension	6.5%
S83 [2_136] S120 [3_116]	49:49:2 MeOH:BA:H ₂ O	-	0.6 0.6	CM (N ₂)/HP CM (N ₂)/HP	100/61 100/65	100/4 100/4	30 30	suspension	29.9% 24.1% (50ml)
S171 [4_153]	49:49:2 MeOH:BA:H ₂ O	-	0.3	CM (N ₂)/HP	100/ 53	100/4	30	clear	10.9% (30ml)
S137 [4_71] trehalose:mannitol (9:1)	80:20 MeOH:BA	-	0.5	CM (N ₂)/HP	100/ 59	100/4	30	clear	N/d
S172 [4_154] trehalose:HP-β-CD (1:1)	80:20 MeOH:BA	-	0.6	CM (N ₂)/HP	100/52	100/4	30	clear	26.6% (30ml)

RAFFINOSE

Title [lab book entry] <i>Notes</i>	Solvent	T.s.c. (% w/v)	Mode (drying gas)/Cyclone	Inlet/Outlet temp (°C/°C)	Aspirator (%) / Gas flow (cm)	Pump setting (%)	Appearance of feed	Yield (volume spray-dried)
S70 [2_117] S70b [2_158] S70r1 [2_179] S70r2 [3_12] S70r3 [3_20] <i>Nz 1.4mm</i> S70r4 [3_22] S70r5 [3_27] S70r6 [3_29] S70r7 [3_40] S70r9 [3_61] S70r10 [4_6]	80:20 MeOH:BA	1	CM (N ₂)/Reg	100/63 100/ 69 100/ 64 100/65 100/64-65 100 /67 100/ 68-66 100 /66 100/ 65 100/65 100/ 67	100/4 100/4 100/4 100/4 100/2 100/2 100/4 100/4 100/4 100/4 100/4	30 50 30 30 30 30 30 30 25 30 30	clear	2.5% (50ml) 2.1% (50ml) 9.4% (50ml) 14.2% (50ml) 26.4% (50ml) 24% (50ml) <i>N/d</i> 27.06%(50ml) <i>N/d</i> <i>N/d</i> 44.12% (500ml)
S70c6 [3_138] S70c6b [3_151] <i>+ 0.2 % coumarin6</i>	80:20 MeOH:BA	1.02	CM (N ₂)/Reg	100 /64 100/65	100/4 100/4	30	clear – yellow/green	12.1% (50ml)
S110 [3_78]	90:10 MeOH:BA	1	CM (N ₂)/ Reg	100 /59	100/4	30	clear	<i>N/d</i>
S111 [3_79]	70:30 MeOH:BA	1	CM (N ₂)/ Reg	100/ 67	100/4	30	clear	8.3% (50ml)
S112 [3_85]	60:40 MeOH:BA	1	CM (N ₂)/ Reg	100/67	100/4	30	coarse suspension	<i>N/d</i> (stopped-nozzle blocking)
S71 [2_119]	50:50 MeOH:BA	1	CM (N ₂)/Reg	100/ 63	100/4	30	coarse suspension	26.7% (50ml)
S88 [2_159]	80:20 MeOH:BA	1	CM (N ₂)/HP	110/ 60	100/4	50	clear	17.4% (50ml)
S89 [2_160]	80:20 MeOH:BA (HPLC)	1	CM (N ₂)/Reg	110/61	100/4	50	clear	2.7% (50ml)
S159 [4_131]	79:19:2 MeOH:BA:H ₂ O	0.5	CM (N ₂)/HP	100/ 58	100/4	30	clear	29% (100ml)

S104 [3-56]	H ₂ O	2.5	OM (Air)/Reg	110 /56	100/4	30	clear	4.5% [25ml]
S101 [3-53]	H ₂ O	2.5	OM (Air)/Reg	120/60-58	100/4	30	clear	22.0% [50ml]
S103 [3-55] S103b [3-187] S103c [4_7] S103d [4_13]	H ₂ O	2.5	OM (Air)/Reg	130/ 70-68 130/66 130/ 58 130/68	100/4	30	clear	29.6% [25ml] 35.3% [50ml] 21.4% [200ml] <i>N/d</i>
S103_sulf101 [3_185] +0.2% <i>Sulforhodamine 101</i>	H ₂ O	2.5	OM (Air)/Reg	130/ 66	100/4	30	clear	44.2% (50ml)
S126 [3_166] +0.2 % coumarin-6	60% EtOH	1	OM (Air)/Reg	100/ 59	100/4	30	clear	25.7% (100ml)

POLYOLS

Title [lab book entry] <i>Notes</i> (mass ratio)	Solvent	Process enhancer (% of t.s.c.)	T.s.c. (% w/v)	Mode (drying gas)/Cyclone	Inlet/Outlet temp (°C/°C)	Aspirator (%) /Gas flow (cm)	Pump setting (%)	Appearance of feed	Yield (volume spray-dried)
S61 [2_93] <i>mannitol</i>	1:7:2 H ₂ O:MeOH: BA	-	0.5	CM (N ₂)/HP	100/ 62-59	100/4	30	suspension	<i>N/d</i> (stopped- nozzle blocking)
S66 [2_106] <i>mannitol</i> <i>filtered feed</i>	1:7:2 H ₂ O:MeOH: BA	-	0.5	CM (N ₂)/Reg	100 /67	100/4	30	suspension	<i>N/d</i> (stopped- nozzle blocking)
S140 [4_76] <i>myo-inositol</i>	H ₂ O	-	2	OM (Air)/Reg	130 /64	100/4	30	clear	31.4% (100ml)
S147 [4_88] <i>myo-inositol</i>	H ₂ O	-	2	OM (Air)/Reg	150/78	100/4	30	clear	58.3% (100ml)
S148 [4_89] <i>myo-</i> <i>inositol:PVP-k15</i> (9:1)	H ₂ O	-	2	OM (Air)/Reg	150/78	100/4	30	clear	35.9% (50ml)



S149 [4_91] <i>myo- inositol:PVP-k15 (4:1)</i>	H ₂ O	-	2	OM (Air)/Reg	130/ 66	100/4	30	clear	27.0% (25ml)
S151 [4_98] <i>myo-inositol : PVP-40 (9:1)</i>	H ₂ O	-	2	OM (Air)/Reg	130/64	100/4	30	clear	14.7% (25ml)
S141 [4_77] <i>myo-inositol</i>	70% EtOH	10 AC	0.5	OM (Air)/Reg	90/ 50	100/4	30	clear	28.4% (100ml)
S145 [4_85] <i>myo-inositol</i>	70% EtOH	-	0.5	OM (Air)/Reg	90/50	100/4	30	clear	28.1% (100ml)
S155 [4_115] <i>myo- inositol:PVP-k15 (3:2)</i>	80% EtOH	10 AC	0.3	OM (Air)/Reg	78 /48	100/4	30	clear	20% (100ml)
S156 [4_116] <i>myo- inositol:PVP-k15 (4:1)</i>	80% EtOH	10 AC	0.3	OM (Air)/Reg	78/47	100/4	30	clear	16.6% (100ml)

LYSOZYME:EXCIPIENT COMPOSITES

LYSOZYME:TREHALOSE – EtOH:H₂O:AC

Title [lab book entry] <i>Notes (mass ratio)</i>	Solvent	Process enhancer (% of t.s.c.)	T.s.c. (% w/v)	Mode (drying gas)/Cyclone	Inlet/Outlet temp (°C/°C)	Aspirator (%) /Gas flow (cm)	Pump setting (%)	Appearance of feed	Yield (volume spray-dried)
*H [2_21] L7651 (1:1)	70% EtOH	25 AC	1	OM (N ₂)/Reg	90/50	100/4	30	cloudy	N/d
S105 [3_68] L6876 (1:1)	70% EtOH	10 AC	0.5	OM (Air)/Reg	78/ 47-44	100/4	30	cloudy	37.3% (100ml)
S100 [3_15] L6876 S100b [3_108] S100c [3_118] S100d [3_120] S100f [3_179] L7651 (1:1)	70% EtOH	10 AC	0.5	OM (Air)/Reg	90/50 90/54 90/52 90/49 90/52	100/4	30	cloudy	20.1% (100ml) 36.3% (200ml) 40.9% (400ml) 36.8% (400ml) 60.1% (400ml)
S107 [3_69] L6876 S107f [3_180] L7651 (1:1)	80% EtOH	10 AC	0.5	OM (Air)/Reg	78/ 48 78/ 48-47	100/4	30	cloudy	50% (100ml) 55.8% (400ml)
S48 [2_24] L7651 (9:1)	70% EtOH	10 AC	0.5	OM (N ₂)/Reg	90/54	100/4	30	cloudy	13.7% (50ml)
S49 [2_25] L7651 (4:1)	70% EtOH	10 AC	0.5	OM (N ₂)/Reg	90 /54-53	100/4	30	cloudy	19.3% (50ml)
S60 [2_89] L7651 (7:3)	70% EtOH	10 AC	0.5	OM (Air)/HP	90/41	80/4	20	cloudy	24.4% (50ml)
S60b [2_90] L7651 (7:3)	70% EtOH	10 AC	0.5	OM (Air)/Reg	90/54-53	100/4	30	cloudy	19.1% (50ml)
S119 [3_105] L6876 (1:4)	70% EtOH	10 AC	0.5	OM (Air)/Reg	90/ 53	100/4	30	cloudy	35.8% (100ml)

