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**STANDARDISATION OF *IN VITRO*
CARBOHYDRATE DIGESTION METHODS FOR
PREDICTING THE RELATIVE GLYCEMIC
RESPONSE TO FOODS**

A thesis presented in partial fulfilment of the requirements for the
degree of doctor of philosophy in the nutritional sciences

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ABSTRACT

Global incidence of type II diabetes is driving the need for communication, via food-labelling, of the likely glycemic effects of foods. *In vivo* methods for measuring the glycemic response are costly, time-consuming and hence unsuitable for routine food analysis. *In vitro* carbohydrate digestion methods offer an alternative to *in vivo* testing. Foods are incubated sequentially with pepsin and pancreatin under simulated *in vivo* conditions and the pattern of sugar release used as a predictor of the food's likely glycemic effect. *In vitro* methods are well-suited to routine food analysis since they are inexpensive, high-throughput and yield highly precise results. Application of *in vitro* technology is hindered by the lack of standardised methodology. Countless *in vitro* methods are described in the literature. All differ in their approach to replicating *in vivo* conditions. It is not known what effect such differences in methodology might exert on relative estimates of glycemic response.

A systematic investigation was undertaken to characterise the relative effect of method variables on subsequent *in vitro* digestion results, using five standard test foods. Variables investigated include mode of comminution, pepsin inclusion versus omission, amylolytic enzyme concentration, incubatum pH and stirring method. A rudimentary framework for a standardised *in vitro* method is proposed. Comminution and stirring were the method factors most influential to *in vitro* starch digestion kinetics. Thus, the standardised method features differing approaches to comminution and incubatum stirring depending on the structural properties of the food to be analysed.

In vitro methods, in their current format, do not account for the effect of gastric emptying rate on the glycemic response. The glycemic response and gastric emptying rate of ¹³C-labelled flatbreads containing either 5, 15 or 30 % fat, known to slow gastric emptying, was measured in ten healthy subjects via a GI test and breath testing. The objective was to obtain *in vivo* data for gastric emptying that might be applied as a correction to parallel *in vitro* digests of the flatbreads improving their predictive power. Gastric emptying rate reduced significantly with increased flatbread fat content. There was no difference in the glycemic response to each flatbread. Due to the lack of glycemic effect *in vivo*, no adjustments to *in vitro* curves could be made.

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Glycemic Carbohydrates: Standardisation of In Vitro Methods

J W Woolnough, J A Monro, A R Bird, C S Brennan

Asia Pacific Journal of Clinical Nutrition

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Simulating Human Carbohydrate Digestion In Vitro: a Review of Methods and the Need for Standardisation

J W Woolnough, J A Monro, C S Brennan, A R Bird

International Journal of Food Science and Technology

Volume 43 Issue 12, Pages 2245 – 2256, 2008

Impact of Guar and Wheat Bran on the Physical and Nutritional Quality of Extruded Breakfast Cereals

M A Brennan, I Merts, J A Monro, **J W Woolnough**, C S Brennan

Starch/Stärke

Volume 60 Issue 5, Pages 248 – 256, 2008

The Effect of a Brief Salivary α -Amylase Exposure During Chewing on Subsequent In Vitro Starch Digestion Curve Profiles

J W Woolnough, A R Bird, J A Monro, C S Brennan

International Journal of Molecular Sciences

Volume 11 Issue 8, Pages 2780 – 2790, 2010

Predicting Glycemic Response In Vitro – Oral Presentation

NZIFST Annual Conference

Wellington, NZ

June 2007

Glycemic Carbohydrates: Standardisation of In Vitro Methods – Oral Presentation
Joint NZ & Australian Nutrition Societies Conference and Annual Scientific Meeting
Auckland, NZ
December 2007

Physiological Determinants of the Glycemic Response to Foods – Oral Presentation
CSIRO Food Futures Flagship Annual Scientific Meeting
Canberra, ACT
March 2010

*A Comparison of the Effect of Comminution Techniques and Stirring Methods on
Subsequent In vitro Starch Digestion Kinetics*
J W Woolnough, C S Brennan, J A Monro, A R Bird
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*The Effect of Fat on Gastric Emptying Rate and the Glycemic Response to Flatbreads:
Reflecting this Effect In Vitro*
J W Woolnough, A R Bird, J A Monro, C S Brennan
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LIST OF ABBREVIATIONS

%dose/h	Per cent recovery of the original ^{13}C dose administered per hour
AACC	American Association of Cereal Chemists
abs	Absorbance
AC	Available carbohydrate
A_{GR}	Amplitude (mmol/L) of the glycemc response
AMG	Amyloglucosidase
ANOVA(s)	Analysis of variance
AUC	Area under the curve
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CPDR	Cumulative percentage of the original dose (^{13}C) recovered
DNS	3,5-dinitrosalicylic acid
DF	Dietary fibre
DP	Degree of polymerisation
DS	Digestible starch
EE	End-over-end inverter
EE, fast	End-over-end inverter, fast treatment (2 rotations every 30 seconds)
EE, slow	End-over-end inverter, slow treatment (1 rotation every 30 seconds)
ESA	Enzyme solution A
FAO	Food and Agriculture Organisation
g	Gram(s)
GGEs	Glycemc glucose equivalents
GI	Glycemc index
GIP	Glucose-dependent insulintropic polypeptide
GL	Glycemc load
GLP-1	Glucagon-like peptide 1
GR	Glycemc response
h	Hour(s)
HI	Hydrolysis index
HPLC	High-performance liquid chromatography
IAUC	Incremental area under the curve
imm	immediate

inv	Invertase
min	Minute(s)
mmol/L	Millimoles per litre
MS	Magnetic stirrer
MS, fast	Magnetic stirring, fast treatment (260 rpm)
MS, slow	Magnetic stirring, slow treatment (130 rpm)
NSP(s)	Non-starch polysaccharides
PG	pregelatinised (starch)
RDS	Rapidly digestible starch
ReS	Reducing sugar(s)
rpm	Rotations per minute
RS	Resistant starch
s	Seconds
sal	Salivary
SD	Starch digestion
SDS	Slowly digestible starch
SE	Standard error of the mean
SEM	Scanning electron microscopy
SWB	Shaking water bath
SWB, fast	Shaking water bath, fast treatment (140 strokes/min)
SWB, slow	Shaking water bath, slow treatment (70 strokes/min)
T ₀	Time zero
T ₂₀	20 min of pancreatic digestion
T ₁₂₀	120 min of pancreatic digestion
T _{1/2GE}	Gastric half-emptying time (min)
T _{GRP}	Time (min) to glycemic response peak
T _{lag}	Gastric emptying lag time (min)
T _{max}	Peak gastric excretion time (min)
TS	Total starch
U	Units (of enzyme)
WHO	World Health Organisation

CHAPTER 1: INTRODUCTION

1.1 DIETARY CARBOHYDRATES

Carbohydrates are a diverse class of biomolecule found abundantly in nature and are the main source of energy in the human diet. The Food and Agriculture Organisation (FAO) estimated that in 1994, 63.2 % of each individual's daily energy intake around the globe came from consumption of dietary carbohydrates (1). The major carbohydrate-containing foods in the human diet are cereals, sweeteners, root crops, pulses, vegetables, fruit and milk products.

When eaten, carbohydrates have been shown to affect satiety (2, 3), blood glucose and insulin levels (4, 5), large bowel habit (6, 7) and large bowel epithelial cell health (8, 9). Consequently, dietary carbohydrates exert major influence on our overall state of health and wellbeing with particular regard to control of body weight, onset of type II diabetes, management of type I and type II diabetes as well as development of cardiovascular disease and bowel cancer.

1.2 CARBOHYDRATE CLASSIFICATION

There are two approaches to the classification of dietary carbohydrates. The primary classification as proposed by the 1997 joint FAO/WHO consultation (1) is based on chemistry. Due to the diversity of physiological properties exhibited by carbohydrates, however, even between carbohydrates of the same chemical class, a secondary classification scheme for dietary carbohydrates, based on nutritional properties has evolved.

1.2.1 Chemical Classification

The chemical approach to classification is based on the degree of polymerisation (DP) and divides carbohydrates into three groups: sugars, oligosaccharides and polysaccharides. A summary of the chemical scheme for classifying carbohydrates is shown in **Table 1.1**.

CLASS	DP	SUBCLASS	EXAMPLES
Sugars	1 - 2	Monosaccharides	glucose, galactose, fructose
		Disaccharides	lactose, sucrose, maltose
Oligosaccharides	3 - 9	Malto-oligosaccharides	dextrins
		Other oligosaccharides	raffinose, stachyose, fructans
Polysaccharides	> 9	Starch	amylose, amylopectin
		Non-starch polysaccharides	cellulose, hemicellulose, pectin

Table 1.1 The major classes of dietary carbohydrates based on their chemical properties. DP, degree of polymerisation. [SOURCE: FAO, Carbohydrates in human nutrition. FAO Food and Nutrition Paper – 66. Report of a Joint FAO/WHO Expert Consultation. Rome, Italy. 1997].

The term sugars encompasses monosaccharides (DP 1) which consist of a single sugar residue, such as glucose and fructose, as well as disaccharides (DP 2), which consist of two sugar monomers linked via a glycosidic bond e.g. sucrose (glucose + fructose) and lactose (galactose + glucose).

Oligosaccharides are short chains of between three and nine glycosidic-linked residues. Malto-oligosaccharides (also termed α -glucans or dextrans) consist of short chains of glucose monosaccharides. Other oligosaccharides include raffinose, stachyose and fructo-oligosaccharides consisting of short chains of combinations of galactose, glucose and fructose monosaccharides.

Polysaccharides are high-molecular weight carbohydrates of DP greater than nine. There are two types of polysaccharide: starches and non-starch polysaccharides (NSP). Starch is the storage carbohydrate of plants such as cereals, legumes and root vegetables and subsequently, is the principal carbohydrate of the human diet. Starch consists exclusively of polymerised glucose monosaccharides occurring in two distinct versions: amylose and amylopectin. Amylose is a non-branching, helical chain of glucose residues (DP $\sim 10^3$) linked by α -1,4 glycosidic bonds whereas amylopectin is much larger (DP $\sim 10^4$ - 10^5) and unlike amylose is highly branched, containing α -1,4 as well as α -1,6 glycosidic bonds at its branch points. Amylose and amylopectin polymers are arranged in a semi-crystalline pattern, packaged within starch granules. The ratio of amylose to amylopectin occurring within starch granules varies with botanical origin, cereal starches for instance typically contain between 15 and 30 % amylose (10).

Non-starch polysaccharides consist of long chains of glycosidic-linked monosaccharides that, unlike starch, are not necessarily exclusively glucose. Cellulose, a linear β -1,4 linked glucose chain (DP 10^3 - 10^6) consists entirely of glucose but because of its β -1,4 linkage is able to form hydrogen bonded multichain linear structures that are strong and highly resistant to degradation. Hemicelluloses are branched heteropolymers of several sugars including xylose, mannose, glucose and arabinose (DP 150-200). Together cellulose and hemicellulose are the most widely distributed NSP occurring in the diet and are major structural components of plant cell walls (10, 11). Due to the common β -linkage between their sugar residues and the fact that sugar

residues are often not glucose, NSPs are not digested by pancreatic α -amylase and brush border enzymes (maltase and dextrinase) in the small intestine which are specific for glucose units linked by α -1,4 and α -1,6 bonds.

1.2.2 Nutritional Classification

Since the chemical divisions of carbohydrate do not reflect their diverse physiological effects when eaten, various terminologies have been developed over time that categorise carbohydrates on a nutritional basis. The major nutritional divisions of dietary carbohydrate are summarised in **Figure 1.1**.

Resistant starch is that portion of starch (as well as the by-products of partial starch digestion e.g. maltose, dextrans) that when eaten, is not absorbed in the small intestine of healthy humans (10). There are four types of resistant starch, distinguishable by the cause of their resistance to digestion: physically inaccessible resistant starch due to encapsulation within the food matrix (RS1), starch granules resistant because they have not been gelatinised during cooking (RS2), retrograded starch (RS3) which forms during heating and subsequent cooling of processed foods, and chemically modified starch (RS4) (1, 10, 12).

Dietary fibre is the term used to describe the dietary carbohydrates that are not digested in the human small intestine, consisting essentially of components of the plant cell wall: cellulose, hemicellulose and pectin (i.e. NSP). Resistant starch, as well as some non-digestible oligosaccharides may sometimes be included in the category of dietary fibre (13, 14) Which carbohydrate components should be included in the definition of dietary fibre has been the subject of debate for years, but NSPs of plant cell wall origin are common to all definitions of dietary fibre, and were the basis of the original definition (15). Dietary fibre enters the large intestine where it is fermented by colonic microflora (16, 17).

The term 'available carbohydrate' defines the fraction of dietary carbohydrate that can be utilised and metabolised by the body (18) i.e. is digestible by human pancreatic enzymes, is absorbed and subsequently metabolised (19). 'Unavailable carbohydrate' on the other hand is that fraction of carbohydrate (including dietary fibre) that reaches

the large intestine and may provide the body with energy (and numerous benefits to gastrointestinal health), not through digestion, however, but via fermentation and absorption of short-chain fatty acids (10). Similar in concept to dividing starch into ‘available’ and ‘unavailable’ are the terms ‘glycemic’ versus ‘non-glycemic’ carbohydrate. Carbohydrate that is hydrolysed in the small intestine by pancreatic α -amylase and which contributes glucose for metabolism is referred to as ‘glycemic carbohydrate.’ Most mono- and disaccharides as well as α -glucan oligosaccharides and all digestible starch are classed as glycemic carbohydrate. Non- α -glucan oligosaccharides, resistant starch, dietary fibre and NSPs that are not hydrolysed in the small intestine do not provide energy to the body in the form of glucose and hence are termed ‘non-glycemic carbohydrate’ (10).

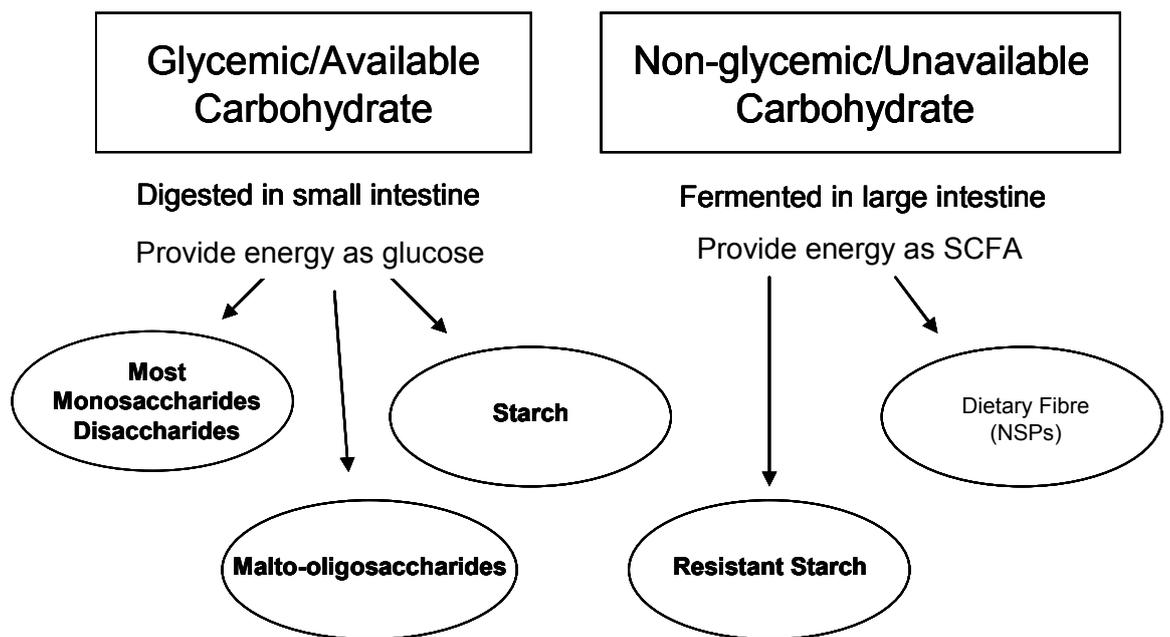


Figure 1.1 The major divisions of dietary carbohydrates based on their physiological or nutritional properties. SCFA, short chain fatty acids.

1.3 CARBOHYDRATE DIGESTION

Carbohydrate digestion is initiated in the mouth where mechanical breakdown of foods by chewing occurs as well as mixing with salivary secretions containing α -amylase. The result is formation of a lubricated bolus suitable for swallowing, and limited hydrolysis of starch to maltose and dextrans (20, 21).

Swallowed boluses are propelled via peristaltic contractions through the oesophagus and into the stomach where gradual mixing with acidic (\sim pH 2) gastric secretions occurs. Pepsin enzyme, secreted by the stomach digests the protein component of the meal. The acidic conditions of the stomach inactivate salivary α -amylase preventing further hydrolysis of starch, although it is likely that a small amount of starch digestion continues within the centre of undisrupted food boluses provided α -amylase is protected from gastric conditions (22). Rhythmic contractions of the stomach wall reduce solid bolus matter into a semi-liquid chyme. At emptying, solid food particles within chyme are of 1 mm diameter or less (23-25). This mechanical aspect of gastric digestion of food greatly potentiates its later chemical digestion in the small intestine through increasing the food's surface area available for interaction with enzyme (24).

Gastric emptying of the meal into the duodenum occurs in periodic spurts of chyme that via duodenal contractions are rapidly mixed with pancreaticobiliary secretions (24) containing sodium bicarbonate, bile salts, proteolytic enzymes, lipases and pancreatic α -amylase (22). As chyme is propelled through the small intestine, pancreatic α -amylase enzyme progressively hydrolyses the α -1,4 glycosidic bonds of starch releasing monosaccharide glucose, maltose disaccharide and dextrin oligosaccharides. Disaccharides (including sucrose and lactose - present depending on the food type) are hydrolysed in turn into their absorbable monosaccharide components (glucose, fructose, galactose) by disaccharidase enzymes embedded within the microvillus brush border of the small intestine wall. Since starch is the major carbohydrate in the human diet, approximately 80 % of monosaccharides present within the small intestine at the end of digestion, and prior to absorption, are glucose (21, 26, 27).

Absorption of glucose from the small intestine lumen across the intestinal wall enterocytes occurs predominantly in the proximal small intestine via sodium-glucose

cotransporters at the luminal membrane and GLUT2 transporters at the basolateral membrane (28) and thence via diffusion into the intestinal villus capillary beds (29). Once in the blood, glucose travels via the portal vein to the liver and then via the systemic circulation to the rest of the body - the glycemic response - where it provides the major energy source for all tissues (20). **Figure 1.2** provides a summary of the major processes underlying carbohydrate digestion in the human upper gastrointestinal tract and generation of a glycemic response.

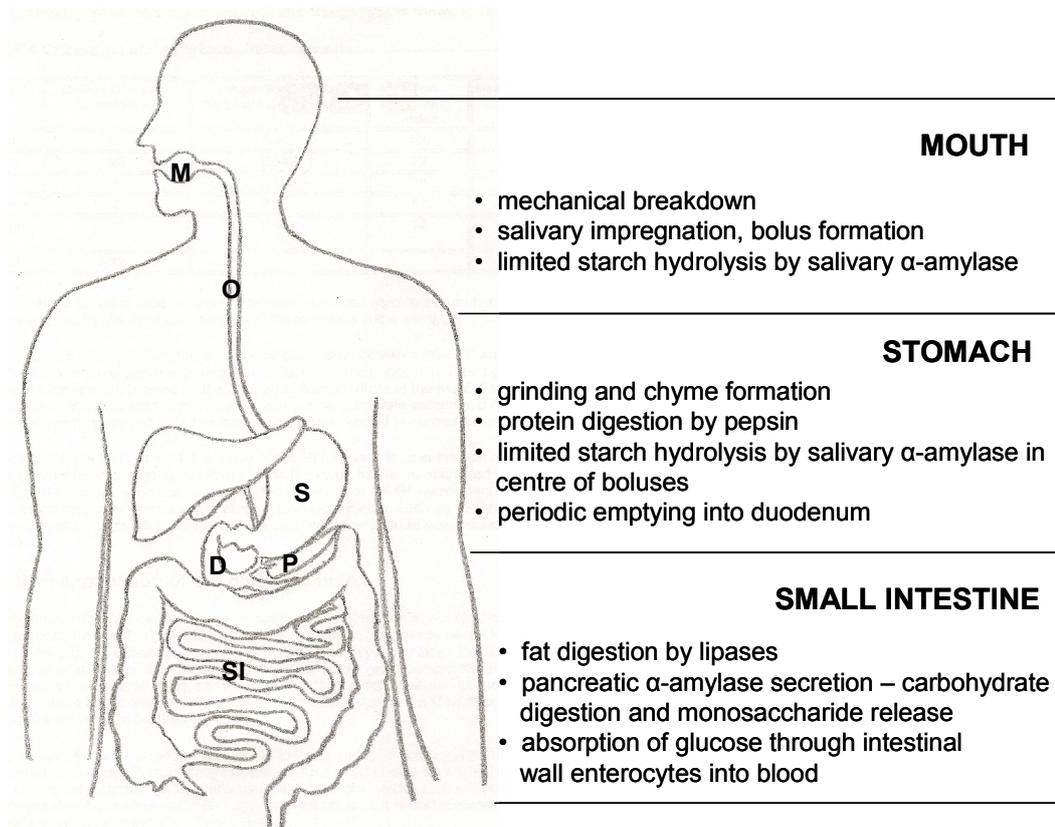


Figure 1.2 The processes underlying carbohydrate digestion and generation of a glycemic response within the human upper gastro-intestinal tract. M, mouth; O, oesophagus, S, stomach; D, duodenum; P, pancreas; SI, small intestine;

1.4 THE GLYCEMIC RESPONSE

The concentration of glucose circulating in the blood during fasting in healthy individuals is maintained at 5.0 - 7.0 mmol/L as a result of homeostatic equilibrium established between the physiological effects of insulin and glucagon hormones. The glycemic response is a disruption of this equilibrium, with elevation of glucose levels in the blood above the normal fasted concentration following consumption, digestion and absorption of a carbohydrate-containing meal. Plotted as a curve over time, the glycemic response is characterised by a blood glucose rising phase, a glucose peak and a glucose disposal/removal phase with subsequent restoration of fasting blood glucose levels (**Figure 1.3**). Depending on many factors, particularly food type, the glycemic response may be gradual in its onset, low in amplitude and prolonged in duration (low glycemic response) or toward the other extreme: rapid in onset, high in amplitude and transient in duration (high glycemic response).

Insulin and glucagon hormones are secreted by the β - and α - cells of the pancreatic islets of Langerhans respectively. Insulin secretion, triggered by rising blood glucose, induces the uptake and hence removal of glucose from the blood by cells, particularly of the liver, muscles and adipose tissue. Glucagon, secreted during periods of fasting and to prevent the onset of hypoglycemia, induces glycogenolysis of glycogen stores in the liver and subsequent release of glucose into the blood (20).

1.5 FACTORS AFFECTING THE GLYCEMIC RESPONSE TO FOODS

Carbohydrate foods differ markedly in their effects on the postprandial glycemic response. The rate at which carbohydrate (mainly starch) digestion occurs in the small intestine is the key determinant of the size and shape of the glycemic response curve (30-32). It follows, then, that any factor that alters the rate and extent to which starch digestion occurs, will subsequently influence the nature of the glycemic response.

Starch digestion rate is affected principally by two broad categories of factors – intrinsic food factors i.e. the physico-chemical properties of foods, and physiological factors – the manner of handling of carbohydrate foods by the gastrointestinal tract, which differs with different food types (32).

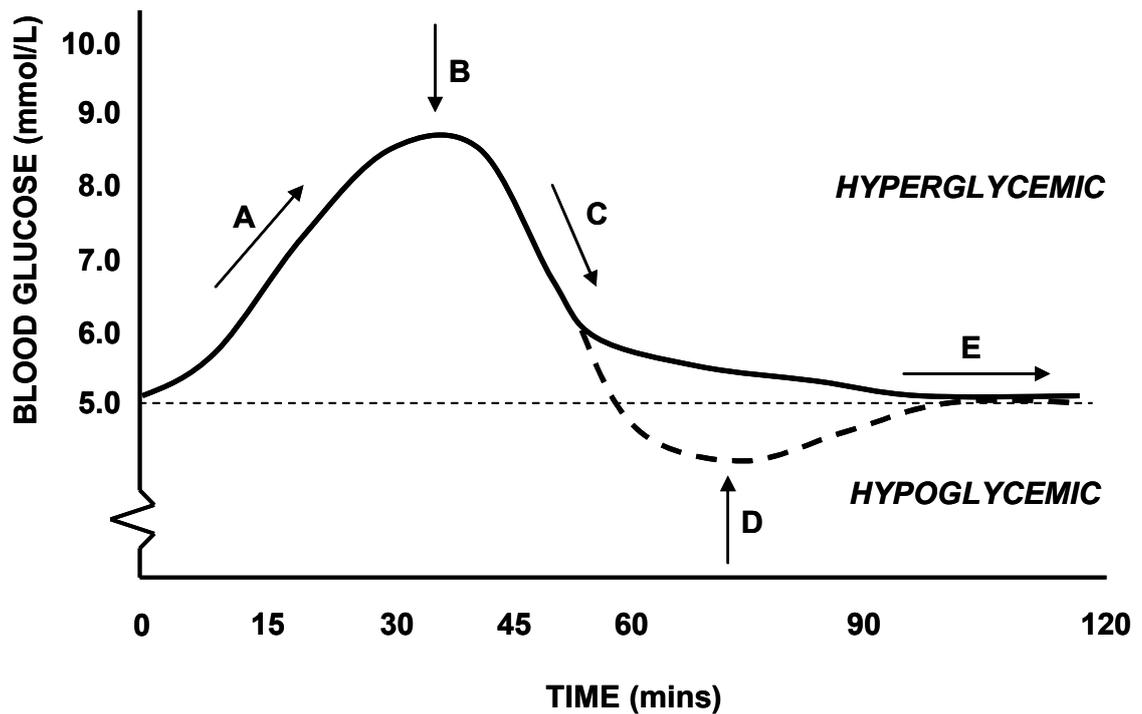


Figure 1.3 The typical appearance of a glycaemic response if measured and plotted as a curve over time following consumption of a carbohydrate-containing meal. The dashed line represents baseline, or fasting blood glucose concentration. A hyperglycaemic state exists when blood glucose concentration is above the normal fasted glucose concentration. Alternatively, a hypoglycaemic state exists when blood glucose concentration falls below the resting glucose concentration. The glycaemic response curve is characterised by an ascending phase (A) where carbohydrate digestion, glucose release and absorption into the blood is ongoing, the glucose response peak (B) where carbohydrate digestion may or may not be complete but physiological mechanisms, namely insulin-induced uptake of glucose by tissues are beginning to take effect and there is net removal/disposal of glucose from the blood, a descending phase (C) where glucose removal from the blood by insulin-mediated tissue uptake is ongoing; there may or may not be a hypoglycaemic phase (D) where blood glucose falls below the original fasted level due to ongoing insulin activity; and restoration of normal/fasted blood glucose concentration (E) where insulin/glucagon equilibrium is re-established.

The botanical origin of the dietary carbohydrate determines the species of sugars present, the types of linkages between sugars, the degree of polymerisation and the ratios of amylose to amylopectin occurring within the starch granule, all of which are factors that affect starch digestibility. Starches high in amylose, for instance, have been shown to digest more slowly than those with reduced amylose. Due to its linear and therefore more compact structure, amylose is hydrolysed more slowly than amylopectin whose branched structure makes it more accessible to amylase (33-35).

Different food processing and preparation techniques cause drastic changes in the structure of foods which in turn affect the digestibility of starch. Disruption of the original botanical structure of carbohydrate foods through, for example, rolling (36), milling (37) and even chewing (38) increases the availability and hence digestibility of starch. Other processing techniques, for example extrusion, can decrease the digestibility of starch in some foods (39, 40) due to the relative impermeability to amylase enzymes of the resulting processed food. Heat processing of carbohydrate foods, particularly in the presence of water leads to disruption of the crystalline structure of starch (gelatinisation) increasing its digestibility (41). Retrogradation on the other hand, is a re-crystallisation of starch (particularly amylose) that occurs to varying extents during subsequent cooling of foods and produces lengths of starch crystal highly resistant to digestion by α -amylase (42, 43)

In mixed foods, the presence of ingredients other than starch, such as fibre, fat or protein can decrease starch digestibility through encapsulation of starch granules (44, 45), formation of starch-lipid or starch-protein complexes reducing availability of starch to amylase (35, 46, 47), or through restricting the degree to which starch gelatinisation occurs during heat treatment (45).

In terms of physiological factors affecting the glycemic response, upper gastrointestinal motility, in particular the rate of gastric emptying is the major influence on the nature of the postprandial blood glucose concentration (28). Since the stomach controls the rate of delivery of nutrients to the duodenum where starch digestion begins in earnest, any change in gastric emptying rate will translate into an altered rate of starch delivery to and digestion in the small intestine, and appearance in the blood as glucose. Studies

have demonstrated a correlation between delayed gastric emptying rate (usually measured as the gastric half-emptying time) and relative reduction in the postprandial glycemic response (48-50). Key mechanisms underlying reduction of gastric emptying rate, in particular as a result of fat ingestion, are explained in part II of chapter 2.

Motility of the small intestine has also been related to the glycemic response. Contractile activity of the small intestine wall governs the transit time of dietary carbohydrates in the small intestine, the degree of mixing that occurs between starch and α -amylase, as well as the extent of contact between digested starch species and the absorptive surface of the small intestinal wall (28). Increased propagating peristaltic contractions of the small intestine wall following ingestion of dietary fibre with the net effect of pushing chyme through the small intestine at a more rapid rate has been linked with a reduction in the postprandial glycemic response (51). Pharmacological inhibition of small intestinal contractions has also been shown to decrease glucose absorption from the small intestine lumen through reducing contact between glucose and the intestine wall (52).

The glycemic response to foods also varies with individual subject. Factors such as age, gender and BMI (53, 54) as well as the individual's health status (insulin resistant, type I or type II diabetic) can affect the nature of the glycemic response following consumption of a carbohydrate-containing meal.

1.6 EXPRESSIONS OF THE GLYCEMIC RESPONSE: DEFINITIONS

Given the multitude of factors that affect the glycemic response to foods and the link between consumption of foods of high glycemic potency and increased risk of development of obesity, type II diabetes and certain cancers (55-60), there is huge interest from scientists and food manufacturers alike in methods for measuring, characterising and expressing the glycemic response to foods and ranking them accordingly. This interest is driven by a consumer market where incidence of obesity and type II diabetes is epidemic and there is increasing awareness of nutritional intervention as a means of preventing and managing such conditions.

The glycemic index (GI) is the most well known and widely used method of classifying and ranking foods based on their postprandial blood glucose raising potency. First published in 1981(61) the GI is defined as the incremental area under the (blood glucose response) curve (IAUC) following consumption of a portion of a test food containing 50 g available carbohydrate expressed as a percentage of the average IAUC response to the same amount of carbohydrate from a reference food (white bread or glucose drink) taken by the same subject on a separate occasion (62). Therefore, GI classifies foods on the basis of glycemic potency of the available carbohydrate in them, determined indirectly from the response to a whole food, and based on “available carbohydrate” measured in standard food analysis, rather than on carbohydrate that is actually available.

GI testing classifies foods into one of three categories depending on their GI value: low GI (GI value ≤ 55), medium GI (56 – 69) and high GI (GI value ≥ 70). Studies have demonstrated a link between consumption of low - medium GI foods and subsequent improvements in fasting glucose, glucose control, LDL cholesterol, glycated haemoglobin and satiety (63-66) in type II diabetic subjects. As a means of measuring and characterising the glycemic response to foods, however, the GI concept has drawn criticism from some, due to, for example, its classifying foods in isolation rather in the context of a mixed meal (67), testing foods on an available carbohydrate instead of a more realistic per serving basis (68) as well as testing on individuals under highly standardised, therefore irrelevant conditions (see section 3.2.3). Furthermore, the expense of testing as well as the need for human ethics approval makes implementation of GI testing in routine glycemic analysis of foods, impractical.

Whereas the GI compares the potential of foods containing the same amount of carbohydrate to raise blood glucose (a comparison of carbohydrate quality), the quantity of carbohydrate consumed during a meal affects the glycemic response also. The concepts of glycemic load (56) and relative glycemic potency (69) were developed, therefore, in 1997 as an extension of GI and combined both carbohydrate quality and quantity in their expression of the glycemic response. Defined as the amount (in grams) of carbohydrate in a given meal multiplied by its glycemic index, the GL provides a measure of the relative glycemic response of a typical serving of food. Foods with a GL ≤ 10 , GL between 11 and 19, and GL ≥ 20 are classified as low, medium or high GL

respectively (70, 71). In healthy subjects, stepwise increases in GL have been shown to produce stepwise increases in both the postprandial glucose and insulin responses (72). Relative glycemic potency was defined as the potential of a food to induce a glycemic response relative to that of an equal weight of glucose, expressed as a percentage, so it is a true glycemic index of food, rather than of carbohydrate in food.

Glycemic impact is a further means of expressing the glycemic response and is defined as the weight of glucose (in grams) that would induce a glycemic response equivalent to that induced by a given amount of food. Unlike GI which compares foods on an equicarbohydrate basis and is insensitive to realistic serving sizes, glycemic impact is measured directly by calculating the weight in grams of glucose required to give the same glycemic response as a serving of food. The comparison between test food and glucose reference is on an equiglycemic basis and the values for glycemic impact are in grams and may be termed glycemic glucose equivalents (GGEs) (68, 73). GGE values are potentially useful in food labelling and communication of the glycemic effects of foods since they are measurable and communicable as a virtual food component on a grams per 100 g of food or grams per serving basis.

Glycemic Index, GL and glycemic impact are all based on the currently accepted methodology for measuring the postprandial glycemic response – calculation of the IAUC over two hours following consumption of test food by volunteers. This measure is expensive to obtain, however, human ethics approval is required and the results often show large intra- and interindividual variability.

The development of *in vitro* digestion methodologies that mimic the digestive processes occurring in the upper gastrointestinal tract, thus providing an indication of the likely kinetics of starch digestion *in vivo*, have given rise to several *in vitro*-based terminologies for expressing the likely glycemic response to foods. *In vitro* digestion methods are gaining favour in routine analysis of the glycemic properties of foods as they are time- and cost-effective and the digestion outcomes are highly reproducible (73). As occurs in glycemic index testing, foods may be ranked in order of likely glycemic potency based on the kinetics of *in vitro* starch digestion.

In 1990 Englyst and Kingman (74) published a method for classifying starch in foods into nutritionally important fractions based on the rate of *in vitro* starch digestion. These fractions were presumed to reflect the likely site, rate and extent of starch digestion *in vivo*. Starch digested to glucose within 20 minutes of pancreatic exposure was termed rapidly digestible starch (RDS), starch digested between 20 and 120 minutes was termed slowly digestible starch (SDS) and that starch remaining undigested after 120 minutes exposure to amylase was termed resistant starch (RS) (**Figure 1.4**). Determined levels of RDS and SDS are used widely in *in vitro*-based glycemic ranking of foods. Studies have demonstrated a link between consumption of foods with high RDS content and a large postprandial glycemic response (75-77) and improvement of postprandial glycemic control following consumption of foods containing higher levels of SDS and RS (78, 79).

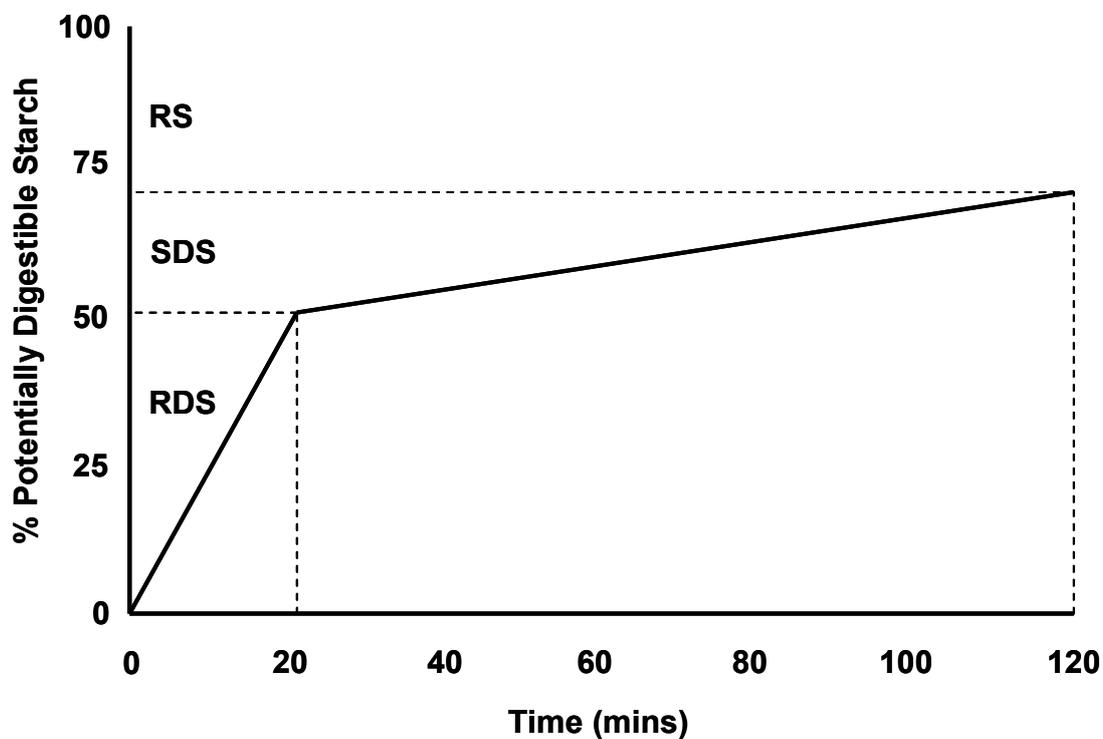


Figure 1.4 A schematic *in vitro* digestion curve for a given test food from which are derived the nutritionally important starch fractions: RDS, SDS and RS. Rapidly digestible starch (RDS) is that starch hydrolysed to glucose by 20 min of *in vitro* pancreatic digestion, slowly digestible starch (SDS) the starch hydrolysed between 20 and 120 min of digestion and resistant starch (RS) the starch that is not digested within 120 min of addition of α -amylase.

CHAPTER 2: LITERATURE REVIEW

PART I:

Simulating Human Carbohydrate Digestion *In Vitro*: A Review of Methods and the Need for Standardisation (80)¹

2.1 INTRODUCTION

Available carbohydrate as defined by the Glycemic Carbohydrate Definition Committee of the AACC is “carbohydrate released from a food by digestion and which is absorbed as monosaccharides and metabolised by the body (73).” The glycemic response is a common measure of the effect a given food’s available carbohydrate content has on postprandial blood glucose concentration. Current global trends relating to Type II diabetes incidence make evermore necessary the capacity to predict with accuracy and precision a given food’s likely glycemic effect. In June 2006 *ad hoc* Glycemic Carbohydrate Definition Committee members (73) expressed concern over the use of *in vivo* measurements for glycemic prediction due to considerable intra- and inter-individual variability in measured postprandial glycemic responses. The committee members recommended developing a robust and standardised *in vitro* method that accurately mimics *in vivo* processes, while measuring a given carbohydrate food’s available and glycemic carbohydrate content as indicative of its likely glycemic impact and effect on glycemic response.

It is the present lack of a robust, validated and standardised *in vitro* carbohydrate digestion method that hinders any large-scale application of the technology to glycemic control via nutrition-labelling in the food industry. To take initial steps toward achieving a standardised method this review compares and contrasts some of the current and commonly referred to *in vitro* carbohydrate digestion methods. Experimental

¹The contents of chapter two, part 1 have been published in the International Journal of Food Science and Technology in the form of a review article but with a research component also (SEE APPENDIX A). To remain consistent with the format of this thesis, only the review section and review-related figures from the published article are included in the present chapter. The research components of the review are included in later chapters of this thesis and referenced accordingly for clarity.

conditions that differ between methods and which may impact on relative carbohydrate digestibility profiles are exposed, thus defining a path toward the development of a robust, reliable and standardised *in vitro* method suitable for large-scale analysis of commercially-available carbohydrate-containing foods.

2.2 BACKGROUND

Current *in vitro* methods for the analysis of the likely glycemic properties of foods have their evolutionary roots in earlier techniques for analysing dietary fibre, total carbohydrate and resistant starch in foods. In 1969, Southgate proposed methods for measuring available (81) and unavailable (82) carbohydrate in foodstuffs by hydrolysing starch with amyloglucosidase and pullulanase. Southgate's method drew criticism, however, due to incomplete removal of starch. Hence in 1982, Englyst *et al* (83) set about developing a quantitative dietary fibre method that incorporated a more effective and specific starch digestion step. In their method, readily digestible starch was hydrolysed with α -amylase and pullulanase, and the portion of starch resistant to α -amylolysis subjected to a more exhaustive hydrolysis with amyloglucosidase. That same year Jenkins *et al* (84) performed a study on the effect of processing on digestibility and the blood glucose response using lentils. A rudimentary *in vitro* assay was performed in parallel using pooled human saliva, and gave results which related positively with blood glucose data.

In 1986 Berry introduced a resistant starch method (85) based on that of Englyst *et al* 1982 (83). Berry's method, however, featured some modifications, namely, removal of Englyst's initial 100 °C heating step to more closely mimic physiological conditions. Subsequently, resistant starch yields were higher. By the early 1990s, published *in vitro* methods, for various different applications, demonstrated a clear trend toward replicating *in vivo* conditions in order to achieve determinations of greater physiological significance. In 1991 Granfeldt and Bjorck (40) outlined a novel *in vitro* method for measuring starch availability in pasta that incorporated a 3-stage protocol simulating the physiological processes occurring in the mouth, stomach and small bowel. By 1992 several such methods had emerged in the literature (86, 87) including that of Brighenti *et al* (88) which was a modification of that of Jenkins' *et al* 1982 method. Usually conducted in parallel with *in vivo* trials, the *in vitro* assays, carried out under simulated

physiological constraints, generated glycemic analytical data that correlated, to varying degrees, with *in vivo* blood glucose results. **Figure 2.1** displays the major *in vitro* carbohydrate digestion methods published in the literature and their evolutionary relationships with preceding methodologies.

2.3 CURRENT *IN VITRO* METHODS

Since the early 1990s to the present day, an array of *in vitro* methods for analysing the digestibility of carbohydrate-containing foods have been reported. While generally there is the common theme exhibited in each of these techniques (the imitation of physiological conditions), the ways in which these conditions are simulated across methods, differ considerably.

2.3.1 Oral Phase

In vivo, the oral phase of digestion entails, primarily, major mechanical disruption of food structure. With mechanical breakdown, there is simultaneous salivary impregnation, a degree of starch hydrolysis by α -amylase, and bolus formation prior to swallowing.

Rather than simulate the *in vivo* oral process, some currently used *in vitro* methods include the actual chewing of sample foods by volunteers. Akerberg *et al* (89), for instance, in measuring the major forms of resistant starch in foods, had a number of volunteers, who, after brushing their teeth, chewed an amount of sample food corresponding to 1 g total starch 15 times for approximately 15 seconds. Similarly, Muir (90), had volunteers chew a prepared sample of food to the stage just before swallowing, at which time the food was removed from the mouth and a sample weighed to contain approximately 0.1 g carbohydrate.

The chewing process has been simulated in some methods through use of sieves of varying gauges (91, 92), mincers (93, 94) or food processors (95). In analyses not necessarily requiring realistic “as eaten” food particle sizes, sample foods may be ground, milled or homogenised (96-98).

Chewing of sample foods by volunteers prior to *in vitro* digestion exposes the foods to some salivary α -amylolysis. In attempting to mimic physiological conditions, several analysts have incorporated a brief α -amylase treatment in their oral phase method. Brighenti simulated the chewing process by extruding sample foods through a Kramer Cell (opening 3 mm) followed by pre-incubation with human salivary α -amylase at pH 6.9 for 5 minutes (99). Lebet (100), with reference to Brighenti's 1992 method, incubated foods with human salivary α -amylase at pH 6.9, 37 °C for 15 minutes. Methods proposed by Urooj (101) and Mishra (102) also included incubation with human saliva or α -amylase respectively.

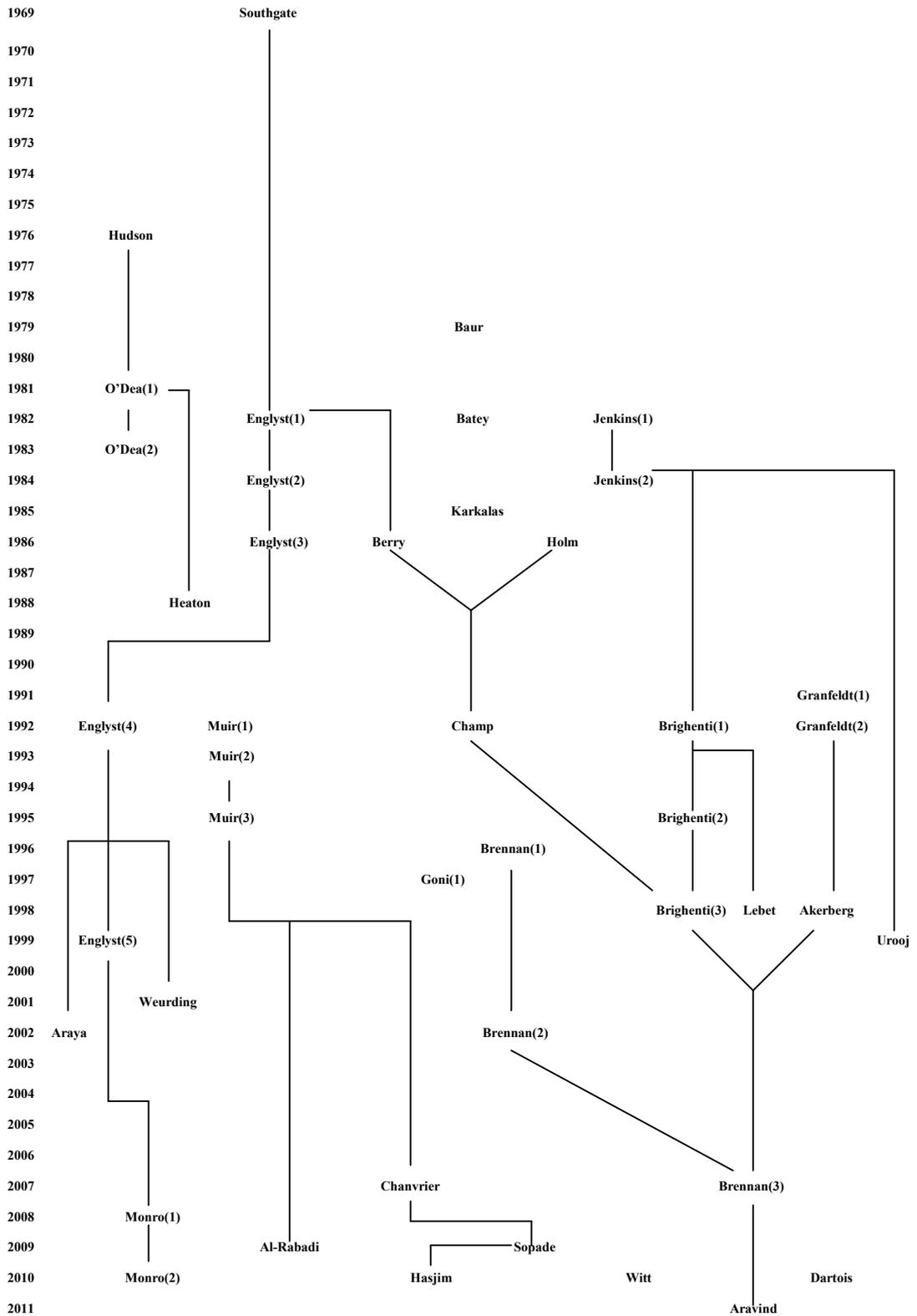


Figure 2.1 Evolutionary relationships of some commonly referred to *in vitro* carbohydrate/starch digestion methods. Connecting lines depict modifications made by analysts to methodology. Methods differ in their application from total carbohydrate and dietary fibre analysis, to quantification of rapidly and slowly digestible and resistant

(Legend to **Figure 2.1** continued) starch fractions. Regardless of the application, the underlying principle is the same – progressive enzymic removal of starch. Earlier methods tended not to observe physiological constraints, whereas the later analytical schemes particularly from 1992 onward, hydrolysed starch under simulated *in vivo* conditions for *in vitro* predictions of a given food's rapidly and slowly digestible and/or resistant starch content. A summary of the experimental details of each method displayed in **Figure 2.1** is provided (in chronological order) in **Table 2.1**.

