Analysis of *in vitro* digestibility of starches and microcapsules: evaluation of retention and release of folic acid in the fortification of foods

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and any editorial work, paid or unpaid, carried out by a third party is acknowledged; and ethics procedures and guidelines have been followed.

Mee-Lin Lim Chai Teo

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Publications and presentations

Most of the work reported in this thesis has previously been presented in the following papers.

**Journal publications**

**Refereed conference proceedings papers**


**Other conference presentations**

Lim Chai Teo M-L and Small D. M. (2012). Microencapsulation of folic acid using rice starch and hydrocolloids. Presented at the AFS Summer School 2012 held in Melbourne from 1st to 3rd February 2012.


Abstract

In the context of the increasing requirements for foods to be fortified with vitamins, particularly folic acid, the relative instability of these essential nutrients is a significant concern. Microencapsulation offers unrealised potential as a means to enhance retention, if the inherent challenges of this approach can be overcome. Currently there is a lack of effective ways to evaluate the release characteristics of microencapsulated materials. Accordingly the objectives of this study have been to investigate the microencapsulation of folic acid and to study the application of \textit{in vitro} digestibility analyses as a means to establish the retention and release properties of the resultant microcapsules.

Procedures for analysis of carbohydrates were validated for use in the study of digestibility and dinitrosalicylic acid reagent was used to measure reducing sugar release. This gave a reliable means of assaying degree of digestion and the results were confirmed by comparisons with HPLC analyses of component sugars. A dialysis model was adapted for evaluation as a way to analyse digestibility and the factors influencing this system were investigated. As starches are potential microencapsulation agents, the focus has been on the \textit{in vitro} digestion of starch granules. The activity of a number of \(\alpha\)-amylase preparations showed significant dependency on the presence of \(\text{CaCl}_2\) while the type of dialysis tubing used did not impart significant effects on results. In a direct comparison of \(\alpha\)-amylases, different rates of reducing sugar were observed in the dialysis model with the animal source giving highest rate followed by bacterial and finally the fungal source. The effects on surface morphology of the granules showed similar patterns of pitting, channelling and endo-corrosion followed by complete collapse of the structure.

The formulation and production of microcapsules by spray drying was investigated with focus on selected binding agents alginate (ALG) and low-methoxy pectin (LMP) in conjunction with rice starch granules. The effect of simultaneously varying the ratio and level of binding agents gave a surface plot that indicated higher folic acid retention with a decrease in LMP. As a means of strengthening the outer shell of the microcapsules, a secondary treatment with \(\text{CaCl}_2\) was applied and generally, a hardening of the microcapsule surface was observed with the environmental scanning electron microscope. The \(\text{CaCl}_2\) treatment time did not affect the folic acid loss while the ratio of binding agent
particularly the sole presence of ALG lead to a higher loss of the core material during hardening. As a compromise between core material recovery and subsequent loss during calcium treatment, a combination of 1% of 1:1 LMP:ALG was shown to give optimum core material retention. When the \textit{in vitro} digestion model was applied to the microcapsules, the release of both reducing sugars and folic acid was significantly reduced for the Ca$^{2+}$ treated microcapsules as compared to untreated controls. Morphologically, both types of samples showed some collapse of structure but a more cohesive cluster was observed from Ca$^{2+}$ treated microcapsules corresponding to enhanced retention of the core material.

These findings demonstrate the potential of the microencapsulation strategy including calcium treatment as an effective way to retain sensitive core materials. This is the first systematic study of an \textit{in vitro} digestion model as an effective means of assessing physiological release of core materials. In addition to contributing to the standardisation of \textit{in vitro} digestibility procedures, the proposed model can now be adapted and extended to evaluation of capsular release of a wide range of food systems.
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<tbody>
<tr>
<td>AACC</td>
<td>American Association of Cereal Chemists International</td>
</tr>
<tr>
<td>ALG</td>
<td>Alginate</td>
</tr>
<tr>
<td>ANZ</td>
<td>Australia New Zealand</td>
</tr>
<tr>
<td>AO</td>
<td><em>Aspergillus oryzae</em></td>
</tr>
<tr>
<td>AOAC</td>
<td>Association of Official Analytical Chemists</td>
</tr>
<tr>
<td>BL</td>
<td><em>Bacillus licheniformis</em></td>
</tr>
<tr>
<td>CD</td>
<td>Carbohydrate digestion (starch and sugar)</td>
</tr>
<tr>
<td>DFE</td>
<td>Dietary folate equivalent</td>
</tr>
<tr>
<td>DNS</td>
<td>Dinitrosalicylic acid</td>
</tr>
<tr>
<td>DP</td>
<td>Degree of polymerisation</td>
</tr>
<tr>
<td>DT</td>
<td>Dialysis tubing</td>
</tr>
<tr>
<td>dwb</td>
<td>Dry weight basis</td>
</tr>
<tr>
<td>ESEM</td>
<td>Environmental scanning electron microscope</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organisation</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration (United States of America)</td>
</tr>
<tr>
<td>FW</td>
<td>Flat width</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HPLC-RI</td>
<td>High performance liquid chromatography – Refractive index</td>
</tr>
<tr>
<td>ICC</td>
<td>International Association for Cereal Science and Technology</td>
</tr>
<tr>
<td>IFT</td>
<td>Institute of Food Technologists (Chicago)</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LMP</td>
<td>Low methoxy pectin</td>
</tr>
<tr>
<td>mM</td>
<td>Milli molar</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut off</td>
</tr>
<tr>
<td>n</td>
<td>The number of replicate analyses used in calculation of individual results</td>
</tr>
<tr>
<td>NA</td>
<td>Not applicable</td>
</tr>
<tr>
<td>NHMRC</td>
<td>National Health and Medical Research Council (Australia)</td>
</tr>
<tr>
<td>NSP</td>
<td>Non starch polysaccharide;</td>
</tr>
<tr>
<td>NTDs</td>
<td>Neural tube defects</td>
</tr>
<tr>
<td>PP</td>
<td>Porcine pancreatin</td>
</tr>
<tr>
<td>RBCs</td>
<td>Red blood cells</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended dietary allowance</td>
</tr>
<tr>
<td>RDI</td>
<td>Recommended daily intake</td>
</tr>
<tr>
<td>RDS</td>
<td>Rapidly Digested Starch;</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reversed phase high performance liquid chromatography</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RS</td>
<td>Resistant Starch</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
</tr>
<tr>
<td>s</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SD</td>
<td>Starch digestion</td>
</tr>
<tr>
<td>SDS</td>
<td>Slowly Digested Starch</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscope</td>
</tr>
<tr>
<td>TS</td>
<td>Total starch</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
</tbody>
</table>
Explanatory notes

The purpose of these notes is to briefly describe the approaches adopted during the preparation of this thesis. They relate to the spelling, calculation of reducing sugars and folic acid, units of measurement, the expression of analytical results, as well as the referencing literature sources:

1. Where alternative spellings are in common use then the British rather than the American approach has been adopted in the text. Examples include words ending with -ise rather than -ize and some technical terms.

2. For the presentation of experimental results, SI units have been used throughout the thesis.

3. Typically calculations relating to reducing sugar and folic acid were expressed on a dry weight basis unless otherwise specified. This was done to ensure that a direct comparison could be performed between results.

4. In the citation and listing of reference and information sources, the current requirements of the American Psychological Association (APA, 6th Edition) have been applied throughout.
Chapter 1

Introduction

The purpose of this chapter is to provide an outline of the research program described in this thesis on the microencapsulation of folic acid and the subsequent release of the core material investigated using an *in vitro* digestion model. This research has been developed on the basis of the following issues:

- Folates are essential to human health, facilitating the single carbon transfer reaction for the synthesis of basic constituents of the nucleic acids, DNA and RNA, which provide the genetic basis of life (Combs, 2008);

- Research over recent decades has shown that low or inadequate folate intakes may be associated with congenital malformations and development of chronic disorders including neural tube defects, megaloblastic anaemia and elevated levels of homocysteine (Boushey, Edmonds & Welshimer, 2001);

- Folates are natural sources with more than 30 different molecular forms having a structure based upon pteroic acid. Folic acid is the simplest form of the folate group of the B vitamins which is prepared synthetically and is more readily absorbed than other folates due to its simpler structure (Gregory, 2008);

- Good natural sources of folates include green vegetables, fruits, berries, liver and dietary surveys in Australia and New Zealand show that cereal grains provide approximately 30% of folate intakes;

- Folates are sensitive to oxidative degradation, pH between 4 and 6, leaching into cooking media and large losses occurs in food processing, preparation and cooking (Gregory, 2008);

- Microencapsulation is a recent and novel technology that has potential growth and significance in the food and ingredient industries and provides an approach which might enhance folic acid content, stability and controlled release in foods;

- One of the significant challenges in evaluating the effectiveness of microencapsulation strategies is in the assessment of release characteristics. One way that this might be
achieved is through the application of procedures used in the measurement of digestibility of foods, including application for glycaemic index (GI);

- The *in vivo* measurement of digestibility and GI is associated with high costs, lengthy time delays as well as a lack of precision;

- Recent studies have considered an alternative approach using *in vitro* measurement of digestibility in relation to its physiological significance. However there have been no systematic investigations of applying these measurements to the release of active agents such as folic acid and other vitamins from microcapsules using an *in vitro* digestion model;

- Currently there is a lack of standardisation of methodologies for the analysis of food digestibility by *in vitro* procedures. Various researchers have used dialysis tubing models and these offer a means of restricting the effects of the viscosity of the dialysate;

Accordingly, this research is based on the need to further investigate and standardise the *in vitro* digestion model for digestibility and then apply the findings to a study of the parameters influencing the release of folic acid from microcapsules. Various strategies have been explored for optimised formulation as well as secondary treatments of microcapsules to enhance retention as well as release characteristics.
Chapter 2

Literature review:
Human digestion, digestive enzymes, native starches and factors to consider for in vitro analysis of digestion

The purpose of this chapter is to provide background and review the relevant scientific literature on human digestion. Among the areas briefly described are digestive enzymes, native starch and starches as an enzymatic substrate as well as factors to consider for in vitro digestion and reducing sugar analytical procedures.

2.1 Explanatory notes

In reference to hydrolytic enzymes mentioned in this study and other relevant enzymes, their common names have been employed. Their respective EC numbers and other referred names are summarised in Table 2.1.
Table 2.1 Names and numbers of selected hydrolytic enzymes involved in human digestion or in the \textit{in vitro} analysis of food digestibility (NC-IUBMB, 2013)

<table>
<thead>
<tr>
<th>Common name</th>
<th>Number</th>
<th>Accepted name</th>
<th>Systematic name</th>
<th>Some alternative name(s) and other notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipase, triacylglycerol</td>
<td>EC 3.1.1.3</td>
<td>triacylglycerol lipase</td>
<td>triacylglycerol acylhydrolase</td>
<td>lipase</td>
</tr>
<tr>
<td>Pepsin</td>
<td>EC 3.4.23.1</td>
<td>pepsin A</td>
<td>not given</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>EC 3.4.21.4</td>
<td>trypsin</td>
<td>not given</td>
<td></td>
</tr>
<tr>
<td>α-Amylase</td>
<td>EC 3.2.1.1</td>
<td>α-amylase</td>
<td>4-α-D-glucan glucanohydrolase</td>
<td></td>
</tr>
<tr>
<td>β-Amylase</td>
<td>EC 3.2.1.2</td>
<td>β-amylase</td>
<td>4-α-D-glucan maltohydrolase</td>
<td></td>
</tr>
<tr>
<td>Chymosin</td>
<td>EC 3.4.23.4</td>
<td>chymosin</td>
<td>not given</td>
<td>rennin</td>
</tr>
<tr>
<td>Glucoamylase</td>
<td>EC 3.2.1.3</td>
<td>glucan 1,4-α-glucosidase</td>
<td>glucan 1,4-α-glucosidase</td>
<td>glucoamylase; amyloglucosidase; γ-amylase</td>
</tr>
<tr>
<td>α-Glucosidase</td>
<td>EC 3.2.1.20</td>
<td>α-glucosidase</td>
<td>α-D-glucoside glucohydrolase</td>
<td>maltase</td>
</tr>
<tr>
<td>Dextrinase</td>
<td>EC 3.2.1.41</td>
<td>pullulanase</td>
<td>pullulan 6-α-glucanohydrolase</td>
<td>bacterial debranching enzyme</td>
</tr>
<tr>
<td>Limit dextrinase</td>
<td>EC 3.2.1.142</td>
<td>limit dextrinase</td>
<td>dextrin 6-α-glucanohydrolase</td>
<td></td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>EC 3.4.21.1</td>
<td>chymotrypsin</td>
<td>not given</td>
<td></td>
</tr>
<tr>
<td>Invertase</td>
<td>EC 3.2.1.26</td>
<td>β-fructofuranosidase</td>
<td>β-D-fructofuranoside fructohydrolase</td>
<td>sucrase</td>
</tr>
<tr>
<td>Sucrose α-glucosidase</td>
<td>EC 3.2.1.48</td>
<td>sucrose α-glucosidase</td>
<td>sucrose-α-D-glucohydrolase</td>
<td>Active on both sucrose and isomaltose</td>
</tr>
<tr>
<td>Lactase</td>
<td>EC 3.2.1.23</td>
<td>β-galactosidase</td>
<td>β-D-galactoside galactohydrolase</td>
<td></td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>EC 3.2.1.21</td>
<td>β-glucosidase</td>
<td>β-D-glucoside glucohydrolase</td>
<td>cellobiase;</td>
</tr>
</tbody>
</table>
2.2 Human digestion

Digestion is the chemical breakdown of food into molecules which are sufficiently small so that they can be absorbed into the blood stream (Zielinski, 2012). In mammals, digestion commences in the mouth where food is broken down physically into smaller pieces through mastication and is mixed with salivary secretion (Lutkić & Votava, 1982). There are various functions of the saliva: it moistens the food and it also contains α-amylase, glycoprotein components and lingual lipase. α-Amylase initiates the breakdown of starch into fragments of various sizes with the ultimate product being the disaccharide, maltose, while lingual lipase initiates the breakdown of lipids into di- and mono-acylglycerols (also known as glycerides). The glycoprotein components of the saliva, when mixed with the food, aid in the formation of a smooth food ball mixture which is often referred to as the bolus. This is then swallowed and passes via the oesophagus to the stomach through peristaltic movement resulting from the contraction and relaxation of the oesophageal muscles.

The strong muscular walls of the stomach churn the bolus with secreted gastric juices which are composed of a mixture of hydrochloric acid (HCl), proteases (chymosin and pepsin) and mucus. The HCl creates an acidic environment that deactivates salivary α-amylase but at the same time provides an ideal acidic pH for activity of the proteases. HCl also aids in denaturing proteins and has an antimicrobial effect. Chymosin clots milk while pepsin breaks down protein. The mucus lubricates the food and also protects the lining of the stomach from any adverse effects of the HCl. Thereon, the food mixture is converted to a porridge-like mixture known as chyme that can be stored in the stomach for a period of up to five hours after which small quantities are gradually released through the pyloric sphincter, passing to the small intestine.

The small intestines are composed of three regions, the duodenum, jejunum and ileum and it is in these that most of the digestion and absorption (about 95%) is carried out (Zielinski, 2012). Digestion takes place primarily in the duodenum and the jejunum while absorption occurs particularly in the ileum. The lining of the duodenum releases hormones that thereon trigger the secretion of bile from the gall bladder, pancreatic juices from the pancreas as well as intestinal juices from the small and large intestines.
The role of bile is firstly to neutralise the acidic chyme and create an alkaline environment thereby facilitating subsequent activity of the enzymes. Bile also includes components that emulsify fats for easier digestion and absorption. Pancreatic juices contain pancreatic α-amylase (also known as pancreatin) which continues the cleavage of long chain oligosaccharides remaining after the partial hydrolysis of the starch and yielding oligosaccharides particularly maltose. Pancreatic α-amylase is 94% similar to salivary α-amylase and has multiple attack mechanisms (Alpers, 2003; Robyt & French, 1967), hence if an adequate amount of pancreatic α-amylase is secreted, digestion of starch can be completed entirely within the small intestines, independent of the action of salivary α-amylase (Smith & Morton, 2010). Following this stage, all of the digestible starch has been hydrolysed to yield approximately 19% glucose, 73% maltose as well as the remaining to malto-oligosaccharides of degree of polymerisation (DP) of up to nine (Lutkić & Votava, 1982). Dextrinase and glucoamylase (Table 2.1) then complete the hydrolysis process so that the oligosaccharides, including maltose, are converted into glucose. Intestinal juices consisting of a number of other digestive enzymes are also involved in the hydrolysis of macronutrients. Maltase, lactase and sucrase break down disaccharides maltose, lactose and sucrose into their component monosaccharides respectively. Simultaneously trypsin and chymotrypsin continue the process of protein digestion.

It is thought that the available carbohydrates are the fastest to be digested followed by proteins and fats (Zielinski, 2012). Micronutrients, consisting of the vitamins and minerals are small enough to be absorbed directly while digested macronutrients are absorbed at different speeds depending on their size. In addition, water-soluble vitamins are absorbed faster than fat-soluble vitamins. Upon absorption, water soluble nutrients pass into the blood stream through the hepatic portal vein and are conveyed to the liver for filtering and nutrient processing as well as removal of toxins. On the other hand, fat-soluble nutrients are absorbed and then pass through the lymphatic system (Collins, 2012).

Once these processes are complete, the residual chyme is low in nutrients and it is then released into the large intestines which are also composed of three main sections, the caecum, colon and rectum. The role of the caecum is simply to collect the chyme, whereas in the colon, the remaining water is absorbed and bacterial action reduces fibre bulk to give
rise to a more compact faecal residue for easy and convenient excretion. The rectum stores the stool until a sufficient quantity has accumulated prior to discharge through the anus.

![Image of the human digestive tract](image)

**Figure 2.1** The human digestive tract (Collins, 2012)

### 2.3 Digestive enzymes in carbohydrate digestion

There is a variety of hydrolytic enzymes in the animal kingdom and some of these act as catalysts in the chemical breakdown of polymeric macromolecules. Enzymes, being protein molecules, are directly affected by a variety of factors particularly temperature and pH, thereby influencing the rate of enzymatic activity. However, in general terms, enzymes from animal sources demonstrate optimal activity at a temperature of 37°C and pH values of approximately 5 (Dona, Pages, Gilbert, & Kuchel, 2010). Other factors including concentration and source may also affect the observed rate of enzymatic activity.

Enzymes may be categorised according to their mode of action where, in the depolymerisation of polysaccharides, they are either endo- or exo-acting hydrolases. Endohydrolases reflect their specificity for random cleavage of the glycosidic linkages towards the centre of the linear polymeric chains, hence resulting in breakdown of large carbohydrate molecules that are not able to diffuse into the cells, into smaller ones that ultimately will be able to traverse the cellular walls. This specific enzyme targets a specific linkage type within the chain and an example of an endo-enzyme is α-amylase (Table 2.1)
which specifically cleaves $\alpha(1\rightarrow4)$ linkages. Exo-hydrolases, on the other hand, target monosaccharide units at the terminal positions of polymeric chains, releasing a monomer or dimer from the non-reducing ends. Two enzymes exhibiting exo-activity and cleaving at the non-reducing ends of starch and oligosaccharide chains are glucoamylase and $\beta$-amylase which give glucose units and maltose units respectively (Dona, et al., 2010).

In relation to the digestion of starches, another issue is that the form of this food component is important. Thus, in plant tissues, native starch is in the granular form and during processing the granules typically lose their integrity and intact structure, by either mechanical damage or as a result of gelatinisation. Both of these types of changes result in molecular starch becoming exposed and more readily attacked enzymatically than intact granules of starch. Whether an enzyme is an endo- or exo-hydrolase, together with the type of starch, determines the physical state of the starch granule post-digestion. This can be investigated by surface analysis of the starch granule using a light microscope or with the higher magnification provided by the scanning electron microscope (SEM).

Generally, enzymatic digestion will result in a “biting appearance” of the starch granule surface in various ways pin-hole, pitting, sponge-like erosion, medium sized holes, single hole in an individual granule and surface erosion. Endo-corrosion can be seen as a result of digestion starting from the surface of the starch granule and gradually making a channel towards the centre of the structure. The presence of numerous channels will weaken the starch granule resulting in breakage and collapse. Exo-corrosion typically involves surface erosion whereby the enzyme may erode the entire surface of the starch granule (Sujka & Jamroz, 2007).

### 2.3.1 $\alpha$-Amylase (Table 2.1)

Whilst salivary and pancreatic $\alpha$-amylases have been thoroughly characterised, there is now extensive literature describing amylases from a variety of organisms (Berry, 1986; Bertoft, Manelius, & Qin, 1993b; Dona, et al., 2010; Gérard, Colonna, Buléon, & Planchot, 2001; Hoover & Zhou, 2003; Kong, Kim, Kim, & Kim, 2003; Lentle & Janssen, 2011; Robyt & French, 1967; Tester, Qi, & Karkalas, 2006). These enzymes are produced from different sources including animal and microbial origins. From studies of the latter, it has been well established that bacterial and fungal organisms typically produce distinct
types of α-amylases to effect extracellular breakdown of starch into soluble glucose and maltose units that are readily absorbed.

In addition to the role of amylases during digestion, increasingly these enzymes are exploited in a range of commercial applications. Amylases from Aspergillus oryzae (a fungal species) are used to delay and control staling in the baking industry, clarify haze from fruit juices and alcoholic beverages as well as for the production of glucose and maltose syrup products (van der Maarel, van der Veen, Uitdehaag, Leemhuis, & Dijkhuizen, 2002). α-Amylase from the bacterium Bacillus licheniformis is commercially used in laundry and dishwashing detergents (Rodriguez, Alameda, Gallegos, Requena, & López, 2006). It has recently been more commonly used in the food sector as a substitute for the enzyme from B. amyloliquefaciens for the production of glucose by enzymatic treatment where a temperature of 95-100°C is applied (van der Maarel, et al., 2002).

The rate of enzymatic hydrolysis of starch may also vary according to the source of the enzyme due to structural genetic variation and hence disparity in relative activity (Tester, et al., 2006). Planchot et al. (1995) have demonstrated that α-amylase from different sources have varying hydrolytic effects when other parameters including concentration and activity per unit mass of protein including the starch source are kept unchanged. Thus, it was found that α-amylase from A. fumigatus showed higher hydrolytic sensitivity towards native starches as compared to α-amylase from porcine pancreatin and Bacillus species. Furthermore, enzymes from the same species but from different strains also demonstrate different hydrolytic activity. A study in which 88 strains of Bacillus spp. were compared for their hydrolytic activity on corn starch was undertaken and variation in enzymatic activity was observed (Dettori-Campus, Priest, & Stark, 1992). It was also concluded that among the strains studied those of B. stearothermophilus and B. amylolyticus yielded the highest enzymatic activity.

2.4 Native starch

Starchy carbohydrates can be classified into two main sources, notably from animal sources and from plant sources. Animals store carbohydrate in the form of glycogen which has a similar structure to that of amyllopectin but has three times as many α(1→6) glucosidic linkages. Plants, on the other hand, typically contain carbohydrates in the form
of starch but also as simple sugars, and a range of other polysaccharides which resist human digestion. The latter category consists of cellulosines and other non starch polysaccharides (NSP) which include pectin, inulin, hemicelluloses and food gums (for example, guar, locust bean, gum Arabic and agar).

Plants generally store native starch in semi-crystalline, stable and highly organised granules that resist hydration and digestion. In so doing, environmental protection is conferred to their food storage from opportunist consumers (for example bacteria, fungi and animals) until it is required by the plant (Lentle & Janssen, 2011). Before animals can digest native starches, three main barriers need to be overcome, namely:

1. The protective structure of the plant tissues. This involves plant cell walls which include lignin as well as various polysaccharides and these impart the structure of the plant particularly the properties of resilience and strength;
2. The barrier at the granule surface; and
3. The obstructive molecular packing of starch polymers within the starch granule (Lentle & Janssen, 2011).

Digestibility can be increased by applying mechanical shearing which is typically seen upon grinding, resulting in disruption of the starch granules and thereby exposing starch molecules. Cooking can also increase digestibility by increasing hydration and hence digestibility.

2.5 Starch granules as a digestive substrate

Postprandial glycaemic responses from starchy foods can be affected by a series of physical and chemical parameters (Björck, 1996). These factors can be considered as a basis for understanding the effect of in vitro digestion on particular substrates and to obtain a desired effect.

2.5.1 Botanical origin

The varying structural features of starch granules from different botanical sources are known to affect both the degree of hydrolysis as well as the mechanism by which this occurs. Hence, the considerable differences in size, composition and microstructure in starch granules are believed to affect digestibility due to their source (Lentle & Janssen,
A classification based upon the intensity and hydrolysis mechanism of granule erosion as well as internal corrosion has been presented by Gallant, Mercier & Guilbot (1972). Individual plant species will exhibit distinct organisational patterns and structures of the amylose and amylopectin fractions within the starch granule thereby affecting the hydrolysis mechanism.

2.5.2 Granule size and shape

Despite the paucity of studies regarding enzymatic hydrolysis in relation to granule morphology, it has been suggested that small granules are more resistant to hydrolysis as compared to larger granules from an evaluation using an SEM instrument (Hoover & Zhou, 2003). This was hypothesised to be the result of higher amylose content in smaller smooth pea granules thereby making them less susceptible to digestion as described in an earlier report (Bertoft, Manelius, & Qin, 1993a). Moreover, this also relates to the general observation that the smaller the particle size, the greater the surface area of the substrate being exposed and hence this might result in a higher rate of digestion. Subsequent studies have appeared to provide confirmatory evidence, as long as the surface of the starch granule lacked a resistant starch layer (Ring, Gee, Whittam, Orford, & Johnson, 1988; Tester, Karkalas, & Qi, 2004). Consequently, it may be more accurate to deduce that the rate of enzymatic activity is more strongly governed by the particle size of the substrate and hence surface area rather than the concentration of enzyme being used (Kong, et al., 2003).

2.5.3 Structure of the starch granule

The structural attributes of granular starch have been extensively studied and reviewed. Starch is made up of two primary polysaccharide fractions: amylose and amylopectin. Amylose is a non-branched linear polysaccharide chain of glucose units linked by α(1→4) glycosidic linkages (Figure 2.2). Amylopectin is also composed of linear polysaccharide chains of α(1→4) linked glucose units but with the addition of branches linked by α(1→6) located at relatively regular intervals on the linear polysaccharide backbone (Figure 2.3). The cluster model of amylopectin is illustrated in Figure 2.4 with clusters of A chains carrying B chains and each amylopectin molecule having a single reducing end at the C chain (Buleon, Colonna, Planchot & Ball, 1998; Coultae, 2009). Amylose and amylopectin also differ in their size with amylose typically having a molecular weight of
0.5 million and a degree of polymerisation in the range of 1,500 to 6,000 while amylopectin is bulkier with a molecular weight of 50 to 500 million and a degree of polymerisation in the millions, depending on the plant species (Thomas and Atwell, 1999).

