

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**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

Massey University
Palmerston North Campus

James William Woolnough

2011

ABSTRACT

Global incidence of type II diabetes is driving the need for communication, via food-labelling, of the likely glycaemic effects of foods. *In vivo* methods for measuring the glycaemic 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 glycaemic 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 glycaemic 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, incubation 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 incubation 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 glycaemic response. The glycaemic 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 glycaemic response to each flatbread. Due to the lack of glycaemic effect *in vivo*, no adjustments to *in vitro* curves could be made.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my supervisors Dr John Monro, Professor Charles Brennan, Dr Tony Bird and Dr Alistair Carr, who, through their expert guidance and input as well as continual patience, understanding and support, have brought me through the entire of this most challenging PhD training exercise. Their rich experience as qualified researchers was certainly a source of inspiration to me as I learned what it was to become a researcher myself. This PhD entailed two migrations for my wife and I and I appreciate the extra moral support that my supervisors afforded us above and beyond what was expected of them as PhD supervisors. Many thanks also go to Dr Damien Belobrajdic and Dr Shusuke Toden who stepped up as unofficial supplementary PhD supervisors, providing me with much valued support, help and advice as well as great friendship, during the later stages of my thesis.

Thank you to Mr Bradley Klingner for providing my wife and I with much practical advice as we prepared to move to Adelaide as well as quickly familiarising me with the CSIRO research laboratories upon my arrival.

The human study comprising the second half of this PhD thesis could not have been conducted if it weren't for the assistance from the professional and very capable staff of the CSIRO nutrition research clinics. Thank you to Mrs Vanessa Courage for helping me in volunteer recruitment and liaison. Thank you to Mrs Lindy Lawson and Mrs Debbie Davies for their excellent blood sampling – not a single sample was missed despite the hectic sampling schedule. I'm immensely thankful to Mrs Sharyn Zrna and Mrs Jennifer Giles who faithfully arrived at work early each test day morning, assisting me in greeting my volunteers, looking after them as they progressed through each clinic visit as well as collecting all the breath samples. Of course a big thank you goes to the volunteers themselves who selflessly devoted much of their time to this study and who were so interested and enthusiastic about the application of the research in which they were involved.

I'd like to thank biostatisticians Mr Duncan Hedderley and Mrs Kylie Lange, who, with much patience, taught me how to conduct all the statistical analyses present in this thesis. I'd also like to thank Dr Zhong Khai Zhou who was more than happy to help me with the scanning electron microscopy of my samples.

The pursuit of this degree would have been all the more challenging if it weren't for the loving support I received from all of my family. My dear wife and friend, Jessica, stood faithfully by me and supported me every single day of this project. At least half of this degree belongs to her. I'd like to thank my parents, Keith and Eveline as well as my parents-in-law, Paul and Gretchen, for all of their support for Jessica and I these last four years.

James Woolnough

Ethics approval for the human study performed during the second phase of this thesis was granted by the CSIRO Human Research Ethics Committee on the 23rd of October, 2008. (Proposal number: 08/09).

Financial support for this research came from GlycANZ – an alliance between CSIRO Food and Nutritional Sciences and the New Zealand Institute for Plant and Food Research (formerly the New Zealand Institute for Crop and Food Research).

TABLE OF CONTENTS

PUBLICATIONS, PRESENTATIONS ARISING FROM THIS THESIS.....	viii
LIST OF FIGURES.....	x
LIST OF TABLES.....	xii
LIST OF ABBREVIATIONS.....	xiii

CHAPTER 1: INTRODUCTION

1.1 Dietary Carbohydrates.....	1
1.2 Carbohydrate Classification.....	1
1.2.1 Chemical Classification.....	1
1.2.2 Nutritional Classification.....	4
1.3 Carbohydrate Digestion.....	6
1.4 The Glycemic Response.....	8
1.5 Factors Affecting the Glycemic Response to Foods.....	8
1.6 Expressions of the Glycemic Response: Definitions.....	11