LYSOZYME:OTHER EXCIPIENT – EtOH:H₂O:(AC)

Title [lab book entry] Notes (mass ratio)	Solvent	Process enhancer (% of t.s.c.)	T.s.c. (% w/v)	Mode (drying gas)/Cyclone	Inlet/Outlet temp (°C/°C)	Aspirator (%) /Gas flow (cm)	Pump setting (%)	Appearance of feed	Yield (volume spray-dried)
S102 [3_54] <i>L6876:raffinose (1:1)</i>	70% EtOH	10 AC	0.5	OM (Air)/Reg	90/54-51	100/4	30	cloudy	13.69% (100ml)
S158 [4_122] <i>L6876:raffinose (1:1)</i>	80% EtOH	10 AC	0.5	OM (Air)/Reg	78/42	100/4	30	cloudy	30.7% (100ml)
S139 [4_75] <i>L6876:inulin (1:1)</i>	70% EtOH	10 AC	0.5	OM (Air)/Reg	90/51	100/4	30	cloudy	37.9% (100ml)
S25 [1_58] <i>L7651:PVP- k15 (9:1)</i>	80%	-	0.3	OM (N ₂)/Reg	78/48	100/4	30	clear	9.56% (50ml)
S26 [1_59] <i>L7651:PVP- k15 (9:1)</i>	80%	10 AC	0.3	OM (N ₂)/Reg	78/47	100/4	30	cloudy	20.49% (50ml)
S138 [4_74] <i>L6876:myo- inositol (1:1)</i>	70% EtOH	10 AC	0.5	OM (Air)/Reg	90/ 52	100/4	30	cloudy	45.8% (100ml)
S152 [4_110] <i>L6876: Myo-inositol:PVP- k15 (5:3:2)</i>	80% EtOH	10 AC	0.5	OM (Air)/Reg	78/48	100/4	30	cloudy	47.0% (100ml)
S153 [4_111] <i>L6876: myo-inositol: PVP- 40 (5:4:1)</i>	80% EtOH	10 AC	0.5	OM (Air)/Reg	78 /47	100/4	30	cloudy	50.4% (100ml)
S154 [4_114] <i>L6876: myo-inositol: PVP- k15 (5:4:1)</i>	80% EtOH	10 AC	0.5	OM (Air)/Reg	78/47	100/4	30	cloudy	48.6% (100ml)

LYSOZYME:CYCLODEXTRIN OR LYSOZYME:SUGAR – MeOH:BA SYSTEMS

Title [lab book entry] <i>Notes</i> <i>(mass ratio)</i>	Solvent ratio	T.s.c. (% w/v)	Mode (drying gas)/Cyclone	Inlet/Outlet temp (°C/°C)	Aspirator (%)/Gas flow (cm)	Pump setting (%)	Appearance of feed	Yield (volume spray-dried)
S11 [1_24][1_37] <i>*L6876: HP-β-CD</i> <i>(4.8:95.2)</i>	1:1	2.1	CM (N ₂)/Reg	100/69	100/4	30	cloudy	39.88% (50ml)
S18 [1_42] <i>*L7651: HP-β-CD</i> <i>(1:4)</i>	1:1	1	CM (N ₂)/Reg	100/67	100/4	30	~cloudy ppt on sides of conical flask	26.05% (40ml)
S18R1 [4_26] LH1 [4_166] <i>*L6876: HP-β-CD</i> <i>(1:4)</i>	1:1	1	CM (N ₂)/HP	100/64 100/59	100/4	30	~clear	68.9% (75ml) 74.5% (50ml)
S129 [4_27] LT1 [4_165] <i>*L7651:trehalose (1:4)</i>	80:20 80:20	0.5 0.5	CM (N ₂)/HP CM (N ₂)/HP	100/61 100/55	100/4 100/4	30 30	~cloudy ~clear	53.9% (150ml) 63.9% (150ml)
S130 [4_28] S130b [4_80] LR1 [4_164] <i>*L7651:raffinose (1:4)</i>	80:20 80:20 80:20	0.5 0.5 0.5	CM (N ₂)/Reg CM (N ₂)/Reg CM (N ₂)/HP	100/67 100/61-63	100/4 100/4 100/4	30 30 30	~cloudy ~clear ~clear	27.9% (150ml) 26.16% (200ml) 53.5% (150ml)
S131 [4_48] S131r [4_79] <i>*L7651:raffinose (3:7)</i>	80:20 80:20	0.5 0.5	CM (N ₂)/Reg CM (N ₂)/Reg	100/68	100/4 100/4	30 30	~cloudy ~clear	7.9% (50ml) 7.0% (50ml)
S142 [4_78] <i>*L7651:raffinose (3:7)</i>	80:20	0.5	CM (N ₂)/HP	100/54	100/4	30	~clear	38.7% (50ml)
S132 [4_49] <i>*L7651:raffinose (1:1)</i>	80:20	0.5	CM (N ₂)/HP	100/62-60	100/4	30	~cloudy	54.2% (40ml)
S134 [4_60] <i>HP-β-CD alone</i>	1:1		CM (N ₂)/HP	100/ 60-58	100/4	30	clear	62.9% (75ml)

**L6876/L7651 first lyophilised from water at pH ~6 (adjusted using NaOH)*

OTHER PROTEINS/PROTEIN:EXCIPIENT COMPOSITES

TRYPSIN SYSTEMS – ETOH:H₂O:AC

Title [lab book entry] Notes (mass ratio)	Solvent	Process enhancer (% of t.s.c.)	T.s.c. (% w/v)	Mode (drying gas)/Cyclone	Inlet/Outlet temp (°C/°C)	Aspirator (%) /Gas flow (cm)	Pump setting (%)	Appearance of feed	Yield (volume spray-dried)
S106 [3_66]	80% EtOH	10 AC	0.5	OM (N ₂) /Reg	78/45	100/4	30	cloudy	12.4% (25ml)
S108 [3_72]	90% EtOH	20 AC	0.5	OM (Air) /Reg	78/44	100/4	30	cloudy	24.9% (50ml)
S109 [3_73]	95% EtOH	40 AC	0.5	OM (Air) /Reg	78 /47	100/4	30	cloudy	22.9% (50ml)
S113 [3_86]	98% EtOH	40 AC	0.5	OM (N ₂) /Reg	78/53	100/4	30	cloudy	18.4% (50ml)
S114 [3_95]	95% EtOH	50 AC	0.5	OM (N ₂) /Reg	78/47	100/4	30	cloudy	13.6% (50ml)
S115 [3_96]	95% EtOH	60 AC	0.5	OM (N ₂) /Reg	78/48	100/4	30	cloudy	6% (50ml)
S116 [3_99] <i>trypsin:trehalose (1:1)</i>	98% EtOH	40 AC	0.5	OM (N ₂) /Reg	78/46	100/4	30	cloudy	17.2% (50ml)
S123 [3_121] <i>trypsin:trehalose (4:1)</i>	95% EtOH	50 AC	0.5	OM (N ₂) /Reg	78/46	100/4	30	cloudy	17.3% (50ml)
S125 [3_159] <i>trypsin:trehalose (9:1)</i>	95% EtOH	50 AC	0.5	OM (Air) /Reg	85/53-51	100/4	30	cloudy	22.8% (50ml)
S170 [4_155] <i>trypsin:trehalose (9:1)</i>	95% EtOH	50 AC	1.0	OM (Air) /Reg	85 /49	100/4	30	cloudy	43.7% (50ml)
S124 [3_122] <i>trypsin:raffinose (9:1)</i>	95% EtOH	50 AC	1.0	OM (Air) /Reg	85/55	100/4	30	cloudy	29.6% (50ml)