**Figure 2.2** The structure of a maltose molecule showing a chain of two glucose units joined by an \( \alpha-(1\rightarrow4) \) glycosidic linkage

**Figure 2.3** The structure of a glucose disaccharide (commonly referred to as iso-maltose) with an \( \alpha-(1\rightarrow6) \) glycosidic linkage
Chapter 2

Amylose

Amylopectin (adapted from Coulttate, 2009)

Figure 2.4 The representative structures of amylose and amylopectin
Polysaccharide chains shown as dark lines and individual glucose units depicted as hexagons.
2.5.3.1 The primary level of granular structure

It has been confirmed that starch granules consist of alternate layers of crystalline and amorphous layers at different order levels (Helbert, Schülein, & Henrissat, 1996; Imberty, Chanzy, Pérez, Buléon, & Tran, 1987). The first starch granule structure level has been described as a series of alternate concentric shells of crystalline and amorphous starch named as ‘growth rings’ towards an off-centred hilum (centre of the growth). These growth rings are typically 120-400nm in thickness and are believed to correspond to diurnal fluctuations in the availability of sugars from photosynthesis (Copeland, Blazek, Salman, & Tang, 2009; Gallant, Bouchet, & Baldwin, 1997; Lentle & Janssen, 2011). Growth rings have been associated with amylopectin molecules being radially orientated in the starch granule with their reducing ends towards the hilum of the starch granule (Coultate, 2009). These concentric domains involve amorphous layers high in $\alpha(1\rightarrow6)$ glycosidic linkages. Crystalline rings are primarily composed of chains having $\alpha(1\rightarrow4)$ glycosidic linkages associating with neighbouring chains and believed to form short double helices. However, little is known of how amylose molecules are arranged within the starch granules although they are assumed to be randomly distributed between the amylopectin molecules and arranged in a radial way towards the hilum as well (Coultate, 2009).

The crystalline shells are further made up of alternating amorphous and crystalline lamellae with a repeat distance of 9-11nm (Copeland, et al., 2009; Lentle & Janssen, 2011). The amorphous regions are believed to be added by plants when glucose is less readily available (Lentle & Janssen, 2011). It also appears that the shells are thinner when they are located closer to the surface due to increasing surface area to be added at a constant growth rate (Gallant, et al., 1997). The surface of the granule is comprised of a crystalline hard shell with pores connected with amorphous channels towards the hilum for “communication”. The functions of these pores are not well understood but it is believed that they may facilitate the passage of cytoplasmic fluids to the centre of the granules (Copeland, et al., 2009). In addition, the pores facilitate the leaching of amylose from a granule as a result of gelatinisation and cooking (Gallant, et al., 1997) and may also aid in the permeation of digestive enzymes and the subsequent leaching of the sugars resulting from hydrolysis (Huber & BeMiller, 2000; Oates, 1997).
2.5.3.2 The secondary level of granular structure

At the secondary level of structure, blocklets are visible by the use of SEM and transmission electron microscopy (TEM) imaging (Gallant, et al., 1997) and are believed to have been formed during the synthesis of the granule. Both the crystalline regions and the amorphous shells are composed of blocklets with the difference that the crystalline blocklets are up to 3-5 times larger in size than the amorphous blocklets. A study carried out by Gallant et al. (1972), showed SEM of residual wheat starch granules with crystalline blocklets of 80-120nm and amorphous blocklets of 25nm in diameter. The blocklets appear to be elongated and do not show any uniformity of size or shape (Gallant, et al., 1997) whereas Lentle and Janssen (2011) reported them to be ‘more or less’ spherical in shape.

The following order of starch granule structure demonstrates that the blocklets are composed of crystalline lamellae separated by amorphous layers with a repeating distance of 9-10nm thick (Copeland, et al., 2009; Jenkins, Cameron, & Donald, 1993; Smith, 2001). It is believed that these lamellae are not always straight, parallel or uniform in size and density. The crystalline lamellae are composed of rows of amylopectin chains packed into a lattice thereby conferring the crystalline characteristics. The amorphous lamellae on the other hand are composed of the side chain (branching points) of the amylopectin chains, amylose chains and amylopectin arranged in a disorderly manner. TEM cross-sectional images have also confirmed the latter observations hence indicating that the lamellae are not continuous crystalline layers (Gallant, et al., 1997). Thus, amorphous areas are present between both the crystalline lamellae and also between amylopectin side chain clusters. Longer amylopectin chains are considered to pass from the crystalline region to the amorphous regions (Tester, et al., 2004) while the inclusion of amylose-lipid complexes in the organisation of the amylopectin chains has also been reported (Blanshard, 1987).

The final level of structure shows the crystal structures of the starch polymorphs. Depending on the different packing of the amylopectin polymorphs, different x-ray diffraction patterns can be observed, as these are commonly designated as being A, B, C or V type patterns.
2.5.4 Amylose and amylopectin content

It has been reported that amylose and amylopectin comprise 98-99% of the dry weight of native starch (Copeland, et al., 2009). Their different concentrations in starch granules have been reported to have an impact on the mechanisms of erosion. In a study carried out by (Planchot, Colonna, Gallant, & Bouchet, 1995), three types of maize starch samples were digested with α-amylase from A. fumigatus and the amylose content of the maize samples was found to result in different corrosion mechanisms.

High-amylose maize showed major endo-erosion observed by trans-sectioned starch granules viewed under TEM. Minor exo-erosion was also seen in the form of rough surface, superficial scratches and large pores. The granules showed unequal degradation with the majority having little or no pitting while a few were highly degraded up to the point of collapse. On the other hand, normal and waxy (high amylopectin content) maize exhibited higher exo-erosion characteristics. Normal maize showed furrowed weakened areas on the surface e.g. pores producing external grooves and internal corrosion channels. Waxy maize was confirmed to have a similar mechanism as for normal maize and there was enlargement of the channels by progressive exo-erosion and then rapid endo-erosion.

The high amylopectin maize sample demonstrated an initial progressive exo-erosion of the surface to allow channelling and hence rapid endo-erosion. On the other hand, high amylose samples gave rise to an extensive endo-erosion of the granules with little exo-erosion. Nevertheless, degradation appears to occur in two main steps (Planchot, et al., 1995). The initial stage involves the enzymes adsorbing onto the surface of the starch granule creating a superficial porosity. Once the enzyme encounters a region which has a less organised structure, degradation takes place, imparting macroporosity with deeper grooves. Thereon, the internal structure of the granule erodes rapidly while erosion of the outer material remains relatively slow. In the case of the high-amylose starch, the enzyme exhibits the ability to penetrate through the resistant structures until endo-erosion takes place rapidly. From this it can be concluded that starch granules consist of superimposed layers of high and low crystallinity with quite different susceptibilities to enzymatic degradation.
Other studies have also confirmed that high amylose starch showed a lesser degree of degradation than waxy starch when digested with $\alpha$-amylase thus providing validation that different mechanisms take place for the same $\alpha$-amylase enzyme (Blazek & Copeland, 2010; Gallant, Mercier, & Guilbot, 1972). These reports also suggest that $\alpha$-amylase favours the degradation of amorphous rings within starch granules.

### 2.5.5 Presence of lipids in starch granules

Lipids are present in small amounts in starch granules, representing approximately 1% of granule weight and primarily in the form of lysophospholipids. It has been reported that their compositional profiles depend on the source of the granules and their presence affects the way in which the starch structures behave (Copeland, et al., 2009; Vasanthan & Hoover, 1992). According to Morrison (1988), there are three types of lipids associated with starch and they can be separated experimentally. These include the lipids inside the granular starch, to be considered as the only true lipid starch, surface lipids which are acquired from the surrounding proteinaceous matrix of the endosperm and non starch lipids which are the membrane and spherosome lipids from starchy endosperms, aleurone layers and the germ regions of a grain. Surface lipids are often components of commercial and laboratory prepared starches, which should not be confused with true lipids. They can be extracted with common fat solvents including diethylether while internal lipids can be extracted with hot aqueous alcohol e.g. 85% methanol or 90% ethanol (Morrison, 1988).

### 2.5.6 Crystallinity of starches

Native starches contain crystal structures due to the packing of the hexagonal chains of amylose into double helices and hence also creating a crystalline region from the linear amylose chains (Tester, et al., 2004; Zobel, Young, & Rocca, 1988). Consequently, starch being a semi-crystalline polymer, shows low and imperfect crystallinity (Frost, Kaminski, Kirwan, Lascaris, & Shanks, 2009). Generally, starch granules consist of components showing crystallinity in the range of 15-45% (Zobel, 1988a).

The procedure used most frequently to measure crystallinity in polymers is wide angle x-ray diffraction (Rabiej, 1991). Based upon their crystalline structures, starches can be classified into three main categories (Table 2.1). The A-type is usually present in cereal...
sources and these demonstrate densely packed crystallites (Imberty, et al., 1987). B-type is the predominant pattern seen in tuber starches. Yet, there are exceptions to the general rule and these include high-amylose maize, barley and wrinkled peas starches that have more than 49% of amylose having B patterns instead of A (Zobel, 1988b). The C-type pattern is present in certain root, seed starches and most leguminous grains. Starches can also demonstrate crystallinity of V pattern associated with A, B and C patterns that generally appears following gelatinisation and in some other native starches (Gallant, Bouchet, & Baldwin, 1997).

Table 2.2  Crystallinity of starch granules showing A, B and C type patterns (adapted from (Zobel, 1988a))

<table>
<thead>
<tr>
<th>Starch</th>
<th>Crystallinity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starches with A pattern</td>
<td></td>
</tr>
<tr>
<td>Oat</td>
<td>33</td>
</tr>
<tr>
<td>Rye</td>
<td>34</td>
</tr>
<tr>
<td>Wheat</td>
<td>36</td>
</tr>
<tr>
<td>Waxy rice</td>
<td>37</td>
</tr>
<tr>
<td>Sorghum</td>
<td>37</td>
</tr>
<tr>
<td>Rice</td>
<td>38</td>
</tr>
<tr>
<td>Corn</td>
<td>40</td>
</tr>
<tr>
<td>Waxy maize</td>
<td>40</td>
</tr>
<tr>
<td>Dasheen</td>
<td>45</td>
</tr>
<tr>
<td>Starches with B pattern</td>
<td></td>
</tr>
<tr>
<td>Amylomaize</td>
<td>15-22</td>
</tr>
<tr>
<td>Edible canna</td>
<td>26</td>
</tr>
<tr>
<td>Potato</td>
<td>28</td>
</tr>
<tr>
<td>Starches with C pattern</td>
<td></td>
</tr>
<tr>
<td>Horse chestnut</td>
<td>37</td>
</tr>
<tr>
<td>Sweet potato</td>
<td>38</td>
</tr>
<tr>
<td>Tapioca</td>
<td>38</td>
</tr>
</tbody>
</table>
The distribution of crystallites in starch granules can be considered as a factor that may affect digestibility (Gérard, et al., 2001). Amorphous regions of high amylose starches show greater hydrolysis by \( \alpha \)-amylase while crystalline areas resist hydrolysis leaving these regions intact (Dona, et al., 2010; Gérard, et al., 2001; Ring, et al., 1988; Tester, et al., 2006). B- and C-types starches depict greater resistance to enzymatic hydrolysis than A-type. Results from Lopez-Rubio et al. (2008) also suggested that there is a positive correlation with increased molecular order and enzymatic hydrolysis in starch. This could imply that resistant starch is not inherently present in native starch granules but is gradually formed due to re-branching of amylose chains as starch is being hydrolysed, thereby increasing crystallinity. As a result, resistance to enzyme hydrolysis can be affected by the competition between the kinetics of enzyme hydrolysis and starch retrogradation kinetics (Dona, et al., 2010).

2.5.7 Gelatinised starch

The state of the starch granule will affect the degree of digestion. Usually, gelatinised starch is regarded as being more readily digested than native raw starch especially if initial rates of amylolysis are compared (Berry, 1986).

2.6 in vitro Evaluation of digestibility

The analysis of total starch (TS) content of cereal grain products has been undertaken by the AOAC Official Method 979.10 using the glucoamylase method (AOAC, 2002d). However, the amount of starch present in foods ingested might not reflect the available carbohydrate to the human body. It has been suggested that food composition tables often have inconsistencies in published values of starch and carbohydrate and metabolisable energy contents (Hudson & Englyst, 1996). in vivo Measurements appear to be able to determine the extent of postprandial glycaemic responses but they can impart considerable discrepancies within and among test subjects as well as being an expensive means for routine food analysis (Brooks, et al., 2006). On the other hand, in vitro measurement of carbohydrate digestibility is being sought as to provide a means of measuring available carbohydrate which implies measuring the glycaemic carbohydrate i.e. carbohydrate able to cause a marked rise in blood glucose level after ingestion of a food (Brooks, et al., 2006).
in vitro Assays of digestibility have been carried out by using robust methods that sometimes include heating at temperatures of up to 100°C (Englyst, Wiggins, & Cummings, 1982). In spite of the satisfactory results obtained, these do not always correspond realistically to the expected physiological situation. In the past few decades, there have been numerous attempts to reproduce the human digestion of starch under laboratory conditions by applying and modifying existing methods to achieve higher physiological significance. Table 2.3 summarises these researches carried out thereby emphasising on the different approaches used to assess in vitro digestibility of starch and starch based products.
### Table 2.3  Summary of in vitro methods carried out and used by other authors (adapted from Woolnough, Monro, Brennan & Bird 2008)

<table>
<thead>
<tr>
<th>Authors</th>
<th>Year</th>
<th>Oral phase</th>
<th>Gastric phase</th>
<th>Intestinal phase</th>
<th>Analysis carried out in</th>
<th>Measured</th>
<th>Foods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Englyst, Wiggins and Cummings</td>
<td>1982</td>
<td>Ball-milled Homogenised</td>
<td>-</td>
<td>α -amylase and pullulanase at 42°C for 16hr.</td>
<td>50mL screw topped glass tubes with magnetic stirrer</td>
<td>NSP</td>
<td></td>
</tr>
<tr>
<td>Jenkins et al.</td>
<td>1982</td>
<td>Ground to a smooth paste</td>
<td>-</td>
<td>Mix with pooled saliva at 37°C for 3hr.</td>
<td>DT for intestinal simulation Diameter: 45mm Length: 13cm MWCO: 12kDa Suspended in stirred beaker.</td>
<td>Rate of SD</td>
<td>Lentils and white bread.</td>
</tr>
<tr>
<td>Jenkins et al.</td>
<td>1984</td>
<td>Crumbed Ground to powder or smooth paste</td>
<td>-</td>
<td>Mix with pooled saliva at 37°C for 3hr.</td>
<td>DT for intestinal simulation Diameter: 45mm Length: 13cm MWCO: 12kDa Suspended in stirred beaker.</td>
<td>Rate of SD</td>
<td>White bread, wholewheat bread, rice, cornflakes, porridge oats, spaghetti, potato, kidney beans, chick peas and lentils</td>
</tr>
<tr>
<td>Granfeldt and Björck</td>
<td>1991</td>
<td>Wet-homogenised Chewed 15 times for 15secs.</td>
<td>Pepsin pH 1.5 at 37°C for 30mins (gentle mixing 4-5 times).</td>
<td>Porcine pancreatic α -amylase pH 6.9 at 37°C for 3hr.</td>
<td>DT for intestinal simulation Diameter: 45mm Length: 13cm MWCO: 12-14kDa Suspended in stirred beaker.</td>
<td>Rate of SD</td>
<td>Pasta.</td>
</tr>
<tr>
<td>Brighenti, Casiraghi and Testolin</td>
<td>1992</td>
<td>0.9% NaCl Extruded through Ottawa Instron cell (diameter 0.6mm) Human salivary α – amylase for 5mins</td>
<td>Hog pepsin pH 2.0 for 1hr</td>
<td>10mM NaCl Hog pancreatic pH 6.9 at 37°C for 5hr.</td>
<td>DT for intestinal simulation MWCO: 6-8kDa Suspended in stirred beaker.</td>
<td>Rate of SD</td>
<td>Spaghetti</td>
</tr>
<tr>
<td>Englyst, Kingman and Cummings</td>
<td>1992</td>
<td>Minced with plates 0.9cm diameter holes Milled. Homogenised.</td>
<td>-</td>
<td>Pancreatin, AMG, invertase pH5.2 at 37°C for 2hr.</td>
<td>Guar gum. Glass balls shaking in water bath</td>
<td>Rate of RDS, SDS and RS</td>
<td>Long grain brown rice, parboiled rice, white spaghetti and sweetcorn.</td>
</tr>
<tr>
<td>Authors</td>
<td>Year</td>
<td>Oral phase</td>
<td>Gastric phase</td>
<td>Intestinal phase</td>
<td>Analysis carried out in</td>
<td>Measured</td>
<td>Foods</td>
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<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>Granfeldt et al.</td>
<td>1992</td>
<td>Chewed 15 times for approximately 15secs.</td>
<td>Pepsin and NaCl pH 1.5 at 37°C for 30mins (gentle mixing 3 times).</td>
<td>Porcine pancreatic α-amylase pH 6.9 at 37°C for 3hr.</td>
<td>DT for intestinal simulation</td>
<td>Rate of SD</td>
<td>A selection of pasta, bread, corn, rice and legumes.</td>
</tr>
<tr>
<td>Muir and O’Dea</td>
<td>1993</td>
<td>Chewing</td>
<td>Pepsin pH 2.0 at 37°C for 30mins</td>
<td>Porcine pancreatin and glucoamylase pH 5.0 at 37°C for 6hr.</td>
<td></td>
<td>Rate of RDS</td>
<td>Rice, cornflakes and chickpeas.</td>
</tr>
<tr>
<td>Brighenti, Pellegrini, Casiraghi and Testolin</td>
<td>1995</td>
<td>Extrusion through Kramer cell (3mm diameter holes) 10mM NaCl Human salivary α-amylase pH 6.9 for 5mins</td>
<td>Hog pepsin pH 2.0 at 37°C for 1hr at</td>
<td>Hog pancreatin pH 6.9 at 37°C for 5hr.</td>
<td>DT for intestinal simulation</td>
<td>Rate of CD</td>
<td>Spelta, spelta flour, rice, parboiled rice, corn, bread, chestnuts, buckwheat, white beans, bean flour, lentils, lentil flour, pasta, broad beans and, and chick peas.</td>
</tr>
<tr>
<td>Goñi, Garcia-Diz, Mañas and Saura-Calixto</td>
<td>1996</td>
<td>Homogenise pH 1.5</td>
<td>Pepsin at 40°C for 60min</td>
<td>α-amylase pH 6.9 at 37°C for 16hr Glucoamylase pH 4.75 at 60°C for 45mins</td>
<td>50mL centrifuge tube Shaking water bath.</td>
<td>RS.</td>
<td>Powders of bean flakes, retrograded amylase, banana flour and bread.</td>
</tr>
<tr>
<td>Goñi, Garcia-Alonso and Saura-Calixto</td>
<td>1997</td>
<td>Homogenised with water or buffer</td>
<td>Pepsin pH 1.5 at 40°C for 1hr</td>
<td>α-amylase pH 6.9 at 37°C Glucoamylase at 60°C for 45mins</td>
<td>Tubes in shaking water bath</td>
<td>Rate of SD</td>
<td>White bread, spaghetti, rice, biscuits, lentils, chick peas, beans, peas, boiled potatoes and crisp potatoes.</td>
</tr>
<tr>
<td>Brighenti, Casiraghi and Baggio (adapted from Champ, 1992)</td>
<td>1998</td>
<td>Minced in waring blender Forced through 1.5mm steel sieve with glass pestle</td>
<td>Pancreatic α-amylase pH 6.9 at 37°C for 16hr Glucoamylase pH 4.5 at 65°C for 90mins</td>
<td>Mixing and shaking</td>
<td></td>
<td>RS and TS.</td>
<td></td>
</tr>
<tr>
<td>Authors</td>
<td>Year</td>
<td>Oral phase</td>
<td>Gastric phase</td>
<td>Intestinal phase</td>
<td>Analysis carried out in</td>
<td>Measured</td>
<td>Foods</td>
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<td>----------------------------------------------------------------</td>
</tr>
<tr>
<td>Englyst et al.</td>
<td>1999</td>
<td>Hand operated mincer (holes 0.9cm diameter), Crushed and Broken up</td>
<td>Pepsin guar gum solution at 37°C for 30mins</td>
<td>Pancreatin, glucoamylase and invertase at pH 5.2</td>
<td>Polyethylene tubes and Glass balls shaking in water bath</td>
<td>Rate of CD</td>
<td>Spaghetti, pearl barley, cornflakes and bread</td>
</tr>
<tr>
<td>Van der Merwe, Erasmus and Taylor (adapted from Granfeldt et al., 1992)</td>
<td>2001</td>
<td>Chewed by researcher 5-7 times in 5-7secs</td>
<td>Pepsin for 30mins</td>
<td>Pancreatic α-amylase</td>
<td>YES</td>
<td>Rate of SD</td>
<td>White maize</td>
</tr>
<tr>
<td>Frei, Sidduraju and Becker (adapted from Gohi, Garcia-Alonso and Saura-Calixto, 1997)</td>
<td>2003</td>
<td>Homogenised for 2mins</td>
<td>Pepsin from porcine gastrine mucosa pH 1.5 at 40°C for 1hr</td>
<td>α-amylase from porcine pancreas pH 6.9 at 37°C for 3hr. Glucoamylase from Aspergillus niger pH 4.75 at 60°C for 45mins.</td>
<td>30mL Erlenmeyer flasks in shaking water baths.</td>
<td>Rate of SD</td>
<td>Six rice cultivars</td>
</tr>
<tr>
<td>Chung, Lim and Lim (adapted from Englyst et al., 1992)</td>
<td>2006</td>
<td>-</td>
<td>Supernatant of pancreatic α-amylase solution and glucoamylase pH 5.2 at 37°C for 2hrs.</td>
<td>Guar gum Test tubes with glass balls in shaking water bath</td>
<td>Rate of SD</td>
<td>Gelatinised and retrograded rice starch</td>
<td></td>
</tr>
<tr>
<td>Koh et al. (adapted from Jenkins et al., 1984).</td>
<td>2009</td>
<td>Cut in small disk using a cork borer</td>
<td>α-amylase from Aspergillus oryzae pH 7.3 at 37°C for 3hr.</td>
<td>DT for intestinal simulation Diameter: - Length: 20cm MWCO: 12-14kDa inverted 3 times and suspended in stirred beaker.</td>
<td>Rate of SD</td>
<td>Rice dough</td>
<td>Wholemeal bread from S. cerevisiae, wholemeal sourdough, white bread from S. cerevisiae and white sourdough</td>
</tr>
<tr>
<td>Scazzina, Del Rio, Pellegini &amp; Brighenti (adapted from Granfeldt et al., 1992)</td>
<td>2009</td>
<td>Dissolved in NaCl</td>
<td>Pancreatic α-amylase pH 6.9 at 37°C for 5hr.</td>
<td>DT for intestinal simulation Diameter: 45mm Length: 13cm MWCO: 3, 6-8 and 12-14,kDa.</td>
<td>Rate of SD</td>
<td>Wholemeal bread from S. cerevisiae, wholemeal sourdough, white bread from S. cerevisiae and white sourdough</td>
<td></td>
</tr>
<tr>
<td>Note</td>
<td>CD</td>
<td>Carbohydrate digestion (starch and sugar)</td>
<td>RS</td>
<td>Resistant starch</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>------</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DT</td>
<td>Dialysis tubing</td>
<td>RDS</td>
<td>Rapidly digested starch</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut off</td>
<td>SDS</td>
<td>Slowly digested starch</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSP</td>
<td>Non starch polysaccharide</td>
<td>TS</td>
<td>Total starch</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>Starch digestion</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Chapter 2
A number of groups of researchers (Brighenti, Casiraghi, & Testolin, 1992) have investigated modifications to the original foundation work described by (Jenkins, et al., 1982). These advances have contributed to providing an approach which is more extensive and realistic for the quantification of readily digestible starch. In the original work, Jenkins et al. (1982) concentrated solely on the effect of pooled human saliva while Brighenti et al. (1992) used a less rudimentary approach by applying human saliva from a commercial source and also incorporated the use of gastric conditions prior to intestinal conditions.

To add to this, Grandfeldt and Björck (1991) were pioneers in separating the in vitro digestion process into three main stages that reflected the actual conditions associated with the three main parts of the human digestive tract. These are the mouth, stomach and lower intestinal tract. The comparative study by these workers outlined the differences encountered following the inclusion of proteolytic enzymes, which was demonstrated to give higher glycaemic response where starch-protein interactions may interfere with amylolysis. There have been numerous variations in the details of in vitro assays being carried out for evaluation of digestibility. This includes the use of different means of simulating the in vivo conditions and these affect the estimated values for the glycaemic impact of a particular food.

The use of dialysis tubing (DT) was also highlighted as a restrictive means of affecting the viscosity of the dialysate and hence the glycaemic response (Granfeldt & Björck, 1991). Other means, for example the addition of guar gum, have also been proposed in order to standardise the viscosity, maintaining the sample in suspension and preventing sedimentation (Englyst et al., 1999).

Nevertheless, in spite of the application of in vitro assays, there is a need for standardisation of methodology. As seen from Table 2.2, there exist discrepancies among work carried out to simulate physiological conditions and inherently resulting in different glycaemic impact estimated for a particular food.
2.7 Summary and conclusion

The application of an *in vitro* assay begins with the understanding of *in vivo* processes and conditions while morphological and structural properties of the substrate can also impart discrepancies in the assay. A variety of techniques have been investigated in recent work and this has emphasised the need for standardisation of method in the aim of more realistically reproducing actual physiological changes resulting from the digestion of starch. Additionally, comparison of different conditions within the same study will contribute to the understanding and hence result in the adequate choice of parameters for the development of a sole *in vitro* digestion assay of starch.
Chapter 3

Literature review:

Microencapsulation

The purpose of this chapter is to provide background and review current knowledge relevant to the research reported in this thesis. Among the areas briefly described are food microencapsulation and its application in the food industry, wall materials available and permitted for microencapsulation purposes, the selection of methods of microencapsulating food ingredients and an appraisal of using spray drying for microencapsulation.

3.1 Food microencapsulation

Microencapsulation is a novel technology that involves minute amounts of a substance that can be solids, liquids or gaseous materials being sealed in capsules of microscopic size (Benita, 1996). Usually, microcapsules range from 1 to 1000 µm in diameter and are most commonly spherical in shape (Augustin, Sanguansri, Margetts, & Young, 2001). In its simplest form, a microcapsule is made up of an internal material known as the core, the internal phase or the fill that will be protected by a distinct and uniform layer known as the wall material, coating, shell, membrane or microencapsulant (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007). However, depending on the method of microencapsulation and the type of materials being used, microcapsules quite often are not in the simple form as described. They will rather consist of several wall layers, have multi-cores, irregular shapes or even form matrices (Gibbs, Kermasha, Alli, & Mulligan, 1999). According to Gharsallaoui et al. (2007), the shape and size of the microcapsules depend on the design, core and wall material being used and the method of encapsulating.