CHAPTER 2: LITERATURE REVIEW

PART I: SIMULATING HUMAN CARBOHYDRATE DIGESTION <i>IN VITRO</i>: A REVIEW OF METHODS AND THE NEED FOR STANDARDISATION.....	16
2.1 Introduction.....	16
2.2 Background.....	17
2.3 Current <i>In Vitro</i> Methods.....	18
2.3.1 Oral Phase.....	18
2.3.2 Gastric Phase.....	22
2.3.3 Small Intestinal Phase.....	23
2.4 The Need for Standardisation.....	30
2.5 Conclusion.....	34
PART II: THE EFFECT OF FAT ON THE GLYCEMIC RESPONSE <i>IN VIVO</i>: REFLECTING THIS EFFECT <i>IN VITRO</i>.....	35
2.6 The Effect of Fat <i>In Vivo</i>.....	35
2.7 The Effect of Fat <i>In Vitro</i>.....	36

2.8	Aligning <i>In Vivo</i> and <i>In Vitro</i> Data	37
------------	---	----

CHAPTER 3: MATERIALS AND METHODS

3.1	Phase 1: <i>In Vitro</i> Digestion Methods Investigation	39
3.1.1	The <i>In Vitro</i> Method Template, Test Foods and Calculations Used.....	39
3.1.2	Method Variables and Conditions Investigated.....	43
3.1.2.1	Mode of Comminution in the Oral Phase.....	44
3.1.2.2	Comparing Chewing and Chopping.....	45
3.1.2.3	Starch-Digesting Capacity of Salivary α -Amylase.....	45
3.1.2.4	Pepsin Inclusion and Omission.....	46
3.1.2.5	Starch-Digesting Capacity of Pancreatin (PG starch).....	48
3.1.2.6	Starch-Digesting Capacity of Pancreatin (test foods).....	48
3.1.2.7	Pancreatin and pH.....	48
3.1.2.8	Method of Stirring.....	49
3.2	Phase 2: Human Glycemic Index (GI) Study	50
3.2.1	Volunteers.....	50
3.2.2	Flatbread Test Foods and Glucose Reference.....	51
3.2.3	Testing Procedure.....	52
3.2.4	GI Calculation.....	52
3.2.5	Measurement of Gastric Emptying Rate.....	53
3.2.6	<i>In Vitro</i> Analysis of Flatbreads.....	54
3.2.7	Scanning Electron Microscopy of Flatbreads.....	54
3.2.8	Assessing the Strength of the Flatbread Starch-Fat Emulsion.....	55
3.3	Statistics	56

CHAPTER 4: RESULTS AND DISCUSSION

4.1	Phase 1: <i>In Vitro</i> Digestion Methods Investigation	58
4.1.1	Mode of Comminution in the Oral Phase.....	58
4.1.2	Comparing Chewing and Chopping.....	64
4.1.3	Starch-Digesting Capacity of Salivary α -Amylase.....	73
4.1.4	Pepsin Inclusion and Omission.....	82
4.1.5	Starch-Digesting Capacity of Pancreatin.....	87
4.1.6	Pancreatin and pH.....	91
4.1.7	Method of Stirring.....	93

4.2	Phase 2: Human Glycemic Index (GI) Study	99
4.2.1	Fat Dose and the Glycemic Response.....	99
4.2.2	Fat Dose and Gastric Emptying Rate.....	105
4.2.3	<i>In Vitro</i> Digestion Analysis of Flatbreads.....	112
4.3	Toward Standardisation	116
CHAPTER 5:	CONCLUSION	120
APPENDICES		124
Appendix A	Literature Review (Part I) Publication.....	124
Appendix B	Starch-Digesting Capacity of Salivary α -Amylase Publication.....	138
Appendix C	CSIRO Human Research Ethics Committee Approval Letter.....	151
Appendix D	Mode of Comminution in the Oral Phase Data.....	153
Appendix E	Comparing Chewing and Chopping Data.....	157
Appendix F	Starch-Digesting Capacity of Salivary Amylase Data.....	161
Appendix G	Pepsin Inclusion and Omission Data.....	167
Appendix H	Starch-Digesting Capacity of Pancreatin Data.....	169
Appendix I	Stirring Method Data.....	173
Appendix J	Glycemic Response/Gastric Emptying Data.....	177
REFERENCES		182