2.3.2 Gastric Phase

Digestion of the protein component of a meal is the fundamental process occurring within the acidic, mobile confines of the stomach. Subsequent periodic emptying of the resultant chyme into the duodenum occurs in a process dependent on several meal factors including food quantity and viscosity (26), (103). A pepsin proteolysis step began to be incorporated into *in vitro* protocols, particularly in total and resistant starch assays, as a physiological way to achieve complete starch digestion through disruption of the protein matrix as well as starch-protein interactions.

Holm's rapid method (104) for the analysis of cereal starches included a 60 minute pepsin incubation occurring at pH 1.5 and 37 °C on a magnetic stirrer. Holm *et al* had demonstrated the previous year (105), a substantial increase in the susceptibility of raw and boiled wheat to α -amylase following pre-incubation with pepsin. Granfeldt's *in vitro* procedure (106) included a pepsin digestion in which chewed samples were expectorated into beakers containing a pepsin solution and incubated for 30 minutes at pH 1.5 and 37 °C. The beakers were mixed gently three times during the incubation. Inclusion of a pepsin step in Granfeldt's method was based on the earlier findings of Holm and Bjorck (107) and Colonna (45) that starch-protein interactions restricted susceptibility of starch to α -amylolysis *in vitro*. Based also on these findings, Brighenti *et al* (88) acidified samples of extruded spaghetti to pH 2.0 with HCl and incubated them with 575 U of hog pepsin/g of starch for 1 hour. Muir and O'Dea's 1992 method featured a 30 minute pepsin incubation at 37 °C, pH 2.0, performed in a shaking water bath. Englyst's 1992 method for the determination of nutritionally important starch fractions did not include a pepsin proteolysis step. Subsequent Englyst methods however such as that published in 1995 (108) and 1999 (93), did include a pepsin step, where samples were incubated with pepsin for 30 minutes at pH 2.0, in a shaking water bath.

Numerous other *in vitro* methods reported throughout the 1990's incorporated a proteolysis step performed under simulated *in vivo* conditions (95-97, 100). A number of published methods, however, did not (92, 94, 101) subjecting foods to an initial chewing step followed immediately by an α -amylolytic (intestinal) digestion.

2.3.3 Small Intestinal Phase

Following emptying through the pyloric antrum into the duodenum, chyme is mixed with the potent α -amylase-containing secretions of the pancreas. Starch is hydrolysed as the chyme is propelled through the small intestine by peristaltic contraction. Enzymes embedded in the brush-border of the small intestine assist with the complete hydrolysis of starch fragments, maltose, fructose and lactose into their constituent monosaccharides. Monosaccharides are subsequently absorbed into the portal blood where they contribute to a glycaemic response.

During the intestinal phase of any *in vitro* scheme the fundamental occurrence is hydrolysis of sample by α -amylolysis within the constraints of physiological parameters. Depending on what the method is trying to measure, samples of hydrolysate might then be taken at specific time points for measurement of the rate of carbohydrate digestion. Samples might be taken only at the end of the hydrolysis period for determination of available carbohydrate, or samples of hydrolysate may not be taken at all, in which case the hydrolysis is performed to remove digestible carbohydrate for isolation of resistant starch and dietary fibre fractions that are subsequently quantified in further digests.

Jenkins *et al* (109) mixed 10 ml of pooled human saliva with samples of bread, cornflakes, potato and legumes. Slurries, placed in dialysis tubing, were suspended in a stirred water bath containing distilled water and incubated at 37 °C for 3 hours after which time 5 ml aliquots of dialysate were taken for sugar analysis. Jenkins *et al* hence measured the total concentration of sugars liberated from the various foods after 3 hours. The quantity of sugars present in dialysate at 3 hours was expressed as a percentage of that for white bread giving each food a digestibility index by which they were ranked. Englyst *et al* (86) digested minced food samples in screw-top tubes with an enzyme mixture containing pancreatin, amyloglucosidase and invertase. Food viscosity was standardised with addition of guar gum powder, and mixing achieved by adding glass balls and shaking the tubes horizontally in a shaking water bath. Hydrolysis proceeded at pH 5.2 and 37 °C over 2 hours with 0.5 ml aliquots being

drawn at 20 and 120 minutes. The principles of Englyst's 1992 method had already been outlined in a 1990 publication (74). Samples of hydrolysate taken at 20 and 120 minutes enabled Englyst to describe fractions of digestible starch as determined by their rate of digestion. Rapidly digestible starch (RDS) was that starch measured as glucose after 20 minutes of digestion, slowly digestible (SDS) that digested between 20 and 120 minutes, and starch not digested by 120 minutes termed resistant starch (RS). *In vitro* derived values for RDS and SDS in foods obtained by Englyst's method reflected the rate of starch digestion *in vivo*. Furthermore, values for RS were similar to the amounts of starch escaping digestion in the small intestine of ileostomates. Englyst's 1990 and 1992 methods for determining nutritionally important starch fractions were an important contribution to the evolutionary development of *in vitro* methods, with the terminology Englyst introduced being widely used since.

In methods containing a gastric phase, intestinal phase digestion is initiated after adding buffer to raise the incubation pH. After neutralising with 4 M NaOH and adding acetate buffer (pH 5.0), Muir *et al* (90), hydrolysed samples of meals differing in resistant starch content with an enzyme mix comprising 10 mg α -amylase and 28 U amyloglucosidase. Samples were incubated in a 37 °C shaking water bath for up to 21 hours to isolate the resistant starch fraction. Muir *et al* achieved a good *in vitro* prediction of the amount of starch in grams that would likely escape digestion in the small intestine when compared with equivalent ileostomate samples. Goni (97) established an intestinal phase incubation pH of 6.9 with addition of Tris-Maleate buffer. Samples were then digested by α -amylase in a shaking water bath (37 °C) with 1 mL aliquots withdrawn every 30 minutes during a 3 hour digestion. A secondary digestion was performed on each 1 mL aliquot with addition of 3 mL sodium acetate buffer (pH 4.75) and 60 μ L amyloglucosidase to hydrolyse starch fragments to glucose. Similar to Englyst's 1992 method, Goni measured the rate of starch digestion in several commonly eaten foods. The amount of starch digested at each time point was expressed as a percent of total starch. Areas under the hydrolysis curves (AUC) for each food were calculated and compared against a reference AUC from white bread, generating a hydrolysis index (HI) for foods. Goni *et al* compared the percent of total starch hydrolysed at different time points for the foods with the GI (glycemic index) values of those foods. A significant correlation was found at 90 minutes of digestion, with Goni

concluding that a measure of *in vitro* glucose release at 90 minutes would provide a good estimate of the GI of foods.

Brighenti *et al* (99) investigated the *in vitro* rate of starch digestion from foods differing in botanical origin and type of processing. After a 1 hour gastric digestion, samples were transferred into dialysis bags, the incubation pH was adjusted to 6.9 with 8 M NaOH and 50 U of hog pancreatin per gram of starch was added. Peristalsis was simulated with insertion of glass balls into and inversion of dialysis tubes every 15 minutes. Hydrolysis proceeded at 37 °C over 5 hours with 1 mL aliquots of dialysate withdrawn every 30 minutes. The rate of starch digestion was measured as the amount of reducing sugars in dialysate aliquots, expressed as a percentage of total available carbohydrate. A sugar diffusion index, based on the rate of maltose diffusion across the dialysis tubing containing either sample or a reference blank was assigned to each food, and the percentage of starch digested from the foods at each time point calculated. For all foods, a reducing sugar index and starch digestion index were generated based on comparison against equivalent reference values from white bread. Good associations were found between the GI of foods and their reducing sugar index at 150 minutes, sugar diffusion index at 270 minutes and starch digestion index at 120 minutes.

Akerberg's 1998 *in vitro* method (89) measured, primarily, the resistant starch content in foods, but allowed parallel determination of potentially available starch as well as dietary fibre. Samples were placed in glass beakers fitted with magnetic stirrers and an exhaustive digestion performed at pH 5 and 40 °C with a pancreatin/amyloglucosidase enzyme mix over a 16 hour period with slow, constant stirring (100 rpm). At the end of the digestion, samples were filtered to separate the liquid and solid components. Glucose from digested starch was measured in the hydrolysate liquid and expressed in grams per 100 grams of total starch. Milling, solubilisation and secondary digestion of solid residue allowed quantification of resistant starch, also expressed on a gram per 100 gram total starch basis. RS measurements were correlated with *in vivo* (ileostomy-based) values of resistant starch for the same or similar foods already available in the literature, where a good correlation was found.

More recently, a modified approach to the analysis of data from *in vitro* digestion has appeared (110, 111) in which a correction is incorporated to allow for the cumulative apparent net disposal of blood glucose that occurs at the same time as the cumulative uptake of carbohydrate from digestion. The aim of the modification was to allow the *in vitro* analysis to be conducted in terms of amounts of different types of foods customarily consumed per eating occasion. As customarily consumed amounts of different foods deliver different glycemic carbohydrate doses, some allowance had to be made for the dose-dependent homeostatic response to increasing carbohydrate load. The allowance for glucose disposal greatly improved the accuracy with which *in vitro* digestions predicted *in vivo* responses to usually consumed food amounts.

A list of the major *in vitro* digestion methods available in the literature, details of experimental conditions used in their three phases of digestion as well as a summary of what each method intended to measure, is provided in **Table 2.1**.

METHOD NAME	YEAR	ORAL PHASE	GASTRIC PHASE	INTESTINAL PHASE	MEASURED	REFERENCE
Southgate	1969	Ground	-	Takadiastase (pH 4.5, 37°C, 18h)	AC	(81)
Hudson	1976	-	-	Inv., AMG (pH 5, 48°C, 16h) End-over-end rotation	AC	(112)
Baur	1979	Ground (60-mesh screen)	-	α -amylase (85°C, 15min), glucoamylase (60°C, 30min)	TS	(113)
O'Dea(1)	1981	Desiccated	-	α -amylase, AMG (pH 5, 50°C, 30min) Shaking water bath	Rate of SD	(114)
Batey	1982	Milled (0.8mm)	-	α -amylase (85°C, 1h), AMG (pH 4.6, 60°C) Manual mixing every 1min	TS	(115)
Jenkins(1)	1982	Ground	-	Human saliva (37°C, 3h) Dialysis bag suspended in stirred water bath	Rate of SD	(84)
Englyst(1)	1982	Ball-milled/homogenised	-	α -amylase, pullulanase (pH 5.2, 42°C, 16h) Magnetic stirring	NSP	(83)
O'Dea(2)	1983	Ground	-	α -amylase, AMG (pH 5, 50°C, 30min)	Rate of SD	(116)
Jenkins(2)	1984	Crumbed/ground	-	Human saliva (37°C, 3h) Dialysis bag suspended in stirred water bath	Rate of SD	(109)
Englyst(2)	1984	Ball-milled/homogenised	-	α -amylase, pullulanase (pH 5.2, 45°C, 16-18h) Magnetic stirring	NSP	(117)
Karkalas	1985	Sieved (0.8mm)	-	α -amylase, (85°C, 30min), AMG (pH 4.6, 60°C, 30min) Glass beads, occasional manual shaking	TS	(91)
Berry	1986	Ground	-	α -amylase, pullulanase (42°C, 16h), AMG (pH 4.5, 65°C, 60min) Shaking water bath	RS	(85)
Englyst(3)	1986	Ball-milled/homogenised	-	α -amylase, pullulanase (pH 5.2, 45°C, 16-18h) Magnetic stirring	RS	(118)
Holm	1986	Milled (0.8mm)	Pepsin (pH 1.5, 37°C, 1h)	Termamyl (100°C, 15min), AMG (pH 4.75, 60°C, 30min) Manual mixing every 5min	TS	(104)
Heaton	1988	-	-	Pancreatin (pH 6.5, 37°C, 3h)	Rate of SD	(119)
Granfeldt(1)	1991	Homogenised/chewed (~15x in ~15s)	Pepsin (pH 1.5, 37°C, 1h)	α -amylase (pH 6.9, 37°C, 3h) Dialysis bag suspended in stirred beaker	Rate of SD	(40)
Granfeldt(2)	1992	Chewed (~15x in ~15s)	Pepsin (pH 1.5, 37°C, 30min)	α -amylase (pH 6.9, 37°C, 3h) Dialysis bag suspended in stirred beaker	Rate of SD	(106)

Champ	1992	Milled/ground/homogenised	-	α -amylase (pH 6.9, 37°C, 16h), AMG (pH 4.5, 65°C, 90min) or just AMG (pH 4.75 60°C, 30min) Mixing/shaking	RS	(98)
Brighenti(1)	1992	Ottawa Instron Cell (0.6mm), sal. A-amylase (pH 6.9, 5mins)	Pepsin (pH 2, 37°C, 1h)	Pancreatin (pH 6.9, 37°C, 5h) Dialysis bag suspended in stirred water bath	Rate of SD	(88)
Englyst(4)	1992	Minced (plate 0.9cm)	-	Pancreatin, AMG, inv. (pH 5.2, 37°C, 2h) Glass balls, shaking water bath	Rate of SD, RS	(86)
Muir(1)	1992	Chewed	Pepsin (pH 2, 37°C, 30min)	α -amylase, AMG (pH 5, 37°C, 6h) Shaking water bath	RS	(87)
Muir(2)	1993	Chewed	Pepsin (pH 2, 37°C, 30min)	α -amylase, AMG (pH 5, 37°C, 21h) Shaking water bath	RS	(120)
Muir(3)	1995	Chewed	Pepsin (pH 2, 37°C, 30min)	α -amylase, AMG (pH 5, 37°C, 15h) Shaking water bath	RS	(90)
Silvester	1995	Milled/crumbed/mashed	Pepsin (pH 2, 37°C, 30min)	α -amylase, AMG, inv. (pH 5.2, 37°C, 2h) Glass balls, shaking water bath	RS	(108)
Brighenti(2)	1995	Kramer Cell (3mm), sal. A-amylase (pH 6.9, 5mins)	Pepsin (pH 2, 37°C, 1h)	Pancreatin (pH 6.9, 37°C, 5h) Glass balls, inversion every 15min	Rate of SD	(99)
Brennan(1)	1996	Domestic Food Processor (30s)	Pepsin (pH 1.5, 37°C, 10min)	α -amylase (pH 6.9, 37°C, 5h) End-over-end rotation	Rate of SD	(95)
Goni	1997	Homogenised	Pepsin (pH 1.5, 40°C, 1h)	α -amylase (pH 6.9, 37°C, 3h), Shaking water bath	Rate of SD	(97)
Brighenti(3)	1998	Sieved (1.5mm)	-	α -amylase (pH 6.9, 37°C, 16h), AMG (pH 4.5, 65°C, 90min) Mixing/shaking	RS, TS	(92)
Akerberg	1998	Chewed (~15x in ~15s)	Pepsin (pH 1.5, 37°C, 30min)	Pancreatin, AMG (pH 5, 40°C, 16h) Magnetic stirring	DS, RS, DF	(89)
Lebet	1998	Ground/milled (0.5mm), sal. A-amylase (pH 6.9, 37°C, 15mins)	Pepsin (pH 2, 37°C, 30min)	Pancreatin (pH 6.9, 37°C, 26h) Magnetic stirring	RS	(100)
Urooj	1999	Ground/powdered (60 mesh)	-	Pancreatin, human saliva (37°C, 3h) Dialysis bag suspended in stirred water bath	Rate of SD	(101)
Englyst(5)	1999	Minced (plate 0.9cm)/crushed/broken	Pepsin-guar gum (pH 2, 37°C, 30min)	Pancreatin, AMG, inv. (pH 5.2, 37°C, 2h) Shaking water bath	Rate of SD	(93)

Weurding	2001	Milled (1mm)	Pepsin (37°C, 30min)	Pancreatin, AMG, inv. (pH 5.2, 37°C, 6h) Shaking water bath	Rate of SD	(96)
Araya	2002	Minced (<1cm)	-	Pancreatin, AMG, inv. (pH 5.2, 37°C, 3h) Glass balls, shaking water bath	Rate of SD	(94)
Brennan(2)	2002	Cut (~1mm ³)	Pepsin (pH 1.5, 37°C, 10min)	α -amylase (pH 6.9, 37°C, 90min) Shaking water bath	Rate of SD	(121)
Brennan(3)	2007	Cut (~1cm ³)	Pepsin (pH 1.5, 37°C, 30min)	α -amylase (pH 6.9, 37°C, 5h) Manual agitation every 15min	Rate of SD	(122)
Chanvriar	2007	Powdered, weak pancreatic amylase (pH 7, 15-20s)	Pepsin (pH 2, 37°C, 30min)	Pancreatin, AMG (pH 6, 37°C, 16h) Shaking water bath	RS	(123)
Monro(1)	2008	Sieved, α -amylase	Pepsin (pH 2.5, 37°C, 30min)	Pancreatin, AMG (pH 6, 37°C, 2h) Magnetic stirring	Rate of SD	(102)
Al-Rabadi	2009	Ground, α -amylase (pH 7, 25°C, 10-15s)	Pepsin (pH 2, 37°C, 30min)	Pancreatin, AMG (pH 6, 37°C, 24h) Shaking water bath	Rate of SD	(37)
Sopade	2009	Ground, α -amylase (15-20s)	Pepsin (37°C, 30min)	Pancreatin, AMG (pH 6, 37°C, 4h) Shaking water bath	Rate of SD	(124)
Monro(2)	2010	Chewed	Pepsin (pH 2.5, 37°C, 30min)	Pancreatin, AMG (pH 6, 37°C, 2h) Intermittent magnetic stirring	Rate of SD – glucose disposal	(110)
Hasjim	2010	α -amylase (15-20s)	Pepsin (37°C, 30min)	Pancreatin, AMG (pH 6, 37°C, 25h) Shaking water bath	Rate of SD	(125)
Witt	2010	α -amylase (30s)	Pepsin (pH 2, 37°C, 30min)	Pancreatin, AMG (pH 6, 37°C, 24h) Shaking water bath	Rate of SD	(126)
Dartois	2010	-	Pepsin (pH 1.2, 37°C, 30min)	Pancreatin, AMG, inv. (pH 6.8, 37°C, 2h) Magnetic stirring	Rate of SD	(127)
Aravind	2011	Cut (0.5/0.25cm slices)	Pepsin (pH 1.5, 37°C, 30min)	α -amylase (pH 6.9, 37°C, 5h) Dialysis bag suspended in shaking water bath	Rate of SD	(128)

Table 2.1 A summary of the experimental conditions used in some of the main *in vitro* digestion methods found in the literature and what each method was designed to measure. Abbreviations: AC, available carbohydrate; AMG, amyloglucosidase; DF, dietary fibre; DS, digestible starch; h, hour(s); inv, invertase; NSP, non-starch polysaccharides; RS, resistant starch; s, seconds; sal, salivary; SD, starch digestion; TS, total starch.

2.4 THE NEED FOR STANDARDISATION

Aside from method application, it is intended that the above comparison would highlight the considerable discrepancy that exists between current *in vitro* protocols in terms of practical methodology. While methods generally encompass an oral, gastric and intestinal phase digestion, performed within physiological constraints, the way in which the methods simulate *in vivo* conditions differ. It is likely that different simulations will give rise to different relative glycaemic impact estimates for a given food. Hence, for the purposes of achieving a standardised method suitable for eventual application to the food industry in nutrition labelling, a systematic investigation of methodological factors that introduce variation in glycaemic estimates must be undertaken.

Actual or simulated chewing techniques used during the oral phase differ between methods, so each method will be digesting foods of differing particle size distribution, depending on the “chewing” process used in the oral phase. Since food particle size is a key factor influencing both *in vivo* and *in vitro* determinations of glycaemic potency (38, 129, 130), it is essential that in the standardised method, sample foods are rendered into “as eaten” particle sizes. Chewing is a subjective process that varies from one individual to another and also between foods. Hence actual chewing of test foods by a cohort of volunteers may introduce variation in the rate and extent of subsequent *in vitro* carbohydrate digestions. This perhaps explains why analysts resort to such simulated chewing techniques as sieves, choppers and mincers, which are more easily standardised and generate hydrolysis curves of greater precision. But do these techniques really achieve particle sizes similar to those achieved by actual chewing, thereby giving physiologically meaningful *in vitro* results? Our group investigated this aspect of the *in vitro* process, testing some commonly used simulated chewing methods using several food types (see section 3.1.2.1). Results (see section 4.1.1) highlighted the need to achieve a standardised method of sample food mechanical breakdown in the oral phase. The ideal “chewing” method, whether it be actual or simulated, will likely depend on interaction of the structural qualities of the food being analysed with the combination of chopping and crushing achieved in human mastication. The method

must be capable of generating hydrolysis curves that consistently correlate with *in vivo* data for the same digested foods. Additionally, since some methods include a brief salivary or other α -amylase exposure during oral phase digestion, the effect of including or omitting such a step on a food's sugar release profile needs characterising before a standardised method can be decided upon.

Some current *in vitro* methods incorporate a gastric phase pepsin digestion whereas others do not. Furthermore, the duration of pepsin digestions used varies between methods from 10 minutes to an hour. What effect will these varying conditions have on glycemic estimates of a given food? Again, using some isolated foods, our laboratory tested the effect of omitting a pepsin digestion on the subsequent rate and extent of starch hydrolysis (see section 3.1.2.4). Observations suggested the necessity of a pepsin incubation when foods to be analysed contain a significant protein component that might otherwise hinder the accessibility of starch to amylolytic enzymes (see section 4.1.4). Certainly to align *in vitro* methodology with *in vivo* processes, a pepsin step should be included in the standardised protocol. However, as the glycemic response to foods usually starts within 10 minutes, and the maximum rate of blood glucose loading is achieved by about 20 minutes of consuming food, the role of pepsin in standard glycemic response measurement is uncertain. The ideal duration of *in vitro* pepsin digestion requires investigation since, *in vivo*, gastric emptying rate differs depending on the food type, quantity and form being digested (131, 132). As opposed to a generic *in vitro* pepsin incubation, it may be that a food or food-group and/or a food-form specific incubation duration will need to be decided on.

There are various additional gastric phase parameters, which currently differ between methods that require standardising. Pepsin activity, pH, incubation temperature and the mode of sample mixing (shaking water bath, incubated vessels stirred magnetically, or with periodic manual stirs etc.) are all parameters that could affect *in vitro* results.

Numerous methodological aspects of the intestinal phase of *in vitro* digestion require standardisation, perhaps primarily the choice of hydrolytic enzyme used (pancreatin or α -amylase), its concentration, and whether amyloglucosidase is simultaneously added to

the digestion medium, added at a later stage in the digestion process, or included at all. Incubation pH will be determined based on the enzyme(s) used, but must also reflect *in vivo* conditions. Pancreatin and α -amylase are optimally active between pH 6 and 7. Therefore, in methods where amyloglucosidase (pH optimum 4 to 5) is added simultaneously with pancreatin or α -amylase, and a pH of approximately 5 is established to accommodate the activity of both enzymes (89, 90), is there really a satisfactory level of enzymic activity, or will *in vitro* results be underestimates due to pH-induced enzyme deactivation? We tested the stability of porcine pancreatin (measured as its capacity to digest 2.5 grams of pregelatinised starch) when incubated across a range of pH values (4.0 – 6.9) commonly used in current methods (see section 3.1.2.7). Pancreatin remained active across a pH range of at least 5 – 7, showing that the enzyme has sufficient robustness to cope with the varying pH values typically used in *in vitro* protocols (see section 4.1.6). For the standardised *in vitro* method, however, the incubation pH required to obtain results of highest physiological significance will need to be identified.

Because amylolysis *in vivo* occurs in the small bowel simultaneously with fat digestion in the presence of bile, should a lipase step be built into the standardised *in vitro* method? Pancreatin enzyme contains lipase, hence methods using pancreatin will inherently incorporate a degree of fat digestion. However, in those methods where α -amylase alone is used as the amyolytic enzyme, and there are no fat-dissolving bile acids added, no fat digestion will take place. The scientific literature demonstrates the role lipid-starch interactions can play in decreasing starch susceptibility to hydrolysis (46, 133). Accordingly, incorporating a fat-digesting step where lipase and bile acids are added, perhaps concurrently with pancreatin, may be one way of improving the predictive power of the standardised *in vitro* method, particularly when carbohydrate foods of high fat content are analysed.

It is well documented that digesta viscosity is a key determinant of the rate of carbohydrate digestion and absorption *in vivo* (103, 134). The effect of viscosity on glycemic response is attributed to slower gastric emptying, reduced access of digestive enzymes to the food bolus in the small intestine and to reduced mixing and/or diffusion due to the viscosity. It is difficult to replicate these complicated physiological processes

in vitro using conventional laboratory equipment, and hence current methods do not account for the effect of food viscosity on the rate of carbohydrate digestion. Englyst *et al* (93) standardised the viscosity of digest samples with addition of guar gum, but this was to achieve better particulate suspension during mixing, and to prevent excessive mechanical grinding of raw starches by the glass balls leading to higher digestibility. The effect of food viscosity on glycemic response *in vivo* could be quantified, however, through a series of clinical measurements, and the quantified effect applied as an algorithmic correction factor to *in vitro* digestibility data. By this approach, sensitivity to the effect of food viscosity on the rate and extent of carbohydrate digestion *in vitro* might be enabled.

What effect, if any, will the mode of sample mixing have on the rate and extent of starch hydrolysis? Current methods use a variety of stirring and agitation techniques and an investigation of their relative effects on hydrolysis rate is required for the identification of the method generating the most physiologically sound results. A further variable that currently differs considerably between methods is the duration of amyolytic incubation. Any standardised method must generate estimates of rapidly and slowly digestible and resistant starch fractions that correlate well with *in vivo* data, but which are also obtainable within a convenient time-span. A food's rapidly digestible starch component is usually hydrolysed and absorbed within the first 15 to 30 minutes of entering the small intestine (26). Hence, in the standardised method, aliquots of digesta for measurement of rapidly digestible starch should occur within this time frame. Similarly, an aliquot that defines starch that is slowly digested, thus isolating starch resistant to digestion, must be taken at a time point giving physiologically-significant estimations of these fractions.

Of equal importance to incubation duration is the concentration of hydrolytic enzyme used. Methods described above differ considerably in their α -amylase or pancreatin enzyme concentrations, with the concentration used generally being reflective of the amount of sample to be digested, as well as the method application and hence incubation duration. Generally, very low concentrations of α -amylase or pancreatin are used, for instance in Akerberg's method (89) only a 0.01 % pancreatin solution is used to hydrolyse 1 to 11 g samples of food. This method measures resistant starch however,

and the hydrolysis is exhaustive, occupying 16 hours. Brennan *et al* (121), on the other hand, used a concentrated 5.1 % α -amylase solution, in which, however, much larger samples (30 g) of food were digested over 90 minutes with the rate of starch breakdown being measured. The Englyst methods for measuring the rate of starch digestion (86, 93) used a 3% pancreatin, 0.5% amyloglucosidase and 1.3% invertase enzyme combination to digest food samples ranging in quantity from 1 to 8 grams. Particularly in methods where the rate of starch digestion is measured, low enzyme concentrations might increase relative measurement variability if the concentrations aren't replicated precisely between different digestion runs. Furthermore, at low enzyme concentrations, variations in sample amount will have greater potential to influence digestion kinetics. Hence the standardised method, like the *in vivo* situation (26), must use an excess of enzyme so as not to become rate-limiting to carbohydrate digestion.

2.5 CONCLUSION

For routine food analyses, *in vivo* methods for estimating glyceic potency are neither time- nor cost-efficient. The use of *in vitro* digestion assays as an alternative or additional support to *in vivo* data has been widely reported in the literature over the past three decades. *In vitro* technology has not been widely applied to routine food analysis in its own right, however, due to the current lack of a robust, reliable and standardised method. Current methods are capable of predicting with reasonable accuracy the likely glyceic properties of a range of foods and food types. Early work in our laboratory, however, has demonstrated that currently-existing variation between methods can introduce significant differences in the estimates of glyceic potency that they yield. Thus, at present, application of non-standardised *in vitro* technology to, for instance, widespread nutrition labelling of food for glyceic communication would be premature. This application, clearly advocated by members of the Glyceic Carbohydrate Definition Committee members, may, however, be attained in the future if an in-depth and systematic investigation of the effect varying methodological conditions have on *in vitro* estimates of glyceic potency is undertaken. This review forms the foundation to such an investigation by comparing some of the current and commonly used *in vitro* methods and identifying key methodological aspects requiring standardisation.

PART II:

The Effect of Fat on the Glycemic Response *In Vivo*: Reflecting this Effect *In Vitro*

2.6 THE EFFECT OF FAT *IN VIVO*

Research conducted over the last twenty years has established a link between addition of fat into a meal and subsequent attenuation in the postprandial glycemic response. Collier and O'Dea, for instance, demonstrated a 50 % reduction in the glucose response IAUC following coingestion of butter with a potato meal, compared to when potato was consumed on its own (135). Jeya *et al* showed that the glycemic response to white bread could be reduced with addition of butter or oil as a spread (136).

Whereas earlier studies investigating the role of fat in decreasing glycemia tended to involve addition of a single large dose of fat to carbohydrate (135, 137-140), more recent work has attempted to characterise the effect, if any, that nutritionally relevant levels of fat exert on the glucose response. Owen and Wolever tested the effect of margarine, applied across the normal range of fat intakes (20 – 45 % total energy) on the glycemic response to white bread and observed a fat-dose-dependent but non-linear relationship (141) where half the effect of adding 40 g fat to the bread was observed when just 5 g fat was added.

The attenuation generally seen in glycemic response when carbohydrates and fat are co-ingested, has encouraged some to claim that incorporation of moderate levels of fat into meals might provide an effective intervention for reducing their glycemic potency - a potentially useful tool for type II diabetics trying to control their blood glucose (137). The disadvantages of such an approach, however, are that fat inclusion in a meal increases the meal's total energy content and has been shown to enhance both the postprandial insulin response (142) as well as postprandial triacylglycerolaemia (143), all of which are unfavourable metabolic responses in type II diabetics. Nonetheless, the fat-inclusion/glycemic response attenuation phenomenon has interested nutritionists sufficiently that considerable ongoing research effort focuses on both characterising the relationship between fat dose and fat type and the glycemic response, as well as

understanding the mechanisms underlying fat attenuation of postprandial glycemia (136, 144-146).

The principal mechanism by which fat decreases the glycemic response to food is through delaying the rate of gastric emptying (138, 144, 147). As mentioned in section 1.5 of chapter 1, any factor (food or physiological) that affects the rate of gastric emptying, will also affect the postprandial glycemic response to a meal through either speeding up or reducing the rate of delivery of carbohydrate to the duodenum, where starch digestion begins. Fatty acids, when present within the small intestine, are a potent stimulator of secretion of, amongst others, the gut hormone glucagon-like peptide 1 (GLP-1) by intestinal mucosal cells, into the circulation (147-149). GLP-1 induces various physiological responses including decreased appetite, decreased glucose production in the liver, increased glucose uptake and storage in muscle and adipose tissue, increased insulin secretion and decreased glucagon secretion from the pancreas and, in particular, a reduction in the rate of gastric emptying (150).

Given that gastric emptying rate is such a major physiological determinant of the glycemic response to foods, *in vitro* digestion methods for predicting the glycemic response have drawn criticism due to their obvious inability to simulate such a complicated physiological process that, *in vivo*, constantly changes with food type (151, 152). Furthermore, since the effect of fat on the glycemic response is mediated through reduction in gastric emptying rate, it can be assumed that *in vitro* digestion methods, in their current format, do not feature any sensitivity to food fat content in their starch digestibility results.

2.7 THE EFFECT OF FAT *IN VITRO*

However, several analysts report an effect of fat on the relative rate of *in vitro* starch digestion. In 1979 Larsson and Miezi demonstrated a 20 % reduction in the rate of *in vitro* starch digestion by α -amylase when potato starch gels were exposed to oleic monoglyceride prior to amylolysis compared with when the starch gels were digested in isolation (153). Holm *et al* reported a similar finding in 1983, also using potato starch and various fatty acids (154). In 1997, Guraya *et al* demonstrated a reduction in the digestion rate of rice starch by up to 33 % when complexed with lipid emulsifier (46),

and in 2000, Crowe *et al* observed up to 35 % reduction in the rate of potato starch digestion when digested in the presence of various fatty acids (133).

Therefore based on these *in vitro* studies, it would appear that a further mechanism by which fat might attenuate the *in vivo* glycemic response is through reducing the accessibility of starch to α -amylase as a result of starch-lipid complex formation, thus slowing its hydrolysis rate. Holm *et al* found that lipid-complexed amylose was hydrolysed and absorbed to the same extent in the small intestine of rats, but at a slower rate (154).

2.8 ALIGNING *IN VIVO* AND *IN VITRO* DATA

Thus while current *in vitro* digestion methods demonstrate some sensitivity to the effect of fat in foods, this sensitivity is limited to effects exerted via starch-lipid complex formation and subsequent reduced enzymic accessibility and not as a result of reduced gastric emptying rate. Fat effect on blood glucose mediated by reduced gastric emptying might be reproducible *in vitro*, however, if a correction factor for relative gastric emptying rate in the presence of varied levels of fat were applied to *in vitro* digestibility data. To date, only one *in vitro*-based study for predicting the glycemic response has ever attempted to incorporate a correction for estimated gastric emptying rate. Van Kempen *et al* showed that *in vitro* starch digestion kinetics accurately predicted portal glucose appearance in pigs up to 8 hours postprandial, once the *in vitro* data had been corrected for gastric emptying time (155).

The objective of the present study therefore is to improve the predictive power of our *in vitro* digestion method by adding to it a correction for gastric emptying rate as influenced specifically by food fat content. To achieve this aim, parallel *in vivo* and *in vitro* studies on a series of equicarbohydrate diets, differing only in their fat content, will be performed to test for initial alignment between the two sets of data. The *in vivo* gastric emptying rate, as influenced by fat content, will also be measured. It is expected that *in vivo*, attenuation of the postprandial blood glucose response will occur in the presence of increasing levels of fat, but that only a small reduction in starch digestion rate, if any, will be observed with increased levels of fat *in vitro*, due to starch-lipid complex formation. Having quantified the effect of fat on gastric emptying rate and

subsequent glycemic response *in vivo*, the intended outcome is elucidation of a correction factor for the effect of fat dose on the *in vivo* glycemic response that can then be used to adjust corresponding *in vitro* data to improve its accuracy and alignment with *in vivo*. In future, accurate *in vitro* prediction of the likely glycemic response to foods varying in their fat content might be achievable by the current approach.

To the author's knowledge, the present *in vivo* study is the first to test more than one level of fat dose on the postprandial glycemic response whilst simultaneously measuring the gastric emptying rate.

CHAPTER 3: MATERIALS AND METHODS

The research comprising this thesis was conducted in two phases and at two locations:

- Phase 1: *In vitro* digestion methods investigation
(New Zealand Institute for Plant and Food Research Limited,
Palmerston North, New Zealand)
- Phase 2: Human glycaemic index (GI) study
(CSIRO Food and Nutritional Sciences, Adelaide, South Australia)

3.1 PHASE 1: *IN VITRO* DIGESTION METHODS INVESTIGATION

3.1.1 The *In Vitro* Method Template, Test Foods and Calculations Used

A systematic investigation of the effect of varying *in vitro* digestion method parameters and conditions on relative measures of *in vitro* starch digestibility, namely levels of RDS and SDS, was carried out using an existing *in vitro* method (102) as a template, and five standard test foods as substrates. The five foods used throughout the investigation were: white bread (*Tip Top, supersoft long rolls*), whole wheat grains (*Claire*), pasta (*Pams lasagne sheets*), chickpeas and potato (*Sebago, loose*). Test foods were selected to reflect a range of different structural/matrix properties (e.g. porous bread, dense pasta), botanical origins (e.g. potato - tubers, chickpea - legumes) and method or degree of processing (e.g. baked bread, extruded pasta). Immediately prior to all *in vitro* digestions the test foods were prepared as follows:

- White bread: crusts removed.
- Wheat grains: boiled for 60 min in an excess of water after an overnight soak (at room temperature).
- Pasta: boiled for 10 min in an excess of water.
- Chickpeas: boiled for 60 min in an excess of water after an overnight soak. Husks removed after boiling.
- Potato: boiled whole for 40 min in an excess of water then peeled.

The *in vitro* digestion method used as a template throughout this investigation and to which systematic modifications were made, is described in **Figure 3.1**.

Reducing sugar concentrations (referred to throughout this thesis as ReS) in the timed aliquots of digesta were measured via colourimetry following reaction with 3,5-dinitrosalicylic acid (DNS). The use of DNS reagent for determination of ReS concentration in hydrolysate was first described by Sumner and Graham (156) and is relatively simple and high throughput. The DNS method used in this thesis is summarised in **Figure 3.2**.

ORAL PHASE

- Press portions of food to be analysed through a sieve of 4 mm gauge.
- Weigh 5.0 g samples of sieved test food into 70 mL plastic specimen pots, inserted to their full depth in a 15-place aluminium heating/magnetic-stirrer block.
- Add 30 mL distilled water to each pot.

GASTRIC PHASE

- Add 0.8 mL of 1M HCl.
- Measure pH and adjust to ~ pH 2.5 with 1 M HCl if necessary. Hold tubes at 37 °C.
- Add 1 mL of a 10 % pepsin/0.05 M HCl solution (*Pepsin EC 3.4.23. from porcine stomach mucosa, Sigma-Aldrich P 7000; 800-2 500 U/mL*) prepared immediately before use.
- Digest for 30 mins at 37 °C with slow, constant mixing (130 rpm).

INTESTINAL PHASE

- Add 2 mL of 1 M NaHCO₃.
- Add 5 mL of 0.1 M sodium maleate buffer, pH 6.0.
- Add 0.1 mL amyloglucosidase (*EC 3.2.1.3. from A niger, Megazyme, E-AMGDF; 3260 U/mL*) to prevent end-product (maltose) inhibition of pancreatic α -amylase.
- Add 5 mL of a 2.5 % pancreatin/0.1 M sodium maleate buffer solution (*Pancreatin EC 232.468.9 from porcine pancreas, Sigma-Aldrich P 7545; 8 x USP specifications*) made immediately before use.
- Make accurately to 55 mL with distilled water.
- Digest for 120 mins at 37 °C with slow, constant mixing (130 rpm).
- During the incubation remove a 1 mL aliquot of digest at 20 and 120 mins, or as required, and add each aliquot to 4 mL absolute ethanol in a separate tube.
- Store tubes at 4 °C until analysis.

Figure 3.1 The *in vitro* carbohydrate digestion method used throughout this thesis as a template, to which systematic modifications were made.

DNS COLOURIMETRIC MEASUREMENT OF REDUCING SUGARS

- Place 0.05 mL of sample (1 mL aliquot of digest in 4 mL ethanol), as well as 5 mg/mL and 10 mg/mL glucose standards and a water blank into a tube.
- To each tube add 0.25 mL of 'enzyme solution A' (ESA) (1 % invertase (*BDH concentrate 39020 3D*) and 1 % amyloglucosidase in acetate buffer, pH 5.2.).
- Mix tubes and rest for 10 mins at room temperature.
- Add 0.75 mL DNS solution (0.5 mg/mL glucose : 4 M NaOH : DNS reagent², mixed in the ratio 1:1:5).
- Cover the tubes and heat for 15 mins at 100 °C.
- Cool the tubes and add 4 mL distilled water.
- Transfer into cuvettes and read absorbance in a spectrophotometer at 530 nm.

Figure 3.2 The method used throughout this thesis for DNS colourimetric measurement of ReS concentration

² DNS reagent: 10 g 3,5-dinitrosalicylic acid + 16 g NaOH + 300 g Na-K tartarate. Make to final volume of 1 L with distilled water. Allow 2 days before use.

The spectrophotometric absorbance (abs) for each digest aliquot was converted into a measure of ReS concentration, expressed as milligrams of ReS per total gram weight of test food (mg/g) using the following formula:

$$\frac{(\text{sample abs} - \text{H}_2\text{O blank abs})}{(10 \text{ mg/ml standard abs} - \text{H}_2\text{O blank abs})} \times 53 \times 10 \times 5 \times \frac{1}{\text{original sample weight (g)}}$$

In the above formula the multiplication by 53 reflects the total volume (mL) of digesta in each pot.³ The multiplication by 10 is to reference the sample reading to the 10 mg/mL glucose standard and the multiplication by 5 to account for the dilution of the 1 mL digest aliquot in 4 mL absolute ethanol.

At the end of all *in vitro* digestions, hydrolysis curves of starch digestion for the five test foods were constructed. Values for RDS and SDS content (in mg/g) for each food type were calculated using the following formulae:

$$\text{RDS} = T_{20} \times 0.9$$

$$\text{SDS} = (T_{120} - T_{20}) \times 0.9$$

T_{20} is the amount of ReS (mg/g) present in the 20 min aliquot and T_{120} is the amount of ReS (mg/g) present in the 120 min aliquot. The 0.9 constant is to convert the weight of free ReS to the equivalent weight of starch.⁴

3.1.2 Method Variables and Conditions Investigated

The key variables and conditions of *in vitro* methodology investigated were:

- The method for replicating chewing – sieving, chopping, mincing
- Whether salivary α -amylase exposure, inherent to chewing pretreatment, affected subsequent *in vitro* digestion rate compared with simulated chewing methods that lack salivary exposure

³ Each digest pot is made up to a final digest volume of 55 mL however a magnetic stirrer, occupying space equivalent to 2 mL water is placed in each digest pot making the actual final digest volume 53 mL.

⁴ A factor of 0.9 is used since the molecular weight of glucose when incorporated into starch is 90% of that of free glucose due to water loss in the condensation reaction.

- Varying the duration of the pepsin (gastric phase) incubation
- Omitting the pepsin incubation
- Pancreatin enzyme concentration
- Intestinal phase (pancreatic) incubation pH.
- Digesta stirring method – magnetic stirring, shaking water bath, end-over-end inversion.

3.1.2.1 Mode of Comminution in the Oral Phase (80)

The five standard test foods were purchased fresh from local markets and prepared as described previously. Following preparation, triplicate 5.0 g samples of each food type⁵ (2.5 g in the case of bread⁶) were digested as described in **Figure 3.1** but after having been either:

- pressed through a sieve of 4 mm gauge (as in **Figure 3.1**)
- chopped 20 times with a handheld domestic chopper appliance (*Zyliss*[®])
- minced (on setting 1) with a domestic electrical mincer (*Kenwood*[®]) fitted with a 9 mm bore plate
- homogenised completely using a homogeniser (*OmniGLH-220*) with a 195 mm × 20 mm saw tooth probe
- or chewed by volunteers.

The *Zyliss*[®] chopper is operated by placing a portion of the food to be chopped in a small round plastic chamber. Above the chamber is a zigzag-shaped stainless steel blade that slices through the chamber, cutting the food, with each depression on the chopper handle. The blade automatically rotates with each chop, ensuring even cutting of the food. In the case of chewing, three volunteers, with normal dentition, were recruited to chew (until they reached the urge to swallow) 5.0 g samples of each food type (2.5 g for bread) after which they expectorated the chewed sample into the digest pots. Volunteers rinsed their mouths thoroughly with 20 mL of distilled water and expectorated the rinse into the digest pots also. Pots containing chewed samples were immediately acidified to gastric pH (~2.5) to stop salivary α -amylase activity.

⁵ Each food type was analysed on separate days.

⁶ Only 2.5 g of bread is used due to its lesser water content relative to the other four test foods that are boiled prior to *in vitro* digestion.

During the intestinal phase of digestion, aliquots of digest were taken for ReS analysis at Time 0 (T_0), then at 10, 20, 40, 60 and 120 minutes post-pancreatin addition. ReS in the aliquots were measured using DNS colourimetry as described in **Figure 3.2**, hydrolysis curves for the five foods constructed and levels of RDS and SDS calculated.

3.1.2.2 Comparing Chewing and Chopping

The five standard test foods were purchased fresh and prepared as described previously. Following preparation, triplicate 5.0 g samples of each food type (2.5 g for bread) were chopped either 20, 30, 40 or 50 times. Due to its soft texture following boiling, potato was chopped either 5, 10, 15 or 20 times. Three volunteers were recruited to chew and expectorate pre-weighed samples of each food as described previously. All foods were then digested *in vitro* as described in **Figure 3.1** with aliquots of digesta withdrawn at T_0 , then at 20, 40 and 120 minutes post pancreatin addition. To demonstrate the relationship between substrate particle size and the rate and extent to which *in vitro* starch digestion occurs, all samples were completely homogenised following withdrawal of the 120 min aliquot. An additional 0.1 mL amyloglucosidase was added and digestion permitted to continue for a further 60 min post-homogenisation. Aliquots of digest were withdrawn at 30 and 60 min post homogenisation (corresponding to 150 and 180 min post pancreatin addition respectively). The 180 min aliquot provides a value for total potentially digestible carbohydrate and the 150 and 180 min values, together, indicate the completeness of digestion. ReS concentration in all aliquots was measured by DNS colourimetry as described in **Figure 3.2**, hydrolysis curves for the five foods constructed and RDS and SDS levels calculated.

3.1.2.3 Starch-Digesting Capacity of Salivary α -Amylase (157)⁷

The five test foods were purchased and prepared as described previously. Following cooking, a subset of each of the foods was chopped 20 times (pasta and wheat, based on the findings of the experiment described in 3.1.2.2 were chopped 47 and 116 times respectively) to replicate chewing. A series of pre-weighed 5.0 g samples of each food

⁷ The experiment detailed in section 3.1.2.3 has been published in the International Journal of Molecular Sciences (SEE APPENDIX B).

type (2.5 g for bread) were chewed by two volunteers until they reached the urge to swallow, expectorated, and then left to sit as chewed boluses for 0, 5, 10 and 15 minutes. To evacuate any remaining food particles, volunteers rinsed their mouths thoroughly with 20 mL distilled water and expectorated the rinse also. Between chewing different food types, volunteers rinsed their mouths thoroughly with water. All samples, whether chewed or chopped, were digested *in vitro* as described in **Figure 3.1**. Aliquots of digesta were withdrawn at T₀, and 10, 20, 40, 60 and 120 minutes after pancreatin addition. ReS concentration was measured by DNS colourimetry as described in **Figure 3.2** except that an additional set of 0.05 mL aliquots were taken from all T₀ digesta samples (chewed and chopped) and no ESA was added to this set, so only free sugars and simple sugars liberated by salivary action were present for measurement. The total starch (TS) content of each food type was deduced from the Australian Food Composition Tables 2006 (158). The percentage of TS hydrolysed by salivary α -amylase to glucose/maltose and dextrans was calculated by converting the TS content (g/100g) to absolute glucose content per 5 g of sample, then dividing the values for glucose (or dextrans) released by salivary action by the absolute glucose content, and multiplying by 100. The experimental design is depicted diagrammatically in **Figure 3.3**.

3.1.2.4 Pepsin Inclusion and Omission (80)

The five test foods were purchased fresh and prepared as described previously. Following sieving, three sets of 5.0 g samples of each food type (2.5 g for bread) were weighed into 70 mL digestion pots divided into three treatment categories: ‘no pepsin,’ ‘pepsin 30 minutes’ and ‘pepsin 60 minutes.’ *In vitro* digestion was performed as described in **Figure 3.1**. In the ‘pepsin 30 minutes’ and ‘pepsin 60 minutes’ categories, 1 mL of the 10 % pepsin (in 0.05 M HCl) solution was added and gastric phase digestion carried out for 30 and 60 minutes respectively. In the ‘no pepsin’ test category, to achieve ionic-equivalence with the other two categories, 1 M HCl and 1 mL of a 0 % pepsin solution (i.e. just 0.05 M HCl) were also added to each digesta pot, but with immediate subsequent addition of the NaHCO₃ and sodium maleate buffers and progression to pancreatin addition. Aliquots of digest were withdrawn from all treatment categories at T₀, and at 20 and 120 minutes following addition of pancreatin. Aliquots were measured for reducing sugar concentration by DNS colourimetry.