TRYPSIN:CYCLODEXTRIN OR TRYPSIN:SUGAR – MeOH:BA:(H₂O) SYSTEMS

Title [lab book entry] <i>Notes (mass ratio)</i>	Solvent	T.s.c. (% w/v)	Mode (drying gas)/Cyclone	Inlet/Outlet temp (°C/°C)	Aspirator (%)/Gas flow (cm)	Pump setting (%)	Appearance of feed	Yield (volume spray-dried)
S133 [4_51] <i>*trypsin:raffinose (1:4)</i>	8:2	0.5	CM (N ₂)/HP	100/64	100/4	30	suspension, no solution in MeOH	26.7% (20ml)
S136 [4_63] <i>*trypsin:raffinose (2:98)</i>	8:2	0.5	CM (N ₂)/HP	100/64	100/4	30	suspension, no solution in MeOH	5.6% (125ml)
S160 [4_132] <i>trypsin:raffinose (1:9)</i>	79:19:2 MeOH:BA:H ₂ O	0.5	CM (N ₂)/HP	100/57	100/4	30	~cloudy	31.3% (100ml)
S166 [4_140] TR1 [4_161] <i>trypsin:raffinose (1:4)</i>	79:19:2 MeOH:BA:H ₂ O	0.5	CM (N ₂)/HP	100/53 100/55	100/4	30	~cloudy Fine ppt	24.9% (100ml) 30.4% (150ml)
S168 [4_148] <i>trypsin:raffinose (1:1)</i>	79:19:2 MeOH:BA:H ₂ O	0.5	CM (N ₂)/HP	100/48-50	100/4	30	~cloudy (coarse ppt)	20.7% (50ml)
S144 [4_84] <i>trypsin:HP-β-CD (1:9)</i>	15:15:1 MeOH:BA:H ₂ O	1.61	CM (N ₂)/HP	100/59	100/4	30	~cloudy	58.6% (31ml)
S164 [4_138] TH1 [4_163] <i>trypsin:HP-β-CD (1:4)</i>	15:15:1 MeOH:BA:H ₂ O	1.61	CM (N ₂)/HP	100/51 100/57	100/4	30	~cloudy ~cloudy (coarse ppt)	63.1% (31ml) 58% (45.5ml)
S169 [4_149] <i>trypsin: HP-β-CD (1:1)</i>	15:15:1 MeOH:BA:H ₂ O	0.81	CM (N ₂)/HP	100/55	100/4	30	~cloudy (coarse ppt)	43.6% (31ml)
S146 [4_87] <i>trypsin:trehalose (1:9)</i>	49:49:2 MeOH:BA:H ₂ O	0.3	CM (N ₂)/HP	100/58	100/4	30	~cloudy	28.7% (100ml)
S165 [4_139] TT1 [4_162] <i>trypsin:trehalose (1:4)</i>	49:49:2 MeOH:BA:H ₂ O	0.3	CM (N ₂)/HP	100/52 100/57	100/4	30	~cloudy	42.4% (100ml) 42.5% (250ml)
S167 [4_147] <i>trypsin:trehalose (1:1)</i>	49:49:2 MeOH:BA:H ₂ O	0.3	CM (N ₂)/HP	100/55	100/4	30	~cloudy (coarse ppt)	36.9% (100ml)

* trypsin initially freeze-dried from water (pH3)

CATALASE

Title [lab book entry] Notes (mass ratio)	Solvent	Process enhancer (% of t.s.c)	T.s.c. (% w/v)	Mode (drying gas)/Cyclone	Inlet/Outlet temp (°C/°C)	Aspirator (%) / Gas flow (cm)	Pump setting (%)	Appearance of feed	Yield (volume spray-dried)
S52 –cat1[2_31]	50mM Phospate Buffer pH7	-	0.1 (Cat.)	OM (Air)/Reg	120/ 62	100/4	30	clear yellow solution	N/d
S135 – cat2 [4_62]	5mM Phospate Buffer pH7	-	0.5 (Cat.)	OM (Air)/Reg	120/58	100/4	30	clear yellow solution	N/d
S53 –cat3 [2_33]	H ₂ O	10 AC	0.25	OM (Air)/Reg	120 /62	100/4	30	clear yellow solution	8.4% (100ml)
S64-cat4 [2_104]	H ₂ O	10 AHC	0.25	OM (Air)/Reg	120/ 65-63	100/~3	20	clear yellow solution	N/d
S56-cat5 [2_53]	50% ETOH	10 AC	0.125	OM (Air)/Reg	78/ 38	100/~3	30	clear yellow solution	7.1% (200ml)
*S99 [2_173] <i>catalase:trehalose</i> (1:1)	H ₂ O	10 AC	0.5	OM (Air)/HP	120/41-37	80-85/4	30	clear yellow solutios	13.69% (100ml)
S161 [4_133] <i>catalase:trehalose</i> (1:9)	49:49:2 MeOH:BA: H ₂ O	-	0.3	CM (N ₂)/HP	100/57	100/4	30	cloudy	38.8% (100ml)
S162 [4_134] <i>catalase:HP-β-CD</i> (1:9)	15:15:1 MeOH:BA: H ₂ O	-	1.61	CM (N ₂)/HP	100/57	100/4	30	cloudy	63.1% (31ml)
S163 [4_135] <i>catalase:raffinose</i> (1:9)	79:19:2 MeOH:BA: H ₂ O	-	0.5	CM (N ₂)/HP	100/51	100/4	30	cloudy	41.3% (100ml)

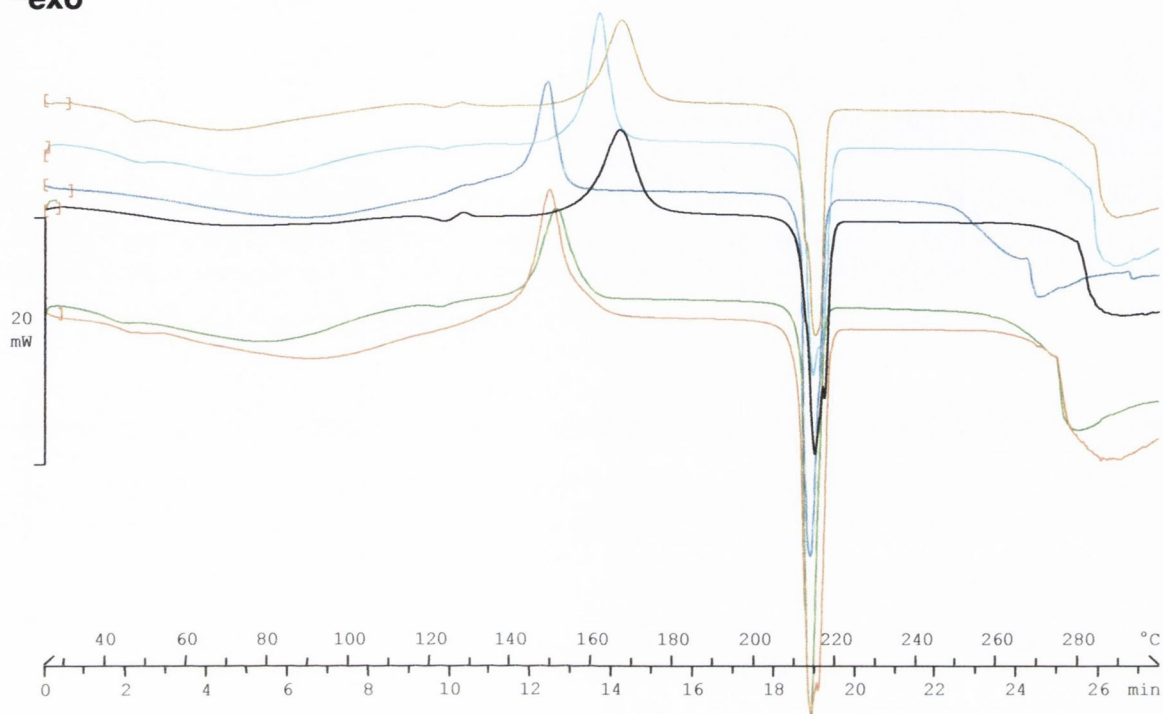
SALMON CALCITONIN:TREHALOSE – MeOH:BA SYSTEM

Title [lab book entry] <i>Notes (mass ratio)</i>	Solvent ratio	T.s.c. (% w/v)	Mode (drying gas)/Cyclone	Inlet/Outlet temp (°C/°C)	Aspirator (%)/Gas flow (cm)	Pump setting (%)	Appearance of feed	Yield (volume spray-dried)
S173 [4_179] <i>SCt:trehalose (1:19)</i>	80:20	0.5	CM (N ₂)/HP	100/57	100/4	30	clear	34.1% (20ml)

APPENDIX 2:

DSC of trehalose NPMPs spray-dried from MeOH:BA showing difference in recrystallisation peaks between batches.

