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Low-Viscosity Soluble Fibres for Individuals with Gastroparesis

A thesis submitted to Western Sydney University

in fulfilment of the requirements for the degree

Doctor of Philosophy (Medicine)

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Statement of Authentication

This thesis document is a requirement for the completion of a Doctor of Philosophy (Medicine) degree at the School of Medicine, Campbelltown Campus at Western Sydney University (WSU). The work presented in this thesis is, to the best of my knowledge, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

Signed:

HARSHA SURESH

March 2022

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I dedicate this document to the endless human pursuit of knowledge; may it never cease. To anyone or anything reading this document in posterity, this is what I have learned: Knowledge is leased from the past and lent to the future; it has no inherent value without observation. Knowledge that is given (numbers, graphs, data) can be taken away at a moment's notice, through circumstance; Knowledge that is earned through trial, error and application (insight) can never be taken from you.

Declaration

The Rotary Club of Devonport, Australian Rotary Health (ARH) and Western Sydney University (WSU) jointly funded my research scholarship for the period of my HDR candidature.

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I. Thesis Abstract

Gastroparesis is an idiopathic gastrointestinal motility disorder which affects around 120,000 people in Australia alone. A majority of sufferers are women (80%), with disease onset usually occurring in teenage or young adulthood. Sufferers of gastroparesis often experience debilitating symptoms such as nausea, vomiting, bloating, post-prandial fullness and delayed gastric emptying. Physicians and dietitians recommend severe dietary restriction for gastroparesis patients in order to minimise their day-to-day symptoms. Patients are told to avoid or minimise all forms of dietary fibre, both soluble and insoluble, because they exacerbate severe symptoms.

A typical healthy adult is supposed to source 25 – 30 grams of fibre from their daily dietary intake. Having a good intake of dietary fibre brings long-term health benefits such as blood glucose regulation, lowered cholesterol and good lower gastrointestinal (colonic) health. On the other hand, a lack of fibre in the diet can cause other gastrointestinal disorders, such as constipation, diarrhoea and irritable bowel syndrome (IBS), which gastroparesis patients suffer at higher rates than the normal population. Therefore, there is a pressing need for the inclusion of dietary fibre in the diet of gastroparesis patients.

This thesis tries to identify suitable "low-viscosity" type soluble dietary fibres in **Chapter 2** using rheology. Among the ten different soluble fibres tested, partially hydrolysed guar gum (PHGG) and gum Arabic displayed the most promising "low-viscosity" characteristics under simulated digestion. Therefore, they were selected for a pilot clinical study analysing their short-term effects in mild-to-moderate symptom gastroparesis patients (no enteral tube feeding).

The pilot study (n = 10) in **Chapter 3** was designed as a crossover study with PHGG and gum Arabic as test fibres, with "high-viscosity" psyllium husk as the positive control and water as the negative

control against a glucose challenge. The pilot study demonstrated that PHGG and gum Arabic were very tolerable, cause very few increases in symptoms (similar to water), while displaying glycaemic index lowering (low-GI) properties similar to the positive control psyllium husk. Therefore, both PHGG and gum Arabic were determined to be viable supplements for a future long-term study in gastroparesis patients.

The monomer components of major polysaccharides in the PHGG, gum Arabic and psyllium husk supplements were characterised in **Chapter 4** using a rapid (21.0 min) HPLC-ESI-Q-ToF-MS method that was specifically developed and validated. In total, separation of nine monomer sugar analytes was achieved using sample derivatization, with five monomers being identified and quantified in PHGG, gum Arabic and psyllium husk. The method displayed very good linearity $R^2 \geq 0.999$ and recoveries (96.22 – 109.49%). The developed method will be very useful for standardisation and labelling of commercial prebiotic soluble dietary fibre supplements.

In summary, a multi-disciplinary approach investigated the rheological behaviour, clinical effects, chemical composition and the potential viability of “low-viscosity” soluble fibre in gastroparesis patients. Future studies based on this thesis can include longer-term clinical investigations (≥ 3 months), with larger cohorts (*n* = 30), more extensive symptom monitoring, an investigation of changes in the faecal microbiome composition, cholesterol, and blood glucose concentrations. Additionally, the variability of monomer composition in “low-viscosity” type soluble dietary fibres and their subsequent effect on the release of beneficial short-chain fatty acids (SCFA) by colonic microbiome fermentation can also be studied.

II. Manuscripts from the Thesis

Chapter 2 has been published in the Q1 (Scimago) Journal *Nutrients.*

Suresh, H.; Ho, V.; Zhou, J.; Rheological Characteristics of Soluble Fibres during Chemically Simulated Digestion and their Suitability for Gastroparesis Patients. *Nutrients* **2020** *Aug 17*, *12*, 8, 2479. doi:10.3390/nu12082479. PMID: 32824535; PMCID: PMC7468937.

Chapter 3 has been published in the Q1 (Scimago) Journal *Nutrients.*

Suresh, H.; Zhou, J.; Ho, V.; The Short-Term Effects and Tolerability of Low-Viscosity Soluble Fibre on Gastroparesis Patients: A Pilot Clinical Intervention Study. *Nutrients* **2021** *Nov 28*, *13*, 12, 4298. doi:10.3390/nu13124298. PMID: 34959850; PMCID: PMC8704257.

Chapter 4 has been published in the Q2 (Scimago) Journal *International Journal of Food Properties*.

Suresh, H.; Mikhael, M.; Zhou, J.; Ho, V.; A HPLC-ESI-Q-ToF-MS Method for the Analysis of Monomer Constituents in PHGG, Gum Arabic And Psyllium Husk Prebiotic Dietary Fibre Supplements. *IJFP* **2022**, *25*, 1, 1650-1667. doi:10.1080/10942912.2022.2096064.

III. List of Presentations & Awards

1. Health Beyond Research & Innovation Showcase, 2019, Sydney, NSW, Australia.
2. 3-Minute Thesis, Runner-up Award, School of Medicine, 2019, WSU, Sydney, NSW, Australia.
3. American College of Gastroenterology Meeting, 2019 (ACG2019), San Antonio, TX, USA.
4. Australian Society for Medical Research (ASMR) Conference, 2021, Sydney, NSW, Australia.
5. Higher Degree Researcher's (HDR) Conference, 2021, GRS, WSU, Sydney, NSW, Australia.
6. Best Clinical Research – Paper Award, School of Medicine, 2022, WSU, Sydney, NSW, Australia.

IV. List of Chapter Sections

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VIII. List of Abbreviations

Low-GI	Glycaemic Index lowering macronutrient
SCFA	Short-chain fatty acids
MW	Molecular weight
DP	Degree of polymerisation
PHGG	Partially hydrolysed guar gum
UV-Vis	Ultra-violet to visible
Da	Dalton
kDa	Kilo-Dalton
Pa	Pascal
kPa	Kilo-Pascal
Dyn	Dyne
nm	Nanometre
cm	Centimetre
mm	Millimetre
pH	Potential of hydrogen
p-value	Probability value
MΩ.cm	Megaohm centimetre
^{o}C	Degree centigrade
h	Hour
min	Minute
s	Second
g	Gram
mg	Milligram

µg	Microgram
L	Litre
mL	Millilitre
µL	Microlitre
kJ	Kilojoule
Mol	Mole
mM	Millimole
w/v	Weight by volume
v/v	Volume by volume
SSF	Simulated salivary fluid
SGF	Simulated gastric fluid
SDF	Simulated digestion fluid
PPI	Proton-pump inhibitor
SD	Standard deviation
RSD	Relative standard deviation
ANMS GCSI-DD	The American Neurogastroenterology and Motility Society Daily Diary
ppm	Parts per million
mMol/L	Millimole per litre
iAUC	Interval area under the curve
PPF	Post-prandial fullness
HPLC	High-performance liquid chromatography
UPLC	Ultra-performance liquid chromatography
CSH	Charged surface hybrid
C18	Carbon-18

MS	Mass Spectrometry
Q-ToF	Quadrupole time of flight
HPLC-ESI-Q-ToF-MS	High-performance liquid chromatography electrospray ionisation quadrupole time of flight mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass to charge ratio
g (Italicised)	Gravitational force
rpm	Revolutions per minute
LOD	Limit of detection
LOQ	Limit of quantitation
C1-C6	Carbon positions on monomer sugar unit

Chapter 1

Introduction

1.1. Gastroparesis: Background and Etiology

"Gastroparesis", which is a combination of the scientific terms "gaster" (stomach) and "paresis" (partial paralysis) is an idiopathic gastrointestinal motility disorder. The primary characteristic of gastroparesis is delayed gastric emptying in the absence of mechanical obstruction, where the breakdown of the food bolus into the particles (with an average size of 2.0 mm) does not occur in a timely manner **[1]**. The pathophysiology of gastroparesis is yet to be determined, and no definitive cause for this debilitating condition has been established. The primary abnormality in gastroparesis is the autonomic neuropathy of the vagus nerve, accompanied by abnormal initiations and conduction of slow wave activity, which are vital for digestion, where on average, gastroparesis patients have fewer slow waves in the stomach compared to the general population (2.4 vs 3.0 cycles per min) **[2,3,4]**. Secondary abnormalities in the autonomic nervous system, enteric neurons, smooth muscle cells and interstitial cells of Cajal (ICC) are strongly linked to the expression of gastroparesis in patients **[5,6,7]**.

The worldwide prevalence of disorders of gut-brain interactions (DGBIs) such as gastroparesis is estimated to be around 40%, using the Rome IV diagnostic criteria **[8,9]**. In Australia alone there are a reported 120,000 sufferers of gastroparesis, an estimated 0.5% of the total population, with a majority of sufferers being female (80%) **[10]**. The onset of gastroparesis typically begins in adolescence, with a substantial number of patients developing gastroparesis after major gastrointestinal trauma such as infection, surgery, or physical injury **[11,7]**. In the USA, literature reports that between 2-4% of the total population may be affected by gastroparesis of varying degrees of severity **[12,13]**. The latter reports could be an over estimation since there is significant overlap and interchangeability in the pathogenic mechanisms and clinical expression of functional dyspepsia and diabetic gastroparesis **[14,15,16]**.

The major etiologies of gastroparesis are idiopathic (36%), diabetic (29%) and post-surgical (13%) **[12,5,17]**. Diabetes mellitus Type-1/Type-2 is the major co-morbidity in gastroparesis followed by

Parkinson's disease (7.5%) and Collagen vascular disease (5%) **[18,19,12]**. A smaller subset of gastroparesis patients suffer from neurological and vascular disorders such as Ehlers-Danlos syndrome, postural orthostatic tachycardia syndrome (POTS) and multiple sclerosis **[20,21,22]**.

1.2. Gastroparesis: Symptoms and Management

Gastroparesis patients experience a range of post-prandial symptoms due to delayed gastric emptying. Symptoms associated with gastroparesis include nausea, retching, post-prandial fullness, early satiety, bloating and distention **[12]**. Nausea, post-prandial fullness and bloating are major symptoms of particular interest since they are the most frequent symptoms after a meal **[4,23,6]**. Since gastroparesis currently has no known cure, symptoms from gastroparesis and other related disorders are managed through a combination of dietary modification, surgeries such as gastric per-oral endoscopic pyloromyotomy (G-POEM), gastric electric stimulation (GES) with standard and novel therapeutics **[24,25,26,27,28]**.

1.3. Dietary Fibres and the Gastrointestinal Function

Dietary fibre is a critical component in a healthy and balanced diet **[29,30,31]**. Dietary fibres are defined by Food Standards Australia New Zealand (FSANZ) as follows:

"Dietary fibre means that fraction of the edible parts of plants or their extracts, or synthetic analogues, that are resistant to the digestion and absorption in the small intestine, usually with complete or partial fermentation in the large intestine. Dietary fibre includes polysaccharides, oligosaccharides (degree of polymerisation >2) and lignins, and promotes one or more of the following beneficial physiological effects: (i) laxation; (ii) reduction in blood cholesterol; (iii) modulation of blood glucose" **[32]**.

This general definition of dietary fibres indicates that there are many different types of dietary fibre with differences in chemistry, solubility, rheological properties, physiological effects and technical applications **[33]**. An adequate daily intake of fibre has been associated with maintenance of good gut health, reduced risk of diabetes, heart disease and colorectal cancer **[34,35,36,37]**. The National Health and Medical Research Council (NHMRC) of Australia recommends that healthy adults should consume between 25-30 g of fibre daily as part of their balanced diet, with a 2-4 g allowance based on sex, age and pregnancy status **[32]**. Adolescents and children are recommended to consume between 14-28 g daily based on their sex and age **[32]**. Most adults, adolescents and children fail to meet their daily dietary fibre intake requirement **[38,39]**.

Shortfalls in dietary fibre intake, based on Burkitt's hypothesis, is associated with poor gastrointestinal health, increased risk of obesity, increased risk of cancer, increased risk of cardiovascular disease, and chronic gastrointestinal disorders such as constipation, diarrhoea and irritable bowel syndrome (IBS) **[40]**. Burkitt's hypothesis was mired in controversy in the initial years after it's proposal (1971). In the years since, the approach used by the hypothesis to investigate gastric diseases based on epidemiological observations and experimentation (i.e., transit studies) is now globally considered a model for public health policy **[40,41]**.

The two primary types of dietary fibre are soluble and insoluble, with both these types having an effect on gastric emptying **[42]**. Both types of dietary fibre are indigestible in the gastrointestinal tract but play an important role in gut health and motility. The addition of either or both insoluble and soluble dietary fibre has been shown to affect the viscosity of small intestinal digesta in simulated gastric conditions *in vitro*, which hypothetically, can have significant physiological implications **[43]**. In addition to this, different types of insoluble and soluble fibres have been speculated to either expatiate or attenuate the bio-availability of antioxidants, dietary lipids, carotenoids and micronutrients from

consumed fruits and vegetables *in vivo*, depending on their macro-molecular configurations, viscosity, and gelling properties **[44,45,46]**.

Most foods such as fruits, vegetables, grains, legumes and mushrooms contain a combination of both insoluble and soluble fibre in varying percentages. Insoluble dietary fibres, in general, have excellent laxative properties, providing "bulking" in the colon during stool-formation **[33]**. The "bulking" ability of insoluble fibre is superior to soluble fibre, especially in the context of constipation relief **[47]**. Insoluble fibres generally, barring a few exceptions like the alkaline-soluble hemicellulose and pectin components, unlike soluble fibres, are not fermentable (i.e., not broken down by gut microbiome) **[48]**. Another notable exception is resistant starch (RS1-RS4), which is known to have many physiological benefits similar to soluble fibre, but is resistant to digestion (i.e., indigestible) in the gastrointestinal tract **[49]**. There are recent arguments in literature that the fermentability of a dietary fibre is more clinically relevant than solubility **[50]**. In diabetic gastroparesis, the incorporation of soluble fibre into fibre-deficient diets could have significant benefits **[51]**. In this context, both insoluble and soluble fibres with higher viscosity are likely to cause severe gastric symptoms in gastroparesis patients **[52]**, while soluble fibres with lower viscosity may theoretically, be more tolerable in gastroparesis patients, providing the benefits of fermentability that insoluble fibres, in general, cannot.

Water-soluble dietary fibres such as psyllium husk, β-glucan, pectin and guar gum, unlike insoluble fibres, become 'sticky' and absorb water in the digestive system to form gel-like substances that aid in regulating blood glucose, attenuating cholesterol and reducing gastroesophageal reflux disease (GORD) symptoms **[53,54,55,56,57]**. The major benefits of soluble dietary includes reduced risk of post-prandial hyperglycaemia & hyperosmolar hyperglycaemic nonketotic syndrome (HNNS), and a prominent role as a glycaemic index (GI) lowering macronutrient (i.e., "low-GI") **[58,59,60,61,62,63]**.

Soluble fibres have also been shown to improve the metabolic profile in Type-1 and Type-2 diabetic patients **[64,65,66]**.

Soluble dietary fibres play a major role as a prebiotic supplement **[67]**. Short-chain fatty acids (SCFA) such as acetate, propionate, and butyrate are released into the colon by the gut microbiome through fermentation, especially by gram-positive bacteria such as *F.prausnitzii*, *Clostridium* cluster XIVa, *Bifidobacterium*. The release of SCFA has been proven to lead to better colonic health, reduced inflammation, insulin sensitivity, renal function and bodyweight control especially in patients with Type-1/Type-2 diabetes **[68,69,70,71,72,73,74,75,76,77,78]**. Supplementation of the soluble dietary fibre partially hydrolysed guar gum (PHGG) alongside routine medication has also been reported to help reduce small intestinal bacterial overgrowth (SIBO) **[79]**. The laxative effects of soluble fibres are more variable with respect to constipation, diarrhoea, and IBS **[80,81,82,83]**, and these effects depend largely on chemical structure and rheological behaviour **[84,53]**.

1.4. The Role of Dietary Fibre in Gastroparesis Patients

Despite known benefits, current dietary recommendations for gastroparesis patients suggests the avoidance or minimisation of all forms dietary fibre **[85]**. Risks for gastroparesis patients include exacerbation of symptoms such as nausea, bloating, abdominal pains and vomiting **[86,87,66,88,89]**. Dietary restrictions increase as gastroparesis patients begin to experience chronic delayed gastric emptying, worsening symptoms and disease progression **[5]**. Certain soluble fibres are shown to cause delayed gastric emptying, and this has been verified using ^{99m}Tc-sulphur colloid radio-labelled meals and gastric emptying scintigraphy **[90]**. As mentioned previously, lack of fibre in a balanced diet, can lead to poor gut health and microbiome dysbiosis **[91]**.

There is a pressing and unmet need for suitable dietary fibre candidates in gastroparesis patients, especially in patients whose disease condition has not progressed to the stage where they

require enteral tube feeding for nourishment **[86]**. An in-depth, multi-faceted approach to potentially suitable dietary modification is necessary due to the limited clinical research investigating the viability of soluble dietary fibre in gastroparesis patients.

1.5. Research Hypothesis, Aims and Outcomes

Hypothesis: Dietary modification using certain types of soluble fibre can improve dietary and clinical outcomes for gastroparesis patients

The research aim of this thesis is to test the above hypothesis by:

1. Investigating the background, causes and etiology of gastroparesis in the literature.

   Rationale: Identify knowledge gaps in literature regarding the use (or lack thereof) of dietary fibres in people with gastroparesis.

2. Study the rheological properties of ten different soluble dietary fibre supplements by:
   a. Characterising their viscoelastic properties using *in* vitro baseline water conditions
   b. Characterising their viscoelastic properties using *in vitro* simulated digestion conditions

   Rationale: To compare and study condition effects in order to select suitable candidate "low-viscosity" type soluble dietary fibres for a pilot clinical study involving gastroparesis patients.

3. Complete a short-term pilot clinical study of "low-viscosity" type soluble dietary fibres in gastroparesis patients by investigating:
   a. Primary endpoint 1: Short-term blood glucose regulation effects
   b. Primary endpoint 2: Symptom effects and tolerability
   c. Secondary endpoint 1: Delays in gastric emptying

Rationale: To assess whether the candidate fibres are suitable for a long-term study to evaluate their longer-term health benefits (or lack thereof) in gastroparesis patients.

4. Develop a validated mass spectrometry (MS) method to identify and quantitate known monomer sugar constituents in the polysaccharide chains of the clinically tested soluble dietary fibre candidates.

Rationale: To provide supplement manufacturers with a rapid, repeatable and reliable method for the labelling of monomer sugars in soluble dietary fibres during commercial manufacturing and batch testing. The MS method will set the groundwork for future clinical investigations exploring the role of monomer composition in SCFA release and colonic health.

5. *Studying the Outcomes*: The research outcomes of this thesis will provide a detailed summary regarding the potential viability of certain "low-viscosity" type soluble dietary fibres in gastroparesis patients. It will provide a knowledge basis for future clinical research involving dietary modification using soluble dietary fibres in people with gastroparesis.

A graphical abstract of the thesis is shown in **Figure 1.1**.

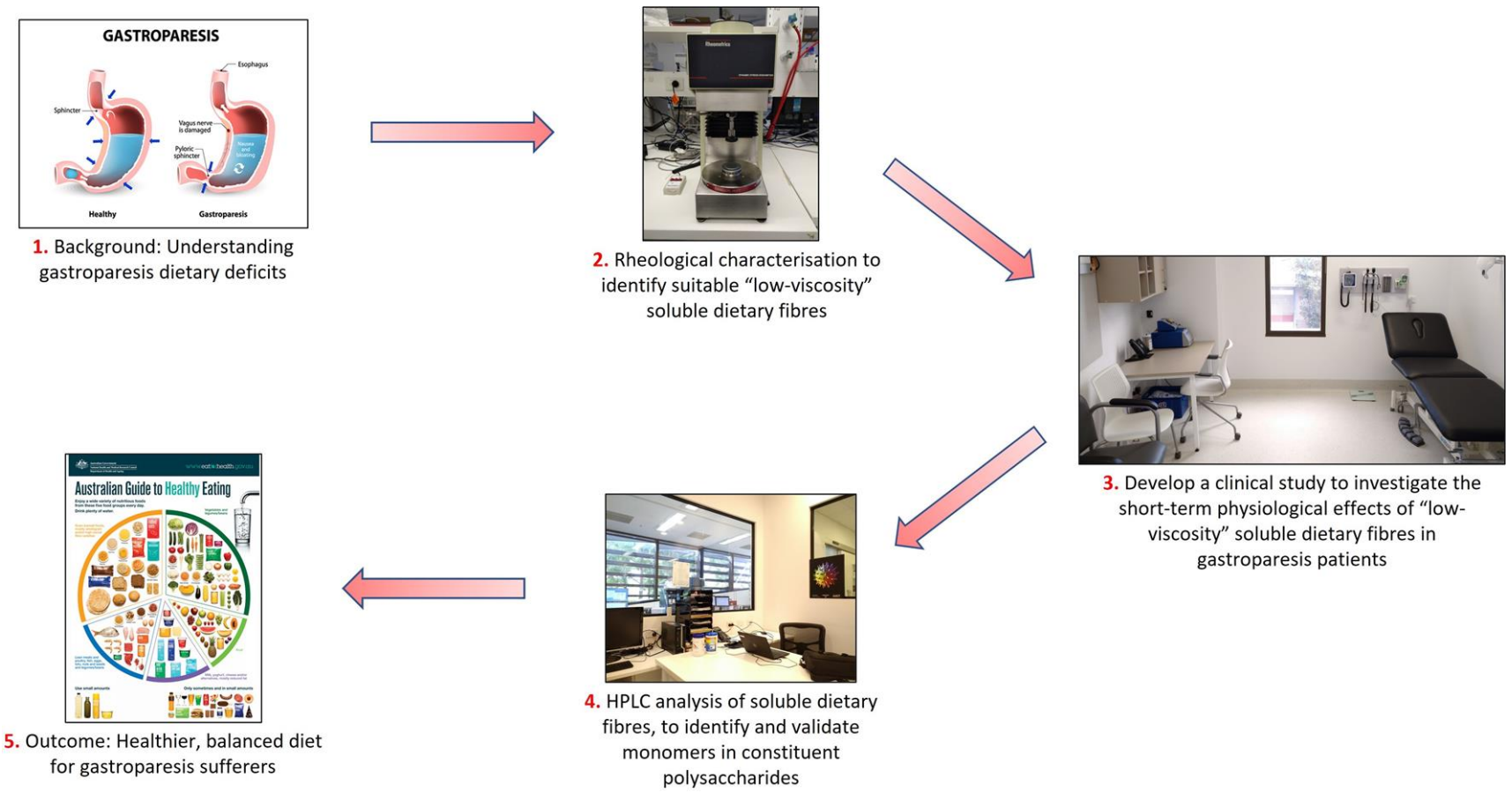


Figure 1.1. A graphical abstract of the research aims investigated in the thesis (graphical assets sourced from Gastroenterology Consultants, San Antonio, TX, USA; Western Sydney University (WSU) campuses, Sydney, NSW, Australia; National Health and Medical Research Council (NHMRC), Australia).

1.6. Rheological Evaluation of Soluble Dietary Fibre

Rheology is the study of the deformation or "flow" of matter when pre-determined amount of force is applied to it **[92]**. The field of rheology has a vast array of applications in food science, particularly in the study of semi-liquid foods and colloids, which are viscoelastic in nature, i.e., possessing the intermediate characteristics of solid and liquid phases, either Newtonian or non-Newtonian in nature **[93]**. The rheological behaviour of a soluble dietary fibre preparation *in vitro* or *in vivo* is primarily dependent on the structural chemistry of constituent polysaccharide components and their interplay with variables such as concentration, temperature and pH **[94,95,96]**. In particular, structural characteristics such as molecular weight (MW), degree of polymerisation (DP), chain length, branching and amphiphilicity greatly influence the extent of hydrogen bonding, covalent bonding and other thermodynamic interactions in solution, which in turn, influence the physical configuration of a polysaccharide in solution **[94,97,98,99]**.

A category of soluble fibres with reduced or low viscosity have recently gained significant clinical interest due to a hypothesised ease of gastrointestinal emptying with fewer accompanied symptoms **[100,101,102]**. Soluble fibres are often diluted, hydrolysed and enzymatically derivatized to reduce or lower the viscosity of food preparations or supplements. A prominent example of this is PHGG, which is the enzymatic hydrolysed derivative of guar gum. Soluble fibres with reduced viscosity have been used to alter the texture, rheology, taste and colour of manufactured food products and prebiotic supplements that assist in glucose regulation, body weight management and gut health **[103,104,105,106]**.

As mentioned earlier, different soluble fibres have different chemical and physical properties, and existing research has shown that varying degrees of physiological effects are produced in consequence **[107,108]**. Therefore, it is important that the rheological properties of a variety of soluble

dietary fibres are characterised simultaneously in simulated gastric conditions *in vitro*. This will enable the identification of suitable candidate soluble fibres with reduced or low viscosity for a pilot clinical study involving gastroparesis patients. An investigation of the rheological properties of ten different commercially available soluble dietary fibre supplements are presented in **Chapter 2**.

1.7. Clinical Investigation of "Low-Viscosity" Soluble Dietary Fibre

After the selection of suitable "low-viscosity" type soluble dietary fibre candidates based on the rheological data, a pilot clinical study was designed to investigate short-term post-prandial effects in gastroparesis patients. As noted earlier in **Section 1.2**, gastroparesis patients may suffer a multitude of severe symptoms when a consumed meal is unsuitable. The design of the clinical study needed to consider the severity of symptoms and the disease progression of the participants in the study **[24]**. To date, there have been no major clinical studies investigating the effects of soluble fibre, "low-viscosity" or otherwise in mild-to-moderate symptom gastroparesis patients (i.e., patients who do not require enteral tube feeding for dietary intake).

The pilot clinical study was designed with defined primary and secondary clinical endpoints, and the nuances of a clinical investigation in a gastroparetic population were considered **[109]**. The clinical design, data collection and analysis of the pilot study investigating the short-term effects of "low-viscosity" soluble fibre in gastroparesis patients is presented in **Chapter 3**.

1.8. Mass Spectrometry Method Development for the Analysis of Monomers in Soluble Dietary Fibre

The chemistry of polysaccharides present in different soluble dietary fibres vary not only in chain length, but also in monomer unit prevalence and composition. As previously mentioned in **Section 1.3**, variations in the constituent monomer components of soluble fibres polysaccharides are known to produce varied physiological and metabolic effects, affecting the proportions of SCFA release in the

colon **[110,111]**. In addition to this, some people with gastroparesis suffer from sugar intolerances and rare genetic disorders such as glucose-galactose malabsorption **[112]**. The gastrointestinal symptoms of people with such disorders or allergies could be worsened by the presence of glucose and galactose monomers commonly found in certain types of soluble dietary fibre.

Monomer analysis, standardisation and labelling in dietary fibre supplements needs to become an essential part of supplement manufacturing and batch processing. A validated monomer analysis method for polysaccharides found in "low-viscosity" soluble fibre supplements would provide manufacturers with a usable tool for batch processing, standardisation and labelling.

The analytical quantification of monomer sugar components in polysaccharides is a time consuming, laborious and potentially expensive process. Phytochemical commercial supplements containing biologically-active molecules such as phenols and flavonoids (< 1200 Da) can be easily extracted using organic/inorganic solvent combinations. Such compounds have detectable chromophores in the UV-Vis fluorescence range (200-750 nm) when analysed using mass spectrometry (MS) and commonly available carbon-18 (C18) columns.

Polysaccharides are much more difficult to extract due to their larger sizes (> 1200 Da), size-similarity and co-solubility with proteins (> 1200 Da) in aqueous phases **[113]**. In addition to this, monomer sugars such as glucose, mannose and others possess epimers (aldohexoses) or stereoisomers with no chromophores in the UV-Vis fluorescence range.

Various analytical techniques such as high-performance liquid chromatography (HPLC) **[114,115,116,117,118,119]**, gas chromatography mass spectrometry (GC-MS) and gas chromatography flame-ionization detection (GC-FID) **[120,121,122,123,124]**, capillary-gel electrophoresis (CE) **[125,126]** and thin-layer chromatography **[127,128,129,130]** have been employed in the detection and analysis of monomer sugar constituents in complex matrices such as mushrooms, plants and herbs.

Specialised detection techniques such as high-performance liquid chromatography refractive index (HPLC-RI) or high-performance liquid chromatography charged aerosol detection (HPLC-CAD) or hydrophilic interaction chromatography evaporative light scattering detection (HILIC-ELSD) can be used without the need to derivatize monomer sugar samples, but such detectors are highly specialised for sugar analysis and expensive to operate exclusively in laboratory settings **[131,132,119,133,134]**. The most sensitive technique among all the above is HPLC, which can assist in the separation of neutral, acidic and basic monomer constituents in complex samples such as dietary fibre supplements **[135]**.

Pre-column derivatization (or labelling) of monomer components creates chromophores, ensuring rapid detection and analysis **[124,116,136]**. Excellent derivatization methods in literature have described the analysis of monomer sugars in the fungi *P. umbellatus* **[114]** and the algae *S. fusiforme* **[115]** using the highly-sensitive HPLC ESI-MS/MS (Electrospray-Ionisation Mass Spectrometry) technique. While these recent methods are very detailed, they are not very rapid (180 min and 70 min respectively). Furthermore, there are few, if any, well validated methods available for the identification and quantification of monomer sugars in polysaccharide containing commercial dietary fibre supplements.

For the all the above reasons, a rapid HPLC method for the simultaneous detection and quantification of monomer sugars found in the polysaccharides of commercial “low-viscosity” soluble fibre supplements was developed. The detailed method validation is presented in **Chapter 4**.

