INVESTIGATING THE BIOPROTECTIVE PROPERTIES OF SUGARS

BY

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Abstract

The purpose of this study was to investigate the stabilizing properties of osmolytes, specifically sugars on biomolecule such as protein. The strategy used in this study involved the utilisation of surfactant-rich micelles; where by the impact sugars have on the free energy of exposure of hydrocarbon groups present within the surfactant micelles was examined. The observation made for sugar-surfactant study was then applied to explain the stabilisation of the native structure and thus the physiologically active form of the protein by sugars. The sugars that have been studied include sucrose, trehalose, maltose, raffinose and mannitol. The surfactants studied were sodium decyl sulphate (SDeS), sodium dodecyl sulphate (SDS) and sodium tetradecyl sulphate (STS).

Tensiometry was used to examine the impact of sugars on the critical micelle concentrations (CMC), Gibbs free energy change of micellization (ΔG_{mic}), surface pressure, surface excess concentration and area occupied per surfactant molecule. The free energy penalty of hydrocarbon chain exposure was obtained from the Gibbs free energy change of demicellization(ΔG_{demic}) which is equal but opposite in sign to the ΔG_{mic}. Measurements were carried out to elucidate the influence of sugar on the aforementioned surfactant properties as a function of increasing sugar concentration. Isothermal titration calorimetry (ITC) was then used to study -sugar surfactant interactions to give enthalpy (ΔH_{mic}) and entropy (ΔS_{mic}) of micellization in addition to CMC and $\Delta G_{mic,}$ thus obtaining a full thermodynamic characterisation, complementing the results obtained by tensiometry.

Tensiometric results revealed that at increasing concentration of sugar, the CMC of the surfactants was decreased and a more negative ΔG_{mic} was obtained. ITC results revealed a similar trend for the effect of sugar on CMC and ΔG_{mic} while the ΔH_{mic} and ΔS_{mic} was increased in the presence of the sugars. The results from surfactant studies suggest an increase in the free energy penalty of hydrocarbon group exposure to the aqueous environment, due to an unfavourable interaction between the hydrophobic groups and the aqueous sugar solution. Consequently, the aggregation process is thermodynamically favoured and more spontaneous in sugar solutions. For instance in SDeS the ΔG_{mic} in water and in sugar solution showed that micellization was more favourable in sugar solution (ΔG_{mic} = -19.14 kJ mol^{-1} at 1.0M Trehalose) than in water (ΔG_{mic} = -18.44 kJ mol^{-1}). In

addition, significant increases in surface pressure of the surfactants in the presence of sugars suggest an enhancement of the surface activity of the surfactants.
Increases in area occupied per surfactant molecules in the presence of sugars suggest increase in the size of the head group area thus, possible interactions between surfactant head group – sugar or sugar-water mediated interactions. Also increases ΔH_{mic} in comparison to lower values of ΔS_{mic} obtained by calorimetry suggest possible hydrogen bonding. In conclusion, surfactant studies suggest that sugars would stabilize biological structures by a combination of both an exclusion from the hydrophobic group due to unfavourable interactions between the hydrophobic groups and possible polar interactions between polar groups.

Differential scanning calorimetry (DSC) was used to study and characterise the effect of the sugars on the thermal stability of RNase *A*. The results revealed an increase the thermal stability of RNase *A* as shown by higher T_m values in the presence of sugars. Results obtained from surfactant studies were then related to DSC results, a linear relationship between the T_m and CMC values suggests a similar mechanism. Hence, though proteins are large complex molecules, their interaction with sugars or other small solutes could be related to simple model systems such as micelles.

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Abbreviations & symbols

Abbreviations	**Meaning**
3-D	three dimensional
Arg	arginine
Asp	aspartame
Ba^{2+}	barium ion
BaCl	barium chloride
Br^-	bromide
CCD	charge coupled device
C_{cell}	cell concentration
Cl^-	chloride
ClO_4^-	perchlorate
CMC	critical micelle concentration
CMP	2'Cytidine monophosphate
CO_2	carbon dioxide
C_{syr}	syringe concentration
DP	degree of polymerization
DNA	deoxyribonucleic acid
DSC	differential scanning calorimeter
EPR	electron paramagnetic resonance
FAO	food and agriculture organization
FT-IR	fourier transform infrared spectroscopy
GdnHCl	guanidium hydrochloride
H_2O	water
HCl	hydrochloric acid
He	helium
His	histidine
HSDSC	highly sensitive differential scanning calorimetry
I^-	iodide
IFT	interfacial tension
ITC	isothermal titration calorimetry
IUPAC	international union of pure and applied chemistry
K^+	potassium ion

Leu	leucine
Lys	lysine
mic	micelle
mon	monomers
MPP	multi-pinned phase
Na^+	sodium ion
NaOH	sodium hydroxide
Ne	neon
NMR	nuclear magnetic resonance
NO_2	nitrogen dioxide
NO_3^-	nitrate
O_2	oxygen gas
RNA	ribonucleic acid
RNase *A*	ribonuclease *A*
SCN^-	thiocyanate
SDeS	sodium decyl sulphate
SDS	sodium dodecyl sulphate
SFT	surface tension
STS	sodium tetradecyl sulphate
TAM	thermal activity monitor
Val	valine
WHO	world health organization
X_{CMC}	CMC in mole ratio

Symbols	**meaning**
f_i	activity coefficient
a_i	activity of any component in the bulk phase
A_o	area per surfactant molecule
N	Avogadro's number
ΔH_{cal}	calorimetric enthalpy change
d / Δ	change
μ_i	chemical potential of any component of the system
μ_m	chemical potential of the surfactant in the micelle phase

θ	contact angle
R^2	correlation coefficient
α	degree of ionisation
D	denatured
T_m	denaturation temperature
x	distance
H	enthalpy
ΔH	enthalpy change
ΔH_{demic}	enthalpy change of demicellization
ΔH_{dil}	enthalpy of dilution
ΔH_{mic}	enthalpy change of micellization
S	entropy
ΔS	entropy change
ΔS_{demic}	entropy change of demicellization
ΔS_{mic}	entropy change of micellization
K_{mic}	equilibrium constant for micelle formation
F	force
R	gas constant
G	Gibbs free energy
ΔG	Gibbs free energy change
ΔG_{demic}	Gibbs free energy change of demicellization
$\Delta G_{D[os]}$	Gibbs free energy change of denatured state in osmolyte
ΔG_{mic}	Gibbs free energy change of micellization
$\Delta G_{N[os]}$	Gibbs free energy change of native state in osmolyte
Q	heat
C_p	heat capacity
Φ	heat exchange within the surroundings
U	internal energy
L	length
x_i	mole fraction of any component in the bulk phase
N	native
P	pressure
Γ_i	surface excess concentration of any component of the system
π	surface pressure

γ	surface or interfacial tension
T	temperature
V	volume
t	time
υ	wavenumber

Dedication

This thesis is dedicated to my beloved Dad and Mum

Let us not become weary in doing good,
For at the proper time we will reap a harvest.
If we do not give up!
Your lives testify.

Chapter 1: Project overview

1.1. Project background

Sugars are generally known for their role as major sources of energy in plant and animal cells[1]. They possess many beneficial properties which make them one of the most studied and widely used classes of compounds in the pharmaceutical and life sciences[1]. They enhance physicochemical properties such as the bulk, body, viscosity and stability of emulsions and foams[1]. They are involved in browning, improving flavour and aroma of food as well as conferring a gamut of desirable textures to the food products[1].

Their role as osmolytes with the ability to stabilise and protect biomolecular systems such as proteins against denaturing conditions such as heat, drying, and presence of urea has generated considerable interest as evidenced by several studies conducted and documented in this area of research over the past eight decades[2-9].

Beilinsson in 1929 first observed the inhibition of thermal coagulation of ovalbumin by sucrose and Ball and co-workers also reported that sugars stabilized proteins against heat denaturation by protecting the macromolecule against loss of solubility and inhibition of heat coagulation [2, 3]. In addition, in the presence of several sugars and polyols the resistance to heat induced denaturation of lysozyme, conalbumin, α-chymotrypsinogen and ribonuclease was increased as observed by an increase in the thermal denaturation temperature (the temperature at which the proteins unfold), suggesting increased thermal stability[10]. Furthermore, the extent of denaturation of ovalbumin in urea solutions was shown to be reduced in the presence of sucrose as reported by Simpson and Kauzman (1953) [11]. More recently, Raman and differential scanning calorimetric studies have revealed that the presence of disaccharides stabilizes the native tertiary structure of lysozyme [12].

The stabilizing effect of these sugars were also observed in anhydrobiotic cells during conditions of dehydration, such as drought and desiccation [13.] Drought and desiccation are distinguished on the basis of critical water levels; drought is characterized by minimal cell water content of 0.3g (H_2O)/g of dry weight while desiccation is characterised by water levels below 0.3g (H_2O)/g of dry weight and further loss of the hydration shell of molecules[13].

The aforementioned conditions causes crowding of cytoplasmic components and increases cell viscosity thus, the chances for molecular interactions that can cause membrane fusion is increased [13]. Sugar molecules are shown to maintain the spacing between phospholipid molecules and prevent liquid-crystalline to gel phase transitions by replacing water in the hydration shells of the cell membranes [13-15]. Studies on isolated liposome suspensions showed that leakage and fusion during drying and freeze drying was inhibited in the presence of sugars [16, 17].

The bioprotective property of sugars has thus been utilized to stabilize various biomolecules in various fields of research as depicted in Table 1.1.

Table 1.1. Industrial applications using the stabilising properties of sugars.

Industry	Application	Bioprotective sugar	Reference
Biotechnology and biopharmaceuticals	Enzymes and other proteins	Trehalose, sucrose, glucose	18-22
	Vaccines and antibodies	Trehalose, sucrose	23, 24
Cosmetics	Liposomes	Trehalose, sucrose	15, 16, 25-27
Cell biology	Membranes & phospholipids	Trehalose, sucrose, maltose	15, 28-34
Microbiology	Bacteria and yeasts	Sucrose, trehalose	14, 26, 28, 35, 36
Cryobiology and medicine	Mammalian blood cells	Sucrose, trehalose, mannitol, sorbitol	33, 37-39

Considering the stabilizing properties of sugars and their wide application in industry, it would be of immense importance to gain a deeper understanding of the modes of action of these molecules.

1.2. Project objectives

This body of work represents an investigative study of osmolytes, principally sugars in terms of their physico-chemical properties and their mode of action on biomolecules such as proteins. To investigate the mode of action of bioprotective sugars on biomolecules,

surfactant-rich micelles were studied to provide evidence for the hypothesis that 'sugars enhance protein stability by increasing the free energy penalty of exposing hydrocarbon residues to aqueous sugar solutions as opposed to water'.

Arising from this, the following objectives have been set which include series of experiments that will be used to provide evidence to substantiate the hypothesis.

- Undertake tensiometric studies examining the impact of sugars on the critical micelle concentrations (CMC) of a homologous series of sodium *n*-alkyl sulphates which include: sodium decyl sulphate (SDeS), sodium dodecyl sulphate (SDS) and sodium tetradecyl sulphate (STS) as a function of increasing sugar concentration. Obtaining the Gibbs free energy change of micellization (ΔG_{mic}) consequently, free energy penalty of hydrocarbon chain exposure.

- Examine the effect of sugars on other physicochemical properties such as conductivity, surface pressure, surface excess concentration and area occupied per surfactant molecules.

- Undertake demicellization experiments of surfactant micelles in water and in the presence of sugars using isothermal titration calorimetry (ITC). Hence, obtaining CMC and a full thermodynamic characterisation complementing results obtained by tensiometry.
- Undertake thermal analysis with the use of differential scanning calorimetry (DSC) to study and characterise the effect of sugars on protein stability and unfolding; hence provide insights into mechanisms of stability by sugars.

Surfactant-rich micelles were used as a model to investigate the effect of sugars on protein stability by examining the impact sugars have on the free energy of exposure of hydrocarbon groups present within the surfactant micelles. Micelles were used because of their simplicity and since protein folding is driven in part by hydrophobic interactions and micelle formation is driven by the same sort of interaction [40, 41]. Therefore, it seems reasonable to extrapolate the findings of the investigation to the impact sugars play in stabilising the native form of a protein.

The sugars that have been investigated in this project are as follows: sucrose, trehalose, maltose, mannitol, and raffinose. Sugars were chosen because of their routine use in

industry, prevalence in nature and the availability of these compounds at high purity. Some of the properties of the aforementioned compounds are given in Table 1.2.

The applications of sugars, their prevalence in nature and stabilizing properties are discussed in detail in chapter two. Surfactants and their properties have also been discussed in this chapter.

The interaction between the sugars and micelles as well as protein were characterised with the aid of biophysical tools such as tensiometry and calorimetry. Chapter three gives a detailed description of the instrumentation, theory and principles of the aforementioned techniques.

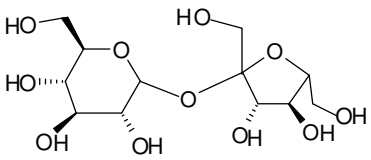
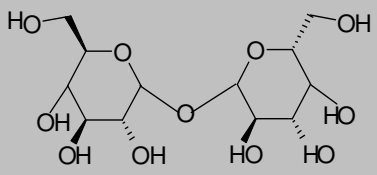
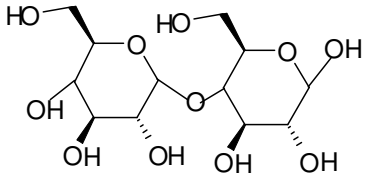
Results of tensiometric studies examining the impact of sugars on the CMC, ΔG_{mic} of the homologous series of sodium *n*-alkyl sulphates and other physicochemical properties such as conductivity, surface pressure, surface excess concentration and area occupied per surfactant molecules are presented in chapter four.

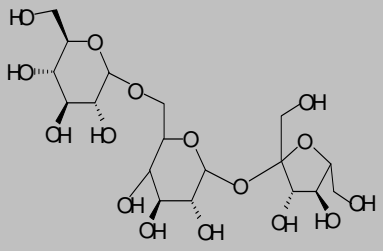
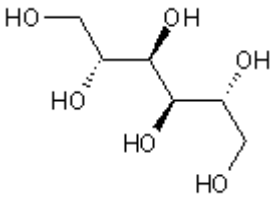
Results of demicellization of surfactant micelles by ITC in water and in the presence of sugars are presented in chapter five.

DSC studies of the effects of the sugars on protein stability were also carried out. The results of these studies are presented in six.

Solid state Raman and FT-IR measurements were carried out to characterise the sugars, with Raman and infra-red spectra recorded for the protonated and deuterated samples of these sugars thus a spectroscopic profile of the investigated sugars is presented in the appendix.

Table 1.2. Physicochemical properties of investigated sugars.

	Name	Structure & IUPAC name	Molecular formulae	Molecular weight(g/mol)	Melting point(^oC)	Solubility
1	Sucrose	 α-D-Glucopyranosyl β –D-fructofuranoside	$C_{12}H_{22}O_{11}$	342.30	185-187	0.30 g/ml (H_2O) at 20^oC
2	Trehalose dihydrate	 .2(H_2O) α- D-Glucopyranosyl-α-D-glucopyranoside	$C_{12}H_{22}O_{11}$ .2(H_2O)	378.33	97-99	0.30g/ml (H_2O) at 20^oC
3	Maltose monohydrate	 .H_20 4-O-α-D-Glucopyranosyl-D-glucopyranose	$C_{12}H_{22}O_{11}$ .H_2O	360.32	102-103	0.20 g/ml (H_2O) at 20 °C

4	Rafffinose pentahydrate	 .5(H_2O) β-D-fructofuranosyl α-D-galactopyranosyl-(1→6)-α-D-glucopyranoside	$C_{18}H_{32}O_{16}$ .5(H_2O)	594.52	78-80	0.10 g/ml (H_2O) at 20°C
6	Mannitol	 Hexan-1,2,3,4,5,6-hexol	$C_6H_{14}O_6$	182.17	165-167	0.18g/ml (H_2O) at 20 °C

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Chapter 2: Osmolytes, sugars and surfactant micelle systems

2.1. Osmolytes

Osmolytes are low molecular weight solutes or metabolites that are produced and used by cells that have been exposed to extreme environmental conditions, in order to maintain cell volume and viability[1, 2]. Examples of such conditions include temperature extremes, dehydration, high extracellular osmotic pressure and high concentration of intracellular urea.

2.1.1. Classes of osmolytes

The major classes of compounds that appear to have been selected in nature to serve as osmolytes are shown in Table 2.1. They can be broadly classified into inorganic osmolytes such as salt ions and organic osmolytes. The organic osmolytes are limited to a few chemical types which include uncharged sugars and polyols, free amino acids and derivatives, methylammonium and urea. They are ubiquitously found in cells of diverse types ranging from halophytic bacteria to mammalian kidney cells.

These compounds are employed as countermeasures against denaturing environmental conditions such as high or fluctuating salinity, drought/desiccation, extreme temperature and hydrostatic pressure. Osmolytes are thus able to protect the structure and maintain the functional activity of biological macromolecules such as proteins, in the presence of the adverse conditions.

In Table 2.1 inorganic ions such as K^+ and Na^+ act as osmolytes in ancient bacteria such as the *Halobacterium sp*, however at high concentrations they have deleterious effects on plant and animal cells [1]. It is paradoxical to classify urea as an osmolyte due to its perturbing effect on macromolecules such as protein, for which it acts a strong denaturant [1,3]. However, in the urea rich cells of cartilaginous fishes the deleterious effect of urea is counteracted by methylamine & trimethylamine-N-oxide (TMAO)[1, 3].

Sugars are of particular interest because they are known as excellent cryoprotectants and lyoprotectants, they are most efficient at protecting proteins and membranes against severe water loss during air or freeze drying[4, 5]

Table 2.1. Classes of osmolytes and their distribution in nature[1].

Class of Osmolyte	Occurrences	Key osmolyte
Sugars/Polyols	**Cynobacteria**	
	Synechococcus sp	Glucosylgycerol
	Fungi	
	Saccharomyces rouxii	Arabitol
	Asteromyces cruciatus	Arabitol,glycerol,mannitol
	Lichens	
	Lichina pygmeae	Mannosidomannitol
	Algae	
	Dunliella viridis	Glycerol
	Chlorella pyrenoidosa	Sucrose
	Vascular plants	
	Gossypium hirsutum L.	Glucose, fructose, sucrose
	Insects	
	Eurosta solidagins(gall fly)	Glycerol, sorbitol
	Crustaceans	
	Artemia salina(brine shrimp)	Trehalose
	Amphibians	
	Hyla versicola(tree frog)	Glycerol
	Mammals	
	Mouse renal cells	Sorbitol, myo-inositol
Amino acids and Amino acid derivatives	**Eubacteria**	
	Salmonella oranienburg	Glutamic acid, proline
	Streptococus feacalis	γ -Amino butyric acid, proline
	Protozoa	
	Miemiensis avidus	Glycine, alanine,proline
	Vascular Plants	
	Spartina towsendii	Betaine
	Aster tripolium	Proline

	Marine Invertebrates *Blanus nubilus* *Eriocheir sinensis* **Amphibians** *Bufo marinus*(marine toad)	 Various amino acids Various amino acids Various amino acids
Urea and Methylamines Urea	**Cartilagenous fishes** *Myxine glutinosa*(hag fish) *Squalus acanthias* (dog fish) *Dystas americana*(ray) **Mollusks** Bulimulus dealbatus **Amphibians** *Scaphiopus couchi* (spadefoot toad)	Urea& TMAO Urea Urea
Inorganic ions	*Halobacterium salinarium*	K^+

2.2. Sugars

According to the IUPAC Gold Book, sugar is a informal term used to describe all monosaccharides and some oligosaccharides[6]. Therefore, the term "sugars" can be used to describe sugar (sucrose) as well as other types of sugars such as glucose, fructose and lactose[7, 8].

A generic term used to describe these compounds is carbohydrates. Carbohydrates are simply defined as hydrates of carbon consisting only of carbon, hydrogen and oxygen in a 1:2:1 atom ratio [7, 9, 10]. This definition although true is exclusive of derived forms of carbohydrate containing other atoms in addition to the aforenamed atoms.

They include substituted carbohydrates in which the hydroxyl and/or hydrogen group is replaced by other groups. Substitution of a hydroxyl group by hydrogen or an amino group to give deoxy- sugars and amino sugars respectively, as well as phospho-sugars which is derived by substitution of hydrogen in hydroxyl group by phosphate groups. These forms of carbohydrates are naturally occurring as well as chemically synthesised [7,10]. Carbohydrates are also found covalently attached to membrane proteins or to membrane lipids termed as glycoprotein and glycolipids, respectively[7, 10].

Therefore, a modern and inclusive definition of a carbohydrate is "a polyhydroxy aldehyde or ketone or a compound that can be derived from them by reduction or oxidation to give sugar alcohols and sugar acids, respectively"[7, 10].

The simplest carbohydrates are monosaccharides (single sugar units) they are aldehydes or ketones with two or more hydroxyl groups. The general chemical formula of an unmodified monosaccharide is $(C{\bullet}H_2O)_n$ the smallest value for n is 3 [7, 10].

Other carbohydrates are composed of monosaccharide units covalently linked together, and break down under hydrolysis [7, 9, 10] These may be classified as disaccharides, oligosaccharides, or polysaccharides, depending on whether they have two, several, or many repeating monosaccharide units [7, 9, 10]. Monosaccharide units are bound together by glycosidic linkages formed via a condensation reaction as shown below, resulting in the loss of water a hydrogen atom from one monosaccharide and a hydroxyl group from the other[7, 10] .

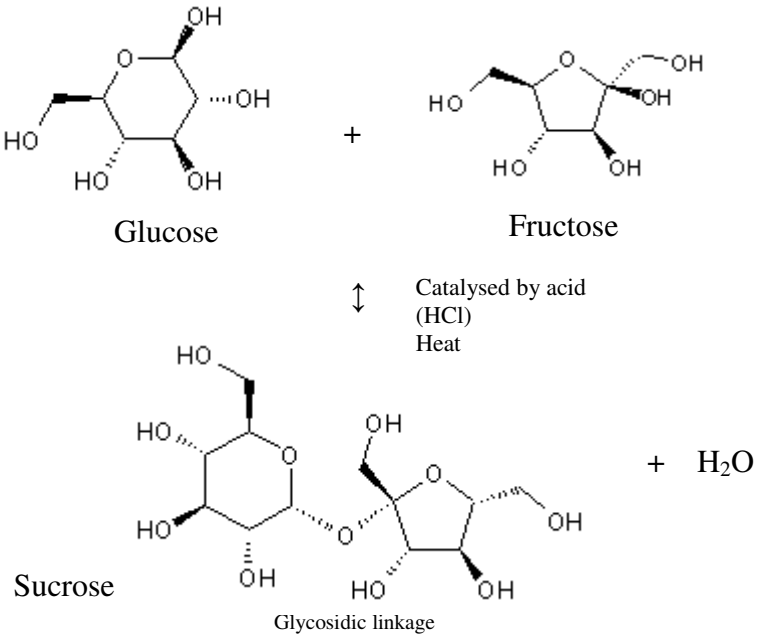


Figure 2.1. Formation of a glycosidic bond.

A useful classification system recommended by the WHO/FAO, groups carbohydrates according to the number of sugar units bonding in the chain or the degree of polymerisation (DP), as shown in table 2.2.

Table 2.2. Classification of carbohydrates[11].		
Class (DP)	Sub-Group	Components
Sugars (1-2)	Monosaccharides	Glucose, galactose, fructose
	Disaccharides	Sucrose, lactose, maltose, trehalose
	Polyols	Sorbitol, mannitol, lactitol, xylitol, erythritol, isomalt, maltitol.
Oligosaccharides (3-9)	Malto-oligosaccharides (α-glucans)	Maltodextrins
	Non α-glucan oligosaccharides	Raffinose, stachyose, fructo and galacto oligosaccharides, polydextrose, inulin
Polysaccharides (≥10)	Starch	Amylose, amylopectin, modified starches
	Non-starch polysaccharides	Cellulose, hemicellulose, pectin, arabinoxylans, β-glucan, glucomannans, plant gums and mucilages, hydrocolloids

2.2.1. Lessons from nature: accumulation of sugars in environmentally stressed organisms

Accumulation of sugars in environmentally stressed organisms have been observed in nature, disaccharides such as sucrose has been observed in higher plants while trehalose has been identified in animals[12-14]. Most notably the anhydrobionts have the unusual property of surviving dehydration for years, with the ability to maintain native cellular structures (membranes and proteins) and their function upon rehydration[15, 16] . An example of such includes *Selaginella lepidophylla*, commonly termed as the desert "resurrection plant" depicted in figure 2.2, in this plant trehalose as opposed to sucrose is accumulated in large quantities apparently as a stress protectant[14, 17, 18]. Other anhydrobiotic organisms include mosses and their spores, fungi, tardigrades, and nematodes[15].

Raffinose and myo-inositol was found located in the chloroplasts of cold-acclimated *Ajuga reptans* leaves. In addition, exposure of mature cotyledonary somatic embryos of *Picea abies* to low temperature (4°C) resulted in the accumulation of raffinose and stachyose[19]. Studies

have reported that stress tolerance is correlated with the observed presence of considerable quantities of non-reducing di- and oligosaccharides [12, 14, 20].

Figure 2.2. *Selaginella lepidophylla*[21]

2.2.2. Application of stabilizing properties of sugars

The stabilizing property of sugars has been applied in the biopharmaceutical industry, where sugars such as trehalose, are routinely used as excipients for protein therapeutics which serve to protect the protein during high temperature processing steps thereby preventing denaturation, precipitation as well as reduce the extent of aggregation[22-25].

In medicine sugars are used as a non toxic cryoprotectant of vaccines and organs for surgical transplants[23, 24].

The possibility of sugars acting as molecular chaperones to assist in preventing protein aggregation, the main cause of illness such as Creutzfeld-Jacob disease and amyloid disorders is being investigated [24].

Protein stabilization still remains a key issue in biotechnology, sugars prevent denaturation and inactivation of enzymes, and they maintain structural conformation and functional activity in solution, frozen and dried states[23, 25].

2.3. Mechanisms of action of sugar osmolytes: current hypothesis

Several mechanisms have been proposed as to how sugars exert their stabilizing effect on proteins. The mechanism differs in the dried and solution states, the modes of action of sugar in solution and the dried state have therefore been described.

2.3.1. Solution state mechanisms

Timasheff and colleagues first proposed a mechanism for the stabilisation effects of sugars in aqueous solution which is based on thermodynamic stabilization of biomolecules. Studies

carried out found that sugars are preferentially excluded from the surface of proteins, which increases the chemical potential of the protein [26-29].

Preferential exclusion of solute or sugars refers to a depletion of solute or sugars near to the surface of the protein[26, 27]. It is a type of interaction that gives a measure of solute composition at the surface of proteins relative to its bulk composition due to different preferences for being near the surface in comparison to water molecules [26, 27]. The condition of preferential exclusion of solutes suggest a consequent enrichment of water near the surface relative to its composition in bulk solution due a preference of water molecules for being near the protein surface, a condition called preferential hydration[27, 28].

Therefore the terms preferential hydration and preferential exclusion of solute can be used interchangeably to describe the solute/solvent distribution with respect to the protein[27,28].

These proposed mechanisms suggest that the degree of preferential exclusion and the increase in chemical potential are directly proportional to the surface area of protein exposed to solvent. Consequently, the system will favour the protein state with the smallest surface area by minimizing the thermodynamically unfavourable effect of preferential sugar exclusion according to the Le Chatelier's principle[27, 29].

This principle states that when a chemical system at equilibrium experiences a change in conditions for example changes in temperature or concentration, the equilibrium shifts to counteract the imposed change and a new equilibrium is established[30].

Therefore for the equilibrium between native and denatured states, the increase in protein chemical potential is greatest for the denatured state, which has a greater surface area [24]. Hence, with preferentially excluded solutes the free energy of denaturation is increased and the native state is stabilized [24].

More recently the osmophobic effect has been used to explain the preferential exclusion of sugars in solution[31, 32]. Bolen and co-workers have described the osmophobic effect as unfavourable interaction between a solvent component (osmolyte) and a protein functional group (the peptide backbone) based on transfer Gibbs free energy measurements of amino acid side chains and peptide backbone from water into a variety of osmolyte solutions [31, 32]. Relative to the native state, denatured protein exposes much more of the protein fabric to solvent. Thus, the denatured state of the protein is more solvophobic (has a greater preferential exclusion) toward osmolyte than the native state, making the Gibbs free energy

change of denatured state $\Delta G_{D[Os]}$ a significantly more positive quantity than Gibbs free energy change of the native state $\Delta G_{N[Os]}$ [31, 32]. The studies suggest that proteins are more stable in the presence of osmolytes than they are in water, because osmolytes raise the free energy of the denatured state far more than they do the native state [30, 31].
Osmolytes have also been shown to protect protein structure in the frozen state by being preferentially excluded from the protein surface [33, 34].

2.3.2. Dried state mechanism

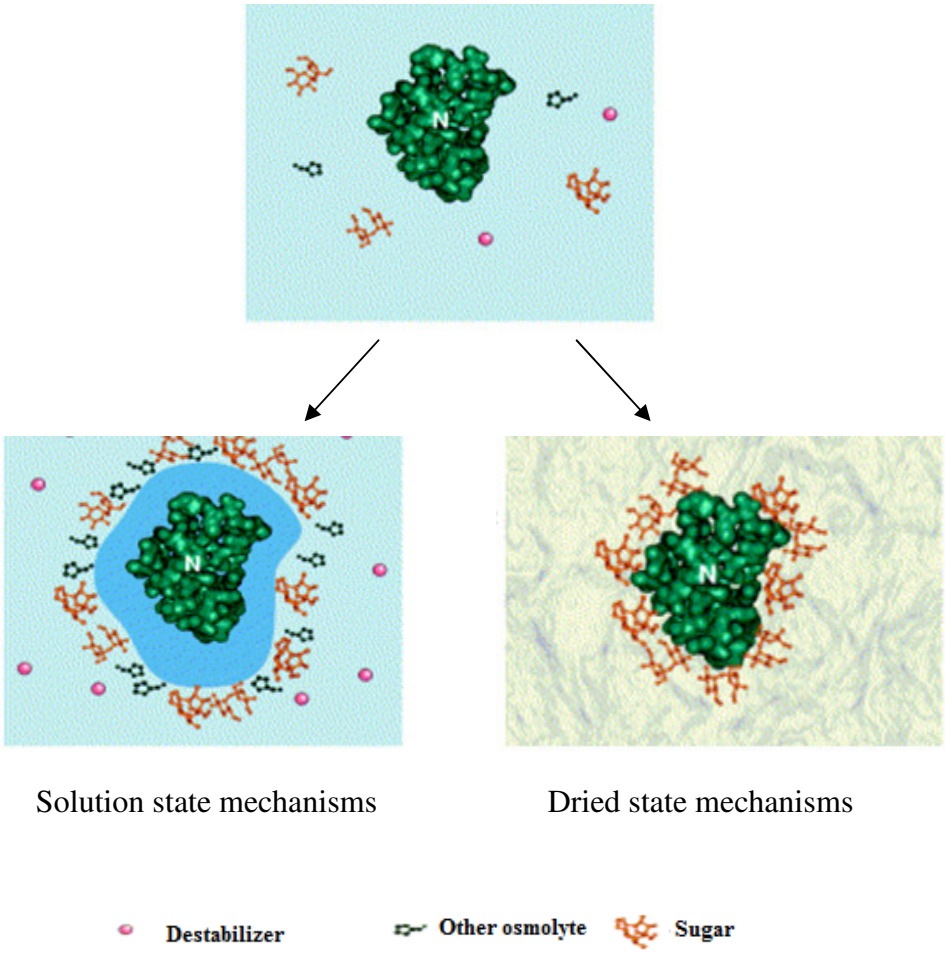
Carpenter and Crowe carried out an infra red spectroscopic study of the interactions of trehalose with dried lysozyme and proposed the water replacement mechanism for stabilization of proteins in the dried state[35]. Sugars were shown to stabilize biomolecules by forming strong hydrogen bonds with polar groups on the biomolecules and thus replace the water molecules that were lost due to drying [35, 36].

In addition, the ability of sugars to form glasses at all water content and temperatures during freeze drying is essential for the stabilization of the biomolecules in the dried state, known as the single amorphous immobilization hypothesis [37-40]

A glass is an amorphous material with characteristics that contrast the properties of crystals such as lack of a defined molecular structure[37, 38]. It does not possess the three dimensional long range order that exist in crystal, and the position of molecules relative to one another is more random, comparable with that of the liquid state[38, 39]. A glass is a physical solid but a supersaturated thermodynamic liquid, with an extremely high viscosity of 10 [12-14] Pa s. Therefore, in the cytoplasm, the viscous nature of glass inhibits diffusional movements of cellular molecules and therefore, any structural alteration would be extremely slow, concurrently achieving stability of the molecules,[38, 39].

In pea seeds with water content below 0.3 g (H_2O)/g dry weight; there exist a dramatic rise in viscosity of the cytoplasm, typical of a glass-forming system [38]. It is suggested that the removal of water induces a supersaturation of the cytosolic components leading to an increase in the cohesive forces between molecules and restriction of the molecular mobility within the cytoplasm[38, 41].

This mechanism involves immobilization of the protein within the glassy matrix, translational motion and relaxation processes are hindered, hence aggregation, unfolding, chemical degradation are prevented [32-35][42, 43]. Figure 2.3 gives a pictorial illustration of protein structure stabilization by sugars in solution state and in the dried state.



In the solution state there is preferential exclusion of the osmolytes from the protein surface which causes preferential hydration[44]. In a completely dried state i.e. below 0.3g (H2O)/ g dry weight, osmolytes other than sugars fail to stabilize the protein [15, 45]. This is so because of the excellent glass forming properties of sugar compared to the other groups of osmolytes[42, 45]. Sugar molecules replace water via hydrogen bonding and thus stabilize the protein in a dry glassy matrix in the cytoplasm of tolerant cell[46]

Figure 2.3. Mode of action of sugars on protein stability in solution and dried state, adapted from[45].

Questions relating to the exact mechanism(s) responsible for stabilizing proteins against freezing, air drying and freeze drying have been raised and investigated[47-50]. Studies have suggested that the mechanisms are not mutually exclusive processes, but linked together to ensure the stabilization of biological structures[51]. The effectiveness of one sugar over the other as a bioprotectant e.g. trehalose as a better lyoprotectant over another dextrose[50, 52] have necessitated exploring the possibility of ranking sugars in order of their stabilizing properties[50, 53].

When a protein unfolds many of the hydrophobic residues buried inside the protein's core are exposed to water. A bioprotective sugar makes this unfolding process energetically less favourable, our conjecture is that this arises because of the unfavourable interaction between the hydrophobic groups and the aqueous sugar solution. In attempting to understand this aspect of the mode of action of bioprotective sugars on biomolecules, the impact that such sugars have on the free energy penalty of hydrophobic group exposure of aqueous surfactant systems was examined. Micelle/surfactant systems and properties are thus described in subsequent sections.

2.4. Surface activity of surfactants

Surfactants are one of the most versatile substances in chemistry with important properties which include[54, 55]

i. Adsorption at interfaces and
ii. Micelle formation in solution

Surfactants have a characteristic molecular structure consisting of a chemical group that has little or no affinity for the solvent and is thus solvent repelling. This is called the "lyophobic group". This group is combined together with a group that has a strong affinity for the solvent and is thus solvent loving, termed as a "lyophilic group". This is known as the amphipathic structure. When the solvent is water or the medium is aqueous then we use the term hydrophobic for the former and hydrophilic for the later groups[56].

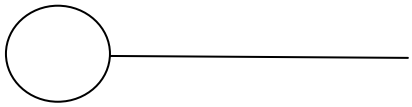


Figure 2.4. Structure of the surfactant.

The above figure is termed as a "head" and a "tail" structure of the surfactant. The head is hydrophilic which is generally depicted as a circle.

The tail may be depicted either as a straight or wavy line and it represents the long hydrophobic hydrocarbon chain.

2.4.1. Adsorption of surfactants at interfaces

A surfactant otherwise known as a surface active agent at low concentrations has the property of adsorbing onto the surfaces or interfaces of a system; they are thus capable of altering the surface or interfacial tensions of such systems[54, 56]. The concentration of surfactant adsorbed at the interface / surface at a given temperature is directly measured by obtaining an adsorption isotherm.

Adsorption isotherm is a direct measurement of the concentration of surfactant adsorbed at the interface as a function of concentration in the liquid phase when equillibrum has been reached at a given temperature[54]. This approach is applicable for a liquid /solid interface, at liquid /air and liquid/ liquid interfaces the direct determination of the amount of surfactant adsorbed per unit area is a difficult process because of the difficulty of isolating the interfacial region from the bulk phases for analysis[54].

Thus an indirect approach by measurement of interfacial /surface tension of surfactant solution as a function of equillibrum concentration of surafctant in the liquid phase is used to calculte the amount of surfactant adsorbed per unit area[54]. From a plot of surface tension as a function of concentration the amount of surfactant adsorbed per unit area is determined by the use of the Gibbs adsorption equation[54].

2.4.1.1. The Gibbs adsorption equation

The Gibbs adsorption equation is fundamental to all adsorption procesess and is given as[57]:

$$d\gamma = -\sum_i \Gamma_i \, d\mu_i \qquad (1)$$

Where $d\gamma$ is the change in surface or interfacial tension of the solvent, Γ_i is the surface excess concentration of any component of the system, $d\mu_i$ is the change in the chemical potential of any component of the system.

At equillibrum between the interfacial and bulk activity

$$d\mu_i = \mathrm{RT}d\ln a_i, \qquad (2)$$

where a_i is the activity of any component in the bulk phase, R is the gas constant and T the absoulte temperature[57].

Thus the Gibbs equation is

$$d\gamma = -RT \sum_i \Gamma_i \; dln\, a_i \qquad (3)$$

$$= -RT \sum_i \Gamma_i \; dln\, x_i f_i \qquad (4)$$

$$d\gamma = -RT \sum_i \Gamma_i \; d(ln\, x_i + lnf_i) \qquad (5)$$

Where x_i is the mole fraction of any component in the bulk phase and f_i its activity coefficient. Therefore for solutions containing solvent and only one solute

$$d\gamma = -RT(\Gamma_1 \; \mathrm{d\ln a_1} + \Gamma_2 \mathrm{d\ln a_2}) \qquad (6)$$

where subscripts 1 and 2 refer to the solvent and the solute respectively

For dilute solutions (10^{-2} M or less) the activity of the solvent and the activity coefficient the solute is considered to be constant and the mole fraction of the solute x_i may be replaced by its molar concentration C_2.

Thus for a completelty dissociating surface active solute in the presence of constant amount of electrolyte we have [58]

$$d\gamma = -RT\Gamma_2 dlnC_2 \qquad (7)$$

$$= -2.303RT\Gamma_2 d\; logC_2 \qquad (8)$$

And for dilute aqueous solutions of ionic surfactant in the absence of other electrolyte the Gibbs equation is given as

$$dy = -2.303\; \chi\; RT\Gamma 2\; d\log C_2 \qquad (9)$$

Where χ is the number of particles per surfactant molecule whose surface concentration change with change in the bulk concentration (C_2) of the surfactant.

For surface active solutes the surface excess concentration Γ_2 can be considered to be equal to the actual surface concentration without significant error[54]. From the surface or interfacial tension data and by the applying of appropriate Gibbs equation the concentration of surfactants at the interface may be calculated[54].

For dilute solutions of nonionic or a 1:1 ionic surfactant in the presence electrolyte with a common non-surfactant ion we have:

$$\Gamma_2 = -\frac{1}{2.303RT}\left(\frac{d_\gamma}{d\ logC_2}\right) \qquad (10)$$

For dilute solutions for a 1:1 ionic surfactant in the absence of any other solutes we have:

$$\Gamma_2 = -\frac{1}{4.606RT}\left(\frac{d_\gamma}{d\ logC_2}\right) \qquad (11)$$

And the surface concentration is obtained from the slope of a plot of γ versus log C_2 at constant temperature. When γ is in mNm^{-1} and R= $8.31Jmol^{-1}K^{-1}$, Γ_2 is given as moles/$1000m^2$. From the surface excess concentration (Γ) the area per molecule (A_o) at the interface in square angstroms is calculated from the relationship[54]

$$A_o = \frac{10^{16}}{N\Gamma_2} \qquad (12)$$

Where N is the Avogadro's number .

The area per molecule (A_o) at aqueous solution interfaces appears to be determined by the cross sectional area of the hydrated hydrophilic group at the interface[54]. An increase in the size of the hydrophlic group results in an increase in A_o and a consequent decrease in Γ.

2.4.2. Micelle formation in aqueous solution

Micelles are aggregated units composed of a number of surfactant molecules in solution[59]. When surfactants are dissolved in water the amphipathic structure of the surfactant induces

an orientation of the molecule at the surface in which its hydrophilic group is in contact with the aqueous phase, and its hydrophobic group oriented away from the aqueous phase predominantly towards air which is essentially non-polar in nature[59, 60]. In figure 2.5 further addition of surfactant causes an increase in its concentration at the air/water surface over its concentration in the bulk solution and thus reduces the surface tension of water consequently, a monolayer of surfactant molecules is formed at the interface [59, 60].

Micelles are formed when all the available surface area of the medium is covered with surfactant molecules such that the addition of any more surfactant molecules onto the sub-face surface will force the molecules to enter the bulk phase[54, 59, 60]. Consequently, they associate to create a new molecular arrangement in the aqueous phase where the hydrophobic groups are oriented within the cluster and the hydrophilic groups are exposed to water in order to minimise their surface free energy[54, 59, 60] . The surfactant concentration at which this phenomenon occurs is called the critical micelle concentration (CMC)[54].

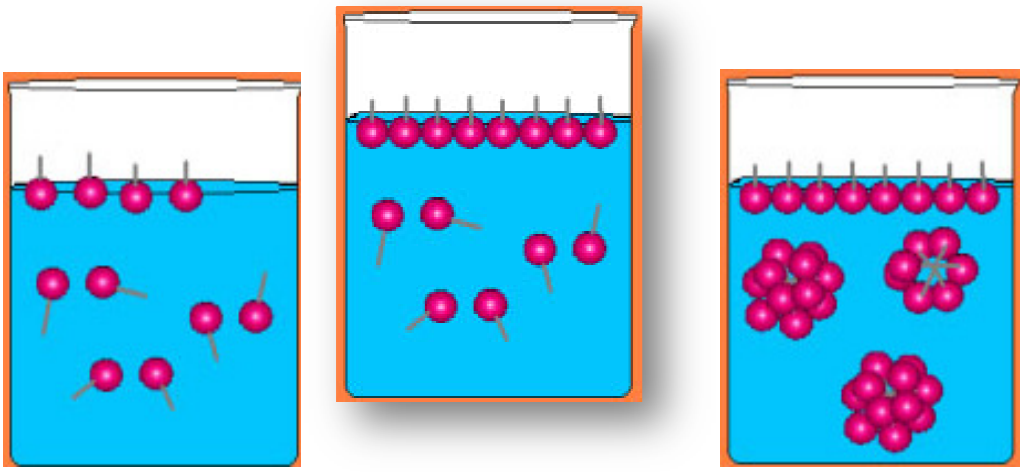


Figure 2.5. Micelle formation, adapted from[61].

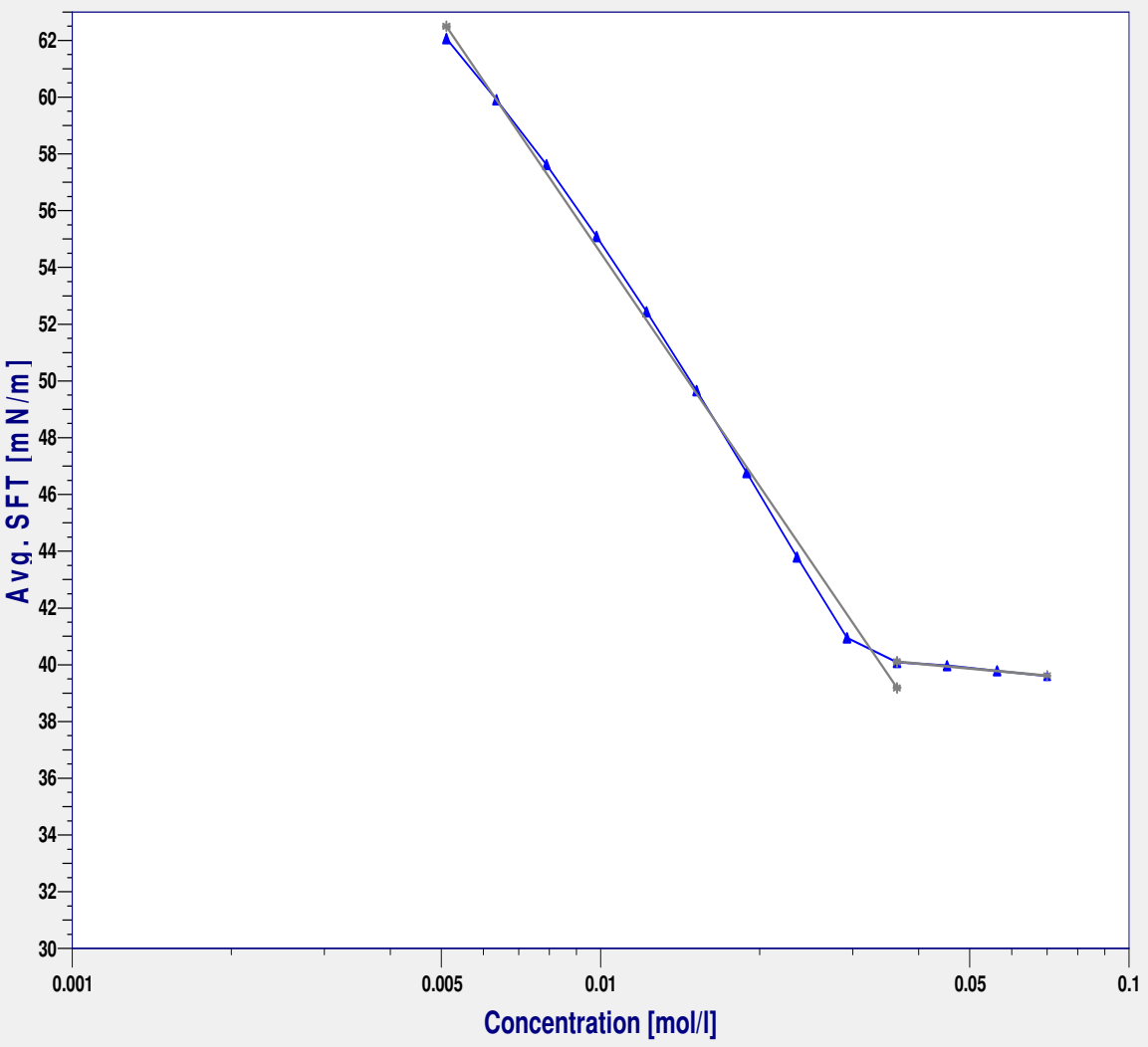


Figure 2.6. Determination of CMC

The CMC may be obtained experimentally by measuring surface tension of the solution with addition of surfactant molecule. The output of the measurement is represented graphically as a plot of surface tension versus surfactant concentration (preferably on a log scale) as shown in figure 2.6 [54, 62]. Surfactants exhibit a specific surface tension curve as a function of the concentration, it consists of a linear concentration dependent section and concentration independent section[62] . In the concentration dependent section the surface tension decreases linearly with the logarithm of surfactant concentration. In the concentration independent section the surface is saturated with surfactant molecules, and a further increase in surfactant concentration is tantamount to no appreciable influence on the surface tension. The position of the CMC is evaluated from the intersection of the two liner sections [62].

The CMC can also be determined by change in other physical properties such as osmotic pressure, detergency and most commonly break in electrical conductivity, light scattering, refractive index versus concentration curves, and others include changes in the spectral characteristics of a particular dye added to surfactant solution[54, 62]. This method is subject to inaccuracies as the presence of the dye stuff might influence the CMC value[54].

Spherical micelles are generally formed to achieve the lowest interfacial area, consisting typically of 30 to 100 surfactant molecules and a typical outer diameter of approximately 3-6 nm[54, 62, 63]. Other geometric shapes can be formed by the association of surfactants molecules such as cylindrical micelles, vesicles and liposomes and non-spherical micelles such as bilayers.

These various geometric shapes are dependent on packing considerations based on geometrical features of a surfactant hence influencing the final shape of the micelles[62] .

The geometric parameters include the optimal head group area (A_h) based mainly on hydrophilic, stearic and ionic repulsions between adjacent head groups trying to enlarge the head group area, tail volume (V_t) which is the volume of hydrocarbon liquid per hydrocarbon molecule and the cone height (L_c) which depends on the effective chain length of the hydrocarbon tail which is slightly less than the radius of the micelle[62, 63].

2.4.2.1. Thermodynamics of micelle formation

It is generally assumed that the onset of micelle formation occurs at a specific concentration, the CMC[54, 64]. The formation of ionic micelles from monomeric ions results from a balance of hydrophobic interactions between hydrophobic groups of the amphipathic micelles and electrostatic interactions between their hydrophilic charged groups as well as with counterions[64, 65]. When a surfactant hydrophobic hydrocarbon tail is exposed to water it does not form a hydrogen bond with it, instead it occupies a cavity in the liquid water structure. On the formation of micelles, the hydrophobic group are removed from the water and the highly organised water structure involved in the cavity breaks up and reverts to normal hydrogen bonded liquid water with an increase in entropy[54, 64].

Theoretical discussion of micelle formation includes the application of two models[54, 64, 66].

i. Mass action model
ii. Pseudophase model

From the law of the mass action the equilibrium between n detergent ions D^{-1} and m counterions C^+ and monodisperse micelles $M^{(n-m)-}$ is given as[54, 66]

$$nD^- + mC^+ \rightleftharpoons M^{(n-m)-} \qquad (14)$$

Where each micelle $[M^{(n-m)-}]$ is assumed to contain n surfactant ions D^- and m free counterions C^+, so that a fraction m/n (known as degree of ionisation α) of the charge of the detergent ions in each micelle is neutralized. From the equation above equilibrium constant for micelle formation is given as

$$K_{mic} = \frac{[M^{(n-m)-}]}{[D^-]^n[C^+]^m} \qquad (15)$$

This model predicts a CMC whereby a relatively rapid increase in $[M^{(n-m)-}]$ occurs over a narrow range of $[D^-]$

From equation (15) the Gibbs free energy change of micelle formation per mole of surfactant monomer is given by the thermodynamic argument as

$$\Delta G_{mic} = -\left(\frac{RT}{n}\right) \ln K_{mic} \qquad (16)$$

Substituting the value of K_{mic} from equation (1) we obtain

$$\Delta G_{mic} = \left(\frac{RT}{n}\right)\{nln\,[D^-] + m\ln[C^+] - \ln[M^{(n-m)-}]\} \qquad (17)$$

At CMC, $[D^-] \cong [C^+] \cong CMC$

$$\Delta G_{mic} = RT(1 + m/n)\ln CMC \qquad (18)$$

The pseudophase model of micelle formation treats micelles as a separate phase distinct from the bulk solvent in which they are present[64, 67]. Micelles differ from a true phase due to the fact that it is not a macroscopic phase they are therefore termed as a pseudophase[64]. In this model micelles are described as dispersible and optically clear solutions, the CMC in the pseudophase theory represents the solubility of the surfactant in the aqueous phase[64].