Δ_{exo}



DSC analysis (25-300 °C at 10 °C/min) of:

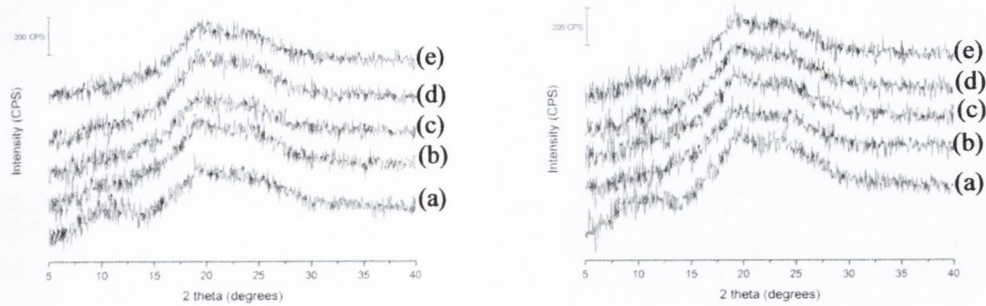
2 batches of spray-dried trehalose from 90:10 MeOH:BA (brown and light blue scans)

2 batches of spray-dried trehalose from 80:20 MeOH:BA (dark blue and black scans)

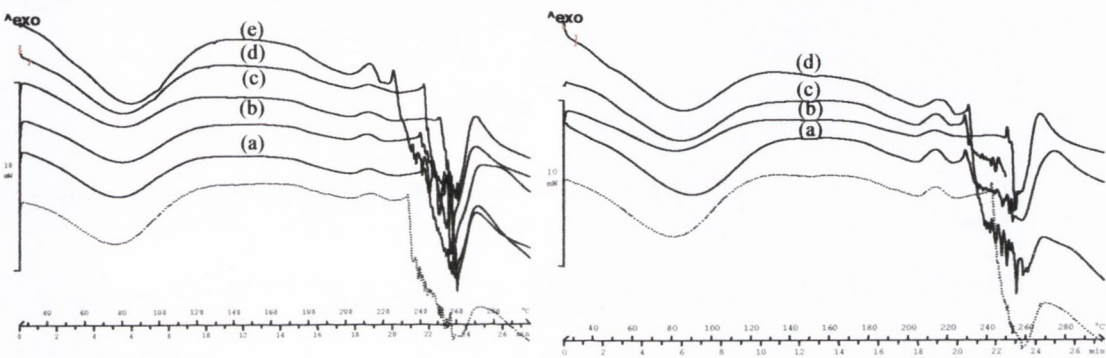
2 batches of spray-dried trehalose from 60:40 MeOH:BA (green and red scans)

APPENDIX 3:

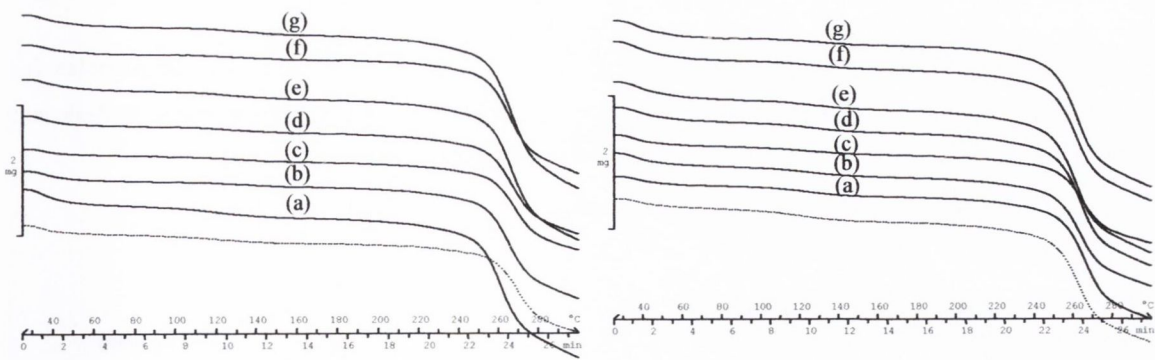
Selected XRD, DSC and TGA of lysozyme:excipients or trypsin:excipient composites on storage stability (Chapters 5, 6)



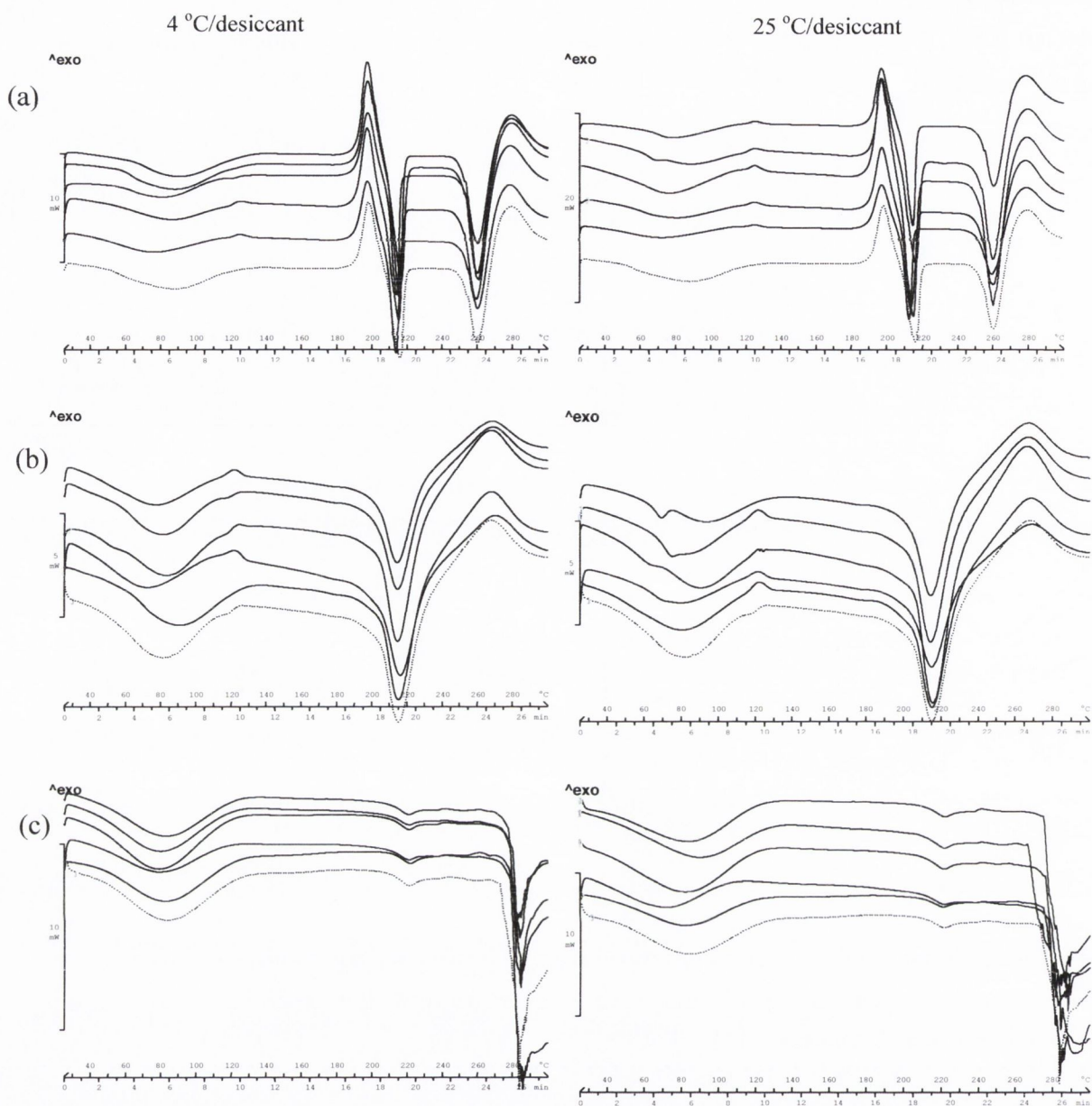
XRD of lysozyme:trehalose (1:1) spray-dried from conditions 1 (left) spray-dried from conditions 2 (right)
(a) week 1 (b) week 2 (c) week 9 (d) week 16 (e) week 20, storage at 4 °C/desiccant



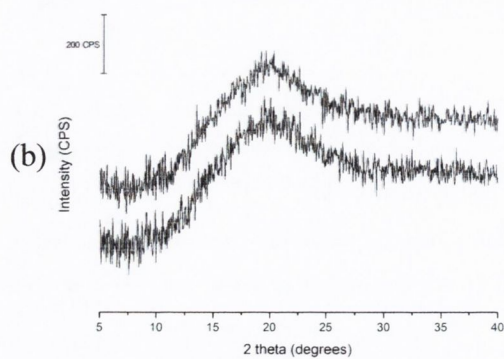
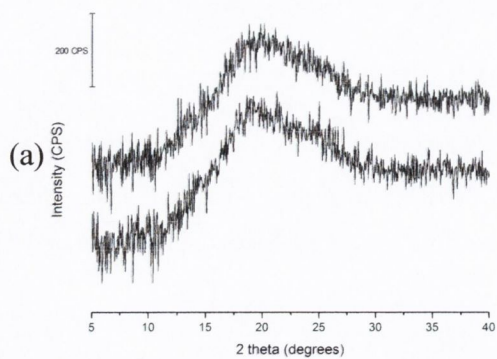
DSC of lysozyme:trehalose (1:1) spray-dried from conditions 1 (left) and spray-dried from conditions 2 (right); broken line – freshly prepared material, solid line after (a) 2 weeks (b) 9 weeks (c) 16 weeks (d), (e) 20 weeks, storage at 4 °C/desiccant

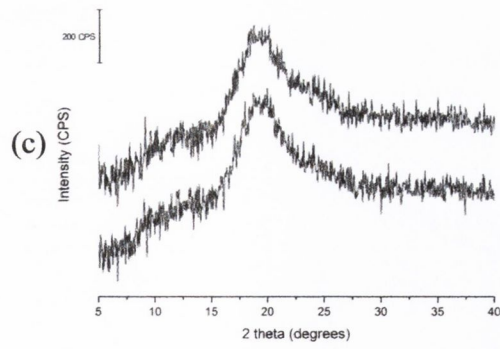


TGA of lysozyme:trehalose (1:1) spray-dried from conditions 1 (left) spray-dried from conditions 2 (right); broken line – freshly prepared material, solid line after (a) 1 week (b) 2 weeks (c) 5 weeks (d) 9 weeks (e) 12 weeks (f) 16 weeks and (g) 20 weeks, storage at 4 °C/desiccant



DSC (day 1, week 1, week 4, week 8, week 12 in ascending order) scans of composite particles (a) lysozyme:trehalose (1:4) (b) lysozyme:raffinose (1:4) (c) lysozyme:HP-β-CD (1:4) stored at 4 °C/desiccant (left trace), 25 °C/desiccant (right trace); broken line shows freshly spray-dried material.