Chapter 2

Rheology

2.1. Introduction and Rationale for Rheology

Based on the arguments set out in **Section 1.6**, the need for the *in vitro* rheological characterisation of certain commonly available soluble dietary fibre supplements is clear. Simultaneous rheological characterisations of multiple soluble dietary fibres can provide critical pre-clinical research information for gastroenterologists, dietitians and food supplement manufacturers. Such an assessment provides options for dietary modification not only in gastroparesis, but also, potentially, for other gastrointestinal conditions. It has been noted in the literature that early satiety, a symptom of gastroparesis, is better correlated with the viscosity of gastric-phase digestion rather than the viscosity of intestinal-phase digestion **[137,138]**. In addition to this, in general, the lack of pre-clinical dietary fibre characterisations have been noted in literature **[139]**.

Inconsistencies in the dietary fibre sample matrix, preparation viscosity, and incorporation methodology can cause unwanted variances during a clinical study, especially in the measurement of satiety-related symptoms. For example, gastroparesis and oesophageal dysmotility have high rates of co-morbidity (60%), where dietary fibres with lower viscosities may be more suitable for clinical investigation when potential adverse symptoms are considered **[91,140]**. In juxtaposition, medium or moderate viscosity dietary fibres are appropriate when a patient only has oesophageal dysmotility **[57]**. Therefore, a rigorous, simultaneous *in vitro* rheological assessment of multiple soluble dietary fibres is a prerequisite before suitable candidate fibres can be administered to gastroparesis patients *in vivo*.

The primary aim of this study was to determine the soluble fibres most suitable for a clinical study in gastroparesis patients. Therefore, the rheological properties of ten different soluble dietary fibres in distilled water (pH 7) and chemically simulated digestion fluid conditions (pH 4 and pH 2). The selected fibres had greatly varied molecular weights (MW), chemical structures, colloidal states and pH affinities. A broad selection of soluble dietary fibres applied a “fishnet” approach to commercially

available supplements to ascertain the optimal candidate test fibres for a clinical study. The detailed quantitative analysis of rheological behaviour and viscosity allowed for the qualitative assignment of the selected dietary fibres into the categories “high-viscosity”, “medium-viscosity” and “low-viscosity”. The optimal “low-viscosity” soluble dietary fibre candidates would demonstrate low yield-point shear stress values at high concentration (1000 mg/mL) in distilled water (pH 7) while showing no significant increase in yield-point shear stress ($p > 0.05$) during chemically simulated digestion fluid conditions (pH 4 and pH 2).

2.2. Materials and Methods

2.2.1. Instrumentation and Equipment

The primary instrument was the Dynamic Stress Rheometer (DSR)™ and CPU from Rheometric Scientific, Texas Instruments (Piscataway, NJ, USA) which included a Neslab RTE11 water bath and fibre Dry™ air-dryer from Pisco (Elmhurst, IL, USA). The 40.0 mm diameter (smooth, chrome-finished, aluminium) bottom plate, the 40.0 mm diameter (smooth, hard-coated, aluminium) upper plate, and the upper plate adapter (hard-coated, aluminium) used during rheological analysis were acquired from Rheometric Scientific, Texas Instruments (Piscataway, NJ, USA).

2.2.2. Chemicals and Reagents

The dietary fibre samples (powdered form) were purchased from various commercial suppliers in Australia and the labelled dietary information on the products is shown in **Table 2.1**. The following chemical standards with reported analytical purity were procured from multiple suppliers. Sodium chloride (99.7%), potassium chloride (99.0%), sodium bicarbonate (99.7%) and hydrochloric acid (32.0%) were purchased from Chem-Supply (Gillman, SA, Australia). Pepsin (99.0%) reference standard was procured from European Pharmacopoeia (Strasbourg, France), and α-amylase (99.0%) was purchased

from Sigma-Aldrich (St Louis, MO, USA). Purified de-ionized water used in the analyses (< 18 MΩ.cm) was obtained from a MilliQ™ Advantage A10 system with a Q-POD from Merck (Darmstadt, Germany).

Table 2.1. Labelled nutritional content of tested dietary fibre supplements.

Product (Name) Qty (per 100 g)	**Commercial Supplier (Name)**	**Total Dietary Fibre (%)**	**Energy (kJ)**	**Carbohydrates, Sugars (g)**	**Protein (g)**	**Total Fat, Saturated Fat (g)**	**Essential Nutrients (mg)***
Guar gum	Ceres Organics	77.3	743.0	0.5, 0.5	4.7	0.3, 0.0	13.0
Iota-carrageenan	The Melbourne Food Ingredient Depot	76.0	1298.0	76.0, 0.0	0.0	0.0, 0.0	640.0
Xanthan gum	Myprotein	62.0	832.0	78.0, 0.0	3.3	0.0, 0.0	0.0
Psyllium husk	SF Health Foods	80.0	802.0	0.0, 0.0	3.0	3.0, 0.0	79.0
Citrus pectin	Lotus Pantry	55.0	1005.0	30.0, 30.0	0.0	0.0, 0.0	1000.0
Beta-glucan	Blooms Health Products	44.0	1251.0	22.0, 0.0	20.0	5.0, 1.0	1088.0
Apple-fibre pectin	Myprotein	40.0	1850.0	90.0, 40.0	0.0	0.0, 0.0	0.0
Inulin	Myprotein	89.0	848.0	8.0, 8.0	0.0	0.0, 0.0	0.0
Gum Arabic	New Directions Australia	80.0	1339.0	80.0, 0.0	0.0	0.0, 0.0	0.0
Partially hydrolysed guar gum (PHGG) (Sunfiber™)	Healthy Origins (Taiyo International, MN, USA)	80.0	1691.0	93.0, 13.0	0.0	0.0, 0.0	0.0

(*) Essential nutrients are the cumulative Na, Mg, Ca, K, Fe and Zn present in the commercial fibre supplement in mg.

2.2.3. Experimental Procedure

The simulated salivary fluid (SSF) and simulated gastric fluids (SGF) were prepared in accordance with the Davis and Minekus methods reported in the literature **[141,142]**. Distilled water (pH 7) was collected in a 1 L Schott bottle. The SSF was prepared by accurately weighing out potassium chloride (0.4 mMol), sodium chloride (0.4 mMol), sodium bicarbonate (5.0 mMol) and α-amylase (2.0% *w/v*) into a 250 mL Schott bottle and made up to volume with 200 mL of de-ionized water. The SGF solutions were prepared by accurately weighing out sodium chloride (34.2 mMol) and pepsin (0.0525% *w/v*) into a 1 L Schott bottle. The SGF solution was adjusted to physiological gastric fluid pH 2 and made to volume with 1 L of de-ionized water. An additional SGF solution was made to pH 4 to the simulate gastric fluid condition of individuals on proton-pump inhibitors (PPI). It has been reported that approximately 60% of gastroparesis patients also suffer gastroesophageal reflux disease (GORD) **[91]**, and are therefore prescribed PPI, which reduces gastric acid production 100-fold and elevates the pH of released gastric fluid from 2 to 4 **[5]**. The SGF prepared at pH 4 was used to accurately represent the amount of gastric fluid released within these patients. The SGF solutions (pH 4 and pH 2) were created by the dropwise addition of dilute hydrochloric acid (6.4% *v/v*) and pH adjustment using a calibrated pH meter (Mettler Toledo, Port Melbourne, VIC, Australia). All the prepared solutions were then sonicated at 60 °C for 30 min and then cooled for 20 min. The sonication and cooling procedures were performed each time the solutions were used for analysis. The solutions were re-sealed and stored in a storage cabinet after each use with a 1-week expiry. Before analysis under the SDF-PPI (simulated digestion fluid proton pump inhibitor at pH 4) and SDF (simulated digestion fluid pH 2) conditions, the SSF and SGF solutions were sealed and placed in a water bath at 37 °C. The experimental rubric for the preparation of the dietary fibre samples is shown in **Figure 2.1**. Under each condition, rheological measurements were taken at 30 min after sample preparation. The sample beakers were sealed in aluminium foil and stored in a fume hood and then re-opened for rheological measurements at 60 min.

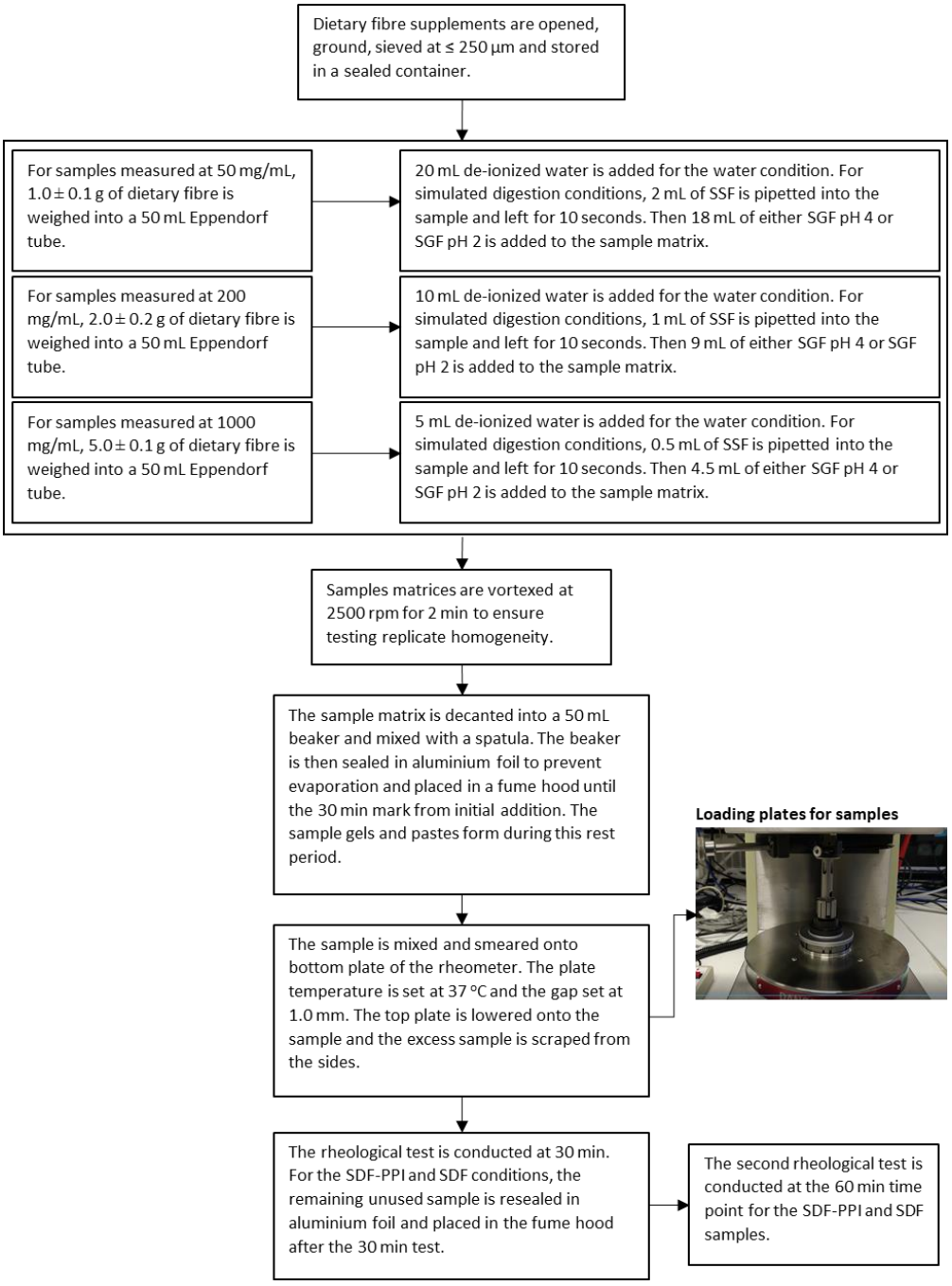


Figure 2.1. Procedural flowchart for sample preparation and rheological analysis. Keywords as follows: SSF (simulated salivary fluid), SGF (simulated gastric fluid), SDF-PPI (simulated digestion fluid proton pump inhibitor at pH 4), SDF (simulated digestion fluid at pH 2).

2.2.4. Rheological Method

The data acquisition and analysis were performed using the RSI Orchestrator v6.5.8 software from Rheometric Scientific (Piscataway, NJ, USA). The sample geometry was initialized using the stored geometry "[Para Plate] 40 mm dia PP Geometry P0019". The smooth parallel-plate geometry was selected for the rheological analysis since a large gap of 0.3–1.0 mm can be used to reduce shear during loading and axial stresses during oscillation, which is proven to be effective for larger particle-size colloidal gels and hydrocolloid pastes formed by dietary fibre preparations **[93,143,144]**. The plate diameter was set at 40.0 mm and the gap between the smooth parallel plates was set to 1.0 mm and auto calibrated. The minimum sample volume was 1.257 cm^3 and the tool serial number was 0019. The pre-defined "[DStresSwp] Dynamic Stress Sweep Test" test setup was used in the method.

2.2.5. Data Acquisition and Analysis

The dynamic stress sweep test was stress-controlled with a frequency of oscillation (ω) of 45.0 rad/s (7.28 Hz) and the sweep mode was linear. The rotation of the smooth parallel plates created the flow behaviour needed to measure the viscoelastic properties of the sample and the oscillation enables destruction free, highly precise movements that are used to measure within the sample's viscoelastic range. The lower plate temperature was set at 37 °C to match standard human biological temperature. The samples were analysed rapidly (1–2 min) to ensure that they did not dry out on the plate, leading to consistent measurement across all sample matrices. The measurement concentration, sample state at measured concentration, rheological behaviour type, shear stress increment, initial applied shear stress (min: 0.078 Pa), final applied shear stress (max: 3901.942 Pa) and measurement time period for each dietary fibre are shown in **Table 2.2**. The target sample concentration for each dietary fibre preparation was incrementally increased from 50 to 1000 mg/mL until a yield-point crossover was observed. For the

sake of analysis, measurements were taken at 50, 200 and 1000 mg/mL concentration thresholds to compare yield points among the selected soluble dietary fibres.

The collected rheological measurements were statistically fitted by the application of Hooke's law for viscoelastic materials **[145]**. The thixotropy of each soluble dietary fibres was determined by the application of the Cox-Merz rule, where the functional dependence of complex viscosity (η*) magnitude is expressed as a function of frequency (ω), which is identical to functional dependence of the steady shear viscosity (η) which is expressed as a function of shear rate (γ) **[146]**. The equation for the Cox-Merz rule **[147]** reads as:

$$|\eta^*(\omega)| = \eta(\gamma) \mid \gamma = \omega$$

After data acquisition, G' (the elastic modulus responsible for energy storage in the sample), G" (the viscous modulus responsible for energy loss in the sample) and Tan (δ) (the loss or damping factor at phase angle δ) were logarithmically plotted using the in-built RSI orchestrator software. At the initial applied shear stress, the behaviour of G' and G" was linear and these moduli were then deformed to produce the G^c (the crossover modulus) as oscillatory shear stress was incrementally increased over time. The yield-point shear stress (τ_y) and G^c at the phase transition (or sol-gel transition) point were interpolated in each plot for each sample where the following mathematical relationship occurs:

$$G' = G'' \mid \text{Tan}(\delta) = 1$$

The complex viscosity (η*) was also plotted against increasing oscillatory shear stress to determine the type of rheological behaviour. The yield points and G^c values were tabulated and analysed using Microsoft Excel (Office 2016). The p-values reported in the results and discussion section were generated using a two-tailed, homoscedastic (two sample equal variance) Student's t-test.

Table 2.2. Measured concentration, colloidal state, rheological behaviour, and method analysis parameters under parallel plate configuration.

Dietary Fibre (Name)	Measurement Concentration (mg/mL)	Sample State at Measured Concentration	Rheological Behaviour at Concentration	Shear Stress Increment (Pa/s)	Initial Applied Shear Stress (Pa)	Final Applied Shear Stress (Pa)	Measurement Time Period (s)
Guar gum	50.0	Colloidal gel	Pseudoplastic	30.0	400.0	1200.0	26.6
Iota-carrageenan	50.0	Colloidal gel	Pseudoplastic	25.0	400.0	1300.0	36.0
Xanthan gum	50.0	Hydrocolloid paste	Pseudoplastic	20.0	50.0	400.0	17.5
Psyllium husk	50.0	Hydrocolloid paste	Pseudoplastic	4.0	1.0	180.0	44.8
Citrus pectin	200.0	Hydrocolloid paste	Pseudoplastic	100.0	1500.0	3900.0	24.0
Beta-glucan	200.0	Hydrocolloid paste	Pseudoplastic	20.0	100.0	900.0	40.0
Apple-fibre pectin	200.0	Hydrocolloid paste	Pseudoplastic	1.0	1.0	100.0	99.0
Inulin	1000.0	Hydrocolloid paste	Pseudoplastic	8.0	20.0	240.0	27.5
Gum Arabic	1000.0	Hydrocolloid paste	Dilatant	0.4	10.0	50.0	100.0
Partially hydrolysed guar gum (PHGG)	1000.0	Hydrocolloid paste	Dilatant	0.4	10.0	40.0	75.0

2.3. Results

2.3.1. Rheological Plots

Rheological analysis of the soluble dietary fibres produced two distinct types of thixotropic phase transitions, pseudoplastic (shear-thinning) and dilatant (shear-thickening), which occur in non-Newtonian type viscoelastic gels and pastes. **Figure 2.2** and **Figure 2.3** show examples of each type of phase transition with arrows indicating the yield point. In both these figures, the elastic G' modulus, the viscous G" modulus, the complex viscosity (η*) and Tan (δ) are tracked as the shear stress is increased. The majority of soluble fibres took the form of a pseudoplastic (shear-thinning) phase transition shown in **Figure 2.2** at the sample concentration when the yield point was achieved, where initially G' is greater than G". As greater oscillatory shear stress (τ) is applied to the sample, G' gradually decreases, and after the yield-point crossover, G" is greater. Once the yield point is crossed, the complex viscosity (η*) decreases rapidly as shown in **Figure 2.2**, indicating a complete breakdown in the molecular associations of the constituent dietary polysaccharides and therefore the sample begins to the "flow" from this point onward with G" dominating the rheological behaviour.

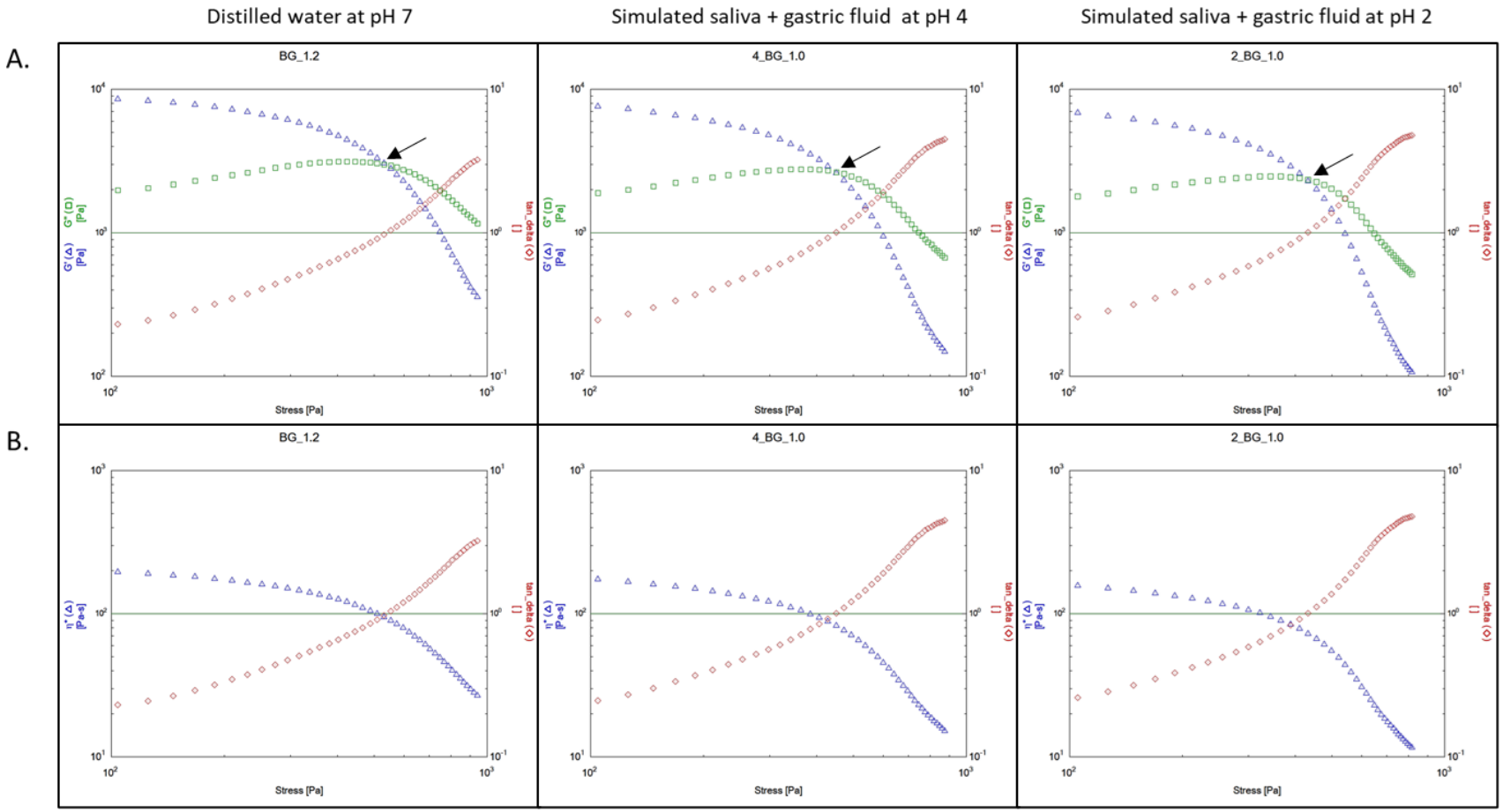


Figure 2.2. An example of pseudoplastic rheological behaviour observed in beta-glucan. Graphs show **(A)** rheological yield points (top half, y-axis left: G' and G" in Pa, y-axis right: Tan (δ), x-axis bottom: shear stress in Pa) and **(B)** complex viscosity (bottom half, y-axis left: η* in Pa.s, y-axis right: Tan (δ), x-axis bottom: shear stress in Pa) plots as shear stress is incrementally increased in distilled water (pH 7), simulated digestions with SDF-PPI (simulated digestion fluid proton pump inhibitor at pH 4) and SDF (simulated digestion fluid at pH 2). The black arrow in each plot indicates the yield point where Tan (δ) = 1. Note that the rheological behaviour does not change under chemically simulated digestion.

For partially hydrolysed guar gum (PHGG) and gum Arabic at 50 mg/mL, G" is greater than G' with no yield point observed in the acquisition range for shear stress (0.078–3901.942 Pa). This type of viscoelastic behaviour at low concentration is almost entirely viscous and Newtonian since the fibre is completely dissolved in solution. This viscous Newtonian behaviour at 50 mg/mL was also found in the dietary fibres which produced pseudoplastic yield-point crossovers at 200 and 1000 mg/mL. As the concentrations of PHGG and gum Arabic preparations were increased to 1000 mg/mL phase transitions and yield points were observed as shown in **Figure 2.3**.

As the shear stress (τ) was increased, the complex viscosity (η*) of the sample increased until a steady-state was reached, indicating complex shear behaviour that is initially dilatant (or shear thickening). The type of behaviour where the G' contribution is higher than the G" contribution is a non-Newtonian, thixotropic characteristic of gum Arabic and PHGG in the distilled water (pH 7), which is consistent with previous rheological studies of these fibres **[148]**. The dilatant rheological behaviour of gum Arabic and PHGG remained relatively consistent during simulated digestion. Representative rheological plots of the eight other soluble dietary fibres are shown in **Appendix A1 – A8**.

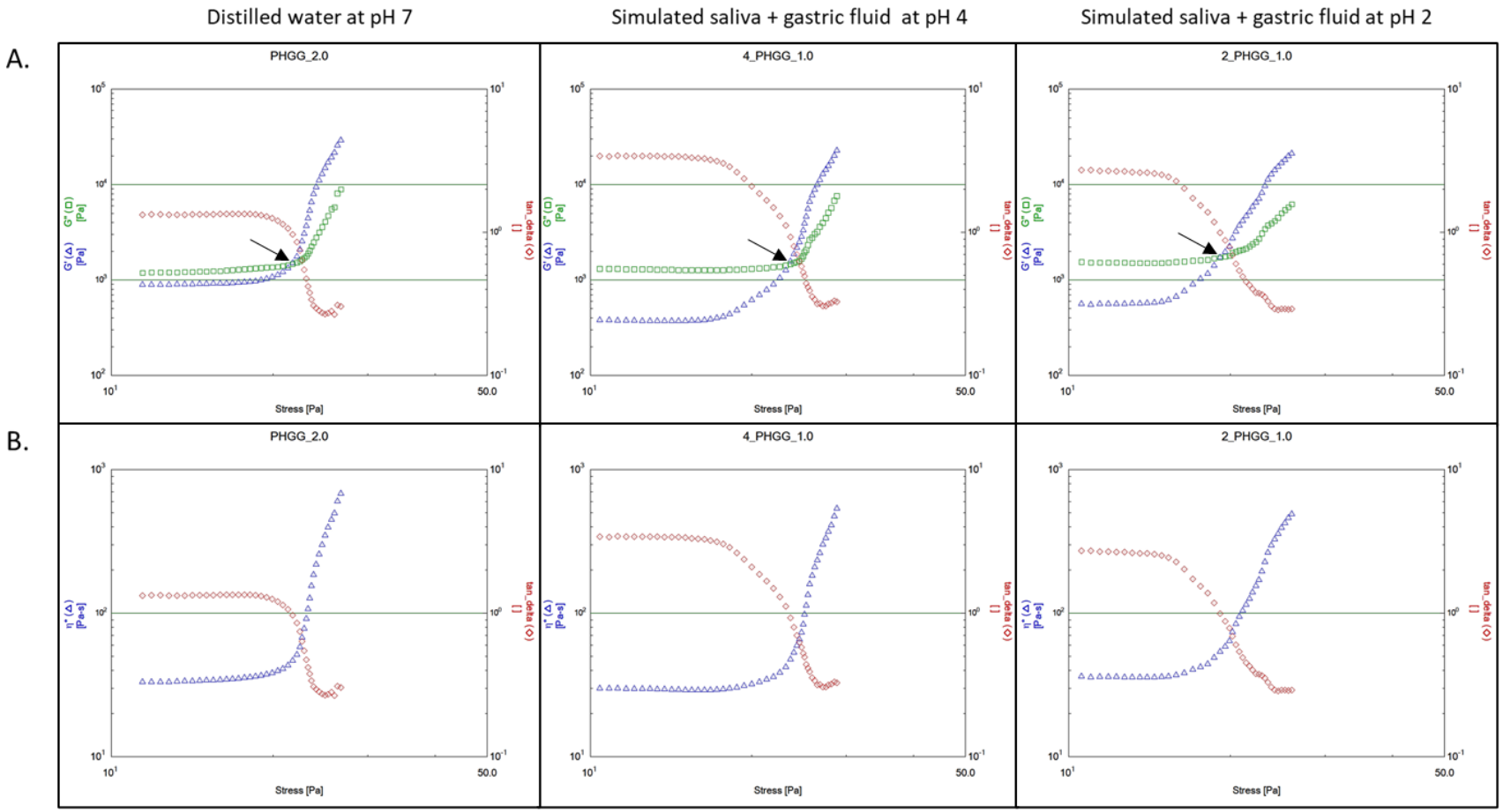


Figure 2.3. An example of dilatant rheological behaviour observed in PHGG. Graphs show **(A)** rheological yield points (top half, y-axis left: G' and G" in Pa, y-axis right: Tan (δ), x-axis bottom: shear stress in Pa) and **(B)** complex viscosity (bottom half, y-axis left: η* in Pa.s, y-axis right: tan (δ), x-axis bottom: shear stress in Pa) plots as shear stress is incrementally increased in distilled water (pH 7), simulated digestions with SDF-PPI (simulated digestion fluid proton pump inhibitor at pH 4) and SDF (simulated digestion fluid at pH 2). The black arrow in each plot indicates the yield point where Tan (δ) = 1. Note that the rheological behaviour does not change under chemically simulated digestion.

2.3.2. Rheological Yield Points

The tabulated summary of the yield-point shear stress (τ_y) and crossover modulus (G^c) values under distilled water and simulated digestion are shown in **Table 2.3**. The sample measurements were acquired from sequential samples prepared in triplicate at both 30 and 60 min. The percentage relative standard deviation (%RSD) values across all measurements for yield shear stress measurements does not exceed ±12.81% (Citrus pectin in SDF-PPI). The %RSD values across all sample measurements for crossover modulus (G^c) does not exceed ±11.80% (Apple-fibre pectin in distilled water). The low %RSD values for both yield-point shear stress and G^c in the measurements across all dietary fibre preparations indicate good method precision, repeatability, and reliability **[93]**.

Among the 10 selected soluble fibres in this study, preparations with yield points at 50 mg/mL are labelled as “high-viscosity”, preparations with yield points at 200 mg/mL are labelled as “medium-viscosity” and preparations with yield points at 1000 mg/mL are labelled as “low-viscosity”. Such broad descriptions are relative terms with respect to possible clinical use, and does not describe the range of distinct rheological properties and behaviours, which is largely dependent on the preparation concentration and chemical composition of a dietary fibre supplement.

Table 2.3. Tabulated summary of yield-point shear stresses (τ_y), crossover moduli (G^c)**,** and percentage relative standard deviation (%RSD) for the dietary fibre preparations in distilled water, and simulated digestion with gastric fluid at pH 4 and gastric fluid at pH 2.