Micellization has been described as a co-operative phenomenon that resembles phase separation such as crystallization, however micellization differs from phase separation in the

sense that micelles are not a macroscopic phase, it is considered as a system consisting of several tens or hundreds of molecules[64, 67]

This model describes the little or no change in the surface tension of surfactant solution above the CMC according to the Gibbs adsorption isotherm. This is due to the formation of a micellar phase since the chemical potential is kept constant irrespective of the amount of material[64, 67].

In the pseudophase model the chemical potential of the surfactant in the micelle phase μ_m can be introduced as a function of T,P and from the equilibrium condition as

$$\mu_i = \mu_m[T, P] \qquad (19)$$

Therefore the Gibbs free energy change of micelle formation (ΔG_m) can be related to the standard chemical potential difference

$$\Delta G_m^\circ = \mu_m - \mu_i \qquad (20)$$

Therefore

$$\Delta G_m^\circ = RTln(x_i) = RTln(x_i)CMC \qquad (21)$$

To apply the mass action model requires the knowledge of aggregation number with its standard deviation, micellar shape and its distribution[64, 66]. Conversely experimental data can be analysed by applying pseudophase model with fewer data than is required for the mass action model. The two approaches are complementary[64, 66, 67].

Other thermodynamic parameters are obtained by the Gibbs-Helmholtz equation[54]

$$\Delta G_{mic} = \Delta H_{mic} - T\Delta S_{mic} \qquad (22)$$

Where ΔH_{mic} is the enthalpy of micellization and ΔS_{mic} is the entropy change of micellization and T is the temperature.

In aqueous medium an increase in the length of the hydrophobic group causes an increase in the value of ΔS_{mic} and usually a smaller decrease in ΔH_{mic} making ΔG_{mic} more negative by about 3KJ per $–CH_2$ group thus favouring micellization[54, 68].

2.4.3. Factors that affect CMC

The value of the CMC in aqueous media is affected by several factors. The factors that markedly affect the CMC include the structure of the surfactant, electrolytes, organic additives and temperature of solution.

2.4.3.1. Surfactant structure

The structure of the surfactant includes the hydrophobic group and the hydrophilic group. In general as the hydrophobic character of the surfactants increases the CMC is decreased[54, 64]. In aqueous medium as the length of the hydrocarbon chain increases the value of the CMC is reduced. A general rule is applied for ionic surfactant, whereby the CMC is halved by the addition of one methylene group to the straight chain hydrophobic group and quartered by addition of two methylene groups[69].

The CMC decreases as the number of carbon atoms in a straight chain hydrophobic group increases to about 16. The CMC reduces less rapidly with increase in the length of the chain and at 18 carbons the CMC value may remain substantially unchanged as a result of coiling of long chains in water[54]. Empirical equations relating the CMC and the number of carbon atoms (N) in the hydrophobic chain of homologous straight chain ionic surfactants in aqueous medium is given by the relationship[69].

$$logC_{CMC} = A - BN \qquad (23)$$

Where A and B are experimentally determined constants for a particular ionic head at a given temperature and at 35^{o}C respectively[69].

2.4.3.2. Electrolytes

The presence of electrolyte causes a decrease in the CMC especially for ionic surfactants. A decrease in the CMC arises mainly from the reduction of the thickness of the ionic atmosphere surrounding the head groups as the electrical repulsion between the ionic heads decreases[65, 70].

2.4.3.3. Organic additives

The presence of organic additive may produce marked changes in the CMC of surfactants in aqueous media. These organic materials have been grouped in two classes, class I and class II based on the how they affect the CMC.

Class I materials affect the CMC by becoming incorporated into the micelles. Members of these groups are generally polar organic compounds such as alcohols and amides, and they tend to decrease CMC by being adsorbed mainly in the outer portion of the micelle close to the water micelle interface[54]. Adsorption of these additives in this manner decreases the work required for micellization and for ionic surfactants by decreasing the mutual repulsion of the ionic heads in the micelles[54].

Class II materials changes the CMC indirectly by modifying the solvent micelle or solvent–surfactant interactions. They increase or reduce the CMC of surfactants at higher bulk concentrations compared to members of class I materials[54].

In aqueous media they act by modifying the structure of water, its dielectric constant or its solubility parameter. They are categorised as water structure breakers or structure makers[54]. Members that increase the CMC of surfactants are said to disrupt the water structure, this may increase the degree of hydration of the ionic group which opposes micellization thereby increasing the CMC[54]. Members of this group include urea, formamide and guanidinium salts. Water structure promoters include sugars such as xylose, fructose.

2.4.3.4. Temperature

The effect of temperature on CMC is complex. As temperature increases the CMC value initially decreases as a result of decreased hydration of hydrophilic group which favours micellization[54]. However a further increase in temperature of the system causes disruption of the structured water surrounding the hydrophobic group an effect which disfavours micelle formation[54]. Hence the relative magnitude of these two opposing effects determines if CMC increases or decreases over a particular range. Reports have shown that for ionics the minimum in the CMC temperature curve is about 25 ^{o}C and 50 ^{o}C for non ionic surfactants[54].

2.5. Micelles, biomolecules and biological structures

Micelles show resemblance to biological structures, in terms of the amphipathic nature of constituent components. It is made up of surfactants with polar (hydrophilic) and non polar (hydrophobic) groups. In aqueous environment its non-polar groups are sequestered in the middle with polar groups facing the aqueous environment forming a colloidal sized cluster. Micelle formation is driven by hydrophobic interactions [60, 62, 63]. This interaction is also present in biological structures as a driving force for the assembly of phospholipids into biological membranes and, in part for the conformation of many proteins.

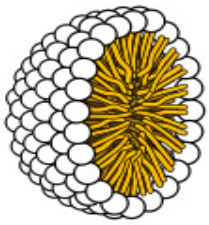


Figure 2.7. A cross section of a spherical micelle[71].

2.5.1. Micelles and membranes: structural similarities

Structurally the membranes of cells are composed of a bilayer comprising phospholipids, lipids and proteins. The bilayer or membrane are of significant importance in biology serving as a barrier in cell separating the interior of the cell from the environment[62]. Bilayers are arranged so that the hydrophilic lipid heads are facing the water on either side of the bilayer and the hydrophobic tails are sequestered in the core of the bilayer[62]. This arrangement is similar to the arrangement found in the micelle; the bilayer has been described as a non-spherical micelle[62].

The hydrophobic interior also prevents diffusion of charged ions and polar molecules from one compartment to another. Protein molecules such as glycoprotein are embed in it, this is made possible by interactions involving the hydrophobic and hydrophilic portions of the protein[62]. These proteins act as channel for the transport of polar molecules and ions across the cell membrane[62].

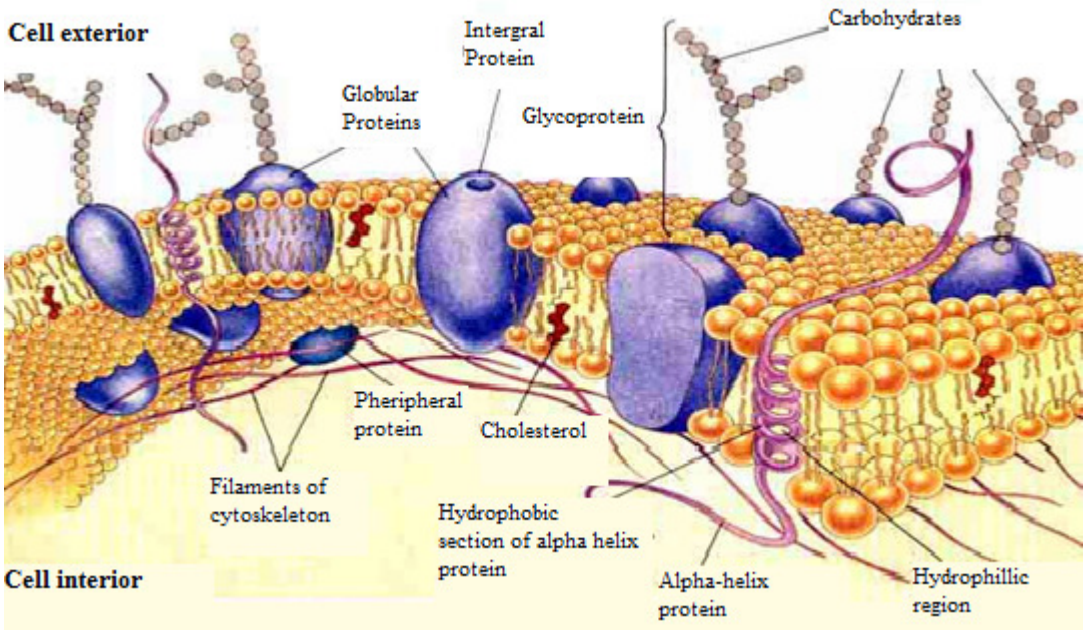


Figure 2.8. A section of phospholipid bilayer of a cell membrane[72].

2.5.2. Micelles and proteins: structural similarities

Proteins are large biological molecule composed of linear sequence of amino acids chain linked together by peptide bonds[73-75]. Proteins contain complex hydrophobic and hydrophilic components, they have a strongly hydrophilic (polar) peptide group and either non-polar (hydrophobic) or polar amino acid side groups[73, 75, 76].

The native conformation of proteins is characterized by tightly folded structure with polar groups on the surface and non-polar groups buried, stabilized by mostly non-covalent forces or interactions mainly categorised as electrostatic and hydrophobic interactions[73-76]. Non-polar hydrophobic amino acid side groups for instance Val and Leu causes the enclosure of these groups into the interior of the protein molecule mainly due to hydrophobic interactions amongst these groups, while polar amino acids such Arg, His, Lys, Asp, and Glu are usually located on the surface of a protein in contact with the aqueous environment[75, 76]. Polar groups tend to extend outward hence forming the protein's surface, while the interior of the protein is largely occupied by non polar side groups[75, 76].

This orientation of polar groups to the exterior and non polar groups to the interior of the protein molecule driven largely by hydrophobic interactions is also evident in the micelles[62]. They are therefore similar to the micelles in this respect, they contain polar and non polar part that arrange into an energetically favourable structure in aqueous environment.

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Chapter 3. Principal experimental techniques: theory, instrumentation and applications

3.1. Introduction

This chapter describes the instrumentation, applications as well as the theoretical underpinnings for the principal experimental techniques used in this project. The data obtained from the use of these techniques have been presented and discussed in subsequent chapters.

3.2. Theory of surface tension and measuring techniques

3.2.1. Definition of surface tension

Surface tension of a liquid (γ) is defined as the force acting parallel to the surface and at right angles to a line of unit length anywhere on the liquid surface [1-3] . The occurrence of surface tension and interfacial tension arise because of the action of intermolecular cohesive forces of attraction [2, 4, 5]. Each molecule is surrounded by other molecules and are all subjected to equal forces of attraction in all directions in the interior of the bulk phase[2, 4, 5]. However, the molecules located at the surface or the liquid/air interface experience an unbalanced attractive force mostly an inward pull and a sideward pull by neighbouring molecules , with no outward pull to balance the inward pull because there are few molecules above the surface [4, 6]. This net inward attraction tends to contract and minimize the area of the surface [4, 7].

3.2.2. Fundamentals of surface tension measurement

The basis of a surface tension measurement can be illustrated by a simple apparatus made up of a loop of wire with a slide wire upon which a film might be formed by dipping into a liquid and stretched by an applied force (F) to a distance (x) as shown in figure 3.1. The force (F) operates along the entire edge of the film and varies with the length (L) of the slide wire.

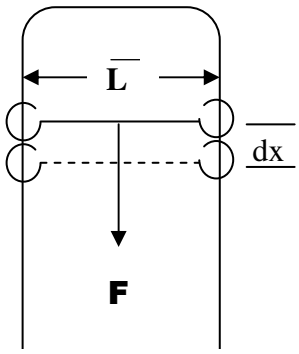

Figure 3.1. A simple apparatus which permits the measurement of surface tension, Adapted from[4].

Therefore, the force per unit length of edge which is the intrinsic property of the liquid surface is measured as the surface tension[4]. Since the film in the figure has two sides, the surface tension measured by this apparatus is given as

$$\gamma = F/2L \qquad (1)$$

Equation (1) defines the unit's surface tension as the force per unit length (N/m or usually mN/m).

The above definition of surface tension is inadequate because it is assumed the liquid has a membrane skin or a tangential force at the surface of the liquid, although there's no such skin or tangential force in real systems[4, 7, 8]. The work done by the system in the above figure is given by the equation[9].

Work = Fdx since F= γ2L from equation 1

$$dW = \gamma\, 2L\, dx = \gamma dA \qquad (2)$$

combining equations 1 and 2 gives

$$\gamma = dW/dA = F/2L \qquad (3)$$

where dx is the change in distance, and dA is change in surface area.

Equation (3) supplies a second definition of surface tension; it is equal to the work per unit area[9]. This equation also defines the surface free energy of a material which is the work required to bring a molecule from the bulk phase to its surface, thereby creating a new surface of $1m^2$ unit area[7, 9]. The surface tension and the surface free energy have been

described as dimensionally equivalent quantities ($N/m=J/m^2$) and numerically equal for pure liquids in equilibrium with their vapour[7].

3.2.3. Liquid surface and interfacial tensions methods

There is no fundamental difference between the terms surface and interface, the word *surface* is used to define the physical boundary of one phase for instance liquid surface[1, 3]. The word *interface* is defined as the physical boundary between two adjacent bulk phases. However in reality, interactions and contact between phases exists such as liquid-air, solid–air except under absolute vacuum conditions for a solid, hence the term interface is preferred [4-6]. Thus in practice, liquid-air interfacial tension instead of surface tension of liquid are measured.
Physical and surface chemists tend to neglect the small difference between air phase and absolute vacuum conditions thus surface and interface are used interchangeably [2,1].

There are many experimental methods for determining the surface /interfacial tension of pure liquids and solutions, these methods have been classified as static or dynamic based on the rate of change of the area of the interface/surface with time. For static methods the interface/surface remains the same while in dynamic methods the surface area changes measurably with time [2,7]. Static methods include the capillary rise, pendant and sessile drop shape techniques as well as the plate and ring methods which can also be classified as detachment methods[7]. Static methods offer greater potential for accurate surface tension measurements in contrast to dynamic methods which offer greater potential for accurate and precise determination of the interfacial tension between two liquids[7]. Examples of dynamic methods are maximum bubble and growing drop pressure techniques. Others include spinning drop, capillary wave and oscillating jet techniques[7]. Some drop volume techniques can also be regarded as a dynamic method where the surface area of the drop changes with flow rate, in which streams of drops of a liquid falls slowly from the tip of a thin glass tube of known radius [6,7].

Surface/ interfacial methods can also be classified according to the type of physical parameter which is being measured [10], as shown in figure 3.2. For instance, the force required to detach a probe (ring /plate) from a surface or interface as used in both the du Noüy ring and Wilhemy plate methods. The latter technique will be described in detail in subsequent sections. Other physical parameters that can be measured are the volumes of falling drops of a liquid detached from a vertically mounted narrow tube[7]. The value of the maximum

pressure needed to push a bubble out of a capillary tube and dimensions such as the shape of a liquid drop suspended from the tip of a thin tube or volumetric syringe. Another is the height of a liquid column in a capillary tube of known radius usually made of glass as in the capillary rise method [7].

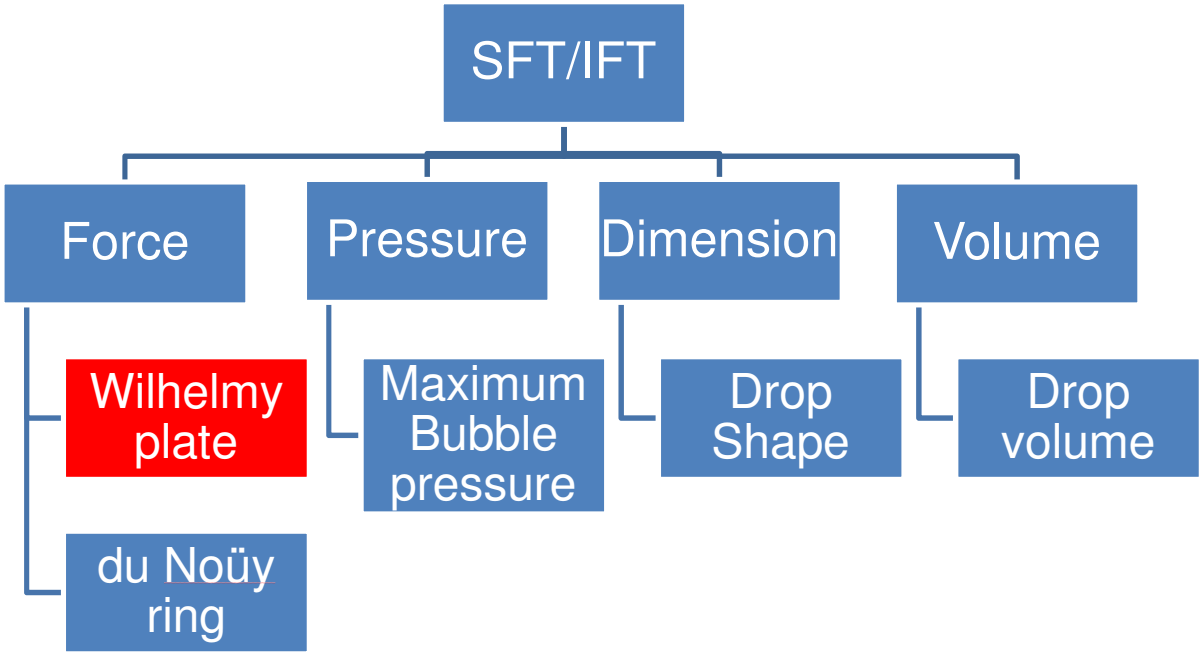

Figure 3.2. Classification of surface / interfacial tension methods based on physical parameters.

In comparison to the other methods, capillary rise method measures the most precise and accurate surface tension values when performed properly i.e. allowing for sufficient equilibration time of the stationary liquid surface [2, 7]. However several difficulties are associated with this method including the ability to obtain a capillary tube with a uniform bore and internal surface that is perfectly wetted by the imbibing liquid.
This method is also tedious and time consuming and is preferably used to measure surface tension of pure liquids [2,7].

Due to improvements in computer controlled instrumentation, other surface tension methods such as ring and plate methods also give high precision and accurate surface and interfacial tension measurements[9]. The plate and the ring methods are one of the most widely used methods in the surface chemistry laboratory due to their ease of use and rapidity [6,7,9] . The Wilhemy plate method immersed at constant depth has been described as a suitable method

for measuring surface tension of solutions with surfactants due their preferential adsorption onto the solid surfaces of glass capillaries, substrates and rings used in other techniques [6,7,9].

3.2.4. Wilhelmy plate method.

This method was first applied in 1863 by Wilhemy, using a lever balance and thin plates called *Wilhelmy plates* usually made of roughened platinum, platinum/iridium, alloy, glass, mica steel or plastic [7, 8]. Other materials used as plates include disposable filter or chromatographic paper. The plate is suspended from a balance and is vertically and partially immersed into the liquid whose surface tension is to be measured as shown in figure 3.3. The liquid is then raised until the contact between the surface or interface is registered[10]. The maximum tension acts on the balance at this instant and the tension is thus calculated from the equation[7].

$$\gamma = F/L.\cos\theta \qquad (4)$$

where: F is the force measured, L is the wetted length and θ is the contact angle, which gives a quantitative indication of wetting of a solid by a liquid[7].

Contact angle less than 90^o indicates that the liquid wets or partially wets the solid, when contact angle is 0, this indicates complete wetting of the solid[7]. A contact angle greater than 90^o represents non wetting of the solid by the liquid[7]. The roughening of the surface improves wettability of the platinum plate such that it is optimally wetted with a contact angle (θ) that is virtually zero (cos (0) = 1).

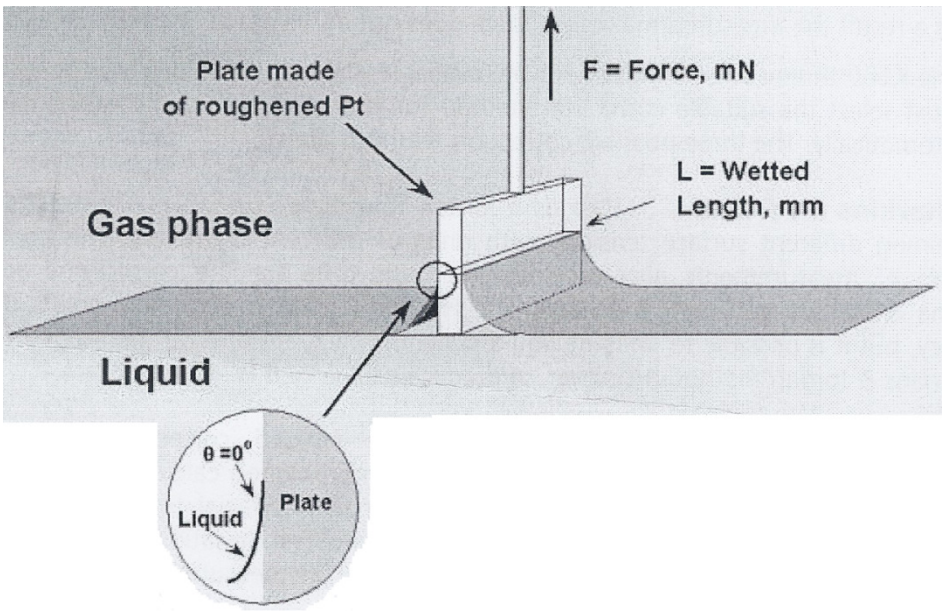

Figure 3.3. Schematic diagram of the plate method[10].

The plate method is advantageous over the ring method because the measured values do not need to be corrected which is in contrast to the ring method[7, 8]. The measured value obtained by the ring method are normally quite high because it also includes the force created by the weight of the lifted liquid lamella, thus the values need to be corrected by a correction factor determined experimentally[7]. In addition discrepancies in the measured and actual value of maximum force that arises as a result of differences in curvature of the film at the inside and outside of the ring, of which the former is greater than the later , are corrected for [7, 8].

Surface tension measurements are exceptionally sensitive to impurities from the surrounding environment thus particular consideration for the need of extreme cleanliness cannot be over emphasized. It's been suggested that touching the surface of 100cm^2 of water with a finger tip deposits enough contamination on the water surface to introduce a 10% error in the value of surface tension[7]. Experiments are thus performed within an enclosed measuring chamber and the measuring probe is flamed before and after measurement of surface tension [7, 8].

3.2.5. K100 Tensiometer

The K100 tensiometer from Kruss (Germany) was used to carry out surface tension studies. This instrument offers a time proven tool in which accurate measurements of the surface tension of a liquid and interfacial tension between two liquids can be obtained.

The K100 determines the surface or interfacial tension with the help of an optimally wettable probe (either a ring or a plate) suspended vertically from a precision balance[10]. The instrument is equipped with a height-adjustable thermostattable sample vessel holder which is used to bring the liquid to be measured into contact with the probe[10]. The measuring parts of the K100 are shown in figure 3.4.

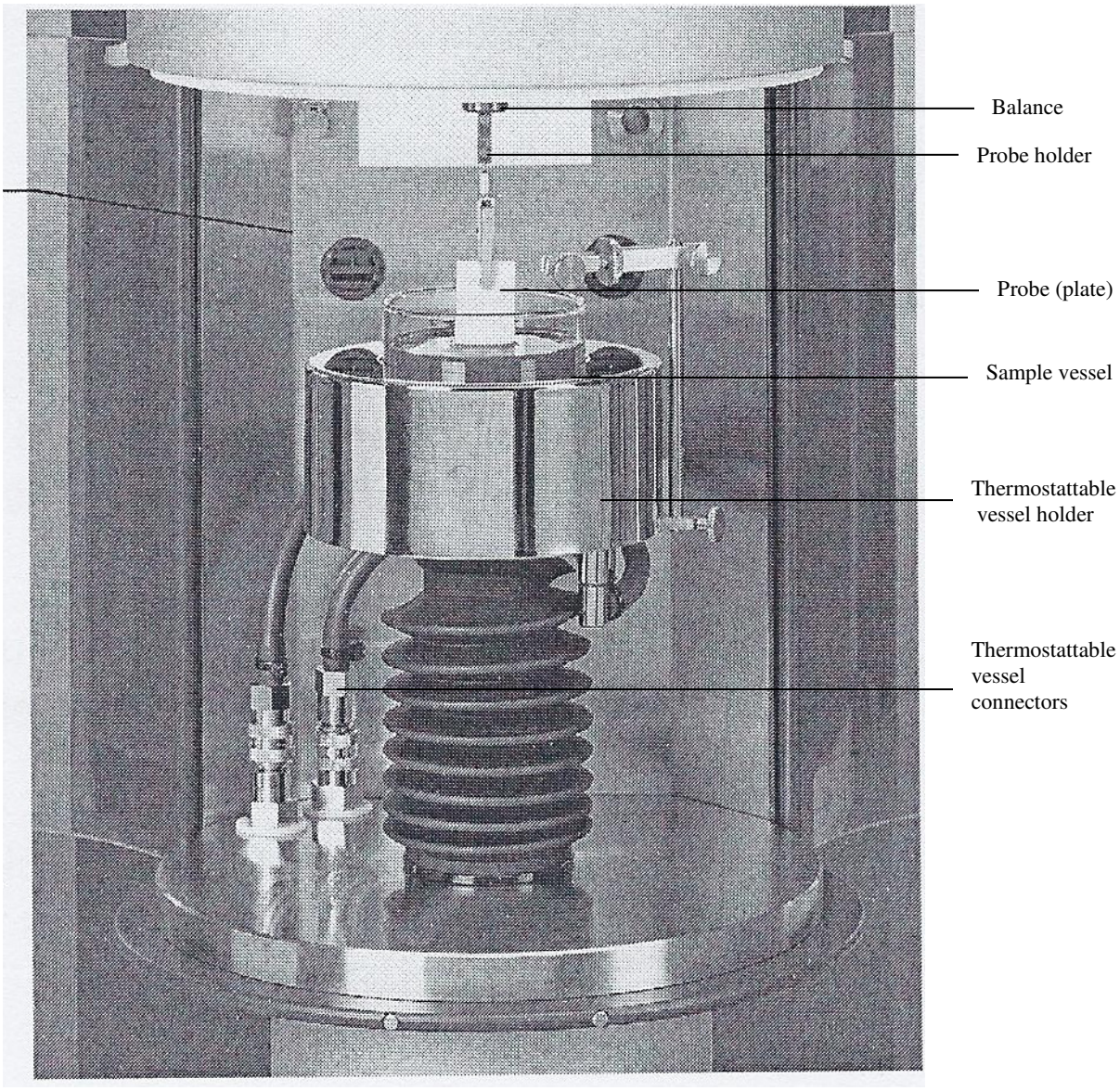

Figure 3.4. Measuring chamber of the Kruss100 tensiometer[10].

A force acts on the balance as soon as the probe touches the surface, and is registered by the tensiometer. The value of the wetted length of the probe and the measured force is used to calculate the surface or interfacial tension. It is required that the probe has a very high surface energy, therefore a platinum iridium alloy is used for the ring and platinum is used for the plate [10].

The temperature of the sample vessel holder of the instrument is thermostatically controlled the K100 is thus able to measure within the temperature range of -10^{o}C up to a maximum of 130^{o}C. For temperatures above 90 ^{o}C oil is used as the thermostat liquid, during high temperature measurements > above 70 ^{o}C the space above the surface gets hot, temperature

variations at the interface causes a measuring inaccuracy which increases as the temperature increases. This error is avoided by thermostatting the gas phase above the interface with the aid of gas thermosetting system[10].

Calibration of the instrument is carried out when the instrument is transported and it is thus done periodically, it involves calibration of the balance. In this process a built in reference is weighed and the variation in measured value is electronically compensated[10].
This instrument also determines the critical micelle concentration (CMC) of surfactants, which is an important parameter for the characterisation of surfactants. Other measurements that can be carried out with the K100 tensiometer include contact angle determination, density of liquids as well as sedimentation and sorption measurements[10].

3.2.5.1. Critical micelle concentration (CMC)

The critical micelle concentration of a surfactant can be defined as the surfactant concentration above which colloidal sized clusters otherwise known as micelles are spontaneously formed in solution, a phenomenon known as micellization[2] . The CMC may be obtained experimentally by carrying out surface tension measurements on a series surfactant solutions of different surfactant concentrations[2, 7]. The output of the measurement is represented graphically as a plot of surface tension against surfactant concentration (preferably on a log scale)[11] evaluation of the CMC from this plot has been illustrated in chapter two. The CMC measurement and evaluation is carried out automatically using the Labdesk software of the K100. The concentration series is generated automatically with a computer controlled Dosimat system from Metrohm, connected to the K100, hence only a stock surfactant solution is prepared[10].

3.2.5.2. K100 and Reverse CMC measurement

The K100 allows CMC measurements to be carried out in the following way, a stock surfactant solution (the surfactant concentration is excess of the CMC) is placed in the measuring vessel[10], the concentration is then incrementally decreased between surface tension measurements by addition of solvent. This method is useful for measuring low CMC values since the method permits low concentration values to be reached through the addition of large amounts of the solvent thereby reducing the error due to dosing small amounts of the sample solution[10].

3.2.5.3. Other important micellization and surface parameters

Gibbs free energy change of micellization ΔG_{mic}

This can be defined by the equation below

$$\Delta G_{mic} = RT.\ In_{(cmc)} \qquad (5)$$

Where R is the gas constant, T is the temperature

A negative free energy change suggests that micellization is favoured[12]

Surface excess concentration (Γ) and **area per molecule** (***Ao***)

Surface excess concentration is a quantity defined by

$$\Gamma i = n^{\sigma}_{i} / A_s \qquad (6)$$

(where $n^{\sigma}i$ is the surface excess (or adsorption) of component i and A_s is the area of the dividing surface or interface.)

For surface active solutes the surface excess concentration can be considered to be equal to the actual surface concentration for dilute surfactant solutions[11]

From the surface excess concentration, the area occupied per surfactant molecule at the interface is calculated from the relationship

$$A_o = 10^{16}/N\Gamma \qquad (7)$$

Where N is the Avogadro's number

The area per molecule at interface provides information on the degree of packing and the orientation of the adsorbed surfactant molecule[11]

Surface pressure (π)

Surface pressure is defined as the change of surface tension caused by addition of a given species to a base solution[11]. When an area of liquid covered with a spread substance is separated from a clean area of surface by a mechanical barrier, the force acting on unit length of the barrier is called the surface pressure.

$$\pi = \gamma^{o} - \gamma \qquad (8)$$

where γ^{o} is the surface tension of the clean surface and γ that of the covered surface.

3.3. Calorimetric analyses

3.3.1. Definition of calorimetry

Calorimetry is the science of measuring the heat (enthalpy) uptake or release in chemical reactions or physical changes[13, 14]. The word calorimeter is derived from the Latin word 'calor', meaning heat[13, 14].

Since most chemical reactions and changes of state involve changes in enthalpy (except the formation of an ideal solution which is enthalpically silent) therefore the use of calorimetry in principle should be of universal applicability provided the enthalpy changes for the reaction can be measured precisely and accurately[15].

Calorimeters are based on direct measurements of intrinsic thermal properties of the samples under investigation. They require no chemical modifications or extrinsic probes as opposed to a wide variety of non-calorimetric methods such as fluorescence and circular dichroism spectroscopy, which indirectly determine thermodynamic parameters from theoretical calculations such as the use of van't Hoff analysis [13, 14]. These types of indirect approach are subject to severe limitations. In addition calorimetric experiments provide fundamental thermodynamic information with careful analysis and interpretation[15].

3.3.2. Types of calorimeter

Calorimeters have been classified into three main groups based on the principle of heat measurement and is depicted in figure 3.5.

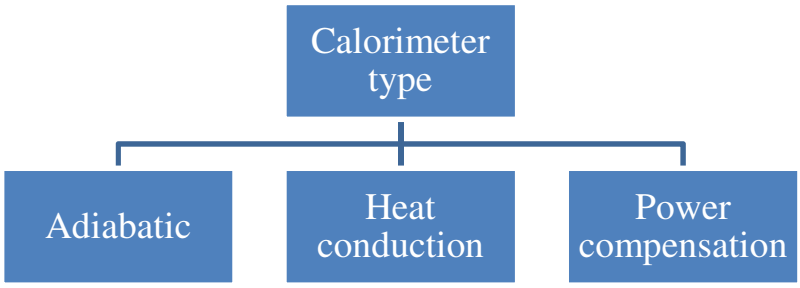

Figure 3.5. Types of calorimeter.

3.3.2.1. Adiabatic calorimeter

In this type of calorimeter the sample is thermally isolated from its surroundings, in other words there is no heat exchange between the calorimetric vessel and its surroundings, thus little or no heat is lost to the environment[13, 16]. However, in principle no calorimeter is truly adiabatic, some heat may be lost by the sample to the sample holder. Adiabatic conditions are achieved by placing an adiabatic shield between the vessel and its surroundings, the temperature difference between the vessel and the shield is kept at zero[13]. The heat which is evolved or absorbed during an experiment is equal to the product between the temperature change and the heat capacity of the calorimetric vessel in an ideal adiabatic calorimeter[15].

3.3.2.2. Heat conduction calorimeter

In contrast to the adiabatic calorimeter, heat generated or absorbed by the sample in the reaction vessel is exchanged with the surrounding[13]. Heat is allowed to flow to or from the surrounding heat sink. Heat flow is achieved by the use of a thermophile sensor positioned between the reaction vessel and the heat sink, usually an aluminium block. The system is designed so that the main path of the flow of heat to or from the reaction/measuring vessel is through the thermopile[13, 15].

3.3.2.3. Power compensation calorimeter

This type of calorimeter utilizes a cooling power to balance the thermal power generated from an exothermic process normally with the use of water or Peltier effect, and for endothermic processes, compensation is achieved by reversing the Peltier effect current or by use of an electrical heater[13, 16].

For all the types of calorimeters the first law of thermodynamics applies, and the same heat balance applies[13, 16].

$$\frac{dQ}{dt} = \Phi + C\,\frac{dT}{dt} \qquad (9)$$

dQ/dt = the rate of heat production by a sample

Φ = the heat exchange within the surroundings

C dT/dt = the rate of heat accumulation of the sample where C is the heat capacity of the sample, and *dT* is the change in temperature during the time *dt*

3.4. Biocalorimetry

Advances in the field of calorimetry in terms of the availability of high sensitivity instrumentation and improvements in computer software permit the study of thermodynamics of biomolecular conformations and interactions in solutions at low concentrations.
Biocalorimetric tools such as isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC) have been utilised in this study.

3.4.1. Isothermal Titration calorimetry (ITC)

ITC is the only technique that allows the precise, accurate and direct measurement without the use of a predetermined model, the enthalpy changes (ΔH) of interaction for most biomolecular processes for example a ligand-receptor binding interaction[15].

ITC is a differential technique that is based upon keeping both a reference and a sample cell at constant and identical temperatures at least $5^{o}C$ above their surroundings[15]. In an ITC experiment, one compound A (titrant) is titrated into another compound B (titrand) and the energy change is measured in the form of heat [12,14]. The measured energy is used as a measure of the extent of interaction between the molecules of both compounds. Compound A is titrated into compound B in the sample cell, with the aid of computer controlled device called a syringe pump, which performs series of injections depending on experimental design[15]. The reference cell is filled with buffer or water and does not play a part in the titration experiment.

Experiments are mostly designed in such a way that the initial injection of component A (usually termed as the titrant) from syringe into component B results in the binding of most of the injected, an AB complex is formed resulting in a maximum production of heat associated with the interaction[15].

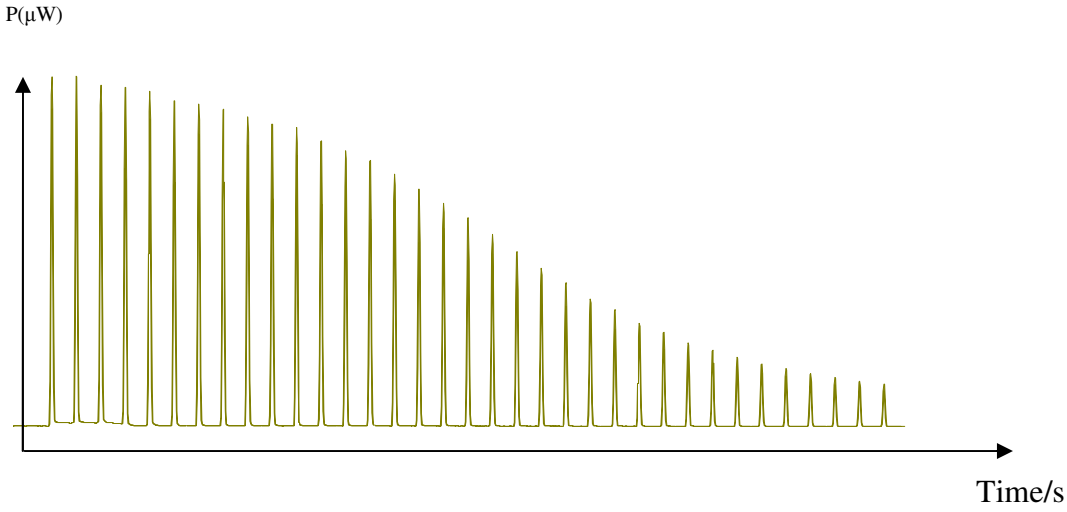

Figure 3.6. Results of titration experiment, showing the heat signal (μW) with time for BaCl and 18 –Crown-ether experiment.

Further injections of component A , will lead to less of component B available for binding hence a reduction in the formation of the complex and associated heats of interaction, eventually all of B is found in a complex and no further heats of binding is observed [12,14]. The heat recorded by the calorimeter could be exothermic (heat is released) or endothermic (heat is absorbed) thus an interaction that is athermal such as processes driven majorly by entropy change will not be detected by the calorimeter [12, 14]. The raw data of a typical ITC experiment is shown in figure 3.6. It shows the results of titrating BaCl into 18-crown ether. Each injection induced a heat flow peak (observed as power in μW) as a function of time.

3.4.1.1. Thermal activity monitor (TAM)

The calorimeter used for ITC study is the TAM 2277 microcalorimeter from Thermometric (Jarfalla, Sweden). The TAM microcalorimeter is a free standing multi channel calorimeter which operates on a heat flow mode and includes a titration unit[17]. These types of instruments are widely used in the field of enzyme kinetics and for the investigation of cell metabolism and cell-ligand (drug) interactions. The TAM microcalorimetric system is composed of a liquid thermostat that allows for a modular system allowing different types of calorimeters to be housed in one instrument. The temperature of the calorimeters is controlled by a high precision liquid thermostat with a precision of $1x10^{-4}$ ^{o}C and with a high accuracy of $\pm$ 0.03^{o}C[17].

The TAM 2277 calorimeter is a twin type calorimeter as shown in figure 3.7 which consists of two sample holders(ampoules) one holding an amount of sample and the other holding an equal amount reference (which is usually an inert sample) the purpose of this is to balance the time constants between the sample and reference sides[17]. The temperature of the sample side is always compared to the temperature of reference side and thermal equilibrium between both the reference and the sample is always maintained. This is achieved by feedback mechanism via a thermopile/ thermocouple system, (Peltier thermopile heat detector) which ensures that heat is retained or supplied to return the temperature of the sample side to that of the isothermal reference side[17]. The electrical energy required to maintain the equality upon injection of a titrant into titrand is measured and converted to a heat of interaction.

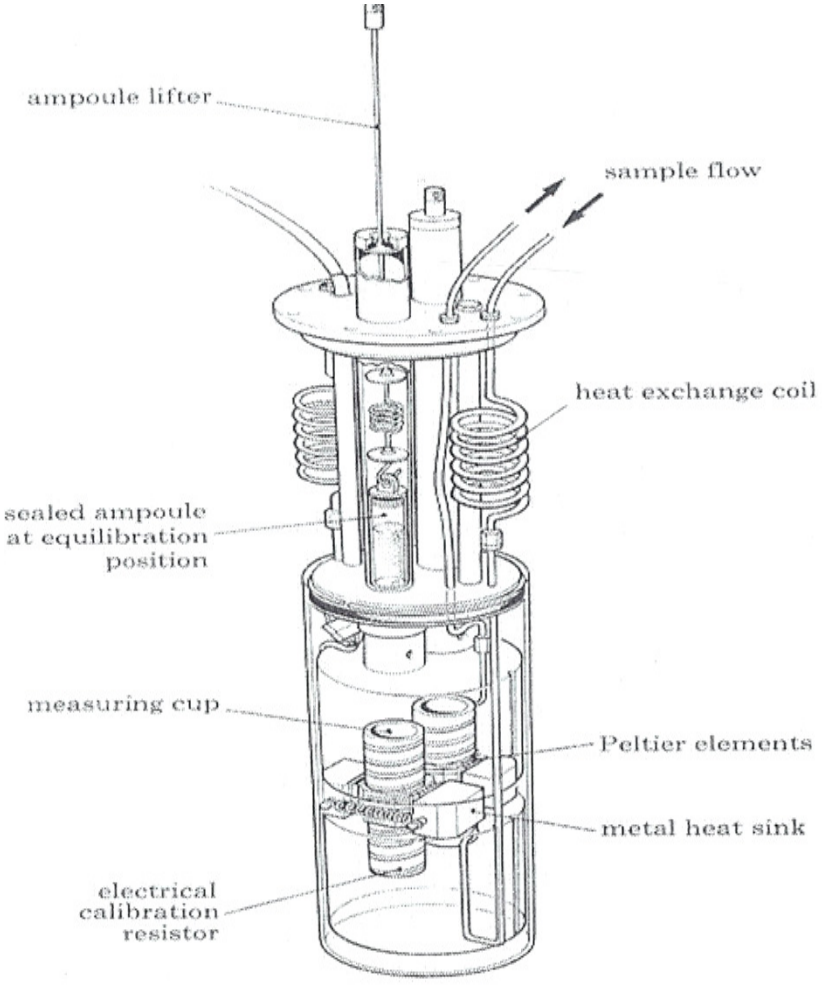

Figure 3.7. The twin type calorimeter of the TAM[17].

On each of the cylinders of the of the twin calorimeter two Peltier detectors are connected in series but in opposition so that the resultant signal of the heat detectors represents the difference in the heat flow between the two measuring cells(figure 3.8)[17]. This is known as a differential signal which directly measures the heat exchange of the sample, thus reflecting the rate of production of heat by the sample.

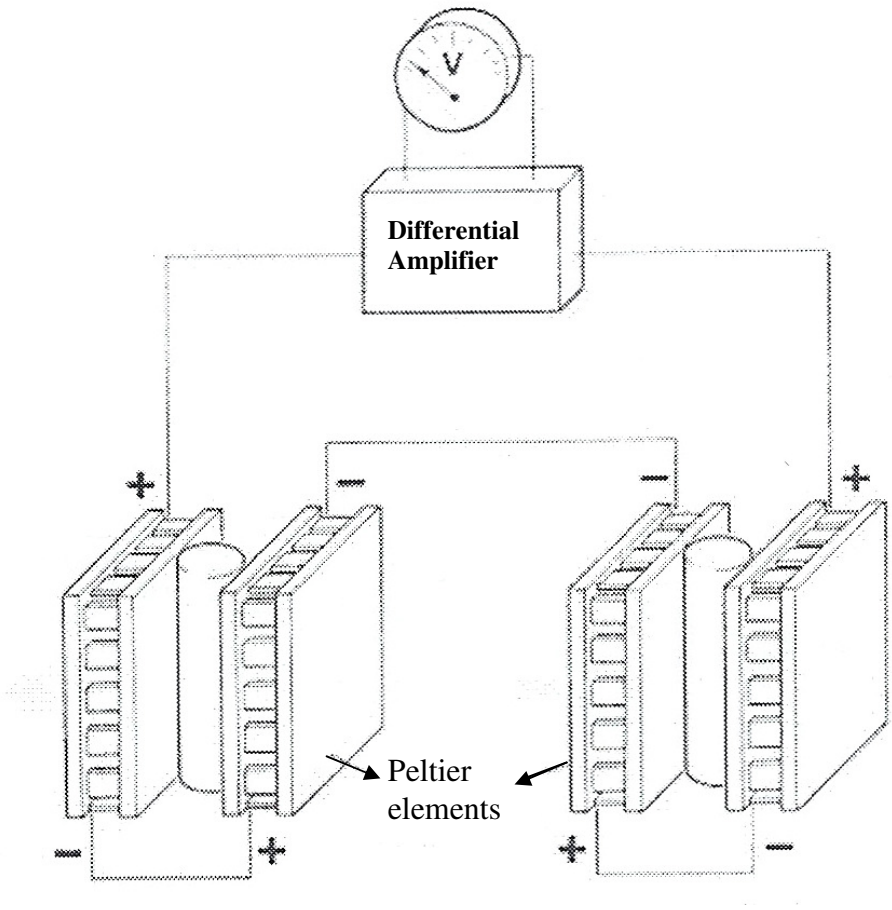

Figure 3.8. Measuring principle of the twin type calorimeter[17].

Calorimetric techniques are prone to systematic experimental errors, some of which are usually caused by contributions from processes accompanying the system under investigation, for instance evaporation, incomplete mixing, non-specific adsorption, all of which are difficult to control[15, 17]. Errors may also be due to the fact that the response time of an injection during a titration experiment is considerably less than the time constant of the measuring system, hence the need for calibration[17].

There is no universal method of calibration, but there are several methods, which can be categorized into electrical and chemical calibration methods.

For the electrical calibration the calorimetric signal is standardised by producing a pulse of an accurately known quantity of heat Q or thermal power dQ/dT with the aid of an electrical heater positioned in or close to the cells[14]. Electrical calibration ensures that the scale used by Digitam (the software programme) and the amplifier are the same[14].

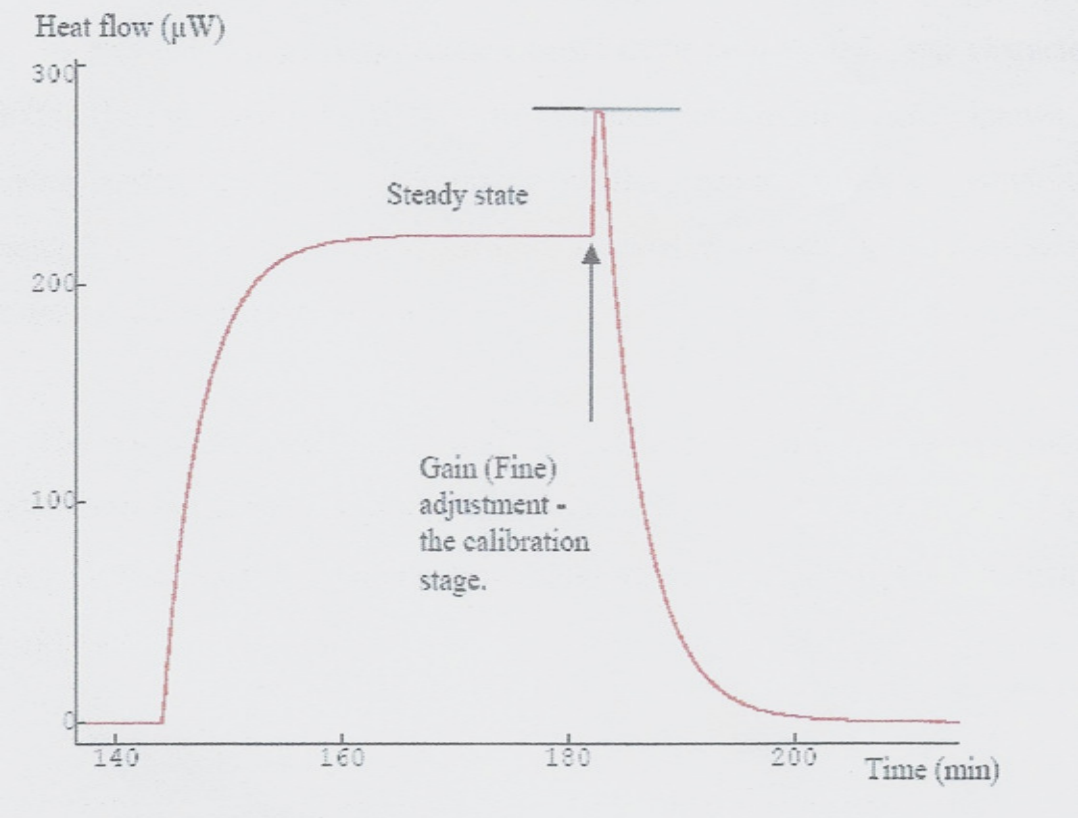

Figure 3.9. Output of an electrical calibration.[17]

Figure 3.9 shows the output of electrical calibration, the electrical heater supplies heat, for an amplifier setting of 280 W, the signal increases and then plateaus after about 20 -30 minutes. When stability is reached the signal is adjusted manually by adjusting the amplifier via the fine gain on the calorimeter unit to the correct value given on the Digitam) [13].

Chemical calibration methods normally include performing a standard calorimetric titration experiment, for example the interaction of HCl with NaOH (care should be taken to avoid contamination with CO_2)[15, 17]. Ligand binding interactions have also been proposed for the chemical calibration such as the inclusion of barium ions Ba2+ to 18-crown-6-ether, for which full thermodynamic parameters have been obtained[15]. Another is the binding of 2'Cytidine monophosphate (CMP) to bovine pancreatic ribonuclease[14].

3.4.1.2. ITC protocol: Demicellization experiments.

ITC has been mostly used to probe the thermodynamics of biomolecular interactions as witnessed by its application to systems that are mostly biological in nature. Table 3.1 gives a of list applications of such nature.

Table 3.1. Applications of ITC.
• Protein-protein, protein-peptide, protein-ligand, nucleic acid-ligand(eg.drug), protein –nucleic acid interactions
• Receptor- ligand interactions
• Structural assembly and functional regulation to molecules
• Higher order nucleic acid structure formation
• Antibody –antigen interactions
• Ligand induced oligimerization
• Cell-ligand interactions
• Metal-ion binding to molecules
• Metal /ligand/macromolecule-model membrane interactions
• pH and structural induced changes in macromolecules
• Interactions of protein domains with macromolecules and ligands
• Carbohydrate binding to other macromolecules

However, ITC has increasingly been applied to colloidal and surfactant micellar systems to probe the aggregation properties such as determination of the CMC and thermodynamics associated with the process by carrying out demicellization/ deaggregation experiments[18, 19].