This technology can be applied as a means of protecting a core ingredient against adverse effects of other food ingredients, food processing and the environment within the food due to the protection afforded by the wall material. Based upon the constitution and nature of the wall material, the release of its contents can be undertaken at controlled rates and under specific conditions (Gibbs et al., 1999a). Microencapsulation has been developed to the stage of being widely used in the pharmaceutical, cosmetic and printing industries (Augustin et al., 2001) and its application became of interest in the food industry in the early 1950’s, when Griffin, (1951) first encapsulated solid oil concentrates (Uddin,
Hawlader & Zhu, 2001). However, it has only been relatively recently that food ingredients have become the focus of attention leading to success in encapsulating and providing enhanced delivery and physical protection from the environment or from undesirable interactions with other food ingredients (Augustin et al., 2001). A summary of recent reports describing encapsulation of food components is presented in Table 3.1.

Table 3.1 Food ingredients successfully microencapsulated (adapted from Kirby, (1991) and Augustin et al. (2001))

<table>
<thead>
<tr>
<th>Type of ingredients</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acids, alkalis and buffers (e.g. citric acid and sodium bicarbonate)</td>
<td>(Abbasi &amp; Rahimi, 2008; Jie, Peng, Chun, &amp; Feng, 2009)</td>
</tr>
<tr>
<td>Agents with undesirable flavours and odours</td>
<td>(Favaro-Trindade, Santana, Monterrey-Quintero, Trindade, &amp; Netto, 2010; Lambert, Weinbreck, &amp; Kleerebezem, 2008)</td>
</tr>
<tr>
<td>Amino acids, peptides and protein</td>
<td>(Ho, Park, Park, &amp; Lee, 2009)</td>
</tr>
<tr>
<td>Antioxidants</td>
<td></td>
</tr>
<tr>
<td>Artificial sweeteners (e.g. aspartame)</td>
<td>(Osorio, Acevedo, Hillebrand, Carriazo, Winterhalter &amp; Morales, 2010; Wang, Lu, Lv, &amp; Bie, 2009)</td>
</tr>
<tr>
<td>Colourants and pigments</td>
<td></td>
</tr>
<tr>
<td>Cross-linking and setting agents</td>
<td></td>
</tr>
<tr>
<td>Enzymes, micro-organisms and bioactive compounds (e.g. probiotic bacteria)</td>
<td>(Martinez-Ferez, 2009; Rokka &amp; Rantamaki, 2010)</td>
</tr>
<tr>
<td>Volatile compounds and essential oils (e.g. omega-3 fatty acids)</td>
<td>(Earnest, Hammar, Munsey, Mikus, David, Bralley &amp; Church, 2009)</td>
</tr>
<tr>
<td>Flavouring agents (e.g. oils, spices, seasoning and sweeteners)</td>
<td>(Cherukuri, Raman, &amp; Mansukhani, 1993; Milanovic, Manojlovic, Levic, Rajic, Nedovic &amp; Bugarski, 2010)</td>
</tr>
<tr>
<td>Instant starches</td>
<td></td>
</tr>
<tr>
<td>Leavening agents</td>
<td>(Janovsky, 1993)</td>
</tr>
<tr>
<td>Lipids (e.g. fish oils, milkfat, vegetable oils)</td>
<td>(Bae &amp; Lee, 2008; Drusch &amp; Mannino, 2009)</td>
</tr>
<tr>
<td>Minerals (e.g. calcium, iron and zinc)</td>
<td>(Jackson &amp; Lee, 1991; Oner, Arcasoy, Kas, &amp; Hincal, 1989)</td>
</tr>
<tr>
<td>Preservatives</td>
<td></td>
</tr>
<tr>
<td>Redox agents (bleaching)</td>
<td></td>
</tr>
<tr>
<td>Fat soluble vitamins and pro-vitamins (e.g. retinol, carotene)</td>
<td></td>
</tr>
<tr>
<td>Water soluble vitamins (e.g. folic acid, thiamin, riboflavin and ascorbic acid)</td>
<td>(Goh, Hau Fung Cheung, Al-Widyan, &amp; Small, 2008; Lim Chai Teo &amp; Small, 2008; Sanyoto, Wijaya, &amp; Small, 2008)</td>
</tr>
</tbody>
</table>
3.2 Applications of food microencapsulation

The main purpose of microencapsulation is to act as a vehicle that conveys control of mass transport in a subsequent environment. The wall material is designed in such a way as to prevent undesired loss of the core through diffusion from within and from the exterior of the microcapsule (Reineccius, 2001). Microencapsulation is used to achieve a series of functions including:

3.2.1 Protecting sensitive and unstable nutrients

Food enrichment and food fortification are commonly applied in order to maintain the nutritional quality of food, to keep nutrient content at a specified level and to minimise the onset of deficiency diseases. Nowadays, though the latter purpose is still of great importance for third world countries, application of food fortification has also broadened with increased interest in its contribution to improving general health (Crane, Wilson, Cook, Lewis, Yetley, & Rader, 1995). In some cases, food fortification has become mandatory as in the case of folic acid fortification in Australia as from September 2009, following the steps taken in USA a few years before (FSANZ, 2009). The use of microencapsulation of micronutrients and its application in food including bread and Asian noodles has been researched (Chung Yew, 2004; Goh et al., 2008; Lim Chai Teo & Small, 2008; Sanyoto et al., 2008). In addition, sensitive compounds such as oleoresins and essential oils can be protected from evaporation, oxidation and chemical reactions through the shielding effect of microencapsulation (Rosenberg, Kopelman, & Talmon, 1990).

3.2.2 Flavour enhancement

Spices impart flavour to a wide range of foods. However the flavouring compounds of freshly ground spices show poor stability as a result of exposure to light, oxygen and heat, causing significant reduction in flavour contribution. Among the various studies carried out on flavour preservation of spices, it has been reported that cardamom oleoresins can be significantly protected from degradation using microencapsulation with a mixture of gum Arabic and modified starch (Krishnan, Kshirsagar, & Singhal, 2005) while cardamom essential oils have been successfully encapsulated with mesquite gum (Beristain, García, & Vernon-Carter, 2001). In the same way, emulsified ethyl butyrate can be encapsulated with gum Arabic, soybean soluble polysaccharide and gelatin (Yoshii et al., 2001) while
flavours can be encapsulated for use in chewing gums allowing for gradual release upon chewing.

3.2.3. Masking of undesirable flavours

Some food ingredients, although conferring desirable nutritional properties, impart undesirable taste. As an example, tuna oil is a rich source of essential omega-3 fatty acids. It is now possible to prepare novel non-fish products containing the desired amounts of omega-3 fatty acids without the drawback of conferring a fishy smell by incorporating of microencapsulated tuna oil into staple foods including bread, (Yep, Li, Mann, Bode, & Sinclair, 2001). The suitability of this approach has been confirmed in a study which showed that only one in seven tasting panellists noticed the fishy smell of microencapsulated fish oil in bread while all seven of them noticed the fishy smell in bread fortified with antioxidant stabilised fish oil (Vilstrup, 1996).

3.2.4. Controlling release rate of the core material to its environment

Encapsulated food ingredients with suitably designed control systems may be released at the desired environment, time and rate (Pothakamury & Barbosa-Cánovas, 1995). By modifying one or a combination of different stimuli including a change in temperature, moisture, pH, the application of pressure (or shear) and the addition of surfactants, release rate of the core material can be controlled. The effectiveness will depend upon the choice of the material so that a desired change in conditions will activate the release mechanism of the core material (Vilstrup, 2001). Some of these mechanisms have been described by Pothakamury & Barbosa-Cánovas, (1995) and include:

1. The liberation of the core ingredients due to diffusion of the core ingredients through the unchanged wall material. This also involves barrier-controlled release where release is dependent upon the concentration difference across the wall material, its thickness and permeability as well as the diffusion coefficient of the core ingredient to the surrounding environment;

2. Biodegradation of the wall material itself through pH, temperature or solvent stimulus;

3. Swelling of the wall material leading to a change from the glassy state to a gel state when swollen with the medium which acts as a penetrant;
4. The action of osmotic pressure whereby the core material is released to the environment due to the high osmotic pressure it exerts inside the microcapsule being greater than that which the wall materials can tolerate. However, this form of release requires that the core material is highly soluble and the wall material water permeable or at least has small openings to allow diffusion of water molecules; and

5. A combined system in which the core material is released as a result of the combination of different mechanisms.

### 3.2.5. Decreasing the transfer rate of core materials to the external environment

Some years ago it was observed that starch granules when sprayed with small amounts of binding agent form spherical aggregates with interconnecting cavities that provide extensive porosity (Zhao & Whistler, 1994). Although there have been relatively few subsequent reports which have further investigated the original finding, it is likely that the porous spheres facilitate protection of the core material while allowing its gradual release. It is also possible that even more protection to the core material might be achieved by coating the spheres with calcium salts of alginate (ALG) or low methoxy pectin (LMP) thereby increasing retention and reduce loss of core material. The encapsulating materials not only act as a shield for the core material but can also allow the sustained release of its content triggered by desired conditions. For example in the chewing gum industry, a non-encapsulated flavour will be rapidly released as compared to the gradual release of an encapsulated flavour coated with wall materials that would have a melting point close to body temperature (Vilstrup, 2001). Other desired sustained release would also include the gradual of release of nutrients along the digestive tract when ingested.

### 3.2.6. Promoting easier handling

If a food ingredient is to be incorporated into a formulation in the liquid form then this often entails challenges due to difficulties in handling. In addition, volatile and gaseous materials are not only difficult to handle but can also readily escape from the food matrix. Volatile compounds have been successfully microencapsulated by spray drying and oil by coacervation (Katona, Sovilj, & Petrovic, 2009; Rosenberg & Sheu, 1996). Conversion of such ingredients into a free-flowing powder or a non-dusting material that can be readily incorporated in a variety of food processing by making it easier and simpler to handle (Vilstrup, 2001).
3.3 Wall materials used in the food industry

The first examples of wall materials used for spray-drying of microcapsules has involved a trial-and-error approach (Gharsallaoui et al., 2007). Different combinations of wall materials were used and the resulting microcapsules evaluated by testing and monitoring their stability under different storage conditions. This provided an evaluation of their controlled release properties, the extent to which the core material is shielded from environmental conditions as well as surface morphology, typically from SEM (Pérez-Alonso, Báez-González, Beristain, Vernon-Carter, & Vizcarra-Mendoza, 2003). Moreover, the extent to which the core materials have been retained reflected their chemical functionality, solubility and diffusion through the wall materials. Thus, the composition of the wall materials is the primary parameter which determines microencapsulation efficiency and the stability of the core components (Gharsallaoui et al., 2007).

The choice of wall materials is crucial in the development of a microcapsule preparation. The individual wall components selected, as well as the combination of these, will determine the particle size of the product, the mechanical strength, the release mechanism of the microcapsules and hence determine their compatibility with the food product and their application (Kirby, 1991). Nevertheless, it is important to mention that no wall material will be able to act as a perfect shield even if the microencapsulation technique was carried perfectly (Thies, 2001). Consequently, a compromise needs to be made in order to decide which imperfection of the wall materials chosen will have least consequence in a specific food system. Moreover, the type of wall material to be used will establish the method of microencapsulation as it will have a significant effect on the process for which it is being used. According to Thies (2001), it would be tedious and almost impossible use a wall material with a processing method for which it is not suited for.

In relation to the selection of suitable wall materials, these will then determine the application of the microcapsules. As such, polysaccharides, being relatively water-soluble, are commonly used to encapsulate water soluble components and non-polar solvents may need to be considered for other food ingredients including fats and oils, fat soluble vitamins and solvent miscible flavours. Given that these wall materials form concentrated solutions with low viscosity, spray drying and pressure extrusion are the processing methods of choice for these microencapsulants. A variety of approved wall materials have
been used in the food industry (Table 3.2) and for many applications, a combination of these would be utilised. Interestingly, gum Arabic remains one of the most popular wall materials for encapsulating food ingredients since it provides adequate viscosity, solubility and emulsification. This is due to the fact that unlike other natural polysaccharides, gum Arabic imparts interfacial activity through the small protein component covalently linked to the main polyasaccharide molecule which allows the formation of stable and fine emulsions (Thies, 2001). Despite these desirable properties, the primary disadvantage remains the expense reflecting the global scarcity (Gibbs et al., 1999). Therefore, there is an ongoing need to seek alternatives and substitutes that will also confer the desirable characteristics of wall materials for microencapsulation.

Table 3.2  Wall materials used in the food industry (adapted from Zhao & Whistler, 1994 and Augustin, Sanguansri, Margetts & Young, 2001)

<table>
<thead>
<tr>
<th>Material class</th>
<th>Examples of encapsulant materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins</td>
<td>Albumin, caseinates, gelatin, gluten, peptides, soy protein, vegetable proteins, whey proteins, zein</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td></td>
</tr>
<tr>
<td>Simple sugars</td>
<td>Fructose, galactose, glucose, maltose, sucrose</td>
</tr>
<tr>
<td>Gums</td>
<td>Agar, ALG, carrageenan, gum acacia (gum Arabic), pectins</td>
</tr>
<tr>
<td>Cellulosic material</td>
<td>Acetylcellulose, carboxymethyl cellulose, cellulose acetate butylate phthalate, cellulose acetate phthalate, ethyl cellulose, methyl cellulose, nitrocellulose.</td>
</tr>
<tr>
<td>Other carbohydrates</td>
<td>Chitosan, corn syrup solids, cyclodextrin, dextrins, dried glucose syrup, maltodextrins, modified starches, starches</td>
</tr>
<tr>
<td>Lipids</td>
<td>Acetoglycerides, beeswax, diacylglycerols, natural fats and oils, fractionated fats, hardened fats, lecithin, liposomes, monoacylglycerols, paraffin, tristearic waxes</td>
</tr>
</tbody>
</table>

When choosing the suitable wall materials, the storage requirements, processing needs, mechanical strength of the microcapsules, compatibility with the target foods, nutritional value as well as the appearance of the resulting microcapsules need to be considered (Brazel, 1999). Consideration is often focussed upon natural polymers as these offer the advantages of being non-toxic, biodegradable and bio compatible (Murano, 1998). The most commonly used natural polysaccharides are obtained from higher plants (cellulose and starch) or from seaweeds (ALG, agar and carrageenan, Table 3.2).
3.3.1. Alginate (ALG) as a wall material

In recent years, algal polysaccharides including ALG have become of considerable importance due to their potential as microencapsulation agents. This reflects their unique gelling properties using a variety of gelling mechanisms, their thermal and chemical stability as well as the ability to form cross-linked networks (Murano, 1998). ALG is one of the most abundant biopolymers after cellulose, providing desirable properties through their ability to readily gel in the presence of calcium ions, often incorporated in the form of CaCl\textsubscript{2} and this occurs readily at room temperature. Various ALG preparations are available and these are resistant to oxygen diffusion and there are no amino acid groups or free amino residues present that could react with the aldehyde groups of flavour compounds. They are also reported to be relatively insoluble in acidic conditions but soluble in neutral and alkaline environments (Thies, 2001).

The mechanical properties of ALG gels depends on the composition of the actual ALG preparation as well as the type and concentration of the cation used for gelation (Brodkorb, 2009; De Vos, De Haan, Wolters, & Van Schilfgaarde, 1996; Simpson, Stabler, Simpson, Sambanis, & Constantinidis, 2004). ALG are a group of linear unbranched polysaccharides which are composed of varying proportions of 1,4-linked \(\beta\)-D-mannuronic acid (M blocks) and \(\alpha\)-L-guluronic acid residues (G blocks) (Gibbs et al., 1999). When suspended in a CaCl\textsubscript{2} solution, electrostatic interactions between the carboxylate group of the G blocks and Ca\textsuperscript{2+} lead to the formation of mechanically stable networks. The binding or junction zone between a number of adjacent G blocks and the Ca\textsuperscript{2+} cations is described by the “egg-box” model due to its structure (Figure 3.1).
Hence, higher proportions of G blocks lead to a stiffer and more porous gel which is able to maintain its integrity for longer periods of time. During the cationic cross-linking, limited swelling and subsequent shrinkage occurs, hence retaining the structural features of the gel (De Vos et al., 1996). On the other hand, higher proportions of M blocks result in formation of softer, less porous gels that tend to disintegrate over time and are strongly affected by swelling and shrinkage during cationic cross-linking (De Vos et al., 1996). Until recently, it was believed that only G blocks provided the desired gel formation attributes of ALG. Recent work has shown that in addition to G blocks, alternating mannuronic and guluronic residues (MG blocks) can also cross-link with calcium ions. Hence, ALG high GG–GG, MG–GG and MG–MG blocks favours desirable gel-formation (Donati, Holtan, Mørch, Borgogna, Dentini, & Skjåk-Bræk, 2005).

ALG forms gels with most di- and multi-valent cations but varying gel strength is obtained with decreasing order Pb^{2+} > Cu^{2+} = Ba^{2+} > Sr^{2+} > Cd^{2+} > Ca^{2+} > Zn^{2+} (Brodkorb, 2009). The use of Ca^{2+} is desirable for its non-toxicity and moderate gel strength. While the proportion and length of glucoronic acid residues determines the overall gel strength, cation concentration can alter the number of alginate strands held together in the “egg-box” model and hence alter the strength of the gel network (De Vos et al., 1996).
3.3.2. Low methoxy pectin (LMP)

In a way similar to that observed for ALG, LMP also possesses the attribute of forming a gel following the “egg-box” model. LMP in which the degree of esterification of galacturonic acid residues is less than 50% generates anionic properties. Though it is a heterogenous polysaccharide, LMP contains linear chains of 1,4-α-D-galacturonic acid residues and these regions are able to cross-link with divalent cations including Ca\(^{2+}\) via their negatively charged carboxylic groups.

3.4. Processing procedures used in preparing food microcapsules

A variety of different methods have been investigated and used for microencapsulation. There are three main steps required in all cases. These are (1) the formation of the wall around the core material, (2) ensuring leakage does not occur and (3) ensuring that undesirable materials are excluded (Gibbs et al., 1999). The choice of microencapsulation technique needs to account for the properties of the core and wall materials, the type of release mechanisms desired, the microcapsule morphology as well as the particle size (Augustin et al., 2001).

3.4.1. Microencapsulation by spray drying

Spray drying is undoubtedly the most commonly used method for microencapsulating food ingredients. It provides an economical and effective way of protecting many desired food ingredients without the need for additional specialised machinery (Gouin, 2004). It is believed to be 30 to 50 times cheaper than freeze drying (Desobry, Netto & Labuza, 1997). However, spray-drying has been described as being wasteful of energy as it is not feasible to fully utilise all of the heat that is channelled through the chamber (Gharsallaoui et al., 2007). The spray drying technique was originally developed for the drying of milk to obtain a fine powder. In milk, the same principles of operation apply as those for the slurry of microencapsulants and wall materials. The fat acts as the core material while the carbohydrates such as lactose and milk protein form the wall material. Carbohydrates confer structure while the proteins provide emulsification and film forming properties (Gharsallaoui et al., 2007).

Microcapsules made by this method require the formation of a solution, emulsion or suspension of the core material(s) in the polymer (wall material) solution that will be used
as the feeding solution. This is then atomised in small droplets in the drying chamber. Common types of atomisers are pneumatic atomiser, pressure nozzle or spinning disks. The type used depends on the inherent characteristics and the viscosity of the liquid feed. It appears that the higher the energy provided by the atomiser and the lower the flow rate, the finer will be the particle size. Hence, process conditions including flow rate have to be appropriately controlled to achieve the desired particle size distribution. However, for all atomisers the objective is the same - increasing the exposed area so that maximum liquid is in contact with the hot air in the chamber. As a result, the evaporation of moisture from the liquid droplets within the chamber of the spray drier is almost instantaneous, thereby creating a fine powder.

There are two main ways in which hot air can be introduced to the chamber, these being either along with the feed (co-current) or against the sprayed fluid (counter-current). As long ago as 1921 Fleming described how, in a co-current process, inlet temperatures of 150 to 220°C will cause immediate evaporation, while the powders will be exposed to mild temperatures of 50-80°C reducing thermal degradation (Gharsallaoui et al., 2007). This contrasts with counter-current processes where thermolabile materials will be more readily affected by exposure to the heat. Yet, the latter method has the advantage that it uses less energy, making it more economical. As opposed to other methods particularly coacervation and phase separation, capsules prepared by spray drying will be composed of multi-cores (as described earlier) rather than of single droplet capsules. They will be composed of hundreds of tiny dispersed core droplets within a polymer matrix (Figure 3.2).

![Figure 3.2 Schematic representation of microcapsules (based on Gibbs et al., 1999)](image)

<table>
<thead>
<tr>
<th>Note</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Single core microcapsule obtained by coacervation</td>
</tr>
<tr>
<td>B</td>
<td>Multi wall microcapsule obtained by subsequent coatings</td>
</tr>
<tr>
<td>C</td>
<td>Multi-core microcapsule obtained by spray drying</td>
</tr>
</tbody>
</table>
3.5. **Summary of other common microencapsulation procedures**

3.5.1. **Coacervation**

Coacervation is the process whereby a core material is dispersed in a solution of wall materials that coacervate around the core material (Augustin *et al.*, 2001). Coacervation effectively involves the interactions of material with the resultant formation of a gel or barrier and can be either simple or complex in nature. The main difference is that it simply utilises a solution of a gelling protein as wall material, with this coacervating around the core material as a result of changes in pH, temperature or ionic salt concentration. In the case of complex coacervation, the wall material will be composed of two oppositely charged ionic compounds which may be proteins and polysaccharides (Vandegaer, 1973).

3.5.2. **Fluidised bed coating**

This technique involves the use of a liquid coating solution or molten wax, fat, protein or carbohydrates being sprayed on a bed of suspended solid core material to form microcapsules (Gouin, 2004). However, this method is limited to the formation of larger microcapsules as compared to other methods, having diameter sizes of at least 200µm. Moreover, agglomeration has been found to be a serious problem if microcapsules less than 100µm are being made (Balassa & Fanger, 1971; Jackson & Lee, 1991).

3.5.3. **Phase separation**

The principle of this method focuses on the fact that a separated phase liquid could wet and surround polar core material. This mobile coating can then be solidified to form capsules by chemical or physical means (Vandegaer, 1973).
3.6 Summary of current knowledge and application of microencapsulation

For many years, microencapsulation has been successfully used in the pharmaceutical industry and its application in the food industry has only been recent. There is choice for methodology and each conveys advantages and disadvantages. Approved and safe wall materials for food application are more limited than in the pharmaceutical industries and there is a need to find alternatives to the expensive and scarce gum Arabic. Hence the application of ALG and LMP show potential notably their ability to cross-link with calcium ions. However, many of the previous reports have been focused mainly on fat soluble rather than water soluble components. There are limited previous publications specifically describing the microencapsulation of folic acid or other water soluble vitamins.
Chapter 4

Literature review:
Folates and folic acid - History, functions, deficiency, availability, stability and analysis

The purpose of this chapter is to give relevant scientific background information and review related to the folates as a vitamin and described in this thesis. Included is the importance of folates, the use of folic acid as a fortificant, stability including susceptibility to particular pH conditions along with an appraisal of extraction and analysis procedures.

4.1 Historical background on folates and folic acid

The existence and role of folates as an essential vitamin was only recognised when the cause of the disease pernicious anaemia was understood. In 1931, Wills identified pernicious anaemia in Indian women and this deficiency was inducible in monkeys given the same diet (Eitenmiller, 2007). This condition was found to be lessened by including yeast or liver extracts into the diet. The unknown anti-anaemia factor was designated as “vitamin M”. Some years later, folic acid was isolated from the leaves of spinach giving rise to its name from the Latin word “folium” (meaning leaves) and was identified as one of the growth factors required by Lactobacillus casei and Streptococcus lactis. It was not until 1945 that folic acid was identified as a cure for megaloblastic anaemia (Combs, 2008; Eitenmiller, 2007).

4.2 The chemistry of folate and folic acid

Folate and folic acid are part of the B-group of vitamins, having a structure based upon pteroyl derivatives to which one or a number of glutamate units have been attached. The term folate should not be used interchangeably with folic acid: folate refers to any molecular form having the physiological function of a folate and derived from natural sources while folic acid is a single molecular form, not found naturally in foods, and of synthetic derivation. All forms of folate have the same core molecular structure (pteroyl acid) as found in folic acid but the minor differences in structure of this core. In addition, the folates will include a side chain of repeating units of glutamate of varying length. Folates therefore can include more than 30 structurally different molecules. The human
body can only absorb folate once the glutamate side chains are cleaved by an intestinal enzyme (commonly known as folate conjugase; EC 3.4.19.9, accepted name: $\gamma$-glutamyl hydrolase) until only one remains (NC-IUBMB, 2013). On the other hand, folic acid is more readily absorbed in the human body than folates due to its simpler structure, with a single glutamate unit not requiring the action of the enzyme prior to absorption (Gregory, 2008)

![Structure of folic acid](https://example.com/structure.png)

**Figure 4.1**  Structure of folic acid (based on Gregory, 2008)

### 4.3 Folate sources and recommended intakes

Generally, good natural sources of folates are green vegetables (including broccoli), leafy greens (particularly spinach), mushrooms, some fruits (orange and berries), legumes (peanuts, cowpeas, peas) and liver. The folates in foods are almost exclusively present in their reduced form as polyglutamyl derivatives of tetrahydrofolic acid ($\mathrm{FH}_4$). Free folate (folyl monoglutamate) is generally rarely found in foods (Combs, 2008).

Dietary surveys in Australia and New Zealand have shown that cereal grains, products and cereal based dishes provide approximately 27% of total folate, while legumes and vegetables contribute 29% and fruits approximately 8-10% intakes (NHMRC, 2006). The recent introduction of enrichment of some foods has resulted in an increased contribution of folate in some foods, particularly orange juice.

It has been reported that folate requirements are affected by several factors such as bioavailability, nutrient interactions, smoking, certain drugs and genetic variations (NHMRC, 2006; van der Put et al., 1995). In addition, the folate vitamers possess differing
bioavailabilities due to their different structures. The more bulky molecules require partial breakdown and hence a longer time to be absorbed through the intestinal linings and used for metabolic functions while folic acid being the simplest form does not require breakdown and will be absorbed directly.

Generally, it appears that the bioavailability of folate will be around 50-60%, folic acid from fortified foods approximately 80-85% and folic acid as a supplement will be 100% bioavailable on an empty stomach (Gregory, 1997; Winkels, Brouver, Siebelink, Katan & Verhoeef, 2007). There is some doubt in this area, although, previous studies demonstrated that folic acid from fortified cereal products have a bioavailability of only 30-60% (Colman, Green & Mets, 1975), more recent findings by Pfeiffer, Rogers, Bailey and Gregory (1997) demonstrated that folic acid is highly bioavailable from fortified cereal grain food products with values being as high as those found for supplements. Consequently, it is believed that cereal grain products are an excellent food carrier for fortification with folic acid (Tamura, 1997). Another recent study also found evidence that folate from foods generally has higher bioavailability than previously thought with aggregate bioavailability of folate from fruits, vegetables and liver being 78% of that found for folic acid (Winkels et al., 2007).