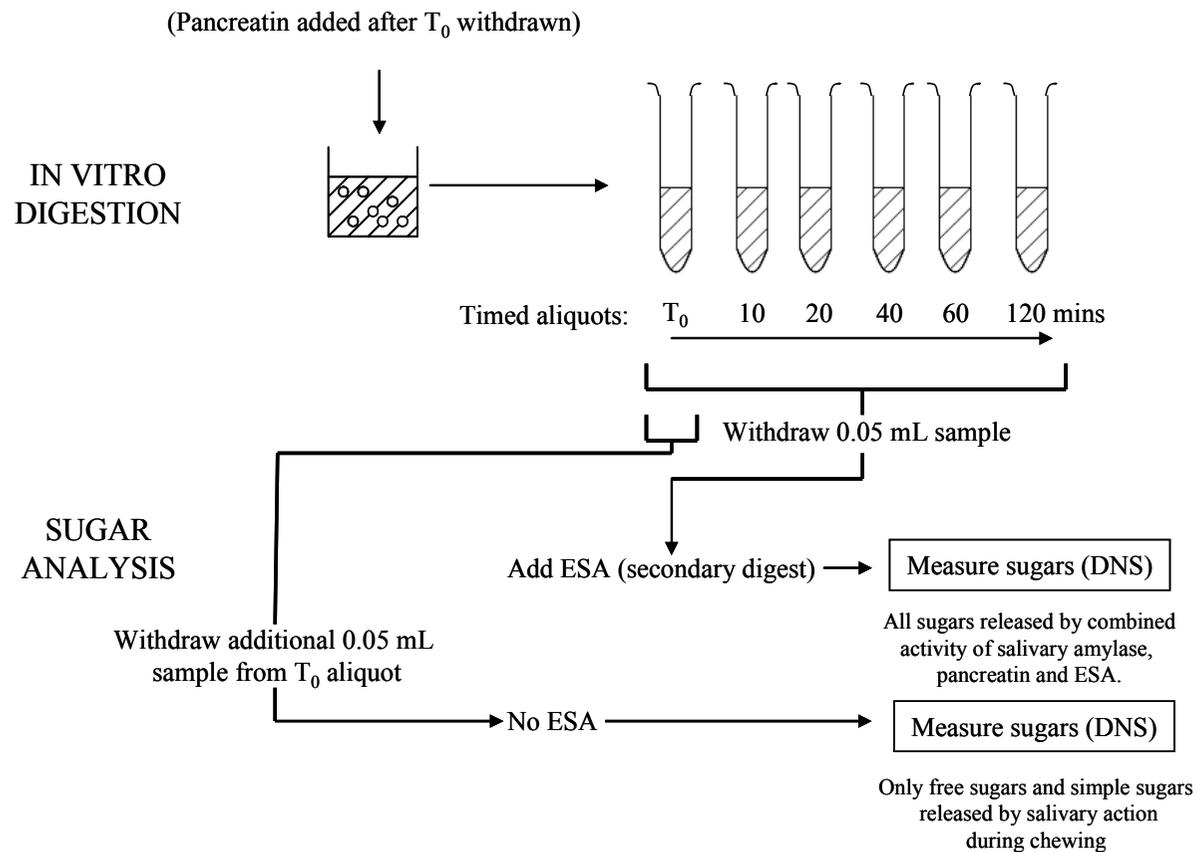


Figure 3.3 Experiment design to assess the starch-digesting capacity of salivary α -amylase. A second sample of the T_0 aliquot of digest was taken and spared the secondary ESA treatment so that only free sugars present in the foods and those released by salivary α -amylase were available for DNS measurement.

3.1.2.5 Starch-Digesting Capacity of Pancreatin (pregelatinised starch)

Samples (2.5 g) of pregelatinised maize starch powder (*BOIIC, 431/9, Penford NZ Ltd.*) were weighed into 70 mL plastic specimen pots and dispersed in 40 mL of 0.1 M sodium maleate buffer (pH 6.0). A 10 % pancreatin (in 0.1 M sodium maleate buffer) stock solution was made up and progressively diluted in appropriate volumes of distilled water to achieve final enzyme concentrations in the digesta pots of 2.0, 1.5, 1.0, 0.5, 0.2, 0.1, 0.02, 0.01, 0.005, 0.0025, 0.001 and 0 %. Following addition of pancreatin, *in vitro* digestion was carried out at 37 °C with slow, constant stirring (130 rpm). Aliquots (1 mL) of digesta were withdrawn at T_0 , and at 10, 20, 40, 60 and 120 minutes post-pancreatin addition. After the 120 minute aliquot was removed, 200 μ L of amyloglucosidase enzyme was added to all digest pots. *In vitro* digestion continued and further digest aliquots (1 mL) were withdrawn 20 and 40 minutes post-amyloglucosidase addition. All digest aliquots were analysed for reducing sugars by DNS colourimetry as described in **Figure 3.2**.

3.1.2.6 Starch-Digesting Capacity of Pancreatin (test foods)

The five test foods were purchased fresh and prepared as described earlier. Following preparation, the foods were pressed through a 4 mm sieve to replicate chewing. Three sets of 5.0 g samples (2.5 g for bread) of each food type were weighed into 70 mL digesta pots and digested initially as described in **Figure 3.1**. At the pancreatin addition step of the intestinal phase, a 10 % pancreatin stock solution (in 0.1 M sodium maleate buffer) was diluted and added to the digesta pots such that the three sets of foods were digested with 1 %, 0.05 % and 0.0025 % pancreatin final concentration respectively. *In vitro* digestion proceeded as described above with aliquots of digest withdrawn at T_0 and at 20 and 120 minutes post-pancreatin addition. At the completion of the digestion, all aliquots were analysed by DNS colourimetry.

3.1.2.7 Pancreatin and pH (80)

Samples (2.5 g) of pregelatinised starch were weighed out in duplicate into 70 mL digesta pots and dispersed in 40 mL of one of the following buffers:

- 0.1 M sodium maleate buffer (pH 6.9)

- 0.1 M sodium maleate buffer (pH 6.0)
- 0.1 M sodium acetate buffer (pH 5.2)
- 0.1 M sodium acetate buffer (pH 5.0)
- 0.1 M sodium acetate buffer (pH 4.0).

A 2.5 % pancreatin enzyme stock solution was made up as described in **Figure 3.1** but the pancreatin was dissolved into a saline solution (distilled water containing 1 % NaCl, 0.2 % CaCl₂) instead of the usual sodium maleate buffer which would otherwise interfere with the final digest pH. Pancreatin was added to all samples after withdrawing a T₀ aliquot. *In vitro* digestion proceeded under the usual conditions with aliquots of digest taken at 10, 20 and 120 minutes post-pancreatin addition. Following the 120 minute aliquot, an additional 2.5 g of pregelatinised starch was added to each digestion pot. Further digesta aliquots were taken at 10, 20 and 60 minutes post starch addition to test for ongoing starch hydrolysis by pancreatin. All digest aliquots were analysed by DNS colourimetry.

3.1.2.8 Method of Stirring

The five test foods were purchased fresh and prepared as described previously. Following sieving, 5.0 g samples of each food type (2.5 g for bread) were weighed out and divided (in triplicate) into three groups, to be digested *in vitro*, using as the stirring device, either:

- a magnetic stirrer - 130 rpm (termed: ‘MS, slow’)
- a shaking water bath - 70 strokes per minute (‘SWB, slow’)
- or an end-over-end inverter positioned inside a heating cabinet - 1 rotation every 30 seconds (‘EE, slow’).

The settings used above were selected as they were the slowest possible speeds at which each device could be operated and were closest to gastric and intestinal mixing. Foods were digested in 50 mL plastic screw-cap tubes when using the shaking water bath and inverter.⁸ In the shaking water bath, tubes were laid horizontally, aligned with the direction of shaking. All apparatus were held at 37 °C and digestions thereafter proceeded as described in **Figure 3.1**. Aliquots of digest for sugar analysis were

⁸ The difference in digesta volume between the screw-cap tubes and digesta pots using in the magnetic stirrer was accounted for in the final reducing sugar concentration calculations.

withdrawn from all samples at 20, 40, 60 and 120 minutes post-pancreatin addition, hydrolysis curves constructed and levels of RDS and SDS calculated.

On a separate day⁹, triplicate samples of each of the foods were again digested *in vitro* as described above, however the speed at which each stirring device was operated, was doubled:

- magnetic stirrer - 260 rpm (termed: 'MS, fast')
- shaking water bath - 140 strokes per minute ('SWB, fast')
- end-over-end inverter - 2 rotations every 30 seconds ('EE, fast').

Digesta aliquots for sugar analysis were again withdrawn at 20, 40, 60 and 120 minutes post-pancreatin addition and hydrolysis curves for the five foods constructed.

3.2 PHASE 2: HUMAN GLYCEMIC INDEX (GI) STUDY

Glycemic index (GI) testing was carried out in the clinical research facility of CSIRO Food and Nutritional Sciences, located in Adelaide, South Australia. The study followed the format of a standard GI test (1, 62). Ethics approval was granted by the CSIRO Human Research Ethics Committee on 23rd October, 2008 (**Appendix C**).

3.2.1 Volunteers

Ten volunteers were recruited from the general public, based on their satisfying the following criteria:

- aged 18 - 60 years
- normal fasting blood glucose concentration (3.5 - 5.5 mmol/L)

Prospective volunteers were excluded from participation if any of the following applied:

- sufferer of type 1 or type 2 diabetes mellitus
- sufferer of a bleeding disorder
- known food allergy, hypersensitivity or intolerance to wheat and/or starchy foods
- taking medications known to influence glucose tolerance (oral contraceptives excepted) such as sulphonylureas, metformin and thiazolidinediones

⁹ Effort was made to exactly replicate the test food preparation conditions between the two different testing days. The same stocks of *in vitro* digestion reagents were used on both testing days.

- persons considered by the investigator to be unwilling, unlikely or unable to comprehend or comply with the study protocol
- participation in another research study within 30 days preceding the start of this study
- history or presence of gastrointestinal, renal or hepatic disease of any cause.

3.2.2 Flatbread Test Foods and Glucose Reference

Three flatbreads, each containing the equivalent of 50 g available carbohydrate and differing only in their fat content, were formulated for use as the diets in the human study. The flatbread as a food format was selected based on its simplicity in terms of preparation time, reproducibility in preparation, minimal ingredients (flour, oil and water) and palatability.

Flatbreads were made the night before consumption, using 68.87 grams of *White Wings* plain flour (corresponding to 50 g available carbohydrate) to which was added either 3.44, 10.33 or 20.66 g of blended vegetable oil (*The Healthy Baker, Manildra Group product code 13100*) resulting in final fat contents of 5, 15 and 30 % (on a dry weight basis) respectively. A dose (1 μL per gram of food) of ^{13}C -labelled octanoic acid (*CLM-293-1 Cambridge Isotope Laboratories Inc.*) was dissolved into the oil of each flatbread prior to mixing with the flour. Boiling water (33 g) was added to each flour-oil mix and the resultant paste kneaded thoroughly into a dough ball which was then covered and left to sit at room temperature for 30 min. After 30 min the dough ball was flattened into an oval-shaped flatbread no thicker than 10 mm and fried on a very hot (210 °C) electric frying pan for 3 min on each side. The flatbreads were allowed to cool, vacuum-sealed in plastic food storage bags and stored at room temperature overnight.

A commercial glucose solution (*Carbotest, Lomb Scientific*) for oral glucose tolerance testing was used as the glucose reference drink throughout the study. The beverages come in 300 mL bottles containing 75 grams of glucose, therefore 100 mL of each bottle was decanted and the remaining 200 mL of solution (containing 50 grams of glucose) served, refrigerated, to the volunteers.

3.2.3 Testing Procedure

Volunteers presented at the clinic once each week for six consecutive weeks after an overnight fast (no food or drink other than water for at least 10 hours prior to each visit). Volunteers were instructed not to consume alcohol during the evening prior to each test and to avoid strenuous exercise on the morning of each test.

Upon arrival at the clinic, volunteers had a cannula (*Surflo I.V. Catheter, Terumo*) inserted into the medial cubital vein of their arm. Two initial samples of blood were collected via the cannula into 2.5 mL blood collection tubes (*Glucose FX NaF/KO, Vacuette*) to establish the baseline (T_0) blood glucose concentration.¹⁰ Volunteers also provided an initial breath sample to establish the baseline (T_0) for $^{13}\text{CO}_2$ in the breath. Breath samples were collected via a straw into 10 mL, glass screw-cap tubes (*Exetainer, Labco Limited*). Volunteers were instructed to firstly exhale, then blow their residual breath through the straw to fill the two 10 mL tubes which were then quickly capped and stored upright at room temperature for later analysis.

Over the six clinic visits, volunteers consumed, in random order, either one of the three flatbreads or the glucose reference drink (tested in each volunteer three times). Volunteers were blinded as to what flatbread they were eating. Ten minutes were permitted for each volunteer to consume the flatbread/glucose drink. Postprandial blood samples for glucose measurement were taken via the cannula at 15, 30, 45, 60, 120 and 180 min after beginning to eat. Duplicate breath samples were collected at 5 min intervals up to 45 min postprandial, and at 15 min intervals thereafter up to 180 min. Volunteers were permitted a 250 mL drink of water at 90 min postprandial.¹¹ At the completion of the 180 min blood and breath-sampling, the cannula was removed and volunteers were provided with a light lunch before departing the clinic.

3.2.4 GI Calculation

¹⁰ The two samples are taken within 5 min of each other, analysed for glucose, and the average result taken as the baseline or fasted blood glucose concentration.

¹¹ The time at which volunteers were permitted a drink was standardised because the rate of gastric-emptying which is likely affected by the timing of fluid intake, was also being measured.

Following collection, blood tubes were centrifuged (2800 rpm, 4 °C, 15 min) and the plasma removed and stored at -80 °C until analysis. Thawed samples (50 µL) of plasma were analysed for glucose concentration (mmol/L) using an automatic analyser (*Hitachi 902 Automatic Analyser*). Mean blood glucose response curves to each flatbread and the glucose reference drink were plotted by calculating the mean blood glucose concentration of all subjects at each time point. The incremental area under the curve (IAUC) up to 120 min¹² (IAUC₁₂₀) for each volunteer, for each flatbread/glucose drink was calculated geometrically using the trapezoid rule, ignoring the area beneath the fasted blood glucose concentration. The IAUC₁₂₀ for each flatbread was expressed as a percentage of the mean IAUC of the three glucose drinks, giving an individual volunteer value of GI for each flatbread. The final GI of each flatbread was determined as the average (\pm SE) of the individual GI values after any outliers were discarded.¹³ An additional “GI” value based on IAUC up to 180 minutes postprandial was also calculated for each flatbread (GI₁₈₀). The IAUC₁₈₀ for each flatbread, for each volunteer, was calculated geometrically and expressed as a percentage of the mean IAUC₁₈₀ of the three glucose drinks. The individual values for “GI” up to 180 minutes were averaged and outliers discarded to obtain GI₁₈₀ values (\pm SE) for each flatbread.

In addition to GI, the mean time (min) to reach the glycemic response peak (T_{GRP}) and mean amplitude (mmol/L) of the glycemic response (A_{GR}) to each flatbread diet and the glucose reference drink was calculated.

3.2.5 Measurement of Gastric Emptying Rate

Breath samples were sent to Adelaide’s Women’s and Children’s Hospital where they were analysed by an isotope ratio mass spectrometer (*ABCA Automated Breath ¹³Carbon Analyser SerCon*) that measures the ratio of ¹³C : ¹²C occurring in the breath sample. A formatted spreadsheet (*Microsoft excel*) converts the ratio values given by the mass spectrometer to percentage recovery per hour of the initial ¹³C dose given (%dose/h) and generates plots against postprandial time for each volunteer and each flatbread diet. The mean cumulative percentage of the administered ¹³C dose recovered

¹² In a standard GI test blood glucose is only measured up to 120 min postprandial not 180 min as in the present study.

¹³ An outlier is defined as the GI value for a particular subject that falls outside 2 standard deviations of the mean of the group of 10 (or more) volunteers.

over time (CPDR) for the three flatbread diets was calculated and plotted. The *excel* program also provides measures of additional gastric emptying parameters including:

- gastric half-emptying time ($T_{1/2GE}$), which is the time taken (min) for 50 % of stomach emptying to occur,
- gastric lag phase (T_{lag}), the time taken (min) for 5 % of stomach emptying to occur,
- peak excretion time (T_{max}), which corresponds to the maximum of the %dose/h curve.

3.2.6 In Vitro Analysis of Flatbreads

The three flatbreads were formulated the night before *in vitro* analyses as described previously, vacuum-sealed, and stored overnight at room temperature. Duplicate samples (2.5 g) of each flatbread type were digested *in vitro* according to the method described in **Figure 3.1**. Aliquots of digest were withdrawn at T_0 and at 10, 20, 40, 60, 120 and 180 minutes post-pancreatin addition and ReS concentrations determined using the DNS method described in **Figure 3.2**. The mean ReS concentration from each duplicate aliquot at each timepoint was calculated and hydrolysis curves for the three flatbreads constructed. The RDS and SDS contents of each of the three flatbreads were determined as described previously.

3.2.7 Scanning Electron Microscopy of Flatbreads

An additional (third) 2.5 g sample of each flatbread type was included in the *in vitro* digestion described above, except that no aliquots of digest were removed for ReS analysis. Rather, particles of flatbread, as it progressed through the *in vitro* digestion, were periodically removed for imaging by scanning electron microscopy (SEM). Flatbread particles were retrieved from *in vitro* digesta at the pre-gastric (immediately prior to pepsin addition) and post-gastric (following pepsin incubation) stages, and at 30, 60, 120 and 180 minutes of pancreatic digestion. In addition, samples of raw flour, raw flatbread (at the dough ball stage, prior to frying), as well as cooked flatbread (post-frying) were collected for SEM.

Upon removal from digest, to prepare for SEM, flatbread particles were immersed in 8 mL of Hepes buffer (2.8 % glutaraldehyde, 0.02 % Triton X-100, pH 7.2) and refrigerated (4 °C) overnight. Samples were then washed three times in 10 mL Hepes buffer with centrifuge cycles of 3000 rpm for 5 min performed between washes. Samples were dehydrated with progressive immersions in 25, 50, 70, 85, 95 and 100 % ethanol, then left to air dry overnight at room temperature. The following day, dried samples were mounted onto metal SEM stubs using double-sided carbon tape. With an automated sputter (*Denton DV-502 vacuum evaporator*) a thin layer of gold was applied to the sample surface and the samples then imaged by SEM (*Philips XL30 FEGSEM*).

3.2.8 Assessing the Strength of the Flatbread Starch-Fat Emulsion¹⁴

Each flatbread type (low, medium and high fat) was prepared as described in section **3.2.2** except that in the present experiment, a fat-soluble, yellow food dye (*0406 Annatto oil soluble, Warner Jenkinson Company, Bronson and Jacobs NZ Ltd.*) was added to enhance the visibility of the fat component within the flatbread food matrix. Flatbreads were sieved as described in **Figure 3.1**, and 5 g samples of each flatbread type weighed in duplicate into 250 mL conical flasks labelled time zero (T₀) and 120 min (T₁₂₀). Samples were hydrated with 5 mL distilled water for 5 min (at room temperature) after which time 10 mL of a 10 % pepsin in HCl solution (**Figure 3.1**) was added to each flask. Samples were gently mixed with 2-3 swirls of the conical flask. T₀ samples were then transferred into labelled 50 mL plastic centrifuge tubes. Complete transfer of the samples was achieved with a 25 mL distilled water rinse. Conical flasks containing the T₁₂₀ samples were placed on an oscillating tray in an incubator set to 37 °C and gently swirled at 100 rpm for 120 min to replicate gastric conditions, after which time the samples were transferred into 50 mL plastic centrifuge tubes along with the 25 mL distilled water rinse.

All centrifuge tubes containing the T₀ and T₁₂₀ samples were centrifuged at a low setting – 700 g, for 5 min in order to separate the non-emulsified fat component from

¹⁴ Section 3.2.8 describes a supplementary experiment that was performed during a brief visit to the laboratories of Massey University's Institute of Food, Nutrition and Human Health. The experiment was conducted after the results of the GI study had been interpreted and it became apparent that starch-fat separation had occurred during gastric digestion. The experiment was not intended to be quantitative, but merely *in vitro*-based confirmation of a suspected *in vivo* phenomenon.

the flatbread food matrix. Following centrifugation all centrifuge tubes were photographed to display the degree of starch-fat separation that had occurred for each flatbread type before and after simulated gastric conditions.

3.3 STATISTICS

All statistical analyses were conducted using *SPSS Statistics 17.0*, *Genstat* and *Microsoft Office Excel 2003* software.

Unless otherwise stated, all *in vitro* hydrolysis curves were analysed with a two-way repeated measures analysis of variance (ANOVA) for comparison of treatments over time as well as comparison of treatments at individual time points. One-way repeated measures ANOVAs were used to compare differences in mean RDS and SDS content of foods between treatments. For *in vitro* experiments investigating the starch-digesting capacity of salivary α -amylase (157), least significant differences (LSDs) for the comparison of different treatments at given time points were calculated.

Mean glycemic response curves to the flatbread diets and glucose reference drink were analysed via a two-way repeated measures ANOVA for comparison of differences between diets over time as well as between diets at each individual time point. Differences in the mean $IAUC_{120}$, $IAUC_{180}$, T_{GRP} and A_{GR} between each diet were compared via a one-way repeated measures ANOVA.

Differences in the mean ^{13}C CPDR curves for the three flatbread diets over time as well as between diets at individual time points were compared via a two-way repeated measures ANOVA. Differences in the mean $T_{1/2GE}$, T_{max} and T_{lag} between the three flatbread diets were compared using a one-way repeated measures ANOVA.

All repeated measures ANOVAs included a bonferroni adjustment for multiple comparisons that maintains statistical significance stringency at a level of 0.05. Significant differences are reported at a significance level of 0.05.

In all bar graphs, different letters denote significant differences between treatments. Where bar graphs are not annotated with such letters, no significant differences between any of the treatments exist.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 PHASE 1: *IN VITRO* DIGESTION METHODS INVESTIGATION

4.1.1 Mode of Comminution in the Oral Phase

In vitro starch digestion curves and levels of the digestible starch fractions RDS and SDS measured in bread, wheat and pasta with each oral phase pretreatment are shown in **Figures 4.1, 4.2, and 4.3** respectively. Graphs for the remaining foods are shown in **Appendix D1/2**. A summary of statistical analysis outcomes for each food type is provided in **Appendix D3**.

In bread, chickpea and potato samples, *in vitro* digestion occurred at a similar rate irrespective of oral phase comminution method used. Typical of high GI foods, the rate of sugar release was rapid in potato and bread samples with ReS levels reaching a plateau within 10 and 40 min respectively. Chickpea on the other hand, with its high DF content restricting starch accessibility to enzyme (159, 160), digested slowly *in vitro*. By T₁₂₀, (the end of *in vitro* digestion), ReS levels continued to rise, indicating incomplete starch hydrolysis. ReS was consistently slightly higher in homogenised chickpea sample compared with the samples comminuted by other means. It is likely that homogenisation of chickpea sample caused greater disruption of chickpea cell walls than the other comminution methods, increasing accessibility of starch to amylase. Importantly, RDS and SDS levels did not differ in any bread, chickpea or potato samples regardless of comminution pretreatment.

Unlike bread, chickpea and potato, wheat and pasta samples exhibited significant differences in the overall rate and extent of starch digestion depending on the mode of oral phase comminution used.

The most rapid and complete starch digestion in wheat occurred in sample that had first been homogenised, with ReS at 20 min (and therefore RDS) significantly higher than that of the other simulated chewing techniques: chopping (p = 0.011), sieving (p = 0.004) and mincing (p = 0.01), which did not differ (**Figure 4.2**). Chewing and

expectation of wheat achieved approximately double the rate and extent of starch digestion compared with simulated chewing methods (chopping, sieving, mincing) which clearly did not achieve the same degree of particle size reduction. The effect of differing particle size as a result of using different comminution methods on starch digestion in wheat, as indicated by relative ReS levels, was preserved across the duration of the *in vitro* digest.

Similar to wheat, starch digestion occurred most rapidly in pasta samples that were homogenised, reducing respectively with mincing, sieving, chewing and chopping pretreatments (**Figure 4.3A**). RDS was 267 mg/g (27 % of the total weight of pasta sample) in homogenised pasta, significantly higher than that measured in minced (233 mg/g), chewed (189 mg/g) and chopped sample (161 mg/g), ($p \leq 0.036$) (**Figure 4.3B**). Unlike wheat, however, the initial effect on starch digestion that the different comminution methods had, did not persist, and there was gradual convergence of ReS concentration measured in respective samples toward the end of *in vitro* digestion at T_{120} .

The comminution method used prior to *in vitro* digestion is important since it determines the particle size at which substrate is presented to the digestive enzymes and thus its susceptibility to digestion (37, 119, 161). The present study indicates, however, that particle size, hence comminution method, is only an influencing factor to *in vitro* starch digestibility for some food types and not others. The key determinant of whether a food type is affected by comminution method, or not, likely lies in the structural properties of the food, in particular, whether or not the particle retains a degree of structural rigidity following oral phase breakdown.

In bread, chickpea and potato *in vitro* digestions, there was no relationship between comminution method and starch digestion rate or extent. Bread, due to leavening, is highly porous and easily permeated by amylase enzyme, irrespective of its particle size (35). Chickpea, following oral phase breakdown and immersion in digesta solution, became friable and disintegrated rapidly, particularly on activation of the magnetic stirrers. Potato sample, regardless of comminution method, clumped together as a pellet

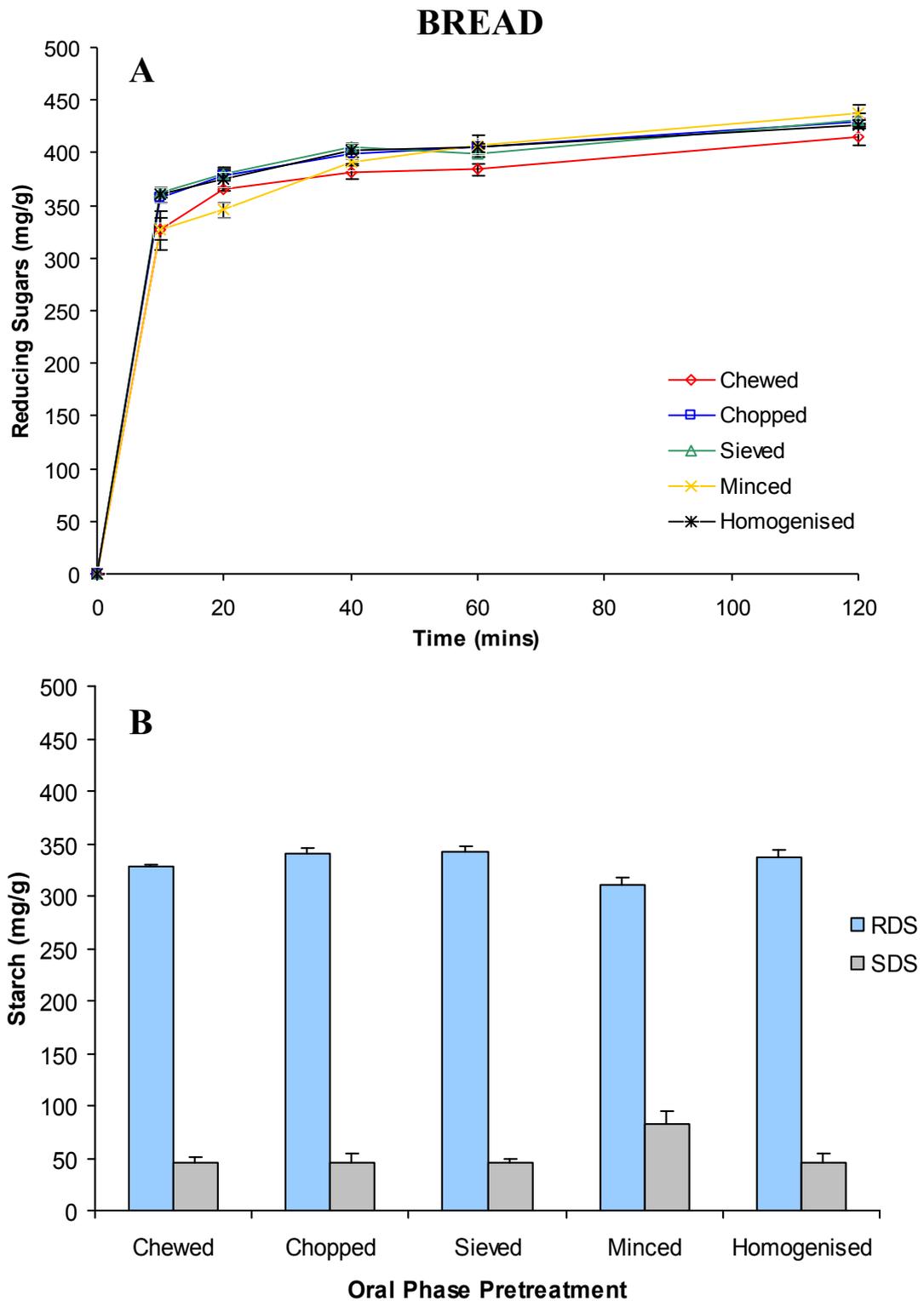


Figure 4.1 (80) Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in bread samples digested *in vitro* following different oral phase pretreatments. Values are means ($n = 3$) \pm SE.

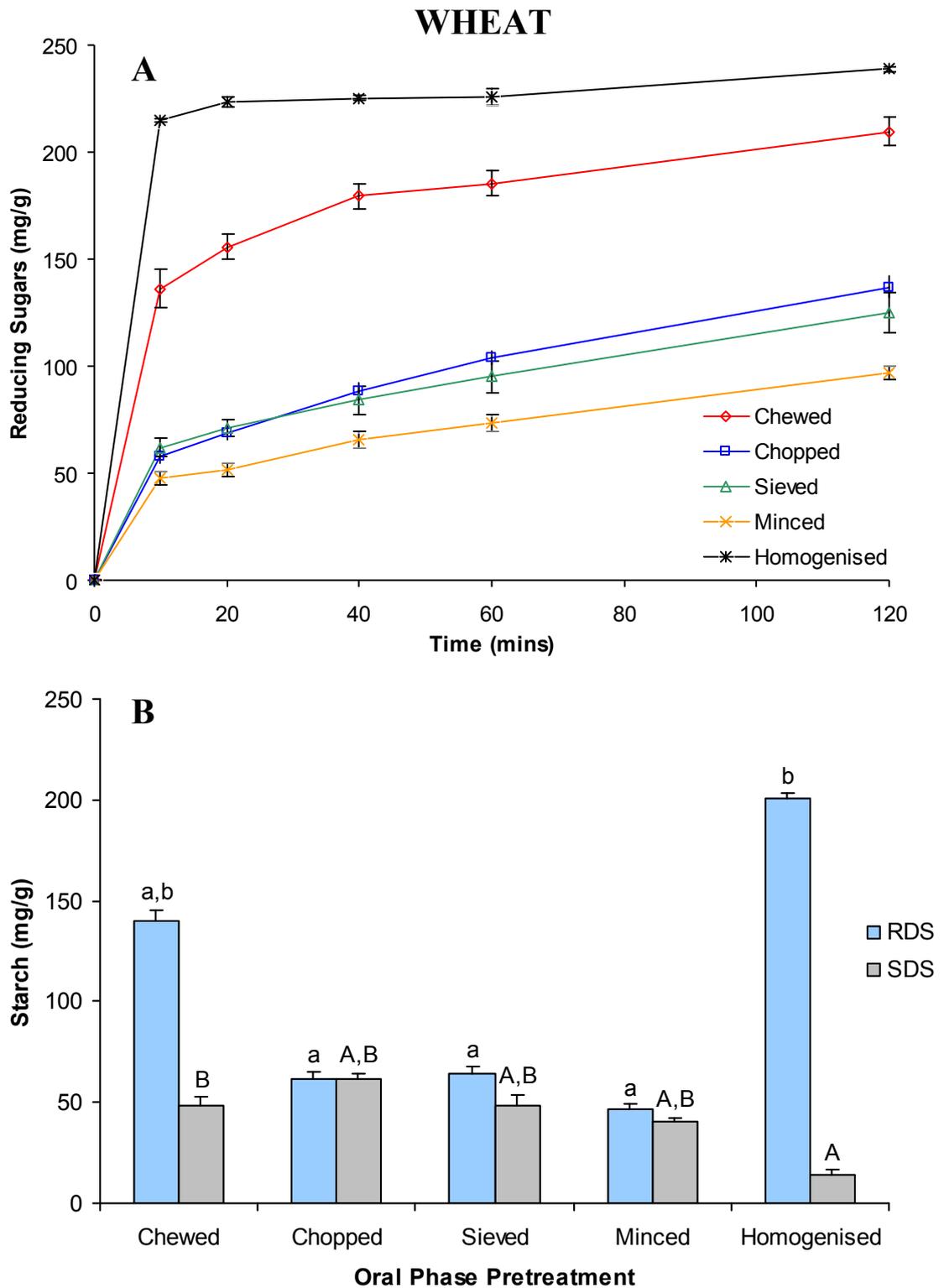


Figure 4.2 (80) Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in wheat samples digested *in vitro* following different oral phase pretreatments. Values are means ($n = 3$) \pm SE

PASTA

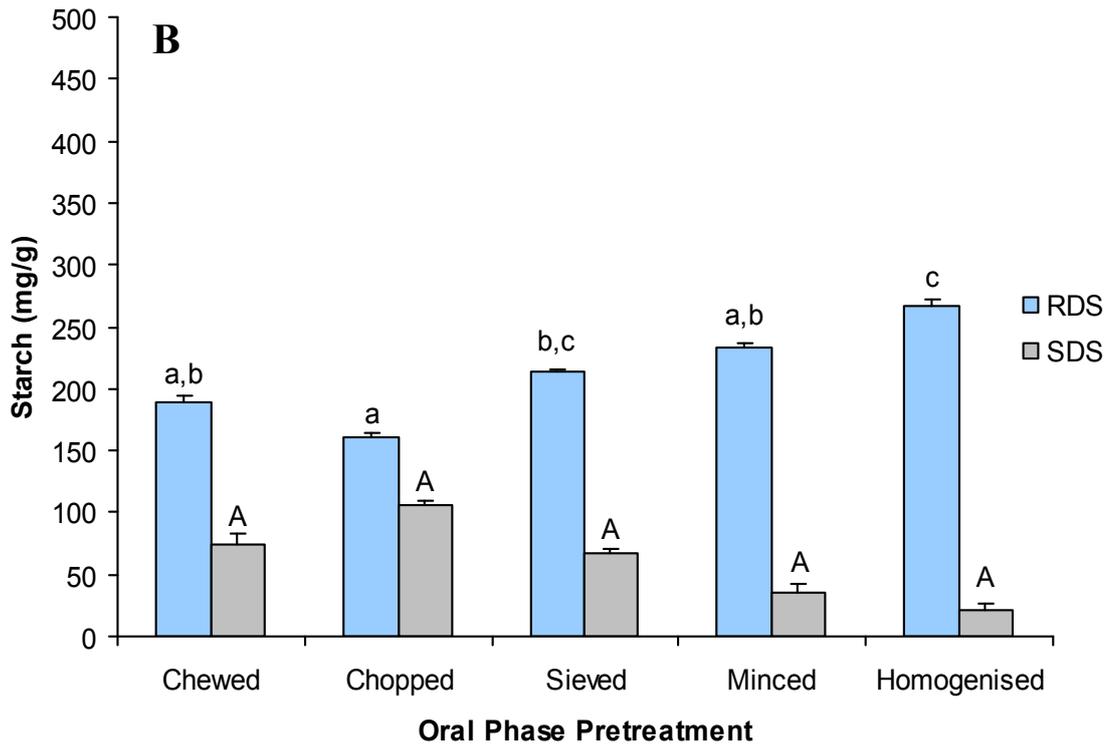
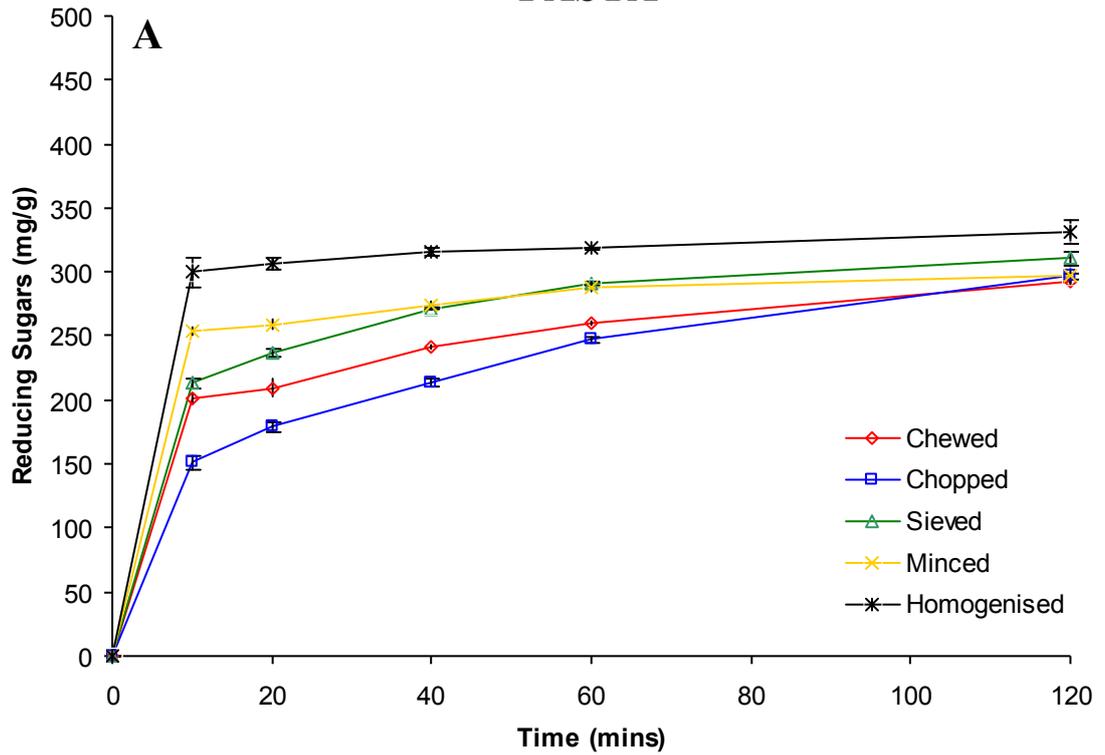


Figure 4.3 (80) Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in pasta samples digested *in vitro* following different oral phase pretreatments. Values are means ($n = 3$) \pm SE.

on the bottom of the *in vitro* digestion pot. Thus with these “soft” food types, any rate-limiting effect on *in vitro* starch digestion that variation in comminution method might have caused, was negated since particle size was either irrelevant (bread), or quickly deviated (chickpea, potato) at digestion onset from that established during oral phase pretreatment. Particles of wheat and pasta on the other hand were well defined and retained some structural rigidity following comminution. Comminuted wheat samples were held intact in digesta by their tough, indigestible bran layer and pasta particles due to their dense protein matrix. Consequently, the oral phase comminution method used and the subsequent unique particle size reduction achieved, influenced the *in vitro* starch digestion kinetics in these foods. So robust were particles of wheat grain, and restrictive to starch digestion was the outer bran layer, that differences in starch digestibility in wheat samples between treatments, persisted to the end of the 120 min *in vitro* digestion.

Chewing and expectoration of sample was adopted by analysts as a technique for achieving realistic, “as eaten” particle size reduction of test food samples prior to *in vitro* digestion (40, 87, 110). Some criticise the use of chewing/expectoration as a pretreatment for *in vitro* digestion however due to the subjective nature of chewing and inconvenience of having to recruit volunteers (86, 162). Thus simulated chewing techniques such as homogenising (97), sieving (92, 102), mincing (86, 94) and chopping (unpublished) are often employed with the advantage being they are convenient to use and achieve standardised particle size reduction, not dissimilar to that of chewing (163).

The present study shows that for softer food types the choice of comminution method used in oral phase pretreatment is inconsequential to subsequent *in vitro* starch digestion dynamics (although this trend needs to be tested across a wider range of foods). Germaine *et al* (164) similarly report no difference in the *in vitro* hydrolysis index of wholegrain bread following either chewing or mincing pretreatment. The present study also highlights the importance of selecting an appropriate comminution method when “hard” foods that remain as defined particles in digesta, are to be analysed. This is illustrated in the *in vitro* digestions of wheat where simulated chewing techniques (chopping, sieving and mincing) achieved only approximately 50 % of the starch digestion that occurred following chewing of sample. Clearly chewing more thoroughly

broke down the wheat grains than did the simulated chewing techniques, causing greater cell damage and rendering the sample more susceptible to pancreatic digestion.

When structurally robust foods are to be analysed, simulated chewing methods should not be used. Rather, the ideal comminution technique is likely a compromise between chewing/expectoration and homogenisation of sample. Chewing and expectoration achieves “as eaten” particles of test food whereas homogenisation liquefies sample just as *in vivo*, the stomach produces a liquid chyme consisting of particles less than 1 mm in diameter prior to gastric emptying into the duodenum (23-25). As much as possible, the standardised *in vitro* digestion method should mimic physiological conditions.

4.1.2 Comparing Chewing and Chopping

In vitro starch digestion curves and levels of the digestible starch fractions RDS and SDS measured in bread, pasta and wheat, with each oral phase pretreatment, are shown in **Figures 4.4, 4.5, and 4.6** respectively. Graphs for the remaining foods are shown in **Appendix E1/2**. A summary of statistical analysis outcomes for each food type is provided in **Appendix E3**.

The inadequacy of chopping as a comminution technique in replicating the particle size reduction achieved by chewing/expectoration of samples of certain food types was demonstrated in the previous experiment. Chopped wheat and pasta samples digested more slowly and less completely than chewed sample, indicating a larger particle size distribution amongst chopped sample compared with chewed (**Figures 4.2/4.3**). The objective of the present experiment, therefore, was to see whether greater alignment of *in vitro* digestion curves, particularly those of wheat and pasta, might be achieved if the number of chops in oral phase pretreatment was increased.

Section **4.1.1** showed that the soft foods: bread, chickpea and potato, digested at a rate unrelated to oral phase comminution method. This again was observed in the present experiment where pretreatment of bread, chickpea and potato samples by chewing or chopping, or by varying the number of chops, had no influence on subsequent *in vitro* digestion curve profiles, or on determined levels of RDS and SDS (see **Figure 4.4, BREAD**).

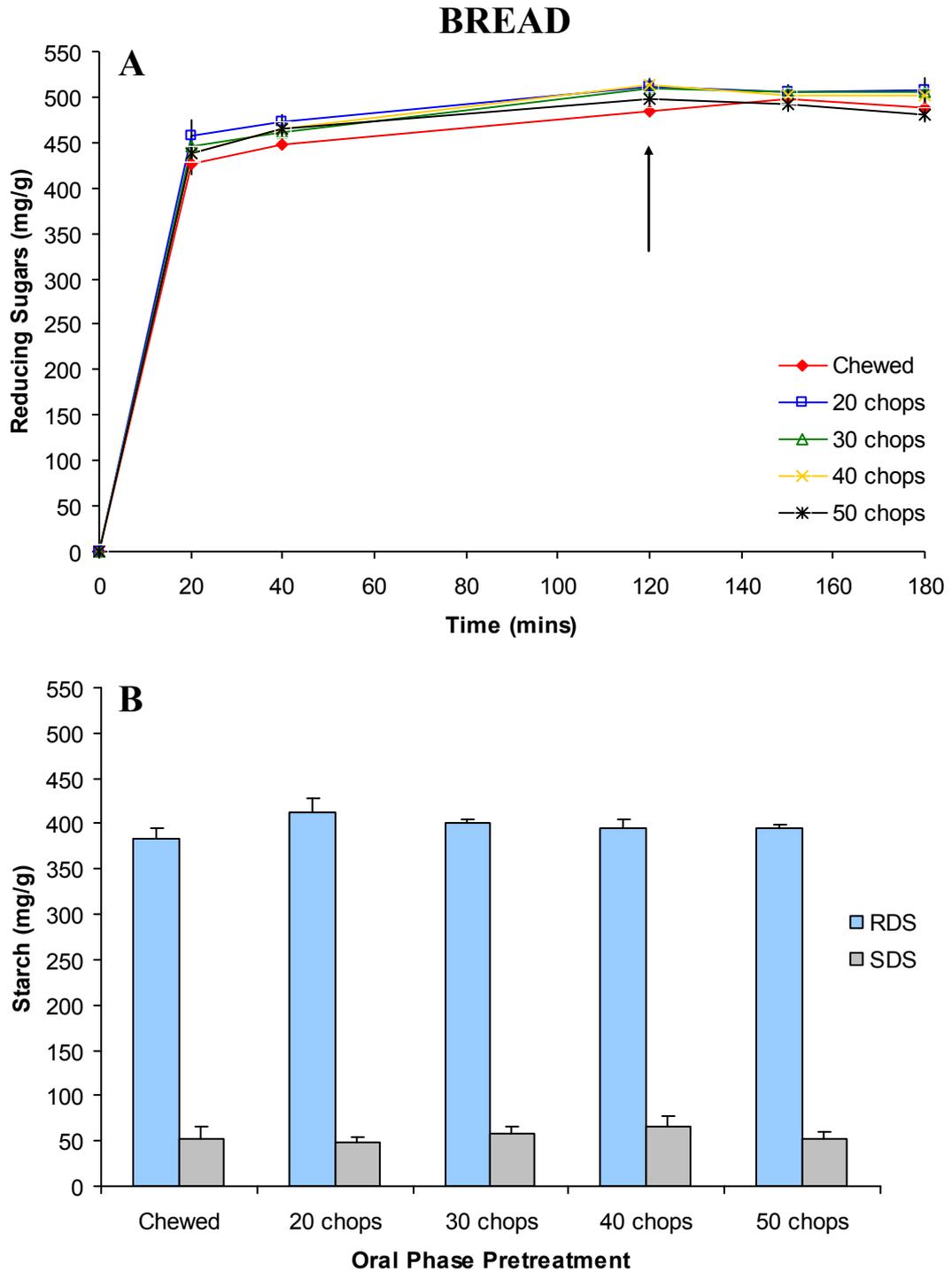


Figure 4.4 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in bread samples digested *in vitro* following different oral phase pretreatments. The arrow denotes the point at which all samples were homogenised. Values are means ($n = 3$) \pm SE.

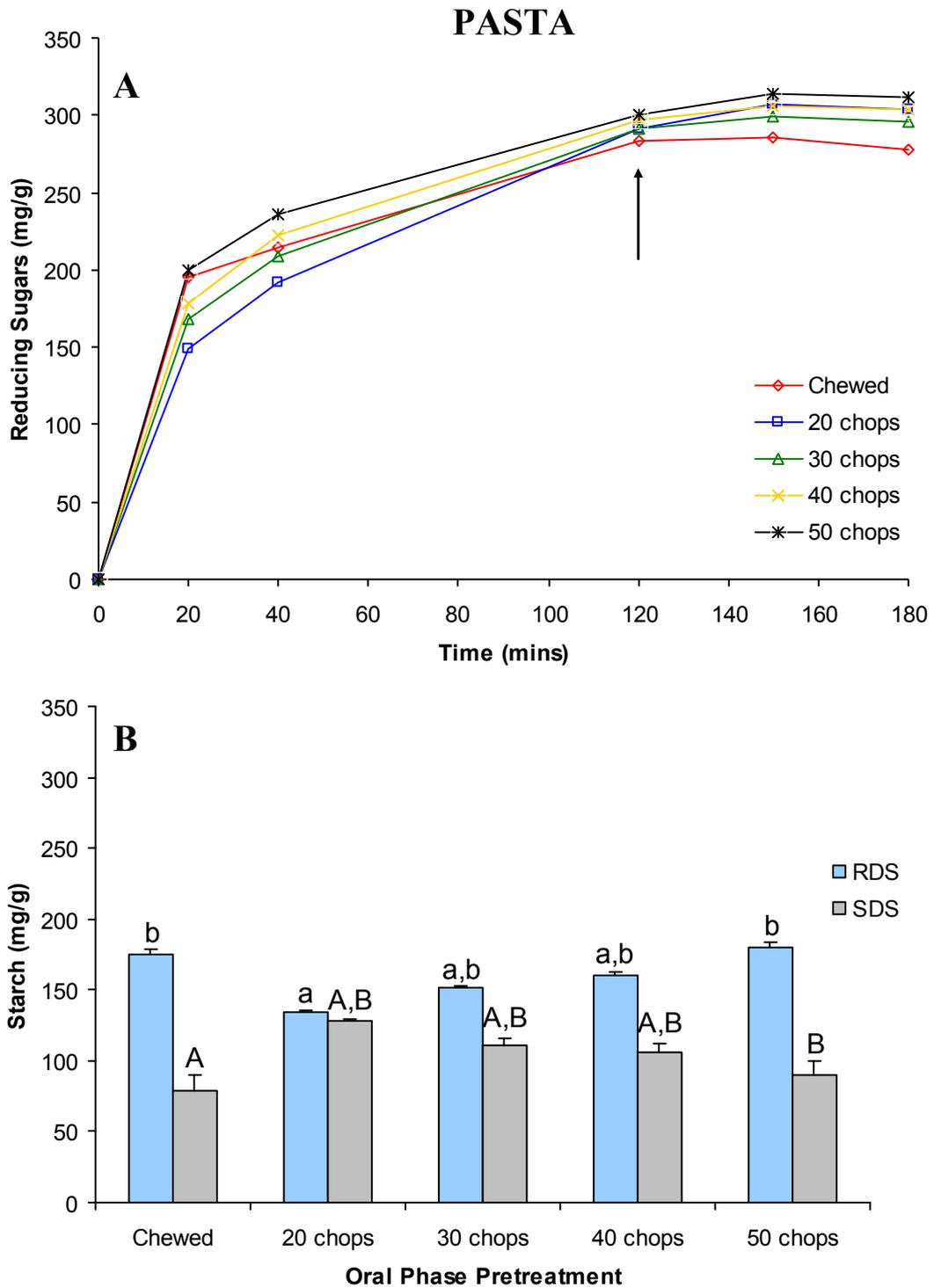


Figure 4.5 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in pasta samples digested *in vitro* following different oral phase pretreatments. The arrow denotes the point at which all samples were homogenised. Values are means ($n = 3$) \pm SE.