A demicellization/deaggregation protocol is performed with an ITC instrument at a constant temperature[19]. The sample cell of the ITC instrument is filled with corresponding buffer solution, while the titration syringe is filled with micellar solution of the surfactant/ detergent

with a concentration much higher than the CMC of the surfactant system i.e. (C_{syr} » CMC) as illustrated in figure 3.10.

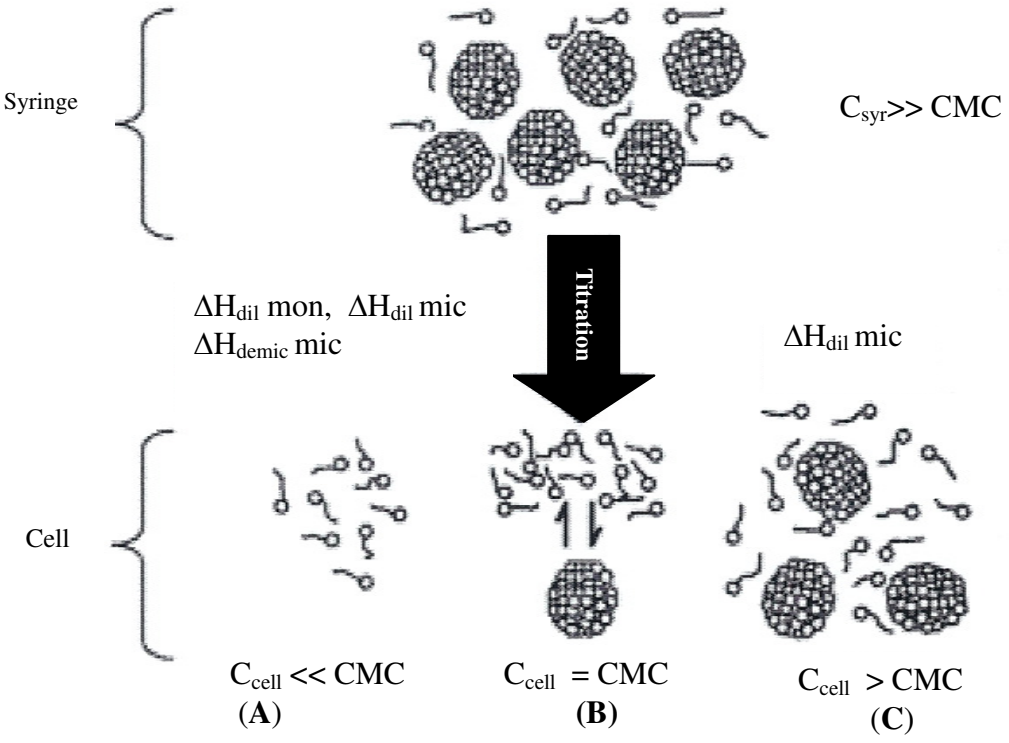

Figure 3.10. Schematic representation of the demicellization experiment[19].

The experiment is then initiated by performing series of injections of the micellar solution in small aliquots of 2 to 10µl into the solution in the sample cell, each injection induces a heat flow as a function of time [19].

At the start of the experiment, the first series of injections represents dilution of the micellar solution in the cell, as a result inducing a demicellization/deaggregation of the micelles as shown in figure 3.10 since the concentration of surfactant in the cell (C_{cell}) is less than the surfactant CMC [18, 19].

With further injections of surfactant solution, the monomer concentration in the cell increases and a concentration is reached where the first micelles/colloidal aggregates are formed, at this point the C_{cell} is equal to CMC [18].

At the end of the experiment the surfactant concentration in micellar form increases (C_{cell} > CMC) the concentration of surfactant in monomer from remains equal to the CMC, and heat of each injection at this stage is dilution of the micellar solution in the cell ΔH_{dil} mic[19].

The reaction heat of each injection is obtained by integration of each heat flow peaks (figure 3.11 A) and plotted as a function of concentration (Figure 3.11 B) from which the CMC and $\Delta H_{demic/mic}$ are obtained.

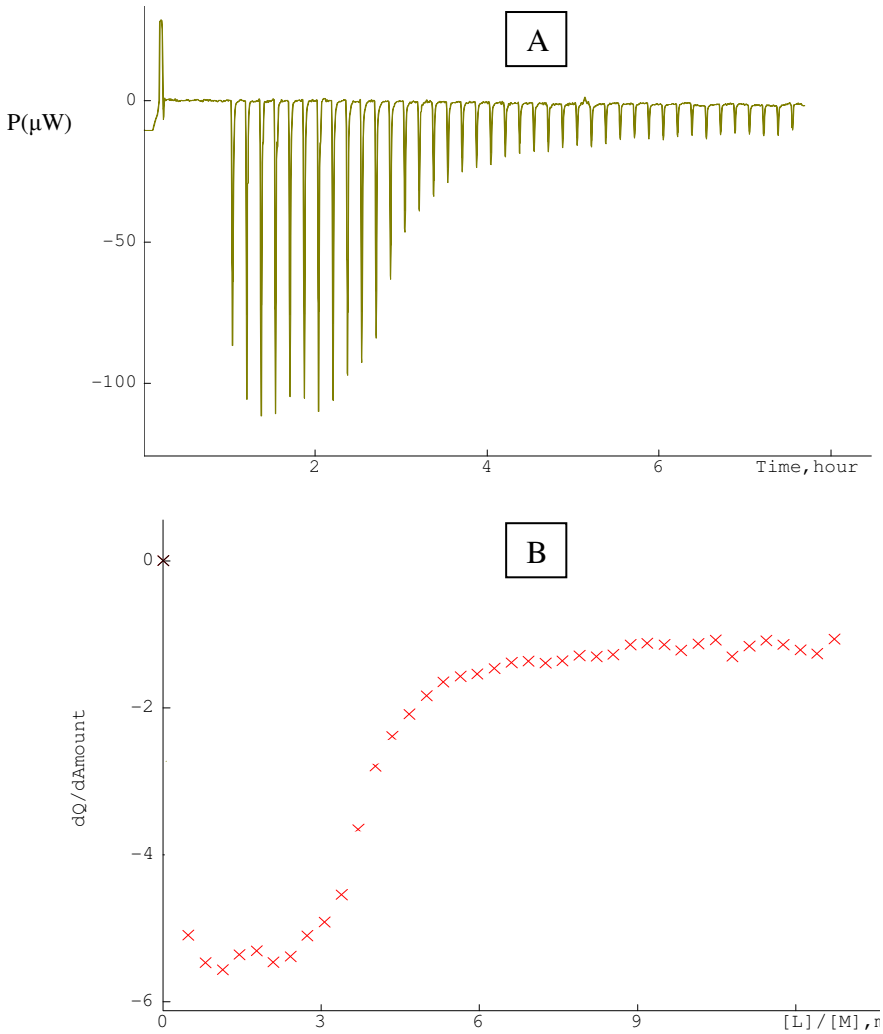

Figure 3.11. Results of demicellization of SDS in sucrose solution (A) raw data, (B) heats of reaction(Q) as a function of surfactant concentration in cell.

Other thermodynamic parameters such as change in free energy and entropy associated with the demicellization can be derived by use of the thermodynamic expression shown below[14]

$\Delta G_{demic} = -RT.\ln CMC = \Delta H_{demic} - T.\Delta S_{demic}$

Where R is the gas constant, T is the temperature, and CMC is the critical micelle concentration expressed in mole fraction.

Thus, a complete thermodynamic profile describing the thermodynamic stability of the formed micelles system can be determined with the use of the ITC.

3.4.2. Differential scanning calorimeter (DSC)

DSC is an experimental technique that measures the power required to maintain an equal temperature between sample and inert reference material while subjected to an identical (heating, cooling or constant) temperature programme[16, 20]. The differences correspond to the apparent heat capacity from which direct information about the energertics of thermally induced processes in a sample can be obtained.

DSC may also used to obtain thermal transition temperatures, such as melting temperatures for samples in solution, solid or mixed phases such as suspensions[16, 20]. DSC can provide useful information on protein stability and protein - ligand interactions, nucleic acid unfolding, and to investigate phase transitions in lipids. DSC has thus become an invaluable method in biophysical chemistry to study macromolecular conformation and interactions[17,21].

The availability of user friendly software and sensitive DSC instrumentation enables small energy changes accompanying a thermal event to be detected for dilute biomolecular solutions[21], this type of differential scanning calorimeter is the focus of subsequent sections.

3.4.2.1. Nano DSC

The calorimeter that was used for the DSC studies is the Nano-DSC from *TA* instruments (Delaware, USA). A schematic cartoon of the capillary design Nano-DSC is shown in figure 3.12. It consists of identical reference (R) and sample (S) cells (0.30ml capacity), the cells are enclosed within an adiabatic shield in which individual cell heaters are located. The cells are pressurized via computer control, as well a temperature program which determines scan rate and controls scan temperature of the shield during the run.

The Nano-DSC operates on power-compensation mode, a Peltier- based solid-state temperature control sensor allows scanning in both heating and cooling directions with equal sensitivity. Power is supplied to the heaters during heating to raise the temperature of the cells at a steady rate whilst the temperature differences (ΔT) between sample and the reference cell as well as the surrounding adiabatic shield (ΔT_2) is monitored[20].

Temperature sensors are located between the sample and reference cell to detect temperature differences between the cells, and if present they are maintained at 0°C by applying the appropriate compensating power through heaters located on the surface of the cells. The power compensation signal is recorded as the calorimetric output.

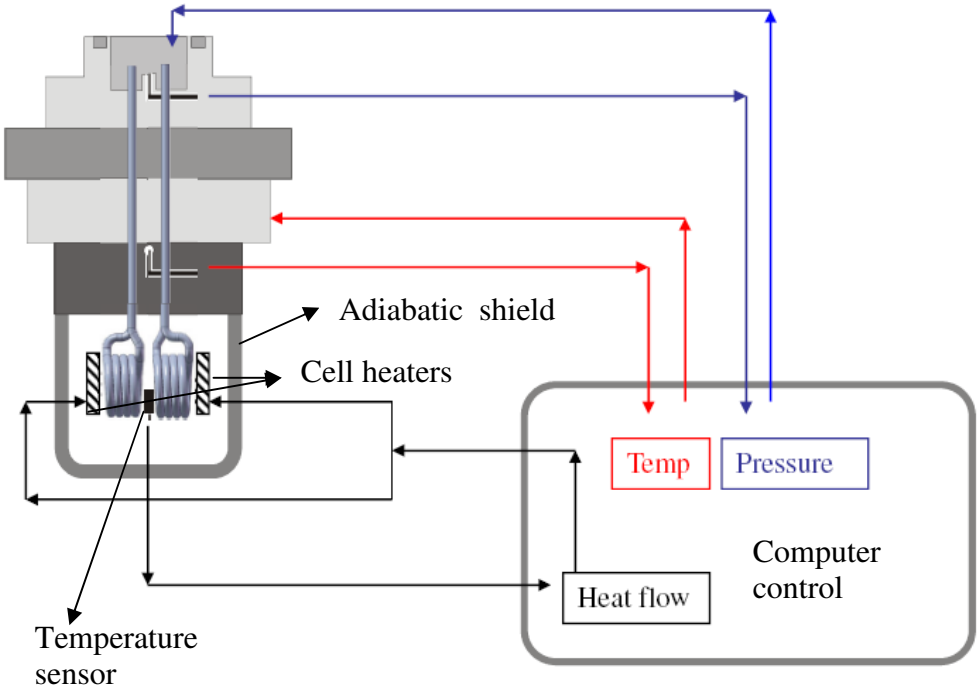

Figure 3.12. Schematic of the Nano DSC[22].

The cell construction for the Nano-DSC used in this study is a capillary type design (figure 3.12), as opposed to the commonly used cylindrical design. It made up of 99.99% Platinum material that is inert to biomaterials, the capillary design is advantageous over the cylindrical design in that it attenuates or delays onset of aggregation until after protein has unfolded. It is also easy to fill and clean.

Other features of the Nano-DSC include:

- A standard operating temperature range between -10 to 130 °C with user-selectable scan rates from 0.001 to 2 °C/min.
- Compact design which requires minimal bench space.
- The adiabatic shield of the N-DSC is enclosed in a vacuum-tight chamber making the instrument less susceptible to environmental changes hence creating an inert environment.

- A built-in, computer-controlled, high-pressure piston which ensures precisely-controlled pressure during scanning.

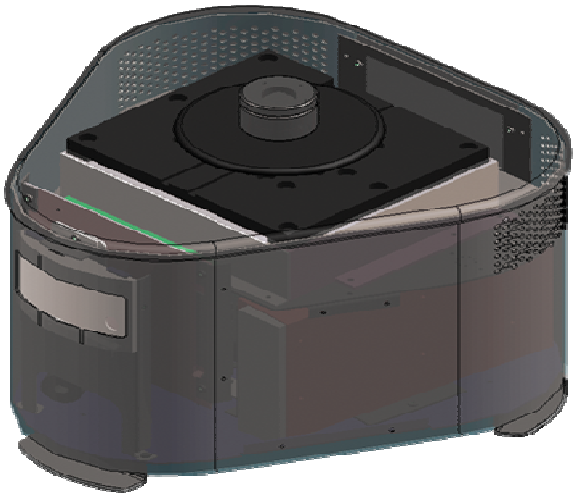

Figure 3.13. Compact design of the Nano –DSC[22].

The Nano-DSC is specifically designed to determine thermal stability and heat capacity of proteins and other macromolecules in dilute solution. The sensitivity of the instrument allows measurement of protein solutions at low concentration with an appreciable signal to noise ratio as seen in the figure 3.14. The calorimeter is able to detect heat signals in dilute solutions ranging from 2ug/ml to 400ug/ml.

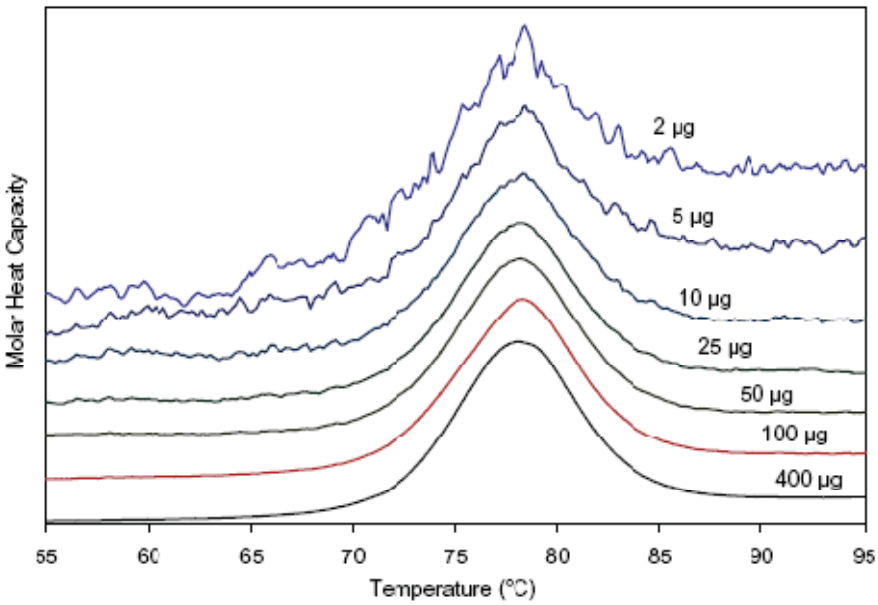

Figure 3.14. DSC scan to show how much protein is required[22].

3.4.3. DSC and application to biological macromolecules

There are four basic types of biological macromolecules they include carbohydrates, lipids, polypeptides (peptides, proteins) and polynucleotides[23]. Peptides and proteins are polymers consisting of chains of amino-acids while polynucleotides (DNA and RNA) are polymers consisting of one or two chains of nucleic acids[23].

The linear sequence of amino acids (peptides and proteins) specifies the biopolymer's 3-D structure, and thus its function. 3-D structure can be determined by X-ray crystallography or NMR spectroscopy, however these structures do not reveal why a linear sequence folds into a specific structure[23, 24].

Since the folding process is dictated by kinetics and thermodynamics, DSC is the most direct approach for determining the thermodynamics of folding, and in comparison to the aforementioned techniques DSC is easy to undertake it requires no immobilization and chemical derivatization [20] Albeit , when used in conjunction with high resolution structural detail, could provide a powerful tool to understand this process at a much deeper level. DSC is thus an invaluable tool with numerous applications to probe biopolymer interactions as shown in the table below.

Table 3.2. DSC and application to biological macromolecules.
• Biopolymer Conformation & solvation
• Biopolymer Stability e.g protein denaturation studies
• Biopolymer Structure e.g. domain organization studies
• Ligand Interactions e.g. drug binding to proteins or nucleic acids
• Membrane Structure e.g lipid bilayers, membrane proteins studies
• Helix-to-coil transitions
• Volumetric properties of biopolymers
• Evaluate effects of formulation additives
• Evaluate relative stability of bioengineered proteins
• Estimate tight molecular binding (up to 10^{20} M^{-1})

3.4.4. Protein denaturation

Linear sequences of amino acids are held together by covalent bonds[23]. The linear sequence folds into a complex three-dimensional (3-D) structure held together by millions of very weak bonds and interactions [24]. The 3-D structure of proteins is stabilized by a delicate balance of non-covalent interactions which includes, hydrophobic interaction, hydrogen bonds, ionic and van der Waals interactions[24]. These interactions are easily disturbed by temperature, ionic strength, pH, detergents or organic solvents, thus denaturing the proteins. Denaturation is classified into two based on reversibility of process.

- Reversible denaturation (unfolding)
- Irreversible denaturation (unfolding) in which aggregation/further unfolding or scrambling of disulfide bonds occurs.

Heat absorption causes the protein atoms to vibrate and move thus disrupting the stabilizing bonds and interactions which eventually lead to unfolding and hydrophobic group exposure to water[23]. Eventually proteins stick together (aggregate) and precipitate, most proteins unfold between 40 – 90 ^{o}C and temperature of unfolding of a protein is characteristic for that protein[24]. Proteins are marginally stable and unfold (denature) easily[25, 26].

3.4.4.1. Two state-model of protein unfolding

The DSC can be used to investigate the thermal unfolding or denaturation of proteins. For a simple globular protein, equilibrium unfolding assumes that the molecule may belong to only one of two thermodynamic states: the folded/ native state (*N*) and the unfolded state (*U*)[27].

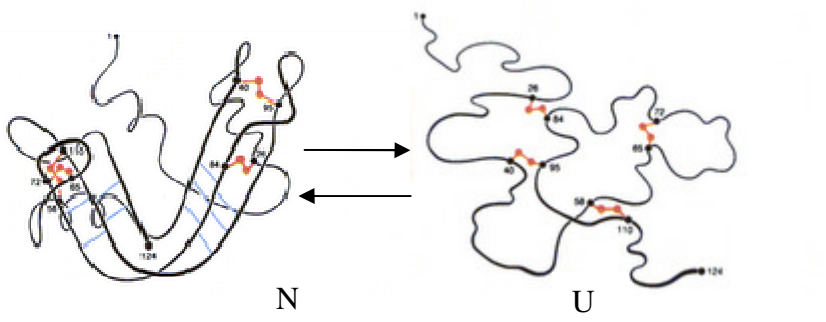

Figure 3.15. Two state model of protein unfolding[27].

The molecule may transition between the native and unfolded states according to a simple thermodynamic model, 'the two state model'. This model of protein folding was first proposed by Anson, however this model is said to only apply to small, single structural

domains of proteins since larger multi structural-domain proteins often exhibit intermediate states[27]. The population of both states is dependent upon temperature[27].

Representative DSC data obtained for the thermal unfolding of a simple globular protein undergoing a two state unfolding transition is shown in the figure 3.16. The thermogram consists of a plot of the excess heat capacity (after subtraction of the baseline reference) of the system as a function of temperature.

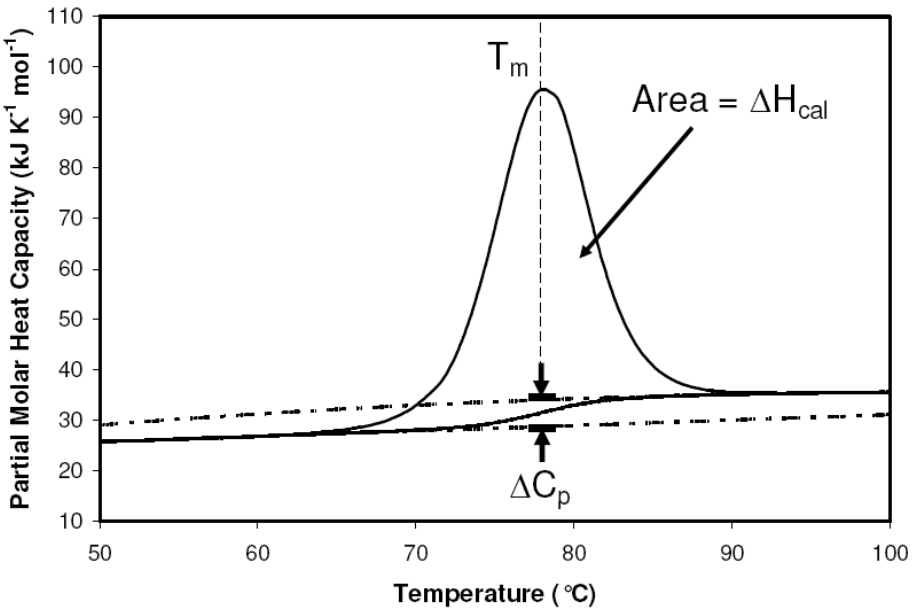

Figure 3.16. A simple unfolding thermogram[22].

It comprises three regions: the pre-transition baseline, the endothermic unfolding transition and the post- transition baseline. The pre- transition baseline is characterised by a slightly positive slope for most proteins, indicating a gradual increase in heat capacity with temperature[20]. With further increase in temperature the protein begins to unfold as more heat energy is taken up and the C_p increases, reaching a peak at approximately the mid-point(T_m) temperature of the process before falling down to the high temperature baseline the post transitional baseline[20]. The post transitional baseline represents the relative heat capacity of the unfolded protein and is normally positive. The integrated area under the calorimetric peak after appropriate baseline correction represents the calorimetric enthalpy (ΔH_{cal})[20].

3.4.6. DSC in Liquid Biopharmaceutical stability studies

DSC has been used in stability studies to determine the thermal stability of proteins[28, 29]. The measured thermal transition ('melting') temperature have been used to evaluate the stability of the proteins[30] whereby increases in T_m are correlated with improved stability[30, 31]. Results from DSC studies on the effect of various additives on the T_m of a cell receptor protein interleukin-1 have been used to predict and the rank order of stability for various additives as shown in the table below[30].

Table 3.3. Effect of additives on the T_m of the IL-1Receptor [30] protein.

	Excipient	Tm (^oC)
Control	-	48.1
Sugars	Mannitol Glucose	46.7 49.6
Polymers / Polyols	PEG (300) Ethanol (low) Ethanol (High)	49.4 48.7 43.8
Salts	NaCl $CaCl_2$	53.1 41.1
Surfactants	Pluronic F68 Tween 80	46.6 45.8
Glucose/NaCl		52.2

3.4.5. Definitions of some thermodynamic parameters

The fundamental thermodynamic parameters namely, enthalpy changes (ΔH), entropy changes (ΔS) and Gibbs free energy change (ΔG) that can be obtained from calorimetry have been defined below.

3.4.5.1. Heat capacity (C_p)

The heat capacity is central to DSC measurements and is fundamental to the derivatization of other thermodynamic quantities. It can be defined as the heat required to raise the temperature of one mole or one gram of the substance by one degree centigrade[20, 32]. The C_p involved in heating from absolute zero to temperature (T) is related to the absolute enthalpies and entropies by the following relationship[20]

$$H = \int_0^T C_p . dT + H_0$$

Where H_0 is the ground state energy at 0K due to chemical bonding and other non-thermal effects.

And from the 2nd law of thermodynamics:

$$dS = \frac{dH}{T} = \left(\frac{C_p}{T}\right).dT$$

Hence:

$$S = \int_0^T \left(\frac{C_p}{T}\right).dT$$

3.4.5.2. Enthalpy (H)

The enthalpy of a system is interpreted molecularly as the total energy taken up plus the work done by the same system[14, 20]:

H = U +PV.

U is the internal energy and PV is the work done by expansion or compression.

The total energy includes all the atomic and molecular motions such as translational, rotational, vibrational as well as energy taken up in changes in the inter and intramolecular bonds[20].

3.4.5.3. Entropy(S)

The absolute entropy is usually described in terms of the degree of molecular disorder[14]. The higher the disorder the higher the entropy and vice-versa, this above definition provides no relation to the heat capacity[14, 20]. A better definition that relates entropy to the heat capacity is 'the multiplicity of ways in which the molecules in a system can take up heat energy without increasing the temperature'[20]. So if a particular system has lots of different ways in which the added energy can be taken up, then higher energy will be needed to raise the temperature of the system and such a system will have a high C_p, the opposite effect is observed for a system with relatively few ways of distributing added heat[20].

3.4.5.4. Gibbs free energy and stability

Free energy describes the overall stability of the system. The enthalpy and entropy is related to the Gibbs free energy change by the following expression.

$$\Delta G = \Delta H - T.\Delta S$$

Chemical stability and equilibrium represents a balance between these two opposing quantities which is the natural tendencies for systems to move to lower energies (decrease in H) and secondly the natural tendency at the molecular level for molecules to explore the multiplicity of states available (Higher S)[20].

The Gibbs free energy tells us how much work must be done to bring about the desired change. If ΔG= negative, changes occur spontaneously, if ΔG = positive, change requires the input of energy and when $\Delta G = 0$ the system is in equilibrium[20].

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Chapter 4: Tensiometric studies examining the impact of sugars on the solution properties of a homologous series of sodium *n*-alkyl sulphates

4.1. Introduction

The sodium-n-alkyl sulphates, are a group of synthetic anionic surfactants which are widely used as standard surfactants in industry as well as in fundamental research in the field of surface and colloidal science[1].

Back et al, (1979) studied the micellization of a series of alkytrimethylammonium bromide surfactants in the presence of sugars and polyols, the critical micelle concentration (CMC) was measured by fluorescence intensity[2]. This method requires the use of dye which might affect the CMC value[3]. Other investigators have also studied the influence of certain organic additives including urea and alcohols on hydrophobic interactions in sodium dodecyl sulphate (SDS) and n-dodecyltrimethyl ammonium bromide(DTAB) micelles by conductimetry [4].

This investigation describes for the first time the influence of sugars on the micellization, thermodynamic and surface active properties of a homologous series of sodium n-alkyl sulphate surfactants as a function of increasing sugar concentration by tensiometry. The homologous series of anionic surfactants which have been studied include sodium decyl sulphate (SDeS), sodium dodecyl sulphate (SDS) and sodium tetradecyl sulphate (STS). Their structures and physicochemical properties are presented in figures and tables 4.1 to 4.3.

The rationale behind the work described in this chapter is to attempt to understand the mode of action of bioprotective sugars on biomolecules by examining the impact that such sugars have on the Gibbs free energy of hydrophobic group exposure of simple systems such as surfactant rich micelles.

When a protein unfolds many of the hydrophobic residues buried inside the protein's core are exposed to water[5]. A bioprotective sugar makes this unfolding process energetically less favourable, our conjecture is that this arises as a result of unfavourable interactions between

the hydrophobic groups and the aqueous sugar solution. The work reported herein suggests that qualitative conclusions might be drawn about hydrophobic interactions in micelles which could be extended to hydrophobic interactions in proteins, and phospholipid bilayers in biological membranes. These insights into sugar-surfactant interactions may perhaps provide a better understanding of the bioprotective properties of sugars.

4.2. Experimental

4.2.1. Materials

SDeS, ≥ 99.0% purity (Sigma Aldrich UK)

SDeS is composed of a linear alkyl chain length of ten carbons atoms (C-10) and a negatively charged sulphate group with sodium ions as counterions. Some other properties are tabulated below.

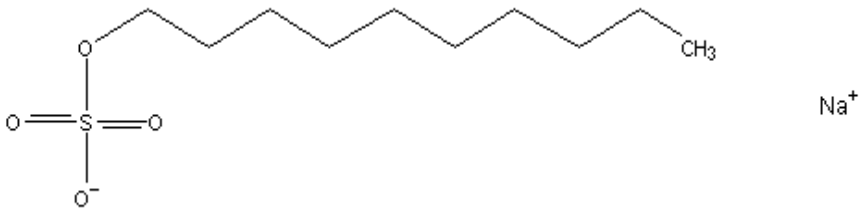

Figure 4.1. Molecular structure of SDeS[6].

Table 4.1. Physicochemical properties of SDeS.

Synonyms	Molecular Formula	Molecular weight (g/mol)[7]	Melting point [7] ^{o}C	Solubility[7]	CMC[8]
Decyl sulfate sodium salt.	$C_{10}H_{21}SO_4^-Na^+$	260.33	144	26.33g/l (in H_2O at 20^{o}C)	33.38mM (in H_2O at 20-25 ^{o}C)

SDS, 99.5% purity (Fissons Plc UK; Lot No. S5202)

SDS is composed of a linear alkyl chain length of twelve carbon atoms (C-12) and negatively charged sulphate group with the sodium ion as the counterions. Some other properties are tabulated below.

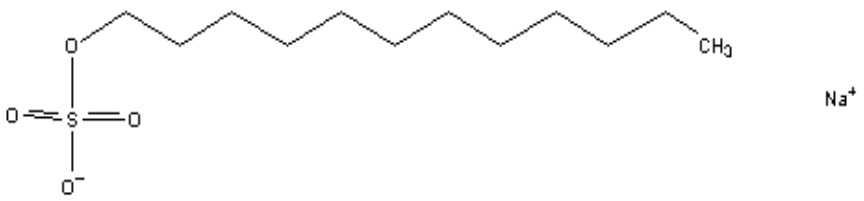

Figure 4.2. Molecular structure of SDS[6].

Table 4.2. Physicochemical properties of SDS.

Synonyms	Molecular Formula	Molecular weight (g/mol)[7]	Melting point[7] ^{o} C	Solubility[7]	CMC [1, 9]
Dodecyl sodium sulfate, Dodecyl sulfate sodium salt, sodium lauryl sulphate, lauryl sulphate sodium salt	$C_{12}H_{25}SO_4^-Na^+$	288.38	204-207	$1.13x10^{-2}$ g/l (in H_2O at 20^{o}C)	7-8mM* (in H_2Oat 20-25 ^{o} C)

* Lower CMC value is due to SDS purity.

STS, 95% purity (Sigma Aldrich UK)

STS is composed of a linear alkyl chain length of fourteen carbon atoms (C-14) and negatively charged sulphate group with the sodium ion as the counterion. Some other properties are tabulated below.

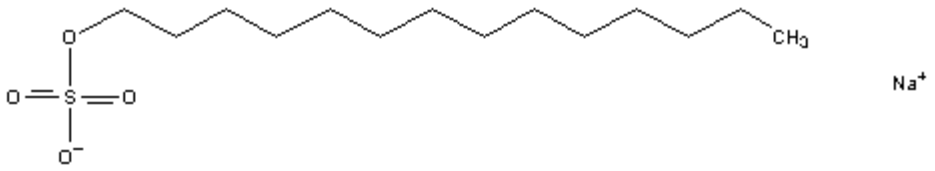

Figure 4.3. Molecular structure of STS[6].

Table 4.3. Physicochemical properties of STS.

Synonyms	Molecular Formula	Molecular weight (g/mol)	Melting point[7] ^{o} C	Solubility[7]	CMC [8]
tetradecyl sulfate sodium salt, Sodium myristyl sulphate.	$C_{14}H_{29}SO_4^-Na^+$	316.43	199	$2.62x10^{-3}$ g/l (in H_2O at 20^oC)	2.84mM (in H_2O at 20-25 ^{o} C)

Other materials include, sucrose ≥99.5%, trehalose dihydrate ≥99.0%, maltose monohydrate 99.0%, raffinose pentahydrate ≥99.0% and mannitol >99.5% which were all obtained from Sigma Aldrich UK. Their physicochemical properties have been given in chapter one. All the chemicals were obtained from commercial and industrial suppliers and used without further purification.

4.2.2. Methods

4.2.2.1. Tensiometry

A Kruss K100 tensiometer with a Wilhemy plate as measuring probe was used to measure surface tension of surfactant solution to an accuracy of $\pm 1 \times 10^{-3}$ mN/m. The instrument was connected to a Julabo heat circulator with a thermostatically controlled temperature bath with a flow rate of 10 dm^3/min to maintain a constant temperature at 25 ± 0.02 °C.

Sugar solutions at various concentrations (0.1 to 1.0M) were prepared by dissolving defined amounts in deionised water (Elga purelab option, ion exchange chromatography) with resistivity of ~15MΩcm and surface tension value of 72.6 mNm^{-1} at room temperature. Surfactant solutions at concentrations above their CMC's were prepared for the three surfactant systems studied; surfactants were dissolved in sugar solutions of the aforementioned concentrations. Samples were weighed to an accuracy of $\pm 1 \times 10^{-4}$g.

The CMC measurement was performed by dilution of the concentrated surfactant solution in the sample vessel (referred to as the base solution) with water or with aqueous sugar solutions (referred to as the added solution). Accurate dilution was achieved using a Metrohm Dosinos unit which performed automatic dosing over a range of surfactant concentration steps (dosing accuracy of ±20µl). This allowed a predetermined volume of water/sugar solution to be added to the surfactant in the sample vessel. The solution was mechanically stirred for 10 to 15 seconds and after a stabilization period; the value of surface tension of the surfactant solution was then measured at each concentration step. Thus a plot of surface tension versus logarithm of surfactant concentration was obtained and the CMC was evaluated.

For the determination of the CMC of the surfactant solution, a reverse CMC measurement was carried out. In this measurement the surfactant solution as opposed to the solvent is put into the sample vessel and diluted with the solvent step by step, micelles in the concentrated solution are thus diluted into surfactant monomers; consequently exposing hydrophobic groups to the aqueous environment. This process was carried out in water and aqueous sugar solutions (figure.4.4). The CMC values in the presence of sugar and in water were obtained

for the three surfactants studied as well as the effect of sugars on the free energy penalty of hydrocarbon group exposure.

The CMC of the surfactants in the presence of 0.1 M solutions of sucrose, trehalose, maltose, raffinose and mannitol, were determined and compared to the CMC of surfactants in deionised water. Further measurements were carried to elucidate the influence of increasing sugar concentration on the CMC.

The surface tensions of pure sugar solutions at the studied concentrations were also measured in order to determine surface pressure.

Experiments were performed under standardized experimental conditions; freshly made samples (≤ 24hrs) were used; the grade and purity of chemicals used were consistent. Experiments were carried out in duplicate and showed good reproducibility.

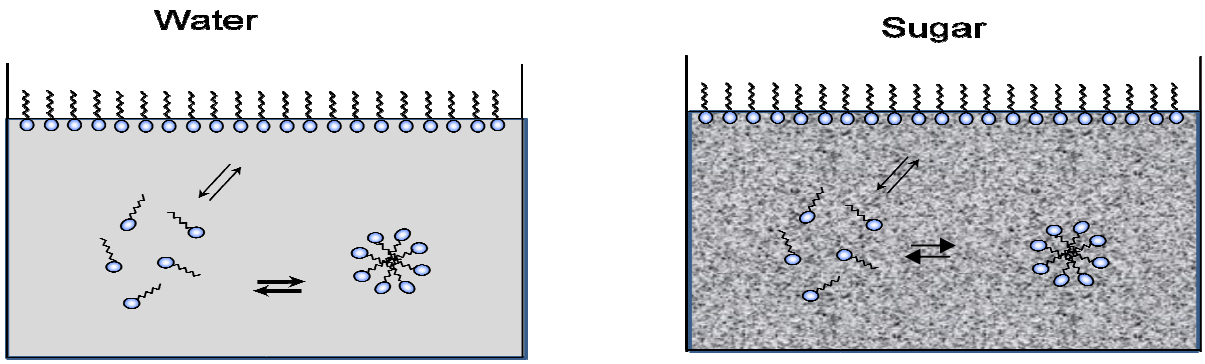

Figure 4.4. Schematic illustration of experiment in water and aqueous sugar solutions.

CMC was determined by the intersection of the baseline of minimal surface tension and the slope where surface tension shows a linear decline (see figure 2.6 in chapter 2). From the CMC the Gibbs free energy change of micellization (ΔG_{mic}) can be obtained from the equation[3].

$$\Delta G_{mic} = -RT \ln X_{CMC} \qquad (1)$$

(The theoretical assumption underpinning this equation has been described in greater detail in chapter two. basically, it is assumed that the micelles form a separate phase, sometimes called a pseudophase.)

The Gibbs free energy change of micellization is equal to Gibbs free energy change of demicellization but opposite in sign.

$\Delta G_{mic} = -\Delta G_{demic}$

The quantity $RT\ln_{cmc}$ is the Gibbs free energy change for forming micelles, thus the lower the CMC, the more negative the ΔG_{mic} and the more stable the micelle at any particular temperature [4].

Surfactant surface parameters such as the surface excess concentration (Γ) and the area per surfactant molecule adsorbed at the interface (A_0) was obtained by utilization of the Gibbs absorption equation as discussed in chapters 2 subsection 2.4.1.1.

4.2.2.2. Conductimetry

The conductivity of SDeS, SDS and STS solution in the absence and in the presence of sugars was measured. The effect of increasing sugar concentrations on conductivity of the surfactants was also investigated. Measurements were carried out with a digital conductivity meter from Hanna Instruments, Podova, Italy.

The conductivity meter was calibrated with standard KCl solutions. All the solutions were prepared by weight in deionised water with an accuracy of ± $1x10^{-4}$g. Solutions of surfactants at concentration between 5 to 10 times their respective CMC's were prepared, the conductance values were measured after thorough mixing. The temperature was kept constant at $25^{o}C \pm 0.02$ with the aid of a water bath.

4.3. Results and discussion

Results representing the micellization of SDeS, SDS and STS in water and in the presence of sucrose are presented in the figures 4.5 to 4.7. These experiments consisted of 20 to 25 surfactant concentration steps at which surface tension was measured ten times; thus obtaining a plot of average surface tension as function of surfactant concentration.

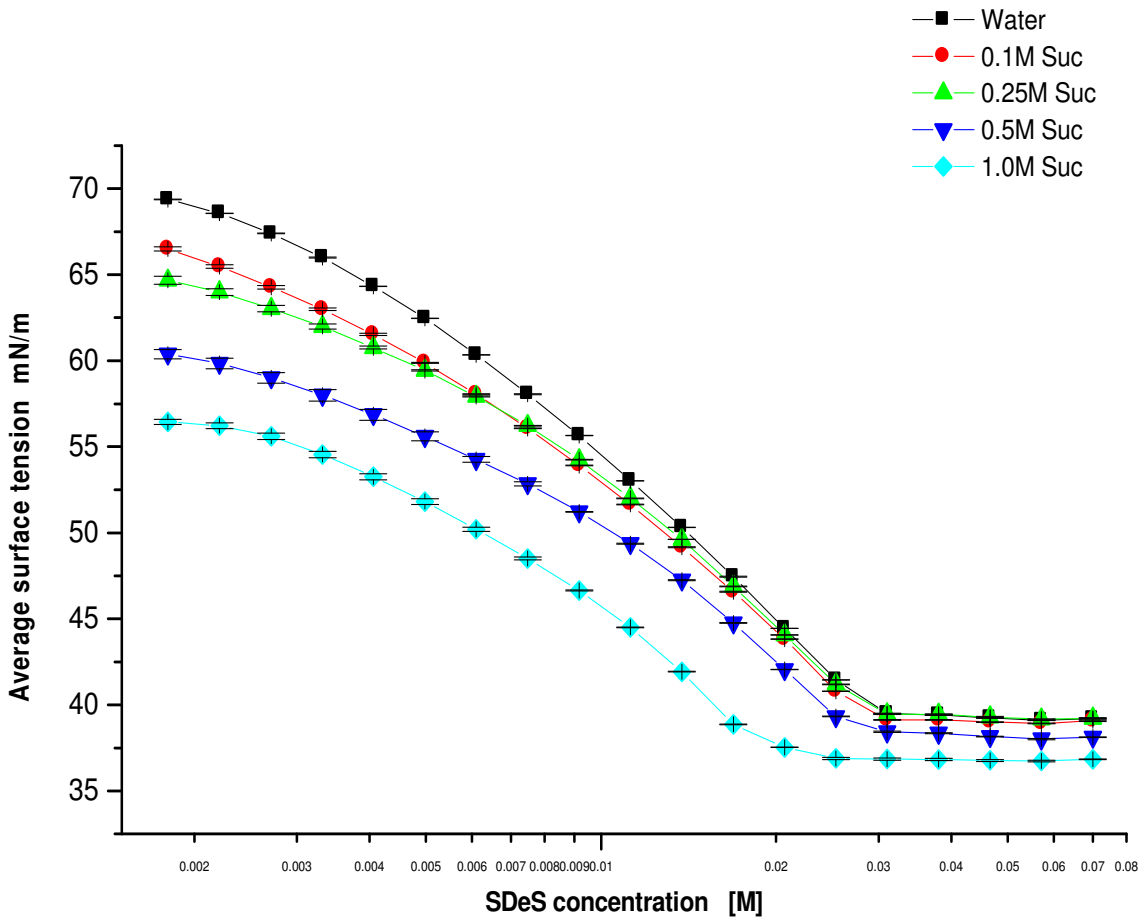

Figure 4.5. Plot of Average surface tension vs log of SDS concentration, showing the effect of sucrose.

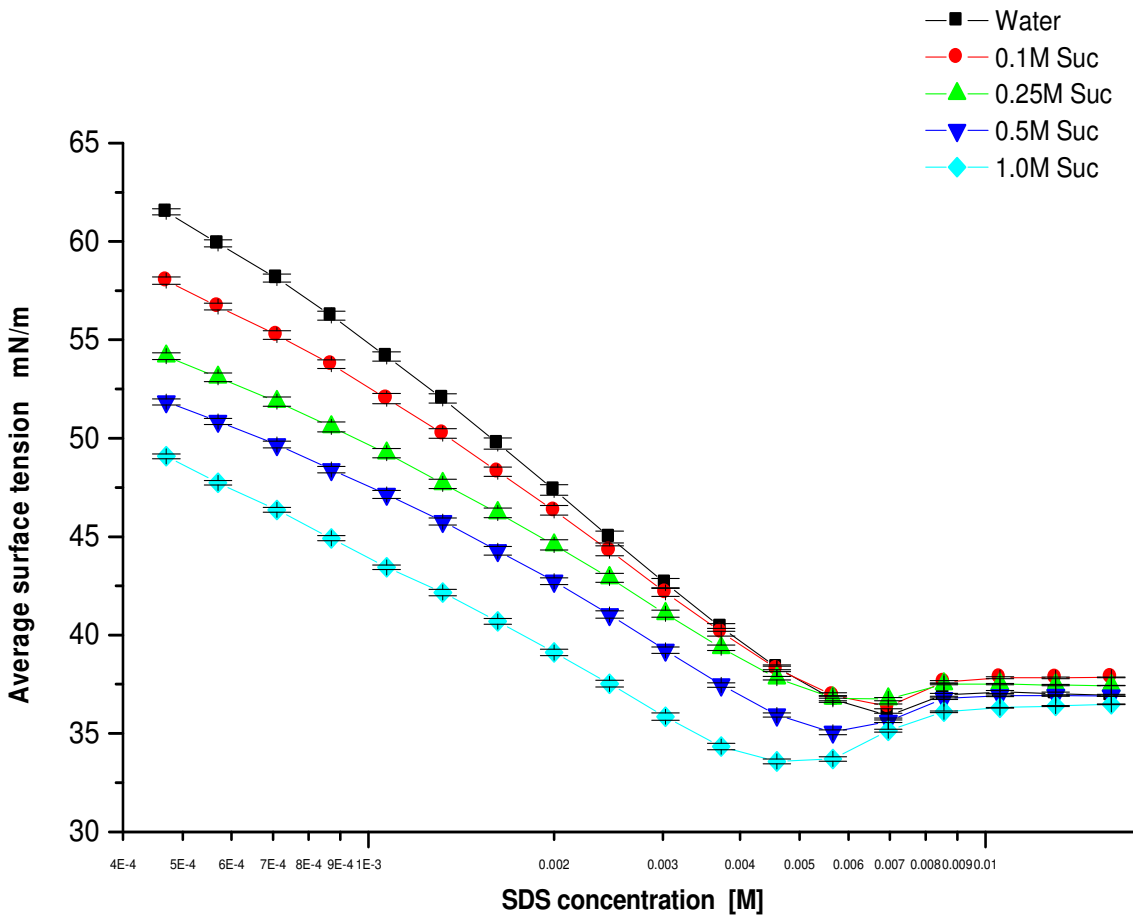

Figure 4.6. Plot of Average surface tension vs log of SDS concentration, showing the effect of sucrose.

Data for SDS shows a surface tension minimum, the surface tension minimum is due to the presence of residual amount of dodecanol. Dodecanol which is a reactant used in the formation of SDS is difficult to completely remove due to complex formation with SDS.

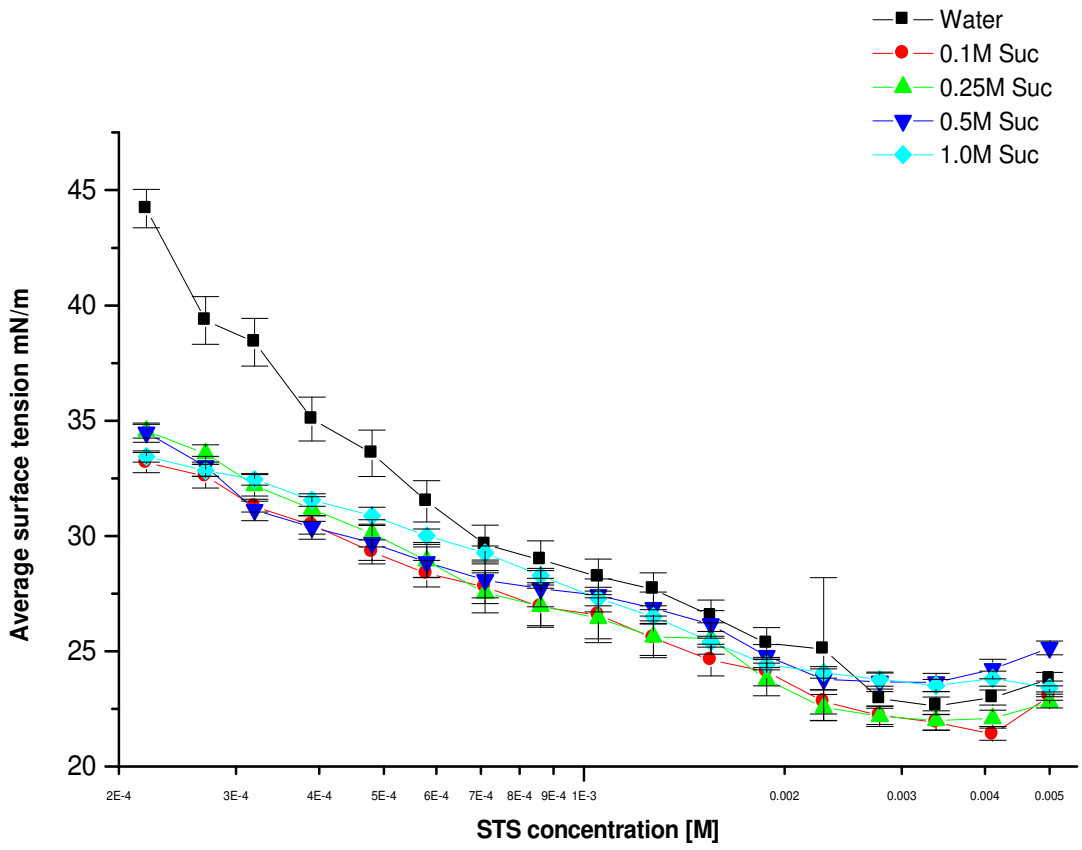

Figure 4.7. Plot of Average surface tension vs log of STS concentration, showing the effect of sucrose.

The results for STS show surface tension scatter, this observation may be due to a reduced solubility in aqueous media at 25°C, and also it is possible that sugars reduce the solubility of STS which complicates matters.

Results showing the tensiometric data for the micellization of the surfactants in the presence of other investigated sugars: trehalose, maltose raffinose and mannitol have been presented at the end of the chapter in section 4.6.

The measured CMC values and evaluated values for $\Delta G_{mic,}$ Γ, A_0 and surface pressure have also been tabulated as shown in section 4.6.

4.3.1. CMC: effect of sugars

The influence of sugars on the CMC of SDeS, SDS and STS as a function of increasing sugar concentration have been summarised in figures 4.8 to 4.12. The results show that the presence of sugars induced a decrease in the CMC for the three surfactants studied relative to their respective CMC's in pure water and as the sugar concentration increased further decreases in CMC were observed. The decrease in CMC was pronounced at higher concentration (1.0M) for all the sugars except raffinose. Micellization experiments using raffinose at higher concentrations were not conducted due to its reduced solubility in water.

The results show a linear dependence of the CMC of SDeS and SDS on sugar concentration, with correlation coefficient (R^2) values ranging between 0.93 to 0.98 for SDeS and 0.65 to 0.88 for SDS (figure 4.8 - 4.12).

The results also showed a linear dependence of the CMC of STS on sucrose concentration, however concentration dependence was observed to a much lesser extent for the other sugars with R^2 values ranging from 0.01 to 0.90 as shown in figures 4.8 to 4.12. These observations may be due to the nature of the STS, which has a longer hydrocarbon chain and lower solubility in aqueous media at 25°C, or its purity (95%) compared to the other surfactants, further investigation is therefore required to substantiate this claim.

