

Competitive Adsorption of Bile Salts and Milk Protein in Oil-in-Water Emulsions

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Abstract

The competitive adsorption of bile salts with milk proteins (whey protein concentrate (WPC) or sodium caseinate (SCN)) was studied in oil-in-water emulsions. The research confirmed previous findings that the degree of displacement of protein depends on the number of hydroxyl groups attached to the sterol ring of the bile salt, but also highlighted the importance of the presence (or absence) and nature of amino acid residues conjugated with the sterol ring. In general, decreasing the number of dihydroxy bile salts such as sodium deoxycholate (NaDC) displaced more protein from the oil droplet surface than trihydroxy bile salts such as sodium cholate (NaC). This is attributed to the greater hydrophobicity of the sterol ring of the NaDC. However, hydrophobicity is not the only factor that determines the ability of the bile salt to displace protein. If sodium taurocholate (NaTC) is used in the competitive adsorption experiments which contains a large charged hydrophilic sulphonate group attached to a taurine residue conjugated to a NaC molecule, the NaTC displaces much more protein from the emulsion droplet surface even though NaTC is less hydrophobic than NaC. A similar effect is seen with sodium glycodeoxycholate (NaGDC) when compared to NaDC. NaGDC is less hydrophobic than NaDC due to the conjugation of a glycine residue, but NaGDC is better at displacing protein from the oil-water interface. This observation is explained in terms of the greater steric hindrance that a conjugated bile salt (NaTC or NaGDC) experiences when it adsorbs to an oil-water interface. The charge amino acid group sits further into the aqueous side of the oil-droplet interface and disrupts the adsorbed protein layer to a greater extent than the non-conjugated bile salts. Another interesting feature of the competitive adsorption was the difference between degree of displacement for the different protein types SCN or WPC. For all bile salts SCN was significantly more resistant to displacement than WPC. The reason for this is not clear but may be due to either the greater surface activity of caseins, or to binding of bile salts to WPC or SCN proteins. To investigate further the adsorption of bile salt in emulsions the surface coverage of the bile salts was determined. Results indicated that NaC adsorb as a monomer over much of the concentration range used, whilst the other three bile salts adsorbed as micelles. The consequences of this for competitive displacement of

protein are discussed. Finally, preliminary experiments were carried out to elucidate the effect of NaC concentration on lipase catalysed hydrolysis of the triglycerides in the emulsion droplets. The release of free fatty acids is observed to increase with increasing NaC concentration in the emulsion. This is discussed in relation to the effect of NaC on the WPC and SCN protein concentration at the droplet surface, and the likely effect of NaC micelles on the rate of lipase activity.

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LIST OF PUBLICATIONS BY THE CANDIDATE

S.R. Euston, W.J. Baird, L. Campbell & M. Kuhns, Competitive Adsorption of Dihydroxy and Tri-hydroxy Bile Salts with Whey Protein and Casein in Oil-in-Water Emulsions, *Biomacromolecules*, **14**, 1850-1858, 2013. DOI: 10.1021/bm4002443

CHAPTER 1 - INTRODUCTION

Obesity is a major problem facing both governments and health services in the western world, and is a contributing factor in a number of health conditions. A report by the World Health Organisation in 2000 (WHO, 2000) listed cardiovascular disease and hypertension; cancer; diabetes mellitus; gallbladder disease; endocrine and metabolic disturbances; osteoarthritis and gout; pulmonary disease and psychological disorders as all having causal links to obesity. The economic costs of obesity are likely to be huge. Cawley & Meyerhoff (2012) have estimated that in 2005 the cost of obesity-related healthcare in the US alone was \$190 billion per annum (21% of total health-spend). In Europe a similar estimate of obesity-related healthcare costs has been made, where in 2006 it was reported that this is somewhere in the region of €59-236 billion per annum (Rettmann, 2006). This is 7-28% of the total healthcare budget of the entire EU member states.

The economic and social effects of obesity are easy to quantify and to state. The causes, however, and therefore the solution are not quite so straightforward. A recent Head to Head debate in the British Medical Journal posed the question “Are the causes of obesity primarily environmental?” Prof. Timothy Frayling of the Peninsular Medical School, University of Exeter, argued in favour of a primarily genetic mechanism (Frayling, 2012). Frayling argues that twin and adoption studies show consistently that genetic factors play a large role in where a person sits on the body mass index scale, and that evidence suggests this is due to variations in the mechanisms of appetite control. Prof. John Wilding of the Dept. of Obesity & Endocrinology, University of Liverpool presented the counter argument, and believes environmental factors to be more important (Wilding, 2012). He attributes this to a number of factors: the fall in the relative cost of food in recent years, particularly energy dense foods; decline in physical activity; and changes in eating patterns that encourage obesity. Wilding (Wilding, 2012) is particularly scathing in his comments on the food industry which he describes as “an unhealthy alliance of producers and marketers”, which he says have “successfully promoted energy dense foods, many of which provide positive reinforcement that increases consumption, effectively producing a “cafeteria diet” for the whole human population – a well

proved way of causing obesity in experimental animals". Unsurprisingly, Wilding (2012) favours schemes whereby food manufacturers agree to reduce the energy content of products and to promote these foods, and if these do not work stronger legislation to enforce the issue. Frayling (2102), perhaps more surprisingly, is in general agreement with this view, since he feels that education programs will be of little use if genetic factors control obesity. He feels that initiatives such as banning the sale of supersized sugary drinks will make genetic factors of less importance, and that this will have a greater effect on obesity levels than education programs.

The food industry is aware that in some quarters they are being at least partly blamed for the obesity epidemic, and are taking steps to produce reduced calorie foods. The problem with this is that the uptake of reduced calorie and in particular reduced fat foods has been low. This is due partly to the view that these foods have inferior organoleptic qualities (McEwan & Sharp, 2000; Hamilton, Knox, Hill & Parr, 2000). If current strategies for fat replacement or low energy density foods production are not producing products of sufficient quality, then other strategies for the formulation of healthier foods should be researched. One such strategy is to try to control or slow the digestion of fats through the intestinal tract so that fats are not adsorbed to any great extent (Maldonado-Valderrama, Gunning, Wilde & Morris, 2010). This would involve finding ways to protect the fats against bile-salt mediated lipase digestion, by reducing the degree of bile-salt induced displacement of protein from the fat-aqueous interface. To achieve this will require significant research effort in a number of areas where there has been limited progress in the past. This will include a more detailed understanding of the surface chemistry, and in particular the competitive adsorption behavior of mixed bile salt-protein systems. This dissertation present results of research which has studied the competitive adsorption of four bile salts, sodium cholate (NaC), sodium deoxycholate (NaDC), sodium taurocholate (NaTC) and sodium glycodeoxycholate (NaGDC). Previous results from our laboratory (Euston, Bellstedt, Schillbach & Hughes, 2011), had shown that NaDC was able to displace more whey protein from the oil-water emulsion droplet interface than NaC at the same molar ratio of protein:bile salt. This difference was attributed to

the higher hydrophobicity of the NaDC molecule due to their being one less hydroxyl group attached to the NaDC.

This project aims to extend the previous research to include other structural differences observed in the bile salt, namely the presence or absence of a conjugated amino acid (taurine or glycine) in the bile salt structure. These are expected to alter the hydrophile-lipophile balance of the bile salt molecules and will influence the way in which they adsorb to the interface and compete for interfacial area with adsorbed protein. The study will compare the ability of the bile salts to displace two milk proteins, whey protein concentrate and sodium caseinate. The whey proteins are globular (Sawyer & Kontopidis, 2000), whilst the caseins are intrinsically disordered proteins (Holt & Sawyer, 1993). The major bovine whey protein, β -lactoglobulin, is known to bind hydrophobic ligands in a specific binding pocket (Sawyer & Kontopidis, 2000), whilst the caseins lack a tertiary structure and if they bind surfactants it will be non-specifically. This is likely to affect the way in which these two proteins interact and compete with bile salts at the oil-water emulsion interface.

In addition to determining whey protein and casein competitive displacement, preliminary studies on a method for the determination of bile salt concentration in the emulsions has been investigated, as an attempt to determine the surface concentration of the bile salts in emulsions. Finally, the effect of bile salt/protein competitive adsorption on pancreatic lipase catalysed hydrolysis of the emulsified oil has also been studied.

Prior to presenting and discussing the results of this project, a brief literature review relating to the relevant background science to this project will be presented.

1.1 Bile Salt Physiology and Structure

Bile salts are the salt form of bile acids. They are a major component of bile which is synthesized in the liver and stored in the gall bladder. The composition of bile is given in Table 1.1. Two primary bile acids, cholic acid and chenodeoxycholic acid are made via the enzymic oxidation of cholesterol (Hoffman, 1994). These are secreted into the gut where some of the cholic and

chenodeoxycholic acid molecules are dehydroxylated by intestinal bacteria to form deoxycholic acid and lithocholic acid. These latter two are called the secondary bile salts.

Component	Composition (%w/w)
Bile salts	67
Phospholipids	22
Protein	4.5
Cholesterol	4
Bilirubin	0.33

Table 1.1 – Composition of bile (data from C.J. O'Connor, R.G. Wallace, Adv. Colloid Interf. Sci. **22**, 1, 1985)

Some re-absorption of these four bile salts into the blood stream occurs. They are taken back to the liver where they are either re-secreted into the intestine or they are conjugated with glycine or taurine and re-secreted. Usually the bile salts structures are found as the sodium salt, which is more soluble than the acid, and collectively they are known as the bile salts. The structure of bile salts is characterized by a sterol ring structure (Figure 1.1) which is derived from cholesterol. The sterol ring is flat in cholesterol, but in bile salts the addition of a hydroxyl group at the 3 position leads to extra steric strain and bending at the interface between the A and B rings of the sterane core. A number of naturally occurring bile salts are found in humans. These differ in structure through the number of hydroxyl groups attached to the sterol ring, the position of the hydroxyls and the presence or absence of conjugated amino acids e.g. glycine or taurine (Figure 1.1) The hydroxyl and CH₃ groups are oriented in opposite directions away from the sterol ring such that the bile salts have a bi-facial structure, with a hydrophilic (OH) and hydrophobic (CH₃) face (Figure 1.2). This contributes to self-association of the bile salts into micelles, and may influence the way in which they adsorb to an oil-water interface.

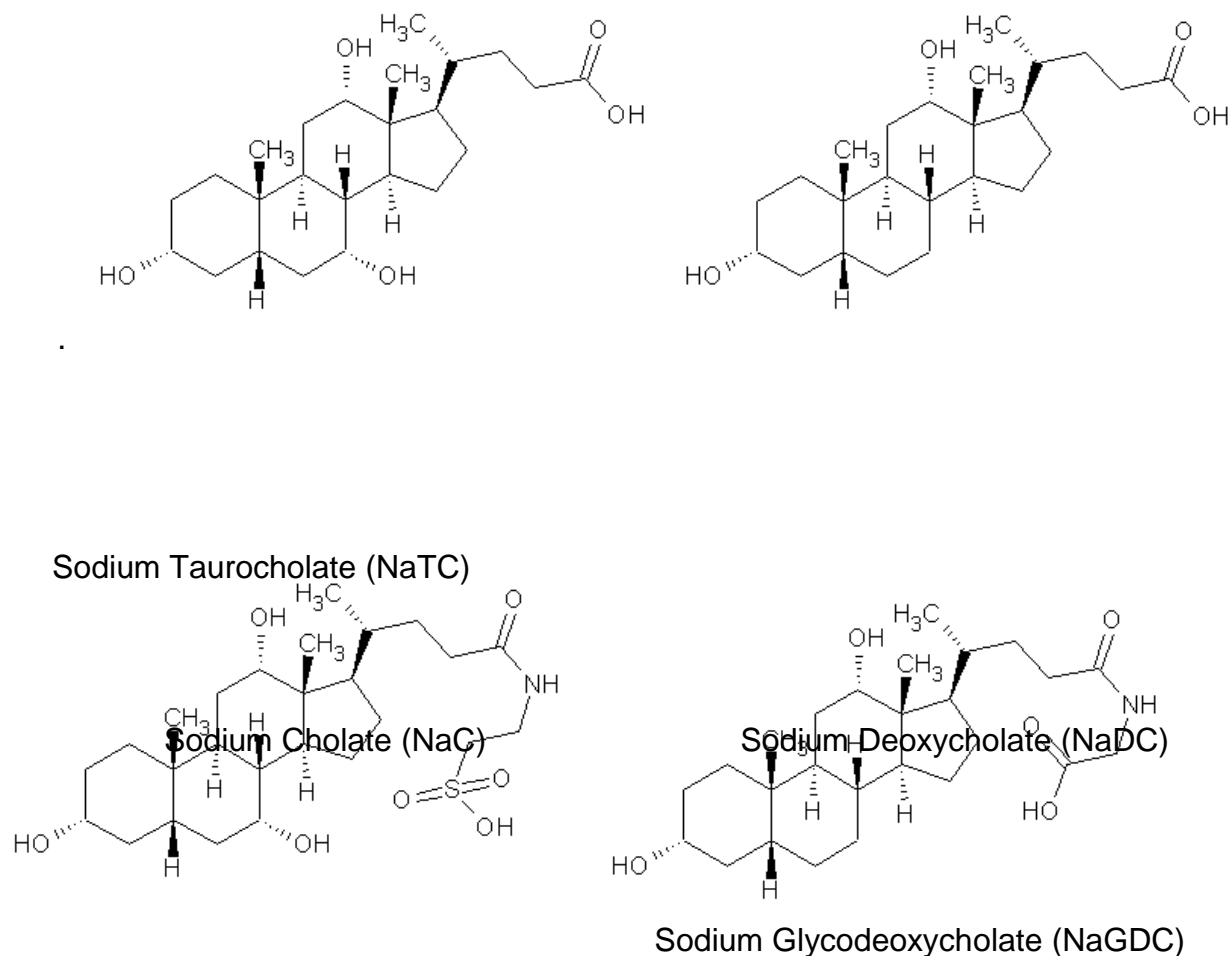


Figure 1.1 – Structure of the four bile salts used in this study. All bile salts contain a sterol ring which is substituted with hydroxyl and methyl groups. NaC and NaTC are trihydroxy bile salts and contain three hydroxyl groups, whilst NaDC and NaGDC are dihydroxy bile salts and lack a hydroxyl group in the 7 carbon position. All bile salts contain a negatively charged head group. In NaC and NaGDC this is a carboxyl group, whilst in NaTC a taurine and in NaGDC a glycine amino acid is joined to the bile salt via an amide bond.

During digestion bile is secreted into the duodenum (upper part of the small intestine) via the bile duct (Figure 1.3). This is the part of the digestive tract where the majority of the fat is digested and bile salts play a crucial role in this process of digesting fat (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011) Bile salts are surfactants (compounds that lower the surface tension between two liquids) that serve a number of functions in fat digestion. They emulsify fat droplets in the intestinal tract liquid which increases the

surface area available for digestion. They displace proteins from the interface of emulsified fat droplets which facilitates the binding of lipase enzymes to the

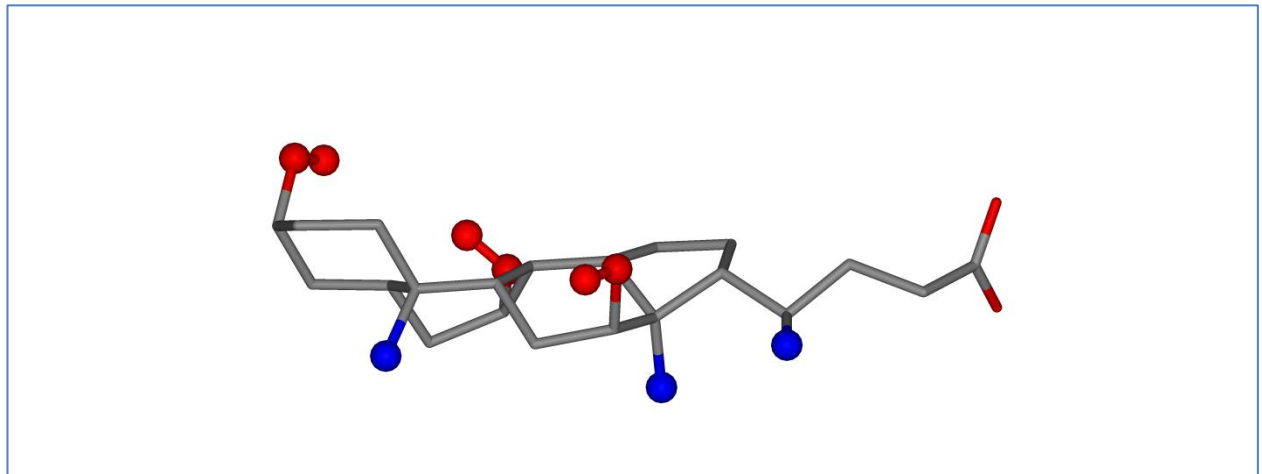


Figure 1.2 – Conformation of NaC showing the orientation of hydroxyl groups (red) and methyl groups (blue) on opposite faces of the sterol ring structure.

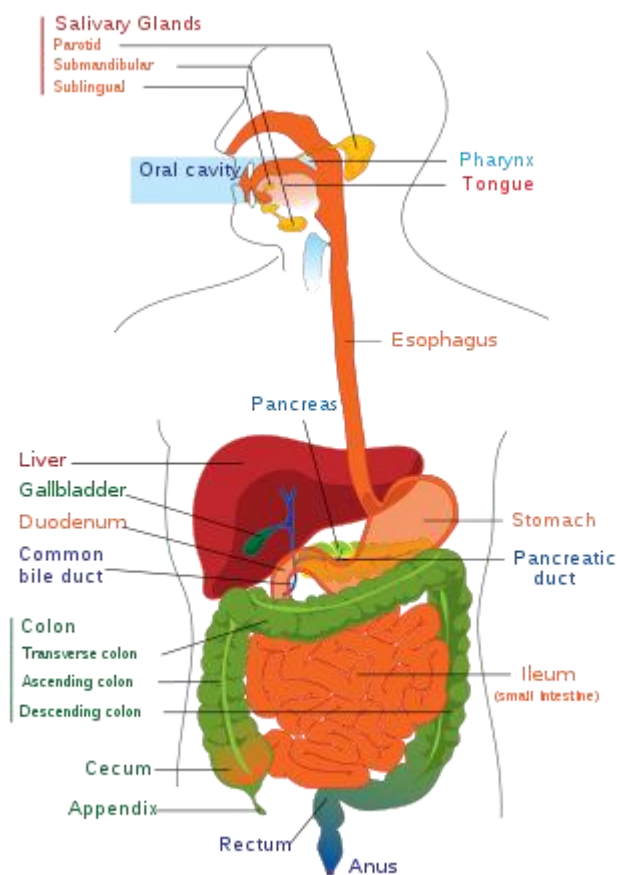


Figure 1.3 – Human digestive system showing connectivity between bile duct and duodenum.

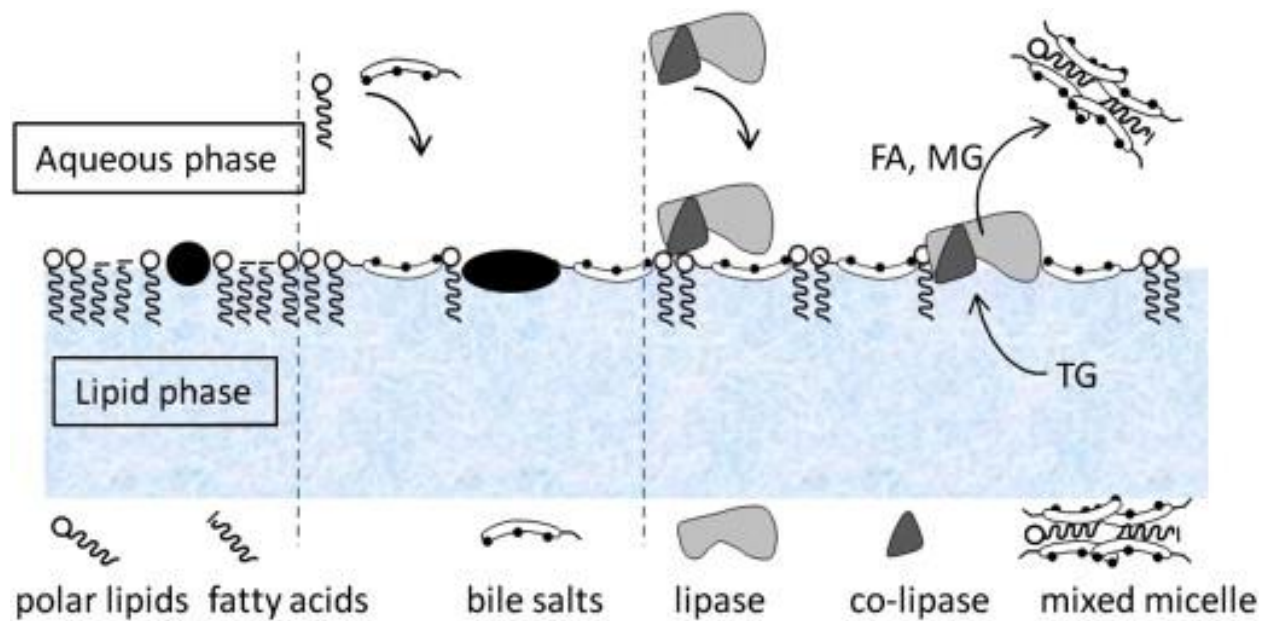


Figure 1.4: Mechanism of duodenal lipolysis. Left: lipid phase is coated by surface active material surviving stomach such as polar lipids, fatty acids, emulsifiers etc. Centre: bile salts and phospholipids secreted into the duodenum adsorb onto lipid surface. Right: pancreatic lipase and colipase adsorb, aided by the interaction with bile salts and hydrolyse triglycerides, which are “solubilised” by bile salts into mixed micelles. Reproduced from Wilde, P.J. & Chu, B.S. Interfacial & colloidal aspects of lipid digestion. *Advances in Colloid and Interface Science*, **165**, 14–22, 2011.

surface of the fat droplet, which in turn makes it easier for the enzyme to hydrolyse triglycerides to fatty acids. Finally, after the fat has been hydrolysed and fatty acids are released the bile salts form micelles which solubilise the fatty acids and transport them to the intestinal wall where they are absorbed by the body. The mechanisms by which bile salts achieve these functions are summarized in Figure 1.4. The surface chemistry of the bile salts is important in all of these functions, and thus it is relevant to consider bile salt physical and surface chemistry in more detail.

