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Development of Biorelevant Simulated Salivary Fluids for Application in Dissolution Testing

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List of Abbreviations

ANOVA	Analysis of Variance
API	Active Pharmaceutical Ingredient
ASD	Artificial Stomach Duodenum (model)
BATA	Brief Access Taste Aversion (model)
BCS	Biopharmaceutical Classification System
BP	British Pharmacopoeia
BSM	Bovine Submandibular Mucin
CMC	Carboxymethylcellulose
FDA	Food and Drug Administration
FIP/AAPS	Federation International Pharmaceutique / American Association of Pharmaceutical Sciences
GI	Gastrointestinal
GLN	Glandosane®
GPCR	G-Protein Coupled Receptors
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
HPLC-UV	High Performance Liquid Chromatography–Ultra Violet (Detector)
HS	Human Saliva
IVIVC	<i>In Vitro–In Vivo</i> Correlation
LOQ	Limit Of Quantification
ODT	Orally Disintegrating Tablet

PBS	Phosphate Buffered Saline
PGM	Porcine Gastric Mucin
PK	Pharmacokinetic
PROP	6-n-propyl-2-thiouracil
PTC	Phenylthiocarbamide
QC	Quality Control
RSD	Relative Standard Deviation
RE	Relative Error
SC	Sildenafil Citrate
SCF	Simulated Colonic Fluid
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SOG	Saliva Orthana® Gel
SOS	Saliva Orthana® Spray
SS	Stimulated Saliva
SSF	Simulated Salivary Fluid
TIM	TNO Intestinal Model
US	Unstimulated Saliva
USP	United States Pharmacopoeia

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Abstract

Conventional adult dosage forms such as tablets and capsules are often not suitable for the paediatric and geriatric population due to either swallowing difficulties or a requirement for tailored dosing to meet individual needs. Alternative oral formulations such as orally disintegrating tablets (ODTs) are available; however these usually require the incorporation of taste masking techniques. One approach to taste masking is to reduce contact between the bitter active pharmaceutical ingredient (API) and taste buds. This may be achieved by hindering release in the oral cavity using reverse enteric polymeric coatings.

In vitro dissolution testing can be employed to elucidate taste masking capability by quantifying release of the API in simulated oral cavity conditions. This provides a robust analytical approach circumventing the expense and ethical challenges associated with human taste testing panels or animal testing. To achieve taste masking, drug release should be below the bitterness threshold concentration of the API. A vast array of dissolution methodologies has been employed in the evaluation of taste masked formulation performance in literature, with little agreement between approaches, and a lack of biorelevance.

For optimal predictability, the dissolution test should be biorelevant and the dissolution media should mimic human saliva as closely as possible. Human saliva is thus a biological fluid of great importance in the field of dissolution testing. However, until now, no consensus has been reached on its key characteristics relevant to dissolution testing. As a result, it is difficult to select or develop an *in vitro* dissolution medium to best represent human saliva.

In this thesis, for the first time, the pH, buffer capacity, surface tension, viscosity and flow rate of both unstimulated (US) and stimulated (SS) human saliva were investigated with a sufficient number of participants to generate statistically meaningful results (Chapter 3). This provides a platform of reference for future dissolution studies using simulated salivary fluids (SSFs).

Additionally, the conversion between US and SS was investigated using mechanical stimulation, and for the first time using an ODT dosage form as a stimulant (Chapter 4). This was in order to ascertain if dissolution testing is necessary in media representing both stimulation states. Furthermore, the characteristics of human saliva were directly compared experimentally to examples of the main types of SSF currently available (Chapter 3). Since the current SSFs were not found to be suitable to represent human saliva according to key characteristics, novel SSFs are proposed in this work (Chapter 5), accompanied by early stage dissolution testing to determine their suitability (Chapter 6).

Chapter 1: Introduction

1.1 Background

N.B. The content of this Chapter of the thesis is modified from the publication by Gittings *et al.*, 2014 [1] in which I was the leading author.

The oral route is by far the most popular route of drug administration due to convenience and thus compliance [2]. However, standard oral tablets or capsules are not suitable for everyone. The paediatric and geriatric population have complex additional needs compared to the adult population. In these groups, swallowing difficulties are common. This results in the insufficiency of oral tablets and capsules for use in these populations and an increased prevalence of alternative oral dosage forms usage [3, 4]. Pharmacokinetic parameters such as volume of distribution, metabolism and clearance may change rapidly in these populations [5, 6], leading to a requirement for more tailored oral dosage forms which can undergo manipulation to meet individual needs [7]. Additionally, in the standard adult population, alternative dosage forms are becoming more popular. For example, orally disintegrating tablets can be taken “on the move” without the requirement for co-administration of water.

There are many alternative oral formulations in existence. In a review written in 2007, there were at least 17 oral formulations listed [8]. These included liquid formulations such as solutions, suspensions, and syrups, as well as tablets and powders for reconstitution into a liquid formulation. Tablet formulations included orally disintegrating tablets, chewable tablets, scored dividable tablets and effervescent tablets. Other formulations such as films, drops, mini-tabs, bulk granules or powders and sprinkle capsules were also detailed.

In the present research, we focus on microparticulates as an alternative dosage form for oral delivery. These are particularly advantageous since they can be formulated into various other dosage forms such as sprinkle formulations, suspensions or more commonly, orally disintegrating tablets (ODTs).

Unlike tablets and capsules, alternative oral formulations tend to be more complex and require advanced taste masking techniques. Many drugs have undesirable organoleptic properties such as a bitter or metallic taste or burning sensation which reduces compliance, resulting in therapeutic failure. In the case of microparticulates, residence time in the oral cavity may be prolonged for a small proportion of particles (particularly after “dry” delivery of an ODT) compared to standard tablets or capsules. This leads to more likelihood of non-compliance due to adverse taste. Taste masking, therefore, could be critical for the therapeutic and commercial success of these microparticulate or alternative oral formulations.

1.2 Taste Masking Techniques

Broadly, approaches to taste masking aim to use strong flavours and sweeteners to overpower the bitter Active Pharmaceutical Ingredient (API), reduce contact between the API and the taste buds, or to reduce release of the API in the oral cavity [9]. Specifically, methods of taste masking include use of flavours and sweeteners [10-14], lipophilic vehicles [15-19], coating with polymers [20-32], carbohydrates [33-36], lipids [37-39] or proteins [9], complexation with cyclodextrins [40-46] or ion-exchange resins [47-60], formation of salts [9], and solid dispersions [61-66]. In practice, combinations of these techniques are often employed. For example in one case, ibuprofen orally disintegrating tablets were manufactured using a lipid matrix, coated with a film forming agent and formulated with a sweetener in order to achieve taste masking [11].

Whilst flavours and sweeteners are straight forward techniques, many excipients are subject to regulatory restrictions which limit their use, particularly in the paediatric population. For example, sucrose is a common sweetener but can cause dental caries, whilst certain flavours have been associated with hypersensitivity, toxicity or allergy and should also be kept to a minimum [8].

When the alternative method of inhibiting contact between API and taste buds by reducing release in the mouth is used, the manufacturing processes become more complex compared to the simple addition of a flavour or sweetener. These require

sophisticated and advanced technologies and are subsequently more costly to develop and manufacture [7].

In addition, flavours and sweeteners, although simple, may not sufficiently mask the taste of extremely bitter compounds. Lipophilic vehicles increase the viscosity in the mouth and coat the taste buds with the oil, surfactant or lipid. On the other hand polymeric, carbohydrate or protein coatings act as a physical barrier surrounding the drug particle. Coatings are commonly used as an initial approach to taste masking and thus are widely used, whereas complexation with cyclodextrins or ion exchange resins is less common. Formation of salts or use of ion exchange resins is particularly suitable for highly soluble, ionisable drugs which form less soluble complexes at salivary pH. However, cyclodextrin complexation is generally reserved for low dose drugs which are shielded from taste buds in the central pore of the cyclodextrin molecule. A detailed review of taste masking technologies for oral pharmaceuticals was carried out by Sohi *et al.* in 2004 which describes these methods in greater depth [9]. More recently, in 2010, Douroumis [67] investigated taste masking technologies specifically for orally disintegrating tablets and thin oral film formulations whereby the merits and drawbacks of such technologies are described. Additionally in 2016, Afriyie, Batchelor and De Matas [68] undertook a critical evaluation of 24 taste masking technologies which were compared based on quality attributes. Despite emerging approaches in the pharmaceutical industry, the authors found that more established techniques such as polymeric coatings showed best results.

In the present research, we use microparticulates intended for oral drug delivery with a view to coating individual particulates using a reverse enteric coating for taste masking purposes. Such reverse enteric coatings are designed to hinder release of the bitter API in the oral cavity, but allow complete and rapid drug release in the acidic environment of the stomach.

Different taste masking techniques and manufacturing parameters can have a great impact on the physicochemical characteristics and performance of the taste masked formulation. Taste evaluation of the masked formulation must be assessed to

guarantee the capability of the taste masking technique. Additionally, one must ensure that the taste masking technique does not affect API bioavailability.

1.3 Taste Evaluation

The most common method of taste evaluation is human taste panels. These are typically small groups (< 20 people) of healthy volunteers who swill the formulation in their mouth for a set time before spitting it out. They then rate the formulation for different attributes based on an intensity scale. Taste panels are usually composed of lay members rather than trained, professional taste testers thus results are subjective with high inter-individual variability [69]. It may be questionable whether results can be translated into the paediatric and geriatric population whose preferences and perceptions of taste may differ. However, due to ethical reasons, paediatric testing is minimal and generally limited to controlled needed clinical studies.

Other *in vivo* tests include animal preference tests, or brief access taste aversion (BATA) tests, where the animal avoids bitter tasting compounds. These are generally based on a “lickometer” model whereby the animal is placed in a chamber with a number of sipper tubes containing the drug in different concentrations. Only one sipper tube is exposed at a time. The number of licks from each sipper tube is counted, and the animal should avoid sipper tubes containing the drug above the bitterness threshold for that API [70, 71].

An additional *in vivo* taste evaluation method is electrophysiological models, where electrodes measure the nerve response to stimuli in an anaesthetised animal. However, these are very rarely used and not documented widely [69]. *In vivo* testing is expensive and subject to ethical considerations and inter-subject variability, therefore *in vitro* taste assessments are becoming increasingly popular.

Recently, there have been several reports of the use of electronic tongues (e-tongues or taste sensors) for taste assessment [12, 19, 23, 29, 37, 41, 52, 72, 73]. These models contain electrochemical sensors which can detect various substances of different tastes and intensities, which generates electrical signals that are interpreted

by the accompanying chemometrics software. One example is the Insent Taste Sensing System TS-5000Z as shown in Figure 1.1.



Figure 1.1: Insent Taste Sensing System TS-5000Z (Insent Intelligent Sensor Technology Inc., Atsugi-Shi, Japan). Outlined by Woertz *et al.* [72].

There are several types of e-tongue in existence, differing by their receptor type and selectivity, required sample properties and handling requirements [69]. An alternative to e-tongues is *in vitro* assay methods which involve measurement of activation of G-proteins found in taste buds based on activation of receptors in an *in vitro* membrane. This method has many limitations outlined elsewhere and is not widely used [69].

Finally, *in vitro* drug release studies (dissolution tests) are employed to evaluate taste masking properties of a formulation. This approach removes the subjectivity associated with *in vivo* taste testing, replacing it with robust analytical data. The amount of drug released in a simulated oral environment is assessed. However, this leads to a requirement for biorelevant dissolution to mimic the oral cavity for accurate predictions. In our case, reverse enteric coatings should completely hinder release of the API in the oral cavity. Therefore the amount of drug released in the dissolution test should be close to zero.

1.4 Dissolution Testing Purpose

With the exception of using flavours or sweetening agents, the aim of taste masking is to reduce or inhibit the interaction between the API and the taste buds [9]. Where hindered release of API in the oral cavity is critical for taste masking, dissolution testing should be carried out to confirm this. Acceptable amounts of drug release in the oral cavity will depend on the bitterness of the API. Highly bitter molecules have lower acceptable limits of release and vice versa, thus there is no set limit of acceptable drug release for all drug candidates. There is also no set Pharmacopoeial dissolution test for taste masked particles. However, the Federation International Pharmaceutique (FIP) and American Association of Pharmaceutical Scientists (AAPS) published joint guidance for the dissolution testing of taste masked polymer coated particles in orally disintegrating tablets (ODT). This guidance recommends the use of a neutral medium where the drug should have less than or equal to 10 % drug dissolved in 5 minutes to achieve taste masking.

It is recommended by the authors that this test is performed by following the same principles of solid oral dosage forms, using a compendial paddle apparatus (usual volume 900 mL and temperature 37 °C) with a suggested agitation rate of 50 rpm. They add that this apparatus may be used to determine the dissolution of either the ODT formulation or the bulk coated granules/powder; however higher agitation speeds may be required for bulk granules due to mounding. No medium specification is detailed other than that it should be neutral, assumingly for biorelevant representation of the oral cavity. It is also not discussed whether the amount of bulk granules or formulation should be increased in line with the non-physiological volume employed in this test.

The acceptance criteria of less than 10 % release largely depends on the bitterness intensity of the API [74]. Taste masking is realistically achieved when API release is minimal and below the bitterness threshold of the API in the oral cavity. This requires an assay for detection of the API from the dissolution media at concentrations below its bitterness threshold, thus the lower limit of quantification of the assay should be considered carefully.

Where hindered release in the oral cavity is desired for taste masking, it is imperative to ensure that the taste masking technique, such as polymer coating, does not inhibit release of the API elsewhere in the gastrointestinal (GI) tract. If dissolution in the GI tract is sub optimal or incomplete, the amount of API absorbed could decrease and its bioavailability or bioequivalence may also be altered. This would result in an altered pharmacokinetic profile for the taste masked formulation which is not well correlated with the profile of the API alone or non-taste masked formulations.

An example of this is the application of coatings as a method of taste masking. These could adversely affect the pharmacokinetic profile of the API by reducing or delaying overall absorption into the systemic circulation. Coatings should ideally hinder release in the oral cavity but allow complete and rapid release and dissolution before the drug reaches the site of absorption.

In the case of reverse enteric coatings of microparticulates, drug release should occur in the acidic environment of the stomach. However, this may be highly dependent on gastric residence time and the prandial state of the individual. In the fed stomach, pH may be raised which may inhibit the release of API from the coated particulate. Thus, the coating material should be selected carefully to avoid this as the choice of polymer is pivotal in obtaining the desired plasma concentration-time profile.

Reverse enteric polymers such as Eudragits® are often employed for taste masking purposes [25, 32, 64, 75-77]. These can selectively swell or dissolve in certain conditions, releasing the drug in specific areas of the GI tract for absorption whilst masking the bitter taste of the drug in the mouth. Eudragit E® is insoluble above pH 5 and does not release the API until reaching the stomach, thus is suitable for taste masking purposes. However, consideration should be given to whether the drug is degraded in the stomach, such as cefuroxime. This undergoes microbial degradation in the stomach, so the system can be combined with other pH sensitive polymers such as Eudragit L® [78]. This is an enteric polymer which does not release the drug until above pH 6, thus protecting the API from degradation in the stomach and allowing release in the early intestine. However, enteric polymers which delay release until the small intestine may not be suitable for drugs with a narrow

absorption window in the upper GI tract [78]. Potential rapid gastric expulsion of dosage forms must also be considered when using pH sensitive coatings in isolation or in combination. Resultantly, polymers should be chosen very carefully and the formulation should be evaluated *in vitro* for dissolution and absorption using biorelevant models to ensure the pharmacokinetic profile of the API is not altered by the coating.

Hoang Thi *et al.* [37] also found the composition of the coating layer to have an impact on the dissolution profile. In this study, acetaminophen particles were coated with sodium caseinate and lecithin in different ratios for taste masking. Both ratios hindered drug dissolution in phosphate buffer pH 7.4 compared to pure drug as shown in Figure 1.2. Whilst this may be suitable for taste masking purposes, it may also reduce the bioavailability and efficacy of the formulation.

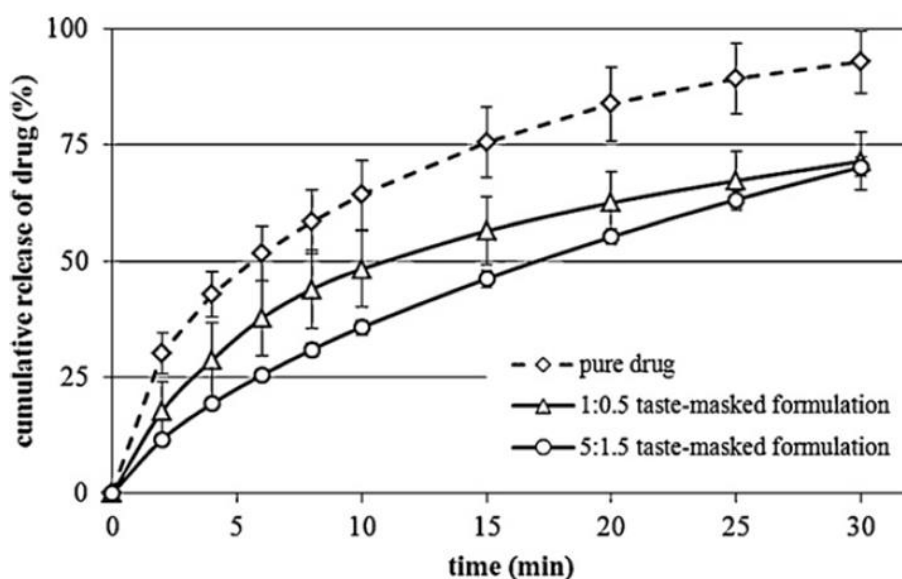
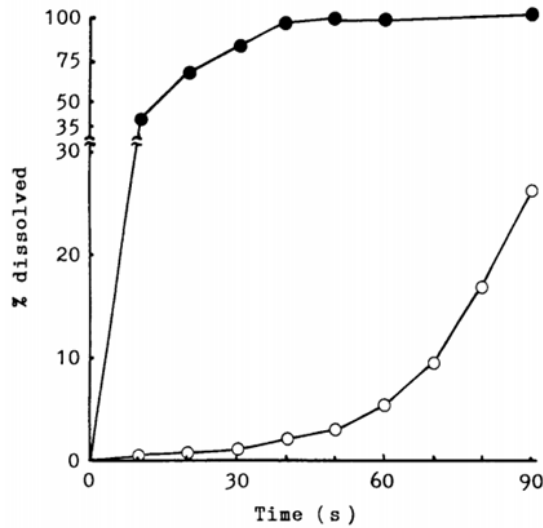


Figure 1.2: Hindered dissolution of acetaminophen from taste masked powders coated with different ratios of sodium caseinate: lecithin. Data obtained using flow-through mini-column apparatus, phosphate buffered saline pH 7.4, 1 mL min⁻¹. Taken from Hoang Thi *et al.* [37].

The very presence of a coating layer, and the composition of the coating and core particles can have a substantial effect on the dissolution and thus absorption and bioavailability of taste masked particles compared to other formulations. This was demonstrated by Shirai *et al.* [33, 35] as shown in Figure 1.3.



●, core fine granules; ○, coated fine granules.

Figure 1.3: The effect of polymer coating on the dissolution of granules of sparfloxacin using paddle apparatus, 50 rpm, 900 mL distilled water. Taken from Shirai *et al.* [35]. Reproduced with permission from Biol. Pharm. Bull. Vol. 16, No. 2. Copyright (1993) The Pharmaceutical Society of Japan.

More recently, it has been demonstrated that the thickness of the coating [21, 22] (Figure 1.4) and the molecular weight of the polymers [36] can also affect dissolution and thus the pharmacokinetic profile.

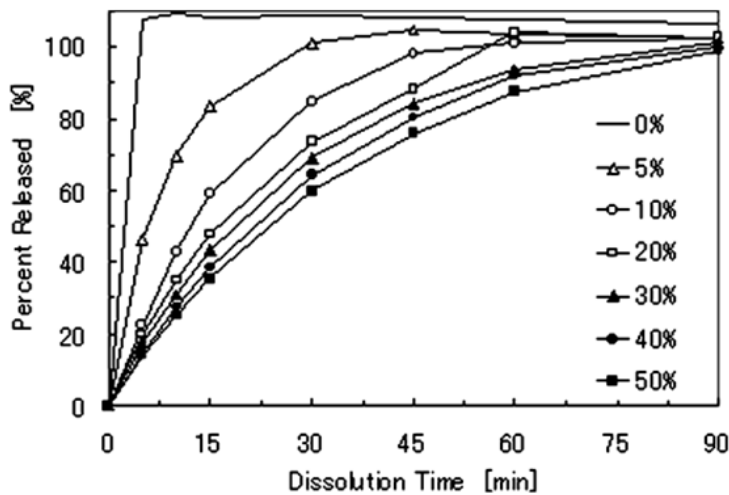


Figure 1.4: Dissolution rate of ibuprofen from core particles with different amounts of film coatings using paddle apparatus, 50 rpm, Japanese pharmacopoeia XV dissolution medium No. 2 pH 6.8, 900 mL. Taken from Hamashita *et al.* [22] Reproduced with permission from Chem. Pharm. Bull. Vol. 56, No. 7. Copyright (2008) The Pharmaceutical Society of Japan.

Of equal importance in coated formulations is the composition of the core material. For example, ibuprofen particles were made using four core components and coated with the same film material [21]. However, dissolution profiles of the four formulations differed as shown in Figure 1.5. The authors found that when dissolution rate was rapid, taste masking was compromised; however, when dissolution rate was slow, bioavailability was compromised. This highlights the importance of developing formulations which efficiently mask the taste sensation of the API without affecting the pharmacokinetic profile.

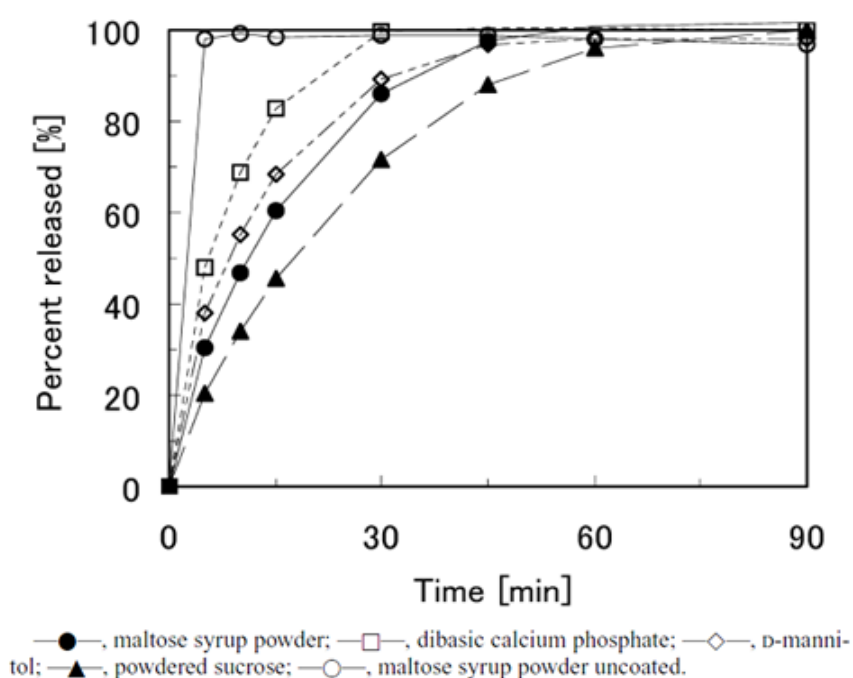


Figure 1.5: Dissolution of ibuprofen from coated particles containing different core materials using paddle apparatus, 50 rpm, Japanese pharmacopoeia XV dissolution medium No. 2 pH 6.8, 900 mL. Taken from Hamashita *et al.* [21]. Reproduced with permission from Chem. Pharm. Bull. Vol. 17, No. 3. Copyright (2007) The Pharmaceutical Society of Japan.

Particles in which alternative methods of taste masking were employed have also been shown to have an impaired or altered dissolution profile as a result of taste masking. Formulations using ion-exchange resins demonstrated dissolution to be dependent on the choice of resin [60], particle size of complexes [59], and ratio of API to resin [57]. In taste masked lipid formulations, the choice of lipid binder and

solubilising agent was shown to affect the dissolution profile of a poorly soluble drug in lipid pellets [15]. In lipid microspheres, the size of the microspheres affected both the rate and extent of dissolution. The composition of fatty acids within the microspheres also effected *in vitro* release rate [17]. Furthermore, the rate and extent of cetirizine release from cross-linked chitosan microparticles was shown to be dependent on chitosan concentration [79].

In our case, taste masked microparticles are often incorporated into ODT formulations. These disintegrate in saliva and are subsequently swallowed, avoiding the requirement to swallow large solid dosage forms. However, this generates potential for administration without water. Such “dry delivery” could affect oropharyngeal and oesophageal transit time as particles may be retained within these regions for extended periods of time. Thus the pharmaceutical industry aims to generate formulations with extended taste masking time. However, this has the potential to affect the dissolution and hence pharmacokinetic (PK) profile and bioequivalence of the formulation. Wilson *et al.* [80] investigated the distribution of a rapidly dissolving formulation of benzodiazepine with a radiolabelled ion-exchange resin. Using dry delivery and normal swallowing, they found that after the 9 minute experiment, 8 % of the resin remained in the glottal area, confirming that taste masking for extended periods of time (perhaps up to 30 minutes to completely avoid adverse taste) is an important consideration for the pharmaceutical industry.

In summary, taste masking techniques have been shown to delay, alter or hinder the dissolution of the API *in vitro*. This may adversely affect the absorption of the API and the PK profile compared to pure drug or non-taste masked formulations. It is therefore important to assess the dissolution of the taste masked formulation for two reasons. Firstly, one can predict the taste masking capability of the formulation by estimating the likely release in the oral cavity. This requires biorelevant models of the oral cavity for accurate prediction of whether drug release is above or below the bitterness threshold of the API. Secondly, one should assess the effect of taste masking on the PK profile using dissolution testing models representing the remainder of the gastrointestinal tract, or crudely, the stomach and small intestine.

The dissolution methodology employed in the evaluation of taste masked formulation is discussed later in this Chapter.

1.5 Anatomy and Physiology of the Oral Cavity

To predict the likely release of API in the oral cavity, dissolution testing should mimic the oral environment as closely as possible. Therefore, one must firstly consider the anatomy and physiology of the oral cavity.

The main anatomical features of the oral region are shown in Figure 1.6 and include the teeth, gingivae, tongue, lips and palate. Many other important anatomical features contribute to this region including a complicated arrangement of muscles, nerves, blood vessels and lymphatic drainage [81-84].

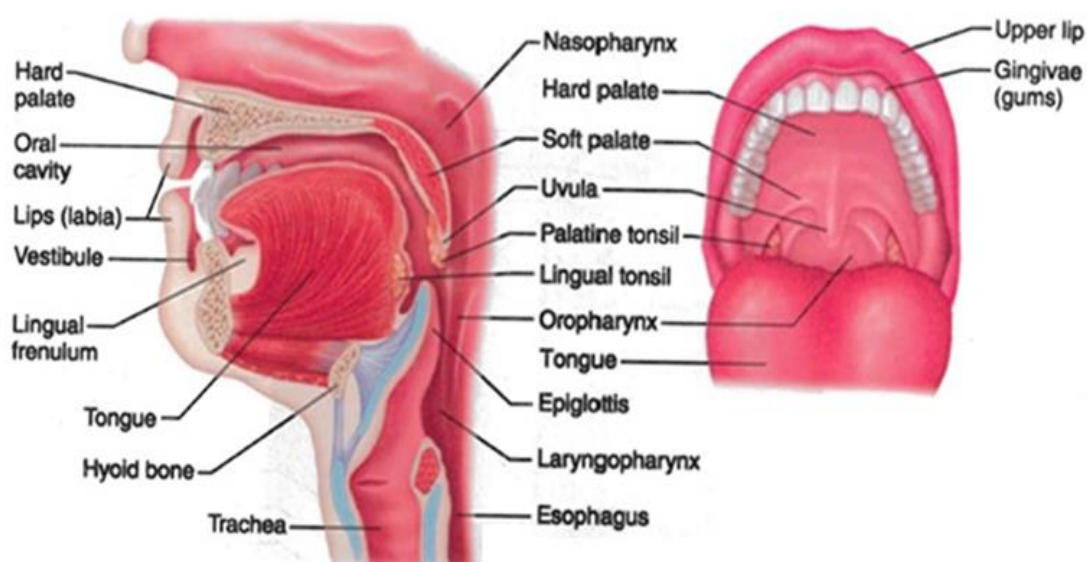


Figure 1.6: The main anatomical features of the oral region [82].

The oral cavity can be further divided into two sections. Firstly, the oral vestibule, situated between the lips or cheeks on one side and teeth and gums on the other and secondly, the oral cavity proper, which is behind the teeth and confined by the palate, tongue and oropharynx.

The oral cavity is covered by a lining known as the oral mucosa. This comprises poorly permeable, keratinized hard palatal and gingival mucosa. It also comprises the more

permeable, non-keratinized sublingual, soft palatal and buccal mucosa. The degree of keratinization affects drug absorption from these sites, along with the thickness of the mucosa, blood flow and saliva flow. For example, the sublingual mucosa is only 100 - 200 μm thick compared to the buccal mucosa which is 500 - 600 μm , so has greater permeability than buccal mucosa, which in turn has greater permeability than keratinised palatal mucosa. However, drugs are quickly removed from the sublingual mucosa in saliva, thus only rapid acting, highly permeable drugs would be absorbed at this site [85-87].

Saliva is generated by three major salivary glands, shown in Figure 1.7, and several minor salivary glands. The major salivary glands, in order of size are the parotid, submandibular and sublingual glands, which together contribute approximately 90 % of total saliva. The remainder comes from minor glands which are located all over the oral cavity with the exception of the anterior hard palate. Salivary glands have a secretory acinus in which isotonic saliva is released. As the saliva travels through the duct of the gland, electrolyte exchange takes place resulting in a hypotonic solution which is released [81, 88]. The excretion process is discussed in more detail in Chapter 3.

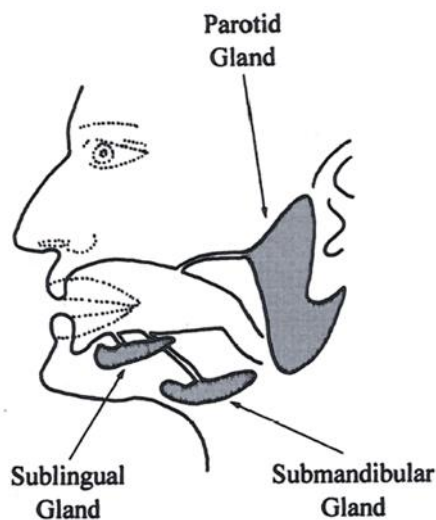


Figure 1.7: Location of the three main salivary glands. Adapted from Moore *et al.* [84].

Unstimulated saliva flow rate has been shown to range between 0.05 - 2.87 mL min⁻¹ with mean values between 0.37 and 0.56 mL min⁻¹ across several studies [89-92]. The volume of saliva present has been stated to range from 0.09 - 1.86 mL, with mean or median values in the range 0.37 - 0.70 mL and only approximately 30 % of saliva being swallowed in each unforced swallow [91, 93, 94]. Additionally, the pH of saliva has been shown to range from 5.45 - 7.8 [89, 90, 92, 95, 96]. The characteristics of saliva, including the pH and flow rate are discussed in more detail in Chapter 3.

Saliva is produced for lubrication of the oral cavity, protection of dentition and soft tissues, digestion of food, anti-microbial purposes and to deliver molecules to the taste buds [97]. Natural saliva is a complex aqueous solution containing 99 % water and a diverse spectrum of inorganic ions, small organic molecules and proteins. The inorganic ions present are bicarbonate and phosphate, which contribute to the buffer capacity of saliva, and electrolytes such as sodium, potassium, magnesium, zinc, calcium, chloride, fluoride, iodide, thiocyanate and nitrates. The small molecules present include steroid hormones, amino acids, glucose, creatinine, and urea. The proteins present include immunoglobulins, mucins (which contribute to the viscosity of saliva), enzymes including lingual lipase and amylase, growth factors and anti-microbial factors such as lactoferrin and lysozyme [97]. As for any biological fluid, the complexity of natural saliva renders it extremely challenging to recreate the exact composition.

1.6 Physiology of Taste and Bitterness Reception

Taste buds are located in circumvallate, foliate and fungiform papillae, shown in Figure 1.8 observed on the tongue and palate. They are comprised of an assembly of taste receptor cells, which have taste receptors for the five taste sensations: sweet, salt, sour, bitter and umami. Umami is a relatively recent discovery and represents tastes associated with the savoury flavours of monosodium glutamate and aspartate. These receptors vary in nature from G-Protein coupled receptors to ion channels, which innervate a series of afferent neurons before reaching higher order brain centres [81, 98].

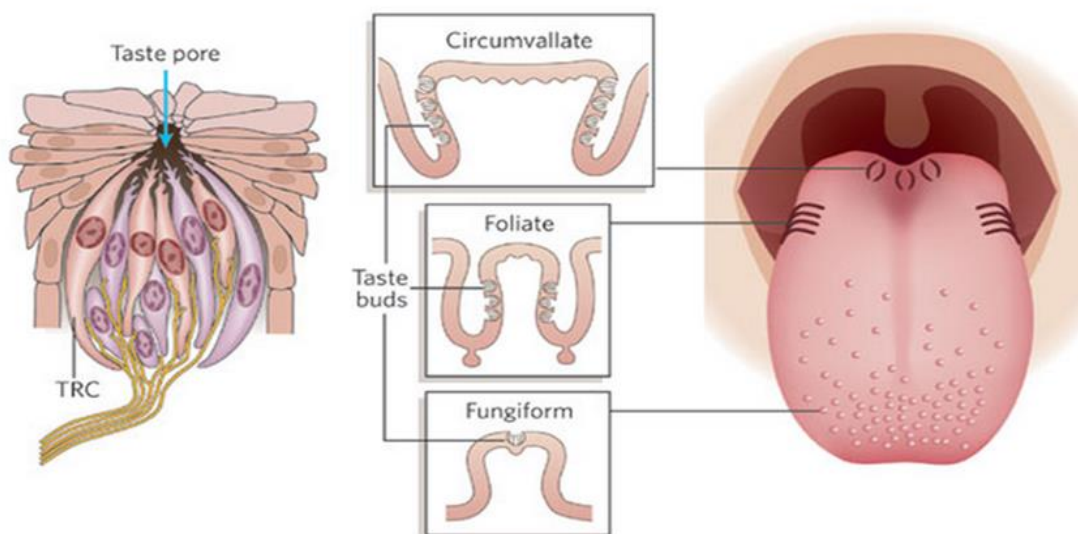


Figure 1.8: The location of taste buds on the tongue and schematical representation of a taste bud. Taken from Chandrashekar *et al.* [98]. (TRC = taste receptor cell).

Each taste sensation aids us in making decisions about the quality of food we ingest. This develops from a very early age since a human baby just a few days old can distinguish between favourable sweet sensations and unfavourable bitter sensations [99]. Substances possessing sweet and umami tastes encourage their own consumption, whilst sour tastes warn us that food may be spoiled or rancid, and salty tastes help to regulate our electrolyte consumption. The bitter taste sensation, associated with numerous APIs, was developed to prevent the ingestion of toxic bitter compounds. Thus it is natural for the patient to reject bitter tasting drugs and imperative that bitter taste is masked.

For many years, it was believed that taste mapping existed on the tongue, whereby each region of the tongue was receptive to only one taste sensation, meaning bitter taste could only be sensed by a certain part of the tongue. However, this theory is now outdated and reception of all five basic tastes is now demonstrated to occur across all areas of the tongue [98]. Therefore, each taste bud contains the receptors for all five taste sensations. However, it is not presently clear how different taste sensations are decoded.

One theory proposes that taste is decoded in the periphery. In this proposal, each taste receptor cell within the taste bud is tuned to detect just one taste sensation

and possesses receptors for that one taste only. Multiple taste receptor cells make up each taste bud, allowing all tastes to be received by a single taste bud.

Another theory proposes that decoding occurs centrally rather than peripherally. This theory is thought to be the prevailing model for nearly three decades [98]. In this theory, a single taste receptor cell expresses receptors for many taste sensations, thus recognising all taste modalities. This combined information is then decoded centrally and interpreted.

The salt and sour tastes are the least well understood in terms of their reception. It is understood that salt reception is mediated via amiloride-sensitive sodium channels. The sour taste is thought to be associated with a member of the transient receptor potential (TRP) ion channel family, PKD2L1, which has been found to be expressed in sour sensitive taste receptor cells not responsive to sweet, umami or bitter tastes [98]. The sweet, umami and bitter tastes are far better understood and are associated with certain G-protein coupled receptors (GPCRs).

The favourable taste sensations are mediated by three GPCRs: T1R1, T1R2 and T1R3. The T1R1+3 receptors form a heterodimer capable of detecting umami tastes, whilst the T1R2+3 receptors form a heterodimer for the detection of sweet tastes. In mice, if either the T1R2 or 3 part of the receptor is knocked out, leaving a homozygous receptor for the remaining part, the mouse suffers a drastic loss of sweet taste sensation [100]. It is interesting to note that the substance itself is not sweet, but the way that the substance is received makes us decide it is sweet. For example, cats possess mutations in the T1R2 receptor gene, causing inactivation of the receptor, resulting in their inability to detect sweet sensations. Similarly, polymorphisms can occur in bitterness receptor genes in humans affecting our perception of bitterness.

The bitter taste is also mediated by GPCRs, T2Rs. The number of T2Rs in the family varies depending on the literature cited. Reviews in 2001 and 2002 describe 24 and 26 T2Rs respectively [99, 100]. A review in 2006 stated there were approximately 30 T2Rs [98], whilst a 2014 review detailed that the number of T2Rs varies from 3 to 49 depending on the species [101]. The large number of receptors allows humans to detect a vast range of potentially toxic substances. However, not all humans would

perceive the same substance to be bitter. Two typical examples are the sensitivity to phenylthiocarbamide (PTC) and 6-n-propyl-2-thiouracil (PROP). In some people, these evoke an intense bitter taste sensation; however, other people fail to detect them as bitter and perceive them as tasteless. These two molecules are detected by T2R38, which has two alleles – one which codes for a PTC/PROP sensitive receptor, and one coding for an insensitive receptor [101]. Polymorphisms have also been found to exist in T2R16, 31 and 43 in humans which no doubt also cause differences in perception of bitterness between individuals [102]. In addition, olfactory and visual information, hunger or satiety, previous exposure and conditioned responses to certain substances may influence individuals' perception of bitterness [98].

Interestingly, bitter taste receptors, T2Rs, have been found in extra-oral locations, including the respiratory system [103, 104] and gastrointestinal tract [102]. These may have some involvement in regulation of digestion and metabolic processes. However, it is not thought that taste buds occur in these locations. The effect of bitter APIs on these taste receptors remains unclear.

1.7 Effect of Age and Gender on Taste Perception and Reception

Weiffenbach, Cowart and Baum [105] stated that early taste threshold studies reported substantial and significant sensitivity decreases with age. They asked 170 participants between the ages of 23 and 88 years old to scale the intensity of four tastant solutions – sweet, sour, salty and bitter. They measured the intra class correlation coefficient, which represents the consistency of participants' responses to each taste. They found that older individuals obtained lower correlation coefficients than younger individuals, indicating a less consistent response to a particular tastant, and a lower ability to detect tastants reliably with increased age.

Similarly, Kennedy *et al.* [106] investigated sweetness detection and recognition thresholds in a young adult population (18 – 33 years) and older adult population (63 – 85 years) and found significant differences in detection and recognition thresholds between the two age groups, with older adults less likely to identify the taste. This may result in pharmaceutical preparations requiring more sweeteners or flavours in

the older population to make them palatable, however, they did not consider bitter taste.

One article by Ng *et al.* [107] investigated the response of different age groups to the savoury flavours of pork and beef and found that taste threshold in the elderly group was significantly higher than middle aged or young adults, and that taste threshold increased with age. Again, this article did not investigate the bitter taste.

Age related changes in perceived sour intensity have been reported [108], as well as a generic taste loss in response to all five basic tastes associated with increased age in one study [109]. Another study agrees with these conclusions and found that increased age resulted in increased recognition thresholds for the four main taste sensations (not considering umami) with significantly higher recognition thresholds in the older group compared to younger adults [110].

Further supporting the phenomenon of age related decline in taste perception of bitter substances is research by Cowart, Yokomukai and Beauchamp [111] who observed an age related decline in the threshold and suprathreshold sensitivity to the extremely bitter molecule quinine, but interestingly not for urea, when younger adults (aged 18 – 38) were compared to elderly adults (aged 65 – 86). This indicates that sensitivity may not be lost to the same degree to all tastant types.

Moreover, Schiffman *et al.* [112] also observed increased detection and recognition thresholds in older adults, compared to younger adults for bitter compounds. Interestingly, they also observed a strong correlation between bitter threshold and the log P of the compound, however, this was not noted at suprathreshold concentrations and may require further investigation.

All of the above studies investigated age related changes in the perception of tastants in elderly adults compared to younger adults. However, these researchers did not consider how taste is perceived by children. There appears to be far fewer studies investigating this in the literature, perhaps due to the ethical issues associated with clinical testing of compounds in children in past years.

To address this issue, research by James *et al.* [113] evaluated the response to different tastants, namely aqueous solutions of sucrose, and three foods: orange juice, custard and shortbread biscuits. Each type of stimulus had five different levels, representing five different levels of sweetness. They compared the different tastants and were asked to estimate the magnitude of each taste sensation. The study comprised of two groups: healthy adults with a mean age of 20.7 years, and healthy children with a mean age of 8.9 years. The results showed a similar response for adults and children to all stimuli with the exception of orange juice, where children showed a reduced response across all concentrations compared to adults. Gender was found to have no effect in the response for the children's response to any of the stimuli. This research indicates that at mid-childhood (8 – 9 years of age), children have a similar response to sweetness magnitude as adults. However, unfortunately, bitter compounds were not evaluated in this research.

Another study [114] conducted a post hoc analysis to investigate the response of children to different types of oral medication. They found that children possessing the bitter sensitive alleles of taste receptor genes were more likely to have taken solid medication rather than a liquid formulation, and preferred higher concentrations of sucrose in their beverages. These findings do not come as a surprise, but do indicate that personalising medicine to reflect children's genotype and thus inherent ability to detect bitter compounds may be an avenue for future paediatric formulation development. However, unfortunately in this study, children's perception of the same bitter compound was not compared to that of adults.

An additional study [115] investigated how children aged from "kindergarten" to "third grade" and adults responded to different spices and found no significant difference between children of any age and adults in response to detection of spices at different concentrations.