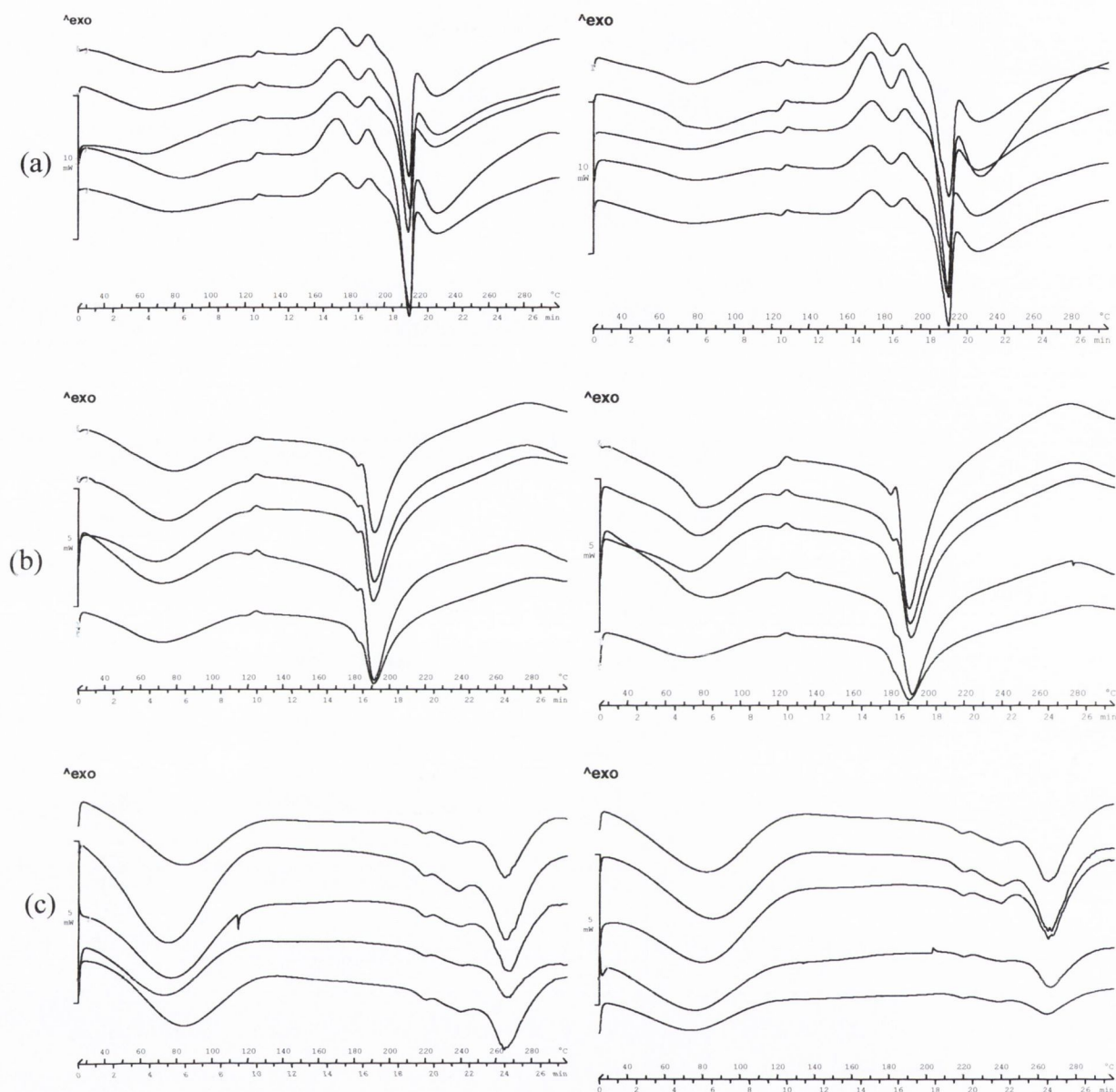




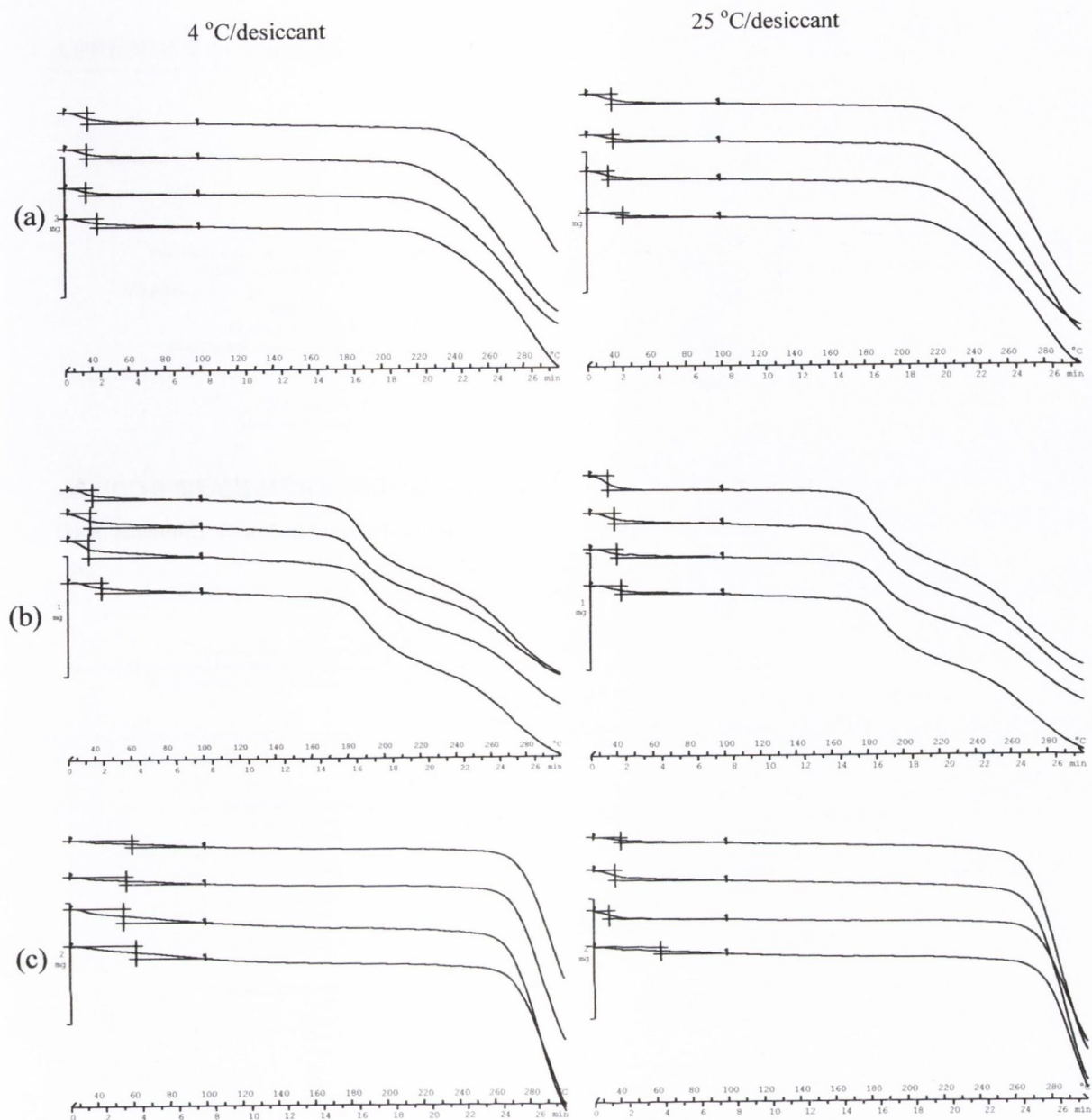
XRD of composite particles (a) lysozyme:trehalose (1:4) (b) lysozyme:raffinose (1:4) (c) lysozyme:HP- β -CD (1:4) at 4 °C/desiccant (top trace), 25 °C/desiccant (bottom trace) after 12 weeks storage