1.2 Bile Salt Micelle Formation

Bile salts are unusual molecules which are made up of flat sterol rings with hydrophilic OH groups on one face and hydrophobic CH₃ groups on the other (Figure 1.2) (Armstrong & Carey, 1982). This structure influences the way in which bile salts behave in solution and the way they adsorb at interfaces. Since

bile salts are amphiphilic i.e. they have distinct hydrophilic and hydrophobic parts, they have a tendency to self-associate in aqueous solution into aggregates called micelles (McClements, 2005). The structure of micelles made from amphiphilic surfactants is well understood, and they are generally considered to exhibit a core-shell type structure where the hydrophobic tails of the surfactants cluster together in spherical micelles, whereas the hydrophilic head groups point outwards into the aqueous phase forming a steric stabilizing layer around the micelle (Figure 1.5). Such micelles show a discrete critical micelle concentration (CMC) above which micelles start to form.

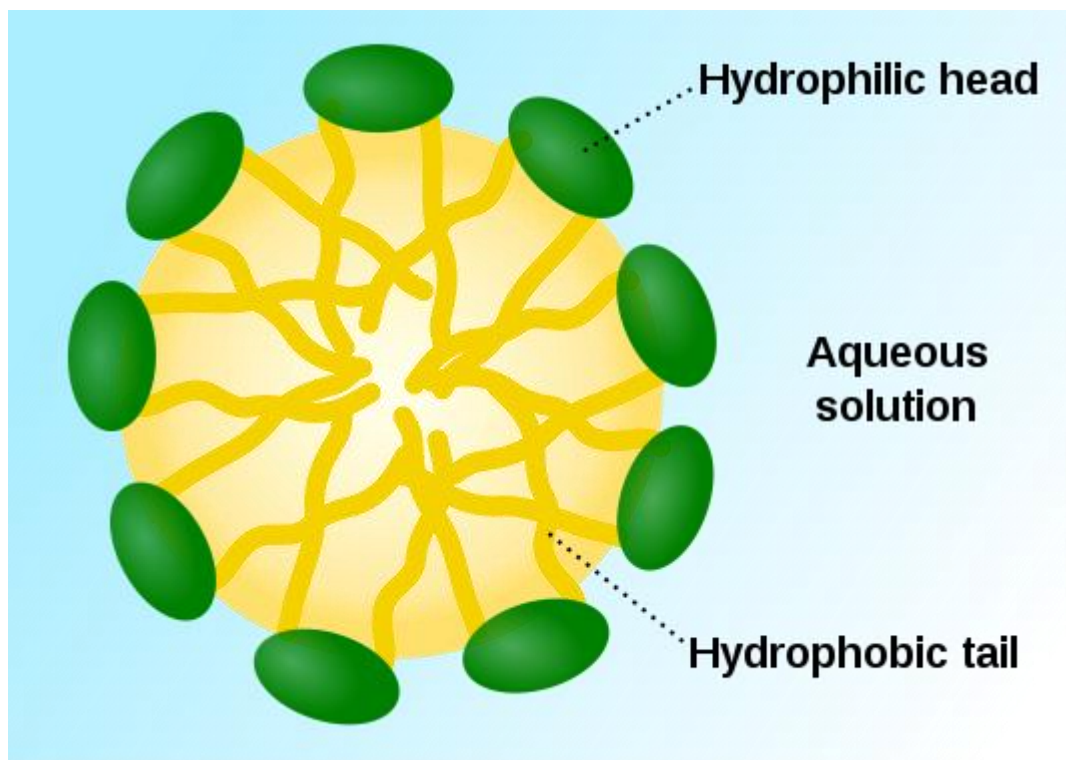


Figure 1.6 – Schematic representation of an amphiphilic surfactant micelle. http://commons.wikimedia.org/wiki/File:Micelle_scheme-en.svg

For bile salts, the formation of micelles is more complicated, and the structure is believed to differ from the spherical model for amphiphilic surfactants (Figure 1.6) (Kawamura, Murata, Yamaguchi, Igimi, Tanaka, Sugihara & Kratochvil; 1989). There are several models for bile salt micelle structure that have been developed over the years. Carey & Small (1972) proposed that bile salts initially form small primary micelles at low bile salt concentration via hydrophobic

association, which then associate into larger secondary micelles (Figure 1.7) at higher bile salt concentrations.

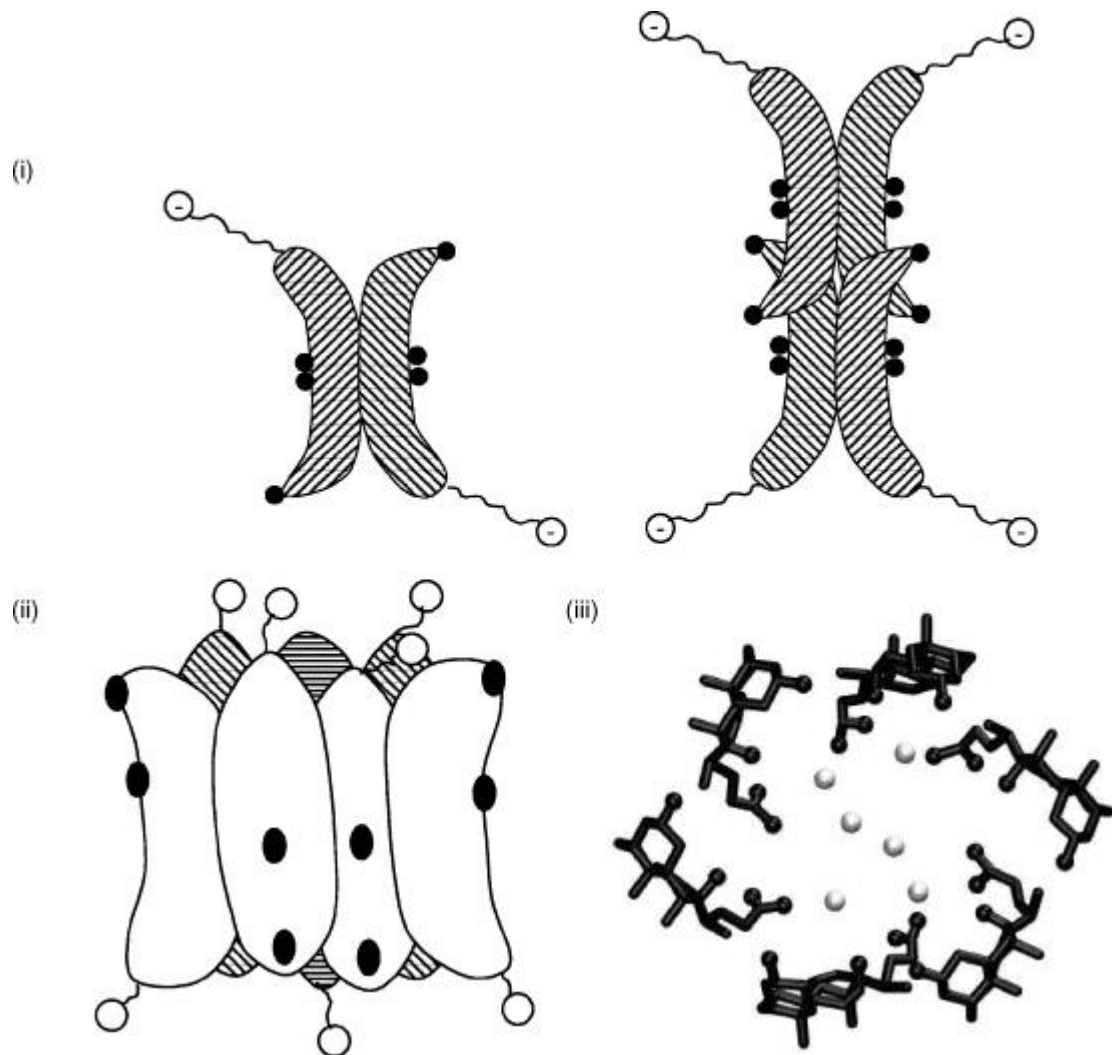


Figure 1.7 - Representations of the literature proposed structures of bile salt micelles: (i) primary and secondary micelles by Carey and Small (1972); (ii) disk-like model by Kawamura et al. (1989), and (iii) helical shaped micelles by Giglio et al. (Conte, Di Blasi, Giglio, Parretta & Pavel, 1984; Esposito, Zanobi, Giglio, Pavel & Campbell, 1987; Esposito, Giglio, Pavel & Zanobi, 1987)). Reproduced from Warren, D.B., Chalmers, D.K., Hutchison, K., Dang, W., Pouton, C.W. *Colloids and Surfaces A: Physicochem. Eng. Aspects*, **280**, 182–193, 2006.

Kawamura, Murata, Yamaguchi, Igimi, Tanaka, Sugihara & Kratochvil (1989) have proposed a second model for bile salt structure based on electron spin resonance (ESR) studies of spin probe immobilization in the hydrophobic core of the micelle. They proposed a disk-like structure (Figure 1.7ii) which allowed for partitioning of the hydrophobic towards the centre of the micelle and

hydrophilic faces of the bile salts towards the aqueous phase. A third model has been put forward by Giglio and co-workers for the structure of NaDC micelles based on a combination of circular dichroism, small angle X-ray scattering, NMR and ESR studies of NaDC micelles (Conte, Di Blasi, Giglio, Parretta & Pavel, 1984; Esposito, Zanobi, Giglio, Pavel & Campbell, 1987; Esposito, Giglio, Pavel & Zanobi, 1987). In this model the deoxycholate anions are arranged in an elongated inverted helical micelle (Figure 1.7iii) where the hydrophobic face is on the outside of the micelle, with the centre filled with cations. The helix is stabilized by a combination of ion-ion, ion-dipole and hydrogen bond interactions. A recent molecular dynamics simulation of the self-association of several bile salts into micelles (Warren, Chalmers, Hutchison, Dang & Pouton, 2007) shows some features that are similar to the primary/secondary micelle model of Carey & Small (1972) and the disk model of Kawamura et al. (1989) but appear to rule out the inverted helix model of Giglio et al. (Conte, Di Blasi, Giglio, Parretta & Pavel, 1984; Esposito, Zanobi, Giglio, Pavel & Campbell, 1987; Esposito, Giglio, Pavel & Zanobi, 1987).

Surfactant micelles are characterized by two parameters; the critical micelle concentration (CMC) and the aggregation number. The CMC is a measure of the concentration at which micelles start to form in a surfactant solution, and is characterized by a change from free surfactant in solution to micellar aggregates. The aggregation number is a measure of the micelle size and gives the average number of surfactant molecules found in the micelle. Bile salts tend to form small micelles because their bifacial amphiphilic structure limits the number that can be packed the micelle. There is also some debate as to whether they have a single CMC, have two CMC's due to a two-stage self-association process or whether they do not have a CMC but exhibit a continuous self-association at all concentrations (O'Connor, Ch'ng & Wallace, 1983). Values for the CMC of bile salts which have been determined using various methods are available in the literature, although there is a lack of agreement between many of these due to differences in experimental methodology and/or difficulties in defining the CMC. Typical CMC and aggregation number values for some of the bile salts are given in Table 1.2.

1.3 Bile Salt Adsorption at Interfaces

Bile salts are amphiphiles and so will adsorb to an interface in such a way as to de-solvate the hydrophobic regions of the molecule. Their adsorption to a number of solid and liquid interfaces has been studied. Bile salt adsorption to solid cholesterol crystal-water and graphite-water interfaces has been measured as a method for determining the hydrophobicity of the molecule. The hydrophobicity is believed to be an indication of the affinity of the bile salt for the surface. The hydrophobicity of the following four bile salts was found to increase in the order NaDC > sodium chenodeoxycholate (NaCDC) >> sodium ursodeoxycholate (NaUDC) > NaC for adsorption on cholesterol crystals and NaDC > NaCDC >> NaC > NaUDC for adsorption on graphite (Sugihara, Hirashima, Lee, Nagadome, Takiguchi, Sasaki & Igimi, 1995; Sasaki, Igura, Miyassu, Lee, Nagadome, Takiguchi & Sugihara, 1995). Early studies of bile salts adsorption to the phospholipid bilayer-water interface suggested that they adopt a conformation where the sterol ring penetrates into the phospholipid phase with only the charged end group in the aqueous phase where it can be solvated by water molecules (Small, 1971). In this conformation the hydrophilic face of the bile salt is in a non-aqueous environment, and Small (1971) hypothesized that to stabilize this conformation the bile salts form dimers and trimers with intermolecular bonds between the hydroxyl groups on the sterol ring. This model for bile salt adsorption is supported by studies of their adsorption at the 1-octanol-water interface (Vadnere & Lindenbaum, 1982). In this study the results are interpreted as indicating the formation of reverse micelles at the 1-octanol-water interface, with the hydrophilic (hydroxyl) faces clustering together whilst exposing the hydrophobic phase to the non-aqueous (1-octanol) phase. A second model for bile salt adsorbed conformation has been proposed based on the bifacial amphiphilicity of the molecule (Ulmius, Lindblom, Wennerstrom, Johansson, Fontell, Soderman & Arvidson, 1982; Fahey, Carey & Donovan, 1995; Wenzel & Cammenga, 1998; Tiss, Ransac, Lengsfeld, Hadvary, Cagna & Verger, 2001).

Bile Salt	CMC (mM)	Aggregation Number
Cholate	4-20 ¹	3-8 ¹ 15-30 ² 3.09-3.35 ³
Deoxycholate	2-5 ¹	6-10 ¹
Taurocholate	3-5 ⁴ 8-12 ⁶	3 ⁵ 2.59 ³
Glycodeoxycholate	3.1 ⁴	
Glycocholate	3 ⁴	
Taurodeoxycholate		24 ³
Sodium dodecyl sulphate	8.27 ⁷	62 ⁸

Table 1.2 - Critical micelle concentration (CMC) and aggregation number for some conjugated and non-conjugated bile salts. The CMC and aggregation number for SDS (sodium dodecylsulphate) is shown for comparison. Data has been collected from the following references. ¹Maldonado-Valderrama et al., (2011), ²Lindman et al., (1980). ³Coello et al. (1996), ⁴Matsuoka et al. (2003), ⁵Mazer et al. (1979), ⁶Meyerhoffer & McGown (1990), ⁷Mandal, Ray & Moulik, (1980), ⁸Turro & Yekta, 1978.

In this the bile salt adsorbs flat at the interface with the methyl substituted hydrophobic face in contact with the oil phase and the hydroxyl substituted hydrophilic face in contact with the water phase where the hydroxyl groups can H-bond with the water. Measurements of the area occupied per bile salt molecule at the interface from surface tension studies have found that bile salts occupy about 1nm² of the interface per molecule. Welzel & Cammenga, (1998) found a value of 1.12 nm² for NaC and Tiss, Ransac, Lengsfeld, Hadvary, Cagna & Verger (2001) values of 1.03 and 1.15 nm² for NaDC and sodium taurodeoxycholate respectively. These are considered to be comparable to the area that a bile salt would occupy if adsorbed flat to the surface and thus support this model for bile salt adsorption.

1.4 Emulsion Formation and Stability

Emulsions are defined as a dispersion of droplets of one liquid phase dispersed in a second liquid continuous phase. In foods two forms of emulsion are found, oil droplets in water (oil-in-water emulsions, o/w) and water droplets in oil (water-in-oil emulsions, w/o). In food systems both types of emulsion can be found, although oil-in-water emulsions are more common. Examples of food emulsions include milk (o/w), cream (o/w), ice-cream (o/w), butter (w/o), margarine (w/o), sauces (o/w), soups (o/w), cake batter (o/w) and mayonnaise (o/w). Formation of emulsified oil droplets is advantageous to the food manufacturer because it allows oil to be mixed into water without it immediately separating. For a stable emulsion to form requires that the droplets are stabilized so that they do not separate into oil and water phases. In food emulsions, stability is imparted via a range of biomolecules that either adsorb to the surface of the droplet, or modify the viscosity of the aqueous phase to slow down separation.

Emulsions are formed by homogenization of oil and water in the presence of protein or other emulsifiers. The homogenization can be achieved using a number of methods including high pressure homogenization, high shear mixing or ultrasonic methods. A common industrial method is the use of the high pressure homogenizer. In this, oil, water and emulsifier are usually pre-mixed to form a coarse emulsion and then forced at high pressure through a narrow gap in the homogenizer valve (Figure 1.8), where the oil is subjected to intense shear and breaks up into small droplets. Then, surface active ingredients such as proteins or low molecular weight emulsifiers adsorb to the surface of the newly formed oil droplets and stabilize them. The size distribution of the emulsion droplets depends on a number of factors, but one of them is how quickly the protein emulsifiers are able to adsorb to the surface of the droplets. Rapid adsorption and stabilization prevents re-coalescence of the droplets, and leads to a smaller droplet size distribution and a more stable emulsion. The structure and stability of the adsorbed protein layer is critical to the stability of the emulsion droplet, and this will be discussed in more detail in a later section.

1.4.1 Protein Adsorption at Fluid Interfaces

When oil is dispersed in water in the absence of an emulsifier there is an unfavourable disruption of hydrogen bonding in the aqueous phase that leads to separation of the oil and water. When a protein adsorbs at an oil-water interface the water molecules in contact with the oil-surface are displaced back into the bulk aqueous phase where they can H-bond with other water molecules. This leads to a lowering of the free energy of mixing of oil and water, and partially offsets the unfavourable free energy of the oil-water interface.

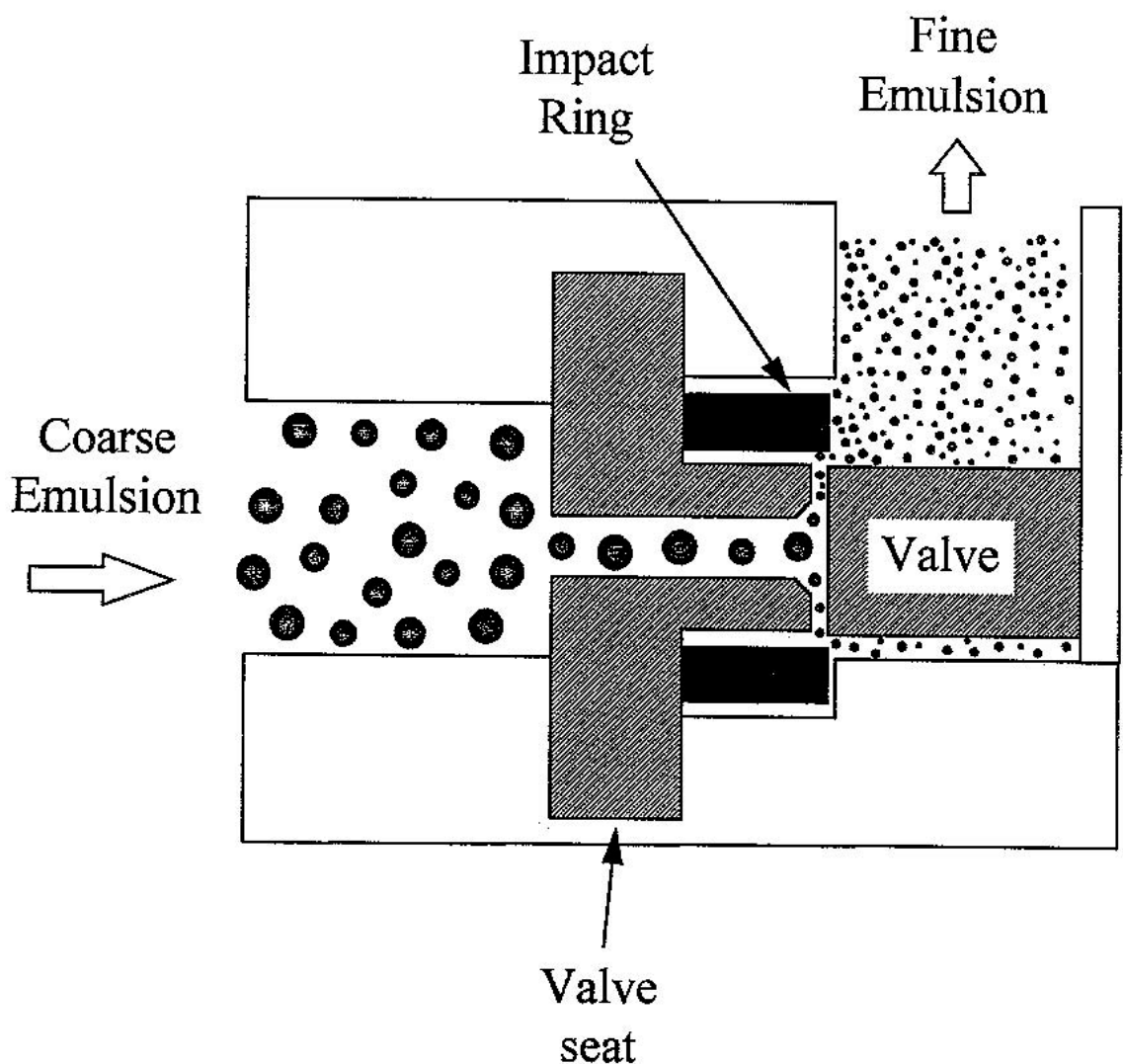


Figure 1.8 – Schematic drawing of the valve assembly of a high pressure homogenizer. As the coarse pre-mixed emulsion is forced at high pressure through the narrow gap of the valve, the fluid velocity increases and creates shear forces that break up the droplets into smaller sizes. Diagram reproduced from McClements (2005).