In general, literature reveals that the elderly population have a poorer perception of taste compared to younger adults, however, this may not affect all taste sensations equally within the same individual. To the best of our knowledge, no research groups compare changes in taste perception over age within the same individual due to

difficulties in experimental design, which would result in very long studies across multiple researchers and years of participant compliance with the trial. Nevertheless, one should compare within subject differences in taste with respect to age in order to take into account genetic polymorphisms. In most of this research, it is not clear if poor taste perception in the elderly group is due to genetic polymorphisms of the taste receptor genes as discussed in Chapter 1, or due to loss of taste perception. Study design should be carefully considered to account for this. In children, fewer studies have been conducted, however response to sweetness in children aged 8 – 9 years, and response to spices in children of a broad age range is comparable to adults. Bitter taste perception requires further assessment in children in comparison to adults. It is also noteworthy that although perception of sweetness or bitterness in children may be comparable to humans, willingness to eat bitter substances may be less in children due to a lack of environmental exposure. As stated previously, tastes allow us to make decisions about the nutrients we intake, with sweet taste encouraging consumption and bitter taste warning of potential toxic compounds. Over time and environmental exposure, adults learn to tell which bitter substances are safe, and to regulate their sweet intake for health reasons. However, children may lack this understanding. Less evidence is available on the effect of gender on taste perception, however, in general, no differences are observed in either the adult or child population.

1.8 Existing Dissolution Testing Methods

Sequential dissolution testing, using more than one medium to simulate the compartments of the GI tract is of high importance for taste masked formulations. One should use a biorelevant oral model to assess taste masking efficiency, followed by a biorelevant model of the stomach and intestine to assess the effect of taste masking on bioavailability of the API. However, very few research groups have employed a sequential dissolution process in the *in vitro* assessment of their taste masked dosage forms [19, 24, 60, 63], and to our knowledge none have employed a suitable sequential process in which the entire GI tract is modelled including the oral cavity.

The British Pharmacopoeia (BP) has recommended dissolution tests for delayed release dosage forms. However, these are not appropriate for taste masked particles as they aim to mimic the stomach and the small intestine with the use of an acid stage followed by a buffer stage, with no consideration of the oral cavity. For coated granules, the reader is directed to dissolution tests for solid dosage forms within the BP, as above [116]. The lack of appropriate pharmacopoeial standard dissolution test for taste masked particles has led to wide variety in the dissolution methods adopted. This project aims to work towards development of an appropriate biorelevant dissolution model for the evaluation of taste masked or alternative oral formulations such as microparticulates. However, we firstly evaluate the existing dissolution methods used in the evaluation of taste masked particles.

1.8.1 Single Dissolution Medium

Some research groups used a single dissolution medium in the *in vitro* release assay for evaluating taste masked particles. Most commonly, phosphate buffer was the medium of choice at pH 6.8 [10, 11, 41, 75, 117-122]. However, phosphate buffers with other pH values in the range 5.6 - 8.0 have also been employed [15, 17, 36, 79]. Water has also been used in some studies [29, 33, 43, 62] and the addition of surfactants to water or phosphate buffer has also been demonstrated. Mizumoto *et al.* added 0.1 % v/v Tween 80®, a non-ionic surfactant to phosphate buffer in order to improve the wettability of the taste masked famotidine particles [123, 124] whilst Hamashita *et al.* used 0.01 % v/v polysorbate 80 in phosphate buffer pH 6.8 [21] and Lee *et al.* used 0.1 % w/v sodium dodecyl sulphate in water [40].

In some of those cases [33, 36, 41, 43, 62, 117, 121], the single medium was used to represent dissolution performance in the small intestine. This was coupled with a different method of taste masking evaluation in most examples such as human taste testing panels. The remaining examples detailed above evaluate the *in vitro* drug release under conditions similar to those observed in the oral cavity, where pH has been shown to be 5.4 – 7.8 [95].

Use of a single medium may enable the researcher to predict taste masking performance by investigating release under simulated oral cavity conditions.

However, in these studies, physiologically relevant volumes were not used. The medium volume in the above examples varied between 100 - 900 mL which may cause an over-estimation of drug release as the volume of saliva in the mouth is typically less than 2 mL [93]. The dose used for *in vitro* dissolution testing was not increased to compensate for the greater than physiological volumes employed in any of these cases. However, if the formulation is designed to be ingested with a glass of water then dissolution testing to reflect intake conditions may be appropriate using volumes of 200 - 300 mL. Additionally, sole use of an oral cavity model does not inform the researcher if the formulation has sufficient dissolution further along the GI tract, and whether the coating, or other taste masking technique allows release of the API for absorption.

It should also be noted that some researchers used a single dissolution test based on gastric conditions. Commonly, 0.1 M hydrochloric acid (HCl) has been employed [13, 56, 57, 64], however 0.07 M HCl [30], simulated gastric fluid (SGF) [59] and HCl with addition of surfactant [16] have also been used. A single dissolution test in a gastric or intestinal medium may inform the researchers about the dissolution of the formulation in that particular location, but is not useful in the evaluation of taste masking.

1.8.2 Dual Dissolution Media

There are numerous examples in the literature where two media have been used to evaluate taste masked particles. In most cases, parallel dissolution tests were undertaken using the paddle apparatus with different media. In such cases, the media was commonly HCl pH 1.2 and phosphate buffer pH 6.8. This can be further divided into cases where the phosphate buffer stage was used to describe the fate of the formulation in the oral cavity [27, 28, 32, 44, 45, 65] or in the small intestine [46, 52, 54, 55]. Oral pH has been demonstrated to be 5.4 - 7.8 and intestinal pH varies between pH 5 - 8 depending on the location and pre- or post-prandial state [95, 125, 126]. Where prolonged periods of time were used for the phosphate buffer stage, results are usually translated to the fate of the formulation in the small intestine.

Water has been used as an alternative to phosphate buffer to represent the oral cavity conditions in some cases [48, 77].

Parallel dissolution testing using acetate buffer pH 4 and phosphate buffer pH 6.8 is also observed within the literature [18, 61, 127]. In these cases the acidic pH can be considered a compromise between fasted pH which has been stated to be between 1 - 3 and fed pH which can reach values of up to 7 [95, 125, 128]. Where parallel tests are undertaken, the possibility of the API dissolving in acidic medium then precipitating out in the higher pH of the intestine is not considered.

In some cases, *in vitro* testing took place in a single dissolution bath with changing media [19, 24, 60, 63]. Sequential dissolution testing was performed using an acid stage (0.1 M HCl) followed by a buffer stage (phosphate buffer pH 6.8) as described in the BP [116] for delayed release oral dosage forms. Such sequential methodologies could be considered superior to alternatives as they are more physiologically representative. Importantly, the above cases do not model the oral cavity and cannot be used to predict taste masking efficacy.

1.8.3 Multiple Dissolution Media

Multiple *in vitro* dissolution tests have been performed on certain taste masked formulations. Ishikawa *et al.* compared taste masked granules at pH 1.2, 5 and 6.8. pH 5 was used to simulate cases in which gastric pH is elevated by drugs or food [76]. Granules were coated with Eudragit® E, a reverse enteric polymer, and dissolution was assessed using basket apparatus containing 900 mL media, 100 rpm. The results showed complete and rapid release of API in the pH 1.2 medium. At pH 5, release was complete but dissolution rate was slower. Finally, at pH 6.8, incomplete release was observed with less than 10 % of API dissolving over 480 minutes.

Similarly, Ostrowski *et al.* compared taste masked enteric coated pellets to a suspension formulation and evaluated them at pH 1.2, 4.5 and 6.8 [20]. Chun and Choi [49] investigated release from an ion exchange complex at pH 2, 4 and 6, whereas Robson *et al.* [38] investigated *in vitro* drug release from fatty acid microspheres at pH 5.9, 6.8, 7 and 8 and Agresti *et al.* [53] used pH 3, 5.5 and 7 to

evaluate release from a drug-peptide complex. Ogata *et al.* [129] used buffers of pH 1.2, 5, 6.8 and water to evaluate their taste masked granules. Chiappetta *et al.* [66] also used water as a dissolution media in the evaluation of their polymeric microparticles, which were analysed in pH 1.5 and 6.8 as well as tap water to simulate intake conditions. Dissolution tests were carried out in parallel in all the above mentioned examples using 500 - 1000 mL media where specified and generally either paddle or basket apparatus. Thus none of these methodologies are biorelevant, particularly when considering the oral cavity dissolution.

1.8.4 Methods Modelling the Oral Cavity

In literature highlighted thus far, several *in vitro* dissolution tests have been performed at oral pH. However, previously discussed examples are not an accurate representation of the oral cavity in terms of volume and agitation. Our attention is now drawn to *in vitro* dissolution testing where the researchers aimed to model the oral cavity in more depth in order to accurately predict taste masking performance.

Lee *et al.* [51] evaluated polymer coated nanohybrid particles of sildenafil at neutral pH in deionised water for two minutes as a model of the oral cavity in addition to testing at pH 1.2 as a gastric model. However, both tests were carried out using 900 mL media and the paddle dissolution apparatus. Such a large volume is not representative of the oral cavity. The dose of the formulation assessed was not increased to compensate for the greater dilution effect. Smaller volumes were used by Guhmann *et al.* [23] who evaluated polymer coated diclofenac particles in 50 mL of simulated salivary fluid (SSF) pH 7.4 which were stirred at 50 rpm over a maximum of 5 minutes and sampled. The composition of SSF used is shown in Table 1.1. Hamashita *et al.* [22] evaluated polymer coated particles in 20 mL Japanese Pharmacopoeia 2nd fluid of pH 6.8 for ten seconds. This contains a 1:1 mixture of phosphate buffer pH 6.8 and water [130]. A sample was placed into a 50 mL beaker and stirred with medium. These models incorporated representative residence times and media for the oral cavity, however, volumes were still excessive.

Table 1.1: Composition of Simulated Salivary Fluid (SSF) used by Guhmann *et al.* [23].

Component	Concentration
Potassium dihydrogen phosphate	12 mM
Sodium chloride	40 mM
Calcium chloride	1.5 mM
Sodium hydroxide	To pH 7.4
Demineralized water	To 1 L

Shukla *et al.* [58] and Sheshala *et al.* [31] assessed taste masked particles in 5 mL of phosphate buffer pH 6.8 by placing the sample into a 25 mL vessel with 5 mL medium and leaving to stand for either 60 or 120 seconds to model the oral cavity. Both groups also assessed the dissolution in HCl, with Sheshala *et al.* additionally testing in 900 mL acetate buffer pH 4.5 and phosphate buffer pH 6.8. Five mL is closer to physiological volumes of saliva, but still excessive. Additionally, no agitation was employed in these examples.

Numerous researchers assessed taste masked particles in 10 mL SSF where samples were placed into the media and shaken for 60 seconds. “Average salivary fluid” at pH 6.8 of unknown origin or composition was used by Patra *et al.* [50] who also performed dissolution testing on etoricoxib ion-exchange complexes in 900 mL 0.1 M HCl. pH 6.8 buffer was used as SSF by Randale *et al.* [25] who additionally used 500 mL SGF without enzymes for evaluation of polymeric metoclopramide microparticles. “SSF” of pH 6.2 was used by Yan *et al.* [26] who also tested polymer coated donepezil particles in 900 mL “SSF”. In this case “SSF” referred to the use of distilled water. Additionally, Khan *et al.* [131] performed dissolution tests on polymeric ondansetron microparticles in SSF pH 6.2 of unknown composition and 500 mL SGF without enzymes. In these cases, shaking the dissolution vessel may not be a reproducible method of agitation and may not accurately simulate the hydrodynamics of the oral cavity.

Simulations of the oral cavity using novel methods of agitation have been developed. Shirai *et al.* [34, 35] performed *in vitro* dissolution testing on taste masked granules

of sparfloxacin by placing a sample of the formulation into a 10 mL syringe and adding 10 mL water. Agitation was provided by revolving the syringe five times in 30 seconds. This method was developed based on the fact that *in vivo* taste tests involved volunteers holding 10 mL of an aqueous solution of the drug in water in the mouth. Similarly, Kondo *et al.* [132] placed a sample of coated paracetamol granules into a 10 mL syringe with 10 mL water. Agitation was provided by ten repeat syringe inversions over 30 seconds.

Perhaps one of the more reproducible and physiologically representative methods adopted for *in vitro* modelling of the oral cavity in the assessment of taste masked particles is that outlined by Thia *et al.* [37]. The authors used a syringe pump connected by tubing to a mini column. The sample was placed in the column with further tubing connecting the distal end of the column to a vessel for sample collection as shown in Figure 1.9. The column was heated to 37 °C with a column heater and phosphate buffer of pH 7.4 was passed through the system at a flow rate of 1 mL min⁻¹. The 1 mL min⁻¹ flow rate has been widely used in saliva flow modelling [133].

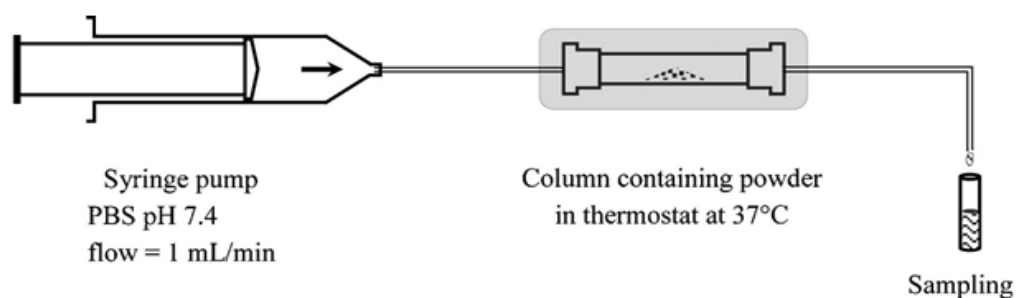


Figure 1.9: Mini column apparatus used by Thia *et al.* [37]. (PBS = phosphate buffered saline).

A similar mini column method, shown in Figure 1.10 was also adopted by Yajima *et al.* [134] for evaluation of the bitterness of clarithromycin dry syrup. Here, phosphate buffer, pH 6.5 filled the column and was then pumped through the column at flow rates of 0.3, 0.5 and 0.7 mL min⁻¹. Better correlation between *in vitro* release and

sensory analysis results was seen for the mini column method than for shaking, inversion or paddle methods suggesting the superiority of this approach in modelling the oral cavity.

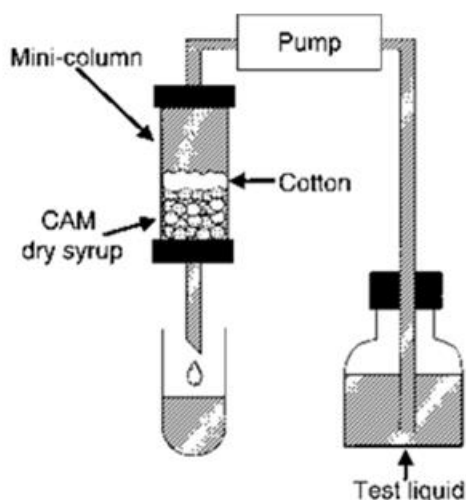


Figure 1.10: Mini column apparatus used by Yajima *et al.* [134]. Reproduced with permission from Chem. Pharm. Bull. Vol. 50, No. 2. Copyright (2002) The Pharmaceutical Society of Japan. (CAM = clarithromycin).

With respect to modelling the oral cavity, no pharmacopoeial recommendations are provided. There is a lack of consensus on the volume, apparatus, media and duration of the dissolution test that should be used, thus a variety of approaches have been adopted. The media representing saliva used in the evaluation of taste masked formulations by *in vitro* dissolution testing have been described in this section. However, it is worthy to note that other artificial salivas are described in the literature or available commercially. These are discussed in more detail in Chapter 3 as they have not yet been used to represent saliva in dissolution testing of taste masked formulations, and many were developed for other applications.

1.9 Limitations of Current Approaches and Future Directions

A vast array of dissolution tests has been executed aiming to predict taste masking capability and/or evaluate subsequent dissolution of taste masked formulations in the GI tract. Little agreement between researchers was observed. A single medium may represent the oral cavity to predict taste masking capability. However this

should mimic the oral environment as closely as possible, which has been achieved in very few of the described examples. It is also apparent that several different media have been used in these dissolution experiments to simulate saliva. Multiple media have been employed in some cases, although these are commonly performed in parallel, providing snapshot information about dissolution in certain conditions but failing to reflect the transit between compartments of the GI tract. Where a sequential dissolution process was carried out, only gastric and intestinal conditions were considered. Importantly, the oral cavity was not considered in these cases.

This introduction has highlighted the need for development of a sequential *in vitro* dissolution model for the evaluation of taste masked formulations. Ideally, this should include a physiologically representative sequential model of the oral cavity, stomach and small intestine. This could be used to evaluate not only the capability of the formulation to mask the taste sensation of the API by hindering release in the oral cavity, but to ensure release is sufficient elsewhere in the GI tract such that the overall pharmacokinetic profile of taste masked formulations is not altered compared to existing formulations. The model should also ensure that no precipitation occurs on transition between compartments. Ideally, the model should have the capability of discriminating between unstimulated and stimulated salivary states and the fed and fasted states. This is particularly pertinent in the case of reverse enteric polymeric coatings for taste masking purposes since the different prandial and stimulatory states may significantly affect drug release from the formulation.

For optimal accuracy of the model in predicting *in vivo* behaviour, physiological conditions should be modelled as closely as possible. Thus consideration should be given towards the relevant environments within the GI tract including the changing pH, agitation and hydrodynamics, volumes and media composition.

An accurate *in vitro* model should also consider the absorption in each compartment as this may reduce the amount of API transferred to the next stage of the sequential dissolution model. Estimating absorption also allows the user to predict the effect of the taste masking technique e.g. coating on the bioavailability and the plasma API concentration-time relationship after dosing.

Taste masked particles are nominally evaluated for *in vitro* dissolution, surface characteristics and *in vitro* or *in vivo* taste testing. Where taste masked formulations have been assessed *in vivo*, in terms of assessment of the effect of taste masking on the pharmacokinetic profile and bioavailability, this has generally been in bioequivalence studies where the formulation is compared to a marketed formulation in the species of choice, as shown in Figure 1.11 [13, 26, 29, 31-33, 35, 43, 48, 51].

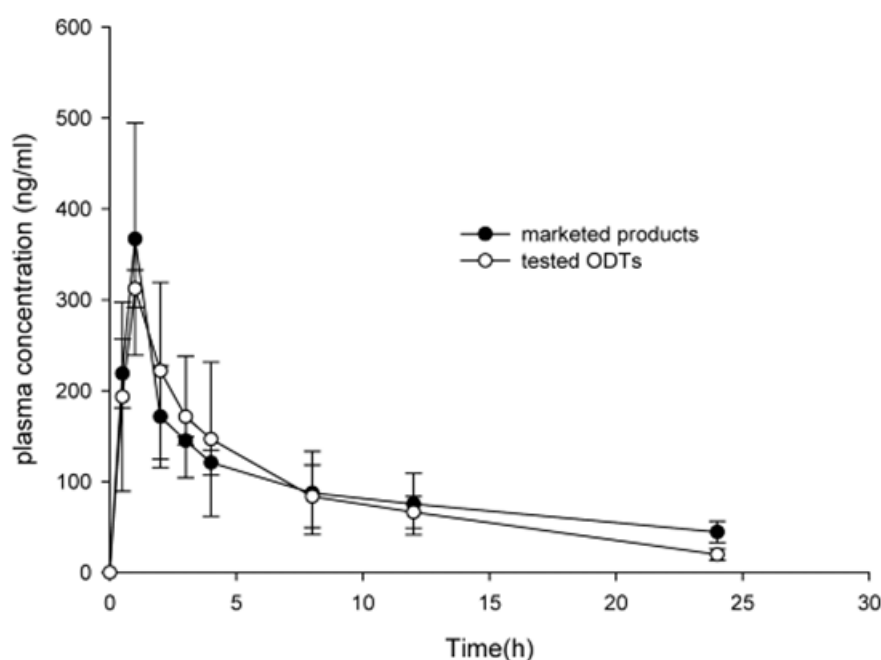


Figure 1.11: Plasma concentration-time graph demonstrating bioequivalence between an orally disintegrating tablet (ODT) comprised of microspheres of donepezil, taste masked by coating with Eudragit EPO[®], and marketed product after oral administration to rats [26]. Reproduced with permission from Biol. Pharm. Bull. Vol. 33, No. 8. Copyright (2010) The Pharmaceutical Society of Japan.

Most published reports achieved bioequivalence with the optimised formulation, reflecting the fact that absorption of the optimised taste masked formulation was comparable to the commercial formulation, and the taste masking technique did not affect the pharmacokinetic profile. The authors of Figure 1.11 assessed the dissolution of the taste masked product in a simulated stomach environment and found that the product had similar dissolution to the non-taste masked, commercial product. Similar dissolution performance *in vitro* suggests that comparable results

are likely to be seen *in vivo* for the two formulations. This was then confirmed using bioequivalence testing, as shown in the Figure. Although *in vivo* bioequivalence testing provides the most accurate assessment of the effect of formulation changes on bioavailability, appropriate sequential dissolution methodology should be able to predict these effects.

Permeability testing is occasionally carried out *in vitro* using cell culture, artificial membranes or *ex-vivo* tissue diffusion in diffusion cells or Ussing chambers [2, 135]. Specific models for estimating permeability in the oral cavity have also been developed and are described elsewhere [133, 136, 137]. However, these are not generally routinely applied to the *in vitro* evaluation of taste masked formulations. Alternatively, *in silico* modelling can be employed to estimate absorption based on the characteristics of the API and dissolution data using computational models [138-142].

The development of dynamic dissolution models, with or without absorption phases is described in detail by McAllister [143]. An example of a model which considers dissolution only is the artificial stomach duodenal model (ASD) [144]. This system comprises two compartments, the stomach and the duodenum, as shown in Figure 1.12. Fluid is pumped into each compartment to mimic flow of secretions. Fluid also flows from the stomach to the duodenum, and exits the duodenum to mimic physiological transfer between compartments. The system is relatively simple and computer controlled, requiring little operator input. It can be used in conjunction with biorelevant media and/or *in silico* predictions of the pharmacokinetic profile [145]. Media composition and volume may be altered to reflect different species, ages and disease states [146]. The ASD model has been used successfully to predict the performance of different crystalline or amorphous forms of an API [146, 147] and the effect of gastric pH variations [145]. Prediction of precipitation on transfer between the two compartments can also be studied using this model [144]. However, the model alone does not incorporate an oral cavity for the consideration of taste masking, nor does it model absorption in any way. Thus the assumption is made that the amount of API dissolved in the duodenal compartment is equal to the overall bioavailability. This is not the case for drugs where permeability and metabolism limit

systemic exposure. It also only models the upper GI tract, whereas many taste masked formulations exhibit delayed release for example due to pH sensitive coatings. This may result in an inaccurate prediction of formulation performance using this system. Thus it is best suited to comparison of immediate release formulations during pharmaceutical development.

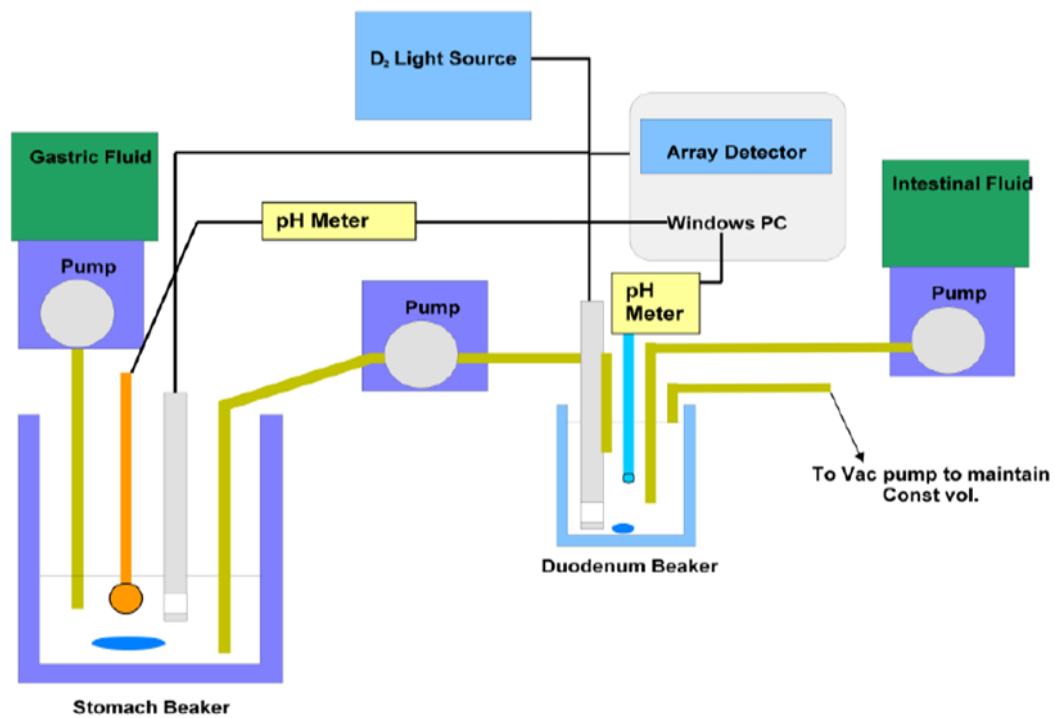


Figure 1.12: Schematic representation of the artificial stomach – duodenum (ASD) model. Taken from Bhattachar and Burns [144].

The most sophisticated and biorelevant model currently in existence is known as TNO Intestinal Model (TIM). This is a dynamic, computer controlled, multi-compartmental, sequential model considering both dissolution and permeability. TIM-1 models the stomach, duodenum, jejunum and ileum, shown in Figure 1.13, whilst TIM-2 models the colon only [148-151]. The two systems can be used together [152]. Both systems incorporate a predetermined pH profile maintained by computer controlled addition of acid or bicarbonate and flexible water filled walls to maintain physiological temperature with alternating water pressure to simulate peristaltic contractions. Valves between compartments can be programmed to mimic the gradual passage of chyme observed *in vivo*. Semi permeable lipid or dialysis membranes are used to

estimate the amount of drug available for absorption. TIM-1 also has computer controlled secretion of enzymes into each compartment. The TIM-2 system has an anaerobic environment maintained by nitrogen flow, where microflora can be cultured and grown prior to the experiment. It is also possible to introduce a solid meal to these models [150, 151].

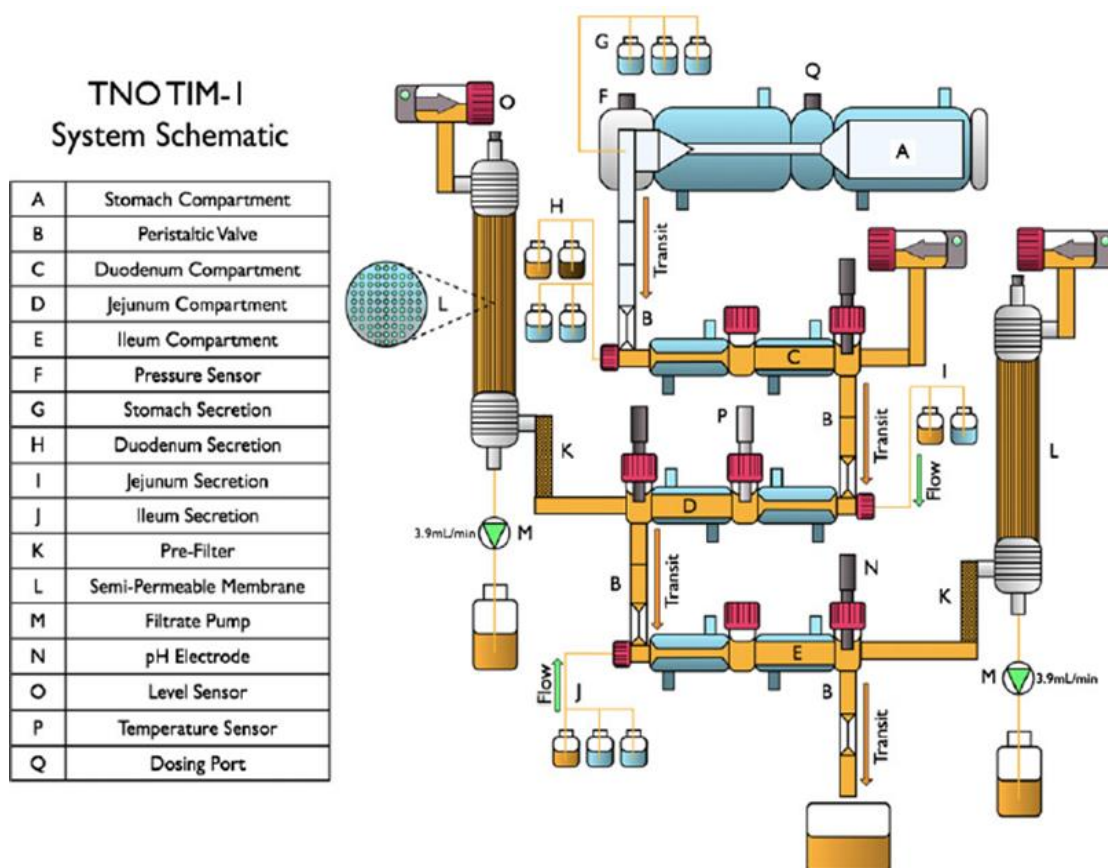


Figure 1.13: Schematic representation of the TNO intestinal model (TIM-1) system. Taken from Dickinson *et al.* [149].

However, these models are not optimal for evaluation of taste masked formulations as, crucially, they do not consider the oral cavity. TIM models are a tool of the pharmaceutical industry, resulting in limited access to them and consequently limited reports of their use. Existing literature generally details the application of these models to Biopharmaceutics Classification System (BCS) [153] class 1 drugs, which are highly soluble, highly permeable drugs. Investigations of the effect of prandial state and formulation have been employed [143, 149, 150, 154-156]. TIM-1 has been used in the successful evaluation of poorly soluble drugs in one report. This report also

describes internal projects in which TIM-1 did not distinguish between tested products, however differences were observed in clinical trials. The authors proposed that this was due to non-biorelevant stomach hydrodynamics and suggested the use of TIM is not appropriate where performance of the formulation is highly dependent on gastric emptying and agitation [149].

Another major limitation of the TIM models is that the semi permeable membranes are non-biological and rely entirely on passive diffusion to predict the amount of drug available for absorption. They cannot claim to predict absorption as they do not account for efflux, active transport or cellular metabolism. The membranes are only present in the jejunal and ileal sections, whereas absorption may occur from any of the compartments *in vivo*. This is particularly important for drugs which are absorbed in the upper small intestine. TIM-1 has been shown to overestimate maximal plasma concentrations in one study based on maximal jejunal dialysis concentrations due to a lack of first pass metabolism and distribution of the drug - factors which are not considered by the TIM-1 system [155].

There have also been reports of extensive plastic binding for some drugs, lowering their recovery. For example, mean recovery of BCS class 1 drug paroxetine was only 75 % [150]. Additionally, the number of replicates of experiments using these models are generally less than 3 due to the lengthy set up time [149]. The complexity of this model suggests that its application is not likely to be in quality control, but may lie in formulation development where differences in predicted pharmacokinetic profiles between formulations can be evaluated [150]. Despite their limitations, the TIM models are very prestigiously designed tools and can be considered highly biorelevant.

Thus a model which employs both a sequential, biorelevant dissolution process and estimation of absorption is needed in order to accurately predict the effect of taste masking on the pharmacokinetic profile of the API. A suitable model, incorporating an oral cavity and remainder of the GI tract with proven *in vitro-in vivo* correlation could reduce variation in the *in vitro* assessment of drugs or taste masked dosage forms, enabling comparison between research and reducing *in vivo* testing. Such a

model could be derived as an extension of an existing model. For example with BP dissolution apparatus 2, a mini paddle apparatus containing SSF could be used to represent the oral cavity with contents subsequently transferred to a larger vessel representing the stomach, followed by a third vessel representing the small intestine. BP dissolution apparatus 3 uses reciprocating cylinders whereby the sample is immersed in a dipping motion into fluids of different composition. An additional cylinder in this apparatus could be filled with a small volume of SSF. BP dissolution apparatus 4 uses a flow through cell whereby different media are passed over the sample within the cell. SSF could be used as the first of a sequence of biorelevant media. The ASD system could be extended by the addition of an oral compartment which flows into the stomach compartment. Alternatively, TIM-1 system could be coupled to a mini column apparatus for more biorelevant conditions than achievable using standard compendial apparatus. In order to also incorporate an absorption phase, dissolution vessels may be combined with cell layers or Ussing chambers. Additionally, dissolution data could be coupled with *in silico* modelling to predict the overall PK profile.

There are numerous opportunities in the development of novel biorelevant technologies for the assessment of taste masked oral formulations. It is perhaps most efficient to begin with a simple approach such as modified compendial apparatus and evaluate the most promising formulations in a more complex model such as the TIM-1 system coupled to an oral model. Whichever approach is adopted should be as biorelevant as practicable to allow for accurate prediction of taste masking efficiency and the effect of taste masking on the PK profile.

In addition to the numerous biorelevant gastrointestinal models in existence as discussed, biorelevant media have also been receiving attention in the biopharmaceutics arena. A vast array of media have been developed representing various biological fluids [157]. Examples of this include simulated gastric fluid, intestinal fluid and colonic fluid (SGF, SIF and SCF respectively), and the prestigiously developed fed and fasted state media representing the gastric and intestinal compartments – fasted / fed state simulated gastric fluids (FaSSGF and FeSSGF), fasted / fed state simulated intestinal fluids (FaSSIF and FeSSIF) and fasted / fed state

simulated colonic fluids (FaSSCoF and FeSSCoF). The FeSSGF and FeSSIF media have also been updated providing snapshot media reflecting the early, mid and late stages after feeding, during which digestion is beginning to take place and composition of fluids alters [128, 158-160]. Such biorelevant simulated gastric and intestinal fluids are widely used in dissolution testing [95, 161-163]. However, simulated salivary fluids have received less attention. This introductory review has demonstrated that no consensus on the choice of model representing the oral cavity, or dissolution media representing human saliva has been reached. This is perhaps because the characteristics of the oral cavity and of human saliva are not well understood. Therefore, the aim of this PhD is to develop a biorelevant dissolution methodology for drugs and dosage forms in the oral cavity. Our focus begins with the selection or development of a suitable biorelevant media representing human saliva. This media could be used in the assessment of taste masked dosage forms to evaluate taste masking efficiency, and coupled with an existing gastrointestinal tract model to predict the effect of taste masking (e.g. polymer coating) on API bioavailability and pharmacokinetics.

Taste masked formulations are most commonly evaluated *in vivo* by taste testing panels. This is subject to inter-individual variation and cannot be extrapolated to the paediatric population. Alternatively, *in vitro* dissolution testing is used to evaluate taste masking capability, by quantifying release in simulated oral cavity conditions. However, little agreement between the *in vitro* dissolution methodologies adopted is observed and most current methodologies are not biorelevant. Additionally, taste masking may affect the absorption and pharmacokinetic profile of the API by hindering dissolution and absorption in the GI tract. Thus dissolution testing should also mimic the rest of the GI tract to ensure the pharmacokinetic profile is not altered by the taste masking technique. Dissolution tests aiming to mimic one or more environment within the remaining GI tract were found to be highly variable, and not physiologically representative. We have identified the requirement for a model which considers both the oral cavity and the rest of the GI tract, ideally considering dissolution and permeability. To our knowledge, there is no such model currently in existence. The stomach and intestine have been extensively modelled, and

prestigiously designed biorelevant media representing these compartments are widely used. However, the oral cavity is less well modelled and there is no consensus on the composition of a media to represent human saliva. The aim of this PhD is therefore to develop a biorelevant dissolution methodology for drugs and dosage forms in the oral cavity. Our focus begins with the selection or development of a suitable biorelevant media representing human saliva.

1.10 Aims and Objectives of the PhD

Aims

1. The selection or development of biorelevant simulated salivary fluid(s) for use as dissolution media.
2. The development of a biorelevant dissolution methodology representing the oral cavity for the *in vitro* assessment of taste masking efficiency.

Objectives

- Investigate the properties of human saliva based on literature
- Characterise human saliva experimentally where literature is inconclusive regarding properties relevant to dissolution
- Evaluate the biorelevance of existing simulated salivary fluids available commercially or outlined in literature
- Select or develop the most biorelevant simulated salivary fluid(s) to be used in dissolution testing
- Confirm the suitability of the chosen media by direct comparison with human saliva for specified parameters, and by comparative dissolution experiments
- Evaluate potential dissolution models and apparatus
- Select and/or develop, and validate, a biorelevant dissolution methodology representing the oral cavity.

Chapter 2: Materials and Methods

2.1 Materials

Glandosane® (Cell Pharm GmbH, Hannover, Germany) was purchased from a local pharmacy. Saliva Orthana® spray and gel were kindly donated by CCMed® (Picket Piece, UK). Note that Saliva Orthana® gel is not currently a marketed product and its composition is confidential information. The full composition of these can be found in Tables 2.1 – 2.3 below.

VitaMelts™ vitamin C orally disintegrating tablets (Bioglan®, UK) were purchased from a local Holland and Barrett store. Xanthan gum, from *xanthanomas campestris*, and Tween 20® were purchased from Sigma-Aldrich® (Gillingham, UK). All other chemicals and solvents were obtained from Fischer Scientific (Loughborough, UK).

Table 2.1: Composition of Simulated Salivary Fluids: Phosphate Buffered Saline

Ingredient	Quantity (per 1000 mL)
Potassium Dihydrogen Orthophosphate	1 g
Dipotassium Hydrogen Orthophosphate	2 g
Sodium Chloride	8.5 g
Deionised Water	To 1000 mL
Sodium Hydroxide or Hydrochloric Acid	Adjust the pH if necessary to pH 6.8

Table 2.2: Composition of Simulated Salivary Fluids: Glandosane® [164]

Ingredient	Quantity (per 50 g aqueous solution)
Carboxymethylcellulose Sodium	0.5 g
Sorbitol	1.5 g
Potassium Chloride	0.06 g
Sodium Chloride	0.0422 g
Magnesium Chloride (6 H ₂ O)	0.0026 g
Calcium Chloride (2 H ₂ O)	0.0073 g
Potassium Monohydrogen Phosphate	0.0171 g

Table 2.3: Composition of Simulated Salivary Fluids: Saliva Orthana® Products

Ingredient	Amount (per 100 mL aqueous solution)
Porcine Gastric Mucin	3500 mg
Methyl-4-hydroxybenzoate	100 mg
Benzalkonium Chloride	2 mg
EDTA Disodium Salt.H ₂ O (E386)	50 mg
H ₂ O ₂	250 ppm
Xylitol	2000 mg
Peppermint Oil	5 mg
Spearmint Oil	5 mg
NaCl	45 mg
KCl	63 mg
CaCl ₂	30 mg
K ₂ HPO ₄	10 mg
KOH	76 mg
Xanthan Gum (in Saliva Orthana® gel)	500 mg

2.2 Ethical Approval

All saliva samples were collected in accordance with ethical approval number R12122013 SoP TTFS, Faculty of Medicine and Health Sciences Research Ethics Committee, Queens Medical Centre, Nottingham University Hospitals. Participation was voluntary and informed written consent was obtained. All data was held in accordance with the Data Protection Act. This included data storage on password protected spreadsheets for all personal information, identification of participants using numbers where possible and back up of all documents on secure servers from the University of Nottingham.

2.3 Human Volunteers

Participants were recruited from the University of Nottingham by email for ease of recruitment and were healthy adult volunteers. Exclusion criteria included chronic or

acute illness in the past 3 months, cold or flu symptoms, oral health concerns and any medication, with the exception of contraception. Participants were asked not to eat, drink, smoke or use oral hygiene for 2 hours prior to donation. In a preliminary literature search, these exclusion factors were reported to affect saliva flow or composition [165]. In addition, other researchers used similar exclusion criteria when collecting human saliva for characterisation, and a period of one hour [90, 96, 166] or two hours [89] after exposure to stimulants was required before human saliva collection. Donations took place at approximately 15:00 hours to avoid diurnal salivary changes. It is thought that saliva flow is lowest during hours of sleep, and has a natural peak flow in the afternoon [165] or during times of stimulation [165, 167]. The time 15:00 hours was chosen for practical reasons to allow sufficient time after exposure to potential stimulants. It has also been reported that circannual cycles of saliva secretion may be present [167], therefore donations all took place within a short period of a few weeks. Participation was voluntary and informed written consent was obtained.

2.3.1 Trial 1

The study group demographics are shown in Table 2.4. The study group was mostly Caucasian (26 of 30 participants). The number of participants required was determined using a power calculation, which is detailed further in section 2.8: Statistical Analysis. The narrow age group recruited was due to recruitment largely from the postgraduate community at the University of Nottingham and is a limitation of this work. However, it is difficult to recruit a “paediatric sample” or “geriatric sample” encompassing all ages within these populations. For example, paediatrics range from pre-term neonates to teenagers, therefore a much larger trial would be required to generate meaningful data for these diverse populations. Nevertheless, future collaborations with Dr. Catherine Tuleu have been discussed and there may be an opportunity for future studies.

Table 2.4: Trial 1 study group demographic data

Total number of participants	30
Age (mean +/- S.D. (range))	26.13 +/- 3.55 (20 - 35)
Age 20 – 27	22
Age 28 – 35	8
Male	13
Female	17

2.3.2 Trials 2 and 3

The study group demographics are shown in Table 2.5. The same participants were used for both Parafilm® and ODT stimulation trials (Trials 2 and 3) to allow for comparison. A smaller group of participants was recruited for this trial according to power calculations.

Table 2.5: Trial 2 and 3 study group demographic data

Total number of participants	10
Age (mean +/- S.D. (range))	26.7 +/- 5.14 (20 - 36)
Age 20 – 27	7
Age 28 – 36	3
Male	4
Female	6
Caucasian ethnicity	5
Oriental ethnicity	5

2.4 Saliva Collection and Characterisation of Flow Rate

2.4.1 Trial 1: Human Saliva Trial

The rationale for collecting human saliva and for selecting the particular parameters to characterise is discussed in Chapter 3. A preliminary literature search indicated that there may be differences in the characteristics of unstimulated and stimulated

saliva. In a review paper of saliva composition and function [165], flow rate was indicated to vary depending on stimulation state. In another review [167], differences in the composition of unstimulated and stimulated saliva were documented. Furthermore, some literature focussed on the characteristics of only unstimulated saliva [89, 92] whilst another review documented the electrolyte composition of unstimulated and chewing stimulated saliva [168]. Therefore, both unstimulated and chewing stimulated saliva were collected.

Participants were asked firstly to donate an unstimulated saliva sample by draining their saliva via a sterile disposable funnel into two 15 mL polypropylene sterile graduated centrifuge tubes (Grenier Bio-One, UK) and one 1.5 mL polypropylene graduated micro centrifuge tube (Sarstedt, UK). Samples were collected in 3 different vessels to allow for separate defrosting for each characterisation. For each type of saliva (US and SS), the following was collected: 10 mL for buffer capacity, 6 mL for viscosity and 1.5 mL for surface tension measurement. This allowed for a slight excess for each measurement. The time taken to donate each volume was recorded using a stopwatch and the exact volume was used to calculate flow rate for each sample.

The methods used for saliva collection in other literature varied slightly. In some cases, saliva was simply expectorated into a vessel such as a cup [92, 169] or wide mouthed test tube [96, 166]. However, these methods do not allow collection into a graduated vessel, which is preferable to accommodate flow rate analysis. Other groups used more invasive techniques such as drawing saliva from under the tongue using a polyethylene catheter attached to a syringe, [170] or instructing the participant to lean forward whilst saliva was drawn into a syringe from under the tongue [171]. These methods collect saliva primarily produced by the sublingual salivary glands and not whole mixed saliva. In addition, the presence of the syringe or catheter in the oral cavity may stimulate salivary flow, and a less invasive procedure would probably be more acceptable to participants. Therefore, the method we employed was in line with other research groups who asked subjects to lean forward and drain their saliva via funnels into graduated centrifuge tubes [89, 172]. This technique benefits from being non-invasive and allowing easy flow rate calculation.