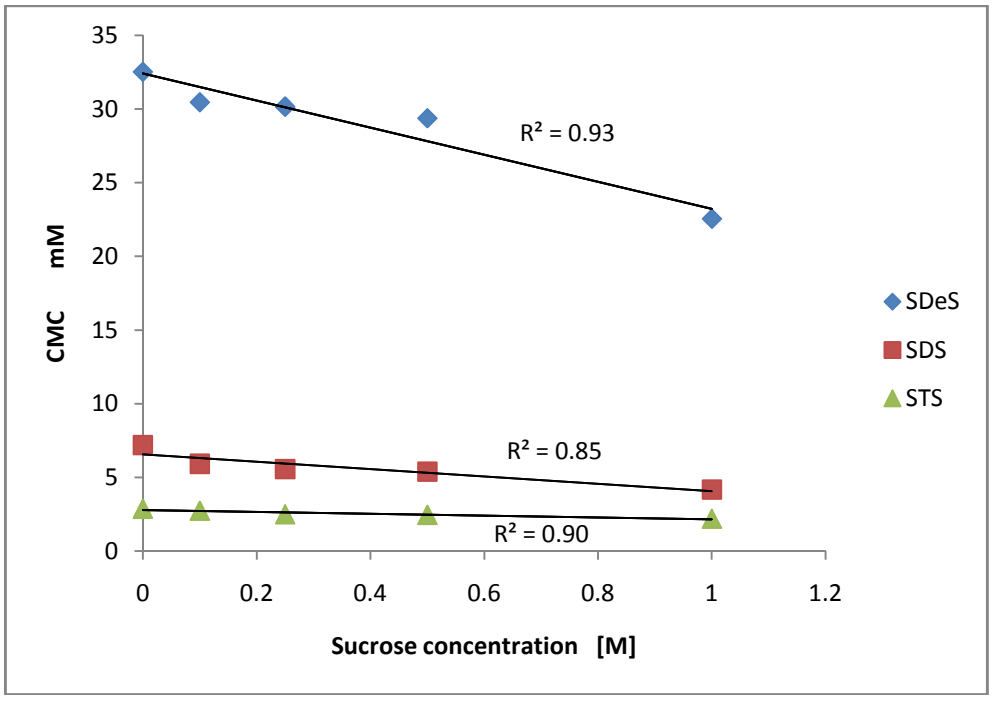

Figure 4.8. Plot of CMC of surfactant; SDeS, SDS and STS as a function of sucrose concentration.

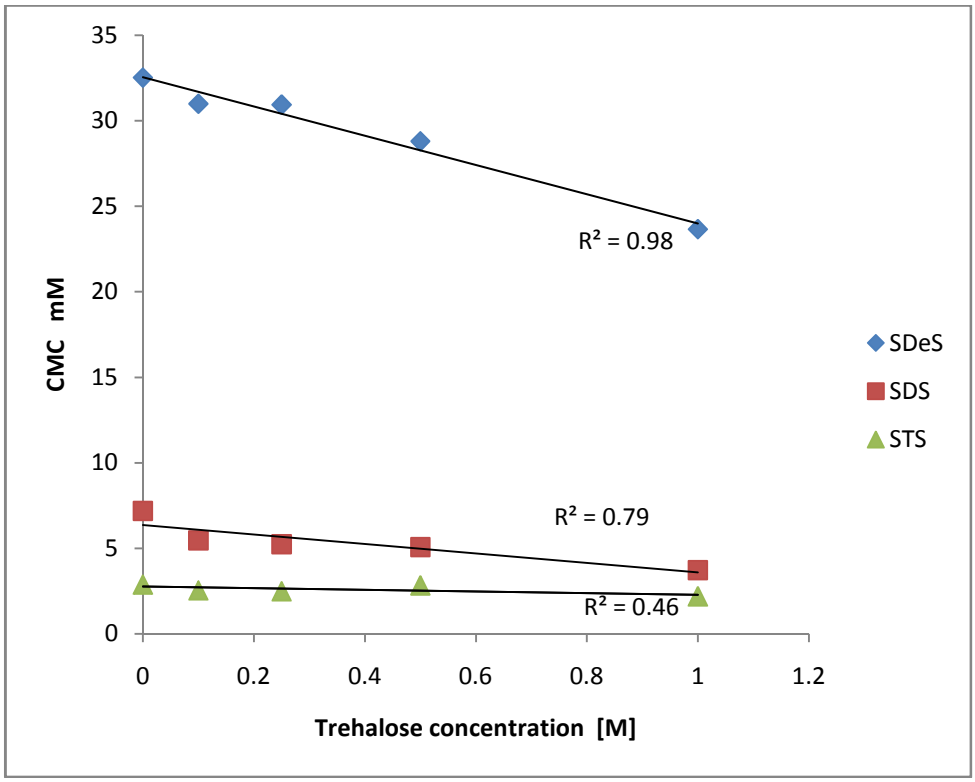

Figure 4.9. Plot of CMC of surfactants; SDeS, SDS and STS as a function of trehalose concentration.

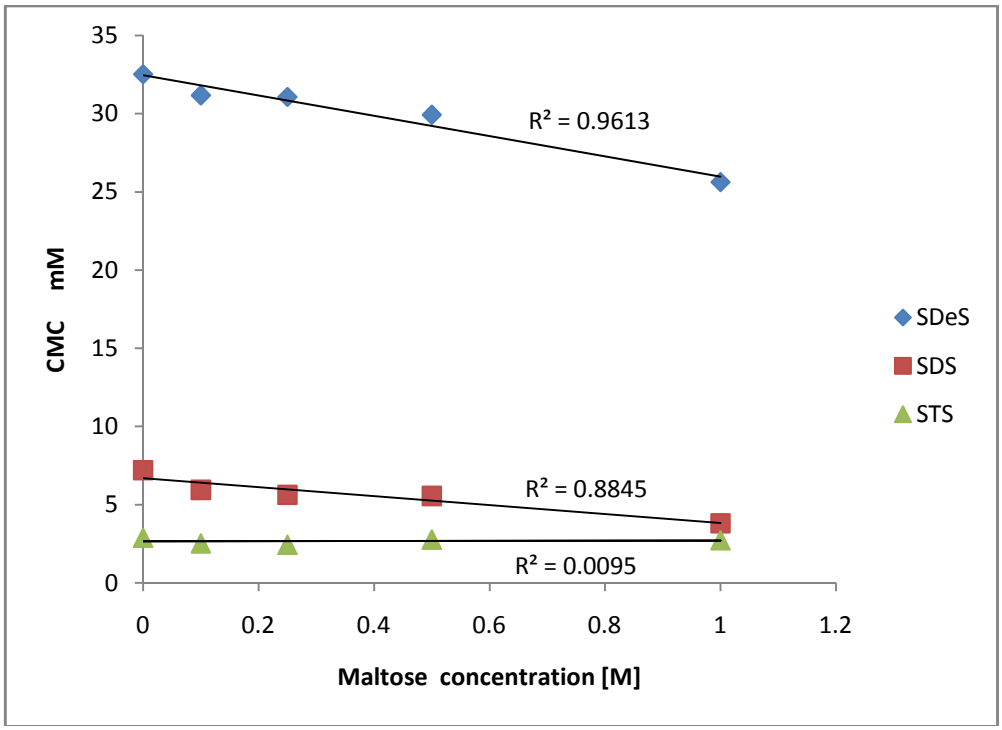

Figure 4.10. Plot of CMC of surfactants; SDeS, SDS and STS as a function of maltose concentration.

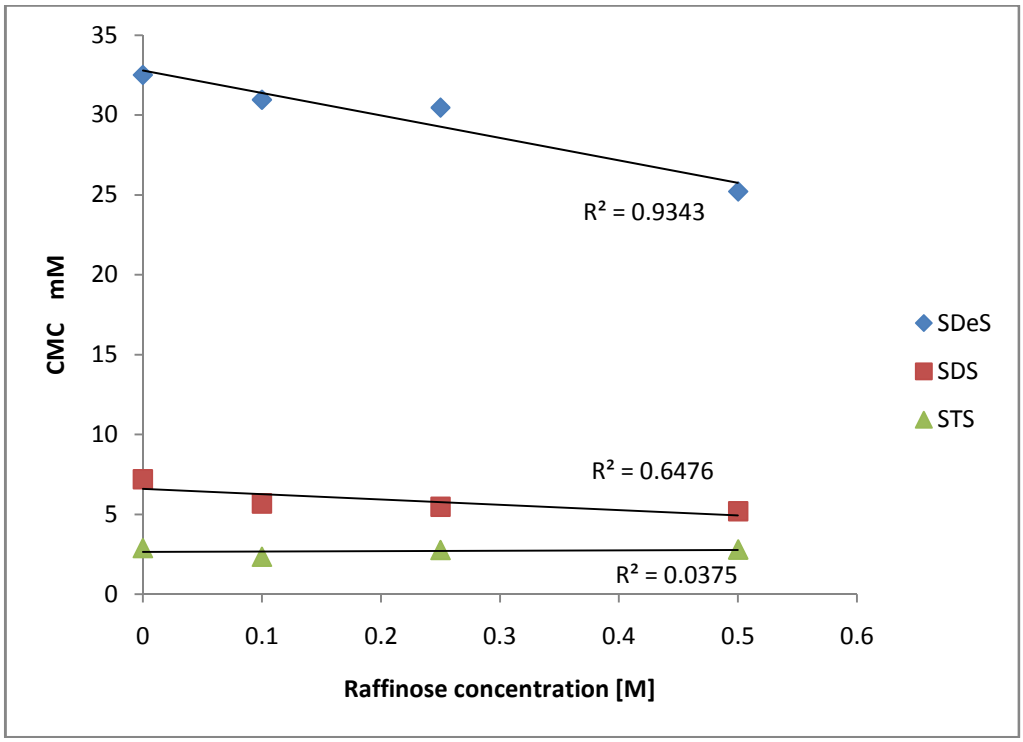

Figure 4.11. Plot of CMC of surfactants; SDeS, SDS and STS as a function of raffinose concentration.

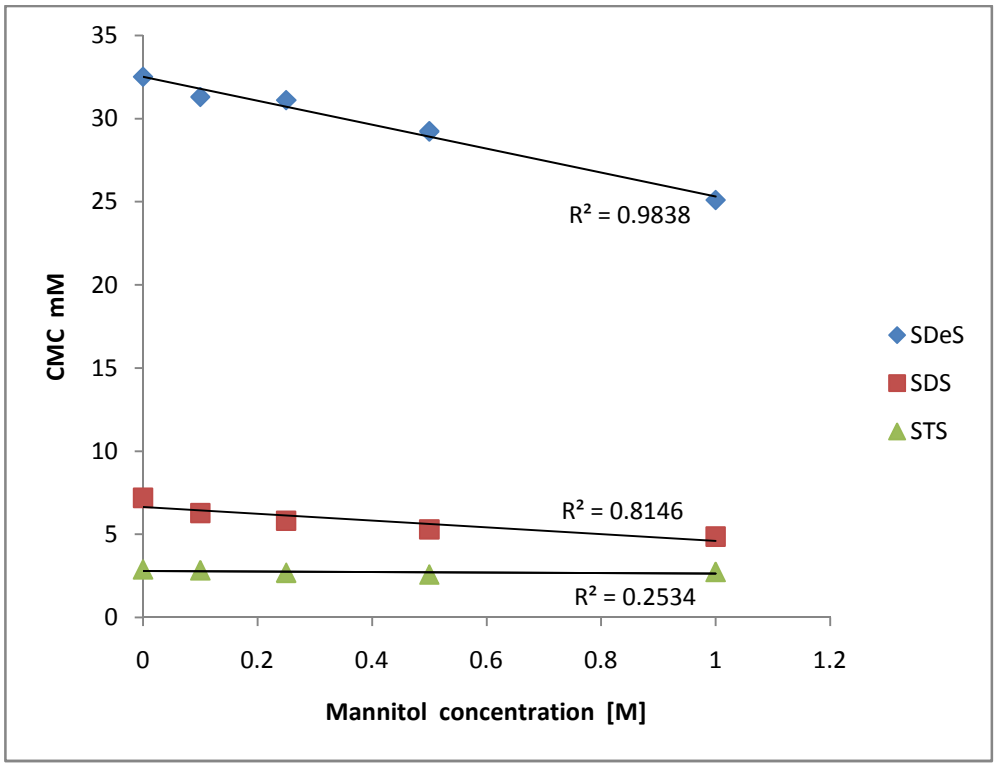

Figure 4.12. Plot of CMC of surfactants; SDeS, SDS and STS as a function of mannitol concentration.

4.3.1.1 Magnitude of decrease in CMC

The results indicate that sugars have an effect on micellization of the surfactants as shown by the decrease in CMC values relative to water. However differences were observed in the extent of decrease in CMC depending on the nature and type of the sugar. The effect of sugar on the micellization between the surfactants also showed differences. Slopes of the CMC vs. sugar concentration plots were obtained and are presented in table 4.4 below.

Table 4.4. Slopes of CMC vs. sugar concentration plots for the homologous series of surfactants (ΔCMC mM/mol of sugar).

Sugar	SDeS	SDS	STS*
Sucrose	9.17	2.50	0.63
Trehalose	8.55	2.75	0.47
Maltose	6.49	2.85	0.04
Raffinose	14.08	3.31	0.21
Mannitol	7.21	2.04	0.15

* The slopes obtained for STS ought to be treated carefully given the line is almost horizontal in comparison to the other surfactants.

For SDeS, from the slopes which is the amount of CMC reduction per mole of sugar, raffinose was observed to induce the most effect on the decrease in the CMC compared to the other sugars (Table 4.4). This was followed by sucrose, trehalose, mannitol and maltose in that order. For SDS, raffinose also showed the most change in the CMC as indicated by the highest value for the slope in comparison to maltose, trehalose, sucrose and mannitol.

For the effect of sugars on the micellization of STS, sucrose showed the most effect on the decrease in the CMC followed by trehalose, raffinose and mannitol; the least effect was observed for maltose (table 4.4). These results suggest a possible difference in stabilizing effect between the sugars.

Effect of sugars on the micellization between the surfactants showed differences, the decrease in CMC was more in SDeS followed by SDS and lastly STS as shown by the higher slope values for SDS in table 4.4. The difference lies in the hydrophobic nature of the tail group, since SDeS is less hydrophobic in nature than SDS; and SDS than STS, changes in the

electrostatic repulsion in the polar groups will be more pronounced with SDeS than it would for a largely hydrophobic surfactant.

The results obtained in this study are in agreement with previous studies carried out by other investigators which showed that the presence of sugars some of which include xylose, fructose, maltose and sucrose may enhance the organisation of the water due the dissolved hydrophobic group, thereby increasing the entropy effect associated with micellization[1, 10, 11]. Thus a lower bulk concentration of surfactant is needed for micelle formation, hence CMC is decreased[1].

The sugar induced decrease in CMC suggests that the surfactant molecules prefer to form aggregates rather than dissolve in the medium, thus the presence of sugar makes the environment more hydrophilic (increase in cohesive energy density)[6] and therefore unfavourable to non polar hydrocarbon groups of the surfactant.

4.3.2. Gibbs free energy change of micellization (ΔG_{mic}): effect of sugars

Micellization of surfactants in sugar was more favourable in sugar solutions as seen by the more negative values when compared to water; and with increasing sugar concentration further negative ΔG_{mic} values were obtained as shown in figures 4.13 to 4.17.

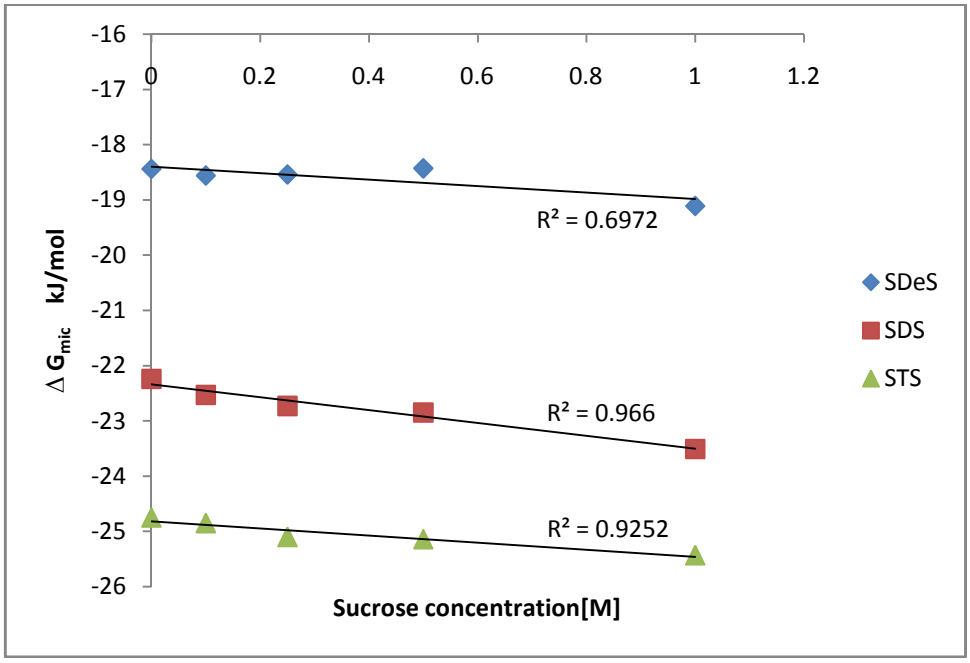

Figure 4.13. ΔG_{mic} of surfactants; SDeS, SDS and STS as a function of sucrose concentration.

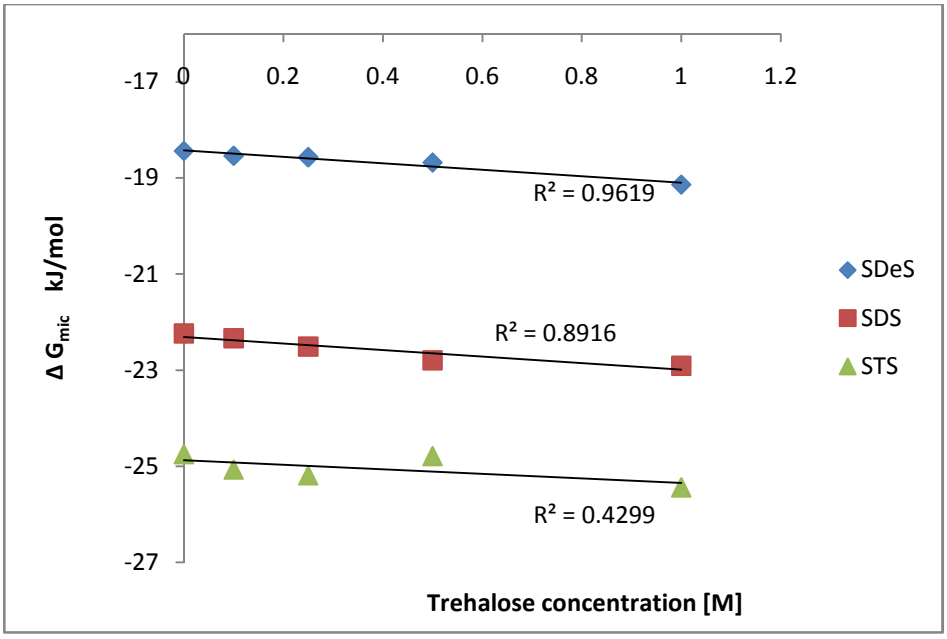

Figure 4.14. ΔG_{mic} of surfactants; SDeS, SDS and STS as a function of trehalose concentration.

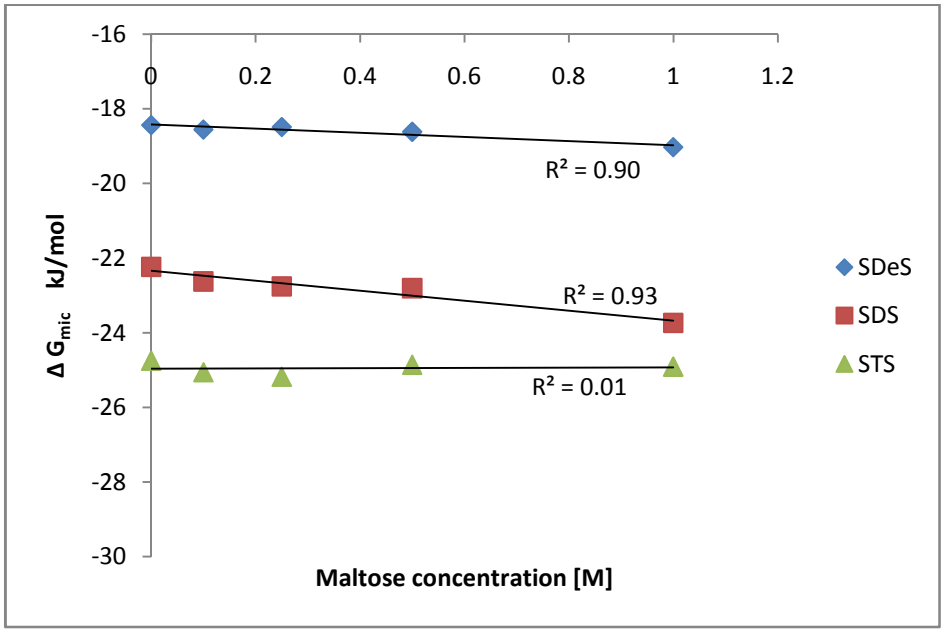

Figure 4.15. ΔG_{mic} of surfactants; SDeS, SDS and STS as a function of maltose concentration.

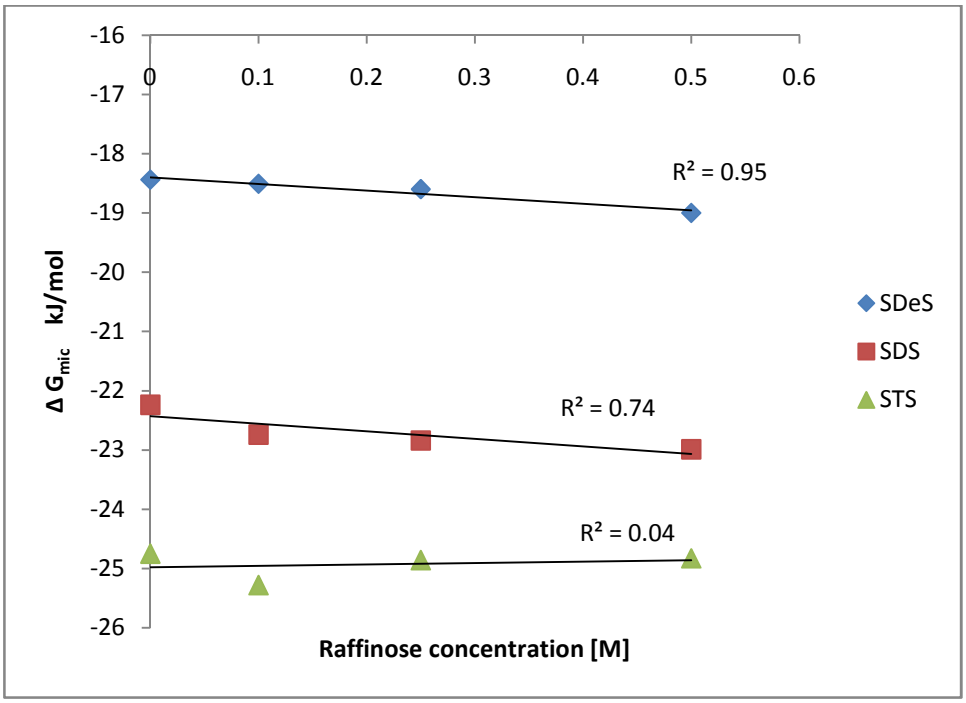

Figure 4.16. ΔG_{mic} of surfactants; SDeS, SDS and STS as a function of raffinose concentration.

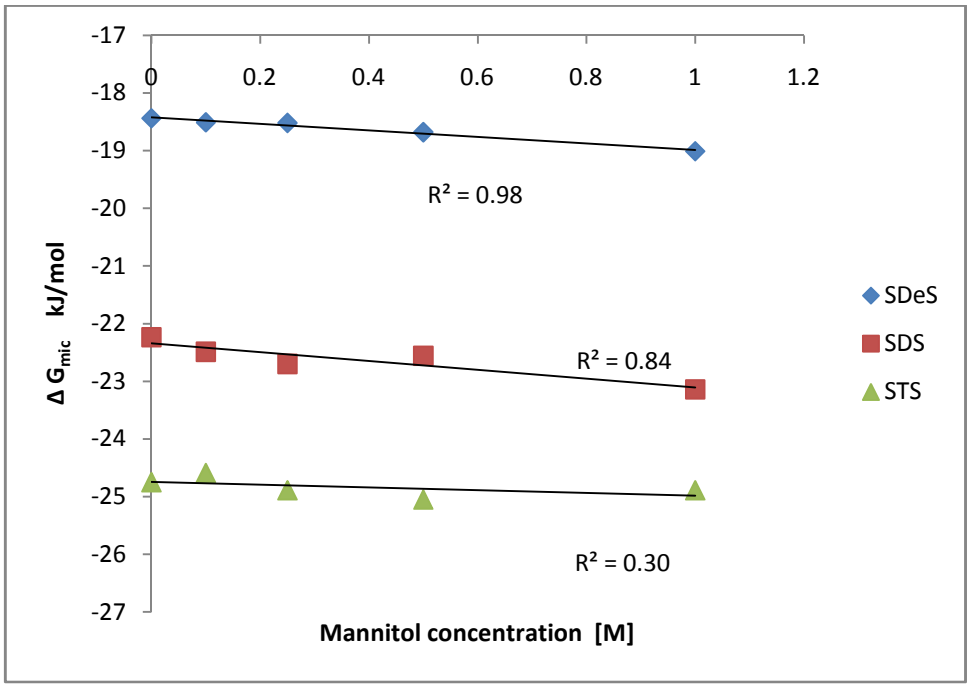

Figure 4.17. ΔG_{mic} of surfactants; SDeS, SDS and STS as a function of mannitol concentration.

Calculating ΔG_{mic} in water and in sugar solution reveals that micellization was more favourable in sugar solution (ΔG_{mic} = -19.14 kJ mol^{-1} at 1.0M trehalose) than in water (ΔG_{mic} = -18.44 kJ mol^{-1}) for SDeS as tabulated in section 4.6.

For the micellization of SDS the ΔG_{mic} obtained in sugar solution was more favourable (ΔG_{mic} = -23.74 kJ mol^{-1} at 1M maltose) than in water (-22.24 kJ.mol^{-1}) as tabulated in section 4.6. A more favourable free energy of STS for the micellization was obtained in sugar solution (ΔG_{mic} = -25.43 kJ.mol^{-1} at 1M sucrose), in comparison to water at (ΔG_{mic} = -24.75 kJ.mol^{-1}) as tabulated in section 4.6. As with the case of CMC, concentration dependence was similarly observed for the ΔG_{mic} values of SDeS and SDS as shown by R^2 values of 0.70 to 0.98 for SDeS and 0.74 to 0.93 for SDS. In STS further increases in the concentration of sugar in the system did not result in a more negative ΔG_{mic} (R^2 between 0.01 to 0.93) due to the fact the increase in sugar concentration did not necessarily result in a further decrease in the CMC.

A more negative ΔG_{mic} value suggests higher ΔG_{demic} values, thus the higher the free energy penalty of hydrocarbon group exposure to aqueous sugar environment. Therefore, micellar phase is favoured as shown by the reduction in CMC which indicates that the concentration of surfactant monomers in solution is reduced. Also the more negative ΔG_{mic} the more stable the micelle is to demicellization at any particular temperature [4, 12.] The results have shown that the hydrophobically driven aggregation of surfactants is energetically more favourable in the presence of sugars; therefore exposure of hydrocarbon groups to the aqueous sugar solution is unfavourable. The results also reveal that sugars enhance hydrophobic interaction as indicated by smaller CMC values obtained for the sugars investigated. It is suggested that these observation arises because of the unfavourable interaction between the hydrophobic groups of the surfactants and the aqueous sugar solution [13].

4.3.3. Adsorption at interface: effect of sugars

4.3.3.1. Surface pressure: effect of sugars

The surface pressure (π) was obtained by measuring the surface tension of the aqueous sugar solution containing surfactant, water and sugar solutions, and the surface pressure of the surfactants was thus calculated from the following equation.

$$\pi = \gamma_o - \gamma$$

(Where γ_o is the surface tension of pure water and γ the surface tension of the aqueous surfactant solutions)

The results obtained showed an increase in the surface pressure of SDeS and SDS as the concentration of sugar was increased as shown in figure (4.18 to 4.22). Thus, in addition to changes in CMC and ΔG_{mic} as a function of increasing sugar concentration, there also exists a functional relationship between surface pressure and aqueous sugar concentration. R^2 values obtained for SDeS ranged from 0.47 to 0.92 and 0.24 to 0.97 for SDS with raffinose and mannitol showing the least effect on the surface pressure as a function of increasing concentration; at 1.0M mannitol a decrease in the surface pressure of SDeS and SDS was observed this is due to the fact that the measured surface tension value of the pure sugar solution was reduced at higher concentration. The measured surface tension values of sugar solutions have been tabulated in section 4.6.

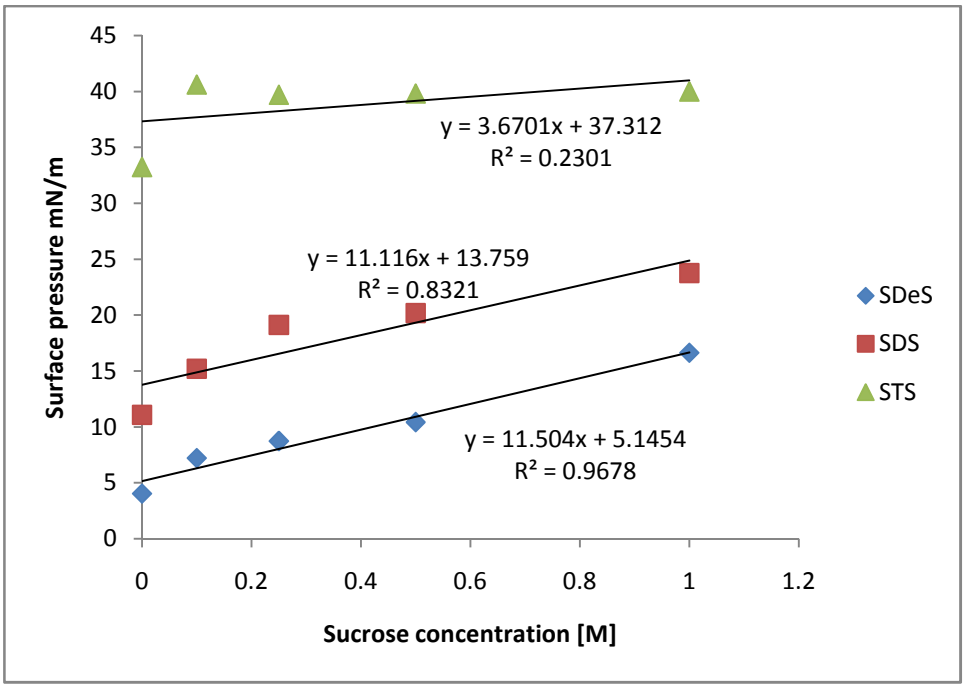

Figure 4.18. Surface pressure of surfactants; SDeS, SDS and STS as a function of sucrose concentration.

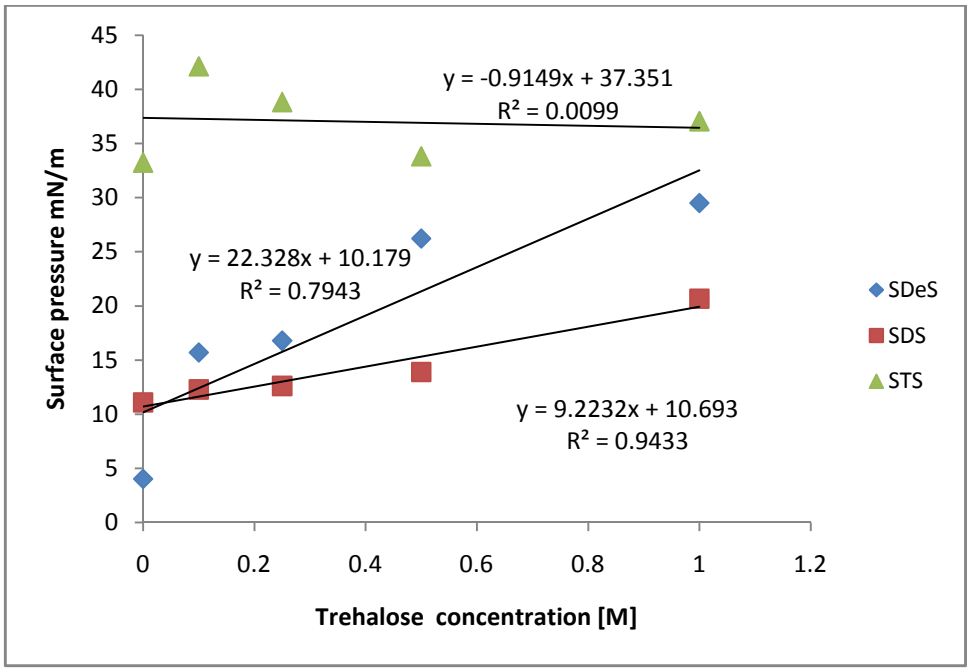

Figure 4.19. Surface pressure of surfactants; SDeS, SDS and STS as a function of trehalose concentration.

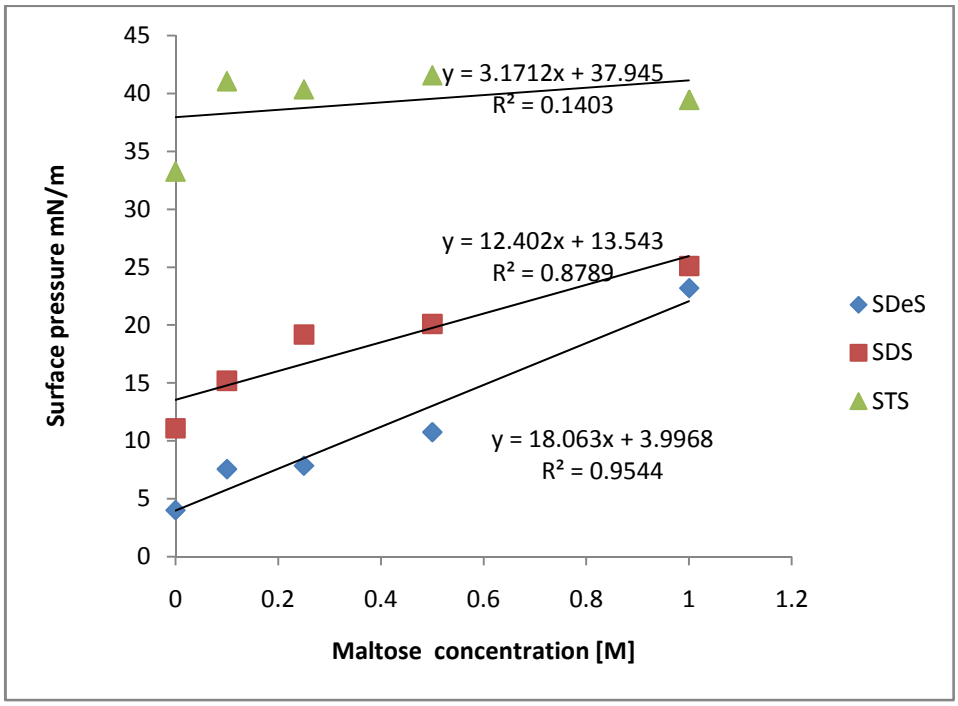

Figure 4.20. Surface pressure of surfactants; SDeS, SDS and STS as a function of maltose concentration.

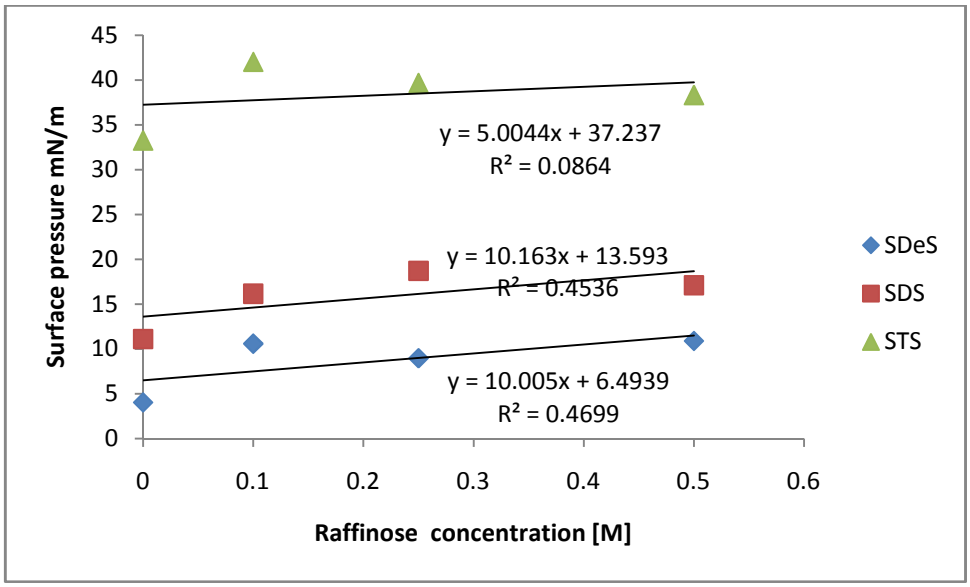

Figure 4.21. Surface pressure of surfactants; SDeS, SDS and STS as a function of raffinose concentration.

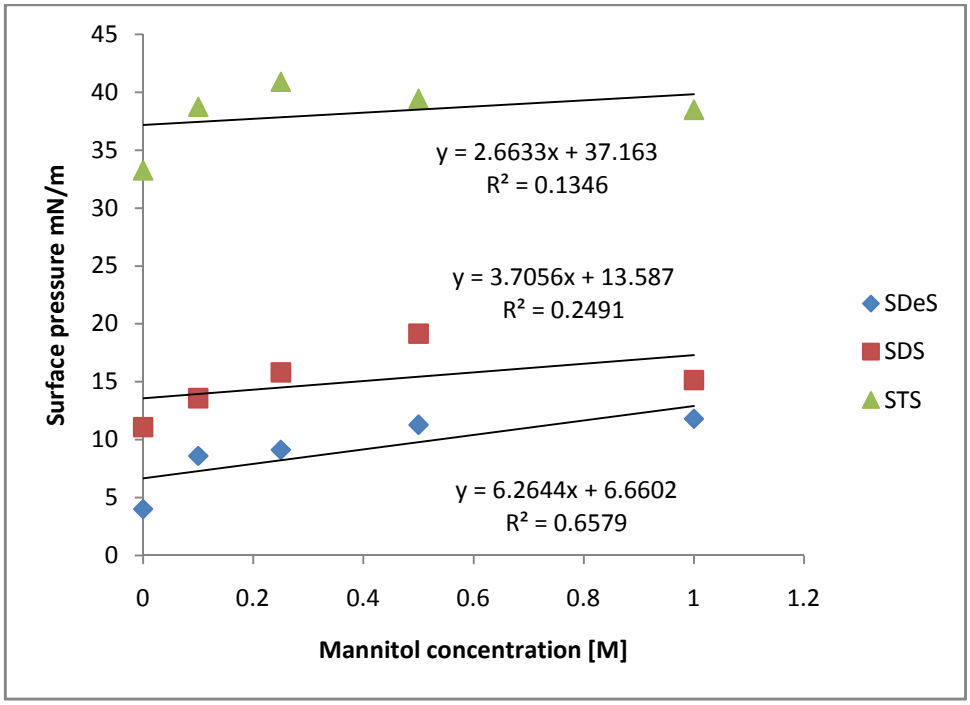

Figure 4.22. Surface pressure of surfactants; SDES, SDS and STS as a function of mannitol concentration.

For STS an increase in the surface pressure in the presence of sugars relative to water was observed. However, further increase in the sugar concentration did not show a linear increase in the surface pressure (figures 4.18 to 4.22) as observed for CMC and ΔG_{mic}.

Surface pressure gives an indication of the tendency of surfactants to adsorb at an interface[1]; increase in surface pressure of surfactant solutions indicates an enhanced surface activity of surfactant molecule. The results obtained for the effect of sugars on the surface pressure was more pronounced for SDeS followed by SDS, and STS as shown by the values of the slope (table 4.5). The results are comparable to their effect on the CMC, as a similar trend was observed for CMC.

Table 4.5. Slopes of surface pressure vs. sugar concentration plots for the homologous series of surfactants (Δ Surface pressure mNm^{-1}/mol of sugar).

Sugar	SDeS	SDS	STS
Sucrose	11.50	11.12	3.67
Trehalose	22.33	9.22	0.91
Maltose	18.06	12.40	3.17
Raffinose	10.01	10.16	5.00
Mannitol	6.26	3.71	2.66

4.3.3.2. Effect of sugars on surface excess concentration (Γ) and the area occupied per surfactant molecule (A_0)

The determination of the Γ and A_0 values reported was carried out by the Labdesk software for the K100 which was based on the equation below

$$\Gamma = \frac{1}{RT} \cdot \frac{d\gamma}{dlnC}$$

derived from the Gibbs adsorption equation.

Thus the Γ was determined from the slope of the plot of γ versus log C_2. At constant temperature when γ is in mNm^{-1} and R= $8.31 Jmol^{-1}K^{-1}$, Γ is given as moles/$1000m^2$. Therefore, the area per molecule (A_0) was calculated from the relationship.

$$A = \frac{10^{16}}{N\Gamma}$$

The presence of sugars induced a decrease in the surface excess concentration of SDeS and SDS relative to water while the opposite effect was observed on the area per molecule as shown in the data obtained. A concentration dependence of A_0 was observed for the aforementioned surfactants as shown in figures 4.23&4.24. The trend in the variation of A_0 with sugar concentration was just opposite to that of Γ.

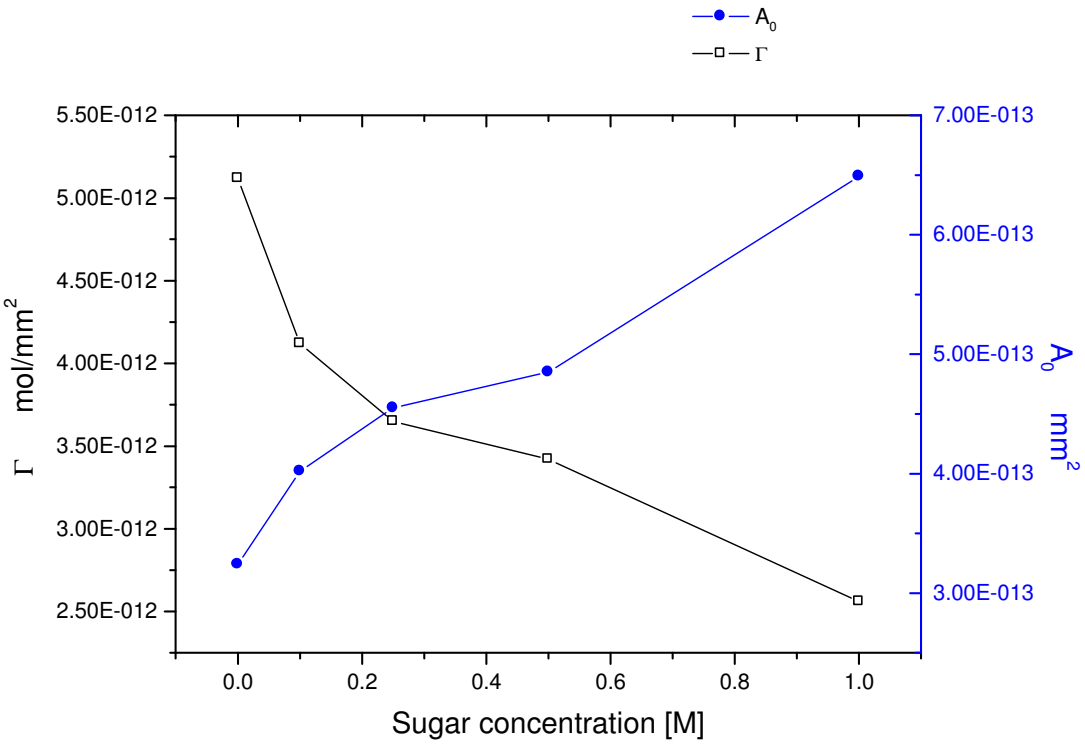

Figure 4.23. Surface excess concentration (Γ) and area per molecule (A_0) of SDeS as a function of sucrose concentration. The graph shows an inverse relationship between these parameters as well as concentration dependence.

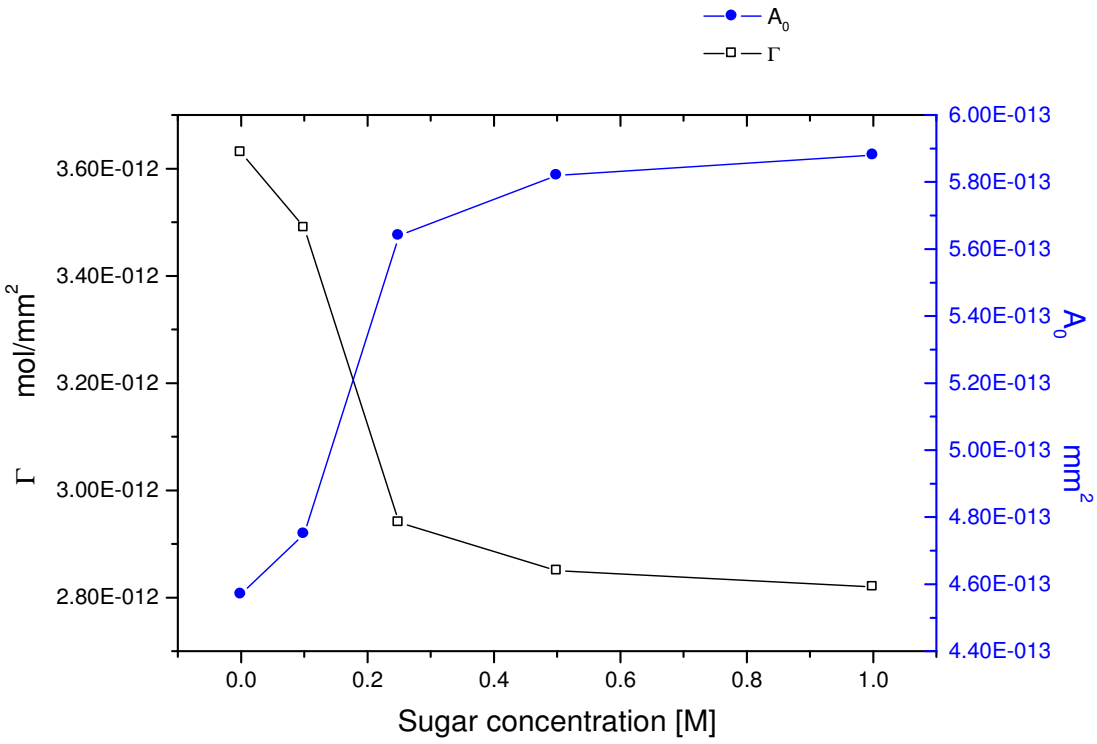

Figure 4.24. Surface excess concentration (Γ) and area per molecule (A_0) of SDS as a function of sucrose concentration. The graph shows an inverse relationship between these parameters as well as concentration dependence.

A non linear concentration dependence of the Γ & A_0 were observed for STS as similarly observed for other measured surfactant properties

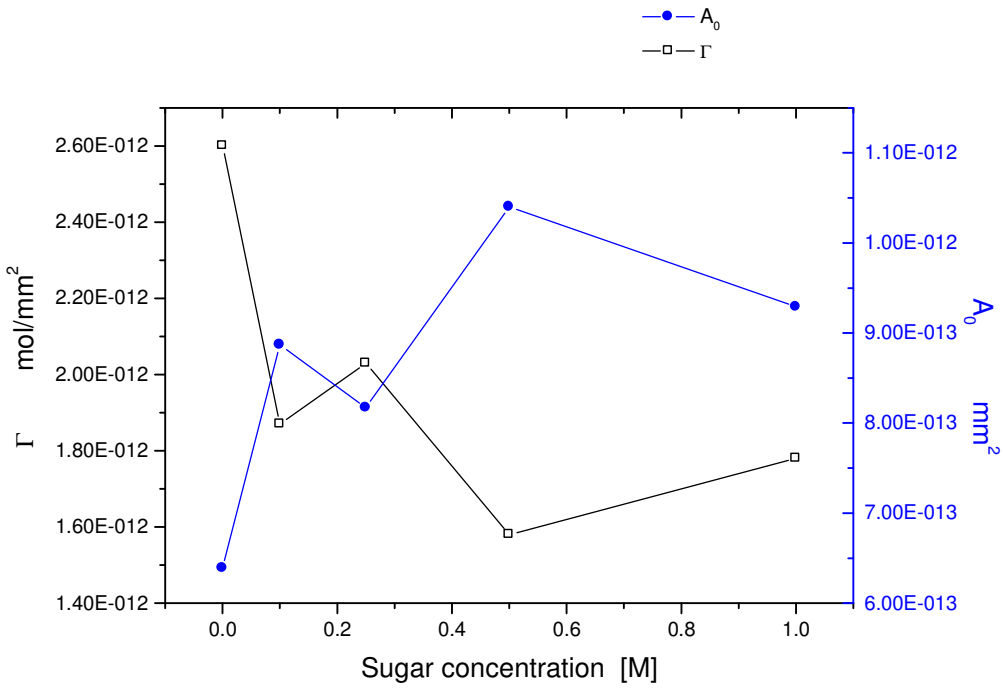

Figure 4.25. Surface excess concentration (Γ) and area per molecule (A_0) of STS as a function of sucrose concentration. The graph shows non concentration dependence.

An explanation for the decrease in the Γ in the presence of sugars may be due to the formation of more hydrogen bonds between water and the sugar molecules[13] hence less surfactant groups at the interfacial layer, thus the added sugar pushes more surfactants into the bulk. The area occupied by a surfactant molecule (A_0) at the surface for ionic surfactants with a single hydrophilic group has been described by Rosen as equivalent to the area occupied by the hydrated hydrophilic head group rather than by the hydrophobic group[1]. In addition, when a second hydratable hydrophilic group is introduced to the molecule, an increase in the A_0 is observed[1].

Larger A_0 could arise because the sugars interact with head groups due to binding of sugars to the polar heads of the surfactants or perhaps the sugars are preferentially encountered in the vicinity of the head groups; that is when the head groups come together they increase the local concentration of sugars relative to the bulk solution.

It is believed that the sugars decrease the electrostatic repulsion between the head groups, and increase the mutual attraction between the head group as a consequence micelles are formed and CMC is decreased[11], conductivity measurements were thus carried out to substantiate this claim.