A further driving force for adsorption involves the hydrophobic amino acid side chains in proteins. When a protein adsorbs at a surface it adopts a conformation where hydrophobic amino acid side chains are oriented so they penetrate the oil side of the interface. Here they can get away from the vicinity of water molecules, and thus they do not disrupt H-bonding of water. This lowers the free energy of mixing further. Similarly, charged and polar amino acids orient themselves into the bulk water phase. Here they are solvated by water molecules, and although they disrupt H-bonding of water, they are able to form H-bonds with water that partially offsets the unfavourable breakage of water structure. The structure adopted by the protein at a surface is influenced by the native state of the protein in solution. In this study we use two protein products; sodium caseinate (SCN) and whey protein concentrate (WPC), that contain proteins that differ in their native state structure. Caseinate consists of four major proteins α_{s1} -, α_{s2} -, β - and κ -casein with a number of other minor constituents also present (Fox & Kelly, 2000). Whey protein concentrate consists mainly of β -lactoglobulin (49%), α -lactalbumin (19%), proteose peptone (20%), bovine serum albumin (6%) and immunoglobulins (8%) proteins (Kilhara & Vaghela, 2000).

Caseins are considered to belong to the class of proteins termed intrinsically disordered or unstructured proteins (Wright & Dyson, 1999). They contain relatively high proportions of the imino acid proline, which is known to disrupt the formation of structure in proteins. As a consequence they contain no tertiary structure and little secondary structure and adopt a random coil conformation in solution. When disordered proteins adsorb to an oil-water interface they spread rapidly at the surface and adopt an open train-loop-tail conformation where hydrophobic amino acids adsorb to the oil surface, with the hydrophobic side chains penetrating into the oil phase, whilst the polar and charged amino acids form tail and loop sections in the aqueous phase where they are able to interact with the water molecules (Figure 1.9). Globular proteins such as the whey protein β -lactoglobulin (β -lac) on the other hand have a compact highly ordered structure held together by a combination of disulphide bonds, hydrophobic association and hydrogen bonds. They possess a significant level of secondary structure (helices and sheets) which are folded to form a compact tertiary structure.

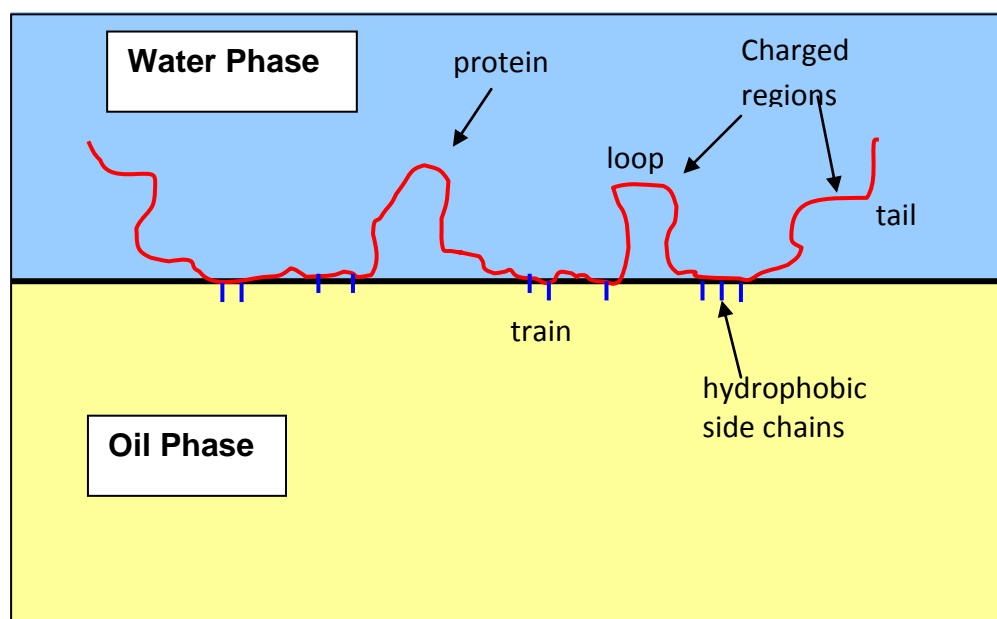


Figure 1.9 – Schematic drawing of the conformation of a disordered protein (e.g. a casein) adsorbed to the oil-water interface.

Often this tertiary structure defines the function of the molecule, as is the case with enzymes. For β -lac (Figure 1.10) the molecule contains a region of 8 β -sheets which are arranged into a β -barrel which defines the position of a binding pocket for hydrophobic ligands (Sawyer & Kontopidis, 2000). One of the suspected biological functions of β -lac is as a lipid transfer protein in the gut of the neonatal calf. When a globular protein adsorbs to an oil-water interface, initially the globular structure is maintained by the forces that stabilize the structure, but with time they will unfold and spread at the interface (Figure 1.11). The conformation adopted by the protein is also dependent on the surface concentration of the protein (Figure 1.12). At low surface concentrations there is more space per protein molecule. The disordered proteins will adopt flat, spread out conformations, whilst the globular proteins will unfold to differing degrees on the surface which depends on their conformational stability. As the concentration at the surface increases the ability of both disordered and globular proteins to spread is reduced. This results in the formation of more loop and tail regions for disordered protein, and less unfolding for the globular protein. At high surface concentrations disordered proteins form highly

extended adsorbed layers, whilst the conformation of globular proteins differ little from the native conformation in solution.

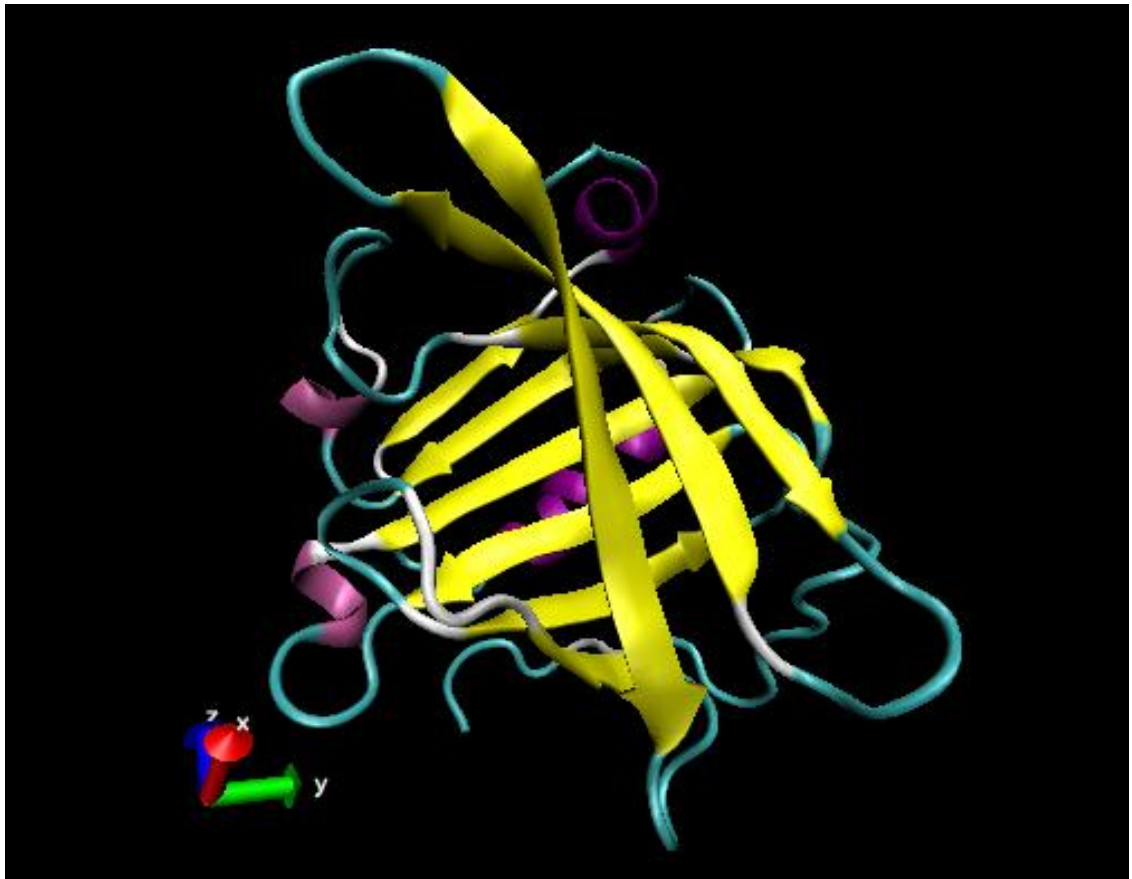


Figure 1.10 – Native conformation of β -lactoglobulin showing the secondary structure (helices = purple, β -sheet = yellow) and the folding of the β -sheet into a β -barrel.

As a result of this conformation the globular biopolymers tend to form relatively thin and compact membranes that have high viscoelasticity and are more resistant to rupture than those formed from random-coil proteins (Dickinson, Murray & Stainsby. 1988).

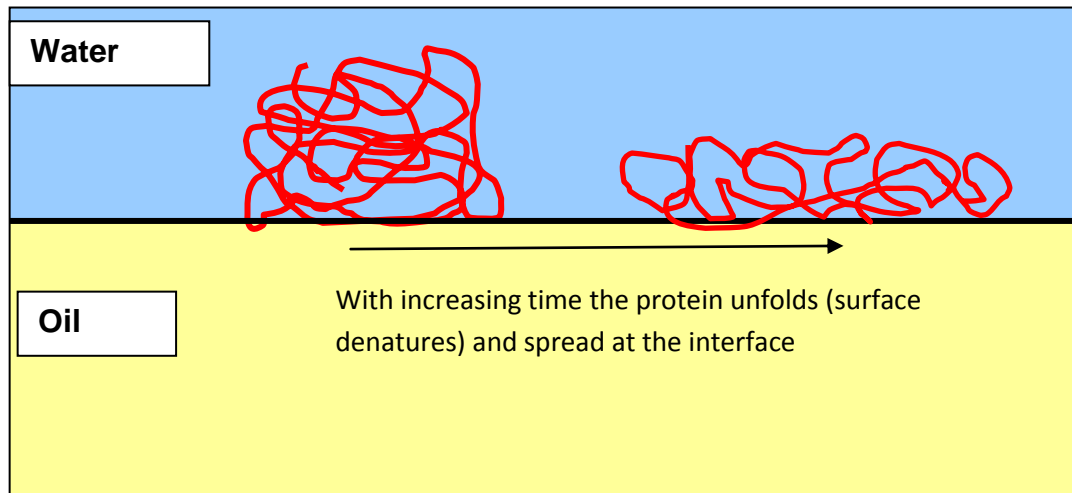


Figure 1.11 – Schematic drawing of the conformation adopted by a globular protein at the time of first adsorption and after a period of time at the interface when it has surface denatured and spread at the surface.

Proteins can stabilise emulsions through two mechanisms. They provide a steric stabilizing layer that prevents emulsion droplets joining together to phase separate, and if the protein is charged there will be electrostatic repulsion between two similarly charged droplets. These repulsive forces have to be balanced against the natural tendency for the atoms in all matter to be attracted to each other through van der Waals forces. It is possible to estimate the attractive and repulsive interactions between pairs of emulsion droplets and to plot this on an interaction energy vs distance diagram. Figure 1.13 is a schematic interaction energy curve for two emulsion droplets interacting via attractive van der Waals forces and repulsive steric interactions. A similar diagram can be drawn for charged emulsions that repel each other through electrostatic mechanisms. The mechanism of steric stabilization is complex. When two emulsion droplets that are surrounded by an adsorbed protein layer approach, the droplets will only repel each other when the two protein layers touch (Figure 1.13). The origin of this repulsive force can be explained in terms of two contributions.

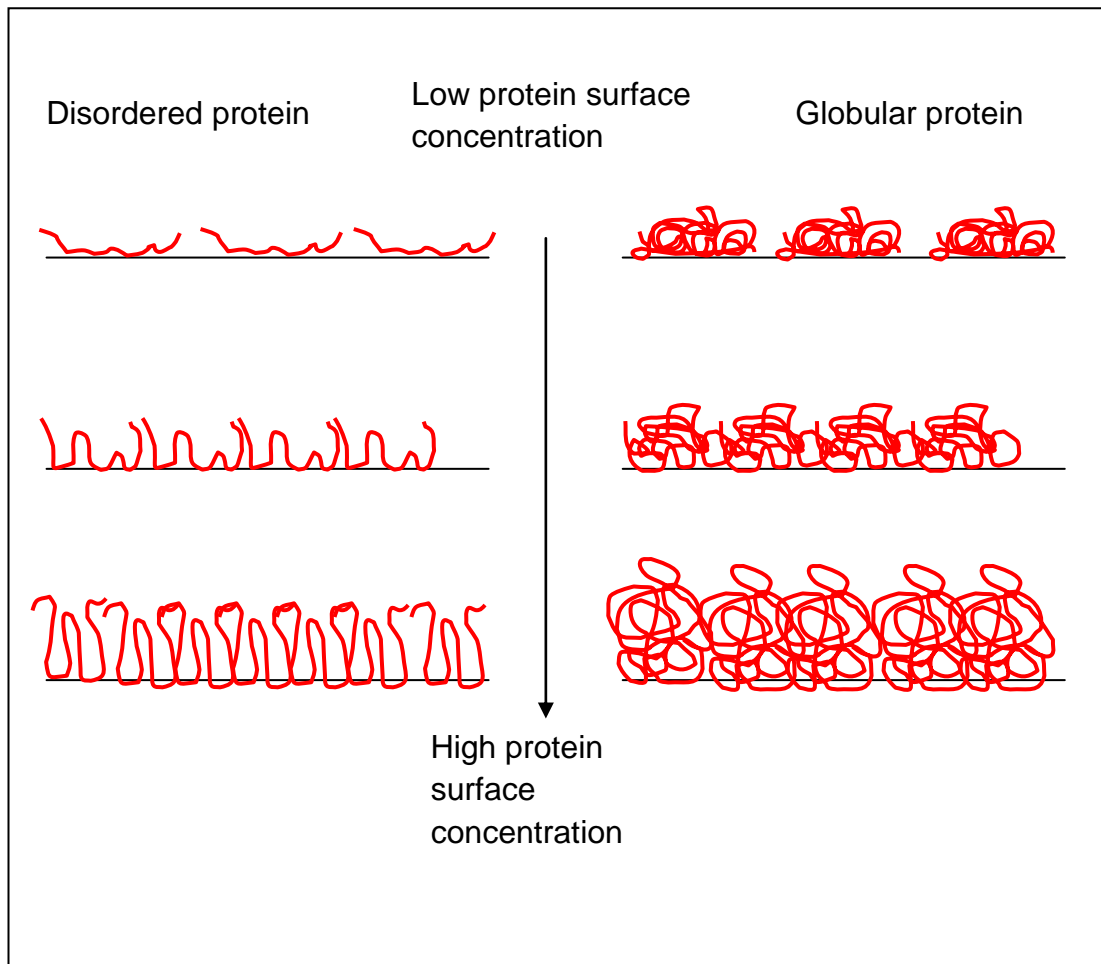


Figure 1.12 – Schematic picture of the conformations adopted by proteins at surfaces for different protein surface concentrations.

When the adsorbed protein layers touch they become compressed. This leads to a reduction in the volume in the adsorbed layer occupied by the protein. Since the protein has less space to occupy the number of conformations it can adopt is reduced and this leads to a decrease in the conformational entropy of the protein chain (Figure 1.14). This decrease in entropy is unfavourable and as a result there is an elastic repulsive force that opposes compression of the layers and pushes them apart.

Steric Repulsion

- Short-ranged

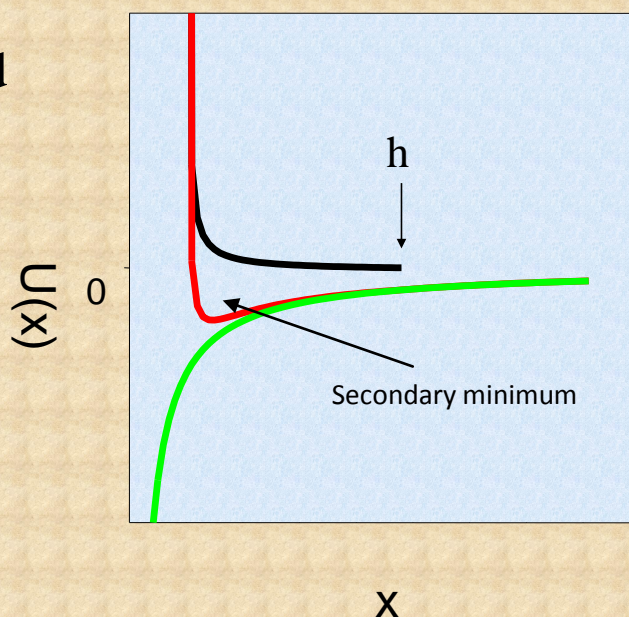
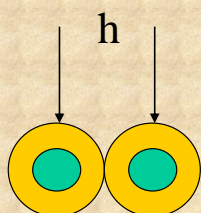


Figure 1.13 – Interaction energy ($U(x)$) vs separation diagram (x) for two emulsion droplets with a steric stabilizing layer. The van der Waals forces (green curve) are attractive and long ranged. The steric repulsive force (black curve) is short ranged and very strongly repulsive at short separations. The steric repulsion is only “felt” by the droplets when the two protein layers touch (a distance h apart). The net interaction energy (the red curve) sometimes has a small attractive secondary minimum where the emulsion droplets become loosely flocculated with each other (see section 1.4.3.1).

A second mechanism also plays a role in steric repulsion. Instead of becoming compressing the two protein layers may cross over and mix (Figure 1.15). Now there is an increase in the protein concentration in the overlap region. In this region the osmotic pressure is higher than in the surrounding solvent, and so there is an osmotic flow of water into the overlap region. This leads to an increase in the volume of the overlap region and the particles are forced apart. In reality both mechanisms will occur together at the same time, and the true situation will be a combination of entropy induced and mixed induced repulsion.

Compression of Adsorbed Layers

Decrease in volume occupied by protein = increased entropy

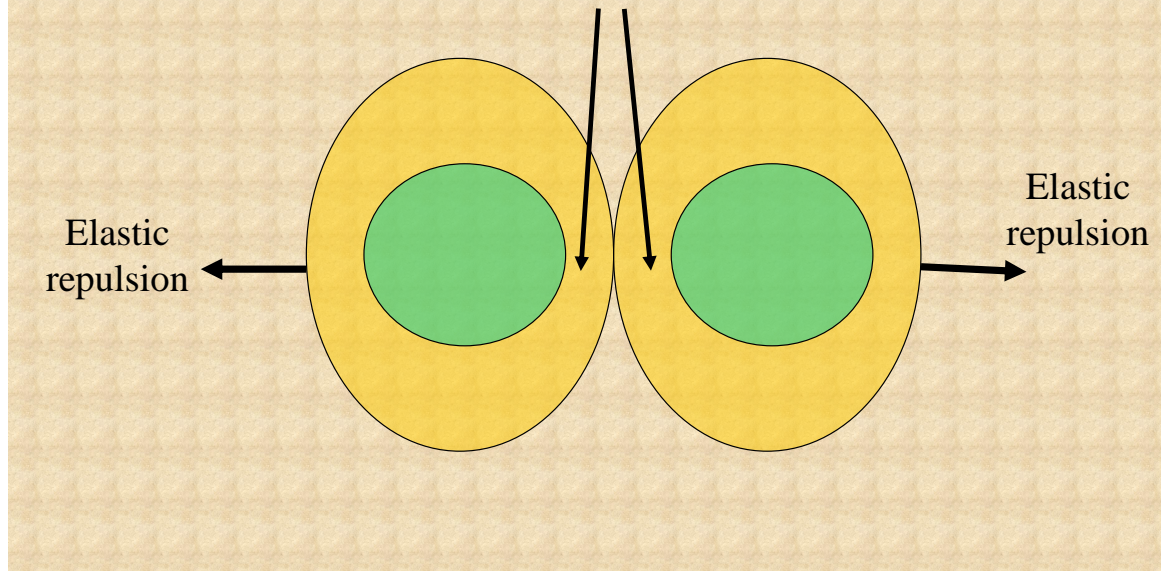


Figure 1.14 – Contribution to steric stabilization due to compression of adsorbed protein layer

1.4.2 Competitive Adsorption

The surface of an emulsion oil droplet in food systems is not stabilised by a single protein type. There are a number of types of surface active molecules found in foods, and these will all compete for area at the emulsion droplet surface with each other. The two main surface active molecules in food emulsions are proteins and low molecular weight surfactants, and there has been a great deal of study of the composition of mixed interfaces of these molecules.