Participants then donated a stimulated saliva sample. For this, they were asked to continually chew a 5 cm x 5 cm piece of Parafilm® and repeat the donations following this stimulation. Stimulation was controlled by regulating the size of the piece of Parafilm®, and the same volume of saliva was collected each time. There are three possible mechanisms of saliva stimulation: mechanical, gustatory and olfactory, with olfactory being the weakest stimulus [167]. Unlike gustatory stimuli, mechanical stimuli can allow reproducible stimulation without tastant molecules remaining in the mouth and activating taste receptors for unknown periods of time or affecting salivary composition and biochemistry. Parafilm® is widely used in literature [173-176] for mechanical stimulation due to its lack of flavour and inert composition. Some research groups have used citric acid as a gustatory stimulant [177-179] however, this affects the pH and buffer capacity of saliva. Additionally, some researchers have used flavoured chewing gums [180, 181], however, this provides both gustatory and mechanical stimulation which is less reproducible than mechanical stimulation alone due to differences in reception and perception of taste amongst individuals [98, 99].

Samples were immediately tested for pH, flash frozen in liquid nitrogen and temporarily placed on dry ice until being transferred to a -80 °C freezer for storage. Samples were labelled as biohazards and stored in the freezer until being defrosted for characterisation. No significant difference in pH was observed between fresh and defrosted samples (paired t-test, N = 60). During storage, samples were required to comply with the Human Tissue Act, thus all samples were logged onto a spreadsheet when they entered storage and when they were removed or destroyed.

It is reported that in untreated human saliva, proteolytic enzymes from white blood cells, oral bacteria and salivary glands can degrade salivary proteins, which reduces saliva viscosity. Thus some researchers choose to centrifuge samples before storage [182]. However, centrifugation may remove some proteins which contribute to salivary viscosity such as mucins. Therefore it is recommended to add protease inhibitors, store samples in the cold and/or reduce the time between collection and measurement instead of centrifugation [182]. Collection of samples on ice and storage at -20 °C or -80 °C can minimise proteolysis. In a review paper evaluating

saliva storage options and their influence on its biochemical and physiochemical properties, storage at -80 °C was found to be preferable to storage at -20 °C as the protein profile of saliva was not altered at the lower temperature. At only -20 °C, the protein composition altered, and this outcome was not improved by adding protease inhibitors. Therefore, our samples were stored at -80 °C. In the same review [182], it was identified that slow freezing and freeze-thaw cycles contributed to protein precipitation, therefore rapid freezing in liquid nitrogen was recommended followed by storage at -80 °C.

Disposal of all saliva samples after analysis was carried out by diluting saliva with disinfectant, sealing and autoclaving according to local procedures.

2.4.2 Trial 2: US to SS Conversion using Parafilm® Stimulation

Participants were asked firstly to donate an unstimulated saliva sample by draining their saliva via a sterile disposable funnel into three pre-weighed 1.5 mL polypropylene graduated micro centrifuge tubes (Sarstedt, UK), each time expectorating their saliva after a 30 s period into a new centrifuge tube for a total of 1.5 minutes. This allowed for three separate samples for analysis and characterisation.

Participants then donated a stimulated saliva sample into pre-weighed 15 mL polypropylene sterile graduated centrifuge tubes (Grenier Bio-One, UK). Larger centrifuge tubes were used as stimulated saliva was expected to have a greater flow rate, and thus a greater volume collected in each time period. For this, they were asked to continually chew a 5 cm x 5 cm piece of Parafilm® [173-176] and repeat the donations during this stimulation. Stimulation was controlled by regulating the size of the piece of Parafilm® as before. Participants expectorated their saliva at 30 s intervals for 30 minutes. The timer was started at the point when Parafilm® was placed into the mouth for chewing, time zero. From zero to 10 minutes, participants advanced onto the next centrifuge tube every 30 s. From 10 to 30 minutes, participants advanced onto the next tube every 2 minutes (whilst still expectorating saliva every 30 s so that flow rate was unaffected).

The experiment was allowed to continue for 30 minutes as this reflects the duration of dissolution testing to be carried out representing the oral cavity. Ultimately, once methodology has been fully established, dissolution testing will be performed with the oral dosage form – multiparticulates (with or without taste masking coating) made into an orally disintegrating tablet (ODT). Although the majority of an ODT is swallowed quickly after disintegration, evidence suggests that some particles remain in the oral cavity for prolonged periods of time. Research by Wilson *et al.* [80] showed that nearly 10 % of an ODT radiolabelled with technetium-99m remained in the oral cavity after 9 minutes. In agreement with Pfizer®, the dissolution test was set to 30 minutes to ensure all ODT particles would be swallowed from the oral cavity and to test the efficiency of taste masking coatings after prolonged oral exposure.

Samples were immediately tested for pH, weighed to determine flow rate (see section 2.4.3 below), then flash frozen in liquid nitrogen and stored at -80 °C until being defrosted for characterisation of buffer capacity. Samples were stored, labelled and disposed of as per section 2.4.1.

2.4.3 Trial 3: US to SS Conversion using ODT Stimulation

Participants donated unstimulated saliva as above. VitaMelts™ vitamin C orally disintegrating tablets (Bioglan®, UK) were divided into quarters using a tablet splitter. Participants placed a quarter ODT on their tongue and allowed to disintegrate. The timer was started when the ODT was placed on the tongue, time zero. Disintegration time was noted and found to have a mean value +/- S.D. of 59.1 s +/- 11.3 s. Participants were asked to swallow three times after disintegration to ensure ODT particles were swallowed. From 1.5 minutes until 30 minutes, participants expectorated saliva every 30 s and donations proceeded as above. Samples were characterised and stored as above.

Vitamin C was chosen as a model ODT since ascorbic acid and citric acid are commonly used as saliva stimulating agents in ODTs, providing gustatory stimulation for enhanced disintegration. Therefore, the active ingredient in the VitaMelts™ ODT (ascorbic acid) is also used as an excipient, making these tablets similar to a placebo. This was a favourable property for gaining ethical approval to use these in our human

clinical trials. In addition, using a specific API may affect the stimulation of saliva, and we wished to investigate only the effect of an ODT in general, not individual APIs, on saliva characteristics. A number of vitamin C ODTs can be sourced online, however, many are only available to order from other countries, or contain more than 100 % of the recommended daily allowance (RDA) of vitamin C, which may affect ethical approval. VitaMelts™ ODTs were readily available and were purchased from a local Holland and Barrett store. These are UK marketed and contain 100 % of the RDA of vitamin C.

The VitaMelts™ ODTs are classified as vitamins, minerals and supplements and do not have to conform to the same regulatory requirements as pharmaceuticals. FDA guidance for Industry suggests ODTs for pharmaceutical use should disintegrate within 30 s [183], however, other authors recommend disintegration should be within less than a minute [184], whilst FIP/AAPS guidance states the more lenient value of less than 3 minutes [74].

However, disintegration time of VitaMelts™ ODTs was towards the longer end of these recommendations according to preliminary experiments in 10 volunteers, with tablet disintegration being between 2 - 3 minutes. The ODTs were therefore divided into quarters yielding mean disintegration time +/- S.D. of 59.1 s +/- 11.3 s, which is more in line with pharmaceutical products.

Participants were asked to swallow their saliva three times after they felt that disintegration was complete. This was to ensure, as best as possible, that ODT particles had been swallowed and did not remain in the oral cavity. The presence of ascorbic acid in saliva may dramatically affect the pH and buffer capacity of saliva. Preliminary trials were carried out in which a VitaMelts™ ODT was placed in deionised water, and our simulated salivary fluids (SSFs), developed as part of the PhD (see Chapter 5). In each media, the pH was reduced by greater than 3 units within the first minute after addition of a whole ODT. Therefore, saliva was swallowed after disintegration to reduce the presence of ascorbic acid in saliva as much as possible.

In a preliminary trial of five participants, the density of US and SS was assessed. A paired t-test showed no significant difference between US and SS, confirming that weight of saliva could be used to calculate flow rate. Density was found to have a mean value of 976.8 mg/mL. All centrifuge tubes were weighed using CPA225D balances (Sartorius, UK). For trials 2 and 3, all centrifuge tubes were weighed before and after the donations. Flow rate was calculated from the weight and density of saliva, and the time period for each sample, measured using a stopwatch.

It was favourable to use weight and density instead of using graduated test tubes (as per the first trial) since samples were collected every 30 s for trials 2 and 3, meaning much smaller volumes were obtained. Although some micro centrifuge tubes are graduated, this is not very accurate as saliva samples contain a liquid phase plus some bubbles as a top layer, which is highly irregular. Therefore, analysing the flow rate by weight and density was considered more accurate.

2.5 Characterisation of pH and Buffer Capacity

2.5.1 Trial 1

2.5.1.1 pH

An S220 seven compact pH/ion meter was used with InLab Science Pro electrode (SI 343 071, Mettler Toledo, Switzerland). The pH meter was accurate to +/- 0.002 pH units and a 3 point calibration was used at pH 4, 7 and 10. The pH of human saliva was measured immediately after collection prior to freezing the samples in liquid nitrogen and storing at -80 °C. The pH was measured in triplicate for each participant for both US and SS.

2.5.1.2 Buffer Capacity

A 4 mL saliva sample was allowed to warm to 37 °C in the test tube. Temperature was maintained using a water bath (beaker) in which the test tube for titration was placed. The beaker was placed on an RCT basic hotplate stirrer (IKA Works GmbH, Germany) with temperature probe. Initial pH was tested using the above electrode. The sample was then titrated with 0.01 M HCl at 37 °C until a decrease in pH of 1 unit was observed. Buffer capacity in mmol H⁺/L saliva was calculated from the volume of

acid added. Stirring speed was set such that the added HCl was adequately mixed throughout the bulk of the sample without forming a vortex. A 100 mm x 23 mm B19/26 glass test tube was used (supplied by Scientific Glassware Supplies, UK). Human saliva was analysed in duplicate for each participant for each type of saliva.

This size test tube was selected for titration because it was the smallest test tube with sufficient diameter to enable the pH electrode to reach the bottom of the tube, whilst larger diameter tubes required a larger sample of saliva for the electrode to be properly submerged in the sample. We aimed to use the smallest amount of saliva possible for the benefit of the trial subjects.

A number of studies have been performed in the literature assessing the buffer capacity of human saliva, with various methodologies. In most cases, the experimental design employed does not allow one to reach quantitative conclusions/comparisons about the actual buffer capacity value or range. Literature values are reported in different ways.

Some research groups have simply quoted the buffer capacity to be high, medium or low, without providing any actual value [185, 186]. In these cases, buffer capacity was determined by measuring the initial pH, adding a known amount of acid and measuring the final pH. The change in pH was measured and based on this buffer capacity was stated as high, medium or low. However, since neither the initial and final pH, nor pH change was quoted one cannot draw direct quantitative comparisons between studies.

Some researchers simply state the bicarbonate concentration of saliva samples to infer buffer capacity [89]. However, since multiple buffers contribute to the buffer capacity of saliva, and the prevalence of each buffer can change depending on the stimulation state [165, 167], this is not entirely appropriate.

Furthermore, in some cases, buffer capacity is quoted in $\text{mmol H}^+ \text{L}^{-1} \text{pH}^{-1}$ [173, 187] assessed by measuring the initial pH and adding acid such that pH decreases by a single unit or known amount. This does allow for quantitative comparison between studies. In addition, this approach has been adopted in the evaluation of buffer

capacity of other gastrointestinal fluids [95, 159, 162, 188-190], allowing for further comparison between the properties of saliva and other fluids. Therefore, this approach was adopted for our research.

Since the bicarbonate buffering system contributes to the buffer capacity of saliva, it is possible that some carbon dioxide escapes the system during the titration as no measures were put into place to seal or close the system during analysis. This could be considered a limitation of the methodology. However, the sampling time was minimal, in the range of a couple of minutes, and samples were defrosted immediately prior to analysis to reduce the effect of CO₂ and loss from the system and resultant pH change.

2.5.2 Trials 2 and 3

2.5.2.1 pH

The pH was measured immediately after sample donation. No water bath was used since samples were freshly donated. An S220 seven compact pH/ion meter was used with an InLab® Ultra-Micro electrode (Mettler Toledo, Switzerland). The pH meter was calibrated as above with the same accuracy. The micro-electrode was selected for trials 2 and 3 since sample volumes were much lower when saliva was donated in just 30 s expectoration periods.

2.5.2.2 Buffer Capacity

A 200 µL saliva sample was allowed to warm to 37 °C in a 1 mL glass vial. Smaller apparatus and sample sizes were used compared to trial 1 due to practical reasons surrounding the smaller sample volume. Temperature was maintained using a polycarbonate water bath with temperature probe in which the glass vial for titration was placed. The water bath was placed on the above RCT basic hotplate stirrer. Initial pH was tested using the micro electrode above. The sample was then titrated as per section 2.5.1.2. Each sample was titrated only once, again due to the low saliva volume produced in 30 s intervals.

2.5.3 Characterisation of Simulated Salivary Fluids

The SSFs characterised were obtained from commercial sources initially, and subsequently our own SSFs were developed in house as part of the PhD. The composition and origin of commercial SSFs was detailed earlier in this Chapter in section 2.1. The rationale for selection of commercial SSFs is discussed in detail in Chapter 3, whilst development and composition of our own SSFs is discussed in Chapter 5.

A 4 mL sample of SSFs was allowed to warm to 37 °C in the test tube and the pH was measured as per section 2.5.1.1. This was also considered the initial pH for buffer capacity measurements, which were performed as per section 2.5.1.2. SSF buffer capacity was measured 5 times for each SSF, thus pH was also measured with 5 replicates for all SSFs.

2.6 Characterisation of Surface Tension

2.6.1 Trial 1 and Characterisation of Simulated Salivary Fluids

A DSA 100 Drop Shape Analyser with DSA 4 software (Kruss GmbH, Germany) using pendant drop method for surface tension analysis with Laplace-Young computational method was employed. Temperature was set to 37 °C using an MB-5 heat circulator (Julabo GmbH, Germany) with water bath. Measurements were taken immediately after droplet formation. Samples were measured with 5 replicates.

A plunger in the syringe holding the sample is depressed and liquid is forced out of the needle causing a drop to be suspended from the needle tip. The shape of the drop depends on the surface tension and the effect of gravity. An image of the drop is recorded from the camera and transferred to the drop shape analysis software. A contour recognition is fitted to the drop, and a mathematical model is fitted to the contour line [191]. The software uses this to calculate the surface tension based on the equation below:

$$\gamma = \frac{\Phi mg}{2\pi r} = \frac{\Phi V \rho g}{2\pi r}$$

Equation 2.1: Calculation of surface tension [2].

Surface tension, γ is related to the acceleration due to gravity, g and mass of the droplet, m . The mass of the droplet is equal to the density of the liquid, ρ which is input into the software after experimental determination, multiplied by the volume of the drop, V . The radius of the needle tip, r is also measured and input into the software, which also uses a correction factor, Φ [2].

This system was selected for practical reasons based on availability of apparatus at the University. It is particularly beneficial as it uses small sample volumes, provides quick, easy and simple measurement of surface tension and has the option for heating the system to physiological temperature. However, the sample is held in a syringe in the vertical position. This can be problematic since saliva is not a solution, but an inhomogeneous mixture of many components. When the sample is orientated vertically in the syringe for a long period of time, heavier components e.g. proteins or particles may drop to the bottom of the syringe and have higher abundance in the first few droplets, which can affect the surface tension measurements. Therefore, five replicates were taken and the surface tension averaged across the droplets to reduce this effect.

In addition, other researchers investigating the surface tension of human saliva using the pendant drop method [192] found that the surface tension decreased as time after droplet formation increased. Therefore, the surface tension was always measured immediately after droplet formation to reduce this variability.

2.6.2 Trials 2 and 3

Surface tension was not measured in trials 2 and 3 since no significant difference between unstimulated and stimulated human saliva was found in trial 1.

2.7 Characterisation of Viscosity

2.7.1 Trial 1 and Characterisation of Simulated Salivary Fluids

A Modular Compact Cone-Plate Rheometer MCR 302 (Anton Paar GmbH, Germany) was used. The cone used was a CP50-2-SN30270 with diameter 49.972 mm, angle 2.016 °, truncation 211 μm . Analysis was carried out at 37 °C. 8 points per decade were used for 3 decades with shear rate increasing logarithmically from 1 - 1000 s^{-1} . A

total of 25 points were made, 1 point per minute. Rheoplus analysis software (Anton Paar GmbH, Germany) was used. The sample volume was 1.2 mL. In a review of the biochemical and physical properties of saliva in 2007 [182], viscosity was measured across shear rates from 1 - 450 s⁻¹. Some studies found that saliva exhibited non-Newtonian behaviour. Therefore, a range of shear rates was used to confirm this. A shear rate of 4 s⁻¹ corresponds to movement of particles across the tongue, 60 corresponds to swallowing and 160 s⁻¹ to speech, whilst shear rates of 10 - 500 s⁻¹ have been proposed to reflect the shear during eating. We therefore used 1 - 1000 s⁻¹ to encompass values that are likely to be present in the oral cavity [193]. The same method was used for all SSFs. Saliva was analysed in triplicate for each participant for US and SS. Each SSF was also analysed in triplicate.

In the development of this method, water was used as a control since it is known to have Newtonian properties. Initially, the shear rate was increased from 0.1 – 1000 s⁻¹ over 5 minutes. However, this gave inconsistent results and indicated non-Newtonian behaviour for water. The shear rate was thus ramped more slowly, over 25 minutes which gave Newtonian results for water. In the low shear rate region, between 0.1 – 1 s⁻¹, the viscosity measurements remained inconsistent. This was thought to be due to the low torque for fluids of low viscosity such as water at low shear rates, thus 1 s⁻¹ was the lowest shear rate used. Only 8 points per decade were taken since increasing the number of points per decade allows less time for each measuring point, and less time for flow to equilibrate at each shear rate, giving unreliable results. These conditions showed a suitable rheological profile for water and were selected for further analysis.

The cone-plate geometry was chosen due to its capability of even shear rate across the whole sample compared to the parallel plate geometry. Truncation of the cone was required due to the presence of particles in biological samples such as human saliva, which can cause friction if the cone tip is not truncated. A 50 mm diameter cone was deemed appropriate since it is mid-range of the available cone diameters, therefore can be used to measure all samples from the low viscosity water / phosphate buffer samples to higher viscosity Saliva Orthana® gel.

This method was found to be reproducible and versatile for analysis of a range of different materials. However, there are some limitations to this method. In the low shear rate region, a slight peak in apparent viscosity was observed for human saliva, perhaps due to inhomogeneous flow. Additionally, for water or phosphate buffer, the first few points were still in the low torque region and may not be considered. Furthermore, at the highest shear rates, turbulent flow caused an apparent increase in viscosity of some samples. The run time was quite long at 25 minutes and due to the absence of any hood around the cone-plate to control the ambient temperature, some evaporation of the fluid occurred from the heated peltier. Finally, the sample volume of 1.2 mL, (determined experimentally once the cone geometry was ascertained) was too high to allow this method to be used for trials 2 and 3 where very low sample volumes were available.

2.7.2 Trials 2 and 3

Viscosity was not characterised for trials 2 and 3 since insufficient volume of saliva was generated during the 30 s intervals for analysis.

2.8 Statistical Analysis

Prior to trial 1, the number of study participants was determined using a power calculation. This was performed using an online power calculator [194]. With 80 % power and a level of significance of $p < 0.05$, a sample size of 8 – 34 participants is sufficient to detect small to very large differences, with sample size being inversely proportional to the difference to be detected.

Following the completion of the experiments, the normal distribution of the results in each group was tested using a D'Agostino & Pearson omnibus normality test. Where two normally distributed groups were compared, a t-test was used (either paired or unpaired). If one or both groups were not normally distributed, a Wilcoxon matched pairs test was used for paired samples, and a Mann Whitney test was used for unpaired samples. Where three or more groups were compared, ANOVA was used if the groups were all normally distributed. A Friedman's test was used where normal distribution was not present for paired (repeated measures) samples, and Kruskal-

Wallis for unpaired samples. $p < 0.05$ was considered significant in all cases. In all Figures, data represents mean \pm S.D.

2.9 Analytical Methodology for Model API

2.9.1 HPLC Methodology and Conditions

A Waters (Milford, USA) 5695 separations module HPLC system with autosampler, quaternary pump and Waters 696 diode array UV detector was employed. A Waters Xterra C18 3.5 μm 2.1 x 100 mm column with guard column and pre-column filter including 0.5 μm stainless steel frit was used. The mobile phase consisted of 0.2 M ammonium acetate buffer pH 7.0 and acetonitrile in a ratio of 56:44, with a flow rate of 0.3 mL/min. This was modified from the methods of Daraghmeh *et al.* [195] and Dinesh *et al.* [196]. The column and sample temperature were 40 °C and 10 °C respectfully and a wavelength of 290 nm was used for stock and biological samples, and 224 for simulated salivary fluids analysis. Injection volume was 20 μL . Run time was 20 minutes with API (sildenafil citrate) and internal standard (bifonazole) retention times of approximately 3.5 minutes and 9.6 minutes respectively. Representative chromatography is shown in Figure 2.1 below.

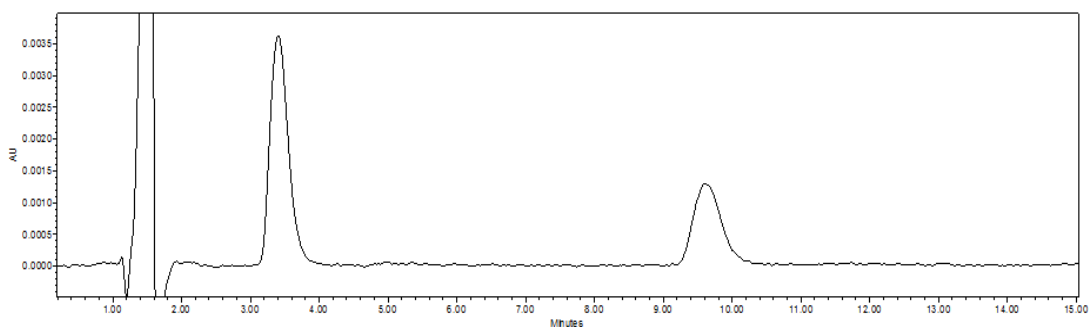


Figure 2.1: Representative chromatography showing stock solution of API (sildenafil, 1 $\mu\text{g}/\text{mL}$ in MeOH) peak at 3.5 minutes and internal standard (bifonazole, 5 $\mu\text{g}/\text{mL}$ in MeOH) peak at 9.6 minutes. Wavelength: 290 nm.

2.9.2 Sample Preparation

2.9.2.1 Stock Solutions

A 100 mg/mL stock solution of API was made in DMSO. Serial dilution with MeOH, by 50 % each time, was performed yielding 16 further stock concentrations for spiking

calibration samples, ranging from 100 mg/ml to 1525.8 ng/mL. A 1 mg/mL solution of bifonazole was made in MeOH and further diluted to 50,000 ng/mL with MeOH. This solution was used as the internal standard.

2.9.2.2 Calibration Solutions

Calibration solutions were generated using the same vehicle as the corresponding analytical samples. 90 μ L of vehicle e.g. saliva was transferred to each glass test tube. These were spiked with 10 μ L of the API stock solutions from section 2.9.2.1. Two separate calibration curves were generated – initially, the highest six concentrations (calibration concentrations 312,500 to 10,000,000 ng/mL), were diluted 100 times with the vehicle e.g. saliva before proceeding further. Subsequently the remaining (lower) concentrations were prepared without dilution.

To all of these, 10 μ L of 50,000 ng/mL bifonazole solution was added, followed by 400 μ L of cold 1:1 acetonitrile / MeOH. Finally, 3 mL of HPLC grade methyl-tert-butyl ether (MTBE) was added. Samples were vortexed at 1000 mot/min for 10 minutes and then centrifuged at 3000 rpm for 10 minutes. 2.7 mL of each supernatant was transferred to a second set of glass tubes and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted with 100 μ L of mobile phase, without buffer (44:56 acetonitrile : HPLC grade water). Samples were then vortexed for a further 10 minutes and centrifuged for a further 1 minute under the conditions previously described before transferring to a HPLC vial. The sample treatment method was modified from Tripathi *et al.* [197], who also added acetonitrile to precipitate proteins of a 100 μ L sample of sildenafil citrate in plasma, followed by liquid–liquid extraction in diethyl ether, evaporation of the supernatant and reconstitution in mobile phase.

2.9.2.3 Dissolution Samples

Development of the dissolution method is described separately in Chapter 6 in more detail. Herein we discuss the sample treatment of dissolution samples for HPLC analysis only. For dissolution samples, the entire contents of each dissolution vial was transferred to a Costar® Spin-X 2 mL polypropylene centrifuge tube with 0.22 μ m

cellulose acetate filter (Corning Inc., Tewksbury, USA) at a set time point and centrifuged for 1 minute at 13,000 rpm. The filtrate was diluted if necessary with plain dissolution media e.g. saliva. 100 μ L was taken and underwent the same sample treatment as per section 2.9.2.2. In each case calibration solutions were made by spiking the same vehicle as the dissolution media.

2.9.3 Validation

The analytical method was validated for limit of quantification (LOQ), intra- and inter-day accuracy and precision and linearity in accordance with FDA guidance [198].

Validation was performed for four different dissolution media: human US and SS, and SSF US and SS. In each media, LOQ and intra- day and inter- day accuracy and precision at three quality control (QC) levels was determined for the model API, sildenafil citrate. For LOQ samples, the acceptance limit was +/- 20 % for accuracy and precision, whereas this limit was 15 % for all QC samples. The validation results are detailed in Table 2.6. LOQ was determined to be within the limits of acceptance in all media at 100 ng/mL. The QC samples tested had concentrations of 312.5, 1,250 and 5,000 μ g for low, medium and high quality control samples respectively.

Table 2.6: Summary of validation data. LOQ = limit of quantification, RSD = relative standard deviation, RE = relative error, QC = quality control, US = unstimulated saliva, SS = stimulated saliva, SSF = simulated salivary fluid.

		LOQ		Lower QC				Medium QC				Higher QC			
				Intra-day		Inter-day		Intra-day		Inter-day		Intra-day		Inter-day	
Media	Sample	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)
Human US	1	6.78	17.35	7.51	1.41	4.14	11.31	3.97	1.95	3.38	-4.37	5.03	-1.19	1.95	-6.50
	2		-1.77		-0.40		6.53		-6.56		1.71		-14.04		-10.87
	3		0.12		8.39		7.57		5.81		-6.74		-9.08		-7.34
	4		-8.59		-0.41		1.57		7.74		-3.60		1.86		-9.55
	5		-3.97		-8.86		5.42		5.74		-2.93		-12.73		-9.17
Human SS	1	5.74	-0.89	5.24	13.71	3.38	-2.48	4.92	5.29	3.17	-7.55	5.73	9.38	2.21	-10.86
	2		5.55		12.70		-6.13		-12.02		-6.29		8.54		-10.69
	3		-4.37		11.20		-4.26		1.39		-10.51		-2.42		-13.81
	4		7.89		7.17		-9.19		-7.53		-2.76		-3.79		-13.99
	5		7.83		-1.47		-1.45		2.61		-8.62		-6.88		-14.82
SSF US	1	6.33	-5.70	4.74	-0.77	1.02	7.16	4.52	-8.08	5.30	-7.37	1.83	5.91	2.58	4.95
	2		-17.86		-10.40		3.04		0.45		6.35		-7.41		5.43
	3		-14.47		-4.55		6.16		-10.22		-5.66		-2.55		9.18
	4		-9.06		0.21		7.14		-12.36		-0.06		6.84		8.58
	5		-16.19		3.35		3.26		-9.65		4.32		5.38		11.94
SSF SS	1	5.23	0.90	4.32	-5.11	1.94	-1.91	6.04	-6.62	2.35	-7.00	2.38	13.76	3.87	-12.53
	2		19.55		-13.26		-2.88		13.42		-7.49		10.06		-11.80
	3		7.68		-14.57		-3.60		9.89		-7.22		0.76		-5.58
	4		2.32		-14.96		-2.82		-5.46		-7.19		-9.73		-9.15
	5		16.87		-13.10		-3.42		-14.50		-4.00		2.45		-5.96

2.9.4 Solubility Testing

The solubility of the model API in water was determined from literature and found to be approximately 3.5 mg/mL, however this value is dependent on the pH of the solution [199, 200]. The solubility in stimulated human saliva was determined experimentally. 50 mg of API powder was stirred in 1 mL of stimulated human saliva from one volunteer for 30 minutes at 37 °C. This was performed in duplicate. This was considered to be far greater than the maximum anticipated solubility in human saliva. This also reflected the greatest possible concentration which could be observed in dissolution testing, since dissolution tests were also performed at 37 °C for 30 minutes. At 30 minutes, samples were diluted 100 times. 100 µL was taken and underwent sample treatment as detailed in section 2.9.2.2. Dissolution experiments in which powdered API was analysed in human US/SS were also indicative of the API powder solubility. However, this preliminary solubility testing was performed prior to these dissolution tests to ascertain expected values for the dissolution experiments and gauge the amount of dilution required such that dissolution samples fall within

the calibration range. The solubility of powdered API in human saliva can be found in Chapter 6.

2.9.5 Method Development

2.9.5.1 Development of Chromatographic Conditions

HPLC System

Method development initially took place on an Agilent (Santa Clara, USA) 1100 series HPLC machine with autosampler, quaternary pump, column heater and diode array detector. However, a hire fee was chargeable to the students using this system. The research group acquired the Waters system detailed in section 2.9.1 which was available free of charge and thus all subsequent work was carried out on this system. In addition, the Waters system has the advantage of a sample chiller and functioning diode array detector (which was not functional in the scanning mode on the Agilent system at the time). No method adjustment was required when switching between the two systems.

Column

Initial method development work was carried out using an ACE (Aberdeen, UK) 3 μm C18 PFP 2.1 x 150 mm column. This column showed slanting of the API peak, and the retention time moved slightly later after each injection causing a marked change in retention time across a long run in the order of minutes, despite prolonged periods of column equilibration with the mobile phase. Efforts were made to reduce this effect by increasing the temperature from room temperature to 50 °C and flow rate from 0.2 mL/min to 0.4 mL/min to yield sharper peaks, but the same effect was still noted.

Method development then continued using a similar column with the same dimensions, but non PFP, however, asymmetrical peaks were observed and changing retention time was still problematic despite prolonged equilibration time. At this point, the Agilent machine was no longer used and all further work continued on the Waters system. A “ghost peak” in the system became problematic with wavelength 258 nm at the same retention time as the API and efforts were made to understand the origin of this peak by changing vial material and storage conditions. Zero volume

injections showed that this peak was to do with the injection system, and varying injection volumes confirmed this as the interfering peak area was proportional to the injection volume. The peak was also not seen on the Agilent system. The injector system was washed with various solvents and a service engineer cleaned the injection system. However, the initial problems of asymmetric peaks and changing retention time continued.

Another column was tested: a Phenomenex (Macclesfield, UK) Luna C18, 3 μm , 2.0 x 100 mm column was tested under the same conditions as the previous columns. The retention time was consistent and area was similar to previous for the API peak. However, the API peak eluted close to the solvent peak, thus different ratios of acetonitrile: water were trialled until 44:56 was found to be optimal. The Phenomenex column belonged to another member of the research group, and could not be used permanently for this research as students do not routinely share columns. Therefore, a column of similar dimensions was then used. This was a Waters Xterra C18 3.5 μm 2.1 x 100 mm column. This column was used for the rest of the analysis.

On one occasion, the system stopped working mid-sample for reasons unknown, leaving a biological sample (human saliva) in the column. This happened whilst the system was operating overnight and the column did not undergo its usual automated washing procedure. When attempts were made to wash the column, the pressure elevated very quickly, probably due to precipitation of the sample in the column. Despite using multiple solvents to try to wash the column, it could not be rescued and the pressure remained very high. Therefore a second Waters Xterra column was used. The column washing cycle was also increased to five hours encompassing both high organic and high aqueous proportions before being stored in a high proportion of organic solvent, to avoid insufficient cleaning after biological samples causing pressure problems in future.

Mobile Phase

The mobile phase initially comprised acetonitrile and water in the ratio 48:52. This was taken from a paper by Dinesh *et al.* [196] who used this mobile phase in the

analysis of sildenafil citrate using reverse phase HPLC. Methanol/water was also tested in various ratios to see if this gave more suitable chromatography. A ratio of 57:43 gave best results for methanol/water; however, chromatography was preferable using acetonitrile: water in the optimised ratio of 44:56.

Peak area and retention time was still somewhat inconsistent for the API using this mobile phase ratio. Some trailing of the peak was also observed. The API, sildenafil citrate, can adopt 6 different ionisation states, ranging from +3 to -1 [201] and thus it was decided to introduce a buffer to eliminate variability in ionisation state. Daraghmeh *et al.* [195] used a 1:1 ratio of ammonium acetate pH 7.0, 0.2 M buffer : acetonitrile in the analysis of sildenafil citrate by HPLC, thus this buffer was used for our research. This largely solved the problem of variability in retention time and peak trailing.

Injection Volume

Injection volume was initially 2 µL. This was so that the minimum possible volume was injected to reduce the likelihood of contaminating the column or causing pressure issues when injecting biological samples. The sample treatment method was optimised, as described in section 2.9.5.2 below, and the injection volume was increased to 20 µL so that a suitable LOQ could be achieved. In addition, an injection volume of 2 µL was below the limit of the machine which is 5 µL and therefore, low injection volume introduced more variability.

Column Temperature

The column temperature was varied at multiple stages during analysis. It was observed that heating the column led to sharper peaks, however, little difference was observed between 40 °C and 50 °C, therefore, the lower of the two values was selected for further analysis.

Sample Temperature

Sample temperature was initially set to 5 °C to reduce the likelihood of any drug degradation during storage in the autosampler. However, the machine struggled to maintain sample temperature at 5 °C and stopped mid-run several times due to

sample temperature being out of range. A temperature of 10 °C +/- 5 °C was therefore selected and proved to be more suitable.

Flow Rate

Flow rate was varied between 0.2 – 0.4 mL/min. A flow rate of 0.3 mL/min was selected as this gave suitably sharp peaks without causing pressure to be too high.

Wavelength

The API, sildenafil citrate, showed two λ_{\max} values at 224 and 292 nm respectively. Analysis was initially carried out using stock solutions at 224 nm as this was the wavelength with the strongest UV absorption. However, when biological samples were tested, interference at 224 nm led us to use 290 nm to reduce noise. When our developed SSFs were tested, interference was observed at 290 nm, perhaps due to the presence of xanthan gum, thus the wavelength for analysis was switched back to 224 nm which yielded a cleaner baseline.

Guard Column and Pre-Column Filter

After the first Waters Xterra column had to be discarded due to high pressure when the machine stopped mid-sample, it was decided to use a guard column to reduce contamination of the main column. Over prolonged periods of analytical testing, the peaks still became broader and had trailing or splitting in some cases. Removal of the guard column and testing of “clean” stocks on the main column revealed there were no problems with the main column and problems were a result of the guard column. This had to be changed approximately every 6 months when peaks began to broaden, split or trail. Exchanging for a new guard column resolved these problems every time. Despite sample treatment being optimised, this is still the case now and perhaps further sample treatment procedure development is necessary. A pre-column filter was also employed to add an additional step to reduce contamination of the guard column; however, the effect of this additional cleaning step is questionable.

2.9.5.2 Development of Sample Preparation and Treatment Procedure

Selection of Internal Standard

A number of internal standards were tested in the development of this method including testosterone, ibuprofen, dextromethorphan, simvastatin, THC (tetrahydrocannabinol), cannabidiol and bifonazole. Bifonazole was found to be the only one with retention time suitable for analysis since it does not elute too close to the solvent or API and does not have a retention time far greater than the API.

Solvent Choice for Stock Solutions

Stock solutions were generally prepared in methanol. The API was found to be more soluble in methanol than acetonitrile or water. In addition, it was hoped that less degradation would occur in methanol than in water. For the 100 mg/mL stock solution, DMSO was used as the solvent since the API was far more soluble in this than methanol.

Protein Precipitation

Protein precipitation alone was initially used as a sample treatment procedure. In this case, a cold solvent was added to precipitate the proteins in human saliva. Numerous solvents were chosen, however, a 1:1 mixture of acetonitrile and methanol yielded the cleanest chromatography. Initially, the solvent was added in a 2:1 ratio of precipitating solvent: saliva. However, this did not yield clean enough samples as over time, pressure began to rise and it was assumed this was due to unclean samples being injected. The ratio was increased to 4:1 precipitating solvent: saliva sample, which yielded better results, however, a continuing increase in pressure over several injections led to the use of liquid – liquid extraction in addition to protein precipitation.

Liquid-Liquid Extraction

Liquid-liquid extraction was employed as a result of rising pressure with simple protein precipitation techniques. A number of extraction solvents were tested including methanol, ethanol, ethyl acetate and methyl-tert-butyl-ether (MTBE) and

hexane. The API recovery was assessed for each one and was found to be greatest for MTBE. Therefore, this was used as the solvent for extraction.

Reconstitution and Dilution

Split peaks were occasionally observed for both the API and internal standard. It was thought that this could possibly be a result of injecting a highly organic sample into a mixed aqueous/organic mobile phase, predominantly aqueous (56 %). Therefore, samples were diluted with water so that the sample vehicle being injected was more consistent with the mobile phase. However, this did not help, and it was likely that the split peaks were due to guard column contamination instead. Samples were reconstituted with mobile phase after liquid-liquid extraction and evaporation so that they were introduced into the system in the same solvents and ratio.

High concentration dissolution samples were diluted prior to sample treatment with fresh dissolution media e.g. saliva to reduce matrix effects and to ensure that they were within the calibration range, as described in section 2.9.2.2.

Use of Two Calibration Curves

It was necessary to use two calibration curves since a wide range of concentrations was expected in dissolution testing. For non-taste masked samples, such as pure API powder or non-coated pellets of API, dissolution values were expected to be very high, towards the solubility of the API. However, for taste masked samples, i.e. pellets coated with a reverse enteric coating to reduce release in saliva or simulated salivary fluids, release was expected to be minimal to zero. Therefore, a wide range of values had to be considered for calibration curves. It was not possible to use one calibration curve since higher values had a greater influence on the equation of the line of best fit, and the curve was found to be non-linear. Therefore, two calibration curves were used. Without dilution, concentrations in the upper calibration range were greater than the maximum limit of quantification and showed rectangular peaks with no correlation between peak area and concentration. The upper calibration curve was therefore diluted 100 fold to generate values within the measureable range of the HPLC-UV detector.

Chapter 3: Characterisation of Human Saliva and Simulated Salivary Fluids

3.1 Introduction

N.B. The content of this Chapter of the thesis is modified from the publication by Gittings *et al.*, 2015 [202] in which I was the leading author.

The oral cavity as a dissolution site is often overlooked due to rapid oral transit as conventional dosage forms are swallowed. However, conventional oral formulations such as tablets and capsules are of limited application in some populations such as paediatrics and geriatrics, and alternative oral dosage forms which may reside in the mouth for a significant time are increasing in popularity [8]. In addition, adult dosage forms which can be taken “on the move”, without the co-administration of water are also gaining interest [203]. Many alternative formulations, such as oral films, sublingual and buccal tablets and orally disintegrating tablets rely on dissolution or disintegration in saliva. On the contrary, taste masked oral dosage forms often aim to reduce drug dissolution in saliva in order to prevent contact between the unpleasant tasting active pharmaceutical ingredient (API) and the taste buds [1]. Saliva therefore plays a critical role in the dissolution and performance of these formulations. However, as highlighted in Chapter 1, there is no consensus on the composition of simulated salivary fluids to represent human saliva in dissolution testing, or the apparatus or model employed. Many of the examples observed in literature are not biorelevant. We therefore aim to develop a biorelevant dissolution methodology for drugs and dosage forms in the oral cavity. Our focus begins with the selection or development of a suitable biorelevant media representing human saliva.

In Chapter 1, we discussed the dissolution media that have been employed in the dissolution assessment of taste masked oral dosage forms. These included water, phosphate buffer and electrolyte mixtures. However, other simulated salivary fluids (SSFs) exist. Five SSFs are proposed in a paper by Marquez, Loebenberg and Almukainzi in 2011 [157] which are simple electrolyte mixtures with or without addition of viscosity modifying mucins and amylase. Additionally, the British National Formulary details artificial saliva preparations used clinically in the treatment of xerostomia [204]. These include a mixture of sprays, gels and saliva stimulating

tablets. In other literature, largely from dental publications, many more SSF compositions can be found. In fact, one review paper from the year 2000 details over 60 different artificial saliva compositions [205]. With such a vast array of simulated salivary fluids to choose from, it is challenging for one to select the most appropriate one for dissolution testing. For best correlation with human saliva, the *in vitro* media should represent the *in vivo* fluid as closely as possible. Thus the most biorelevant SSF should be selected. In order to select the media which mimics human saliva the closest, one first needs to understand the key characteristics of human saliva.

A number of parameters can be considered as highly influential on dissolution. The pH, buffer capacity and surface tension have been identified as some of the most important factors [206]. Additionally, viscosity is considered in many cases [207]. Furthermore, Wang *et al.* described biorelevant dissolution and suggested consideration of pH, buffer capacity, surface tension and viscosity of the medium to be paramount for biorelevant dissolution testing (along with non-medium related hydrodynamic factors such as volume, flow, agitation and apparatus) [208]. The importance of these particular parameters is evident as similar approaches have been adopted in the characterisation of other gastrointestinal fluids, leading to the development of other simulated biological fluids [95, 189, 190, 209-212]. By investigating the same characteristics, one is also able to compare saliva to other biological or simulated biological fluids.

A literature search was carried out to investigate the pH, buffer capacity, surface tension, viscosity, and, to aid model development in later stages, flow rate for human saliva and SSFs. This allows one to draw direct comparisons between the characteristics of human and artificial saliva, thus supporting the selection of the most appropriate SSF.

The pH of a dissolution medium is important since it affects ionisation of the API, according to the Henderson-Hasselbalch equation, and ionisation is directly linked with the aqueous solubility of an API [2]. Of equal importance therefore is the ability of the medium to resist changes in pH as an acidic or basic drug begins to dissolve, i.e. the medium's buffer capacity. This was demonstrated by Tsume *et al.* [213] who

performed dissolution experiments in media of different buffer capacities with the acidic drug ibuprofen and found that when the buffer capacity was low, the pH decreased to a greater extent as dissolution proceeded, which hindered the rate and extent of further dissolution.

The pH of human saliva has been described previously, with varying results in the wide range of 5.3 to 7.8, depending on the stimulation state [95, 192]. The findings of our preliminary literature search are shown in Table 3.1. As seen in this Table, in most studies, either unstimulated saliva (US) or stimulated saliva (SS) was investigated, but not both [90, 176]. We noted at this point that there may be significant differences in the characteristics of human saliva relevant to dissolution in different stimulation states. Additionally, the only study in which the pH of both types of saliva was investigated experimentally, by Bardow *et al.* [173] had only a small number of participants, and focussed on just two salivary characteristics relevant to dissolution testing - the pH and buffer capacity. With so little consensus on the pH of human saliva, and limitations in methodology or study design employed, we decided to design and carry out our own characterisations of human saliva. We therefore aim to characterise the pH of both US and SS, as well as other key parameters within the same sample.