Dietary Fibre (Name)	Sample Concentration (mg/mL)*	Rheological condition (pH; time point)**									
		Distilled Water (pH 7; 30 min)		Simulated Digestion (pH 4; 30 min)		Simulated Digestion (pH 2; 30 min)		Simulated Digestion (pH 4; 60 min)		Simulated Digestion (pH 2; 60 min)	
		τ_y (Pa) ± (%RSD)	G^c (Pa) ± (%RSD)	τ_y (Pa) ± (%RSD)	G^c (Pa) ± (%RSD)	τ_y (Pa) ± (%RSD)	G^c (Pa) ± (%RSD)	τ_y (Pa) ± (%RSD)	G^c (Pa) ± (%RSD)	τ_y (Pa) ± (%RSD)	G^c (Pa) ± (%RSD)
Guar gum	50.0	994.51 (4.56)	1573.43 (5.46)	1011.12 (6.01)	796.77 (4.31)	946.93 (2.14)	810.08 (9.31)	1057.50 (3.02)	872.16 (7.47)	975.59 (5.91)	807.69 (7.28)
Iota-carrageenan	50.0	700.08 (4.30)	819.82 (2.18)	758.00 (0.95)	2050.50 (7.79)	1232.03 (4.16)	782.68 (8.07)	921.82 (1.85)	1480.77 (9.95)	1068.17 (7.18)	528.68 (5.28)
Xanthan gum	50.0	156.89 (7.16)	114.16 (7.48)	160.99 (6.63)	125.16 (9.63)	180.37 (3.94)	129.33 (4.41)	174.83 (1.47)	139.27 (11.63)	183.16 (9.40)	128.65 (11.64)
Psyllium husk	50.0	56.31 (6.78)	134.31 (3.12)	51.38 (9.49)	117.06 (4.04)	80.85 (4.19)	119.94 (0.88)	62.70 (9.90)	116.84 (3.11)	104.49 (10.24)	123.44 (1.66)
Citrus pectin	200.0	3049.43 (5.36)	2043.93 (6.72)	2848.50 (8.41)	2234.63 (1.99)	2996.10 (9.38)	2036.47 (6.30)	2725.07 (12.81)	1578.80 (1.97)	2911.83 (3.50)	1743.17 (8.00)
Beta-glucan	200.0	544.58 (6.08)	2938.67 (1.22)	454.57 (3.41)	2653.37 (2.88)	419.02 (7.57)	2427.70 (9.47)	614.19 (3.88)	2952.63 (1.11)	545.38 (5.28)	2784.37 (6.67)
Apple-fibre pectin	200.0	23.27 (8.49)	343.67 (11.80)	19.29 (2.53)	226.21 (11.16)	22.99 (4.88)	295.24 (5.07)	39.27 (6.56)	451.10 (9.76)	36.51 (2.13)	385.13 (10.23)
Inulin	1000.0	47.36 (4.64)	98.01 (9.52)	103.87 (7.40)	151.04 (9.14)	105.15 (6.37)	143.09 (8.68)	123.48 (0.94)	222.70 (1.07)	126.41 (4.22)	197.24 (1.03)
Gum Arabic	1000.0	31.76 (7.94)	794.02 (6.41)	36.09 (5.39)	805.88 (2.53)	33.83 (4.36)	815.66 (4.55)	35.66 (8.82)	784.39 (8.11)	31.15 (8.08)	893.63 (5.75)
PHGG	1000.0	20.01 (7.52)	1460.33 (3.79)	21.99 (9.00)	1504.47 (4.17)	20.55 (8.78)	1699.07 (2.86)	23.51 (4.28)	1522.40 (3.29)	21.22 (2.30)	1904.53 (10.41)

(*) Fibre concentration in solution was increased when no crossover modulus (G^c) was observed in the 0.08–3901.01 Pa shear stress (τ) acquisition range; (**) Measurements taken at each condition with n = 3 replicates for yield shear stress (τ_y) and crossover modulus (G^c) in Pa with ± %RSD at the yield point (sol-gel transition point).

For the soluble dietary fibre preparations measured at 50 mg/mL, as shown in **Figure 2.4**, under distilled water conditions, guar gum exhibits the highest yield-point shear stress (994.51 Pa), and psyllium husk exhibits the lowest (56.31 Pa). At 30 min, no significant differences in shear stress yield points were observed between simulated digestions (pH 4) and in distilled water ($p > 0.05$), apart from iota-carrageenan ($p = 0.03$). Under simulated digestion (pH 2), psyllium husk (80.85 Pa) and iota-carrageenan (1232.03 Pa) display significantly increased yield stress ($p < 0.01$) along with xanthan gum ($p < 0.05$), while no changes were observed in guar gum. At 60 min for both simulated digestive conditions, changes in yield stress were observed for psyllium husk and iota-carrageenan ($p < 0.05$) while no major changes were observed for xanthan gum and guar gum ($p > 0.05$).

When the sample concentration was increased to 200 mg/mL as shown in **Figure 2.5**, yield points were observed for citrus pectin, apple-fibre pectin and beta-glucan. Under distilled water (pH 7), citrus pectin exhibited the highest yield-point shear stress (3049.43 Pa), and apple-fibre pectin exhibited the lowest yield shear stress (23.27 Pa). At 30 min under simulated digestive conditions, citrus pectin did not show any major changes in yield-point stress ($p > 0.05$) , while apple-fibre pectin showed a minor decrease in yield-point shear stress in simulated digestion at pH 4 ($p = 0.03$). Beta-glucan showed a stepwise decrease in yield-point stress from distilled water (pH 7) to simulated digestion at pH 4 ($p < 0.05$) and then pH 2 ($p = 0.01$). At 60 min, citrus pectin exhibited no significant changes in yield-point stress ($p > 0.05$) while beta-glucan and apple-fibre pectin exhibited a significant increase in yield-point stress compared to the previous time point ($p < 0.01$).

At 1000 mg/mL as shown in **Figure 2.6**, yields were then observed for inulin, gum Arabic and PHGG, with pseudoplastic rheological behaviour in inulin and dilatant behaviour in gum Arabic and PHGG. In distilled water (pH 7), inulin displayed the highest yield-point shear stress (47.36 Pa) and PHGG displayed the lowest (20.01 Pa). At 30 min in simulated digestion, no significant changes in yield-point

stress were observed for either gum Arabic or PHGG compared to water ($p > 0.05$). On the other hand, inulin exhibits a significant increase in yield-point stress in simulated digestions at pH 4 and pH 2 ($p < 0.01$), relative to water. At 60 min, the yield-point stress changes were not significant for PHGG and gum Arabic ($p > 0.05$), while there was a significant increase in yield-point stress for inulin in both simulated digestion solutions compared to its 30-min time point ($p = 0.01$).

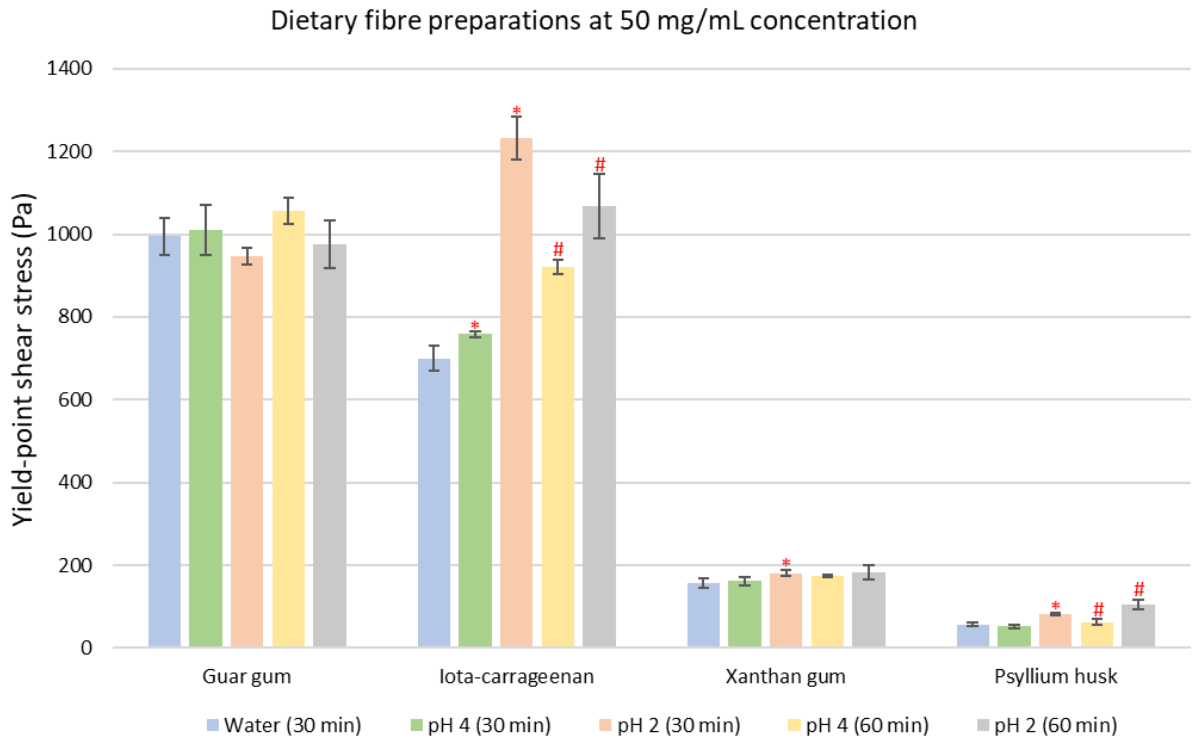


Figure 2.4. Comparisons of yield-point shear stresses of four 'high-viscosity' fibres; guar gum, iota-carrageenan, xanthan gum, and psyllium husk at concentration of 50 mg/mL. Test conditions are in distilled water (pH 7), and simulated digestion at pH 4 and pH 2. The data tip (*) indicates significant difference between the distilled water condition and the simulated digestion condition (pH 4 or pH 2) at 30 min. The data tip (#) indicates significant difference between the simulated digestion condition at 30 min (pH 4 or pH 2) and its corresponding simulated digestion condition at 60 min. The significance level is $p \leq 0.05$ for both (*) and (#).

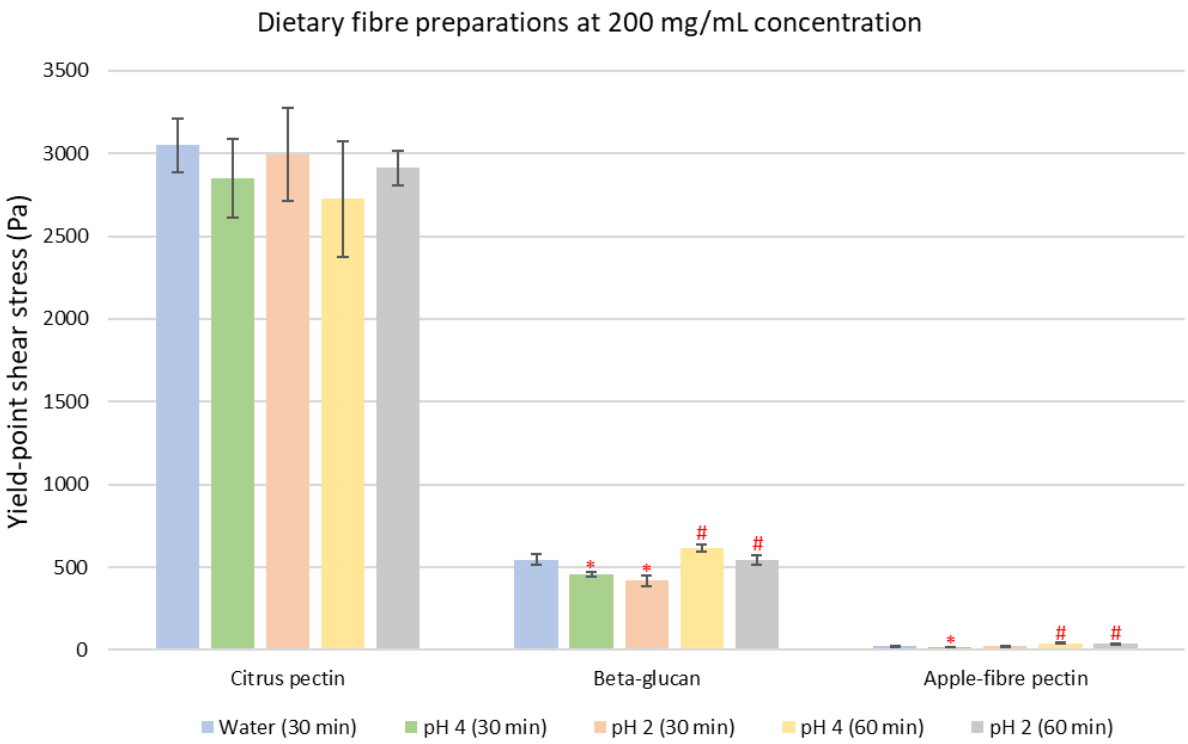


Figure 2.5. Comparisons of yield-point shear stresses of three 'medium-viscosity' fibres; citrus pectin, beta-glucan, and apple-fibre pectin at 200 mg/mL. Test conditions are in distilled water (pH 7), and simulated digestion at pH 4 and pH 2. The data tip (*) indicates significant difference between the distilled water condition and the simulated digestion condition (pH 4 or pH 2) at 30 min. The data tip (#) indicates significant difference between the simulated digestion condition at 30 min (pH 4 or pH 2) and its corresponding simulated digestion condition at 60 min. The significance level is $p \leq 0.05$ for both (*) and (#).

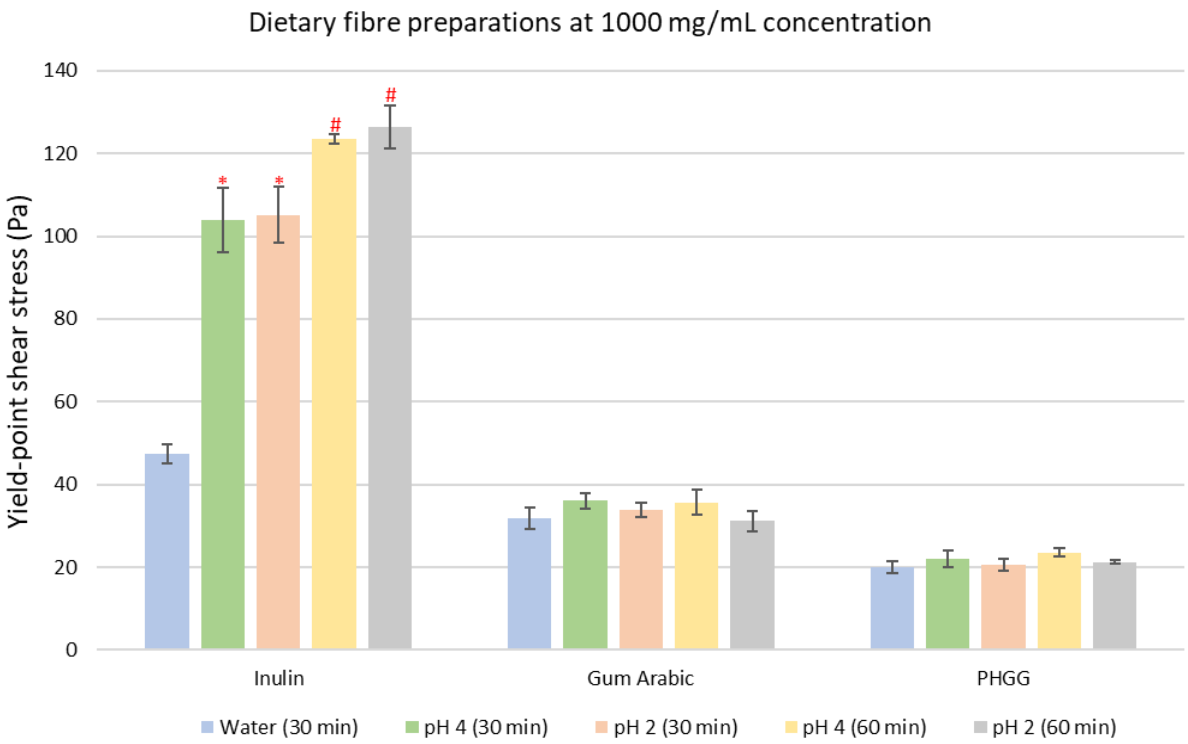


Figure 2.6. Comparisons of yield-point shear stresses of three 'low-viscosity' fibres; inulin, gum Arabic, and PHGG at 1000 mg/mL. Test conditions are in distilled water (pH 7), and simulated digestion at pH 4 and pH 2. The data tip (*) indicates significant difference between the distilled water condition and the simulated digestion condition (pH 4 or pH 2) at 30 min. The data tip (#) indicates significant difference between the simulated digestion condition at 30 min (pH 4 or pH 2) and its corresponding simulated digestion condition at 60 min. The significance level is $p \leq 0.05$ for both (*) and (#).

2.4. Discussion

The rheology of 10 commercially available soluble dietary fibres were comprehensively studied under neutral (distilled water, pH 7) and simulated digestion conditions. The study identified gum Arabic and PHGG as two promising candidates for gastroparesis patients based on their low-viscosity behaviour in both distilled water and simulated digestion conditions.

In general, the rheological parameters of yield-point shear stress and complex viscosity (η*) are affected by the chemical structure, preparation concentration, pH and the presence of cations at steady biological temperature (37 °C). Due to these factors, significant variability in rheological properties were observed between our ten soluble dietary fibres. Fibres such as guar gum and psyllium husk required relatively high shear stresses to achieve their yield points at 50 mg/mL, suggesting that a large amount of mechanical force is required to breakdown their molecular associations. The physiological digestion of food bolus in the stomach requires the breakdown of particles to an average size of 2.0 mm, which is required for transit through the pylorus **[149,150,151]**. The shear stress requirement for a yield point in dietary fibre preparations can be analogous to the mechanical force needed by the stomach (2000 dyn/cm^2 or 200 Pa in a normal stomach) to churn and breakdown the molecular associations of the dietary fibre **[152]**. When gastric motility is impaired or absent (as is the case in gastroparesis), fibres with high yield points may not be sufficiently broken down, which leads to delayed gastric emptying and associated symptoms **[153]**.

It can be ascertained from **Figures 2.4 – 2.6** that the chemical structure and composition of dietary fibres play an important role in the shear stress requirements for a yield point in distilled water (pH 7). Guar gum and PHGG are good examples to demonstrate this, as PHGG is a short chain enzymatically hydrolysed (short chain (3 – 8) + medium chain (9 – 30) units in a 1:7 ratio) version of the guar gum polysaccharide (~200 units), which consists of a mannose backbone and a galactose side chain

(2:1). PHGG shows a far lower yield-point stress and lower viscosity than guar gum, demonstrating that shortening the length of major polysaccharides in a dietary fibre directly affects the rheological properties **[154]**. It must be noted that hydrolysing guar gum into PHGG also changed the sample state from a colloidal gel into a hydrocolloid paste. Such de-gelling helps lower the viscosity of PHGG and allows it to be incorporated into yogurts in order to reduce viscosity and improve texture quality **[155]**.

The Gum Arabic dietary polysaccharide has a large molecular weight of 240–580 kDa but has been shown to demonstrate low-viscosity rheological behaviour in distilled water (pH 7) and in various dietary preparations **[156]**. This rheological behaviour is due to the extensively branched arabinogalactan polysaccharide which consists of a backbone of (1,3)-linked β-d-galactopyranosyl units, a side chain of between two to five (1,3)-linked β-d-galactopyranosyl units which is joined to the backbone with (1,6)-linkages and monomer residues of rhamnose, arabinose, glucuronic acid. The proportion of the amphiphilic micellar structured arabinogalactan protein (AGP) complex in gum Arabic has also been shown to be correlated with increased shear-thinning thixotropy and a reduction in viscosity **[157]**. This effect is due to AGP micelles in solution being disrupted by the application of steady shear stress. When the steady shear stress is ceased, the AGP polysaccharide returns to its original micelle configuration **[157]**.

Significantly different yield points were observed in pectin dietary fibres from apple and citrus as seen in **Figure 2.5**. This discrepancy may be due to varying proportions of high-methoxyl and low-methoxyl type pectin polysaccharides, such as homogalacturonan, xylogalacturonan, apiogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II **[158]**. It should be noted that pectins are known to decrease in solubility as the pH is decreased, where the COO^- ionization in solution is gradually reduced **[158]**. Differences in the total dietary fibre content (apple fibre 40% vs citrus fibre 55%) in their respective supplements, as reported in **Table 2.1** may also contributed to the observed discrepancy.

It has been reported in the literature that the rheological properties of some water-soluble polysaccharides (such as, pectins, gums, mucilages) are affected by the adjustment of pH and the presence of cations and temperature **[159]**. In this study, the presence of H^+, Na^+ and K^+ cations within the simulated gastric fluids are the primary factors that affect molecular associations of the fibre polysaccharides, but this effect is greatly dependent on whether the cations present in solution can disrupt and rearrange the default thermodynamic configuration of a constituent polysaccharide **[160]**.

In the polysaccharides of gum fibres (gum Arabic, PHGG, guar gum, xanthan gum) lowered pH and increased cations seems to have little to no effect on the yield-point shear stress requirements or viscosity due to the hydrogen and covalent bonding in micellar configurations being stable and undisrupted **[161,162]**. The pectin-type dietary fibres (apple-fibre and citrus pectin) were similarly unaffected by the simulated gastric conditions, but these pectin dietary fibres did not form the colloidal gels reported in the "egg-box" model **[163,164]** and instead formed hydrocolloidal pastes, which may have affected their rheology **[163]**.

It has been reported that the rheological properties of high-methoxy pectins (like apple-fibre and citrus pectin) are largely unaffected by the decrease in pH and increased cation presence and are reported in the literature to be more affected by substances like the food additive gelatine **[158]**. It should be noted that the solutions used in our experiments contained little to no free Ca^{2+} ions, which are required for pectin to form the colloidal gels found in the "egg-box" model where the Ca^{2+} ions interact with COO^- ions forming sliding sheets of polysaccharide chains, which stack over each other and result in increased viscosity **[158]**. Pectin gelation at low pH (pH 3) is greatly dependent not only on the presence and binding of Ca^{2+} ions to COO^- groups on the pectin polysaccharide, but also on hydrophobic interactions and the formation of hydrogen bonds in solution **[158]**.

Fibres such as iota-carrageenan, inulin, beta-glucan and psyllium husk display significant changes in yield-point shear stress in simulated digestion conditions when compared with distilled water. For the iota-carrageenan polysaccharide, the mechanism of gel formation and solubility is primarily affected by the presence of the OSO_3^- ester sulphate group on its O-3-substituted β-d-galactopyranosyl and O-4-substituted 3,6-anhydro-α-d-galactopyranosyl dimer backbone with a double helical configuration **[165]**. The iota-carrageenan variety is more sulphated than kappa-carrageenan, forming a soft gel rather than the rigid gel formed by kappa-carrageenan **[166]**. The abundance of free cations in solution results in cationic interaction where the sulphate ester groups on the crosslinked double helix configuration are aggregated, especially by K^+. The sol-gel transition points after such interactions have been studied in the literature using the photon transmission technique **[167]** and are shown to increase gel formation, decrease solubility and significantly increase yield-point shear stress.

Inulin is a linear short chain β-2,1 fructan polysaccharide with variable degrees of polymerisation. The alpha-inulin variety used in this study is water soluble at room temperature unlike delta-inulin, which is insoluble at temperatures below 40 °C. Inulin polysaccharides are of low molecular weight, with a range of 0.6 kDa to 7.2 kDa, leading to low yield-point stress values in distilled water (pH 7) **[168]**. Similar to iota-carrageenan, inulin also exhibited increased yield-point shear stress due to its six-turn helical configuration of the alpha-inulin polysaccharide in aqueous solution, which is also affected by the aggregation of cations to the helix in a manner very similar to iota-carrageenan **[169,170,171]**. Although inulin has relatively low viscosity in distilled water (pH 7), as previously mentioned, the significant increase in viscosity under simulated digestion is not ideal for gastroparesis patients. Given the delayed gastric motility characteristic of gastroparesis, inulin may hypothetically be exposed to gastric fluids for over 4 h in these patients, resulting in greatly increased viscosity and likelihood of associated symptoms. In addition, inulin can cause severe reactions in people with fructan

allergies and was, therefore, excluded from any potential clinical study involving gastroparesis patients **[172,173]**.

Psyllium is an anionic mucilage polysaccharide with many COO^- groups and a great deal of electrostatic repulsion, which causes the polysaccharide strands to expand and become interpenetrated. The microstructure of psyllium is extensively porous in distilled water and in alkaline conditions (pH > 7). When the pH is sufficiently decreased, the net intermolecular electrostatic repulsion in the polysaccharide is reduced causing the colloidal material to become more rigid, increasing the yield-point shear stress and increasing viscosity **[174]**. Such intermolecular associations explain the increased yield-point stress of psyllium at low pH solutions.

Beta-glucan is a water-soluble mucilage polysaccharide with repeating β-D-glucose monomer units with (1,3) and (1,4) glycosidic linkages that can be branched **[175]**. Literature investigations of beta-glucan report that the rheological viscosity is increased under acidic conditions and decreased under alkaline conditions **[176]**. This runs counter to the result obtained in this study, where the yield-point shear stress values and viscosity after yield are reduced more when pH is lowered in simulated digestion conditions. Such an effect may be due to an unknown effect of cations in solution since their effects on beta-glucan has not been investigated extensively. The reduced viscosity may also be due to the low percentage of total dietary fibre present in beta-glucan (44%) and molecular interactions with other constituents within the supplement. The other polysaccharides present in the beta-glucan supplement (22%) and their intermolecular interactions with the beta-glucan (22%) polysaccharide in aqueous solution may also have affected the rheology when the pH was reduced.

Simulated digestions were also carried out over the course of 60 min, and iota-carrageenan, psyllium husk, beta-glucan, apple-fibre pectin showed significant increases in yield-point shear stress compared to their 30 min time point results. The primary factors playing a role in the yield point

increase are the water-binding ability of constituent polysaccharides and the syneresis effect. The binding of water by dietary fibres can be done through anionic interactions, ionic interactions involving carboxyl groups with cations, strong and weak types of hydrogen bonding, hydrophobic interactions involving the formation of water clathrates on gel surfaces, and the enclosure of water through capillary action **[177]**. Some soluble dietary fibres experience rapid syneresis during the rest or stabilization period where the sample matrix expels water bound to the constituent polysaccharides. This causes colloidal gels and hydrocolloid pastes to become brittle and dried out, increasing the yield-point shear stress and the viscosity, though this process is reversible **[178]**. Therefore, the water-binding capacity of soluble dietary fibres and any observed syneresis during rheological measurements are largely dependent on the chemical structure and thermodynamic configuration of constituent polysaccharides in aqueous solution.

There are some limitations in this study. While the rheological properties of the dietary fibres under neutral and simulated digestive conditions were studied, the direct physiological effects of these fibres in the upper gastrointestinal tract have not been evaluated clinically. Though the yield-point shear stress values provide valuable rheological information for pre-clinical evaluation, they may not be an accurate representation of the peristaltic forces in the stomach. Evaluation of the physiological effects requires either a simulated stomach like a SIMulator Gastro-Intestinal (SIMGI) compartment **[179]** or clinical study involving human participants. Despite these limitations, the rheological data presented in this research can prove useful for physicians and dietitians as a pre-clinical assessment of the suitability of certain dietary fibre supplements for patients with upper gastrointestinal disorders such as gastroparesis where peristaltic (stomach) activity is greatly reduced or impaired.

In summary, the analysed data demonstrates the rheological heterogeneity within a range of soluble dietary fibres. It can be stated based upon the *in vitro* modelling that digestion may have a

significant impact on dietary fibre viscosity and should be taken into consideration when evaluating clinical suitability for patients with gastric motility disorders. The data in this chapter provides robust evidence for the potential suitability and tolerability of PHGG and gum Arabic in patients with gastroparesis. Both PHGG and gum Arabic demonstrate low yield-point shear stresses in both distilled water (pH 7) and simulated digestion fluid (pH 4 and 2) conditions with no significant change ($p > 0.05$) at high concentration (1000 mg/mL). Based upon this result, PHGG and gum Arabic were selected as candidate “low-viscosity” test fibres at therapeutic concentration (50 mg/mL) for a pilot clinical study involving gastroparesis patients.

Chapter 3

Clinical Study

3.1. Introduction and Clinical Endpoints

In **Section 1.7**, the fundamental rationale for a pilot clinical study investigating the effects of "low-viscosity" soluble dietary fibres in gastroparesis patients was explained. In **Section 2.4**, the "low-viscosity" type, linear, low molecular weight partially hydrolysed guar gum (PHGG) and the "low-viscosity" type, branched, medium molecular weight gum Arabic soluble dietary fibres demonstrated rheological characteristics that indicated suitability for clinical investigation in gastroparesis patients. The next logical step after this finding was to evaluate the performance of these low-viscosity dietary fibres in individuals with gastroparesis *in vivo* i.e., in a clinical setting. A pilot clinical study was designed to determine the short-term blood glucose regulation and tolerability of PHGG and gum Arabic in a small cohort (n = 10) of gastroparesis patients.

The first primary endpoint of this study was to analyse changes in the blood glucose regulation following a glucose challenge in patient-rated gastroparesis symptoms. The second primary endpoint of this study was to analyse changes in gastric symptoms and meal tolerability. The secondary endpoint of this study was the measurement of the "starting" or "reaching" of mouth-to-caecum transit. The test fibres were compared against a negative control (water) and positive control (psyllium husk) in their abilities to modulate blood glucose concentration following a glucose challenge. "High-viscosity", high molecular weight psyllium husk is a commonly prescribed fibre supplement, and its physiological effects have been investigated extensively in clinical studies **[180,181]**. Similarly, there have been clinical investigations of the physiological effects of low-viscosity PHGG **[182,183,47]** and gum Arabic **[184,185,186,187]** but not in a gastroparetic cohort.

Pin-prick blood glucose concentrations were recorded at 30-min intervals during the study. Gastroparesis symptoms were monitored using a patient-rated validated questionnaire, The American Neurogastroenterology and Motility Society Daily Diary (ANMS GCSI-DD) for gastroparesis patients

[188,189,190,191]. Breath testing was used as an estimator of the "reaching" or "starting" of oral-caecal transit **[192]**, in order to identify alterations following dietary fibre consumption.

3.2. Materials & Methods

3.2.1. Study Design & Premises

The pilot clinical study was designed as a randomised controlled crossover study with four arms that studied the short-term effects of low-viscosity dietary fibre meals during a 3-hour period in patients with mild-to-moderate symptom gastroparesis. The randomised controlled crossover study design in a small number of participants was ideally suited as a baseline for future investigations. By studying three different fibres and the negative control in the same participant (crossover), normalizing the collected data (thereby reducing variability), the blood glucose changes, and symptom effects caused by the dietary fibre interventions become comparable both within the same participant (internal control) and among other participants (mild-to-moderate symptom gastroparesis patients).

The study lasted four sessions for each participant with water (negative control) as the first meal. Participants waited for a minimum 1-week in-between each session, assigned as the washout period between test meals **[193]**. In weeks 2, 3 & 4 the intervention order of the dietary fibre test meals of psyllium husk (positive control), PHGG (test fibre 1) and gum Arabic (test fibre 2) were randomised. Participants were given 10 g of a dietary fibre test meal and were allowed to end the meal when their tolerable meal amount was reached. The consumed amount of each meal varied from participant to participant and was measured. The PHGG (Sunfiber™) supplement was purchased from Healthy Origins, Australia. Gum Arabic was purchased from New Directions, Australia. Psyllium husk was purchased from SF Health Foods, Australia. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Western Sydney University Human Research Ethics Committee (HREC) of Western Sydney University (WSU) under the ethics reference number H12254. The study is

retrospectively registered at the Australian New Zealand Clinical Trials Registry (ANZCTR) with the registration number ACTRN12621001646831. The workflow of the clinical study progression is shown in **Figure 3.1**. The participant information sheet and consent form used in this study are available for perusal in **Appendix B1** and **Appendix B2**, respectively.