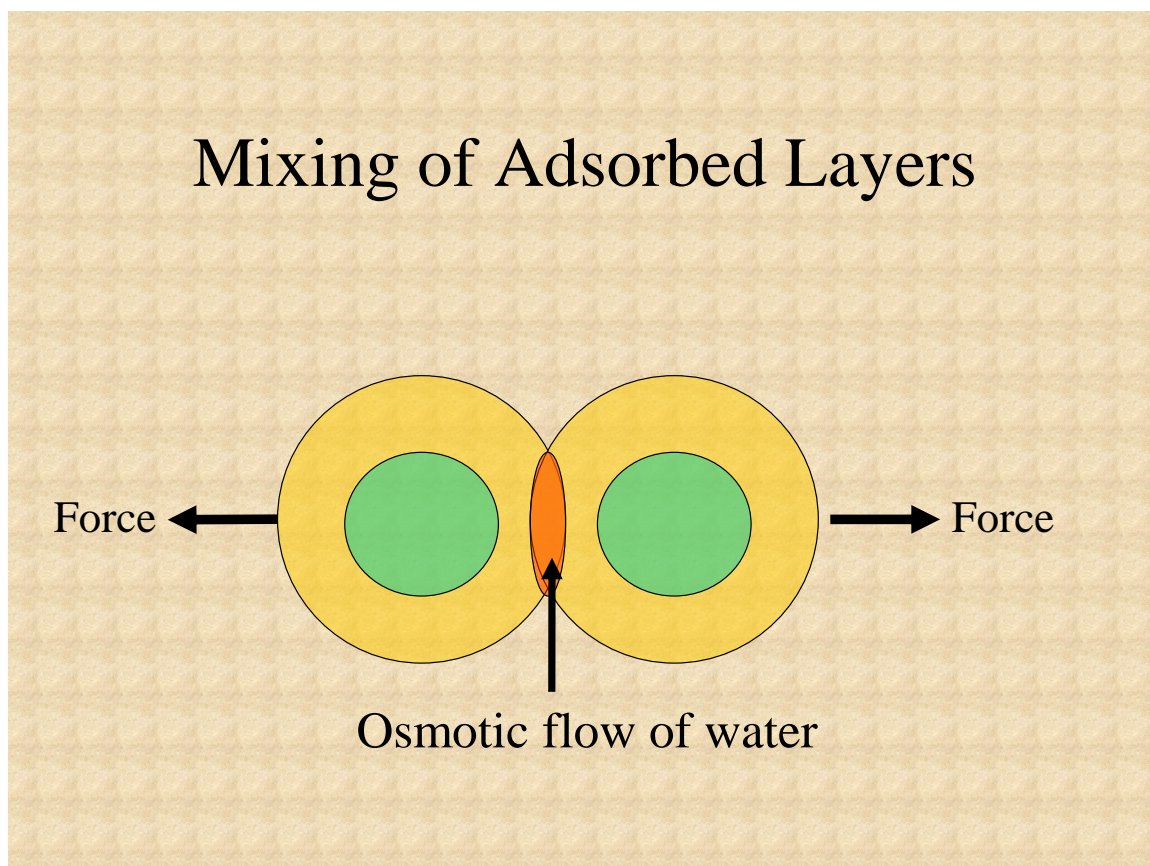


Figure 1.15 - Contribution to steric stabilization due to mixing of adsorbed protein layers.

1.4.2.1 Competitive Adsorption of Mixed Proteins

For the proteins used in this study (the caseins and whey proteins) it has been shown that emulsions made with equimolar mixtures of α_{s1} - and β -casein lead to adsorbed layers around the emulsion droplets that contain more β - than α_{s1} -casein (Dickinson, Rolfe & Dalgleish, 1988). When emulsions were made with either α_{s1} - or β -casein which were then mixed with a solution of the other second casein, exchange between the adsorbed protein and non-adsorbed protein occurred and some of the adsorbed protein was displaced from the droplet interface (Dickinson, Rolfe & Dalgleish, 1988). It was found however, that β -casein was always found at the interface at a higher concentration irrespective of whether it was used to make the initial emulsion or not. Dickinson et al. (1988) explained this as being due to the greater surface activity of the β -casein compared to the α_{s1} -casein. The whey proteins β -lac and α -lactalbumin (α -lac) have also been studied with respect to their competitive adsorption. The

ability of one whey proteins to displace the other is limited in extent, and the rate of exchange is slow compared to the rapid exchange seen with the caseins (Dickinson, Rolfe & Dalgleish, 1988). The whey proteins are also not able to displace β -casein from the oil droplet surface due to their lower surface activity, although β -casein is able to displace both the whey proteins (Dickinson, Rolfe & Dalgleish, 1990)

More recent studies on competitive adsorption in emulsions made with sodium caseinate or whey protein concentrate have confirmed the preferential adsorption of β -lac over α -lac, but have found that at caseinate concentrations greater than 2% (w/w) in the emulsion α_s -casein was found in a slightly higher proportion and β -casein a slightly lower proportion than was present in the caseinate powder (Ye, 2008).

1.4.2.2 Competitive Adsorption of Proteins and Surfactants

Proteins will also compete for interfacial area with low molecular weight surfactants. The concentration of protein required to saturate an oil-water interface is lower than is required for a low-molecular weight surfactant (Dickinson, 1993). As a result of this in mixed protein-surfactant layers the protein will predominate at low surfactant concentrations, but at higher concentrations more efficient surfactant packing at the surface will lead to a lower interfacial tension for the surfactant which will displace the protein from the interface (Nylander, Arnebrant, Bos & Wilde, 2008). The effectiveness of a surfactant at displacing a protein depends on a number of factors, including the relative hydrophobicity/hydrophilicity of the surfactant, whether they are charged or non-charged and whether or not they interact with the protein molecule at the interface. Water soluble surfactants, particularly non-ionic ones, are better than oil-soluble ones at displacing milk proteins from the emulsion oil droplet surface (Dickinson, 2001). Charged surfactants, such as the anionic SDS form interfacial complexes with protein and require higher surfactant concentrations than non-ionic surfactants for complete displacement of protein (Dickinson & Woskett, 1989). The mechanism of displacement of protein by surfactant has been studied extensively over the past fifteen years and has led to the now well accepted orogenic displacement model (Mackie, Gunning, Wilde & Morris,

1999). In orogenic displacement surfactants are believed to adsorb into gaps in the protein adsorbed layer. These act as nucleation points for surfactant domains which grow bigger as more surfactant adsorbs. The growing surfactant domains increase the surface pressure on the adsorbed protein such that it is forced to occupy an ever decreasing space at the interface. Eventually the layer is compressed to the point at which it fractures and the protein lifts away from the surface.

1.4.2.3 Competitive Adsorption of Bile Salts with Proteins

Bile salts are anionic surfactants, although with a more complex structure than most low molecular weight surfactants. Their adsorption at interfaces has already been discussed in section 1.3, but they have also been shown to compete for interfacial area with proteins and to displace the protein from the interface. The displacement of β -lac by NaTC and NaGDC has been studied using atomic force microscopy (Maldonado-Valderrama, Gunning, Wilde & Morris, 2010) and has been demonstrated to occur by the same orogenic mechanism. Euston et al. (Euston, Bellstedt, Schillbach & Hughes, 2011) have measured the degree of displacement of WPC by NaC and NaDC at the emulsion droplet interface. The degree of displacement is greater for the dihydroxy NaDC than the trihydroxy NaC, with this being explained by the greater hydrophobicity of the sterol ring of NaDC. Euston et al. (2011) also studied the adsorption of the NaC and NaDC molecules to a decane-water interface using molecular dynamics simulation. In this study they found that the neither the NaC nor NaDC molecules adopted a flat conformation at the interface, but both inserted the sterol ring into the decane phase. The sterol ring of the bile salts was oriented at an angle of about 49° to the normal of the interface, i.e. it was half way between an upright conformation and a flat conformation. In simulated concentrated bile salt layers, both the NaC and NaDC formed clusters at the interface that may have a reverse micelle structure. This structure is closer to that proposed by Small (1971).

1.4.3 Emulsion Instability

When oil and water are mixed they will rapidly de-mix unless they are stabilised in an emulsified form. By adsorbing protein to the surface of the emulsified oil droplets they are trapped in a kinetically stable state, and may remain in this state for some time. When formulating food emulsions, a more stable emulsion will give a longer shelf life to the product. Thus, an understanding of emulsion stability, the mechanisms of destabilization and how this can be influenced through emulsion formulation is critical for the food emulsion manufacturer.

There is always a tendency for oil and water to separate even when the oil is in an emulsified form. This occurs because of the effect that dispersed oil droplets have on the structure of water, where they disrupt the natural hydrogen-bonded network in liquid water. Emulsions destabilize via several mechanisms (McClements, 2005). Of these the main mechanisms observed in food emulsions are flocculation, coalescence, and creaming, with a fourth mechanism, Ostwald ripening being uncommon for triglyceride oil emulsions. Flocculation and creaming are reversible, the droplets only being loosely associated with each other, whilst coalescence is an irreversible merging of more than one droplet into a single droplet. Although the reversible forms of emulsion destabilization do not lead directly to an irreversible breakdown in emulsion structure, they are highly important because flocculated or creamed emulsions have a higher tendency to coalesce.

Coalescence of emulsions occurs when two emulsion droplets merge together to form a single droplet. The net result is that the average size of the droplets increases. Coalescence is important in food emulsions because once two or more droplets have joined together they cannot be separated without the input of extra energy (e.g. re-homogenization). Eventually, if coalescence proceeds far enough a separate oil and water phase can form.

Creaming represents the tendency for emulsion droplets to rise to the top of a container due to the effects of gravity. Emulsion droplets have a lower density than water and so will tend to move upwards under gravitational forces. The velocity (v_s) at which a droplet will rise can be estimated from Stokes law (equation 1.1),

$$v_s = \frac{2a^2 \Delta\rho g}{9\eta} \quad (1.1)$$

where a is the droplet radius, $\Delta\rho$ the density difference between the oil droplet and water, g the gravitational constant and η the viscosity of the aqueous phase. Stokes equation does make many simplifying assumptions that limit the accuracy of any predictions, but it does predict that smaller droplets will be more stable to creaming, and that increasing the viscosity of the aqueous phase decreases the rate of creaming. This explains why homogenized milk does not form a cream layer, and why polysaccharides thickeners are used as stabilisers in emulsion systems.

Flocculation in emulsions can come about through a number of mechanisms, namely secondary minimum flocculation, bridging flocculation and depletion flocculation. Secondary minimum flocculation occurs when the balance of attractive van der Waals forces and repulsion steric or electrostatic forces between two droplets is such that a weak attractive energy minimum occurs in the interaction energy plot for two interacting emulsion droplets (Figure 1.13).

Bridging flocculation (sometimes called homogenization clustering) occurs when emulsions are made with insufficient protein to fully stabilise the oil-water interface of the droplets. In this case the protein can adsorb to the surface of one or more droplet, and these droplets become joined together (Figure 1.16).

Depletion flocculation, on the other hand, is an osmotic effect that occurs in mixtures of particles and relatively large molecules such as polysaccharides. Polysaccharides of a particular size range can become excluded from the gap between two approaching emulsion droplets due to their large size (Figure 1.17). The osmotic pressure in this exclusion zone is then lower than in the rest of the aqueous phase, since osmotic pressure is proportional to the concentration of dispersed macromolecules. Therefore you get an osmotic flow of water out of the gap into the bulk of the aqueous phase. The result is a net force bringing the particles together.

If emulsion droplets are flocculated through any of the mechanisms above they are weakly held together in aggregates and can be re-dispersed by simple shaking or mixing.

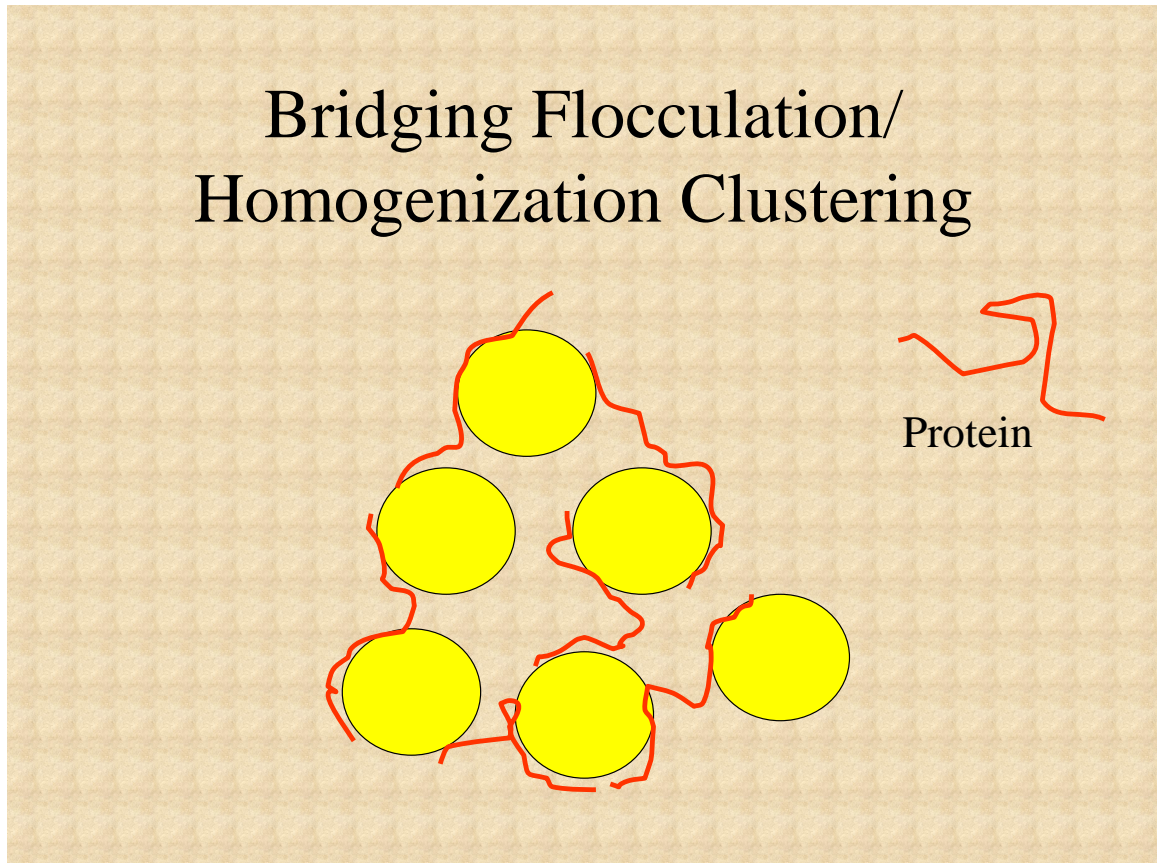


Figure 1.16 – Bridging flocculation in emulsions

However, in the flocculated form the aggregated droplets behave as if they are large particles, and thus they will cream more rapidly and rise to the top of the emulsion. This tends to give an increased rate of coalescence, simply because the droplets are physically much closer together in the flocculated and creamed states. So although flocculation in itself is not a major problem, it does accelerate the rate of irreversible coalescence and is therefore to be avoided if a stable emulsion is desired.

Depletion Flocculation

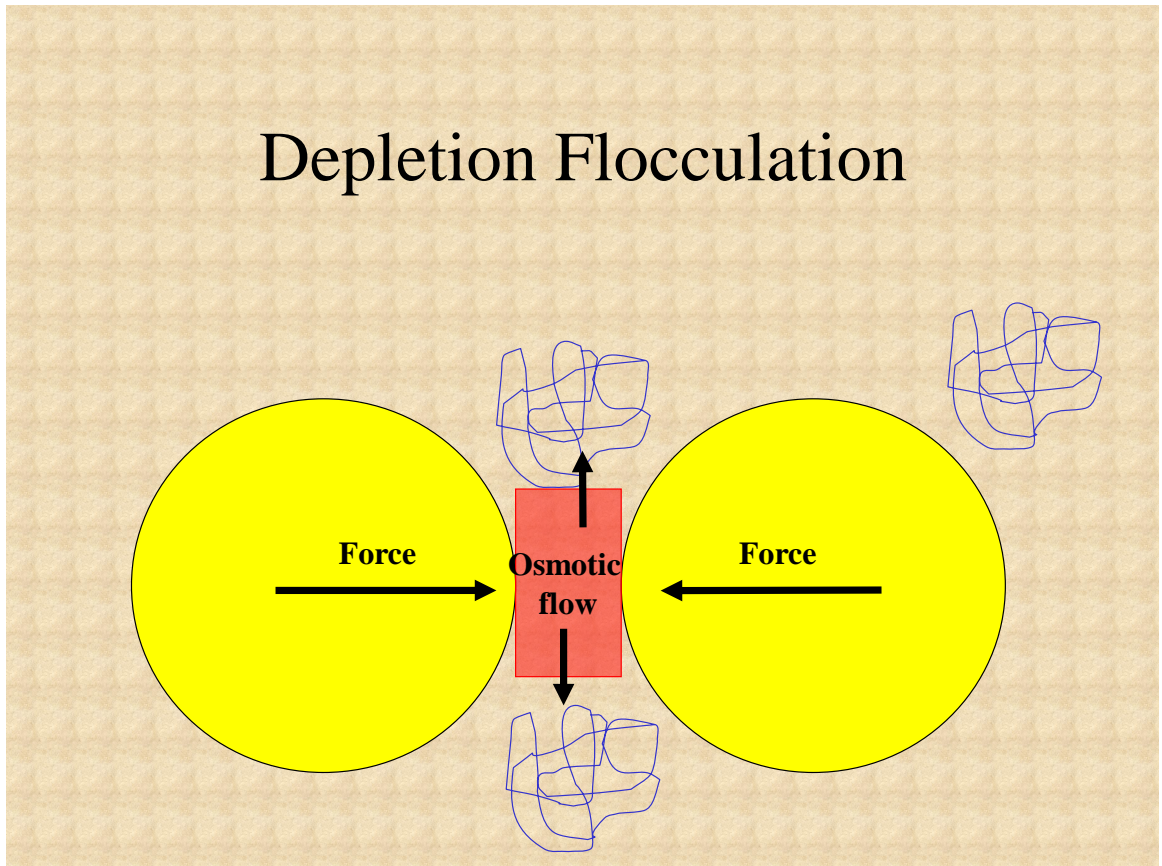


Figure 1.17 – Depletion flocculation in emulsions

1.4.3.1 Changes in Emulsion Stability during Digestion

Digestion is a complex process whereby ingested food is broken down into smaller molecules that are easily absorbed into the body. Digestion does not only take place in the digestive tract, but starts in the mouth where food is subjected to shear, and enzymes which cause structural and chemical changes in the food. For food emulsions these will affect the stability of the emulsion. Recent research has looked at how the structure and stability of protein stabilized oil-in-water emulsions changes when they are subjected to in vitro models for digestive tract conditions. The results of these studies are reviewed briefly in the following sections.

1.4.3.1.1 Changes in the Mouth

The first changes to emulsified fat droplets occur in the mouth, and are the result of chewing and subsequent mixing with saliva. The addition of saliva to oil-in-water emulsions stabilized by whey protein isolate and sodium caseinate has been shown to lead to two forms of destabilization. Flocculation of the fat droplets occurs and is thought to be due to the highly glycosylated, negatively charged mucins in saliva that are able to adsorb to and bridge between positively charged emulsion droplets (Vingerhoeds, Blijdenstein, Zoet, van Aken, 2005; Silletti, Vingerhoeds, Norde & van Aken, 2007a; Silletti, Vingerhoeds, Norde & van Aken, 2007b; Sarkar, Goh & Singh, 2009). Coalescence of fat droplets has also been observed for fat droplets in the mouth, which is thought to be caused by shear-induced interactions (Dresselhuis, de Hoog, Cohen Stuart, Vingerhoeds & van Aken, 2008).

1.4.3.1.2 Changes in the Stomach

After being swallowed the emulsified fat droplets enter the stomach. In the stomach the emulsified fat is subject to highly acidic conditions ranging from pH 1 to 3. In addition to being acidic the digestive juices also contain pepsin and gastric lipase enzymes, salts, and mucins. These conditions could affect the emulsion in a number of ways, for example hydrolysis of interfacial protein layers by pepsin, screening of interfacial charge by ions or alteration of charge balance through pH changes, and interactions between mucins and interfacial proteins (Singh & Sarkar, 2011).

Studies have shown that for lactoferrin and β -lactoglobulin-stabilised emulsions (Sarkar, Goh, Singh & Singh, 2009) pepsin hydrolysis of the adsorbed protein layer is the main driving force in destabilization of emulsified fat droplets. Hydrolysis of the protein layer by pepsin causes flocculation and some coalescence of fat droplets, which is most likely caused by a loss of positive charge on the droplet surface and weakening of the adsorbed layer. The peptides that remain at the interface are unable to provide sufficient electrostatic repulsions and/or steric effects. (Sarkar, Goh, Singh & Singh, 2009) Furthermore it was also found that the action of pepsin in hydrolyzing the adsorbed protein layer was accelerated in the presence of high salt

concentrations (Sarkar, Goh & Singh, 2010). Although β -lactoglobulin is resistant to pepsin attack in its native state in aqueous solutions it is susceptible when it is adsorbed to the oil droplets of an emulsion, presumably because the adsorbed conformation is more open than the native state which exposes the peptic cleavage sites for proteolysis, (Sarkar, Goh, Singh & Singh, 2009) In another study of the gastric digestibility of β -casein and β -lactoglobulin stabilized emulsions it was found that β -casein digested twice as fast as β -lactoglobulin, which caused great instability of the emulsion. (Macierzanka, Sancho, Mills, Rigby & Mackie, 2009) Again, this is most likely explained by the differences in the interfacial conformation adopted by the disordered casein and the globular β -lactoglobulin.

1.4.3.1.3 Changes in the Small Intestine

After passing through the stomach the emulsion enters the small intestine. It is here where the gall bladder excretes digestive enzymes and bile via the bile duct into the duodenum. In the duodenum the majority of the fat is digested and absorbed. Bile salts play a major role in this process. The digestibilities of emulsions stabilized by β -casein or β -lactoglobulin have been compared using *in vitro* gastrointestinal models with and without the addition of bile salts and phosphatidyl choline (Macierzanka, Sancho, Mills, Rigby & Mackie, 2009). β -lactoglobulin stabilized emulsions were found to be destabilized primarily by competitive adsorption between the protein and bile salts and or phosphatidyl choline while the primary destabilizer of β -casein emulsions was gastric hydrolysis of the adsorbed casein layer by pepsin (Macierzanka, Sancho, Mills, Rigby & Mackie, 2009). From a separate study of competitive adsorption of β -lactoglobulin and bile salts at the air-water and oil-water interfaces using *in vitro* duodenal model it was concluded that β -lactoglobulin was completely displaced by the bile salts (Maldonado-Valderrama, Woodward, Gunning, Ridout, Husband, Mackie, Morris, & Wilde, 2008).