Because of the differing bioavailabilities of the folates and folic acid, the term dietary folate equivalent (DFE) is used to assess a defined diet containing both (NHMRC, 2006). Thereon,

\[
1\mu g \text{(DFE)} = 1\mu g \text{food folate} \\
= 0.5\mu g \text{folic acid on an empty stomach} \\
= 0.6\mu g \text{folic acid with meals or as fortified foods}
\]

The recommended intakes for folates vary based on different individual needs. These values are termed differently in different countries but primarily represent the minimum daily intake of this vitamin. For example, in Australia it is defined as the recommended daily intake (RDI) while in USA, it is known as the daily reference intake (DRI). Nevertheless, similar intakes are recommended with adults requiring 400µg (DFE), pregnant women 600µg (DFE) and lactating mothers 500µg (DFE) (NHMRC, 2006).
4.4 Folate stability

Most forms of folate, with the exception of folic acid and 5-formyl-FH4, are readily oxidised and so there is a susceptibility to oxidation during processing and storage especially due to heat, light and metal ions (Combs, 2008). However, the degree of glutamation does not appear to have an impact on folate stability (Gregory, 2008). The evidence is that folic acid is more stable than other forms of folate and this is one of the main reasons for its use for fortification (Eitenmiller, 2007). The increased stability of folic acid in comparison with other folates has been reported to apply at both ambient and elevated temperatures (Bui, Small & Coad, 2013).

As the folates are water soluble, this vitamin is also susceptible to leaching into the cooking media. Consequently relatively large amounts of naturally occurring folates in foods can be readily lost during food processing and preparation (Gregory, 2008). The extent of these loses will vary according to the nature of specific food matrices, oxygen availability, the chemical environment, the extent of heating and the types of folate vitaminers present in the food (Stea, Johansson, Jägerstad, & Frølich, 2007). A recent study demonstrated that irrespective of the order of broccoli being crushed or blanched, the same level of total folates was noted but the folates profile was different (Munyaka, Oey, Verlinde, Van Loey, & Hendrickx, 2009).

Based upon previous studies, it is thought that folate stability can be enhanced within particular buffered pH conditions and maximum stability is reached at pH values within the ranges of 1-2 and 8-12. At pH 4-6, folates are typically very unstable and it is also believed that, even in favourable pH zones, the tetrahydrofolate form can be unstable (Bui, Small & Coad, 2013). The presence of metal ions including Fe(II) in the environment is also crucial, as well as traces of Cu(II) present as contaminants in phosphate salts, will increase the rate of oxidation of folates. These problems can be overcome by the addition of reducing agents particularly in the form of either citrate ions or ascorbic acid. Despite the relative instability of folates when they are present in dry foods they are quite stable particularly in the absence of light and oxygen (Eitenmiller, 2007).
4.4 Functions of folates

Folates act as coenzymes in the metabolism of amino acids, nucleotides and the formation of the primary donor for biological methylation by accepting or donating single carbon groups during molecular re-arrangement reactions (Combs, 2008). Accordingly, folates are necessary for DNA synthesis, purine synthesis as well as interconversion of some amino acids. Therefore, folates are essential for metabolic roles in human growth and development including in the assistance of cell repair, replacement and division to take place at all stages of life.

Folates also contribute to the functioning of vitamin B12, assisting in the formation of ‘haeme’ (the organic structure with iron found within haemoglobin protein structures) and is necessary in the formation of red and white blood cells. However, the use of folate supplements can mask the presence of macrolytic anaemia caused by a deficiency in vitamin B12. Failure to identify vitamin B12 deficiency can lead to neuropathy (Brouwer & Verhoef, 2007; Combs, 2008).

4.5 Folate deficiency

Folate is required for normal human growth and development. It is needed for the formation of healthy red blood cells (RBC) and failure to do so can lead to the production of abnormally larger RBC with shorter lifespan. As a consequence of insufficient RBC, body tissues and organs may not receive sufficient oxygen. This condition is known as megaloblastic anaemia and symptoms include mood disturbances, panic attacks, psychotic reactions, paranoia, confusion, fatigue, insomnia and depression (Alpert & Fava, 1997; Fafouti, Paparrigopoulos, Liappas, Mantouvalos, Typaldou, & Christodoulou, 2002; Rawalpally, 2000).

The role of folate as a coenzyme in nucleotide metabolism is crucial in the prevention of abnormalities in early neural embryonic development. Insufficient folate can specifically lead to the risk of malformations of the embryonic brain and/or spinal cord and these problems are collectively referred to as neural tube defects (NTD) (Boushey, Edmonds, & Welshimer, 2001; Combs, 2008). Spina bifida, a type of NTDs arises during low folate consumption where neurolation is incomplete. Neurolation is a critical, complex developmental process in all vertebrate embryos during which the flat neural plate
Chapter 4

transforms into a tube that becomes the brain cranially and the spinal cord caudally. It has been suggested that spina bifida arising from folate deficiency is due to failure of closure of specific multi-sites of the neural tubes (van Allen, Kalousek, Chernoff, Juriloff, Harris, & McGillivray, 1993). These earlier findings have received considerable attention and have been confirmed by subsequent research (Ahmad & Mahapatra, 2009).

Folate is also believed to reduce the risks various disease conditions. Among these are cardiovascular diseases including coronary heart disease and stroke and certain cancers such as cervical, colon, brain and lung, cognitive diseases or mental conditions including Alzheimer's disease, age-related dementia or cognitive decline and depression (Alpert & Fava, 1997; Francis, 1999; McIntosh & Henry, 2008; Wang, et al., 2012).

4.6 Folic acid fortification and strategies

According to Locksmith & Duff (1998), three approaches can be undertaken to increase vitamin intakes including folates. These are:
1. The consumption of fresh vegetables and legumes,
2. Fortifying staple foods and
3. Increasing consumption of folic acid supplement.

Fortification being the addition of micronutrients to foods so as to restore levels of those present before processing, offers the health benefit of increasing nutrient intake without relying on individual supplementation practices (Hunt & Dwyer, 2001).

As a means of reducing the instance of NTDs, fortification of folic acid mainly in flour and cereal products have been made mandatory in USA, Canada, Mexico, Chile, Hungary, Australia and New Zealand. Various counties have put in place different level of fortification as shown in Table 4.1. Controversies still prevail in the effectiveness of the fortification strategies in increasing consumption of folic acid and the level of fortification. While most of the countries mandate a minimum level, only Australia and New Zealand have enforced a maximum level. As a consequence, many manufacturers in other countries notably USA often incorporate overages of nutrients so as to meet the minimum required levels throughout the shelf-life of their products (Rader, Weaver & Angyal, 2000). In spite of the benefits of food fortification, over consumption of folic acid of more than 1mg/day may mask vitamin B12 deficiency (Hunt & Dwyer 2001; Locksmith & Duff, 1998).
Table 4.1  Folic acid fortification levels (Bower & Stanley, 2004; Chowdhury, Marriott & Small, 2003; Oakley Weber, Bell & Colditz, 2004)

<table>
<thead>
<tr>
<th>Countries</th>
<th>Folic acid fortification levels (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chile</td>
<td>2.2</td>
</tr>
<tr>
<td>Canada</td>
<td>1.5</td>
</tr>
<tr>
<td>USA</td>
<td>1.4</td>
</tr>
<tr>
<td>Australia &amp; New Zealand</td>
<td>2 to 3</td>
</tr>
</tbody>
</table>

4.7 Analytical methods of folates

4.7.1 Folate extraction

Both free folates and total folates can be assayed. The latter requires an additional extraction of the bound folates most commonly by folate conjugase (pteroylpoly-γ-glutamyl hydrolase), an enzyme able to cleave the glutamate groups, prior to folate assay (Tamura, 1998). Following the distinction between free folates and total folates, the application of a trienzyme treatment for a more realistic extraction has been reported. This involves the inclusion of protease and α-amylase with folate conjugase so as to release bound folates from protein and carbohydrate matrices which significantly gives higher values (Bui & Small, 2007; Pfeiffer et al., 1997; Tamura, Mizuno, Johnston, & Jacob, 1997).

4.7.2 Microbial assay

Traditionally, analysis of folates by microbial assay is the official method recognised by the Association of Official Analytical Chemists, method 992.05 (AOAC, 1990) and the American Association of Cereal Chemists, method 86-47.01 (AACC, 2000). It is the preferred method primarily due to its ability to determine mono-, di- and tri- glutamate forms of folate (Han & Tyler, 2003). This assay responds to the bacteria, Lactobacillus casei spp. rhamnosus (ATCC 7469), which demonstrates growth promoting characteristics to folates. Other micro-organisms have also been used namely Enterococcus hirure (ATCC 8043) and Pediococcus cerevisae (ATCC 808 1) but L. casei spp. rhamnosus still shows highest preference as it responds almost equally to the widest variety of folate derivatives (Eitenmiller, 2007; Tamura, 1998). Once a very long and tedious assay, the introduction of cryo-frozen L. casei spp. rhamnosus, a 96-well plate and a microplate reader has reduced
cost and time consumption while improving reproducibility (Arcot & Shrestha, 2005; Tamura, 1998). Nevertheless, analysis time is still one of it major drawbacks.

### 4.7.3 Other alternatives

By comparison, other instrumental assays including the use of HPLC, CE, UPLC and LC-MS offers potential as they are not as labour intensive, can be fully automated while providing reliable separations (Edelmann, Kariluoto, Nyström, & Piironen, 2012; Hau Fung Cheung, Hughes, Marriott, & Small, 2009; Jastrebova, Witthöft, Grahn, Svensson, & Jägerstad, 2003; Lindeberg, 1996; Phillips, Ruggio, & Haytowitz, 2011; Ruggeri, Vahteristo, Aguzzi, Finglas, & Carnovale, 1999; Vahteristo, Lehikoinen, Ollilainen, & Varo, 1997).

Reversed phase HPLC (RP-HPLC) has been commonly applied to the analysis of B-group vitamins, particularly folates (Viñas, López-Erroz, Balsalobre, & Hernández-Córdoba, 2003). Most commonly, the use of C18 columns and reversed-phase in combination with ion-pair techniques have been used to the separation of folates from cereal foods (Osseyi, Wehling, & Albrecht, 1998). The primary advantage of LC is its ability to quantify specific folate forms often not possible with other methods (Eitenmiller, 2007). The low concentrations of folates in most foods emphasises the need of sensitive analytical tools to detect amounts in ppm, As folic acid and folates do not naturally fluoresce, the use of the UV detectors has been generally used even though derivatisation of folic acid is possible (Arcot & Shrestha, 2005). Couple with MS detection, LC-MS provides both specificity and sensitivity while allowing accurate data including quantification of coenzyme constituents. Unfortunately, its cost remains its major drawback (Eitenmiller, 2007).

### 4.8 Summary of current knowledge

Folates exist as many forms of vitamins and their high susceptibility to environmental conditions can result in significant loss of this essential vitamin. An understanding of these parameters must be considered in the aim of food fortification as a means of reducing the incidence of NTDs. There is evidence of insufficient folate intake in developing countries while excessive intakes of this B vitamin are controversial. Even though traditional method of analysis using a microbial assay has been popular, other analytical ways of analysis particularly instrumental analysis have proven potential.
Chapter 5
Summary of background and description of the project aims

The purpose of this chapter is to summarise the context in which this project has been developed and to describe the aims of the research program.

5.1 Summary of current situation and significance of the project

Microencapsulation has demonstrated potential in the protection of sensitive nutrients including water soluble vitamins. A variety of wall materials have been researched and shown promise in various food applications and food matrices including Asian noodles and bread. Recent research showed that when these microcapsules are subjected to controlled storage trials they demonstrated significantly enhanced retention properties thereby validating the effectiveness of the specific wall material combinations applied and the microencapsulation technique. The microcapsules produced by spray drying from starch based wall materials also promote easier handling of the desired core material as well as its even distribution throughout the food matrix.

The protection of the core material (in this study, folic acid) is one of the primary objectives of microencapsulation. The ultimate release of folic acid under the desired conditions is also of equal importance and should work alongside core material protection. Thus, microcapsules that show high protection would be ineffective if its core material cannot be released at controlled conditions. Hence, the release of folic acid from the ingested microcapsules would aid in the understanding of the bioavailability of the protected core material. Although the principles of release were the subject of various reviews in the past, there have been very few recent reports describing practical approaches to the evaluation of release properties of microcapsules.

5.2 Hypothesis

The research reported in this thesis has been based upon the hypothesis that the release characteristics of microcapsules can be evaluated using in vitro procedures adapted from those used to estimate the digestibility of foods.
5.3 Project aims

The aim of this project has been to investigate and apply digestibility analysis procedures to starch based systems along with the evaluation of release characteristics of microcapsules.

The specific objectives have included:

1. To compare and optimise procedures for the evaluation of the release of reducing sugars including DNS reagent method, Nelson and Somogyi method and HPLC-RI assay.

2. To investigate the factors influencing the rates of digestion of starch based materials in a dialysis model system. These laboratory conditions are designed to mimic the main stage of starch digestion in the human digestive tract i.e. at the intestinal stage.

3. To validate the procedures that allow for the in vitro evaluation of native starches that form the basis of the wall material applied during microencapsulation.

4. To determine the optimum treatment approaches for the microencapsulation of core material, using folic acid, based on recent findings that hydrocolloid agents and calcium can provide protection to folic acid.

5. To apply the in vitro digestion model to microcapsules containing folic acid and to evaluate the usefulness of this approach in the investigation of release characteristics of core material from ingested microcapsules.
Chapter 6
Materials and methods

The purpose of this chapter is to describe the chemicals, reagents, equipment and methods used during this study. This includes procedures applied in the preparation and sampling of microcapsules, extraction methodology, the assay of folic acid and reducing sugars as well as in vitro digestion procedures along with details of calculations.

6.1 Materials

The chemicals including enzyme preparations and vitamins used in product formulations and analytical procedures were of analytical grade or of the highest purity available, unless otherwise specified. Vitamins were used in the current study as chemical standards for analytical purposes as well as for spiking and fortification purposes. Details for chemicals used in the study are presented in Table 6.1. Dialysis tubing (DT) was from cellulose membrane and detailed in Table 6.2. The details of all chemicals and reagents used in this study are presented in a series of five tables as follows: general chemicals (Table 6.1), dialysis tubing (DT, Table 6.2), carbohydrate compounds used as analytical standards (Table 6.3), α-amylase preparations (Table 6.4) and staining and embedment materials (Table 6.5). The items of equipment used, together with the details of manufacturers and model numbers are presented in Table 6.6. The HPLC instrumentation, columns and ancillary items are described in Table 6.7. Details of consumables used are listed in Table 6.8.
### Table 6.1  Details of chemicals and suppliers

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Chemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ajax Fine Chem, Australia</td>
<td>Acetone (A6), copper sulphate pentahydrate (171-500G), Dipotassium hydrogen orthophosphate (2221), D-glucose anhydrous (0908212), ethanol absolute (214-UN 1170), glacial acetic acid (A1), sodium arsenate pentahydrate (D3247), sodium hydroxide (482), starch maize (1537 73236), starch potato (1534 92476)</td>
</tr>
<tr>
<td>Starch Australasia Ltd.,</td>
<td>Wheaten cornflour (TDT-121-01)</td>
</tr>
<tr>
<td>Australia</td>
<td></td>
</tr>
<tr>
<td>BDH Chemicals Ltd., England</td>
<td>2-methylpropan-1-ol (iso-butyl alcohol) (27505), maltose (29131), Amberlite resin IR-45 (OH) (55004), Amberlite resin IR-120(H) (55001) and sodium bicarbonate (10397)</td>
</tr>
<tr>
<td>Chem supply, Australia</td>
<td>Anhydrous sodium carbonate (SL099), calcium chloride fused dehydrate (93264 10002320), potassium di-hydrogen orthophosphate (93264 10005592), sodium potassium (+) - tartrate (Rochelle salt) (PL 086)</td>
</tr>
<tr>
<td>CP Kelco, Genu, Denmark</td>
<td>Pectin type LM-104 AS(2200-18)</td>
</tr>
<tr>
<td>M &amp; B Laboratory Chemicals</td>
<td>Ammonium molybdate (A222/18/66-1)</td>
</tr>
<tr>
<td>Merck Pty. Ltd., Germany</td>
<td>Acetonitrile (100030), sodium chloride (610241)</td>
</tr>
<tr>
<td>Rowe Scientific, Australia</td>
<td>Anhydrous sodium sulphate (CS13354)</td>
</tr>
<tr>
<td>Sigma-Aldrich, Australia</td>
<td>Alginic acid sodium salt from brown algae (A2033) Dipotassium hydrogen orthophosphate (P8281), folic acid (F7876), L-ascorbic acid (A5960), starch from rice (S7260), 3,5 – dinitrosalicylic acid (D0550),</td>
</tr>
<tr>
<td>Penford Australia Ltd.,</td>
<td>Maltodextrin Fieldose 17 (F17)</td>
</tr>
<tr>
<td>Australia</td>
<td></td>
</tr>
</tbody>
</table>

**Note** Description presented as chemical name (product number, batch or lot number)
### Table 6.2  Details of dialysis tubing

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Product description (Lot No)</th>
<th>MWCO (kDa)</th>
<th>FW (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cole Parmer, Australia</td>
<td>Spectra/Por ® (132724)</td>
<td>3.5</td>
<td>45</td>
</tr>
<tr>
<td>Science Supply Australia</td>
<td>Tubing dialysis (453105)</td>
<td>12</td>
<td>42</td>
</tr>
<tr>
<td>Cole Parmer, Australia</td>
<td>Membra-cel MD36 x 100CLR</td>
<td>20</td>
<td>42</td>
</tr>
<tr>
<td>Cole Parmer, Australia</td>
<td>Spectra/Por ® Biotech (131342)</td>
<td>20</td>
<td>16</td>
</tr>
</tbody>
</table>

**Note**

MWCO = Molecular weight cut off
FW = Flat width

### Table 6.3  Details of standards used in carbohydrate analysis

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Product description (Cat no)</th>
<th>Purity</th>
<th>Moisture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigma-Aldrich, Australia</td>
<td>Maltotriose (M8378)</td>
<td>≥95%</td>
<td></td>
</tr>
<tr>
<td>Supelco Analytical, USA</td>
<td>Isomaltotriose (47884)</td>
<td>99.0%</td>
<td>5.89%</td>
</tr>
<tr>
<td>Supelco Analytical, USA</td>
<td>Maltotetraose (47877)</td>
<td>96.0%</td>
<td>6.00%</td>
</tr>
<tr>
<td>Megazyme, Ireland</td>
<td>Glucosyl-Maltotriose (O-GMT)</td>
<td>~95%</td>
<td>2-3%</td>
</tr>
<tr>
<td>Supelco Analytical, USA</td>
<td>Maltopentaose (47876)</td>
<td>96.0%</td>
<td>5.44%</td>
</tr>
<tr>
<td>Supelco Analytical, USA</td>
<td>Maltohexaose (47873)</td>
<td>92.0%</td>
<td>5.00%</td>
</tr>
<tr>
<td>Supelco Analytical, USA</td>
<td>Maltoheptaose (47872)</td>
<td>94.0%</td>
<td></td>
</tr>
</tbody>
</table>

### Table 6.4  Details of α-amylase preparations

<table>
<thead>
<tr>
<th>Suppliers</th>
<th>Product description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Megazyme Ltd., Ireland</td>
<td>α-Amylase from porcine pancreatin (E-PANAA)</td>
</tr>
<tr>
<td>Sigma-Aldrich, Australia</td>
<td>α-Amylase from <em>Aspergillus oryzae</em> (A-6211)</td>
</tr>
<tr>
<td></td>
<td>α-Amylase from <em>Bacillus licheniformis</em> (A-4551)</td>
</tr>
</tbody>
</table>
### Table 6.5  Details of staining and embedding materials

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Product description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro Sci Tech, Australia</td>
<td>Procure S12 (Epon S12 substitute) (060110), NMA (Nadic Methyl Anhydride) (060329), BDMA (Benzylidimethylamine) (050804)</td>
</tr>
<tr>
<td>Merck, Germany</td>
<td>Agar extra pure, fine powder (1.016151000)</td>
</tr>
<tr>
<td>BDH Chemicals Ltd., England</td>
<td>Periodic acid 50 per cent (29460), thiose-micarbazide (TSC) (30420)</td>
</tr>
<tr>
<td>Johnson Matthey, England</td>
<td>Silver nitrate</td>
</tr>
</tbody>
</table>
### 6.2 Apparatus and auxiliary equipment

#### Table 6.6 Description of equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer</th>
<th>Model no</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH meter</td>
<td>Hanna Instrument Ltd., US</td>
<td>HI 8520</td>
</tr>
<tr>
<td>Overhead stirrer</td>
<td>Laboratory Supply P/L, Australia</td>
<td>0-10 LFA 0-318</td>
</tr>
<tr>
<td>Hot plate with magnetic stirrer</td>
<td>Industrial Equipment &amp; Controls Pty. Ltd., Australia</td>
<td>CH 2092-001</td>
</tr>
<tr>
<td>Masterflex® peristaltic pump</td>
<td>Barnant company division of Cole Parmer Instrument, US</td>
<td>7512-35</td>
</tr>
<tr>
<td>Analytical balance Adventurer Pro</td>
<td>Ohaus Corporation, US</td>
<td>Model: AR2140</td>
</tr>
<tr>
<td>Spray dryer, type Minor</td>
<td>Niro Atomiser, Denmark</td>
<td>1902</td>
</tr>
<tr>
<td>Spray dryer rotatory atomiser</td>
<td>Niro Atomiser, Denmark</td>
<td>CK 929</td>
</tr>
<tr>
<td>SPI-Module™ Sputter Coater Module</td>
<td>SPI Supplies, US</td>
<td>11430</td>
</tr>
<tr>
<td>Environmental scanning electron microscopy (ESEM)</td>
<td>FEI Company, US</td>
<td>FEI Quanta 200</td>
</tr>
<tr>
<td>Transmission electron microscope (TEM)</td>
<td>JEOL, USA</td>
<td>JEOL 1010</td>
</tr>
<tr>
<td>Particle size analyser Mastersizer X</td>
<td>Malvern Instruments, UK</td>
<td>MSX025A</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Eppendorf, Germany</td>
<td>5810R</td>
</tr>
<tr>
<td>Oven</td>
<td>Memmert, GmbH, Germany</td>
<td>Type: UML 500</td>
</tr>
<tr>
<td>Freeze dryer</td>
<td>Operon freeze dryer, Korea</td>
<td>FDB-5503</td>
</tr>
<tr>
<td>Rotary evaporator</td>
<td>Haidolph Laborota 4000 Efficient</td>
<td>31784501-316686</td>
</tr>
</tbody>
</table>
### Table 6.7  Description of equipment and columns used in HPLC analysis

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer</th>
<th>Model no</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Folic acid analysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPLC</td>
<td>Agilent, USA</td>
<td>HP series 1100</td>
</tr>
<tr>
<td>LiChroCART®</td>
<td>Merck Pty. Ltd., Australia</td>
<td>Lot L54000717 No: 511191</td>
</tr>
<tr>
<td>LiChrospher® RP-18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Column 5µm, 125mm x 4mm</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Reducing sugars analysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPLC</td>
<td>Waters Associates</td>
<td>18314</td>
</tr>
<tr>
<td>Differential Refractometer</td>
<td>Waters Associates</td>
<td>R401</td>
</tr>
<tr>
<td>LiChroCART®</td>
<td>Merck Pty. Ltd., Germany</td>
<td>Lot L321017 No: 821531</td>
</tr>
<tr>
<td>LiChrospher® RP-18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Column 5µm, 125mm x 4mm</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 6.8  Description of consumable items

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer</th>
<th>Cat no</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micropipette 1-10mL</td>
<td>Eppendorf, Germany</td>
<td>022472208</td>
</tr>
<tr>
<td>Micropipette 100-1000µL</td>
<td>Eppendorf, Germany</td>
<td>2540039</td>
</tr>
<tr>
<td>Micropipette 20-200µL</td>
<td>Eppendorf, Germany</td>
<td>022472054</td>
</tr>
<tr>
<td>Nylon filter 0.45µm pore size</td>
<td>LabServ, Biolab, New Zealand</td>
<td>SE4M019100</td>
</tr>
</tbody>
</table>
6.3 Laboratory procedures for reducing sugar analysis

6.3.1 DNS reagent method

Preparation of DNS reagent (adapted from Southgate, 1991)
3,5-Dinitrosalicylic acid (10g) was dissolved in warm NaOH (1M, 400mL). Sodium potassium tartrate (300g, Rochelle salt) was dissolved in warm Milli-Q water (300mL). Both solutions were cooled, then mixed and made up to 2L with Milli-Q water. This solution stored for up to six months in a dark bottle at ambient temperature.

Preparation of glucose standard solutions
Glucose stock solution (100mM) was prepared freshly with Milli-Q water from which a working solution of 1mM was made. For preparation of a standard curve, aliquots of the working solution were taken as described in Table 6.9.

Table 6.9 Graduated standard solution preparation

<table>
<thead>
<tr>
<th>Test tube no</th>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of working solution (mL)</td>
<td>0.50*</td>
<td>0.75</td>
<td>1.00</td>
<td>1.25</td>
<td>1.50</td>
<td>1.75</td>
<td>2.00</td>
</tr>
<tr>
<td>Volume of water</td>
<td>1.50</td>
<td>1.25</td>
<td>1.00</td>
<td>0.75</td>
<td>0.50</td>
<td>0.25</td>
<td>0.00</td>
</tr>
<tr>
<td>Concentration (mM)</td>
<td>0.25</td>
<td>0.38</td>
<td>0.50</td>
<td>0.63</td>
<td>0.75</td>
<td>0.88</td>
<td>1.00</td>
</tr>
<tr>
<td>Concentration - blank (mM)</td>
<td>0.00</td>
<td>1.30</td>
<td>0.25</td>
<td>0.38</td>
<td>0.50</td>
<td>0.63</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Note B Blank
* An additional 0.50mL of glucose solution was added to blanks, standard solutions and samples to compensate for the loss of reducing sugar when Rochelle salt is added.

Preparation of oligosaccharide standard solutions
Stock solutions of glucose, maltose, maltotriose, iso-maltotriose, maltotetraose and glycosyl maltotriose were prepared (10mM) with Milli-Q water from which serial dilutions of 0.0 to 0.9mM were made. Blank solution and all dilutions contained an additional 0.1mM of reducing sugar incorporated as described in Table 6.9.

Method adapted from Miller (1959) and Southgate (1991)
DNS reagent (2mL) was added to blank, standard solutions and samples. Each tube was vortexed, heated at 100°C for 15min and cooled down under tap water to standardise the
effect of temperature on the absorbance of coloured reaction products (Brodersen and Ricketts, 1949). The solutions were transferred to 10mm disposable cuvette and absorbance was recorded at 540nm ($A_{540}$) with slit width 0.06mm.

**Preparation of standard curve**

The standard curve was plotted using Microsoft Office Excel® and the scatter option, with $A_{540}$ as y axis and concentration of the respective reducing sugar (mM) as x-axis. A linear regression equation of the form $[y = mx + c]$ typically gave the best statistical fit and the equation as well as the $R^2$ value were recorded. The latter value was then considered and the analyses were repeated if the value was lower than 0.98.