The participants' weight and height were measured using a Marsden M-100 clinical scale from Marsden, UK. The glucose measurements were performed using a FreeStyle Optium Neo™ blood glucose and ketone probe meter from Abbott Laboratories, USA. Test strips, glucose and ketone calibration solutions for the probe meter were also purchased from Abbott Laboratories, USA. Participants were given a glucose challenge (unflavoured 50 g in 300 mL) Glucoscan™ glucose drinks from Cleanaway Daniels, Australia. In addition, Actilax™ (lactulose, 3.3 g/5 mL) (Chemist's Warehouse, NSW, Australia) was used to determine the "reaching" or "starting" of mouth-to-caecum gastric transit through its initial breakdown and gas production in the caecum. The symptom severity was measured using the ANMS GCSI-DD validated survey **[194]**, which is recognized by the Food & Drug Administration (FDA) as one of the most rigorous measures of patient-rated gastroparesis symptoms **[191]**.

The breath hydrogen (H_2) and methane (CH_4) values monitored for indications of the start of mouth-to-caecum transit, were measured using a Quintron Breath Tracker™ instrument purchased from Quintron, USA. The 750 mL breath test bags, the injection tube Drierite™ (98% $CuSO_4$, 2% $CoCl_2$) desiccant, the instrument drying desiccant SivRite-4™, and the QuinGas-3™ (150 ppm H_2, 75 ppm CH_4, 6.2 ppm CO_2) calibration gas were also purchased from Quintron, USA. The instrument was calibrated and cycled at full and half volume before use. Breath-blows with CO_2 concentration higher than 6.0 ppm were discarded and the breath-blows were repeated to ensure a valid result. Pin-prick blood glucose, patient-rated ANMS GCSI-DD symptoms and breath test measurements were taken at baseline (0 min, pre-glucose challenge), and at 30 min intervals after test meal consumption and post-glucose challenge.

The study was conducted at the designated clinical rooms of the MacArthur Clinical School, Western Sydney University (WSU), in Campbelltown, NSW 2560, Australia.

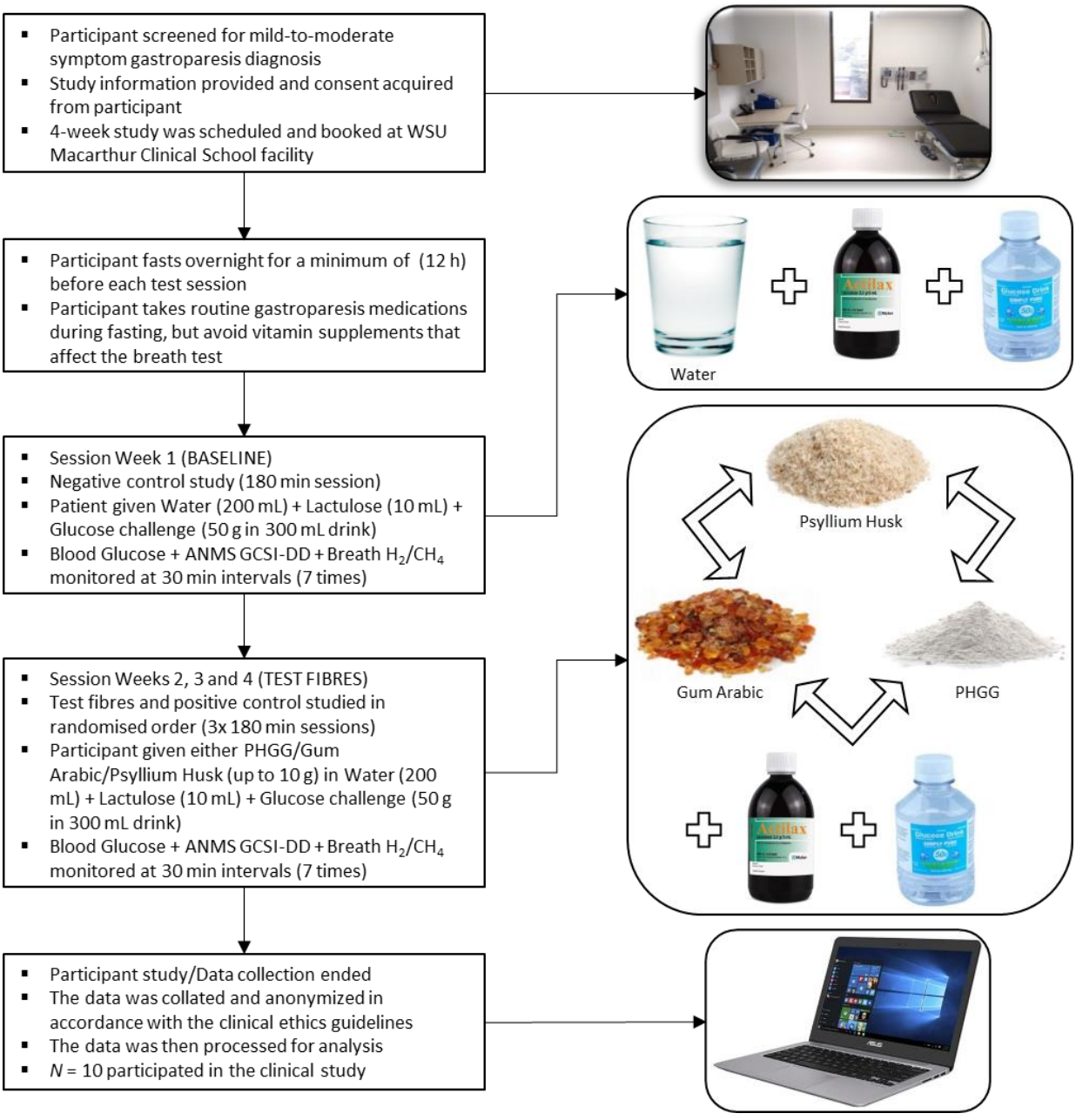


Figure 3.1. Procedural flowchart for the clinical study.

3.2.2. Participant Diagnosis & Eligibility

All potentially eligible participants (both female and male) in this study were diagnosed within the preceding five years. Gastric scintigraphy or a gastric emptying study is currently considered the "gold standard" for gastroparesis diagnosis by physician **[195]**. Eligible participants in this study met the scintigraphic criteria for gastroparesis diagnosis, which is defined as the minimum of 10% retention of solid food contents at 4 hours after the consumption of a Tc-99m radio-labelled meal **[195]**. It should be noted that all participants in this clinical study had completed a scintigraphy study no more than 12 months prior to their participation, in order to either confirm or re-confirm the severity of their delayed gastric emptying and gastroparesis symptoms. The eligible participants' gastroparesis-related symptoms during the period of the clinical study were being managed with routine medications prescribed for gastroparesis patients **[196]**.

3.2.3. Inclusion & Exclusion Criteria and Risks

The inclusion criteria permitted participants aged 18 years or older (adults), who were able to provide written consent. Participants diagnosed with idiopathic gastroparesis or diabetic gastroparesis (Type-1/Type-2) were eligible to participate in the study. Participants excluded from the study included pregnant women, people who could not provide self-consent, and those diagnosed with co-morbidities related to gastroparesis excepting Type-1/Type-2 diabetes mellitus. Participants diagnosed with celiac disease were also excluded, due to the use of wheat-based products in this clinical study **[197]**. Individuals with severe gastroparesis requiring enteral feeding tubes were excluded from this study due to the risk of tube blockage.

3.2.4. Participant Recruitment

Participants were recruited through a recruitment poster on the Gastroparesis Australia support group website. Potential participants were also recruited though word-of-mouth information provided by their physician. Interested participants were screened through an online questionnaire on the Gastrointestinal Motility Disorders Unit website. After screening, investigators made contact to explain the study and provided them with the Participant Information Sheet. The tests were conducted at MacArthur Clinical School, Campbelltown, NSW, Australia, where the participant was provided with the consent form before commencement. General information about study participants is provided in **Table 3.1**. Overall, *n* = 10 participants were recruited into this clinical study, all of them female. This number was determined according to the power calculation for the most similar reported in literature, where the primary endpoint was the measurement of the short-term post-prandial glycaemic response. The detailed summary of this study is shown in **Table 3.2.** The power calculation was determined using the sample size calculator from ClinCalc **[198]**.

Table 3.1. General summary of study participants.

Demographic Information (Units)	Value
Number of Participants	10
Gender (Female/Male)	10/0
Age (Years, Mean ± SD)	38.5 ± 17.8
BMI (Index, Mean ± SD)	29.3 ± 11.7
Etiology of Gastroparesis (Idiopathic/Diabetic)	7/3

Table 3.2. Summary of study used for sample size power calculation (PC).

Primary End-point	Study Information	Reported Values [b]	Sample Size [a]
Short-term post-prandial glycaemic response	- Pastors et al. 1991 **[180]** - Crossover study - Psyllium group (*n* = 18) - Placebo group (*n* = 18) - Dosage 6.8 g psyllium (twice a day; breakfast and dinner) - Peak blood glucose measured	*Breakfast (mMol/L):* Control: 7.02 ±0.62 PHGG: 6.03 ±0.65 *Dinner (mMol/L):* Control: 3.76 ±0.42 PHGG: 2.98 ±0.42	*PC Breakfast:* 7 per group *PC Dinner:* 5 per group

(a) Sample size calculations are based on alpha value of 0.05 (Type I error) and power of 80% (Type II error). (b) When SD values were not provided in the publication, an SD of ±20% was used for power calculation.

3.2.5. Data Curation & Analysis

Microsoft Excel (Office 2016) was used to analyse and generate usable data from collected pin-prick blood glucose measurements, patient-rated ANMS GCSI-DD symptom scores and breath test measurements. Dataset normalization of the 4-week study of each participant enabled direct comparisons of changes in blood glucose and symptoms among all participants in the dataset. The statistical power of normalization enhanced the data analysis, since the effect of major variables such as co-morbidities, medication and gender were carefully controlled in this study. It should also be noted that participants with co-morbidities typically associated with gastroparesis other than Type-1/Type-2 diabetes were excluded, and routine medications for each participant was maintained across all four test weeks during the fasting period (12 hours prior to the start of each test session), further minimizing dataset variability.

Normalization of the collected glucose data was performed by subtracting the measured, pre-meal blood glucose (at 0 min) from the blood glucose measurements of subsequent data points (post-meal, 30 min – 180 min). Similarly, the ANMS GCSI-DD scores were also normalized using “Mean of

Mean" ANMS GCSI-DD scores obtained by subtracting the baseline symptom scores (0 min) from summed mean scores (30 min – 180 min) for each symptom post-meal. Standard deviation (SD) values were calculated for each mean normalized data point. For clarity and brevity, the 9-symptom GCSI scorecard was truncated into three representative symptom composites, which conforms with published reports using the validated ANMS GCSI daily-diary **[191]**. The three composites used in this analysis are Composite (1) for Nausea/Retching related symptoms, Composite (2) for Post-prandial fullness (PPF)/Early Satiety related symptoms and Composite (3) for Bloating/Distension related symptoms.

The Food & Drug Administration (FDA) has stated in a recent guidance report (2018) that "Bloating" as a symptom can be deemed too similar to "Post-prandial fullness" and can be removed the GCSI daily-diary **[194]**. For the purposes of this study, the patient-rated "Bloating" related Composite (3) was included as an exploratory item. All stated two-tailed, homoscedastic p-values in this text were generated using the Student's t-test in R version 4.0.3 and R Studio (Graphic User Interface). The baseline "stats", "utils", "methods", "graphics", "datasets" and "grDevices" R packages were used.

3.3. Results

3.3.1. Blood Glucose Monitoring

The mean normalized blood glucose peak values and the interval area under the curve (iAUC) for the ten participants in the study are summarised in **Table 3.3**. It is very important to note that participants on average, were only able to tolerate 4.13 g of a psyllium husk meal as opposed to 7.99 g of a PHGG meal and 7.57 g of a gum Arabic meal. The participants in general, were not able complete their assigned dietary fibre meals (10 g) and this played an important role in determining the usefulness and relevance of a soluble dietary fibre to a gastroparesis patient.

It can be seen in **Figure 3.2** that the mean normalized blood glucose peaked at 60 min for water (5.9 mMol/L). This can be contrasted to the lower mean normalized blood glucose peaks at 30 min for the test fibres PHGG (3.9 mMol/L), gum Arabic (4.1 mMol/L) and test fibre psyllium husk (3.9 mMol/L). When PHGG and gum Arabic are compared to the positive control meal psyllium husk (3.9 mMol/L) at 30 min, no significant increase or decrease in blood glucose is observed ($p > 0.05$). Following blood glucose peaks at the 30 - 60 min time-points, mean blood glucose begins to gradually reduce during the 90 - 120 min interval. The normalized blood glucose values at 90 - 120 min time-points range from 0.7 – 2.6 mMol/L, with minor differences observed between the test fibres and the negative control. At the 150 – 180 min time-points, the test fibres (PHGG & gum Arabic) return to baseline faster (at 150 min) as opposed to the slower return to baseline observed for the negative control (water) and positive control (psyllium husk) at 180 min. The test fibres (PHGG & gum Arabic) "dip" slightly below baseline blood glucose at 180 min (-0.7 mMol/L). While severe hypoglycaemia (blood glucose < 2.0 mMol/L) can be a significant risk in diabetic gastroparesis patients **[199]**, the lowest measured blood glucose value among the ($n = 10$) participants was 2.9 mMol/L, which occurred during a participant's water meal (negative control) session. None of the participants reported any symptoms typically related to hypoglycaemia **[200]**.

The low-GI capabilities of all three test fibres are summarised by the 1-hour iAUC values of PHGG (169.50 mMol.min/L), gum Arabic (184.93 mMol.min/L) and psyllium husk (169.13 mMol.min/L) when compared to water (241.83 mMol.min/L), as shown in **Table 3.3**. It should also be noted that total 2-hour iAUC values of the dietary fibres PHGG (309.00 units), gum Arabic (322.29 units) and psyllium husk (325.50 units) are still very similar to each other and are not significantly different ($p > 0.05$). This 1-2-hour post-prandial period is less important since blood glucose typically returns to fasting (overnight, 12 h) level post-peak, in the 2-3-hour monitoring period **[201]**.

At the 3-hour time point, as expected, all test meals returned to their pre-meal baseline with a negligible change in iAUC. Interestingly, both the test fibres PHGG and gum Arabic return to baseline levels at 150 min in contrast to the 180 min mark observed for psyllium husk and water. This observed discrepancy was not significant when compared statistically ($p > 0.05$). In $n = 5$ participants the "monophasic" single glucose peak pattern of a water meal (negative control) was converted into "biphasic" double glucose peaks when they were given the dietary fibre test meals of PHGG, gum Arabic and psyllium husk (positive control).

Table 3.3. Blood glucose parameters including the mean normalized blood glucose values at 30 min intervals, the interval area under the curve (iAUC), and the time to baseline (TTB) shown for *n* = 10 participants in the study.

Test Meal (In 200 mL water)	**Average meal consumed** (g) ± (SD)	**Mean Normalized Blood Glucose Differences** (mMol/L) ± (SD), **Interval area under the curve or iAUC** (mMol.min/L), **Time to baseline or TTB** (min)								
		At 30 min	**At 60 min**	**iAUC (1 h)**	**At 90 min**	**At 120 min**	**iAUC (2 h)**	**At 150 min**	**At 180 min**	**TTB min**
Water (Negative control)	0 (0)*	5.1 (2.8)	5.9 (4.9)**	241.83	2.3 (0.8)	1.5 (1.4)	420.83	0.6 (2.1)	0.1 (1.8)	180
Psyllium husk (Positive control)	4.13 (1.18)	3.9 (0.6)**	3.5 (1.9)	169.13	2.6 (2.2)	1.8 (2.3)	325.50	0.7 (2.1)	0.0 (1.0)	180
PHGG (Test fibre 1)	7.99 (1.92)	3.9 (1.5)**	3.5 (1.8)	169.50	2.2 (2.0)	1.5 (1.4)	309.00	0.0 (1.6)	-0.7 (1.2)	150
Gum Arabic (Test fibre 2)	7.57 (1.54)	4.1 (1.7)**	4.0 (1.6)	184.93	2.2 (2.1)	0.7 (1.9)	322.29	-0.3 (1.7)	-0.7 (0.6)	150

(*) All participants the 200 mL water meal (i.e., no test fibre). (**) Indicates the time and mean normalized concentration for the glucose peak in each test meal.

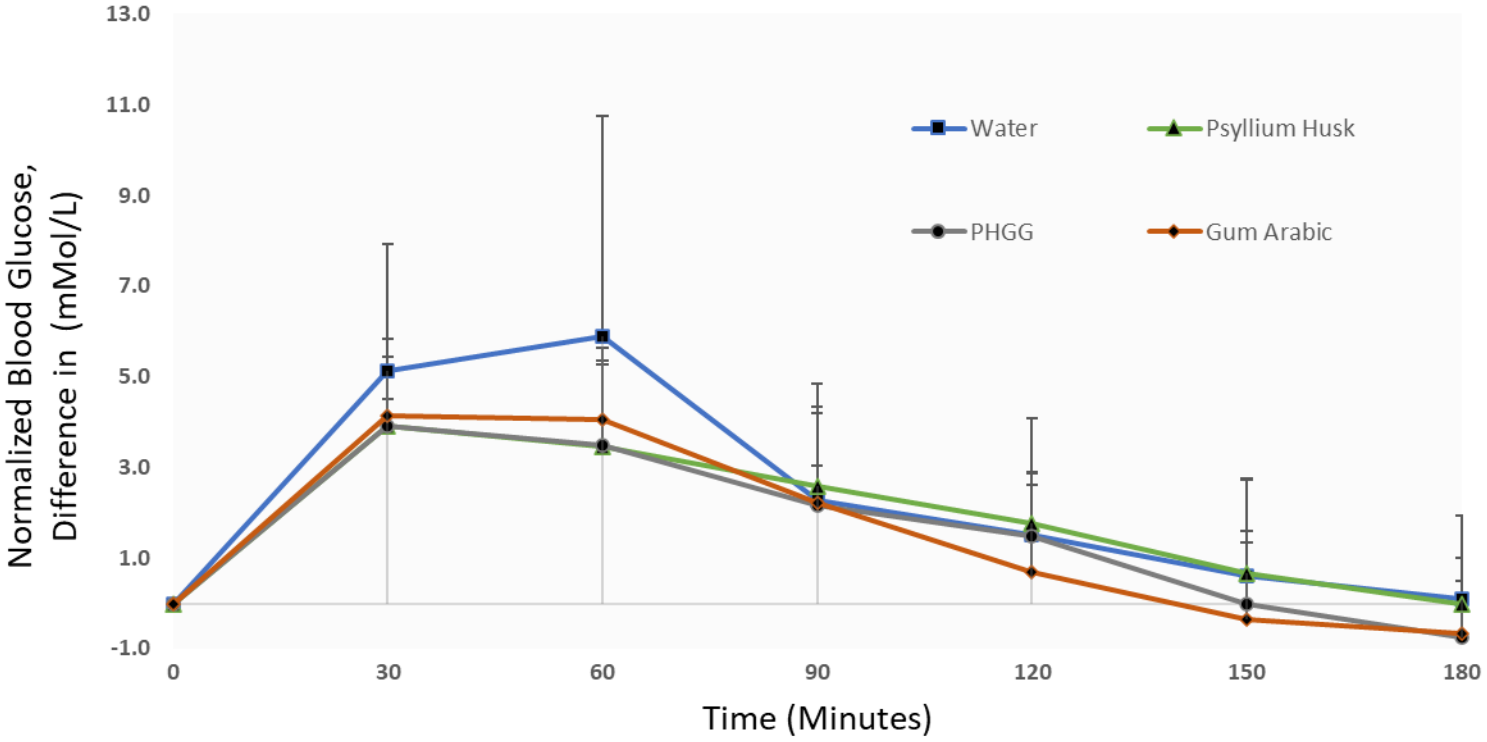


Figure 3.2. Changes in blood glucose concentration of participants (*n* = 10) given test meals of water (negative control), psyllium husk (positive control), PHGG (test fibre 1), and gum Arabic (test fibre 2).

3.3.2. ANMS GCSI-DD Monitoring

The summarised, mean normalized ANMS GCSI-DD scores across ten participants are shown in **Table 3.4**. Eight significant correlations, in total, are observed when comparing the composite symptom scores among ten participants. There are significant comparisons when comparing individual symptoms across the four test meals, but these are truncated into the composite scores due to the limited sample size of participants (n = 10). Significant correlations among individual symptoms are also shown in **Table 3.4** but are not discussed in any great detail.

When Nausea-related (1) composite scores are compared, it is clear that the negative control water (-0.22) slightly reduced nausea when compared to the baseline measurement. The positive control psyllium husk (0.51) displays significantly increased Nausea-related symptoms compared to water (-0.22), with p = 0.05. This result is entirely expected since psyllium husk is a "high-viscosity" dietary fibre. What was unexpected was the noticeable increase in Nausea-related symptoms from the PHGG meal (0.85), although this increase is not significant when compared to water (-0.22), with p > 0.05. It is important to note that there is a large standard deviation (SD) of 1.30 for the Nausea-related composite of PHGG. This informs us that some participants experienced increased Nausea-related symptoms after a PHGG meal, while others did not, resulting in the noticeably large standard deviation.

When the mean of the "highest" or "worst" patient-rated symptom scores ("Mean of Max" scores, not shown in **Table 3.4**) of PHGG (1.88) and gum Arabic (0.71) for the "Nausea" symptom are compared, a statistically significant difference is observed (p < 0.05). This indicates that for PHGG, the "Nausea" symptom is disproportionately causing an increase in the Nausea-related composite with "Vomiting" and "Retching" contributing minimally. Gum Arabic (0.07) caused little to no increase in Nausea-related symptoms when compared to the negative control water (-0.22), with p > 0.05.

Four significant differences are observed when comparing the PPF-related (2) scores among the four test meals. In comparing PPF-related symptoms, the negative control water meal (-0.31) was significantly lower than positive control psyllium husk (0.91) with $p = 0.01$. The water meal (-0.31) also had lower PPF-related symptoms when compared to gum Arabic (0.57), with $p = 0.01$. PHGG (0.15) shows little significant change when compared to the negative control water (-0.31), with $p > 0.05$. Both gum Arabic (0.57) and PHGG (0.15) demonstrated lower PPF-related symptoms when compared to positive control psyllium husk (0.91), with $p \leq 0.05$. This data reveals that when PPF-related symptoms like “Stomach Fullness”, “Early Satiety”, “Post-prandial Fullness” and “Loss of Appetite” are considered, the effects of a PHGG meal was comparable to drinking a “glass of water”.

When Bloating-related composite scores are compared, three significant changes are observed. A highly significant difference is observed when negative control water (-0.17) is compared to positive control psyllium husk (1.40), with $p < 0.01$. This result was entirely expected, since the higher-viscosity positive control meal was expected to increase Bloating-related symptoms, especially compared to the negative control. PHGG (0.40) demonstrated a slight increase in Bloating-related symptoms, although no significant correlations are observed when compared to other meals, with $p > 0.05$.

Gum Arabic (0.31) displays significantly reduced Bloating-related symptoms when compared to the positive control psyllium husk (1.40), with $p < 0.05$. Interestingly, when gum Arabic (0.31) is compared to water (-0.17), a correlated symptom increase can be observed, with $p = 0.05$. This correlation might have occurred due to the consistently higher “Bloating” scores of gum Arabic (0.50) compared to PHGG (0.44) and water (-0.17) across all $n = 10$ participants. Even still, the observed Bloating-related symptom increases are very small in comparison to the symptom increase observed in the positive control.

Table 3.4. Mean normalized ANMS GCSI-DD scores across all time-points for *n* = 10 participants in the study, with Composite (1) score for (Nausea/Vomiting); Composite (2) score for (Post-prandial Fullness/Early Satiety) and Composite (3) score for Bloating/Distension.

		Mean Normalized ANMS GCSI-DD Scores ± (SD) (*n* = 10)				
Symptom Subscale (Composite No.)	**GCSI-DD (Symptom)**	**Baseline Mean (Pre-meal scores)***	**Water (Negative control)**	**Psyllium Husk (Positive control)**	**PHGG (Test fibre 1)**	**Gum Arabic (Test fibre 2)**
Nausea / Vomiting (1)	Nausea	1.55 (1.15)	0.04 (1.22)	0.90 (0.73)	1.00 (1.41)	0.12 (0.72)
	Retching	0.45 (0.83)	-0.31 (1.03)	0.04 (0.63)	0.71 (1.47)	0.05 (0.13)
	Vomiting	0.40 (0.81)	-0.37 (1.11)	0.60 (1.16)	0.85 (1.33)	0.05 (0.13)
Post-prandial Fullness / Early Satiety (2)	Stomach Fullness	2.43 (1.91)	0.17 (1.22)	1.40 (1.22)	0.35 (1.04)	0.40 (0.95)
	Early Satiety*	3.35 (0.91)	-0.78 (0.97)	0.88 (0.64)**	-0.38 (1.69)	0.43 (0.98)
	Post-prandial Fullness*	3.83 (0.73)	-0.22 (0.83)	0.25 (0.46)	0.00 (0.53)	0.29 (0.76)
	Loss of Appetite	2.58 (1.82)	-0.41 (0.92)	1.10 (1.51)**	0.63 (1.02)**	1.17 (1.52)**
Bloating / Distension (3)	Bloating	2.10 (1.68)	-0.17 (0.70)	1.52 (1.14)**	0.44 (0.94)	0.50 (0.64)**, ***
	Belly Visibly Larger	1.88 (1.77)	-0.17 (0.60)	1.27 (1.09)**	0.35 (0.71)	0.12 (0.44)***
Composite Scores	Composite (1)	0.80 (1.06)	-0.22 (1.10)	0.51 (0.48)**	0.85 (1.30)	0.07 (0.29)
	Composite (2)	3.04 (1.50)	-0.31 (0.65)	0.91 (0.70)**	0.15 (0.75)***	0.57 (0.64)**, ***
	Composite (3)	1.99 (1.68)	-0.17 (0.56)	1.40 (1.09)**	0.40 (0.82)	0.31 (0.48)**, ***

(*) Reported mean baseline scores (0 min) are patient-rated (*n* = 10), before each study session and not normalized, with early satiety and post-prandial fullness measured once immediately after the meal. (**) Indicates significant difference ($p \leq 0.05$) vs water (negative control). (***) Indicates significant difference ($p \leq 0.05$) vs psyllium husk (positive control).

3.3.3. Breath Hydrogen/Methane Monitoring

Among ten participants, only five showed any signs of "reaching" or "starting" mouth-to-caecum transit within the 180 min time frame, which can be identified as a greater than 6 ppm but lesser than 12 ppm increase in breath hydrogen and methane (H_2 + CH_4) levels from the baseline measurement taken at 0 min. This is the standard ppm guideline range for the start of mouth-to-caecum transit of the indigestible lactulose sugar. It should be noted that the breath tests measured the "reaching" or "starting" time-point of gastric transit not its completion or "ending" (≥ 90% gastric emptying) time-point. This result is not entirely unexpected, since most gastroparesis patients show signs of delayed mouth-to-caecum transit in the longer, 4-hour gastric scintigraphy studies **[202]**.

One participant was able to show signs of mouth-to-caecum transit commencement of all four meals, water (at 150 min), PHGG (at 180 min), gum Arabic (at 180 min) and psyllium husk (at 180 min). Two other participants showed signs of mouth-to-caecum transit of the water meal at 150/180 min with transit for PHGG and gum Arabic starting at 180 min. Two participants showed signs of mouth-to-caecum transit of PHGG at 180 min but did not show signs of transit for water, gum Arabic or psyllium husk within 180 min. The participant who was able to show signs of mouth-to-caecum transit for all four test meals was the only one that showed any signs of psyllium husk transit (at 180 min).

Three participants co-produced H_2 and CH_4, while seven participants exclusively produced H_2 with < 1 ppm CH_4 produced across all measurements. Two participants produced greater than 12 ppm H_2 + CH_4, a positive indicator for SIBO, but these specific participants were already diagnosed with SIBO (Small Intestinal Bacterial Overgrowth) **[203]** by their physician. It must be noted that the breath test was not used to diagnose SIBO among any of the participants. The breath testing information is summarised in **Table 3.5**.

Table 3.5. General data produced by the breath test measurements.

Demographic Information	**Participants (out of 10)**
Number of Participants	10
H_2 producer	7/10
H_2 + CH_4 producer	3/10
SIBO (Already diagnosed)	2/10
Mouth-to-Caecum Reaching / Starting Transit (6 ppm < Breath < 12 ppm, within 180 min)	**Participants (out of 10)**
Any sign of transit	5/10
Water meal	3/10
Psyllium Husk meal	1/10
PHGG meal	4/10
Gum Arabic meal	2/10

3.4. Discussion

In summary, the short-term physiological effects of soluble dietary fibres were comprehensively studied in ten mild-to-moderate symptom gastroparesis patients. This pilot clinical study demonstrates that low-viscosity soluble dietary fibres PHGG, and gum Arabic have blood-glucose regulation properties and are tolerable for patients with mild-to-moderate symptom gastroparesis.

The blood glucose data suggests that all three dietary fibres, in general, are able to “hold” more glucose from the glucose challenge during the 1st post-prandial hour compared to the no-fibre water meal. The iAUC results suggest that PHGG and gum Arabic are comparable to psyllium husk at blood glucose regulation. Lower iAUC values were observed for all three dietary fibres compared to the negative control water, due to their ability to “trap” a lot of glucose until the further release of glucose for absorption in the small intestine, beyond the 3-hour monitoring period of this clinical study **[204]**.

This is an important development, since it has been shown in previous randomised clinical studies that consumption of "high-viscosity" dietary fibres such as guar gum, β-Glucan and psyllium husk have been shown to result in improved blood glucose regulation and reduced insulin resistance in Type-2 diabetic patients **[205]**. The follow-on benefit of this regulation is improved long-term insulin control, reduced risk of post-prandial glycaemia, significantly reduced inflammation, and better metabolic health **[206,207,208,209]**.