In addition to measuring the breakdown of β -casein and β -lactoglobulin stabilized emulsions during digestion there have been a number of studies conducted to better understand how the interface of emulsions affects the rate of lipid digestion. (Mun, Decker, Park, Weiss & McClements, 2006; Mun, Decker & McClements, 2007; Gargouri, Julien, Bois, Verger & Sarda 1983; Armand, Borel, Ythier, Dutot, Melin & Senft, et al., 1992) These studies used

pancreatic lipase in *in vitro* intestinal models and found that the amount of fatty acid after 2 hours of hydrolysis of whey and caseinate stabilized emulsions were similar; however the whey stabilized emulsions were more unstable. With the addition of bile salts to the same model system lipid hydrolysis is greatly increased. (Mun, Decker & McClements, 2007) Protonated free fatty acids released during hydrolysis limit the amount of lipid digestion at high concentrations. It was concluded that surface active monoglycerides and diglycerides played a major role in limiting lipid digestion as they would displace bile salts and other surface active molecules from the droplet surface. Another study found that lactoferrin- and β -lactoglobulin-stabilised emulsions underwent a significant degree of coalescence on the addition of physiological concentrations of pancreatin and bile salts. (Sarkar, Horne & Singh, 2010)

CHAPTER 2 - MATERIALS & METHODS

2.1 Materials

The whey protein concentrate was a gift from Arla Foods, Denmark, and the sodium caseinate a gift from Kerry Ingredients, Ireland. The protein content of the WPC and SCN were measured by Kjeldahl nitrogen analysis and found to be 87% and 91% (dry weight basis) respectively. Vegetable oil (rapeseed oil) was purchased from a local supermarket and used un-purified. Bile salts (NaC, NaDC, NaTC and NaGDC), porcine lipase and acetone were purchased from Sigma Aldrich Chemical Company, Poole, Dorset. All chemicals were Analar grade.

2.2 Methods

All experiments were repeated in triplicate.

2.2.1 Preparation of Oil-in-Water-Emulsions

Oil-in-water emulsions were made at a total protein content of 1% (w/w), 20% oil (w/w) using the following procedure. Protein (5.75 g of WPC or 5.55g SCN, sufficient to give 1% (w/w) protein in the final emulsion) was added to 394.25g of Milli-Q water, and dissolved by stirring for 30 mins at room temperature. One hundred grams of vegetable oil was added to the protein solution and the solution was mixed for 30 seconds using an Ultra Turrax high speed mixer (IKA, Germany) to produce a coarse emulsion. The pre-mixed emulsion was passed through a high pressure valve-type APV 2000 homogenizer (APV, Denmark) at 200 bar pressure, and recycled for 10 minutes to ensure complete homogenization of the oil phase. The particle size distribution of the oil droplets in the emulsion was measured using a Malvern Mastersizer 2000 (Malvern Instruments, Malvern, UK), assuming an oil-phase refractive index of 1.456, an aqueous phase refractive index of 1.33 and an oil droplet absorbance of 0.00.

2.1.2 Preparation of Bile Salt Solutions

Bile salt solutions of concentration ranging from 0.2 to 2.0% (w/v) were made up in 25mL volumetric flasks using milli-Q water. Milli-Q water was used instead of a buffer due to the finding in previous studies (Euston, Bellstedt, Schillbach & Hughes, 2011) that NaDC had a tendency to precipitate in the presence of buffer salts. Four bile salts were used in this project: NaC, NaTC, NaDC, and

NaGDC. To determine the competitive adsorption of bile salts with proteins at the oil-water emulsion droplet interface the following procedure was carried out.

2.1.3 Competitive Adsorption of Bile Salts and Protein

Twenty-five mL of emulsion and 25mL of the bile salt solution were mixed together and allowed to stand for 2 hours. This gave a final bile salt concentration in the emulsions in the range 0.1-1.0% (w/w) and a final emulsion protein and fat content of 0.5% and 10% respectively. Previous studies had shown that the displacement of protein by NaC and NaDC is complete by 2 hours (Euston, Bellstedt, Schillbach & Hughes, 2011). The mixture of bile salt solution and emulsion were then centrifuged in a Beckman Avanti J26-XP centrifuge at 25000 rpm, 4 °C using a JA25.50 rotor (Beckman-Coulter, High Wycombe, UK) which separates the oil phase droplets from the aqueous phase. The aqueous phase supernatant was removed from the centrifuge tube and the protein content in the aqueous phase of the emulsion determined by Kjeldahl nitrogen analysis. The protein adsorbed to the oil-droplet surface was then calculated as the difference between the total protein added and that in the aqueous phase. The surface coverage was calculated from the original protein content of the emulsion, the mean emulsion droplet diameter ($d_{3,2}$), and the adsorbed protein content using equation (1) below.

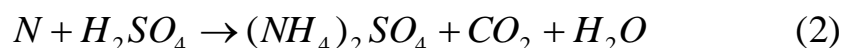
$$\frac{c_{\text{total}} - c_{\text{aqueous}}}{\rho_{\text{oil}} - \rho_{\text{aqueous}}} \Phi_{\text{oil}} = \frac{4}{3} \pi d_{3,2}^2 \Gamma$$

In this equation c_{total} and c_{aqueous} are the total protein concentration of the emulsion and the concentration of protein in the centrifugally separated aqueous phase, ρ_{oil} and ρ_{aqueous} the densities of the oil and aqueous phase, Φ_{oil} the mass fraction of oil in the emulsions and $d_{3,2}$ the surface mean diameter of the oil droplets obtained from the Malvern Mastersizer.

2.1.4 Determination of Crude Protein by Kjeldahl Analysis

Ten mL of aqueous subphase from the centrifugally separated emulsions was digested by boiling the sample in concentrated sulphuric acid in the presence of

a selenium catalyst. This converts the nitrogen in the protein to ammonium sulphate. The unbalanced reaction equation for this is;



Excess NaOH is added to the digested protein sample which releases ammonia from the ammonium sulphate i.e.,



The ammonia is recovered from the digestion by distillation of the sample into a boric acid solution. The ammonia is trapped as a complex with the boric acid, and partially neutralises the acid;



An indicator is used which changes colour from grey to blue due to the partial neutralization of the acid. The solution is then titrated with sulphuric acid until the blue colour of the indicator returns. The volume of sulphuric acid required for this change to occur is related to the nitrogen content and hence the protein content using the equation;

$$\% Nitrogen = \frac{Titre \text{ in mL} \times 14.007 \times 100}{mg \text{ of Sample}} \quad (5)$$

For conversion of % nitrogen to % protein the % nitrogen is multiplied by a conversion factor equal to 6.38, which reflects the proportion of nitrogen in the protein.

2.1.5 Determination of Bile Salt Surface Coverage in Emulsions

The bile salt concentration in the aqueous phase was determined using a colorimetric method proposed by Urbani & Warne (2005). This method involves dehydration of the ring hydroxyl groups followed by rearrangement to a yellow coloured pigment (Fini, Fazio, Tonelli, Roda & Zuman, 1992) that can be

determined spectrophotometrically by measuring absorbance at 389nm. A generalised reaction scheme for the formation of the yellow coloured product from sodium cholates is shown in Figure 2.1.

Standard curves of absorbance at 389nm against bile salt concentration were made using the following procedure. Solutions of each bile salt in milli-Q water were made up in the concentration range 0 – 0.05% (w/w) at 0.005% intervals for NaC and NaTC and 0 - 1.0% (w/w) (0.1% intervals) for NaDC and NaGDC. The solutions were made in triplicate. Then 50µL of the bile salt solution was reacted with 800 µL of concentrated sulphuric acid in a 1mL Eppendorf tube in a fume cupboard. The mixed bile salt acid solution was left to stand for 30 mins to allow time for the reaction to occur and the yellow colour to develop, after which the absorbance was measured at 389nm for each replicate using a Genesys 6 UV/Vis spectrophotometer (Thermo Scientific, UK). The standard curves obtained from these measurements are shown in Figures 2.2-2.5. Initially we made up NaDC and NaGDC solutions in the same concentration range as for NaC and NaTC. The absorbance of these solutions was, however, low and as a consequence we repeated the standard curve at twenty times higher concentration to get the standard curves in Figures 2.3 and 2.5. The standard curves of the NaC and NaTC (Figures 2.2 and 2.4) were very similar, as were those of NaDC and NaGDC (Figures 2.3 and 2.5).

To try to understand what was causing this difference in the absorbance of the NaC/NaTC and NaDC/NaGDC the adsorption spectra of NaC and NaDC were measured using a scanning spectrophotometer (Figure 2.6). The spectrum for NaDC was shifted such that the maximum absorbance was no longer at 389 nm but was closer to 300nm. This was most probably due to differences in the structure of the complex formed for trihydroxy bile salts (NaC and NaTC) and for dihydroxy bile salts (NaDC and NaGDC). The reaction between bile salts and sulphuric acid is believed to start on the hydroxyl groups attached to the sterol ring. The hydroxyl groups are first protonated (Fig. 2.1b) and then dehydrated to form a carbocation (Fig. 2.1c). The carbocation then dissociates to form an olefin (Fig. 2.1d). NaC has three hydroxyl groups, and can form a trienylic carbocation and subsequently an olefin with 3 double bonds. Dihydroxy bile salts such as NaDC can only form olefins with 2 double bonds. It is likely that these will rearrange to give molecules with conjugated double bonds that are

coloured. Since NaC and NaDC will form coloured compounds with differing numbers of double bonds there adsorption spectra will be different.

With hindsight, it would have been better to have used 300 nm as the absorbance wavelength for NaDC (and probably also for NaGDC) for determination of the concentration in the emulsion. However, this experiment was carried out after the bile salt concentration in the emulsions had been determined and insufficient time was available to repeat the experiments with a different absorption wavelength. It is believed that the results for concentration of the NaDC and NaGDC are still valid since a linear relationship between concentration and absorbance was observed. It is considered, however, that the larger error bars seen in the NaDC and NaGDC standard curves are most likely due to the measurements not being carried out at an optimal absorbance wavelength.

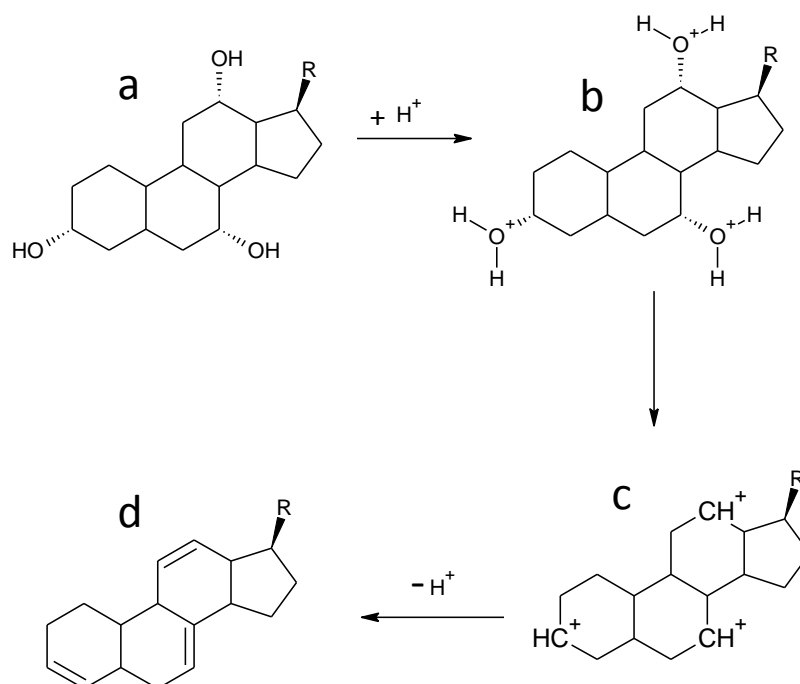


Figure 2.1 – Reaction of NaC with concentrated sulphuric acid.

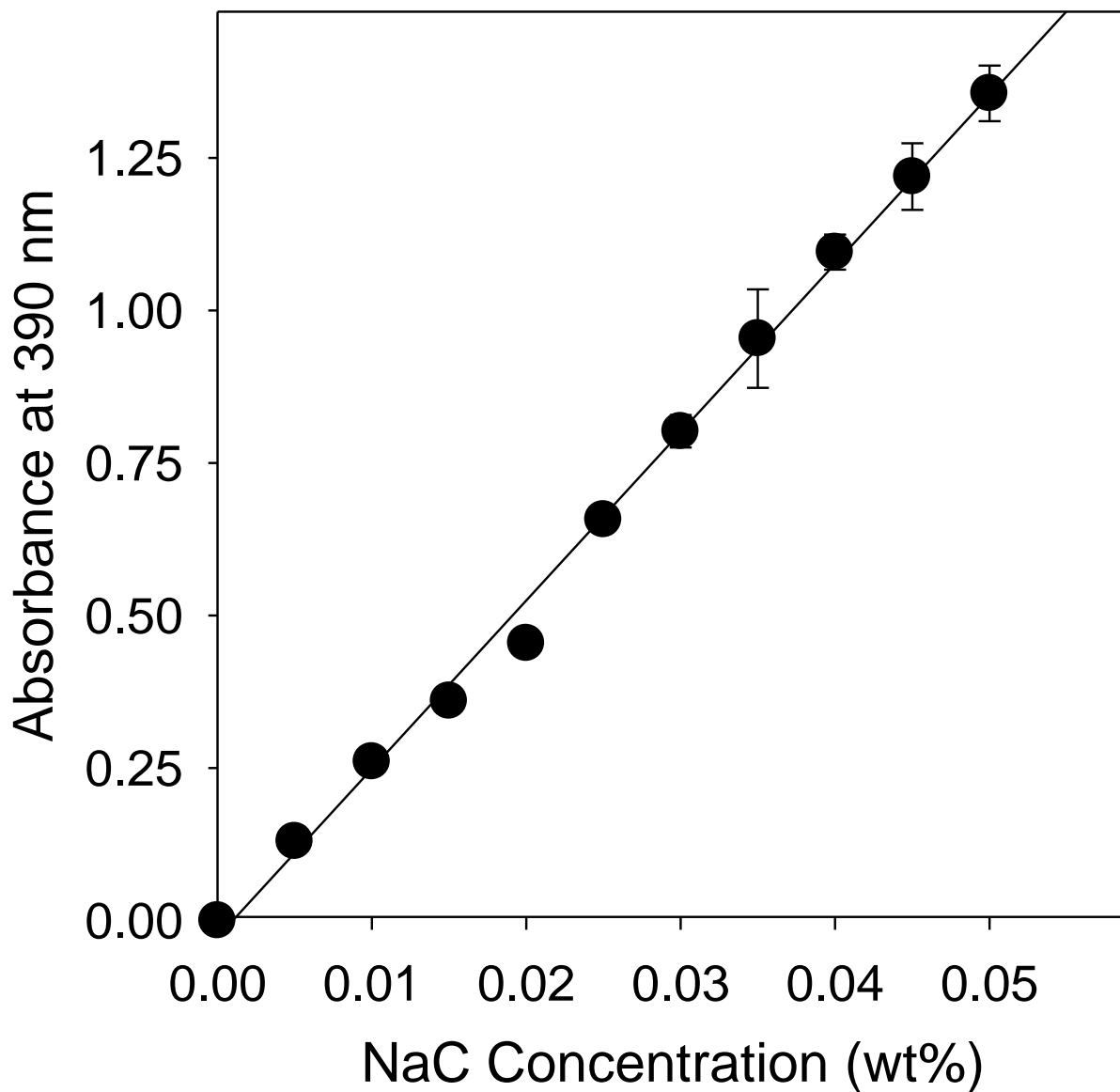


Figure 2.2 – Standard curve for NaC absorbance against concentration plotted over the linear range.

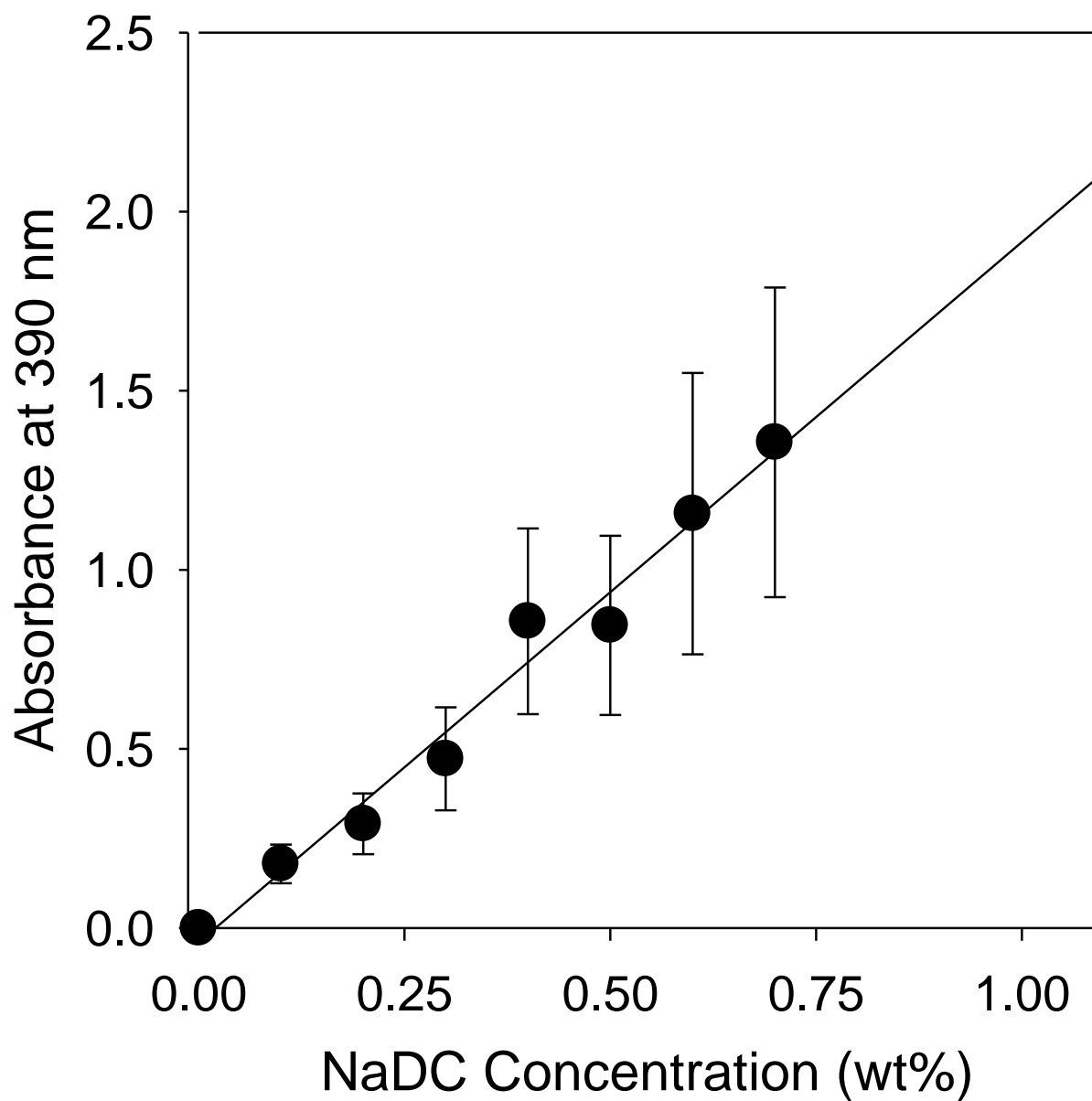


Figure 2.3 - Standard curve for NaDC absorbance against concentration plotted over the linear range.