Results obtained for measurement of conductance values of SDeS, SDS and STS in the presence of sugar as a function of sugar concentration reveal a decrease in conductivity as depicted in figure (4.26 to 4.28). The data showed uncertainty values of ≤ ±0.03 mS/cm

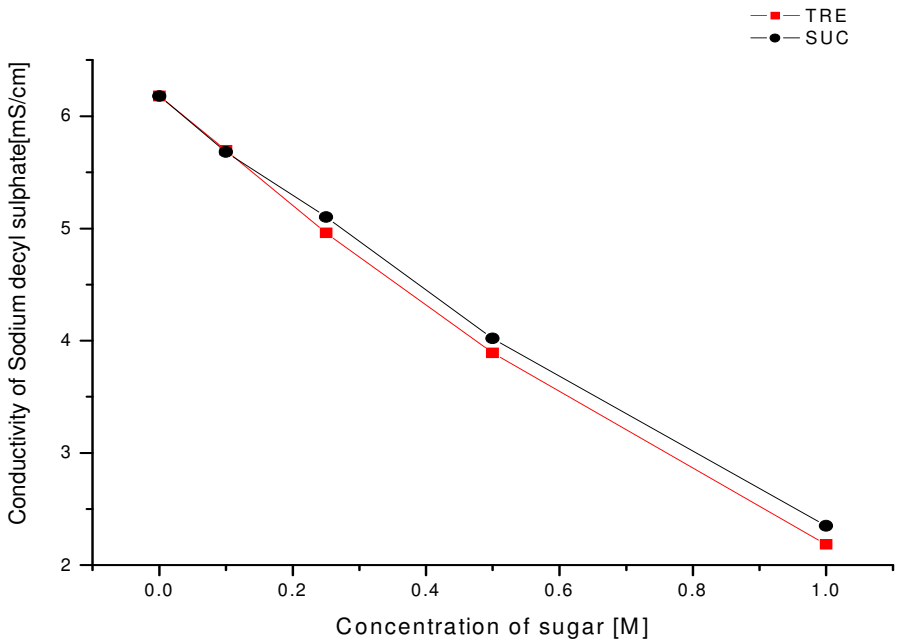

Figure 4.26. Conductivity of SDeS as a function of sugar concentrations, for trehalose and sucrose.

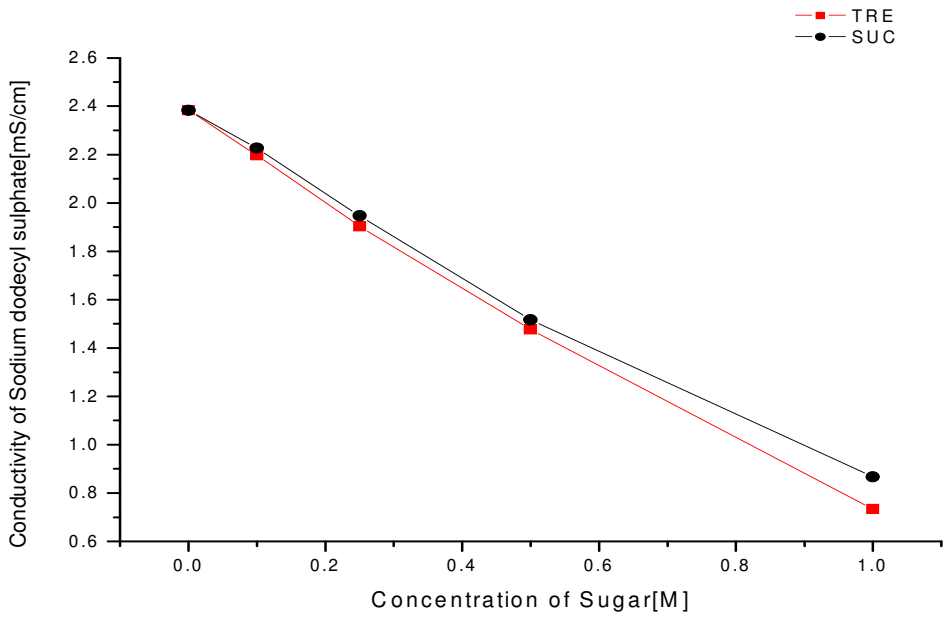

Figure 4.27. Conductivity of SDS as a function of sugar concentrations, for trehalose and sucrose.

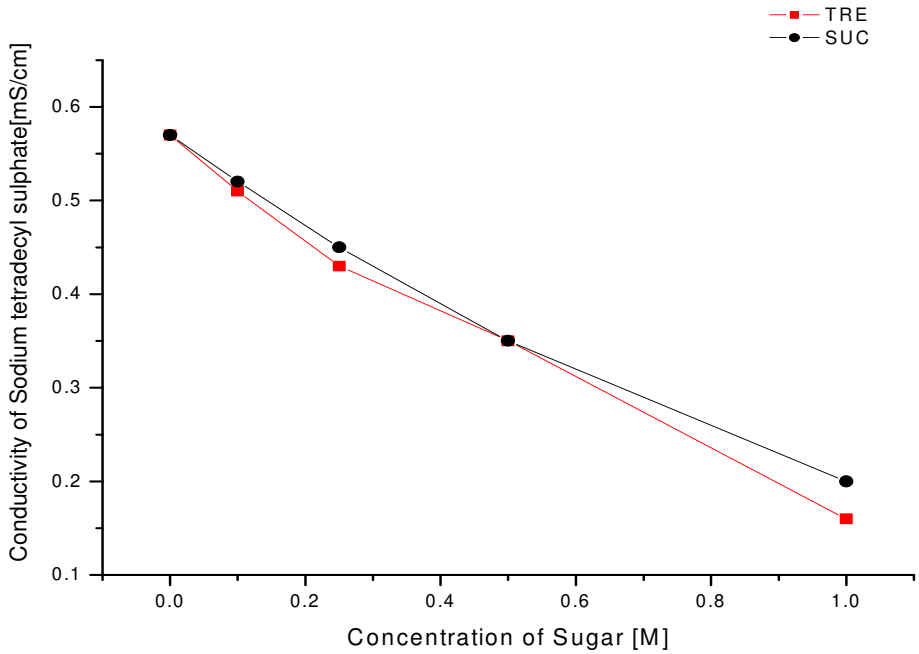

Figure 4.28. Conductivity of STS as a function of sugar concentrations, for trehalose and sucrose.

Sugars appear to reduce the degree of dissociation of the surfactant molecules; this could be probably due to sugar modification of the solvent properties. Studies by other investigators report decreases in the dielectric constant of water in the presence of sugars[14], this thus explains the effect of sugars on conductivity of the surfactants with respect to its effect on enhancing the attraction between the head groups. However a probable explanation for the conductivity results could be due to the viscosity of the solutions, viscosity goes up as sugar concentration increases hence influencing the dissociation process.

4.5. Concluding Remarks

The effect of various sugars (sucrose, trehalose, maltose, raffinose and mannitol) on the CMC and surface parameters of SDeS, SDS and STS was successfully studied and recorded. The results reveal that as the concentration of sugar increases the CMC of the surfactants studied decreases, and a more negative ΔG_{mic} was obtained, hence indicating that, the hydrophobically driven aggregation into micelles is energetically more favourable in the presence of sugars. The results also indicate an increased free energy penalty of exposing hydrocarbon residues to aqueous sugar solutions as opposed to water due to unfavourable interaction between the hydrophobic groups and the aqueous sugar solution.

However it was observed that the effect was not overly large, the presence of the sugars did not produce a significant increase in the $\Delta G_{mic.}$ Nevertheless, this raises the thought provoking question as to whether we should expect or even desire a large effect, and the implication for protein stability, if this is the case. For instance, if we stabilise a protein structure too much does it lose it is biological function?

The measurement of other surface parameters such as the area per molecule, surface excess concentration and surface pressure and the effect of sugars on these parameters were also investigated. The results indicate that the sugars influence interactions present at the interfacial layer suggesting probable interactions between polar groups as indicated by increase in the area occupied per molecule of the surfactants in the presence of sugars, solvent modification by enhancing hydrogen bonding network, and influence on the electrostatic forces.

Measurements of CMC also allow qualitative conclusions to be drawn about hydrophobic interactions in micelles which could be extended to hydrophobic interactions in proteins, and phospholipid bilayers in biological membranes. In understanding the effect of sugar on biomolecules it is important to see the effect of sugar on the forces responsible for the stability of micelles. Micelles are said to be stabilized by both the mutual attraction between the hydrophilic head groups (which is electrostatic in nature) and hydrophobic interactions between the hydrophobic tail groups[1]. Since the results shows sugars have their effect on both the head groups and tail group, they therefore act by a combined effect of enhancement of the aforementioned interactions.

These observations are very interesting and complicated and require further investigation. Analysis of co crystals of sugar and surfactant molecules by single crystal x-ray diffraction will provide complementary data to help elucidate the plausible interaction between head group and sugar; however it should be taken into account that solid state is not a true representation of the system due to the absence of the solvent. A possible method that could give more information on sugar surfactant interactions is calorimetry; hence further studies of sugar-surfactant interactions have been investigated by this method and results of this study have been reported in the subsequent chapter.

The results also suggest a difference in stabilizing effect between isomeric sugars. Differences were observed in the extent of reduction of CMC, this indicates that sugars have varying reductive effects on CMC. Studies suggest that the extent of their effect differs with their molecular structure, and how they interfere with or affect the hydrogen bond network of water, as observed in Raman spectroscopy [15-17].

4.6. Data obtained for the micellization of SDeS, SDS and STS by tensiometry.

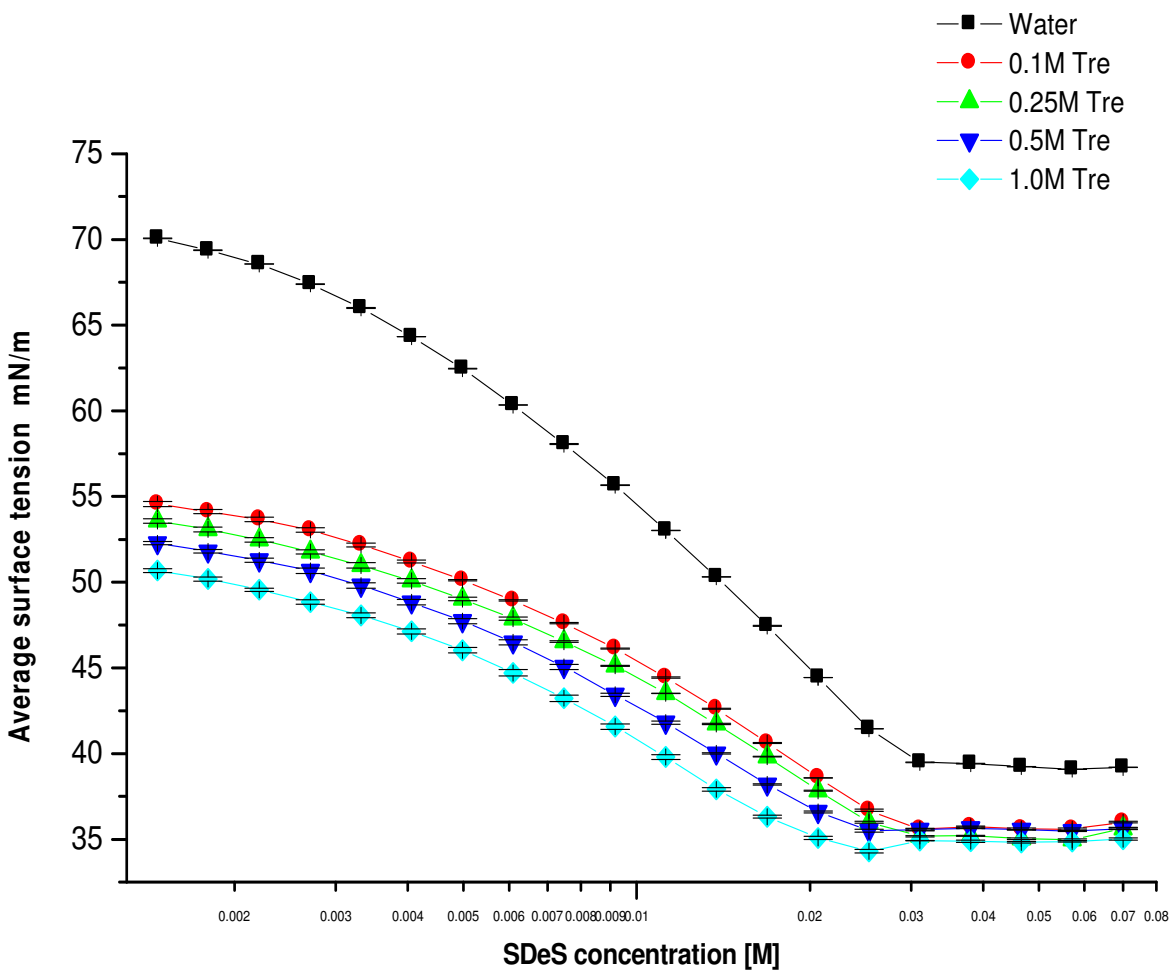

Figure 4.29. Plot of Average surface tension vs log of SDeS concentration, showing the effect of trehalose.

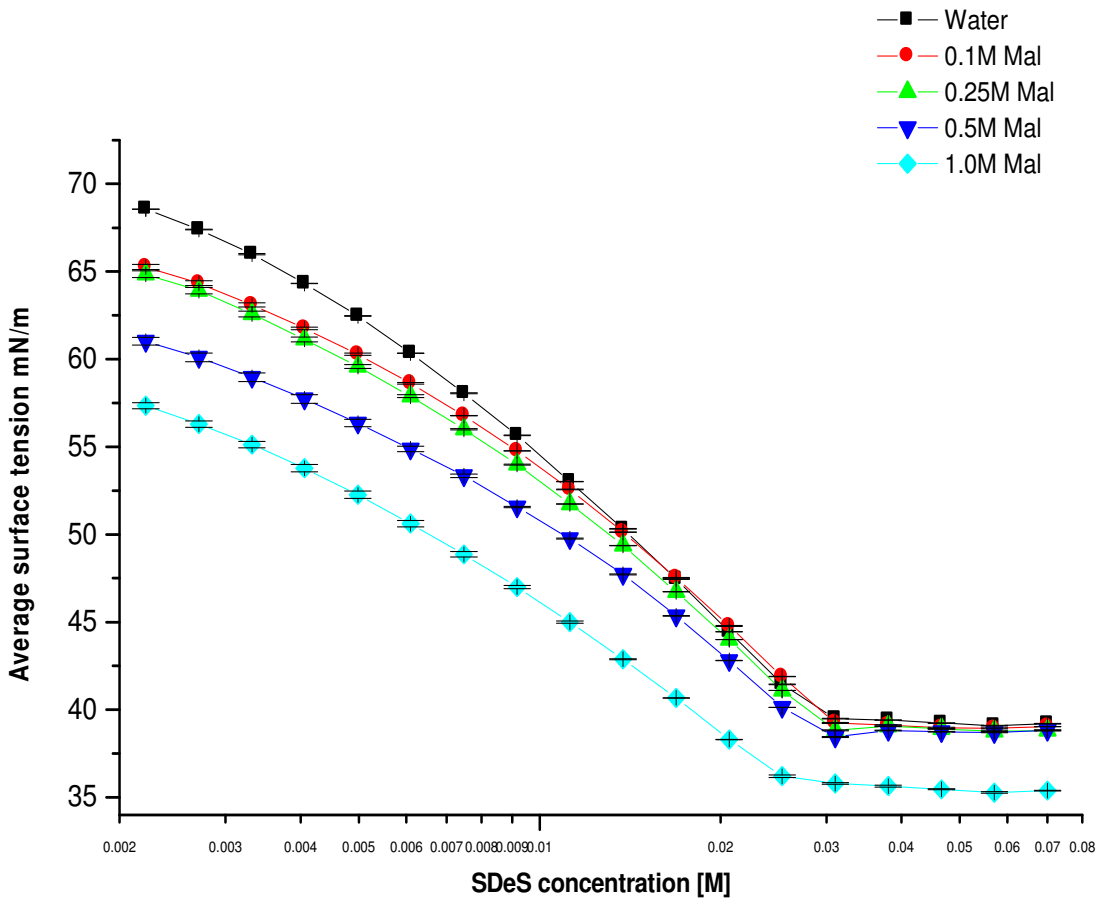

Figure 4.30. Plot of Average surface tension vs log of SDeS concentration, showing the effect of maltose.

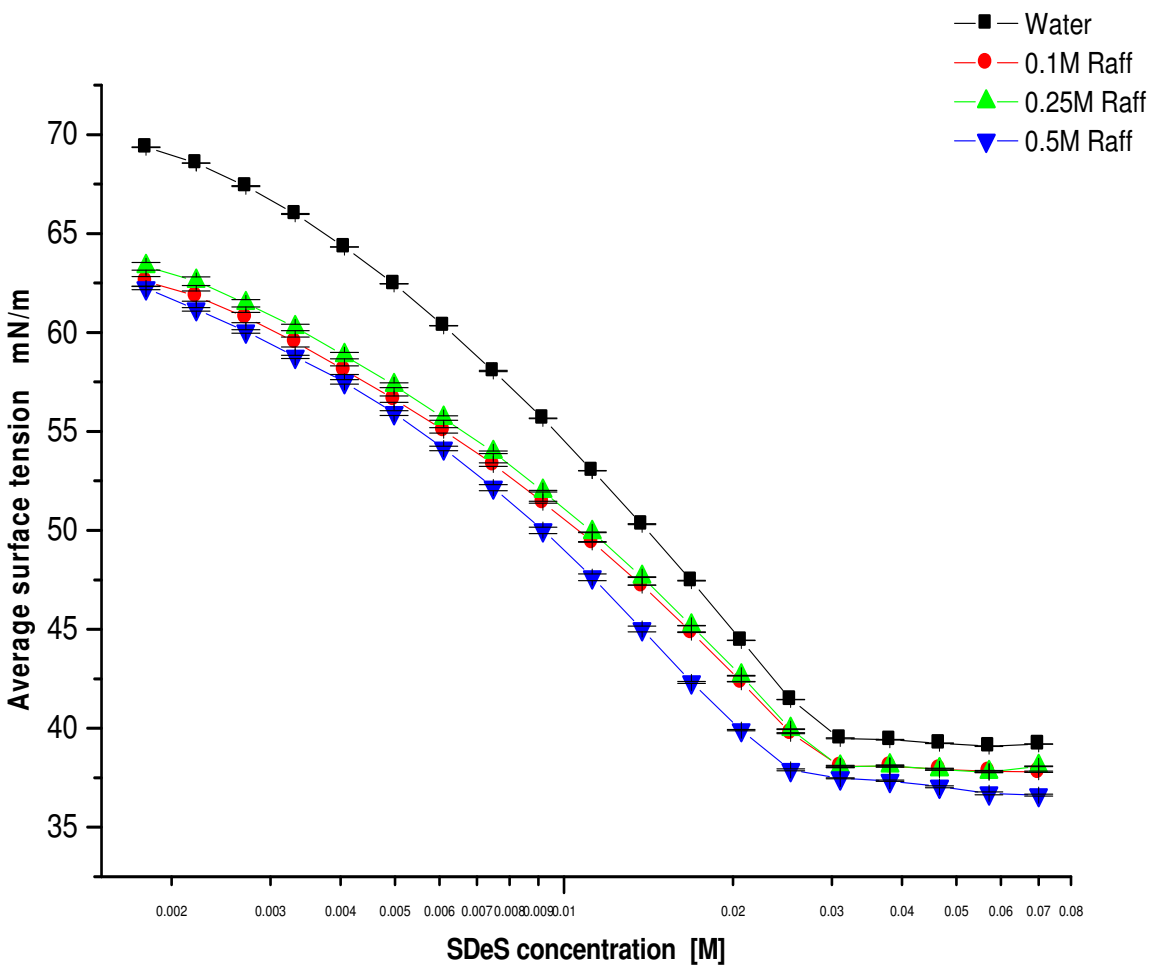

Figure 4.31. Plot of Average surface tension vs log of SDeS concentration, showing the effect of raffinose.

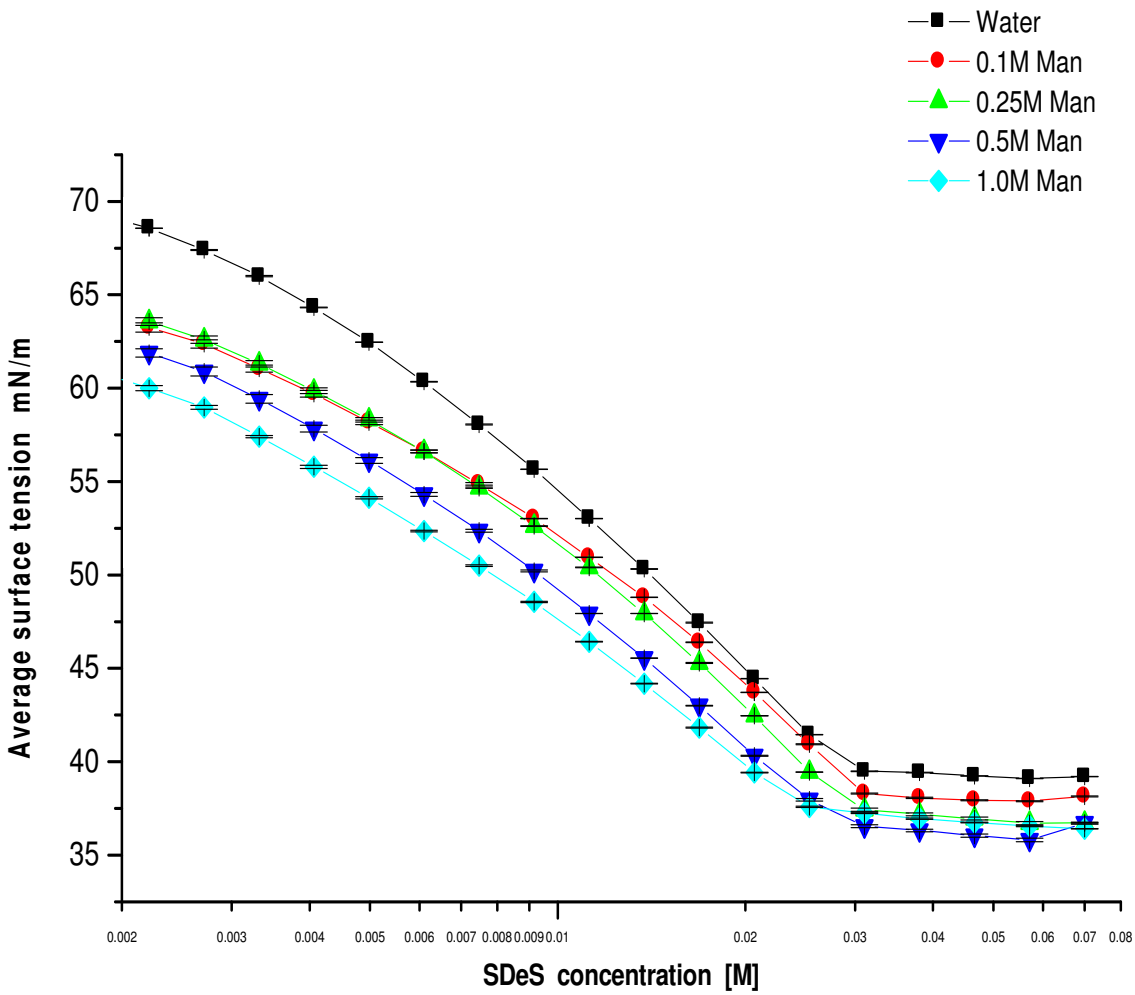

Figure 4.32. Plot of Average surface tension vs log of SDeS concentration, showing the effect of mannitol.

Table 4.6. The effect of investigated sugars on the surface and micellization parameters of SDeS.

Sugar concentration [M]	Critical micelle concentration (CMC) (mM)	Surface excess concentration $x10^{-12}$ mol/mm^2	Area per molecule $x10^{-13}$ mm^2	Gibbs free energy of micellization (kJ/mol)	Surface Pressure (mN/m)
0(water)	32.51	5.12	3.24	-18.44	4.03
Sucrose					
0.1	30.45	4.98	3.33	-18.56	7.21
0.25	30.15	5.09	3.26	-18.54	8.73
0.5	29.36	4.58	3.63	-18.43	10.42
1.0	22.54	3.95	4.21	-19.11	16.62
Trehalose					
0.1	30.98	4.12	4.02	-18.54	19.19
0.25	30.93	3.65	4.55	-18.57	24.79
0.5	28.79	3.42	4.85	-18.68	26.21
1.0	23.66	2.56	6.49	-19.14	29.49
Maltose					
0.1	31.17	5.43	3.06	-18.56	7.57
0.25	31.06	4.99	3.33	-18.49	7.86
0.5	29.92	4.73	3.51	-18.62	10.76
1.0	25.62	4.43	3.74	-19.03	23.18
Raffinose					
0.1	30.96	4.36	3.81	-18.51	10.59
0.25	30.47	4.73	3.51	-18.60	8.97
0.5	25.22	4.76	3.48	-19.00	10.89
Mannitol					
0.1	31.30	5.39	3.08	-18.51	8.61
0.25	31.11	5.04	3.30	-18.52	9.14
0.5	29.23	4.42	3.75	-18.68	11.30
1.0	25.10	4.45	3.73	-19.01	11.81

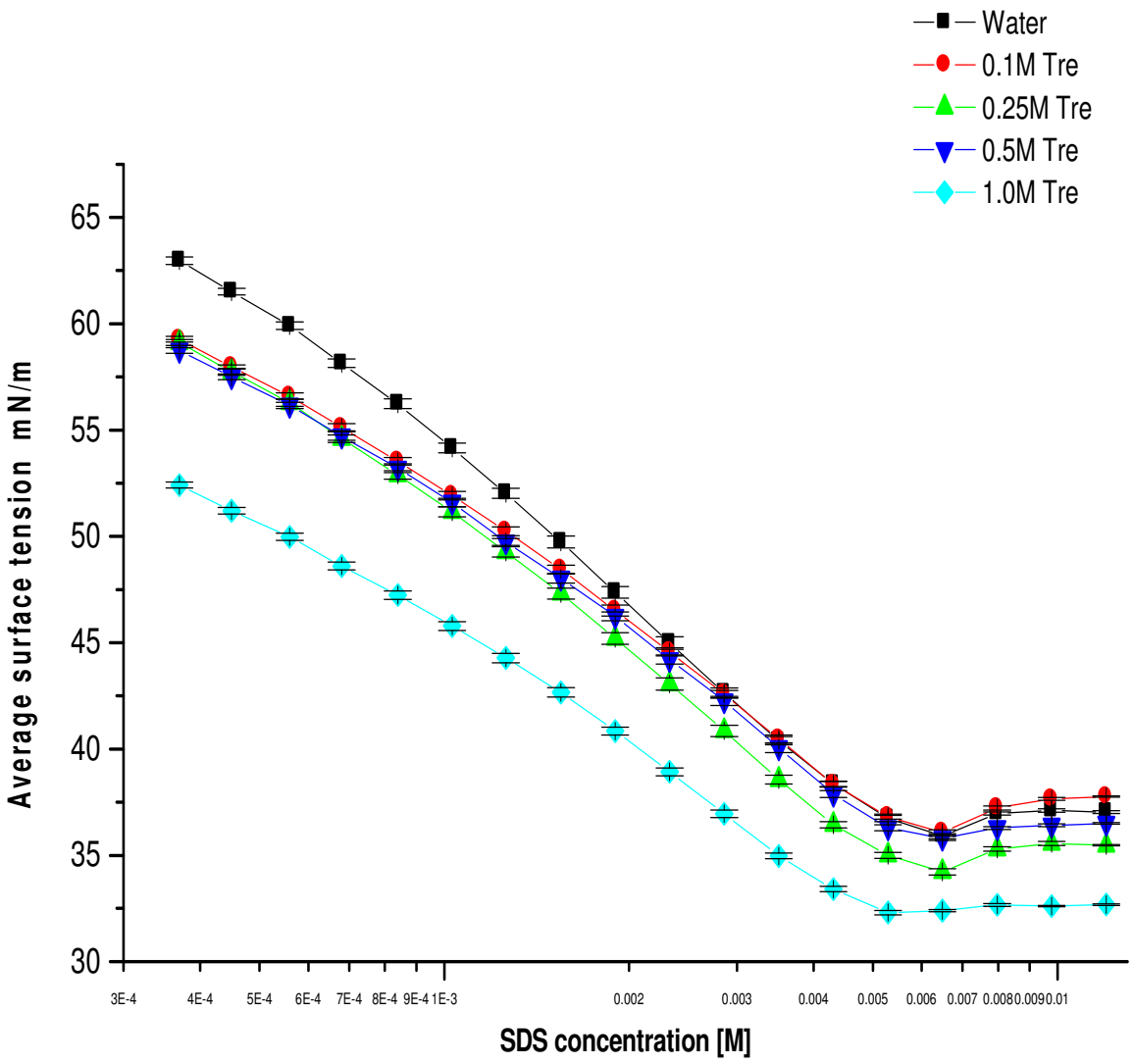

Figure 4.33. Plot of Average surface tension vs log of SDS concentration, showing the effect of trehalose.

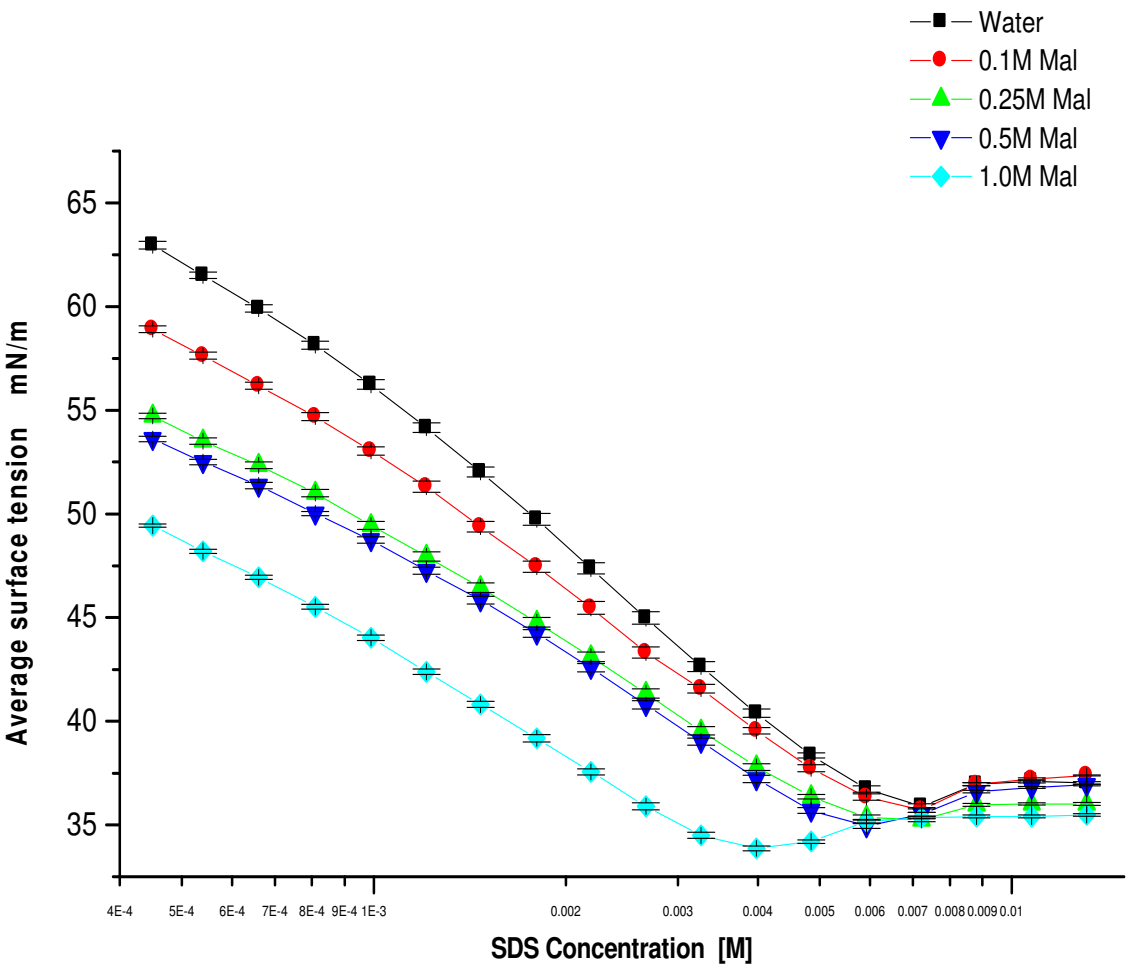

Figure 4.34. Plot of Average surface tension vs log of SDS concentration, showing the effect of maltose.

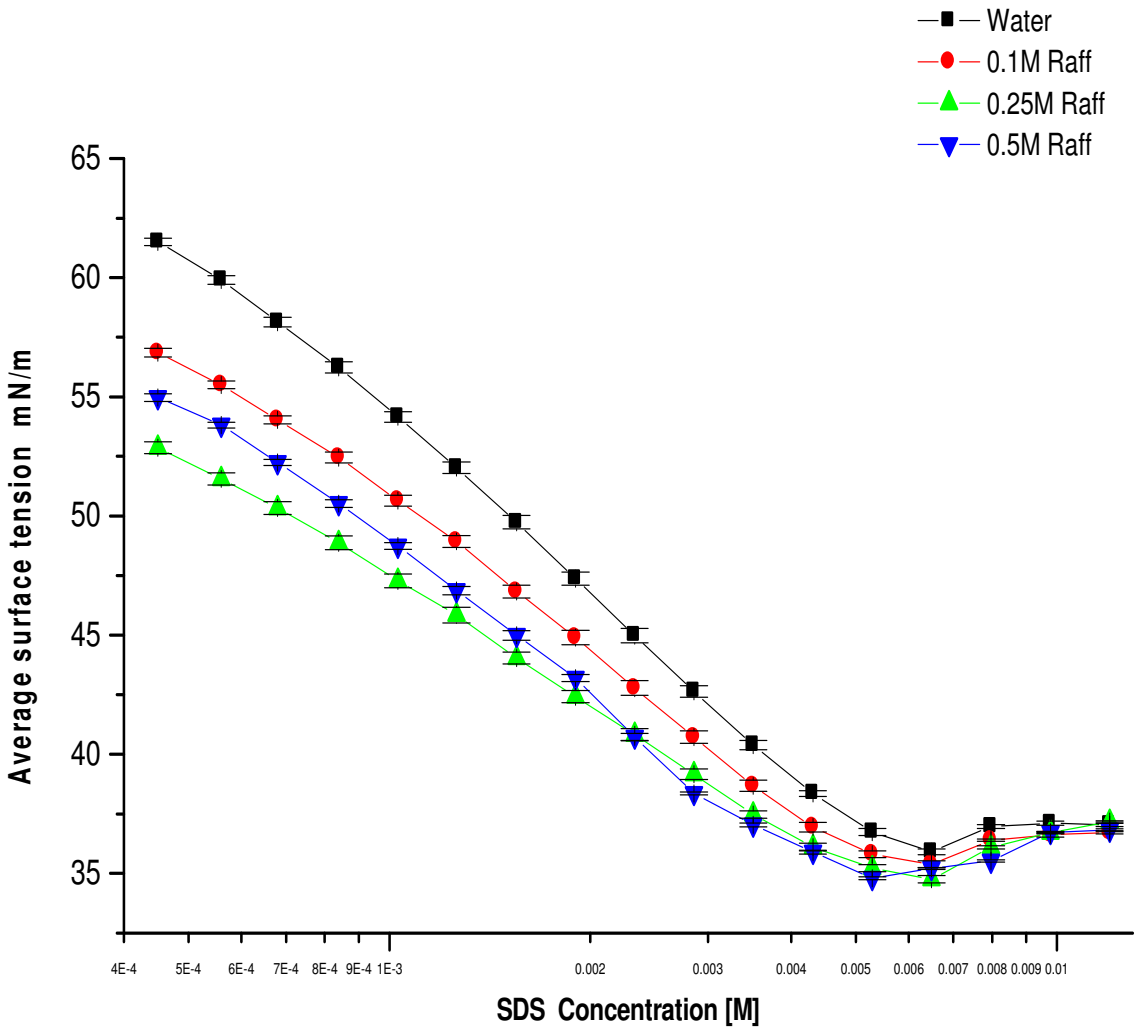

Figure 4.35. Plot of Average surface tension vs log of SDS concentration, showing the effect of raffinose.

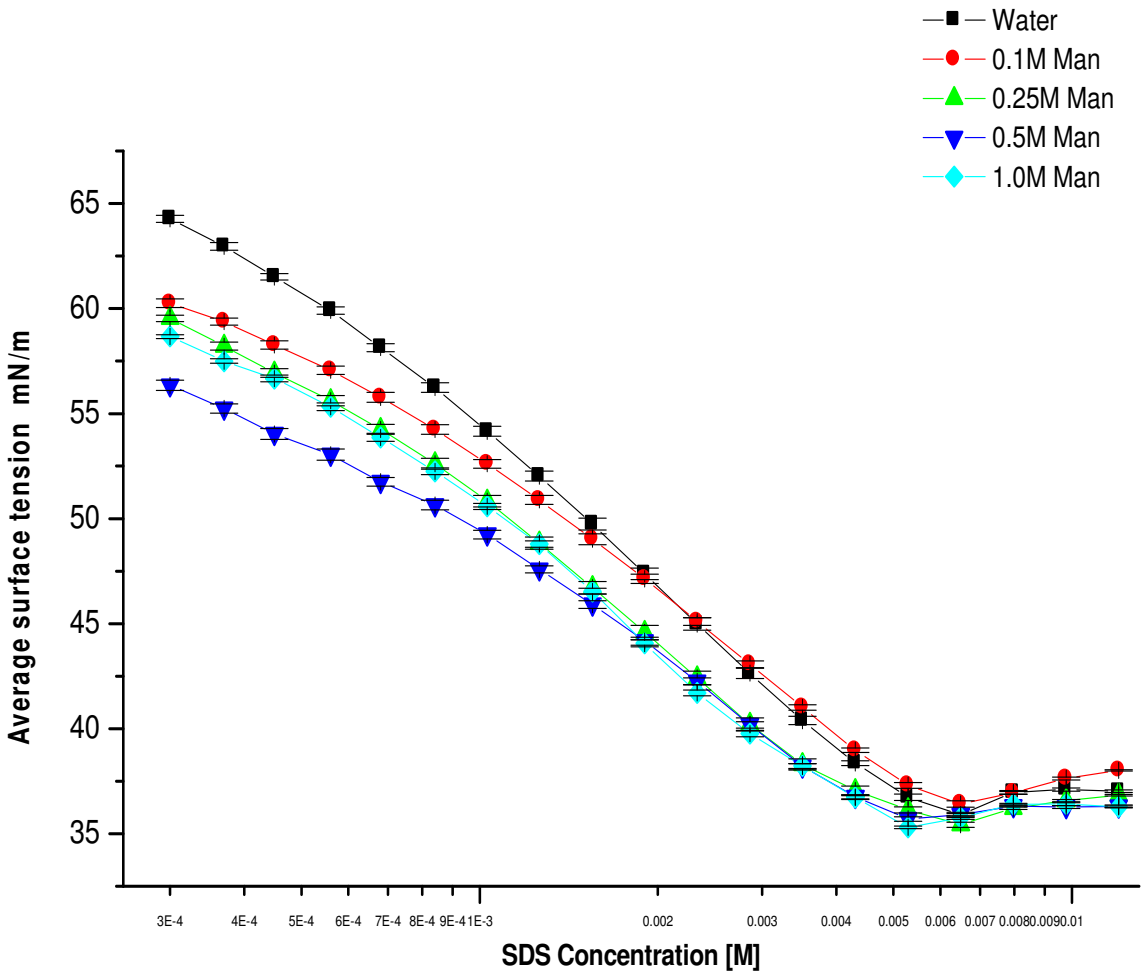

Figure 4.36. Plot of Average surface tension vs log of SDS concentration, showing the effect of mannitol.

Table 4.7. The effect of the investigated sugars on the surface and micellization parameters of SDS.					
Sugar Concentration [M]	Critical micelle concentration(CMC) [mM]	Surface excess concentration $x10^{-12}mol/mm^2$	Area per molecule $x10^{-13}mm^2$	Gibbs free energy of micellization KJ/mol	Surface pressure mN/m
0(water)	7.19	3.63	4.57	-22.24	11.09
Sucrose					
0.1	5.92	3.49	4.75	-22.53	14.67
0.25	5.56	2.94	5.64	-22.73	18.54
0.5	5.39	2.85	5.82	-22.85	20.19
1.0	4.17	2.82	5.88	-23.51	23.76
Trehalose					
0.1	5.89	3.73	4.45	-22.34	12.28
0.25	5.76	3.94	4.21	-22.51	12.61
0.5	5.50	3.71	4.47	-22.80	13.90
1.0	5.39	3.11	5.34	-22.91	20.65
Maltose					
0.1	5.94	3.38	5.49	-22.63	15.19
0.25	5.62	3.31	5.02	-22.76	19.19
0.5	5.55	3.18	5.22	-22.81	20.10
1.0	3.81	2.87	5.77	-23.74	25.09
Raffinose					
0.1	5.68	2.67	6.21	-22.74	16.14
0.25	5.47	2.91	5.71	-22.84	18.69
0.5	5.19	3.25	5.10	-22.99	17.09
Mannitol					
0.1	6.27	3.52	4.71	-22.49	13.59
0.25	5.81	3.29	5.04	-22.70	15.80
0.5	5.30	3.16	5.26	-22.56	19.15
1.0	4.86	3.85	4.31	-23.14	15.15

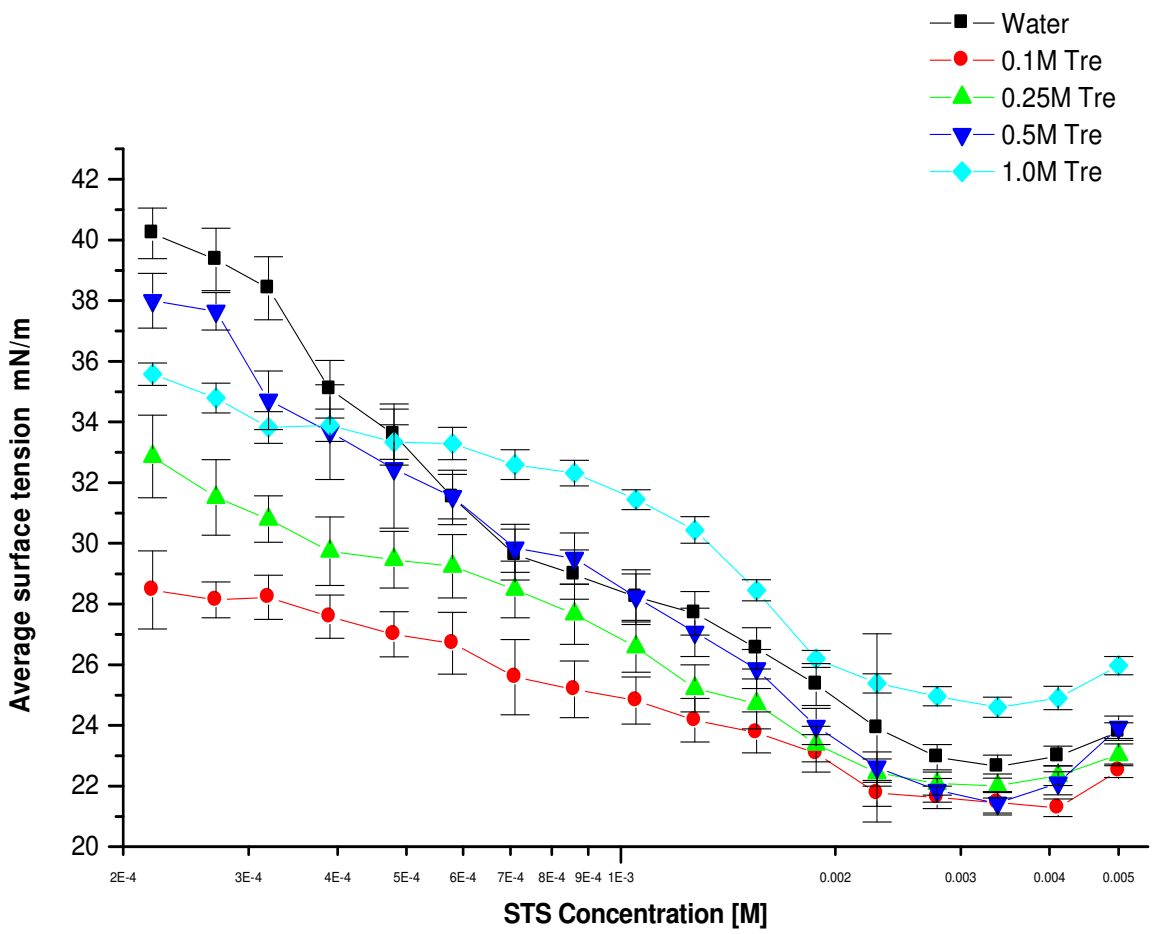

Figure 4.37. Plot of Average surface tension vs log of STS concentration, showing the effect of trehalose.

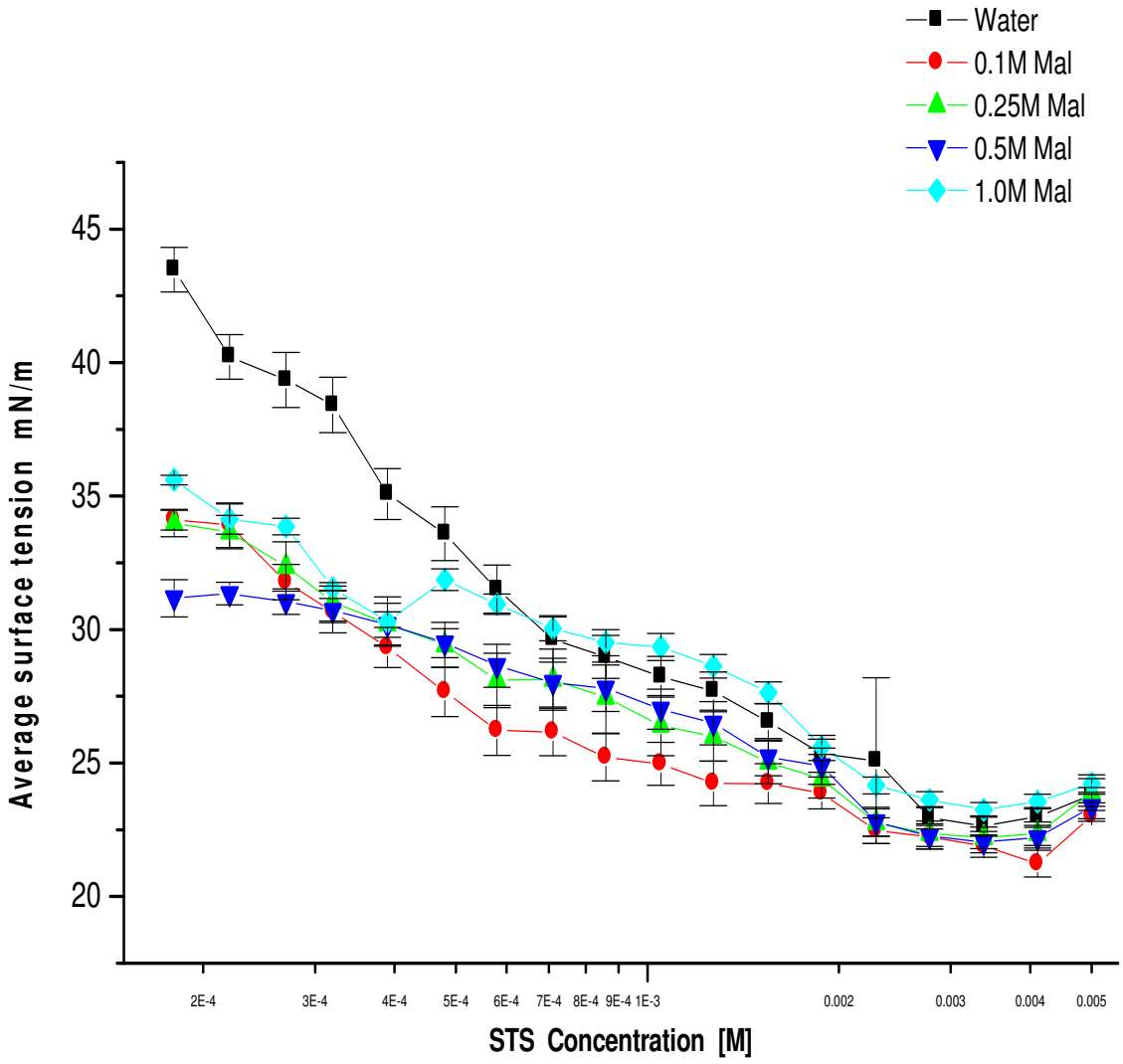

Figure 4.38. Plot of Average surface tension vs log of STS concentration, showing the effect of maltose.

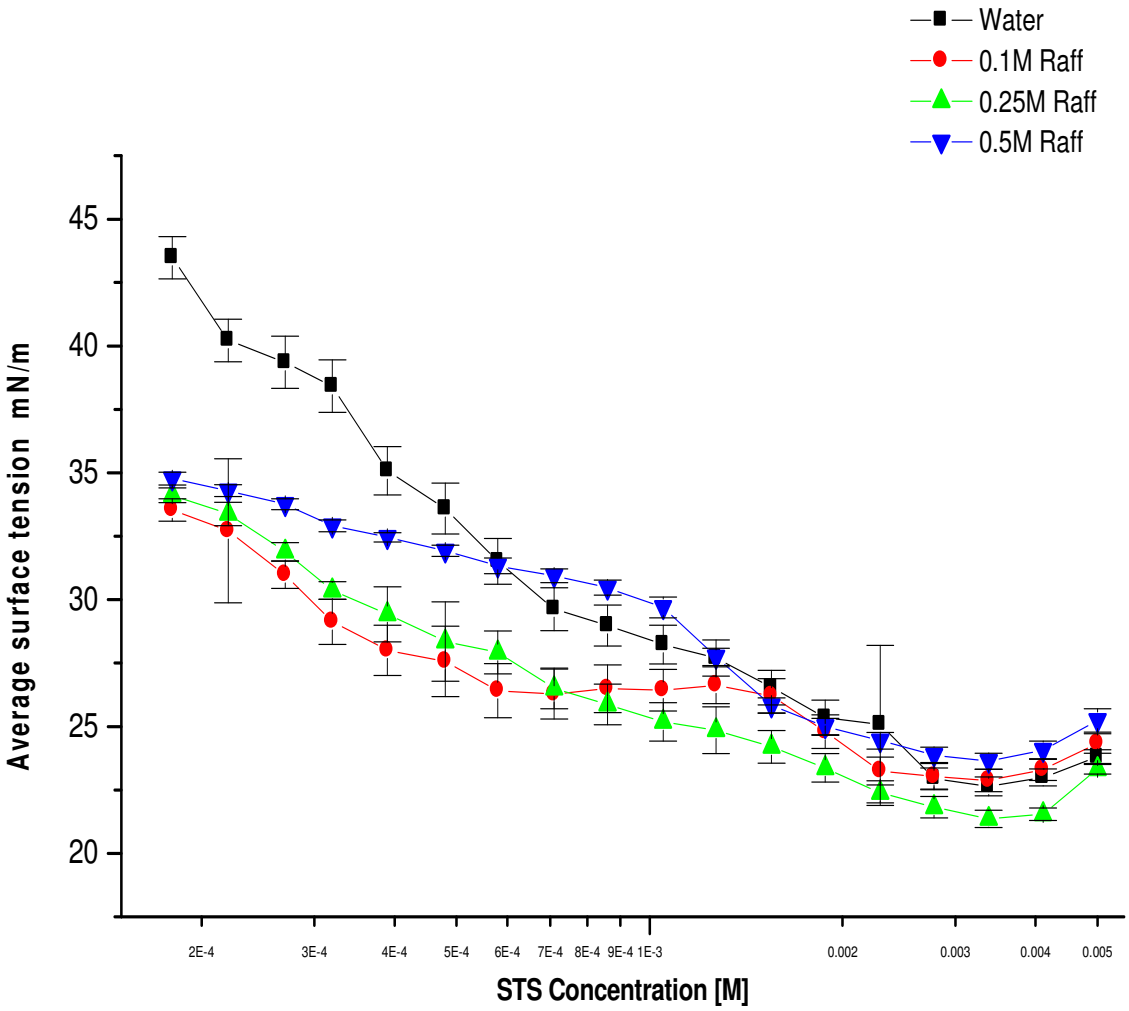

Figure 4.39. Plot of Average surface tension vs log of SDS concentration, showing the effect of raffinose.