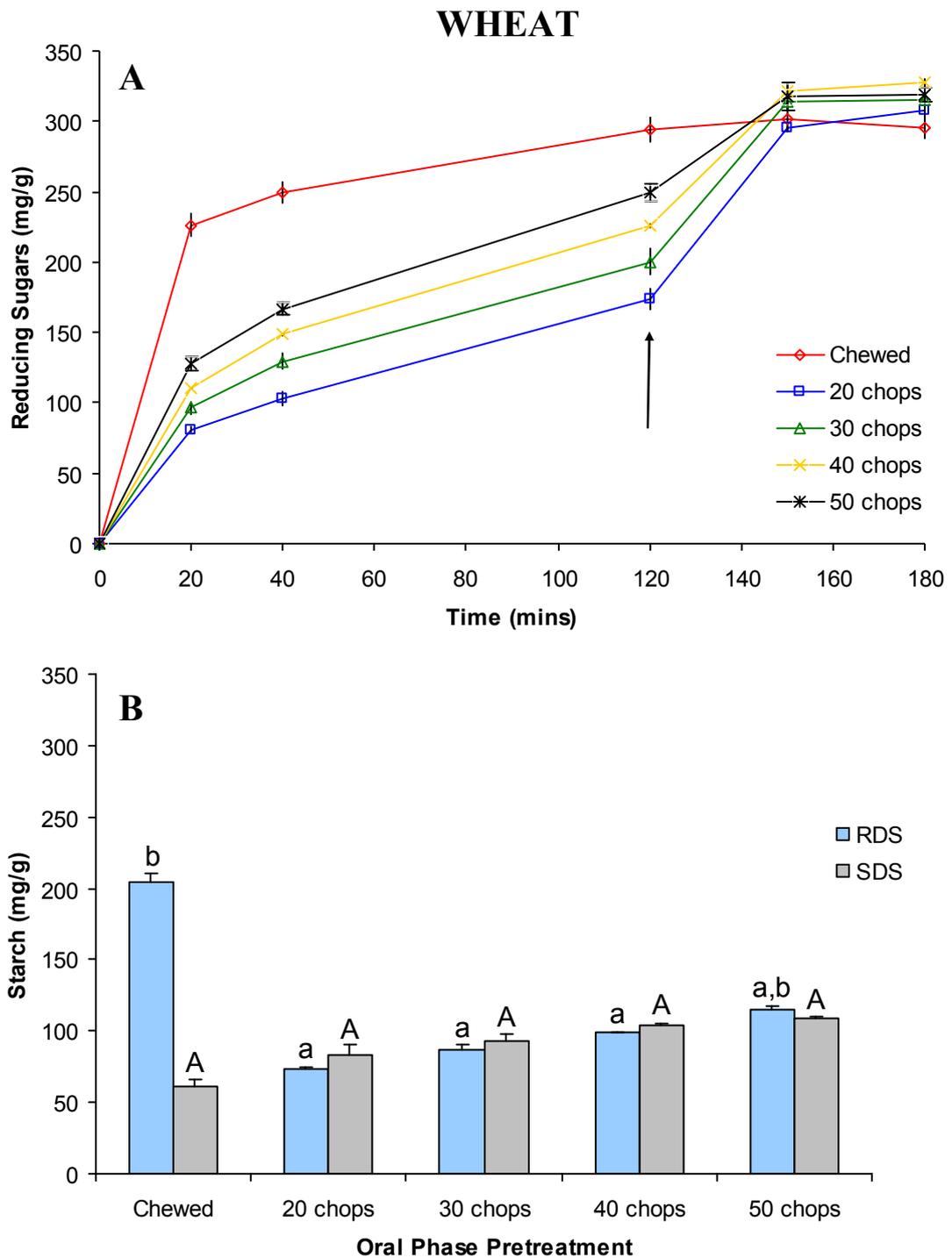


Figure 4.6 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in wheat samples digested *in vitro* following different oral phase pretreatments. The arrow denotes the point at which all samples were homogenised. Values are means ($n = 3$) \pm SE.

Pasta and wheat samples, forming more structurally robust particles following comminution, displayed sensitivity to the number of chops applied prior to *in vitro* digestion. Predictably, the slowest starch digestion from pasta occurred in samples that had been chopped 20 times prior to digestion (**Figure 4.5A**). Thereafter, there was an apparent stepwise increase in the initial rate of starch digestion with increased number of chops. At 20 min pancreatic digestion, there was higher ReS in pasta sample chopped 50 times compared with sample chopped 20 times ($p = 0.041$). Increasing the number of chops helped to align digestion curves from chopped sample with that of chewed. Levels of ReS released by 20 min digestion of chewed sample lay between that of pasta chopped 40 and 50 times. Compared with sample chopped 20 times, RDS was higher in sample chopped 50 times ($p = 0.041$) and in sample that had been chewed ($p = 0.027$) (**Figure 4.5B**). Beyond T_{20} , there was a gradual convergence of *in vitro* digestion curves as ongoing pancreatic digestion eroded the effect of differing particle sizes between pasta samples. By T_{120} digestion was mostly complete since homogenisation of samples (indicated by the arrow on **Figure 4.5A**) did not result in any further substantial ReS release.

Similar to pasta, starch digestion rate in wheat progressively increased with number of chops applied, however, the most rapid and complete starch digestion was still measured in wheat sample that had been chewed. At 20 min of pancreatic digestion, ReS was higher in chewed wheat sample compared with samples chopped 20 times ($p = 0.016$), 30 times ($p = 0.005$) or 40 times ($p = 0.04$) (**Figure 4.6A**). RDS was 204 mg/g in chewed wheat sample, almost double that measured in sample chopped even 50 times (115 mg/g) (**Figure 4.6B**). Clearly, increasing the number of chops applied to even 50 was insufficient to align subsequent *in vitro* digestion curves with those of chewed wheat sample. This result demonstrates the obvious effectiveness with which chewing breaks down the wheat grain structure, compared with chopping pretreatment. Whereas the chopper merely cuts the wheat grain, chewing incorporates a cutting and grinding/crushing component (with the molar teeth), causing substantially greater structural damage and particle size reduction and enhanced *in vitro* starch digestibility. It is interesting that *in vitro* digestion curves from chopped pasta could be aligned with those of chewed when the number of chops was increased, whereas those of chopped wheat samples could not. This could be attributed to the fact that pasta is a processed

food product which, particularly following extrusion, features a homogenous food matrix consisting of damaged wheat endosperm cells and exposed starch granules encapsulated within protein. Wheat grains on the other hand, were in their natural botanical state immediately prior to *in vitro* digestion. Starch granules remain enclosed within intact endosperm cells which themselves are protected by the bran layer. Subsequently, either significantly more chops than 50 are required to align *in vitro* digestion curves with those of chewed wheat, or a different simulated comminution method altogether, that incorporates a grinding component needs to be employed.

Homogenisation of all wheat samples at T_{120} (indicated by the arrow in **Figure 4.6A**) illustrates how much starch remains trapped and undigested within chopped wheat particles (RS1) compared with chewed. Immediately following homogenisation there is a rapid increase in ReS, matching the level already released from chewed sample.

Figure 4.7 and **Figure 4.8** show plots of RDS measured in pasta and wheat samples following the different chopping pretreatments. In both food types, there is an approximate linear relationship between the amount of chops applied prior to *in vitro* digestion, and the level of RDS measured at T_{20} . By further plotting, on each axis, the amount of RDS measured in pasta and wheat samples following chewing, and pinpointing the intercept between this value and the trend line of number of chops vs. RDS, the theoretical number of chops that should be applied to each food type in order to produce the same value for RDS as chewed, is deduced. For pasta, the theoretical number of chops to be applied is 47. RDS measured in chewed wheat samples lay well outside the range of RDS values measured in chopped samples. Thus a large extrapolation was required, indicating that 116 chops of wheat grain samples would produce the same subsequent measure of RDS as if the wheat were chewed. (The large extrapolation most likely makes this value unreliable). Nonetheless, the values: 47 chops for pasta, and 116 chops for wheat grain were tested in a further *in vitro* digestion (see section **4.1.3**) to see whether close alignment of subsequent *in vitro* digestion curves with those of chewed sample might be achieved.

This and the previous experiment demonstrate the significant influence that different oral phase comminution methods can have on subsequent *in vitro* starch digestion kinetics. This is an important finding since indicators such as HI, or the digestible

starch fractions: RDS and SDS, used to rank foods based on their likely glycemic response, are all derived from the *in vitro* starch hydrolysis curve. Thus the choice of one comminution method over another can potentially shift the glycemic ranking of a given analysed food. When test foods (particularly non-homogenous foods) anticipated to form robust, defined particles in digesta are to be analysed, careful consideration must be taken by the analyst as to the choice of comminution method used in oral phase pretreatment. The present study shows that there are some food structural types (wheat grain) for which simulated chewing techniques do not achieve particle size reduction similar to that of chewed sample. In these cases, it is recommended that, despite the inconvenience, chewing/expectoration pretreatment of sample is employed prior to *in vitro* digestion.

The present study also shows that when softer foods that do not form defined particles in digesta are to be analysed, the choice of comminution method is made redundant. In this case, chewing and expectoration, or any simulated chewing technique may be employed as pretreatment and subsequent *in vitro*-derived indicators of likely glycemic impact will not be affected.

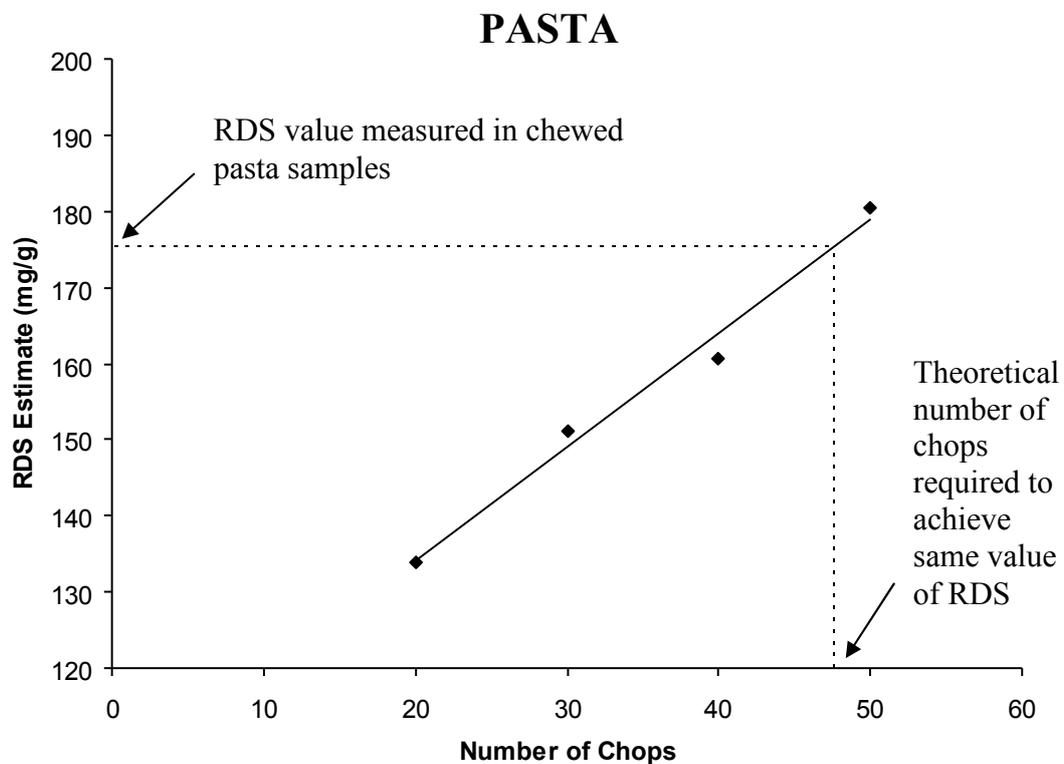


Figure 4.7 Values for RDS in pasta samples chopped 20, 30, 40 or 50 times prior to *in vitro* digestion, when plotted, assumed an approximately linear relationship ($r^2 = 0.9849$). Chewing of pasta samples produced an RDS value of 175 mg/g (see **Figure 4.5B**) which when plotted on the same axes (dashed line), indicate that 47 chops of pasta sample prior to *in vitro* digestion would produce the same value for RDS.

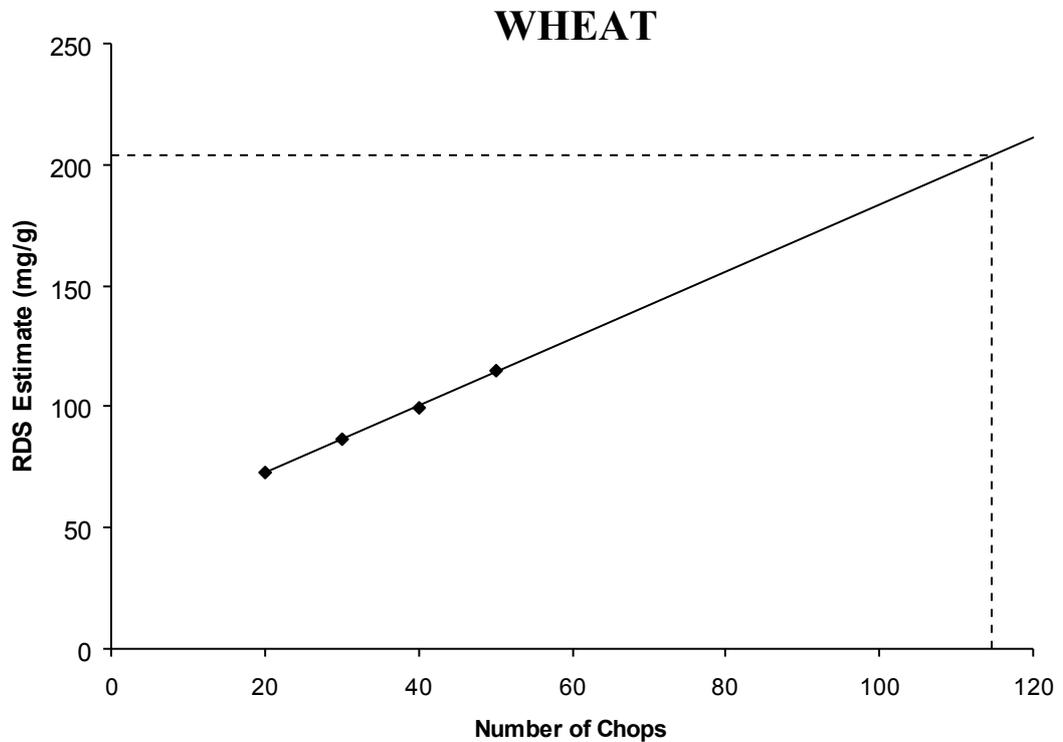


Figure 4.8 Values for RDS in wheat samples chopped 20, 30, 40 or 50 times prior to *in vitro* digestion, when plotted, assumed an approximately linear relationship ($r^2 = 0.9978$). Chewing of wheat samples produced an RDS value of 204 mg/g (see **Figure 4.6B**) which when plotted on the same axes (dashed line), indicate that 116 chops of wheat sample (admittedly a large extrapolation) prior to *in vitro* digestion would produce the same value for RDS.

4.1.3 Starch-Digesting Capacity of Salivary α -Amylase (157)

Hydrolysis curves for bread and pasta following chopping and chewing are shown in **Figure 4.9** and **Figure 4.10** respectively. Hydrolysis curves for wheat, chickpea and potato samples are shown in **Appendix F1-3**. Concentrations of free sugars, glucose and dextrans measured in T_0 aliquots of bread and pasta are shown in **Figure 4.11** and for wheat, chickpea and potato in **Appendix F4-6**. Values for RDS and SDS starch fractions in bread following the different treatments are shown in **Figure 4.12**. Digestible starch fraction data for the remaining foods is not shown.

The percentage of total starch hydrolysed by salivary amylase to glucose (no ESA treatment) and to glucose plus dextrans (ESA treatment) in each chewed treatment category for each food type is shown in **Tables 4.1** and **4.2** respectively.

There was a significant increase in the amount of ReS (glucose) in T_0 (no ESA) samples from chewed bread, wheat, pasta and potato compared with the corresponding chopped samples of these foods ($p \leq 0.017$). There was no difference in ReS concentration between chopped and chewed T_0 (no ESA) chickpea samples. Prolonged salivary exposure (5, 10 or 15 min) did not contribute to increased amounts of glucose appearing in T_0 (no ESA) samples in any of the foods studied.

A secondary digest of T_0 samples from chickpea using ESA did not cause a significant increase in ReS regardless of whether the chickpea was chopped or chewed and regardless of the duration of post-chewing salivary exposure. Chopped samples of the remaining foods also showed no significant increase in ReS at T_0 following a secondary ESA digest when compared with respective T_0 (no ESA) samples. A secondary ESA digest of T_0 samples from chewed pasta, bread, wheat and potato, however, did elicit significantly greater measures of ReS when compared with their respective T_0 (no ESA) samples ($p \leq 0.001$). For pasta and bread, this significant effect was further increased with prolongation of salivary exposure.

Any significant difference in ReS measured between chopped and chewed T_0 samples

TABLE 4.1

FOOD	Ch	Ch + 5 min	Ch + 10 min	Ch + 15 min
Chickpea	1	1	4	3
Bread	4	10	7	8
Pasta	11	13	16	15
Wheat	17	19	17	19
Potato	15	25	27	24

TABLE 4.2

FOOD	Ch	Ch + 5 min	Ch + 10 min	Ch + 15 min
Chickpea	0	2	2	2
Bread	13	23	34	38
Pasta	15	28	25	24
Wheat	27	28	26	28
Potato	20	39	43	38

Table 4.1/4.2 Percentage of total starch hydrolysed to glucose (**4.1**) or to glucose plus dextrins (**4.2**) by salivary α -amylase during chewing. Percentages are rounded to the nearest whole number. Ch, chewed.

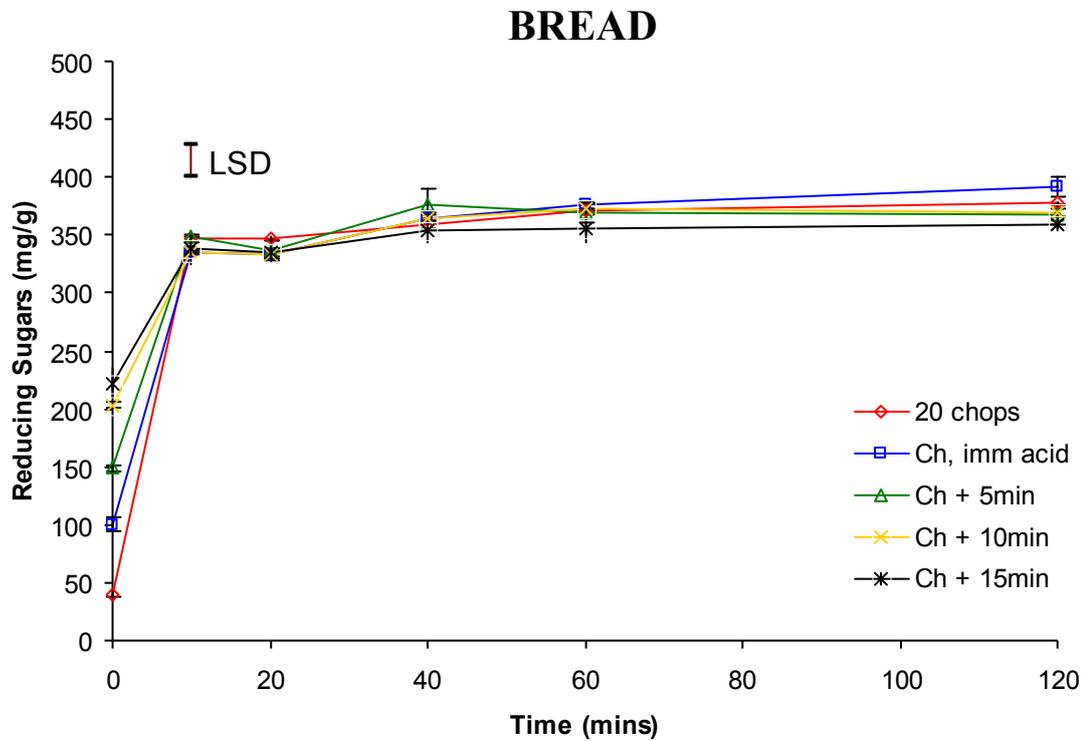


Figure 4.9 Starch hydrolysis curves for bread digested *in vitro* following differing levels of exposure to salivary amylase during the oral phase. Ch, chewed; imm, immediate. Values are mean ReS ($n = 2$) \pm SE. LSD = 29.

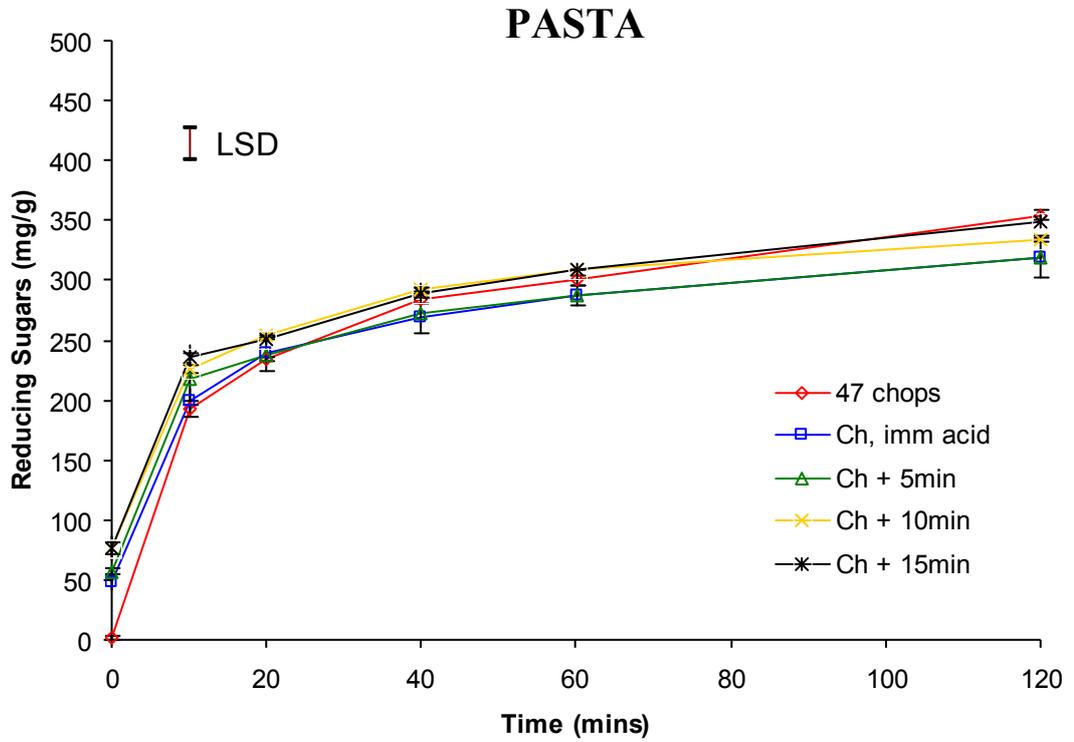


Figure 4.10 Starch hydrolysis curves for pasta digested *in vitro* following differing levels of exposure to salivary amylase during the oral phase. Ch, chewed; imm, immediate. Values are mean ReS (n = 2) ± SE. LSD = 28.

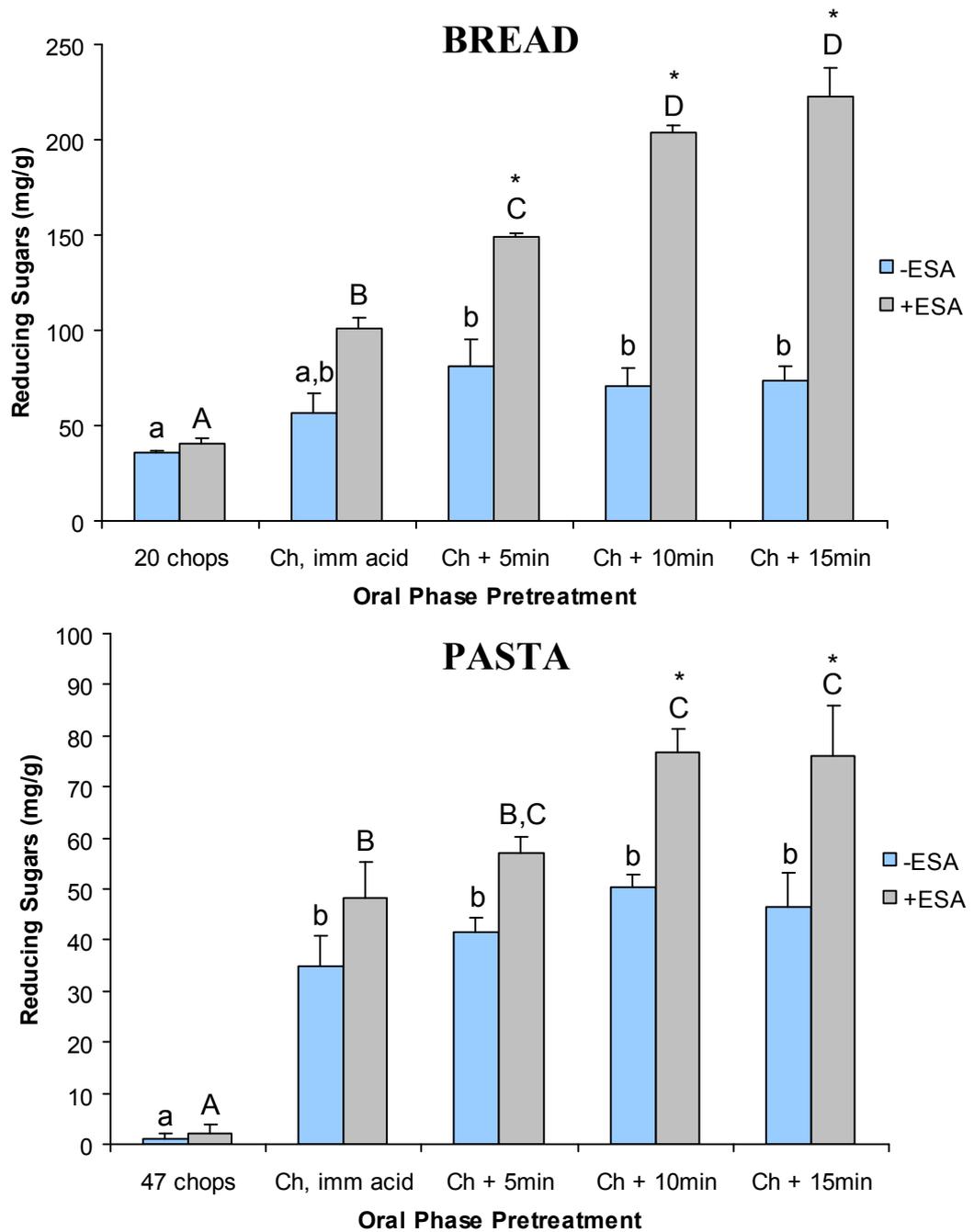


Figure 4.11 ReS released by salivary amylase action during chewing (chewed treatments) or freely occurring in bread and pasta (chopped treatment) and measured in T_0 aliquots prior to pancreatin addition. The T_0 aliquot is subsampled and ReS measured either directly (-ESA) or subjected to a secondary ESA digestion (+ESA) prior to measurement of ReS. Different letters above corresponding bars between treatments denote a significant difference. An asterisk (*) denotes a significant difference between ‘-ESA’ and ‘+ESA’ subsamples within each treatment. Ch, chewed; ESA, enzyme solution ‘A’; imm, immediate. Values are mean ReS ($n = 2$) + SE.

was lost within 10 min of pancreatic *in vitro* digestion (20 min for pasta) as indicated by LSD values. No interaction between pretreatment and digestion time was found for chickpea and potato samples. Bread, however, showed differences in hydrolysis curve pattern both between chopped and chewed samples ($p < 0.001$) as well as among the chewed ($p < 0.001$). Wheat showed a significant lower ReS average when chopped ($p < 0.001$) and a different pattern between chopped and chewed ($p < 0.001$). Pasta showed differences in pattern between the chopped and chewed samples ($p < 0.001$).

During chewing and subsequent pre-gastric bolus resting, salivary α -amylase demonstrated varying capacity to digest starch from the test foods into at least short chains of partly digested starch (dextrins) and in some cases to glucose monosaccharide in a manner dependent on food type and duration of salivary exposure. For instance, salivary amylase digested 27 % of the total starch in potato to glucose monosaccharide and up to 43 % of total starch to glucose and/or dextrins when left to incubate for 10 min following chewing (**Tables 4.1/2**). A key finding was that despite the hydrolysis of sometimes substantial percentages of total starch to glucose and dextrins that occurred in some foods with pre-exposure to salivary α -amylase by chewing, the subsequent rate and extent of *in vitro* starch digestion from the foods, as indicated by hydrolysis curve profiles, when compared with those of the saliva-free, chopped samples, was unaffected. Simulated intestinal digestion rapidly obscured any differences in glucose released due to different durations of salivary digestion before initiation of the pancreatic digestion. This is important because *in vitro* estimates of a given food's carbohydrate digestive properties such as the starch fractions RDS, SDS and RS are commonly based on 20 min and 120 min periods of digestion (86, 93), shown here to not be affected by even prolonged (15 min) salivary pre-exposure (**Figure 4.12**). These results show a point of robustness in *in vitro* methodology and indicate that for the sake of convenience it is not necessary to exactly replicate salivary α -amylase action that occurs during normal chewing. Simple simulated chewing techniques may be used provided they achieve identical particle size reduction as chewing, and the transient influence of salivary α -amylase may be ignored. The slight differences in hydrolysis pattern observed with bread and pasta are not likely a result of salivary influence but of small dissimilarities in relative particle size distributions occurring between chopped and chewed samples as well as between different chewed samples of each test food.

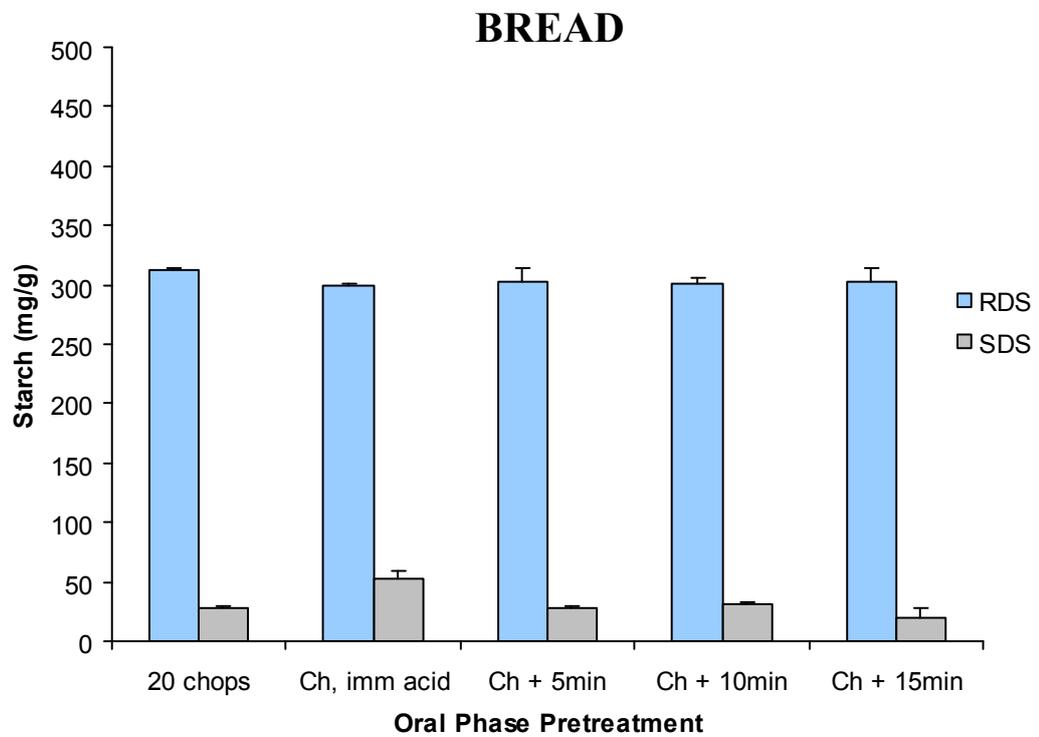


Figure 4.12 Levels of RDS and SDS measured in bread digested *in vitro* following differing levels of exposure to salivary amylase during the oral phase. Ch, chewed; imm, immediate. Values are means (n = 2) + SE.

Differences between chewed and chopped samples after 20 min of pancreatic digestion persisted in whole wheat (**Appendix F1**) showing that it is necessary to carefully replicate chewed food structure for some food types. Wheat samples that had been chopped 116 times gave significantly lower measures of ReS than chewed samples across the entire duration of *in vitro* digestion. Differences of this magnitude are not likely to have been caused by salivary action in chewed samples releasing sustained higher levels of glucose, but are more likely a result of differences in particle size affecting the overall rate and extent of starch digestion in wheat. *In vitro* experiments discussed in section 4.1.2 indicated that 47 chops (instead of the standard 20 for the softer foods) would adequately simulate chewing in terms of particle size reduction for pasta and that wheat grains would require 116 chops. These assumptions proved accurate for pasta, but not for wheat, as shown in **Appendix F1** which shows the overall rate of *in vitro* digestion was greatly retarded following chopping, due most likely to chopped wheat particles being, on average, much larger than chewed wheat particles. This particular result for wheat highlights the importance of achieving “as eaten” particle sizes following simulated chewing, especially when structurally robust foods are being analysed. Chewing involves a combination of both cutting and crushing forces, whereas chopping only involves cutting. Thus, despite the wheat being cut into quite small particles, it is likely many cells remain unruptured and hence resistant to digestion.

It was expected that exposure to salivary α -amylase in chewed samples would result in increased measures of glucose at T_0 compared with chopped samples. While there was a trend for this to occur, a significant effect in the case of chewing and immediate acidification was only observed in pasta (the significant effect observed in wheat having to be discredited due to the failure of chopping to accurately replicate chewing for this food type). Prolonged pre-gastric exposure to salivary amylase (5 min) was required before a significant increase in glucose was measurable at T_0 in white bread and potato. In none of the foods did pre-gastric exposure to salivary amylase of greater than 5 min duration result in higher levels of glucose at T_0 . It may be that the overall duration of exposure to saliva was the rate-limiting factor for glucose release and that further prolongation of exposure beyond 15 min would have resulted in higher levels of glucose being released in the foods studied. Equally likely, however, is the possibility that

enzyme specificity of salivary α -amylase to just $\alpha,1-4$ glycosidic bonds (165) limits further release of glucose from the foods regardless of whether exposure time is increased. This is perhaps demonstrated in the case of pasta and white bread, where increased exposure to saliva generated increased measures of partially-digested starch fragments yet the amounts of glucose being measured over this time period remained the same.

The concentration of ReS measured at T_0 of pancreatic digestion in chopped samples, free of amylolytic enzyme – salivary amylase or pancreatin, and when ESA treatment is omitted – can be said to be the amount of free sugar occurring in that food. It follows, then, that any increase in sugars in corresponding T_0 readings for chewed samples will have occurred as a result of starch being digested by salivary α -amylase during chewing. This increase in sugars (glucose) hence defines a portion of “extremely rapidly digestible starch” that is hydrolysed by salivary α -amylase during the brief chewing cycle and measurable as simple reducing sugars at T_0 . The partly digested fragments of starch, measured after a secondary ESA digestion could also be included in this definition of extremely rapidly digestible starch, since they too are released by salivary action. Certainly a portion of starch that is rapidly digested by saliva during chewing, and perhaps even in transit through the stomach, would contribute to the almost immediate glycemic response to food ingestion seen in fasted subjects (166, 167), since this portion is already in the liquid phase of digesta upon arrival in the duodenum. Salivary amylase may continue to digest starch whilst in the stomach, as long as it is protected from the acid environment by being encapsulated in a bolus of food. It is this possibility that lends some physiological relevance to our leaving chewed boluses to sit for up to 15 min before progressing to the acidic gastric phase of *in vitro* digestion.

The activity of salivary α -amylase during chewing contributes to up to 43 % of the total starch in foods being hydrolysed to simple sugars (glucose) and short chains of partly digested starch (dextrins). The degree of digestion that occurs depends on food type and duration of salivary exposure. Initiation of simulated intestinal digestion by adding pancreatin rapidly overwhelms any effect of saliva, such that the relative profiles of sugar released from foods during *in vitro* digestion, from which estimates of their glycemic potency are derived, do not differ significantly, regardless of the degree of

salivary pre-exposure. Therefore, in *in vitro* digestion methods where chewing is simulated, a salivary α -amylase enzyme step need not be included. The fraction of starch that is hydrolysed to glucose and dextrans by salivary action during the oral phase may be termed “extremely rapidly digestible starch” and will contribute to the fraction responsible for the rapid onset of glycemic response following ingestion of food.

4.1.4 Pepsin Inclusion and Omission

Hydrolysis curves for pasta, chickpea and potato samples and determined levels of RDS and SDS following *in vitro* digestion, with or without a gastric phase pepsin incubation, are shown in **Figures 4.13, 4.14, and 4.15** respectively. Graphs for bread and wheat are shown in **Appendix G1/2**.

This experiment should have been conducted with more than one sample per treatment so that the results could be more than merely observational. Nonetheless, the characteristic precision of *in vitro* digest measures means that the results of this study can be interpreted with some confidence.

A weak trend for increased starch digestion with inclusion and prolongation of a pepsin incubation step was observed in samples of pasta, chickpea and potato, as indicated by higher levels of ReS at 20 min. This trend was most pronounced in pasta samples where RDS content increased from 13 % with no pepsin incubation to 18 % following 60 min of pepsin exposure (**Figure 4.13B**). Digestion of starch from bread and wheat samples appeared to occur at a rate unrelated to whether a gastric digestion was included or not. Inconsistent with the hypothesis of this experiment – that starch digestion would be enhanced with prolonged exposure to pepsin, ReS was highest in wheat sample exposed to pepsin for 30 min compared with other treatments (**Appendix G2**). It is possible that contamination of sample by some means, or sampling error, could have lead to this higher than expected measure.

Inclusion of a pepsin incubation in *in vitro* digestion should improve starch digestion in the subsequent intestinal phase, since protein that might otherwise hinder amylase access to starch granules is removed (47, 168). The capacity for protein to retard starch

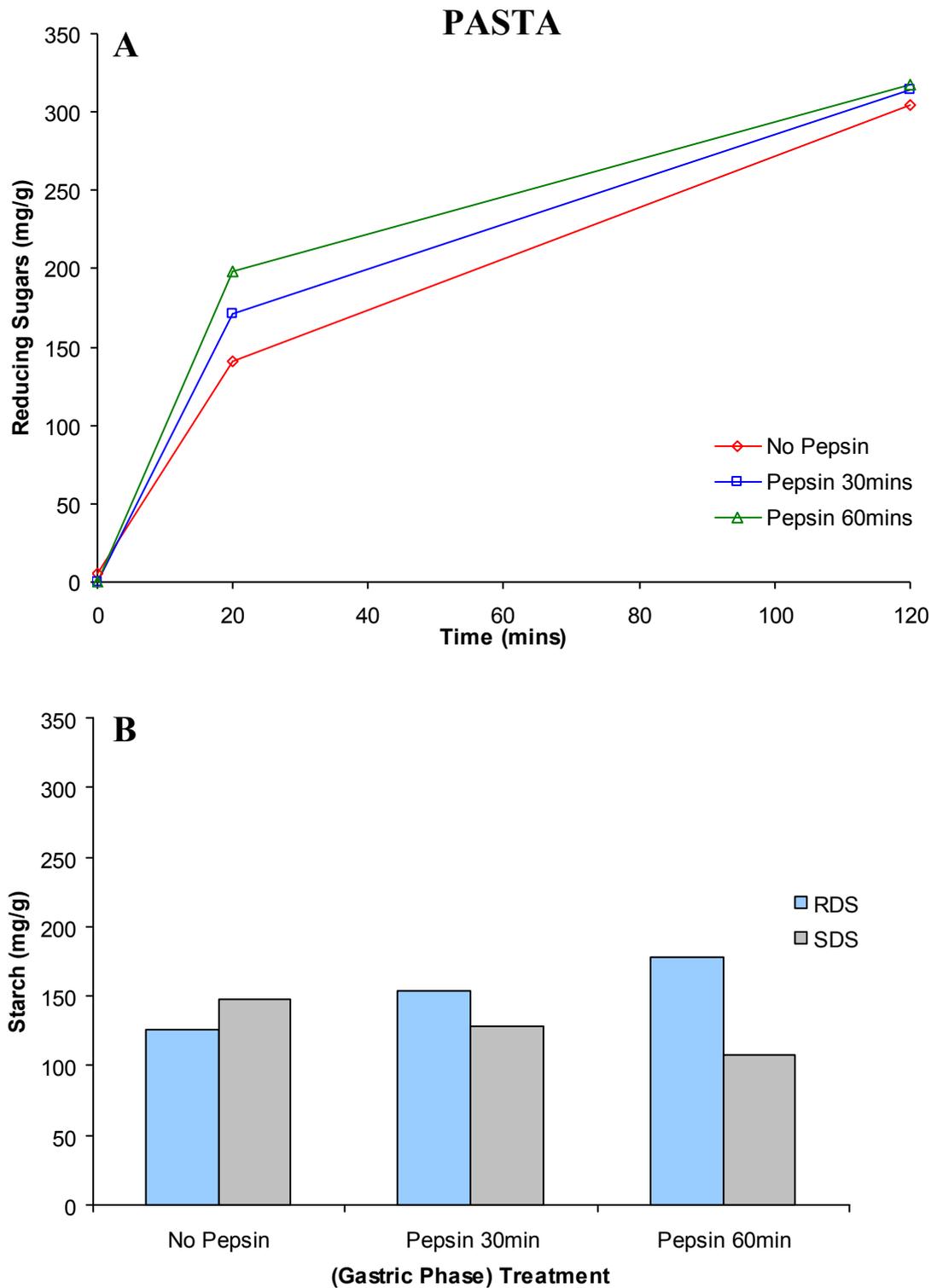


Figure 4.13 (80) Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in pasta samples digested *in vitro* following differing levels of exposure to pepsin during the gastric phase.

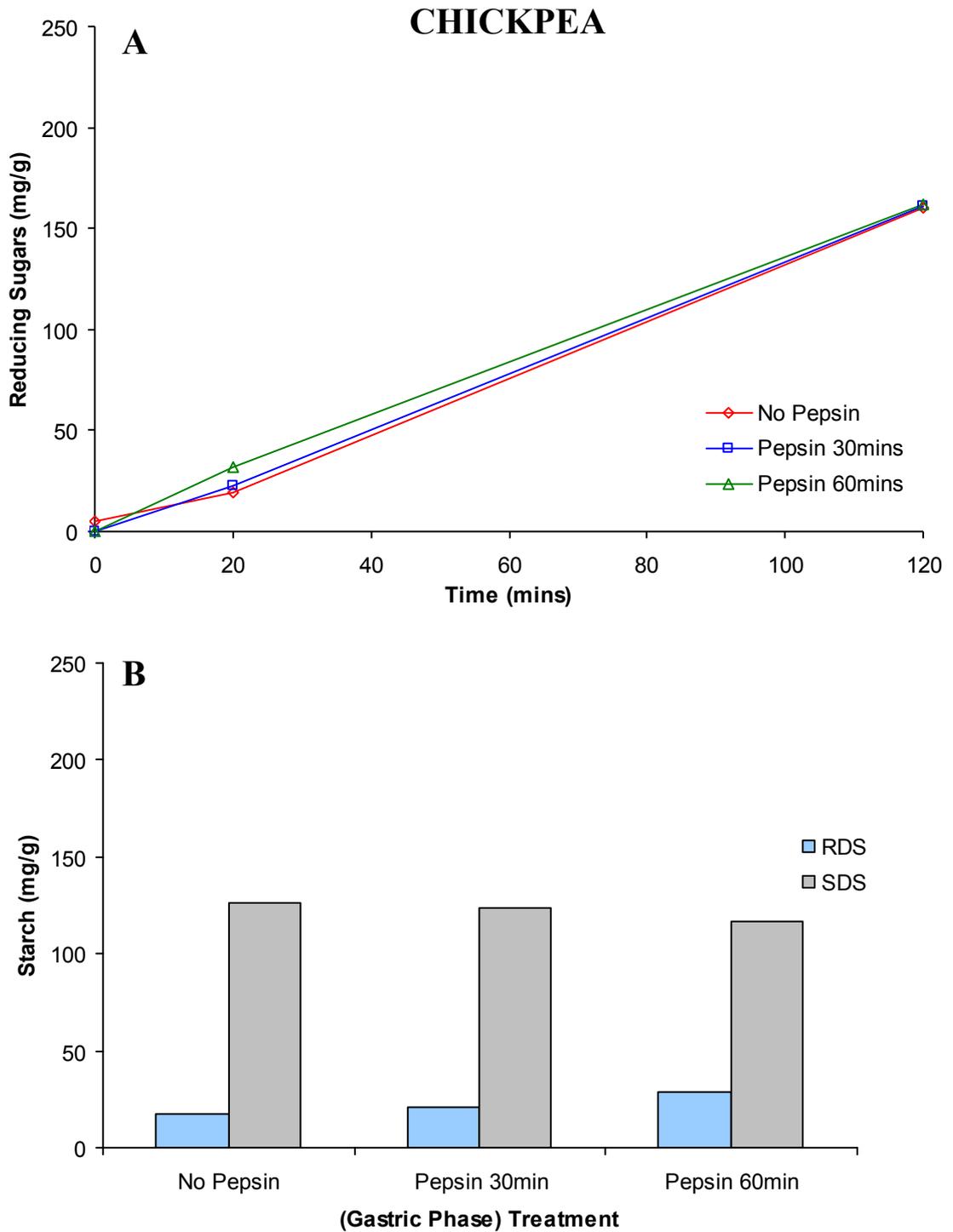


Figure 4.14 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in chickpea samples digested *in vitro* following differing levels of exposure to pepsin during the gastric phase.