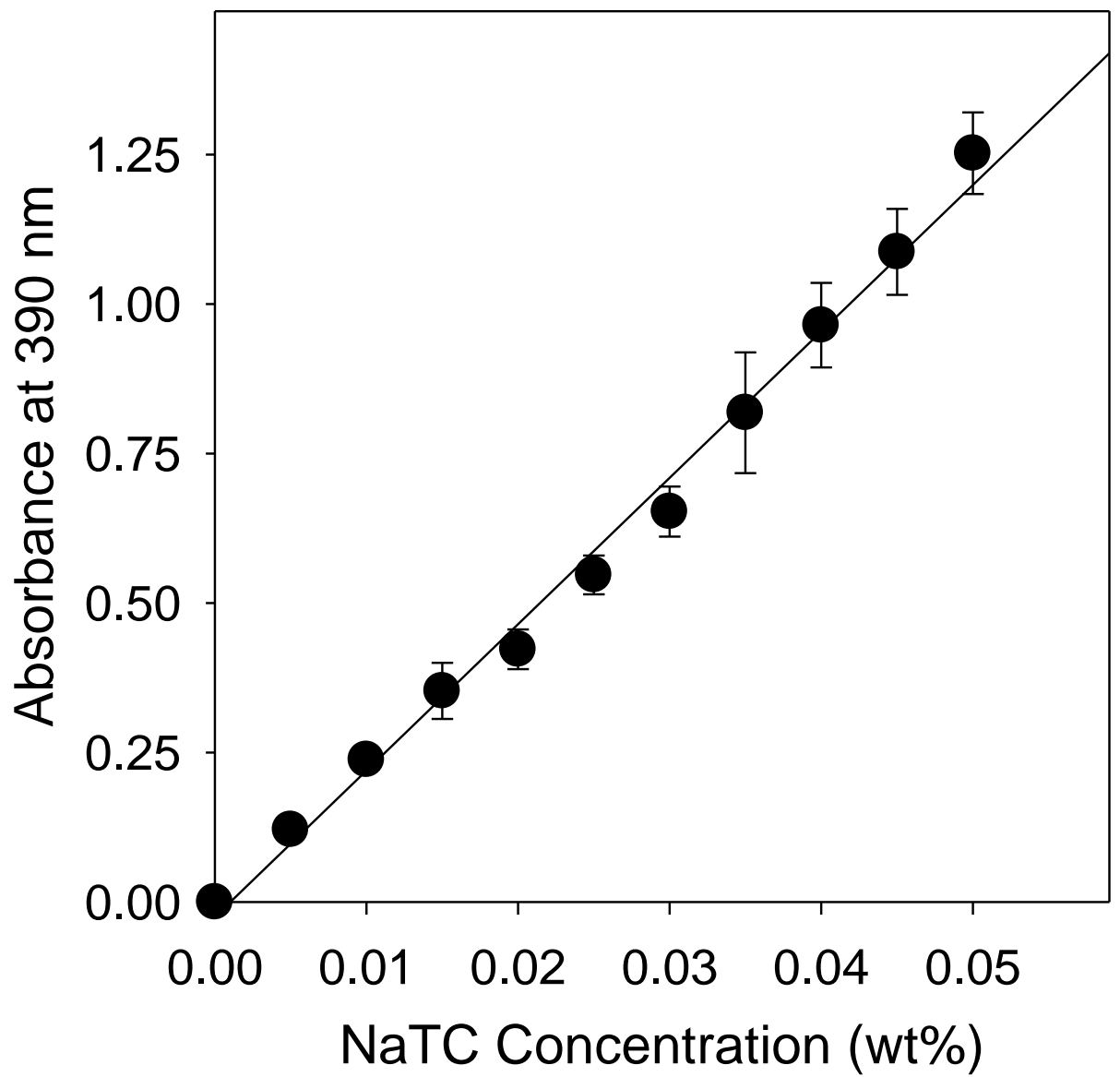


Figure 2.4 - Standard curve for NaTC absorbance against concentration plotted over the linear range.

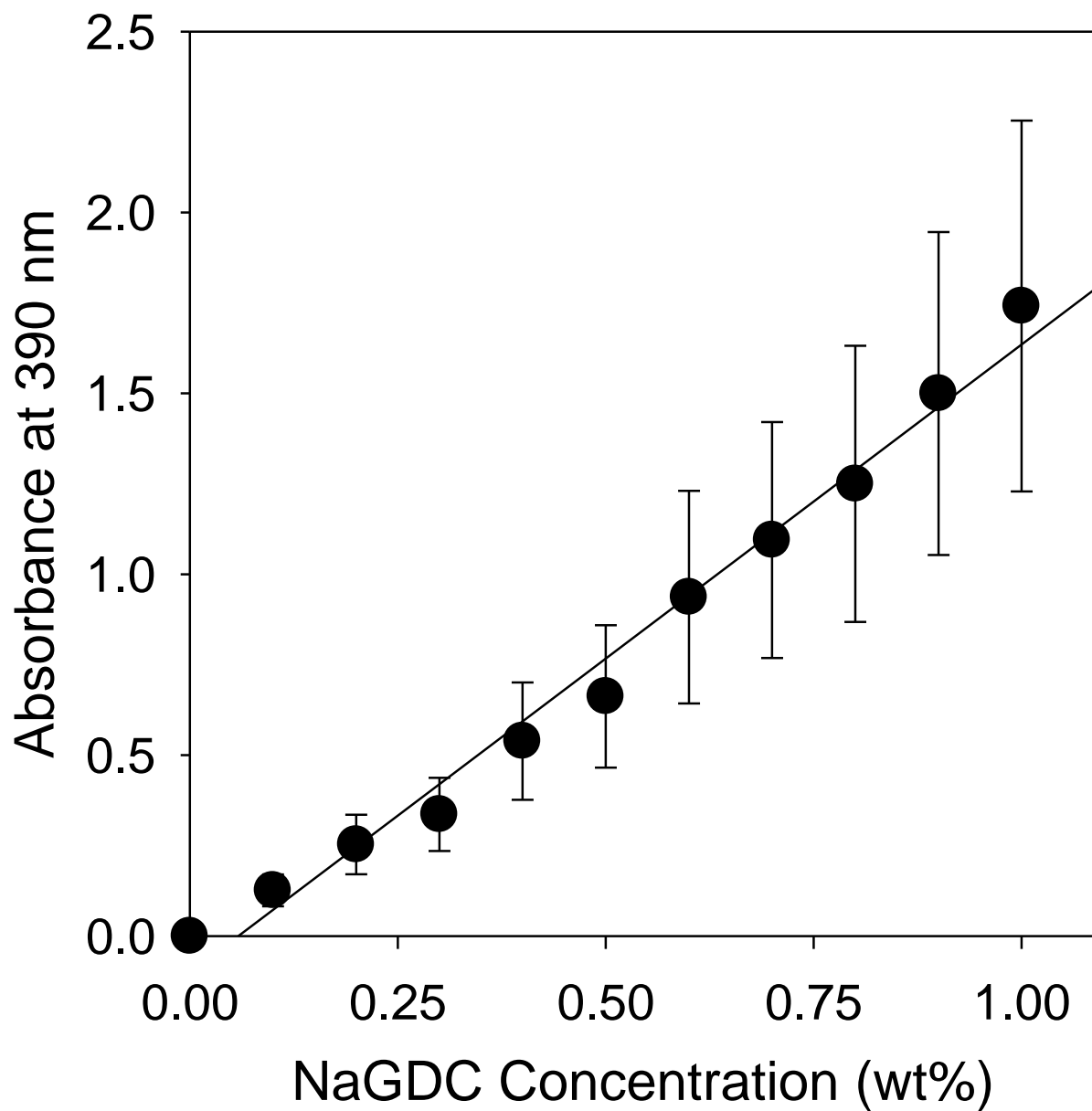


Figure 2.5 - Standard curve for NaGDC absorbance against concentration plotted over the linear range.

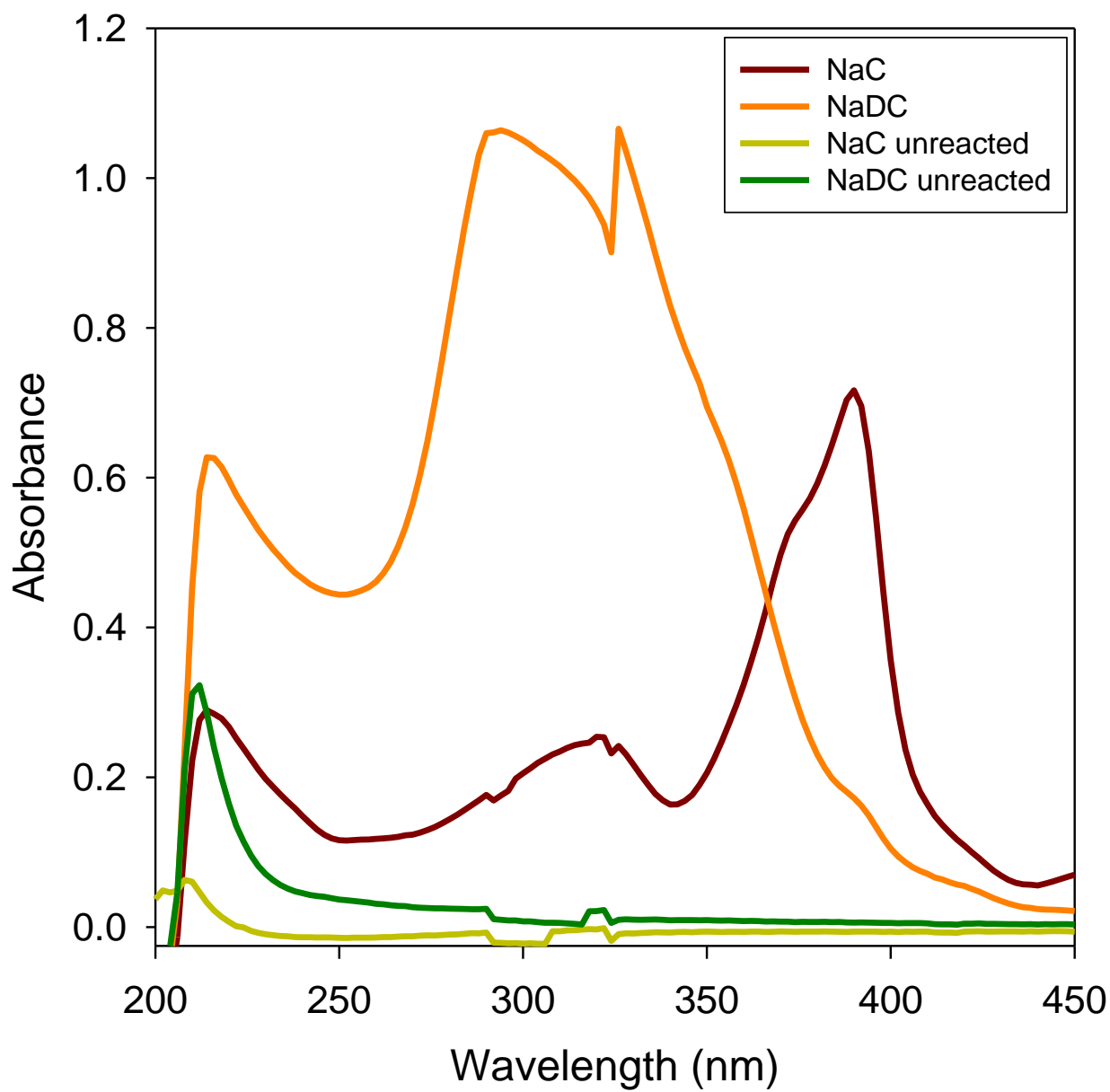


Figure 2.6 – UV-Visible Spectra for reacted and un-reacted NaC and NaDC.

To determine the surface coverage of bile salts at the emulsion droplet interface the following procedure was used. One mL of the aqueous subphase from NaC and NaTC containing emulsions, from the same centrifugally separated emulsions as used to determine protein surface coverage, was diluted to 50mL with Milli-Q water in a volumetric flask. This was found to give bile salt concentrations in the linear range of the standard curves for these two bile salts. For NaDC and NaGDC no dilution of the aqueous subphase was required. Then 50ul of the diluted emulsion aqueous phase was reacted with 800 uL of concentrated sulphuric acid, left to stand for 30 mins and the absorbance measured at 389nm. The bile salt concentration in the aqueous phase sample was determined by comparison to the appropriate standard curve of the bile salt used, and by taking account of any dilution of the aqueous phase that was required. From this the concentration of surface adsorbed bile salt was determined by difference and the surface coverage calculated using equation (1) above.

The centrifugal separation of the aqueous phase of the emulsions usually leaves some residual very small emulsion droplets in suspension. In addition, the aqueous phase will also contain residual protein that is not adsorbed to the oil droplet surface. Therefore we carried out few preliminary experiments to verify that protein did not adsorb in the same region as the coloured sulphonated bile salt derivatives and that the small oil droplets did not scatter light and interfere with the absorbance measurements. To determine the effect of protein on the bile salt determination a solution of 1% (w/w) WPC or SCN protein in Milli-Q water was made and diluted into 5 samples ranging from 0.1% protein to 0.5% protein. Fifty μ L samples of the protein solutions were mixed with 800 μ L of sulphuric acid, left to react for 30 mins and the absorbance measured at 389nm. The absorbance values were close enough to zero that it was determined that the effect of protein would be insignificant when measuring absorbance for the bile salt solutions. To determine whether protein would interfere with the determination of bile salts concentration, the test was repeated with the same concentrations of protein but a known concentration of NaC was added to the mixture. The absorbance values for each of the samples were in the expected range of values for the NaC concentration on the standard curve, and we concluded from this that any interactions between the protein and NaC

that might occur did not interfere with the bile salt assay. Finally, to test if the small residual oil droplets would interfere with the determination of bile salt concentration the following experiments were carried out. A 0.1% (w/w) NaC solution was made and this was used to make 5 diluted samples ranging from 0.01% to 0.05% (w/w) concentration of NaC. The bile salt solution was diluted with the aqueous phase of an emulsion (not containing bile salt) that had been centrifuged. These samples were then reacted with sulphuric acid and the absorbance measured at 389nm. The concentration of NaC determined from the standard curve was within statistical limits for the method, and we concluded that the emulsion droplets did not interfere with the bile salt assay.

2.1.6 Effect of Bile Salt on Lipase Activity in Emulsions

In order to further understand the role of bile salt in the digestion of fat an experiment was carried out to determine the effect of changing bile salt concentration on lipase activity. WPC emulsions were made using the method detailed in section 2.1.1. Emulsion samples were made by first diluting 15mL of WPC emulsion with 15mL of milli-Q water in 100mL conical flasks. The pH of the emulsion samples was then adjusted to pH 7 using 1 M NaOH. A solution containing 60 mg pancreatic lipase, a known mass of NaC and 7.5 mL of milli-Q water was added to each sample. Two sets of replicates were prepared from duplicate emulsions. The samples were placed immediately in a shaking water bath at 37 °C. Flasks were removed at time periods of 10 min, 20 min, 30 min, 1 hour, 1.5 hours, and 2 hours. After removal of an emulsion sample from the water bath, 5mL of acetone was added to the sample to stop the lipase reaction. Then 5 drops of phenolphthalein indicator (in ethanol) was added and the sample was titrated using 0.1 M NaOH until it was a medium pink colour. This procedure estimates the free fatty acids released via lipase action. The experiment was repeated for added NaC in the mass range 0.03-0.3g at intervals of 0.03g. In addition two controls were used in this experiment. In the first control 15mL of emulsion sample was diluted with 15mL of milli-Q water, adjusted to pH 7. A further 7.5 mL of Milli-Q water was added and the emulsion was heated in the water bath at 37 °C. No bile salt or lipase was added to these solutions. They were then sampled and titrated in the same way as described above. For the second control the 15mL of emulsion sample was diluted with

15mL of milli-Q water, adjusted to pH 7, and 60 mg pancreatic lipase and 7.5 mL of milli-Q water was added to the diluted emulsion samples, but no NaC, prior to them being placed in the water bath. They were then titrated in the same way as described above.

CHAPTER 3 - RESULTS & DISCUSSION

3.1 Competitive adsorption of WPC or SCN with bile salts

When bile salts are added to protein stabilised emulsions they displace the protein from the oil-water interface (Figure 3.1 and Figure 3.2). The degree of displacement depends on the concentration of the bile salt, the type of protein (WPC or NaC) and the bile salt type. For WPC emulsions (Figure 3.1) at low protein to bile salt molar ratio (M_R) up to $M_R \approx 20$ the order of changing ability to displace bile salts is $\text{NaC} \approx \text{NaTC} < \text{NaDC} < \text{NaGDC}$. As the concentration of the bile salt is increased further above $M_R = 20$ the surface coverage of WPC reaches a plateau for NaC and NaDC containing emulsions, but continues to decrease for NaTC and NaGDC emulsions. Above $M_R = 60$ (Figure 3.1) the order of ability to displace WPC now becomes $\text{NaC} \approx \text{NaDC} < \text{NaTC} < \text{NaGDC}$. Only the NaGDC appears able to completely displace WPC from the oil-water emulsion droplet interface. A similar trend is observed for SCN stabilized emulsions (Figure 3.2), where at M_R below 30 the order of ability to displace casein is also $\text{NaC} \approx \text{NaTC} < \text{NaDC} < \text{NaGDC}$ and above $M_R = 30$ $\text{NaC} < \text{NaDC} < \text{NaTC} < \text{NaGDC}$. It might be expected that the ability to displace the protein from the interface would be linked to the hydrophobicity of the bile salts. The order of hydrophobicity (Heuman, 1989; Donovan, Jackson, Carey, 1993) for the four bile salts studied is $\text{NaTC} < \text{NaC} < \text{NaGDC} < \text{NaDC}$, which is clearly not the same as the order in which they displace the proteins. Hydrophobicity does play a role in determining displacing ability, since the dihydroxy bile salt NaDC is more hydrophobic and better at displacing protein than the trihydroxy bile salt NaC. However, both NaTC and NaGDC are more efficient at displacing protein from the interface than NaC and NaDC, and thus have a greater displacing ability than their relative hydrophobicities would suggest. It must be concluded from this that the conjugated amino acid also plays a role in determining bile salt surface activity. At least a partial explanation for this may be found in the work of Mohapatra & Mishra (2011). They have studied the mechanism of incorporation of bile salts into dipalmitoyl- and dimyristoyl-phosphatidyl choline (DPPC and DMPC) vesicles. They found that the presence of a conjugated amino acid affects the way in which the sterol ring is inserted between the phospholipid acyl chains in the bilayer and that the order of bile salt induced hydration of the lipid bilayer is $\text{NaDC} > \text{NaC} > \text{NaGDC} > \text{NaTC}$. In

a similar way to the results presented in this thesis for displacement, the hydration of the bilayer does not follow the order of bile salt hydrophobicity. It would be expected that the more hydrophobic bile salts would find it easier to penetrate into the bilayer structure. The authors explain this as being due to a steric contribution from the conjugated amino acid of NaGDC and NaTC. NaC is less hydrophobic than NaGDC, but lacks an extended charged amino acid group. The NaC can penetrate into the bilayer membrane structure, inserting the sterol rings between the lipid chains, with less steric hindrance from the charged group than occurs with NaGDC (and NaTC) which have larger charged side chains (Figure 3.3). A similar mechanism may partly explain the differences in protein displacing ability of the bile salts. The NaGDC and NaTC have more of the bile molecule sitting in the water phase, and this will interfere more with the protein adsorbed layer.

In Figure 3.4 the displacement of SCN and WPC by the bile salts is compared. The surface coverage of SCN is slightly lower than for WPC in the absence of bile salts ($2.34 \pm 0.18 \text{ mg.m}^{-2}$ for SCN compared to $2.55 \pm 0.16 \text{ mg.m}^{-2}$ for WPC). The surface coverage's obtained with the SCN and WPC emulsions in the absence of surfactant in this study are comparable to those observed in other studies (Euston & Hirst, 1999). When bile salts are added to the protein-stabilised emulsions, however, the WPC is clearly more easily displaced than the SCN for all bile salts and at all molar ratios studied. There are several reasons why this may be the case. SCN is more surface active than WPC and thus you might expect more surfactant to be required to displace SCN than is needed for WPC (Ridout, Mackie & Wilde, 2004). The globular proteins in WPC (particularly β -lac) may bind hydrophobic surfactants in a lipophilic binding pocket, and this is known to alter the surface activity of the protein (Bos, Nylander, Arnebrant & Clark, 1997). It is not possible at this stage to determine whether either mechanism explains the differences in displacement between SCN and WPC. There is conflicting evidence for the binding of bile salts to proteins in WPC. Whereas binding of NADC to bovine serum albumin has been demonstrated (Makino, Reynolds & Tanford, 1973) others researchers have carried out surface tension experiments that suggest binding of NaTC and NaGDC to β -lac does not occur (Maldonado-Valderrama, Woodward, Gunning, Ridout, Husband, Mackie, Morris & Wilde, 2008).

3.2 Bile salt surface coverage

The surface coverage of the four bile salts as a function of bile salt concentration is shown in Figure 3.5. The surface coverage of bile salt is determined in emulsions stabilized by both WPC and SCN. When the Γ vs bile salt plots were compared for these two systems the results were very similar, and so they have been combined into a single plot in Figure 3.5 for each of the four bile salts. This suggests that binding of bile salt to the protein does not occur. Indeed there is little evidence for binding of bile salts to either the proteins in WPC or SCN, although there have been few studies of binding and it cannot be ruled out completely. The low surface coverage (Γ) attained for NaC compared to the other three bile salts is noticeable immediately on inspection of Figure 3.5. The curve for NaC increases up to a plateau value of $\Gamma = 0.55 \pm 0.10 \text{ mg m}^{-2}$ for bile salt concentrations in the approximate range 4-20 mM, and then Γ starts to increase as the bile salt in the emulsion rises above this concentration (Figure 3.5a). For NaDC (Figure 3.5b) the Γ increases with increasing bile salt concentration up to 10 mM where a plateau is observed at $\Gamma = 2.11 \pm 0.10 \text{ mg m}^{-2}$ which is significantly higher than the plateau for NaC. Above about 20mM NaDC the surface coverage increases again. NaTC shows slightly different adsorption behaviour (Figure 3.5c). The surface coverage of NaTC remains low until a bulk concentration of just above 5 mM is reached (Figure 3.5c). Above this Γ increases rapidly, but unlike NaC and NaDC no evidence of a plateau in Γ is seen over the concentration range of NaTC added to the emulsions. NaGDC appears to exhibit similar adsorption behaviour to NaDC (Figure 3.5d). The surface coverage increases linearly up to a bulk NaGDC concentration of about 12.5 mM, and then plateaus. The average surface coverage in the plateau region is $\Gamma = 2.11 \pm 0.24 \text{ mg m}^{-2}$ the same as for the NaDC.