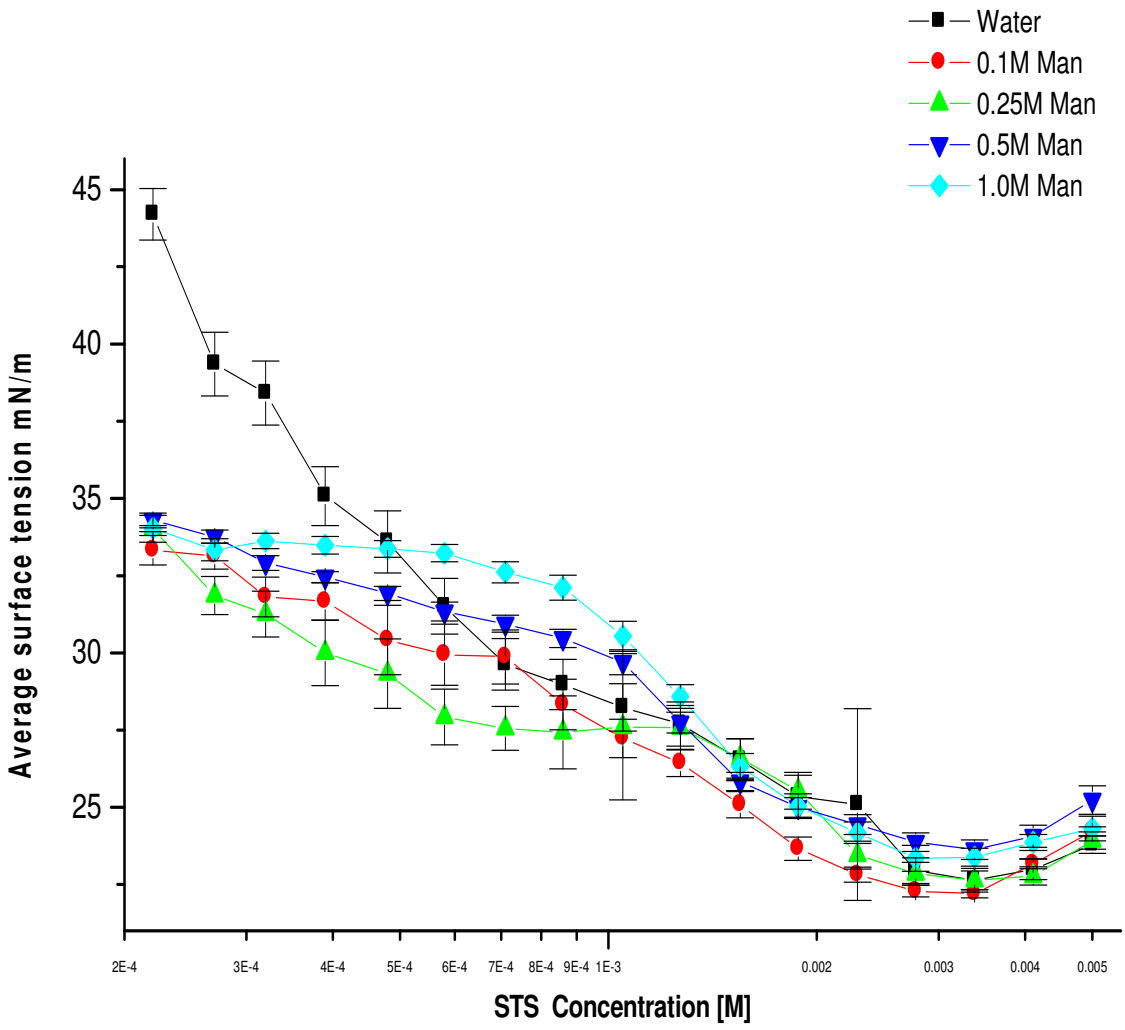

Figure 4.40. Plot of Average surface tension vs log of STS concentration, showing the effect of mannitol.

Table 4.8. The effect of investigated sugars on the surface and micellization parameters of STS.

Sugar Concentration [M]	Critical micelle concentration(CMC) [mM]	Surface excess concentration $x10^{-12}$mol/mm^2	Area per molecule $x10^{-13}$mm^2	Gibbs free energy of micellization KJ/mol	Surface pressure mN/m
0(water)	2.88	2.60	6.39	-24.75	33.24
Sucrose					
0.1	2.74	1.87	8.87	-24.85	40.61
0.25	2.50	2.03	8.17	-25.10	39.70
0.5	2.46	1.58	1.04	-25.14	39.81
1.0	2.20	1.78	9.29	-25.43	39.99
Trehalose					
0.1	2.54	1.46	1.13	-25.07	42.11
0.25	2.49	1.94	8.55	-25.19	38.83
0.5	2.83	2.50	6.62	-24.79	33.82
1.0	2.19	3.05	5.44	-25.44	37.06
Maltose					
0.1	2.52	1.40	1.18	-25.06	41.04
0.25	2.43	2.30	7.22	-25.18	40.33
0.5	2.76	2.22	7.48	-24.86	41.55
1.0	2.71	2.56	6.47	-24.91	39.43
Raffinose					
0.1	2.34	3.01	5.51	-25.28	42.00
0.25	2.76	1.54	1.07	-24.86	39.66
0.5	2.79	1.98	8.35	-24.83	38.30
Mannitol					
0.1	2.83	3.73	4.44	-24.59	38.73
0.25	2.73	2.56	6.47	-24.89	40.88
0.5	2.58	1.99	8.33	-25.05	39.42
1.0	2.73	1.98	8.34	-24.89	38.47

Table 4.9. Surface tension values of water and aqueous sugar solutions.	
Solution	Surface tension [mN/m]
water	72.67±0.09
Sucrose[M]	
0.1	72.68 ±0.75
025	72.72 ± 0.80
0.5	72.04± 0.80
1.0	72.84± 0.92
Trehalose[M]	
0.1	70.25±0.75
0.25	70.35±0.90
0.5	71.47±0.99
1.0	71.86±0.90
Maltose[M]	
0.1	72.82±0.75
0.25	72.70±0.90
0.5	72.60±1.42
1.0	73.29±0.90
Raffinose[M]	
0.1	72.43±0.80
0.25	71.56±1.34
0.3	72.06±0.79
Mannitol[M]	
0.1	71.86±0.80
0.25	72.73±1.42
0.5	73.18±1.40
1.0	71.81±0.92

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Chapter 5: Micellization of sodium dodecyl sulphate in aqueous sugar solutions by Isothermal Titration Calorimetry

5.1. Introduction

Isothermal titration calorimetry (ITC) is a powerful method for the investigation of self assembly in association colloids, such as micelles[1]. In addition the thermodynamic parameters that characterise the stability of micellar systems can be determined by ITC through investigation of demicellization/disaggregation processes[1, 2].

Several researchers have used calorimetric methods to study the micellization of alkytrimethylammonium bromides in water as a function of temperature[3-6], this method can also be used to study interactions between surfactants and molecules such as lipids[7] and with drug molecules such as simvastatin[8].

Blume and co-workers have carried out extensive studies revealing the thermodynamic description of the micellization of several surfactant systems in aqueous salt solutions as well as micelle interaction with lipid vesicles by ITC [3, 9-11].

However there is little in the literature describing the micellization/demicellization behaviour of surfactants in various aqueous sugar solutions. In this study ITC was used to follow the micellization of the anionic surfactant sodium dodecyl sulphate (SDS) in aqueous sugar solutions. The experimental data was then used to obtain the CMC and other thermodynamic parameters such as the enthalpy (ΔH_{mic}), entropy (ΔS_{mic}),Gibbs free energy change of micellization(ΔG_{mic}).

Thus a comprehensive thermodynamic profile for surfactant/sugar interactions was obtained that show the effect of sugars on the thermodynamic stability of the micelles. And from these investigations into sugar–surfactant interactions we may also obtain insights into the molecular understanding of the bioprotective nature of the sugars.

5.2. Experimental

5.2.1. Materials

SDS; 99.5% purity was obtained from Fissons plc, sucrose ≥ 99.5%, trehalose dihydrate ≥ 99.0%, maltose monohydrate 99.0%, raffinose pentahydrate ≥ 99.0% and mannitol > 99.5% purities were obtained from Sigma Aldrich UK.

Deionised water (Elga purelab option, ion exchange chromatography) with resistivity of ~15MΩcm and surface tension value of 72.6 mNm^{-1} at room temperature was used.

All the chemicals were obtained from commercial and industrial suppliers and used without further purification.

5.2.2. Method

5.2.2.1. Sample preparation

Sugar and SDS solutions were prepared by weighing to an accuracy of $\pm 1 \times 10^{-4}$g and dissolved in deionised water. In addition, an aqueous sugar-SDS solution was prepared by dissolving defined amounts of surfactant into various sugar solutions between 0.1- 1.0M concentrations.

Freshly prepared samples were degassed for 10-15 minutes to ensure the removal of air bubbles prior to analysis with the calorimeter, thus preventing unstable and noisy baseline that can arise if air bubbles are present in the samples.

5.2.2.2. Isothermal titration calorimetry

The ITC experiments were performed at 25^{o}C with a thermometric TAM 2277 microcalorimeter (Thermometric. Jarfalla, Sweden) equipped with a titration unit. The calorimeter works under heat conduction conditions. Therefore the heat associated with a sample will be exchanged with the surroundings, resulting in a constant temperature in the sample cell as described in detail in chapter 3. The temperature of the calorimeter was controlled by a thermostat with high accuracy of 0.03^{o}C and also a high precision liquid thermostat of 1×10^{-6} ^{o}C.

The amplifier was set to 30 μW and the system was electrically calibrated before the experiment. A reference experiment using an inclusion complex of barium chloride in crown

ether was carried out in order to validate that correct electrical calibration had been performed on the calorimeter[12].

The titration syringe was loaded with SDS or SDS-sugar solution and then titrated into the calorimeter sample cell containing water or sugar solutions respectively. The reason sugar was included in the syringe was to eliminate the heat of dilution of sugar solution that results due to titrating surfactant solution into sugar solution. The reference cell was filled with deionised water. The experiments were designed so that the process of demicellization occurred during the titration process. This was achieved by using an aqueous surfactant concentration far in excess of the CMC.

Raw ITC curve showing the heat flow per injection were obtained. Data was analysed by the Digitam software for ITC as provided by Thermometric, in which reaction enthalpy (H) were determined by integrating every peak of the heat flow curve over time after a subtraction of appropriate control experiment. Enthalpograms were obtained which is a plot of reaction enthalpy (H) against total surfactant concentration in the reaction cell. The experiments were carried out in duplicate to monitor and ensure reproducibility. The surfactant solution was titrated first into water, then into aqueous sugar solutions of varying concentrations of the following sugars: Sucrose, trehalose, maltose, mannitol and raffinose.

The CMC and ΔH_{mic} were directly measured as described in the next section.

5.3. Results and Discussion

5.3.1. Demicellization experiment of SDS in water and aqueous sugar solution.

Figures 5.1A & B show the typical ITC curves obtained for the dilution of 174 mM SDS solution in water and sugar solutions at 25^{o}C. The titration experiment consisted of 40 injections of 5μL aliquots of a micellar solution into a 5ml ampoule containing 2.7ml of water or sugar solution. Each injection produced a heat flow peak observed as power (μW). The titration curve can be classified into three concentration ranges[3] for figure 5.1B this can be easily seen.

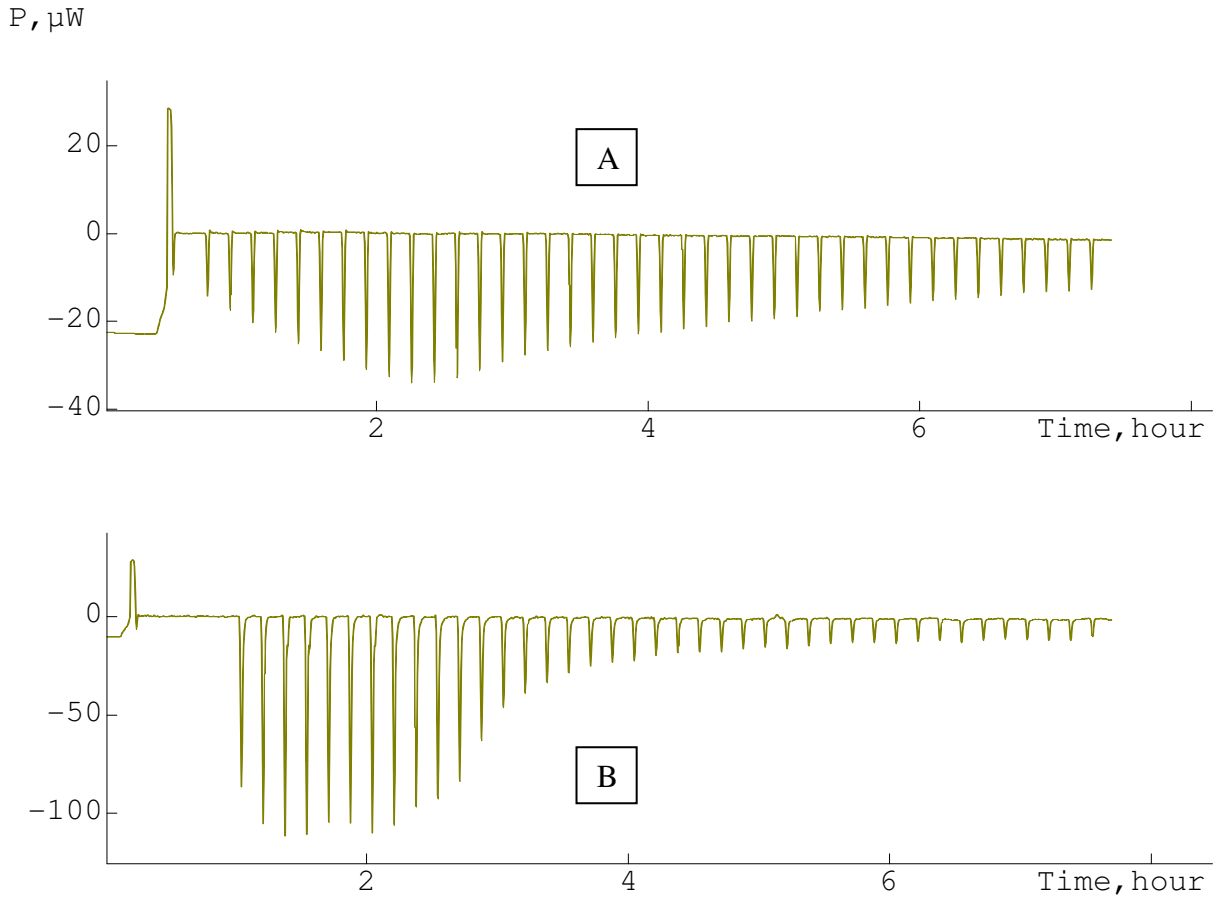

Figure 5.1. ITC titration curve showing heat flow (power in µW) versus time , of 174mM micellar SDS in steps of 40 x 5 µl (A) in water and in (B)1M sucrose solution.

The first concentration range was observed during the first few injections in which the concentration of the surfactant in the sample ampoule remained below the CMC. Large almost constant enthalpic effects (recorded as power change per injection in µW) were observed due to demicellization of the micelles, dilution of the micelles and resultant monomers.

The second region is characterised by a rapid decrease in the observed heat effects, this observation indicates that the surfactant concentration is approaching the CMC of the surfactant in the sample ampoule.

The third concentration region occurs after the CMC is reached in the sample ampoule, and in this region a constant power change per injection is observed. It reflects only heat effects due to the dilution of micelles as a result of further addition of a concentrated micellar solution to the existing micelle solution in the sample ampoule. At which point total demicellization

cannot be assured and the measured power reduces and become constant at the end of the experiments.

Thus, a plot of integrated heat of injection versus concentration yields an S-shaped enthalpogram with plateaux before and after the CMC.

It is immediately apparent that for the titration of SDS in water the behaviour exhibited does not follow the ideal case described above. The shape of the curve is asymmetric, an explanation for this behaviour is due to the fact that during titration the ionic surfactant is fully dissociated in the premicellar region[3]. The presence of a large number of counter ions ions (Na^+) has been suggested to be the likely cause for the observed difference in behaviour. The SDS molecules will dissociate to a large degree in water, forming dodecyl sulphate ions and counter ions (Na^+) but to a much lesser degree in sugar solutions, the sugars interact with the charged group and possibly charged micelles[3, 8]. This claim is also supported by the decrease in conductivity observed for SDS in the presence of sugars as reported previously in chapter four.

The reaction enthalpy was obtained by integration of the peaks as plotted and a function of surfactant concentration in the cell as shown in the figure below.

The CMC was evaluated at the surfactant concentration in which the enthalpy of dilution versus total surfactant concentration curve showed an inflection. It is also precisely determined as the peak maximum of the first-derivative of the reaction heat with respect to total surfactant concentration[3].

The difference in heat between the plateaux gives ΔH_{mic} (which is equal and opposite to the enthalpy of demicellization[3], ΔH_{demic}) as shown in figure 5.2.

For an accurate determination of ΔH_{mic}, the monomer concentration in the syringe has to be taken into account, the contributions from the heat of dilution of monomer ($\Delta H_{mon,dil}$) and micelles($\Delta H_{mic,dil}$) is also subtracted from the observed enthalpy $\Delta H_{obs.}$

The $\Delta H_{mic,\ dil}$ is due to the dilution of micelles and it corresponds to the enthalpy values of the last few injections[3, 9].

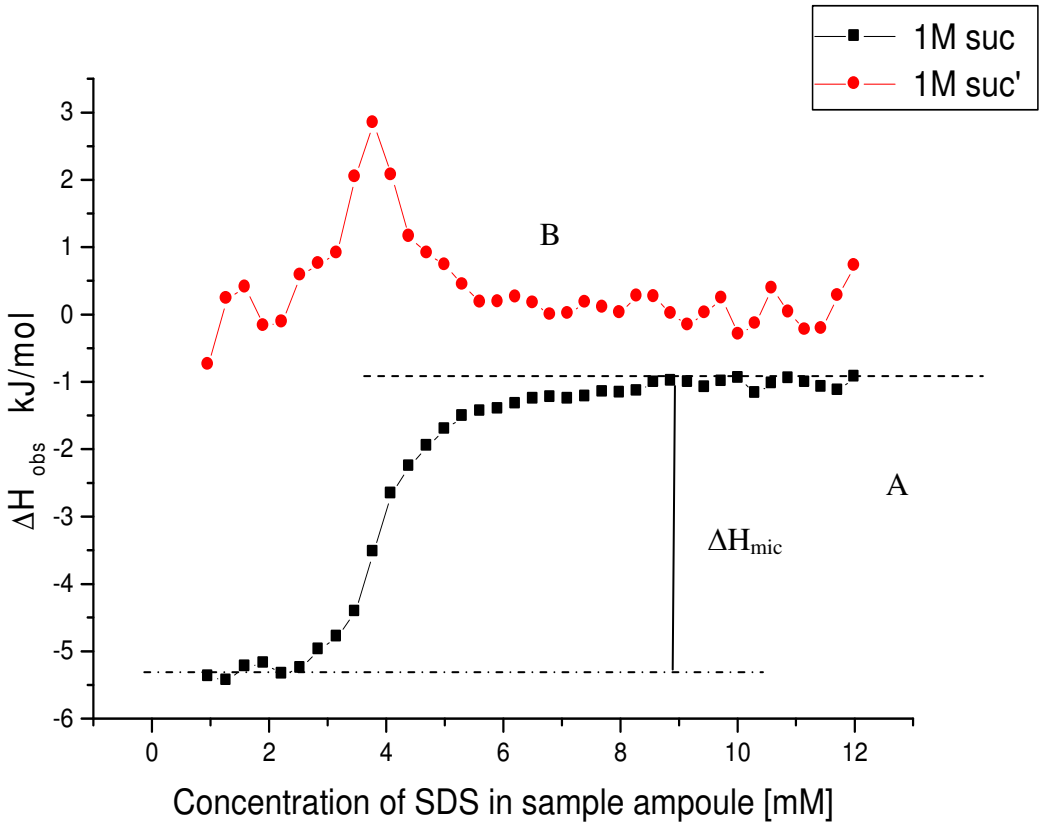

Figure 5.2. Enthalpograms observed for dilution of SDS into 1M sucrose solution A (shown in ■) and the first derivative plot (B) of A (as shown in ◆).

According to the pseudophase description of micelle formation, the Gibbs free energy of micellization (ΔG_{mic}) for the transfer of 1 mol of monomer to the micellar state for ionic surfactants is given by[13]

$$\Delta G_{mic} = RT \ln X_{CMC}$$

Where X_{CMC} is the CMC in mole fraction units

The entropy change ΔS_{mic} was calculated using the Gibbs-Helmholtz equation

$$T\Delta S = \Delta H_{mic} - \Delta G_{mic}$$

Thus a full thermodynamic characterisation of SDS demicellization in water and in the sugar solutions is presented in the tables 5.1.

Table 5.1. CMC and thermodynamic parameters ΔH_{mic} , ΔS_{mic} , $T\Delta S_{mic}$, ΔG_{mic} , and ΔG_{demic} of SDS in water and in the presence of various sugar at different concentrations.

		CMC [mM] ±0.4	ΔH_{mic} kJ.mol^{-1} ±0.1	ΔG_{mic} kJ.mol^{-1} ±0.4	ΔS_{mic} kJ.mol^{-1}.K^{-1} ±0.1	$T\Delta S_{mic}$ kJ.mol^{-1} ±0.1	ΔG_{demic} kJ.mol^{-1} ±0.4
	Water	**7.43**	**0.7**	**-22.10**	**0.076**	**22.81**	**22.10**
	Sugar Concentration [M]						
Sucrose	**0.1**	**6.01**	**0.95**	**-22.64**	**0.079**	**23.59**	**22.64**
	0.25	**6.65**	**1.60**	**-22.39**	**0.080**	**23.99**	**22.39**
	0.5	**6.81**	**2.50**	**-22.34**	**0.083**	**24.84**	**22.34**
	1	**3.81**	**4.27**	**-23.81**	**0.094**	**28.08**	**23.81**
Trehalose	**0.1**	**5.91**	**0.80**	**-22.68**	**0.078**	**23.48**	**22.68**
	0.25	**5.92**	**0.95**	**-22.68**	**0.079**	**23.63**	**22.68**
	0.5	**5.78**	**1.77**	**-22.75**	**0.082**	**24.52**	**22.75**
	1	**5.30**	**4.77**	**-22.99**	**0.093**	**27.76**	**22.99**
Maltose	**0.25**	**5.61**	**1.60**	**-22.81**	**0.081**	**24.41**	**22.81**
	0.5	**6.45**	**3.32**	**-22.48**	**0.087**	**25.80**	**22.48**
	1	**4.10**	**5.45**	**-23.62**	**0.098**	**29.07**	**23.62**
Raffinose	**0.1**	**5.91**	**0.89**	**-22.68**	**0.079**	**23.57**	**22.68**
	0.25	**5.96**	**2.25**	**-22.66**	**0.084**	**24.91**	**22.66**
	0.3	**5.20**	**2.28**	**-23.00**	**0.085**	**25.28**	**23.00**
Mannitol	**0.1**	**5.01**	**0.48**	**-23.10**	**0.079**	**23.58**	**23.10**
	0.25	**6.81**	**0.95**	**-22.33**	**0.078**	**23.28**	**22.33**
	0.5	**4.70**	**2.13**	**-23.26**	**0.085**	**25.39**	**23.26**

The results show a decrease in the CMC of SDS in sugar solutions in comparison to water, significant decrease in the CMC of SDS at 1M sugar concentration was observed for maltose, sucrose, and trehalose. Decrease in CMC values obtained by ITC in the presence salts has also been reported by other investigators [9-11]. An explanation given for the reduction in CMC is that additional salt increases the counterions concentration thereby increasing the counterions binding. Consequently, the electrostatic repulsions between the head groups of the ionic surfactant molecules are reduced. Thus the micellization process becomes thermodynamically more favourable in the presence of counterions, as observed by lower ΔG_{mic} values [14]. Likewise decreases in the values of CMC obtained in the presence of additional sugars suggest the sugars might be inducing counterions binding and inhibiting

counterions dissociation. This is supported by conductivity studies carried out in chapter four which showed reduced conductance values in the presence of sugars suggesting a reduction in the degree of dissociation and mobility of ionic groups. Increased counterions binding would therefore lead to a reduction in the electrostatic repulsions between the surfactant monomers, hence favouring micellization. These results were comparable with results obtained by tensiometric investigation.

However the CMC values obtained by ITC were different from the CMC results obtained by tensiometry. The CMC obtained for tensiometry are lower than CMC obtained by calorimetry, this is probably due to dodecanol in SDS which causes a surface tension minimum.

Furthermore, calculating the ΔG_{mic} for SDS micellization in water and in sugar solution reveals that micellization is more favourable in sugar solution ($\Delta G = -23.81$ kJ mol^{-1} for 1M sucrose) than in water ($\Delta G = -22.10$ kJ mol^{-1}).

The more negative ΔG_{mic} indicates a larger driving force for micellization and a greater propensity to form micelles. As a result, the aggregation process is thermodynamically favoured and more spontaneous in sugar solutions. This suggests that there is a higher free energy penalty associated with hydrocarbon chain exposure to sugar solutions as compared with water.

The results show that in the presence of sugars the ΔH_{mic} was increased. A further increase was observed with increasing sugar concentration (0.1M to 1.0M). The micellization enthalpy of SDS becomes more positive as presented in figure 5.3. A positive value for enthalpy change indicates that micellization of SDS is endothermic.

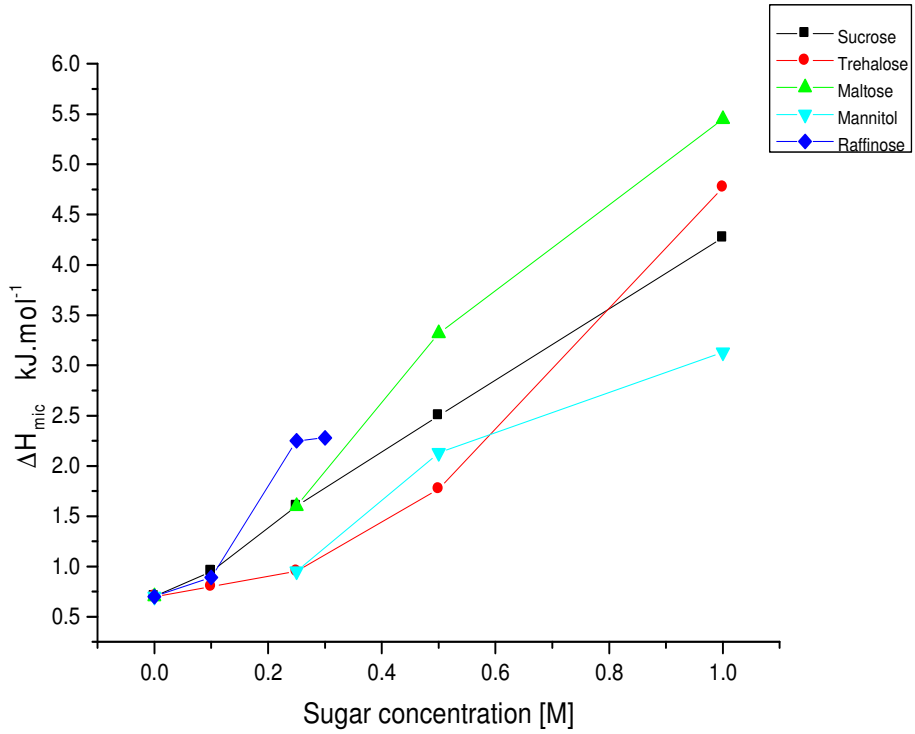

Figure 5.3. The effect of increasing sugar concentration on the ΔH_{mic} for various sugars studied.

Since the ΔH_{demic} is equal and opposite to the ΔH_{mic} , the ΔH_{demic} of SDS would be exothermic.

The results obtained for the ΔS_{mic} in water solution gives a positive value and in the presence of sugar solutions the value becomes more positive as presented in table 5.1.

At 1.0M sucrose the ΔS_{mic} is 0.094 $kJ.mol^{-1}K^{-1}$ in contrast to ΔS_{mic} in water which is 0.076 $kJ.mol^{-1}\ K^{-1}$.

Hydrophobic interactions have been described as the major driving forces for micelle formation; during the micellization process water molecules in hydration shells around the hydrophobic parts of the monomeric amphiphiles are released. This is reflected by positive entropy values of micellization[15, 16]. The results show the presence of sugars increase the entropy values in a concentration dependent manner. This suggests that sugars would enhance the hydrophobically driven aggregation of surfactant monomers into micelles.

In addition to hydrophobic interactions, other forces such as attractive and repulsive electrostatic interactions between their hydrophilic charged head groups, and with and

between the counterions also contribute to the formation of micelles[16]. Furthermore, hydrogen bonding is said to occur between water and some surfactant molecules as evidenced by studies of the ionic surfactant benzyl (2-acylaminoethyl) dimethyl ammonium chloride which suggest that the presence of amide group may form direct and/or water mediated intermolecular hydrogen bonds[17]. In addition, a recent study on the effect of propanediol-1, 2 on the micellization of F127 surfactant have shown that inter-molecular hydrogen bonds occur between water and propanediol-1, 2[18].

The increase in enthalpy in the presence of sugar would therefore suggests that more bonds are being formed due to contributions from a number of events such as polar interactions (surfactant head group and sugar, sugar –water hydrogen bonding) and possible non-polar interactions (surfactant chain–surfactant chain, and surfactant chain–sugar)[8, 19]. It has been suggested that the hydrogen bonds could also be responsible for a lower degree of freedom and in turn, a lower value of ΔS_{mic} [18]. This is a possible explanation of lower values of ΔS_{mic} obtained in comparison to the enthalpy values in this study.

The results show that sugars can modify the structure of the solvent by forming hydrogen bonds with the water molecules, thereby contributing to micelle formation and thus establishing a relationship between the solvent structure and the stability of micelles. This is comparable to studies which have shown that the stabilizing effect of sugars is due to their effect on the structure and properties of water , which in turn may enhance hydrophobic interactions in proteins[20].

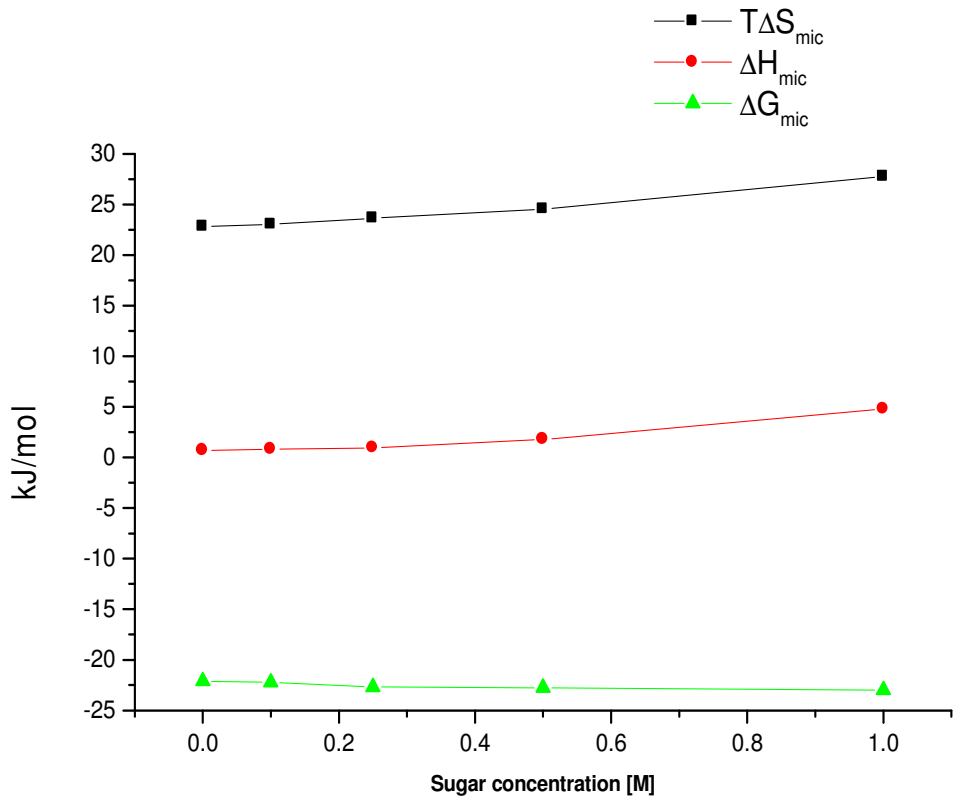

Figure 5.4. Entropy change of micellization (ΔS_{mic}) , Enthalpy change of micellization (ΔH_{mic}) and Gibbs free energy change of micellization (ΔG_{mic}) of SDS as a function of sugar (trehalose) concentration. The graph shows concentration dependence for these parameters.

From Figure 5.4, a dependence on sugar concentration is observed for the changes in entropy, enthalpy and Gibbs free energy. The entropy contribution usually dominates the micellization process in aqueous surfactant solutions, with the enthalpy playing a minor role ($T\Delta S_{mic} > \Delta H_{mic}$). The results obtained indicate a higher entropic contribution in comparison to the enthalpic contribution during micelle formation. The presence of sugar is shown to enhance both entropic and enthalpic effects.

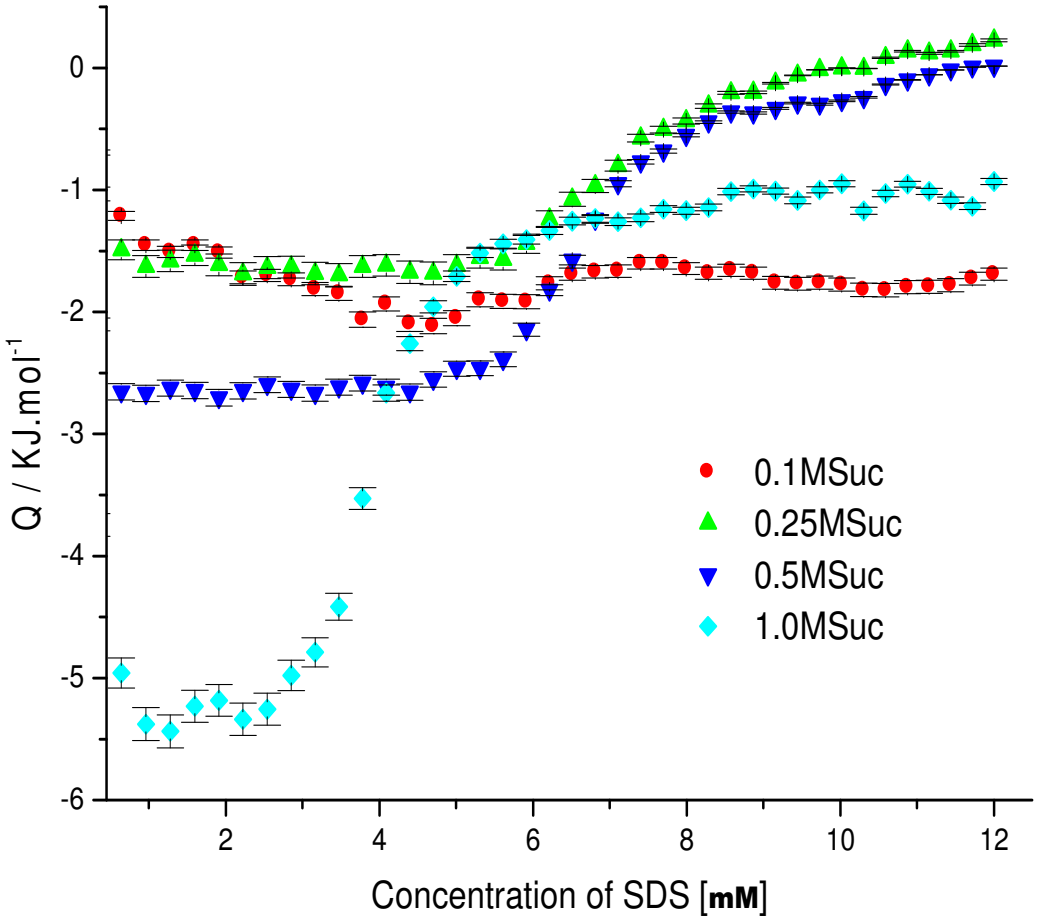

Figure 5.5. Plots of reaction enthalpy per injection vs. final concentration of surfactant solution in the calorimetric ampoule, as observed due to the demicellization of a micellar SDS solution into sucrose solution. The graph shows the effect increasing sugar concentration on the CMC and ΔH_{mic} of SDS.

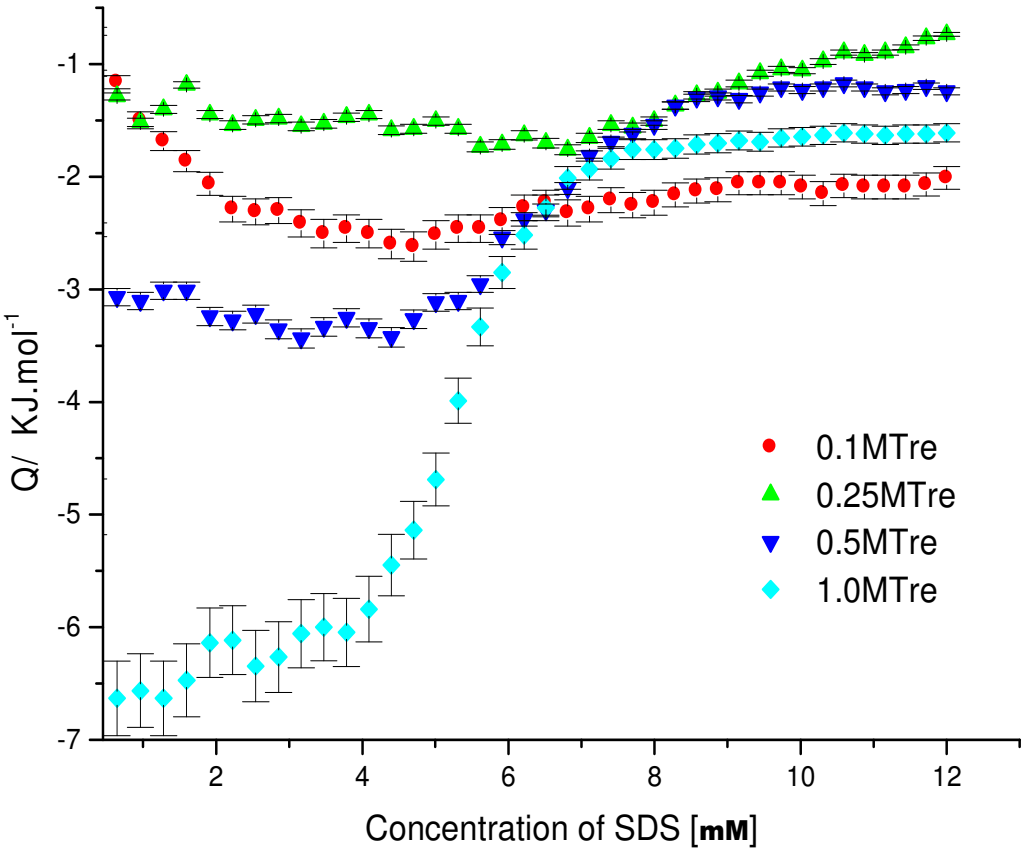

Figure 5.6. Plots of reaction enthalpy per injection vs. final concentration of surfactant solution in the calorimetric ampoule, as observed due to the demicellization of a micellar SDS solution into trehalose solution. The graph shows the effect increasing sugar concentration on the CMC and ΔH_{mic} of SDS.

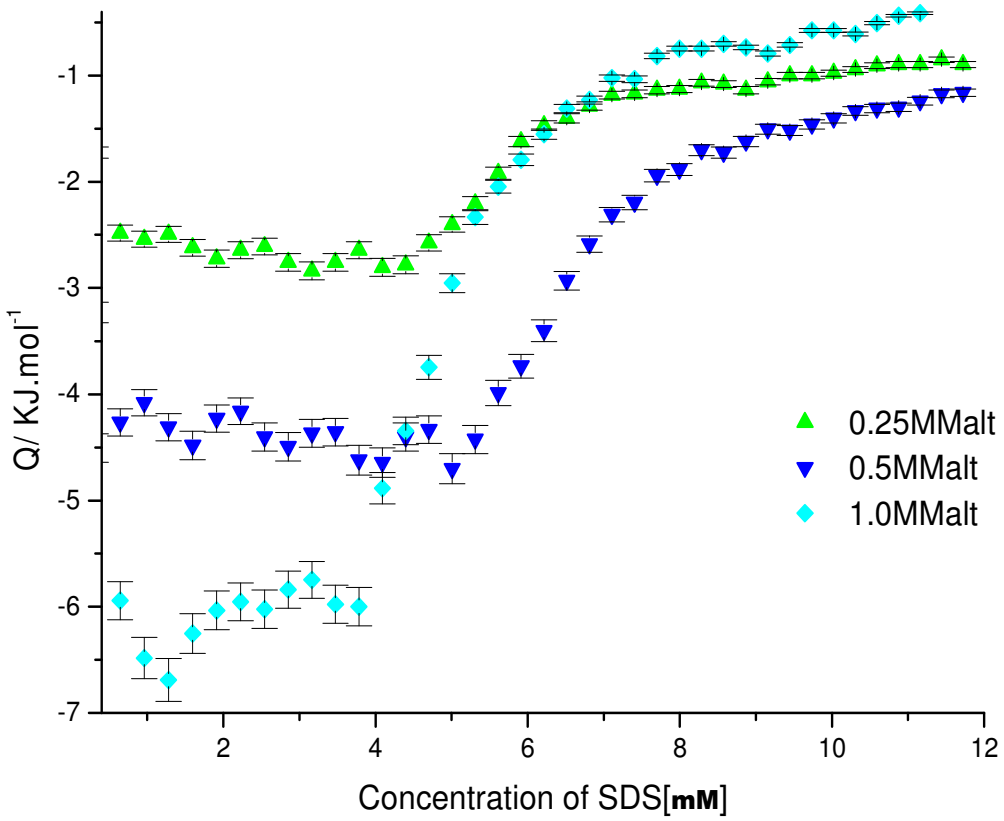

Figure 5.7. Plots of reaction enthalpy per injection vs. final concentration of surfactant solution in the calorimetric ampoule, as observed due to the demicellization of a micellar SDS solution into maltose solution. The graph shows the effect increasing sugar concentration on the CMC and ΔH_{mic} of SDS.

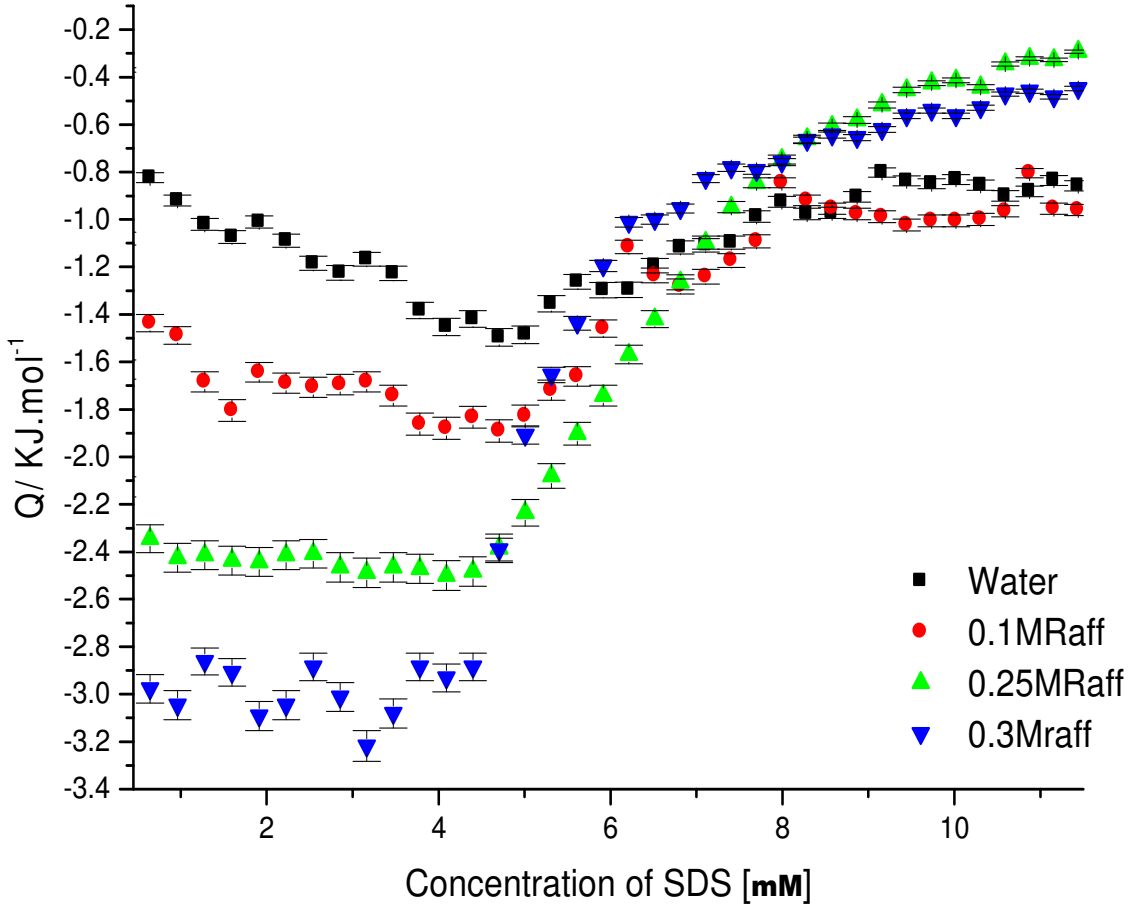

Figure 5.8. Plots of reaction enthalpy per injection vs. final concentration of surfactant solution in the calorimetric ampoule, as observed due to the demicellization of a micellar SDS solution into raffinose solution. The graph shows the effect increasing sugar concentration on the CMC and ΔH_{mic} of SDS.

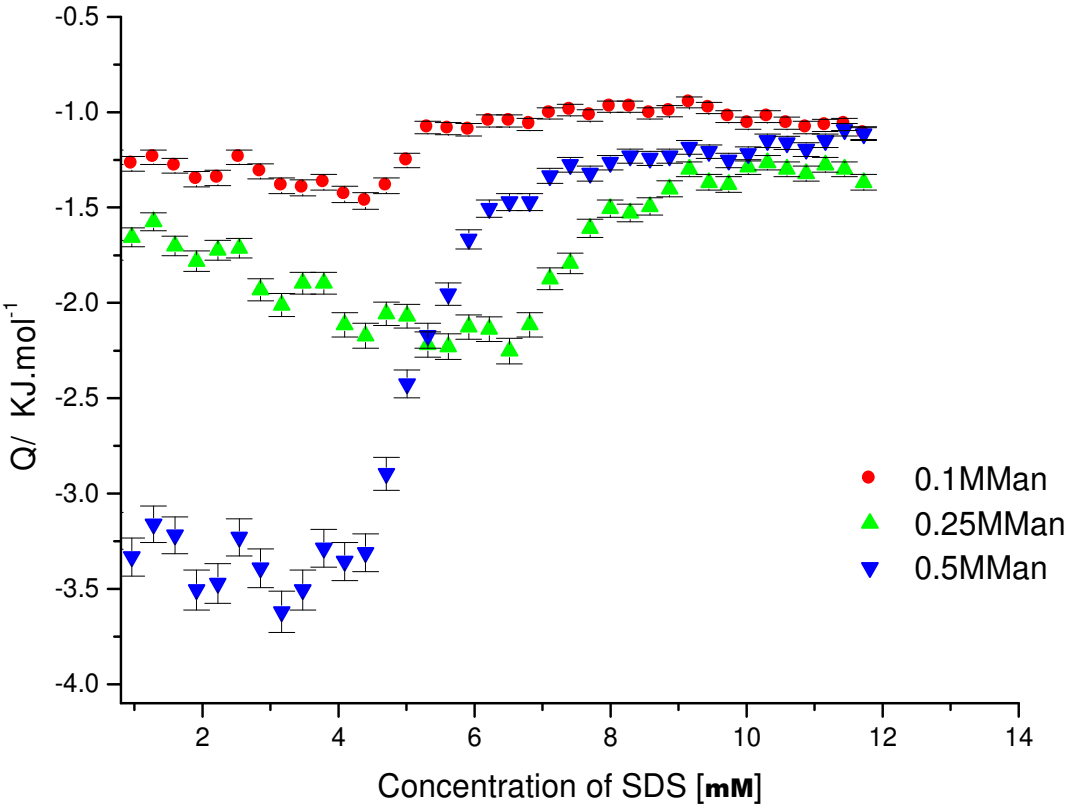

Figure 5.9. Plots of reaction enthalpy per injection vs. final concentration of surfactant solution in the calorimetric ampoule, as observed due to the demicellization of a micellar SDS solution into mannitol solution. The graph shows the effect increasing sugar concentration on the CMC and ΔH_{mic} of SDS.

The scatter in the data at high sugar concentrations might be due to the increase in viscosity of the solutions (figures 5.5 to 5.9). Increase in the viscosity of the solutions as the sugar concentration goes up suggests that heat transport to the sensors may become more difficult. This increase in viscosity also supports the notion of greater degrees of intermolecular interaction presumably through hydrogen bonding.

5.4. Concluding Remarks

The demicellization of SDS in water and the influence of sugar at different concentration were studied by ITC. Experiments allowed the direct determination of the CMC values, and ΔH_{mic} from which other thermodynamic parameters were calculated. The results showed that the CMC of SDS was reduced at higher concentrations of sugar.