**6.3.2 HPLC-RI**

**Preparation of standards**

D-glucose and oligosaccharide stock standards (5% m/v) were prepared with Milli-Q water and aliquots were frozen to prevent contamination. Prior to use, individual portions were thawed with intermittent shaking for 20min and injected into the HPLC-RI in parallel with each batch of samples run. Retention times of standards were recorded.

**HPLC-RI conditions**

Reducing sugar analysis using HPLC-RI was carried out with a Waters HPLC equipped attached to a differential refractometer. Separations were performed on a LiChroCART ® column (particle size 5µm, length 100mm and internal diameter 4mm). Mobile phase was Milli-Q water with a flow rate of 1mL/min. Peak identification was based on the retention time against standards injections.

**Sensitivity of detector relative to glucose standard (IUPAC, 1998)**

The validity of the aliquots of standard solutions was assessed by calculating the relative detector response factor ($f$) of the frozen glucose sub-standard over three different days against freshly made D-glucose standard. This determines the sensitivity of the detector with regards to a specific standard substance. Response factor was calculated by the following equation:
\[ f_i = \frac{A_i}{A_{st}} \times f_{st} \]

Where:

- \( f_i \) = Response factor of sample
- \( f_{st} \) = Response factor of standard with an arbitrary value of 100 was taken
- \( A_i \) = Peak area of sample
- \( A_{st} \) = Peak area of standard

**Linearity of standard (ICH, 1996)**

Glucose standard was used to confirm the linear response of glucose concentration against peak area. A stock solution of 100mM was prepared from which dilutions of 1, 2, 3, 4, 5, 6, 8 and 10mM were made. Milli-Q water was used as a blank. Linearity was evaluated by visual inspection of a plot of peak area as a function of glucose concentration using Microsoft Excel. From the linearity relationship, a regression line by least squares was calculated using Statistical Package for Social Sciences (SPSS).

**Determination of the detection limit (ICH, 1996)**

The detection limit of the HPLC-RI system was determined based on the signal-to-noise ratio. This method was chosen as it can be applied to analytical procedures which exhibit baseline noise. Slope-to-noise ratio was determined according to the approach described by Agilent Technologies Inc. (2008) using a blank solution. The difference between the maximum and minimum peak to peak noise of seven contiguous segments was determined. Each segment had 10% overlap and the seven consecutive values peak to peak noise were averaged to obtain the slope to noise ratio. Triplicates were performed and the detection limit and quantitation limit were calculated using the following formulas:

\[ DL = 3 \times S/N \]
\[ QL = 10 \times S/N \]

Where:

- \( DL \) = Detection limit
- \( S/N \) = Slope to noise ratio
- \( QL \) = Quantitation limit
6.3.3 Other methods – The modified method of Nelson and Somogyi (Mcclear & Glennie-Holmes, 1985)

Solution A
Anhydrous sodium carbonate (25g), potassium sodium tartrate (25g), sodium bicarbonate (20g) and anhydous sodium sulphate (200g) were dissolved in distilled water, filtered and adjusted to 1L. The solution was stored at >20ºC. If solution formed sediment after a few days, it was filtered without any adverse effect on the use of the reagent.

Solution B
Copper sulphate pentahydrate (30g) was dissolved in distilled water, concentrated sulphuric acid (4 drops) was added and the solution made up to 200mL. Solution B was stored in a stoppered dark glass bottle.

Solution C
Ammonium molybdate (50g) was dissolved in 900mL distilled water and concentrated sulphuric acid (42mL) was added. Sodium arsenate pentahydrate (6g) was dissolved in distilled water (50mL), added to the previous solution and made up to 1L. The mixed solution was incubated at 55ºC for 25-30min with occasional stirring to prevent sedimentation and then stored in a dark bottle.

Solution D (prepared fresh daily)
Solution D was prepared fresh daily at a ratio of 1:25 (solution B: solution A).

Solution E
Solution E was prepared by carrying out a 10-fold dilution of solution C. (e.g. solution C (5mL) made up to 50mL)

Preparation of glucose/ maltose standards
Stock solution and working solution of either glucose or maltose were prepared as described in section 6.3.1 for the DNS method. Standard solutions of 0.1, 0.2, 0.3, 0.4 and 0.5mM were prepared from the working solution.

Method (Mcclear & Glennie-Holmes, 1985)
Solution D (0.25mL) was added to sugar solution (0.3mL) in test tubes and incubated at 100ºC for 20min. The samples were cooled under running cold tap water and solution E
(3.0mL) was added. The mixtures were vortexed, left to stand for 10min before being read at 520nm ($A_{520}$).

6.4 Microencapsulation

6.4.1 Slurry preparation

A series of spray drying trials was carried out to assess the effect of changes in formulation on the particle size, morphology and folic acid release of the microcapsules. The formulations are presented in Table 6.10. Binding agents (0.2-8% w/w with respect to rice starch) comprising of a combination of the hydrocolloids ALG and LMP were hydrated with Milli-Q water by heating the mixture to 50ºC, cooled to room temperature and stirred constantly overnight. Rice starch (6.25-30% w/v) was dispersed in Milli-Q water using an overhead stirrer with gentle heating to 45ºC. The hydrated hydrocolloids were also heated to 45ºC and combined with the rice slurry for at least 30min to ensure homogeneity. The slurry was cooled to room temperature and pH adjusted to 8.0 with 1M KOH. Folic acid (0.5g) was added and the slurry covered with aluminium foil and mixed for at least 10min before the pH was readjusted to 8.0 with 1M KOH and the slurry fed to the spray dryer.

Table 6.10 Formulation of microcapsules

<table>
<thead>
<tr>
<th>Binding agents Type</th>
<th>Binding agents % (w/w)</th>
<th>Rice starch % (w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALG</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>LMP</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>1:1 ALG:LMP</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6.25</td>
</tr>
</tbody>
</table>

* Based on 500mL of Milli-Q water
6.4.2 Spray drying

The spray dryer was pre-equilibrated with an inlet temperature of 120°C and outlet temperature of 80-85°C. The slurry was fed at a constant flow rate of 7mL/min using a peristaltic pump and the atomiser was spun at a pressure of 5kg/cm². The slurry was kept under constant stirring using a stirring rod to prevent the settling of the rice starch. The spray dried microcapsules were collected in a pre-weighed glass jar. At the end of the run, the spray dryer chamber was brushed to collect microcapsules that had adhered to the inner surface side of the chamber walls and this material was stored in a separate glass jar. Yield was calculated using both glass jars as follows:

\[
\text{Yield (\%)} = \frac{\text{Weight of microcapsules}}{\text{Weight of rice starch}^* + \text{binding agents}} \times 100
\]

* Expressed as a dry weight basis

The sealed glass jars containing the microcapsules were immediately covered with aluminium foil and stored at ambient temperature until further analysis.

6.4.3 Calcium treatment of microcapsules

Microcapsules (4.0g) were suspended in CaCl₂ (25mL of 1M, 0.55M and 0.1M) for 1h at ambient temperature. Calcium coated microcapsules were vacuum filtered through a sintered glass filter disc with porosity grade 3 (pore size 15-40µm). Filtrates were retained for subsequent analysis of folic acid by RP-HPLC. Microcapsules were rinsed with 50mL ethanol followed by 50mL acetone prior to drying to 40°C for 1h. Samples were transferred to glass vials covered with aluminium foil and stored at ambient temperature until further analysis.

6.5 Analysis of folic acid

6.5.1 RP-HPLC

Folic acid recovery from microcapsules

Microcapsules and Ca-coated microcapsules (0.1g) were suspended in phosphate buffer (25mL of 0.1M) with L-ascorbic acid (0.1%) at pH 8.3 in a beaker covered with aluminium foil. The suspension was stirred continuously at constant speed with a magnetic stirrer for 30min for the microcapsules and 1h for Ca-coated microcapsules. The microcapsule
suspensions were filtered through 0.45µm syringe filters into amber vials and the filtrate analysed for folic acid by RP-HPLC with standard folic acid solutions in the range of 0 to 80ppm.

**Folic acid standard solution preparation**

Folic acid stock solution (400mg/L) was prepared in K₂HPO₄ (0.1M, pH 8.3) with L-ascorbic acid (0.1% m/v). The stock solution was stored in a bottle wrapped with aluminium foil at 4°C for up to three months. Working solution of 40mg/L was prepared freshly on the day of analysis for each run from which standard solutions (0-80mg/L) were made with 0.1M K₂HPO₄ buffer with 1% L-ascorbic acid. A linear calibration curve with a zero intercept was computed for each sample set.

**RP-HPLC conditions (Hau Fung Cheung, Morrison, Small, & Marriott, 2008)**

Folic acid assay was carried out using an HP series 1100 system. 20µL of sample was injected and separation was carried out using a LiChroCART® LiChrospher® RP-18 column (125mm length, 4mm internal diameter and 5µm particle size) controlled to a temperature of 25°C. Isocratic elution was set up with 90:10 of 1% (v/v) acetic acid:acetonitrile as mobile phase and a flow rate of 1mL/min. Detection was performed using a UV detector set at 280±2nm.

**General procedures applied in analysis of folic acid**

Due to the sensitivity of folic acid, all procedures involving the vitamin were performed in subdued light. All glassware was covered with aluminium foil and stoppered or amber glassware were also used wherever possible. Unless otherwise indicated, all steps in analytical methods were performed without delay.

**Validation of folic acid analysis method**

During the development and establishment of the methods, the initial approach was to measure standard solutions of folic acid. Moreover, the extraction procedures were validated with the addition of a spike with appropriate amounts of the standard folic acid prior to extraction. Recovery was determined as follows:

\[
\text{Recovery (\%)} = \frac{\text{Folic acid in spiked sample} - \text{Folic acid in unspiked sample}}{\text{Folic acid added in spiked sample}} \times 100
\]
6.5.2 Absorbance spectrum of folic acid

An initial verification of the folic acid stock solution prior to sample runs was carried out by obtaining the absorbance spectra of a known concentration of folic acid solution at pH 7.0 in conjunction with the following equation:

\[ c = \frac{A}{\varepsilon \times l} \]

Where:

- \( A \) = Absorbance at \( \lambda_{\text{max}} \) 282nm and 350nm
- \( \varepsilon \) = Molar absorptivity (mol/L) of 27000 at \( \lambda_{\text{max}} \) 282nm and 7000 at \( \lambda_{\text{max}} \) 350nm (Dawson, Elliot, Elliott & Jones, 1991)
- \( c \) = Concentration of folic acid
- \( l \) = Length of solution the light passes through denoted as 1cm

6.6 \textit{in-vitro} Digestion of starch and starch based microcapsules


A 1L beaker containing phosphate buffer (800mL of 0.02M), NaCl (0.03M) and, when needed, CaCl\(_2\) (0.03M) at pH 6.9 was equilibrated at 37ºC and stirred with a magnetic stirrer (Bernfeld, 1955, Jenkins \textit{et al}.., 1984 and Woolnough \textit{et al}.., 2008). DT (15cm lengths) was soaked and washed gently with deionised water. The tubing was tied at one end and the sample (2g, starch or microcapsule) was added (Koh \textit{et al}.., 2009 and Scazzina \textit{et al}.., 2009). Phosphate buffer (10mL of 0.02M), NaCl (0.03M) and, when needed, CaCl\(_2\) (0.03M) was transferred into the tubing (Koh \textit{et al}.., 2009). \( \alpha \)-Amylase (5mL of 250, 1000 or 5000 Sigma units) was added. The DT was sealed at the other end, inverted repeatedly to achieve uniform mixing, attached to the end of a long glass rod with rubber bands and placed into the pre-equilibrated beakers for 3h (Patty, 2009). An aliquot (0.5mL) from the dialysate (outside the DT) was taken prior to DT insertion and immediately after insertion for time 0 and thereafter every 30min for 3h.

Reducing sugar quantification by DNS reagent method adapted from Miller (1959) and Southgate (1991)

Dialysed aliquots were pipetted into a test tube containing glucose solution (0.5mL of 1mM) and quantification was carried out as per the DNS reagent method described in section 6.3.1.
6.6.2 Determination of reducing sugar release by DNS reagent method

Rate of reducing sugar release
The concentration of reducing sugar was calculated using the regression equation for the standard curve (expressed in mM). A scatter plot was computed with time (min) as x axis and release of reducing sugar (mM) as y axis. Typically a linear regression line was obtained in the form of \( y = mx + c \) with \( m \), the gradient taken as the comparative rate.

Reducing sugar release post in vitro digestion
From the equation obtained from rate of reducing sugar release referred above, reducing sugar release (\( y \), mM) after 3h of digestion was calculated by substituting \( x \) by 180min. Then, release was calculated as follows:

\[
\text{Reducing sugar (mMoles/g starch)} = \frac{y \times 0.8 \times M_w \times W_s}{1000}
\]

Where:
- \( y \) = Reducing sugar release after 180min
- 0.8 = Conversion to 800mL of dialysate
- \( M_w \) = Molecular weight of reducing sugar (e.g. glucose = 180.16)
- \( W_s \) = Weight of starch sample digested adjusted to dry weight basis
- 1000 = Conversion of mMoles to moles

Calculation of reducing sugar release to a dry weight basis
The results obtained for contents of each of the folic acid and reducing sugar release from starch samples were routinely adjusted by calculation to a dry weight basis. The purpose was to facilitate the direct comparison of the results particularly for different sample types. Starch and microcapsules samples were tested for moisture content and then the following general equation was applied:

\[
\text{Weight of starch/microcapsules (adjusted to a dry weight basis)} = \frac{\text{Weight of starch/microcapsules (as is basis)}}{\frac{100}{100 - \text{actual moisture content of sample}}}
\]
6.6.3 Determination of reducing sugar release post in-vitro digestion by HPLC-RI

Sample preparation
Dialysate samples were concentrated 15 fold using a rotary evaporator set at 60°C. For reducing sugar analysis inside the DT, freeze dried samples stored at -18°C (different from freeze dried samples used for morphological analysis) were dispersed in 5mL of Milli-Q water. Contents of DT were treated with a mixture of adsorbent resins (2g of 1:1 Amberlite resin IR-45(OH): Amberlite resin IR-120(H)) for 30min prior to being filtered through at 0.45µm syringe filter while the concentrated dialysate samples were treated twice with the adsorbent resin mixture. The samples (20µL) were then injected in the pre-equilibrated HPLC-RI.

Calculations
The amount of reducing sugars released from the samples was calculated using the external standards. At both the start and end of a series of HPLC measurements, at least a replicate analysis of the standard test solutions was performed and the average peak areas were calculated. These were then compared with those of the sample test solutions. For this comparison, the weighed starch sample adjusted to a dry weight basis, the concentration factor and molecular weight of individual reducing sugar were considered.

Reducing sugar (mMoles/g starch) = \[ \frac{P_s \times M_{st} \times V_d}{P_{st} \times V_{st} \times M_w \times CF \times 1000} \]

Where:

- \( P_s \) = Peak areas of reducing sugars sample test solution
- \( P_{st} \) = Peak areas of reducing sugar in standard test solution
- \( V_{st} \) = Volume of standard test solution
- \( M_{st} \) = Mass of standard taken for analysis
- \( M_w \) = Molecular weight of reducing sugar
- \( V_d \) = Volume of dialysate (800mL)
- \( CF \) = Concentration factor
- 1000 = Conversion of moles to mMoles
Validation of concentration method

Concentration method was validated by concentrating a known concentration of a product similar to the dialysate profile and maltodextrin Fieldose 17 (M17, 0.5% m/v) was chosen. Then, the concentration factor was calculated using peak areas for maltose as follows:

\[
\text{Concentration factor} = \frac{\text{Mean peak area of concentrated sample}}{\text{Mean peak area of original sample}}
\]

Verification of the effect of adsorbent resins on buffer adsorption and oligosaccharides recovery

To verify the effectiveness of the adsorbent resins in removing the interference peak from the phosphate buffer, four solutions of M17 were prepared as presented in Table 6.11 and, when required, treated with the adsorbent resins as described in section 6.3.3 (sample preparation). The solutions were filtered through a 0.45µm pore size nylon syringe filter and analysed using the HPLC-RI system and oligosaccharides recovery was determined.

Table 6.11 Preparation of M17 solutions

<table>
<thead>
<tr>
<th>Solution no</th>
<th>Diluent</th>
<th>Concentration (% m/v)</th>
<th>Adsorbent resin treatment carried out?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Milli-Q water</td>
<td>10</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Phosphate buffer (0.02M) containing NaCl (0.03M) andCaCl₂ (0.03M)</td>
<td>10</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

6.7 Starch morphology prior to and upon digestion by α-amylase

6.7.1 ESEM

Immediately after 3h of digestion, the contents of the DT were transferred into 250mL round bottom flasks with several rinsing steps to ensure complete transfer, sealed with aluminium foil and frozen using liquid nitrogen. The samples were freeze dried overnight and transferred to an airtight container. Then, each sample was mounted on a copper stub with double-sided carbon tape and gold coated with a gold sputter coater. The stubs were secured onto the ESEM stand and examined at an accelerating voltage of 5–7kV and probe current of \(6 \times 10^{-11}\) under low vacuum. In spite of viewing under low vacuum which leads
to minimal heat damage of the starch granules, gold coating was found necessary to achieve images of higher resolutions.

6.7.2 TEM

Method (adapted from Planchot et al. (1995) and Thiery (1967))

Agar (3% w/v) was heated to 60ºC until dissolved, cooled to 50ºC and transferred to petri dishes to form a thin layer. The agar was cooled to approximately 45ºC and the sample (native starch or freeze dried digested samples) was scattered on top of the agar. A second thin layer of agar was added and the plates inverted and left to cool until the agar had set. Cubes of approximately 1mm³ were cut with a metal scalpel.

Sample staining

Samples within the agar cubes were treated with periodic acid (1% w/v) for 30min followed by several washes with distilled water (2 quick and 3×10min). They were then treated with saturated thiose-micarbazide (TSC, 10% in distilled water or 1% in acetic acid) for 30-40min followed by washes in acetic acid (10%, 2 rapid and 3×20min). Samples were then washed with decreasing concentrations of acetic acid (5% and 2%) followed by distilled water (2 rapid and 3×20min). The agar cubes were then treated with AgNO₃ (1% w/v, PATAg treatment) for four days in the dark. After the staining process, the samples were filtered from the AgNO₃ and washed with an ethanol series (four times) and left to dry on filter paper in a desiccator overnight. For control, periodic acid or TSC was omitted.

Embedment in Procure S12 (Epon S12 substitute)

Resin was prepared with PROCURE S12 (9g, resin), NMA (7.5g, hardener) and BDMA (0.3g, accelerator). The materials were mixed by tilting the container back and forth for 1min to prevent the introduction of air bubbles. Silicone moulds were half-filled and resins and baked at 60ºC for 50-60h. Then the cured resins were removed from the oven and cooled. The stained samples were placed top of the cured resin and were carefully covered with raw resin mixture and baked again at 60ºC for a further 50-60hours. Excess resin mixture was stored in a stoppered PVC container at 4ºC for up to one week.
Cross-sectioning by ultramicrotomy and TEM conditions (Hagler, 2007)

Before the embedded samples could be sliced, excess resin around the samples was trimmed using a metal scalpel. The embedded sample was secured into the Leica Ultracut microtome and a glass knife set at 45° was used to cut away excess resin from the cutting face until the sample just protruded. The glass knife was replaced with a diamond knife and the collection boat was filled with distilled water. Cutting speed of 0.5mm/s was set and sections of less than 0.1µm were obtained and verified by the interference colours observed in the floating sections. Slices were scooped and transferred to strong mesh copper grid and air dried overnight. The mounted grids were examined using a JEOL 1010 TEM with accelerating voltage set at 100kV.

6.8 Particle size analysis by laser diffraction adapted from (Cornell, Hoveling, Chryss, & Rogers, 1994)

6.8.1 Laser diffraction settings and definitions

Presentation: the mastersizer involves two main stages in the measurement of a sample. Firstly, it captures the scattering of light patterns of the particular sample i.e. carries out a measurement. Secondly, the data measured is interpreted by a predetermined presentation which involves predicting scattering pattern of the sample from theoretical particles. There are three presentations available from the Mastersizer X.

2$S$D – This represents the measurement in the simpler Fraunhofer model.

2OHD – This is a standard wet presentation that assumes that the sample is dispersed in water and hence takes an in between value for refractive index and adsorption of the sample.

2RHA – this is a standard dry presentation similar to 2OHD but assumes that the sample is suspended in air instead.

Analysis refers the choice of analysis mode. A polydisperse model was selected as this model does not assume anything about the shape of the result graph. Other models are also available including multimodal model which assumes that there will be one or more distinct peaks in the resulting graph thereby indicating several distinctive sizes of particles. On the other hand, a monomodal model assumes there will be only one distinctive peak and hence only one size of particle.
**Obscuration** is a measure of light lost through the incorporation of the sample thereby aiding in controlling the concentration of the sample when added to the dispersant. An obscuration of 10-30% is ideal.

**Residual** indicates how well the data analysed was fitted to the measurement data. A good fit would have result in a residual of less than 1%. A residual above 1% would indicate incorrect presentation of data.

**Distribution** indicates the type of distribution the analysis has used. Fundamentally, the Mastersizer carries out a volume distribution. Yet, the volume distribution can be modified (modification) to a surface area, number or length distribution through a mathematical process which will inherently impart higher error to the original results.

**Concentration** is calculated through the Beer-Lambert law which states that there is a linear relationship between absorbance and concentration of an absorbing species. It follows the equation \( A = \varepsilon \times b \times c \), where \( A \) is the Absorbance, \( \varepsilon \) the wavelength-dependent absorptivity coefficient, \( b \) is the path length, and \( c \) is the analyte concentration.

**Statistics of the distribution**
Along with the setting available, statistical results of the distribution can be obtained. These are:
\( D(v, 0.1) \) - the size of particles for which 10% of the sample is below this size.
\( D(0, 0.5) \) - the size of particles for which 50% of the sample is below and above this size. It is also known as the Mass Median Diameter (MMD).
\( D(0, 0.9) \) - the size of particles for which 90% of the sample is below this size.
\( D(4, 3) \) - is the volume mean diameter.
\( D(3, 2) \) - is the surface area mean diameter also known as Sauter mean.

**Uniformity** refers to the absolute deviation from the mean.

**SSA** stands for Specific Surface Area calculated by dividing the total area of the particles by the total weight.
Span is a measurement of the width of the distribution and the smaller the span, the narrower the width. It is calculated as follows:

\[
\frac{D(0, 0.9) - D(0, 0.1)}{D(0, 0.5)}
\]

6.8.2. Analysis of starch samples (wheat, maize, rice and potato)

Particle size analysis was carried out using a Mastersizer X equipped with a 45mm lens with a reading size range of 0.1 to 80µm. Swelling of starch particles was considered and preliminary trials (not mentioned in the thesis) has shown comparable results between Milli-Q water and iso-butanol as dispersant. Hence, the flowing cell was rinsed several times with the dispersant, Milli-Q water. Starch samples were mixed with a spatula at least 20 times. The system was aligned and a background reading was taken before each sample was analysed. Duplicate samples were suspended in the dispersant and the amount of sample was adjusted so that an obscuration close to 30% was obtained. Duplicate readings were taken for each analysis.

6.8.3 Analysis of Microcapsules

The same procedure was followed as for starch samples but a stirring cell was used instead of a flowing cell. iso-Butanol was used as the dispersant to minimise microcapsule dissolution and the Mastersizer X was equipped with a 100nm lens with a reading range of 0.5 to 180µm. Duplicate sub-samples were analysed and in addition with duplicate readings taken for each treatment or batch of samples.

6.8.4 Evaluation and interpretation of results

The information obtained from the light scattering of the samples was collected and evaluated using the Malvern software data system Version 3.10. A polydisperse analysis model, a standard-wet presentation (2OHD) and a volume distribution were chosen. A residual of <1% was confirmed to indicate correct presentation of data. The median (D(0,0.5) and uniformity (absolute deviation from the median), span and graph were presented unless otherwise specified.
6.9  Granule size separation by sedimentation adapted from Dhital, Shrestha and Gidley (2010))

Wheat starch samples were separated into two main fractions based on a sedimentation procedure and using the pipette technique. Starch (20g) was weighed and made up to 1L with Milli-Q water. Using a hand stirrer, the starch was dispersed with lateral movement based on Stokes law for 30s. The mixture was then left undisturbed for 61min and the top 10cm of the mixture was decanted using a 10mL pipette connected to a vacuum inlet and an adjustable Drechsel head. The remaining mixture was filled to 1L again and the procedure was repeated 13 to 15 more times, until the top layer was clear. The decanted mixture was left to settle overnight until all starch was deposited. Water was decanted and the remaining mixture was frozen with liquid N₂ and freeze dried overnight. Samples were dispersed again in Milli-Q water and checked for particle size using the Mastersizer X equipped with a 45mm lens. The sedimentation time based on Stokes law was determined by the following equation:

\[ t = \frac{18 \eta h}{g(\rho_s - \rho_w)X^2} \]

Where:

- \( t \) = Sedimentation time (s)
- \( \eta \) = Viscosity of water at 20°C (1.003 ×10⁻³ Pa s)
- \( h \) = Sedimentation height (10⁻¹m)
- \( g \) = Acceleration due to gravity (9.8m/s²)
- \( \rho_s \) = Density of starch (1500kg/m³)
- \( \rho_w \) = Density of water (998.23kg/m³)
- \( X \) = Particle diameter (10⁻⁵ m)
6.10 Other tests

6.10.1 Moisture content (Method 925.10, AOAC (2002a))

The moisture content of native starch, starch fractions, microcapsules and food products was carried out using the air oven method. Empty dishes and lids were placed in the pre-equilibrated air oven at 130±3°C for 1h. The dishes were immediately placed into an air-tight desiccator until cooled to room temperature (30min). Samples (2g) in duplicate were weighed into each empty metal dish before the dish containing the sample (uncovered) and the lids (separately) were returned to oven for 1h. Dishes were covered while still in the oven and transferred to a desiccator and weighed soon after reaching room temperature (45min cool down). The heating, cooling and weighing steps were repeated until no further change in mass. The moisture content for native starch, starch fractions, microcapsules was calculated using the equation as follows:

\[
\text{Moisture content (\%)} = \frac{\text{Loss in weight of sample upon drying (g)}}{\text{Initial weight of sample (g)}} \times 100
\]

6.10.2 pH (Method 945.42, AOAC 2002c)

The samples were prepared as per section 6.8.1. pH determination was carried out following the AOAC method 945.42 which referred to AOAC method 943.02 (AOAC 2002b). Samples (10.0g, in duplicates) were weighed into a dry Erlenmeyer flask and 100mL of recently boiled distilled water cooled to 25°C was added. The flask was shaken until particles are evenly suspended and mixture free of lumps. The mixture was digested for 30min with frequent shaking and left to stand for 10min. The supernatant was decanted into a 250mL beaker and immediately measured for pH using a pre-calibrated pH meter.