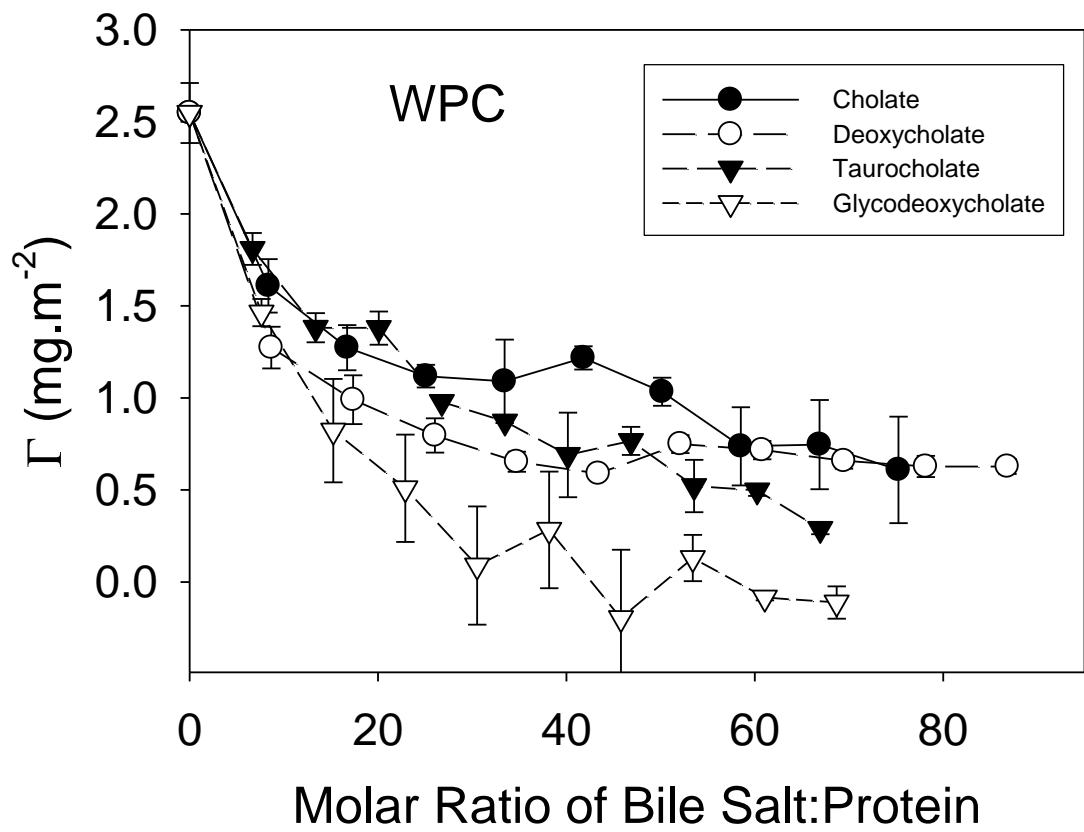


Figure 3.1 – Surface coverage as a function of bile salt:WPC molar ratio (M_R) for the four bile salts used in this study.

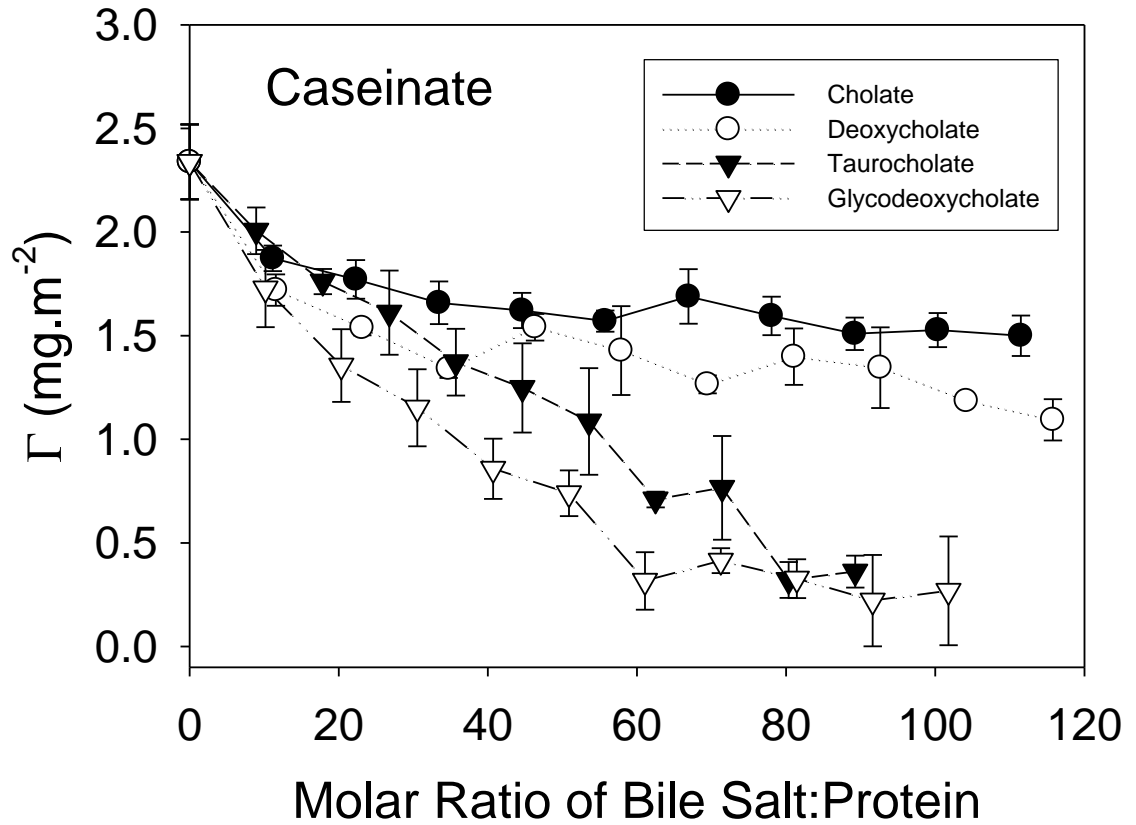


Figure 3.2 – Surface coverage as a function of bile salt:SCN molar ratio (M_R) for the four bile salts used in this study

An estimate of the area occupied per bile salt can be obtained from the measured surface coverage. For NaC a plateau surface coverage of 0.55 mg.m^{-2} is reached above 4 mM L^{-1} bile salt concentration. The number of moles (N_m) of NaC at the surface is therefore,

$$N_m = \frac{0.55 \times 10^{-3}}{MW_{NaC}} = \frac{0.55 \times 10^{-3}}{430.55} = 1.28 \times 10^{-6} \text{ Mm}^{-2} \quad (3.1)$$

where MW_{NaC} is the molecular weight of NaC. Multiplying N_m by Avogadro's number gives the number of NaC molecules at the surface,

$$\text{Number of NaC molecules} = N_m \times N_{Av} = 1.28 \times 10^{-6} \times 6.022 \times 10^{23} = 7.69 \times 10^{17} \quad (3.2)$$

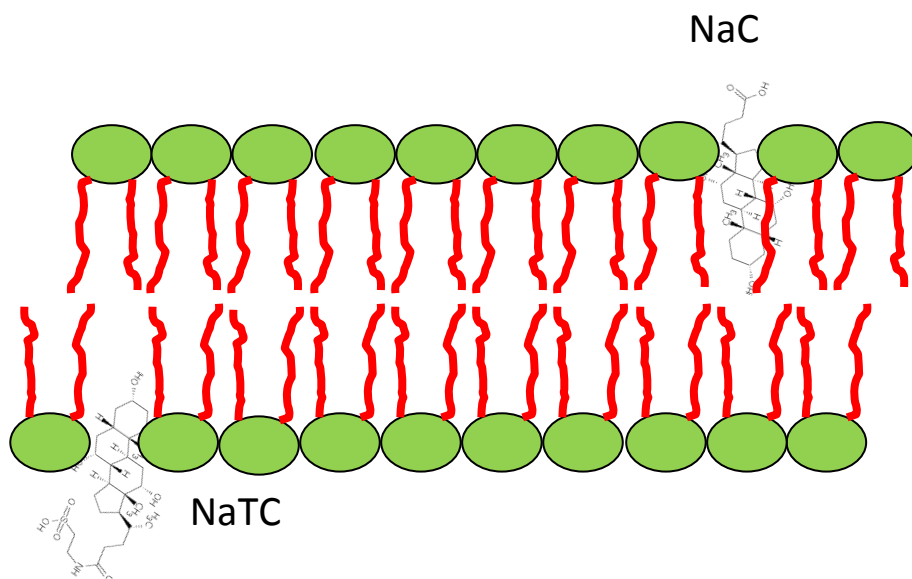


Figure 3.3 - NaC is able to fit into a phospholipid bilayer membrane more easily than NaTC due to a lower degree of steric hindrance from the carboxyl hydrophilic group compared to the more bulky sulphonate group of NaTC.

This is the number of molecules per square metre of surface, so the reciprocal of this number gives the area per molecule,

$$\text{Area per molecule} = \frac{1}{7.69 \times 10^{17}} = 1.30 \times 10^{-18} \text{ m}^2 = 1.30 \text{ nm}^2 \quad (3.3)$$

The calculated molecular area of $1.30 \pm 0.20 \text{ nm}^2$ agrees well with a molecular area of 1.12 nm^2 estimated by Welzel & Cammenga (1998) for NaC in a densely packed adsorption layer and with the molecular area for cholic acid monolayers at collapse pressure (ca 1.08 nm^2) obtained by Miyoshi et al. (1992).

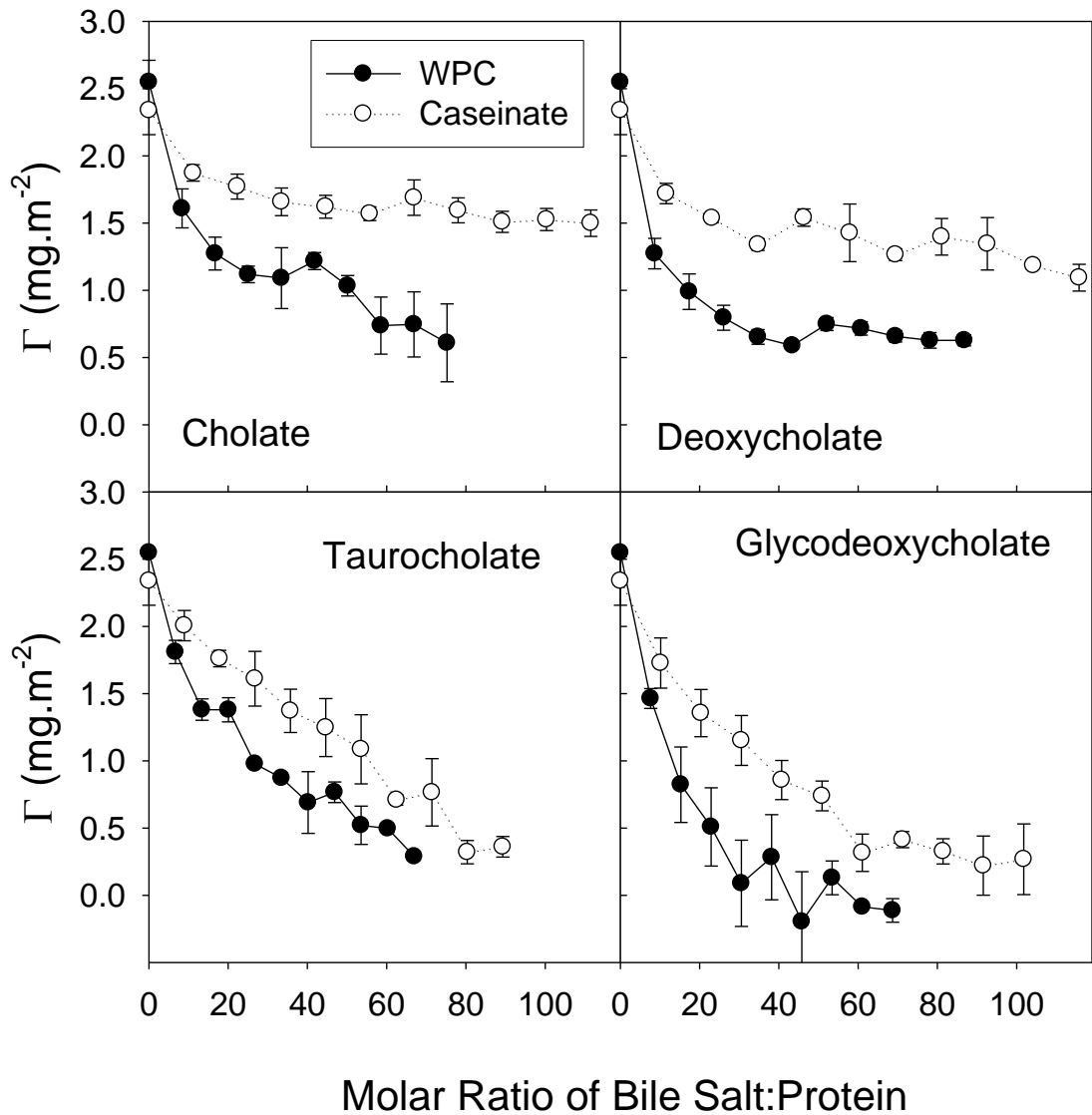


Figure 3.4 – Comparison of the effect of four different bile salts on the protein surface coverage in WPC and SCN emulsions.

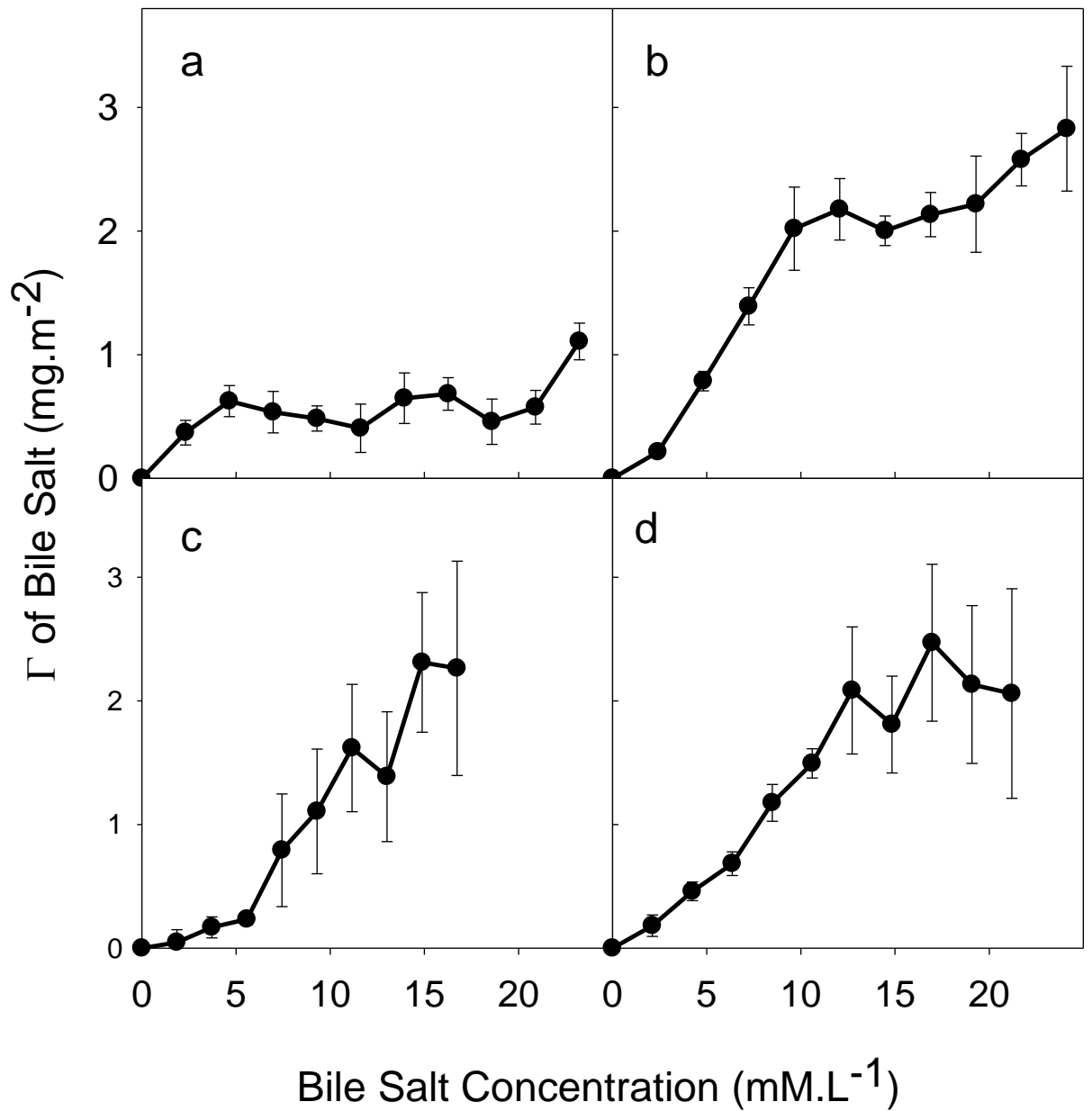


Figure 3.5 – Bile salt surface coverage in emulsions as a function of bile salt bulk concentration. (a) NaC; (b) NaDC; (c) NaTC; (d) NaGDC.

For NaDC and NaGDC the surface coverage increases up to about 2.11 mg.m^{-2} . This corresponds to an area per molecule of $0.33 \pm 0.02 \text{ nm}^2$ for NaDC and $0.37 \pm 0.02 \text{ nm}^2$ for NaGDC. Given that the molecular area occupied per bile salt is not believed to vary a great deal between the bile salts (Tiss, Ransac, Lengsfeld, Hadvary, Cagna & Verger, 2001) the much higher Γ for NaDC, NaTC and NaGDC, and the four times lower molecular area suggests that either they adsorb as micellar aggregates rather than individual molecules, or they adsorb as individual molecules but then aggregate at the surface to form micelle-like structures. One possible reason for this could be that the critical micelle concentration (CMC) for NaC is in fact at the high end of the range quoted (20mM) and so the NaC concentrations we are using are submicellar. This means that the NaC adsorbs as individual molecules for most of the concentration range. The other bile salts, on the other hand, are present at concentrations above their CMC for most of the concentration range used in the experiments, and thus adsorb as micelles which explains the higher surface concentration. Subuddhi & Mishra (2007) have studied the adsorption of NaC and NaDC to the surface of dipalmitoyl phosphatidylcholine (DPPC) vesicles. They found that NaDC caused significant structural changes in the DPPC membrane at concentrations well below its CMC of 6 mM, whilst the interaction of NaC with the membrane was much lower below its CMC and only significant above the CMC. Subuddhi & Mishra's results suggest that the micellar state of the bile salts is important in the interaction with hydrophobic surfaces. The results presented in Figure 3.5 for NaC show an increase in Γ for bulk concentrations above 20mM which is at the upper end of the range for CMC of NaC quoted in the literature (Chapter 1, section 1.2). This suggests that the plateau region in the Γ vs bile salt concentration plot (Figure 3.5a) for NaC corresponds to adsorption of individual NaC molecules at sub-micellar concentrations to form a saturated monolayer. Once the concentration of NaC increases above the CMC (here estimated to be about 20mM) micelles form and these start to adsorb to the oil-water interface and the Γ of NaC starts to increase. This is summarised schematically in Figure 3.6. The CMC of the other bile salts is lower, in the range 2-5mM for all of the other three, and therefore micelle adsorption occurs at lower bulk bile salts concentrations for these, and leads to a greater increase in Γ .

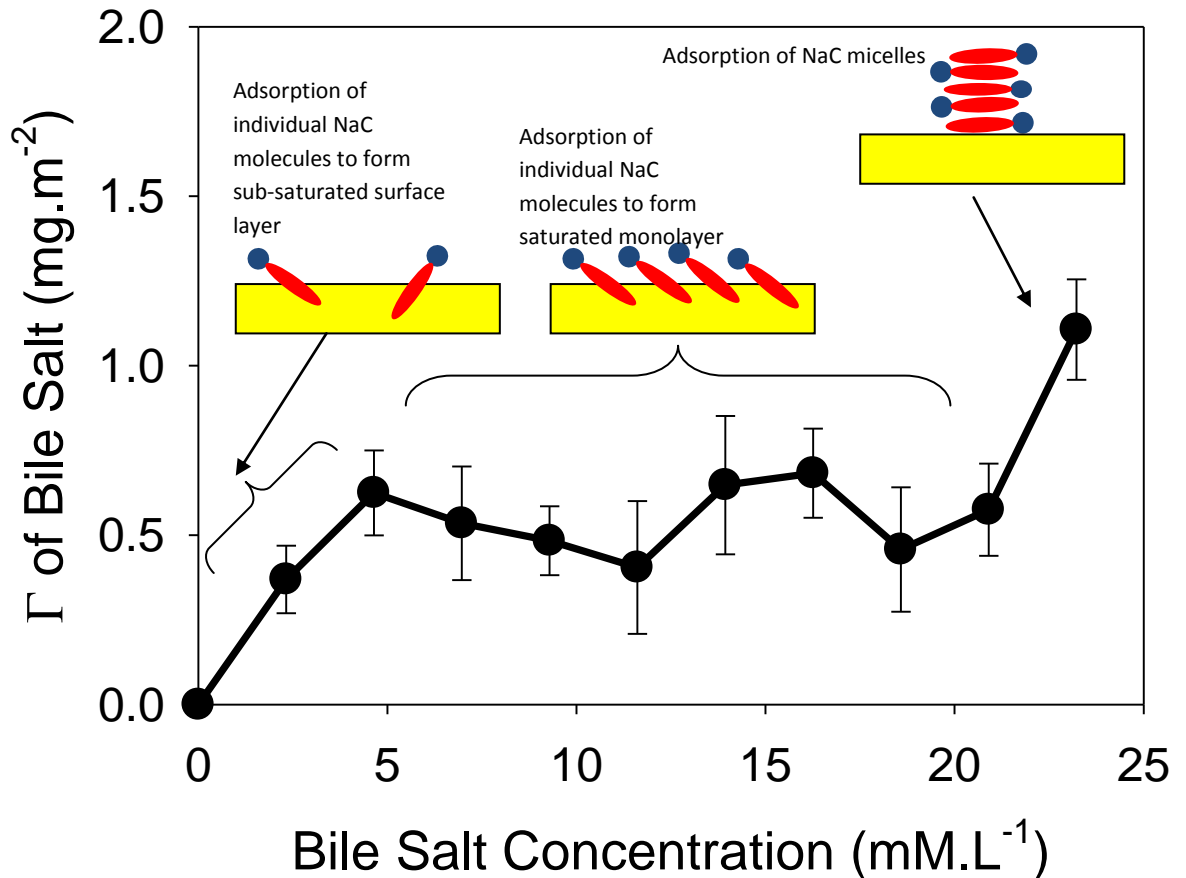


Figure 3.6 – Surface coverage plot for NaC showing the hypothesized adsorbed bile salt adsorbed layer structure in different regions of the graph.