Table 3.1: pH of human saliva from literature values. (US = unstimulated saliva, SS = stimulated saliva).

Reference	Type of Saliva	Measure Quoted	Value	Standard deviation	pH Range
Aframian, Davidowitz and Benoliel [90]	US	Mean (N = 50)	6.78	0.04	6.24 – 7.36
Fenoll-Palomares <i>et al.</i> [89]	US	Mean (N = 159)	6.79	0.29	5.86 - 7.54
Shpitzer <i>et al.</i> [96]	US	Median (N = 25)	6.4	-	5.5 – 7.3
Kazakov <i>et al.</i> [192]	US	Mean (N = 142)	6.77	0.33	6.13 – 7.53

Reference	Type of Saliva	Measure Quoted	Value	Standard deviation	pH Range
Emekli-Alturfan <i>et al.</i> [92]	US	Mean (N = 11)	7.4	0.44	-
Bardow <i>et al.</i> [173]	US	Mean (N = 20)	6.8	0.3	-
	SS	Mean (N = 20)	7.2	0.2	-
Whelton [168]	US	Mean (review)	7.04	0.28	-
	SS	Mean (review)	7.61	0.17	-
Madsen <i>et al.</i> [214]	SS	Mean (N = 12)	6.83	0.4	-
Christersson <i>et al.</i> [176]	SS	Mean (N = 3)	7.4	-	-
Kalantzi <i>et al.</i> [95]	All	Mean (review)	-	-	5.45 – 7.8
Patel, Liu and Brown [136]	All	- (review)	-	-	5.5 - 7.0
Humphrey and Williamson [167]	All	- (review)	-	-	5.3 - 7.8

Buffer capacity has been investigated in numerous studies. However, as discussed in Chapter 2, in most cases the experimental design employed does not allow one to draw conclusions about the actual buffer capacity value or range. Literature values are reported in different ways. Some research groups have simply quoted the buffer capacity to be high, medium or low, without providing any actual value [185]. Thus one cannot draw direct comparisons between studies. Some researchers simply state the bicarbonate concentration of saliva samples to infer buffer capacity [89].

Furthermore, in some cases, buffer capacity is quoted in $\text{mmol L}^{-1} \text{pH}^{-1}$ [173, 187]. The lack of similarity in experimental design has led to inconclusive findings regarding the buffer capacity of saliva. This research aims to address these issues by assessing the buffer capacity of saliva using similar experimental design to that used for other gastrointestinal fluids [159, 162, 189, 190] to allow for comparison.

Viscosity is another key parameter affecting dissolution. A high viscosity medium would increase the thickness of the boundary layers (h) and decrease the diffusion coefficient (D) according to the Noyes – Witney dissolution model, thus reducing the drug dissolution rate compared with a medium of lower viscosity [215]. Despite viscosity of stimulated and unstimulated whole human saliva being evaluated by several research groups, no consensus has been reached on human saliva viscosity due to differences in experimental conditions. For example, in a review by Schipper *et al.* [182] viscosity of unstimulated whole saliva was found to be 1.5 - 1.6 mPa.s over a shear rate of 1 - 300 s^{-1} in one study [216]. However another study found it to range from 3.8 to 8.8 mPa.s at a single shear rate of 90 s^{-1} [172] and a viscosity of 100 mPa.s was recorded at a shear rate of 0.02 s^{-1} in another study [217] within this review. Research groups used different shear rates, temperatures and types of rheometer and often small sample sizes. This research aims to address these issues by using physiological temperature and assessing viscosity across a wide range of shear rates.

It is well known that the surface tension of the medium also affects the rate of dissolution [211]. A high interfacial tension reduces wetting of the drug particles and reduces the rate of dissolution. Wetting can be improved by the addition of surfactants, reducing interfacial tension and increasing the rate of dissolution, and it is a common practice to add surfactants to dissolution media [218]. Although many studies have investigated the film forming properties of saliva, as well as salivary pellicle thickness and composition [219], few studies have focussed on the surface tension of whole human saliva [192] and none have investigated the unstimulated vs. stimulated surface tension of whole saliva. Literature regarding the surface tension of saliva uses variable experimental designs including different temperatures and sites in the oral cavity, and often small or non-specified numbers of participants [171,

220]. Further clarification of this parameter is therefore required, using a sufficient number of samples and physiologically relevant temperature.

Despite not directly affecting media choice and composition, salivary flow rate is an important factor when developing a biorelevant dissolution model [208]. The volume available for dissolution, or flow rate, should reflect physiological conditions since this affects the concentration gradient of solvated API molecules and saturation of the bulk fluid. Salivary flow rate has been investigated; however, most groups investigated either US [90] or SS [221] as shown in Table 3.2. Since inter-individual variation is so vast in these studies, flow rate should be considered for US and SS in the same individual to allow accurate comparison of stimulation states.

Table 3.2: Flow rate of human saliva from literature values. (US = unstimulated saliva, SS = stimulated saliva).

Reference	Type of Saliva	Measure Quoted	Value (mL/min)	Standard deviation	Range (mL/min)
Aframian, Davidowitz and Benoliel [90]	US	Mean (N = 50)	0.37	0.21	0.05 – 0.95
Fenoll-Palomares <i>et al.</i> [89]	US	Median (N = 159)	0.48	-	0.1 – 2.0
Rudney, Ji and Larson [91]	US	Mean, Median (N = 128)	0.56, 0.41	0.41	0.10 – 2.87
Emekli-Alturfan <i>et al.</i> [92]	US	Mean (N = 11)	0.52	0.38	-
Hershkovich and Nagler [166]	US	Mean, Median (N = 90)	0.34, 0.24	-	0.04 – 1.5

Reference	Type of Saliva	Measure Quoted	Value (mL/min)	Standard deviation	Range (mL/min)
Del Vigna de Almeida <i>et al.</i> [165]	US	Range (review)	-	-	0.1 – 0.35 Hyposalivation if < 0.1
	SS	Range (review)	-	-	0.7 – 3.0 Hyposalivation if < 0.7
Bardow <i>et al.</i> [173]	US	Mean (N = 20)	0.55	0.19	-
	SS	Mean (N = 20)	1.66	0.67	-
Humphrey and Williamson [167]	US	Mean (review)	0.3		Hyposalivation if < 0.1
	SS	Max. (review)	7.0	-	-
Inoue <i>et al.</i> [175]	US	Mean (N = 51)	0.43	0.23	-
	SS	Mean (N = 51)	1.71	0.87	-
Whelton [168]	US	Mean (review)	0.32	0.23	-
	SS	Mean (review)	2.08	0.84	-
Rantonen [222]	US	Range of means (review)	0.17 – 0.39	0.16 – 0.23	-
	SS	Range of means (review)	1.49 – 1.87	0.6 – 0.92	-

Reference	Type of Saliva	Measure Quoted	Value (mL/min)	Standard deviation	Range (mL/min)
Delvadia <i>et al.</i> [133]	SS	Mean (review)	0.9	0.094	-
Erdem, Yildiz and Erdem [221]	SS	Mean (N = 40)	1.41	0.50	-
Schipper <i>et al.</i> [182]	All	Range (review)	-	-	0.2 – 7.0

No single SSF has been evaluated for all of these parameters in the literature, with a single characteristic being reported for a specific SSF in many cases. Despite the wide range of artificial salivas (SSFs) available, these have generally been developed for different applications other than dissolution testing. For example, artificial salivas are used clinically for the treatment of xerostomia. Consequently, the film forming and lubricating properties of such formulations are of paramount importance, but perhaps the buffer capacity for example may have been overlooked. Electrolyte solutions are often used for dental applications such as erosion studies, and do not consider the viscosity or surface tension. Whilst it is possible that some of the 60 compositions in the review article may be appropriate for dissolution studies, information regarding parameters key to dissolution testing is not available [205]. It is also difficult to select the single most appropriate SSF without a clear understanding of human salivary characteristics.

The aim of this work was therefore to characterise stimulated and unstimulated human saliva for the key characteristics relevant to dissolution to provide a platform of reference for the future selection or development of oral dissolution media that would be representative of human saliva. The saliva flow rate was assessed in this work to aid development of oral dissolution models. Age and gender related differences were also investigated for each parameter. To the best of our knowledge, this is a first work in which the key parameters relevant to drug dissolution - pH, buffer capacity, viscosity, surface tension and flow rate - are assessed simultaneously

for both stimulated and unstimulated whole human saliva with a sufficient number of participants to draw statistically meaningful conclusions.

In addition, to aid selection or development of a suitable SSF to represent human saliva, we characterised SSFs under the same conditions as human saliva to allow for direct comparison. There is a vast array of SSFs available for analysis. However, they can broadly be categorised into three main types: simple electrolyte solutions, SSFs containing the viscosity modifying polymer carboxymethylcellulose, and SSFs containing viscosity modifying mucins. We selected one SSF from each class for characterisation: phosphate buffered saline from the British Pharmacopoeia [223], Glandosane® as a carboxymethylcellulose containing SSF, and Saliva Orthana® spray which contains mucin. These SSFs were selected to represent each category since their full quantitative composition was available to us. CCMed® supplied Saliva Orthana® spray free of charge and also requested that we characterise Saliva Orthana® gel, which has the same composition as the spray except for the addition of xanthan gum in the gel. This is also a first work in which SSFs have been compared to both unstimulated and stimulated human saliva for the four key characteristics relevant to drug dissolution.

3.2 Aims

The aims of this chapter are:

- To characterise both stimulated and unstimulated human saliva from the same participants for key characteristics relevant to dissolution, with a sufficiently large sample to draw statistically meaningful conclusions
- To understand how age and gender influence salivary characteristics
- To evaluate the suitability of existing SSFs to represent human saliva

3.3 Methods

All methods relevant to this Chapter are detailed in Chapter 2. Refer to sections 2.1 to 2.8 including specific sections regarding “Trial 1”.

3.4 Results

3.4.1 pH

3.4.1.1 Human Saliva

SS had a higher pH than US (Figure 3.1) and a statistically significant difference was observed between the two groups according to a paired t-test ($p < 0.0001$).

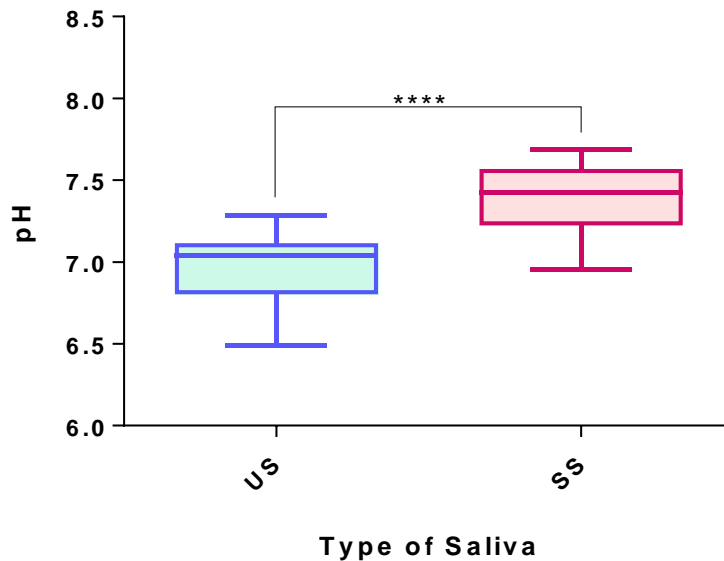


Figure 3.1: pH of US and SS. Box represents median value, 25th and 75th percentile. Whiskers represent maximum and minimum values. N = 30, triplicate. (US = unstimulated saliva, SS = stimulated saliva). **** significant difference ($p < 0.0001$, paired t-test). The saliva of the investigator (relevant for Chapter 6) had mean pH values of 6.8 and 7.3 for US and SS respectively.

No significant difference in pH was observed between males and females for either US or SS. Similarly, no significant difference in pH was observed between age groups for US or SS (Table 3.3).

Table 3.3: pH of Human Saliva. (US = unstimulated saliva, SS = stimulated saliva).

	US pH Mean (S.D.)	SS pH Mean (S.D.)
All participants (N = 30)	6.97 (0.20) ^a	7.40 (0.21)
Male (N = 13)	7.02 (0.23)	7.40 (0.16)
Female (N = 17)	6.93 (0.17)	7.39 (0.25)
Age 20 - 27 (N = 22)	6.97 (0.18)	7.40 (0.21)
Age 28 - 35 (N = 8)	6.98 (0.25)	7.40 (0.20)

All measurements are in triplicate. ^a significantly different to SS ($p < 0.0001$, paired t-test).

3.4.1.2 Simulated Salivary Fluids

The pH of SSFs was measured and results are shown in Figure 3.2 below. Due to the limited number of batches, an ANOVA statistical test across all groups is not appropriate as results represent one batch of each SSF with the exception of PBS where N = 5. Therefore, each SSF was compared as a control group to human saliva in both the stimulation states.

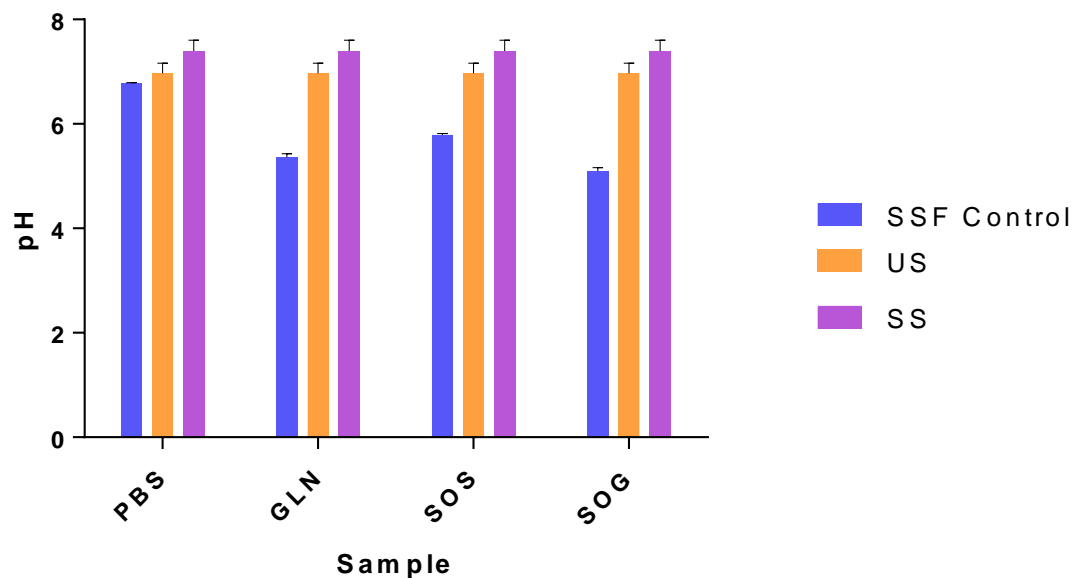


Figure 3.2: pH of SSFs and human saliva. Data represents mean +/- S.D. N = 30 for human saliva, triplicates, N = 5 for PBS, quintuplicate, otherwise N = 1, quintuplicate. (PBS = phosphate buffered saline, GLN = Glandosane®, SOS = Saliva Orthana® spray, SOG = Saliva Orthana® gel, US = unstimulated saliva, SS = stimulated saliva, SSF = simulated salivary fluid).

3.4.2 Buffer Capacity

3.4.2.1 Human Saliva

The buffer capacity was found to be significantly different for US and SS (paired t-test, $p < 0.0001$), with SS having a much greater buffer capacity, as shown in Figure 3.3.

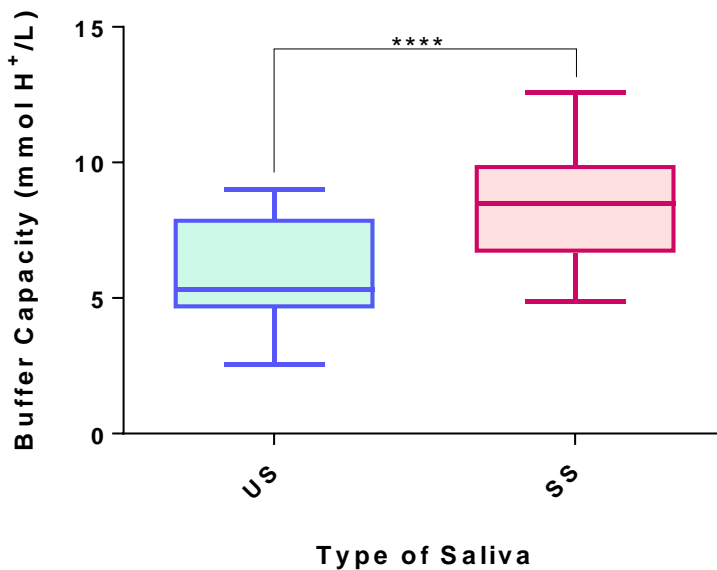


Figure 3.3: Buffer capacity of US and SS. Box represents median value, 25th and 75th percentile. Whiskers represent maximum and minimum values. N = 30, duplicates. (US = unstimulated saliva, SS = stimulated saliva). **** significant difference ($p < 0.0001$, paired t-test). The saliva of the investigator (relevant for Chapter 6) had mean buffer capacity values of 3.8 and 8.3 mmol H⁺/L for US and SS respectively.

No significant difference in buffer capacity was observed for US between males and females. However, a significant difference in buffer capacity was observed for SS between males and females (unpaired t-test, $p < 0.05$). No significant difference in buffer capacity was observed between different age groups for US or SS (Table 3.4).

Table 3.4: Buffer Capacity of Human Saliva. (US = unstimulated saliva, SS = stimulated saliva).

	US buffer capacity (mmol H ⁺ /L) Mean (S.D.)	SS buffer capacity (mmol H ⁺ /L) Mean (S.D.)
All participants (N = 30)	5.93 (1.78) ^a	8.41 (2.02)
Male (N = 13)	6.60 (1.73)	9.39 (1.31) ^b
Female (N = 17)	5.42 (1.72)	7.66 (2.18)
Age 20 - 27 (N = 22)	5.83 (1.76)	8.44 (2.25)
Age 28 - 35 (N = 8)	6.21 (1.99)	8.31 (1.32)

All measurements are in duplicate. ^a significantly different to SS ($p < 0.0001$, paired t-test) ^b significantly different to SS female ($p < 0.05$, unpaired t-test).

3.4.2.2 Simulated Salivary Fluids

The buffer capacity of the SSFs varied greatly, with PBS and SOG appearing the most similar to human saliva values according to characterisations on a limited number of batches, as shown in Figure 3.4 below. PBS did not have a statistically significant difference to SS but was found to be significantly different to US according to ANOVA ($p < 0.05$). As with other characterisations, statistical analysis could not be used to compare the other SSFs with human saliva at this stage since only one batch was analysed. However, Glandosane[®] and SOS showed distinct differences in buffering capability compared to human saliva. All of the SSFs are based on a phosphate buffer and not bicarbonate.

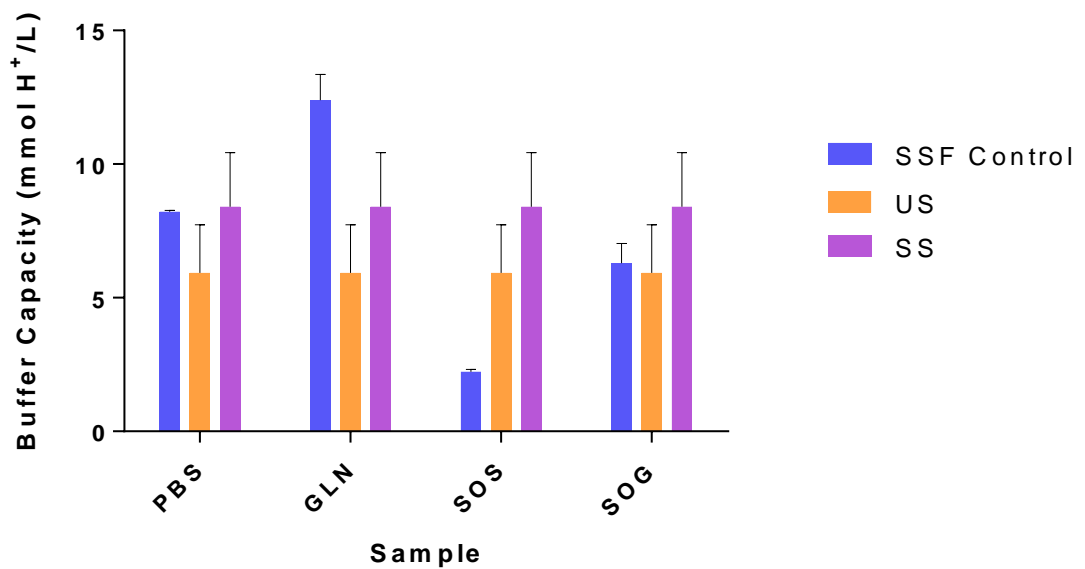


Figure 3.4: Buffer capacity of SSFs and human saliva. Data represents mean +/- S.D. N = 30 for human saliva, duplicate. N = 5 for PBS, quintuplicate, otherwise N = 1, quintuplicate. (PBS = phosphate buffered saline, GLN = Glandosane[®], SOS = Saliva Orthana[®] spray, SOG = Saliva Orthana[®] gel, US = unstimulated saliva, SS = stimulated saliva, SSF = simulated salivary fluid).

3.4.3 Surface Tension

3.4.3.1 Human Saliva

The surface tension for US and SS are shown in Figure 3.5. The surface tension of US was very similar to SS, with no significant difference observed between the two types of saliva (paired t-test). Note the variability between individuals in surface tension of saliva was very low.

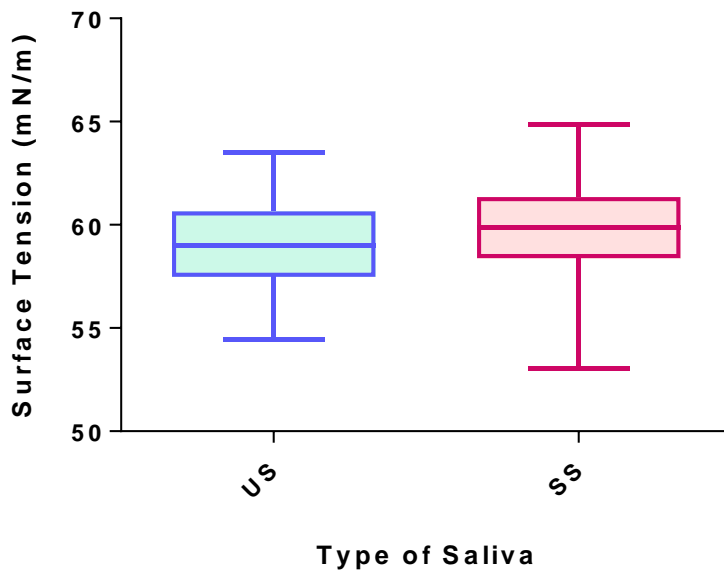


Figure 3.5: Surface tension of US and SS. Box represents median value, 25th and 75th percentile. Whiskers represent maximum and minimum values. N = 30, quintuplicate. (US = unstimulated saliva, SS = stimulated saliva). No significant difference in surface tension between US and SS (paired t-test). The saliva of the investigator (relevant for Chapter 6) had mean surface tension values of 58.8 and 59.4 mN/m for US and SS respectively.

The surface tension of human saliva (Table 3.5) showed no significant difference between males and females for US or SS. In addition, no significant difference in surface tension of human saliva was observed between different age groups for US or SS.

Table 3.5: Surface Tension of Human Saliva. (US = unstimulated saliva, SS = stimulated saliva).

	US Surface Tension mN/m Mean (S.D.)	SS Surface Tension mN/m Mean (S.D.)
All participants (N = 30)	58.98 (2.18)	59.69 (2.71)
Male (N = 13)	58.71 (2.06)	59.19 (3.43)
Female (N = 17)	59.18 (2.30)	60.07 (2.03)
Age 20 - 27 (N = 22)	58.86 (2.13)	59.49 (2.34)
Age 28 - 35 (N = 8)	59.30 (2.40)	60.22 (3.67)

All measurements are in quintuplicate. No significant difference between US and SS for all participants. No significant differences observed between males and females, or between age groups for US or SS.

3.4.3.2 Simulated Salivary Fluids

The SSFs were compared as a control group to human saliva. The results are shown in Figure 3.6 below. PBS was compared to US and SS using an analysis of variance (ANOVA) test. A statistically significant difference was observed for PBS compared to both US and SS ($p < 0.05$). However, at this time, we are unable to perform statistical tests for other SSFs due to the low number of batches assessed. Preliminary data suggests Saliva Orthana® gel was the most different to human saliva and no particular SSF was a clear closest match.

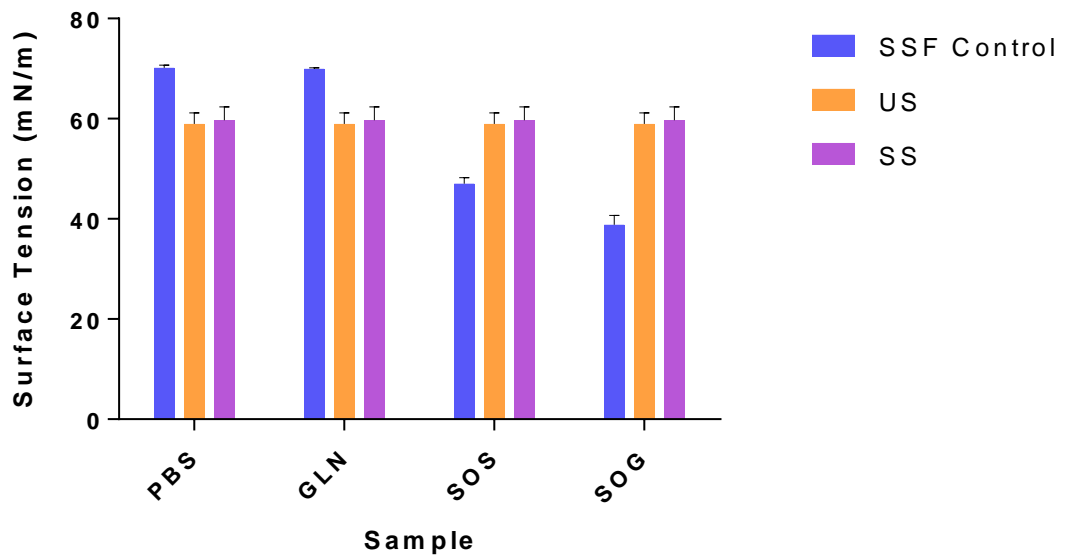


Figure 3.6: Surface tension of human saliva and SSFs. Data represents mean +/- S.D. N = 30 for human saliva, N = 5 for PBS, otherwise N = 1, all quintuplicate. (PBS = phosphate buffered saline, GLN = Glandosane[®], SOS = Saliva Orthana[®] spray, SOG = Saliva Orthana[®] gel, US = unstimulated saliva, SS = stimulated saliva, SSF = simulated salivary fluid).

3.4.4 Viscosity

3.4.4.1 Human Saliva

The viscosity of US and SS are described in Figure 3.7. SS was shown to have a lower viscosity, and a statistically significant difference in viscosity was observed between US and SS at every shear rate recorded with $p < 0.0001$ (Wilcoxon matched pairs test).

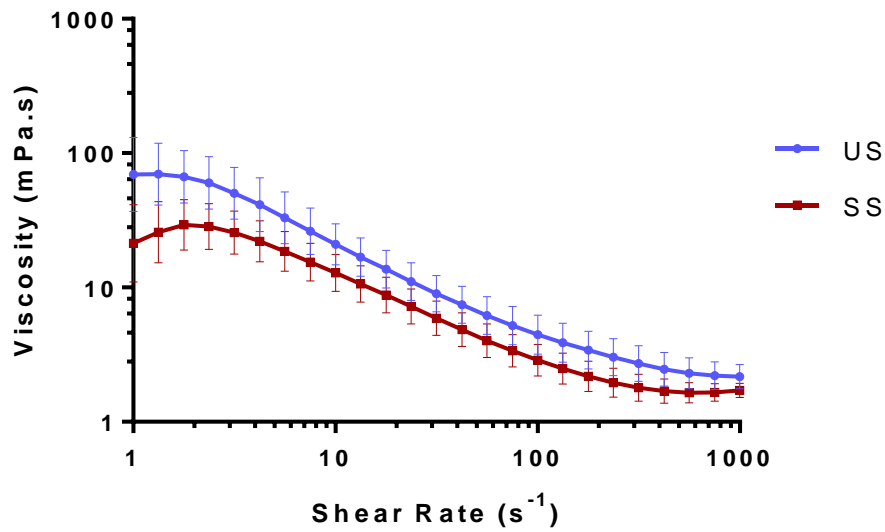


Figure 3.7: The viscosity of US and SS at different shear rates. Data represents mean +/- S.D. N = 30, triplicates. (US = unstimulated saliva, SS = stimulated saliva). A statistically significant difference in viscosity was observed between US and SS at every shear rate recorded ($p < 0.0001$, Wilcoxon matched pairs test). The saliva of the investigator (relevant for Chapter 6) had mean viscosity values within one standard deviation of the mean for both US and SS respectively.

A statistically significant difference (Mann Whitney test, $p < 0.05$) in US viscosity was observed between males and females at 5 shear rates in the lower shear rate range, with male saliva showing higher viscosity (Figure 3.8 A). This trend appears to continue across the remainder of the viscosity profile. Practically no difference was observed for SS between male and female groups, with a statistically significant difference (Mann Whitney test, $p < 0.01$) observed at just one shear rate (Figure 3.8 B).

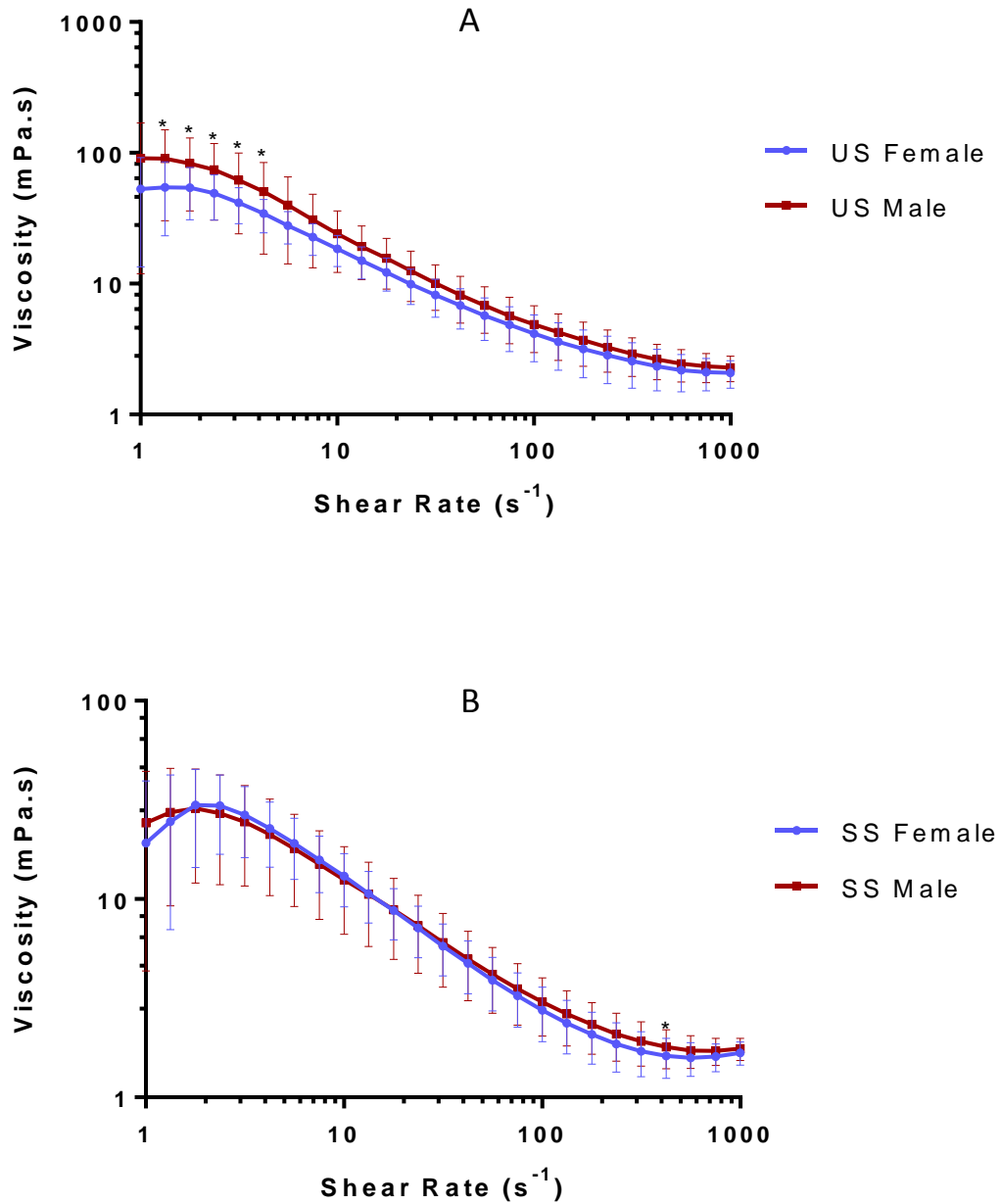


Figure 3.8: The viscosity of US (panel A) and SS (panel B) for males (N = 13, triplicates) and females (N = 17, triplicates) at different shear rates. Data represents mean +/- S.D. (US = unstimulated saliva, SS = stimulated saliva). * significant difference between males and females (Mann Whitney test, $p < 0.05$).

The viscosity of US was significantly higher for the age group 28 - 35 compared to 20 - 27 at 3 shear rates (Mann Whitney test, $p < 0.05$, Figure 3.9 A). This trend also appears to continue across the rest of the viscosity profile. For SS, no significant difference was observed between the two age groups (Figure 3.9 B).

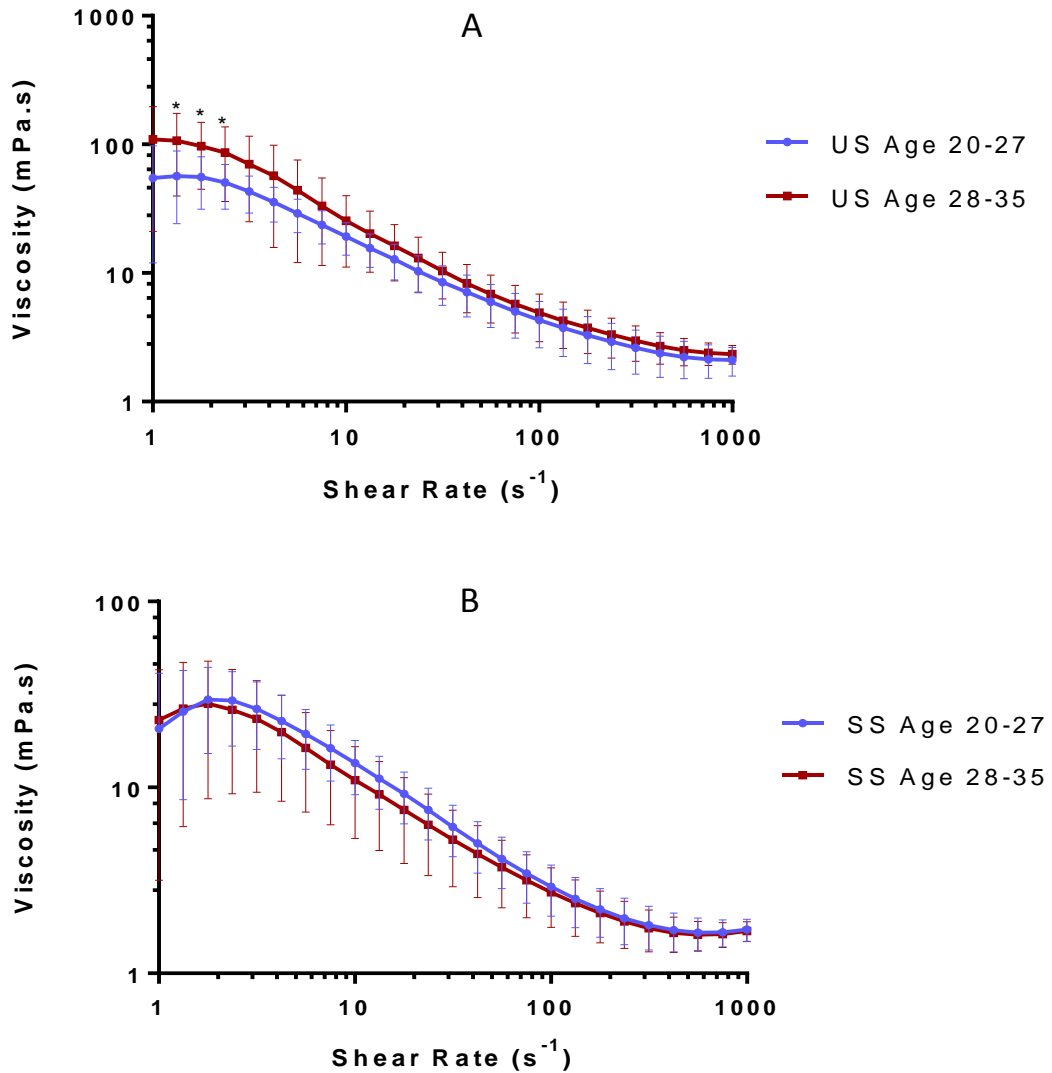


Figure 3.9: The viscosity of US (panel A) and SS (panel B) for participants age 20 - 27 (N = 22, triplicates) and age 28 - 35 (N = 8, triplicates) at different shear rates. Data represents mean +/- S.D. (US = unstimulated saliva, SS = stimulated saliva). * significant differences between age groups (Mann Whitney test, $p < 0.05$).

3.4.4.2 Simulated Salivary Fluids

The four SSFs were compared with US and SS under the same conditions. The SSFs demonstrated a variety of behaviours, some being Newtonian (PBS, SOS) and some being Non-Newtonian (SOG, Glandosane®) in which shear thinning is observed. Glandosane® results were not plotted due to the large variability in results, probably due to shearing when sprayed from its container. As seen in Figure 3.10 below, no particular SSF was a close match for human saliva, indicating the need for development of a novel biorelevant SSF. Water was also plotted as a control.

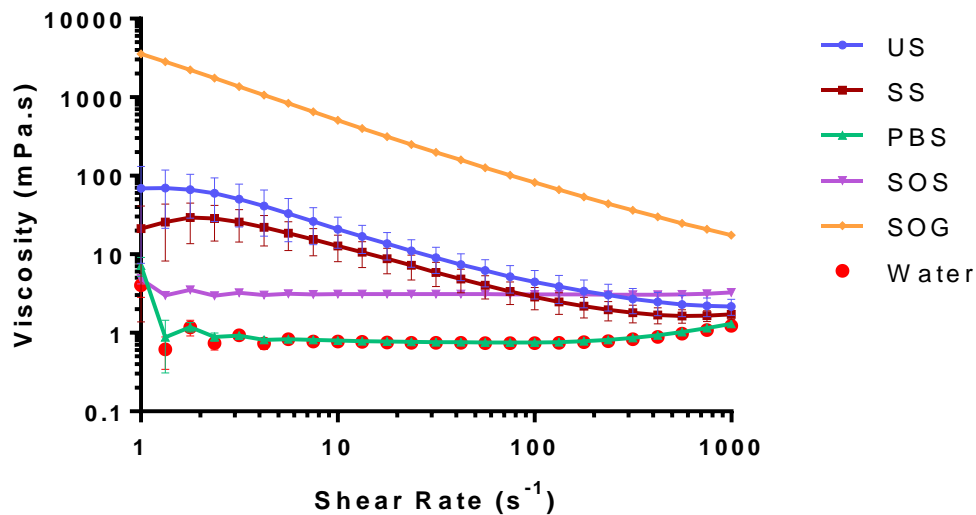


Figure 3.10: SSF and human saliva viscosity. Data represents mean +/- S.D. N = 30 for human saliva, triplicates, N = 5 for PBS, triplicates, otherwise N = 1, quintuplicate. (PBS = phosphate buffered saline, SOS = Saliva Orthana® spray, SOG = Saliva Orthana® gel, US = unstimulated saliva, SS = stimulated saliva, SSF = simulated salivary fluid).

3.4.5 Flow Rate

As anticipated, the flow rate of SS was significantly greater than US, shown in Figure 3.11 (paired t-test, $p < 0.0001$).

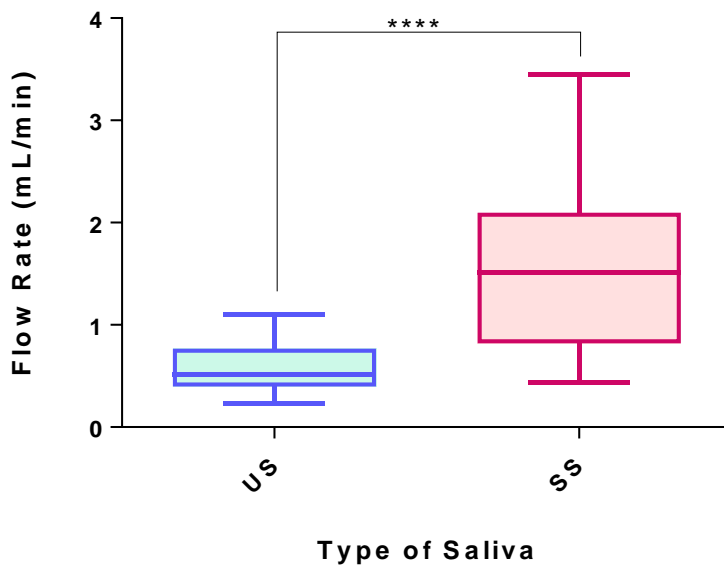


Figure 3.11: Flow rate of US and SS. Box represents median value, 25th and 75th percentile. Whiskers represent maximum and minimum values. N = 30, triplicates. (US = unstimulated saliva, SS = stimulated saliva). **** significant difference ($p < 0.0001$, paired t-test). The saliva of the investigator (relevant for Chapter 6) had mean flow rate values of 0.5 and 1.4 mL/min for US and SS respectively.

No significant difference in flow rate was observed between males and females for US or for SS. Similarly, no significant difference in flow rate was observed between age groups for US or SS (Table 3.6).

Table 3.6: Flow Rate of Human Saliva. (US = unstimulated saliva, SS = stimulated saliva).

	US flow rate (mL/min) Mean (S.D.)	SS flow rate (mL/min) Mean (S.D.)
All participants (N = 30)	0.58 (0.24) ^a	1.51 (0.72)
Male (N = 13)	0.65 (0.20)	1.60 (0.63)
Female (N = 17)	0.52 (0.26)	1.44 (0.79)
Age 20 - 27 (N = 22)	0.57 (0.22)	1.46 (0.66)
Age 28 - 35 (N = 8)	0.61 (0.28)	1.64 (0.88)

All measurements are in triplicate. ^a significantly different to SS ($p < 0.0001$, paired t-test).