A "monophasic to biphasic" peak transition was observed in five participants after the consumption of dietary fibres used in this study, irrespective of type. This phenomenon might have occurred due to the "two-phase" controlled release of insulin, which is closely regulated by single β-cells, pancreatic islets, and the whole pancreas **[210]**. The first release phase of insulin occurs within the 10-min post-prandial time-period followed by a long second phase of 2-3-hours **[211]**. Even though the "monophasic to biphasic" trend is not significant enough to be apparent in **Figure 3.2**, its observance in half the participants of this study is a positive development, since biphasic peaks are associated with higher insulin sensitivity, and the lowered incidence of impaired glucose tolerance and metabolic syndrome **[212]**.

While the blood glucose effects of all three dietary fibres are very similar, it is important to note that participants were able to tolerate much less psyllium husk in comparison to PHGG and gum Arabic. A smaller meal of psyllium husk was able to have an effect size similar to PHGG and gum Arabic, despite its lower tolerability. All three soluble dietary fibres (unlike insoluble dietary fibres) are able to form gels in the pH 2.0 – 4.0, HCl rich environment of the adult human stomach **[42]** and are successfully able to "trap" and gradually release glucose through entanglement and molecular interactions **[213]**. High-viscosity psyllium husk's outsized effect was likely due to its better gelation ability *in vivo* compared to low-viscosity PHGG and gum Arabic **[43]**. Still, larger meals of PHGG and gum Arabic fibre will be able to

affect glucose and lipid metabolism in the small intestine, and release more beneficial short-chain fatty acids (SCFA) downstream in the caecum section of the large intestine, which is predicated by the gut microbiota composition, prevalence and fermentability **[214]**.

The ANMS GCSI-DD symptom monitoring scorecard provides a great deal of insight into the post-ingestion response of the test fibres PHGG and gum Arabic when compared to psyllium husk (positive control) and water (negative control). As noted in **Section 3.3.1**, patients were able to tolerate a larger amount of PHGG and gum Arabic compared to psyllium husk, and this must be taken into consideration when comparing the post-prandial changes in symptoms. Nausea-related symptoms are intimately connected to gastric motility, with symptom relief achieved through the prokinetic medication **[215]**, and the drinking of water, especially cold water, which is known to greatly reduce nausea **[216]**. While it can tentatively be stated that gum Arabic is better than PHGG when Nausea-related symptoms are compared, literature-reported side effects of PHGG such as nausea, have always been incidental and not concrete **[106,82]**. The Nausea-related increase observed in PHGG may be related to hyperosmia (smell sensitivity) and can be overcome by selective flavour modification **[217]**.

The superior performance of PHGG in the PPF-related metric compared to gum Arabic may be due to the structural chemical differences of PHGG (linear, short-chain) and gum Arabic (branched, long-chain), both of which as previously mentioned, are “low-viscosity” soluble dietary fibres **[154,156]**. The mechanistic causes of PPF-related symptoms in gastroparesis patients remains elusive, and speculated causes include damage to the vagus nerve, autoimmune causes or viral vectors **[218]**. The data presented in this study reveals that when considering PPF-related symptoms like “Stomach Fullness”, “Early Satiety”, “Post-prandial Fullness” and “Loss of Appetite”, the symptom effects of PHGG are comparable to a “glass of water” in mild-to-moderate symptom gastroparesis patients. This clinical

study also demonstrates that the low-viscosity soluble dietary fibres PHGG and gum Arabic cause very little Bloating-related symptom increases, especially compared to high-viscosity psyllium husk **[219]**.

When assessing the breath test results, it was apparent that some participants were able to show signs of "starting" mouth-to-caecum transit in either or both of PHGG and gum Arabic within 180 min, but delayed transit was universally observed in participants consuming the psyllium husk meal. It is well known in literature that "high-viscosity" gel forming dietary fibres tend to increase the viscosity of stomach fluid during digestion, thereby delaying gastric emptying **[54]**. Based on this data, it can be tentatively stated that the low-viscosity soluble test fibres PHGG and gum Arabic do not seem to impede mouth-to-caecum transit as much as high-viscosity psyllium husk, though no definitive conclusions can be made.

There were prominent limiting factors that affected this study. The primary limiting factor was the sample size of n = 10, even though that number is sufficient for a pilot clinical study, as shown in **Table 3.2**. The crossover design and the use of normalization, as previously stated, largely mitigates this limitation and pro-vides significant statistical power in the generated dataset, despite the small sample size. While there have been other pilot clinical investigations of soluble dietary fibre with larger participant cohorts ($10 \geq n \geq 30$) **[180,181,182,183,184]**, none have been investigated in gastroparesis patients. These studies primarily dealt with gastric conditions where the physiological effects of dietary fibres have been investigated more extensively, such as constipation, diarrhoea, IBS and diabetes. These reported studies involved the direct comparison of one soluble fibre against a negative control (placebo) **[180,181,182,183,184]**, in contrast to the two candidate soluble fibres (PHGG, gum Arabic) compared simultaneously against a positive control (psyllium husk) and a negative control (water) in this study.

Another limiting factor was the use of a glucose probe monitor instead of a continuous glucose monitor, which could have produced more discrete information. A glucose probe meter was selected

due to ease of use, patient comfort and convenience in comparison to a continuous blood glucose monitor. In a potential longer-term study, continuous blood glucose measurement could be employed at the beginning and end of a monitoring period, where the observation duration might be a few months **[220]**. The longer-term effects on gastric emptying and digestive behaviour *in vivo* could also be studied using highly sensitive gastric emptying scintigraphy, post-prandial plasma paracetamol monitoring and ^{13}C breath tests at the beginning and end of a future study, rather than employing the less sensitive hydrogen and methane breath test **[202,221,222]**.

The lack of clinical studies besides a recent case study investigating dietary fibre in relation to diabetic gastroparesis, necessitated the need for rigorous and cautious data collection **[223]**. The encouraging performance of the low-viscosity soluble fibres PHGG and gum Arabic in this pilot clinical study can be used as the basis for a future study investigating their longer-term effects in a larger cohort ($n \geq 30$) of gastroparesis patients. The long-term tolerability, glucose regulation benefits, metabolic effects, and the soluble fibre-mediated changes in gut microbiota composition and its associated release of beneficial SCFA would be of significant interest for individuals with gastroparesis.

In summary, the data presented in this clinical study demonstrates that the “low-viscosity” soluble fibres PHGG and gum Arabic may be viable dietary supplements for mild-to-moderate symptom gastroparesis patients. Glucose monitoring indicates that PHGG and gum Arabic have GI lowering properties comparable to “high-viscosity” psyllium husk. The ANMS GCSI-DD data indicates both test fibres are more tolerable, and cause far fewer symptoms compared to psyllium husk. In some participants, PHGG and gum Arabic did not impede mouth-to-caecum transit as much as psyllium husk, if the start of mouth-to-caecum transit was observed during the monitoring period. Future studies will involve the investigation of longer-term physiological and metabolic effects of both low-viscosity soluble test fibres in the diet of gastroparesis patients.

Chapter 4

Mass Spectrometry

4.1. Introduction and Method Validation Parameters

In **Section 1.8** the utility and benefit in developing a rapid, repeatable and reliable validated method for the analysis of monomer sugars was discussed. An ideal high-performance liquid chromatography (HPLC) method of a phytochemical sample or commercial dietary supplement possess these four characteristics in simultaneous combination: A large number of monitored target analytes (> 5 analytes), a short run time (<60 min), a repeatable and reliable extraction method (n > 3 replicates), and an inexpensive and easily accessible method of analysis. HPLC methods reported in literature are either rapid (< 60 min), using specialized instrumentation and techniques (HPLC-RI, HPLC-CAD, HILIC-ELSD) **[131,132,119,133]** or have very long run-times (> 60 min) **[114,115]** using the comparatively cheaper and more versatile technique HPLC-ESI-MS/MS. A small literature review of six recent mass spectrometry methods that identified and analysed monomers constituents of polysaccharides in various sample matrices is shown in **Table 4.1**.

The primary aim of this study was to develop and validate a rapid method for the simultaneous targeted analysis of nine monomer sugars in commercial dietary fibre supplements. HPLC coupled with electrospray-ionisation quadrupole time-of-flight tandem mass spectrometry detection (HPLC-ESI-Q-ToF-MS) was identified as the most suitable technique for the rapid analysis of monomer sugar units. MS/MS detection in combination with ToF and multiple reaction monitoring (MRM) provides simultaneous quantitation of target analytes that co-elute at the same retention time but have different *m/z* precursor masses. The fragmentation of precursor *m/z* ions into product *m/z* through collision induced dissociation (CID) provides analyte identity confirmation by comparison of relative ion intensities of standards and samples.

While the MS/MS technique is able to pick up analytes that have no chromophores, due to its high selectivity and sensitivity, common monomer sugars are often stereoisomers or epimers of each

other, possessing exact masses, making peak separation and identity confirmation very difficult and time consuming using conventional ToF and MS/MS. An ideal means of separation would be the derivatization (or chemical labelling) of the monomers using 1-phenyl-3-methyl-5-pyrazolone (PMP), which well suited for the simultaneous analysis of acidic (uronic acids), neutral and basic monomer sugars due to its ability to derivatize under mild conditions, causing no inadvertent isomerization during sample preparation **[224,225,226]**.

Derivatization improves sensitivity and column binding due the increased hydrophobicity of the PMP-adduct in comparison to the original monomer. When a suitable column such as the charged surface hybrid (CSH C18) column is employed, rapid analyte separation can be achieved for PMP-labelled monomers. In addition to these advantages, PMP-labelling of monomers produces derivatives that are detectable at 245 nm (UV-Vis range). The chemical structures of the major polysaccharides found in partially hydrolysed guar gum (PHGG), gum Arabic and psyllium husk and are shown in **Figure 4.1**, **Figure 4.2** and **Figure 4.3** respectively (sketched using ChemDraw Ultra version 12.0.2.1076).

The following method parameters were measured in order to validate the method developed and presented in this chapter:

1. ***Accuracy*** – Is the "trueness" of the agreement between the results of the proposed method and the results of an established reference method derived from standard analytical references. The accuracy of the method is measured using the recoveries at three spiking levels (50%, 75% & 100%).
2. ***Precision*** – The intra-day laboratory precision is the repeatability and reliability of the proposed method. The precision of the method is represented by the standard deviation (SD) and relative standard deviation (RSD) of analyte concentration and retention time.

3. ***Linearity*** – The linearity of the method is determined by its ability to produce results that are directly, or by means of defined mathematical transformations, proportional to the analyte concentration. The linearity of the method is represented by the R^2 value of the analyte standard curve.
4. ***Detection limit*** – The detection limit of the method is represented by the limit of detection (LOD) and the limit of quantitation (LOQ). The limit of detection (LOD) is three times the standard deviation (SD) and the limit of quantitation (LOQ) is ten times the standard deviation (SD) for each analyte in the validation sample, which is injected multiple times (replicates).
5. ***Stability*** – The stability of the method measures the decomposition limit of analyte within the sample when undergoing storage under controlled conditions as a function of time. The stability is measured as the time-point at which a ≥ 2% decrease in analyte peak area is observed when the sample is stored at 4 ^{o}C, wrapped in aluminium foil (to reduce photodecomposition).

When the above five criteria are met, the developed method is considered to be validated for intra-laboratory use in commercial PHGG, gum Arabic and psyllium husk supplements. Individual monomer analyte concentrations measured using the exact validated method can also be listed on batch product certification by manufacturers. For the stated reasons, a rapid pre-column derivatized HPLC-ESI-Q-ToF-MS method for the analysis of monomer constituents in PHGG, gum Arabic and psyllium husk prebiotic dietary fibre supplements was developed.

Table 4.1. A literature review of six recent methods analysing monomers constituents in sample matrices containing polysaccharides.

Author [Reference]	Technique & Column	Sample Matrix	Monomers Analysed	Precision & Run Time
Tihomirova *et al.* **[131]** (un-derivatized)	High-Performance liquid chromatography refractive index (HPLC-RI); COL–AMINO (150 x 4.6 mm) column	Hay samples: Hydrolysed Hay	Arabinose, Xylose, Fructose, Mannose, Galactose, Glucose (6 in total)	$R^2 \geq 0.999$; Recovery: 28.0 – 96.0%; Run Time: 7.0 min
Slawinska *et al.* **[132]** (un-derivatized)	High-performance liquid chromatography charged aerosol detector (HPLC-CAD); Shodex Asahipak NH2P-50 4E (4.6 x 250 mm x 5 µm) column	Mushroom samples: *A. bisporus* *P. ostreatus*	Arabinose, Fructose, Mannitol, Glucose, Sucrose, Trehalose (6 in total)	$R^2 \geq 0.99$; Recovery: 96.0 – 101.0%; Run Time: 22.0 min
Barzen-Hanson *et al.* **[119]** (un-derivatized)	High-Performance liquid chromatography high-resolution MS (HPLC-HRMS); (4.6 x 100 mm x 3.5 µm) XBridge™ Amide column	Monomer digestion in minimal-nutrient growth medium (MNGM) by *P. protegens*	Xylose, Fructose, Mannose, Glucose, Maltose (5 in total)	$R^2 = 1.00$; Recovery: Not reported; Run Time: 18.0 min
Crha *et al.* **[133]** (un-derivatized)	Hydrophilic interaction liquid chromatography evaporative light-scattering detector (HILIC HPLC-ELSD); HALO Penta-HILIC, (150 x 4.1 mm x 2.7 µm) column	Dietary supplements: Energy drinks, gels, teas	Fructose, Glucose, Sucrose, Isomaltose, Maltodextrins (5 in total)	$R^2 \geq 0.99$; Recovery: 97.7 – 103.8%; Run Time: 30.0 min
Guo *et al.* **[114]** (derivatized)	High-performance liquid chromatography time of flight MS (HPLC-ToF-MS); Shimpack VP-ODS C18, (250 mm x 4.6 mm x 5 µm) column	Fungus sample: *P. umbellatus*	Mannose, Glucosamine, Lyxose, Erythrose, Glucuronic acid, Galacturonic acid, Glucose, Galactose, Xylose, Fucose (10 in total)	$R^2 \geq 0.999$; Recovery: 96.1 – 103.7%; Run Time: 180.0 min
Wu *et al.* **[115]** (derivatized)	High-performance liquid chromatography MS (HPLC-MS); Agilent Zorbax XDB-C18 column (250 x 4.6 mm x 5 µm) column	Algae sample: *S. fusiforme*	Mannose, Glucose, Galactose, Xylose, Fucose (5 in total)	$R^2 \geq 0.9999$; Recovery: 87.1 – 103.0%; Run Time: 70.0 min

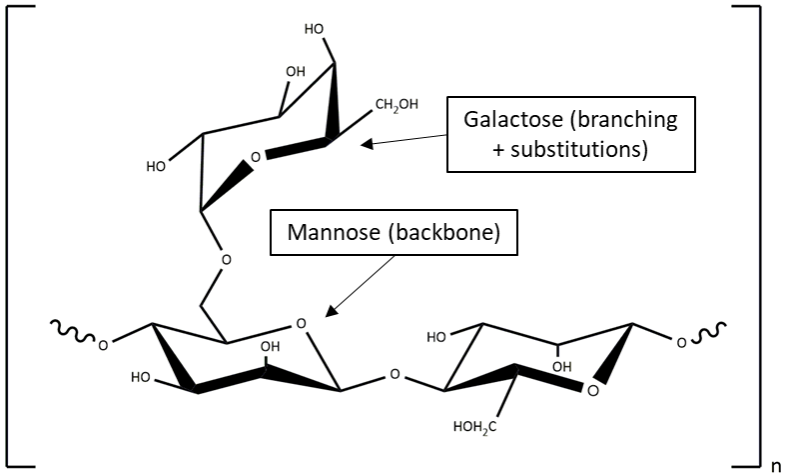


Figure 4.1. Structure of the main guar gum polysaccharide (galactomannan). PHGG is the enzymatic hydrolysed derivative of guar gum.

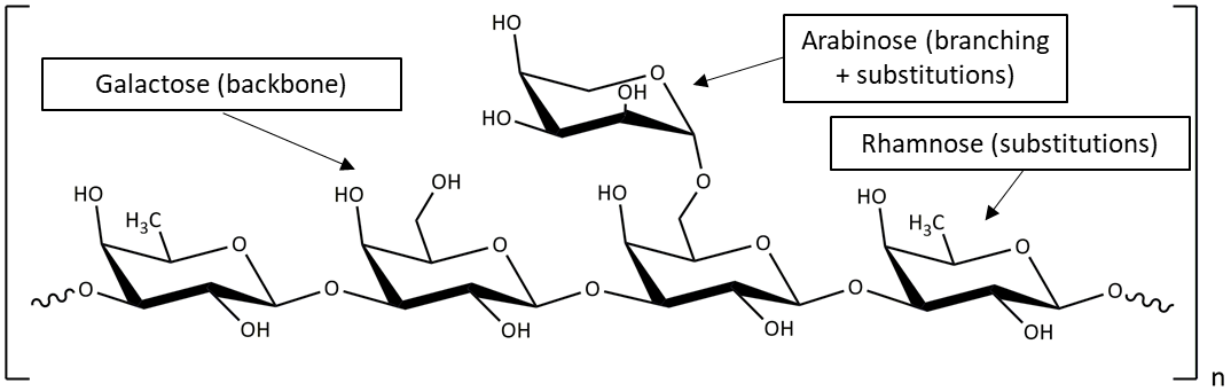


Figure 4.2. Structure of the main gum Arabic polysaccharide (arabinogalactan).

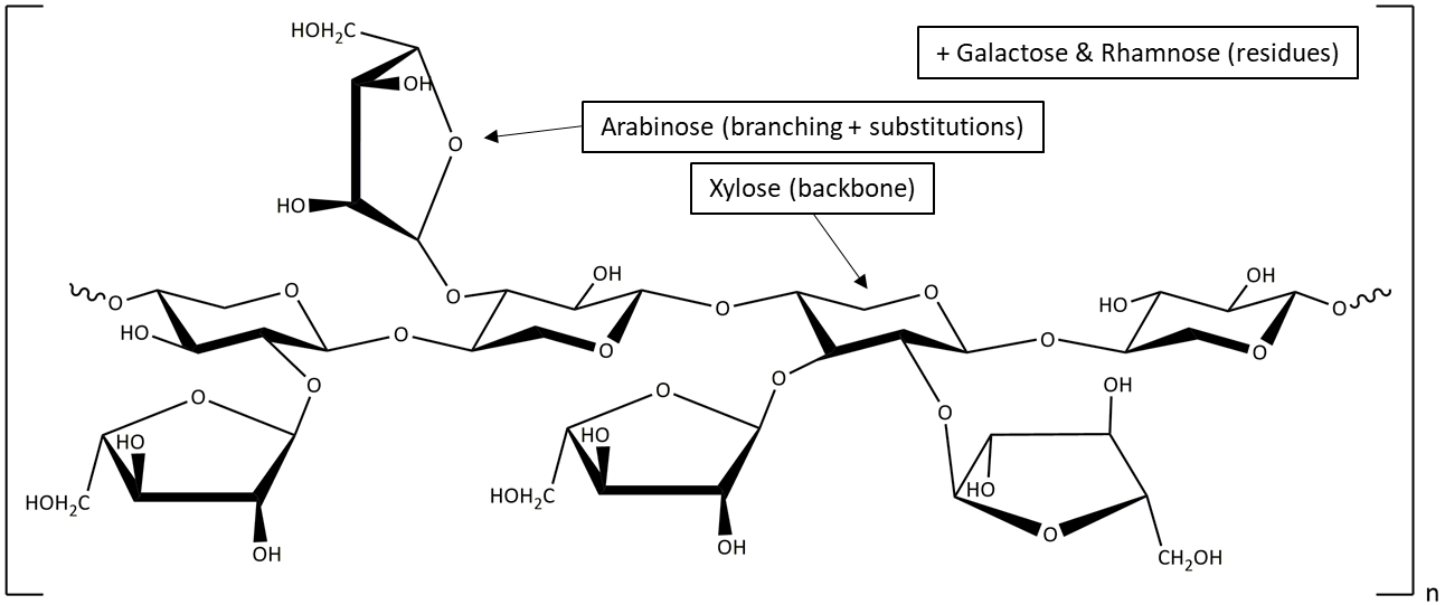


Figure 4.3. Structure of the main psyllium polysaccharide (arabinoxylan).

4.2. Methods

4.2.1. Instrumentation

A Waters SYNAPT G2-Si Q-ToF (Quadrupole Time-of-Flight) UPLC (Ultra Performance Liquid Chromatography) system (Waters Corporation, Milford, MA, USA) coupled to a hybrid quadrupole mass spectrometer was used for rapid method development and validation. A picture of the machine is shown in **Figure 4.4**. Analyte separation was achieved using a Waters ACQUITY™ UPLC CSH (Charged Surface Hybrid) C18 Column (130Å, 1.7 µm, 2.1 mm X 100 mm packing) coupled with ion mobility separation. Injection was performed using a Waters ACQUITY™ UPLC H-Class autosampler. A positive electrospray ionisation ((+)/(-) ESI-MS/MS) scanning mode and argon collision gas were used.

All samples, reagents and analytical standards used in extraction, hydrolysis and derivatization were weighed out using a Mettler Toledo Analytical Balance ME204 (Mettler Toledo, Port Melbourne, VIC, Australia). A Vacuubrand PC 3001 Vario™ speedy vacuum concentrator with a CVC 3000 controller (BrandTech Scientific, Essex, CT, USA), a Dynamica Velocity 14R refrigerated centrifuge (Dynamica Scientific, Livingston, UK), liquid nitrogen storage, and other assorted laboratory equipment including glassware were used during extraction and sample preparation. All experiments were performed in the PC2 (Physical Containment Level 2) laboratory facilities at the School of Medicine, Campbelltown Campus, Western Sydney University (WSU, Sydney, Australia).

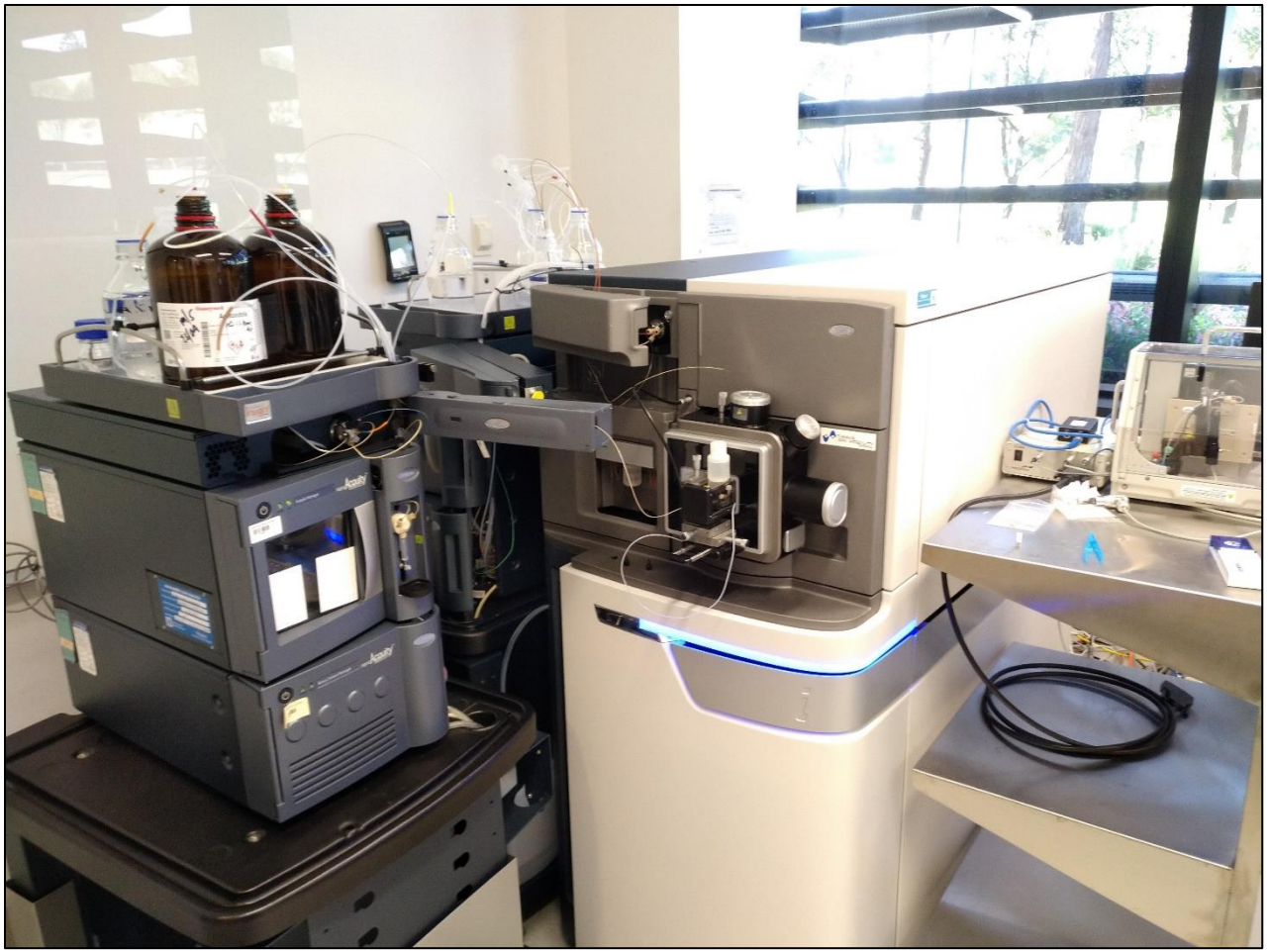


Figure 4.4. Waters SYNAPT G2-Si Q-ToF MS machine used for analysis and method validation.

4.2.2. Reagents, Chemicals, and Samples

Reagent grade ethanol (95%), acetone (95%), n-butanol (98%), chloroform (98%), ammonia (4 M in methanol), trifluoroacetic acid (TFA) (99%), glacial acetic acid (100%), phosphorous pentoxide (99%) and formic acid (98%) were obtained from Sigma-Aldrich (St Louis, MO, USA). De-ionized water used during extraction and reverse-phase chromatography (> 18 MΩ.cm) was obtained from a Milli-Q® Advantage A10 system with a Q-POD™ from Merck Millipore (Darmstadt, Germany). HPLC grade acetonitrile solvent was purchased from Honeywell (Honeywell International, NC, USA). The nebulizing gas argon, and other gases, including nitrogen, used in analytical method development and MS analysis were provided by Coregas (Coregas, Sydney, NSW, Australia).

The derivatization reagent 1-Phenyl-3-methyl-5-pyrazolone (PMP) (99.0%), also known as edaravone, was obtained from Sigma-Aldrich (St Louis, MO, USA). The analytical standards of the monomer sugars glucose, galactose, arabinose, rhamnose, glucuronic acid, galacturonic acid, mannose, xylose and fucose (all ≥ 99.0%) were purchased from Sigma-Aldrich (St Louis, MO, USA). The chemical structures of the monomer sugar standards are shown in **Figure 4.5**. The partially hydrolysed guar gum (PHGG) (80%) brand name Sunfiber™ (Taiyo International, Minneapolis, MN, USA), gum Arabic (80%) (New Directions Australia, Australia) and psyllium husk (80%) (SF Health Foods, Australia) dietary fibre supplements were purchased from local suppliers in Australia.

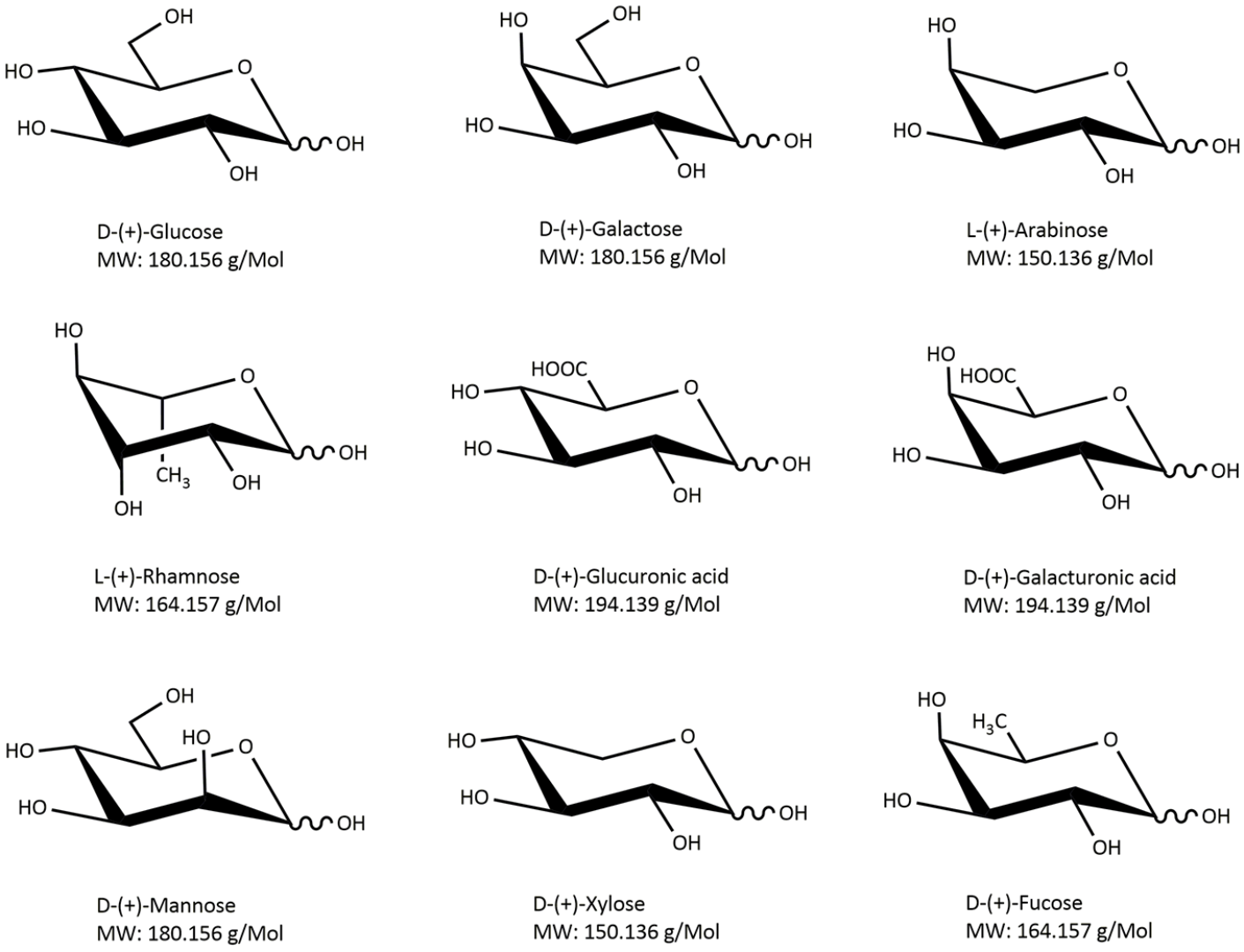


Figure 4.5. Structures of the nine monomer sugars (chair form) purchased as analytical standards for method validation.