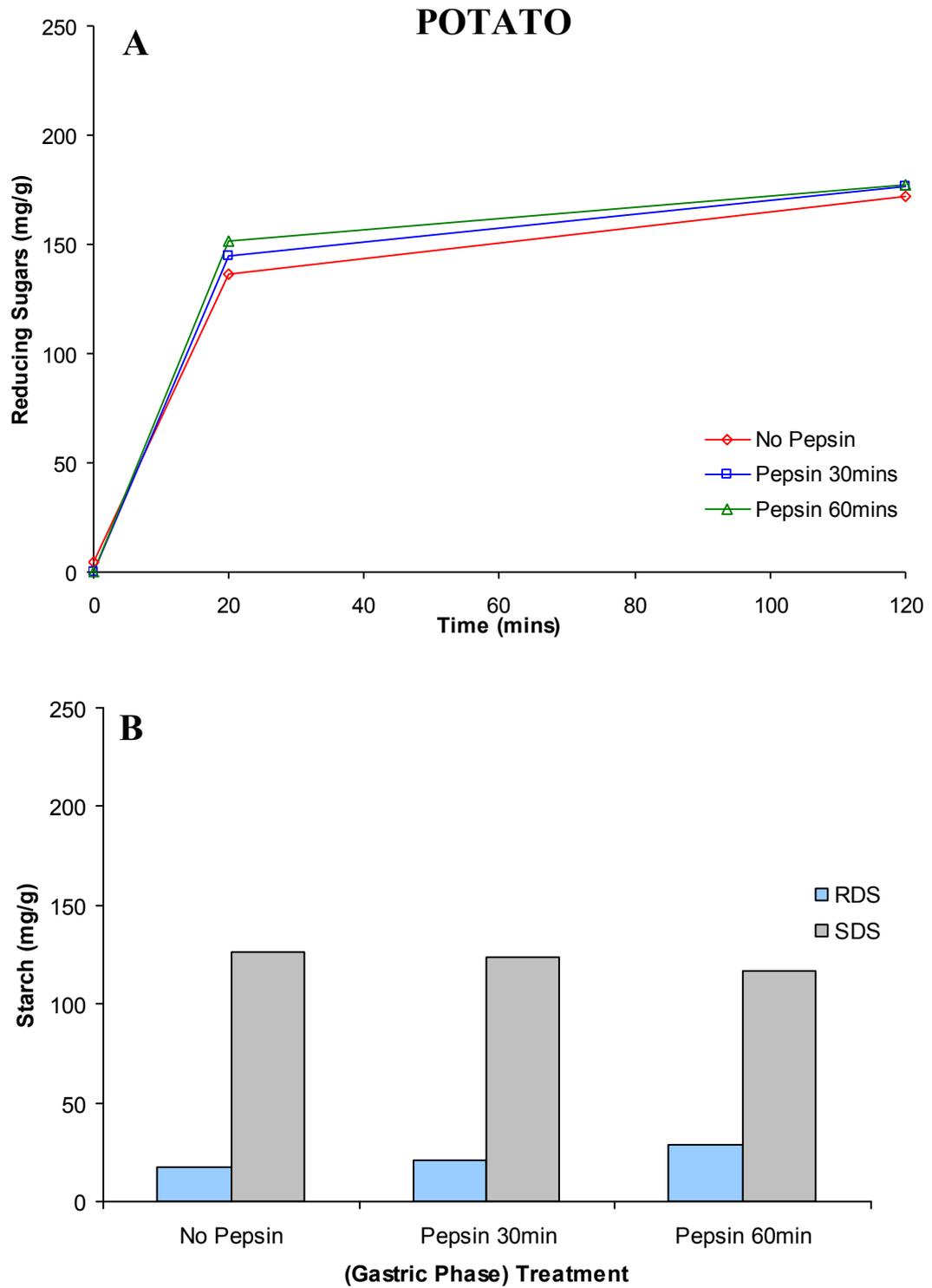


Figure 4.15 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in potato samples digested *in vitro* following differing levels of exposure to pepsin during the gastric phase.

digestion, however, is likely dependent on several food factors including botanical origin of the food, method and degree of processing as well as particle size of the substrate. White bread consists of 7.7 % protein (158), is made from highly refined flour and with baking, becomes highly porous. Following such substantial processing, it is likely that any surviving protein matrix offers little to no protection of highly exposed starch to amylase enzyme, which easily permeates the food (35). Thus omission of the pepsin incubation does not result in retardation of starch digestion rate. It is interesting, then, that no pepsin effect was seen in whole wheat grain samples either, – the same ingredient as used in white bread, but subject to minimal processing. Perhaps the prolonged cooking time (60 min) required of the wheat grains before *in vitro* digestion, denatures the protein content (11.4 %) such that, particularly following sieving, little encapsulation of starch granules remains (158, 169, 170).

Following extrusion, pasta structure is dense, restricting permeability of amylase enzyme and starch granules are embedded within a protein matrix comprising approximately 12 % of the weight of pasta (45, 158, 171). The increase in starch digestibility observed in the present study, with exposure of pasta particles to pepsin, likely results from an increase in the porosity (thus permeability) of the pasta particle as well as increased accessibility of starch granules to enzyme due to proteolysis of the protein matrix.

The pancreatin enzyme used during intestinal phase digestion (*EC 232.468.9 from porcine pancreas, Sigma-Aldrich P 7545; 8 x USP specifications*) contained residual protease enzyme. It is possible that this protease contributed to some protein digestion in all treatments, including those where a gastric digestion was omitted, thus masking the effect of pepsin inclusion and omission in the foods studied. This would help to explain why chickpea, which had the highest protein content of the foods studied, 19.7 % (158) appeared to digest only marginally faster when incubated with pepsin for 60 min. Use of a pure α -amylase enzyme solution (contains no proteases) during the intestinal phase of *in vitro* digestion, such as is used in other methods (92, 122), would have perhaps been more appropriate for this particular experiment.

The present experiment was conducted since there are some current *in vitro* methods that incorporate a pepsin step (93, 110) whereas others do not (92, 94, 101). The

subsequent effect, if any, this discrepancy in methodology might have on relative *in vitro* results has not been characterised. This study indicates that for most food structural types, inclusion or omission of a gastric phase pepsin step has little to no effect on subsequent *in vitro* starch digestion (at least when pancreatin enzyme is used, which contains residual proteases). There are some food types, however, for example pasta, for which a pepsin incubation is necessary to improve starch digestibility. For this reason, and to help align *in vitro* digestion with physiological conditions, it is recommended that a gastric phase pretreatment feature in the standardised *in vitro* digestion method.

4.1.5 Starch-Digesting Capacity of Pancreatin

Hydrolysis curves and digestible starch fractions measured in pregelatinised starch and bread samples following *in vitro* digestion using progressively decreasing concentrations of pancreatin enzyme are shown in **Figures 4.16** and **4.17** respectively. Graphs for the remaining four test foods are shown in **Appendix H1-4**.

As with the previous experiment, this *in vitro* digestion would have been better performed had there been more than one sample per treatment. Consequently the results presented are observational.

ReS release appeared to occur at a similar rate from PG starch samples digested with between 2 % and 0.1 % pancreatin enzyme final concentration. In samples where enzyme concentration used was less than 0.1 %, there was an apparent stepwise reduction in both the rate and extent of starch digestion, indicative of enzyme saturation. Addition of 200 μ L amyloglucosidase at T₁₂₀ (indicated by the arrow in **Figure 4.16A**) caused an immediate increase in ReS released from these samples and subsequent convergence of hydrolysis curves with those of PG starch digested using stronger concentrations of pancreatin. RDS levels were similar in PG starch samples digested with between 2 % and 0.1 % pancreatin, reducing thereafter in samples digested with weaker enzyme (**Figure 4.16B**).

The pancreatin enzyme concentrations: 1, 0.05 and 0.0025 % were used in the *in vitro* digestions of the five test foods since they exhibited varied capacity to digest PG starch

samples. In each of the five foods tested, starch digestion occurred most rapidly in the presence of 1 % pancreatin, reducing progressively with enzyme concentration. There was minimal starch digestion measured in chickpea samples incubated with 0.0025 % pancreatin (**Appendix H3**). Even white bread, perhaps the most easily digested of the foods studied, showed reduction in starch digestion (and corresponding RDS levels) in the presence of weaker pancreatin solutions (**Figure 4.17**).

Pregelatinised starch was used initially in this experiment since it is pure enzyme substrate with minimal features that might otherwise interfere with starch digestibility. Clearly, if a given enzyme concentration becomes rate-limiting to starch digestion using isolated starch as substrate, it will also be rate-limiting to digestion of more structurally complicated test foods that contain, in addition to carbohydrate, other macronutrients. It is interesting that 100 % recovery of starch as glucose was not achieved in any of the PG starch samples. This is not as a result of pancreatin enzyme specificity for α -1,4 glycosidic bonds since addition of AMG (that hydrolyses both α -1,4 and α -1,6 bonds) at T₁₂₀ did not elicit higher ReS in samples that had already plateaued (172). Perhaps a proportion of the PG starch (approximately 20 %) becomes resistant to digestion as a result of starch retrogradation following gelatinisation in manufacture, although this fraction seems quite high. In PG samples digested in an excess of pancreatin (≥ 0.1 %) there appears to be a fraction of starch digested more slowly, appearing as ReS between 10 and 60 min post-pancreatin addition. This portion of starch may digest more slowly due to maltose inhibition of α -amylase (162) as well as gradual dispersal within the digest medium of PG starch granules which tended to clump initially.

The results of this experiment are important since they demonstrate the effect of using too weak an enzyme solution on *in vitro*-derived indicators of a food's likely glycemic response, such as RDS. If insufficient concentration of amylolytic enzyme is utilised, there is rate-limitation of starch digestion and subsequent underestimation of glycemic response. Furthermore, low enzyme concentrations increase relative measurement variability if concentrations aren't replicated precisely between different digestion runs. *In vivo*, pancreatic enzyme secretions are concentrated (26). Although increasing the concentration of enzyme used in *in vitro* digestion adds to the total cost of the assay, as

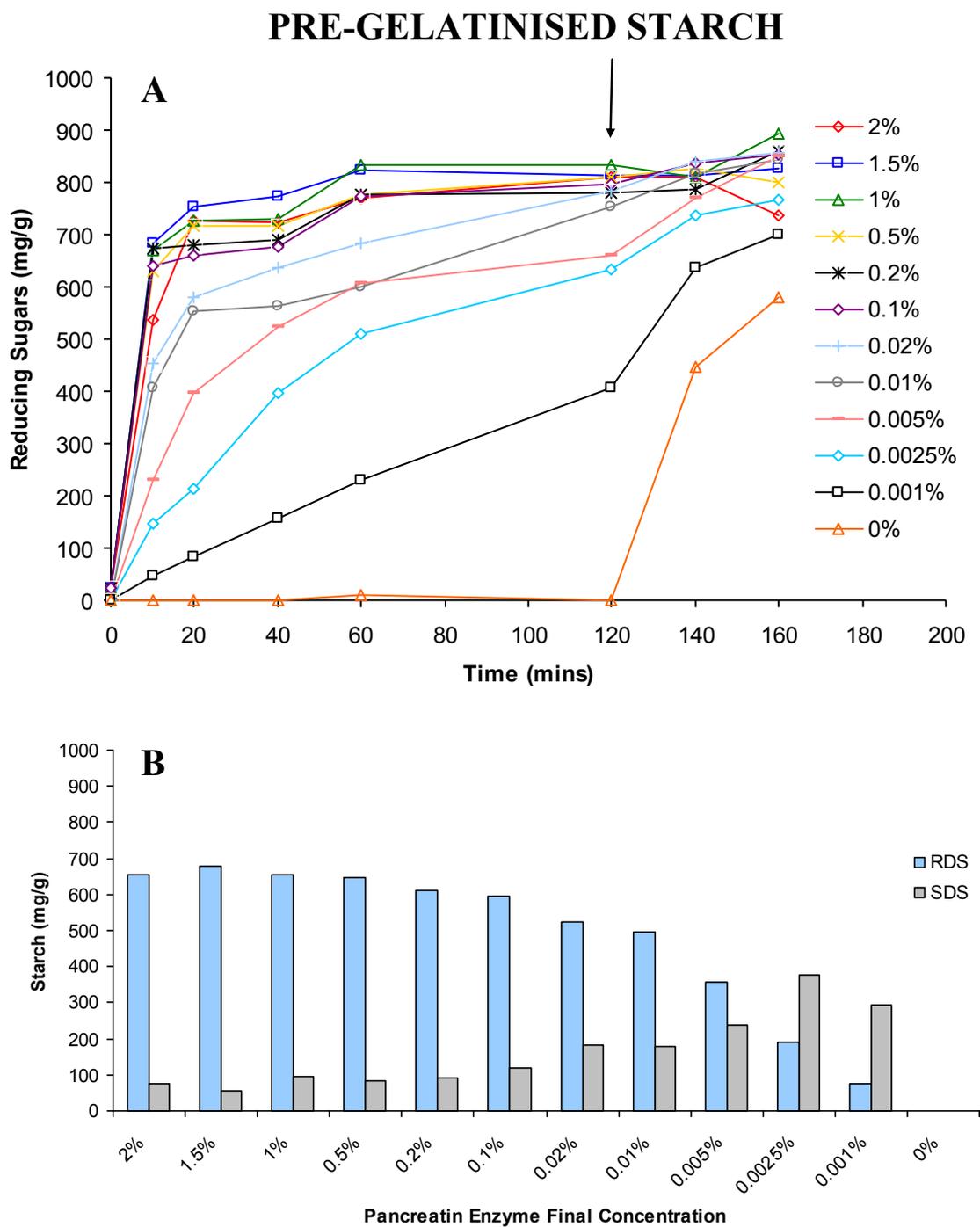


Figure 4.16 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in pregelatinised maize starch samples digested *in vitro* using progressively weaker final concentrations of pancreatin. The arrow denotes the point at which 200 μ L of amyloglucosidase enzyme was added to all samples.

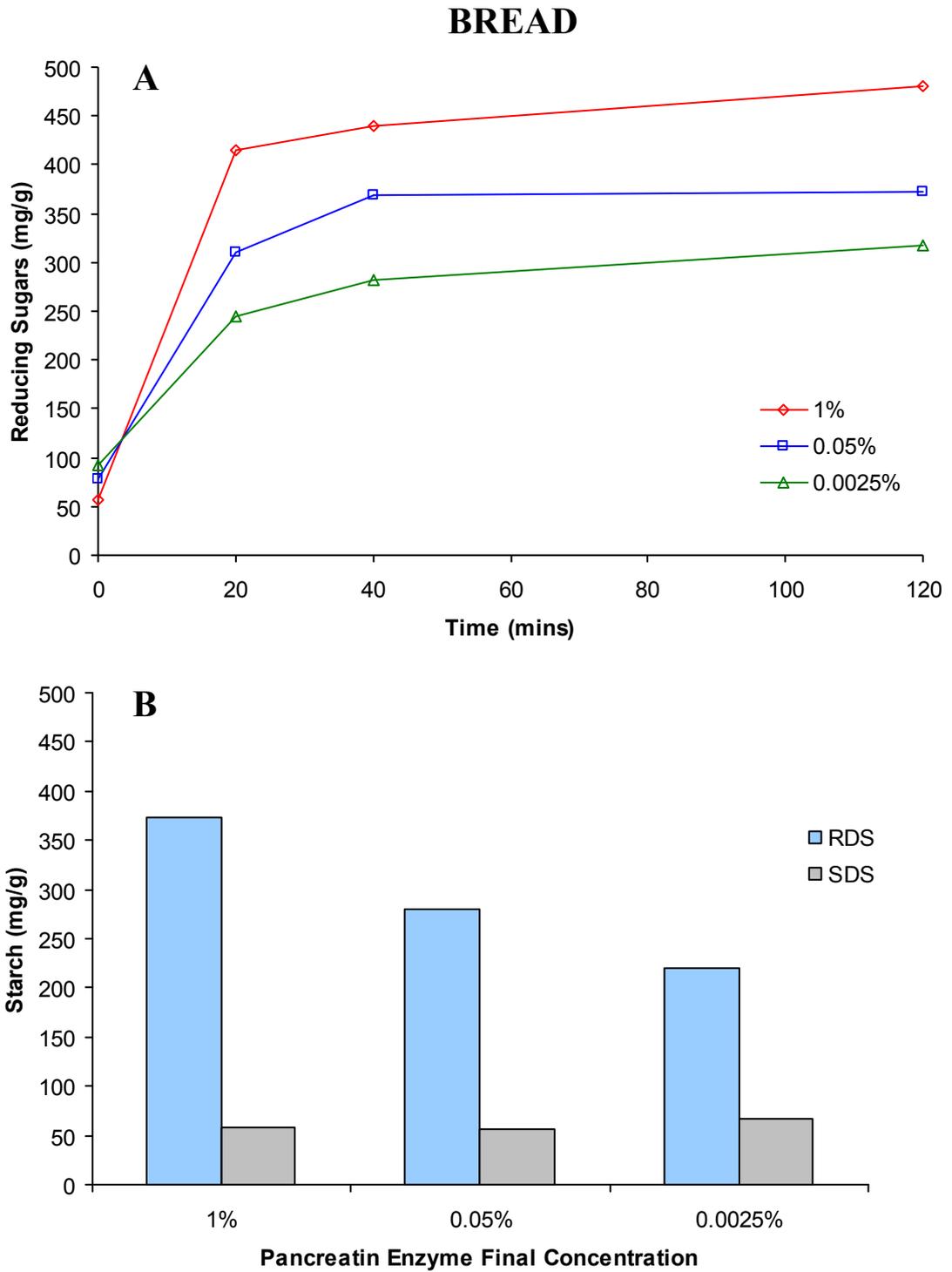


Figure 4.17 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in bread samples digested *in vitro* using differing final concentrations of pancreatic enzyme.

much as possible, *in vivo* conditions should be replicated. The present study shows, however, that pancreatin has sufficient efficacy for starch such that enzyme concentration can be substantially reduced (20-fold in this experiment) before rate-limitation of starch digestion occurs. McCleary and Monaghan report a similar finding when comparing the effect of pancreatin concentration albeit on *in vitro* estimates of RS (162). The present study indicates a pancreatin final concentration of between 0.2 and 0.5 % should be adequate to ensure robust and maximum rate starch digestion.

4.1.6 Pancreatin and pH

Hydrolysis curves and digestible starch fractions measured in PG starch samples digested with pancreatin in varied pH conditions (4.0 – 6.9) are shown in **Figure 4.18**. There was a pH treatment \times time effect ($p < 0.001$). *In vitro* digestion of the first 2.5 g of PG starch occurred at a similar rate irrespective of the digesta pH. ReS concentration did not differ between any of the treatments up to and including T₁₂₀. Upon addition of the second batch of 2.5 g PG starch to the digesta of each treatment (indicated by the arrow in **Figure 4.18**), a second starch hydrolysis curve, of similar amplitude to the first, was generated in samples incubated at pH between 5.0 and 6.9. At 130 min of pancreatic digestion, ReS was higher in samples incubated at pH 5.0 compared with those incubated at pH 6.9 ($p = 0.041$). No further starch digestion was measured in the pH 4.0 treatment when the second dose of PG starch was added, indicating pancreatin enzyme deactivation at some point between T₁₀ and T₁₂₀. Importantly, RDS and SDS fractions from the starch samples did not differ with incubatum pH.

The optimum pH for pancreatin enzyme activity is 7.0 which is also the pH in the human small intestine (26, 162). The present study shows, however, that pancreatin remains stable and capable of digesting starch at incubatum pH as low as 5.0, even beyond 120 min. This finding helps to validate previous *in vitro* methods (89, 90, 93, 94) where pancreatic digestion was conducted at pH \sim 5.0 to accommodate for simultaneous AMG activity - optimal at pH 4.5 and incorporated to replicate the action of intestinal brush-border enzymes that hydrolyse starch fragments to glucose (21, 162). McCleary and Monaghan (162) criticise these methods since they found that pancreatic

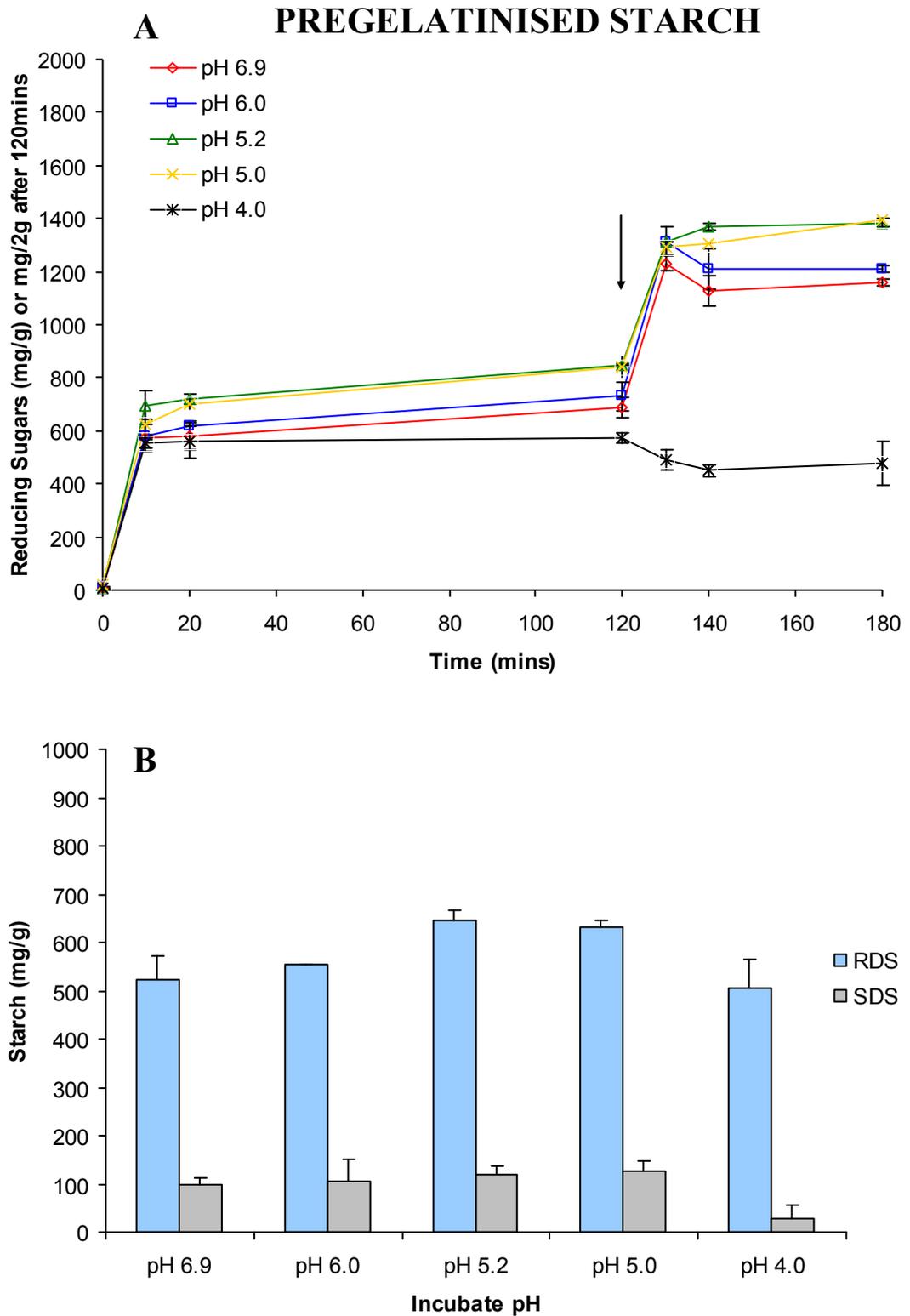


Figure 4.18 (80) Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in pregelatinised maize starch samples digested *in vitro* in incubatum of varied pH. The arrow denotes the point at which an additional 2.5 g of pregelatinised starch was added to the digest. Values are means (n = 2) ± SE.

α -amylase was unstable at pH 5.2, with more than 95 % inactivation within 1 hour - a finding that contradicts those of the present study. McCleary preincubated enzyme in buffers of varying pH, however, before subsequently diluting and testing enzyme activity on starch substrate, whereas the present study exposed enzyme to both substrate and incubation pH simultaneously – a more physiologically relevant means of testing enzyme stability. Furthermore, McCleary used pure α -amylase enzyme instead of crude pancreatin which is perhaps more susceptible to pH-induced deactivation.

In vitro methods for measuring starch digestibility are typically short duration (120 min). The present study indicates that pancreatin, even in incubation of pH as low as 5.0, remains active beyond this time frame and relative starch digestion kinetics are not affected. Thus in the standardised method, lowering of intestinal phase incubation pH below the optima for pancreatin (7.0), to accommodate for AMG activity, seems permissible.

4.1.7 Method of Stirring

Hydrolysis curves and digestible starch fractions for bread, potato and pasta samples following *in vitro* digestion using different stirring apparatus are shown in **Figures 4.19, 4.20 and 4.21** respectively. Hydrolysis curves for wheat and chickpea are shown in **Appendix I1/2**. A summary of statistical analysis outcomes for each food type is provided in **Appendix I3**.

Bread samples digested at a similar rate regardless of stirring method or stirring speed, and subsequently RDS and SDS levels did not differ between treatments (**Figure 4.19**). Similarly, wheat and chickpea samples digested at a rate independent of stirring method and speed except for ‘MS, fast’ samples which in both food types, digested more rapidly and completely than the other treatments. Consequently, RDS was higher in ‘MS, fast’ wheat samples compared with ‘EE, slow’ samples ($p = 0.011$) and RDS was higher in ‘MS, fast’ chickpea samples compared with ‘SWB, fast’ samples ($p = 0.037$).

In vitro starch digestion kinetics differed in samples of potato (**Figure 4.20A**) and pasta (**Figure 4.21A**) depending on the stirring method used. Generally, starch digestion was most rapid in these foods when using the magnetic stirrer, reducing thereafter with

shaking water bath treatment and end-over-end inversion. Pasta RDS was higher in 'MS, fast' sample compared with 'SWB, fast' and 'EE, slow' samples ($p \leq 0.018$) (**Figure 4.21B**). Differences in potato digestion curve profiles between treatments did not translate into differences in determined levels of RDS and SDS (**Figure 4.20B**). Doubling the speed of apparatus operation did not necessarily increase starch digestion rate in potato and pasta samples. There was a slight increase in potato RDS with fast operation of the SWB and EE inverter compared with slow operation (**Figure 4.20B**), similarly in 'MS, fast' and 'EE, fast' pasta samples compared with corresponding slow treatments (**Figure 4.21B**), although none of these increases were significant.

Bread's porous structure renders the food highly susceptible to digestion (35). Just as this factor has been attributed to bread's digesting at a rate unrelated to comminution method, it likely also forms the basis of the lack of effect seen presently, when comparing stirring methods and speeds. Following sieving pretreatment, the well-defined wheat particles and friable chickpea particles, suspended readily in digesta, showing no tendency for clumping. Consequently, all stirring methods achieved adequate dispersal of substrate within the pancreatin-containing digesta, regardless of speed setting, and *in vitro* starch digestion proceeded at a similar rate in all treatments except 'MS, fast.' Attrition of particles by the magnetic stir bar operating at its high speed setting (260 rpm), probably contributed to the increased starch digestion measured in these samples.

Samples of pasta and potato appeared to clump more readily in the digesta medium, compared with the other test foods. Potato samples, in particular, tended to form plugs at the bottom of digestion pots that did not dislodge with shaking water bath and inverter stirring. Perhaps the degree of substrate clumping that each respective stirring method permits to occur in digesta is the key determinant of subsequent *in vitro* starch digestibility. Clearly, with substrate clumping, starch digestion will be reduced due to decreased substrate exposure to enzyme. Clumping also introduces a source of variation, decreasing precision of digestibility results between different *in vitro* runs. In comparing stirring and shaking treatments in *in vitro* determinations of RS, McCleary and Monaghan recorded the greatest discrepancy between treatments when analysing substrates that plausibly would clump, such as starch powder, pasta and green banana as opposed to beans, where differences were smaller (162). Perhaps recognising the

potential for substrate clumping in shaking water bath incubation, several analysts place glass balls in the digestion pots to improve sample mixing (94, 99, 108).

The present study shows that the choice of stirring method affects the rate and extent of *in vitro* starch digestion in some food types, however the effect is not substantial. *In vitro* digestions of foods that tend to clump in solution seem more susceptible to influence by stirring method, although this hypothesis needs to be tested across a wider range of substrates. *In vivo*, small intestinal contractions ensure rapid and complete bolus breakdown and thorough mixing with pancreatic secretions (173, 174). This should be mimicked in the standardised *in vitro* digestion method, with robust stirring of samples. In this respect, magnetic stirring of samples is the most appropriate method of stirring since it consistently achieved the highest digestion in all foods studied. Also, the magnetic stirring bar, rotating at the bottom of the digestion pot, helps to prevent clumping and sedimentation of sample seen to occur in shaking and inversion methods. Magnetic stirring also holds several other important advantages over shaking water bath and inverter treatments. Samples that are stirred magnetically are easy to aliquot – a feature that makes simultaneous assaying of large numbers of samples, under time constraints, much easier. Shaking water baths and inverters, on the other hand, require sealed digestion pots that must be removed from the apparatus then opened for each aliquot, interrupting the *in vitro* digestion in the process. End-over-end inversion is particularly inconvenient since it requires a large incubator to house the inverter, perhaps explaining why this method of stirring is rarely used (95, 112).

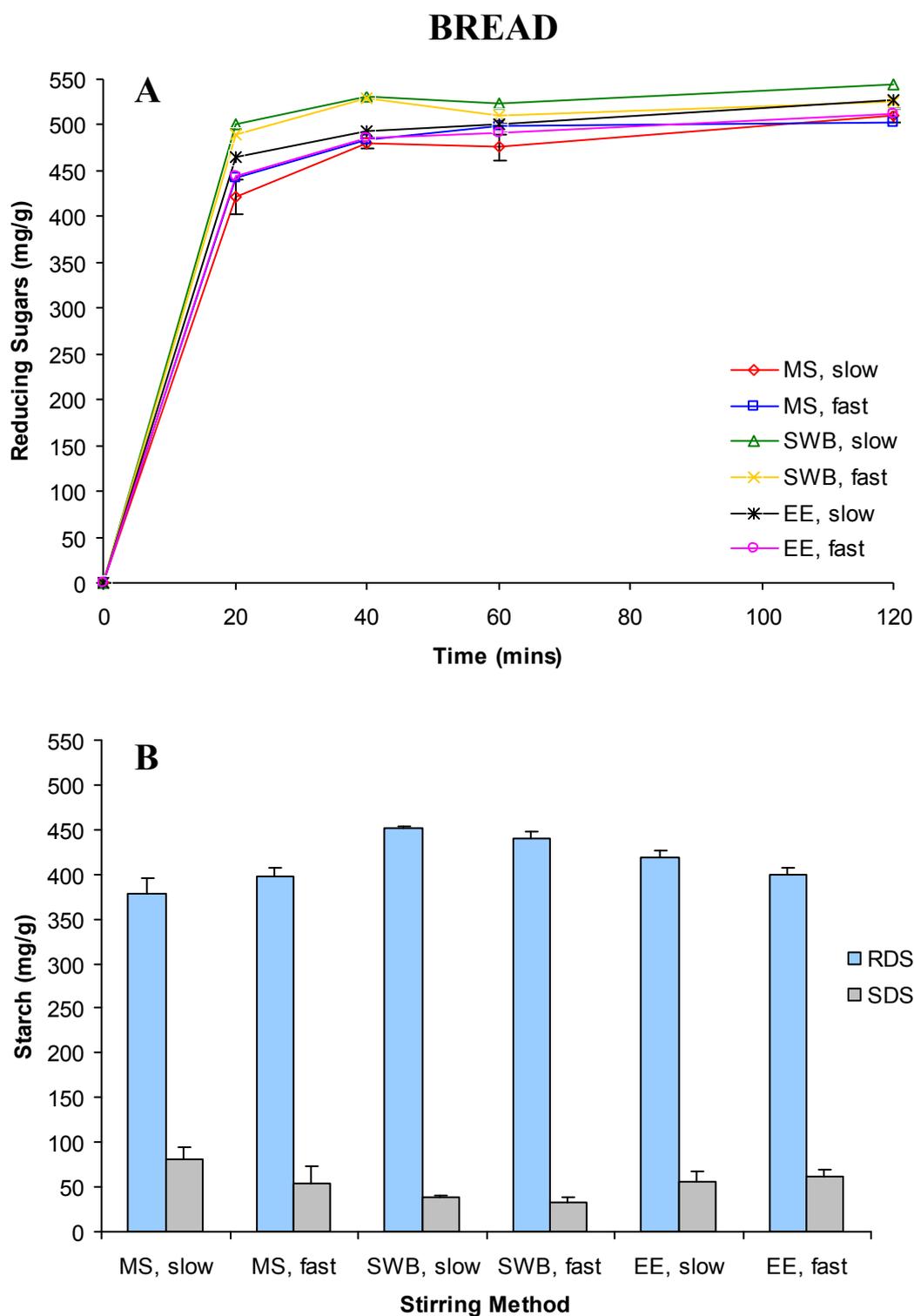


Figure 4.19 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in bread samples digested *in vitro* using different incubatum stirring/agitation apparatus, set at slow and fast speeds. MS, magnetic stirrer; SWB, shaking water bath; EE, end-over-end inversion. Values are means ($n = 3$) \pm SE.

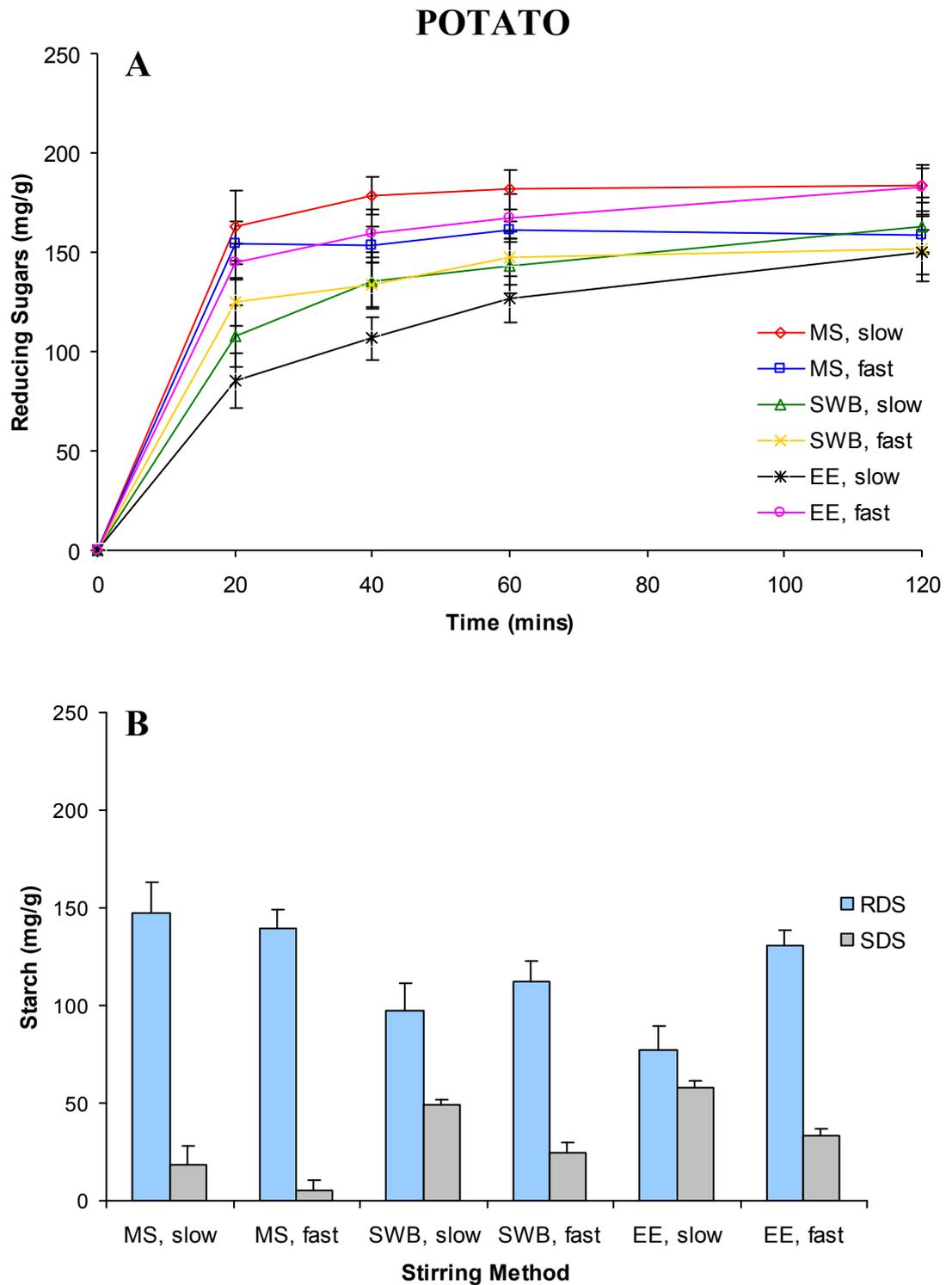


Figure 4.20 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in potato samples digested *in vitro* using different incubatum stirring/agitation apparatus, set at slow and fast speeds. MS, magnetic stirrer; SWB, shaking water bath; EE, end-over-end inversion. Values are means ($n = 3$) \pm SE.

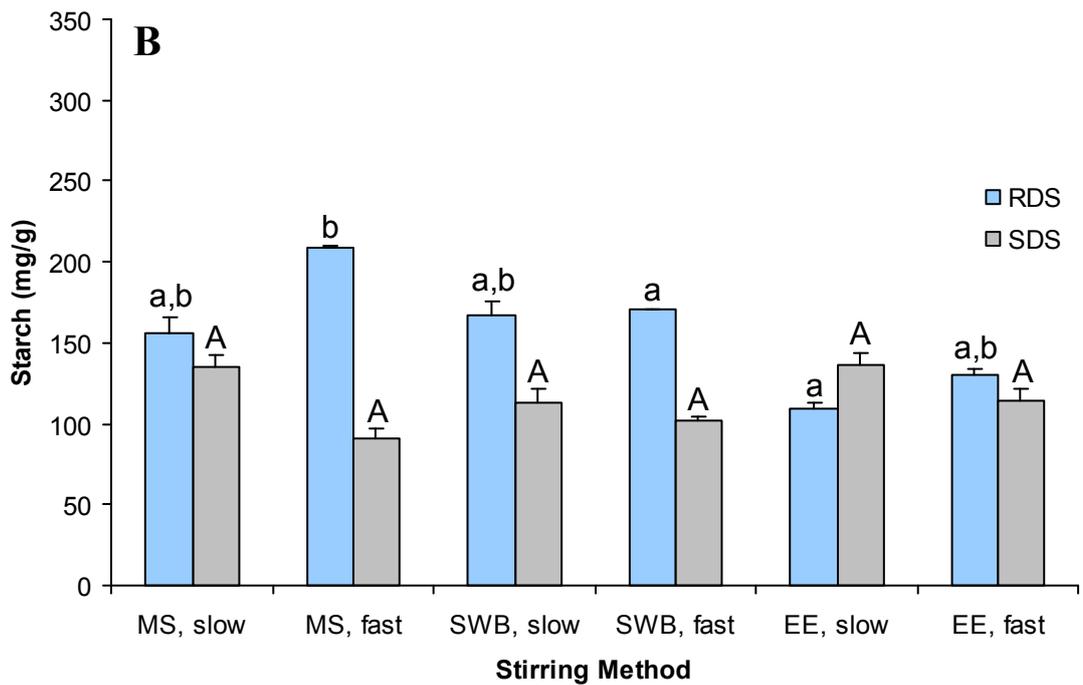
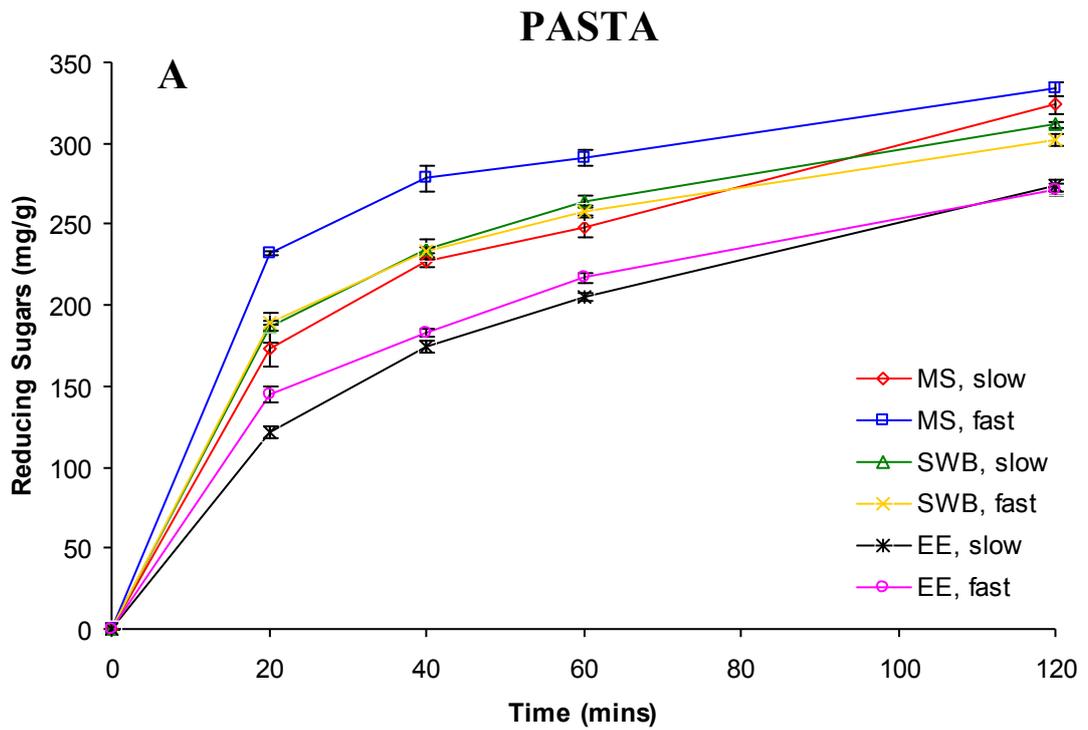


Figure 4.21 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in pasta samples digested *in vitro* using different incubatum stirring/agitation apparatus, set at slow and fast speeds. MS, magnetic stirrer; SWB, shaking water bath; EE, end-over-end inversion. Values are means ($n = 3$) \pm SE.

4.2 PHASE 2: HUMAN GLYCEMIC INDEX (GI) STUDY

4.2.1 Fat Dose and the Glycemic Response

The mean plasma glucose response curves to the three flatbreads and glucose reference drink are shown in **Figure 4.22**. A complete summary of the entire glucose data is provided in **Table 4.3**.

There was a significant flatbread diet \times time effect ($p < 0.001$). There was no difference in mean plasma glucose concentration at each timepoint for the three flatbreads across the entire duration of testing. Following consumption of the glucose reference drink, plasma glucose was higher at 15 min compared with the low ($p < 0.001$), medium ($p < 0.001$), and high fat flatbreads ($p < 0.001$), was higher at 30 min than the low ($p = 0.005$), medium ($p = 0.048$) and high fat flatbreads ($p = 0.002$), and remained higher than the high fat flatbread at 45 min ($p = 0.025$). Plasma glucose was lower after consumption of the reference drink at 120 min compared with the medium ($p = 0.032$) and high fat ($p = 0.038$) flatbreads and lower than the low ($p = 0.016$), medium ($p = 0.006$) and high fat ($p = 0.043$) flatbreads at 180 min postprandial.

There was no difference in the time taken for the glucose response curve for each flatbread as well as for the reference drink to reach its peak (T_{GRP}) (**Appendix J1**). The glucose response peaked higher after consumption of the reference drink compared to the low ($p = 0.001$), medium ($p = 0.02$) and high fat ($p = 0.002$) flatbreads which did not differ in their glucose peak amplitude (A_{GR}) (**Appendix J2**).

There was no difference in the mean IAUC (either up to 120 min or 180 min postprandial (**Appendix J3**)) of the glycemic responses to the three flatbreads. The $IAUC_{120}$ of the glycemic response to the reference drink was greater than that of the low fat ($p = 0.004$) and high fat flatbreads ($p = 0.023$) and the $IAUC_{180}$ of the reference drink was greater than that of the low fat flatbread ($p = 0.006$).

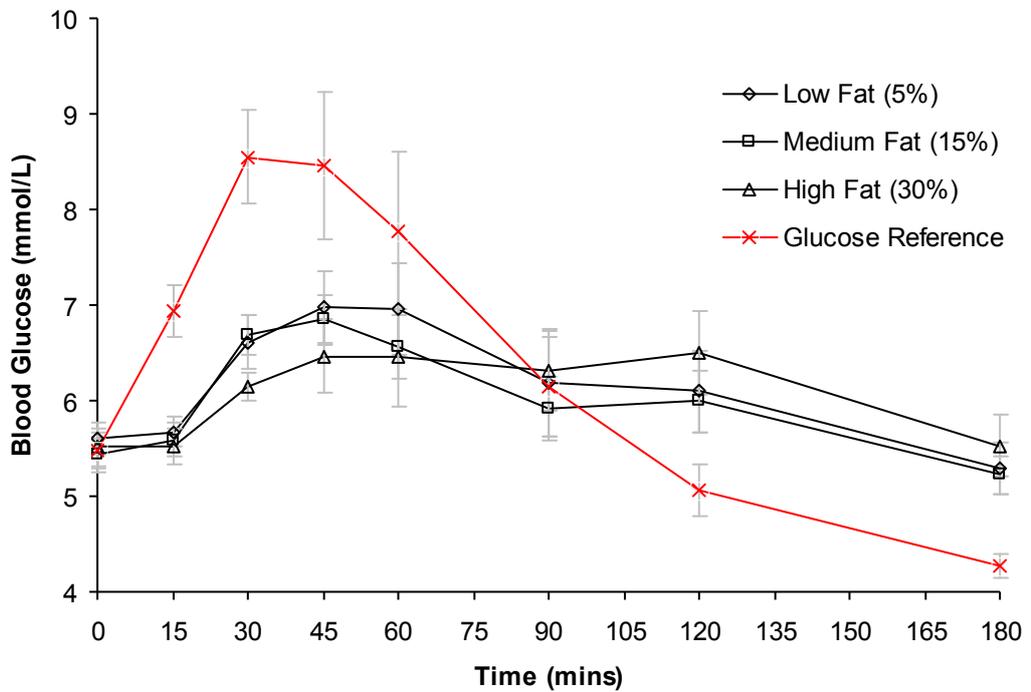


Figure 4.22 Blood plasma glucose levels measured over 180 min following consumption of one of three flatbreads (differing in fat content) and an equicarbohydrate glucose reference drink. Values are mean plasma glucose ($n = 10$) \pm SE.

Diet	Low Fat		Medium Fat		High Fat		Glucose Reference	
Available Carbohydrate (g)	50		50		50		50	
Fat (g)	3.44		10.33		20.66		-	
Fat % (dry weight basis)	5		15		30		-	
Glucose (mmol/L)	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Fasting	5.61 ^a	0.17	5.43 ^a	0.18	5.51 ^a	0.20	5.48 ^a	0.19
15 min	5.68 ^a	0.16	5.59 ^a	0.17	5.52 ^a	0.20	6.94 ^b	0.27
30 min	6.61 ^a	0.28	6.69 ^a	0.21	6.14 ^a	0.14	8.55 ^b	0.50
45 min	6.98 ^{a,b}	0.37	6.85 ^{a,b}	0.26	6.46 ^a	0.38	8.46 ^b	0.77
60 min	6.96 ^a	0.47	6.56 ^a	0.33	6.46 ^a	0.51	7.78 ^a	0.83
90 min	6.18 ^a	0.56	5.92 ^a	0.33	6.31 ^a	0.42	6.14 ^a	0.53
120 min	6.1 ^{a,b}	0.42	5.99 ^a	0.33	6.5 ^a	0.43	5.06 ^b	0.27
180 min	5.3 ^a	0.27	5.22 ^a	0.20	5.53 ^a	0.33	4.26 ^b	0.12
Time to Glucose Peak (min)	57 ^a	9.17	52.5 ^a	9.81	70.5 ^a	11.63	41.5 ^a	4.78
Amplitude of Glucose Peak (mmol/L)	2.04 ^a	0.27	1.87 ^a	0.25	1.71 ^a	0.27	3.65 ^b	0.49
iAUC up to 120 mins (mmol x min/L)	106.57 ^a	25.72	99.47 ^{a,b}	21.16	91.51 ^a	20.84	200.76 ^b	42.12
iAUC up to 180 mins (mmol x min/L)	129.75 ^a	32.57	121.98 ^{a,b}	26.89	127.58 ^{a,b}	33.16	207.75 ^b	42.89
GI	48 ^a	6.38	58 ^a	9.11	41 ^a	12.24	100 ^{NA}	-

Table 4.3 A summary of the entire mean glucose response data collected following consumption of the three flatbreads differing in fat content. Different letters denote a significant difference between treatments.