3.5 Discussion

3.5.1 pH

The pH of SS was found to be significantly higher than US. This can be attributed to differences in electrolyte composition, including a greater bicarbonate concentration in SS [168]. The pH of saliva is modified as it moves through the duct system within salivary glands by the secretion and reabsorption of electrolytes, depicted in Figure 3.12. Initially, an isotonic fluid is released in the secretory acinus. As fluid travels along the duct, reabsorption of some ions such as sodium and chloride, and secretion of others such as bicarbonate and potassium occurs, until a hypotonic solution is released from the duct [167].

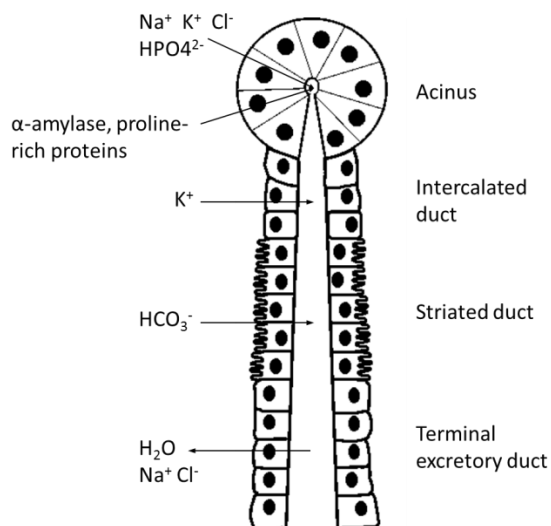


Figure 3.12: Structure of a single salivary gland acinus and duct showing ion movement. Modified from Gibson and Beeley [97].

Each acinus may contain only serous cells, mucous cells or both. Serous secretions are rich in electrolytes and enzymes, whereas mucous secretions are rich in glycoproteins. The parotid gland has predominantly serous secretion. Upon stimulation of saliva, there is a greater parotid gland output, thus a greater release of bicarbonate rich serous secretion. This coincides with a lower mucin concentration for SS which also affects viscosity, as discussed in section 3.3.4 [167].

Literature stating the pH of human saliva reports variable values that range from 5.3 to 7.8 depending on the stimulation state [90, 176, 192]. A detailed analysis of

literature values for the pH of human saliva was provided in Table 3.1 which can be used for comparison with our results. We found US and SS to be within the literature range, with mean values of 6.97 and 7.40 for US and SS respectively (range US: 6.49 – 7.28, range SS: 6.96 – 7.69). It thus would be advisable to select or develop artificial salivas as a dissolution media with pH values to reflect these findings.

The pH of phosphate buffer was unsurprisingly closest to that of human saliva. PBS was compared as a control group to US and SS using ANOVA. It was found only to be significantly different to SS ($p < 0.05$). This is because after reviewing the literature, pH 6.8 was chosen since this was within literature values for human saliva. PBS was made up in the laboratory to this pH. Saliva Orthana[®] Gel, being an unlicensed, unmarketed product at the moment does not have any literature in which the pH has been characterised. According to the manufacturers of Saliva Orthana[®] products (CCMed), both products should be in the pH range 6.5 - 7.5. However, we found the pH values to be lower than this, with the gel being the lowest at a mean pH of 5.12 and spray at a mean pH of 5.91. Other research groups have characterised the pH of Saliva Orthana[®] Spray. Christersson *et al.* [88] found it to be 5.7, whilst Madsen *et al.* [87] found it to be 5.9. Thus it is reassuring to know that other research groups also found the pH to be outside the manufacturer's specification. Characterisation of several batches of each SSF may help us to understand the variability in these commercial products. Finally, Glandosane[®] pH is reported to be 5.75 [63], however we reported the mean pH value to be 5.40. Difficulties in the analysis of Glandosane[®] were encountered due to it being contained in a pressurised vessel, and sprayed propelled by carbon dioxide, which may explain the slightly lower pH in our characterisation compared to manufacturers reports. This spraying of Glandosane[®] also caused difficulties in the rheological analysis as discussed in section 3.3.4.

To the best of our knowledge, the pH of saliva is not known to directly affect reception or perception of bitter tastant molecules directly. However, it is reported to affect sourness perception. Sour tastant molecules are often weak acids, with the degree of acidity being proportional to the proton concentration. In stimulated saliva, a greater bicarbonate proportion is present, thus increasing the pH and buffer capacity of stimulated saliva. This counteracts the acidity of solutions containing sour,

acidic components. Therefore, an increasing salivary pH has been reported to correlate with a diminished sour taste perception [224].

The data shows notable differences in pH for all SSFs when compared to US and SS. However, we were unable to confirm these statistically except for PBS which was not found to be significantly different to US, but was for SS. The Saliva Orthana® products were donated free of charge by the supplier, who were unable to provide sufficient batches to allow for statistical analysis. Based on pH alone, PBS appeared to be the most suitable SSF to represent human saliva in dissolution testing. However, all other parameters should be considered together.

3.5.2 Buffer Capacity

The greater buffer capacity of SS can also be attributed to the higher bicarbonate concentration. Bicarbonate contributes approximately 80 % of the overall buffering capacity of human saliva [225], and is found in higher concentrations in SS due to the higher proportion of parotid gland secretions [185]. It should be noted that unlike pH which was measured immediately upon collection, buffer capacity was measured after flash freezing and short term storage at -80 °C. The bicarbonate buffer is a dynamic system and in liquid saliva samples, carbon dioxide may be lost from the system. Although we do not anticipate the buffer capacity to alter as a result of freezing, this could be considered a limitation of the study. However, as discussed in Chapter 2, no significant difference was observed in the same sample when the pH was compared before and after freezing, storage and defrosting.

A direct comparison with other literature is challenging due to methodological differences. Nevertheless, the approach used here was also used by Bardow *et al.* [173] who found the buffer capacity to range from 3.1 to 6.0 mmol H⁺/L of saliva in US and 3.3 to 8.5 mmol H⁺/L of saliva in SS depending on the pH. This is comparable to our values, since we found mean values to be 5.93 and 8.41 mmol H⁺/L of saliva for US and SS respectively. In both cases, SS buffer capacity is higher than US. Despite methodological differences, this was also true for other literature [185]. However, we found buffer capacity to be highly variable for both US and SS, with relative standard deviation being 30.29 % and 24.08 % for US and SS respectively. This demonstrates a

high inter-individual variation, which should be taken into account when designing a dissolution medium.

The buffer capacity of the SSFs varied greatly, with PBS and SOG appearing the most similar to human saliva values according to characterisations on a limited number of batches. PBS did not have a statistically significant difference to SS but was found to be significantly different to US according to ANOVA ($p < 0.05$). As with other characterisations, statistical analysis could not be used to compare the other SSFs with human saliva at this stage. However, Glandosane® and SOS showed distinct differences in buffering capability compared to human saliva. Interestingly, all of the SSFs are based on a phosphate buffer and not bicarbonate. Additionally, buffer capacity of SSFs does not appear to be well documented in literature.

3.5.3 Surface Tension

The mean value for US was 58.98 mN/m whilst SS was slightly higher at 59.69 mN/m. Literature reports variable values, however our results are similar to some other research groups. For example, Kazakov *et al.* [192] measured the surface tension of US at room temperature and found that it ranged from 68.7 to 44.9 mN/m depending on the time after surface formation, with highest values being obtained at 1 s after surface formation, and lowest values representing time infinity after surface formation. Kirkness *et al.* found US surface tension to have a more similar mean value to us at 57.4 mN/m in their year 2000 study [220], and 57.7 mN/m in 2005 [170]. However, these articles did not specify the number of samples or participants tested. Furthermore, for SS, Madsen *et al.* [214] found human saliva to have an equilibrium surface tension of 41.83 mN/m, whilst Christersson *et al.* [176] found it to range from 56.2 mN/m at 30 s after surface formation to 48.5 mN/m at 600 s after surface formation.

The exact composition of surface-active molecules responsible for the interfacial tension of saliva remains unclear. However, proteinaceous and glycoproteinaceous material has been attributed with surface activity according to numerous studies investigating the composition of salivary pellicle [226, 227] or salivary film formation [176, 228]. In particular, proline rich proteins are thought to be present at these

interfaces [229, 230]. There is also some suggestion of “surfactant associated proteins A, B, C and D” in saliva, which are not structurally or functionally described [231]. Moreover, lipidic material such as phospholipids, fatty acids and triglycerides are known to be present which may also play a role [192, 232]. Since we found no significant difference between US and SS, it is likely that the surface active components of human saliva remain approximately constant, regardless of the stimulation state. Therefore, SSFs representing US and SS should have the same surface tension as each other, and as human saliva.

Characterisation of our chosen SSFs’ surface tension is not well documented in literature, with Saliva Orthana® spray receiving the most attention. Madsen *et al.* [87] also characterised the equilibrium surface tension of PBS and Saliva Orthana® spray and found them to be 69.7 and 31.3 mN/m respectively. These values are slightly lower than those observed under our conditions, probably due to the time after surface formation. Meanwhile, Christersson *et al.* [88] characterised Saliva Orthana® spray and found at 30 s after surface formation, the surface tension was 41.9 mN/m, which is quite similar to our findings. Some researchers also characterised carboxymethylcellulose containing solutions, however, due to the differing electrolyte and carboxymethylcellulose content compared to Glandosane®, direct comparisons of the surface tension cannot be made [88, 89]. Based on surface tension alone, it is difficult to conclude which of the SSF best reflects the properties of human saliva, however, SOG appears to be the poorest match.

3.5.4 Viscosity

Human saliva was found to be non-Newtonian across the range of shear rates applied. As discussed in Chapter 2, the shear rates tested are likely to be in the range observed in the oral cavity since it has been suggested that a shear rate of 4 s^{-1} corresponds to the movement of particles across the tongue whilst 60 s^{-1} and 160 s^{-1} correspond to swallowing and speech respectively [193, 217]. Furthermore, shear rates between 10 and 500 s^{-1} have been proposed to mimic the range of shear rates in the mouth during eating [233]. US was shown to have a higher variability, with a

greater relative standard deviation observed for US than SS at all shear rates measured, with the exception of the very lowest shear rate.

SS's lower viscosity is proportional to its higher flow rate, leading to an increased aqueous content, and a lower concentration of mucins - glycoproteins with a polypeptide backbone and oligosaccharide side chains which are thought to be responsible for the viscosity of saliva [234]. It has been suggested that this is due to SS originating predominantly from different salivary glands compared to US [172]. SS has been suggested to have a larger proportion of parotid secretions. However, mucins are mainly secreted from the sublingual, submandibular and palatal glands [172]. Indeed, it is well documented that secretions from the main salivary glands have differing mucin proportions and thus differing viscosities. In some cases, parotid saliva has actually been shown to demonstrate Newtonian behaviour, further reinforcing the link between mucin presence and shear thinning behaviour [182, 235].

In human saliva, there are two main types of mucin present: a high molecular weight (MW) mucin, MUC5B (MW 2 - 40 MDa), and a low molecular weight mucin, MUC7 (MW approx. 150 kDa). The molecular structure of mucin is discussed in detail elsewhere by Haward *et al.* [20] One study investigated which of these types of mucin is responsible for modifying the viscosity of saliva. They established that MUC5B concentration increased linearly with viscosity, but MUC7 did not, thus it is likely that MUC5B is responsible for the viscosity of saliva [22].

The results obtained in this study correspond well with other reports regarding the viscosity of US and SS since other research groups found SS to be of lower viscosity [175, 235]. The actual viscosity values for US and SS in literature vary depending on the type of viscometer, shear rates and temperature used. However, similar to other reports [234-236], we also observed non-Newtonian behaviour for human saliva. This is thought to be attributed to the destruction of the mucin networks within the samples which undergo breakdown upon shearing [182].

It is clear from the graph that, as expected, PBS behaved similarly to water. The closest matches to human saliva were the Saliva Orthana® products. The gel also

contains xanthan gum, but is otherwise identical to the spray. This alters the viscosity profile from one demonstrating Newtonian behaviour to one of non-Newtonian nature. This indicates that xanthan gum is the component responsible for the shear thinning behaviour of the gel, although literature clearly describes the shear thinning properties of mucins [69, 74, 78, 82], which are present in both formulations. However, the rheological properties of mucins largely depend on the origin and type of mucin used. The mucin used in Saliva Orthana® products is porcine gastric mucin (PGM), 3.5 % (35 mg/mL). This has been shown to demonstrate a linear viscosity at all concentrations tested in one study (2, 4 and 8 % w/v), exerting Newtonian behaviour over a range of shear rates. On the other hand, in the same study, bovine submandibular mucin (BSM) (2 %) and 1:1 mixtures of PGM/BSM (2 %/2 %) showed clear shear thinning behaviour with a similar profile to whole human saliva [74]. In accordance with our findings, another study showed that Saliva Orthana® spray is Newtonian with approximately the same viscosity as observed under our conditions [69]. However, the authors of this paper also found both BSM and PGM solutions to demonstrate some degree of shear thinning behaviour at 5 mg/mL or less. Interestingly, the vehicle in which the mucins were dispersed made a difference to their results and may explain differences between this research and others.

The differential effect on viscosity described earlier between MUC5B and MUC7 [78], coupled with the origin of the mucin effecting its rheological behaviour further reinforces the concept that mucins of different types have different effects on viscosity and should be selected very carefully in the design of a novel SSF.

Glandosane® showed a huge variation in viscosity profiles, particularly below a shear rate of 100 s^{-1} . Above this shear rate, viscosity became more constant, showing a linear profile with approximate viscosity of 4 mPa.s. This may be due to spraying before analysis, which is a high shear procedure and may cause some breakdown of the carboxymethylcellulose (CMC) polymer chains. Carboxymethylcellulose sodium is responsible for the viscosity of Glandosane® and is known to be non-Newtonian with viscosity being proportional to the concentration of CMC and temperature [83]. Glandosane® and other SSFs containing 1 % CMC were analysed by Vissink *et al.* [74]

and were found to be slightly shear thinning and therefore non-Newtonian with viscosity decreasing from 48 mPa.s to 25 mPa.s over the range 0.0175 - 94.5 s⁻¹.

None of the SSFs investigated were found to be suitable to represent human saliva based on their viscosity. In the development of a biorelevant SSF which better represents human saliva, it may be prudent to investigate combinations of mucin (perhaps of differing origins), xanthan gum or carboxymethylcellulose at different concentrations.

3.5.5 Flow Rate

The increased flow rate for SS results from the parasympathetic response to Parafilm® chewing which increases saliva output from the salivary glands, in particular the parotid gland. Inter-individual variability was high, with relative standard deviation being 41.0 and 47.5 % for US and SS respectively. In this study, US flow rate ranged from 0.23 – 1.10 mL/min with a mean value of 0.58 mL/min whilst SS flow rate ranged from 0.43 – 3.45 mL/min with a mean value of 1.51 mL/min.

Literature is also highly variable, with one study finding a maximum US flow rate of 2.87 mL/min [91], whilst mean SS flow rate was quoted to be just 0.9 mL/min in another study [133]. Across literature, salivary flow rate has been quoted to range from 0.05 to 7.0 mL/min [90, 222]. Literature values were previously detailed in Table 3.2 and can be used for comparison with our findings.

It is known that saliva undergoes diurnal changes in flow rate [222], and since a higher flow rate was associated with a higher pH, higher buffer capacity and lower viscosity in our study, the time at which saliva is collected may affect many of the other salivary parameters investigated. Thus, the time of collection was controlled and 3pm was chosen for practical reasons.

There are three main mechanisms of salivary stimulation: mechanical, gustatory and olfactory [167]. Dissolution testers should consider whether the dosage form may stimulate saliva. Crucially, the presence of a dosage form in the oral cavity such as an orally disintegrating tablet or oromucosal formulation may stimulate the release of saliva and therefore it may be prudent to consider both US and SS when modelling

the oral cavity. Given that the flow rate and many other parameters are so variable for human saliva, this reinforces the requirement to model both the US and SS state since a single set of test conditions is unlikely to represent the range of salivary scenarios observed.

Decreased salivary flow and dry mouth rate have been reported to be associated with taste abnormalities and taste dissatisfaction [237-239]. This is probably related to saliva's action as a solvent for the dissolution of tastant molecules, and a carrier of such molecules to taste bud sites. Low salivary flow rates may provide less solvent for dissolution, and less mobility to the taste buds, therefore reducing the ability to taste the substance [224]. Low salivary flow rate is also sometimes associated with the sensation of a burning mouth – something which is occasionally attributed to treatment with certain APIs, thus it may not always be an iatrogenic phenomenon [239]. However, many other factors can result in taste abnormalities, such as iron deficiency, oral candidiasis, psychiatric stress, depression, presence of dentures covering entire hard palate, inadequacy in chewing, certain diseases and a number of medications, which should also be taken into account [237, 238, 240].

3.5.6 Effect of Age and Gender on Salivary Parameters

We observed significant gender and age related differences in viscosity of US in the low shear rate region wherein viscosity was found to be higher for males and the older age group (even with the relatively narrow age range of volunteers). This low shear rate region may require further investigation as key differences between demographic groups are only seen in this region. Furthermore, when designing biorelevant dissolution media, this low shear rate region should be modelled accurately. Little is understood about the effect of age and gender on saliva viscosity. Humphrey and Williamson [167] claim that mucin concentrations decrease with age, but also state that secretory hypofunction is not a normal age related phenomenon.

No differences were observed in surface tension for any demographic group. Similarly to viscosity, little research has been carried out in this area. Kazakov *et al.* [192] found that equilibrium surface tension decreased with age, whereby age 5 - 9 > 10 - 15 > 40 - 55 years. However, surface tension in the over 55 years group began to

increase so a linear relationship with age was not established. The effect of gender was also not considered in that study.

Conversely, extensive literature exists detailing the influence of age or gender on flow rate. Despite this, age and gender related effects remain unclear due to conflicting reports [165]. In this study, no significant differences in flow rate were observed between males and females, or between the two age groups. Accordingly, other researchers also found flow rate was not affected by age [241] or gender [173]. However, some literature suggests that female gender correlates with lower flow rate [89, 242] which may be attributed to smaller salivary glands and a lower body mass index (BMI) [243, 244]. If this is true, it could be extrapolated from this that paediatrics or elderly people of low BMI may also have a smaller body mass and therefore smaller salivary glands and a lower flow rate. However, this is not proven by literature. Additionally, increased age has been reported to correspond with lower flow rates in some cases [89, 245]. In a review by Whelton [168], decreased salivary flow in older patients is described as being secondary to disease or medication rather than directly due to aging, and total flow is considered to be independent of age.

No significant differences in pH or buffer capacity were found for any demographic group in this study except for SS buffer capacity, which was found to be higher for males than females. This is in agreement with Wikner and Soder [246] who found females had a lower SS buffer capacity. Fenoll-Palomares *et al.* [89] also found no significant differences in pH, and higher bicarbonate concentration in men than women. However, their findings were based on US only. Conversely, another report states gender had no effect on buffer capacity [173]. pH has been described as higher for males in some studies [242]. Additionally, literature describing the effect of age on pH [242] and buffer capacity [247] reaches no consensus. A review of paediatric gastrointestinal physiology data relevant to oral drug delivery [248] states the pH of human saliva across different age groups to be 7 for neonates (0 - 27 days), 7.1 for children (2 - 11 years), 7.4 for adolescents (12 - 18 years) and 6 - 7.4 for adults. This does not show any particular trend in saliva pH values across different ages. Additionally, the wider range stated for the adult population could perhaps be due to this population receiving the most extensive characterisation of salivary pH values.

In this research, for the first time the effect of both age and gender on salivary key parameters for dissolution testing was investigated. The age and gender related differences observed were not as distinct as the differences between US and SS. Therefore, the development of two different biorelevant dissolution media representing US and SS is strongly recommended, whilst age and gender related differences should be kept in mind and may require further investigation. This is particularly prudent since taste masked and alternative oral formulations are most commonly used in the paediatric and geriatric population. To note, a limitation of this study is the relatively narrow age range employed. This is a result of recruiting unpaid volunteers from within the University. Further investigations of these key parameters in human saliva in a wider age range would be necessary in order to confirm trends seen in the data. However, it is worthy to note that should the paediatric and geriatric population be investigated, it would be inappropriate to make conclusions about each of those populations as a single group compared to the adult population. For example, a single “paediatric dissolution media” or “paediatric model” is unadvisable since a neonate differs greatly in physiology, body mass and pharmacokinetics to an infant or teenager, and salivary parameters may vary greatly too.

3.6 Conclusions

US and SS were found to be significantly different to each other for pH, buffer capacity and flow rate, with SS being higher for these characteristics. No significant difference was seen between US and SS for surface tension. SS had lower viscosity with significant differences between US and SS observed across all shear rates measured. US and SS were both found to be non-Newtonian. Significant age and gender related differences were observed in some parameters but were not as distinct as differences between US and SS and may require further investigation. None of the four simulated salivary fluids characterised in this study were found to represent human saliva adequately based on key parameters relevant to dissolution. These SSFs represent the three main types of artificial saliva available. Therefore the development of a novel, biorelevant SSF is indicated.

These findings can be used as a platform of reference for the development of future dissolution media representing human saliva. Since SS was found to be significantly different to US for all of the assessed characteristics except surface tension, this suggests the potential requirement for the development of two different biorelevant dissolution media: one representing US with a lower pH and buffer capacity but higher viscosity, and one representing SS with a higher pH and buffer capacity, but lower viscosity.

Chapter 4: Conversion from Unstimulated Saliva to Stimulated Saliva in Human Volunteers

4.1 Introduction

Biorelevant dissolution tests simulating the oral cavity can be used to assess the taste masking efficacy or dissolution of alternative oral dosage forms such as microparticulates or ODTs as discussed in Chapter 1.

If the amount of API released in a simulated oral environment is below its bitterness threshold, taste masking is achieved. This provides a robust, reproducible, analytical approach which circumvents issues associated with *in vivo* taste testing such as cost and ethical considerations. However, there are no pharmacopoeial recommendations for dissolution testing methodology or choice of media for the assessment of taste masked formulations [116].

For optimal predictability, the dissolution media should resemble human saliva as closely as possible. Until recently, no consensus had been reached on the key properties of human saliva that are likely to affect dissolution. We recently observed significant differences between US and SS for pH, buffer capacity, viscosity and flow rate. This suggested the requirement for SSFs representing both stimulation states. However, despite an array of SSFs being available, to our knowledge, no SSFs currently represent the US and SS states for parameters likely to influence dissolution such as pH, buffer capacity, surface tension and viscosity.

It could be argued that the presence of a dosage form in the oral cavity may immediately stimulate saliva, and that saliva may remain in the stimulated state for the likely duration in which particles reside in the oral cavity. However, to our knowledge, the effect of placing a dosage form in the oral cavity on the stimulation of saliva has never been reported in literature. Thus testing in only SS could be appropriate. Consequently, to confirm the requirement for development of dissolution media representing both US and SS, the conversion from the unstimulated state to the stimulated state with respect to time was investigated.

Two different types of stimulation were evaluated: prolonged mechanical stimulation using Parafilm[®], in line with previous research [202], and a single gustatory stimulant in the form of an ascorbic acid ODT in order to better represent the presence of a dosage form in the oral cavity. The rationale for the selection and type of ODT is described in Chapter 2, section 2.4.3. Rationale for experimental design is also discussed in Chapter 2.

The parameters assessed were similar to those characterised in our previous research. Surface tension was not assessed in line with previous results as this was shown to remain constant irrespective of the stimulation state. Assessment of viscosity required a greater volume than the microliter amounts available at short time intervals after stimulation. Therefore, the pH, buffer capacity and flow rate were assessed in the unstimulated state, and with respect to time after stimulation commenced.

4.2 Aims

The aims of this chapter are:

- To investigate the effect of mechanical and dosage form (ODT) stimulant exposure on the characteristics and stimulation state of human saliva with respect to time
- To evaluate if development of biorelevant SSFs representing both stimulation states is necessary

4.3 Methods

All methods relevant to this Chapter are detailed in Chapter 2. See sections 2.1 to 2.8 including specific sections regarding “Trials 2 and 3”.

4.4 Results

4.4.1 Characterisation of Unstimulated and Parafilm[®] Stimulated Saliva over Time (Trial 2)

4.4.1.1 pH

The pH of unstimulated human saliva was compared with Parafilm® stimulated saliva collected over a 30 minute period of continuous masticatory stimulation. The pH of SS was consistently raised compared to US values, and continued to increase over the 30 minute stimulation period (Figure 4.1). Each SS point was compared to US as a control group. Significant differences between US and SS were observed from 5 minutes (Friedman’s test with Dunn’s multiple comparisons test).

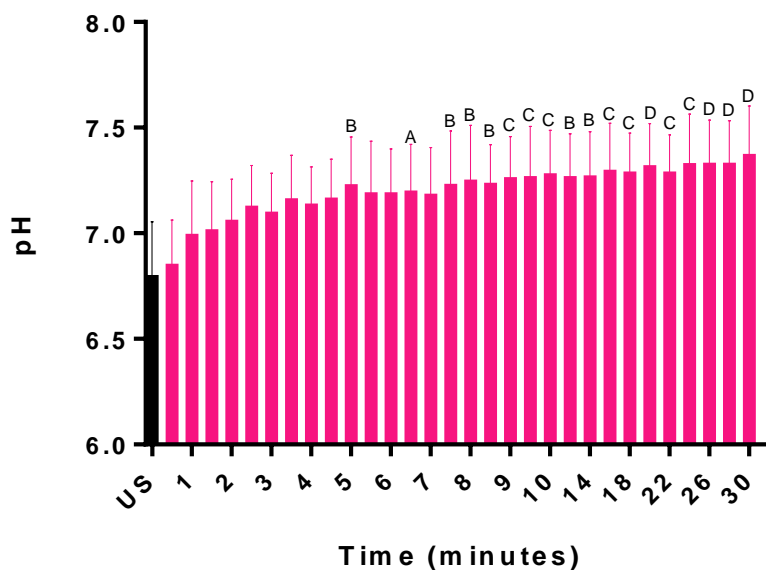


Figure 4.1: The pH of unstimulated human saliva and Parafilm® stimulated human saliva over 30 minutes of continuous masticatory stimulation. Data represents mean +/- S.D. N = 10 participants, US saliva triplicates, SS saliva single measurements. Friedman’s test compared each SS point to US control, with Dunn’s multiple comparisons test. A: $p < 0.05$, B: $p < 0.01$, C: $p < 0.001$, D: $p < 0.0001$. (US = unstimulated saliva, SS = stimulated saliva).

In order to more clearly see the variability and difference between the US value and each SS value, this is also presented as an X-Y plot as shown in Figure 4.2. Variability appears quite consistent across all SS time points. Some SS points show error bars in the negative range, indicating that for initial SS time points, the pH was sometimes lower than US mean value.

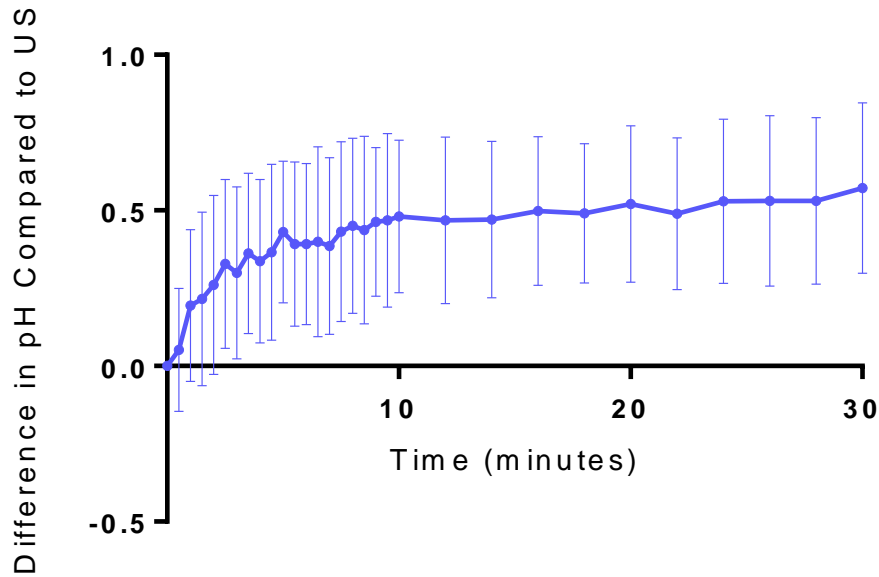


Figure 4.2: The difference in pH between US (time zero) and each time point of Parafilm® stimulated human saliva over 30 minutes of continuous masticatory stimulation. Data represents mean +/- S.D. N = 10 participants, US saliva triplicates, SS saliva single measurements. (US = unstimulated saliva, SS = stimulated saliva).

4.4.1.2 Buffer Capacity

The buffer capacity of unstimulated human saliva was compared with Parafilm® stimulated saliva collected over a 30 minute period of continuous masticatory stimulation. The buffer capacity of SS was consistently raised compared to US values. An initial peak in buffer capacity was seen in the first 2.5 minutes of stimulation, followed by a steady decrease toward but not returning to US values over 30 minutes (Figure 4.3). Each SS point was compared to US as a control group. Significant differences between US and SS were observed within the first 2.5 minutes of stimulation (ANOVA with Dunnetts's multiple comparisons test).

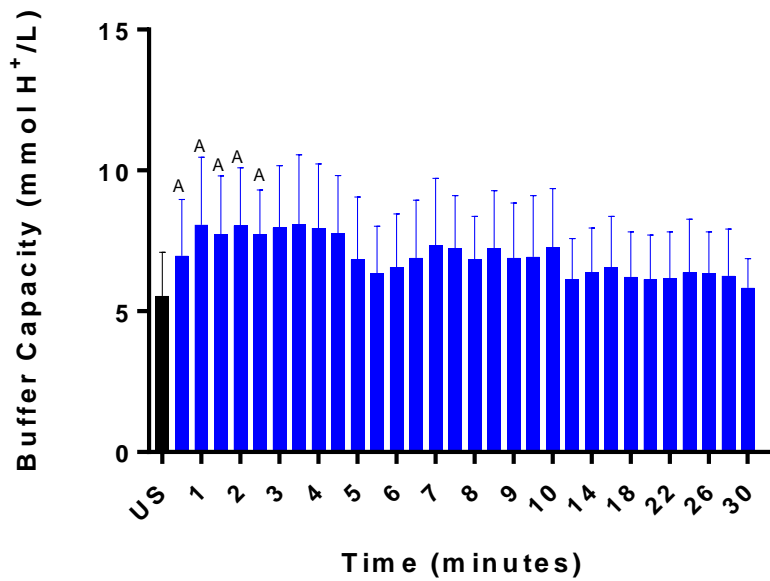


Figure 4.3: The buffer capacity of unstimulated human saliva and Parafilm® stimulated human saliva over 30 minutes of continuous stimulation. Data represents mean +/- S.D. N = 10 participants, US saliva triplicates, SS saliva single measurements. ANOVA compared each SS point to US control, with Dunnetts's multiple comparisons test. A: $p < 0.05$. (US = unstimulated saliva, SS = stimulated saliva).

Similarly to pH, in order to more clearly see the variability and difference between the US value and each SS value, this is also presented as an X-Y plot as shown in Figure 4.4 below. Variability appeared greatest for SS samples taken in the first 10 minutes after stimulation commenced. Many error bars indicate negative values, showing that buffer capacity was below the US value in some individuals, particularly from 12 – 30 minutes. However, mean values remained elevated compared to US values across the 30 minute period.

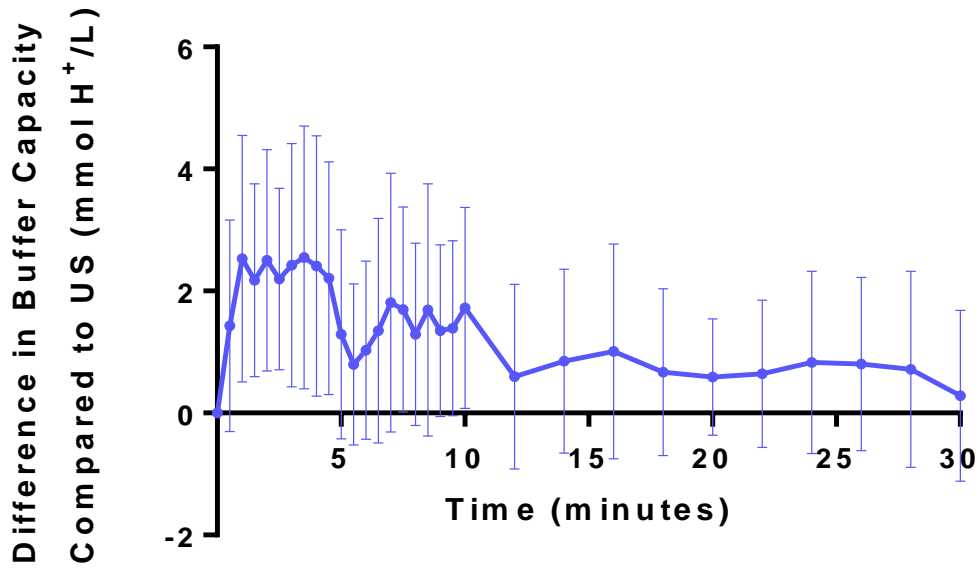


Figure 4.4: The difference in buffer capacity between US (time zero) and each time point of Parafilm® stimulated human saliva over 30 minutes of continuous masticatory stimulation. Data represents mean +/- S.D. N = 10 participants, US saliva triplicates, SS saliva single measurements. (US = unstimulated saliva, SS = stimulated saliva).

4.4.1.3 Flow Rate

The flow rate of unstimulated human saliva was compared with Parafilm® stimulated human saliva collected over a 30 minute period of continuous masticatory stimulation. The flow rate of SS was consistently raised compared to US values. Highest flow rate values were observed within the first 10 minutes, with some SS points showing statistical significance compared to US values (Friedman's test with Dunn's multiple comparisons test). From 10 – 30 minutes, flow rate remained elevated compared to US values, however no significant differences were observed (Figure 4.5).

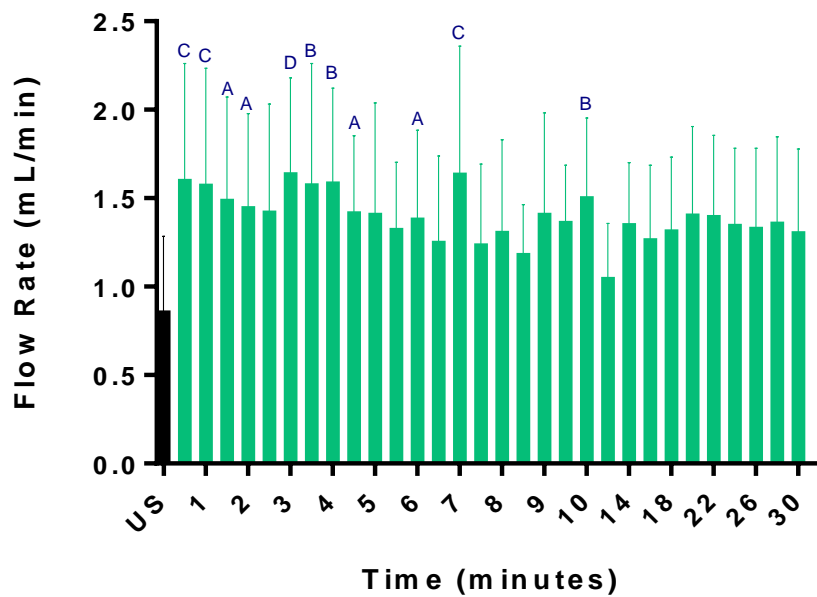


Figure 4.5: The flow rate of unstimulated human saliva and Parafilm® stimulated human saliva over 30 minutes of continuous stimulation. Data represents mean +/- S.D. N = 10 participants, US saliva triplicates, SS saliva single measurements. Friedman's test compared each SS point to US control, with Dunn's multiple comparisons test. A: $p < 0.05$, B: $p < 0.01$, C: $p < 0.001$, D: $p < 0.0001$. (US = unstimulated saliva, SS = stimulated saliva).

In line with previous parameters, in order to more clearly see the variability and difference between the US value and each SS value, this is also presented as an X-Y plot as shown in Figure 4.6 below.

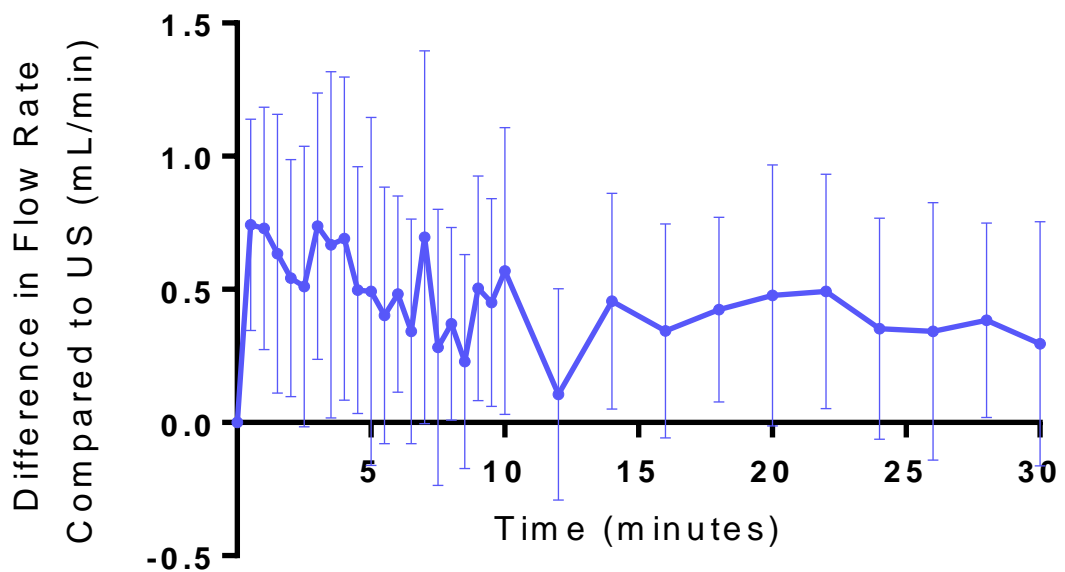


Figure 4.6: The difference in flow rate between US (time zero) and each time point of Parafilm® stimulated human saliva over 30 minutes of continuous masticatory stimulation. Data represents mean +/- S.D. N = 10 participants, US saliva triplicates, SS saliva single measurements. (US = unstimulated saliva, SS = stimulated saliva).

4.4.2 Characterisation of Unstimulated and ODT Stimulated Saliva over Time (Trial 3)

4.4.2.1 pH

The pH of unstimulated human saliva was compared with ODT stimulated human saliva collected over a 30 minute period. Stimulation was provided by a single short stimulus as the mean ODT disintegration time was 59.1 s. The pH of SS was initially raised compared to US values, however continued to decrease toward but not reaching US values over the 30 minute experiment (Figure 4.7). Each SS point was compared to US as a control group. Significant differences between US and SS were observed within the first 10 minutes. (Friedman's test with Dunn's multiple comparisons test).

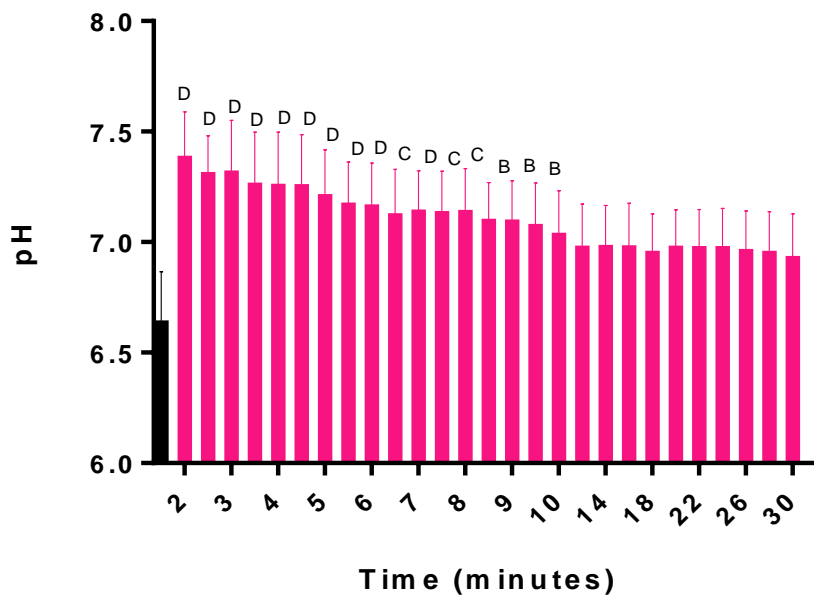


Figure 4.7: The pH of unstimulated human saliva and orally disintegrating tablet stimulated human saliva. Data represents mean +/- S.D. N = 10 participants, US saliva triplicates, SS saliva single measurements. Friedman's test compared each SS point to US control, with Dunn's multiple comparisons test. B: $p < 0.01$, C: $p < 0.001$, D: $p < 0.0001$. (US = unstimulated saliva, SS = stimulated saliva).

In order to more clearly see the variability and difference between the US value and each SS value, this is also presented as an X-Y plot as shown in Figure 4.8. Variability appears quite consistent across all SS time points. The data points on this graph are not connected between time zero (US value) and the first SS time point, during which time, the ODT was administered. Participants allowed the ODT to disintegrate then swallowed their saliva three times to ensure all acidic components of the ODT were removed from saliva by swallowing. Thus one cannot extrapolate the pH in the intervening period.

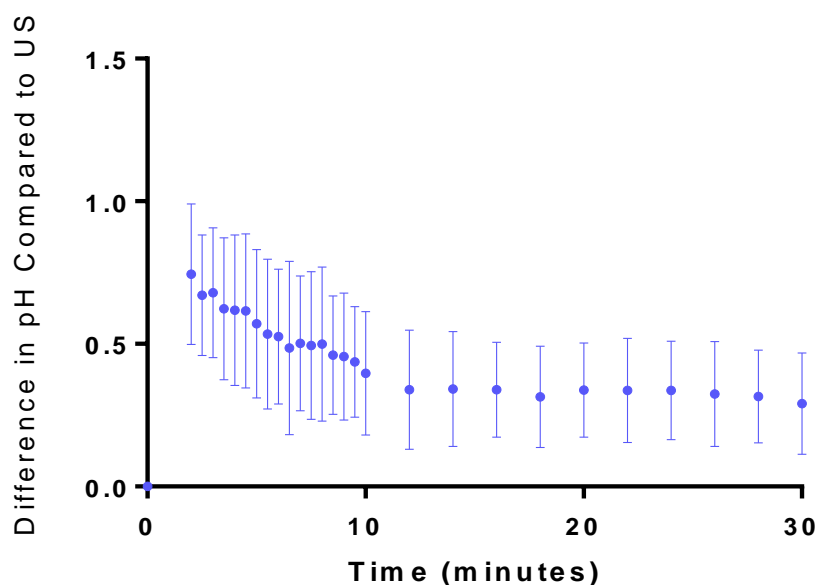


Figure 4.8: The difference in pH between US (time zero) and each time point of orally disintegrating tablet stimulated human saliva over 30 minutes after stimulation. Data represents mean \pm S.D. N = 10 participants, US saliva triplicates, SS saliva single measurements. (US = unstimulated saliva, SS = stimulated saliva).