4.2.3. Extraction and Purification of Polysaccharide

Polysaccharide extraction and purification from the commercial supplements prior to MS analysis was based on methods reported in literature **[227,123]**. The removal of protein and fat constituents from the supplements prior to MS analysis eliminates other complications involving analytical method development (i.e., the selection of suitable analysis columns).

Samples (1 g ±0.001 g each) of the powdered dietary fibre supplement were finely ground using a mortar and pestle and sieved (≤ 200 µm) in 250 mL conical flasks. 30 mL of de-ionized water was added, and each flask was sealed with two layers of aluminium foil and autoclaved for 1.5 h at 121 ^{o}C. Water soluble polysaccharides were extracted into the supernatant. The flasks were then cooled to room temperature 25 ^{o}C for 30 min. The supernatant was then carefully pipetted out into 50 mL Eppendorf tubes and centrifuged at 4000 rpm (2683 × *g*) for 10 min. The aqueous supernatant was then precipitated with 120 mL 95% ethanol (supernatant : ethanol (1:4)) in a 250 mL conical flask with slow addition and stirring. The precipitate was then filtered in a Buchner flask (with a 0.45 µm Whatman paper) and washed with acetone. The "wet" precipitate was then sealed in a 50 mL Eppendorf tube and placed in a refrigerator (4.0 ^{o}C) for 24 h to complete the precipitation reaction. The precipitate was then removed and pelleted in a centrifuge at 4000 rpm (2683 × *g*) for 40 min. The crude polysaccharide pellet was then dissolved in de-ionized water (10 mg/mL). Protein in the crude polysaccharide pellet was removed by the addition of ¼ volume of Sevag reagent (n-butanol : chloroform (1:4)) using the Sevag method for protein removal in polysaccharide samples **[228]**. The protein gel layer was removed, and the process was repeated twice. The de-proteinated polysaccharide aqueous layer was then desiccated for 48 h using phosphorus pentoxide. After desiccation, the polysaccharide pellet was freeze-dried using liquid nitrogen in a freeze-drier and stored at 4.0 ^{o}C.

4.2.4. Hydrolysis, Derivatization and Preparation of Calibrated Stock Solution

Analytical standards (2 mg ±0.1 mg each) of the glucose, galactose, arabinose, rhamnose, glucuronic acid, galacturonic acid, mannose, xylose and fucose were carefully weighed into clean 10 mL glass vials. 5 mL of ammonia was then pipetted into each vial and the stock solution (400 µg/mL) was sonicated for 10 min at 40 °C and cooled at room temperature (25 °C) for 5 min. The stock was then serially diluted to 80 µg/mL using 4 mL of ammonia. This monomer stock solution was then PMP-labelled and derivatized using an existing method described in literature **[114,115]**.

The derivatization procedure is described as follows. 100 µL of analytical calibrated stock (80 µg/mL) was added to a clean 10 mL glass vial. **Table 4.2** shows the serial dilutions of the initial stock for the generation of the calibration curve. 100 µL of PMP was then added and the solution was mixed. The calibrated stock solution was allowed to react on a hot plate for 30 min at 40 °C, cooled to room temperature and neutralized with 20 µL of 1% glacial acetic acid. This was followed by the addition of 1.5 mL of de-ionized water and 1.5 mL of chloroform. The immiscible two-layer solution was shaken vigorously, and the cap was opened repeatedly to release any gas pressure formed during mixing. The top aqueous layer (1.72 mL) and bottom chloroform layer (1.5 mL) solution were allowed to rest for 10 min under a fume hood. The aqueous layer, containing the PMP-labelled monomers was then carefully pipetted out into a 1.7 mL Eppendorf tube for vortexing (1 min) and micro-centrifugation at 12000 rpm (1610 *g*) for 5 min. The aqueous layer was diluted by ¼ (concentration after dilution: 0.5 µg/mL -> 0.025 µg/mL) and then transferred into clean slit septa HPLC vials for overnight refrigeration storage (4 °C) and mass spectrometry (MS) analysis. The derivatization reaction for mannose is shown in **Figure 4.6**.

Table 4.2. Serial dilutions for the construction of the calibration curve.

Concentration (µg/mL)	Stock Volume (µL)	Ammonia Volume (µL)
2.0	43.00	57.00
1.5	32.25	67.25
1.0	21.50	78.50
0.5	10.75	89.25
0.25	5.40	94.60
0.10	2.15	97.85

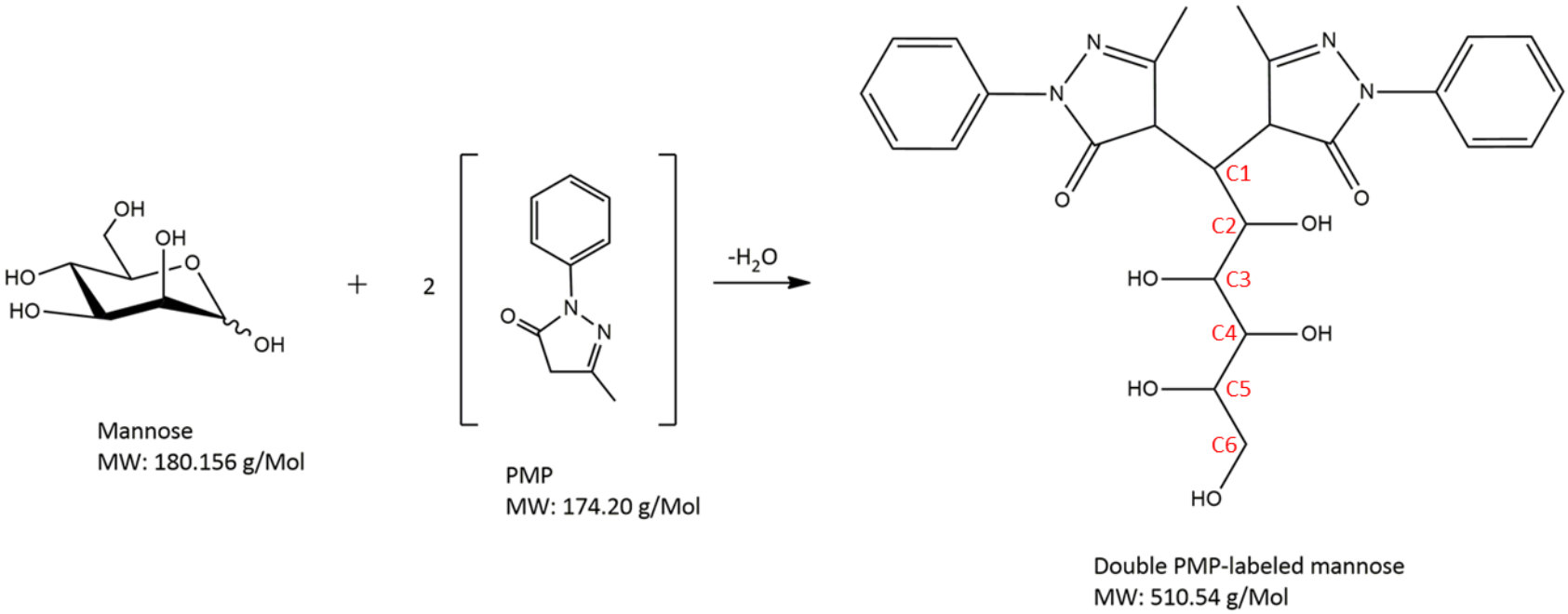


Figure 4.6. Derivatization reaction of mannose with PMP resulting in the PMP-labelled mannose derivative (chromophore). Carbon positions are labelled C1, C2 etc., on the PMP-labelled mannose derivative.

4.2.5. Hydrolysis and Derivatization of Purified Polysaccharide

1.2 mg (±0.1 mg) of PHGG polysaccharide, 2.1 mg (±0.2 mg) of gum Arabic polysaccharide and 1.1 mg (±0.1 mg) of psyllium polysaccharide were carefully weighed out into three separate 1.7 mL Eppendorf tubes. 700 µL of 4M TFA was added to each tube and sealed at room temperature for 15 min. 175 µL of de-ionized water was then added. The sample tubes were then sealed and placed in a 120 ^{o}C boiling water bath for 2 h to allow hydrolysis to occur. At the 2-hour time point, a further 350 µL of de-ionized water was added to each tube and then placed back in the water bath for 1 h. At the 3-hour time point, the sample tubes were removed from the water bath, cooled back to room temperature (25 ^{o}C) and micro-centrifuged at 12000 rpm (1610 *g*) for 10 min. The samples were then evaporated overnight (4 h) in 1.7 mL Eppendorf tubes using a vacuum concentrator.

The samples were then dissolved in 1 mL of ammonia and vortexed for 2 min. 10 µL of stock was transferred into a clean glass vial and 990 µL of ammonia was then added to produce the sample stock solution (1/100 serial dilution). The derivatization procedure for the polysaccharide samples is the exactly as described in **Section 4.2.4**, except in this case, the water-layer was not diluted by ¼ in the penultimate step of the procedure, before the transfer of the layer into a clean slit septa HPLC vial.

4.2.6. Recovery Solution Preparation

To determine the extraction efficiency of monomers in this method, careful amounts of each dietary polysaccharide sample were weighed out in the manner described in **Section 4.2.5.** 10 µL of a sample-specific stock solution (i.e., containing monomers only found in each dietary fibre polysaccharide sample) was added to create the 100% spike level. 5 µL of stock solution was added for the 50% spike level and 7.5 µL of stock solution was added for the 75% spike level. The spiking solutions were then evaporated using a nitrogen gas manifold in the fume hood. Hydrolysis and derivatization were

performed as described in **Section 4.2.5.** Seven replicates were used for each spike level to obtain average recoveries.

4.2.7. HPLC-ESI-Q-ToF-MS Conditions and Data Analysis

The PMP-labelled monomers were separated using a CSH C18 column attached in series with the ToF (Time of Flight) detector. The autosampler injection volume for each sample was 1.0 µL. The mobile phase flow rate was set at 0.5 mL/min. The sample pre-injection temperature was set at 4 °C, with the column temperature set at 35 °C. The weak and strong volumes for the autosampler wash in-between injections was 600 µL and 200 µL, respectively. Each run was 21.0 min long including a 1.5 min wash. The mobile phase gradient program for the method is shown in **Table 4.3**. All standards and samples were injected in triplicate.

Table 4.3. Mobile phase gradient program for the LC-ESI-MS/MS method.

Time (min)	**% Water (A) (with 0.1% *v/v* formic acid)**	**% Acetonitrile (B) (with 0.1% *v/v* formic acid)**
Initial	95.0	5.0
1.50	95.0	5.0
12.50	75.0	25.0
16.50	70.0	30.0
17.50	5.0	95.0
19.50	95.0	5.0
21.00	95.0	5.0

The ESI (Electro-spray Ionisation) source conditions were set with the desolvation gas (nitrogen) at 800 L/h, the source block temperature at 120 °C and the desolvation temperature at 500 °C. The collision gas flow (argon) was set at 0.15 mL/min. Pressure was maintained in the column with the high-pressure warning limit set at 15000 psi. The MS inter-scan delay was set at 0.005 s with both polarity

mode delay and enhanced inter-scan delay set at 0.02 s. The extractor voltage was set at 3V, and the cone gas flow (L/h) was set to OFF status. The capillary voltage for the positive (+) ESI scanning mode was 0.50 kV.

Multiple reaction monitoring (MRM) dwell times (0.05 s for all reaction channels) were auto-calculated by the in-built MassLynx™ v4.2 software and data points of interest were carefully selected for the peak determination. Any *m/z* ions below 1200 Da in size were scanned. In total, four reaction channels for the ions *m/z* 525.21, *m/z* 511.23, *m/z* 495.24 and *m/z* 481.23 were monitored down to *m/z* 175.09. Tuning for optimal cone voltages and collision energies was performed by the IntelliStart™ microfluidics system built into the SYNAPT G2-Si mass spectrometer. In the MRM of all four channels, the cone voltage was set at 30 V, and the collision energy was set at 25 eV. Multiple *m/z* product ions were identified for each target analyte for identity confirmation in both the analytical standard and the samples. The European Commission Directorate for Agriculture guidelines of a minimum of two product ions from a precursor ion with matching intensities between standard and sample peaks were to be met for all target analytes **[229]**.

Initial analysis of MS fragmentations and chromatographic data was performed using the MassLynx™ software v4.2 (Waters Corporation, Milford, MA, USA). Integrated peak area values and retention times were obtained from chromatographic data using the in-built TargetLynx™ software v4.2. All method validation parameters such as the standard curve, analyte concentrations, LOD, LOQ and spiked recoveries of target analytes were calculated using Microsoft Excel (Office 2016).

4.3. Results

4.3.1. MS Identity Confirmation and Precision

Of the nine monomer analytes scanned for in the samples, only five were found among the dietary fibre polysaccharides. Method validation parameters including linear range, linearity, precision, LOD, LOQ and stability calculated from $n = 7$ replicates are shown in **Table 4.4**. Excellent linearity ($R^2 > 0.999$) for all analytes are observed within their respective linear ranges. The standard curve for each analyte in the calibrated stock solution is shown in **Appendix C1**.

The precision RSD for the analytes found in the samples are ≤ 5%, which is excellent. Glucose, glucuronic acid, galacturonic acid and fucose were not present in any of the three polysaccharide samples, therefore the precision measurements for these analytes was not possible. For all the standards and samples, the extraction and instrumental standard deviation (SD) are the two primary contributors to the relative standard deviation (RSD) observed in the analyte sample concentrations and peak retention times. The method precision shown in **Table 4.4** is an aggregate of both the extraction and instrumental precision, with the instrumental precision contributing to one-third of the SD. Analytes found within the sample were stable for 72 h when stored at 4 ^{o}C, wrapped in aluminium foil (≤ 2% degradation in peak area). The stability of analytes present only in the calibrated stock solution, and not within any of the three samples was higher at 96 h (in the mixed standard).

Table 4.4. Precision of quantitation.

Analyte	Linear Range (µMol/L) [d]	Linearity (R^2)	Precision [a]		LOD (mg/g) [b]	LOQ (mg/g) [c]	Stability (h)
			Amount (mg/g) ± % RSD	RT (min) ± SD			
Glucose	0.56-11.10	0.9990	N.P.	12.92 ±0.01	N.P.	N.P.	96
Galactose (*)	0.56-11.10	0.9995	425.03 ±0.69	12.83 ±0.02	8.86	29.53	72
Arabinose (**)	0.67-13.32	0.9992	658.82 ±2.10	13.05 ±0.02	41.42	138.07	72
Rhamnose (**)	0.61-12.18	0.9991	31.91 ±1.32	12.62 ±0.01	1.26	4.20	72
Glucuronic acid	0.52-10.30	0.9991	N.P.	13.04 ±0.01	N.P.	N.P.	96
Galacturonic acid	0.52-10.30	0.9994	N.P.	13.15 ±0.02	N.P.	N.P.	96
Mannose (*)	0.56-11.10	0.9993	269.04 ±1.52	12.06 ±0.02	12.24	40.79	72
Xylose (***)	0.67-13.32	0.9991	723.89 ±1.03	13.18 ±0.01	22.47	74.91	72
Fucose	0.61-12.18	0.9990	N.P.	13.32 ±0.02	N.P.	N.P.	96

(*) Validated in PHGG, (**) Validated in gum Arabic, (***) Validated in psyllium husk, (N.P.) Not present in samples. (a) Average, standard deviation (SD) and relative standard deviation (RSD) calculated from *n* = 7 replicates injected in triplicate. (b) The limit of detection (LOD) is three times the standard deviation (SD) for each analyte in the corresponding validation sample. (c) The limit of quantitation (LOQ) is ten times the standard deviation (SD) for each analyte in the corresponding validation sample. (d) The calibration range for all target analytes was 0.1 – 2.0 µg/mL, shown here in µMol/L units, accounting for the different molecular weights of individual monomers.

For all five analytes found in the samples, the relative intensity ratios of three product ions from the $[M + H]^+$ precursor *m/z* ion were measured. The most useful product ions were formed by C2-C3 (*m/z* 373) and C5-C6 (*m/z* 271) cleavages, and by $[M + H – PMP – 2H_2O]^+$ and $[M + H – PMP – 3H_2O]^+$ fragmentations which varied depending on the precursor mass. In **Figure 4.7**, which compares the MS/MS breakdowns for galactose in the mixed standard and the PHGG sample, it can be observed that the *m/z* 175 $[PMP + H]^+$ fragment is of higher relative intensity than the precursor ion $[M + H]^+$. Such a phenomenon occurs due to the breakdown of the precursor into the major PMP fragment during MS/MS. Fragments from the C4-C5 cleavage (*m/z* 241), the C2-C3 cleavage (*m/z* 217), and the C1-C2 cleavage (*m/z* 187) were also observed, but the ionic masses for these *m/z* fragments were common across all monomer sugars and not distinct, therefore, they were ignored for the purpose of identity confirmation. The MS/MS breakdown comparisons for the four other monomer sugars identified within the supplements is shown in **Appendix C2 – C5**. It can be observed in **Table 4.5** that all *m/z* fragments used for identity confirmation are well within the tolerances described by the European Commission Directorate for Agricultural guidelines **[229]**, providing good identity confirmation for monomers found in the polysaccharide samples.

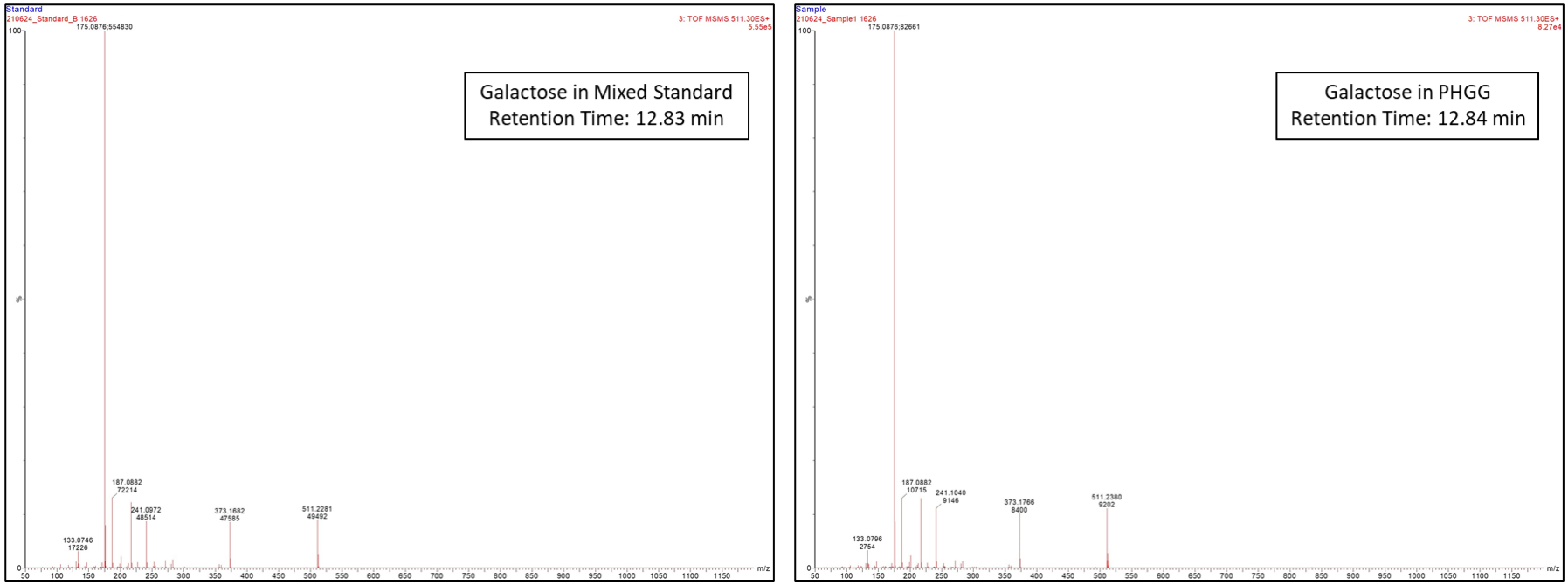


Figure 4.7. Comparison of MS/MS spectra of the galactose peak in the mixed standard (calibrated stock solution) and the galactose peak in the PHGG supplement.

Table 4.5. Labelled MRM fragmentations of analytes in tested dietary fibre supplements.

Analyte	Precursor & Product Ion Fragmentations [a, b]	*m/z*	Relative Intensity		Tolerances		
			Standard (%)	Sample (%)	Relative Difference ±(%) [d]	Permitted Tolerance ±(%) [e]	Pass/Fail
Glucose	$[M + H]^+$	511.2183	100	N.P.	-	Base peak	N/A
Galactose (*)	$[M + H]^+$	511.2281	100	100	-	Base peak	N/A
	C2-C3 cleavage – H_2O	373.1682	96.1	91.3	4.8	20	Pass
	$[M + H – PMP – 3H_2O]^+$	283.1098	17.6	11.3	6.3	30	Pass
	Previous – C5-C6 cleavage [c]	271.1122	16.1	12.8	3.3	30	Pass
Arabinose (**)	$[M + H]^+$	481.2203	100	100	-	Base peak	N/A
	C2-C3 cleavage – H_2O	373.1682	127.5	130.1	2.6	20	Pass
	$[M + H – PMP – 2H_2O]^+$	271.1122	62.5	45.2	17.3	20	Pass
	$[M + H – PMP – 3H_2O]^+$	253.0989	62.9	52.7	10.2	20	Pass
Rhamnose (**)	$[M + H]^+$	495.2361	100	100	-	Base peak	N/A
	C2-C3 cleavage – H_2O	373.1682	98.5	103.5	5.0	20	Pass
	$[M + H – PMP – 2H_2O]^+$	285.1261	52.6	49.8	2.8	20	Pass
	$[M + H – PMP – 3H_2O]^+$	267.1159	74.7	78.1	3.5	20	Pass
Glucuronic acid	$[M + H]^+$	525.2108	100	N.P.	-	Base peak	N/A
Galacturonic acid	$[M + H]^+$	525.2108	100	N.P.	-	Base peak	N/A
Mannose (*)	$[M + H]^+$	511.2380	100	100	-	Base peak	N/A
	C2-C3 cleavage – H_2O	373.1766	100.7	83.4	17.3	20	Pass
	$[M + H – PMP – 3H_2O]^+$	283.1172	20.0	17.1	2.9	25	Pass
	Previous – C5-C6 cleavage [c]	271.1122	30.6	25.9	4.7	25	Pass
Xylose (***)	$[M + H]^+$	481.2203	100	100		Base peak	N/A
	C2-C3 cleavage – H_2O	373.1766	101.7	107.3	5.6	20	Pass
	$[M + H – PMP – 2H_2O]^+$	271.1122	57.0	52.7	4.3	20	Pass
	$[M + H – PMP – 3H_2O]^+$	253.1058	54.0	60.1	6.1	20	Pass
Fucose	$[M + H]^+$	495.2361	100	N.P.	-	Base peak	N/A

(*) Validated in PHGG, (**) Validated in gum Arabic, (***) Validated in psyllium husk, (N.P.) Not present in samples, (N/A) Not applicable. (a) The precursor ion for each analyte shown as $[M + H]^+$. (b) Carbon atoms to be labelled C1, C2 etc., to the terminal carbon, top to bottom, as shown in **Figure 4.6**, with carbon cleavage, PMP and H_2O fragmentations occurring as indicated in the table. (c) $[M + H – PMP – 3H_2O – C6]^+$ where the terminal $2H_2O$ molecules re-attach on carbons C2 and C3. (d) Relative difference = [intensity of sample - intensity of analytical standard) / (intensity of analytical standard)] × 100. (e) Maximum permitted tolerance from the European Commission Directorate for Agricultural guidelines **[229]**.

4.3.2. Chromatographic Data, Recoveries and Analyte Concentrations

Chromatograms for the calibrated stock solution, including the four scanned MRM channels and the total ion chromatogram (TIC) are shown in **Figure 4.8**. Representative chromatograms for the PHGG, gum Arabic and psyllium husk method validation samples are shown in **Figure 4.9**. As reported in **Table 4.6**, good average recoveries were observed for the five monomer analytes that were quantified in three method validation samples (96.22 – 109.49%). The 100% spike level produced slightly higher recoveries than the 50% and 75% levels, but this can be attributed to the higher proportion of glassware adsorption of analytes at the 50% and 75% spike levels **[230]**. Average recovery RSD values were ≤ 10% for most target analytes. The recovery RSD for galactose in PHGG was slightly higher at 10.63%, but this is still an acceptable value (≤ 15%). The concentration of the five analytes measured in the three dietary fibre polysaccharide samples are shown in **Table 4.7**. As expected, galactose and mannose was present in PHGG, galactose, arabinose and rhamnose was present in gum Arabic, arabinose and xylose were present in psyllium husk. Galactose and rhamnose residues were detected in psyllium husk, but the concentration of these residues were well below the LOQ.

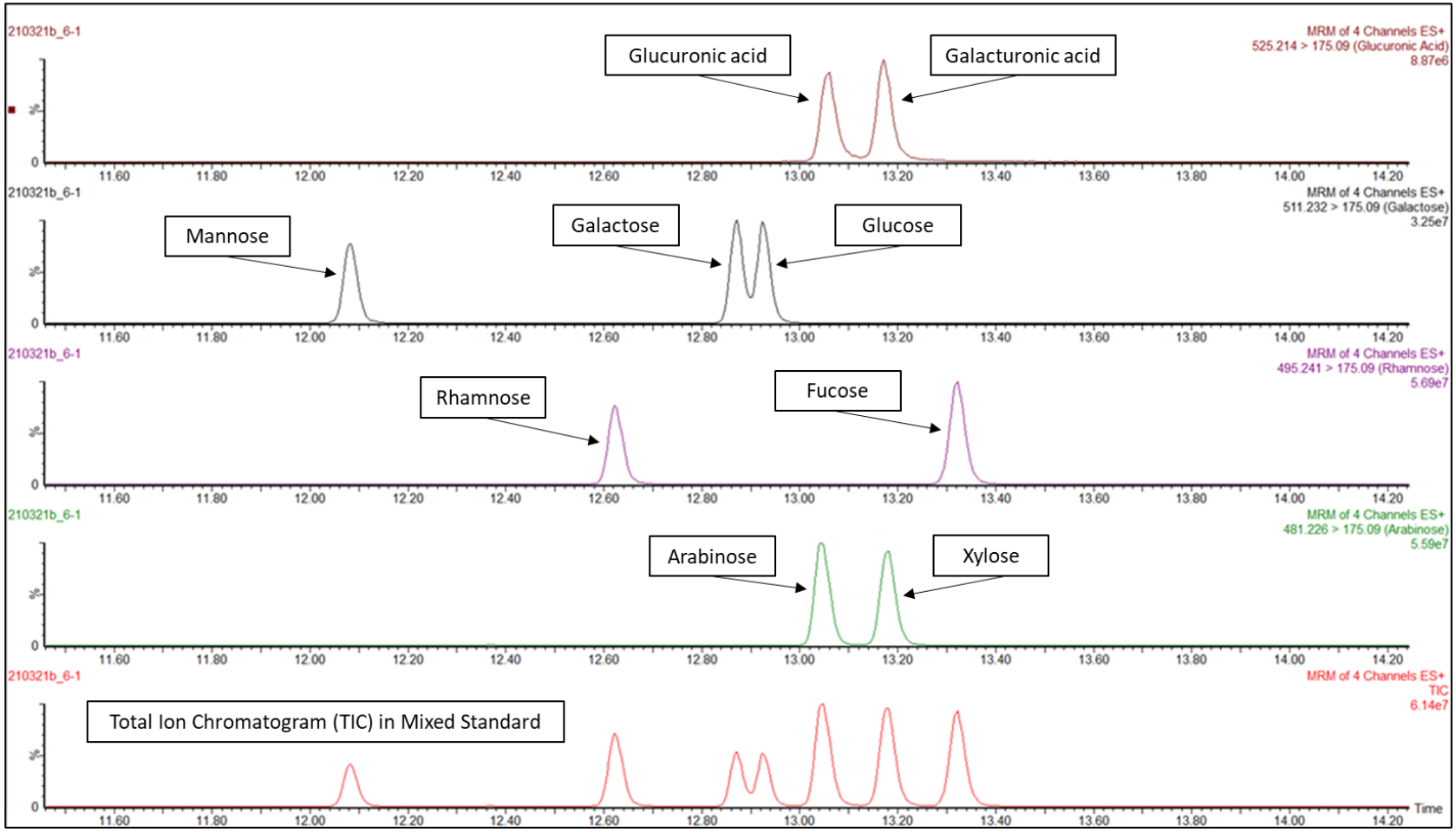


Figure 4.8. Representative HPLC-ESI-MS/MS chromatograms of the target analytes in mixed standard (calibrated stock solution). MRM channels at *m/z* 525, *m/z* 511, *m/z* 495 and *m/z* 481 shown along with total ion chromatogram (TIC).