6.11 Duplication and presentation of analytical results

In the analysis of samples for vitamin content, moisture content, median particle size and reducing sugar content and where duplicate analyses was performed, the results have been presented as mean values ± their relative standard deviation (RSD) or coefficient of variability. These calculations were carried out using Microsoft® Excel 2000 software. The coefficient of variability of a series of values was also calculated using the following formula:
RSD (%) = \frac{SD}{mean} \times 100

6.12 Statistical analysis

SPSS version 17.0 was used for all statistical analysis. Independent samples t-test was used to compare the mean scores of two different conditions. One-way analysis of variance (ANOVA) at 5% level of significance (p<0.05) was applied. Where significance difference was observed, a post-hoc test using Tukey’s test was applied to compare multiple results.
Chapter 7

Results and discussion:
Development and validation of sugar analysis procedures

The purpose of this chapter is to provide a very brief background on the selection of methods applied for the measurement of reducing sugars and the specific challenges associated with the analyses. In addition, the results obtained during the evaluation and validation of procedures for sugar analysis are discussed. These include the use of the Nelson and Somogyi procedure, the DNS reagent method as well as HPLC with RI detection (HPLC-RI).

7.1 Introduction

The analysis of malto-sugars can be performed by a variety of methods depending on the required outcome. Generally, methods including DNS reagent as well as Nelson and Somogyi are applied to determine the total amount of sugars based on their reducing properties. Thus, these methods can only be used for quantification of reducing sugars. However, with the popularity of instrumental assays particularly HPLC, identification of sugars as well as quantification is made possible with satisfactory analyte separation. The validation of HPLC procedures also involves the minimisation of challenging interference and reducing run times.

7.2 DNS reagent method

This method involves a redox reaction whereby the aldehyde groups of aldose sugars are oxidised to their respective sugar acids and other products while 3,5-dinitrosalicylic acid is reduced to 3-amino, 5-nitrosalicylic acid under alkaline conditions (Figure 7.1). The product of this reaction, being a reddish brown colour forms the basis of quantitative analysis of reducing sugar by a spectrophotometric assay (Coultate, 2009). The reaction is believed to occur through a series of side reactions, being dependent on the exact type of reducing sugar as well as the conditions. Hence, a number of short chain carboxylic acids are also produced (Coultate, 2009). Different reducing sugars result in different colour intensities, making it necessary to calibrate for each of them (Miller, 1959).
Preparation of DNS reagent and optimisation of reaction as described by Miller (1959) includes Rochelle salt (sodium potassium tartrate) to stabilise colour intensity by preventing dissolution of oxygen. However, its addition interferes with the protective action of sulphites. This problem can be solved by adding a small, known amount of glucose into each analysis tube, that is, blanks, standards and samples or by adding the Rochelle salt after colour development (Miller, 1959).

### 7.2.1 Analytical curve with DNS reagent

In the initial stages of this investigation, the use of DNS reagent was trialled and a typical standard curve is presented in Figure 7.2. It was consistently observed that an exponential trend rather than a linear relationship was obtained in the early experiments. Based upon previous reports, the problem of the non-linear curve could be resolved by modifying the procedure whereby an extra 0.5mL of the working solution was added to the blank and all of the standard solutions (Miller, 1959). The resultant analytical curve (Figure 7.3) then followed a linear relationship with $R^2$ greater than 0.99. The preparation of the curve was repeated many times and it was consistently observed that there was a high degree of linearity as well as consistency from analysis to analysis over a period of a number of weeks. Therefore the use of a one-point calibration curve was adopted for the further analysis using this procedure. However, it is emphasised that due to the stock preparation of DNS reagent and the degradation of the oxidising properties of the DNS reagent over time, calibration was carried out at the same time and under the same conditions for each analysis.
Figure 7.2  Analytical curve of D-glucose by DNS reagent obtained in initial trials, demonstrating a lack of linearity
Note  Each point is an average of 3 determination between runs

Figure 7.3  Corrected analytical curve of D-glucose by DNS reagent in which a fixed aliquot of glucose was incorporated into each tube
Note  Each point is an average of 3 determinations
7.2.2 The application of DNS reagent to the analysis of oligosaccharides

In the context of the plan to apply the procedure to partial hydrolysates of starch, the response of the DNS reagent to increasing degree of polymerisation (DP) was investigated. Equimolar concentrations of a series of reducing sugars were used as these contained the same amount of reducing ends from the sugar solutions as opposed to a mass per volume concentration whereby higher sugar solutions would contain fewer reducing ends. The results obtained in this study (Figure 7.4), showed that different colour intensities were obtained when comparing glucose, maltose, maltotriose and maltotetraose responses, thereby supporting recent claims of a stronger colour reaction to DNS reagent with increasing DP. The current data corroborates those of (Saqib & Whitney, 2011) who highlighted a similar pattern of a higher colour intensity from disaccharides when compared to monosaccharides.

In the original report, Miller (1959) suggested that such an effect could be due to the some of the higher oligomers being hydrolysed under the influence of the DNS reagent in conjunction with the heating process. From their findings, Saqib and Whitley (2011) observed that even though the selected disaccharides had different monomers and bonding, similar response was still obtained thereby leading them to question the hypothesis of Miller. From this current study of malto-oligosaccharides, this pattern continued with increasing DP up to four. Thus, the colour intensity from DNS reagent is not strongly impacted by the nature of the reducing sugar but rather by its DP, consistent with the earlier hypothesis proposed by Miller (1959).
Figure 7.4  DNS reagent response to increased DP of malto-oligosaccharides

When comparing equimolar isomeric form of maltotriose (DP3, Figure 7.5), a lower response rate was observed with iso-maltotriose (DP3) as opposed to linear maltotriose (Figure 7.6). Instead, a branched DP4 (glucosyl maltotriose, Figure 7.7) showed higher colour intensity with DNS reagent in comparison to linear DP4 (maltotetraose). Thus, the different structure of oligosaccharides also affects the response to DNS reagent. It is therefore emphasized that as the reaction of the DNS reagent with reducing sugars is not a one step reaction but rather a succession of reactions. Thus, depending on the structure of the reducing sugar, a different degree of oxidation was achieved reflecting varying degrees of partial hydrolysis of the reducing sugars and hence the stability of the end products.

Figure 7.5  The structure of malto sugars with DP3
A: Maltotriose  B: Iso-maltotriose (joined by an α-(1→6) glycosidic linkage)
Figure 7.6  DNS reagent response to for malto-oligosaccharides of DP3 and 4
Note  Each point is an average of 3 determination

Figure 7.7  The structure of glucosyl maltotriose (DP4)

7.3  Comparison of DNS method with the modified Nelson and Somogyi method

Another traditional reducing sugar method that has found application in research is that originally described by Nelson (1944) and Somogyi (1937, 1952) and utilised by McClear and Glennie-Holmes (1985). Standard curves were analysed using this and the DNS procedure and the results are compared in Figure 7.8. A lower slope was consistently obtained with the Nelson and Somogyi method (McClead and Glennie-Holmes, 1985) as compared to the DNS method. It is noted that whereas a small amount of glucose was required in the DNS procedure to achieve linearity of the standard curve, this was not necessary for the Nelson and Somogyi method. However, the preparation of only one
The DNS reagent makes the DNS reagent method more convenient as compared to the preparation of five solutions (solutions A, B, C, D and E) for the modified Nelson and Somogyi method.

![Graph comparing Nelson and Somogyi method and DNS reagent method](image)

**Figure 7.8** Comparison of Nelson and Somogyi method and DNS reagent method

### 7.4 Introduction to the analysis of sugars using HPLC

Over the decades, a wide range of methods has been developed and applied for the determination of sugars in foods. In the early days, as attempts were made to separate individual sugar components, open column chromatography, paper chromatography and thin layer chromatography were trialled and found to be labour intensive, with varying degrees of usefulness reported for the various approaches. More recently, automated HPLC, GC (Gas chromatography) and HPCE (High pressure capillary electrophoresis) have become popular reflecting reduced turn around times as well as greater specificity and precision especially for quantitative analysis (Peris-Tortajada, 2000; Hau Fung Cheung, Marriott & Small, 2007). In recent years, HPLC has become the favoured instrumental method of sugar analysis over GC techniques partly due to the omission of a derivatisation step required for GC (Molnár-Perl, 2000; Ruiz-Matute, Hernández-Hernández, Rodríguez-Sánchez, Sanz, & Martínez-Castro, 2011; Sanz & Martínez-Castro, 2007), higher recoveries with greater accuracy and shorter analysis times (Peris-Tortajada, 2000). However, GC continues to be favoured for monosaccharides analysis and has the potential advantage of providing differentiation between α and β anomers.
7.4.1 HPLC-RI

Traditionally, quantitative analysis of sugars involved the colorimetric assay from reaction with chemicals e.g. anthrone using UV detection. Now, most commonly, refractive index (RI) detectors or refractometers are employed without the need to chemically modify the sugar samples. According to Peris-Tortajada (2000), the challenge does not reside in the HPLC separation which can be generally achieved successfully but rather from the sample preparation, especially in highly processed foods where it might be difficult to obtain suitable concentrations of the analyte with minimal interference. Polar molecules including phospholipids, shorter chain fatty acids and amino acids account for most of the interference with the column and hence an appropriate extraction and clean-up is necessary. It is also important that the correct extracting solvent is chosen thereby allowing the solubilisation of the analyte(s) with minimal solubilisation of the interfering non-analytes. In this context, the most widely used extractants include ethanol and water as well as mixtures of these, although for increased selectively methanol is used for larger carbohydrates.

Nevertheless, in the case of high interference, complementary sample pre-treatment is required including liquid-liquid extraction, protein precipitation, ultrafiltration, dialysis, the use of solid phase extraction (SPE) e.g. sep-pak cartridges, ion exchangers and column switching (Peris-Tortajada, 2000; Turnell & Cooper, 1987).

RI detection is the most widely used detection for carbohydrates, partly because they are universal and useful for analytes without strong UV chromophores, fluorophores or electrochemical or ionic activity. As such, RI detectors are relatively non-specific and have comparatively lower sensitivity, although it has been reported that sensitivity can be greatly enhanced by adequate sample preparation (Peris-Tortajada, 2000). It has often been observed that changes in temperature and pressure can cause baseline instability. In addition, the composition of solutes affects detection and so, for example, salts would elute peaks which might interfere with the peaks of interest. Furthermore, as RI detection involves a reference cell, i.e. the measurements are of the difference in RI between the reference cell containing the solvent and the sample cell, gradient elution cannot be carried utilised.
7.4.2 Optimisation of HPLC-RI

Validity of standards

In establishing HPLC procedures for use in the current study various steps were taken to establish the validity of the approach. Firstly, due to the number and relative expense of some of the standards involved, a stock solution of each of these was prepared and subsamples stored at -18ºC. The reliability of this approach was assessed based on trials in which subsamples were thawed, run on the HPLC and the response factor of the thawed glucose standard compared against freshly prepared solutions were assessed over three consecutive days. The results are reported in Table 7.1, and on this basis, D-glucose solutions were recovered with a response factor \( \bar{f} \) of greater than 98% hereby validating the freezing, storage and thawing process of D-glucose standard solution. An independent-samples t-test was also performed and it was confirmed that stored substandard were not significantly different from fresh D-glucose standards \( (p=0.9) \) with the magnitude of the differences in the mean being very small (estimated square=0.009). It was therefore concluded that the approach of storing prepared standard solutions in the frozen form was appropriate and this was applied throughout the course of the current study.

Table 7.1 Assessment of use of thawed standard solutions of D-glucose

<table>
<thead>
<tr>
<th></th>
<th>D-Glucose</th>
<th>Concentration(^1) (% m/v)</th>
<th>Mean(^2) peak area</th>
<th>SD (peak areas)</th>
<th>RSD</th>
<th>( \bar{f} )(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 1</strong></td>
<td>Fresh</td>
<td>0.506</td>
<td>61157.0</td>
<td>4.2</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thawed</td>
<td>0.502</td>
<td>61170.0</td>
<td>530.3</td>
<td>0.9</td>
<td>100.0</td>
</tr>
<tr>
<td><strong>Day 2</strong></td>
<td>Fresh</td>
<td>0.510</td>
<td>60366.0</td>
<td>677.4</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thawed</td>
<td>0.502</td>
<td>59414.5</td>
<td>405.2</td>
<td>0.7</td>
<td>98.4</td>
</tr>
<tr>
<td><strong>Day 3</strong></td>
<td>Fresh</td>
<td>0.502</td>
<td>57656.5</td>
<td>208.6</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thawed</td>
<td>0.502</td>
<td>58187.5</td>
<td>58.7</td>
<td>0.1</td>
<td>100.9</td>
</tr>
</tbody>
</table>

Note 1 Concentration of solution as prepared  
2 Mean was obtained from duplicate analyses  
3 Response factor of thawed standard against freshly made standard solution
7.4.3 Linearity of standard curve for HPLC of glucose

A series of dilutions of D-glucose solution was prepared and these confirmed the linearity of the response and that the Beer-Lambert law is followed (Figure 7.9). Based upon the linearity consistently observed for the linearity of D-glucose, a single known concentration of standards was used for characterisation of the samples for each run. The elution of D-glucose occurred between 1.03 to 1.07 min with satisfactory baseline (Figure 7.10).

![Linearity Graph](image)

**Figure 7.9** A typical analytical curve obtained for D-glucose run on HPLC

Note: Each point is an average of 3 determinations

![Chromatogram](image)

**Figure 7.10** The chromatographic patterns for D-glucose standards on HPLC for concentrations of up to 10 mM
7.4.4 Identification of malto-oligosaccharides during HPLC

When various standard oligosaccharides were run on HPLC, the chromatograms obtained from the standard solutions were suitable for identification of samples containing mixtures of sugars, based upon their retention times (Figures 7.11 and 7.12). With the specific column used for this analysis, separation of oligosaccharides was achieved with longer retention times corresponding to an increase in DP. Peak shape was particularly satisfactory for DP 1 to 3. However, DP4 (maltotetraose) and DP5 (maltopentaose) gave double peaks while DP6 and DP7 had broader peaks. It has been reported that double peaks can be due to flow disturbances as a consequence of the presence of voids in the column, the solution following different paths, a poorly packed bed, high pH of the silica packing material or partially plugged frit (Agilent Technologies, 2008). As the double peaks were not observed for DP<4, it is likely that the bulkier molecular structure of the solution adhered poorly with the packing material resulting in the solution reaching the stationary phase at different times as if two injections took place and hence, depicting double peaks, one slightly delayed relative to the other.

Figure 7.11 Superimposed chromatographic patterns for various standard sugars run separately on HPLC

Note 1 Concentration used was 0.5% (m/v) for each sugar
2 Each peak is an average of 3 determinations injected twice between runs
3 Average retention time is represented in brackets for each sugar
Figure 7.12  Chromatographic data for standard sugars showing the DP of each standard

Note 1  Concentration used was 5mM for each sugar
2  Each peak is an average of 3 determinations injected twice between runs
3  Each standard was injected individually

A more likely explanation is that the double peaks observed in some cases could be caused by the presence of one or more other isomeric forms of the oligosaccharide, and that the standard did not solely contain linear maltotetraose. This would be consistent with the delayed retention time of the second peak similar to the pattern observed in the case of the two DP3 sugars maltotriose and iso-maltotriose (Figure 7.13). Thus, to elucidate this explanation, a solution of the DP4 isomer, glucosyl-maltotriose was chromatographed under the same conditions and compared with the linear form of the molecule (Figure 7.14).
Figure 7.13 The chromatographic patterns for DP3 oligosaccharides
Note 1 0.5% (m/v) standard sugar solutions were injected
2 Each peak is an average of 3 determinations

Figure 7.14 The chromatographic patterns for DP4 oligosaccharides
Note 1 0.5% (m/v) standard sugar solutions were injected
2 Each peak is an average of 3 determinations

Figure 7.14 clearly demonstrates that glucosyl maltotriose eluted at a later time than maltotetraose but did not show the double peak obtained from the maltotetraose chromatograms. Therefore, the maltotetraose standard solution appears unlikely to contain glycosyl maltotriose in sufficient amounts to be readily detected by the HPLC system. In addition, even though a broad peak was obtained for glycosyl maltotriose consistent with the hypothesis regarding adhesion to the stationary phase of the column, a distinct high peak (2.13min) preceded by a smaller one (1.9min) was observed. The purification of
higher DP of malto sugars is laborious and difficult thereby possibly causing a degree of contamination with other isomeric forms of the reducing sugar. Thus, it remains possible that the secondary peak obtained at 1.96min in the standard maltotetraose could indicate the presence of the third isomeric form of DP4, maltosyl maltose (Figure 7.15).

![Structure of maltosyl (1→6) maltose](image_url)

**Figure 7.15  Structure of maltosyl (1→6) maltose**

### 7.4.5 The detection and quantitation limits for the HPLC system

One of the parameters in validating a method is the determination of its sensitivity by calculating its detection and quantitation limits. Detection limit provides an estimate of the minimum amount of the analyte that can be realistically detected while the quantitation limit estimates the minimum reportable concentration of the analyte from the specific analysis. In so doing, a higher confidence of reportable values can be achieved particularly when assessing low concentration of analytes. Detection limit was evaluated based upon the measurement of the slope to noise ratio of blank solutions as described by Agilent Technologies Inc. (2005). A typical measurement is shown in Figure 7.16. From the chromatogram, the detection limit was 109.9 ± 6.4mV equivalent to a peak area of 819 and concentration of 0.37mM with respect to glucose. Quantitation limit was 366.3 ± 6.4mV equivalent to a peak area of 2729 and concentration of 1.27mM with respect to glucose. Hence, a peak area of less than 800 was considered as non detectable while a peak area of less than 2700 was not quantifiable.
Figure 7.16  Slope to noise (S/N) ratio

Note 1 Chromatogram of blank sample (Milli-Q water)
2 Red arrow shows measurement of the 2nd segment of S/N ratio
3 Seven consecutive segments were averaged

7.5 Use of resin beads to remove phosphate buffer and salts

A further issue which became relevant during the trials carried out in this study was that of interference in the chromatographic patterns of some samples due to the presence of ionic materials. This problem arose when buffering species were incorporated into digestion systems for the purpose of controlling conditions during enzymatic hydrolysis of starch components. Under the conditions adopted for some HPLC-RI, it was found that phosphate ions eluted at 0.9min while glucose eluted at 1.0min. The presence of both resulted in co-elution of the peaks thereby hindering the identification or over-estimation of the amount of D-glucose present in dialysate solutions which contained phosphate buffer. Consequently, the use of resin beads (H⁺) and (OH⁻) was considered in order to remove the phosphate salts so that interference in the analysis of D-glucose could be achieved.

The Amberlite resins used were made from a polymeric composite of styrene and divinylbenzene (DVB) which account for the majority of the material used for this purpose as this material has proven the most physical and chemical stability (Dow Liquid Separations, 2002b). Prior to use, the polymeric resin is converted to an ion-exchange resin by the addition of functional groups that would pick up unwanted ions. These include sulphonates (strong acid resins), carboxylic groups (weak acid resins), quarternary amines.
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(strong base resins) and tertiary amine groups (weak base resins) (Dow Liquid Separations, 2002a). The resin beads possess a macroporous structure that allows for ion exchange to occur inside as well as on the surface of the beads (Dow Liquid Separations, 2002b). Thus, the microporous structure facilitates the adsorption as well as the subsequent desorption of a targeted species depending on the type of the functional groups and the environment in which it is being applied (Dow Liquid Separations, 2002a).

Strong base resins (Amberlite IR-45(OH)) contain exchangeable OH\(^-\) anions which give the resins the ability to behave as an insoluble but very reactive base. This resin was used to exchange the OH\(^-\) ion for the anions in the buffer solution i.e. PO\(_4^{3-}\) and Cl\(^-\). Synergistically, a strong acid resin (Amberlite IR-120(H)) was added to exchange H\(^+\) for cations present in solutions with minimal disruption to the pH of the solutions. The use of these two resins effectively removes the salt present in the solutions, as interfering ions adsorb to the beads by exchanging with H\(^+\) and OH\(^-\) ions.

In order to evaluate the use of these resin materials and any potential effect they might have upon the quantitation of components in enzymatic hydrolysates and digested materials, a series of experiments were designed. For these, a commercial maltodextrin was selected and this was Fieldose 17 (M17) reflecting the fact that it was expected to contain a similar profile of oligomers as the dialysates to be analysed in subsequent phases of this study. To ascertain the efficiency of the resin beads and that they do not interfere with the recovery of D-glucose, an independent samples t-test was used to compare the mean scores of D-glucose recovery between M17 in water (Figure 7.15A) and M17 in water following treatment with resin beads (Figure 7.15B).

It is firstly observed that there was little difference in the pattern of elution for the maltodextrin samples before and after treatment with resins. In addition, statistical data shows that there no significant difference in the mean contents for D-glucose in the two samples prepared without resin (M=10767, SD=476) and with resin beads (M=10375, SD=587; t(4)=0.900, p=0.419). As there was no significant effect of the resin beads on the recovery of D-glucose, a further evaluation was carried out on the use of the phosphate buffer, NaCl and CaCl\(_2\) as these were to be used in the subsequent digestion analyses. Accordingly, M17 was also analysed without (Figure 7.17C) and with the use of resin
beads (Figure 7.17D) and the resultant chromatograms showed the disappearance of the phosphate peak at 0.9 min following treatment. This appeared to facilitate the identification as well as quantitation of D-glucose in these solutions.

However, to ascertain that all of the phosphate is removed thereby eliminating the possibility that even small amounts of phosphate might be present and would co-elute with the D-glucose peak, a further comparison was undertaken to compare recoveries of D-glucose from M17 in water (Figure 7.17B) and M17 in phosphate buffer with resin beads (Figure 7.17D). The results of this showed that there was no significant difference in the mean for D-glucose prepared in water (M=10375, SD=587) and in phosphate buffer with resin bead treatment (M=9896, SD=603; t(4)=0.985, p=0.380).

**Figure 7.17** Use of resin on reference M17 chromatograms

A 10% M17  
B 10% M17 + resin  
C 10% M17 in phosphate buffer  
D 10% M17 in phosphate buffer + resin
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7.6 Summary of findings on sugars analysis

Several methods have been considered in the measurement of sugars. Simple colorimetric assay including DNS reagent provides a quick and easily accessible means of determining sugar content while the HPLC method also offers potential. Now that these assays have been validated, their relevance in quantifying the products of digestion from starch and starch based microcapsules in the next stages of this research can be applied and hence compared.
Chapter 8

Results and discussion:
Enzymatic hydrolysis of starch and factors influencing an \textit{in vitro} dialysis model

The purpose of this chapter is to describe and discuss the results obtained when evaluating procedures for the \textit{in vitro} assay of starch digestion. The parameters considered included the addition of CaCl$_2$, the concentration and source of \(\alpha\)-amylase as well as the characteristics of the dialysis system used.

8.1 Introduction to the use of amylase preparations for digestibility studies

There has been considerable interest in the use of \textit{in vitro} procedures for the investigation of digestion. This reflects the significance of digestive processes to health and wellbeing and accumulating evidence that postprandial glucose levels are significant determinants of health. Whilst \textit{in vivo} investigations may have value, these are also expensive and accordingly there has been increasing interest in the potential of \textit{in vitro} techniques for analysis and study of digestibility (Hur, Lim, Decker & McClements, 2011). In recent reviews (Woolnough \textit{et al.}, 2008), it has been emphasised that there continues to be a lack of standardisation of approach which is limiting progress in applying the published procedures and interpreting the results.

In developing procedures and the approach to be used for the purposes of the current study careful consideration has been given to the many options described in previous reports (Brighenti \textit{et al.}, 1995, Englyst, & Macfarlane, 1986, Dona \textit{et al.}, 2010, Gan \textit{et al.} 2009, Jenkins \textit{et al.}, 1984, Koh \textit{et al.}, 2009, Patty 2009, Scazzina \textit{et al.} 2009, Tester \textit{et al.}, 2006 and Woolnough \textit{et al.} (2008). The focus of the current study has been restricted to aspects of starch digestion and their measurement and so, for example, there has been no attempt to include proteolytic enzymes or to consider the hydrolysis of dietary components other than starch. A further aspect is that, \textit{in vivo}, starch digestion may occur both prior to the food reaching the stomach as well as in the small intestine. It has been reported that the majority of the starch hydrolysis and absorption occurs at the intestinal stage of digestion and estimates are that the proportion is as high as 95% (Zielinski, 2012). It is appreciated that the use a complex mixture of enzymes is more often realistic as one nutrient is often
affected by the digestion of other nutrients (Boisen & Eggum, 1991). However, the use of a single purified enzyme rather than a complex biological mixture facilitates the standardisation of in vitro digestion models thereby making comparisons more consistent (Coles, Moughan & Darragh, 2007, Hur et al., 2011). On this basis, the approach adopted here has been designed around this latter stage of digestion.

Among the different approaches undertaken to assess starch digestibility, the use of DT has been popular as a means to mimic the lower digestive tract (refer to Table 2.3 in Chapter 2). This approach is commonly referred to as the dialysis model, and while not fully representing what might take place in vivo, this does provide a convenient system that has been widely adopted. For the current purpose, it confers the advantage of mimicking in vivo digestion while providing a restrictive means of affecting the viscosity of the dialysate and hence the glycaemic response.

A diagram of the arrangement used as the dialysis model for this study is presented in Figure 8.1. A series of investigations were then designed to clarify the significance of selected parameters relevant to this research and these are now described in the following sections.

Figure 8.1  Schematic representation of the dialysis model

1. Magnetic stirrer
2. Beaker containing dialysis buffer at pH 6.9
3. DT containing buffer, α-amylase and starch or microcapsule sample
4. Glass rod holding dialysis tubing
5. Immersion heater with circulating pump, set to 37°C
6. Stirrer bar under constant stirring
8.2 Effect of CaCl₂ on α-amylase activity

It has been documented that regardless of the source, α-amylase activity may be dependent on the presence of Ca\(^{2+}\) in the surrounding buffer system, particularly in terms of stability, due to the presence of bound Ca\(^{2+}\) within the molecular structure of the enzyme. Accordingly, in applying the dialysis model system, it was considered important to evaluate the addition of Ca\(^{2+}\) into the solutions, both within the DT, as well as outside, which is designated as the dialysate. Three widely used sources of α-amylases were selected for evaluation and these were compared on the basis of their measured activities. In addition, two different levels of activity were trialled and samples of the dialysate were analysed for reducing sugar content by the DNS procedure validated in the earlier phase of the study. Samples were taken at regular intervals over a period of three hours and this approach was adopted as previous studies investigating starch fractions have used a single time assay (Englyst, Kingman & Cummings, 1992, Miao et al., 2010, Rodriguez et al., 2006). Throughout these trials, unmodified native wheat starch was used as substrates.