PUBLICATIONS, PRESENTATIONS ARISING FROM THIS THESIS

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

Volume 16 Supplement 3, Page S47, 2007

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
2011 – yet to be submitted

*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
2011 – yet to be submitted

LIST OF FIGURES

- Figure 1.1 Nutritional divisions of carbohydrates
- Figure 1.2 Carbohydrate digestion in the human upper gastrointestinal tract
- Figure 1.3 A typical glycemic response curve
- Figure 1.4 Nutritionally important starch fractions
- Figure 2.1 Evolutionary relationships of common *in vitro* digestion methods
- Figure 3.1 The *in vitro* carbohydrate digestion method template
- Figure 3.2 Method for DNS measurement of reducing sugars
- Figure 3.3 Experiment design to assess the starch-digesting capacity of salivary α -amylase
- Figure 4.1 Mode of comminution in the oral phase – BREAD *in vitro* digestion
- Figure 4.2 Mode of comminution in the oral phase – WHEAT *in vitro* digestion
- Figure 4.3 Mode of comminution in the oral phase – PASTA *in vitro* digestion
- Figure 4.4 Comparing chewing and chopping – BREAD *in vitro* digestion
- Figure 4.5 Comparing chewing and chopping – PASTA *in vitro* digestion
- Figure 4.6 Comparing chewing and chopping – WHEAT *in vitro* digestion
- Figure 4.7 Aligning digestion curves for PASTA following chewing or chopping
- Figure 4.8 Aligning digestion curves for WHEAT following chewing or chopping
- Figure 4.9 Salivary amylase pre-exposure – BREAD *in vitro* digestion
- Figure 4.10 Salivary amylase pre-exposure – PASTA *in vitro* digestion
- Figure 4.11 *In vitro* T₀ aliquots following salivary pre-exposure – BREAD/PASTA
- Figure 4.12 Salivary amylase pre-exposure – BREAD digestible starch fractions
- Figure 4.13 Pepsin inclusion and omission – PASTA *in vitro* digestion
- Figure 4.14 Pepsin inclusion and omission – CHICKPEA *in vitro* digestion
- Figure 4.15 Pepsin inclusion and omission – POTATO *in vitro* digestion
- Figure 4.16 Starch-digesting capacity of pancreatin – PG starch *in vitro* digestion
- Figure 4.17 Starch-digesting capacity of pancreatin – BREAD *in vitro* digestion
- Figure 4.18 Pancreatin and pH – PG starch *in vitro* digestion
- Figure 4.19 Stirring method – BREAD *in vitro* digestion
- Figure 4.20 Stirring method – POTATO *in vitro* digestion
- Figure 4.21 Stirring method – PASTA *in vitro* digestion
- Figure 4.22 Flatbread glycemic response curves

- Figure 4.23 Flatbread GI values
- Figure 4.24 Flatbread CPDR curves
- Figure 4.25 Flatbread gastric half-emptying times
- Figure 4.26 Illustration of fat separation from flatbread matrix
- Figure 4.27 Flatbread *in vitro* digestion curves
- Figure 4.28 SEM images of flatbreads during *in vitro* digestion
- Figure 4.29 Framework for the standardised *in vitro* method

LIST OF TABLES

Table 1.1	Chemical divisions of dietary carbohydrates
Table 2.1	Summary of the main <i>in vitro</i> starch digestion methods
Table 4.1	Total starch hydrolysed to glucose by salivary amylase
Table 4.2	Total starch hydrolysed to glucose plus dextrans by salivary amylase
Table 4.3	Summary of glucose response data
Table 4.4	Summary of gastric emptying data

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 _{1/2} GE	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