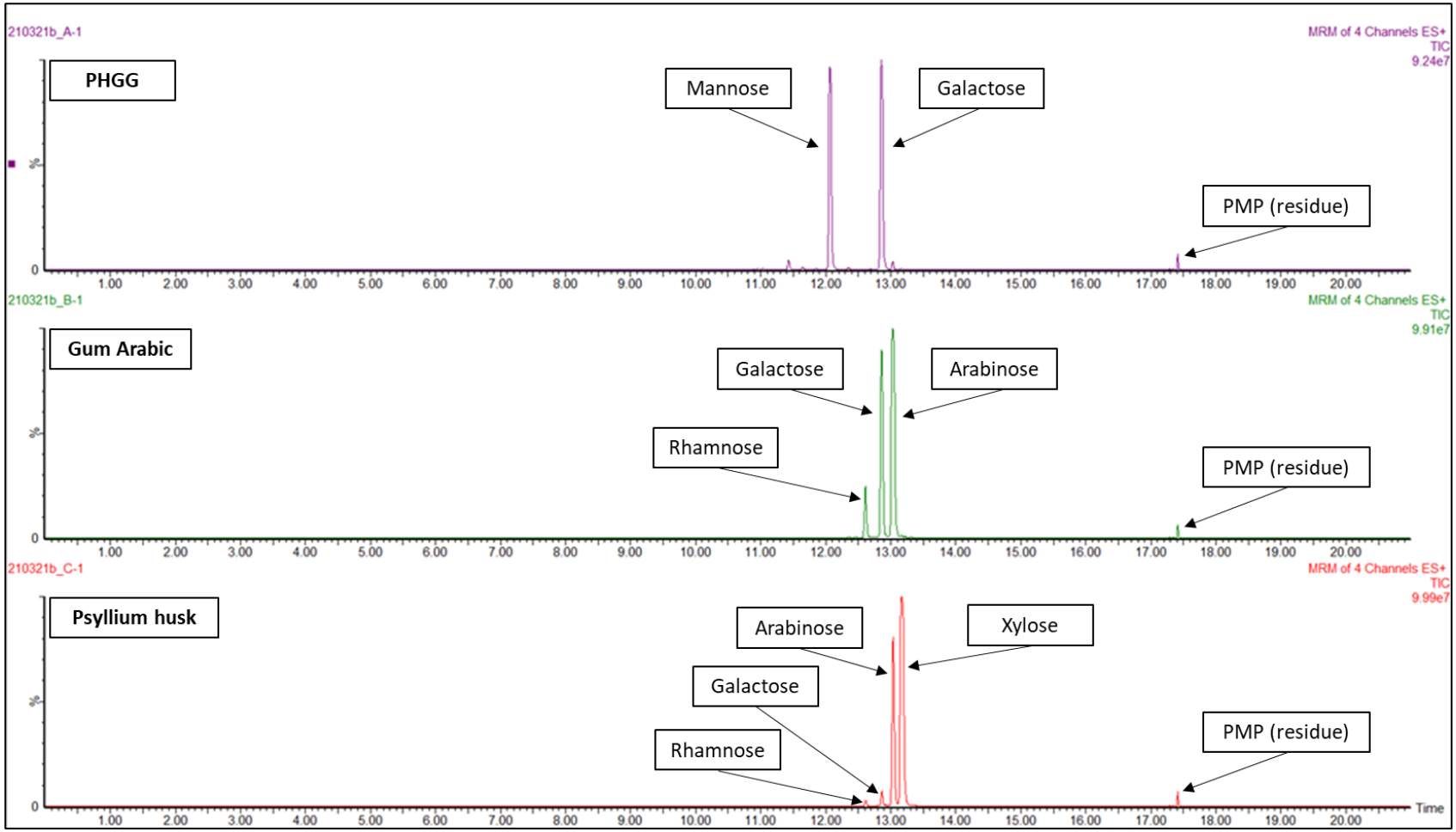


Figure 4.9. Representative HPLC-ESI-MS/MS chromatogram for analytes found in the purified polysaccharide of PHGG, gum Arabic and psyllium husk samples. Note the common PMP residue at 17.42 min in all three samples. The run is 21 min long, including a 1.5 min terminal wash phase.

Table 4.6. Table of analyte recoveries.

Analyte	Spike Levels [a]						Cumulative Results	
	Level 1 (50%)		Level 2 (75%)		Level 3 (100%)			
	% Recovery	% RSD	% Recovery	% RSD	% Recovery	% RSD	% Average Recovery [b]	% RSD
Galactose (*)	95.73	13.15	92.85	9.66	100.07	9.08	96.22	10.63
Arabinose (**)	100.54	5.25	110.13	5.75	117.80	6.39	109.49	5.80
Rhamnose (**)	103.09	4.63	110.40	9.12	112.50	4.42	108.66	6.05
Mannose (*)	101.46	13.20	109.11	10.14	115.92	6.02	108.83	9.78
Xylose (***)	92.45	6.94	103.50	1.94	100.10	2.59	98.68	3.82

(*) Validated in PHGG, (**) Validated in gum Arabic, (***) Validated in psyllium husk. (a) % Recovery ± % RSD calculated from *n* = 7 replicates injected in triplicate. (b) Average recovery of all three spiking levels ± % RSD.

Table 4.7. Concentration of target analytes in samples.

Analyte	Analyte Concentration (mg/g) ± SD [a]				
	Galactose	Arabinose	Rhamnose	Mannose	Xylose
PHGG (*)	425.03 ±2.95	N.P.	N.P.	269.04 ±4.08	N.P.
Gum Arabic (**)	141.19 ±2.24	658.82 ±13.81	31.91 ±0.42	N.P.	N.P.
Psyllium husk (***)	< LOQ	135.64 ±2.18	< LOQ	N.P.	723.89 ±7.49

(*) Validated sample PHGG, (**) Validated sample gum Arabic, (***) Validated sample psyllium husk, (N.P.) Analyte was not present in the validation sample, (LOQ) Analyte was present in sample but below limit of quantitation. (a) Analyte concentration calculated from *n* = 7 replicates injected in triplicate.

4.4. Discussion

A rapid pre-column derivatized 21 min method was developed for the quantification of five monomer constituents in PHGG, gum Arabic and psyllium husk commercial supplements. During extraction optimisation, solvent combinations such as methanol/water and dichloromethane/water were tested, but ethanol/water combinations consistently produced the highest amounts of precipitate. Besides its greater yield, ethanol is more suitable as a food-grade extraction solvent when compared against methanol or acetonitrile. During the gradient program optimisation, several analytical columns with C18 and amide stationary phases were tested, but the CSH C18 column consistently produced optimal analyte resolution, separation and the shortest method run times. The MS/MS conditions for the identification and confirmation of the selected monomer sugars was optimized and developed on an ad hoc basis, for the HPLC-ESI-Q-ToF instrument without relying exclusively on literature sources.

When compared to the methods reported in **Table 4.1**, the method presented in this chapter is a considerable improvement. The first four un-derivatized methods use highly specialised detectors and machines, where there are increased maintenance costs and potentially less frequent machine use over time. In the first method reported in **Table 4.1**, the recoveries were very poor (28.0 – 96.0%), and quantification could not be achieved for their reported analytes. In the next three un-derivatized methods ~5-6 monomers were analysed, which is similar to the five monomers quantified in this method, in a very similar time frame (18 – 30 min).

When compared to the pre-column derivatized HPLC-ESI-ToF-MS / HPLC-MS methods Guo *et al.* **[114]** and Wu *et al.* **[115]**, the method presented here is more than three times faster (21.0 min), using the HPLC-ESI-Q-ToF-MS technique for the not only the quantitation of five monomers, but for the identification and separation of nine monomers. The analyte recoveries (96.22 – 109.49%) of the quantitated monomers in PHGG, gum Arabic and psyllium husk also compares favourably against

reported un-derivatized methods. In addition to this, the large number (nine) of monomer analytes identified and separated here can be used as the basis for the development and validation of polysaccharides in dietary fibre supplements other than PHGG, gum Arabic and psyllium husk.

Therefore, the method presented here represents a considerable saving in run time, mobile phase use and other costs and expenses for manufacturers looking to standardise polysaccharide constituents in batches of commercial dietary fibre supplements. Standardisation and labelling by manufacturers will provide adequate clarity about product efficacy and allergen warnings to consumers purchasing commercial prebiotic dietary fibre supplements.

As expected, mannose and galactose were identified in the partially hydrolysed guar gum polysaccharide (galactomannan). What was unexpected was the higher amount of galactose (425.03 mg/g) in comparison to mannose (269.04 mg/g). This result is curious, since guar gum, the dietary fibre from which PHGG is enzymatically derived, is known to have a 1:2 ratio of galactose to mannose **[100]**. This result suggests that the enzymatic hydrolysis of guar gum by Endo-β-D-mannanase breaks the mannose backbone more frequently than the galactosyl side chain residue. The conventional assumption is that the proportional monomeric composition of guar gum is very similar to PHGG, but this result seems to suggest otherwise.

In gum Arabic, arabinose (658.82 mg/g) predominated greatly over the concentration of galactose (141.19 mg/g) and rhamnose (31.91 mg/g) residues. This result is unsurprising since gum Arabic polysaccharide arabinogalactan is extensively branched and these branches primarily consist of arabinosyl side chains **[231]**. Residues containing galacturonic and glucuronic acid have been reported in gum Arabic, but neither of these uronic residues were found in the dietary fibre supplement.

Psyllium husk is less extensively branched and primarily consists of a xylose backbone with some residues of arabinose and galactose. Xylose (723.89 mg/g) is present in large quantities, accompanied by

noticeable arabinose (135.64 mg/g). Smaller residues of galactose and rhamnose were detected as shown in **Figure 4.9**, but both these monomers were below the LOQ. This result conforms with what has been reported in literature regarding the structure of arabinoxylan **[232]**.

In theory, any the different monomer chemical composition of PHGG (galactomannan), gum Arabic (arabinogalactan) and psyllium (arabinoxylan) polysaccharides found in the analysed dietary fibre supplements may have an effect on the gut microbiome fermentability and the subsequent release of short-chain fatty acids (SCFA) in the caecum section of the colon, but this speculated effect has not been comparatively and quantitatively studied in literature **[233,234,235]**. Therefore, the variability of monomer composition in commercial dietary fibre polysaccharides may have significant implications for manufacturer claims of action against gastrointestinal disorders such as constipation, diarrhoea and IBS.

There are some limitations to the method presented here. Only five monomers were quantified in the dietary fibre supplements of PHGG, gum Arabic and psyllium husk. There may have been other monomer residues (fructose, erythrose, lyxose etc.) present in the samples that were not analysed in this method. Furthermore, method validation for different dietary supplements using this method as a basis might require further analyte identification, separation and method development. Another unavoidable limitation of this method is the need for the additional derivatization step during sample preparation, but fortuitously, that step is a very short one (~1-2 hours).

In summary, A rapid 21.0 min HPLC-ESI-Q-ToF method with MS/MS was developed and validated for the identification and quantitation of five monomer analytes found in PHGG, gum Arabic and psyllium husk commercial dietary fibre supplements. Expected monomer units were identified and quantified in PHGG (galactose, mannose), gum Arabic (arabinose, galactose, rhamnose) and psyllium husk (arabinose, xylose). Although some other residues were reported in existing literature, they were either not present or below the limit of quantitation (LOQ).

The developed method provides manufacturers a rapid, repeatable and reliable method for the labelling of monomer sugars in soluble dietary fibres during commercial manufacturing and batch testing. The MS method will also set the groundwork for future clinical investigations exploring the role of monomer composition in SCFA release and colonic health.

Chapter 5

Summary Discussion and Conclusion

5.1. Discussion and Conclusion

The combined research aim of this thesis was to investigate whether beneficial dietary modification using soluble dietary fibre was viable in patients with gastroparesis. To find an answer to this question, three different investigative approaches were required, namely, rheological analysis, mass spectrometric monomer characterisation, and clinical investigation in a pilot cohort of gastroparesis patients. A multidisciplinary approach was required since there are current knowledge gaps regarding the potential viability of soluble fibres in patients with gastroparesis.

It was quite clear from the rheological analysis **Chapter 2** that among the ten different soluble fibres tested, partially hydrolysed guar gum (PHGG) and gum Arabic displayed the target viscoelastic properties that could be described as “low-viscosity”. These two fibres seemed to require very little application of force to “flow” and remained completely dissolved at the therapeutic concentration (50 mg/mL) for a potential clinical study. Both PHGG and gum Arabic displayed very similar “low-viscosity” properties despite possessing vastly different polysaccharide structures, where PHGG is a short chain and linear gum polysaccharide, and gum Arabic is a long-chain and branched gum polysaccharide. Different molecular mechanisms lead both PHGG and gum Arabic to the state that can be described as “low-viscosity”, but in structural and chemical terms, they are simply different.

It can be observed in the clinical study **Chapter 3**, that for the most part, “low-viscosity” PHGG and gum Arabic are much better tolerated in mild-to-moderate gastroparesis patients, compared to the “high-viscosity” soluble fibre psyllium husk. Both PHGG and gum Arabic display the characteristic glucose regulation effect very similar to psyllium husk, while demonstrating significantly fewer negative symptoms when compared to the negative control, water. It must be noted that the patients were able to tolerate more (and similar) amounts of PHGG (7.99 g) and gum Arabic (7.57 g) on average, compared

to psyllium husk (4.53 g), and this variability could have significant implications, especially for colonic health.

In **Chapter 4**, the composition and prevalence of the monomer units in PHGG and gum Arabic were analysed and quantified rigorously not only to assist in the commercial standardisation of those supplements, but also in aiding the understanding of how monomer composition affects potential physiological effects in the lower gastrointestinal tract. It can be ascertained from the mass spectrometry (MS) analysis that not only are the constituent monomers in PHGG (galactose, mannose) and gum Arabic (arabinose, galactose and rhamnose) different, the concentrations of these monomers are also different. A lay observer might think given the chemical differences, that the immediate upper gastrointestinal effects on gastroparesis patients might also be somewhat different.

Soluble fibre, besides its role as a glycaemic index lowering (low-GI) macronutrient in the upper gastrointestinal tract, plays a major role in the lower gastrointestinal tract (the colon) as a prebiotic. A recent study has reported that people with gastroparesis suffer from slow transit constipation at higher rates (64.7%) compared to the normal population (28.1%) **[236]**. Around 30% of gastroparesis patients who require enteral tube feeding also suffer from chronic diarrhoea, and this number goes up further, to 80%, when these patients are admitted to the intensive care unit (ICU) **[237]**. Gut dysbiosis (imbalance of the gut microbiome) is also speculated to play a major role in both constipation and diarrhoea with complex cellular and molecular pathways being involved **[16]**. Due to these significant co-morbidities, gastroparesis patients also suffer from increased rates (24%) of anxiety/depression **[10]**. The common factor to unlocking potential solutions to all the above problems might be something as simple as adding soluble fibre to the diet, that is actively avoided by the gastroparetic population.

Soluble fibre supplementation cannot cure gastroparesis, but it can have a role to play in their health and well-being. As reported in **Chapter 1**, soluble fibres in general, are well known to have

varying degrees of beneficial effects with regards to constipation, diarrhoea and IBS. There are many literature reports of certain soluble fibres being effective against both constipation and diarrhoea at the same time, which can be confusing for physicians and dietitians looking for potential solutions **[42,60]**.

For example, the consensus opinion in literature is that the relief effect of PHGG against diarrhoea is confirmed **[238]**, while there are also studies that show that PHGG provides relief in patients with constipation dominant irritable bowel syndrome (IBS-C) **[80,239]**. The known mechanism of action for PHGG in relieving diarrhoea is by fermentation of PHGG by the gut microbiome, which releases short-chain fatty acids (SCFA) that are absorbed by the colonic lumen, which then stimulates sodium-dependent water absorption via cyclic AMP-independent processes (i.e., osmosis) **[240]**. It is well known that the soluble fibre mediated mechanism of constipation relief is through the modulation of osmotic pressures in the colon and stool softening (i.e., by water "holding" capacity), and that the structural chemistry and gel-forming ability of a specific soluble fibre in the colon directly affects its laxative ability **[47,53]**. The simultaneous mechanism of PHGG action against constipation through stool softening, and breakdown by SCFA digestions seem quite contradictory and counterintuitive, therefore, another factor may be playing a role.

This may be where the gut microbiome itself plays a huge role in the physiological effectiveness of a soluble fibre. There are many genera of organisms that live in the gut, prominent among these are *Bacteroides*, *Firmicutes*, *Akkermansia*, *Bifidobacterium*, *Faecalibacterium* and *Lactobacillus* which constitute well over 80% of the total gut microbiome **[70]**. The varied chemical structures in soluble fibres (PHGG vs gum Arabic) in interplay with the variability and prevalence of these many genera in individual gut environments may be producing varied physiological effects in different people. This is one of the reasons why the thorough chemical characterisation of monomers constituents in the physiologically relevant "low-viscosity" PHGG and gum Arabic in **Chapter 4** is of vital importance.

The three major factors that affect the efficacy of both PHGG and gum Arabic in relation to diarrhoea, constipation and IBS are the chemical structure (chain length, branching), the monomer constituents (galactose, mannose, arabinose etc.) and the gut microbiome composition. For example, gum Arabic might actually be better at treating constipation than PHGG in some people because of microbiome variation and the long and branched structure of gum Arabic, which may be harder to ferment than PHGG **[241]**. On the other hand, PHGG has been reported to be good at constipation (in combination with diarrhoea) relief because the genera most responsible for SCFA release through PHGG fermentation, the butyrate-producing *Bifidobacterium*, might be either absent or at very low prevalence in certain types of patients **[103]**.

PHGG-induced microbiota changes have been suggested to improve slow-transit constipation as a result of reduced intra-lumen pressure, but this theory remains controversial **[242]**. Therefore, there could be a large gradient of effectiveness in PHGG and gum Arabic with respect to constipation, diarrhoea and IBS, where the reasons for effectiveness or ineffectiveness of either of these fibres as a treatment might not be immediately apparent or obvious.

In summary, **Chapter 2** and **Chapter 3** demonstrate the viability and short-term tolerability of "low-viscosity" soluble fibre in gastroparesis patients, where both PHGG and gum Arabic have very similar immediate beneficial effects. In **Chapter 4** the monomer constituents of the polysaccharides in these "low-viscosity" soluble fibres were quantified as a precursor for future investigations. These future investigations will explore the different effects that PHGG and gum Arabic produce in the lower gastrointestinal tract, which might help not only people with gastroparesis, but also people who suffer from other gastrointestinal motility disorders, either in combination with, or without gastroparesis.

5.2. Future Work

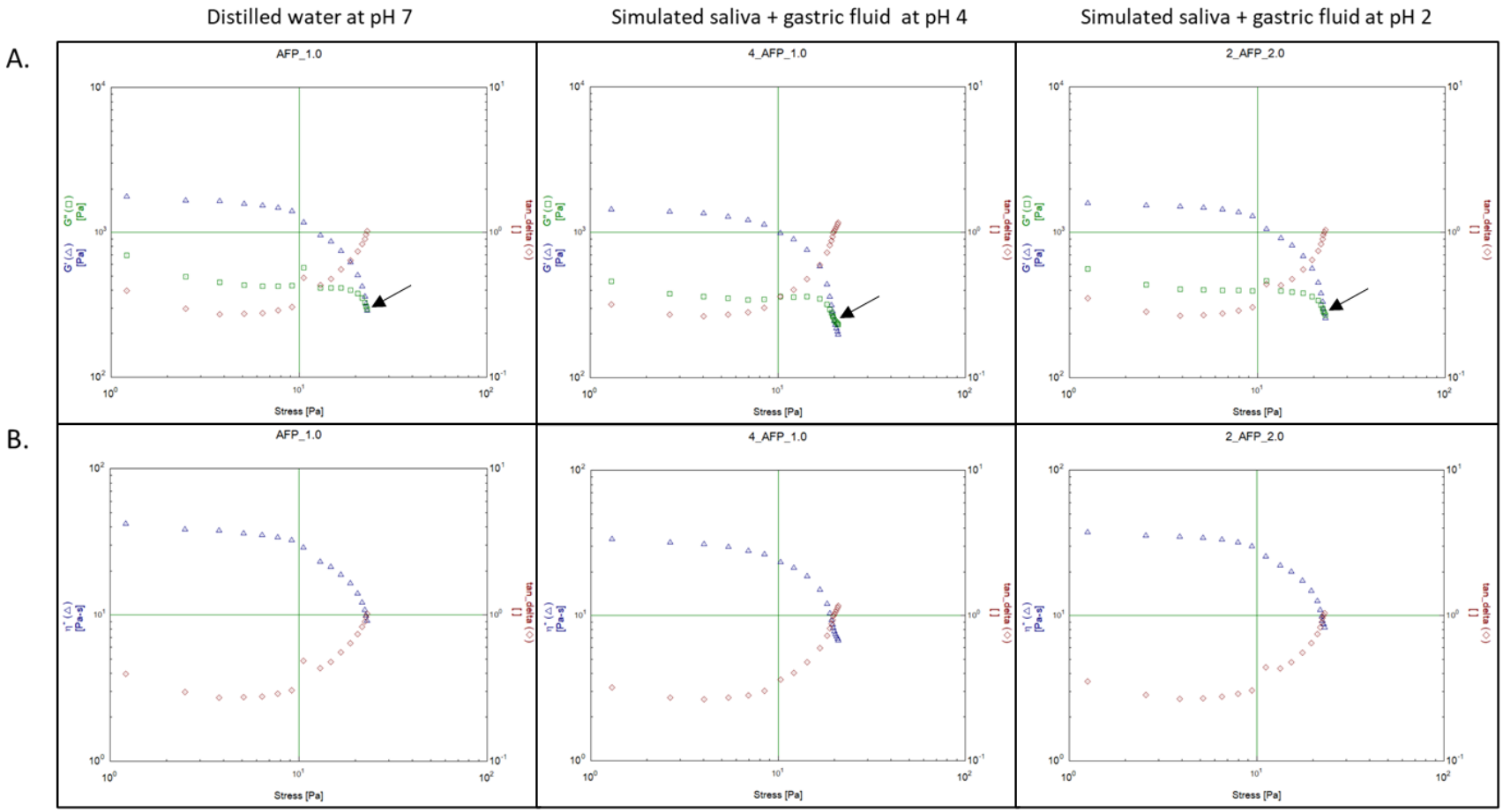
While the viability and short-term tolerability of "low-viscosity" PHGG and gum Arabic in gastroparesis patients has been established by this thesis, long-term clinical investigations are required due to the lack of existing literature. While the fibre supplement delivery method presented in this thesis is adequate (fibre dissolved in water), other delivery methods such as packaged dietary fibre jellies, where the taste and texture can be modified, could be explored for a longer-term study. A longer-term study could also investigate using blood panels (long-term resting blood glucose, insulin, cholesterol, C-reactive protein), stool samples and gut microbiome composition (with 16S rRNA or whole genome sequencing) that were not explored in this study.

Recent case studies have also explored the effectiveness of "low-viscosity" soluble fibres in reducing the occurrence of constipation and diarrhoea in gastroparesis patients with dysphagia, requiring enteral tube feeding **[223]**. Supplementation of dietary fibre in enteral tubes comes with its own unique set of challenges, such as avoiding tube blockages, but this topic will be worth exploring once the long-term effects of PHGG and gum Arabic are better understood. Other possibilities for future work includes the designing of new types of soluble dietary fibre supplements, the development of more comprehensive mass spectrometry methods, and the exploration of other potential benefits of "low-viscosity" soluble fibres in gastrointestinal disorders such as dysphagia.

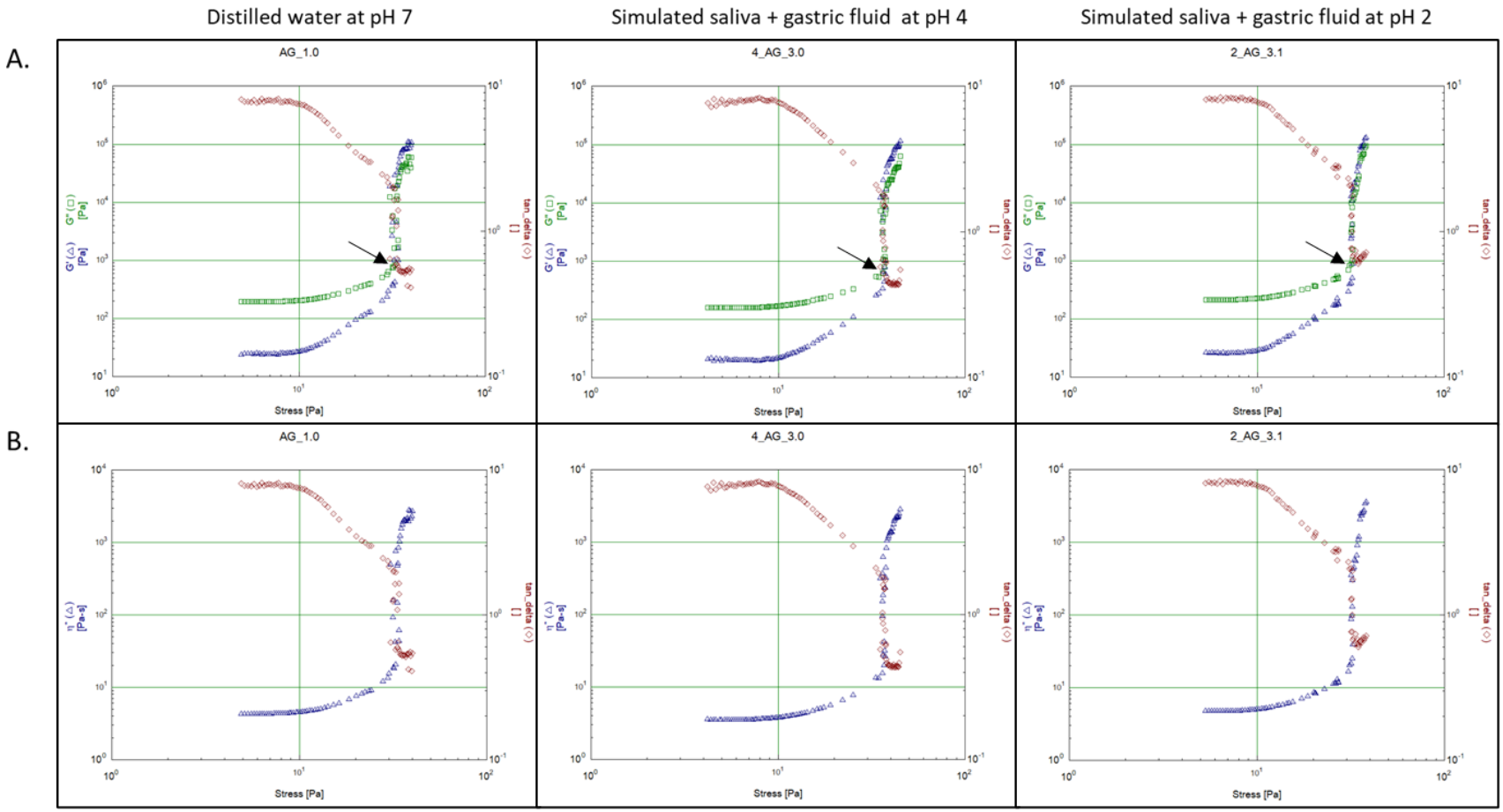
The ultimate purpose of exploring the role and usefulness of "low-viscosity" type soluble dietary fibre in this thesis, is to provide enough evidence for its eventual inclusion in the treatment and management protocols for gastroparesis, thereby, becoming a valuable resource for both physicians and dietitians.

Appendix

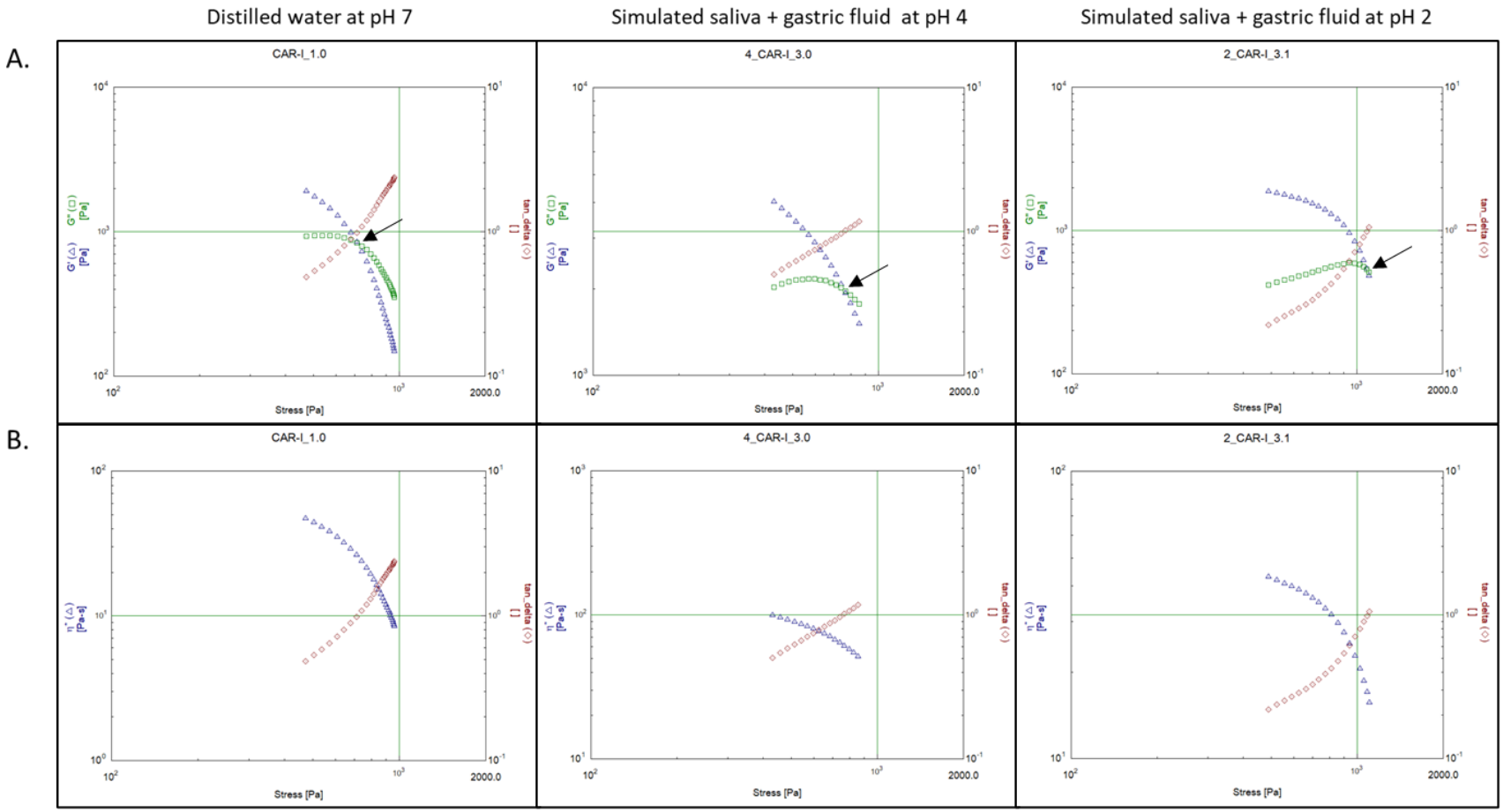
Appendices A, B & C



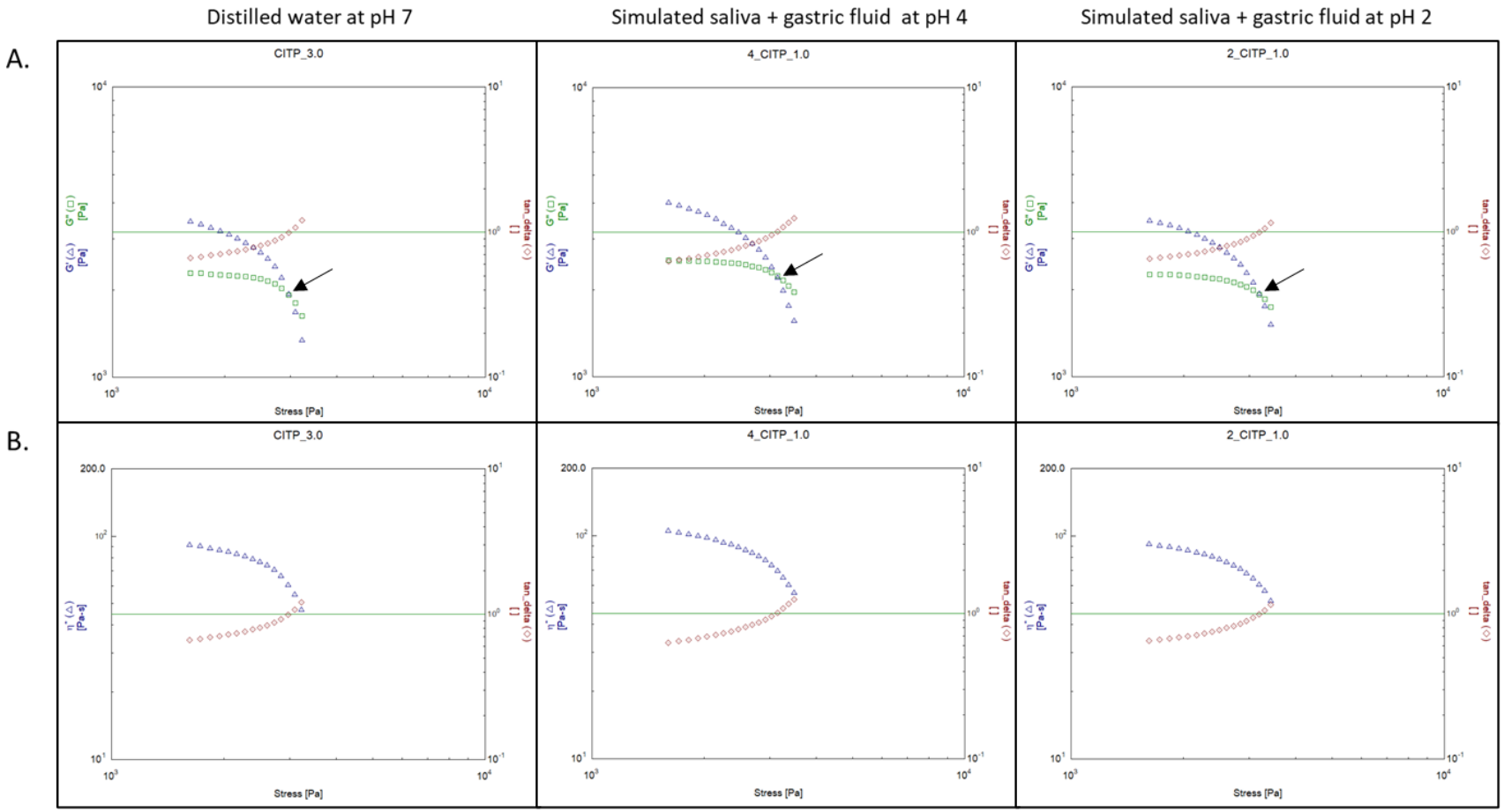
Appendix A1. An example of pseudoplastic rheological behaviour observed in apple-fibre pectin. Graphs show **(A)** rheological yield points (top half, y-axis left: G' and G" in Pa, y-axis right: Tan (δ), x-axis bottom: shear stress in Pa) and **(B)** complex viscosity (bottom half, y-axis left: η* in Pa.s, y-axis right: tan (δ), x-axis bottom: shear stress in Pa) plots as shear stress is incrementally increased in distilled water (pH 7), simulated digestions with SDF-PPI (simulated digestion fluid proton pump inhibitor at pH 4) and SDF (simulated digestion fluid at pH 2). The black arrow in each plot indicates the yield point where Tan (δ) = 1. Note that the rheological behaviour does not change under chemically simulated digestion.