4 °C/desiccant

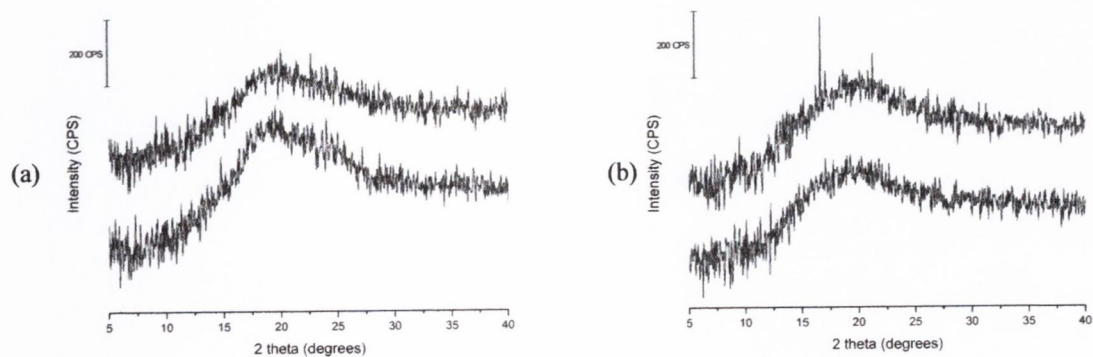
25 °C/desiccant

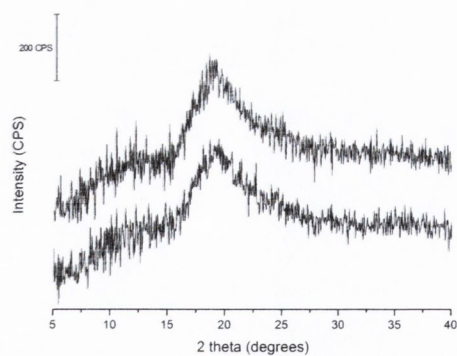


DSC (day 1, week 1, week 4, week 8, week 12 in ascending order) scans of composite particles (a) trypsin:trehalose (1:4) (b) trypsin:raffinose (1:4) (c) trypsin:HP- β -CD (1:4) stored at 4 °C/desiccant (left trace), 25 °C/desiccant (right trace).



TGA (week 1, week 4, week 8, week 12 in ascending order) scans of composite particles (a) trypsin:trehalose (1:4) (b) trypsin:raffinose (1:4) (c) trypsin:HP-β-CD (1:4) stored at 4 °C/desiccant (left trace), 25 °C/desiccant (right trace).





XRD traces of 1:4 mass ratio composites (a) trypsin:trehalose (b) trypsin:raffinose (c) trypsin:HP- β -CD, after storage for 12 weeks at 4 °C/desiccant (top trace), and at 25 °C/desiccant (bottom trace). (Siemens XRD used)

APPENDIX 4: Example of Andersen Cascade impactor calculation

Calculation of Fine Particle fraction, Mass Median Aerodynamic Diameter (MMAD) and Geometric Standard Deviation
S129_3 (BCA method:microplate)

	Weight (mg)	Percentage
Empty Weight of Capsule	43.872	
Full Weight of Capsule (half filled with powder)	68.84	
Weight of Powder in the Capsule	24.968	
Weight of powder in Capsule + Device after experiment	5.05	
Emitted Dose	19.92	79.77
Emitted Dose (recovered)	20.02	80.17
Fine Particle Fraction/Respirable Fraction		62.47
Dose accounted for		100.40

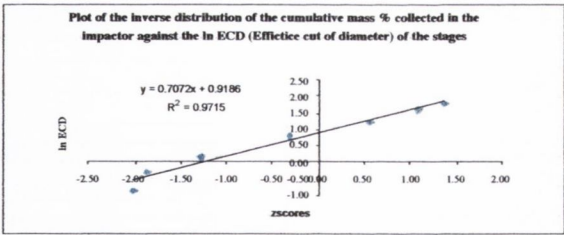
Flow rate (L/min)	50.8
Time per actuation (s)	4.9

Andersen Cascade Components	Mass (mg)
Mouth piece adaptor, Induction port	5.23
Stage -1	0.69
Stage -0	0.59
Stage 1	0.81
Stage 2	2.18
Stage 3	4.98
Stage 4	4.08
Stage 5	1.01
Stage 6	0.13
Filter	0.32
Total Mass of Powder delivered to apparatus (A)	20.02
Mass of drug recovered from the impactor stages beneath the uppermost stage (Stages -1 to F) (B)	14.78
Mass of drug captured in the fine particle size range (Stages 1-F) (R)	13.50

Calculation of Mass Median Aerodynamic Diameter and the Geometric Standard Deviation

Andersen Cascade Components	ECD (Effective Cut-Off Diameter)	Stage collection (%)	Cumulative (%)
Filter	0.43	2.14	2.14
Stage 6	0.76	0.87	3.01
Stage 5	1.20	6.84	9.85
Stage 4	2.28	27.59	37.44
Stage 3	3.59	33.67	71.11
Stage 2	5.11	14.73	85.85
Stage 1	6.30	5.49	91.33
Stage -0	9.78	3.97	95.30
Stage -1		4.70	100.00

Andersen Cascade Components	Cumulative (%)	zscore	ECD	ln(ECD)
Filter	2.14	-2.02	0.43	-0.83
Stage 6	3.01	-1.88	0.76	-0.27
Stage 5	9.85	-1.29	1.20	0.18
Stage 4	37.44	-0.32	2.28	0.83
Stage 3	71.11	0.56	3.59	1.28
Stage 2	85.85	1.07	5.11	1.63
Stage 1	91.33	1.36	6.30	1.84
Stage -0	95.30	1.67	9.78	2.28
Stage -1	100.00			

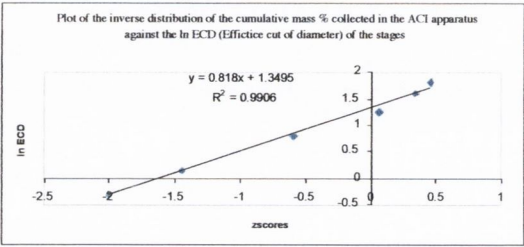


	Coefficients
Intercept	0.919
Slope	0.707
MMAD	2.506
GSD	2.028

Calculation of the fine particle fraction and particle fraction < 3micrometre

Andersen Cascade Components	ECD (Effective Cut-Off Diameter)	Stage collection	Cumulative (%)
Filter	0.43	1.583314831	1.58
Stage 6	0.76	0.639709147	2.22
Stage 5	1.20	5.052453375	7.28
Stage 4	2.28	20.37355684	27.65
Stage 3	3.59	24.86956039	52.52
Stage 2	5.11	10.88199378	63.40
Stage 1	6.30	4.053341474	67.45
Stage -0	9.78	2.93	70.38
Stage -1		3.47	73.85
Mouth Piece, Induction Port		26.15	100

Andersen Cascade Components	Cumulative (%)	zscore	ECD	ln(ECD)
Filter	1.58	-2.148596361	0.43	-0.833066628
Stage 6	2.22	-2.009722012	0.76	-0.27345084
Stage 5	7.28	-1.45557744	1.20	0.178534284
Stage 4	27.65	-0.593299423	2.28	0.825161449
Stage 3	52.52	0.063173623	3.59	1.277146572
Stage 2	63.40	0.342482107	5.11	1.630786613
Stage 1	67.45	0.452482585	6.30	1.841082021
Stage -0	70.38	0.535455906	9.78	2.280448681
Stage -1	73.85	0.638844345		
Fine particle fraction	62.47	0.317772509	5	1.609437912
Particle fraction < 3 micrometre	37.95	-0.306708694	3	1.098612289

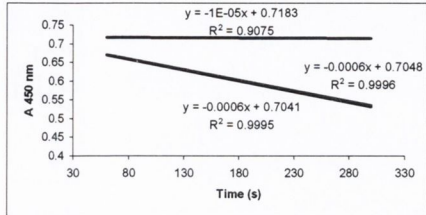


APPENDIX 5: Example of lysozyme activity assay calculation

01/08/2007 s18r1

Time (s)	A 450nm		
	Blank	Test1	Test2
0			
30	0.717	0.691	0.691
60	0.718	0.672	0.672
90	0.717	0.652	0.654
120	0.717	0.635	0.635
150	0.716	0.617	0.619
180	0.716	0.6	0.602
210	0.716	0.583	0.586
240	0.715	0.566	0.569
270	0.715	0.549	0.553
300	0.715	0.532	0.536
330	0.716	0.515	0.519