4.4.2.2 Buffer Capacity

The buffer capacity of unstimulated human saliva was compared with ODT stimulated human saliva collected over a 30 minute period. The buffer capacity of SS was initially raised compared to US values, however it continued to decrease toward but not reaching US values over the 30 minute stimulation period (Figure 4.9). Each SS point was compared to US as a control group. Significant differences between US and SS were observed within the first 9.5 minutes. (ANOVA with Dunnett's multiple comparisons test).

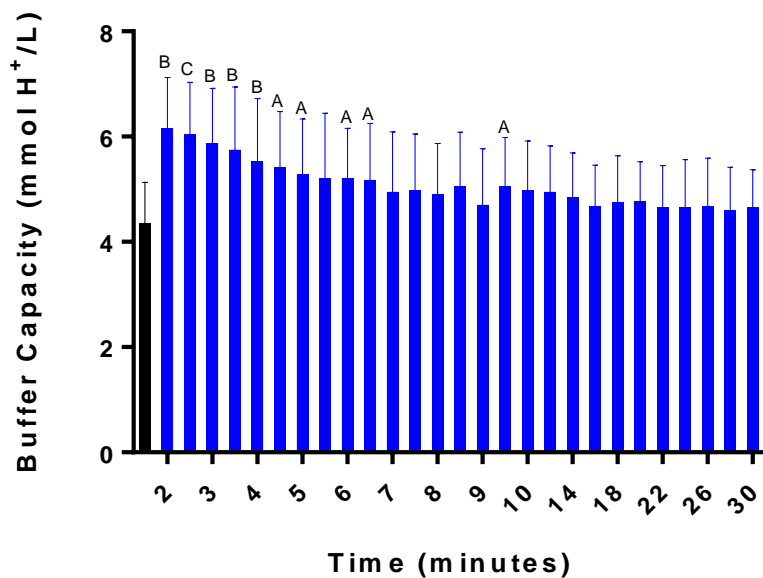


Figure 4.9: The buffer capacity of unstimulated human saliva and orally disintegrating tablet stimulated human saliva. Data represents mean +/- S.D. N = 10 participants, US saliva triplicates, SS saliva single measurements. ANOVA test compared each SS point to US control, with Dunnett's multiple comparisons test. A: $p < 0.05$, B: $p < 0.01$, C: $p < 0.001$. (US = unstimulated saliva, SS = stimulated saliva).

This data was also presented as an X-Y plot (Figure 4.10) for reasons detailed above. Similarly to Figure 4.8, the time points were not connected as discussed earlier. Variability appears greater at earlier SS time points compared to later ones in the profile. Error bars show negative values for some SS time points, indicating that some individual values were lower than the US mean value at these data points.

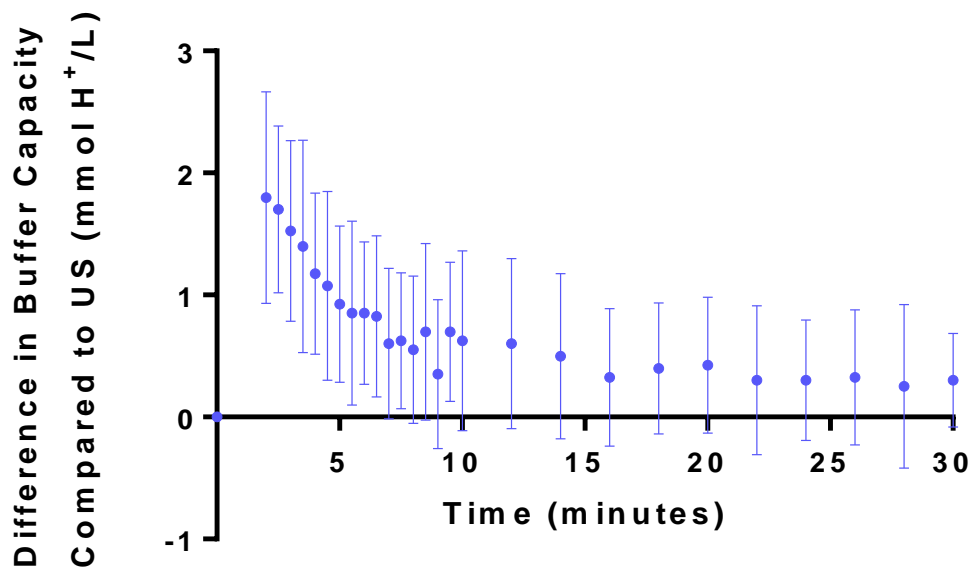


Figure 4.10: The difference in buffer capacity between US (time zero) and each time point of orally disintegrating tablet stimulated human saliva over 30 minutes after stimulation. Data represents mean +/- S.D. N = 10 participants, US saliva triplicates, SS saliva single measurements. (US = unstimulated saliva, SS = stimulated saliva).

4.4.2.3 Flow Rate

The flow rate of unstimulated human saliva was compared with ODT stimulated human saliva collected over a 30 minute period. The flow rate of SS was initially raised compared to US values, with significant differences between SS and the US control group in the first 3 minutes (Friedman's with Dunn's multiple comparisons test). From 3.5 to 30 minutes, SS flow rate remained elevated compared to US, however, significant differences between US and SS were only seen at 5 points during this time period (Figure 4.11).

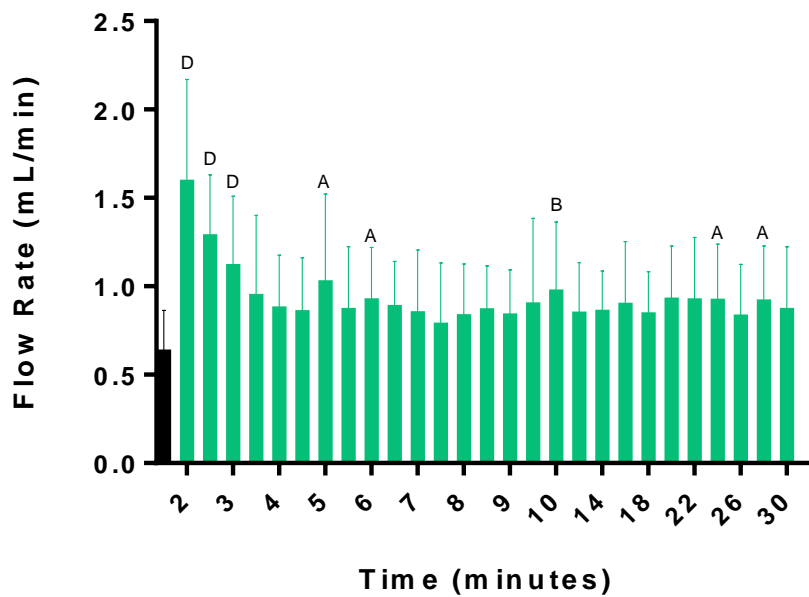


Figure 4.11: The pH of unstimulated human saliva and orally disintegrating tablet stimulated human saliva. Data represents mean \pm S.D. N = 10 participants, US saliva triplicates, SS saliva single measurements. Friedman's test compared each SS point to US control, with Dunn's multiple comparisons test. A: $p < 0.05$, B: $p < 0.01$, D: $p < 0.0001$. (US = unstimulated saliva, SS = stimulated saliva).

As with previous Figures, this data is now presented as an X-Y plot (Figure 4.12). Similarly to Figure 4.10, variability appears greater at earlier SS time points compared to later in the profile. Error bars show negative values for some SS time points, as they did for Figure 4.10, indicating that some individual values were lower than the US mean value at these data points.

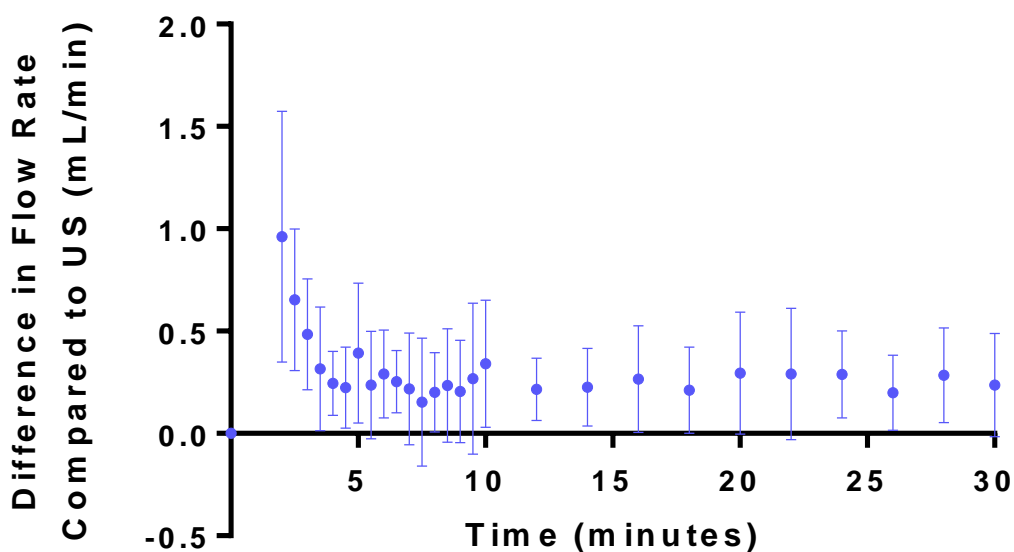


Figure 4.12: The difference in flow rate between US (time zero) and each time point of orally disintegrating tablet stimulated human saliva over 30 minutes after stimulation. Data represents mean +/- S.D. N = 10 participants, US saliva triplicates, SS saliva single measurements. (US = unstimulated saliva, SS = stimulated saliva).

4.5 Discussion

4.5.1 Characterisation of Unstimulated and Parafilm® Stimulated Saliva over Time

It could be argued that when a dosage form is placed in the mouth, saliva is immediately stimulated and may remain in the stimulated state for the likely duration that taste masked particles would reside in the oral cavity. Thus dissolution testing in SS alone would be indicated. To this end, the rate of conversion of saliva from US to SS was investigated, and properties were monitored for a 30 minute period of stimulation to assess whether they returned to US during this time. It is common practice to achieve stimulation of saliva by asking participants to chew on Parafilm®, an inert material, and to control this stimulation using a set sized piece of Parafilm® [173-175]. Thus, participants were asked to donate a US sample and SS samples collected over 30 minutes of continuous masticatory stimulation. Samples were characterised for pH, buffer capacity and flow rate.

The pH of SS was consistently greater than US and continued to rise over the 30 minute experiment. Significant differences between the US control group and SS appeared after 5 minutes of stimulation and the level of significance increased as stimulation continued. In previous research (Chapter 3) [202], the pH of SS was found to be significantly greater than US. This was attributed to the different glandular composition of SS, originating largely from the parotid gland, which releases a bicarbonate rich secretion, leading to higher pH values in this stimulation state.

Numerous other researchers have investigated the effect of masticatory stimulation on the pH of saliva. A mixture of flavoured chewing gums and unflavoured chewing gum bases have been used as the stimulant [180, 249-251]. It must be noted that those using flavoured gums involve both gustatory and mechanical stimulation, and therefore cannot be compared directly to Parafilm® (mechanical) stimulation alone. Other researchers have reported pH showing an initial peak in the first few minutes, then decreasing slightly over time toward, but not returning to US state. In these cases, SS pH remained significantly higher than US throughout the experiment [180, 249, 251]. Dawes and Kubeineic [180] compared unstimulated saliva with chewing gum stimulated saliva using peppermint and fruit gum. SS pH was found to be significantly higher from 2 minutes to 2 hours compared to US for both gums. For peppermint gum, an initial peak and gradual decrease was observed, whereas with fruit gum, an initial decrease due to release of acids was observed followed by a steady increase throughout the 120 minute experiment. Polland, Higgins and Orchardson [249] used mint flavoured chewing gum and found that pH showed peak levels in the first 6 minutes, then remained statistically greater than US during the 90 minute experiment. They also administered a fresh piece of chewing gum every 30 minutes for 90 minutes. Similarly, peak pH values were observed within the first 6 minutes of administration of each new piece of gum, with peak values rising a little every time a new piece was given. SS pH remained significantly greater than US at all time points in this research.

In our case, pH continued to rise throughout the whole 30 minute stimulation period. These findings are in accordance with literature since pH remained elevated above US values for the whole experiment. However literature reports an initial peak, which

was not observed in our experiments. It is unclear why this increasing trend was observed in our study and not others, but this could possibly be related to the release of some components from the Parafilm®. Despite the manufacturers describing it as odourless and colourless [252], the exact composition is not divulged and may have affected the study.

The buffer capacity showed a similar initial peak and slightly reducing trend to that described in literature which was discussed previously surrounding the pH. Buffer capacity of SS showed an initial increase, with significant differences observed between the US control group and SS in the first 3 minutes of stimulation. The buffer capacity then decreased toward, but did not return to US values over the 30 minute stimulation period. In previous research (Chapter 3) [202], the buffer capacity of SS was significantly greater than US. Again, this is attributed to the presence of higher amounts of bicarbonate. The buffer capacity of SS was consistently greater than that of US, but only significantly different in the first 3 minutes. The changing buffer capacity over time as saliva becomes stimulated is not described in literature. However, it would be expected to follow a similar pattern to pH. Literature describing the pH is in accordance with the trend observed for buffer capacity data [249, 251].

Flow rate was found to be a highly variable parameter with significant differences between the US control group and SS in the first 10 minutes of stimulation. It then remained elevated above US values and did not return to US values over the 30 minute experiment, although no significant differences were observed between US and SS from 10 - 30 minutes. Many studies of the effect of mechanical stimulation on saliva investigated flow rate as an outcome. In all cases, the flow rate demonstrated an initial peak [180, 181, 249, 251, 253-255], occurring in the first minute in many cases [181, 253-255], followed by a gradual decrease to levels higher than US flow rate over the length of the experiment (up to 120 minutes), or to a plateau above US values [180]. Rosenhek, Macpherson and Dawes [255] also compared unflavoured chewing gum base with flavoured chewing gum and found the same flow rate profile was observed for both types of stimulant, whereby an initial increase in the first two minutes was followed by a decrease to a plateau above US values for the remainder of the 20 minute experiment.

Interestingly, in two cases, a fresh piece of gum was provided at set times. One study gave a fresh piece of gum at 30 and 60 minutes and found flow rate showed a further peak within the first two minutes of each new piece of gum [249]. In another study, a second piece of gum was administered at 90 minutes and flow rate also increased further above the already stimulated flow rate [180].

After the initial peak and raised phase, the subsequent reduction in buffer capacity and flow rate over time compared to US could possibly be explained by a reduction in the size of the piece of Parafilm® and it becoming less “chewy” over time, reducing the size of the stimulus. It has been reported by Kjeilen *et al.* [177] that increasing the frequency, force and number of teeth involved in chewing increased parotid secretions, therefore a reduction in the salivary output would be expected if the size of the Parafilm® decreased. Additionally, Rosenhek, Macpherson and Dawes [255] reported that flow rate increased as weight of gum increased, thus if the Parafilm® decreased in size, salivary output would also be expected to. It may also be possible that under prolonged and continuous mechanical stimulation, the response to the stimulant becomes desensitised.

4.5.2 Characterisation of Unstimulated and ODT Stimulated Saliva over Time

It was considered that continuous masticatory stimulation over 30 minutes could be not representative of the stimulation of saliva provided when an oral dosage form is administered e.g. an orally disintegrating tablet. In this case, short term stimulation of saliva occurs. Thus, an orally disintegrating tablet was administered as the stimulant, and the trial was repeated as before, with characterisation of US, and SS at set times after stimulation. Citric and ascorbic acid are often excipients in ODT formulations, included to stimulate the production of saliva and to provide a pleasant taste [256]. Thus an ascorbic acid ODT with no active pharmaceutical ingredient was used as a placebo ODT.

The release of ascorbic acid from the ODT results in a decrease of salivary pH, and inability to accurately titrate saliva to assess buffer capacity. In another study, citric acid was used as a stimulant and the decrease and recovery of pH was measured over time [257]. However, in this study, participants were asked to swallow their

saliva three times after disintegration of the ODT to ensure that particles of the ODT were swallowed and would not affect subsequent analysis.

In this trial, all parameters showed an initial increase with significant differences between the US control group in the first 10 and 9.5 minutes for pH and buffer capacity respectively. Flow rate showed an initial increase with significant differences in the first 6 minutes. Then values remained at an elevated plateau for flow rate, with some further significant differences observed between US control group and SS at later time points. However, for pH and buffer capacity, values reduced towards without reaching US values over the 30 minute experiment.

To our knowledge, this is the first time in which stimulation of saliva with respect to time has been investigated with a realistic method of stimulation representing dosage form administration, thus we cannot compare this data to literature. However, short term gustatory stimulation has been used in the literature, commonly using citric acid application to the tongue. The changes in pH and buffer capacity with respect to time after gustatory stimulation are not well documented in literature; however a number of studies investigated changes in the flow rate after short-term gustatory stimulation with citric acid.

In general, the flow rate increased in the first minute after stimulation, but returned to US values and demonstrated a rapid recovery compared to continuous masticatory stimulation. Morimoto *et al.* [178] investigated the effect of citric acid stimulation on parotid gland size. The parotid gland is known to be responsible for the majority of stimulated saliva secretions [202]. They found that mean time to maximum duct area was 69 s +/- 29 s after stimulus application, and time taken to return to 50 % of pre-stimulation size was 156 s +/- 61 s. Therefore, flow rate and parotid output was shown to be quickly induced and demonstrated a rapid recovery to pre-stimulation conditions. Tanaka *et al.* [258] performed a similar experiment on both the parotid and submandibular glands and found that parotid gland maximum duct area was observed within 60 s and submandibular within 120 s, confirming the rapid induction of salivary flow. Millward *et al.* [257] observed peak parotid flow 1 minute after citric acid stimulation, with full recovery to baseline US levels within 6 minutes, whilst

Duran *et al.* [259] observed peak salivary flow within 30 s of citric acid stimulation, and recovery to US levels within two minutes. Additionally, de Mata *et al.* [260] compared two acid containing lozenges and found peak flow rates within 5 minutes of administration, with flow rates returning to baseline within 20 minutes. The time taken for lozenge dissolution could be responsible for slightly longer flow induction and recovery times in this study compared to other literature.

In all cases, the results of this trial are in agreement with literature with respect to the initial peak in each parameter and subsequent reduction. However, unlike literature, a return to US values was not seen for any parameter during the 30 minute time period. The slight prolonged elevation of each parameter above US levels (although not always significantly different to US values) may possibly be explained by prolonged stimulation of, and desensitisation of taste receptors. Despite participants being asked to swallow saliva to ensure all particles of the ODT are removed from the oral cavity, it is possible that some particles remained in the oral cavity at very low concentrations at taste bud sites. This may have caused prolonged stimulation of the taste receptors, resulting in a response over time that slowly declines, which would describe the pattern observed in this study [261].

The observation of significant differences between US and SS at different times for each parameter with Parafilm® stimulation and with perhaps the more realistic ODT stimulation confirms that dissolution testing in SS alone is not sufficient as saliva does not remain in the stimulated state throughout these experiments. Thus dissolution testing of taste masked formulations is recommended in simulated salivary fluids representing both the US and SS states.

4.6 Conclusions

The requirement for development of a simulated salivary fluid representing both US and SS was investigated. Salivary parameters were characterised with respect to time after Parafilm® and ODT stimulation. Significant differences between the US and SS states appeared at different times after stimulation for different parameters. Saliva did not remain in the stimulated state for the whole duration of the two experiments as significant differences between US and SS disappeared over time for most

parameters. Thus, it is recommended to perform dissolution testing of taste masked oral dosage forms in simulated salivary fluids representing both the unstimulated and stimulated states to confirm taste masking is achieved.

Chapter 5: Development and Proposal of Biorelevant Simulated Salivary Fluids

5.1 Introduction

The choice of media to represent human saliva in dissolution tests has been a matter of great interest. The media should represent human saliva as closely as possible in order to best predict the dissolution behaviour of drugs or dosage forms in the oral cavity. Our previous research also confirmed that dissolution media should be developed representing both the unstimulated and stimulated states.

There are a number of simulated salivary fluids (SSFs) available for dissolution testing, as discussed in Chapter 3. Crucially, to our knowledge, there are no SSFs in existence representing both the salivary stimulation states. Existing SSFs can be categorised into three main groups: 1 - simple electrolyte mixtures such as phosphate buffered saline, 2 - those containing the viscosity modifying polymer carboxymethylcellulose, such as Glandosane[®], and 3 - those containing viscosity modifying mucins such as Saliva Orthana[®] products. In Chapter 3, these were characterised using the same methodology as human saliva to allow for a direct comparison. Unfortunately, none of the three classes were found to be suitable to represent human saliva based on key parameters relevant to dissolution. There is therefore a requirement for novel, more biorelevant SSFs representing both the unstimulated and stimulated human salivary states to be developed.

In the present research, for the first time, biorelevant SSFs representing both the unstimulated and stimulated states of human saliva are proposed for use as dissolution media.

5.2 Aims

The aims of this Chapter are:

- To develop novel biorelevant simulated salivary fluids representing both the unstimulated and stimulated states for application in dissolution testing

- To evaluate the suitability of these to represent human saliva by direct comparison with human saliva based on key characteristics relevant to dissolution

5.3 Methods

All methods relevant to this Chapter are detailed in Chapter 2. In particular, refer to section 2.1 for materials and 2.5 – 2.7 for characterisation of pH, buffer capacity, viscosity, and surface tension. These were characterised according to methodology for “Trial 1”. Human saliva data is described in Chapter 3, and methods can be found in Chapter 2 for human saliva characterisation. Refer also to Chapter 2, section 2.8 for statistical analysis.

5.4 Results

5.4.1 Development of Simulated Salivary Fluids

5.4.1.1 Comparison of Glandosane[®] made up from its component parts to Human Saliva and PBS, and Analysis of the Effect of Carboxymethylcellulose on SSF Characteristics.

In Chapter 3, Glandosane[®] was characterised and compared to human saliva. However, measurements were found to be erratic due to it being a spray, propelled by carbon dioxide from a pressurised container. This may have influenced the pH measurements as CO₂ left the sample, and made measurements of viscosity incomprehensible due to the high shear exerted on the sample during its removal from the container prior to rheological analysis. The development of a biorelevant SSF therefore began with the manufacture and characterisation of Glandosane[®] in our laboratory from its component parts to assess its similarity to human saliva. The quantitative composition of Glandosane[®] is publicly available [164] and is listed in Chapter 2, Table 2.2.

Glandosane[®] contains 1 % w/w of the viscosity modifier carboxymethylcellulose sodium (CMC). In order to assess the suitability of CMC as a viscosity modifier, and its effect on the pH, buffer capacity and surface tension of a potential SSF solution, concentrations between 0 – 5 % w/w CMC were assessed. These varying

concentrations of CMC were characterised in Glandosane[®] made from its component parts in our laboratory and also in phosphate buffered saline (PBS) from the British Pharmacopoeia [223] as vehicles. These solutions were compared to each other and to human saliva. Characterisation of each parameter followed the same methodology as for human saliva, detailed in Chapter 2, section 2.5.1 to allow for a direct comparison.

The pH of PBS / CMC solutions was found to be approximately the same (pH 6.8) irrespective of CMC concentration. This was slightly lower compared to human US values and substantially lower than human SS values. The pH of Glandosane[®] made up from its component parts (GLN) increased as the concentration of CMC increased. However, pH values were all lower than that of PBS / CMC solutions and therefore less suitable to represent human saliva than PBS / CMC solutions as shown in Figure 5.1.

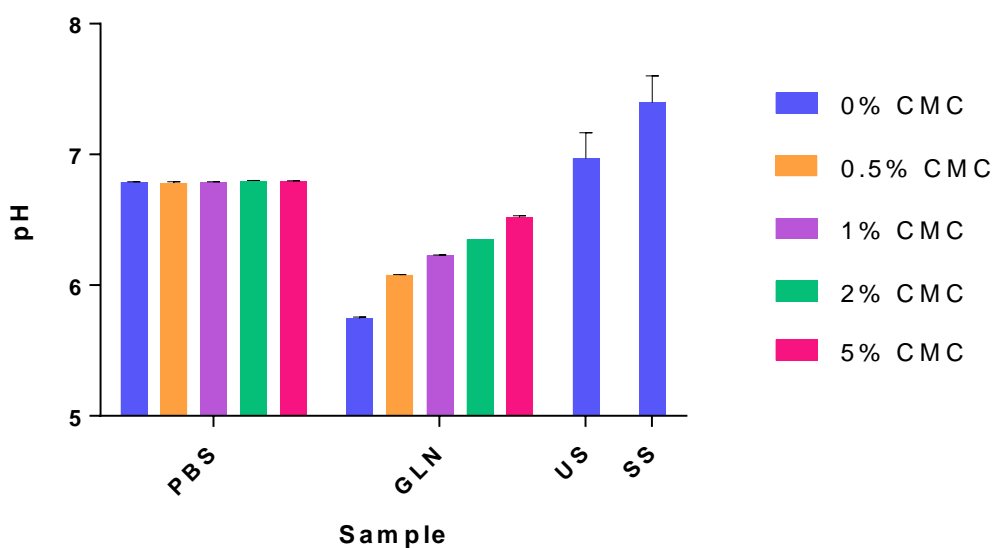


Figure 5.1: The pH of potential SSFs containing differing carboxymethylcellulose (CMC) concentrations expressed as % w/w compared to unstimulated and stimulated human saliva. Data represents mean +/- S.D. N = 30, quintuplicate for US and SS, N = 5, quintuplicate for PBS and GLN. (PBS = phosphate buffered saline, GLN = Glandosane[®] made up from its component parts, US = unstimulated human saliva, SS = stimulated human saliva (data from Chapter 3)).

The buffer capacity of PBS / CMC solutions was found to increase slightly as CMC concentration increased. These values were slightly higher than human US values but similar to human SS values. The buffer capacity of Glandosane® made up from its component parts (GLN) also increased as the concentration of CMC increased. However, these buffer capacity values were all much lower than that of human US, except for 5 % CMC, and thus less suitable to represent human saliva than PBS / CMC solutions as shown in Figure 5.2.

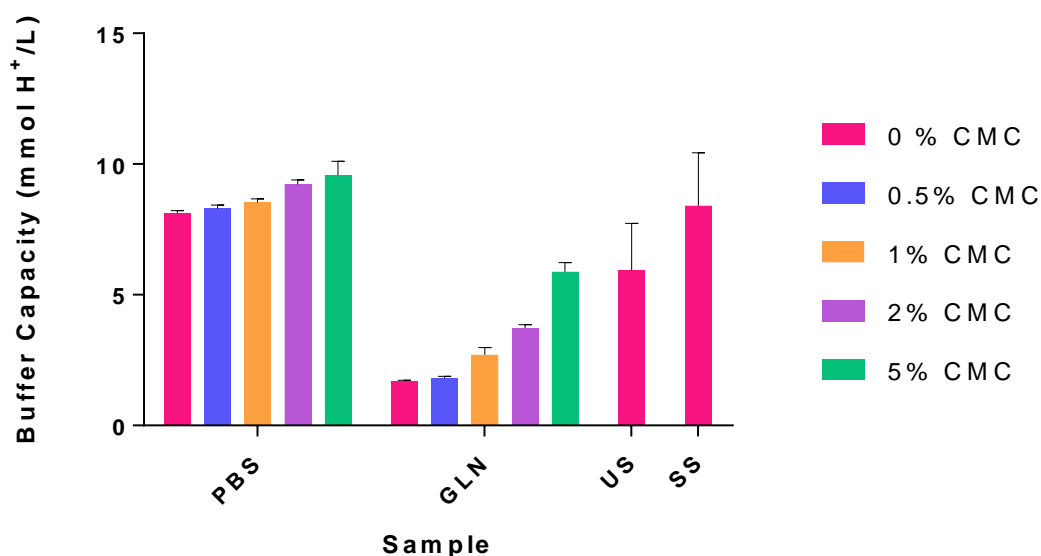


Figure 5.2: The buffer capacity of potential SSFs containing differing carboxymethylcellulose (CMC) concentrations expressed as % w/w compared to unstimulated and stimulated human saliva. Data represents mean +/- S.D. N = 30, duplicate for US and SS, N = 5, triplicate for PBS and GLN. (PBS = phosphate buffered saline, GLN = Glandosane® made up from its component parts, US = unstimulated human saliva, SS = stimulated human saliva (data from Chapter 3)).

The surface tension of both the PBS / CMC and GLN / CMC solutions changed very little as CMC concentration increased as shown in Figure 5.3. Additionally, all values were found to be much higher than human US and SS values and neither would be suitable to represent human saliva without the addition of a surfactant.

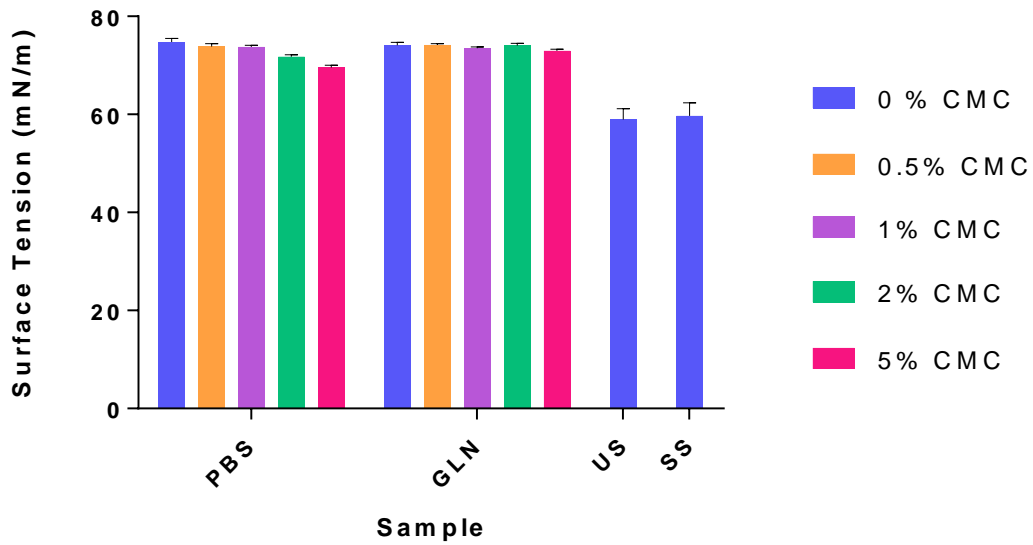


Figure 5.3: The surface tension of potential SSFs containing differing carboxymethylcellulose (CMC) concentrations expressed as % w/w compared to unstimulated and stimulated human saliva. Data represents mean +/- S.D. N = 30, quintuplicate for US and SS, N = 5, quintuplicate for PBS and GLN. (PBS = phosphate buffered saline, GLN = Glandosane® made up from its component parts, US = unstimulated human saliva, SS = stimulated human saliva (data from Chapter 3)).

The viscosity of PBS / CMC solutions was found to be very similar to that of GLN / CMC at each CMC concentration, and the rheological profiles almost overlap. However, none of the solutions were found to be a good match to human saliva. Human saliva shows considerable shear thinning behaviour; whereas the CMC solutions showed very little shear thinning except at the higher 5 % concentration. However, even at this concentration, it was not found to exhibit suitable rheological behaviour to represent human saliva as shown in Figure 5.4.

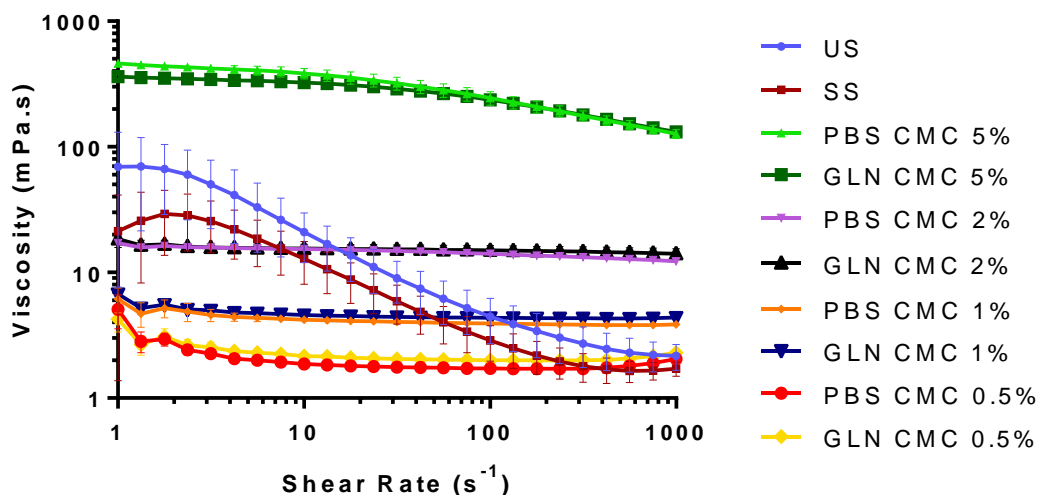


Figure 5.4: The viscosity at different shear rates of potential SSFs containing differing carboxymethylcellulose (CMC) concentrations expressed as % w/w compared to unstimulated and stimulated human saliva. Data represents mean +/- S.D. N = 30, triplicates for US and SS, N = 5, triplicates for PBS and GLN. (PBS = phosphate buffered saline, GLN = Glandosane® made up from its component parts, US = unstimulated human saliva, SS = stimulated human saliva (data from Chapter 3)).

5.4.1.2 Choice of Viscosity Modifier

It was clear from the rheological analysis of carboxymethylcellulose solutions that CMC is not an appropriate viscosity modifying agent since it did not exhibit the required extent of shear thinning necessary to represent the highly shear thinning human saliva. Other viscosity modifiers were therefore investigated.

Acacia is a component of one of the commercially available artificial saliva formulations detailed in the British National Formulary [204] used clinically for the treatment of xerostomia. It has also been reported to have shear thinning properties [262, 263]. The viscosity of PBS with differing concentrations of acacia was therefore evaluated. Unfortunately, the solutions did not demonstrate shear thinning behaviour and thus acacia is not a suitable choice of viscosity modifier to use in simulated salivary fluids, as shown in Figure 5.5.

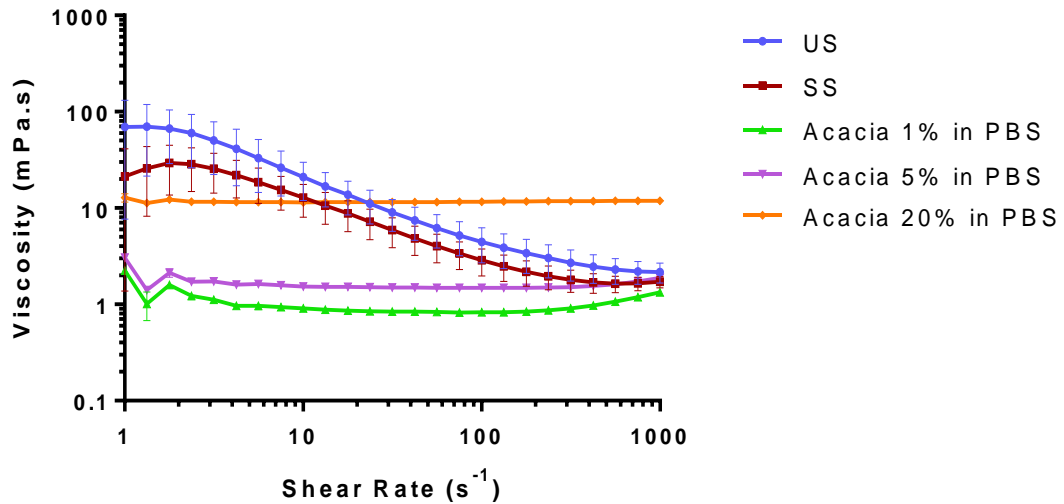


Figure 5.5: The viscosity at different shear rates of potential SSFs containing differing acacia concentrations expressed as % w/v compared to unstimulated and stimulated human saliva. Data represents mean \pm S.D. N = 30, triplicates for US and SS, N = 5, triplicates for acacia samples. (PBS = phosphate buffered saline, US = unstimulated human saliva, SS = stimulated human saliva (data from Chapter 3)).

Bovine submandibular mucin (BSM) has also been reported to have shear thinning properties in some literature as discussed in more detail in Chapter 3, section 3.3.4. We therefore evaluated the viscosity of two concentrations of BSM in PBS. However, as seen in Figure 5.6, shear thinning was not demonstrated under our conditions and this was also not found to be a suitable viscosity modifier for SSF solutions.

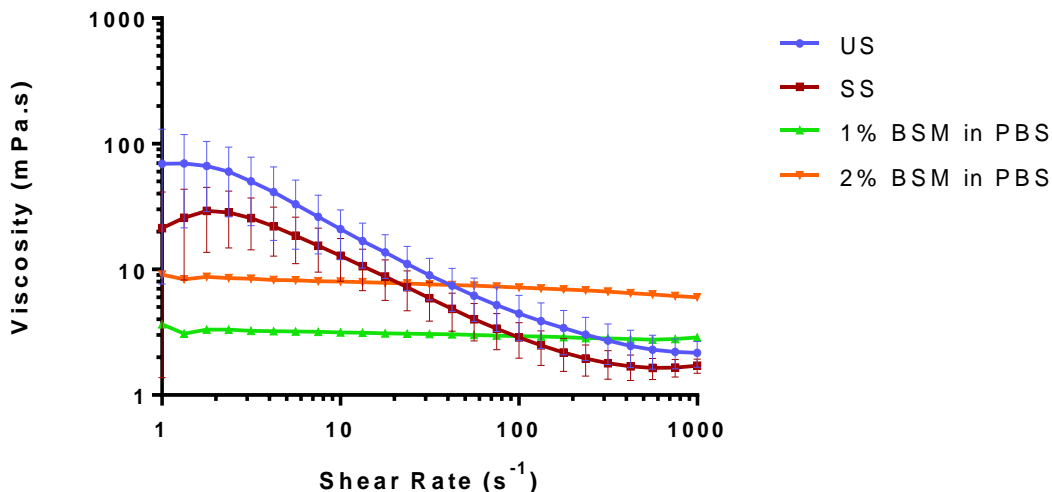


Figure 5.6: The viscosity at different shear rates of potential SSFs containing differing bovine submandibular mucin concentrations expressed as % w/v compared to unstimulated and stimulated human saliva. Data represents mean +/- S.D. N = 30, triplicates for US and SS. N = 1, triplicates for BSM samples. (PBS = phosphate buffered saline, BSM = bovine submandibular mucin, US = unstimulated human saliva, SS = stimulated human saliva (data from Chapter 3)).

Xanthan gum is a viscosity modifying component of the Saliva Orthana[®] gel characterised in Chapter 3. We observed clear shear thinning behaviour for Saliva Orthana[®] gel. This was attributed solely to the presence of xanthan gum, since the Saliva Orthana[®] spray did not exhibit shear thinning behaviour, and the only difference in composition between the two formulations is the presence of xanthan in the gel. However, the viscosity of the gel, which contains 0.5 % w/v xanthan gum was much higher than that of human saliva. Xanthan was therefore investigated in more depth as a viscosity modifier at concentrations of 0.05 – 0.15 % w/v.

All concentrations of xanthan gum in PBS showed distinct shear thinning behaviour comparable to that of human saliva, as seen in Figure 5.7. A concentration of 1 % was found to be within the range of values seen for human saliva since it sits inside the error bars, which represent standard deviation. Although the gradient of the viscosity profile is not an exact match, xanthan gum was found to be the most suitable viscosity modifier for use in SSFs to represent human saliva out of all options tested. Therefore, further work continued with xanthan gum.

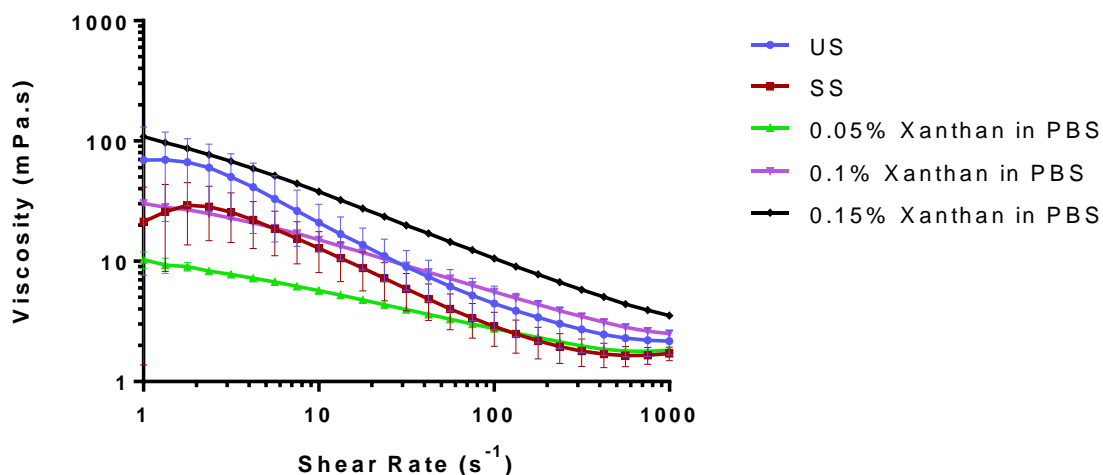


Figure 5.7: The viscosity at different shear rates of potential SSFs containing differing xanthan gum concentrations expressed as % w/v compared to unstimulated and stimulated human saliva. Data represents mean \pm S.D. N = 30, triplicates for US and SS. N = 5, triplicates for xanthan samples. (PBS = phosphate buffered saline, US = unstimulated human saliva, SS = stimulated human saliva (data from Chapter 3)).

5.4.1.3 Choice of Buffer

Human saliva is made up of three buffering systems: bicarbonate, phosphate and protein buffers [182]. The prevalence of each buffer changes with respect to the stimulation state. Bicarbonate is present in greater quantities in stimulated saliva, as discussed in Chapter 3, and is the predominant buffer in stimulated saliva. However unstimulated saliva is predominantly buffered by the phosphate buffering system.

Bicarbonate buffers have not been used in any of the SSFs detailed in literature or commercially available. Due to the escape of carbon dioxide from the system, which constantly changes the pH unless measures are taken to avoid this, it is a challenging buffer to use from practical considerations. Phosphate buffer was therefore selected for all future experimentation. Additionally, where a buffering system is employed in SSFs from commercial sources or literature, phosphate buffer is also the buffer of choice.

Until now, our characterisations have focussed on the use of phosphate buffered saline BP pH 6.8 as this appears in many of the SSFs from the literature (see Chapter

1). However a number of other phosphate buffers are detailed in both British Pharmacopoeia (BP) [264] and United States Pharmacopoeia (USP) [265] without the high, non biorelevant concentrations of sodium chloride. British Pharmacopoeial phosphate buffer [264] is manufactured from a disodium hydrogen phosphate solution and a citric acid solution, mixed in differing proportions to generate buffers of different pH, as seen in Table 5.1. In this case, the pH is varied by changing the ratio of salt present.

Table 5.1: Phosphate buffer solutions modified from British pharmacopoeia. Quantities provided for 100 mL buffer.

pH of Buffer	Volume Na₂HPO₄ solution, mL (28.36 g/L Na₂HPO₄)	Volume citric acid monohydrate solution, mL (21 g/L C₆H₈O₇·H₂O)
7.0	82.4	17.6
7.4	91.3	8.7

However, USP phosphate buffer [265] is made by placing 50 mL of a 0.2 M monobasic potassium phosphate solution into a 200 mL volumetric flask, adding the specified volume of the sodium hydroxide solution from Table 5.2, and then adding water to volume. In this case, the pH is varied by changing the amount of NaOH added in the final step.