The results are presented in Figure 8.2 and it was firstly observed that for each of the time courses of reaction, a relatively high correlation coefficient was found, indicating a linear rate of reaction. By adding Ca\(^{2+}\) for each of the three enzymes, there was a significant increase in the rate of release of reducing sugars for all combinations except for 1000U from BL and 5000U from AO (One-way ANOVA, \(p<0.05\)). This indicates that generally for the three sources of the enzyme the addition of Ca\(^{2+}\) influences the activity measured using the dialysis system. It was found that the α-amylase from BL showed less dependency on Ca\(^{2+}\) for activity although with an increase to 5000U, the rate of release for this enzyme was significantly dependent on CaCl₂ (\(p<0.05\)) thus suggesting higher dependency on CaCl₂ when a greater concentration of α-amylase from BL is used.

The observations from amylase from BL confirms previous reports regarding this enzyme (Wong & Robertson, 2003). Structural Ca\(^{2+}\) bound to α-amylase is related to stability and activity while dependency of α-amylase on Ca\(^{2+}\) differs from α-amylase isoforms (Seo, et al., 2008). Other studies showed that secondary binding sites from barley α-amylase showed a decrease in their thermal stability at lower Ca\(^{2+}\) concentrations and conformational stability is lowered by substituting certain side chains that bind to or are close to structural Ca\(^{2+}\) (Seo, et al., 2008).
Figure 8.2 Effect of addition of CaCl₂ on release of reducing sugars from three α-amylase source at two concentrations

Note Each point is an average of 2 determinations
In the current study, calculated $R^2$ values have been used as an indication for the line of best fit and the high $R^2$ show a strong linear relationship between time and release of reducing sugars. Yet, in general terms, lower $R^2$ values were obtained when in vitro digestion was carried without CaCl$_2$ again indicating a dependency on CaCl$_2$ for $\alpha$-optimal amylase activity. Despite the fact that this approach has not previously been reported for digestibility analyses, based upon the current observations, it was decided that for all subsequent in vitro digestion analyses, these would be carried out with the inclusion of CaCl$_2$ in both the buffer solutions used within and outside the DT.

### 8.3 Effect of DT on the release of sugars over time

As a means of standardising the dialysis model, the effect of different types of DT was assessed. While a review in published literature has shown an inconsistency in the MWCO, length and FW of the DT (Jenkins et al., 1982; Granfeldt & Björck, 1991; Brighenti, et al., 1992) the purpose here has been to assess these different characteristics while keeping all other conditions unchanged.

As wide a range as possible of types of DT useful for this study was procured from various suppliers and these were compared directly using the dialysis model system and wheat starch in conjunction with PP. The results are presented in Figure 8.3 and confirmed a strong correlation and high degree of linearity for the rates of reducing sugars released from wheat starch over time. A further series of analyses involved the recovery of all of the solid material retained within the DT and the weight of these was compared following freeze drying on a dry weight basis. The results for each type of DT are presented in Table 8.1.

It was firstly observed that the rate of sugar released was apparently higher with a smaller FW. It is also noted that DT with FW 16mm required a longer strip for the in vitro digestion. Higher sugar release might have been expected on the basis that a smaller FW will result in high rate of osmosis or that the low molecular weight hydrolysis products had a shorter distance to travel to reach the membrane and hence allowing more to pass through into the dialysate. However, the differences due to the width of tubing were not statistically significant ($p<0.05$). Similarly, FW of DT did not impart a significant difference in the amount of recovered starch from inside the DT ($p<0.01$) thereby
providing further confirmation that similar amounts of digesta passed through the DT membrane (Table 8.1).

![Graph showing release of reducing sugars](image)

**Figure 8.3** Effect of FW and MWCO of DT on release of reducing sugars

Note 1 Each point is an average of 2 determinations
Note 2 5000U of α-amylase PP was used with CaCl₂

**Table 8.1** Recovered starch (%) from inside of different types of DT

<table>
<thead>
<tr>
<th>MWCO (kDa)</th>
<th>FW (mm)</th>
<th>16</th>
<th>42-45</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>74.5 ± 0.3</td>
<td>74.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>77.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>3.5</td>
<td>-</td>
<td>81.6 ± 1.7</td>
<td></td>
</tr>
</tbody>
</table>

Note 1 Each point is an average of 2 determinations ± RSD
Note 2 Recovery expressed on a dry weight basis

With regards to MWCO, no significant differences were observed with the exception being for a MWCO of 3.5kDa which gave a lower rate \(p<0.05\), Figure 8.2). Similarly, MWCO appears to have no significant effect on the amount of digested material retained by the DT membrane, apart from 3.5kDa which had a significantly higher retention than for DT with
a MWCO of 20kDa (Table 8.1, \(p<0.01\)). DT with MWCO 3.5kDa hindered the dialysis of dextrin components of high DP in sufficient amounts to be readily assayed by the DNS reagent method. In the case of DT with MWCO 12kDa, even if some of the higher DP of malto sugars did not dialyse through the membrane, the amounts were low enough so that similar rate was obtained as for DT with MWCO 20kDa. DT provides similar restrictive effects that can be dependent to MWCO and FW for the range of materials used in this study. Accordingly, DT MWCO 12 or 20kDa and FW 42-45mm can be utilised in the \textit{in vitro} system for evaluating glycaemic response and hence subsequent trials have employed the application of DT with MWCO 12kDa and FW 42mm unless otherwise specified.

### 8.4 Effect of source of \(\alpha\)-amylase

The effect of the source of the enzyme in the dialysis model was assessed and the three were obtained from a bacterial source (BL), a fungal source (AO) and an animal source (PP). The rates of sugar release were compared directly using the dialysis model and the results are shown in Figure 8.4. PP gave the highest rate of release followed by \(\alpha\)-amylase from BL and AO respectively and all three results were significantly different from the other two \((p<0.05)\). The current findings agree with previous work by Planchot \textit{et al.} (1995) who also reported lower starch granule degradation from \textit{Bacillus} sp. as opposed to PP. Robyt & French (1967) confirmed that different \(\alpha\)-amylase exhibit varying degrees of multiple attack mechanism hence resulting in different degree of digestion when comparing PP and AO activities. They also highlighted the fact that the degree of attack depends on the reaction conditions.

In this study, the difference between the apparent rates of release probably reflects the effects of pH upon the enzymes. It has been reported that crystalline PP has a similar hydrolytic mechanism, a pH optimum of 6.9 and a requirement for Cl\(^-\) activation as human pancreatic \(\alpha\)-amylase (Bernfeld, 1955; Robyt & French, 1967). On the other hand, \(\alpha\)-amylase from AO requires more acidic conditions for optimal activity as compared to human \(\alpha\)-amylase thereby explaining the lower activity of this enzyme source using this DT model. \(\alpha\)-amylase from thermophilic BL has optimal activity close to pH 6.9 (pH 7 to 9) but requires higher temperature of 90\(^\circ\)C (Morgan & Priest, 1981). Hence, in relation to the three \(\alpha\)-amylases, those from BL and AO have been found here to provide similar hydrolytic rates which are significantly lower than for PP under the conditions used which
have been chosen to replicate those of the section of gastrointestinal tract where most starch is believed to be digested.

![Graph showing the effect of α-amylase source on release of reducing sugars]

**Figure 8.4  Effect of α-amylase source on release of reducing sugars**

Note 1 Each point is an average of 2 determinations
2 *in vitro* digestion was carried out with 5000U of α-amylase with CaCl₂

### 8.5 Effect of concentration of enzymes

As a further evaluation of the three enzyme sources, a series of concentrations of each was evaluated using the dialysis model and the data is compared in Figure 8.5. As expected, the rate of release of reducing sugars increased with an increase in α-amylase concentration with PP being significantly different for all concentrations used (p<0.01). For the bacterial (BL) and fungal (AO) preparations, the rates obtained for 250U and 1000U of α-amylase were not significantly different (p<0.01) probably reflecting the lower rates found for these under the conditions of the dialysis model. This may indicate the need for a minimum concentration of α-amylase required for the significant release of reducing sugars using this dialysis model. Consequently zero order kinetics was not followed for the lower concentrations in the series of data obtained. Based on these results, subsequent work in chapters 9 and 10 have applied α-amylase of a concentration of 5000U.
Figure 8.5 Effect of concentration of three different α-amylase source on release of reducing sugars

Note 1 Each point is an average of 2 determinations

2 in vitro digestion of wheat starch was carried out in the presence of CaCl_2
Following *in vitro* starch digestion using the dialysis model, further analyses were carried out in investigating and understanding the action of α-amylase on native starch. These included the surface and internal changes occurring on the morphology of the starch granules (section 8.6) using an ESEM and TEM including the identification of the resulting products (section 8.7) from the digestion model using HPLC-RI.

### 8.6 ESEM

SEM applies beams of high speed electrons directed towards the sample thereby giving a topographic surface through electrical conductivity. However, food systems including starch are non-conductive and will lead to accumulation of static electric charge resulting in noisy images. They may be coated with a metal including gold (the most commonly used), platinum, gold-palladium alloy, tungsten and graphite using a sputter unit. Thus, coating will make the sample electrically conductive resulting to more detailed images with higher resolution. However this additional sample preparation may impart starch granule damage and hence the alternative solution of using a low vacuum environment (LVSEM) could permit the viewing of uncoated starch granules believed to represent the true starch morphology (Baldwin, Davies & Melia, 1997). Yet, trials from this study have resulted in the higher prevalence of swelling and deformation of uncoated starch granule samples when viewed at high magnifications under low vacuum. Consequently, based on findings in this study and from other authors, starch samples were gold coated and viewed under low vacuum to achieve sharper images.

#### 8.6.1 Morphological changes upon *in vitro* digestion

ESEM images showed that α-amylase from the three sources carried out similar exo corrosion patterns on wheat starch granules (Figure 8.6). α-Amylase appears to favour attack at the outer crease of the starch granule rather than at other sites on the surface. This might suggest a weakening of the starch granule from the characteristic outer crease. Uneven amylolysis within the distribution of starch granules was also observed with the larger granules of the bimodal wheat starch distribution being more visually attacked than the smaller granules. This is further discussed in section 9.3.

Over time, surface pitting of the starch granules was observed leading to an increase in numbers of attack points and widening until a sponge-like structure was obtained. These
findings are similar to some previous observations (Buttrose, 1960; Copeland, Blazek, Salman, & Tang, 2009; Sujka & Jamroz, 2007; Tester et al., 2004; Zhang, Ao, & Hamaker, 2006). It has been suggested that the surface of native starches is made up of the crystalline hard shell with pores connected with amorphous channels towards the hilum, the central point of the starch granule where the concentric layers amylose and amylopectin fractions are deposited. It is believed that they may facilitate the passage of digestive enzymes including α-amylase and the subsequent leaching of the resultant sugars from hydrolysis (Huber & BeMiller, 2000; Oates, 1997) as well as leaching of amylose during the changes associated with gelatinisation and cooking (Gallant et al., 1997). Thus, pitting as a result of α-amylase is suggested to be dependent on the surface pores of the native starch and hence the starting point for the morphological changes arising from digestion thereby making it easier for α-amylase molecules to progress through the starch granule.
In the current study, further investigations were pursued in order to assess whether the presence of pitting led to the formation of internal channels. For this a series of cross-sections of the digested wheat starch were prepared and typical examples of the images obtained are shown in Figure 8.7. These confirm that channels appear to originate from the regions of pitting. The digested starch granules also undergo endo-corrosion such that following extended digestion the inner structure of the granule largely disappears and there is marked emptiness. In Figure 8.7, cross-sectional images of different starch granules demonstrate the different stages of endo-corrosion, from pitting to internal channel formation, further endo-corrosion and then to the eventual collapse of the starch structure.
When compared to the cross-section of undigested wheat starch (Figure 8.8), the latter depicts an even internal structure with no apparent distinct characteristics. However, \textit{in vitro} digestion of the wheat starch in this study as well as from other authors exposed the inherent presence of growth rings (Copeland, \textit{et al.}, 2009; Gallant, \textit{et al.}, 1997; Lentle & Janssen, 2011). These growth rings are described as a series of alternate concentric shells of crystalline and amorphous starch towards an off-centred hilum. The amorphous rings are believed to be high in $\alpha$-(1\,$\rightarrow\,$6) glycosidic bonds while the crystalline areas are higher in $\alpha$-(1\,$\rightarrow\,$4) glycosidic bonds (Coultate, 2009). $\alpha$-Amylase unable to cleave $\alpha$-(1\,$\rightarrow\,$6) glycosidic bonds will thereby cause the crystalline shells to be more susceptible to digestion while remaining amorphous areas remains undigested. This lead to the creation of the initial appearance of ‘teeth’ surfaces (Figure 8.9). With advanced digestion and later
in time, the growth rings in layers are even more exposed. In this study, a more intensive digestion conditions with a concentration of 5000U of PP, lead to the leaching of amylose fragments as a result of amylolytic cleavage and is observed by the ‘glued-like’ appearance in Figure 8.10).

Figure 8.8  ESEM image of a cross section of undigested wheat starch

Figure 8.9  TEM image of digested maize starch
8.7 Sugar profile of digested starch

After the application of the \textit{in vitro} digestion model, the identification of the digestion products were warranted and hence carried out using the HPLC-RI. As such, these findings could lead to a better understanding of the mechanism of the dialysis model, determining the extent of the digestion and comparing the outcomes of different \textit{in vitro} digestion parameters.

When analysing the sugar profile of the \textit{in vitro} digestion (Table 8.2), PP resulted in the production of glucose, maltose and maltotriose which were clearly identified by the HPLC system (Figure 8.11). DP4 (glucosyl maltotriose) was identified but did not give a clear separation from the following sugar. DP5 and DP6 did not exhibit similar retention times as the standards used but their presence can be approximated by their closeness to the retention times. This implies the production of larger fragments of malto-sugars with different conformation structures. Indeed, $\alpha$-amylase cleaves $\alpha(1\rightarrow4)$ bonds that form the linear structure of starch molecules. This enzyme unable to cleave $\alpha(1\rightarrow6)$ that creates the branched side of the starch molecule will therefore result in a higher concentration of branched fragments.

From the sugar profile of the dialysate, similar sugar proportions were observed as in the contents inside of the DT but only for sugars up to DP3. This suggests that glucose,
maltose and maltotriose i.e. malto-sugars up to DP3 were small enough to dialyse through the DT. When comparing sugar profiles from the three sources of \( \alpha \)-amylase (Figure 8.11, 8.13 and 8.15), consistency was achieved in the sugar profile being dialysed through the DT i.e. glucose, maltose and maltotriose. However the ratio each sugar was different. PP gave higher glucose and lower maltotriose than with \( \alpha \)-amylase from BL. As a higher reducing sugar release rate was obtained with PP, it is believed that more maltotriose was broken down into glucose units with 5000U of PP than with BL. In the same lines, \( \alpha \)-amylase from AO which gave lower release rate provided similar sugar profiles as PP but with lower response. Moreover, though PP and \( \alpha \)-amylase from AO appear similar in sugar profile suggesting a similar area of attack on the substrate. PP being very similar to human amylase which has similar specific activity as to AO even though the end products are of higher molecular weight (Bernfeld, 1955). \( \alpha \)-amylase from BL did not produce the DP4 and DP5 found with PP and \( \alpha \)-amylase from AO. Thus, suggesting a different attack site on the polysaccharide chain.

Table 8.2  Reducing sugar profile of \( \alpha \)-amylase dialysate by HPLC-RI

<table>
<thead>
<tr>
<th>Sugar</th>
<th>( \alpha )-Amylases</th>
<th>PP</th>
<th>BL</th>
<th>AO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \text{SR}_{\text{time}} ) (min)</td>
<td>( \text{MR}_{\text{time}} ) (min)</td>
<td>mMoles/ g starch*</td>
<td>( \text{SR}_{\text{time}} ) (min)</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.055</td>
<td>1.061</td>
<td>0.60</td>
<td>1.065</td>
</tr>
<tr>
<td>Maltose</td>
<td>1.200</td>
<td>1.201</td>
<td>0.55</td>
<td>1.259</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>1.411</td>
<td>1.416</td>
<td>0.10</td>
<td>1.576</td>
</tr>
<tr>
<td>Isomaltotriose</td>
<td>1.490</td>
<td>-</td>
<td>-</td>
<td>1.696</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>1.25</td>
<td></td>
</tr>
</tbody>
</table>

Note  
\( \text{SR}_{\text{time}} \): Retention time for standard solution  
\( \text{MR}_{\text{time}} \): Mean retention time of sample  
* dry weight basis
Figure 8.11  **Sugar profile of dialysate after 3hr in vitro digestion with PP**

Note 1  Each point is an average of 2 determinations

2  *in vitro* digestion was carried out with 5000U of α-amylase with CaCl$_2$

Figure 8.12  **Sugar profile inside DT after 3hr in vitro digestion with PP**

Note 1  5000U of α-amylase with CaCl$_2$ was used

2  Duplicate analysis was carried out
Figure 8.13  **Sugar profile of dyalisate after 3hr *in vitro* digestion with BL.**

Note 1  Each point is an average of 2 determinations

2  *in vitro* digestion was carried out with 5000U of α-amylase with CaCl₂

Figure 8.14  **Sugar profile inside DT after 3hr *in vitro* digestion with BL.**

Note 1  5000U of α-amylase with CaCl₂ was used

2  Duplicate analysis was carried out
Figure 8.15  **Sugar profile of dyalisate after 3hr *in vitro* digestion with AO**

Note  
1. Each point is an average of 2 determinations.
2. *in vitro* digestion was carried out with 5000U of α-amylase with CaCl₂.

Figure 8.16  **Sugar profile inside DT after 3hr *in vitro* digestion with AO**

Note  
1. 5000U of α-amylase with CaCl₂ was used.
2. Duplicate analysis was carried out.
8.8 Comparison of DNS method and HPLC-RI for reducing sugar analysis

All analyses investigating the release of reducing sugars were carried out by DNS method. However, this assay as mentioned previously was only able to quantify the total amount of reducing sugars independent of the DP of the sugars. While HPLC-RI was applied to identify the type of reducing sugars obtained as a result of in vitro digestion, quantification of reducing sugars from both assays were compared (Table 8.3). Independent t-test was carried out to determine statistical difference in the two methods and there was no significant difference in the release of reducing sugars analysed by HPLC-RI or DNS method for wheat samples digested by PP and α-amylase from AO ($p=0.384, 0.072$). However, HPLC and DNS method results were significantly different for α-amylase from BL ($p=0.04$). This could be explained by the error associated with the fact that HPLC chromatograms depicted a higher amount of maltose present in the digesta while glucose was used for determining total reducing sugar released by α-amylase from BL.

Table 8.3 Reducing sugar from of α-amylase dialysate

<table>
<thead>
<tr>
<th>α-Amylase source</th>
<th>PP</th>
<th>BL</th>
<th>AO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DNS - Dialysate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total reducing sugars</td>
<td>1.28 ± 0.02</td>
<td>0.65±0.05</td>
<td>0.46±0.05</td>
</tr>
<tr>
<td><strong>HPLC - Dialysate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0.60 ± 0.05</td>
<td>0.13 ± 0.02</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>Maltose</td>
<td>0.55 ± 0.06</td>
<td>0.21 ± 0.01</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>0.103 ± 0.004</td>
<td>0.102 ± 0.002</td>
<td>0.012 ± 0.004</td>
</tr>
<tr>
<td>Total reducing sugars</td>
<td>1.251 ± 0.001</td>
<td>0.44 ± 0.04</td>
<td>0.29 ± 0.04</td>
</tr>
</tbody>
</table>

Note 1 mMoles/ g starch expressed on a dry weight basis
2 Data represented as mean ± SD of duplicate analyses

8.9 Summary of findings on digestibility analysis

From the results obtained in this research, several factors were confirmed to affect the dialysis model. Hence in all subsequent phases of this research project, CaCl$_2$ was included in the buffer solution for consistent enzyme activity while 5000U of α-amylase from PP,
giving a higher response, was used in the dialysis model. While the results showed that the type of DT did not impart significant difference, tubing having a 12kDa MWCO with 42mm FW was used throughout for consistency. Though, it may be difficult to define the exact *in vitro* digestion conditions, results and morphological changes from the dialysis model in this study are consistent with previous work reported on the digestion of starch without the use of a dialysis model. It is also appreciated that more research is needed to assess the advantages and disadvantages of the chosen conditions in this study and the importance of *in vitro – in vivo* correlations in digestion models.
Chapter 9

Results and discussion:
Application of an *in vitro* dialysis model on native starches from different botanical sources and effect of granule size

The purpose of this chapter is to report and discuss results obtained from the application of the previously standardised *in vitro* dialysis model on native starches from different botanical sources. This chapter also includes the effect of particle size on the release rate of reducing sugars during digestion using wheat as a model. Thus determining if these two factors also contribute to different outcomes.

9.1. Particle size analysis

Laser diffraction was applied as a means of determining particle size of the different native starches used in this study and hence determining if a relationship between particle size and degree of digestion occurs. Laser diffraction applies the Mie theory whereby the way light is scattered, passes through or is adsorbed by spherical particles. By knowing the way the particles scatter light and by applying the Mie Theory, it is impossible to determine the particle size of a specific sample. However, interpretation of results needs to assume that all samples are of spherical shapes and this method is less accurate for non-spherical particles.

9.2  *in vitro* Digestion of native starches from different botanical origin

A range of different starch sources was also analysed upon digestion with porcine pancreatin and the data indicates significantly different rates of hydrolysis thereby suggesting variations in starch hydrolysis ($p<0.01$, Figure 9.1). Rate of reducing sugar release was in the following order wheat > rice > maize > potato starches with wheat giving the highest rate. This agrees with previous studies carried out and reflecting the varying organisation and structural differences in amylase and amylopectin fractions of starches dependent on botanical origins.
Figure 9.1 Release rates of native starches from in vitro digestion

Note 1 5000U PP was used
2 Mean ± 95% CI of two determinations is represented

Moreover, the degree of surface pores on starch granules could also play a role in the susceptibility of the starch granules being liable to digestibility as they are believed to aid in the permeation of digestive enzymes and the subsequent leaching of the sugars (Huber & BeMiller, 2000; Oates, 1997). In some previous reports potato starch granules were found to have low porosity suggesting a higher resistance to enzymatic action (Juszczak, Fortuna, & Krok, 2003). In addition, potato starch has been reported to possess a rougher surface than of wheat starch there again resulting in higher resistance the in vitro digestion (Baldwin et al., 1997)

When the samples were viewed under the ESEM, similar morphology as to wheat starch with pin-holes was observed after digestion (Figure 9.2). Formation of channels was more prominent in granules of maize starch while those of potato showed lesser exo-corrosion. Digestion of bimodal wheat starch appeared to be visually more prominent on the larger granule fraction than the smaller one. Nevertheless, further investigation was carried out and discussed in section 9.3 whereby this was not the case.
Figure 9.2  ESEM images of native starches before and after *in vitro* digestion

A, E  Wheat starch  
B, F  Maize starch  
C, G  Rice starch  
D, H  Potato starch
The rate of release of reducing sugars also appears to relate to the median granule size analysed using Malvern Mastersizer X with rice starch: 9\(\mu\)m, maize starch: 18 \(\mu\)m wheat starch giving two main median populations of 4 \(\mu\)m and 22 \(\mu\)m, and potato starch: 37 \(\mu\)m (Figure 9.3). Even though the total median particle size was in the following order rice<maize<wheat<potato starches, wheat starch gave a higher release of reducing sugars than rice starch. It is believed that the bimodal starch has accredited a higher digestion rate imparted by the smaller starch fraction. This is further elaborated in section 9.3.

Figure 9.3 Particle size of native starches from
A Rice  B Maize  C Wheat  D Potato
Note  Mean ± 95\%CI of two determinations

9.3 Rate of digestion as a function of granule particle size

To determine whether rate of digestion is dependent on particle size with the exclusion of the disparities caused by botanical origins, the bimodal wheat native starch was separated by sedimentation technique. Hence, the resulting two main fractions were digested using the *in vitro* digestion model.
Wheat starch sample was confirmed having two clearly differentiated populations from ESEM images (Figure 9.4) and from laser diffraction of the separated starch granules <10μm and >10μm in diameter (Figure 9.5). From the sedimentation technique, a recovery of 92.6 ± 0.8% starch (expressed on a dry weight basis) was obtained. The two primary populations (<10μm and >10μm, Table 9.1) as well as the original unseparated wheat sample were digested and the data for release of reducing sugars was plotted (Figure 9.6).

Figure 9.4  ESEM image of undigested wheat starch
Figure 9.5  Particle size distribution of starch fractions after sedimentation

Table 9.1  Composition of wheat starch based on particle size

<table>
<thead>
<tr>
<th>Granule diameter</th>
<th>% weight ± RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10µm</td>
<td>29.2 ± 1.6</td>
</tr>
<tr>
<td>&gt;10µm</td>
<td>70.8 ± 0.7</td>
</tr>
</tbody>
</table>

Note 1  Weight calculated on a dry weight basis
Note 2  Mean of three determinations
In this study, wheat starch granules of <10µm showed significantly higher rates of reducing sugar release than the granules >10µm ($p<0.05$). This confirms previous observations associated with greater surface area of the substrate exposed to enzymatic attack due to the smaller particle size and hence the higher the rate of digestion. As the small and larger fractions of starch granules were subjected to similar treatment conditions during the separation process, it is likely that a direct comparison of rates can be made. The results obtained in the current do not preclude the possibility that the surfaces of some of the starch granules may have included a layer of resistant starch (Ring et al., 1988; Tester et al., 2004).

The rate of reducing sugar release of the original starch sample was not significantly different from that of either of the wheat starch fractions (<10µm and >10µm, $p<0.05$). Less than one third of the starch granules were <10µm in the original starch sample thereby resulting in these having a minimal effect on the overall rate of release of reducing sugars during digestion.

Morphological changes upon *in vitro* digestion showed a change from an initial smooth surface on the undigested starch granules to patterns of exocorrosion on digested
samples. Typically, similar surface pitting in conjunction with internal channels giving rise to a sponge-like appearance were observed from the original wheat starch and with the granules >10µm in size. These observations are similar to those described by other researchers (Blazek & Copeland, 2010; Buttrose, 1960; Tester, et al., 2004).

In the original wheat sample, uneven amylolysis within the distribution of starch granules was observed with the larger granules of the bimodal wheat starch distribution being more obviously subjected to hydrolytic action. This may indicate that smaller granules are more resistant to hydrolysis than larger granules based on observations of the ESEM images as discussed by other researches (Copeland et al., 2009; Hoover & Zhou, 2003; Tester, et al., 2004). However, when <10µm digested granules were viewed in the ESEM, similar pitting were found even though damaged starch granules appear to be more sparsely distributed (Figure 9.7A). Moreover, in one of the images of digested granules <10µm, there appears to be a relatively large amount of amorphous material from the partial breakdown of granular starch and which was not readily dialyzable. This is consistent with a greater release of molecular starch during digestion as well as a higher digestion rate than the other samples and hence confirms the higher rate of reducing sugar release seen from Figure 9.6.