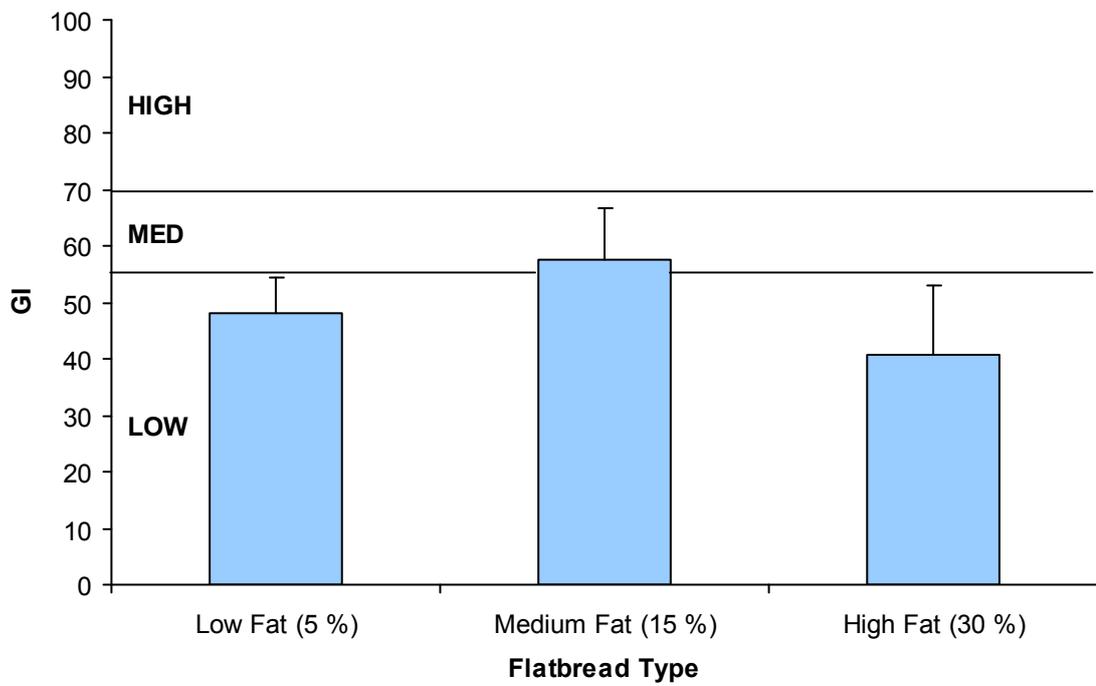


Figure 4.23 Glycemic index (GI) values for each of the three flatbreads differing in their fat content. Values are means of individual subject-determined GI values ($n = 10$) + SE.

The GI values for the low, medium and high fat flatbreads respectively were 48 (low GI), 58 (medium GI) and 41 (low GI) (**Figure 4.23**). Differences between GI values for each flatbread type were not significant.

It was expected that as the level of fat inclusion increased, the postprandial glycaemic response to the flatbreads would reduce proportionally, particularly in amplitude. As can be seen in **Figure 4.22**, however, the three mean glycaemic response (GR) curves to each respective flatbread type displayed only very weak features of fat-induced attenuation, of no statistical significance. For instance, the low fat flatbread GR peaked slightly higher than that of the medium and high fat flatbreads (**Appendix J2**), and the GR to the high fat flatbread peaked slightly later than the lower fat breads, at approximately 70 min postprandial (**Appendix J1**). Based on statistical outcomes, however, the conclusion of the present study is that fat does not attenuate the glycaemic response, a finding that disagrees with the current general consensus in the literature.

Published studies reporting a significant attenuation of GR by fat generally did so after testing on foods that were readily digestible and highly glycaemic (i.e. high GI) e.g. white bread (136) or mashed potato (147). In such cases, fat has increased “opportunity” to attenuate the glycaemic response since the GR in the absence of fat is high and there is considerable “room” on the GR curve, particularly in terms of its amplitude, for fat to exert its attenuating effect. Correspondingly, any effect of fat on GR will be more easily measured as well as differentiated statistically from the other treatments. In this respect and in the present study where the objective was to demonstrate a fat-induced attenuation in GR, and furthermore, quantify this effect, the flatbread as a food format for *in vivo* carbohydrate delivery was perhaps a less than ideal choice. Whereas the GR curve following consumption of the glucose reference drink was very typical of a highly glycaemic food (high amplitude, transient duration, rebound hypoglycemia), the GR curves of the three flatbreads are characteristic of slowly digested carbohydrate foods - the curves are low in amplitude, prolonged in their duration, and almost biphasic in nature. **Figure 4.23** shows that the GI of the lowest fat (5 %) flatbread, which theoretically, should have been the most glycaemic, was only 48 – a low GI classification. Thus the flatbreads had very little blood glucose concentration raising potential in the first place, even when they contained very little fat. Nonetheless,

this was not known at the outset of the study, and the flatbread format was selected as it met the other desired characteristics of the test diet, namely:

- minimal presence of other ingredients/macronutrients, allowing maximal interaction between starch and fat,
- easily and reproducibly prepared,
- serving size was still appropriate after achieving equivalence to 50 g available carbohydrate content,
- palatable.

It is possible that low starch gelatinisation levels as a result of cooking the flatbreads with little water, as well as the dense nature of the flatbread food matrix were the two key food factors contributing to the relatively low digestibility of the diets in the present study. Both of these food factors have been shown in other studies to reduce the *in vitro* starch digestibility and/or the relative postprandial glycaemic response (45, 175). Inherently, the flatbreads also featured a large proportion of crust shown previously to contribute to slower digestion characteristics (176).

Worth noting on the graph of glycaemic response curves for the glucose reference and the three flatbreads (**Figure 4.22**) is the proximity of each curve relative to the typical amplitude of their standard error bars. For example, the glycaemic response curves for the glucose reference (GI 100) and the high fat flatbread (GI 41) are, at best, only separated by approximately 2.5 mmol/L units of blood glucose (at 30 min postprandial), and yet, some standard error bars are as high as 1 mmol/L. In other words, the glycaemic response data contain considerable noise and with such close proximity of even the control glycaemic response curve to those of the test foods, a significant stepwise attenuation in blood glucose with multiple, increasing levels of fat was always going to be difficult to measure. The typically high variability of physiological measures even when performed under highly standardised conditions such as in glycaemic index testing is a common complaint when using *in vivo* systems (73, 177, 178). Note also that the final GI classifications assigned to each flatbread type following *in vivo* testing, don't actually differ significantly from one another despite the low and high fat flatbreads being classified low GI and the medium fat flatbread falling into the medium GI category. There seems to be no stipulation or requirement stated in the Australian

Standard for glycemic index testing of foods that GI values deduced for a particular type of food differ significantly from one another before they become valid and used to rank foods based on their relative glycemic potency (62).

4.2.2 Fat Dose and Gastric Emptying Rate

Curves for the cumulative percentage of the ^{13}C dose recovered (CPDR) over the postprandial testing period for the three flatbreads are shown in **Figure 4.24**. A summary of all gastric emptying rate data is provided in **Table 4.4**.

There was a significant flatbread diet \times time effect ($p < 0.001$). CPDR recovery was lower for the high fat flatbread compared to the low and medium fat breads at 30 min ($p = 0.01$; $p = 0.004$ respectively), 45 min ($p = 0.014$; $p < 0.001$ respectively), 60 min ($p = 0.008$; $p = 0.001$ respectively), 90 min ($p = 0.009$; $p = 0.008$ respectively) and 120 min postprandial ($p = 0.02$; $p = 0.05$ respectively) and lower than the low fat bread at 180 min postprandial ($p = 0.043$).

The gastric half-emptying time ($T_{1/2\text{GE}}$) was longer for the high fat flatbread compared to the low fat ($p = 0.024$) bread. There was no difference in $T_{1/2\text{GE}}$ between the low and medium fat breads, or between the medium and high fat breads (**Figure 4.25**). It took longer to reach T_{max} after consuming the high fat bread compared with the low and medium fat breads ($p = 0.02$) which did not differ (**Appendix J4**). There was no difference in lag time (T_{lag}) following consumption of the low and medium fat breads. T_{lag} increased after consumption of the high fat bread compared with the low ($p = 0.011$) and medium fat ($p = 0.005$) flatbreads (**Appendix J5**).

Thus, while no measurable relationship between fat content and glycemic response was observed, a significant retardation of gastric emptying rate occurred with increasing levels of fat. Whereas the gastric half-emptying time for the low fat flatbread was 86 min, half-emptying time for the medium flatbread was 109 min increasing to 128 min for the high fat flatbread (**Figure 4.25**). The mean gastric lag time (the estimated time at which emptying of the flatbread meal from the stomach into the duodenum begins) increased from 47 min (low fat) to 60 min with the high fat flatbread (**Appendix J5**). Percentage recovery of the original ^{13}C dose administered progressively decreased

across the postprandial breath testing period with increased fat dose, indicating a slower overall gastric transit time of the higher fat content flatbreads (**Figure 4.24**).

Comparing the glycemic response curves for the three flatbreads with the gastric emptying rate data, it is apparent that there is discrepancy between the timing of emptying of the flatbread meal from the stomach and the timing of onset of the glycemic response. **Figure 4.24** shows that appreciable traces of ^{13}C octanoic acid in the breath, indicating presence of the flatbread meal in the small intestine, did not begin to be measured until approximately 30 – 40 min postprandial. Yet the onset of the glycemic response for each flatbread type was measurable within 15 to 30 min postprandial, and by 45 min – the supposed approximate time at which emptying of the low fat flatbread from the stomach should have only just begun (T_{lag}), the three GR curves were at their peak.¹⁵ Since the carbohydrate component of the flatbreads elicit the glycemic response, and the ^{13}C -octanoic acid marker for measuring gastric emptying rate is solubilised into the fat component of each flatbread, it follows that discrepancy between the timing of glycemic response onset and gastric emptying rate indicates separation of the carbohydrate and fat components of the flatbreads whilst in the stomach. The early onset of the glycemic response for each flatbread type relative to their predicted gastric emptying rate points to the passage first of the carbohydrate portion of each flatbread from the stomach into the small intestine, followed by the fat portion. **Figure 4.26** illustrates the propensity with which fat leached out of even the low fat flatbread matrix when incubated under simulated gastric conditions, helping to confirm the likelihood that fat-starch separation occurred whilst in transit in the stomach.

The subjects remained in a seated upright position throughout the postprandial testing period. Fat, being the least dense ingredient in the flatbreads and evidently poorly emulsified with the carbohydrate, likely floated in the proximal stomach whilst the heavier carbohydrate was emptied into and subsequently digested in the small intestine. Posture has been shown in other studies to influence the distribution of oil within the stomach (179). It has also been shown previously that the effectiveness of fat in decreasing the gastric emptying and subsequent digestion rate of carbohydrate depends

¹⁵ The GR curve for the high fat flatbread actually exhibited a second, slightly higher peak at 120 min postprandial.

on the sequential timing of fat and carbohydrate administration, with fat exerting a greater effect when ingested prior to the carbohydrate meal (138, 147). This is because the slowing of gastric emptying by fat is dependent on the action of gut hormones (including GLP-1) which are only released upon arrival of digested fatty acids within the small intestine, with the entire feedback process taking 30 to 40 mins to achieve (147). Hence, in the present study where fat was emptied into the small intestine after the carbohydrate, it follows that the reduction in gastric emptying rate induced by fat will have a lesser effect on the digestion rate of carbohydrate and subsequent glycemic response.

The findings of other published studies indicate that a stable emulsion between starch and fat is not a prerequisite for fat-induced attenuation in glycemic response. Gentilcore *et al* (147) as well as Jeya *et al* (136) demonstrated significant reductions in glycemic response when testing oil added to mashed potato and margarine/oil added to white bread respectively. It is reasonable to expect that separation of fat and starch would have occurred in the stomach for these food types in the same manner as the flatbreads used in the present study. Perhaps the fundamental difference between the diets used in published studies where a fat effect was observed and the flatbreads used in this study where a fat effect was not observed, lies in the relative digestibility and glycemic potency of the test food. Mashed potato and white bread are rapidly digested once in the small intestine and accordingly are classified as high GI foods (152). Hence, fat-induced reduction in gastric emptying rate likely becomes the rate-limiting step in the generation of a glycemic response and subsequently a relationship between fat content and glycemic response is measurable. The flatbreads used in this study, on the other hand, were inherently poorly digestible. Carbohydrate digestion in the small intestine was likely the rate-limiting factor in generation of the glycemic response. Any alteration to gastric emptying rate through varying the fat dose in each flatbread would therefore have little to no effect on their subsequent glycemic response.

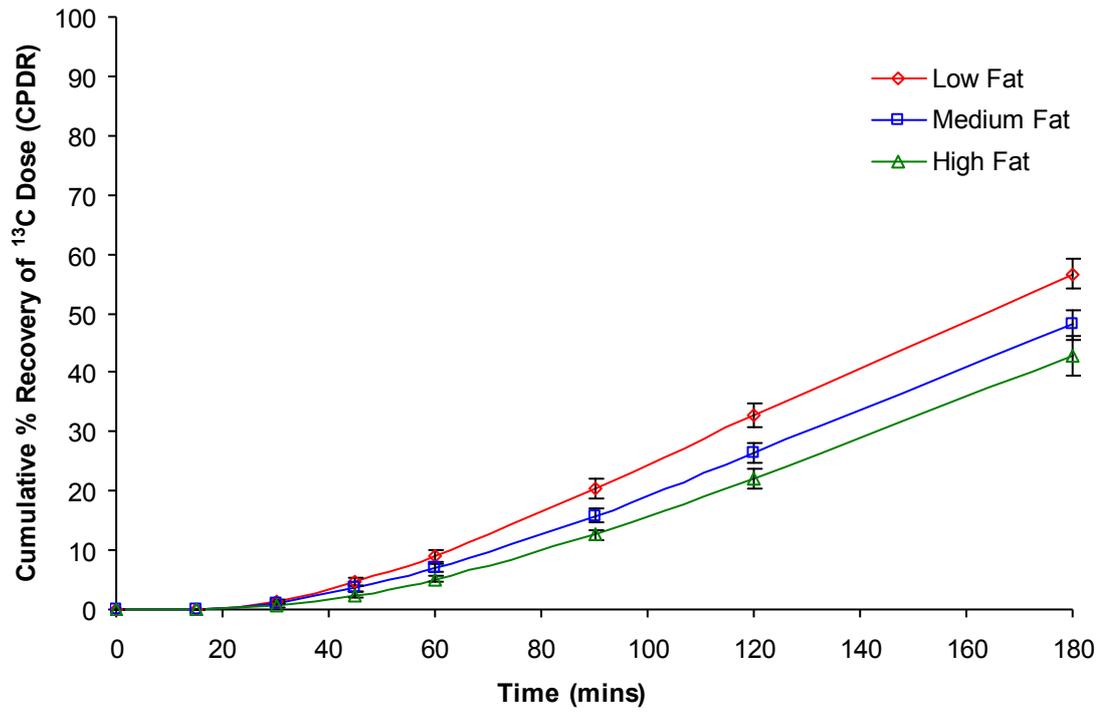


Figure 4.24 Cumulative percentage of the original administered ¹³C dose recovered (CPDR) via the breath over the 180 min following consumption of each of the three flatbreads. Values are means (n = 10) ± SE.

Diet	Low Fat		Medium Fat		High Fat	
	Mean	SE	Mean	SE	Mean	SE
% of ¹³ C Dose Recovered						
Fasting	0 ^a	0	0 ^a	0	0 ^a	0
15 min	0.09 ^a	0.06	0.11 ^a	0.02	0.01 ^a	0.05
30 min	1.44 ^a	0.23	1.15 ^a	0.18	0.64 ^b	0.21
45 min	4.60 ^a	0.60	3.61 ^a	0.45	2.50 ^b	0.41
60 min	9.05 ^a	0.98	6.99 ^a	0.75	5.12 ^b	0.58
90 min	20.34 ^a	1.66	15.84 ^a	1.20	12.62 ^b	0.90
120 min	32.74 ^a	2.12	26.31 ^a	1.63	22.07 ^b	1.72
180 min	56.55 ^a	2.48	48.09 ^{a,b}	2.56	42.70 ^b	3.30
Half-Emptying Time (min)	85.92 ^a	6.27	108.99 ^a	7.54	127.50 ^b	9.34
tmax (min)	112.52 ^a	5.12	127.28 ^a	5.22	142.67 ^b	5.91
tlag (min)	47.42 ^a	2.77	52.74 ^a	2.81	59.76 ^b	2.56

Table 4.4 A summary of the entire mean gastric emptying rate data collected via breath testing following consumption of the three flatbreads that differed in their fat content. Different letters denote a significant difference between treatments.

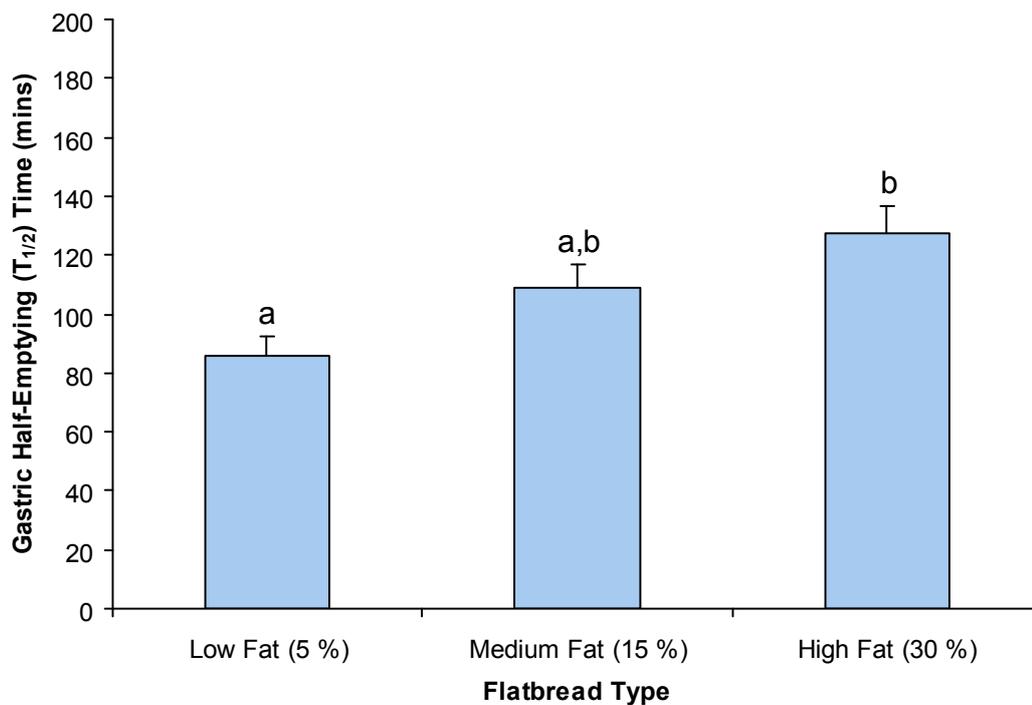


Figure 4.25 Gastric half-emptying ($T_{1/2}$ GE) time for the three flatbreads. Different letters denote a significant difference between treatments. Values are means ($n = 10$) + SE.

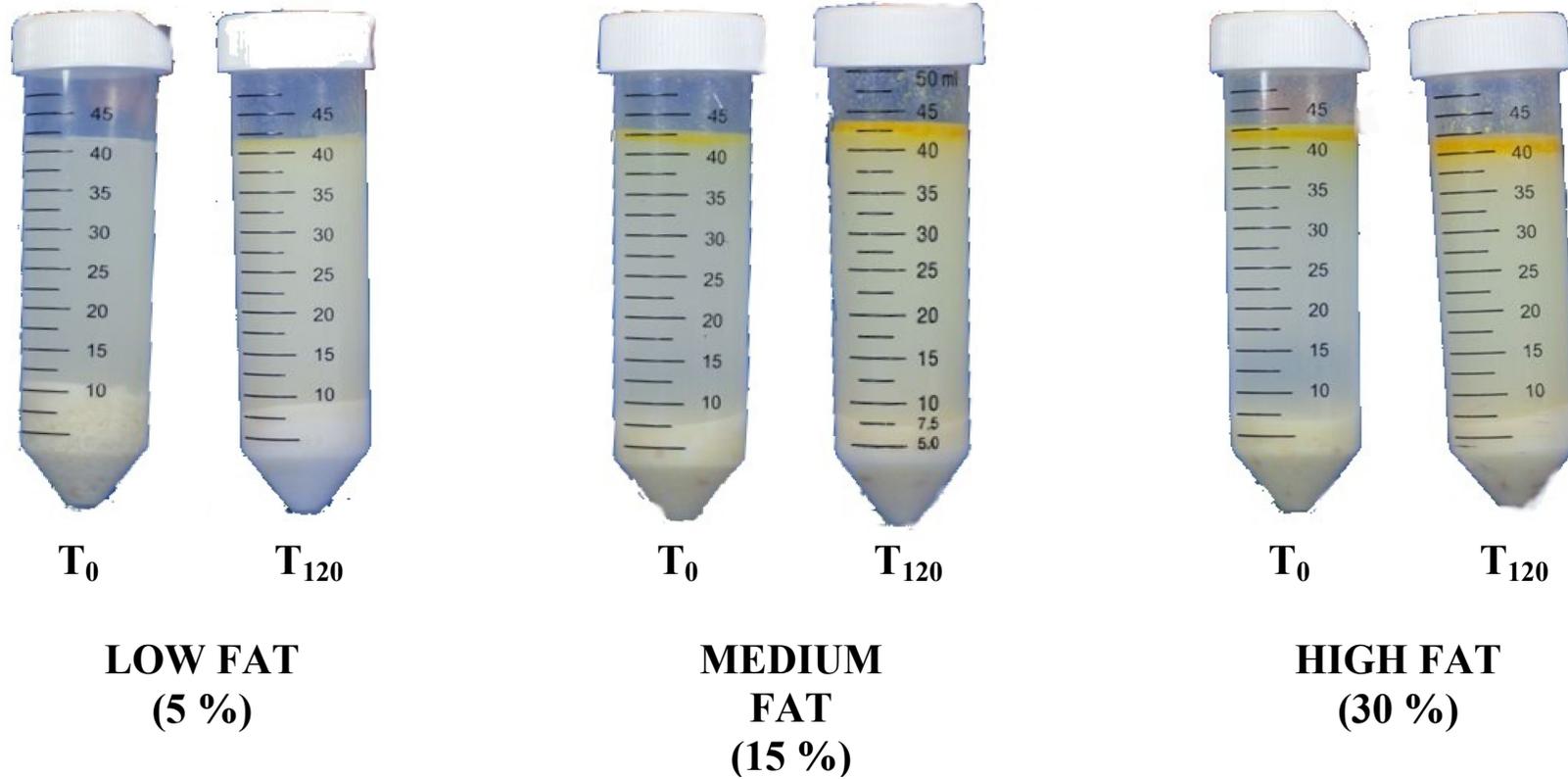


Figure 4.26 Demonstration of the fat (stained yellow) separation from the remainder of the flatbread food matrix (white sediment) that occurred under simulated gastric conditions.

4.2.3 In Vitro Digestion Analysis of Flatbreads

Hydrolysis curves and levels of the digestible starch fractions RDS and SDS for each flatbread type following *in vitro* digestion are shown in **Figure 4.27**. There was no flatbread diet × time effect in the *in vitro* analysis of the flatbreads, nor was there a diet effect. Relative ReS concentration between the three flatbreads did not differ at any timepoint throughout the duration of *in vitro* digestion. RDS and SDS levels were not affected by fat content.

If any attenuation in the *in vitro* digestibility of the flatbreads had been observed with increasing fat content, it was believed that the major mechanism underlying such attenuation was decreased accessibility of starch granules to amylase enzymes as a result of encapsulation within fat. Due to the ease with which fat was found to leach out of the flatbread matrix, however, (illustrated in **Figure 4.26**) it is not surprising that no relationship was observed between fat content and *in vitro* starch digestion rate.

The objective of the present study was to quantify the effect of fat on the glycemic response *in vivo* - exerted via variation in gastric emptying rate, and to apply this effect as a correction factor to corresponding *in vitro* data to improve its subsequent predictive capability. Unfortunately, the anticipated *in vivo* fat attenuation effect, for the reasons described previously, was not observed in this study. Furthermore, the apparent separation of the ¹³C-labelled fat and starch components of each flatbread test food, while in transit in the stomach, makes redundant the measure of the rate of gastric emptying, since this measure is no longer coupled with the timing of the passage of carbohydrate from the stomach into the small intestine and glycemic response onset. Direct ¹³C-labelling of the carbohydrate component of the meal e.g. through use of ¹³C-enriched durum wheat, as opposed to labelling of a separate macronutrient (fat), also present in the meal but which subsequently separated from the carbohydrate, would have been a better approach in this regard. The *in vivo* study failed to provide the data necessary for deduction of a correction factor for the effect of fat on glycemic response and subsequently, no adjustments to the corresponding *in vitro* hydrolysis curves can be made.

The GI study classified the flatbreads either as low (low fat, high fat flatbreads) or medium GI (medium fat flatbread), i.e. slowly digested and contributing to a relatively low glycemic response. The *in vitro* digestion curves for each flatbread, on the other hand, imply rapid and easy digestion of starch, with high levels of RDS measured (mean = 332 mg/g) and only small amounts of SDS (mean = 71 mg/g). In fact, *in vitro* digestion curves for the flatbreads are not dissimilar to those of white bread (see **Figure 4.4**) - a high GI food (152). Thus while the flatbreads, regardless of their fat content, digested slowly *in vivo*, they digested rapidly *in vitro*, the *in vitro* digestion method used giving an apparent over prediction of their glycemic potency compared with the *in vivo* response actually measured.

SEM images presented in **Figure 4.28** illustrate the rapid rate at which starch from the flatbreads was digested *in vitro*. At the post-gastric digestion phase, starch granules (S), though damaged from cooking, are abundant and remain relatively intact. By 30 min of pancreatic exposure, however, virtually all starch granules are hydrolysed, irrespective of flatbread fat content, and only fragments of bare cell wall material (CW) remain.

There are several possible factors that could contribute to the apparent *in vitro* over prediction of flatbread starch digestibility observed in this study. Firstly is the delay in glycemic response onset that occurs *in vivo* as a result of gastric transit time and gastric emptying (an adjustment for which this study was attempting to achieve). Monro *et al* (110) found that the glycemic response to a range of foods typically began after a 10 min delay postprandial, and that if corresponding *in vitro* digestion curves were similarly adjusted by 10 min, better alignment between *in vivo* measures and *in vitro* prediction was achieved. A similar adjustment seems to apply in the present study also. Approximately 80 – 90 % of all starch hydrolysis from the flatbreads occurred within the first 20 min of *in vitro* digestion (**Figure 4.27**). *In vivo*, maximum glycemic loading from each flatbread (the steepest ascending section of each GR curve) occurred between 15 and 45 min postprandial (**Figure 4.22**). Thus, if the flatbread *in vitro* digestion curves were shunted 15 – 20 min along the x (time) axis, the ascending phases of each *in vivo* (glucose absorption) and *in vitro* (glucose release) curve for the flatbreads would coincide in their timing.

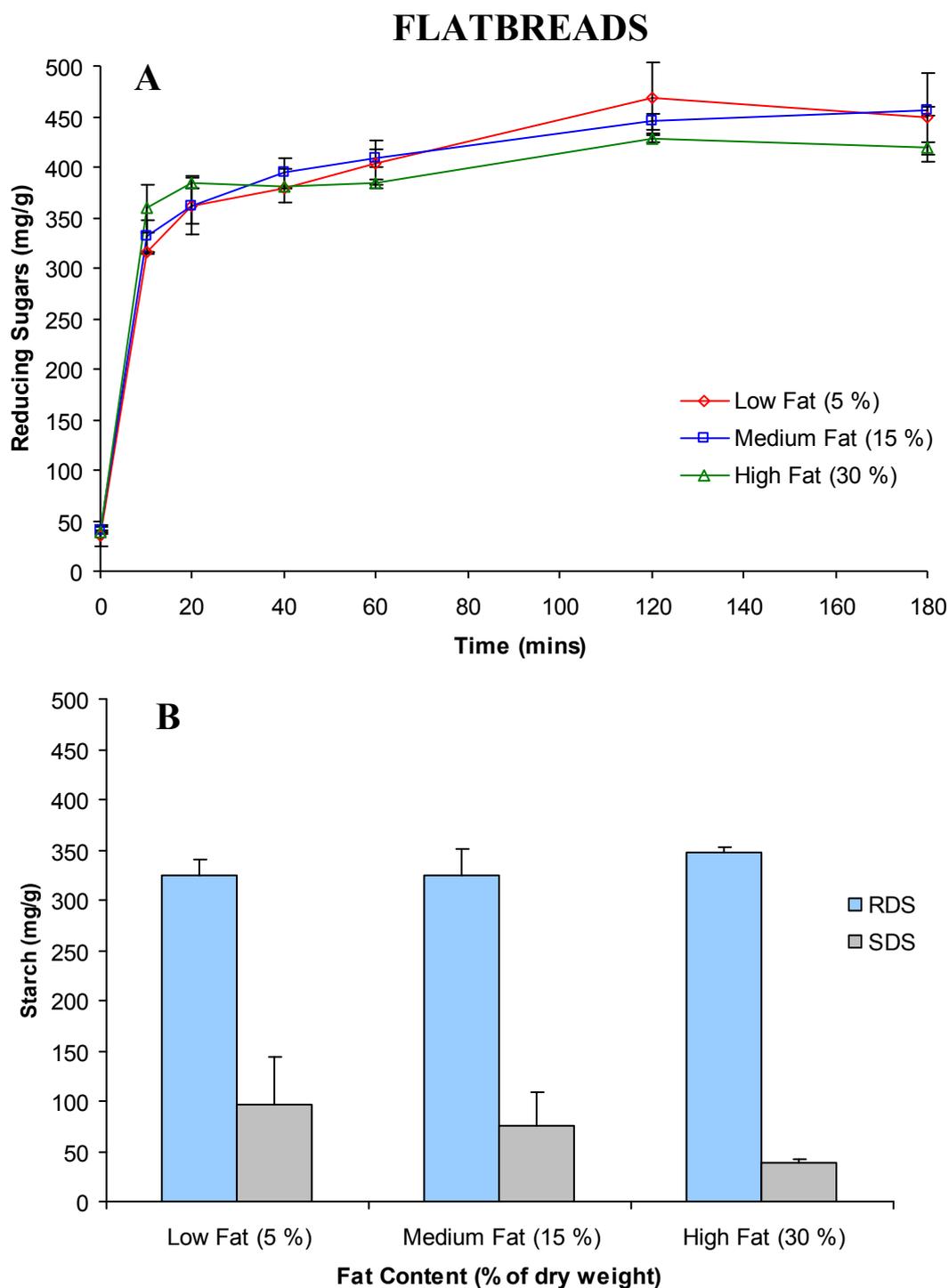
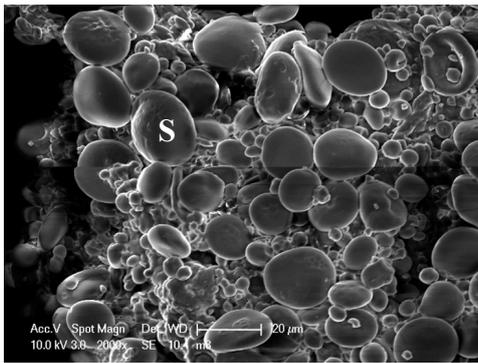
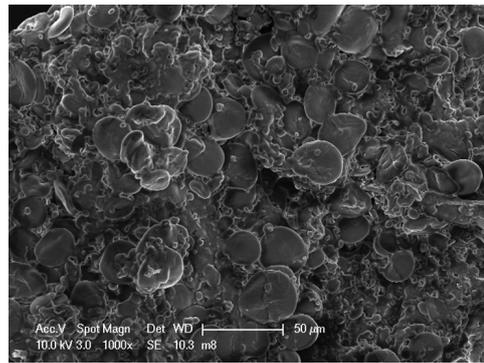


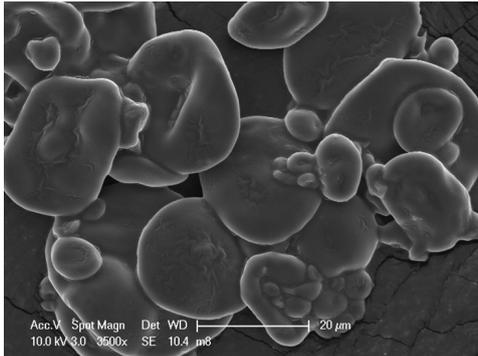
Figure 4.27 Starch hydrolysis curves (A) and relative RDS and SDS levels (B) measured in the three flatbreads following *in vitro* digestion. Values are means (n = 2) + SE.



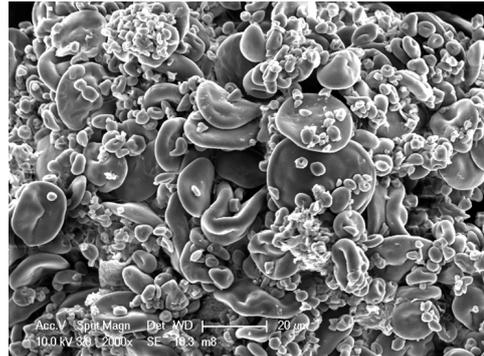
RAW 15 % Fat



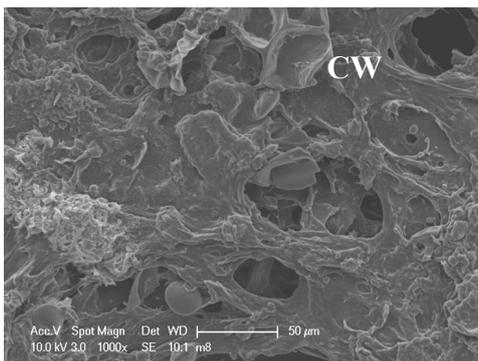
COOKED 5 % Fat



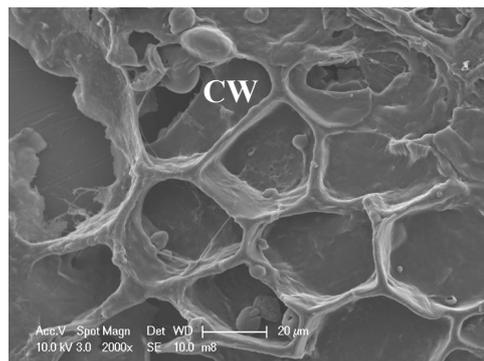
PRE-GASTRIC 30 % Fat



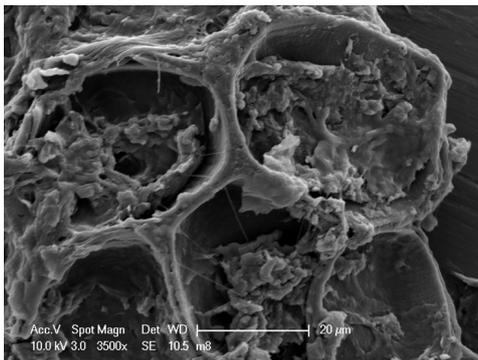
POST-GASTRIC 30 % Fat



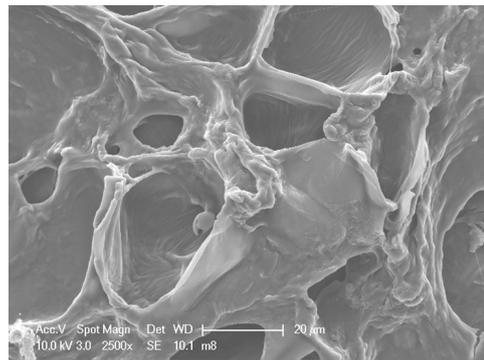
30 MIN 5 % Fat



60 MIN 5 % Fat



120 MIN 15 % Fat



180 MIN 15 % Fat

Figure 4.28 SEM images of raw and cooked flatbread samples as well as at different stages of *in vitro* pancreatic digestion. S, starch granules; CW, cell wall.

Digesta viscosity has been shown to affect the rate of starch digestion both *in vivo* (180-182) and *in vitro* (127, 183, 184). *In vivo*, increased viscosity delays gastric emptying (185, 186) and reduces gut motility (51, 187). Increased viscosity also slows glucose diffusion to the absorptive gut wall (95, 188) and, in both *in vivo* and *in vitro* situations, reduces accessibility of amylase enzyme to starch (95, 189). Since the flatbreads were initially ingested without a beverage¹⁶, it is most likely they formed a very viscous chyme, *in vivo*, contributing to their low/medium GI classification for the reasons just mentioned. In the *in vitro* digestions of the flatbreads, however, 2.5 g samples of each flatbread were digested in the presence of 53 mL of an enzyme/buffer solution. This, combined with the friable nature of the flatbreads and the “aggressive” magnetic stirring technique used, meant that the flatbreads quickly disintegrated into a very watery digesta, easily permeated by amylase and from which glucose rapidly diffuses. Thus the higher *in vitro* indication of flatbread starch digestibility compared with the measured *in vivo* glycemic response could have resulted, in part, from relative differences in digesta/chyme viscosity.

Finally there is the consideration that *in vitro* digestion curves are cumulative measures of ReS (mainly glucose) released over time, whereas *in vivo* curves are measures of glucose appearance in the blood, out from which, however, glucose is continually being removed by cellular reuptake. Adjusting the *in vitro* hydrolysis curves for the flatbreads by a factor representing the rate at which glucose is removed from the blood *in vivo* would likely help to align the two sets of data. Such an approach has been attempted recently in the literature with promising results (110).

4.3 TOWARD STANDARDISATION

Having outlined the major differences between current *in vitro* digestion methods, compared their relative effect on starch digestion curve profiles and validated such method parameters as incubatum pH and enzyme concentration using a range of test foods, a rudimentary framework for a standardised *in vitro* carbohydrate digestion method is proposed, outlined in **Figure 4.29**. The method features different treatments

¹⁶ Volunteers were not permitted a drink (250 ml of water) until 90 min postprandial.

of test foods, depending on the structural properties of the food, as well as the anticipated behaviour of the food particle once in the digesta medium.

Comminution technique used in the oral phase was identified as the *in vitro* method variable that exerted greatest influence on subsequent *in vitro* starch digestion rate and extent. Whether or not comminution technique affected starch hydrolysis depended on the rigidity of particles of test food once in digesta. Thus, in the standardised method there are two approaches to oral phase pretreatment of test foods. Porous, easily permeated foods, or foods anticipated by the analyst to likely form poorly defined particles, may be comminuted using any simulated chewing technique convenient to the analyst. Structurally robust foods, however, that form well-defined, rigid particles in digesta, should not be subject to simulated chewing methods which won't adequately achieve particle size breakdown resembling that of chewed. Rather, these foods, despite the inconvenience, should be chewed and expectorated in order to achieve physiologically-relevant, "as eaten" particles. The transient effect of salivary α -amylase pre-exposure in chewed samples compared with those comminuted artificially, may be ignored, since pancreatin exposure will rapidly obscure differences in levels of digested starch measured at T_0 . Following comminution, it is recommended that a generous quantity of test food is subjected to *in vitro* digestion (we use 5.0 g) to ensure adequate sampling of the entire particle size distribution achieved by the comminution method and thus greater reproducibility between different *in vitro* runs.

Inclusion of a gastric phase pepsin incubation doesn't seem to improve subsequent starch digestion during the intestinal phase, at least when pancreatin is used as the amyolytic enzyme. Nonetheless, a 30 min, pH 2.0, 37 °C gastric phase features in the standardised *in vitro* template to mimic *in vivo* conditions.

The intestinal phase pancreatic digestion may be conducted across a range of pH values (5.0 – 7.0) to accommodate for simultaneous AMG activity. Pancreatin enzyme should remain stable and active even at incubation pH of 5.0 for at least 2 hours. Amyolytic enzyme concentration should be in excess to that required (≥ 0.2 %) in order to prevent rate-limitation of starch digestion and underestimation of digestibility. Stirring of samples during the gastric and intestinal phase digestions is carried out via magnetic stirrers, since this achieves robust mixing of sample as well as giving easy access to

samples for rapid digesta aliquoting. Shaking water bath mixing is permissible, however, if the analyst anticipates the test food(s) will disperse easily in the digestion medium without clumping and few samples and/or aliquots of samples are required.

This proposed standardised method is a framework and as such requires refinement. Consideration needs to be given to the ratio of sample to digesta solution, for example, as this will affect the viscosity and therefore potentially the digestibility of sample as well as the effectiveness of stirring if the digesta is viscous. However, the major method parameters have been addressed. The principle underlying this method is that no method parameter would contribute to rate-limitation of starch digestion, such that the profiles of hydrolysis curves are truly reflective of that food's digestion characteristics.

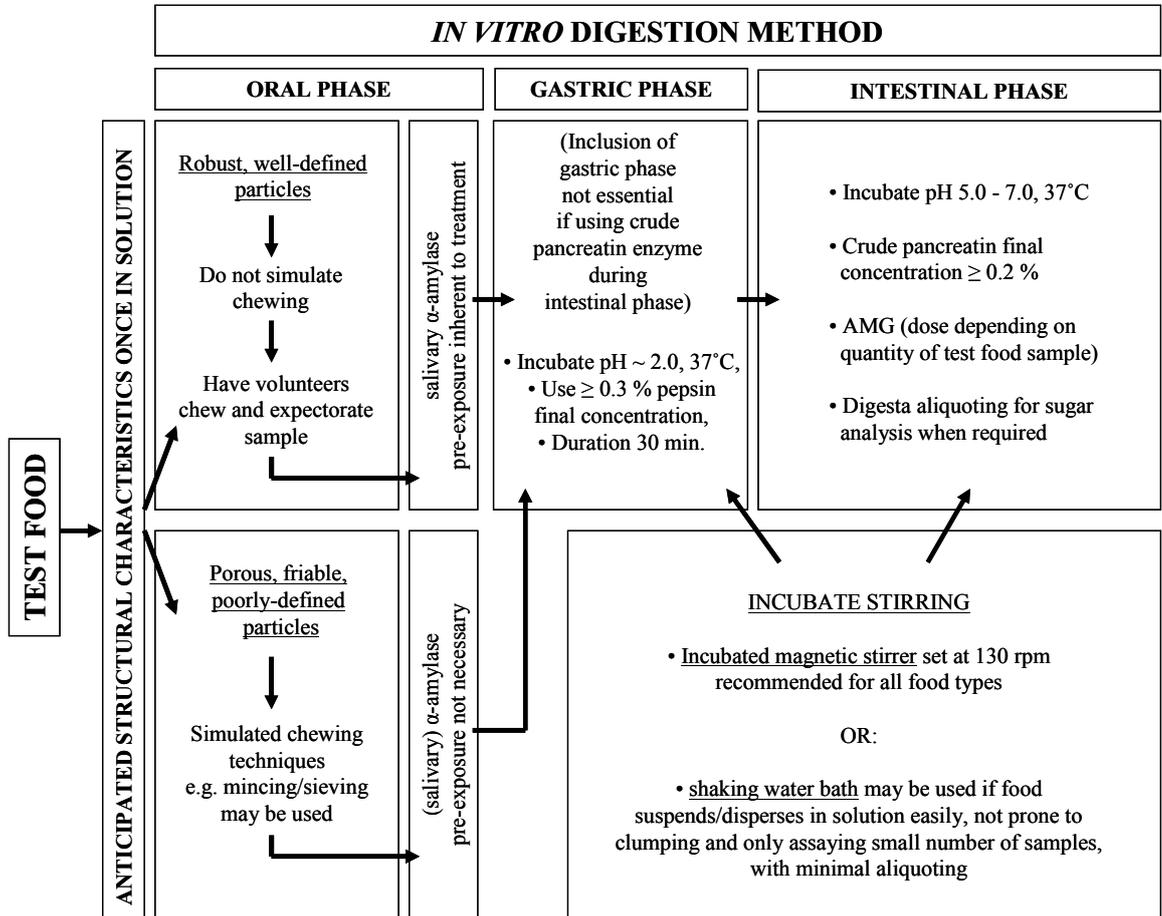


Figure 4.29 The proposed basic framework for the standardised *in vitro* carbohydrate digestion method for prediction of relative glycemic response to foods.

CHAPTER 5: CONCLUSION

Rising global incidence of insulin resistance and type II-diabetes is driving the need for communication to consumers, via food-labelling, of the likely relative glycaemic impact of commonly-consumed, carbohydrate-containing foods, to enable informed food choices and better management of postprandial blood glucose levels. Traditionally, *in vivo*-based methodologies have been utilised toward this purpose. The glycaemic index (GI) test is perhaps the most widely-used *in vivo* method for ranking foods based on their glycaemic potency. *In vivo* techniques are costly and time-consuming, however. They require ethical approval and recruitment of volunteers and usually yield blood glucose results exhibiting considerable inter- and intraindividual variability. Thus, *in vivo* techniques, such as glycaemic index testing, are unsuitable for large-scale application in routine food analysis.

There is growing interest amongst food manufacturers, technologists and analysts in the potential for *in vitro* carbohydrate digestion methods to predict the likely glycaemic response to foods. *In vitro* digestion methods are well-suited to routine food glycaemic analysis since they are high-throughput, inexpensive and highly precise. Subsequently, countless *in vitro* methods have been developed over the past twenty years. Methods differ considerably, however, in their comminution pretreatment of test food, whether a pepsin incubation is included and of what duration, the type and concentration of amylolytic enzyme used as well as other method variables such as incubation pH, temperature and mode of stirring. It is not known what effect, if any, such differences in methodology exert on relative *in vitro* measures of starch digestibility.

In 2006, an ad hoc Glycaemic (Net) Carbohydrate Definition Committee, appointed by the AACC, recommended the use of *in vitro* digestion technology toward routine glycaemic analysis of foods and communication of likely glycaemic response via food-labelling. However, it is the present lack of a validated and standardised *in vitro* carbohydrate digestion method that prevents such routine and large-scale glycaemic analysis and labelling of foods from being realised.

This thesis takes steps toward achieving a standardised *in vitro* carbohydrate digestion method through conductance of a systematic investigation of method variables and their relative impact on *in vitro* results - namely levels of the digestible starch fractions RDS and SDS, used to rank foods based on their likely glycemic impact. The major method variables investigated include: oral phase comminution method and salivary amylase pre-exposure, inclusion versus omission of a pepsin incubation (of varied duration), and incubation stirring method. Fundamental method parameters such as incubation pH, enzyme concentration and incubation duration were also validated in their use and a rudimentary framework for the standardised *in vitro* digestion method proposed. Whether foods were chewed or comminuted by simulated chewing methods and the method of sample stirring were the factors that introduced the greatest variation in *in vitro* results. Subsequently, the proposed standardised method features alternative oral phase pretreatments as well as stirring techniques depending on the structural characteristics of the food to be analysed. Used correctly, the method should achieve robust digestion and precise analysis of all types of test food.

Aside from the lack of standardisation, *in vitro* digestion method technology faces criticism due to its inability to account for the effect of gastric emptying rate – a complex physiological process that constantly varies with food type and is heavily influential to glycemic response. In the present thesis, a GI test was performed using a simple flatbread food model that varied in fat content – a factor known to attenuate the glycemic response by reducing gastric emptying rate. Through measurement of gastric emptying rate and glycemic response to each flatbread, the objective was to obtain a correction for gastric emptying that might be applied to parallel *in vitro* analyses of the flatbreads to improve their glycemic predictive power. *In vivo*, a significant reduction in gastric emptying rate was measured with increased levels of fat; however, there was no measured effect of fat on glycemic response. Suspected separation of fat from the carbohydrate component of each flatbread whilst in the stomach, as well as low glycemic potency of even the low fat flatbread, are the two factors believed to have contributed to the lack of any effect of reduced gastric emptying rate on glycemic response. Unfortunately, since no fat effect on glycemic response was measured *in vivo*, no adjustments to parallel *in vitro* digestion curves to improve their predictive power could be made. *In vitro* starch digestion of the flatbreads was rapid, implying that they were high GI, thus overestimating their glycemic effect compared with *in vivo*.

The lack of a correction for gastric emptying rate as well as the low viscosity of the *in vitro* digests are likely the two factors contributing to this apparent overestimation.

The systematic investigation of *in vitro* method parameters carried out in this thesis, utilised only five standard test foods. While these foods are reflective of a range of botanical origins, structural properties and GI values, it would be interesting to broaden the present systematic investigation by retesting some of the key method variables on a greater range of foods. The conclusion that oral phase comminution method is only a determinant of *in vitro* starch digestion kinetics when “hard foods” forming rigid, defined particles are analysed, for example, needs to be rigorously tested with many more supposedly “hard foods” as well as “soft foods,” digested using the standardised *in vitro* method. Certainly *in vitro* digestions of a much greater range of foods would help to define “hard foods” versus those that are “soft foods,” - an important distinction particularly since the proposed standardised method requires that the analyst make this classification of their test food prior to *in vitro* digestion. Similarly, the effect of the different stirring methods on *in vitro* digestion kinetics and the conclusion that magnetic stirring or shaking water bath treatments may be used when test foods that disperse easily in solution without clumping, should be tested and validated across a greater range of food types.

Throughout this thesis all aliquots of digesta from *in vitro* digestions were analysed via DNS colourimetry which, although sufficient for RDS and SDS determination, merely provides a quantitative, non-specific measure of reducing sugars. Using more specific endpoint techniques such as HPLC, species of sugars and dextrans released during progressive *in vitro* starch digestion could be identified. It would be interesting to compare what effect different *in vitro* method conditions (such as comminution and stirring method) had on relative distributions of these partially-digested species at different time points of *in vitro* digestion. Comparisons between distributions of these species occurring *in vitro* could also be made with *in vivo* animal models or in human ileostomate subjects, to see how closely *in vitro* starch digestion kinetics, at the molecular level, mirror those occurring *in vivo*.