The presence of sugar tends to affect the energertics of micellization of SDS in a concentration dependent manner. A decrease in the value of ΔG_{mic} indicates a larger driving force for micellization suggesting an unfavourable free energy of demicellization or exposure of hydrophobic groups to aqueous solutions. The entropy of micellization was increased in the presence of sugar which is indicative of an enhancement of hydrophobically driven micellization process. The increase in enthalpy in the presence of sugar would therefore suggests more possible hydrogen bonding between surfactant head group and sugar, this also indicates the change of solvent composition by formation of hydrogen bonding between sugar and water. ITC results thus support findings of tensiometric studies that sugars exhibit their effect on both the head and tail groups. Therefore, sugars would stabilize biological structures by a combined effect of an unfavourable free energy of exposure of hydrophobic groups to aqueous solutions and their interactions with the polar groups of biomolecules.

A better understanding of sugar –surfactant interactions obtained from ITC thermodynamic data provide an opportunity to understand the mechanism of protein stabilization by sugars.

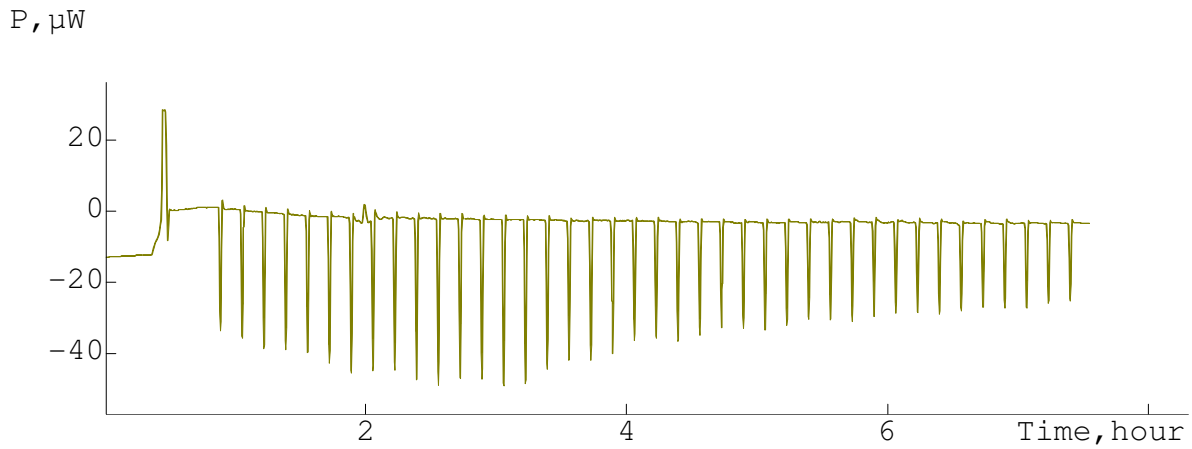

Figure 5.10. ITC titration curve showing heat flow (power in µW) versus time , of 174mM micellar SDS in steps of 40 x 5 µl (0.1M trehalose solution).

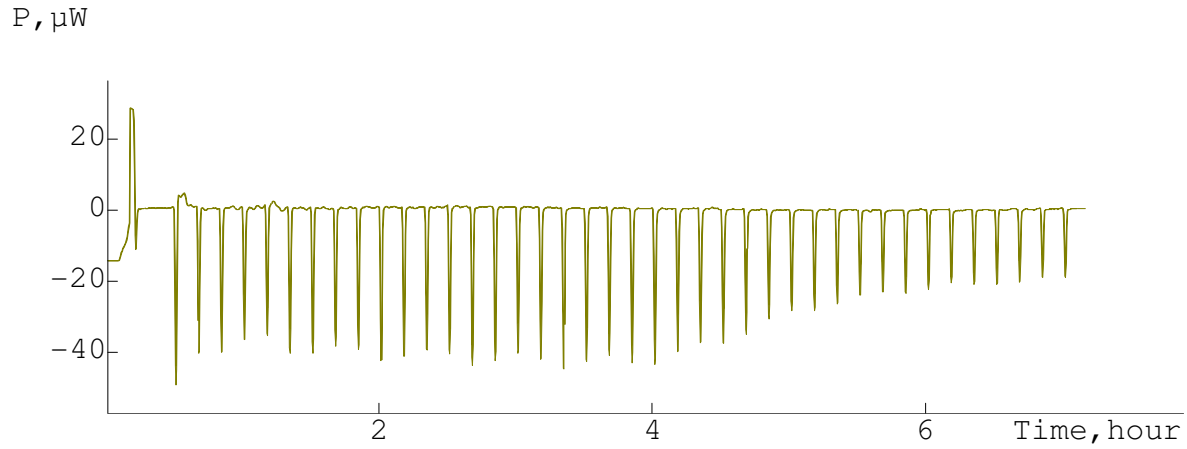

Figure 5.11. ITC titration curve showing heat flow (power in µW) versus time , of 174mM micellar SDS in steps of 40 x 5 µl (0.25M trehalose solution).

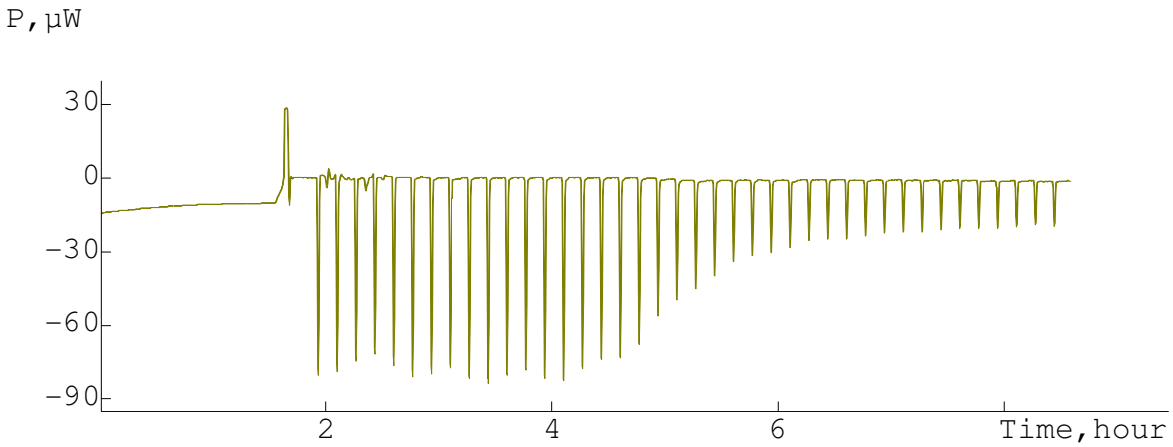

Figure 5.12. ITC titration curve showing heat flow (power in µW) versus time , of 174mM micellar SDS in steps of 40 x 5 µl (0.5M trehalose solution).

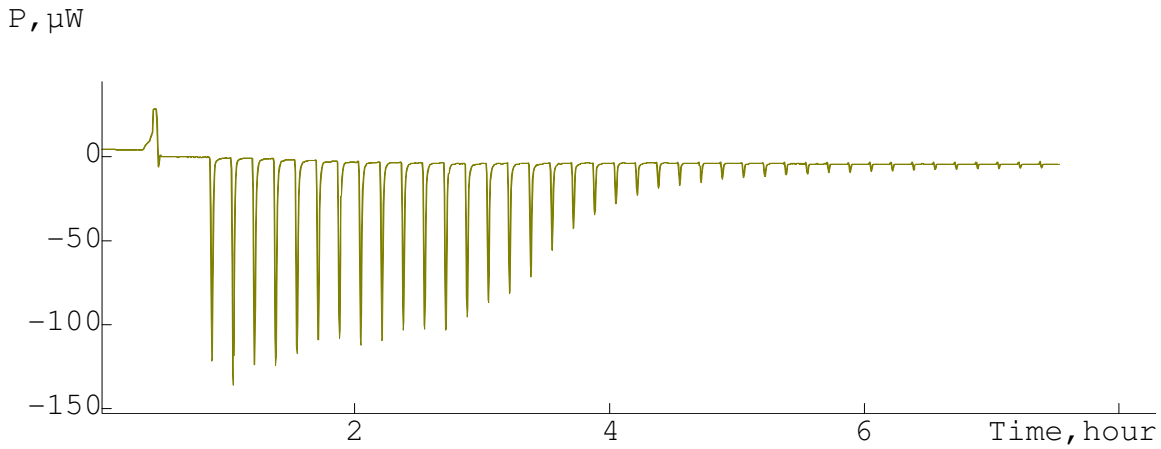

Figure 5.13. ITC titration curve showing heat flow (power in µW) versus time , of 174mM micellar SDS in steps of 40 x 5 µl (1M trehalose solution).

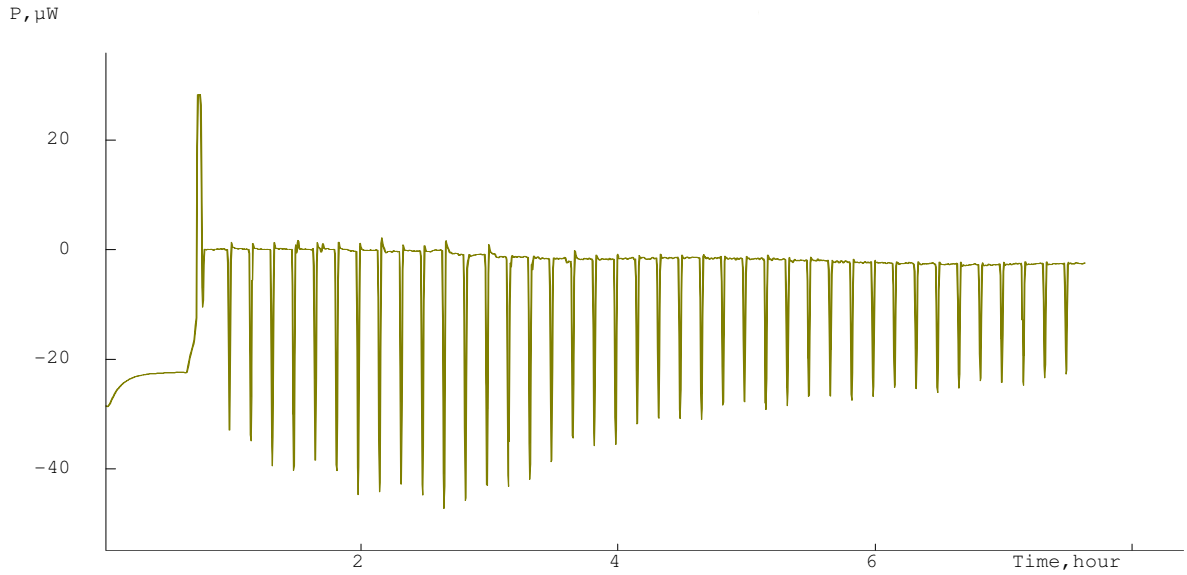

Figure 5.14. ITC titration curve showing heat flow (power in µW) versus time, of 174mM micellar SDS in steps of 40 x 5 µl (0.1M Sucrose solution.)

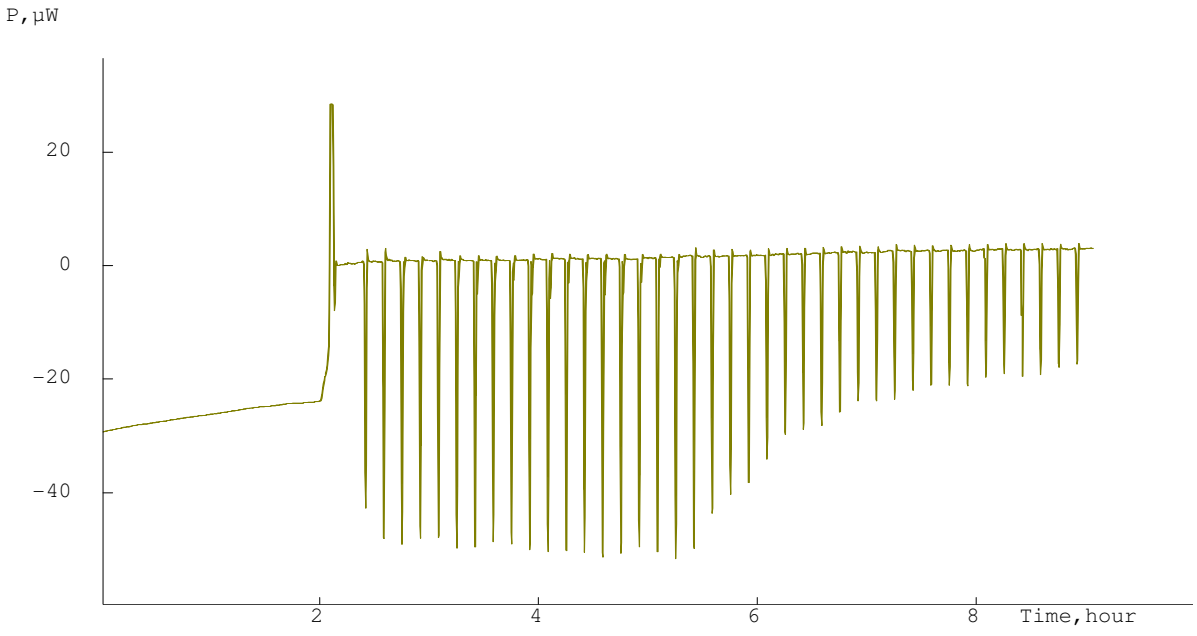

Figure 5.15. ITC titration curve showing heat flow (power in µW) versus time , of 174mM micellar SDS in steps of 40 x 5 µl (0.25M Sucrose solution).

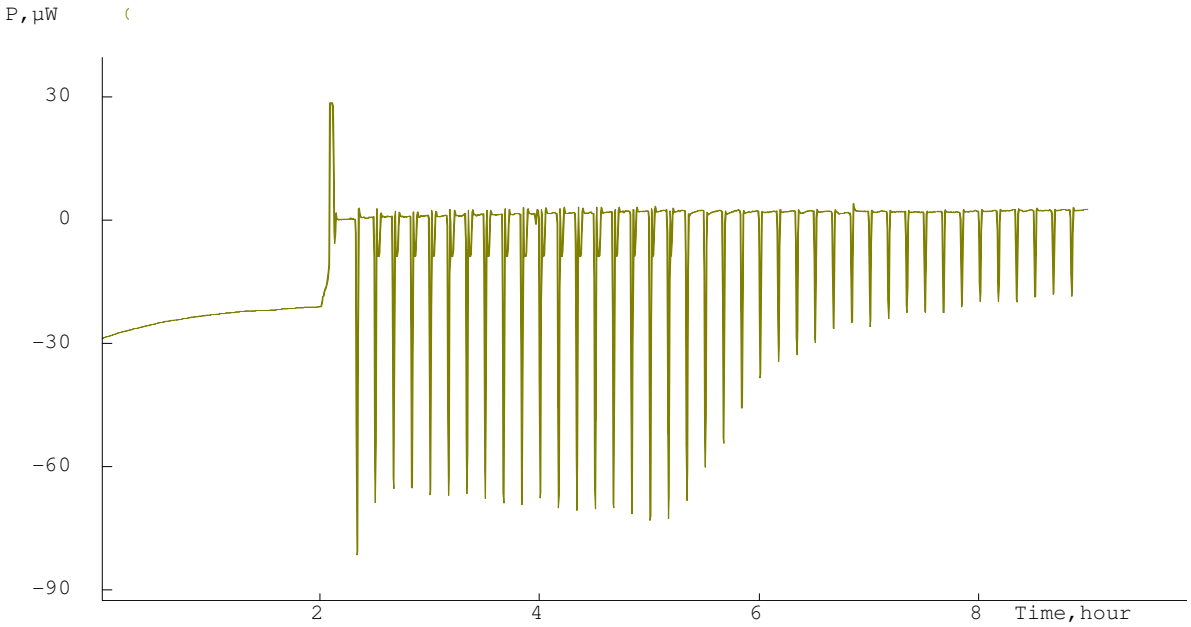

Figure 5.16. ITC titration curve showing heat flow (power in µW) versus time , of 174mM micellar SDS in steps of 40 x 5 µl (0.5M Sucrose solution).

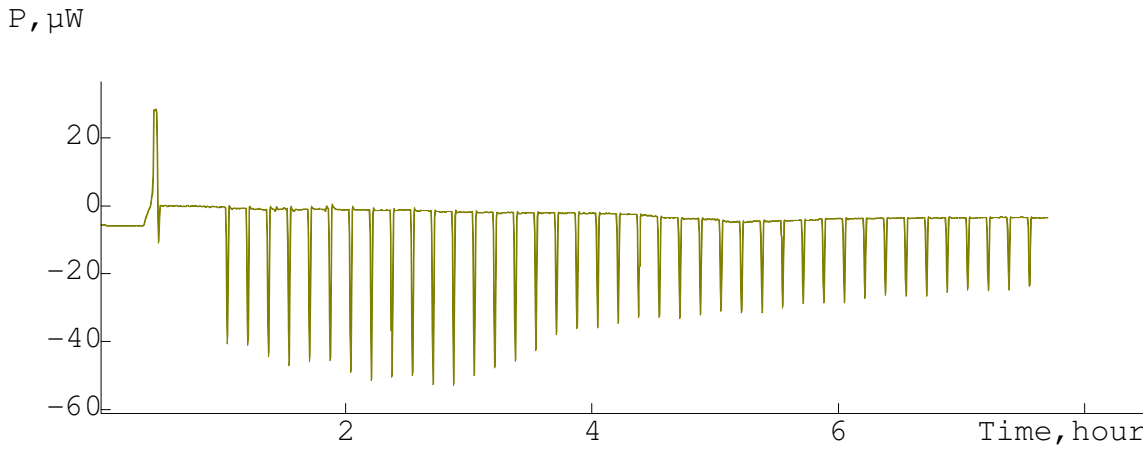

Figure 5.17. ITC titration curve showing heat flow (power in µW) versus time , of 174mM micellar SDS in steps of 40 x 5 µl (0.1M maltose solution).

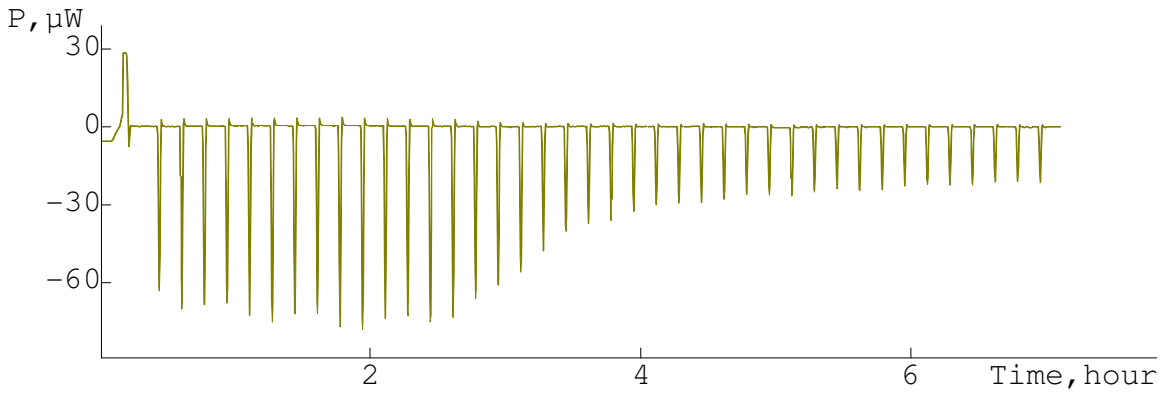

Figure 5.18. ITC titration curve showing heat flow (power in µW) versus time , of 174mM micellar SDS in steps of 40 x 5 µl (0.25M maltose solution).

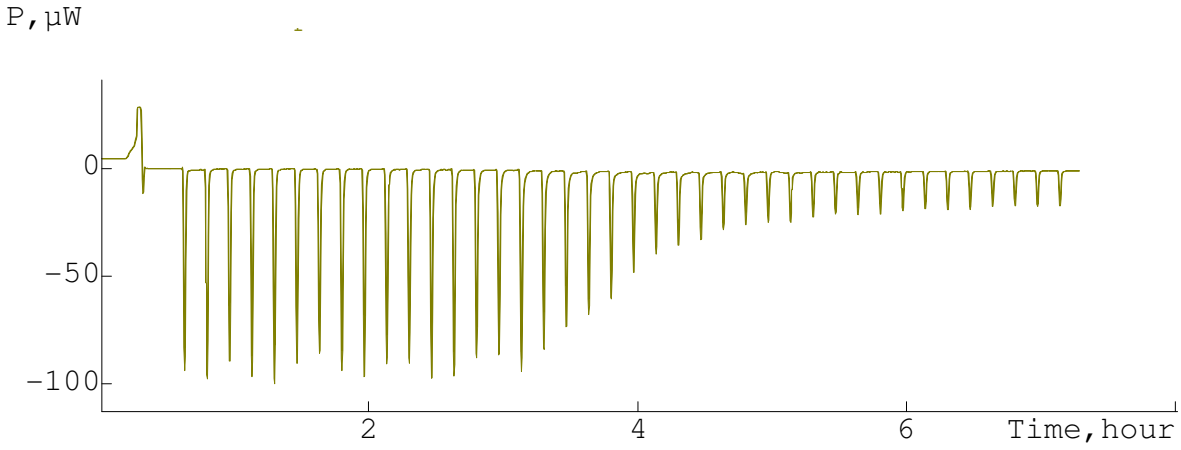

Figure 5.19. ITC titration curve showing heat flow (power in µW) versus time , of 174mM micellar SDS in steps of 40 x 5 µl (0.5M maltose solution).

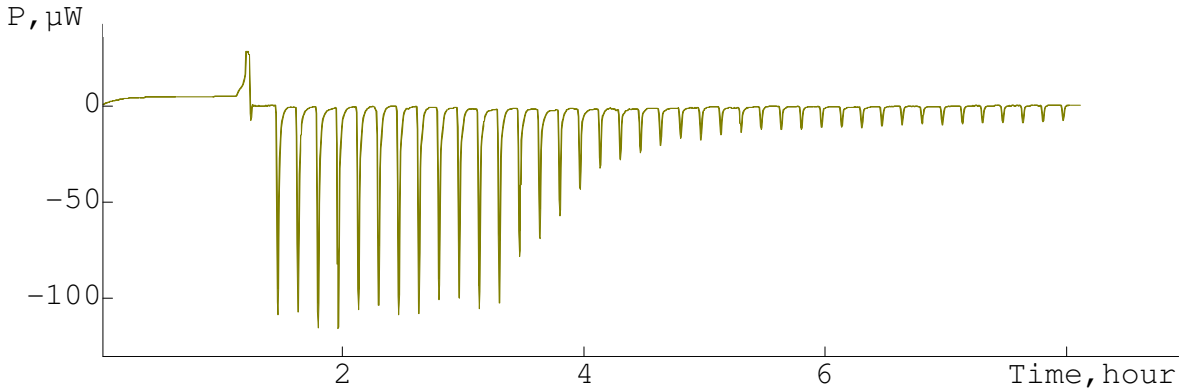

Figure 5.20. ITC titration curve showing heat flow (power in µW) versus time, of 174mM micellar SDS in steps of 40 x 5 µl (1M maltose solution).

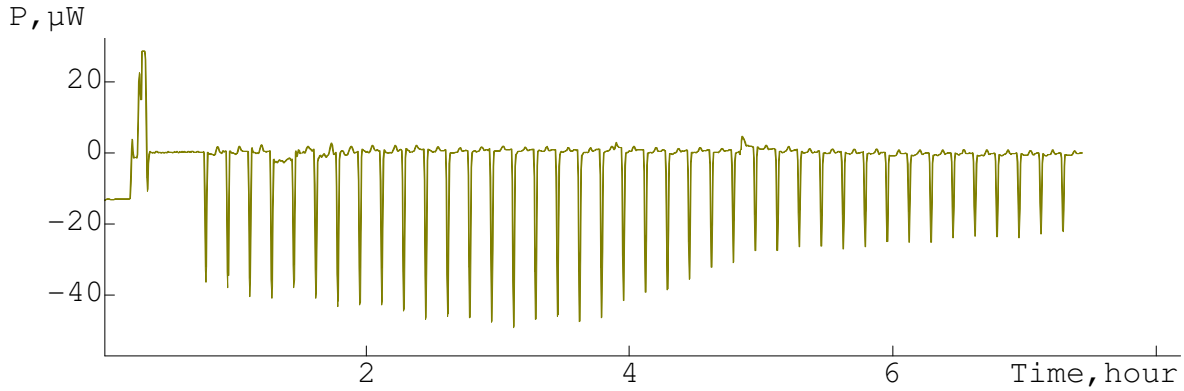

Figure 5.21. ITC titration curve showing heat flow (power in µW) versus time , of 174mM micellar SDS in steps of 40 x 5 µl (0.1 M raffinose solution).

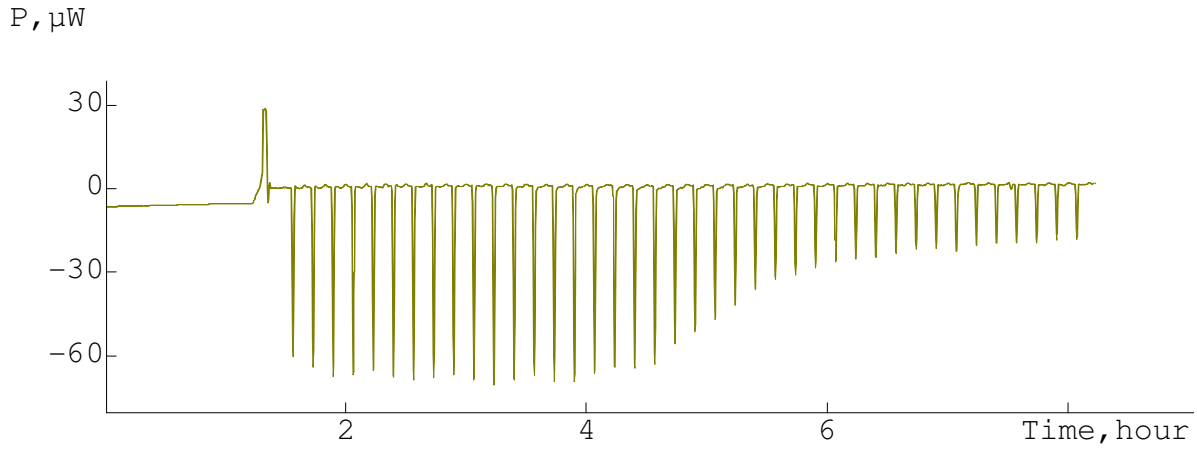

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Figure 5.22. ITC titration curve showing heat flow (power in µW) versus time , of 174mM micellar SDS in steps of 40 x 5 µl (0.25M raffinose solution).

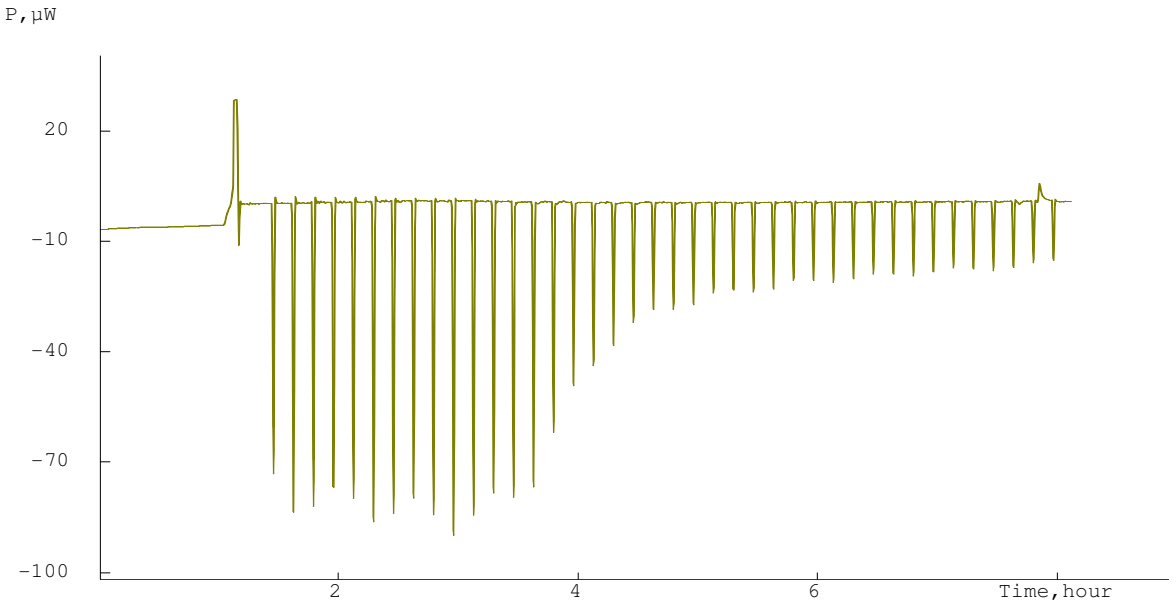

Figure 5.23. ITC titration curve showing heat flow (power in µW) versus time , of 174mM micellar SDS in steps of 40 x 5 µl (0.3M raffinose solution).

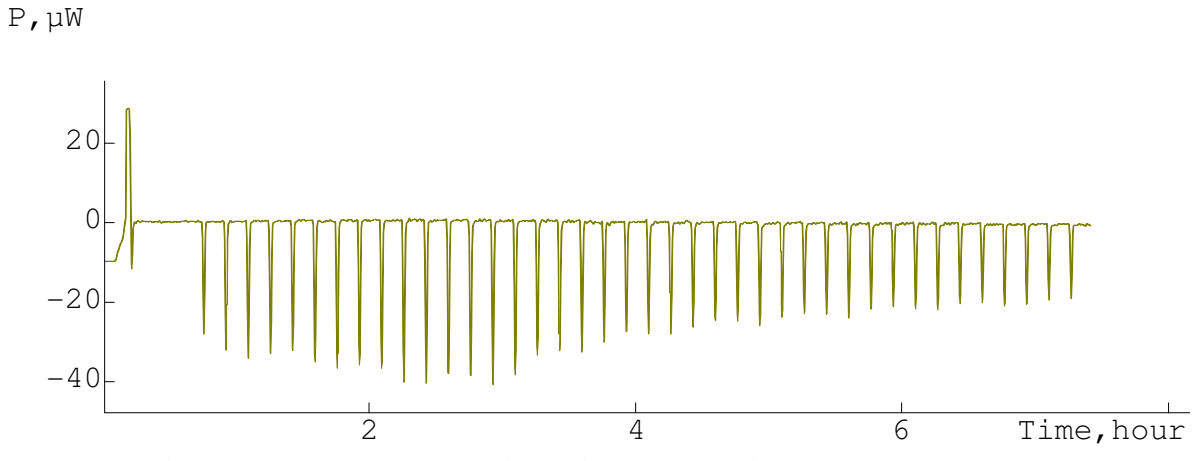

Figure 5.24. ITC titration curve showing heat flow (power in µW) versus time, of 174mM micellar SDS in steps of 40 x 5 µl (0.1M mannitol solution).

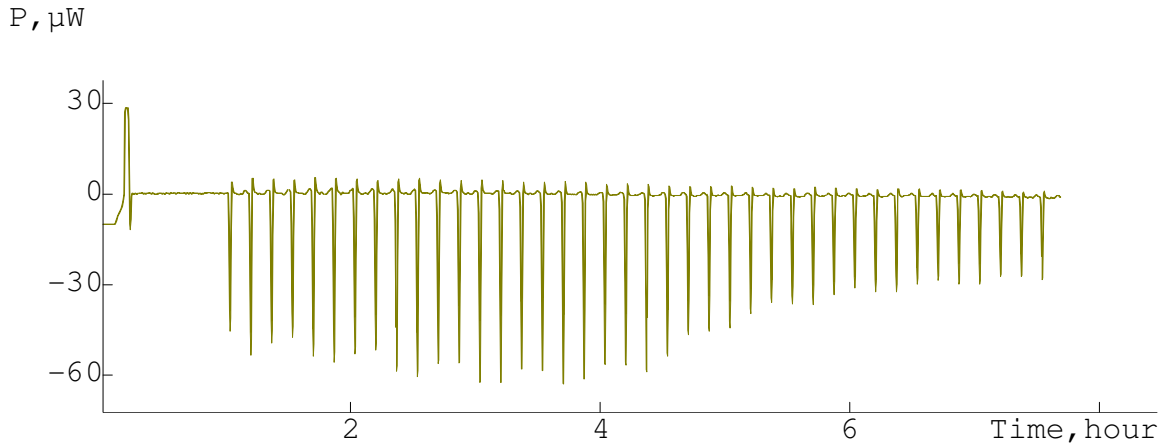

Figure 5.25. ITC titration curve showing heat flow (power in µW) versus time, of 174mM micellar SDS in steps of 40 x 5 µl (0.25M mannitol solution).

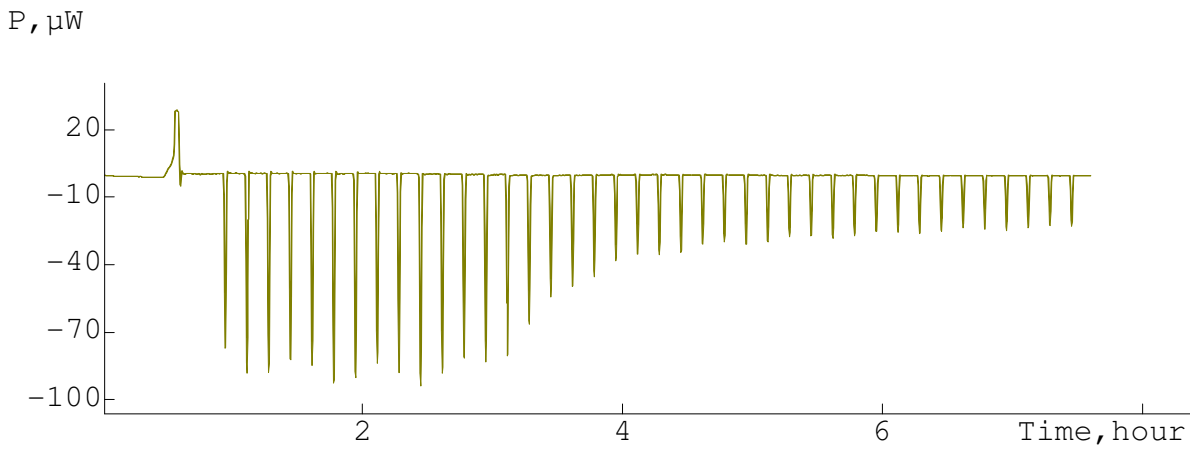

Figure 5.26. ITC titration curve showing heat flow (power in µW) versus time, of 174mM micellar SDS in steps of 40 x 5 µl (0.5M mannitol solution).

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Chapter 6: Differential scanning calorimetry (DSC) studies of Ribonuclease A in aqueous sugar solutions

6.1. Introduction

The availability of highly sensitive differential calorimetry (HSDSC) instrumentation permits the study and monitoring of the thermal stability of biopolymers such as proteins and nucleic acids in dilute solutions[1-5]. HSDSC is thus routinely applied in liquid biopharmaceutical formulations for optimization and stability studies[5-9].

The native conformation of proteins are only slightly stable, and in most cases they are easily denatured under various environmental stresses such as an increase or sometimes decrease in temperature and changes in pH and ionic strength[10, 11]. Proteins are also denatured by addition of urea, guanidium hydrochloride (GdnHCl) and chaotropic salts of the following ions SCN^- , I^- , Cl^-, ClO_4^-, Br^- , NO_3^- [12-15].

In addition, freeze drying or freeze thawing of protein formulations for long term storage is a potential source of denaturation due to the exposure of the proteins to high/low temperature and pressure which is inherent in the process [16-21].

Therefore one strategy widely used in the biopharmaceutical industry to prevent denaturation as well as aggregation of proteins is the inclusion of additives such as sugars / polyols, certain amino acids, and some amine in formulations to stabilize these molecules [2, 22-25]. Studies have reported that only sugars such as disaccharides effectively protect proteins from drying and freeze drying damage[19, 26, 27]. These properties of sugars have allowed the development of strategies for the preservation and long term storage of biotherapeutics. As recently reported in the literature, a mixture of sucrose and trehalose was shown to stabilize vaccines at tropical temperatures[28].

HSDSC have been used in this study to investigate the changes in the denaturation temperature(T_m) of Ribonuclease *A* (RNase *A*) in the presence of various sugars. The sugars

that were studied include the mannitol, sucrose, trehalose, and raffinose. The effect of the aforementioned sugars on the T_m of RNase *A* has thus been presented and discussed.

RNase *A* is an important endonuclease responsible for the cleaving of RNA[29]. The desirable properties of RNase *A* make it a protein of choice for stability studies; it is small, stable, and easily purified, and has been widely studied as a model protein for the development of spectroscopic methods for assaying protein structure, which includes absorbance, circular dichroism/optical rotary dispersion, Raman, EPR and NMR spectroscopy[29].

6.2. Experimental

6.2.1. Materials

RNase *A* from bovine pancreas ≥ 90%, sucrose ≥ 99.5%, trehalose dihydrate ≥ 99.0%, raffinose pentahydrate ≥ 99.0% and mannitol > 99.5% were obtained from Sigma Aldrich UK.

Buffer: A 20mM sodium phosphate buffer was prepared from monobasic sodium phosphate and dibasic sodium phosphate[30].

6.2.2. Method

6.2.2.1. Protein preparation

A stock solution of 1mg/ml RNase solution was prepared by weighing to an accuracy of $\pm 1 \times 10^{-4}$g and dissolved in 20mM phosphate buffer (pH 7.0). Protein was used as received.

Mixture of RNase A- sugar solutions were then made by dissolving defined amounts of sugar by weight (weighing accuracy of $\pm 1 \times 10^{-4}$g) to portions of stock protein solution to give a series of samples containing 0.2-0.9M sugar.

Identical concentrations of sugar in buffer alone were also prepared for use as reference. All reference and sample solutions (Buffer, RNase *A*, and RNase *A*/sugar mixture) were degassed with a TA degassing station at 25 ^{o}C under (559mmHg) for 10mins prior to loading to the DSC to minimise gas bubble formation during scanning as the solubility of dissolved gases (O_2,NO_2) is decreased with increasing temperature. This process is necessary in order to prevent the acquisition of noisy heat capacity data as abrupt changes in heat capacity can result from bubble formation.

6.2.2.2. DSC

HSDSC experiments were performed with NanoDSC from TA Instruments (Newcastle, Delaware, USA) equipped with a capillary cell as described in larger detail in chapter 3. The sample and reference cells were loaded with degassed buffer solution , cells was pressurized to 3atm, and a buffer baseline scan was collected over a temperature range of 20 – 80^{o}C at a scan rate of 1^{o}C/min. The calorimeter was allowed to cool down and the sample cell was refilled with protein sample/protein sugar mixture, sample was scanned under conditions identical to the foregoing experimental setup. Scans were repeated to check for reversibility and reproducibility. Routine cleaning of the cells using SDS was carried out between separate sample scans in order to obtain reliable DSC data.

Raw data from the DSC were processed with the NanoAnaylse software for Nano DSC and it involved a subtraction of buffer baseline, concentration normalization.

6.3. Results/Discussion

The figure below is a DSC thermogram showing the excess heat capacity (after subtraction of instrumental baseline and concentration normalization) as a function of temperature. The thermogram exhibits a single symmetrical peak for the denaturation transition, also known as the mid-point temperature of the transition (T_m).

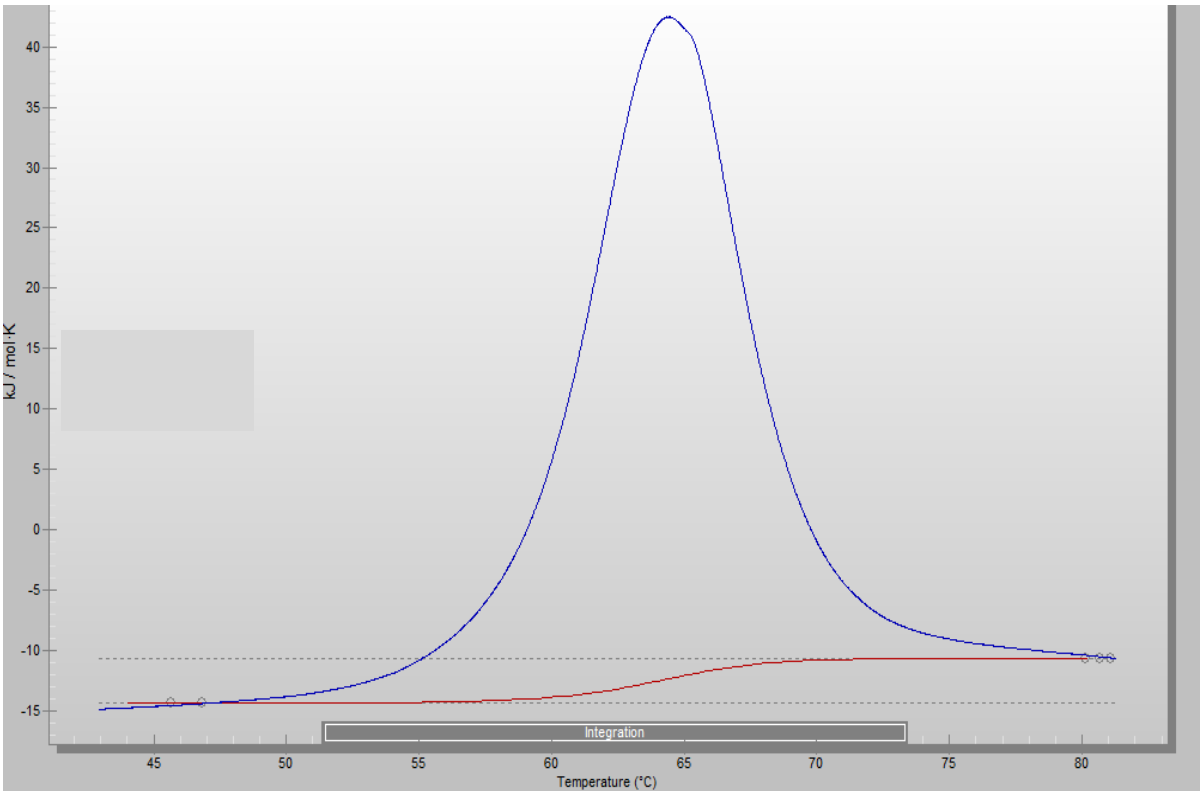

Figure 6.1. DSC thermogram showing the unfolding transition of RNase *A*.

The denaturation of native RNase *A* undergoes a cooperative two state unfolding transition that is characterized by a single transition as shown in figure 6.1. The unfolding temperature (T_m) of RNase *A* at pH 7 was observed at 63.27 ^oC this value compares with previously published literature values [31].

Figures 6.2 to 6.5 illustrates the effect of sugars, mannitol, sucrose, trehalose, and raffinose at increasing concentrations on the thermal transition temperature of RNase at pH 7. In the presence of sugars the T_m shifts to higher temperatures in a concentration dependent manner. The R^2 data obtained for the sugars showed good correlation coefficient values of approximately 0.97 for mannitol, 0.99 for trehalose and sucrose and 0.98 for raffinose. In DSC the resulting thermal transition ('melting') temperature is routinely used to evaluate the stability of the biopolymer and increases in T_m correlated with improved stability[23]. The use of DSC can be used evaluate effects of formulation additives, and thus evaluate relative stability of bioengineered proteins.

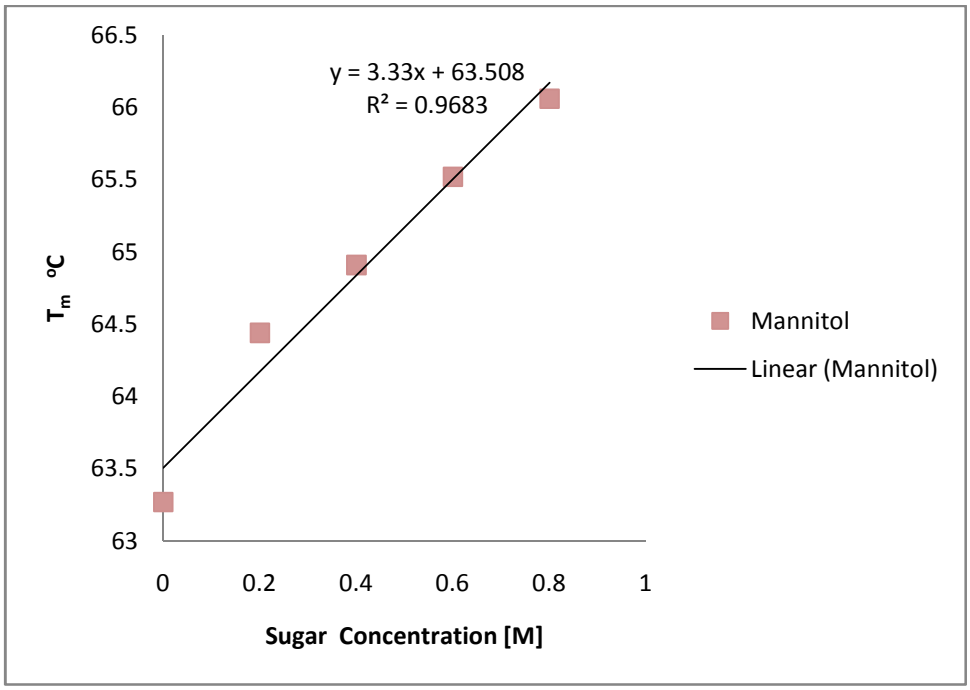

Figure 6.2. T_m of RNase *A* as a function of mannitol concentration.

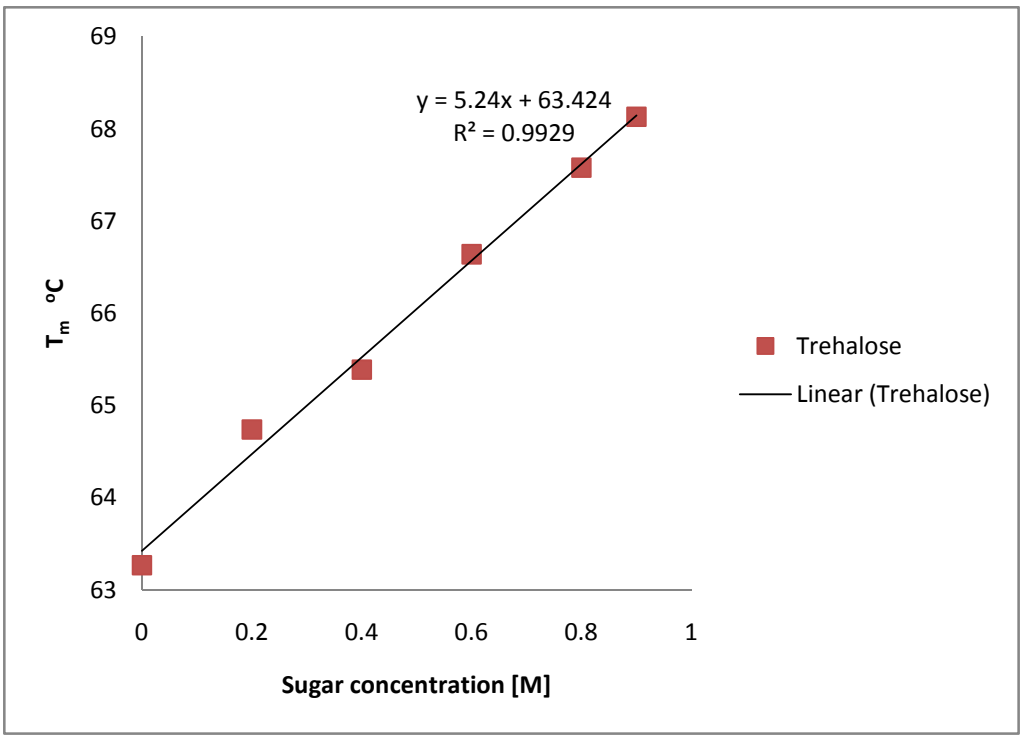

Figure 6.3. T_m of RNase *A* as a function of trehalose concentration.

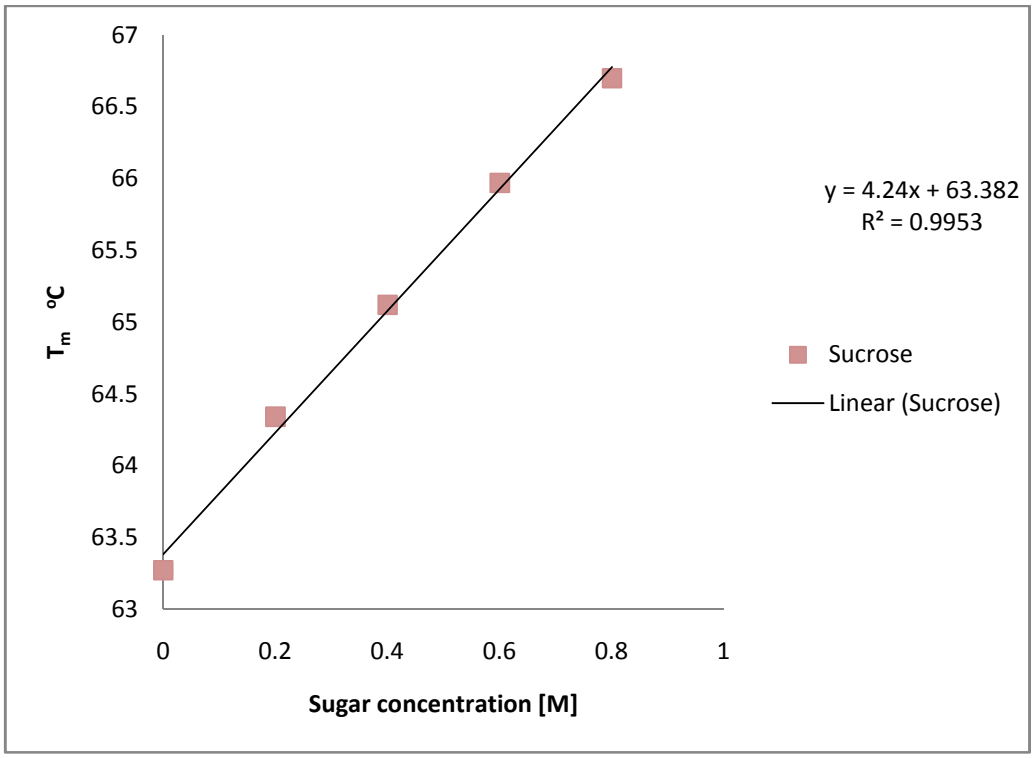

Figure 6.4. T_m of RNase *A* as a function of sucrose concentration.