SLOPE -1.22222E-05 -0.00057833 -0.00056

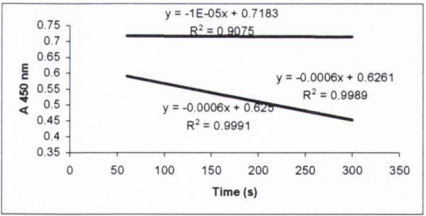


T	Δ A 450nm/min			
	Blank	Test1	Test2	Average
0				
30-60	-0.001	0.019	0.019	0.019
60-90	0.001	0.02	0.018	0.019
90-120	0	0.017	0.019	0.018
120-150	0.001	0.018	0.016	0.017
150-180	0	0.017	0.017	0.017
180-210	0	0.017	0.016	0.0165
210-240	0.001	0.017	0.017	0.017
240-270	0	0.017	0.016	0.0165
270-300	0	0.017	0.017	0.017
300-330	-0.001	0.017	0.017	0.017
average Δ A 450nm/30s	0.000222222	0.0176	0.0172	0.0174
st.dev.	0.000666667	0.001074968	0.001135	0.000937
average Δ A 450nm/min (lin)	0.00075	0.035	0.034	0.0345
st.dev.	0.000517549	0.001069045	0.001069	0.000845
units/ml		342.5	332.5	337.5
units/mg solid		42812.5	41562.5	8437.5
units/mg protein (as per BCA)				39706

01/08/2007 s18r1

Time (s)	A 450nm		
	Blank	Test1	Test2
0			
30	0.717	0.614	0.614
60	0.718	0.594	0.593
90	0.717	0.574	0.574
120	0.717	0.556	0.556
150	0.716	0.537	0.538
180	0.716	0.52	0.521
210	0.716	0.503	0.504
240	0.715	0.487	0.488
270	0.715	0.47	0.471
300	0.715	0.454	0.456
330	0.716	0.438	0.439

SLOPE -1.22222E-05 -0.00058000 -0.00057

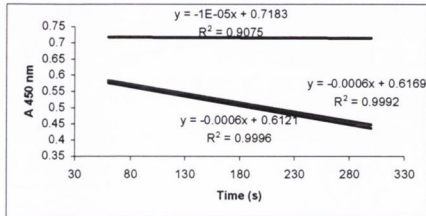


T	Δ A 450nm/min			
	Blank	Test1	Test2	Average
0				
30-60	-0.001	0.02	0.021	0.0205
60-90	0.001	0.02	0.019	0.0195
90-120	0	0.018	0.018	0.018
120-150	0.001	0.019	0.018	0.0185
150-180	0	0.017	0.017	0.017
180-210	0	0.017	0.017	0.017
210-240	0.001	0.016	0.016	0.016
240-270	0	0.017	0.017	0.017
270-300	0	0.016	0.015	0.0155
300-330	-0.001	0.016	0.017	0.0165
average Δ A 450nm/30s	0.000222222	0.0176	0.0175	0.01755
st.dev.	0.000666667	0.001577621	0.00165	0.001571
average Δ A 450nm/min (lin)	0.00075	0.035	0.03425	0.034625
st.dev.	0.000517549	0.001414214	0.001246	0.001308
units/ml		342.5	335	338.75
units/mg solid		42812.5	41875	8468.75
units/mg protein (as per BCA)				38494

01/08/2007 s18r1

Time (s)	A 450nm		
	Blank	Test1	Test2
0			
30	0.717	0.602	0.598
60	0.718	0.584	0.578
90	0.717	0.567	0.56
120	0.717	0.55	0.54
150	0.716	0.53	0.524
180	0.716	0.514	0.505
210	0.716	0.497	0.488
240	0.715	0.481	0.471
270	0.715	0.465	0.453
300	0.715	0.449	0.437
330	0.716	0.433	0.422

SLOPE -1.22222E-05 -0.00056500 -0.00058833



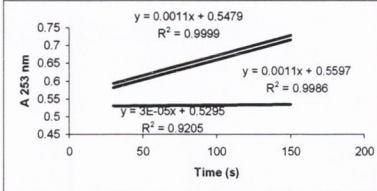
T	Δ A 450nm/min			
	Blank	Test1	Test2	Average
0				
30-60	-0.001	0.018	0.02	0.019
60-90	0.001	0.017	0.018	0.0175
90-120	0	0.017	0.02	0.0185
120-150	0.001	0.02	0.016	0.018
150-180	0	0.016	0.019	0.0175
180-210	0	0.017	0.017	0.017
210-240	0.001	0.016	0.017	0.0165
240-270	0	0.016	0.018	0.017
270-300	0	0.016	0.016	0.016
300-330	-0.001	0.016	0.015	0.0155
average Δ A 450nm/30s	0.000222222	0.0169	0.0176	0.01725
st.dev.	0.000666667	0.001286684	0.001713	0.001087
average Δ A 450nm/min (lin)	0.00075	0.03375	0.03525	0.0345
st.dev.	0.000517549	0.001356203	0.001408	0.000802
units/ml		330	345	337.5
units/mg solid		41250	43125	8437.5
units/mg protein (as per BCA)				37088

APPENDIX 6: Example of trypsin activity assay calculation

05/06/2007 s124

Time (s)	A 253nm		
	Blank	Test1	Test2
0			
30	0.53	0.581	0.591
60	0.532	0.616	0.629
90	0.532	0.649	0.663
120	0.533	0.683	0.694
150	0.534	0.716	0.727
180	0.534	0.75	0.761
210	0.534	0.781	0.791
240	0.534	0.79	0.798
270	0.535	0.79	0.798
300	0.535	0.79	0.798
330	0.536	0.79	0.798

SLOPE 2.57143E-05 0.00112286 0.00111905

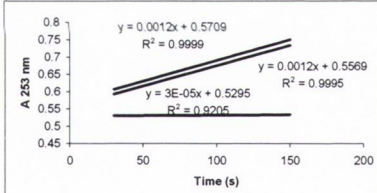


T	Δ A 253nm/min			
	Blank	Test1	Test2	Average
0				
30-60	-0.002	-0.035	-0.038	-0.0365
60-90	0	-0.033	-0.034	-0.0335
90-120	-0.001	-0.034	-0.031	-0.0325
120-150	-0.001	-0.033	-0.033	-0.033
150-180	0	-0.034	-0.034	-0.034
180-210	0	-0.031	-0.03	-0.0305
210-240	0	-0.009	-0.007	-0.008
240-270	-0.001	0	0	0
270-300	0	0	0	0
300-330	-0.001	0	0	0
average Δ A 253nm/min (t150-30)	-0.002	-0.0675	-0.068	-0.06775
st.dev. (t150-30)	0.000816497	0.000957427	0.002944	0.001797
units/ml				-328.75
units/mg solid				-6736.68
units/mg protein (as per BCA)				-8539

05/06/2007 s124

Time (s)	A 253nm		
	Blank	Test1	Test2
0			
30	0.53	0.607	0.593
60	0.532	0.642	0.628
90	0.532	0.679	0.663
120	0.533	0.715	0.697
150	0.534	0.75	0.736
180	0.534	0.782	0.769
210	0.534	0.806	0.796
240	0.534	0.806	0.799
270	0.535	0.806	0.799
300	0.535	0.806	0.799
330	0.536	0.806	0.799

SLOPE 3E-05 0.00119667 0.00118333

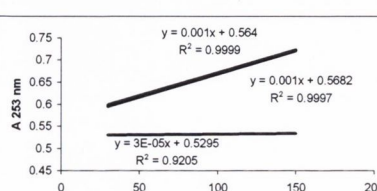


T	Δ A 253nm/min			
	Blank	Test1	Test2	Average
0				
30-60	-0.002	-0.035	-0.035	-0.035
60-90	0	-0.037	-0.035	-0.036
90-120	-0.001	-0.036	-0.034	-0.035
120-150	-0.001	-0.035	-0.039	-0.037
150-180	0	-0.032	-0.033	-0.0325
180-210	0	-0.024	-0.027	-0.0255
210-240	0	0	-0.003	-0.0015
240-270	-0.001	0	0	0
270-300	0	0	0	0
300-330	-0.001	0	0	0
average Δ A 253nm/min (t150-30)	-0.002	-0.0715	-0.0715	-0.0715
st.dev. (t150-30)	0.000816497	0.000957427	0.002217	0.000957
units/ml				-347.5
units/mg solid				-7120.902
units/mg protein (as per BCA)				-8455

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Time (s)	A 253nm		
	Blank	Test1	Test2
0			
30	0.53	0.596	0.6
60	0.532	0.626	0.63
90	0.532	0.658	0.66
120	0.533	0.69	0.692
150	0.534	0.721	0.724
180	0.534	0.753	0.756
210	0.534	0.783	0.786
240	0.534	0.805	0.81
270	0.535	0.805	0.81
300	0.535	0.805	0.81
330	0.536	0.805	0.81