In vitro carbohydrate digestion methodology for prediction of the relative glycemic response will never be widely accepted until its two major drawbacks are addressed: its

inability to account for varying gastric emptying rate as well as the effect of viscosity on the glycemic response *in vivo*. The work in this thesis attempted to build a correction for gastric emptying rate into a parallel *in vitro* digestion, but for the reasons described previously, was unsuccessful. Research effort needs to be focused on characterising the role of gastric emptying in the subsequent glycemic response, as well as identifying the key food factors that alter gastric emptying. An algorithmic correction for gastric emptying rate, used to adjust *in vitro* digestion curves to improve their predictive power, should be obtainable if a pattern for food factors (such as fat content) and their effect on gastric emptying and glycemic response is established. Perhaps direct ¹³C-labelling and measurement of the gastric emptying of the carbohydrate component of the test meal (instead of fat for instance) and utilisation of continuous blood glucose monitoring devices, (instead of intermittent blood sampling as per GI testing protocol) would be a more robust means of obtaining this data. A similar correction for viscosity could be obtained using animal models or human ileostomate subjects where intestinal chyme viscosity, following consumption of a range of food types is related to the glycemic response and parallel *in vitro* digestion curves adjusted accordingly.

Finally, if, in future, *in vitro*-based definitions of the likely relative glycemic response to foods do get utilised in food labels, it is important that such information, related solely to the glycemic effect of the food, be considered not in isolation but in conjunction with the full spectrum of other nutrient values (e.g. fat, protein and energy content) such that only foods that are truly nutritious and beneficial to health are recommended for consumption on a regular basis.

APPENDICES

APPENDIX A Literature Review (Part I) Publication



MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: James Woolnough

Name/Title of Principal Supervisor: Dr Alistair Carr

Name of Published Research Output and full reference:

Simulating human carbohydrate digestion: a review of methods and the need for standardisation. *International Journal of Food Science and Technology* 2008, 43, 2245-2256.

In which Chapter is the Published Work: Chapter 2

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 100% and / or
- Describe the contribution that the candidate has made to the Published Work:

James
Woolnough

Digitally signed by James Woolnough
DN: cn=James Woolnough, o, ou,
email=james.woolnough@gmail.com, c=AU
Date: 2011.11.29 14:25:59 Z

Candidate's Signature

29/11/2011

Date

Dr Alistair Carr

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ou=FNHI, email=j.carr@massey.ac.nz,
c=NZ
Date: 2011.11.30 09:17:16 +1300'

Principal Supervisor's signature

30/11/2011

Date

APPENDIX B Starch-Digesting Capacity of Salivary α -Amylase Publication



MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

**STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS**

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: James Woolnough

Name/Title of Principal Supervisor: Dr Alistair Carr

Name of Published Research Output and full reference:

The Effect of a Brief Salivary Alpha Amylase Exposure During Chewing on Subsequent In Vitro Starch Digestion Curve Profiles. International Journal of Molecular Sciences 2010, 11, (8), 2780-2790.

In which Chapter is the Published Work: Chapters 3 and 4

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 100% and / or
- Describe the contribution that the candidate has made to the Published Work:

James
Woolnough

Candidate's Signature

Digitally signed by James Woolnough
DN: cn=James Woolnough, o=Massey University,
email=james.woolnough@gmail.com, c=AU
Date: 2011.11.29 14:36:13 Z

29/11/2011

Date

Dr Alistair Carr

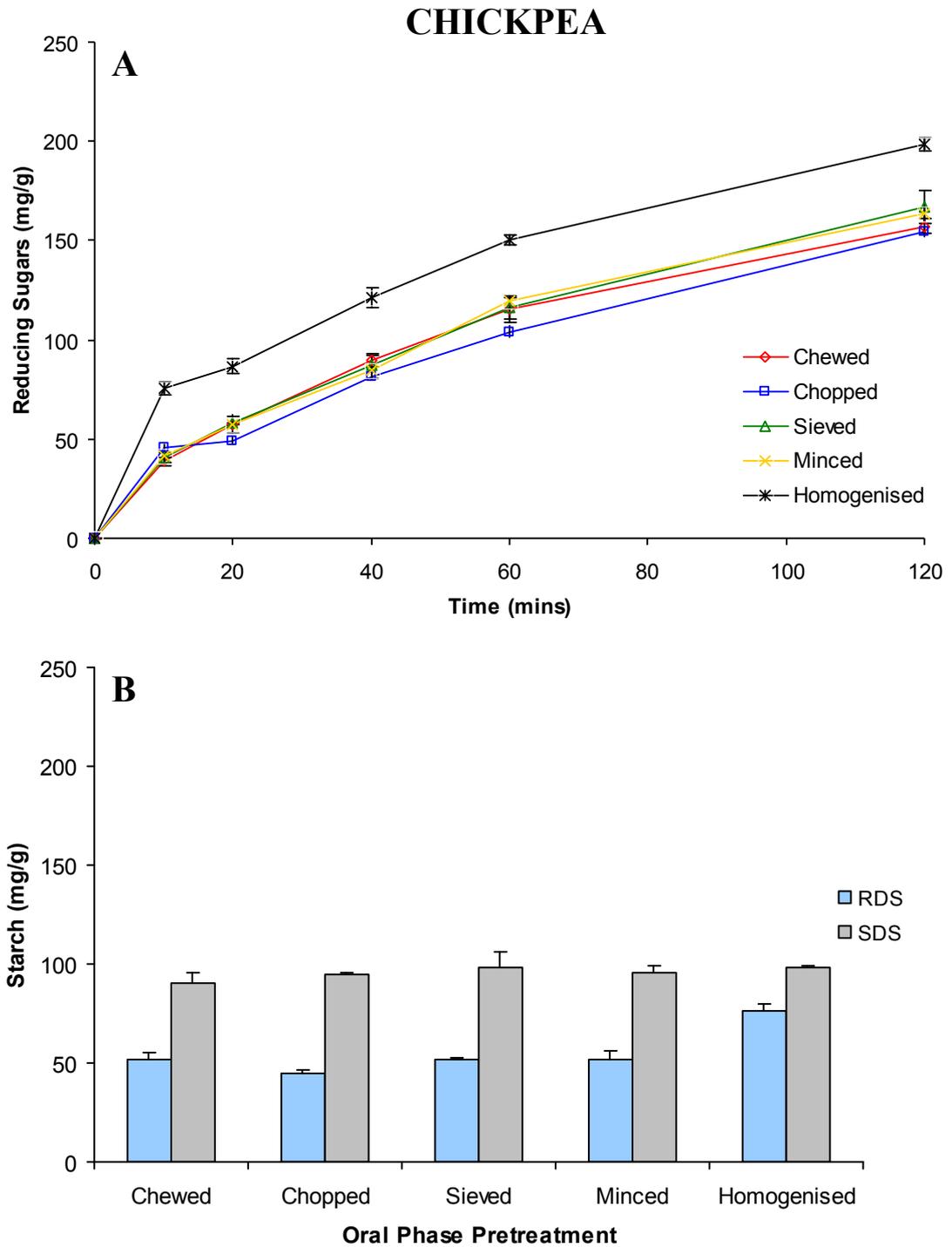
Principal Supervisor's signature

Digitally signed by Dr Alistair Carr
DN: cn=Dr Alistair Carr, o=Massey University,
ou=FNH, email=j.carr@massey.ac.nz,
c=NZ
Date: 2011.11.30 09:18:46 +1300

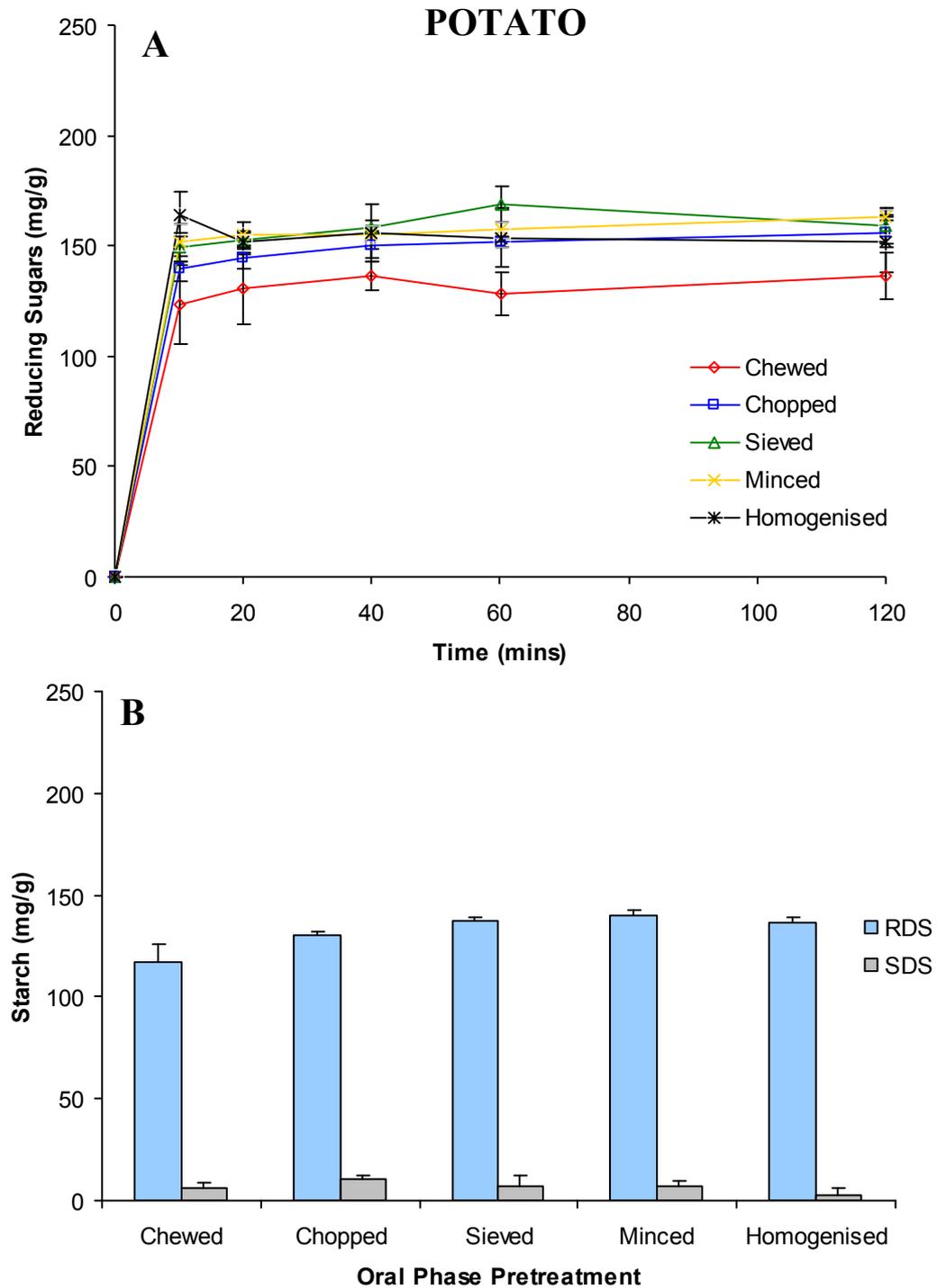
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Date

APPENDIX C **CSIRO Human Research Ethics Committee Approval Letter**



Appendix D1 (80) Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in chickpea samples digested *in vitro* following different oral phase pretreatments. Values are means (n = 3) ± SE.



Appendix D2 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in potato samples digested *in vitro* following different oral phase pretreatments. Values are means (n = 3) ± SE.

APPENDIX D3 Mode of Comminution in the Oral Phase – Statistical Analysis

Overview

There was a treatment \times time effect for bread ($p = 0.007$). ReS concentration at each timepoint did not differ between pretreatment methods across the duration of pancreatic digestion, except at 40 min where there was higher ReS in homogenised bread samples compared with chewed ($p = 0.015$). Measures of bread RDS and SDS content did not differ with oral phase pretreatment method.

There was a treatment \times time effect for wheat ($p < 0.001$). At 10 min of pancreatic digestion, ReS was higher in homogenised wheat samples compared with chopped ($p = 0.005$), sieved ($p = 0.008$) and minced samples ($p = 0.004$). At 20 min of digestion, ReS was higher in homogenised samples compared with chopped ($p = 0.011$), sieved ($p = 0.004$) and minced ($p = 0.01$). At 40 mins of digestion ReS was still higher in homogenised wheat compared with chopped ($p = 0.011$), sieved ($p = 0.022$) and minced samples ($p = 0.002$) and remained higher than chopped ($p < 0.001$) and minced ($p = 0.007$) samples at 60 min and at 120 min digestion ($p = 0.015$; 0.008 respectively). ReS was higher in chewed wheat samples compared with chopped at 20 ($p = 0.023$), 40 ($p = 0.045$), 60 ($p = 0.023$) and 120 min ($p = 0.015$) of pancreatic digestion. The RDS measure from homogenised wheat was higher than that of chopped ($p = 0.011$), sieved ($p = 0.004$) and minced ($p = 0.01$). There was more SDS in chewed wheat samples compared with homogenised ($p = 0.02$).

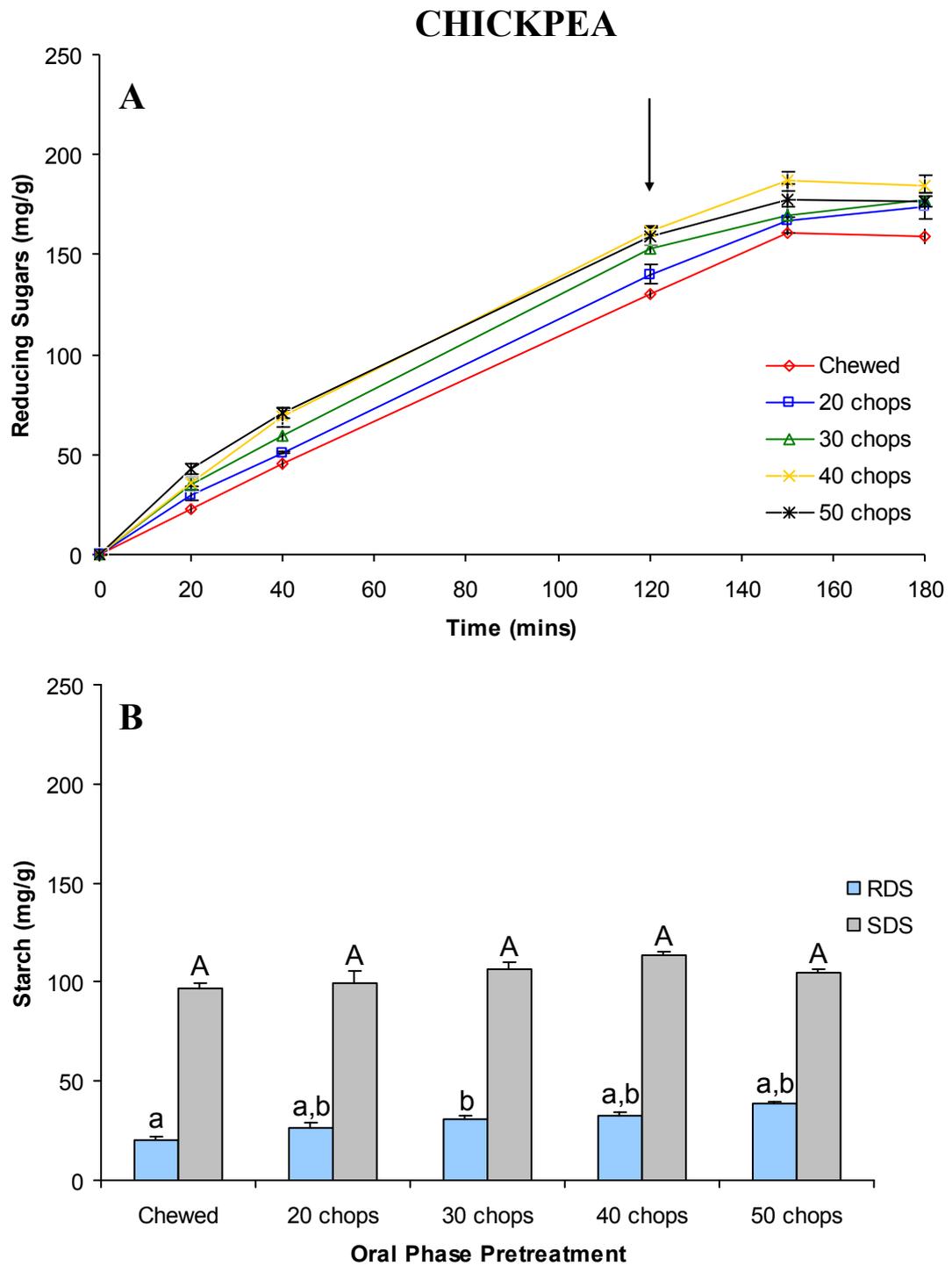
There was a treatment \times time effect for pasta ($p < 0.001$). At 10 min of digestion, ReS was lower in chopped pasta compared with chewed ($p = 0.036$), sieved ($p = 0.004$), minced ($p = 0.027$) and homogenised samples ($p = 0.023$). ReS from chopped pasta remained lower than sieved at 20 ($p = 0.02$) and 40 min ($p = 0.038$) and lower than homogenised samples at 20 ($p = 0.035$) and 60 min ($p = 0.009$). ReS was higher in minced pasta compared with chewed at 10 min of digestion ($p = 0.02$). There was higher ReS in homogenised pasta compared with chewed ($p = 0.009$) and minced samples ($p = 0.036$) at 20 min of digestion. ReS was lower in chewed samples than in sieved ($p = 0.021$) and homogenised ($p = 0.024$) at 40 min of digestion and remained lower than homogenised pasta ($p = 0.008$) at 60 min of digestion. There was less ReS

APPENDIX D3 (Continued)

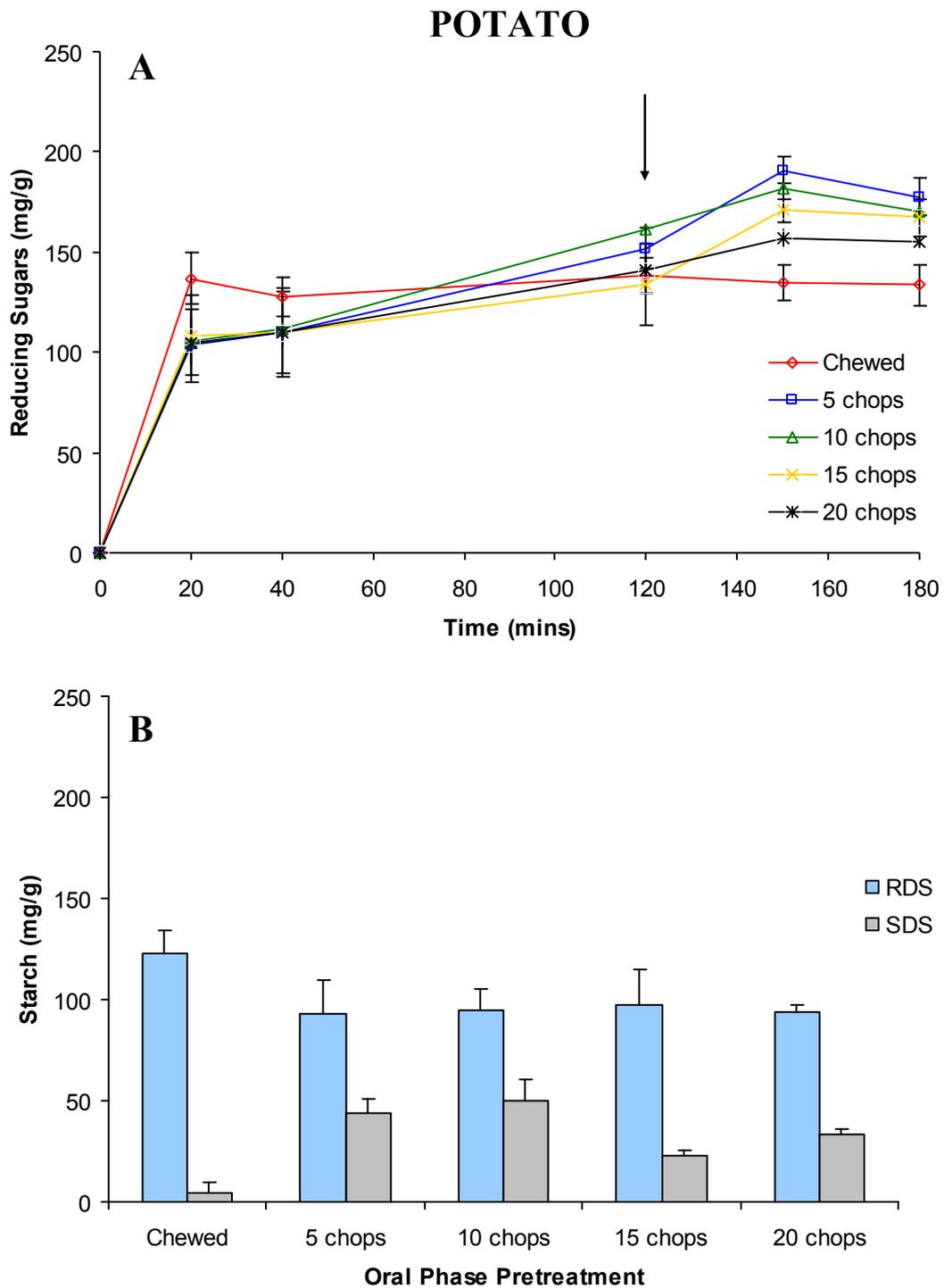
in minced samples at 40 min of digestion compared with homogenised ($p = 0.004$). RDS was higher in homogenised pasta compared with chewed ($p = 0.009$), chopped ($p = 0.035$) and minced samples ($p = 0.036$). RDS was higher in sieved pasta compared to chopped ($p = 0.02$). SDS did not differ with oral phase pretreatment method.

There was a treatment \times time effect for chickpea ($p < 0.001$). At 10 min of pancreatic digestion, there was higher ReS in homogenised chickpea samples compared with minced ($p = 0.036$). At 120 min of digestion ReS was higher in homogenised sample compared to chewed ($p = 0.007$). Oral phase pretreatment method did not affect subsequent determinations of chickpea RDS or SDS content.

There was a treatment \times time effect for potato ($p < 0.001$). ReS did not differ between pretreatment methods at any time point throughout the *in vitro* pancreatic digestion. The different pretreatment methods did not affect measures of potato RDS or SDS.



Appendix E1 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in chickpea samples digested *in vitro* following different oral phase pretreatments. The arrow denotes the point at which all samples were homogenised. Values are means (n = 3) ± SE.



Appendix E2 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in potato samples digested *in vitro* following different oral phase pretreatments. The arrow denotes the point at which all samples were homogenised. Values are means ($n = 3$) \pm SE.

APPENDIX E3 Comparing Chewing and Chopping – Statistical Analysis Overview

No treatment × time effect was observed for bread, nor was there a significant treatment effect. ReS did not differ between pretreatments at any timepoint throughout the pancreatic digestion. Measures of bread RDS and SDS content were not affected by whether the bread was chewed or chopped or by the number of chops.

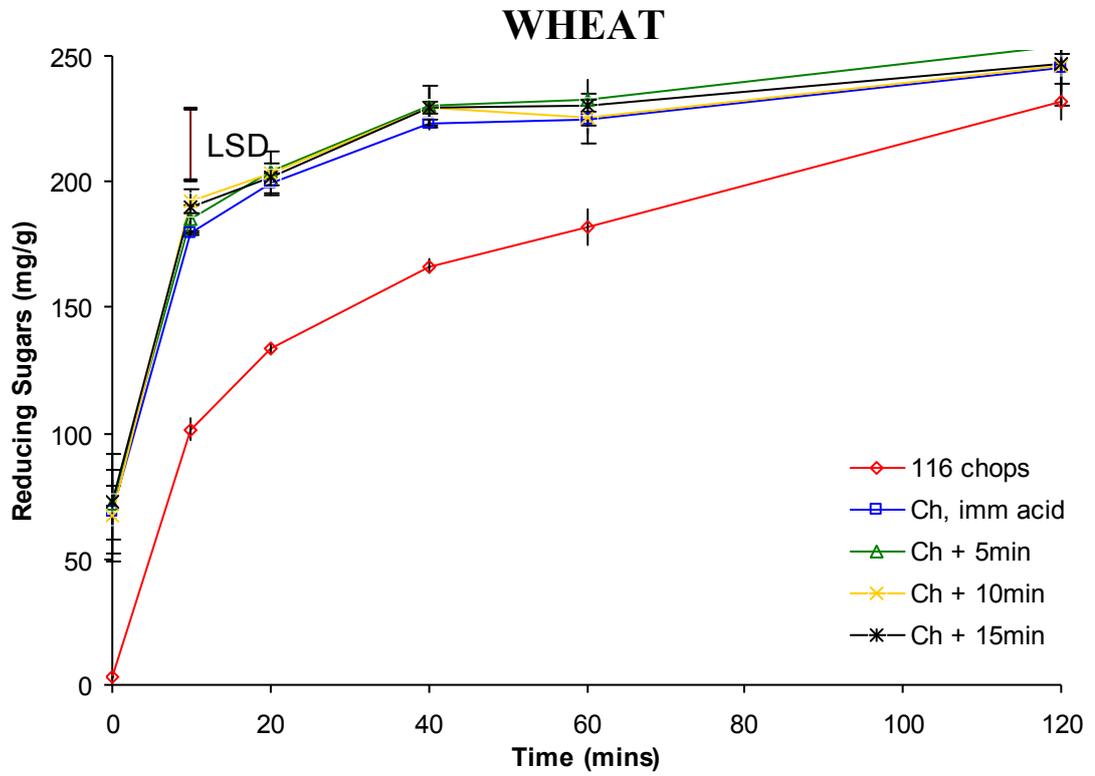
There was a treatment × time effect for wheat ($p < 0.001$). At 20 min pancreatic digestion, there was higher ReS in chewed wheat sample compared with samples chopped 20 times ($p = 0.016$), 30 times ($p = 0.005$) or 40 times ($p = 0.04$). There was higher ReS in wheat chopped 40 times compared to wheat chopped 20 times ($p = 0.042$). ReS in chewed wheat was higher at 40 min digestion compared with wheat chopped 30 times ($p = 0.009$). ReS in chewed wheat was higher than in wheat chopped 30 times at both 40 min ($p = 0.009$) and 120 min ($p = 0.002$) of digestion. Following homogenisation, for the remainder of *in vitro* digestion, there were no differences in reducing sugar levels between treatments. RDS was higher in chewed wheat compared with wheat chopped 20 ($p = 0.016$), 30 ($p = 0.005$) or 40 times ($p = 0.04$). Wheat SDS measures did not differ with oral phase pretreatment.

There was a significant treatment × time effect for pasta ($p < 0.001$). At 20 min of pancreatic digestion there was lower ReS in pasta chopped 20 times compared with chewed pasta ($p = 0.027$) and pasta chopped 50 times ($p = 0.041$). RDS was higher in chewed samples ($p = 0.027$) as well as in samples chopped 50 times ($p = 0.041$) compared with pasta chopped 20 times. SDS was higher in pasta chopped 50 times compared with chewed pasta ($p = 0.026$).

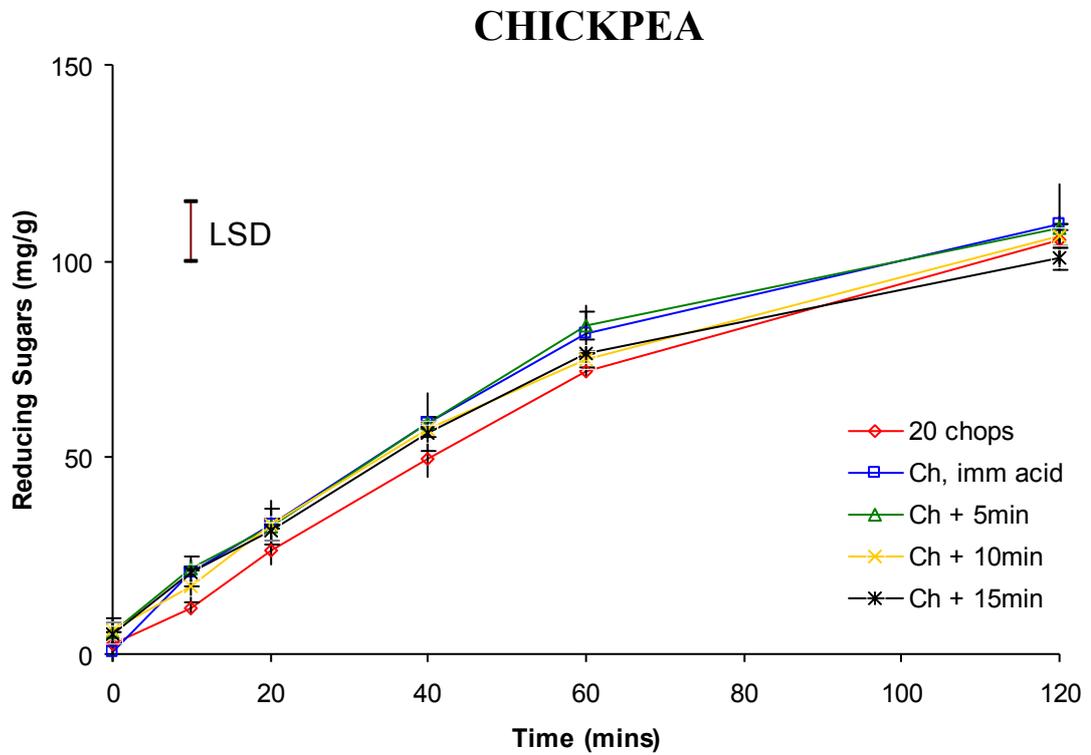
There was a significant treatment × time effect for chickpea ($p < 0.001$). At 20 min of digestion, ReS was higher in chickpea chopped 30 times compared with chewed sample ($p = 0.048$). At 120 min of digestion, ReS was higher in samples chopped 50 times compared with chewed sample ($p = 0.012$). Chopping chickpeas 30 times resulted in a higher measure of RDS compared with chewing chickpea ($p = 0.048$). SDS values did not differ with chickpea pretreatment method.

APPENDIX E3 (Continued)

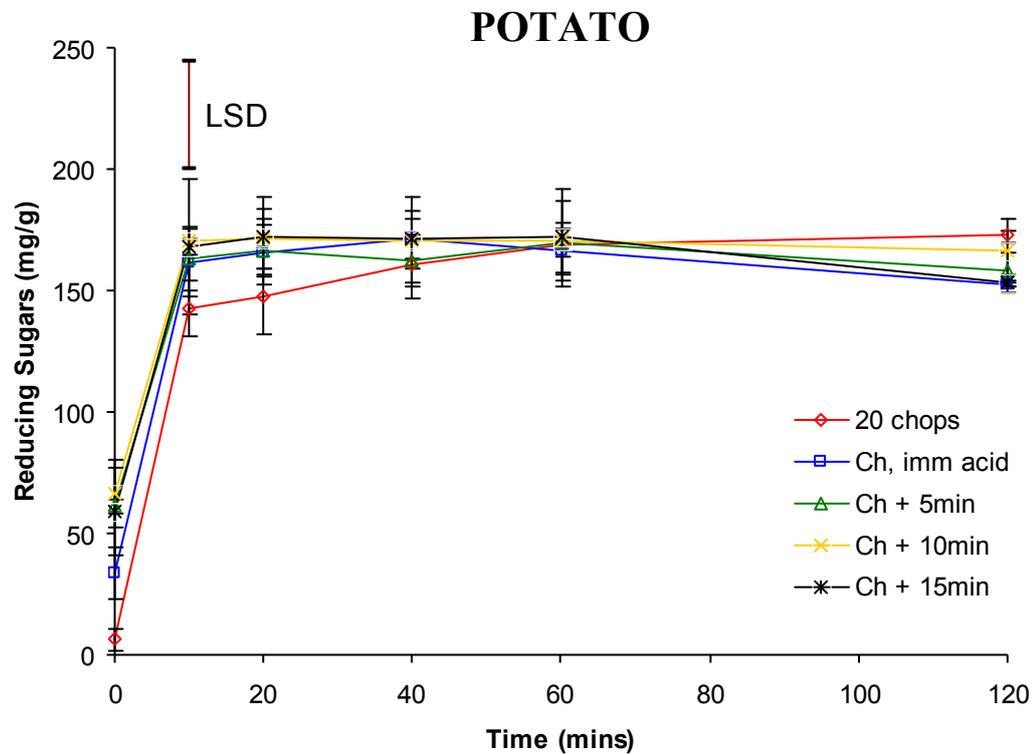
There was a significant treatment \times time effect for potato ($p = 0.003$). There was no difference in the levels of ReS between the different treatments until 120 min of digestion where ReS was in potato samples chopped 5 times compared with those chopped 20 times ($p = 0.043$). RDS and SDS values for potato did not differ with oral phase pretreatment.



Appendix F1 Starch hydrolysis curves for wheat digested *in vitro* following differing levels of exposure to salivary amylase during the oral phase. Ch, chewed; imm, immediate. Values are mean ReS (n = 2) ± SE. LSD = 29.

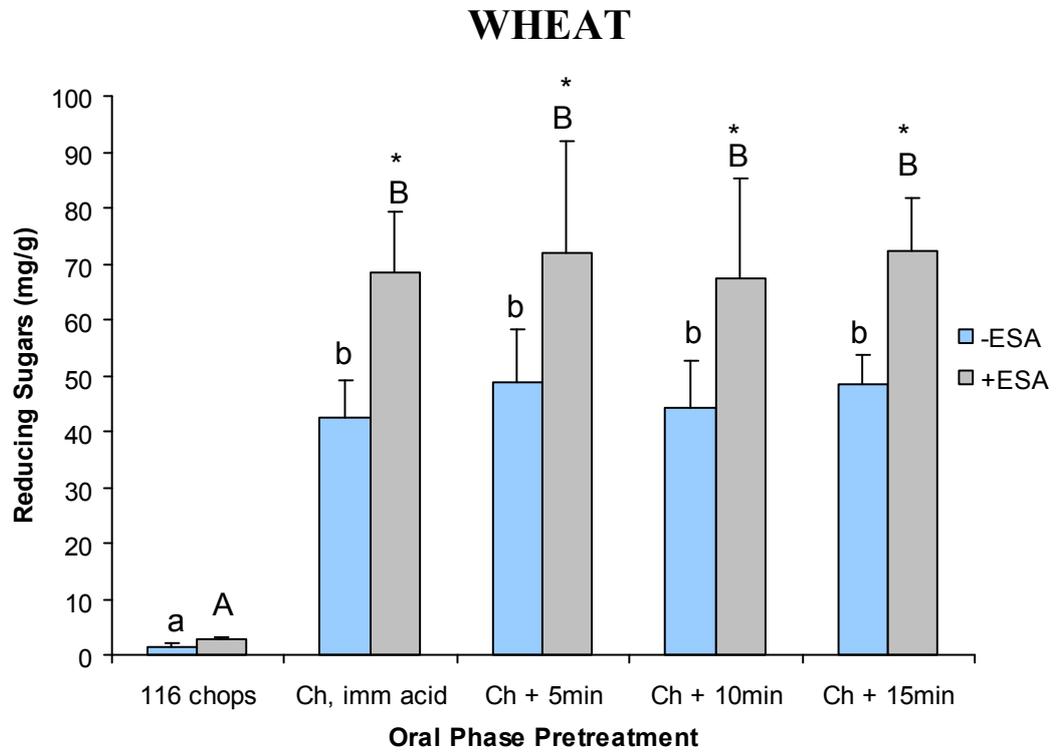


Appendix F2 Starch hydrolysis curves for chickpea digested *in vitro* following differing levels of exposure to salivary amylase during the oral phase. Ch, chewed; imm, immediate. Values are mean ReS (n = 2) ± SE. LSD = 15.



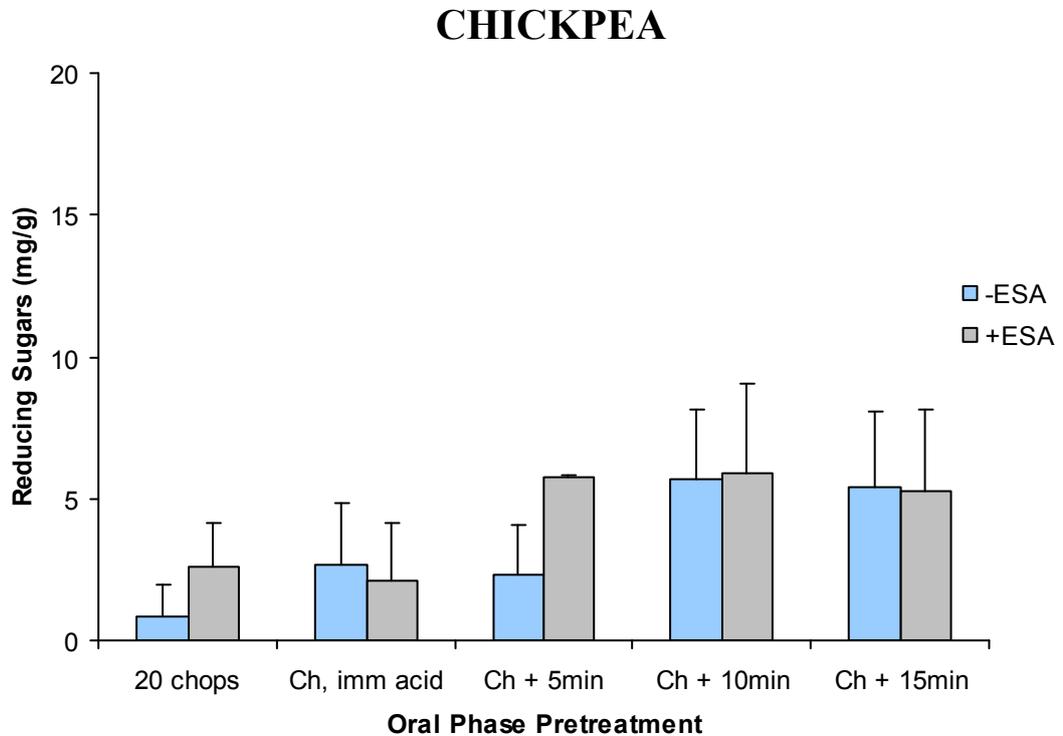
Appendix F3 Starch hydrolysis curves for potato digested *in vitro* following differing levels of exposure to salivary amylase during the oral phase. Ch, chewed; imm, immediate. Values are mean ReS (n = 2) \pm SE. LSD = 44.

Aliquots



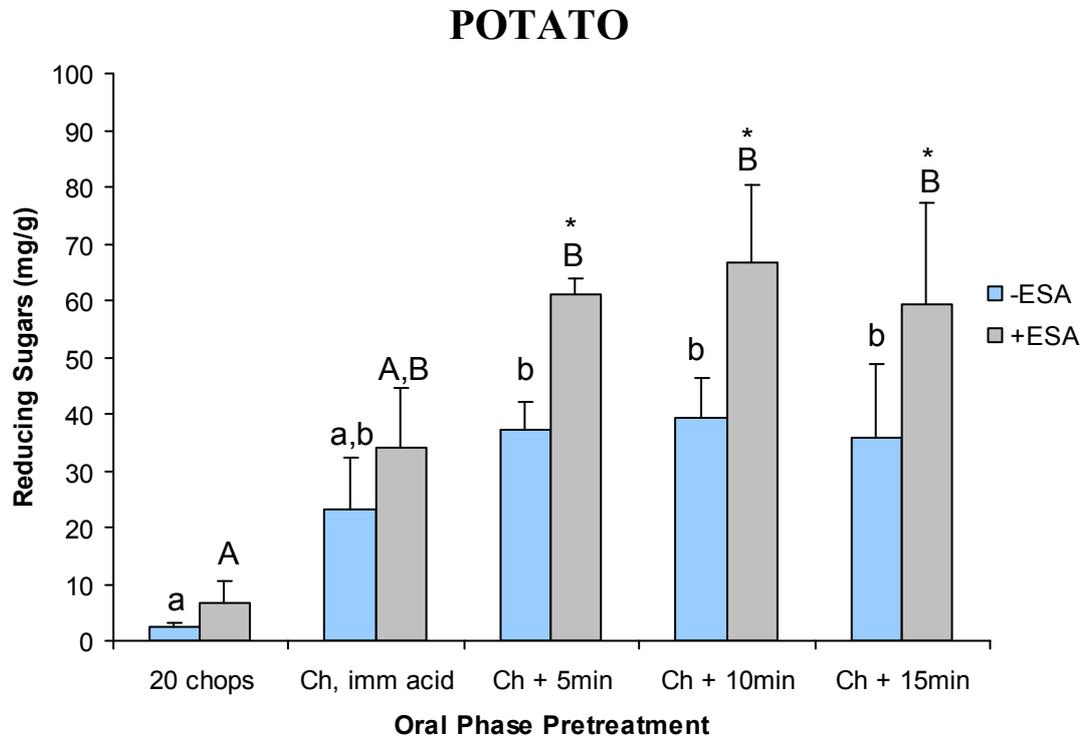
Appendix F4 ReS released by salivary amylase action during chewing (chewed treatments) or freely occurring in wheat (chopped treatment) and measured in T₀ aliquots prior to pancreatin addition. The T₀ aliquot is subsampled and ReS measured either directly (- ESA) or subjected to a secondary ESA digestion (+ ESA) prior to measurement of ReS. Ch, chewed; ESA, enzyme solution ‘A’; imm, immediate. Values are mean ReS (n = 2) + SE.

Aliquots



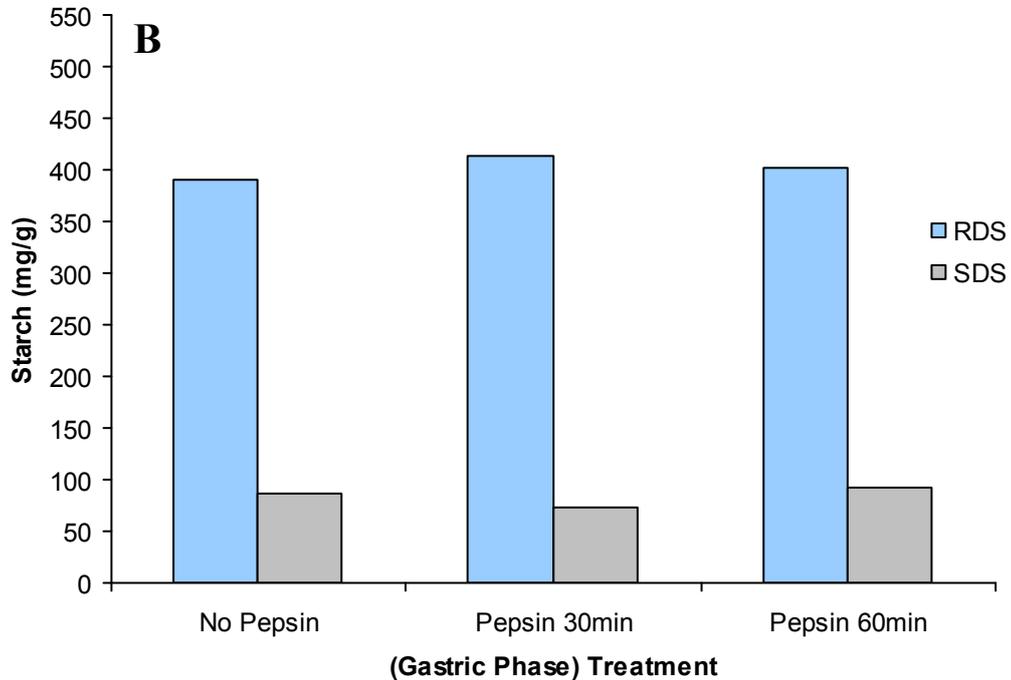
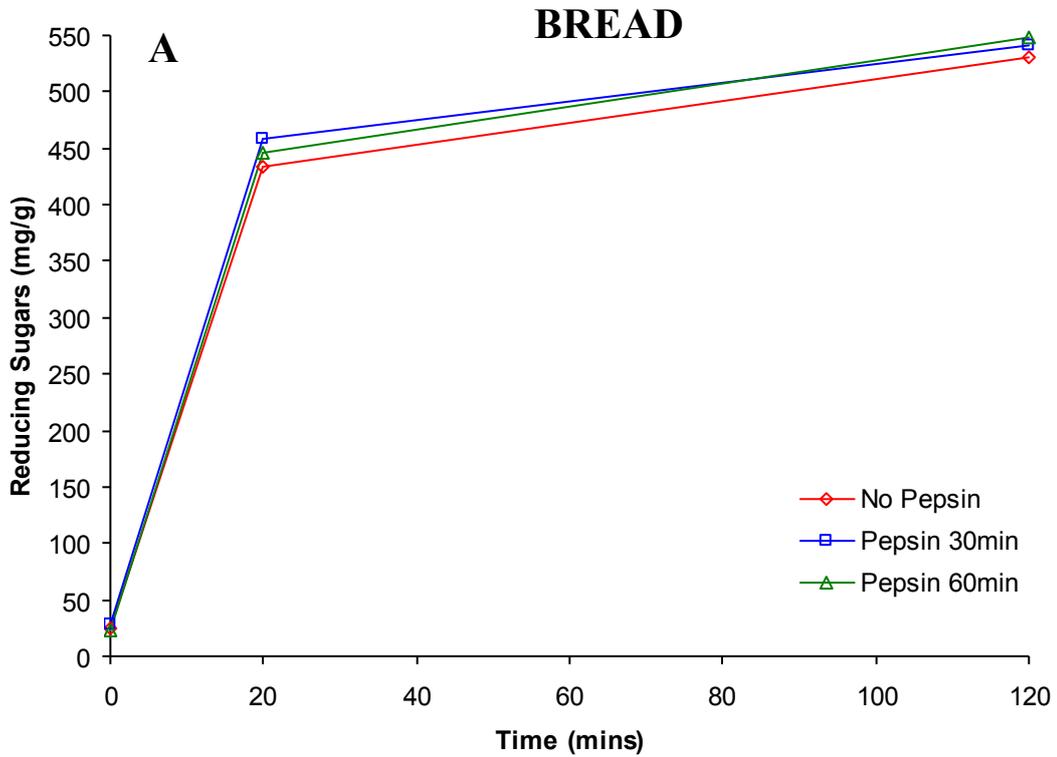
Appendix F5 ReS released by salivary amylase action during chewing (chewed treatments) or freely occurring in chickpea (chopped treatment) and measured in T₀ aliquots prior to pancreatin addition. The T₀ aliquot is subsampled and ReS measured either directly (- ESA) or subjected to a secondary ESA digestion (+ ESA) prior to measurement of ReS. Ch, chewed; ESA, enzyme solution ‘A’; imm, immediate. Values are mean ReS (n = 2) + SE.