If the surface coverage of WPC and SCN are compared to the NaC surface coverage then an interesting difference is observed. For WPC the surface coverage of protein continues to decrease in the plateau region of Γ for NaC, whilst the surface coverage for SCN also reaches a plateau value. So for WPC something other than the concentration of the NaC at the interface must be affecting the protein adsorption. This may suggest that for WPC there is an interaction between non-adsorbed NaC and the protein molecules, which contributes to their displacement from the interface, but this interaction is absent with the caseins.

3.4 Lipase Activity

In addition to removing proteins and peptides from the oil-water interface, which allows lipase to easily adsorb to the emulsion droplet surface, bile salts also play other roles in digestion. Some lipases are bile-salt-dependent, i.e. they are activated by bile salt, whilst the micellar form of bile salts play a role in encapsulation and transport of free fatty acids. Bile salts will form mixed micelles with free fatty acids, which are auto-inhibitory to lipases activity, thus maintaining or improving the enzyme function.

To investigate the effect of bile salts on lipase activity, emulsions were prepared with different concentrations of NaC. These emulsions were then treated with a fixed concentration of lipase enzyme and the enzyme activity determined in terms of the release of free fatty acid (see Chapter 2, section 2.1.6). There was only sufficient time to carry out preliminary experiments. These are summarized in Figure 3.7, where the free fatty acid content of the emulsions has been followed for a period of two hours at 37 °C for systems containing different NaC concentrations. A control sample with no added lipase and no NaC was also heated at 37 °C, and the free fatty acid content for this sample was subtracted from the results for lipase and NaC containing emulsions. For clarity, not all results obtained in this study are shown in Figure 3.7, but the omitted results also follow the same trend. All data at different NaC concentrations in Figure 3.7 can be fitted to a power law of the form,

$$[FFA] = kt^n \quad (3.4)$$

where [FFA] is the free fatty acid concentration, t is the time and k and n are constants. Fitting of the experimental data to equation (3.4) was carried out by non-linear regression using SigmaPlot 8. Graphs showing the closeness of fit between equation 3.4 and the experimental data are given in Appendix 1.

Addition of NaC to the WPC stabilized emulsion increases the release of free fatty acids (Figure 3.7). This observation is in agreement with the results of Mun, Decker & McClements (2007) who have shown that the release of free fatty acids during lipase digestion of both SCN and WPC stabilized emulsions increases in the presence of bile salt extract.

For enzyme controlled reactions it is common to quote the maximum rate of the reaction (V_{max}), which occurs in the early stages of the reaction, to describe the kinetics. In this case, it is not possible to determine V_{max} so we have chosen to determine the rate of the reactions at an arbitrary time of 1minute (V_{1min}). Since the fit of the experimental data to the power law of equation (10) is very close for all curves (Appendix 1) the rate has been determined by differentiating equation (3.4) and setting $t = 1$, i.e.,

$$\frac{d[FFA]}{dt} = knt^{n-1} \quad (3.5)$$

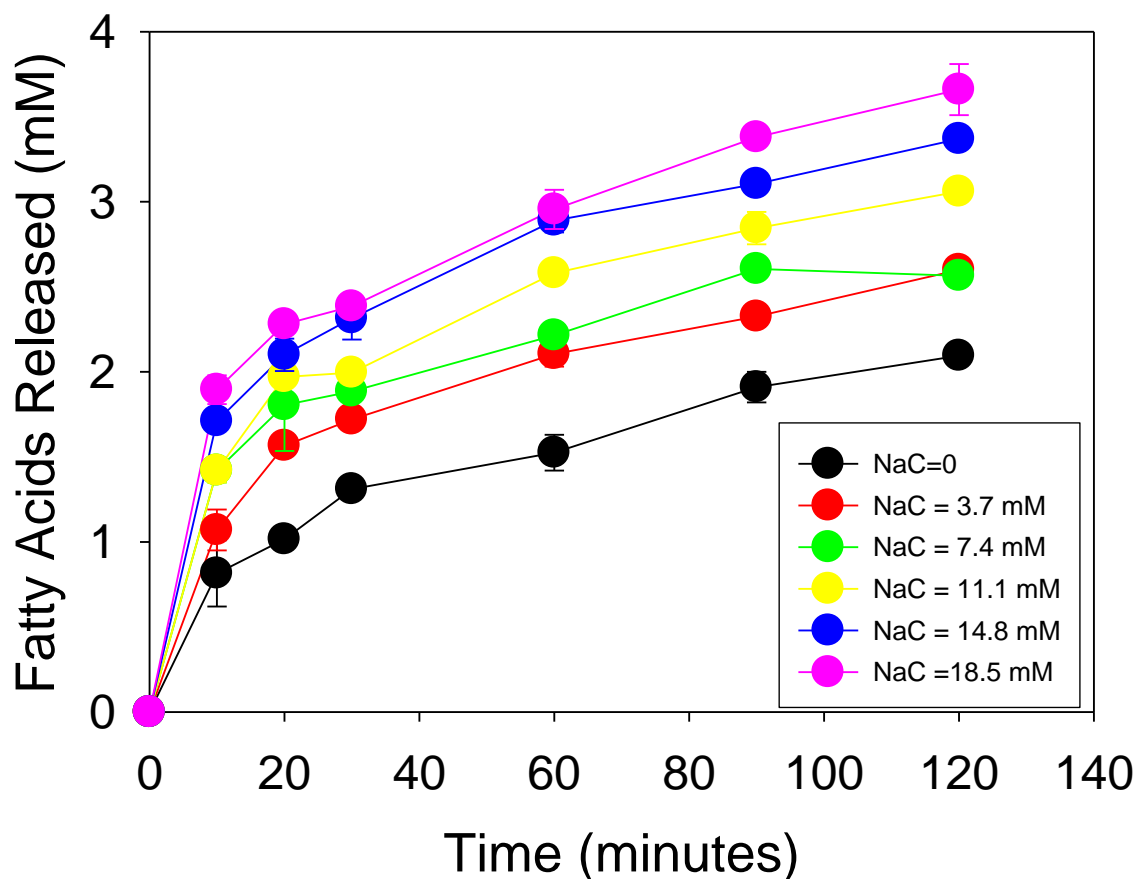


Figure 3.7 – Lipase catalysed release of free fatty acids from a WPC stabilised oil-in-water emulsion at different NaC concentrations. The free fatty acid content of a control emulsion containing no lipase or NaC was constant at 0.66 ± 0.05 mM over the two hour period, and this was subtracted from the results in Figure 3.7.

In other words $V_{1\min}$ is equal to kn , and can be determined from the fit to the data. Figure 3.8 shows a plot of $V_{1\min}$ against NaC concentration, and demonstrates a linear relation between the reaction rate and NaC content over the concentration range used. The data in Figure 3.8 suggest that the presence of NaC in the emulsion increases the activity of the lipase enzyme. There could be several explanations for this.

- (1) The NaC displaces protein from the interface and makes it easier for the lipase to adsorb to the emulsion droplet surface and hydrolyse the triglyceride to release free fatty acids.
- (2) The NaC interacts with the enzyme directly and stimulates the activity of the lipase.

The NaC forms mixed micelles with the free fatty acids produced. Free fatty acids are normally auto-inhibitory to the lipase enzyme, and reduce lipase activity most likely via a competitive inhibition mechanism. When incorporated into micelles the free fatty acids are not able to bind to and inhibit the enzyme. The actual mechanism is likely to be a combination of more than one of the proposed explanations. In Figure 3.9 the lipase activity is compared to the surface coverage of protein and with the surface coverage of bile salt in the emulsions. The lipase activity increases as the protein surface coverage which suggests that explanation (1) above will contribute to the increase in lipase activity. Comparing to the bile salt surface coverage, the lipase activity continues to increase even when the bile salt reaches monolayer coverage. This may suggest that the non-adsorbed bile salt contributes to the lipase activity, although since the NaC concentration is below the CMC mixed micelle formation with liberated FFA's is unlikely to be the cause of this.

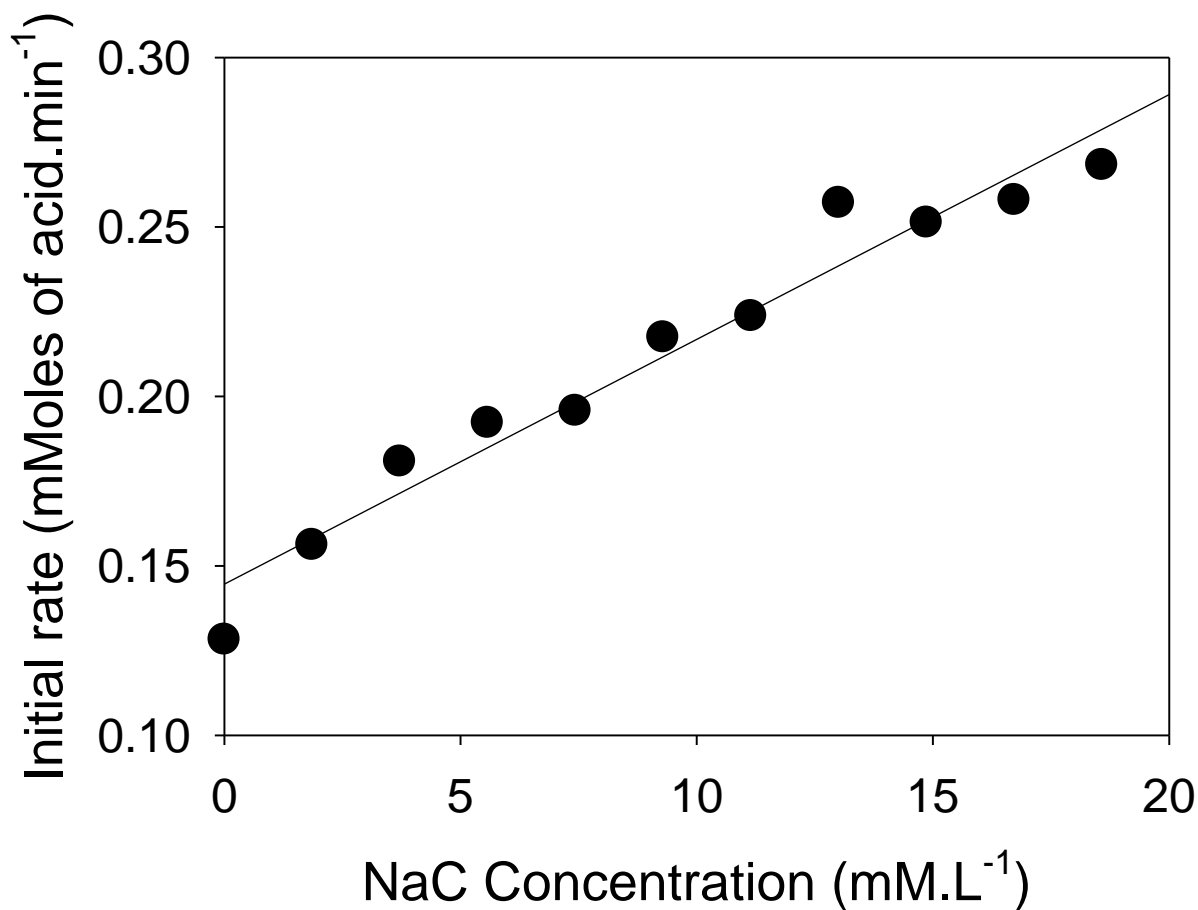


Figure 3.8 – Estimated initial rate of lipase catalysed breakdown of triglycerides in WPC stabilised oil-in-water emulsions reaction in the presence of varied concentrations of NaC.

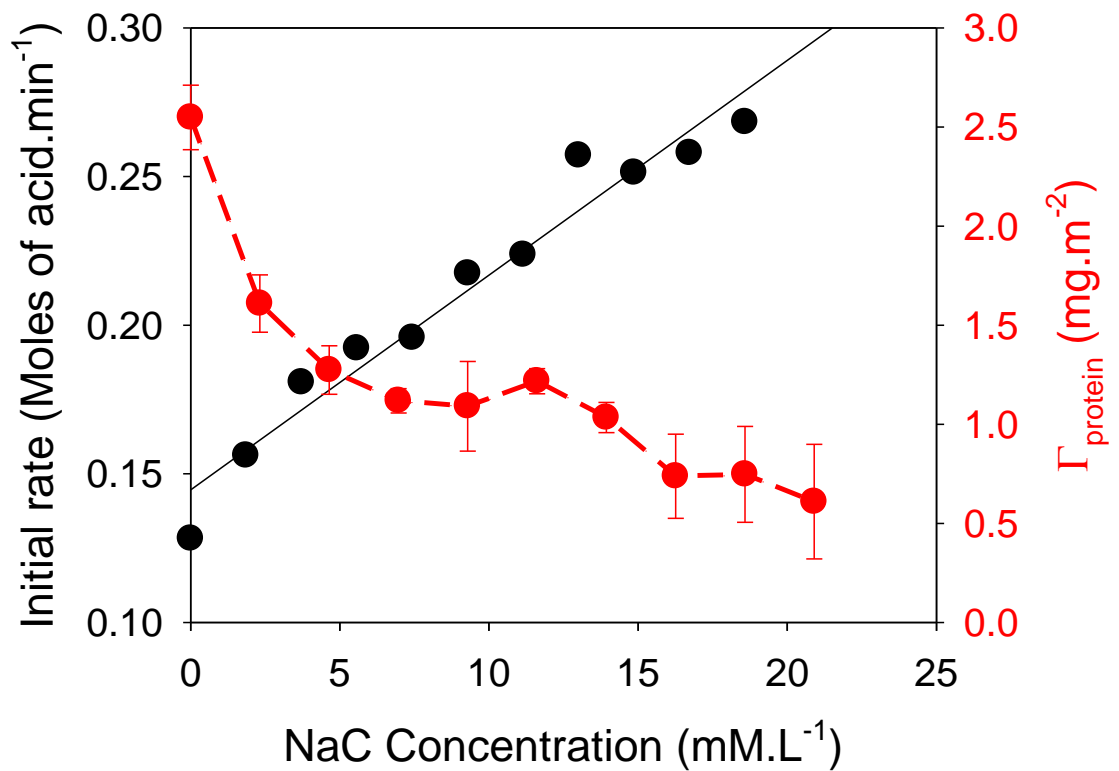
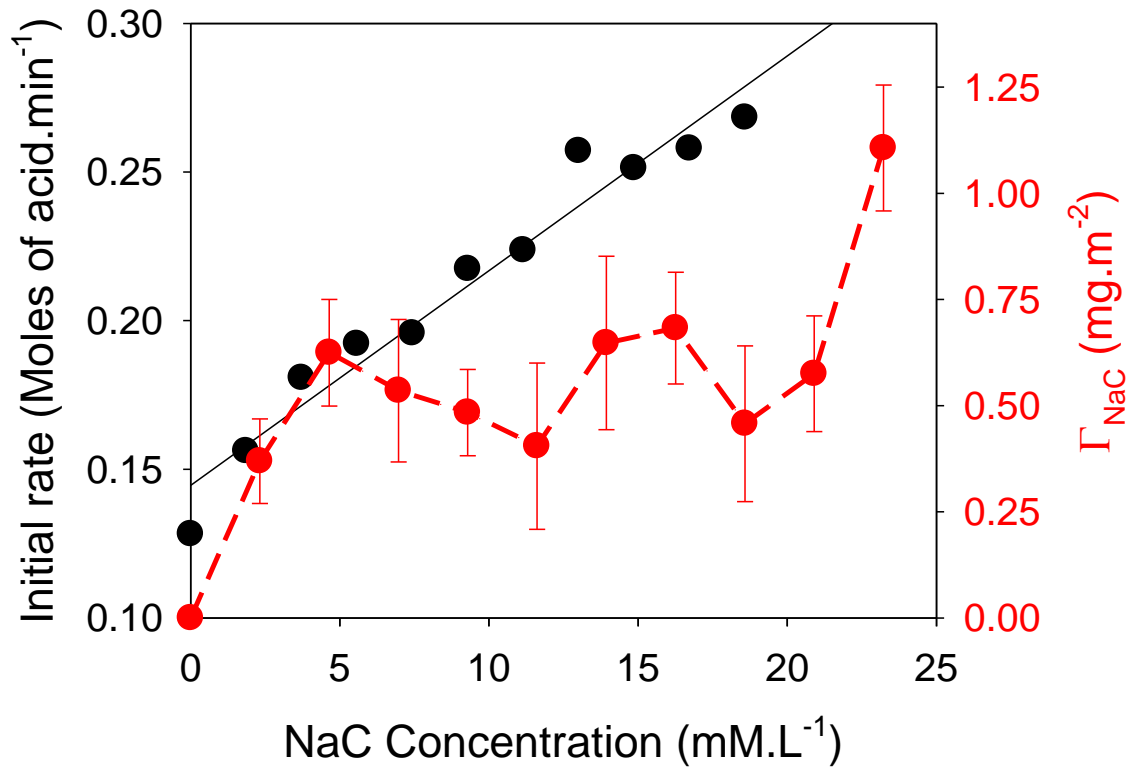


Figure 3.9 – Comparison of the rate of the lipase reaction at 1 minute with the surface coverage of NaC and WPC at different NaC concentrations.

CHAPTER 4 - CONCLUSIONS AND FURTHER WORK

The ability of bile salts to displace protein from an oil-water emulsion droplet interface is a complex process and depends on the nature of the bile salt (number of hydroxyl groups, presence of conjugated amino acid); the nature of the protein; the physical chemistry of the bile salt (CMC) and probably many other factors not studied in this dissertation. The number of hydroxyl groups on the sterol ring determines the hydrophobicity of the bile salt. Intuitively, one would expect the more hydrophobic bile salts to adsorb more strongly to the interface and to displace protein more efficiently, but the real situation is more complex. For NaC and NaDC which only differ by a single hydroxyl on the sterol ring, the more hydrophobic NaDC is in general more efficient at displacing protein. When a conjugated amino acid is added to the bile salt e.g. addition of a taurine to NaC to make NaTC and a glycine to NaDC to make NaGDC, although this reduces the overall hydrophobicity of the bile salt, they become much better at displacing protein from the emulsion droplet surface. In this case the charged group of the bile salt is moved further from the hydrophobic sterol ring and this alters the conformation that the bile salt adopts at the interface. The competitive adsorption of the individual bile salts with SCN and WPC will also be complicated by the solution behavior of the bile salts, and particularly their tendency to form micelles in solution. Measurements of the surface coverage of the bile salts strongly suggest that only NaC is found at the interface as individual molecules, and that the other bile salts adsorb predominantly as micellar aggregates. The consequence of this to the competitive adsorption with proteins is not yet clear.

4.2 Further Work

There are a number of directions which this research could take from this point forwards, and these are discussed below.

(1). Determination of bile salt binding to whey proteins and caseins

It is not clear if bile salts bind to whey proteins or caseins, either at a specific binding site or non-specifically. Binding studies, using for example equilibrium dialysis (Takikawa, Sekiya, Yamanaka & Sugiyama, 1995) or affinity

chromatography methods (Winzor, 2004) will help to resolve this. If it is found that binding of bile salt to the proteins is significant, then this is likely to change their surface activity and may partly explain the difference in displacing ability between the bile salts, and the difference in displacement of SCN and WPC.

(2). Further optimization of the bile salt assay.

The standard curves for the dihydroxy bile salts (NaDC and NaGDC) are subject to greater potential error particularly at higher bile salt concentrations. This must be due to the differences in absorption spectrum observed for the trihydroxy and dihydroxy bile salts. It may be possible to improve the accuracy of the method for NaDC and NaGDC by using an absorption wavelength closer to 300nm, which is closer to the maximum in the NaDC absorption spectrum. This is close to 280 nm, the absorbance maximum for the amino acid tryptophan, which is used as a method for quantifying protein. In this case care would need to be taken to optimize the method to minimize interference from non-adsorbed protein.

(3). Repeat lipase experiments for NaDC, NaTC and NaGDC.

The other bile salts have differing CMC's and effects on the surface coverage of protein. This will be seen in the dependence of the rate of lipase catalysed triglyceride hydrolysis.

(4). Bile salt/WPC or SCN/lipase competitive adsorption studies

It would be interesting to study the competitive adsorption of WPC with lipase, lipase with bile salt and the WPC/lipase/bile salt system. The question that it would be useful to answer is why does the rate of triglyceride hydrolysis increase as bile salt concentration increases when you might expect lipase to be displaced from the oil-water emulsion droplet interface?

(5). Mixed bile salt – protein competitive adsorption.

Bile salts are found as a mixture in the human gut. It therefore makes sense to study how they interact with each other to influence competitive adsorption of in emulsions.

(6). Mixed bile salt effect on lipase activity.

It also makes sense to study the effect of mixed bile salts on lipase activity in emulsions.

(7). Repeat work on other bile salts which are positional isomers of NaC etc.

Bile salts also exist as isomers where the hydroxyl groups occupy different positions on the sterol ring. These have the same hydrophobicity but differ in some chemical properties and so may differ in their ability to displace proteins from the emulsion droplet interface.

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APPENDIX 1

Data for the release of free fatty acids from WPC stabilised oil-in-water emulsions at different NaC concentrations. The curve in each figure is a fit to equation (1).