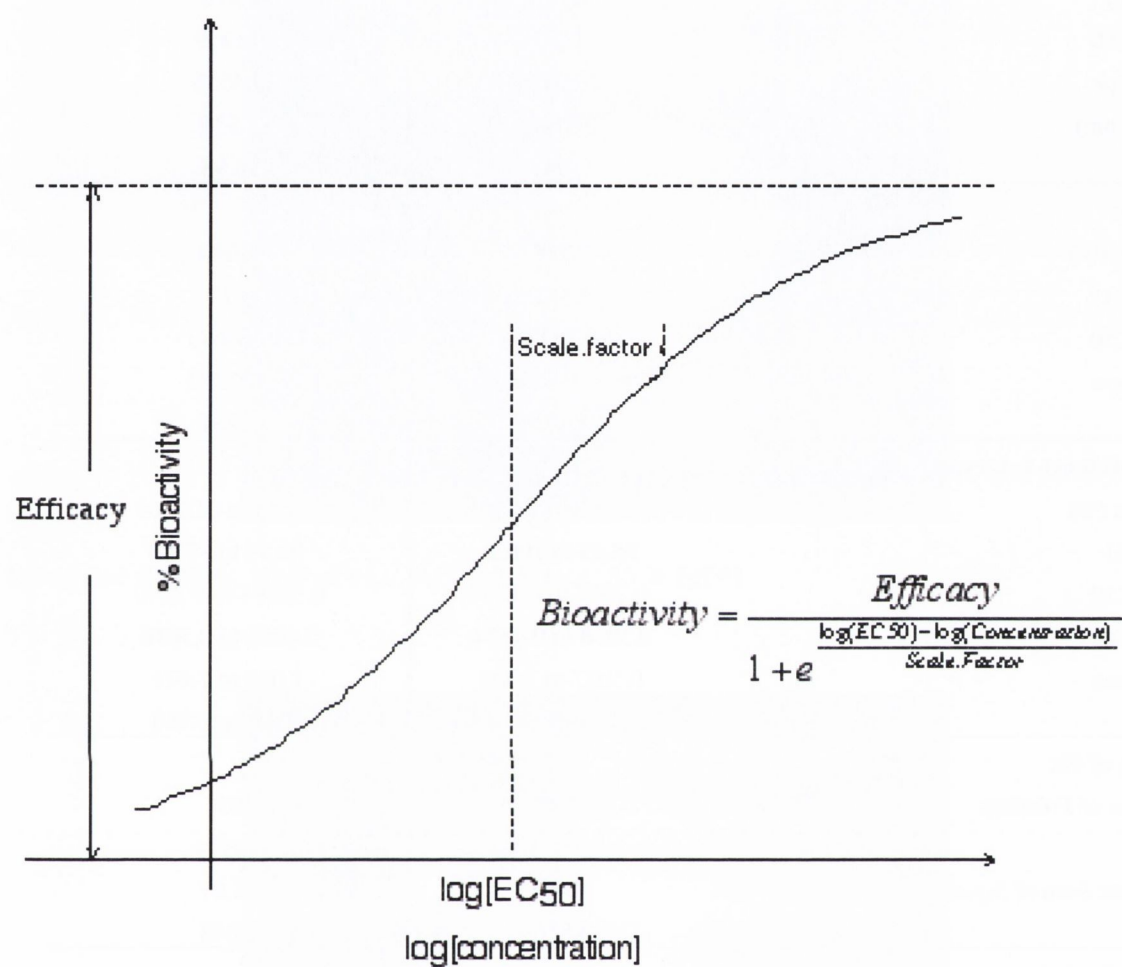
SLOPE 3E-05 0.00104667 0.00103333



T	Δ A 253nm/min			
	Blank	Test1	Test2	Average
0				
30-60	-0.002	-0.03	-0.03	-0.03
60-90	0	-0.032	-0.03	-0.031
90-120	-0.001	-0.032	-0.032	-0.032
120-150	-0.001	-0.031	-0.032	-0.0315
150-180	0	-0.032	-0.032	-0.032
180-210	0	-0.03	-0.03	-0.03
210-240	0	-0.022	-0.024	-0.023
240-270	-0.001	0	0	0
270-300	0	0	0	0
300-330	-0.001	0	0	0
average Δ A 253nm/min (t150-30)	-0.002	-0.0625	-0.062	-0.06225
st.dev. (t150-30)	0.000816497	0.000957427	0.001155	0.000854
units/ml				-301.25
units/mg solid				-6846.591
units/mg protein (as per BCA)				-7825

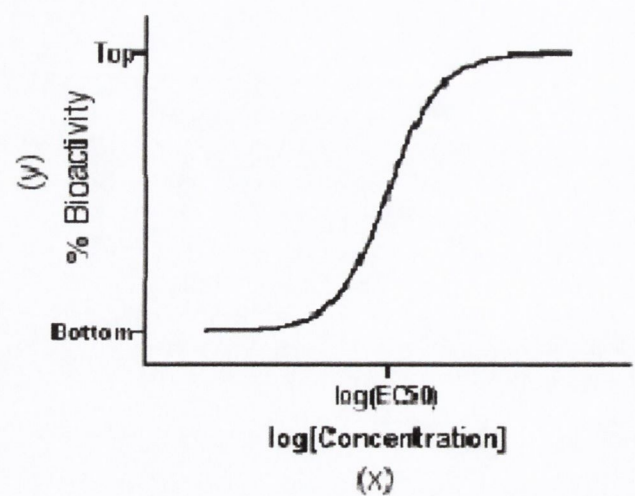
APPENDIX 7:

Parameters in the SSlogis model used for non-linear regression fitting of the calcitonin activity data
– Dr. Sinead Ryan (coefficients and 95 % confidence intervals are given in Chapter 6).



Results and model parameters, from re-plotting the data using dose response curve (variable slope), using GraphPad5 Prism Software Inc.

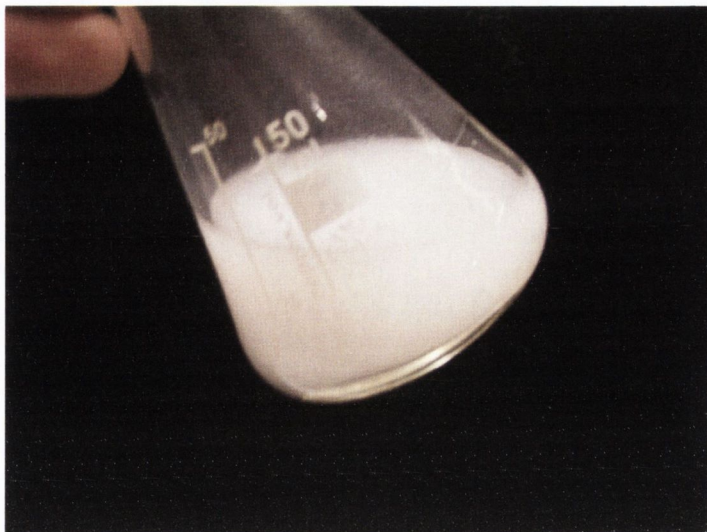
log(agonist) vs. response - Variable slope	SCT	SCT:Trehalose
Best-fit values		
Bottom (%)	-3.085	-2.215
Top (%)	100.9	94.76
LogEC50	-0.08718	0.1259
HillSlope	0.3816	0.4556
EC50 (nm)	0.8181	1.336
Span	104	96.98
Std. Error		
Bottom (%)	2.718	1.084
Top (%)	2.562	1.17
LogEC50	0.1022	0.04693
HillSlope	0.03664	0.02224
Span	4.533	1.864
95% Confidence Intervals		
Bottom (%)	-8.709 to 2.539	-4.458 to 0.02764
Top (%)	95.63 to 106.2	92.34 to 97.18
LogEC50	-0.2987 to 0.1244	0.02884 to 0.2230
HillSlope	0.3058 to 0.4574	0.4096 to 0.5016
EC50 nm	0.5027 to 1.332	1.069 to 1.671
Span	94.64 to 113.4	93.12 to 100.8
Goodness of Fit		
Degrees of Freedom	23	23
R ²	0.9911	0.9974
Absolute Sum of Squares	334.9	95.97
Sy.x	3.816	2.043
Number of points		
Analysed	27	27



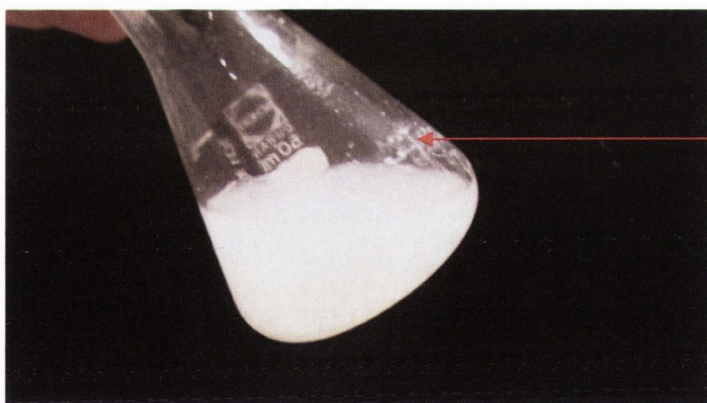
$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\text{LogEC50} - X) \cdot \text{HillSlope}}}$$

APPENDIX 8:

Phase separation of lysozyme and trypsin in EtOH:H₂O:AC systems producing NPMPs following spray-drying



Lysozyme (L7651), 10 % of t.s.c. AC, 0.5 % t.s.c, 80 % EtOH



Trypsin (93610), 50 % of t.s.c. AC, 0.5 % t.s.c. 95 % EtOH; red arrow shows deposition of some coarser material on the sides of the feed vessel