Figure 9.7  ESEM images of small and large wheat starch granules following digestion
Note that samples were digested for three hours and different magnifications were used for the two images
A  <10µm  B  >10µm
9.4 Summary of findings

Results in this chapter demonstrated different outcomes when the *in vitro* dialysis model was applied on different types of native starches. This study confirms that particle size is a determining factor that affects the different rates of digestion obtained for starches from different botanical sources. It is therefore emphasised that the choice of starches for use as a substrate and its description as well as its homogeneity are important factors that need to be accounted for especially when doing comparative studies. Caution also needs to be taken when comparing results from other studies as similar starches may contain different particle size distribution based on the maturity of the plant from which the starch is extracted.
Chapter 10

Results and discussion:
Microencapsulation of folic acid and the application of an *in vitro* dialysis model to capsule release characteristics

The purpose of this chapter is to report and discuss results on the microencapsulation of folic acid by spray drying using native rice starch and a combination of hydrocolloids. The optimum wall material combination has been evaluated in relation to maximum folic acid retention. Then, the *in vitro* dialysis model has been applied for the microcapsules as measure of the release of the core material.

Microencapsulation was applied as a means to protect a particular core material. In this study, folic acid was used as a core material example and hence its stability and release was analysed. The wall material of microcapsules was designed so as to prevent the diffusion of material either from within or from the exterior into a microcapsule, hence acting as a barrier only allowing diffusion under controlled conditions (Reineccius, 2001). Pilot scale spray drying of folic acid with rice starch and a combination of ALG and LMP was carried out based on previous literature reviews and research studies (Augustin, *et al*., 2001; Gharsallaoui *et al*., 2007; Madziva, Kailasapathy, & Phillips, 2005, Madziva, Kailasaphy & Philips, 2006; Zhao & Whistler, 1994) and recent work carried out at RMIT University (Hau, 2008). Rice starch was chosen due to its smaller particle size distribution. This has the advantage of providing good capsular integrity while producing microcapsules sufficiently small to prevent grittiness when included in flour based products as well as providing a more complete coverage of the core material. Spray drying was chosen as the method of encapsulation due its popularity in the microencapsulation field including its economical and flexible aspects and giving dry and stable products.

10.1 General characteristics of microcapsules

ALG and LMP gave microcapsules with relatively uniform spherical shape and typical yields of 75% were obtained. During capsule production, a slight inlet temperature variation was also noted with minimal effects on the resulting products. ESEM images of the resultant microcapsules are presented in Figure 10.1.
Figure 10.1 The effect of wall material concentration: ESEM images at 2500× magnification

A  0.2% ALG-LMP  B  0.5% ALG-LMP  C  1% ALG-LMP
D  8% ALG-LMP    E  1% ALG       F  1% LMP
The ESEM images do not show any morphological differences between the microcapsules based on type, ratios and level of binding agents. In some cases, incomplete hollow microcapsules were formed thereby possibly indicating that the rice starches bounded by the hydrocolloids form a single outer shell with an empty interior (simple form).

To assess this statement, a sample of the microcapsules was frozen with liquid N\textsubscript{2} and sliced with a scalpel in an attempt to provide a clear cut and minimise collapse of the microcapsules. ESEM images showed and confirmed that the microcapsules are not hollow inside but rather exhibit a multi-core form system (Figure 10.2). It is hence suggested that the core material is dispersed throughout the microcapsules being entrapped within the hydrocolloids both on the outer surface and in the interior of the microcapsules.

![Figure 10.2 ESEM images of cross sections of microcapsules](image)

**Figure 10.2**  **ESEM images of cross sections of microcapsules**

Microcapsules prepared from 1% 1:1 ALG:LMP binding agent
A  at 2000X  
B  at 5000X

**10.2   Effect of ratio of binding agents**

Three microcapsules formulas were produced based on a combination of ALG and LMP as binding agents including 1% ALG, 1% 1:1 ALG:LMP and 1% LMP. Microcapsules produced were kept in air tight containers covered in Al foil until they were further analysed.
10.2.1 Particle size distribution

Ratios of binding agents did not significantly differ in median particle size ranging from 25 to 30µm \((p<0.05)\). Particle size distribution was also very similar irrespective of the type of binding agent when used at the same concentration (Figure 10.3). Generally, a polydisperse and non-skewed distribution was obtained.

![Particle size distribution](image)

**Figure 10.3** Particle size distribution of microcapsules based on ratio of binding agents

Data represents mean of duplicates with RSD as error bars

1% of total binding agent was used for each combination

10.2.2 Folic acid recovery

When comparing folic acid recovery based on the ratio of binding agents, results were significantly different except between LMP and 1:1ALG:LMP trials \((p<0.05, \text{ Figure 10.4})\). Higher recoveries were obtained with LMP only or with the addition of LMP to the binding agents. Hence, a higher core material retention was obtained with the addition of ALG whereby the folic acid remained embedded in the ALG/rice starch matrix. The addition of LMP resulted in the weakening of the matrix thereby allowing more of the core material to be released during extraction.
10.3 Effect of level of binding agents

From section 10.2.2, 1:1 ALG:LMP was reported as providing the higher folic acid recovery and hence, the effect of level of binding agent was carried out based on a 1:1 ratio. Batches of microcapsules were prepared using a combination of 1:1 ALG:LMP at 0.2, 0.5, 1 and 8% with respect to rice starch. Similarly as in section 10.2, microcapsules were kept in air tight containers covered in Al foil until they were further analysed.

10.3.1 Particle size distribution

The different levels of binding agents produced a significant variation in median particle diameters ranging between 25 and 34μm. Median particle size was significantly higher with an increase in binding agents from 0.5 to 8% and also from 1 to 8%, but not from 0.2 to 8% (p<0.05, Figure 10.5). Patterns of particle distribution showed a decrease in particle size when levels of binding agents are increased from 0.2 to 1% but particle size significantly increased when binding agents were increased to 8%. However, when examined under the ESEM, microcapsules appear to be at its smallest with 8% of binding agents but with the presence of agglomerated microcapsules. Agglomeration due to the noticeably higher viscosity of the slurry mixture prior to spray drying resulted in an increase in the overall particle size distribution of the 8% batch. One of the limitations of laser diffraction involves the assumption that the sample is isotropic and spherical in shape.
based on the Mie theory. Agglomeration created clusters of anisotropic particles thereby shifting the particle size distribution. Therefore, an increase in binding agent levels resulted in a decrease in particle size distribution.

![Particle Size Distribution of Microcapsules based on Levels of 1:1 ALG:LMP](image)

**Figure 10.5** Particle size distribution of microcapsules based on levels of 1:1 ALG:LMP
Data represents mean of duplicates with RSD as error bars
1% of total binding agent was used for each combination

### 10.3.2 Folic Acid Recovery

Following particle size distribution analysis, microcapsules were thereon extracted for folic acid recovery. Figure 10.6 shows that with an increase in the level of binding agent, lower folic acid was recovered. Folic acid recovery was significantly lower from 0.2 to 8% and from 0.5 to 8% of binding agents \( p<0.05 \). Thus, increasing level of binding agent resulted in higher retention of folic acid within the microcapsules embedding materials.
To determine the simultaneous effect of both binding agents ALG and LMP on microencapsulation of folic acid, a factorial design was carried out using the Minitab software and the following trials were carried out in the specified order (Table 10.1).

Table 10.1  Factorial design of ALG and LMP on folic acid recovery

<table>
<thead>
<tr>
<th>Std order</th>
<th>Run order</th>
<th>Centre point</th>
<th>% ALG</th>
<th>% LMP</th>
<th>% binding agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>1.0</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>0</td>
<td>0.5</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>1</td>
<td>1.0</td>
<td>0.0</td>
<td>1.0</td>
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<tr>
<td>3</td>
<td>4</td>
<td>1</td>
<td>0.0</td>
<td>1.0</td>
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<tr>
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<td>5</td>
<td>1</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

The microcapsules were thereon extracted for folic acid and results were presented using a surface plot from Matlab (Figure 10.7). In this study and in agreement with previous work (Hau, 2008; Madziva et al., 2005), the addition of LMP showed higher recovery of folic acid than when ALG was used alone. At all levels of ALG (From 0.0 to 1.0), when LMP was increased, an increase in folic acid recovered was obtained. Thus, confirming that LMP showed higher recovery of the core material than ALG.
10.5 Calcium treatment of microcapsules

In an attempt to decrease the release of the core material from the microcapsules, an external coating with Ca\(^{2+}\) was carried out. This approach has been based around the earlier findings which showed a strengthening of the network and a reduction in swelling and hydration thereby decreasing the release of core material (Hau, 2008). This arises from the ability of ALG and LMP (high Ca\(^{2+}\) binding) to cross link with Ca\(^{2+}\) to form a stable, rigid and water insoluble network through the ‘egg box’ model. For the capsules treated in the current study, a comparison of the treated and untreated microcapsules is shown in Figure 10.8, with the Ca\(^{2+}\) coated microcapsules showing a tightening of the outer surface of the microcapsules. Based on the combinations of microcapsules produced, 1% LMP (1P30RS), 1% ALG (1A30RS) and 1% 1:1 ALG:LMP (1PA30RS) were chosen for further experiments to establish suitable calcium treatment conditions.
The three microcapsules were treated with 1M CaCl₂ for a duration of 30min or 1hr. The filtrates were analysed for folic acid loss during calcium treatment and the resulting Ca²⁺ microcapsules were extracted for folic acid content (Figure 10.9). The results are represented in Figure 10.8. The outcome showed that the duration of the calcium treatment did not have a marked effect on the recoveries of folic acid. However, the binding agents of the microcapsules led to different core material loss during treatment and hence the recovery of the resulting calcium coated microcapsules. Microcapsules containing ALG only showed highest leaching out of the core material during the treatment. The addition of LMP as binding agent lead to a reduction of folic acid loss by 20% while LMP only showed the lowest folic acid loss. These results indicate that ALG showed a slower rate of
hardening with Ca\(^{2+}\) than LMP. Even though more folic acid is retained with ALG than LMP, the secondary coating treatment leads to a higher loss with ALG. Yet, part of the folic acid loss can be attributed to the presence of surface folic acid including unencapsulated folic acid in the sample. Hence, data showed that the presence of LMP reduces losses of the core material during the calcium treatment as an attempt to reinforce the outer shell of the microcapsules. Also, it appears that increasing the level of binding agent can increase core material retention but similar losses of folic acid were reported from previous studies (Hau, 2008).

*Figure 10.9  Folic acid recovered during and after Ca\(^{2+}\) treatment*

The simultaneous effect of binding agent composition and concentration of Ca\(^{2+}\) was also assayed (Figure 10.10). The surface plot showed a lower folic acid recovery with a lower concentration of CaCl\(_2\). Initial studies used a calcium treatment with 1M of CaCl\(_2\) as the basis of the calcium treatment. Yet, the saturation of Ca\(^{2+}\) in solution resulted in the slow migration of Ca\(^{2+}\) to the ALG/LMP thereby resulting in the slower hardening of the microcapsules and throughout the matrix. Thus leading to higher loss of the core material
before hardening is accomplished. With lower Ca\textsuperscript{2+} concentrations, there is greater binding with ALG/LMP and hence gelling is faster. The outer shell surface hardens before more of the core material is leached out during the calcium treatment. Further work is warranted to determine the optimum Ca\textsuperscript{2+} concentration and hence minimising core material loss. Even though previous studies suggested higher concentrations of Ca\textsuperscript{2+} leads to higher number of alginate strands held together in the “egg-box” model giving stronger gel network (De Vos et al., 1996), an excessive amount affects the rate of hardening and may have an undesirable effect.

![Figure 10.10 Simultaneous effect of binding agent composition and Ca\textsuperscript{2+} concentration](image)

**Figure 10.10 Simultaneous effect of binding agent composition and Ca\textsuperscript{2+} concentration**

10.6 *in vitro* Digestion of microcapsules

In order to evaluate the dialysis model system as a means of studying release characteristics, microcapsules with or without Ca\textsuperscript{2+} coating were digested using the *in vitro* digestion model using dialysis tubing and folic acid recovered from the dialysate was plotted against time over three consecutive hours (Figure 10.11). Results show a significant difference in the rate of core material release from Ca\textsuperscript{2+} treated microcapsules and the untreated samples. Untreated microcapsules showed similar core material release rate for the first hour irrespective of the choice of the binding agent. After two hours of digestion a
clear separation in the rate of release is observed as the starting folic acid in the microcapsules retained in the Ca$^{2+}$ treated microcapsules varied. The \textit{in vitro} digestion model provided a gradual release of the core material primarily imparted the slowing effect of diffusion of the active agent through the physical barrier provided by the dialysis tubing.

On the other hand, the digestion of the Ca$^{2+}$ microcapsules provided a significantly slower rate than the untreated microcapsules. In comparison, the calcium treatment greatly reduced the core material release rate. It henceforth suggested that the $\alpha$-amylase activity were hindered by the calcium coating and hence reducing release of the core material. When reducing sugar release was assayed, a lower rate was again obtained between the treated and untreated microcapsules thereby confirming a slower digestion rate with the calcium coated microcapsules.

![Figure 10.11 Release of folic acid from microcapsules using \textit{in vitro} digestion model](image)

\textbf{Figure 10.11 Release of folic acid from microcapsules using \textit{in vitro} digestion model}

Data represent duplicates with RSD as error bars

Digestion carried out with 5000U of porcine pancreatin

The morphology of the microcapsules after digestion was also viewed from the ESEM and both types of microcapsules showed marked disintegration after the digestion and loss of their spherical shape. A closer examination showed pitting on the non-calcium treated
microcapsules similar to native rice starch. Calcium treated microcapsules showed fewer traces of signs of pitting and erosion but a more compact structure bound by the calcium ALG/LMP, hence still allowing the containment of the core material. However, it is also possible that the solubility of folic acid might be affected by the higher concentration of calcium inside the dialysis tubing thereby hindering its passage through the dialysis tubing.

![Figure 10.12 Digested 1%PA30%RS microcapsules](image)

- **A** Untreated
- **B** Calcium treated

### 10.7 Summary of findings

From the results obtained in this study, the choice of binding agents to produce the microcapsules was significant in core material retention. Even though ALG demonstrated higher folic acid retention, subsequent treatment with Ca$^{2+}$ lead to a higher amount of the core material being leached from ALG high microcapsules. Consequently, a combination of ALG and LMP was the optimum core material for providing retention in this study. Moreover, the subsequent calcium treatment of the microcapsules resulted in a significant reduction in core material release in vitro thereby confirming the desired effect of this treatment. It is however recognised that further studies are necessary to further understand the mechanism of the slower core material release.
Chapter 11

General conclusions and recommendations for further research

The purpose of this chapter is to summarise and discuss the results obtained during the current study and draw final conclusions. In addition recommendations are made for further research into procedures for *in vitro* digestion and their application to food systems as a means of evaluating release characteristics of microcapsules.

11.1 Analysis procedures for starch digestion

A series of approaches to the analysis of starch hydrolysis products were set up and trialled during the preliminary phase of the current study. Colorimetric methods were compared, particularly the Nelson-Somogyi and the DNS procedures for measuring reducing sugars. DNS provided repeatable and consistent results that were sufficiently sensitive for the purposes of this study. Comparisons were also made of the response factors for a variety of standards including glucose and oligosaccharides that might be expected in the hydrolysates of starch as a result of enzymatic hydrolysis. Some variation was found in the standard curves when these different simple sugars were used as standards.

Further analyses involved the use of HPLC-RI as a means of separating the lower molecular weight hydrolysis products from starch. The procedures were found to be reliable and provided sufficient separation to enable quantitation of the oligomers up to those having an apparent degree of polymerisation of approximately seven in starch hydrolysates. The detection and quantitation limits for glucose were established and the validity of deionisation techniques for the removal of buffer components in hydrolysates was also investigated using polymeric Amberlite resins.

11.2 Evaluation of parameters for the dialysis model system

In the context of the hypothesis of the current study that the use of digestibility analysis may provide a means of assessing the release characteristics of microcapsules, the carbohydrate analysis by DNS and HPLC-RI developed in the initial stages of this work were applied. Consideration of previous research indicated that DT can be used to simulate the lower digestive tract where a large proportion of starch digestion probably occurs.
Accordingly, a dialysis system was evaluated in terms of its potential for investigating the digestion of starch and starch microcapsules. In order to validate the procedure and to understand the effects of a number of variables on the apparent rates of starch hydrolysis, a series of comprehensive investigations were carried out.

The rates of hydrolysis were compared for $\alpha$-amylase preparations from a number of sources. For each of these, linear relationships were consistently observed when the release of reducing sugars was measured over time periods of up to three hours. It was found that the incorporation of low concentrations of calcium ions into the buffering solutions used in the dialysis model had a significant effect upon the measured rates of starch hydrolysis. In addition, differences in rates were found when the $\alpha$-amylase from pancreatic sources was compared with those of microbial origins, reflecting the pH conditions utilised in the dialysis system and reflecting those in the lower digestive tract.

Again there has been a lack of standardisation in the specific conditions and parameters applied to digestibility studies using the dialysis approach and so a comparison was made between the MWCO and dimensions of the DT used. No significant effects were observed in these trials.

### 11.3 Comparisons of starch digestion in the dialysis model system

SEM was used to observe the effects of starch digestion on its morphology by recovering the undigested material from within the DT followed by freeze-drying. TEM was also applied to the samples to investigate internal digestion characteristics and the patterns found were related to the results obtained from HPLC-RI analyses of the hydrolysates after three hours of digestion. The predominant hydrolysis products were found to be glucose, maltose along with smaller amounts of maltotriose. In addition, the same observations were made for $\alpha$-amylases from each of the three sources studied here. Smaller amounts of some higher oligomers were also found in the chromatograms but remained inside the DT and did not traverse the dialysis membrane. Moreover, when the total reducing sugar contents of the dialysate were directly compared with the results from HPLC-RI analyses, the sum of the contents of glucose, maltose and maltotriose were the same as the results obtained using the DNS reagent.
11.4 Application of the dialysis model to a study of starch digestion

On the basis that the model system validated in the earlier phases of this study was found to be robust, it was applied to a comparative evaluation of starch digestion. For this, granular starch from various botanical sources was analysed using porcine pancreatin and SEM was also applied to observe changes occurring during the digestion process. When the results were related to the particle size distribution, the hydrolysis rates did not systematically reflect the measured differences in granule size from the selected botanical starches. The relative rates were highest for wheat, followed by rice, maize and potato starches.

In a further application of the dialysis model, the relative rates of hydrolysis of the two distinct size ranges of the bimodal population of wheat starch granules were investigated. Samples of granules were prepared using a sedimentation technique and the size distributions confirmed by laser scattering. Although not detailed in the thesis, analyses were carried out to confirm that the levels of starch damage were sufficiently low to be undetectable. The rate of digestion of the small granule fraction was significantly higher than that found for those having larger diameters.

11.5 Microencapsulation strategies for enhancement of folic acid retention

Spray drying was used for the preparation of microcapsules in conjunction with a matrix of rice starch granules and the hydrocolloids ALG and LMP as binding agents. The optimum approaches for microencapsulation were studied and higher recoveries of folic acid were obtained when lower concentrations of the hydrocolloids were used. In addition, LMP and combinations of LMP and ALG provided higher recoveries than when ALG alone was trialled.

Calcium treatment of the prepared microcapsules was evaluated as it was expected that the calcium ions would effectively cross-link with the hydrocolloids and enhance the integrity and stability of the wall material of the capsule. SEM images indicated that calcium treatment was useful as a means of strengthening the barrier between the materials encased within capsules and the exterior conditions depicted by a hardening of the surface of the microcapsules.
11.6 Application of the dialysis model for determining release characteristics of starch based microencapsulated folic acid

The validated dialysis model from the preliminary steps of this study was objectively applied to the starch based microcapsules with folic acid as core material. The rates of release of folic acid from calcium treated and control samples of microcapsules were significantly different. This demonstrates the novelty of this study, where for the first time, the dialysis model might be usefully applied to simulate the rates of release of microcapsule contents within the digestive system. Its application provides significant potential in the controlled release studies of microcapsules as it appears to simulate the *in vivo* conditions of the human digestive tract.

11.7 Conclusions

The final conclusions of this study are summarised here:

1. An *in vitro* digestion model using DT has been evaluated and optimised with respect to types of DT used, the addition of CaCl2 for enzyme optimised activity, source of α-amylase, concentration of the enzyme used, the effect of granule size on digestion rate and its effect on starch botanical origin;

2. Assessment of digestion was carried out by a DNS reagent assay as well as the use of HPLC-RI. Both methods give comparable results with HPLC-RI providing the identification of products of digestion;

3. The *in vitro* digestion DT model allowed the passage of malto-sugars of DP up to three i.e. glucose, maltose and maltotriose and isomaltotriose while malto-sugars with higher DP did not dialyse;

4. A sedimentation technique was successfully applied in separating the two main starch fragments from the bimodal wheat starch based on granule size. It was confirmed that the smaller starch granules were digested at a faster rate than those of larger size;

5. Degree of digestion of starch is dependent on the botanical source of the starch as it is dependent on granule size. Generally, signs of pitting, channelling, endo-corrosion and granule collapse were observed in the ESEM micrographs while cross-sectional
images by both the ESEM and TEM strengthened the evidence of the channelling and teething resulting from $\alpha$-amylase attack on native starches. Sample preparation, dying and embedment of digested starch for TEM are long and tedious while sectioning sample for the ESEM included a degree of randomness but provided images of high quality;

6. Microencapsulation of folic acid by spray drying provided both high yield and high recoveries with uniformly fine particles. Ratio and levels of binding agents were assessed;

7. Higher folic acid retention is obtained with an increase in ALG and a decrease in LMP but using ALG only as binding agent leads to significant losses of folic acid during calcium treatment of the microcapsules. Hence, a combination of 1:1 LMP:ALG provides the optimum folic acid retention during the production of calcium coated microcapsules;

8. Lower levels of binding agents result in higher loss of folic acid while higher levels of up to 8% resulted in a more viscous slurry and agglomerated microcapsules with higher moisture content. Hence, a combination of 1% of 1:1 LMP:ALG is found to produce microcapsules with optimum core material retention;

9. Calcium treatment of microcapsules significantly reduces core material release as a result of the cross-linking of the hydrocolloid components when the in vitro digestion model was applied. $\alpha$-Amylase activity was reduced as a result of the calcium treatment on the microcapsules, confirmed by a lower release of reducing sugars. In addition, calcium treated microcapsules showed fewer signs of pitting and erosion but a more compact structure bound by the complex formed between calcium and ALG/LMP, hence facilitating the containment of the core material.

In conclusion, a series of comprehensive studies towards the standardisation of the in vitro digestion of native starches have provided useful insight and understanding. Its innovative application on starch based microcapsules provides a reliable platform for the use of in vitro approaches to evaluation of controlled release of the core material. The results have demonstrated the production of microcapsules with enhanced retention of folic acid. This
study has been applied to the microencapsulation of folic acid but also offers potential for other water soluble and sensitive core materials.

11.8 Possible areas for future research

The broad aim of this research was to evaluate, validate and apply an *in vitro* digestion model. This has been considered as a means of studying the digestibility of starch granules by focussing upon native granular starches. In particular the intestinal stage of human digestion was modelled because it has been suggested that this is the site of digestion for most of the starch that we ingest. On the basis of the results obtained in the current study, there are a number of areas of research that are now recommended for further investigation.

Although it has been suggested that starch is primarily being digested within the small intestine, it appears that there have been relatively few studies on the relative contributions of the oral, gastric and intestinal phases. It would therefore be useful if the *in vitro* procedures available could be applied to such a study. This would extend the model system used in the current study but should also incorporate the validated modifications established for intestinal digestion described in this thesis. This recommendation would extend our knowledge of the *in vitro* digestion to the oral and gastric stages of the human digestive system which would account for a more in-depth study of the *in vitro* digestion of native starches from start to end points. A further aspect here is that such work is likely to be relevant to our understanding of other food components which might influence starch digestion as well as their significance for microcapsules prepared from materials other than starch.

A further addition to the work described here would be to investigate the effects of the additional use of the secondary enzyme amylglucosidase in the digestion model. This enzyme is usually involved in the complete digestion of starch to glucose following the action of α-amylase and would further develop our understanding of digestion and the model system described here.

In relation to the digestion of starches from different botanical sources, the current work demonstrated the significant influence of granule size, both for a variety of starch sources
as well as within the bimodal distribution that is characteristic of wheaten starch. However, other factors such as surface characteristics including the presence of surface pores and starch crystalline fractions may also have contributory roles. Accordingly it is desirable that additional investigations consider these factors as determinants affecting the digestion rate of native starches.

Regarding the *in vitro* digestibility model, the current work provides evidence of the usefulness of this approach for studying release properties of sensitive materials for which it is required during food digestion. Although the trials needed would present a variety of challenges, the ultimate value of this simplified approach and the model system would involve a direct comparison involving clinical trials and human subjects.

Microencapsulation of folic acid using LMP and ALG as binding agents in conjunction with rice starch offer potential for folic acid protection and its controlled release within the digestive tract. The inclusion of a secondary treatment with calcium demonstrated the reinforcement of the microcapsule structure and hence slower release of folic acid upon digestion. There are a number of aspects of microencapsulation that were beyond the immediate scope of the current study and these warrant attention. Examples include a systematic comparative evaluation of different sources of pectins and alginates as it is known that source as well as treatments of these materials can influence their properties. It is therefore likely that the effectiveness of microencapsulation including the calcium treatment might be influenced. Other aspects of encapsulation that may be important are the inclusion of antioxidant materials that may have a protective effect upon the core material as well as varying pH because this is known to be a significant determinant of the properties of the hydrocolloids used here, along with the stability properties of folic acid.

Based upon the potential attributes of the microcapsules developed and described here, another area that will require ongoing study is their ultimate application in a selection of different food matrices. These should be selected to encompass a range of food types. These might be chosen to represent, for example, a variety of cereal products with different pH as well as water activity and moisture contents. It is certainly possible that the combination of binding agents giving highest retention properties might be different for different foods. Accordingly a variety of trials should be designed to evaluate these aspects.
The particular active agent studied here has been folic acid, reflecting its importance for expecting mothers and their unborn infants, as well as the wider population. In the context of the global efforts to enhance human wellbeing, to ensure the adequacy of intakes of essential nutrients and to develop fortification strategies for these purposes, the models and approach described in this thesis can also be extended to other sensitive micronutrients. This includes a number of the other vitamins that are known to be relatively unstable during processing, transport and storage of foods. Another micronutrient that has been identified as representing a problem with widespread deficiency as well as challenges in food fortification is iron. Although it must be recognised that different procedures may be necessary for different food components, the adaptation of the encapsulation procedures reported here may prove useful not only for water-soluble vitamins as well as those that are fat-soluble and mineral nutrients for which encapsulation is required.

Similarly a variety of functional ingredients might be investigated. Among these are antioxidant compounds and the many phytochemicals that are increasingly the subject of study as many of these might also provide strong benefits for health and wellbeing. Again the benefits of these dietary components may be enhanced if they require protection or gradual release is important.

Finally, it is our earnest hope that the work reported here may provide a strong foundation upon which future research may be built. Furthermore may the adaptation and extension of the strategies developed in the current study have significant benefits to nutrition and well-being of the many individuals who make up our world’s growing population.
References


References


doi: http://dx.doi.org/10.1016/j.tifs.2005.03.013


References


References


References


standardisation. *International Journal of Food Science and Technology, 43*(12), 2245-2256.