Aliquots

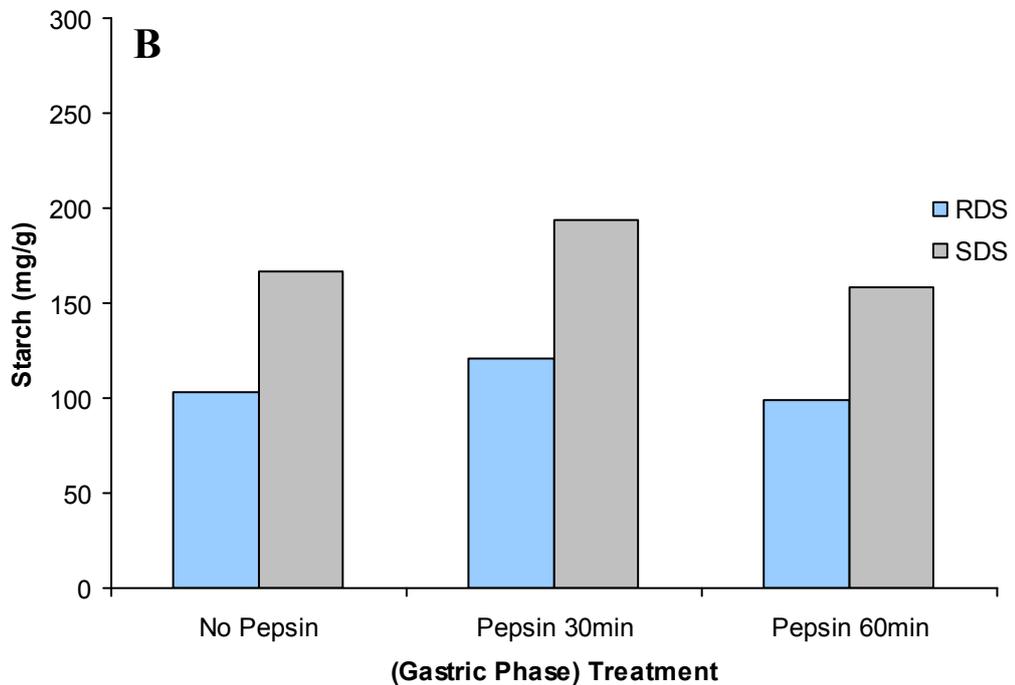
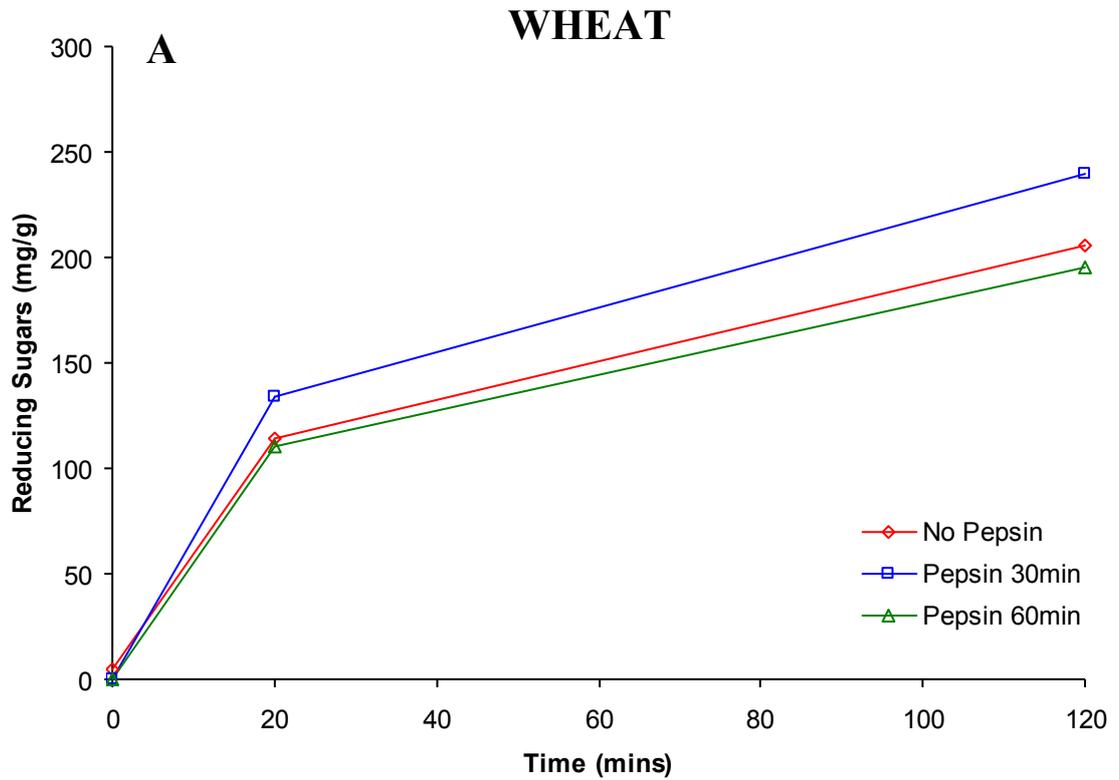


Appendix F6 ReS released by salivary amylase action during chewing (chewed treatments) or freely occurring in potato (chopped treatment) and measured in T₀ aliquots prior to pancreatin addition. The T₀ aliquot is subsampled and ReS measured either directly (- ESA) or subjected to a secondary ESA digestion (+ ESA) prior to measurement of ReS. An asterisk (*) denotes a significant difference between ‘-ESA’ and ‘+ESA’ subsamples within each treatment. Ch, chewed; ESA, enzyme solution ‘A’; imm, immediate. Values are mean ReS (n = 2) + SE.

APPENDIX G1 Pepsin Inclusion and Omission – Bread

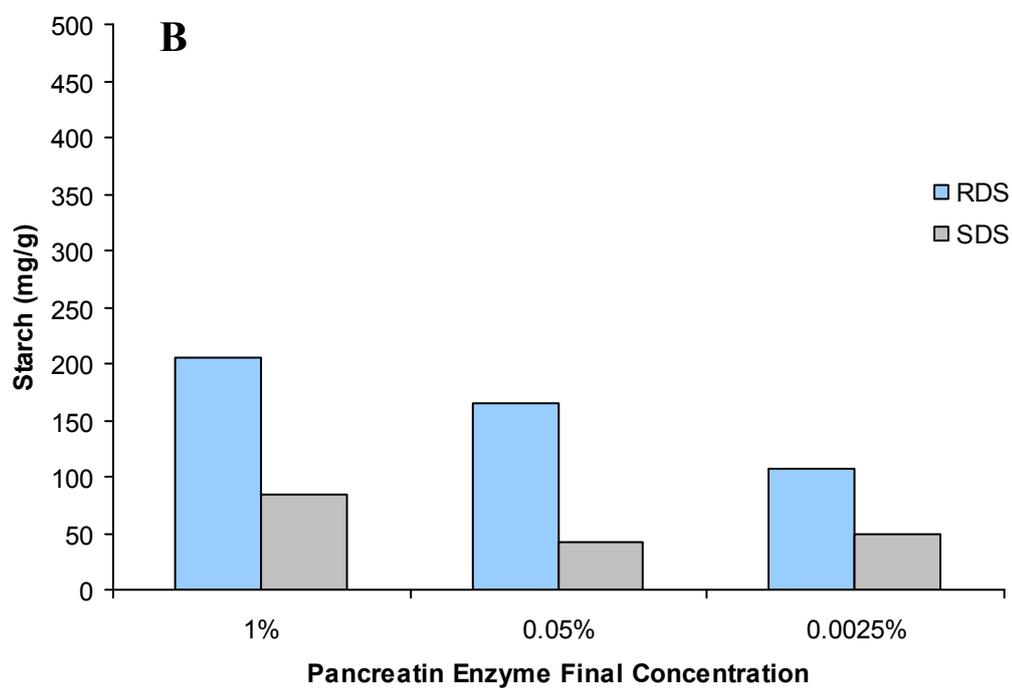
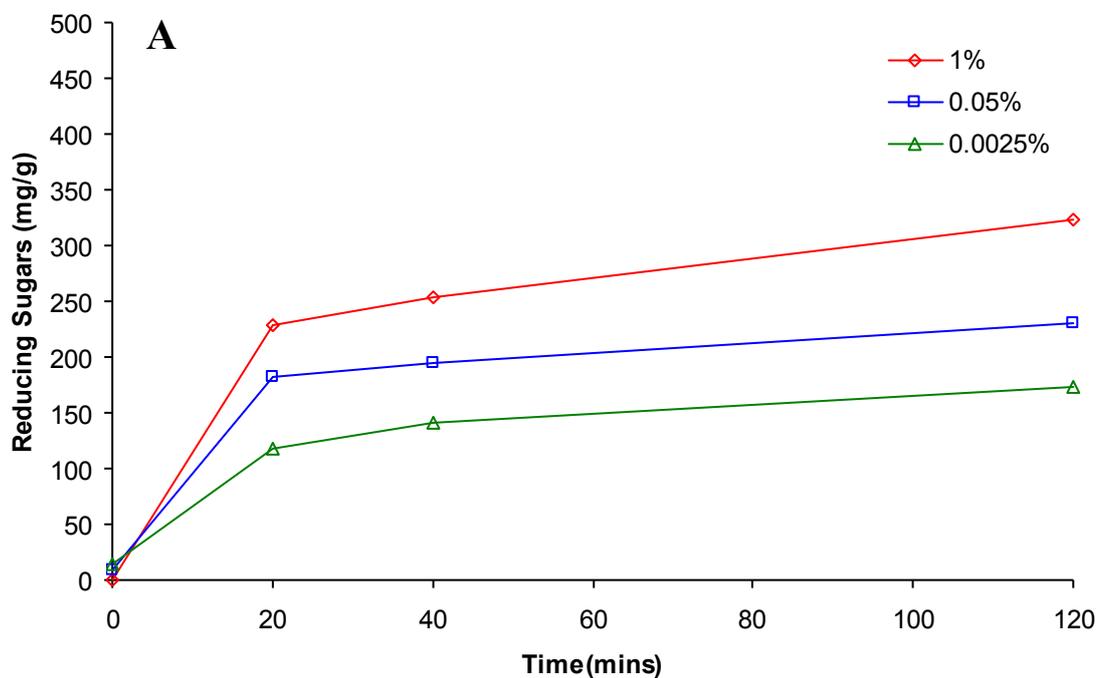


Appendix G1 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in bread samples digested *in vitro* following differing levels of exposure to pepsin during the gastric phase.

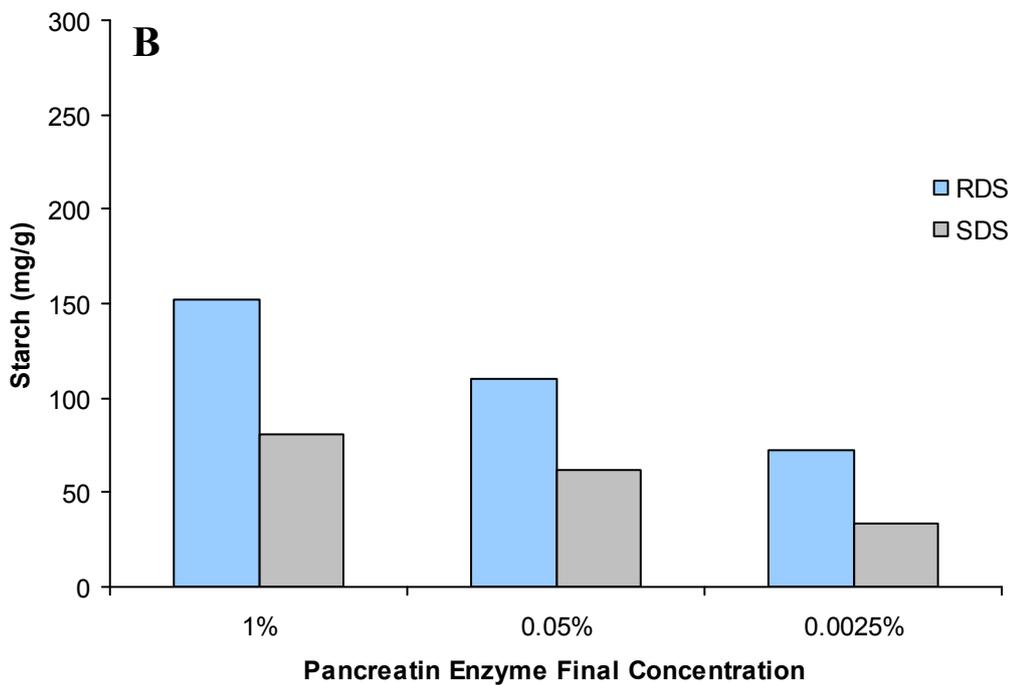
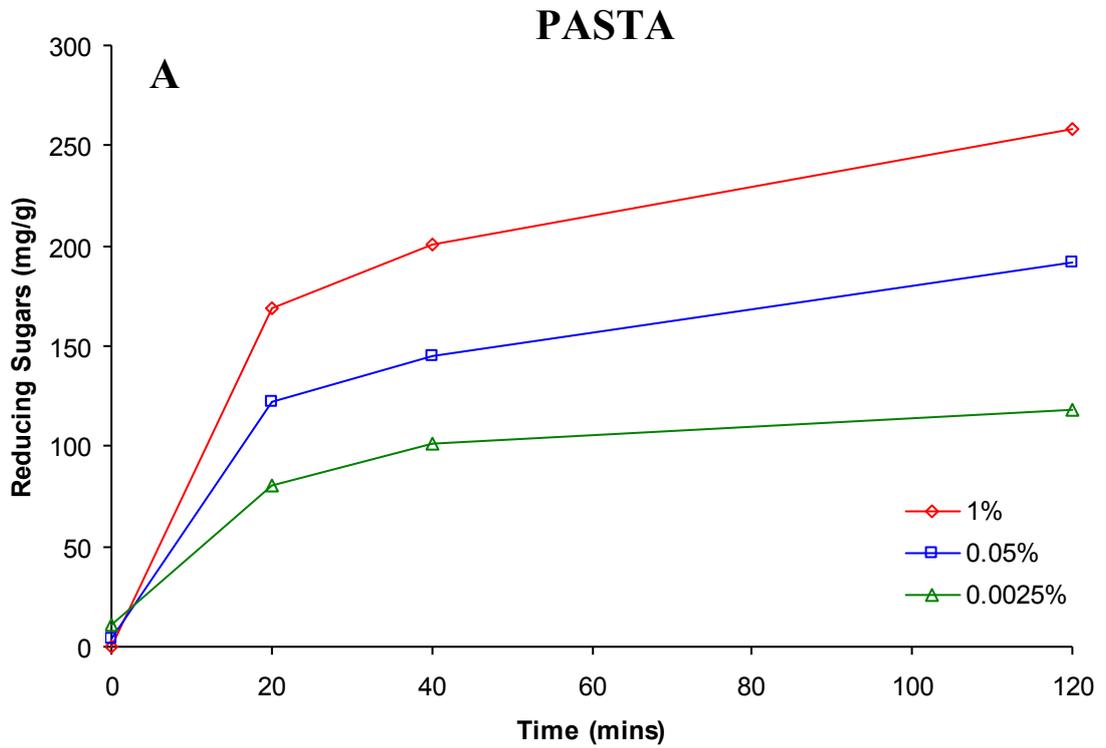


Appendix G2 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in wheat samples digested *in vitro* following differing levels of exposure to pepsin during the gastric phase.

WHEAT

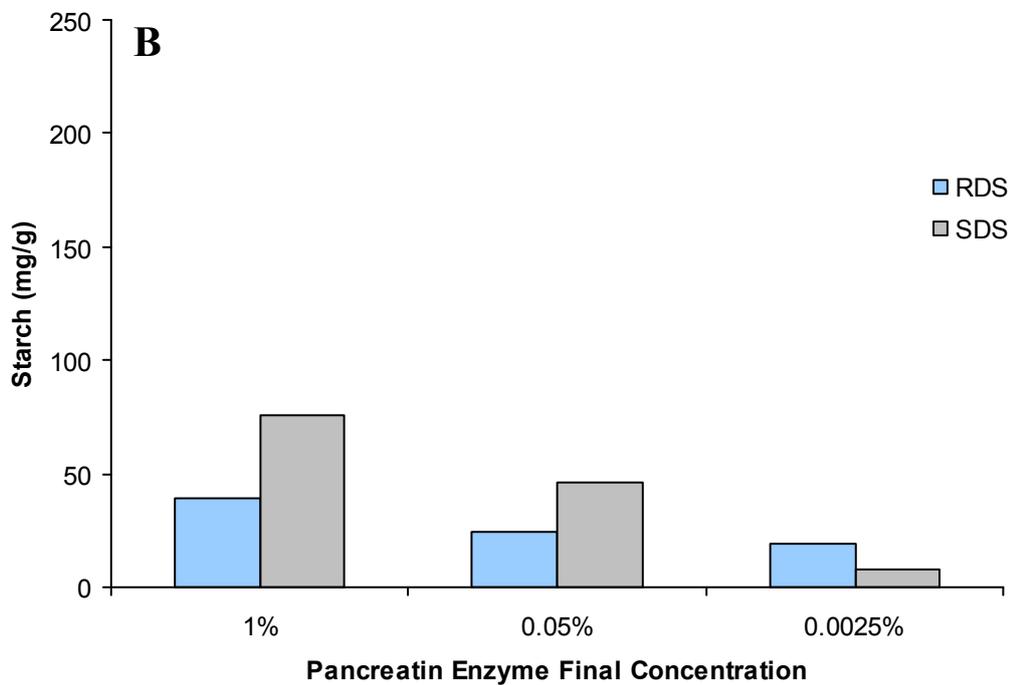
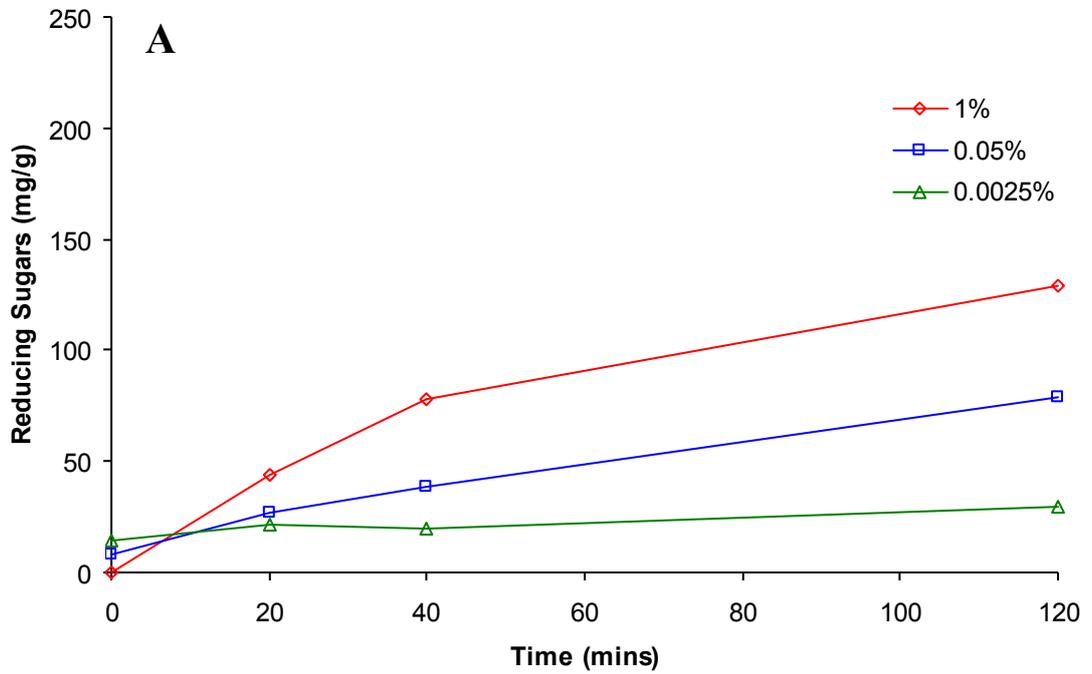


Appendix H1 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in wheat samples digested *in vitro* using differing final concentrations of pancreatin enzyme.

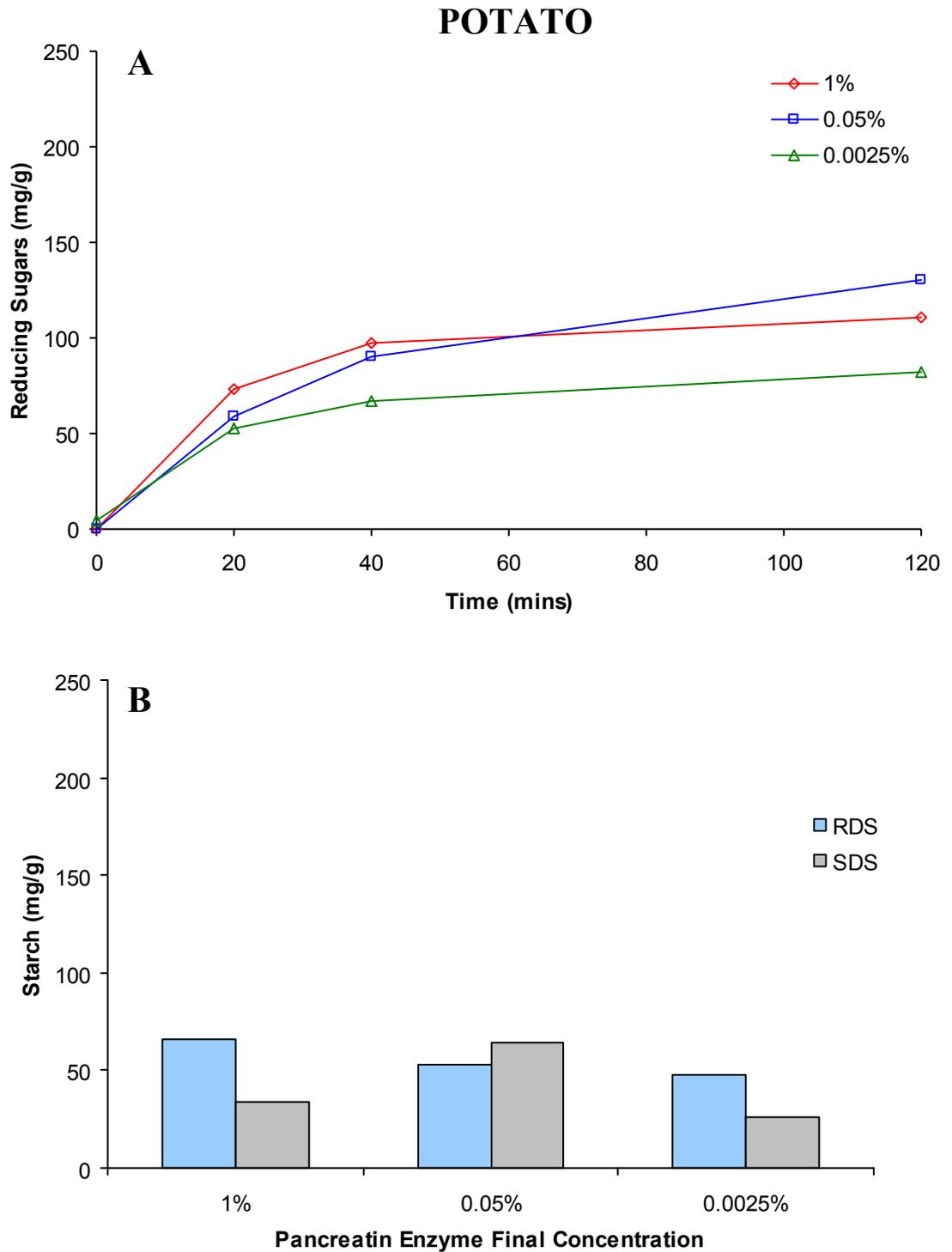


Appendix H2 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in pasta samples digested *in vitro* using differing final concentrations of pancreatin enzyme.

CHICKPEA

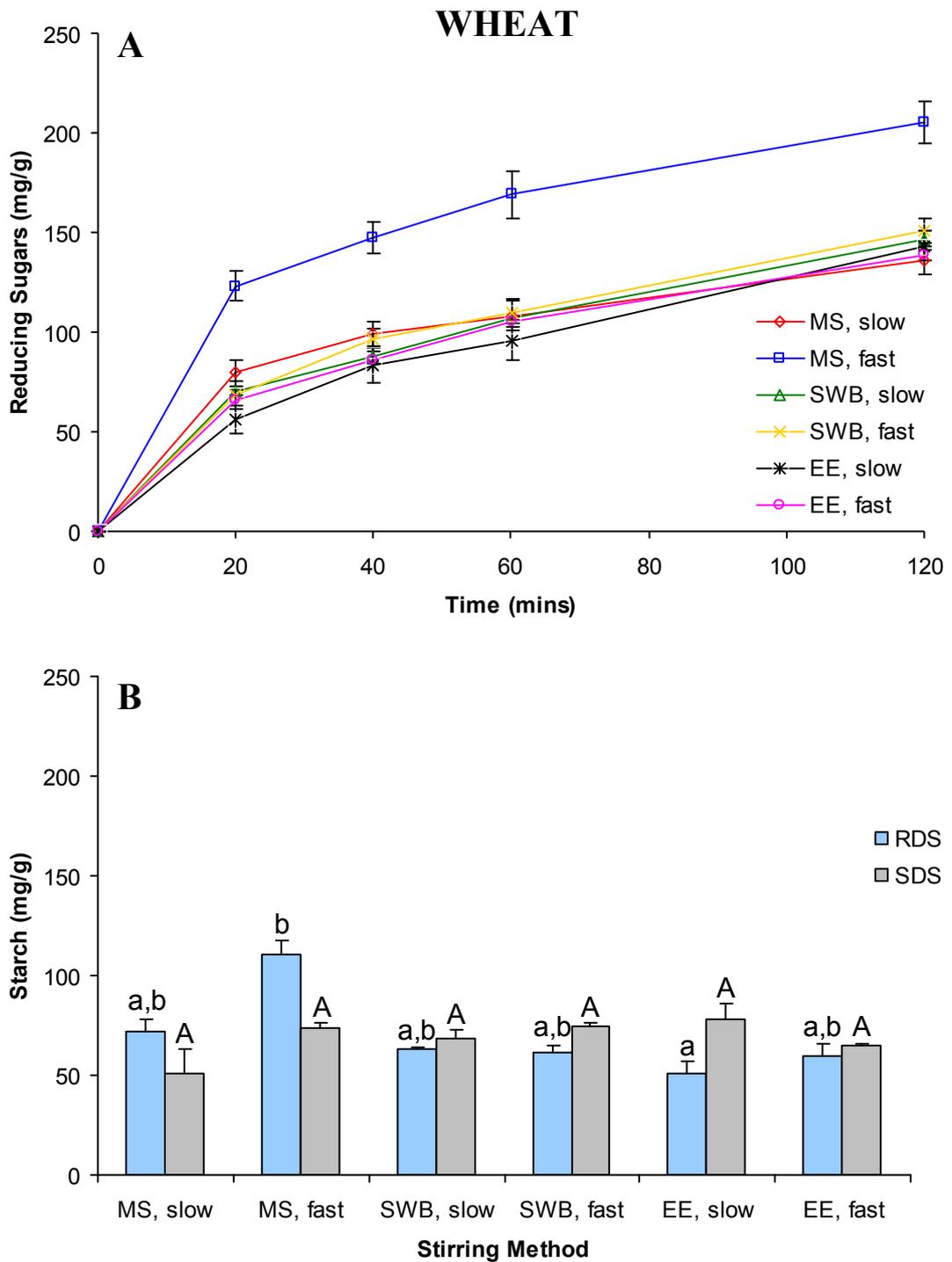


Appendix H3 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in chickpea samples digested *in vitro* using differing final concentrations of pancreatin enzyme.

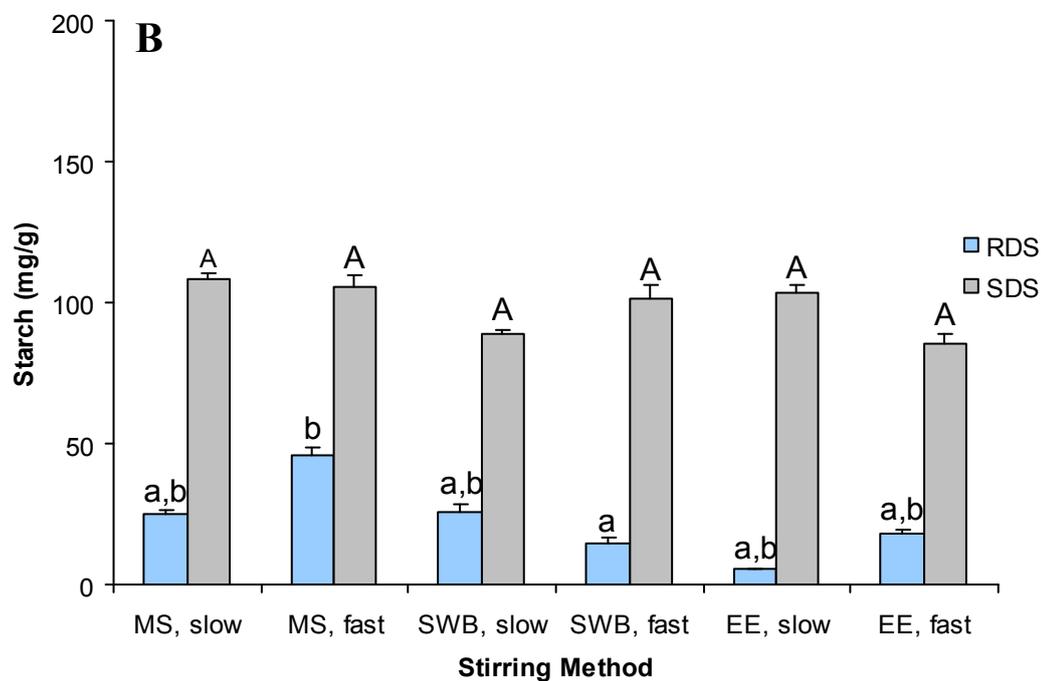
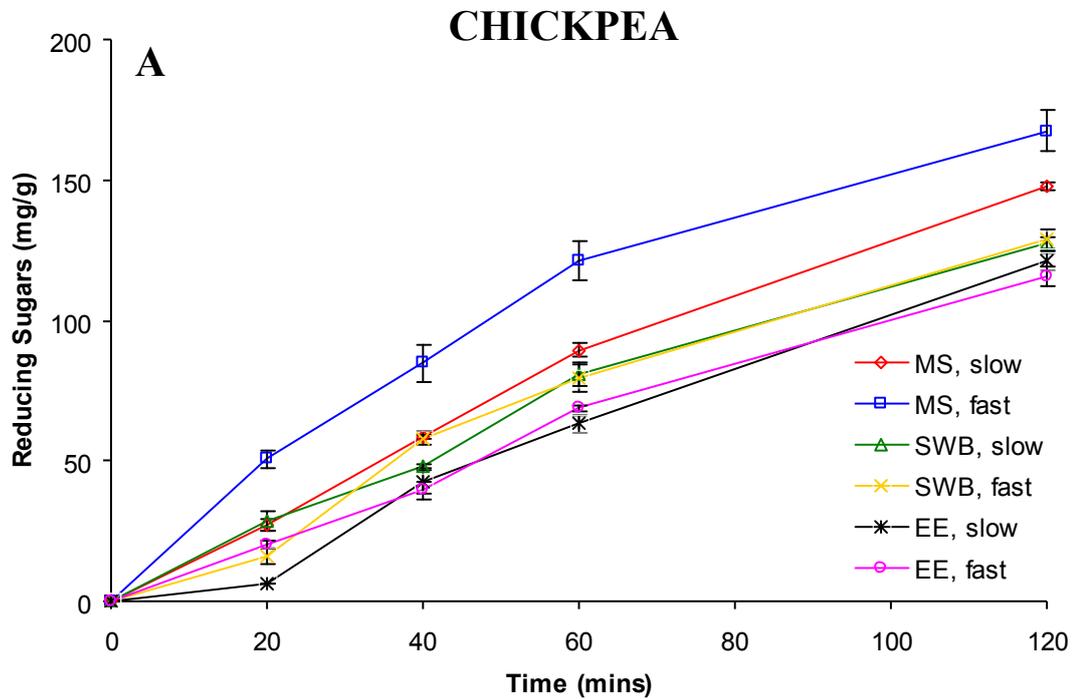


Appendix H4 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in potato samples digested *in vitro* using differing final concentrations of pancreatin enzyme.

APPENDIX II **Stirring Method – Wheat**



Appendix II Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in wheat samples digested *in vitro* using different incubatum stirring/agitation apparatus, set at slow and fast speeds. MS, magnetic stirrer; SWB, shaking water bath; EE, end-over-end inversion. Values are means (n = 3) ± SE.



Appendix I2 Starch hydrolysis curves (A) and relative RDS/SDS levels (B) measured in chickpea samples digested *in vitro* using different incubatum stirring/agitation apparatus, set at slow and fast speeds. MS, magnetic stirrer; SWB, shaking water bath; EE, end-over-end inversion. Values are means (n = 3) ± SE.

APPENDIX I3 **Stirring Method – Statistical Analysis Overview**

There was a treatment × time effect for bread ($p = 0.001$). ReS levels did not differ between treatments across the entire digestion except at 40 min where ReS was higher in SWB slow samples compared with EE slow ($p = 0.018$). RDS and SDS determinations did not differ with stirring method or speed.

There was a treatment × time effect for wheat ($p < 0.001$). ReS levels did not differ between treatments across the entire digestion except at 20 min where ReS was higher in MS fast samples compared with EE slow ($p = 0.011$). RDS was higher in MS fast wheat samples compared with EE slow samples ($p = 0.011$). Wheat SDS values did not differ with method or speed of stirring.

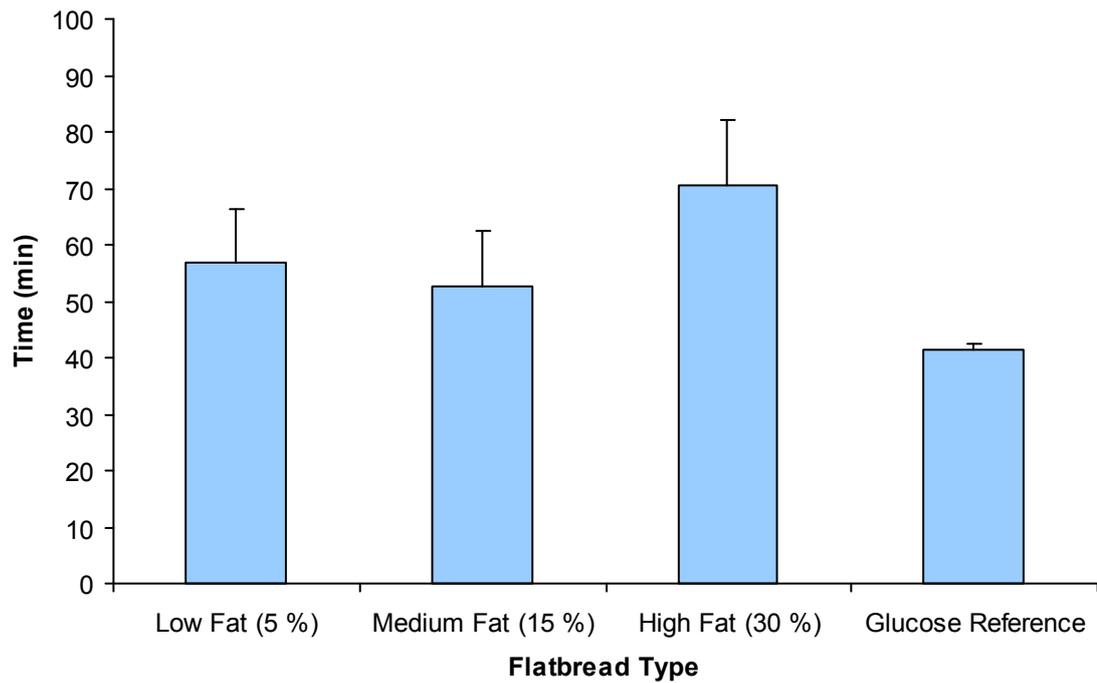
There was a treatment × time effect for pasta ($p < 0.001$). At 20 min of digestion, ReS was higher in MS fast samples compared with both EE slow ($p = 0.01$) and SWB fast samples ($p = 0.018$). At 40 min of digestion, ReS was higher in MS slow samples compared with EE slow ($p = 0.016$), and ReS was higher in SWB fast samples compared with EE fast ($p = 0.016$). At 60 min of digestion, ReS concentration was higher in MS fast samples compared with SWB fast ($p = 0.025$). At 120 min of pancreatic digestion, ReS was higher in MS fast samples compared with EE slow ($p = 0.009$). RDS was higher in MS fast samples compared with EE slow ($p = 0.01$) and SWB fast ($p = 0.018$). SDS was not affected by stirring method or stirring speed.

There was a treatment × time effect for chickpea ($p < 0.001$). At 20 min digestion, ReS was higher in MS fast samples compared with SWB fast ($p = 0.037$). At 40 min digestion, ReS was higher in MS slow samples compared with SWB slow ($p = 0.012$). At 60 min digestion, ReS was higher in SWB slow chickpea samples compared with EE slow ($p = 0.041$). Chickpea RDS was higher in MS fast samples compared with SWB fast ($p = 0.037$). SDS was not affected by stirring method or speed.

There was a treatment × time effect for potato ($p < 0.001$). At 40 min of pancreatic digestion, ReS concentration was lower in EE slow potato samples compared with MS slow ($p = 0.05$), SWB fast ($p = 0.023$) and EE fast samples ($p = 0.016$). By 60 min of digestion, ReS was still lower in EE slow samples compared with MS slow ($p = 0.019$)

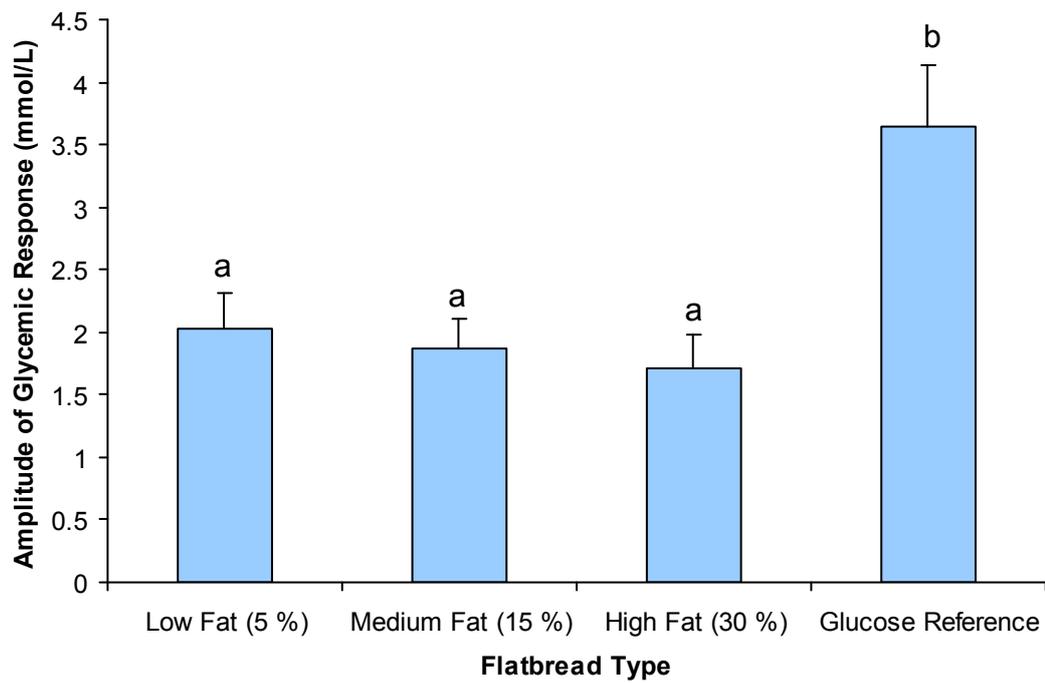
and EE fast ($p = 0.004$). RDS and SDS measures did not differ with method or speed of stirring.

APPENDIX J1 Time to GR Peak Following Flatbread Consumption

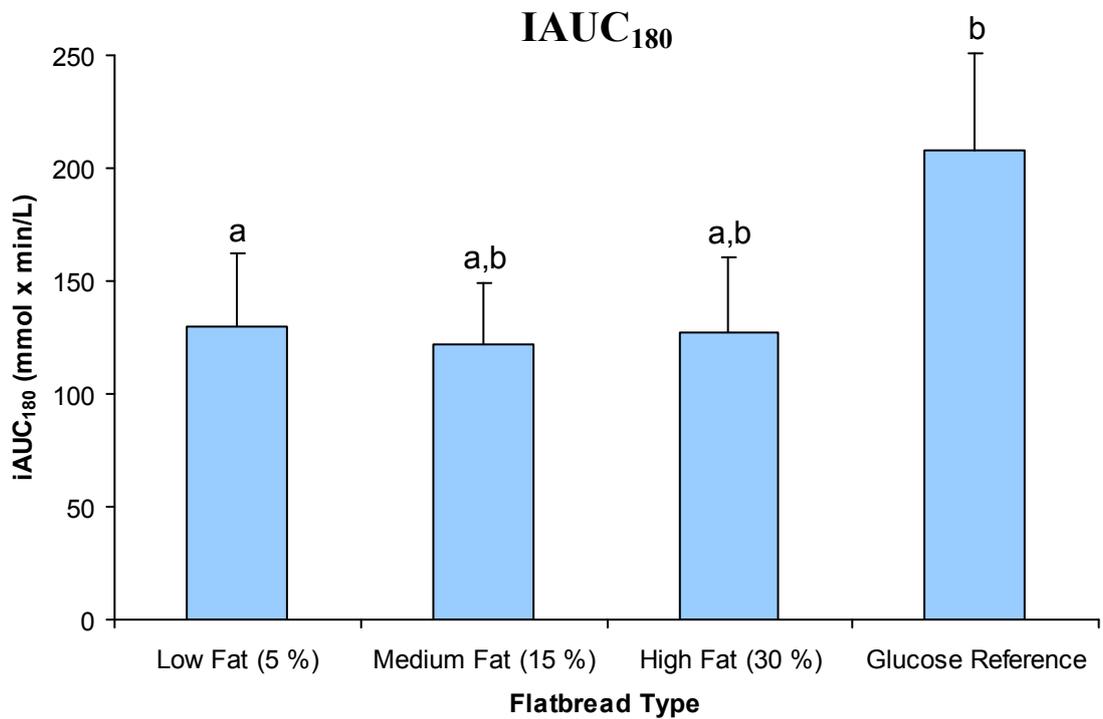
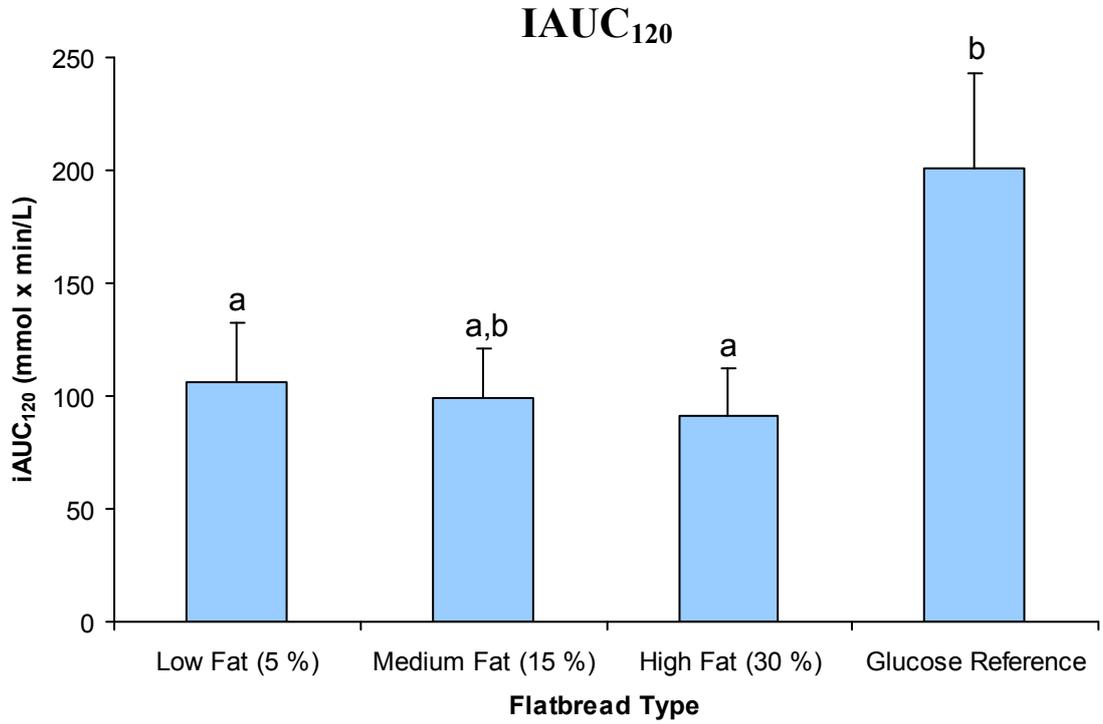


Appendix J1 Time taken for the blood plasma glucose response to reach its peak (T_{GRP}) following consumption of the three flatbreads and the glucose reference drink. Values are means ($n = 10$) + SE.

APPENDIX J2 Amplitude of the GR Curve Following Flatbread Consumption

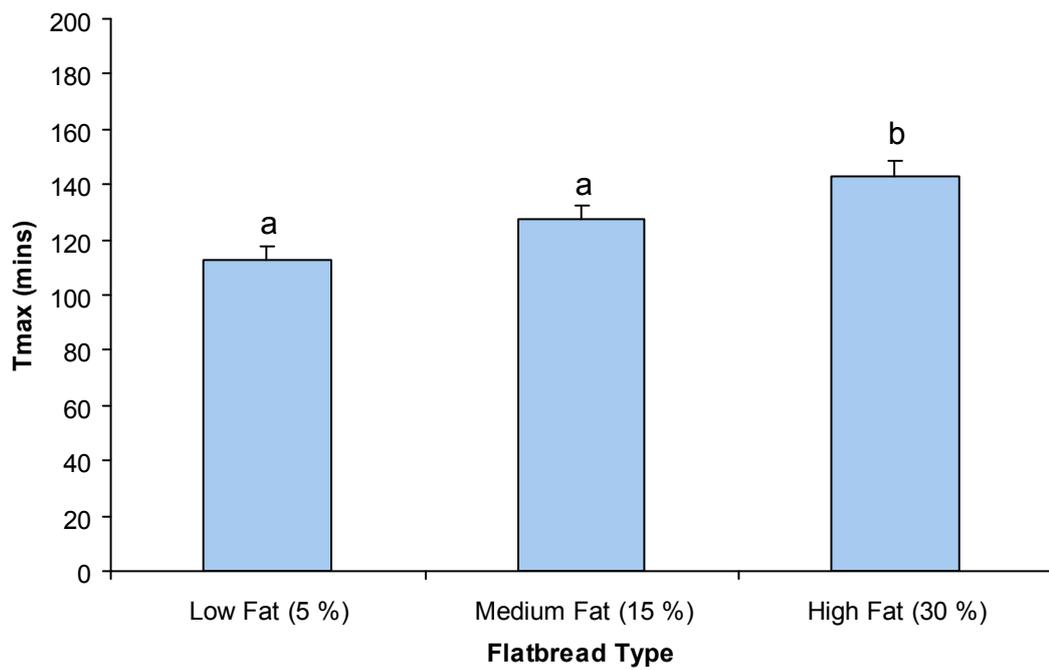


Appendix J2 Amplitude of the glucose response peak (A_{GR}) following consumption of the three flatbreads and glucose reference drink. Different letters denote a significant difference between treatments. Values are means ($n = 10$) + SE.



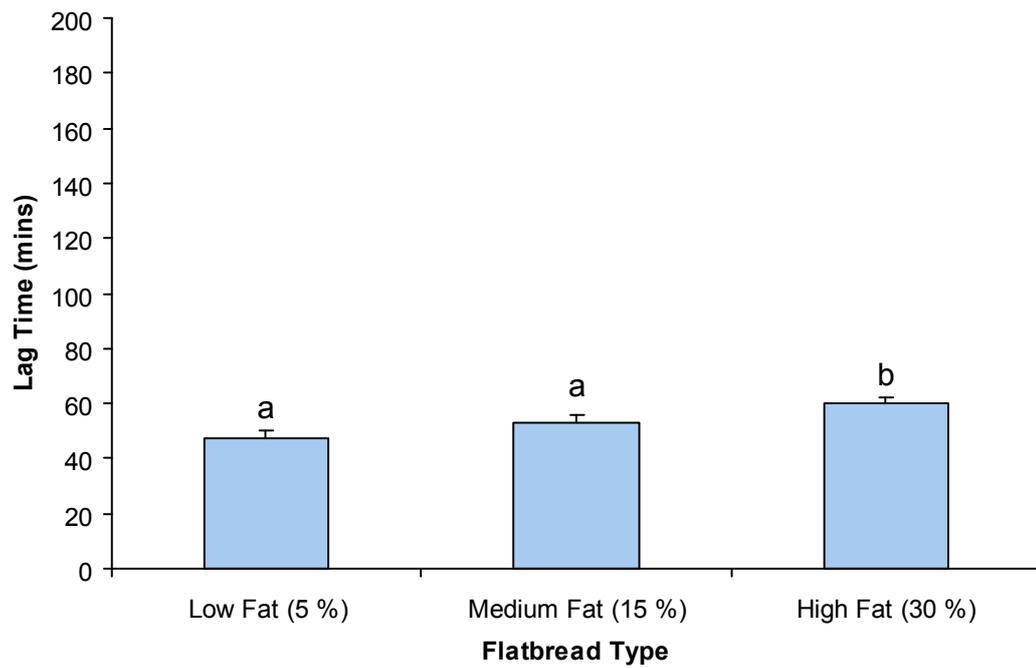
Appendix J3 IAUC_{120/180} of the GR curves following consumption of the three flatbreads and glucose reference drink. Different letters denote a significant difference between treatments. Values are means (n = 10) + SE.

APPENDIX J4 Peak ^{13}C Excretion Time for Each Flatbread



Appendix J4 Peak ^{13}C excretion time (T_{\max}) for each flatbread type. Different letters denote a significant difference between treatments. Values are means ($n = 10$) + SE.

APPENDIX J5 Gastric Emptying Lag Time for Each Flatbread



Appendix J5 Gastric emptying lag time (T_{lag}) for each of the three flatbreads. Different letters denote a significant difference between treatments. Values are means ($n = 10$) + SE.

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