Table 5.2: Amount of NaOH required to make 200 mL of phosphate buffer with the pH specified in the Table. Modified from the United States Pharmacopoeia.

pH of Buffer	7.0	7.4
mL 0.2 M NaOH	29.1	39.1

The buffer capacity of both BP and both USP buffers was determined with and without the addition of 0.1 % xanthan. The pH values of 7.0 and 7.4 were based on our previous findings from Chapter 3 which state that the mean pH of unstimulated and stimulated human saliva was found to be 7.0 and 7.4 to one decimal place respectively.

The addition of xanthan did not affect the pH during the manufacture of the buffers, nor did it appear to have any effect on the buffer capacity of the solution, as seen in Table 5.3. BP buffers were found to have approximately 10 times the buffer capacity of human saliva, and USP buffers approximately 4 times the buffer capacity of saliva since values for human saliva were 5.9 and 8.4 mmol H⁺/L respectively. Buffer capacity was also much greater for BP phosphate buffers than USP buffers. These would therefore require dilution before they could be considered sufficient to represent human saliva in SSFs.

Table 5.3: Buffer capacity of BP and USP buffers with or without the addition of 0.1 % xanthan gum. (BP = British pharmacopoeia, USP = United States pharmacopoeia).

Buffer	pH	Xanthan (Y/N)	Run	Buffer capacity (mmol H ⁺ /L)	Mean buffer capacity (mmol H ⁺ /L)
BP	7.0	Y	1	85.00	86.25
			2	87.50	
		N	1	82.50	80.00
			2	77.50	
	7.4	Y	1	75.00	77.50
			2	77.50	
		N	1	77.50	76.25
			2	82.50	
USP	7.0	Y	1	22.50	21.88
			2	21.25	
		N	1	22.50	21.88
			2	21.25	
	7.4	Y	1	25.00	25.25
			2	25.50	
		N	1	25.00	21.38
			2	23.75	

5.4.1.4 Modifying the Rheological Properties

The rheological profile of phosphate buffered saline with 0.1 % xanthan gum is a reasonable match for human saliva as seen previously in Figure 5.7. However, a steeper gradient of the profile would be ideal since it currently encompasses viscosity values across both human US and SS, and does not closely represent either of the stimulation states. We investigated the effect of changing the pH and salt composition of the buffer on the gradient of the rheological profile, with a view to obtaining a steeper gradient for the xanthan containing buffer solution such that it can represent US or SS individually.

USP buffers of varying pH within the range of human saliva were used to ascertain the effect of pH on the rheological profile of xanthan gum containing buffer solutions. This is because the pH of USP buffers can be altered by simply adding more NaOH without affecting the phosphate concentration. Very little difference was observed between profiles as seen in Figure 5.8, thus pH did not appear to affect viscosity.

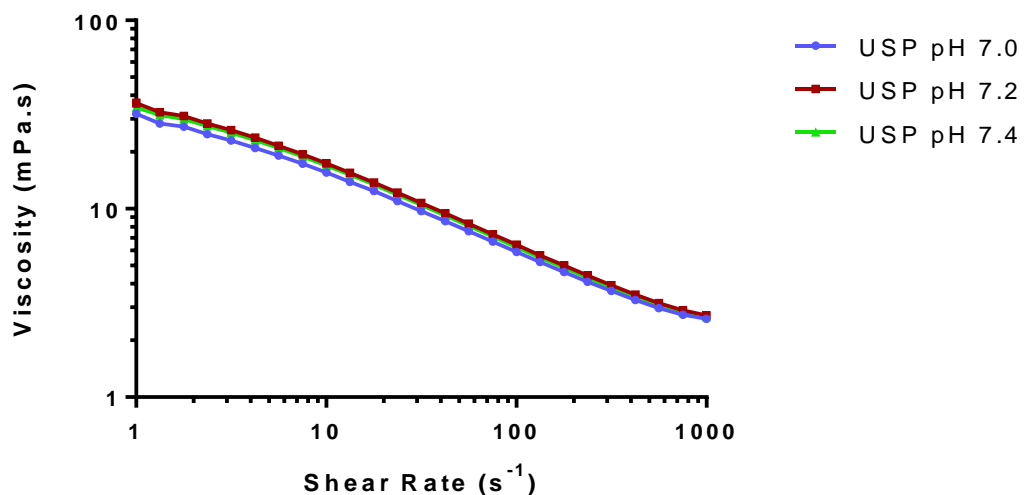


Figure 5.8: The viscosity at different shear rates of potential SSFs containing 0.1 % w/v xanthan gum in USP phosphate buffer solutions. Data represents mean \pm S.D. N = 5, triplicates. (USP = United States pharmacopoeia).

BP buffers of varying pH within the range of human saliva were used to ascertain the effect of salt concentration on the rheological profile of xanthan gum containing buffer solutions. This is because the pH of BP buffers is altered by changing the phosphate and citrate concentrations. Very little difference was observed between profiles as seen in Figure 5.9, thus salt concentration did not appear to affect viscosity.

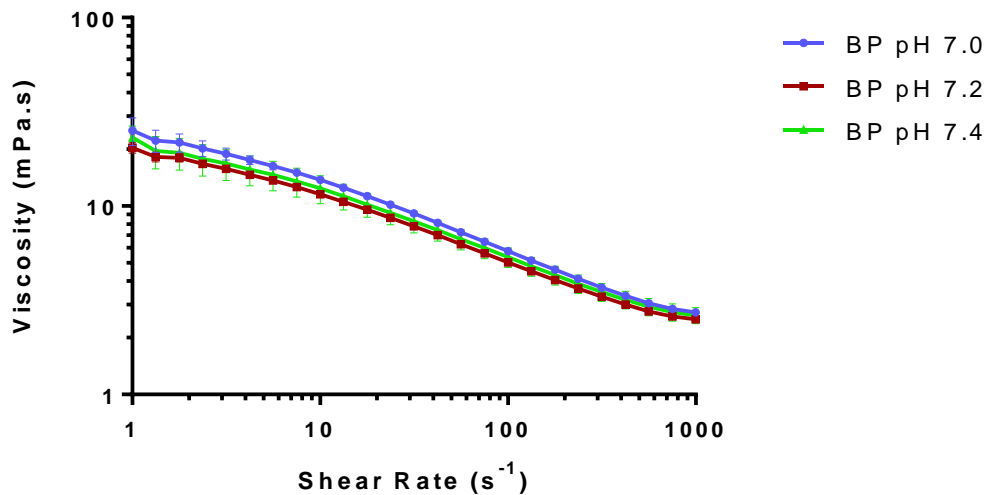


Figure 5.9: The viscosity at different shear rates of potential SSFs containing 0.1 % w/v xanthan gum in BP phosphate buffer solutions. Data represents mean +/- S.D. N = 5, triplicates. (BP = British pharmacopoeia).

These two data sets were then overlaid with human saliva data from Chapter 3 in order to assess how well these solutions represent human saliva, and the differences between them, as seen in Figure 5.10 for BP buffer solutions and USP buffer solutions respectively. BP buffer / xanthan solutions had a very slightly lower viscosity compared to USP buffer / xanthan solutions. This meant the BP solutions were always within the error bars of at least one subtype of human saliva, whereby error bars represent standard deviation. However, USP solutions were a good match for human US, but were of very slightly higher viscosity than SS in the high shear range, making BP solutions marginally more suitable when 0.1 % xanthan is used.

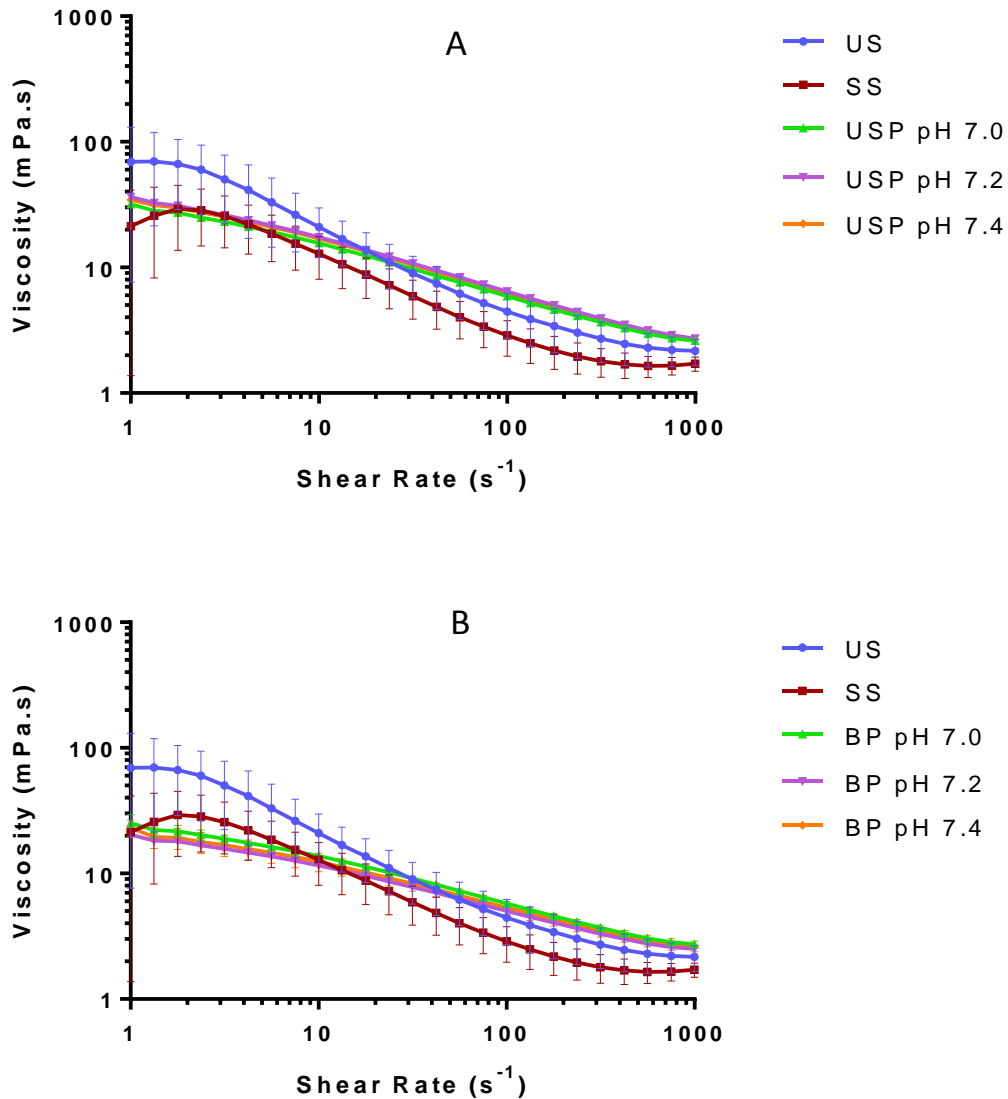


Figure 5.10: The viscosity at different shear rates of potential SSFs containing 0.1 % w/v xanthan gum in USP phosphate buffer solutions (Panel A) and in BP phosphate buffer solutions (Panel B). Data represents mean \pm S.D. N = 5, triplicates for potential SSFs. N = 30, triplicates for US and SS. (BP = British pharmacopoeia, USP = United States pharmacopoeia, US = unstimulated human saliva, SS = stimulated human saliva (data from Chapter 3)).

5.4.1.5 Choice of Surfactant

We observed in Table 5.3 that the addition of xanthan did not appear to affect the buffer capacity of phosphate buffer solutions. It was also observed during manufacture that no change to the pH was observed upon addition of xanthan gum. We therefore investigated the surface tension of xanthan-containing solutions based

on both BP and USP phosphate buffers to assess their suitability to represent human saliva based on surface tension. No significant difference was observed between the surface tension of the four samples (ANOVA and Holm-Sidak's multiple comparisons test). All samples had a higher surface tension than human saliva which was 58.98 mN/m and 59.69 mN/m for US and SS respectively. Therefore a surfactant is required regardless of which type of buffer is selected. However, BP values were slightly lower and thus slightly closer to human saliva values (Figure 5.11).

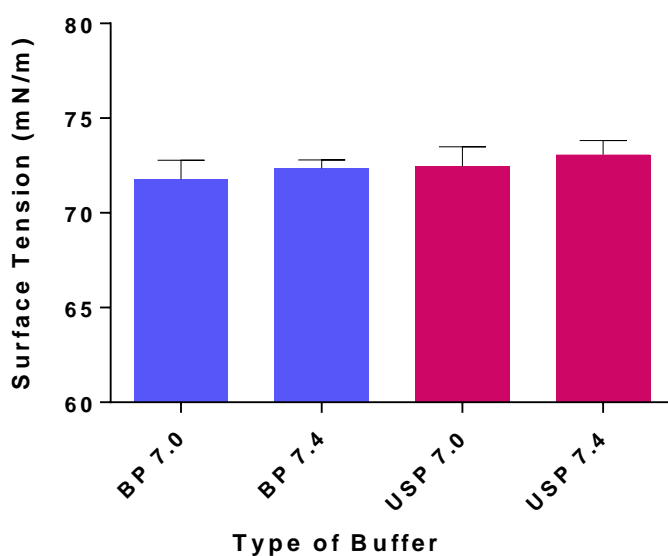


Figure 5.11: Surface tension of phosphate buffers of different pH containing 0.1 % xanthan gum. Data represents mean +/- S.D. N = 5, quintuplicate. (BP = British pharmacopoeia, USP = Unites States pharmacopoeia).

Neither type of buffer was found to be a suitable match for human salivary buffer capacity without dilution. Additionally, neither buffer affected the rheological profile or the surface tension of xanthan solutions. BP buffers were a very slightly better match to human salivary values for viscosity as BP buffer values were within the error bars at both extremes of shear rate for US and SS. BP buffers also exhibited slightly closer surface tension values to human values without surfactants. Therefore, BP buffers were chosen for further work. The amount of dilution required for each buffer to have a similar buffer capacity to human saliva was determined experimentally by dilution of the buffer with deionised water and analysis of buffer

capacity. The exact composition of the final buffer solution is given shortly in the summary of SSF composition. All further work in this Chapter now relates to diluted BP buffers as the base component of developed SSFs.

Since the surface tension of all xanthan containing phosphate buffers was greater than that of human saliva, (Figure 5.11), it was identified that a surfactant is required to reduce surface tension to physiological values. Two surfactants were tested which are used in other biorelevant media: lecithin and Tween 20[®] [218, 266-268]. To solutions of diluted BP phosphate buffer pH 7.2 plus 0.1 % xanthan was added varying concentrations of Tween 20[®] or lecithin, and the surface tension was assessed. Tween 20[®] demonstrated a more linear, predictable relationship between concentration and surface tension as shown in Figure 5.12 than lecithin in Figure 5.13, and thus Tween 20[®] was used for further development of biorelevant simulated salivary fluids.

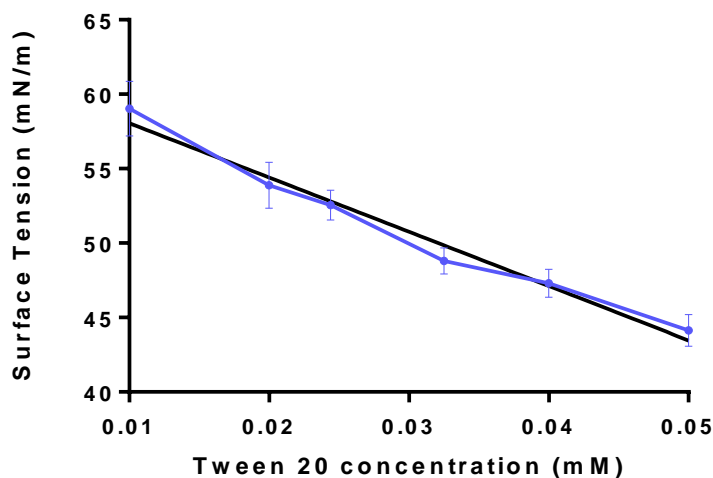


Figure 5.12: Surface tension of diluted phosphate buffer pH 7.2 containing 0.1 % xanthan gum and varying concentrations of Tween 20[®]. Data represents mean +/- S.D. N = 2, quintuplicate. Linear regression line indicated.

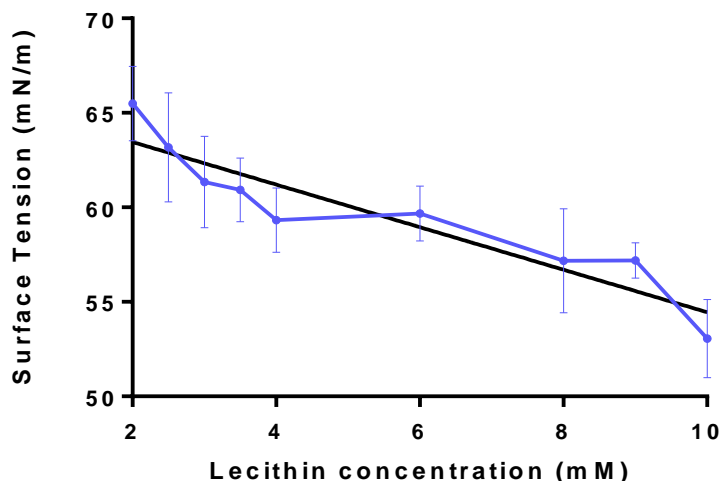


Figure 5.13: Surface tension of diluted phosphate buffer pH 7.2 containing 0.1 % xanthan gum and varying concentrations of lecithin. Data represents mean +/- S.D. N = 2, quintuplicate. Linear regression line indicated.

The surface tension of human saliva was found to be 58.98 and 59.69 mN/m for human US and SS respectively, with no significant difference in surface tension between the two stimulation states. Thus a concentration of 0.01 mM was employed since this concentration provided the closest surface tension compared to human salivary values.

5.4.1.6 Evaluation of the Effect of Tween 20[®] on Viscosity

The addition of Tween 20[®] did not affect the pH of the solutions. Being a non-ionic surfactant, it was also not expected to affect buffer capacity. However, it is a viscous solution and may affect the viscosity of the solution of buffer and xanthan. Therefore the viscosity was re-assessed after the addition of Tween 20[®]. The viscosity of buffer solutions containing 0.1 % xanthan gum and 0.01 mM Tween 20[®], as shown in Figure 5.14, Panel A, was slightly raised compared to Figure 5.10, Panel B, which shows BP buffer solutions containing 0.1 % xanthan gum without Tween 20[®]. It was therefore decided to reduce the concentration of xanthan gum from 0.1 % to 0.09 % (Figure 5.14, Panel B) and 0.08 % (Figure 5.14, Panel C). The xanthan concentration of 0.08 % appeared to give the closest results compared to those of human saliva, thus this concentration was used for future work.

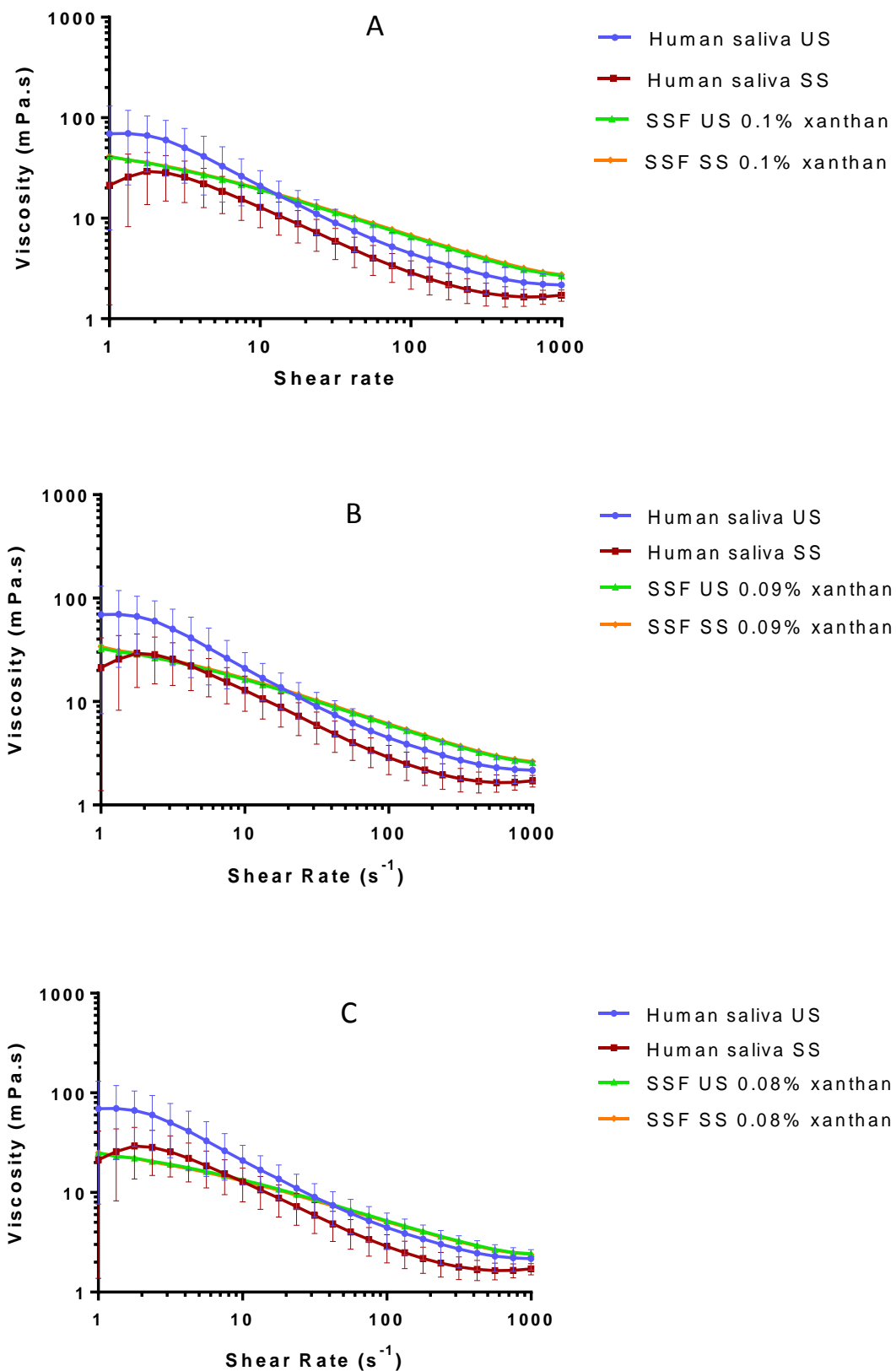


Figure 5.14: The viscosity at different shear rates of potential SSFs containing 0.1 % w/v (Panel A), 0.09 % (Panel B) and 0.08 % (Panel C) xanthan gum in diluted BP phosphate buffer solution plus 0.01 mM Tween 20®, compared to human US and SS.

Data represents mean +/- S.D. N = 30, triplicates for US and SS. N = 3, triplicates for potential SSFs. (US = unstimulated human saliva, SS = stimulated human saliva (data from Chapter 3), SSF = simulated salivary fluid).

5.4.1.7 Summary of Developed Simulated Salivary Fluids' Composition

The most suitable composition to represent human saliva based on pH, buffer capacity, surface tension and viscosity was found to be BP phosphate buffer, diluted with deionised water as determined experimentally, with 0.08 % xanthan gum and 0.01 mM Tween 20®. The final composition of the developed SSFs is summarised in Table 5.4.

Table 5.4: US and SS composition for 100 mL of SSF. (US = unstimulated saliva, SS = stimulated saliva, BP = British pharmacopoeia).

Component	US	SS
BP pH 7.0 buffer (Table 5.1)	7.692 mL	-
BP pH 7.4 buffer (Table 5.1)	-	9.009 mL
HCl (1 M)	To pH 7.0	To pH 7.4
Tween 20®*	5.6 µL	5.6 µL
Xanthan gum**	80 mg	80 mg
Deionised water	To 100 mL	To 100 mL

* Tween 20® from Sigma Aldrich, MW 1228, density 1.095 g/mL ** Xanthan gum from Sigma Aldrich, viscosity 800 - 1200 mPa.s for 1 % solution

5.4.2 Characterisation of Developed Simulated Salivary Fluids and Comparison to Human Saliva

5.4.2.1 pH

Five batches of US and SS SSF were made and characterised for pH, buffer capacity, surface tension and viscosity, and compared to human saliva characteristics from Chapter 3.

The pH of unstimulated and stimulated human saliva was compared to developed simulated salivary fluids (Figure 5.15). As described in Chapter 3, significant

differences were observed between human US and SS. No significant difference was observed between human saliva and SSF for either stimulation state. However, significant differences were observed between US SSF and SS human saliva, and between US human saliva and SS SSF (Kruskal-Wallis and Dunn's multiple comparisons test).

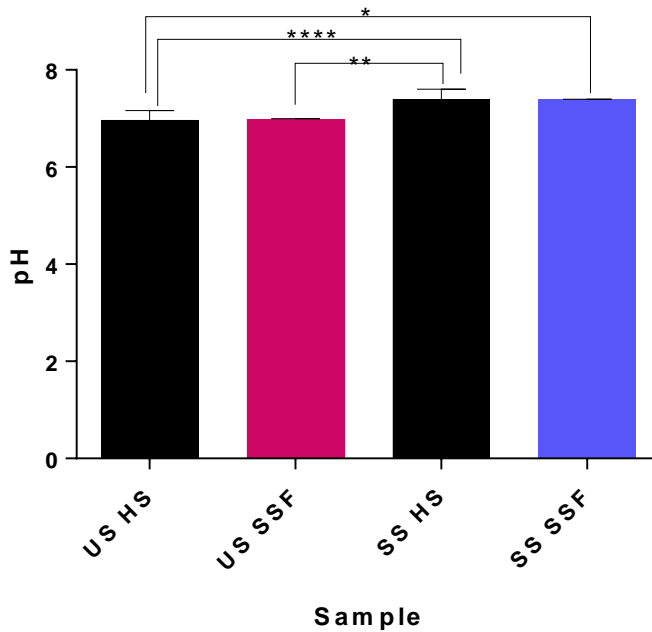


Figure 5.15: The pH of unstimulated and stimulated human saliva and simulated salivary fluids. Data represents mean +/- S.D. N = 30 for human saliva, triplicates. N = 5 for SSFs, triplicates. (US = unstimulated saliva, SS = stimulated saliva, SSF = simulated salivary fluids, HS = human saliva). * significant difference (Kruskal-Wallis and Dunn's multiple comparisons test, $p < 0.05$) ** significant difference (Kruskal-Wallis and Dunn's multiple comparisons test, $p < 0.01$) **** significant difference (Kruskal-Wallis and Dunn's multiple comparisons test, $p < 0.0001$).

5.4.2.2 Buffer Capacity

The buffer capacity of unstimulated and stimulated human saliva was compared to developed simulated salivary fluids (Figure 5.16). As described in Chapter 3, significant differences were observed between human US and SS. No significant difference was observed between human saliva and SSF for either stimulation state. However, significant differences were observed between US SSF and SS human saliva,

and between US human saliva and SS SSF (ANOVA and Holm-Sidak's multiple comparisons test).

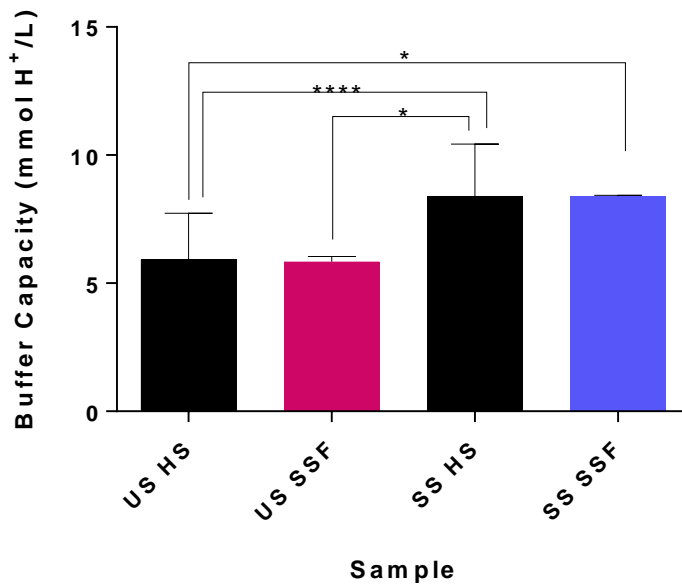


Figure 5.16: The buffer capacity of unstimulated and stimulated human saliva and simulated salivary fluids. Data represents mean +/- S.D. N = 30 for human saliva, triplicates. N = 5 for SSFs, triplicates. (US = unstimulated saliva, SS = stimulated saliva, SSF = simulated salivary fluids, HS = human saliva). * significant difference (ANOVA and Holm-Sidak's multiple comparisons test, $p < 0.05$) ***** significant difference (ANOVA and Holm-Sidak's multiple comparisons test, $p < 0.0001$).

5.4.2.3 Surface Tension

In our previous research, no significant difference was observed between US and SS human saliva (Chapter 3) [202]. In the present work, no significant differences were observed between human saliva and SSFs for either stimulation state (ANOVA and Holm-Sidak's multiple comparisons test), (Figure 5.17).

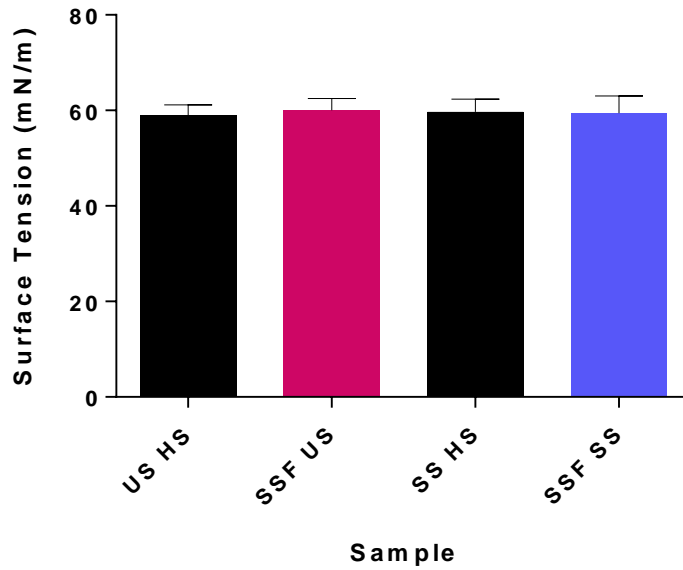


Figure 5.17: The surface tension of unstimulated and stimulated human saliva and simulated salivary fluids. Data represents mean +/- S.D. N = 30 for human saliva, triplicates. N = 5 for SSFs, triplicates. (US = unstimulated saliva, SS = stimulated saliva, SSF = simulated salivary fluids, HS = human saliva).

5.4.2.4 Viscosity

Human saliva demonstrated a shear thinning pattern with unstimulated saliva showing significantly higher viscosity at every shear rate as described in Chapter 3. The viscosity of human saliva was compared to simulated salivary fluids (Figure 5.18). SSFs also demonstrated a shear thinning rheological profile similar to that of human saliva. The amount of xanthan in both SSFs was constant since minor increases or decreases in xanthan concentration resulted in SSF viscosity outside of the standard deviation of human saliva, indicated by error bars on the graph, in either the high or very low shear rate regions.

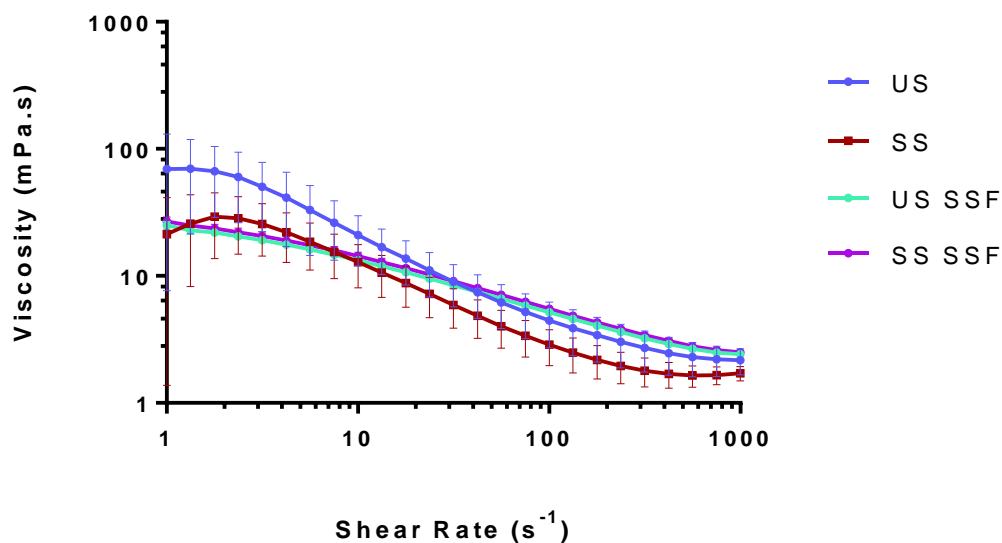


Figure 5.18: The viscosity of unstimulated and stimulated human saliva and simulated salivary fluids. Data represents mean +/- S.D. N = 30 for human saliva, triplicates. N = 5 for SSFs, triplicates. (US = unstimulated saliva, SS = stimulated saliva, SSF = simulated salivary fluid).

5.5 Discussion

In our previous research (Chapter 3) [202], we identified significant differences between human US and SS for salivary characteristics likely to affect dissolution. Many SSFs are available including over 60 in literature [205], plus several clinical formulations for treatment of xerostomia [204]. Broadly, these can be classified into 3 main groups, as discussed in Chapter 3. Previously, we took one example from each group and characterised these under the same conditions as human saliva to allow for a direct comparison. Unfortunately, none were found to be suitable to represent human saliva based on key characteristics for dissolution (Chapter 3). This is likely to be because they were developed for other reasons e.g. to relieve xerostomia, in which case the film forming properties are very important, or for dental applications whereby characteristics relevant to dissolution are perhaps less important. Additionally, none of the SSFs available were found to represent the two salivary states (US and SS) which have significantly different parameters. In this work, we developed SSFs representing both the US and SS states based on characteristics relevant to dissolution testing.

SSF development began with the characterisation of Glandosane[®] made up from its component parts, and of phosphate buffered saline plus CMC, both with varying concentrations of CMC. This is because it had previously not been possible to reliably characterise Glandosane[®] due to it being a spray, propelled by CO₂ under high shear from a pressurised container. Despite this, CMC-containing SSFs represent one of the three main categories of SSF and therefore should be considered in more detail.

Interesting trends were observed when Glandosane[®] made from its component parts was compared to PBS/CMC containing solutions, both with varying CMC concentrations. The pH of PBS did not change, and the buffer capacity changed only a little, regardless of the CMC concentration. However, for Glandosane[®] the pH and buffer capacity demonstrated a profound increase as the concentration of CMC increased.

An explanation for this could be because PBS has a greater buffer capacity than Glandosane[®] at all concentrations, as seen by Figure 5.2 Therefore, the addition of CMC (as the sodium salt, carboxymethylcellulose sodium) was well buffered in PBS and thus did not change the pH. However in Glandosane[®], the buffer capacity is less and the addition of CMC sodium increased the pH. Carboxymethylcellulose sodium contains sodium ions to neutralise the charge of the ionised carboxylic acid groups (COO⁻Na⁺). In solution this can dissociate to become COO⁻ and Na⁺. The pH of the solution may increase as the COO⁻ groups accept a proton, and as the Na⁺ groups form NaOH. The buffer capacity of both types of solutions increased as CMC sodium concentration increased, possibly due to the increase in ionisable species present.

PBS/CMC solutions were found to be more suitable than Glandosane[®] solutions to represent human saliva for pH. Little difference was observed between the two in their suitability to represent human saliva based on buffer capacity as neither was found to be a close match to human values. CMC was not found to alter surface tension considerably with similar results from both PBS and Glandosane[®] containing CMC solutions. This suggests that the CMC is not a surface active molecule. PBS solutions demonstrated a slightly closer surface tension to human salivary values. Crucially, CMC did not demonstrate sufficient shear thinning to represent human

saliva, and therefore further work with CMC-containing solutions was ceased, and the search for a more suitable viscosity modifier began.

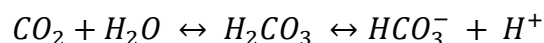
PBS gave more promising results than Glandosane® made up from its component parts, based on pH and surface tension, and no clear preference between the two was observed for buffer capacity and viscosity. Therefore the analysis of other viscosity modifiers took place in PBS.

Acacia, bovine submandibular mucin and xanthan were investigated since these have been used as viscosity modifiers in other SSF formulations [204, 240, 243]. Unfortunately, the rheological profiles of acacia and BSM demonstrated Newtonian behaviour under our conditions and thus were not found to be suitable. However, only one batch of BSM was analysed at two concentrations, in triplicate, in this study due to the high costs associated with working with *ex vivo* products. Ideally, further batches should be evaluated to fully confirm this rheological behaviour.

Xanthan gum showed promising results, demonstrating clear shear thinning similar to the extent observed for human saliva, and was therefore selected for further research as it was the most suitable candidate. Xanthan gum is a naturally occurring, biodegradable, anionic polysaccharide containing glucose, mannose, potassium glucuronate, acetate and pyruvate with a molecular weight of 2 – 20 MDa [269]. It is known to have non-Newtonian, shear thinning rheology. This is thought to be due to changes in conformational status of the polymer due to shear flow. In aqueous solution, xanthan gum may be regarded as highly extended worm like chains interacting by non-covalent association, such as hydrogen bonding, to develop a weak gel network. In times of shear flow, disentanglement of the polymer chains occurs, accompanied by alignment of the chains in the direction of flow, leading to a lower viscosity [270, 271]. Additionally, in preliminary testing, shear thinning was found to be reversible with xanthan gum solutions exhibiting complete and rapid recovery.

Our attention then focussed on the selection of a suitable buffer. Phosphate buffered saline contains higher concentrations of sodium chloride than those present in human saliva [168, 223] and is thus not the most suitable choice of buffer solution.

Bicarbonate is one of the major buffers present in saliva, and is the most prevalent buffer in stimulated saliva. However loss of carbon dioxide from the buffer solution results in changes in pH unless measures are taken to prevent this. This is due to the equilibria shown in equation 5.1.



Equation 5.1: Bicarbonate buffer system equation

In order to prevent this escape of CO₂ from the system, the system can be sealed. However, this would mean not only that the buffer should be made in a sealed environment and kept in a sealed container, but dissolution vessels would also need to be sealed. A certain amount of CO₂ could escape into any airspace in the vessel, therefore ideally, the vessel should be hermetically sealed with no airspace in the vessel, or air removed to create a vacuum. An alternative approach is to use a pH stat. This is a system in which CO₂ is sparged into the system in order to maintain a pre-determined pH, and can be automated or controlled manually [272-274]. Due to the complexity of working with bicarbonate buffers, phosphate buffers were selected for further investigation instead.

Two types of phosphate buffer were compared for their buffer capacity: USP and BP buffers. Interestingly, both were found to have a far greater buffer capacity than human saliva, with BP buffers being approximately ten times, and USP buffers being approximately four times the buffer capacity of human saliva. To our knowledge, this finding has not been reported previously. This is highly impactful, since a number of research groups have used phosphate buffers without dilution to represent human saliva [10, 11, 15, 17, 75, 79, 118-120, 122], perhaps leading to inaccurate dissolution profiles not representative of *in vivo* dissolution. BP buffers were selected for further research for reasons detailed in the results section. However, these were diluted with deionised water such that the buffer capacity reduced to values in line with human salivary values.

We have now identified that BP buffers, diluted with deionised water provide suitable pH and buffer capacity to represent both the unstimulated and stimulated

human salivary states. We had previously found that addition of 0.1 % xanthan gum yielded a rheological profile reasonably similar to human saliva. However, a steeper gradient of this profile would allow us to tailor this to both stimulation states. According to literature [269, 270], the viscosity of xanthan solutions depends on the pH and concentration of salts. Attempts were made to alter the gradient of the SSF viscosity profile to better match that of human saliva by altering the electrolyte concentration and pH using different BP and USP buffers. However these were unsuccessful. Thus the concentration of xanthan gum remained constant for both US and SS states. This was because minor adjustments in xanthan concentration resulted in SSF viscosity values outside of the standard deviation range of human saliva in either the high or very low shear rate regions.

The surface tension of diluted BP buffers with 0.1 % xanthan was found to be elevated compared to human saliva. Thus Tween 20[®] was added, at a concentration of 0.01 mM. This is well below the critical micelle concentration (0.06 mM) and achieved the desired reduction in surface tension to values representing human saliva. Since the surface tension of human saliva was found to remain constant irrespective of stimulation state in Chapter 3, the same amount of Tween 20[®] was added to both US and SS SSFs. Tween 20[®] is a non-ionic surfactant composed of polyethylene glycol sorbitan monolaurate. It has a molecular weight of approximately 1228 Da and takes the form of a viscous liquid [275]. Consequently, after the addition of Tween 20[®], a minor reduction in the concentration of xanthan was made, with 0.08 % xanthan gum found to be most suitable to represent human saliva rheology.

Five batches of the final composition of developed US and SS SSF were made, containing diluted BP buffers with the addition of xanthan gum and Tween 20[®]. These were characterised for key parameters likely to affect dissolution: pH, buffer capacity, viscosity and surface tension. These SSFs were found to have no significant difference to human saliva for any parameter for US and SS respectively. This confirms their suitability to represent human saliva in dissolution testing, and is the first time whereby SSFs representing both US and SS are proposed.

5.6 Conclusions

To address the lack of appropriate dissolution media for evaluation of taste masked formulations, two simulated salivary fluids were developed. Previous research demonstrated significant differences between unstimulated and stimulated human saliva. In this work, for the first time, SSFs representing human saliva in both the US and SS states are proposed. No significant difference was observed between the SSF and human saliva for each stimulation state. This confirms the potential of these SSFs to represent human saliva in dissolution testing.

Chapter 6: Dissolution methodology of model API in human saliva and novel simulated salivary fluids

6.1 Introduction

In previous research, we identified significant differences between US and SS human salivary characteristics. Due to the lack of biorelevance of currently available SSFs and the absence of SSFs representing both stimulation states independently, we developed novel simulated salivary fluids representing both unstimulated and stimulated human saliva. These were characterised under the same conditions as human saliva and were found to be suitable to represent human saliva based on parameters relevant to drug dissolution.

However, it is important to assess how well dissolution of drugs and dosage forms in the developed, novel SSFs corresponds to the same drugs and dosage forms in human saliva. Only then can one truly understand the suitability of the developed novel media to represent human saliva in dissolution testing.

Many dissolution tests have been carried out in simulated salivary fluids as discussed in Chapter 1, section 1.7.4. However, the majority of these works did not use biorelevant apparatus, with a suitably low media volume to represent human saliva. Additionally, we have already discussed the limitations of current media choices, highlighting the inadequacy of current dissolution methodologies. Furthermore, there are no clear pharmacopoeial recommendations for dissolution tests representing the oral cavity.