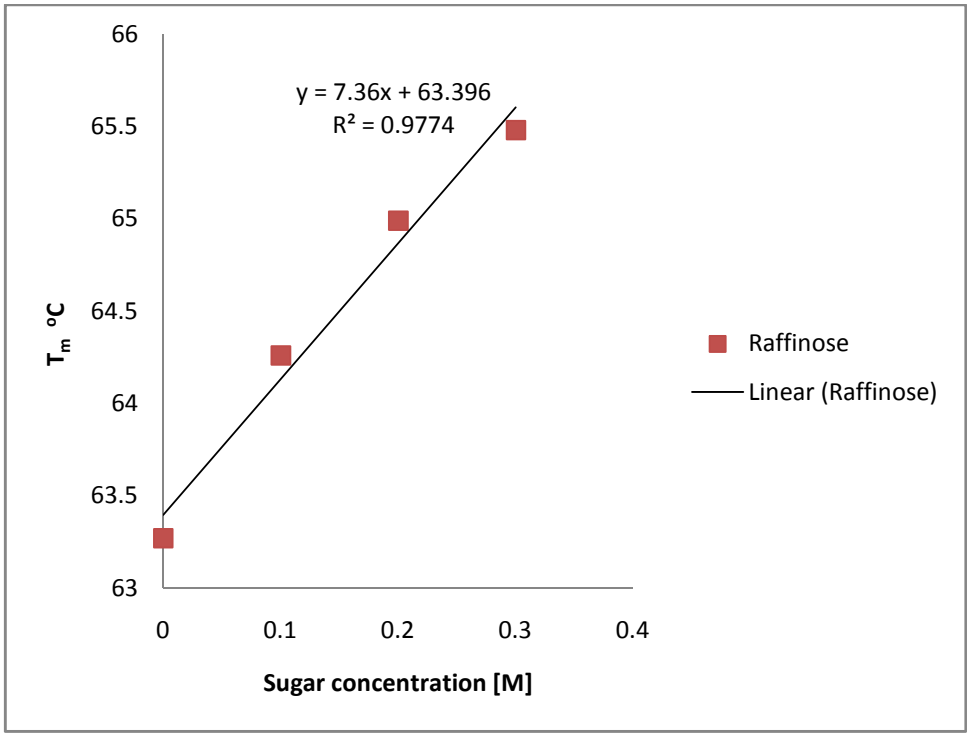

Figure 6.5. T_m of RNase *A* as a function of raffinose concentration.

Table 6.1. The thermal transition temperatures of RNase *A* in the presence of different concentration of sugars.

Sample	T_m ^oC	ΔT_m
Control	63.27	0
RNase A + Trehalose[M]		
0.2	64.74	0.96
0.4	65.39	1.61
0.6	66.64	2.86
0.8	67.58	3.80
0.9	68.13	4.35
RNase A + Sucrose[M]		
0.2	64.34	0.56
0.4	65.12	1.34
0.6	65.97	2.19
0.8	66.70	2.92
RNase A + Raffinose[M]		
0.1	64.26	0.48
0.2	64.99	1.21
0.3	65.48	1.70
RNase A + Mannitol [M]		
0.2	64.44	0.66
0.4	64.91	1.13
0.6	65.52	1.74
0.8	66.06	2.28

The transition temperatures of RNase *A* in presence of different concentrations of mannitol, trehalose, sucrose, and raffinose are shown in Table 6.1. The magnitude of the change in T_m (ΔT_m) varied with the sugar. At 0.2M concentration of sugars, raffinose appears to induce the most change in the value of the T_m and at much higher concentration of sugar (0.8M), trehalose was observed to cause the highest increase in T_m in comparison to mannitol and sucrose (table 6.1). At 0.8 M sugar concentration, the T_m shifted to a maximum value of 66.70 ^oC and 66.06 ^oC for sucrose and mannitol, respectively. For trehalose the T_m increased to 67.58 ^oC from a control of 63.27 ^oC. Trehalose has been described as a better stabilizer due to its significant effects on the structure and properties of water compared to other sugars and polyols [32-34]

In the case of raffinose the T_m increased to a maximum value of 65.48 in the presence of 0.3M, higher concentration of raffinose was not conducted due to limited solubility of raffinose.

The data obtained from DSC can utilized to predict the rank order of stabilizing properties for various additives [35]. In comparing the slopes (table 6.2) obtained from the T_m versus sugar concentration plot, it observed that sugars increase the thermal unfolding temperature Tm ; thus the thermal stability of RNase *A* in the order :

Raffinose > trehalose > sucrose > mannitol i.e. The thermal stability of the protein increases with increasing the number of monosaccharides unit.

Table 6.2. slopes of the T_m of RNase *A* versus sugar concentration plot (ΔT_m °C/mol of sugar concentration).

Sugar	Slope
Mannitol	3.33
Sucrose	4.24
Trehalose	5.24
Raffinose	7.36

The results suggest differences in the magnitude of their stabilizing effect between the sugars. Such differences have been attributed to differences in their molecular structure and the extent to which they would affect the structure of water[23].

The results clearly indicate an increased thermal stability of RNase *A* in the presence of these sugars as indicated by the increases in T_m. The higher the T_m, the more stable the molecule These observations supports several studies which have also shown an increase in the stability of proteins in solution in the presence of sugars and polyhydric alcohols [22,23, 36-39] .

6.3.1.Micellization studies: application of sugar effect to protein

The result of micellization studies indicates that an unfavourable interaction exist between the hydrophobic groups of the surfactants and the sugars as shown by decrease in CMC values and increase in free energy penalty of hydrocarbon exposure when compared to water. The forgoing discussion of the effect of sugar albeit to a qualitative extent is applicable to proteins, thus an unfavourable interaction between exposed non polar groups and sugars would increase the tendency of the groups to enter into the interior of the protein, enhancing

hydrophobic interactions, consequently enhancing the stability of the proteins in the sugar solution. Sugar will thus reduce the extent of thermal denaturation as seen by higher T_m values.

Earlier studies by Lee and Timasheff showed an increase in T_m with sucrose concentration, it was suggested that stabilization was due an increase in the cohesive force of water by sugar molecules as result of the strong interaction between water and sucrose thus excluding the protein from the system thereby stabilizing the folded native state of the protein[22]. The same effect of sugar on solvent system was observed in the aqueous surfactant system as reported in chapter four where sugars where shown to increase the cohesive energy density.

Figure 6.6 shows a linear relationship between the T_m and CMC values this suggesting a similar mechanism based on the solvent modification by sugars.

The stabilizing effect of the sugars against heat denaturation can thus be described as a non-specific process largely attributed to their effect on water structure, which in turn may enhance hydrophobic interactions in the protein [23, 40].

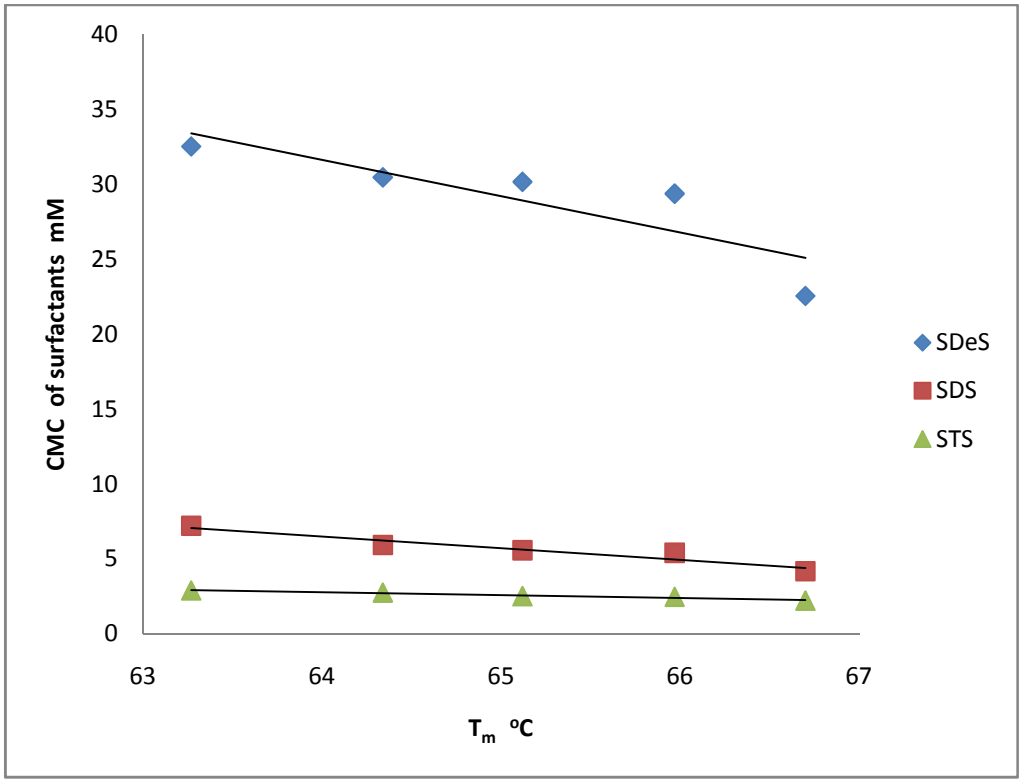

Figure 6.6. CMC of Homologous series of surfactants and T_m of RNase *A* : effect of sucrose.

6.4. Concluding Remarks

DSC studies of RNase *A* in presence of different sugars used in this study such as sucrose, trehalose, raffinose and mannitol increased the thermal stability in a concentration dependent manner thus, protein stability depends linearly on the osmolyte concentration.

The extent of T_m increase of RNase *A* varied with the different sugars studied. The increase in T_m suggests that the native/folded from of the protein is more stable in sugar solution as seen by the increase in the onset temperature of unfolding. Since the folded state of protein is stabilized in the presence of sugar, the exposure of hydrophobic group in the protein of an unfolded state is unfavourable in the presence of sugar.

These results also suggest that, though all the sugars used in this study tend to stabilize the protein, the extent to which an individual sugar confers stability is different depending upon the identity and concentration of the sugar used. This observation may be due to the extent of the structuring of water in presence of these sugars [23].

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Chapter 7. Summary and Future work

7.1. Summary

This body of work is an investigative study probing the stabilising properties of sugars with an ultimate aim of increasing the knowledge base in this area of research. The ability of sugars to stabilize the native conformation of biomolecules is a well established phenomenon[1-4] and several studies have been conducted in this area of research with several mechanisms put forward[5-11], however a universal interpretation of the mechanism of stabilization is still elusive[12].

A full understanding of how these compounds protect biomolecules would help in development of stability protocols in biopharmaceutical drug development, thus allowing for optimized manufacturing procedure during pre- formulation and formulation stages of drug development.

During the course of this research study several physicochemical techniques were used to investigate and characterise the following sugars: sucrose; trehalose; maltose; raffinose and mannitol. Spectroscopic profiles for the aforementioned sugars were obtained by the use of techniques such as FT-IR and Raman spectroscopy.

The interaction between the sugars and simple surfactant systems was characterised by tensiometry in an attempt to throw more light into the mechanism of stability of sugars. Tensiometric studies examining the impact of sugars on the CMC and ΔG_{mic} of a homologous series of sodium *n*-alkyl sulphates which include: sodium decyl sulphate (SDeS), sodium dodecyl sulphate (SDS) and sodium tetradecyl sulphate (STS) as a function of increasing sugar concentration was carried out. The effect of the sugars on other physicochemical properties such as conductivity, surface pressure, surface excess concentration and area occupied per surfactant molecules were also examined.

In the presence of the sugars, decrease in CMC was observed as well as a more negative ΔG_{mic}. The observed increase in the free energy of exposure of hydrocarbon groups suggests an unfavourable interaction between the aqueous sugar solutions and hydrocarbon groups.

Significant increases in surface pressure of the surfactants in the presence of sugars were also observed suggesting an enhancement of the surface activity of the surfactants. Increase in the size of the head group area as the concentration of sugar increased indicated possible interactions between the surfactant head group and sugar, via hydrogen bonding or dipole-dipole interactions. These interactions as a consequence decreased the electrostatic interactions between surfactant heads and enhanced micelle formation. This claim was substantiated by decreases in conductivity of the surfactants and reported decrease in dielectric constants in the presence of the sugars. In addition, surfactants-sugar interactions studied by calorimetry showed an increase in ΔH_{mic} in comparison to lower values of ΔS_{mic} thus showing formation of bonds due to contributions from a number of events such as polar interactions of surfactant head group – sugar and sugar-water.

The tensiometric studies shows that the sugars are excluded from the hydrophobic tail group but probably interact with the head group. The balance between contact interaction and exclusion due to unfavourable interactions is shown to stabilize the micelles.

Demicellization experiments of surfactant micelles in water and in the presence of sugars by ITC complemented results obtained by tensiometry. The results showed an increase in the entropy that favours micellization and the free energy of cost of hydrocarbon group exposure. The results suggest sugars enhanced the hydrophobic effect and increased the thermodynamic stability of micelles. The results were is in good agreement with the tensiometric study.
It might be argued that the observed sugar induced changes in the solution properties of surfactant such as surface pressure and CMC may be due to trace surfactant impurities found in the sugars thus affecting the results obtained by tensiometry which is susceptible to minor impurities. However, this is not the case, the reason being that the results obtained by tensiometry are consistent with observation obtained by calorimetric method. Besides, surface tension values measured in the presence of sugars showed little effect on the surface tension of water – an increase in surface tension of water was reported, which was in good agreement with results shown by other investigators.

DSC was used to study and characterise the effect of the investigated sugars on the transition temperature (T_m) of *RNAse A*. The results obtained showed the sugars increased the thermal

stability of *RNAse A* as shown by an increase in the T_m in the presence of the sugars. The T_m also shifted progressively to higher temperatures as a function of sugar concentration.

The forgoing discussion of the effect of sugars used in the surfactant studies suggest that sugars would stabilize proteins by producing an increase in the free energy of denaturation due to unfavourable interaction between the hydrophobic groups on the protein and the sugars when the protein unfolds. Thus the native, unfolded or compact state of the protein would be favoured in the presence of sugars.

One common theme observed in the results for all the studies carried out is an unfavourable interaction between the sugar and the hydrophobic groups as seen by the increase in the free energy of hydrocarbon group exposure. The increases in the free energy would thus favour a more compact state of the protein (unfolded /native) or micellar state in surfactants.

It is our conjecture that while proteins are large complex molecules, their interaction with sugars or other small solutes could be related to simple model systems such as micelles, these insights into sugar-surfactant interactions could perhaps give a better knowledge of stabilizing properties of sugars.

7.2. Future work

These observations are very interesting and complicated and further investigation is therefore required in the following areas:

- Analysis of co crystals of sugar and surfactant molecules by single crystal x-ray diffraction will provide complementary data to help elucidate the plausible interaction between head group and sugar.
- Further demicellization studies with ITC on the following surfactants: sodium decyl sulphate, sodium tetradecyl sulphate, and cationic surfactants dodecyl trimethylammonium bromide and tetradecyl trimethyl ammonium bromide. Consequently, the ΔG_{mic} with increasing chain length can be calculated, in effect predicting the contribution of alkyl group to the stability of protein.
- To examine the possibility of performing solution state Raman on other sugars which have not been previously published in literature or novel cryoprotectants.

- DSC studies of R*Nase A* as well as other proteins giving an extensive thermodynamic profile showing the effect of sugars on parameters such as and heat capacity of denaturation(ΔC_{pd}).
- DSC studies of the effect of sugars on micellization of surfactants including non ionics.
- Obtain surface acessible surface area calculations for surfactants with use of molecular modelling programs such as Arguslab, Chimera or Phyton molecular viewer in the presence of the sugars studied consequently comparing the transfer free energies of the surfactants in the presence of sugars with the $\Delta\Delta G$'s for R*Nase A*

7.3. References

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8. Appendix

8.1. Spectroscopic profile of the investigated sugars: Sucrose, Trehalose, Maltose, Raffinose, Mannitol.

Solid state Raman and FT-IR measurements were carried out to characterise the sugars, with Raman and infra-red spectra recorded for the protonated and deuterated samples of these sugars.

A Perkin Elmer Paragon 1000 FT-IR spectrophotometer was used to measure the infrared spectra of protonated and deuterated samples of trehalose, sucrose and maltose in the solid state. The samples were examined as pressed KBr disks, this was performed at 10 scans with a resolution of $2cm^{-1}$ in the 450-4000 cm^{-1} range. A dry nitrogen gas purge was maintained in the sample compartment to facilitate a simpler background subtraction.

The Raman spectra was obtained with a LabRam Raman spectrometer (Horiba Jobin Yvon) equipped with a He/Ne laser using excitation at 632.18 nm wavelength and a power of 8mW. The spectrometer is equipped with a 1800 line mm^{-1} holographic grating, a holographic notch filter, a Peltier-cooled CCD (MPP1 chip) for detection and an Olympus BX40 microscope. Raman spectra of solid state samples were collected, at room temperature, on a microscope slide using a microscope objective of x100 magnification to focus the laser beam. A back scattering (180^{o} excitation and collection) geometry was used in all experiments. The Raman instrument was calibrated using the ν_1 line of silicon at 520.7 cm^{-1}. Centering of the silicon line was checked by using the frequencies of the principal lines of a neon lamp.

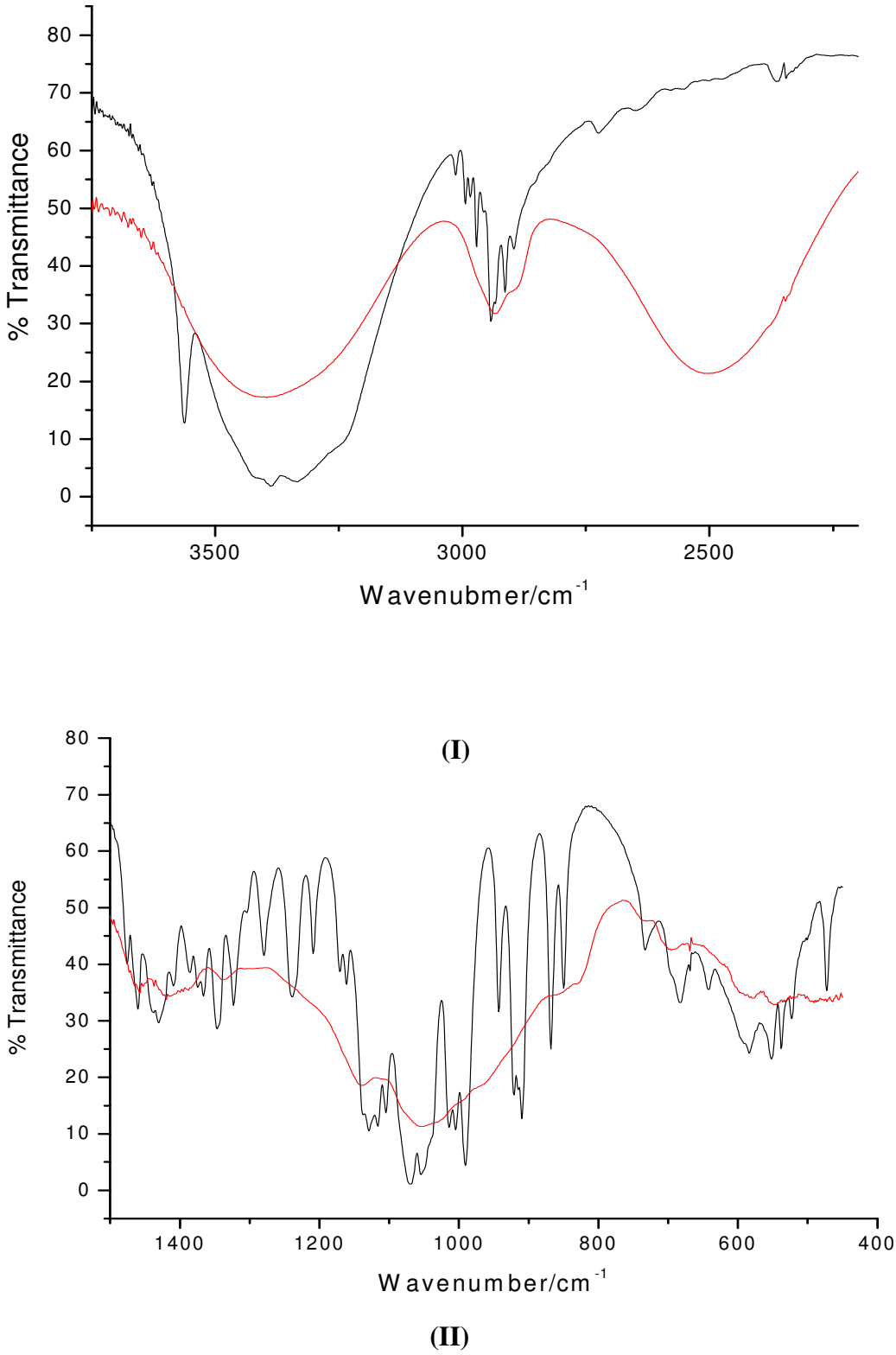
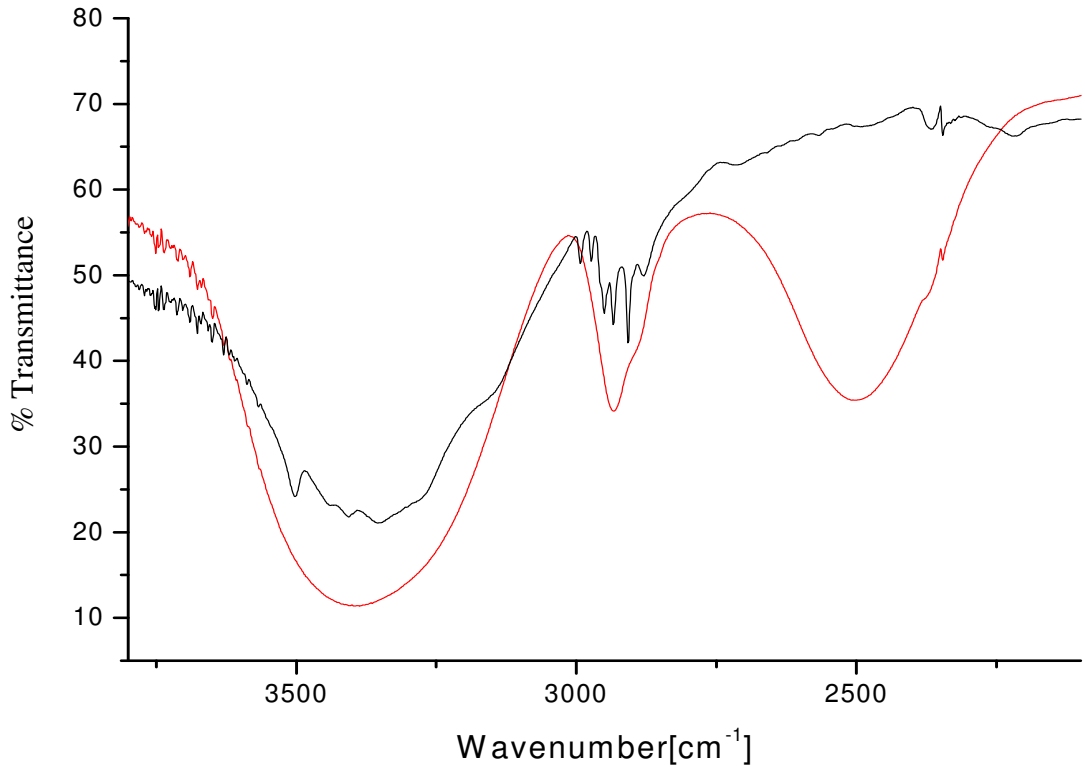
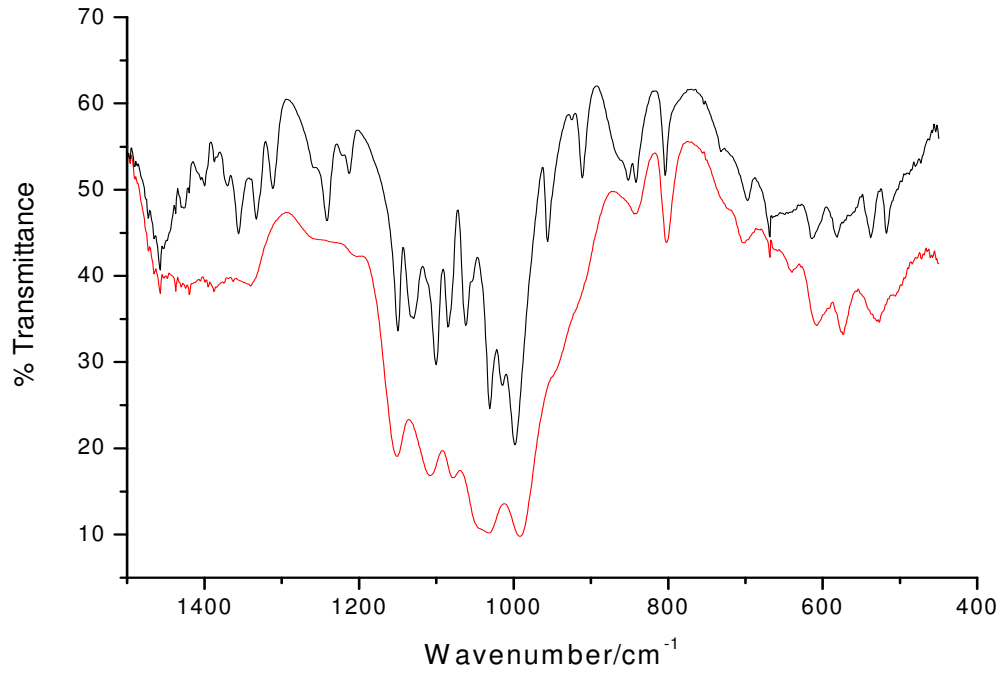


Figure 8.1. IR spectra of protonated (black) and deuterated (red) sucrose. Spectral window 3600-2000 cm^{-1} (**I**) and spectral window 1500-400cm^{-1} (**II**).

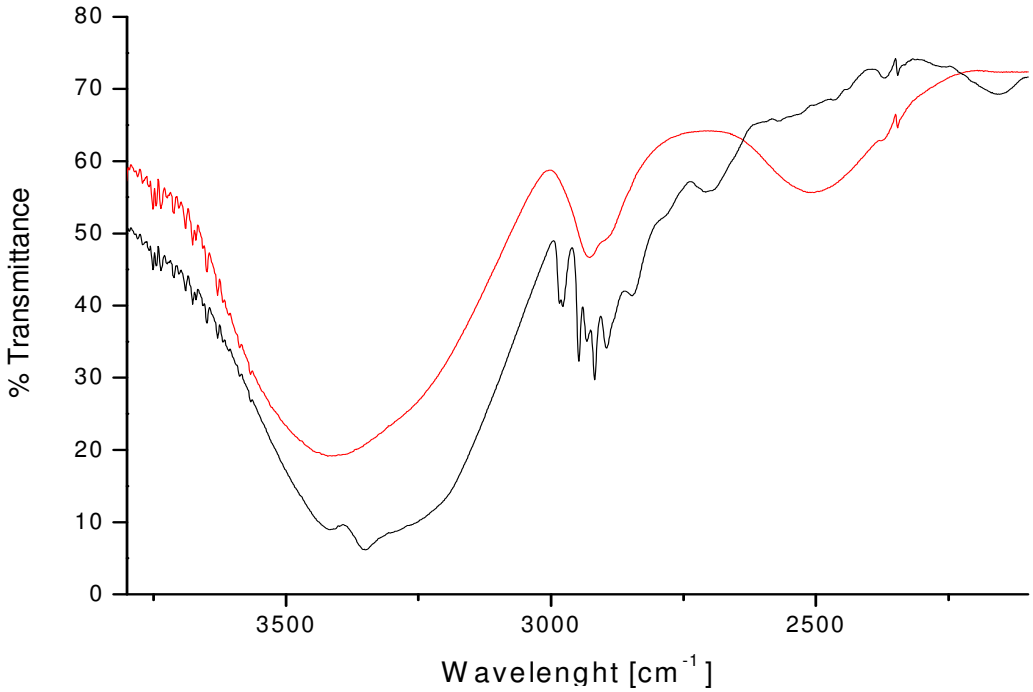


(I)

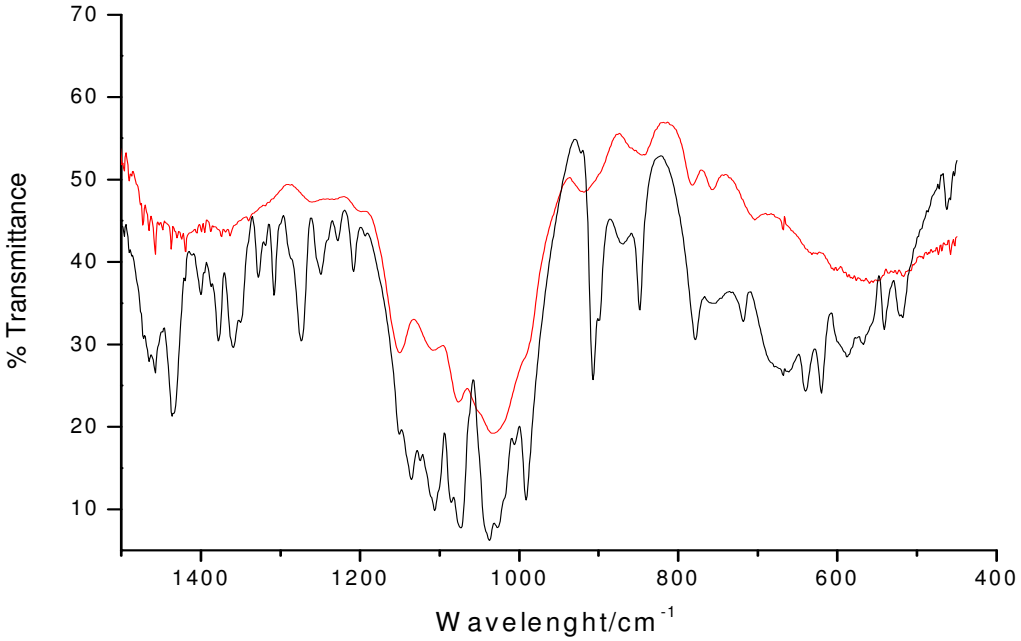


(II)

Figure 8.2. IR spectra of protonated (black) and deuterated (red) trehalose. Spectral window 3600-2000 cm^{-1} (**I**) and spectral window 1500-400cm^{-1} (**II**).



(I)



(II)

Figure 8.3. IR spectra of protonated (black) and deuterated (red) maltose. Spectral window 3600-2000 cm^{-1} (**I**) and spectral window 1500-400cm^{-1} (**II**).

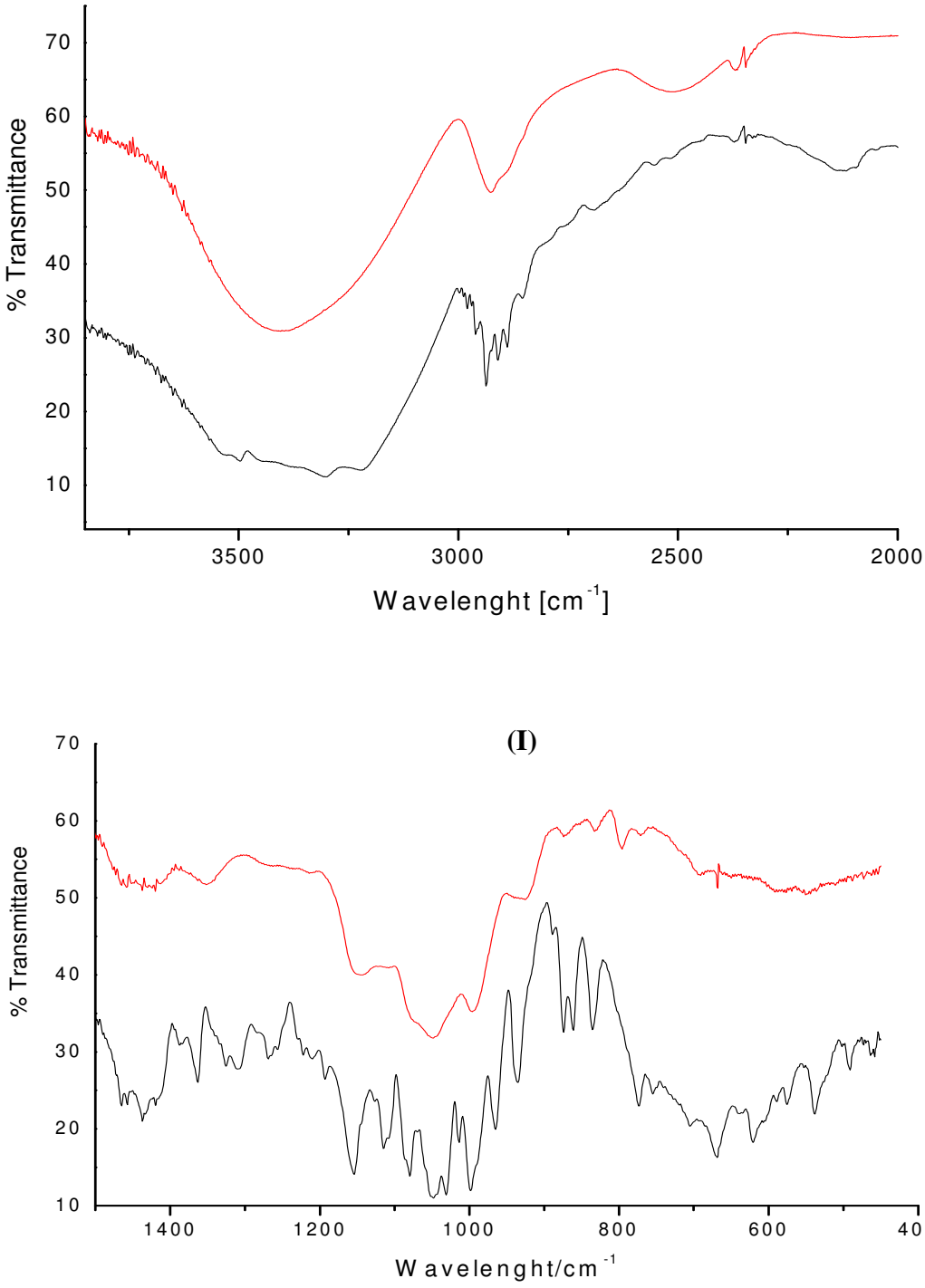
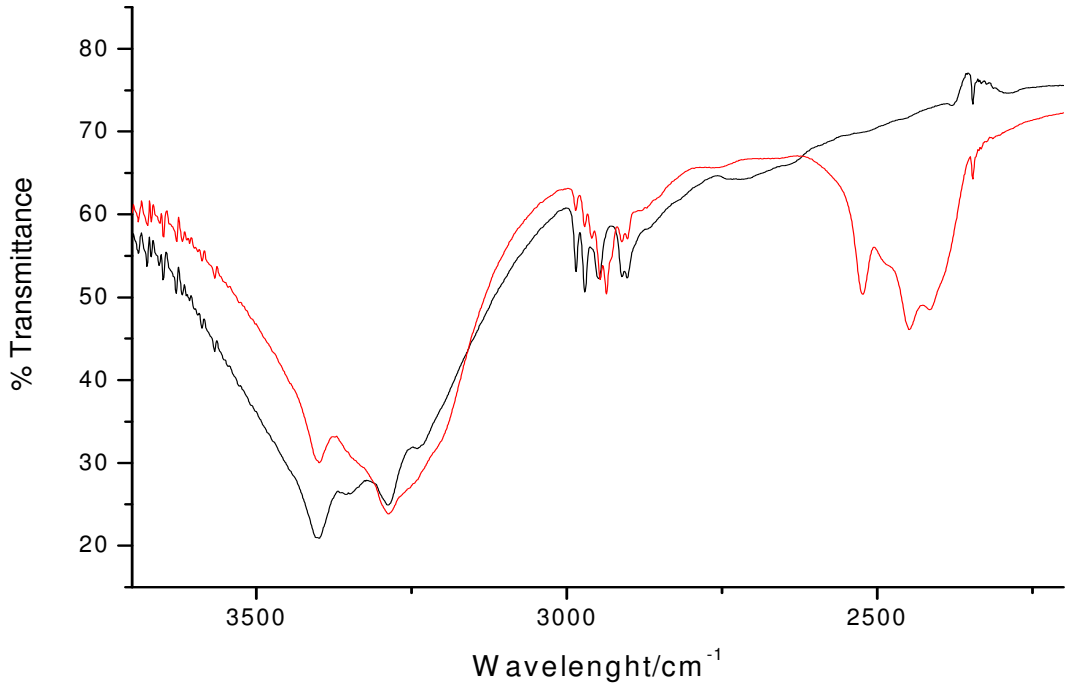
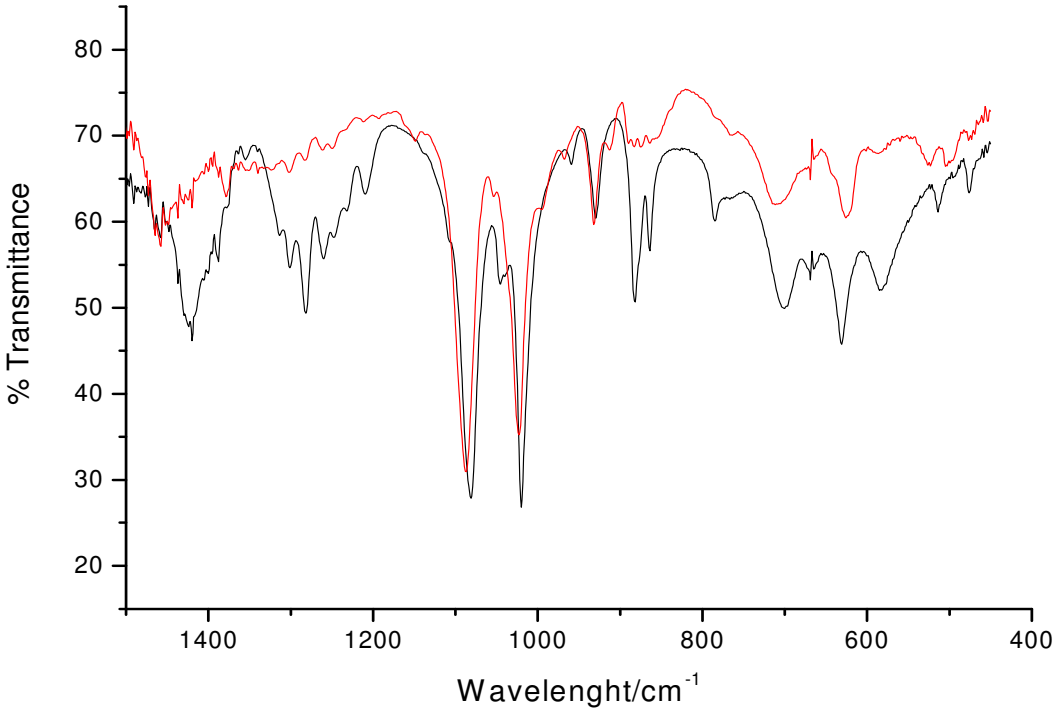


Figure 8.4. IR spectra of protonated (black) and deuterated (red) raffinose. Spectral window 3600-2000 cm^{-1} (**I**) and spectral window 1500-400cm^{-1} (**II**).



(I)



(II)

Figure 8.5. IR spectra of protonated (black) and deuterated (red) mannitol. Spectral window 3600-2000 cm^{-1} (**I**) and spectral window 1500-400cm^{-1} (**II**).

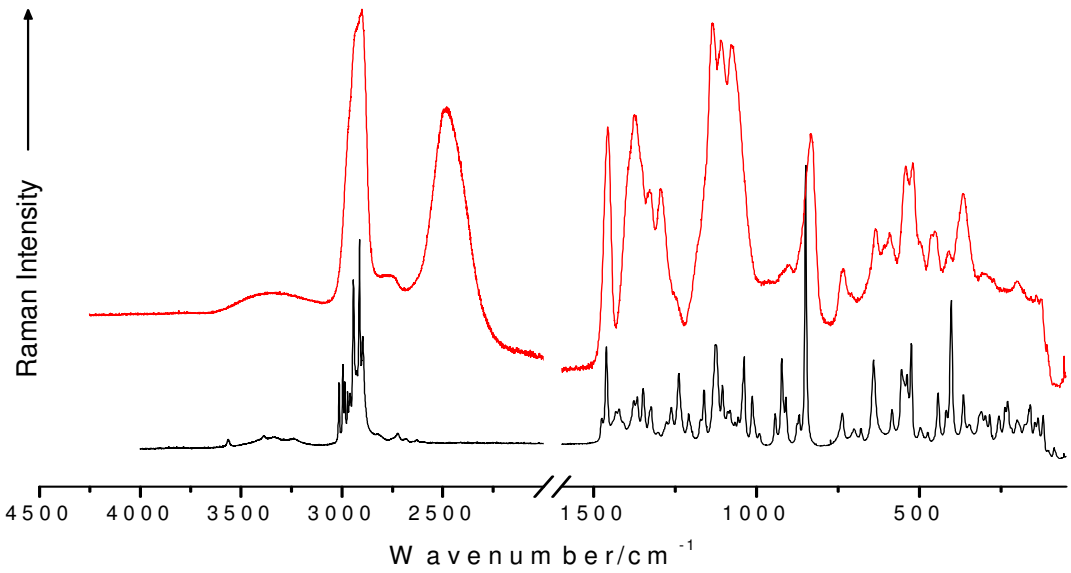


Figure 8.6. Raman spectra of protonated (black) and deuterated (red) sucrose.

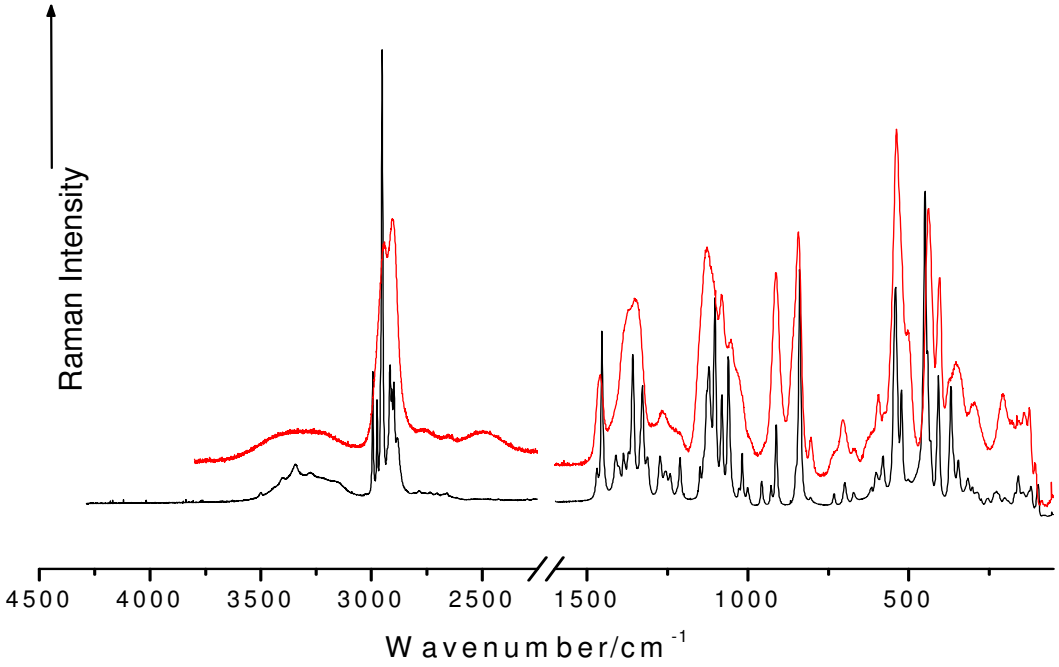


Figure 8.7. Raman spectra of protonated (black) and deuterated (red) trehalose.

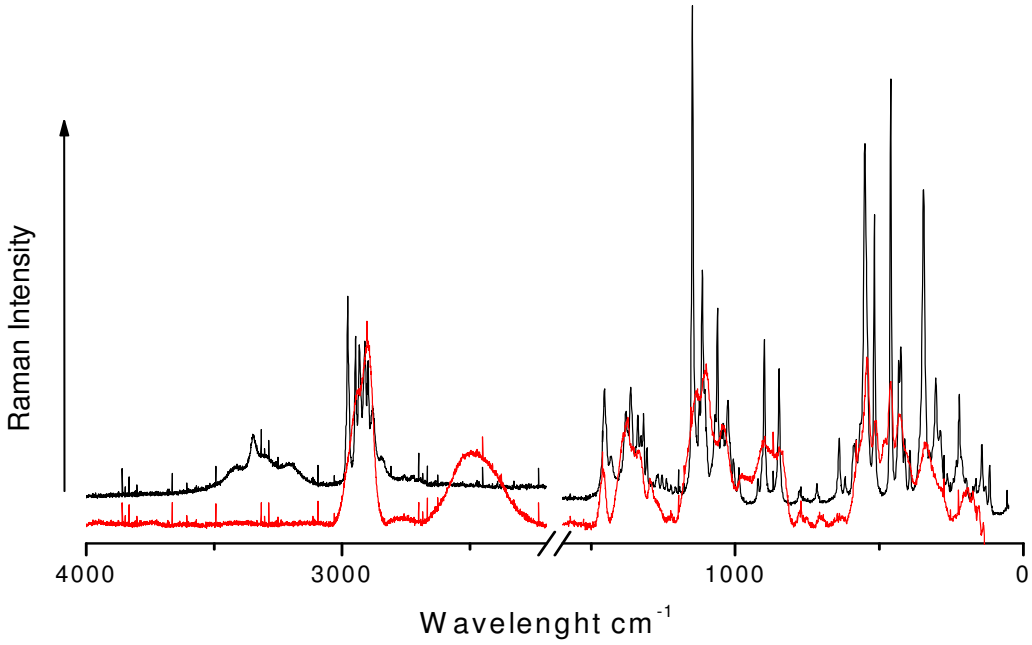


Figure 8.8. Raman spectra of protonated (black) and deuterated (red) maltose.

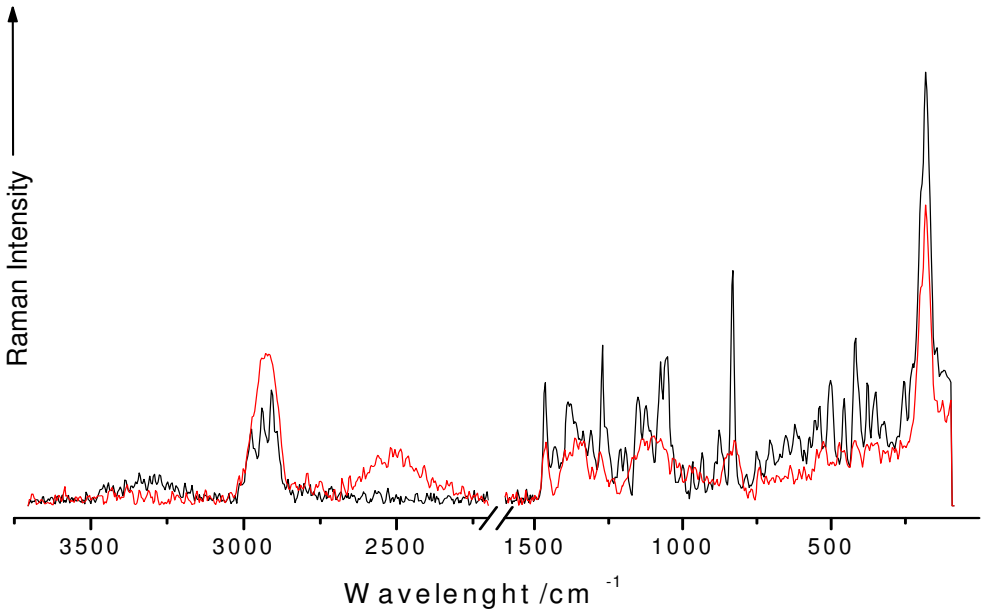


Figure 8.9. Raman spectra of protonated (black) and deuterated (red) raffinose.

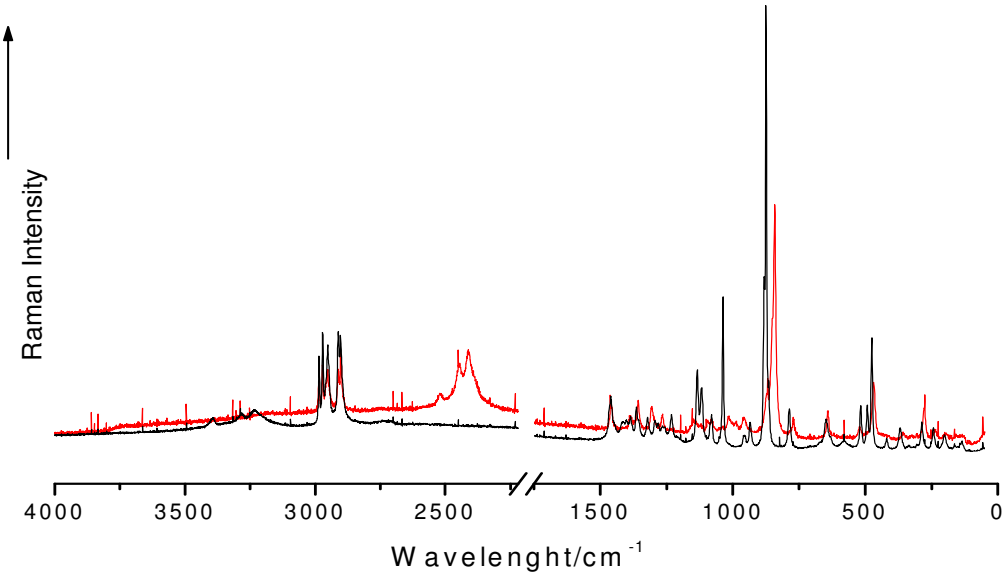


Figure 8.10. Raman spectra of protonated (black) and deuterated (red) mannitol.

8.2. Presentations and publications

8.2.1. Presentations

Ehiwe, T.I (Aug 2009) Study of bioprotective sugars: lessons from micelle model systems. *International Union of Pure and Applied Chemistry (IUPAC) Congress*. Glasgow, Scotland.

Ehiwe, T. I.(Jul 2008). Tensiometric studies of micellar systems in aqueous sugar solutions. *Poster presentation at RSC Industry Tour of Pfizer, Kent, UK*

Ehiwe, T. I.(Jul 2008). Tensiometric studies of micellar systems in aqueous sugar solutions. School of Science research day. University of Greenwich.

Ehiwe, T.I.(Jul 2007). Sweet success: probing bioprotective sugars. Spotlight on research day: School of Science. University of Greenwich

8.2.3. Publications

Ehiwe, T.I., Chowdhry, B.Z and Leharne, S.A.(2011). Tensiometric studies of micellar systems in aqueous sugar solutions. *J.Phys.Chem* Manuscript in preparation.

Ehiwe, T.I., Chowdhry, B.Z and Leharne, S.A.(2011). Demicellization behaviour of sodium dodecyl sulphate micelles in the presence of sugar by ITC**.** *J.Phys.Chem* Manuscript in preparation.

Ehiwe, T.I., Chowdhry, B.Z and Leharne, S.A.(2009) Study of bioprotective sugars:lessons from micelle model systems. *Book of Abstracts IUPAC Congress*.