We therefore designed and employed a simple, biorelevant dissolution methodology in the assessment of drug and dosage form dissolution in both human and simulated saliva. To our knowledge, this is a first work in which human saliva itself has been used as a dissolution media, since all other researchers employed simulated salivary fluids, as discussed in Chapter 1. It is therefore also a first work in which dissolution in human saliva is directly compared with simulated salivary fluids, in both the unstimulated and stimulated states.

6.2 Aims

The aims of this Chapter are:

- To evaluate the dissolution of a model API in human saliva in both the US and SS states using a biorelevant methodology
- To evaluate dissolution of the same model API in the novel, developed SSFs representing the US and SS states
- To evaluate the suitability of the novel, developed SSFs by comparison of dissolution performance with human saliva

6.3 Methods

Materials

Sildenafil citrate was used as a model API due to its bitter characteristic. Sildenafil citrate powder and pellets were kindly manufactured and donated by Pfizer® (Sandwich, UK). Uncoated sildenafil citrate pellets consisted of sildenafil citrate, microcrystalline cellulose and polyplasdone in the ratio 60 : 30 : 10. The pellets had a size range of 180 – 425 µm and a sphericity value of > 90 % according to in house testing at Pfizer®.

The pellets were manufactured using Glatt® controlled pelletisation system (CPS) technology. During this process, powdered API, microcrystalline cellulose and polyplasdone are loaded into the CPS chamber by spraying in at the side. Then rotation of the base plate rotor begins to blend the powders. Water is then sprayed from the central rotating spray rotor nozzle. The droplets travel in a horizontal direction into the wall of powder bed created by rotation of the angled base plate (45 degree angle) and baffles direct particles back into the central zone. Once the pellets are formed and the process is complete, they are subsequently removed from the CPS chamber and dried using either a tray drier or fluid bed drier system.

A taste masking coating can then be added to the particles using a fluid bed coating process - this is a separate machine and not also a function of the Glatt® CPS system.

Methods

Some methods for this Chapter are described in Chapter 2 - specifically, refer to section 2.8 for statistical analysis and 2.9 for analytical method development, sample preparation development and final analytical methodology for sildenafil citrate.

With the exception of Figure 6.7 and 6.8 whereby $N = 3$, all dissolution tests were performed such that $N = 5$ (five separate dissolution tests) with results analysed by HPLC in triplicate. Human saliva was obtained from a single volunteer (the investigator) according to the methods detailed in Chapter 2, section 2.4.1. Composition of novel SSFs is detailed in Chapter 5, section 5.3.1.7, Table 5.4.

Dissolution methodology used is detailed below.

Development of dissolution methodology

In the literature, as discussed in Chapter 1, section 1.7.4, various apparatus have been employed to represent the oral cavity; however none were found to accurately represent the volume of saliva available in the oral cavity. Examples included use of 900 mL of water in a paddle dissolution apparatus [51], a beaker of 50 mL of electrolyte solution as an SSF [23], a beaker of 20 mL phosphate buffer [22], and beakers with 5 mL phosphate buffer [31, 58]. Some perhaps more biorelevant attempts included placing a dosage form into a 10 mL syringe with 10 mL of water and either inverting or revolving the syringe by hand for 30 seconds before analysis of the concentration of API [34, 35, 132]. All of these methods used far greater volumes than the volume of saliva present in the oral cavity and were not appropriate.

An early-stage, simple biorelevant dissolution methodology was developed. A mid-range dose of the API (sildenafil citrate, SC) was selected for dissolution studies, which was 50 mg [204]. In one study, the volume of saliva in the oral cavity available for dissolution was found to be a mean of 1.19 mL and 0.96 mL for males and females respectively before swallowing, reducing to a mean value of 0.82 and 0.60 mL after swallowing for males and females respectively [93]. In another study, saliva volume was determined in 128 healthy young adults of mixed genders and was found to have

a mean value and standard deviation of 0.46 +/- 0.31 mL [91]. Therefore a value of 1 mL of saliva as a dissolution medium was deemed appropriate. When 50 mg SC was placed into 1 mL of human saliva in a glass vial, this resulted in a wet mass of API with no distinct liquid phase from which to take samples at different time points. This simple approach was thus not suitable.

It was therefore decided to perform multiple small scale dissolution tests inside 1.5 mL Waters® glass HPLC vials placed in a water bath, held by a plastic rack within the water bath, on a magnetic stirrer at 37 °C, using a biorelevant volume of media i.e. 50 mg API in 1 mL media, which equates to 10 mg API in 200 µL media. The stirring speed was set such that adequate mixing of the contents of the vial was observed visually, without the formation of mounding of particles or vortexes of the liquid phase. Due to the inability to sample from the liquid phase when this ratio of API/media is used as discussed above, dissolution was carried out in several individual vials, with each vial allowing dissolution to proceed for a set time. At the set time point, the entire contents of the dissolution vial was transferred to a filtered centrifuge tube and centrifuged for 1 minute at 13,000 rpm. This separated the undissolved API, which was retained in the filter from the dissolved API in the filtrate. The filtrate was then treated and analysed by HPLC to determine the API concentration at each time point, as described in Chapter 2, section 2.9.

A limitation of our chosen methodology is that it does not represent the flow of saliva and the removal of particles by swallowing. Two research groups [37, 134] employed a mini column apparatus whereby the sample was placed into a column with phosphate buffer passed through the column and dissolution assessed over time. However, this method does not accurately represent swallowing where the majority of particles and a large proportion of the volume of saliva would be removed from the oral cavity in the first few seconds by swallowing. Another option is to use a USP 4 apparatus, which is a flow through cell with similar principle to the mini column apparatus. This was available to us; however the minimum flow rate achievable was 2 mL/min which is higher than the stimulated flow rate as seen in Chapter 3. This system, as well as the mini column systems, requires the cell to be wetted with dissolution media, and the system to be primed before use by passing media through

the cell. With a highly soluble drug, this priming sequence may allow substantial dissolution and the test results could be invalid. If these “flow-through” methods are used in future, this disadvantage must be taken into account in the experimental design.

Additionally, a limitation of the analytical methodology employed in the analysis of human saliva dissolution samples was observed. The guard column required regular changing, after just a few months of use. Despite employing protein precipitation and liquid-liquid extraction in the sample treatment, over time the peaks became broader and/or split. This was resolved by changing the guard column indicating an accumulation of contaminants over time. Further investigation into the sample treatment is required to overcome this.

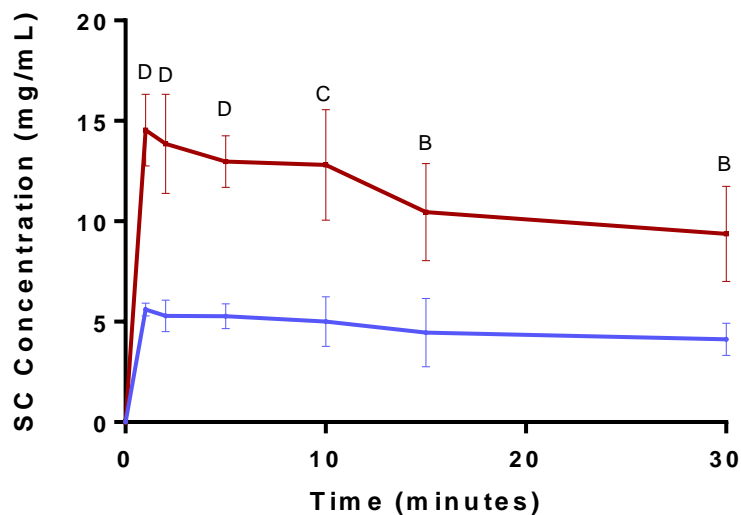
Evaluation of the effect of varying pH and buffer capacity on dissolution

The pH was varied within the range of human saliva by 0.4 pH units above and below the usual value for each type of SSF by the addition of citric acid or sodium hydroxide to the SSF. The value of 0.4 units was chosen since this represents the difference in mean pH between human US and SS.

The buffer capacity was varied by doubling or halving the amount of the BP “concentrated” buffers used in the manufacture of each SSF (Table 5.4). This yielded “high, medium and low” buffer capacity SSFs likely to be within the maximum range of buffer capacity values observed in human saliva.

6.4 Results

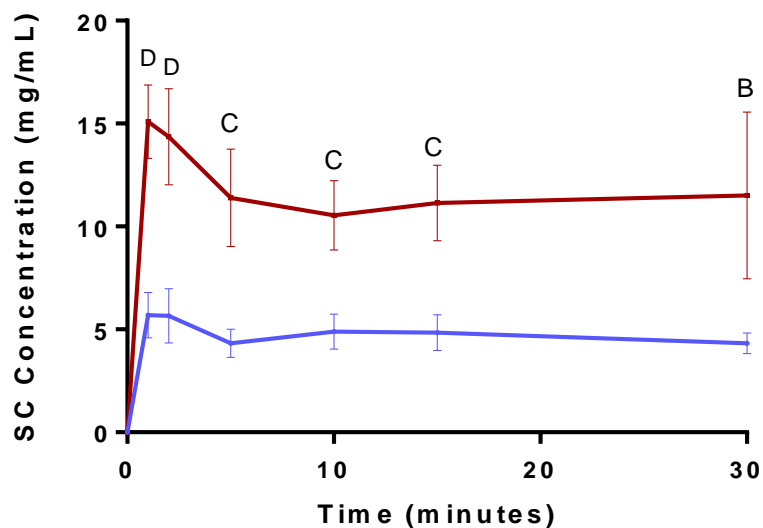
Initially, the dissolution of sildenafil citrate powder, a model bitter API, was assessed in human saliva. The rationale for selection of sildenafil citrate as a model API is discussed later. The dissolution of sildenafil citrate (SC) powder was evaluated in unstimulated and stimulated human saliva. A significant difference in SC dissolution in unstimulated saliva compared to stimulated saliva was observed at every time point as seen in Figure 6.1 (unpaired t-test, $p < 0.01$).



— SC powder dissolution in US — SC powder dissolution in SS

Figure 6.1: Dissolution of sildenafil citrate (SC) powder in human saliva. Data represents mean \pm S.D. N = 5, triplicate. (US = unstimulated saliva, SS = stimulated saliva). Significant difference between US and SS at every time point, unpaired t-test, B: $p < 0.01$, C: $p < 0.001$, D: $p < 0.0001$.

The powdered API has to be manufactured into a dosage form before administration. In our case, the dosage form we are evaluating is pellets of sildenafil citrate. The dissolution of sildenafil citrate (SC) pellets was evaluated in unstimulated and stimulated human saliva. A significant difference in SC dissolution in unstimulated saliva compared to stimulated saliva was observed at every time point as seen in Figure 6.2 (unpaired t-test, $p < 0.01$).



— SC pellets dissolution in US — SC pellets dissolution in SS

Figure 6.2: Dissolution of sildenafil citrate (SC) pellets in human saliva. Data represents mean \pm S.D. N = 5, triplicate. (US = unstimulated saliva, SS = stimulated saliva). Significant difference between US and SS at every time point, unpaired t-test, B: $p < 0.01$, C: $p < 0.001$, D: $p < 0.0001$.

The above two Figures were overlaid to allow the reader to observe the similarity between the dissolution profiles of SC powder and pellets (Figure 6.3). No significant difference was observed between powder and pellets within each stimulation state (ANOVA and Holm-Sidak's multiple comparisons test). Further work therefore continued with the pellets as a formulation.

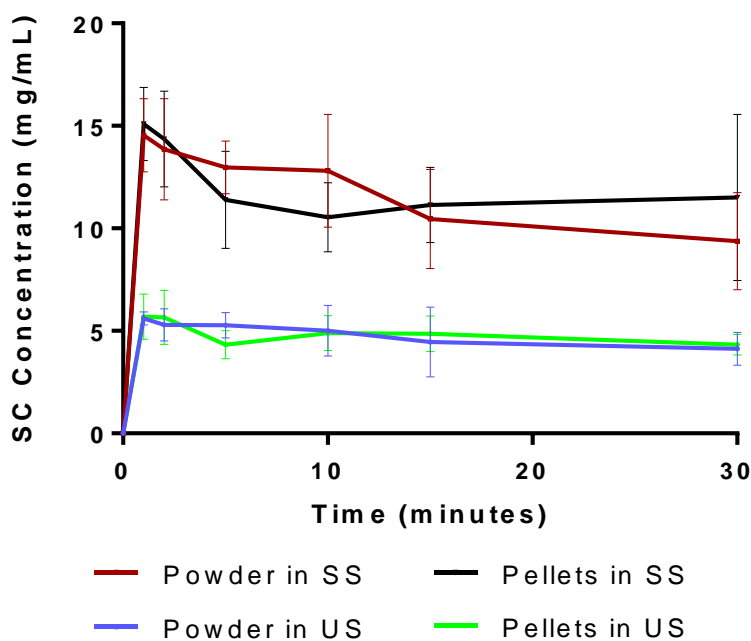


Figure 6.3: Dissolution of sildenafil citrate (SC) powder compared to pellets in human saliva. Data represents mean +/- S.D. N = 5, triplicate. (US = unstimulated saliva, SS = stimulated saliva).

The dissolution of SC pellets was then investigated in the developed, novel SSFs from Chapter 5 representing both the unstimulated and stimulated states as shown in Figure 6.4. No significant difference was observed in dissolution of pellets in unstimulated SSF compared to stimulated SSF (unpaired t-test).

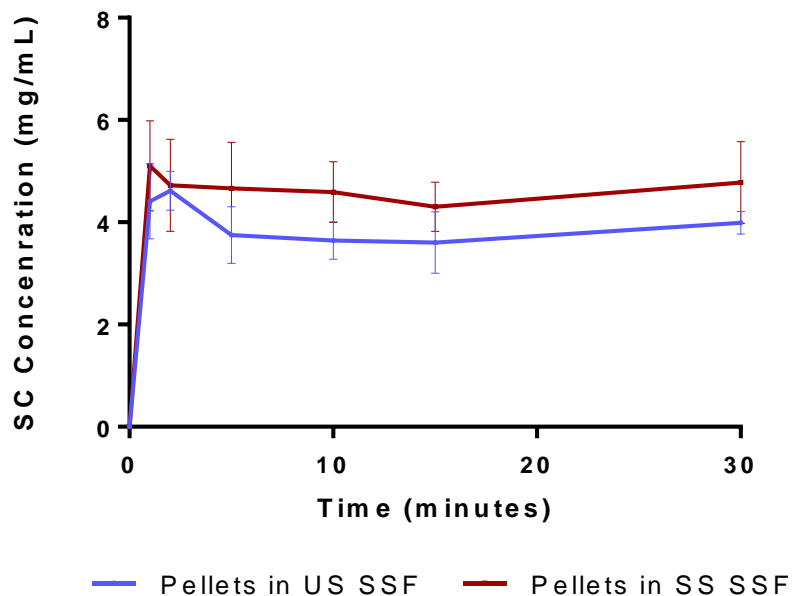


Figure 6.4: Dissolution of sildenafil citrate (SC) pellets in simulated salivary fluids. Data represents mean \pm S.D. N = 5, triplicate. (US = unstimulated saliva, SS = stimulated saliva, SSF = simulated salivary fluid).

Figure 6.4 was then combined with Figure 6.2 to allow the reader to observe the similarity between the dissolution profiles of SC pellets in human saliva and SC pellets in the developed, novel SSFs (Figure 6.5). ANOVA and Holm-Sidak's multiple comparisons test was carried out which showed no significant difference between human US and SSF US, confirming the suitability of the developed novel SSF US to represent human US in dissolution testing, at least for sildenafil. However, a significant difference was observed at every time point between human SS and SSF SS ($p < 0.001$), indicating the developed novel SSF SS is not suitable to represent human SS, at least for sildenafil. No significant difference was observed between SSF SS and either SSF US or human US, thus the developed, novel SSF SS is more similar to human US.

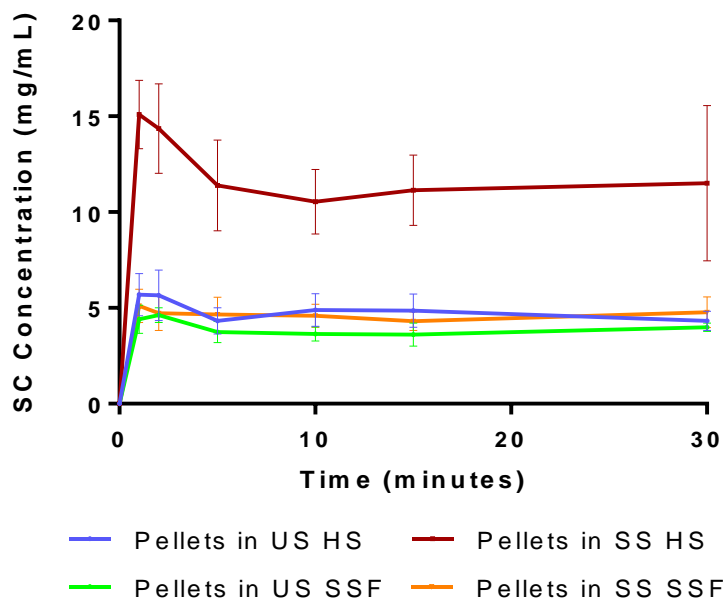


Figure 6.5: Dissolution of sildenafil citrate (SC) pellets in human saliva and simulated salivary fluids. Data represents mean \pm S.D. N = 5, triplicate. (US = unstimulated saliva, SS = stimulated saliva, HS = Human Saliva, SSF = simulated salivary fluid). SS human saliva significantly different to all other dissolution profiles at every time point (ANOVA and Holm-Sidak's multiple comparisons test, $p < 0.001$).

Since the developed, novel SSF stimulated saliva (SSF SS) was found not to be suitable to represent human SS, we investigated which parameters were most influential on dissolution of SC pellets in SSF SS. Firstly, the effect of viscosity on dissolution was evaluated by performing dissolution tests in SSF SS with and without the addition of xanthan gum, as seen in Figure 6.6. No significant difference was observed between dissolution in SSF SS with vs. without xanthan gum, at four of the six time points. Only the samples taken at 1 minute and 10 minutes were found to be significantly different (unpaired t-test, $p < 0.05$).

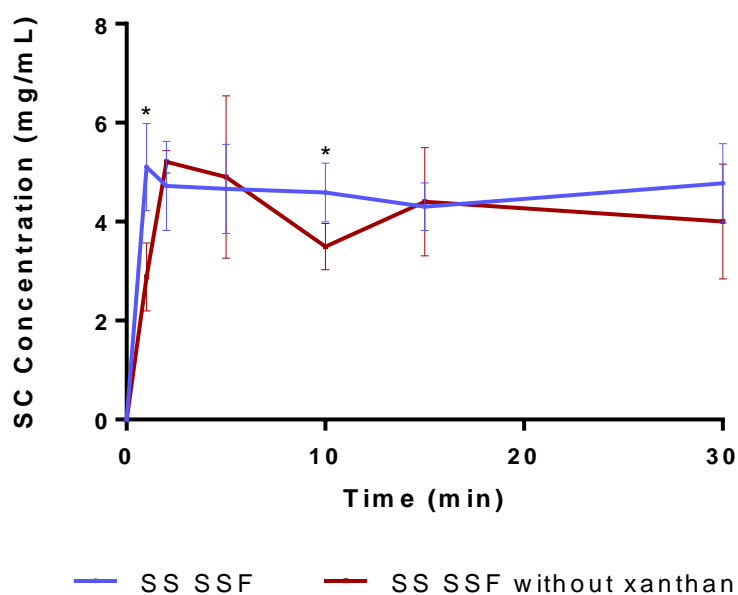


Figure 6.6: Dissolution of sildenafil citrate (SC) pellets in SSF SS with and without xanthan. Data represents mean \pm S.D. N = 5, triplicate. (SS = stimulated saliva, SSF = simulated salivary fluid). * significant difference observed between profiles at time points 1 and 10 minutes only (unpaired t-test, $p < 0.05$).

The effect of pH on dissolution of SC pellets was investigated. pH was varied within the range of human saliva and the effect of changing pH was evaluated in both US and SS SSFs. In Chapter 3, human US was found to have a mean pH of 7.0, and human SS of 7.4 to one decimal place. The developed SSFs were thus designed to have a pH of 7.0 and 7.4 for US and SS SSF respectively, whereby the difference between the two is 0.4 pH units. For each SSF, the pH was raised and lowered by 0.4 pH units compared to the usual value by the addition of citric acid or sodium hydroxide. The effect of this change in pH on the dissolution of SC pellets was determined. Although some significant differences were observed as shown in Figure 6.7 (ANOVA and Holm-Sidak's multiple comparisons test, $p < 0.05$), no clear trend was apparent and the effect of pH within this narrow range on dissolution remains unclear.

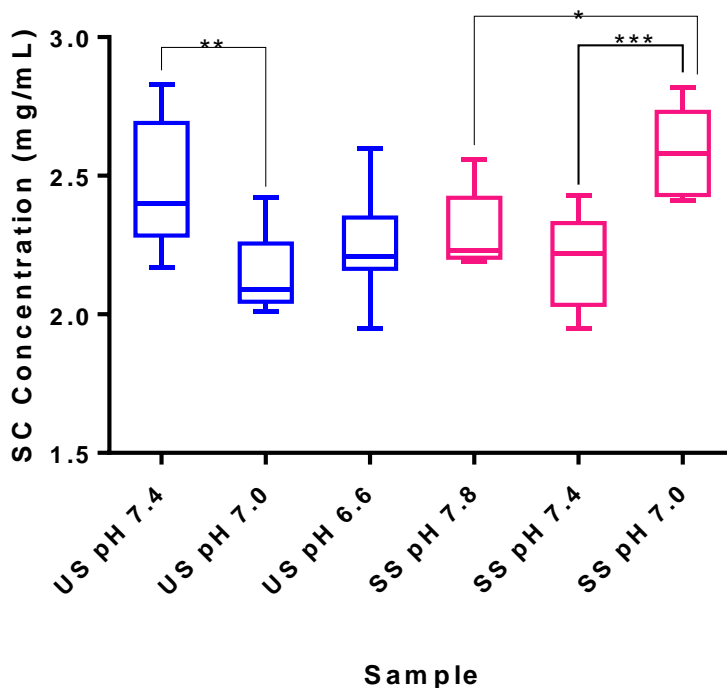


Figure 6.7: Dissolution of sildenafil citrate (SC) pellets in simulated salivary fluids of varying pH. Box represents median value, 25th and 75th percentile. Whiskers represent maximum and minimum values. Data represents samples taken after 30 minutes of dissolution. N = 3, triplicate. (US = unstimulated saliva, SS = stimulated saliva). * significant difference (ANOVA and Holm-Sidak's multiple comparisons test, $p < 0.05$), ** significant difference (ANOVA and Holm-Sidak's multiple comparisons test, $p < 0.01$), *** significant difference (ANOVA and Holm-Sidak's multiple comparisons test, $p < 0.001$).

The effect of buffer capacity on dissolution of SC pellets was also investigated. SSFs are made using British pharmacopoeial phosphate buffers of pH 7.0 and 7.4, diluted with deionised water to reduce the buffer capacity. The amount of “concentrated” BP buffer used was doubled (high buffer capacity), and halved (low buffer capacity) as well as being tested at the usual value (medium buffer capacity) in each type of SSF. Buffer capacity was thus varied and the effect was evaluated in both US and SS SSFs. No significant difference was observed between high and medium buffer capacity, however, low buffer capacity SSF had significantly higher dissolution than high or medium, in both SSFs as seen in Figure 6.8 (ANOVA and Holm-Sidak's multiple comparisons test, $p < 0.001$).

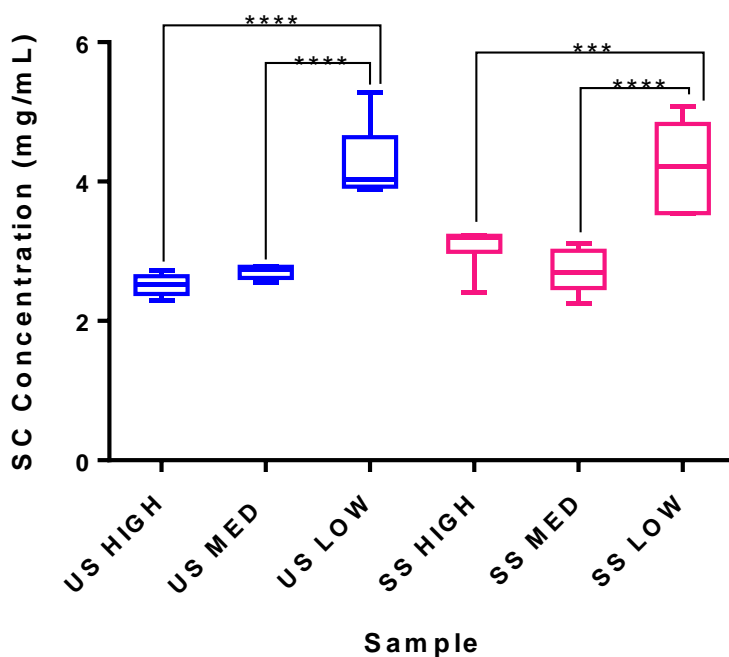


Figure 6.8: Dissolution of sildenafil citrate (SC) pellets in simulated salivary fluids of varying buffer capacity. Box represents median value, 25th and 75th percentile. Whiskers represent maximum and minimum values. Data represents samples taken after 30 minutes of dissolution. N = 3, triplicate. (US = unstimulated saliva, SS = stimulated saliva). *** significant difference (ANOVA and Holm-Sidak's multiple comparisons test, $p < 0.001$), **** significant difference (ANOVA and Holm-Sidak's multiple comparisons test, $p < 0.0001$).

6.5 Discussion

In this research, for the first time, the dissolution of a drug and dosage form was evaluated in both US and SS human saliva and in novel simulated salivary fluids representing US and SS human saliva. This was in order to understand how demonstrative the novel SSFs are in terms of dissolution performance compared to the biological fluids they represent.

Sildenafil citrate is a typically bitter substance and was selected as a model API for this reason. Taste masking of sildenafil citrate formulations has been reported in literature [51, 276, 277] and therefore dissolution testing representing the oral cavity can be employed to evaluate taste masking efficiency *in vitro*. In our case, we aimed to use reverse enteric coatings applied directly to sildenafil pellets to achieve taste

masking. These are designed not to release the API at oral pH but to allow complete release of the API at gastric pH. Dissolution testing representing the oral cavity is therefore required to ensure that release of the sildenafil citrate is minimal and below the bitterness threshold of the API in the oral cavity.

The bitterness threshold of sildenafil citrate is not documented in published literature currently. However, unpublished research [278] from the PhD of Jessica Soto at University College London investigated sildenafil citrate taste using both human taste testing panels and the brief access taste aversion (BATA) model [71] in rats. It was observed that the bitterness threshold was 1.99 mM in rats and 1.58 mM in humans. Release of sildenafil citrate in the oral cavity from taste masked microparticulates should thus be less than 1.58 mM (2.37 µg/L) for taste masking to be achieved. Additionally in Soto's research [279], sildenafil citrate bitterness was compared in both humans and rats to that of quinine and was found to be only slightly less bitter than the characteristically bitter quinine which has a bitterness threshold of between 0.873 – 1.052 mM [280-282]. When non-taste masked pellets of sildenafil citrate were evaluated for their dissolution in human saliva, the concentration of sildenafil was much greater than the bitterness threshold from Soto's work in both unstimulated and stimulated conditions. This confirms that taste masking is required for compliance.

Although sildenafil citrate is most commonly used for treatment of erectile dysfunction, it is also indicated in pulmonary hypertension [204]. For this application, it can be prescribed to the paediatric and geriatric population. These populations often cannot tolerate oral tablets and require alternative formulations, which are generally taste masked to increase compliance. We aimed to evaluate dissolution of taste masked and non-taste masked microparticulates (pellets) of this API, however the taste masked dissolution tests find their place in the future work section.

Once the dissolution methodology had been determined, dissolution testing commenced with evaluation of powdered SC and a microparticulate (pellet) formulation of SC in human saliva. Pellets can be uncoated or taste-masked reverse enteric coated pellets. These can then be incorporated into an ODT, sprinkle

formulation or suspension. It is most likely that an ODT would be the final formulation. However, we evaluated the dissolution of the pellets alone. Unfortunately, dissolution of coated pellets was not possible and is a consideration for future work. No significant difference between the powdered API and pellet formulation was found in either human US or SS, indicating the pellets formulation could be used for future dissolution testing. Drug loading of the pellets was taken into account.

Significant differences were observed in the dissolution of both powder and pellets between unstimulated and stimulated human saliva. This finding is as expected since significant differences in many characteristics relevant to dissolution – pH, buffer capacity, viscosity and flow rate were observed in Chapter 3. This confirms that these characteristics are highly likely to be influential on dissolution and that there is indeed a requirement to develop novel SSFs representing both types of human saliva.

The dissolution of SC pellets was then evaluated in the novel SSFs. Unfortunately dissolution was not found to be significantly different between the two novel SSFs, and when compared to human saliva, both SSFs were similar to human US with no significant difference between either novel SSF and US, but both being significantly different to SS. Thus the novel US SSF was found to be suitable to represent human US, but the novel SS SSF was not found to be suitable to represent human SS in dissolution testing.

It is worthy to note that a limitation of this work is that human data was obtained from the saliva of a single individual. The single individual demonstrated salivary parameters close to the mean for each parameter in Chapter 3 and was not an outlier or extreme result in the earlier trial. The mean values for this individual are stated in each corresponding Figure legend to allow for comparison to whole sample results. In most cases, with the exception of US buffer capacity (whereby the value is within 2 standard deviations of the mean), the mean values of the individual's saliva used in this Chapter are within one standard deviation of the mean for all parameters. Nevertheless, dissolution testing in human saliva should be repeated to ensure the same results are observed in multiple people's pooled saliva. Five separate

dissolution tests were carried out for each dissolution profile thus $N = 5$, and results were analysed by HPLC in triplicate. An additional limitation of this work is that only a single API was evaluated due to time restrictions. In order to make robust conclusions about the suitability of developed SSFs to represent human saliva, this work should be repeated with multiple APIs.

In Chapter 3, four factors were found to be significantly different between human US and SS – pH, buffer capacity, viscosity and flow rate. Flow rate was not a factor for the dissolution methodology we employed. However, the novel SSFs were of different pH and buffer capacity. They also had a viscosity modifier, xanthan gum. The effect of pH, buffer capacity and viscosity was therefore assessed in order to understand which factor is most influential on dissolution, and how to develop the novel SS SSF such that it is more representative of human saliva.

The effect of viscosity was assessed by performing dissolution tests in the standard novel SS SSF, and in SS SSF without the addition of xanthan gum. Dissolution of the pellets was only found to be significantly different at 2 of the 6 time points and the presence or absence of xanthan gum did not have a significant effect on dissolution across the whole profile. Thus viscosity was not thought to be a highly influential parameter on dissolution in this case.

When the pH was varied by ± 0.4 units from the usual value for each SSF, although some significant differences were observed in dissolution of pellets at different pH values, no clear trend was observed indicating how the SSFs could be modified to improve dissolution. Therefore small changes in pH were not considered to be a major contributor to dissolution performance.

The effect of varying the buffer capacity of SSFs was also evaluated for its influence on dissolution. Buffer capacity was found to have a clear effect on the dissolution of pellets, with similar results being observed for US and SS SSF. As the buffer capacity decreased, dissolution increased significantly. This was contrary to expectations as it was thought that the higher the buffer capacity, the more of the basic sildenafil could dissolve without changing the pH and hindering further dissolution, as described in Chapter 3, section 3.1. This interesting result may be caused by the combination of

using a phosphate – citrate buffer and dissolving a citrate salt of the API, meaning that the lower the buffer capacity, the less citrate is present and the less dissolution is hindered by the presence of a common ion. This theory should be evaluated in future work for example by using a different salt of the API or a different type of buffer. This is discussed in more detail in Chapter 7.

6.6 Conclusions

Dissolution testing of sildenafil citrate was carried out for the first time in both US and SS human saliva. Dissolution was found to be significantly different between US and SS human saliva as predicted by differences in key characteristics for dissolution in Chapter 3. To investigate the suitability of novel SSFs to represent human saliva, dissolution testing was also carried out in SSFs representing US and SS. The novel SSF representing US was found to be a good match to human US with no significant differences between these two, at least for sildenafil citrate. However, the novel SSF representing SS was not found to be suitable as it was significantly different to human SS. The most influential parameter on dissolution testing in this case appears to be buffer capacity. The presence of a citrate salt of the API and citric acid in buffer may have hindered dissolution. Further work is required to address these issues and develop a more biorelevant SSF SS.

Chapter 7: Conclusions and Future work

7.1 Conclusions and Implications

A number of findings presented in this PhD thesis are of great significance and have been reported here for the first time. In Chapter 1 [1], we highlighted the requirement for a biorelevant oral dissolution model, using a biorelevant media choice and volume to represent human saliva that can be coupled to gastrointestinal models for assessment of taste masked or alternative dosage forms. No such model is currently in existence.

In Chapter 3 [202], we demonstrated the inadequacy of current literature surrounding the properties of human saliva. For the first time, human saliva was characterised for pH, buffer capacity, surface tension, viscosity and flow rate in both the unstimulated and stimulated states with a sufficient number of participants to generate statistically meaningful results.

In addition, we characterised an example from the three main types of SSF and compared these under the same conditions to human saliva. This comparison had not previously been carried out based on all four parameters for each SSF. None of the SSFs were found to be suitable to represent human saliva. This implies that a novel SSF should be developed and existing ones should not be considered as biorelevant SSFs.

Our research showed significant differences in pH, buffer capacity, viscosity and flow rate between the unstimulated and stimulated salivary states, based on the same sample of participants. This has not been reported elsewhere. The implications of this finding are that dissolution media representing human saliva (SSFs) should represent both the unstimulated and stimulated salivary states. However, no SSFs are currently in existence representing both stimulation states.

The characterisation of human saliva for parameters key to dissolution [202] also provides a platform of reference for other research groups working with human saliva as a dissolution medium, or developing or selecting an SSF for use in dissolution testing.

In Chapter 4, we stimulated human saliva with Parafilm® and with an orally disintegrating tablet and assessed the conversion between stimulation states with respect to time. Although this had been performed before with unflavoured chewing gums and citric acid solutions as discussed in Chapter 4, it was a first work in which an ODT itself had been used to stimulate saliva.

With both methods of stimulation, human saliva did not remain in the stimulated state (significantly different to US) for the whole test period for all parameters. The implications of this finding reinforce the requirement to perform dissolution tests for dosage forms in the oral cavity in media representing both US and SS human saliva.

As mentioned previously, no SSFs are in existence representing both stimulation states. Therefore in Chapter 5, for the first time, SSFs representing both unstimulated and stimulated human saliva were developed. These were characterised under the same conditions as human saliva for key parameters relevant to dissolution and found not to show any significant differences between the novel SSF and human saliva for each parameter, in each stimulation state. This implies their suitability to represent human saliva in dissolution testing based on key parameters.

In Chapter 6, dissolution testing was carried out in US and SS human saliva and significant differences were seen in dissolution between the two stimulation states. This further reinforces that dissolution testing should be carried out in SSFs representing both stimulation states. To the best of our knowledge, no dissolution tests have been performed in human saliva, particularly in both the stimulation states.

Dissolution testing of the same formulation was also carried out in the developed, novel SSFs. The novel SSF US was found to be suitable to represent human saliva with no significant difference in dissolution between the SSF US and human US. However, significant differences were observed between SSF SS and human SS, thus SSF SS was not found to be suitable. The implications of this are that whilst further dissolution testing can now be carried out in SSF US, further work is necessary to improve the biorelevance of SSF SS before it can be proposed as suitable to represent human SS.

An additional interesting finding involved the discovery that BP and USP phosphate buffers have approximately 10 and 4 times the buffer capacity of human saliva as described in Chapter 5. This implies that other research groups should think carefully before simply choosing a compendial phosphate buffer to represent human saliva, and should consider a more biorelevant alternative. Many research groups have used compendial buffers and may have overestimated drug dissolution compared to that in human saliva.

Finally, we also found that saliva as a matrix for dissolution required meticulous sample treatment prior to HPLC analysis with both protein precipitation and liquid-liquid extraction. However this was not sufficient and the guard column became contaminated after a few months of use. This has not been reported before by other research groups using similar extraction methods for saliva. This implies that human saliva is a particularly complex matrix and additional steps may be required in the sample treatment process. It is surprising that saliva appears to be a more challenging matrix than plasma, which should be taken into consideration when future analytical methods are being developed for determination of drugs in saliva by our and other research groups.

When considering the original aims of the PhD (Chapter 1), we can conclude that our first aim – the selection or development of biorelevant simulated salivary fluid(s) for use as dissolution media - has largely been met. We have developed biorelevant media representing the two stimulation states of human saliva and found them to compare well with human saliva based on key characteristics relevant to dissolution. However, dissolution experiments whereby these artificial media were directly compared to human saliva has revealed opportunities for future development.

Our second aim - the development of a biorelevant dissolution methodology representing the oral cavity for the *in vitro* assessment of taste masking efficiency - has also partially been met. We have a very early stage model encompassing (aside from media parameters) biorelevant volume, temperature and residence time. However, further developmental work may be required to improve the model.

7.2 Future Work

7.2.1 Short term

In Chapter 6, the dissolution of sildenafil citrate powder and pellets was assessed in human saliva. Although each dissolution test was performed five times, this was from the saliva of a single individual. Therefore all dissolution testing in human saliva should be performed in the saliva of several other people, or in pooled saliva from a number of volunteers to ensure that the same trend is observed.

The novel SSF SS was found not to be representative of human saliva, at least for sildenafil citrate. Repeating the human saliva experiments in pooled saliva from multiple volunteers may affect this. However, evaluation of the effect of pH and buffer capacity of the SSF on dissolution was repeated only three times and should be repeated at least two more times such that $N = 5$ for more meaningful statistical comparisons. This will help to confirm what steps to take next.

If the same trend in results is still observed when $N = 5$ as above, future work should focus on improving the biorelevance of the SSF SS. In Chapter 6, a lower buffer capacity was found to result in greater SC dissolution, possibly due to the presence of less common ion (citrate). To confirm if this theory is true, the same experiment could be performed using a different salt of the API, sildenafil. In addition, the phosphate – citrate buffer could be changed to a simple phosphate buffer without the citrate component. Alternatively, a different API could be evaluated instead.

Unfortunately, it was only possible to perform dissolution testing using a non-taste masked pellet formulation in this research. It is highly important to evaluate the taste masked, reverse enteric coated formulation in addition, since the dissolution methodology was developed to support *in vitro* determination of taste masking efficiency by quantifying drug release from taste masked formulations in simulated salivary fluids. Where a taste masked formulation is evaluated, drug release is anticipated to be minimal in the oral cavity and the limit of detection may need to be reviewed. Analysis of very low concentrations may require the use of mass spectroscopy since this is a more sensitive technique than HPLC-UV.

The bitterness threshold of each API should be determined experimentally *in vivo*, if unknown. This result should be related back to the amount of drug released from the taste masked microparticulates. This will ascertain how *in vitro* release compares to the bitterness threshold, in order to understand the taste of the formulation and efficiency of taste masking. *In vivo* testing could be carried out using the BATA model [279] in rodents or using human taste testing panels [69].

7.2.2 Medium term

In order to ensure the dissolution methodology is robust and consistent, multiple APIs should be investigated, and all dissolution tests performed with sildenafil citrate in both human saliva and simulated salivary fluids should be repeated with additional APIs to validate the methodology.

In addition, full validation of the HPLC methodology should be performed for each API in each media according to FDA guidance [198]. We also described in Chapter 6 a limitation of the HPLC methodology being that the guard column requires replacement after a few months of use as peaks become broader and show shoulders. This indicates a need for further development of the sample treatment procedure to improve the purity of the samples, which should also be investigated.

In Chapter 3, we characterised SSFs from the three main classes and concluded that they were unsuitable to represent human saliva based on key parameters likely to affect dissolution. For completeness, dissolution testing of at least one API should be carried out in these and compared to human saliva and the novel SSFs to confirm that they do indeed demonstrate differences in dissolution and are unsuitable to represent human saliva, and that novel biorelevant SSFs are more suitable.

In Chapter 6, the limitations of using a flow through cell (USP 4) or mini column apparatus were described, such as the need to prime the system and wet the contents of the cell before analysis. The methodology for using these systems could be investigated in more detail to understand how much effect this priming has on overall drug dissolution and whether this can be circumvented or accounted for in some way.

7.2.3 Long term

A limitation of the human clinical trial in which characteristics of human saliva were evaluated (Chapter 3) is the relatively narrow age range of the population (aged 20 – 35). The most likely recipients of taste masked, multiparticulate formulations are those with swallowing difficulties and a requirement for tailored dosage forms to meet individual needs such as the paediatric and geriatric population. All work in this thesis is based on the salivary characteristics of a healthy, young adult population and such salivary characteristics may change greatly in paediatrics or geriatrics. The trial should therefore be repeated in people of the target age range.

One of the key issues highlighted in Chapter 1 is that there are currently no models in existence that combine an oral cavity (to assess taste masking efficiency) with a full sequential gastrointestinal model (to ensure that the taste masking technique does not affect the pharmacokinetic profile or bioavailability). Therefore, once the oral cavity dissolution methodology is established and validated, with biorelevant media representative of both human saliva stimulation states, the oral dissolution model should be coupled to a gastrointestinal model. A number of options were discussed in more detail in Chapter 1, section 1.8 for combining methodologies, such as using a flow through cell and passing different media through the cell, or transferring the contents of an individual dissolution vessel representing oral dissolution into an artificial stomach duodenum (ASD) model or TNO intestinal model (TIM) gradually to simulate swallowing. Ideally this process should be automated for convenience. Any model that is developed demonstrating a full sequential dissolution process simulating the multiple compartments of the gastrointestinal tract should be fully validated using *in vitro*–*in vivo* correlation to confirm its predictability, using multiple APIs.

Once a fully validated model is developed, this could be used to evaluate the effect of different reverse enteric coating materials, thicknesses and porosities. The effect of particle sphericity on dissolution of taste masked microparticulates could also be assessed.

7.3 Final Remarks

The key aspects of this research are the comprehensive characterisation of human saliva, leading to the discovery that human saliva exists in two distinct and significantly different stimulation states. Additionally, the discovery of the unsuitability of existing SSFs to represent human saliva for dissolution testing based on key characteristics relevant to dissolution, and the lack of SSFs representing the two stimulation states. Finally, the first steps in the development and evaluation of novel SSFs representing unstimulated and stimulated human saliva.

This research provides a platform of information regarding the properties of human saliva, and the commencement of research working towards development of a more biorelevant dissolution media representing human saliva. This research may be continued to develop SSFs fully representative of human saliva in both stimulation states for dissolution testing. Should such media have proven *in vitro*–*in vivo* correlation (IVIVC) in dissolution performance, this may vastly reduce the use of expensive and ethically challenging *in vivo* taste evaluation using BATA models or subjective human taste testing panels.

In addition, the use of SSFs representing US and SS with proven IVIVC could reduce variability in media used to evaluate taste masked or alternative formulations. Such a reduction in variability of methodology would allow for comparison between different formulations or APIs in the same media. Dissolution testing in these media could provide a robust, analytical approach to taste evaluation via quantitative analysis of the concentration of API in simulated salivary fluids and may become an industry standard dissolution methodology or pharmacopoeial recommendation in future.

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