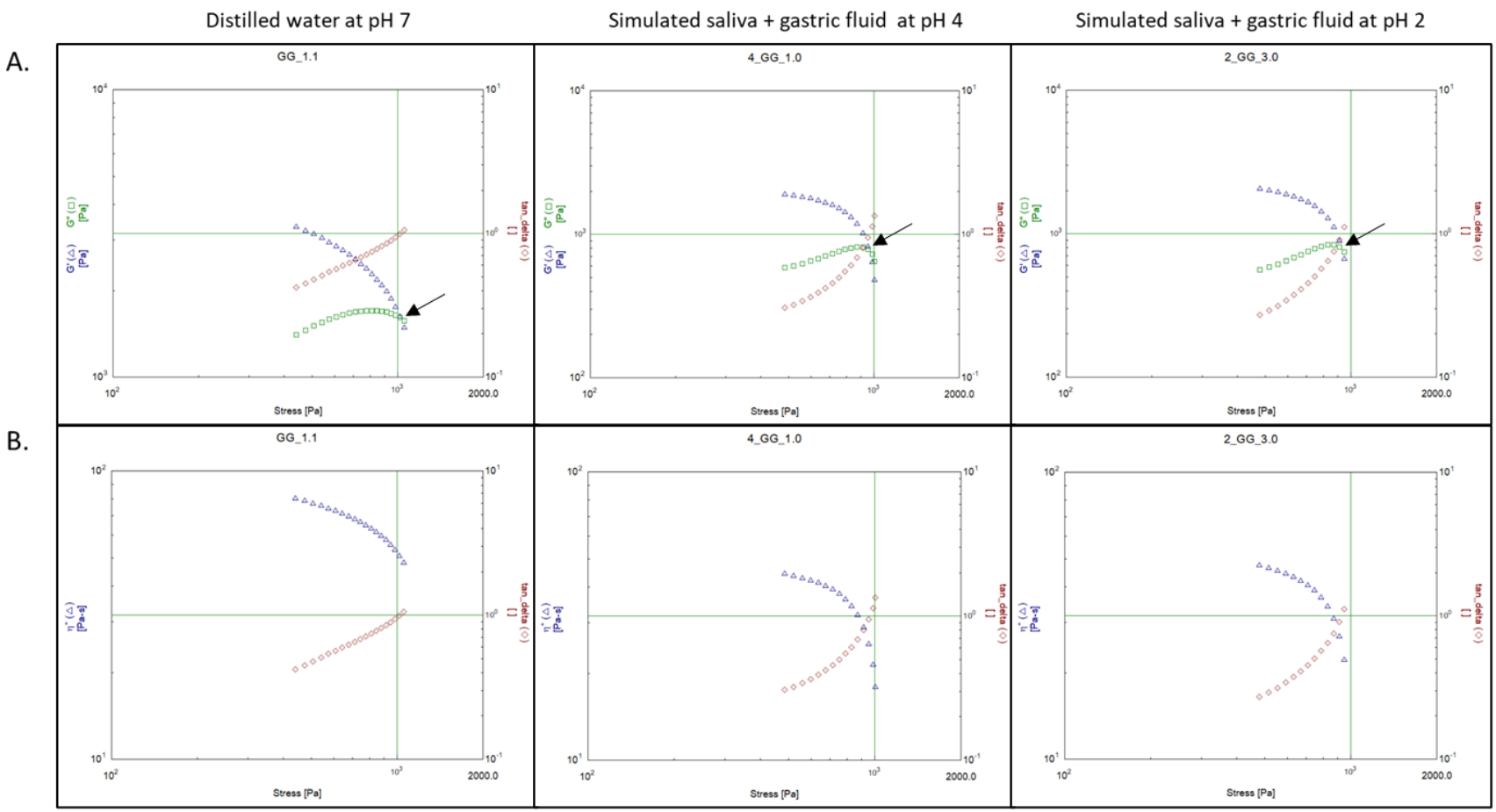
Appendix A2. An example of dilatant rheological behaviour observed in gum Arabic. Graphs show **(A)** rheological yield points (top half, y-axis left: G' and G" in Pa, y-axis right: Tan (δ), x-axis bottom: shear stress in Pa) and **(B)** complex viscosity (bottom half, y-axis left: η* in Pa.s, y-axis right: tan (δ), x-axis bottom: shear stress in Pa) plots as shear stress is incrementally increased in distilled water (pH 7), simulated digestions with SDF-PPI (simulated digestion fluid proton pump inhibitor at pH 4) and SDF (simulated digestion fluid at pH 2). The black arrow in each plot indicates the yield point where Tan (δ) = 1. Note that the rheological behaviour does not change under chemically simulated digestion.



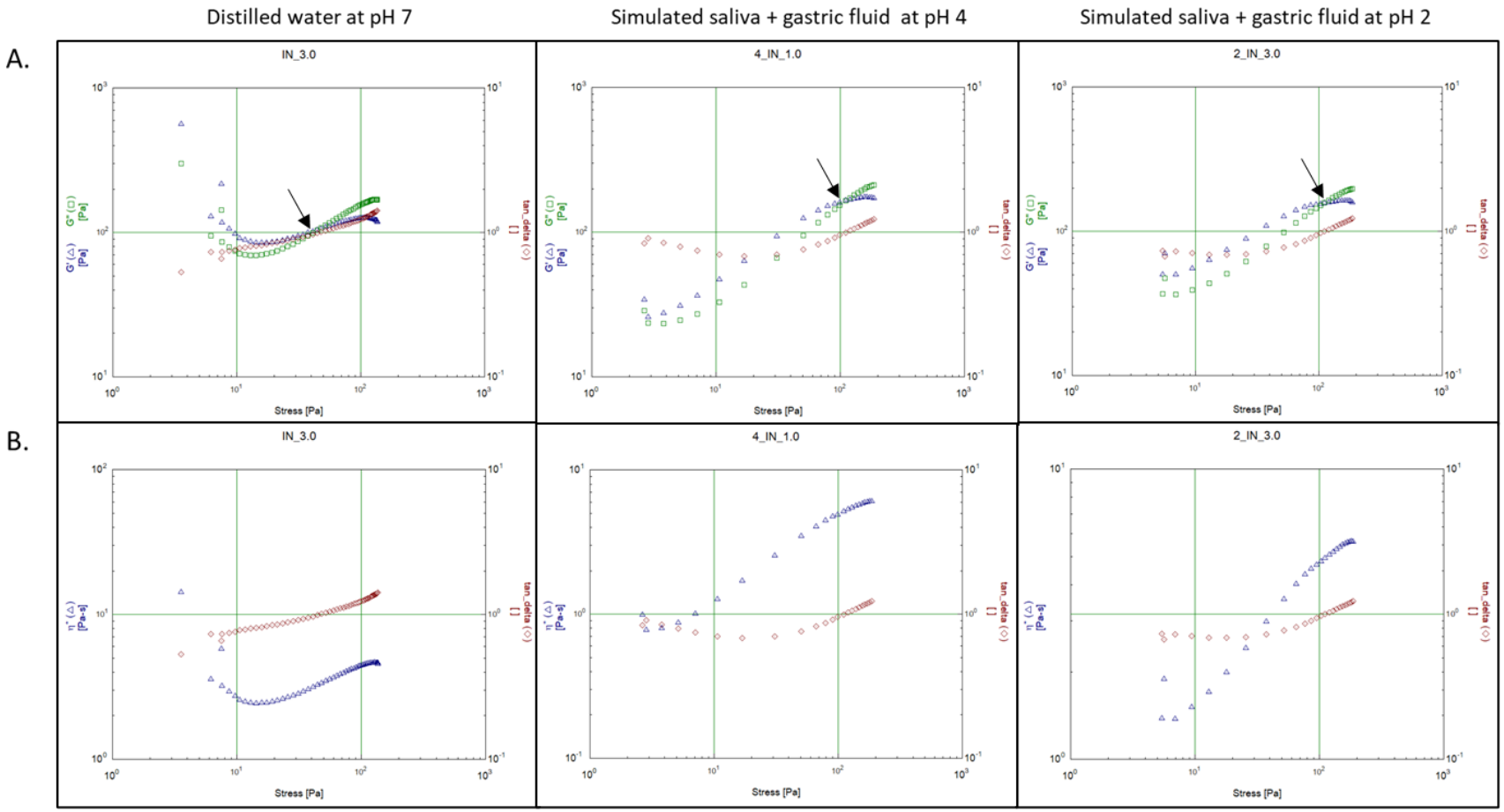
Appendix A3. An example of pseudoplastic rheological behaviour observed in iota-carrageenan. Graphs show **(A)** rheological yield points (top half, y-axis left: G' and G" in Pa, y-axis right: Tan (δ), x-axis bottom: shear stress in Pa) and **(B)** complex viscosity (bottom half, y-axis left: η* in Pa.s, y-axis right: tan (δ), x-axis bottom: shear stress in Pa) plots as shear stress is incrementally increased in distilled water (pH 7), simulated digestions with SDF-PPI (simulated digestion fluid proton pump inhibitor at pH 4) and SDF (simulated digestion fluid at pH 2). The black arrow in each plot indicates the yield point where Tan (δ) = 1. Note that the rheological behaviour does not change under chemically simulated digestion.



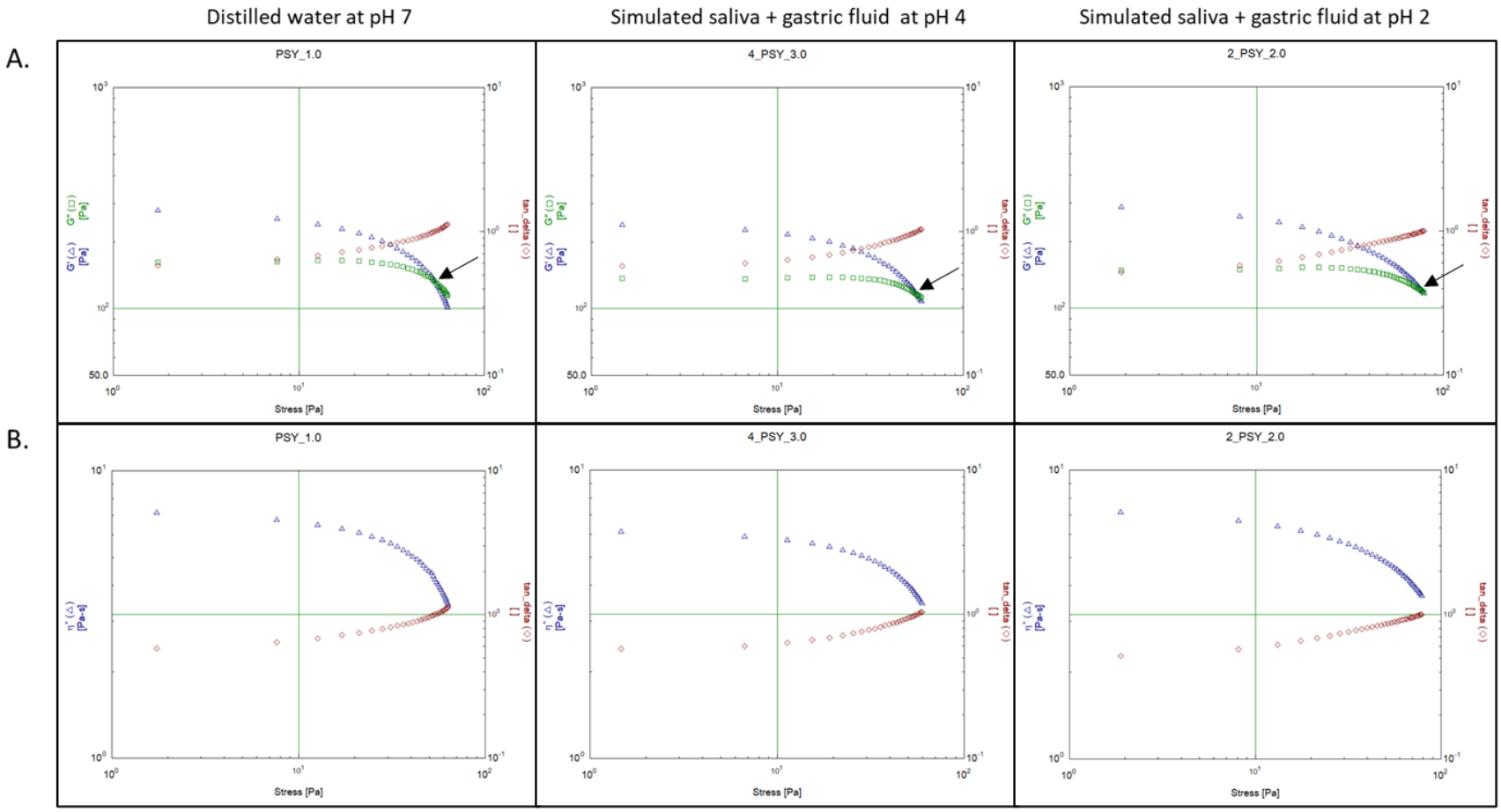
Appendix A4. An example of pseudoplastic rheological behaviour observed in citrus pectin. Graphs show **(A)** rheological yield points (top half, y-axis left: G' and G" in Pa, y-axis right: Tan (δ), x-axis bottom: shear stress in Pa) and **(B)** complex viscosity (bottom half, y-axis left: η* in Pa.s, y-axis right: tan (δ), x-axis bottom: shear stress in Pa) plots as shear stress is incrementally increased in distilled water (pH 7), simulated digestions with SDF-PPI (simulated digestion fluid proton pump inhibitor at pH 4) and SDF (simulated digestion fluid at pH 2). The black arrow in each plot indicates the yield point where Tan (δ) = 1. Note that the rheological behaviour does not change under chemically simulated digestion.



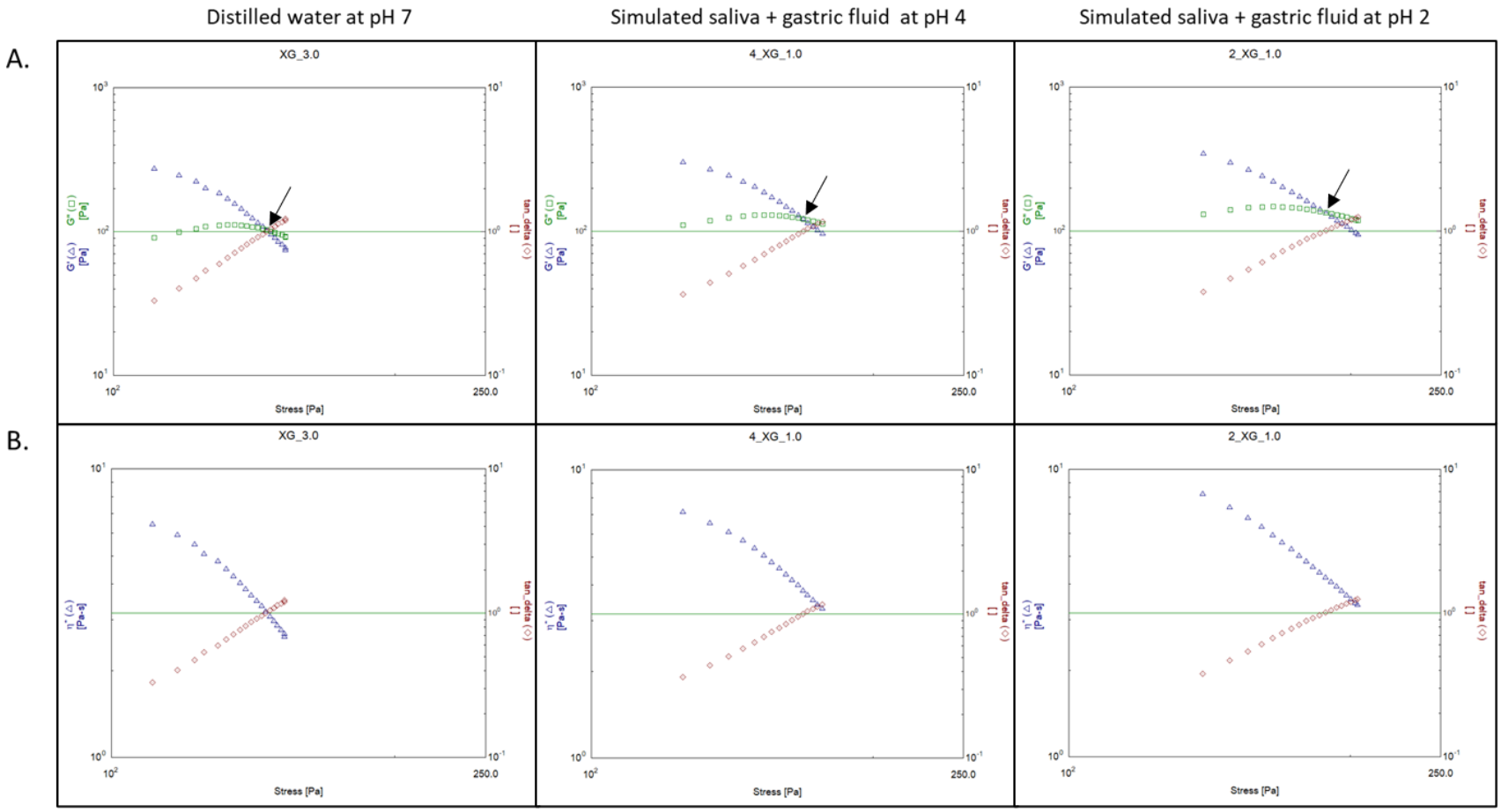
Appendix A5. An example of pseudoplastic rheological behaviour observed in guar gum. Graphs show **(A)** rheological yield points (top half, y-axis left: G' and G" in Pa, y-axis right: Tan (δ), x-axis bottom: shear stress in Pa) and **(B)** complex viscosity (bottom half, y-axis left: η* in Pa.s, y-axis right: tan (δ), x-axis bottom: shear stress in Pa) plots as shear stress is incrementally increased in distilled water (pH 7), simulated digestions with SDF-PPI (simulated digestion fluid proton pump inhibitor at pH 4) and SDF (simulated digestion fluid at pH 2). The black arrow in each plot indicates the yield point where Tan (δ) = 1. Note that the rheological behaviour does not change under chemically simulated digestion.



Appendix A6. An example of pseudoplastic rheological behaviour observed in inulin. Graphs show **(A)** rheological yield points (top half, y-axis left: G' and G" in Pa, y-axis right: Tan (δ), x-axis bottom: shear stress in Pa) and **(B)** complex viscosity (bottom half, y-axis left: η* in Pa.s, y-axis right: tan (δ), x-axis bottom: shear stress in Pa) plots as shear stress is incrementally increased in distilled water (pH 7), simulated digestions with SDF-PPI (simulated digestion fluid proton pump inhibitor at pH 4) and SDF (simulated digestion fluid at pH 2). The black arrow in each plot indicates the yield point where Tan (δ) = 1. Note that the rheological behaviour does not change under chemically simulated digestion.



Appendix A7. An example of pseudoplastic rheological behaviour observed in psyllium husk. Graphs show **(A)** rheological yield points (top half, y-axis left: G' and G" in Pa, y-axis right: Tan (δ), x-axis bottom: shear stress in Pa) and **(B)** complex viscosity (bottom half, y-axis left: η* in Pa.s, y-axis right: tan (δ), x-axis bottom: shear stress in Pa) plots as shear stress is incrementally increased in distilled water (pH 7), simulated digestions with SDF-PPI (simulated digestion fluid proton pump inhibitor at pH 4) and SDF (simulated digestion fluid at pH 2). The black arrow in each plot indicates the yield point where Tan (δ) = 1. Note that the rheological behaviour does not change under chemically simulated digestion.



Appendix A8. An example of pseudoplastic rheological behaviour observed in xanthan gum. Graphs show **(A)** rheological yield points (top half, y-axis left: G' and G" in Pa, y-axis right: Tan (δ), x-axis bottom: shear stress in Pa) and **(B)** complex viscosity (bottom half, y-axis left: η* in Pa.s, y-axis right: tan (δ), x-axis bottom: shear stress in Pa) plots as shear stress is incrementally increased in distilled water (pH 7), simulated digestions with SDF-PPI (simulated digestion fluid proton pump inhibitor at pH 4) and SDF (simulated digestion fluid at pH 2). The black arrow in each plot indicates the yield point where Tan (δ) = 1. Note that the rheological behaviour does not change under chemically simulated digestion.

Appendix B1. Participant information sheet (PIS) used in clinical study.

Would you like to participate in a research study?

We are recruiting volunteers with gastroparesis to participate in a dietary trial of low viscosity soluble fibres at the newly established Western Sydney University, Macarthur Clinical School.

You may contact our investigator Harsha for more information about this trial.

You can contact **Harsha or Jerry**
Email (Anytime): 17271790@student.westernsydney.edu.au
Phone (Office Hours 9-5): (02) 4620 3865

Benefits of low viscosity soluble fibre in gastroparesis patients

Project Summary

Soluble fibres are an essential part of a healthy diet. When mixed with liquids, soluble fibres form a gel-like substance which allows for slow release of ingested sugars and a low glycaemic index (low GI). In patients with gastroparesis, the high-viscosity of soluble fibre can further increase digestion time and associated symptoms. A range of low-viscosity fibres may offer the some of the benefits without an increase in symptoms. The study is being conducted by Dr Jerry Zhou (Clinical Researcher), Dr Vincent Ho (Gastroenterologist) and Mr Harsha Suresh (PhD student) at the School of Medicine, Western Sydney University.

How is the study being paid for?

School of Medicine, Western Sydney University

Rotary Health Australia/Rotary Devonport Scholarship

What will I be asked to do?

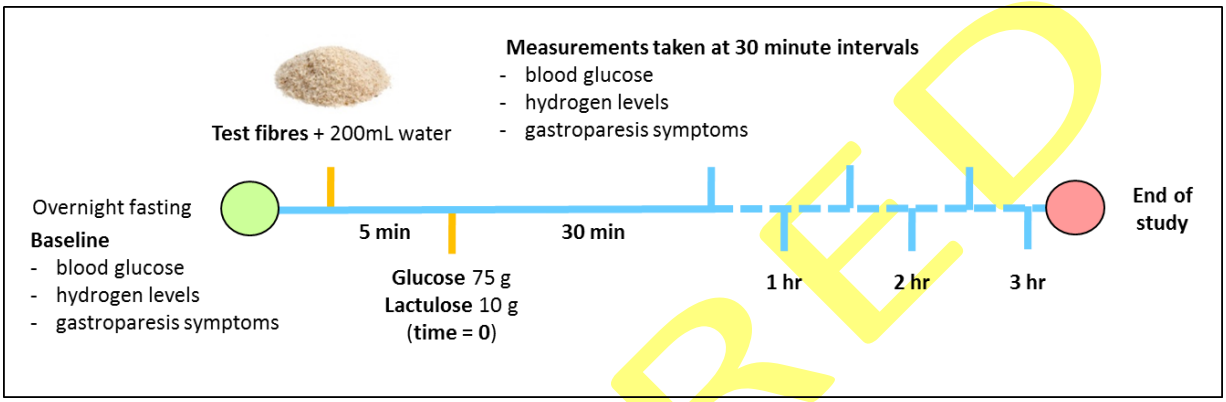
The total study will consist of four 3 hour visits, ideally over a 4 week period. Your blood glucose and gastric transit times will be measured using a portable glucose monitor and hydrogen breathalyser, respectively at 30 min intervals. The hydrogen breath analyser measures hydrogen levels in exhaled breathe. You will be asked to exhale into a collection bag periodically throughout the test. Hydrogen is produced by the gut bacteria at the end of the small intestine when they encounter sugars. By observing increase in hydrogen we can calculate the transit time from mouth to large intestine.

You will also be required to fill out a symptom severity form at the same time in accordance with the Gastroparesis Cardinal Symptom Index (GCSI). These tests are not invasive and require minimal effort

from the participant. The researchers may ask you general questions relevant to this study (age, gender, BMI, medical history, exercise and diet scheme).

For this study, there will be four tests spread out over consecutive weeks. A test can be re-scheduled if it is missed during that week due to reasons such as sickness, time conflict or emergencies. The trial will be randomised, you will not know which fibre or control is being tested until the information is collected for analysis.

No prescriptions or medicines are required during the test. The procedure will take around 3 hours to complete and you may bring laptops, books, or phone during the intervals to pass the time.



What benefits will I, and/or the broader community, receive for participating?

There are no direct benefits or incentives to the participants. At the end of this study, the benefits of low-viscosity fibre at managing blood glucose and gastroparesis symptoms will be shared with the participants. The results of this research will also contribute towards understanding dietary options in gastroparesis patients and further medical knowledge.

Will the study involve any risk or discomfort for me? If so, what will be done to rectify it?

Serious complications are extremely rare during these tests and Dr. Ho will be available nearby during any complications or emergencies.

Complications may include:

Bloating & Nausea – Bloating can occur during the test and may be severe. In such cases, the attending physician will be contacted. Nausea can happen during the test and if there is a serious reaction, the test will be stopped and the attending physician informed.

Hyperglycaemia – This is major risk factor for type 1 and type 2 diabetes patients who are consuming a sugar test meal, though it is a rare occurrence. You may be recommended to an endocrinologist by Dr. Ho for insulin therapy.

Hyperosmolar hyperglycaemic nonketotic syndrome (HHNS) – Is extremely rare and happens when your blood glucose levels suddenly spike. You will be taken to an emergency facility at nearby Campbelltown Hospital if this occurs during a test.

You can reduce your risk of complications by carefully following your doctor's instructions for preparing for a prolonged blood glucose test, such as fasting and eschewing certain medications.

How do you intend to publish or disseminate the results?

All aspects of the study, including results, will be strictly confidential and only the investigators in this form will be able to access the information about the participants. A report of the study may be submitted for research publication and the participants will not be identifiable in the report.

Will the data and information that I have provided be disposed of?

Please be assured that only the researchers will have access to the raw data you provide. During analysis and publication the data will not be identifiable. All information will be held for a maximum of 15 years before being properly disposed of.

Can I withdraw from the study?

Participation is entirely voluntary, if you do sign the form and wish to withdraw any time later, your data will be removed and excluded from the study. Whatever your decision, please be assured that it will not affect your medical treatment or your relationship with medical staff.

What if I require further information?

When you have read this information Harsha Suresh will discuss it with your further and answer any questions you may have.

What if I have a complaint?

If you have any complaints or reservations about the ethical conduct of this research, you may contact the Ethics Committee through Research Engagement, Development and Innovation (REDI) on Tel +61 2 4736 0229 or email humanethics@westernsydney.edu.au.

Any issues you raise will be treated in confidence and investigated fully, and you will be informed of the outcome.

If you agree to participate in this study, you may be asked to sign the Participant Consent Form. The information sheet is for you to keep and the consent form is retained by the researcher/s.

This study has been approved by the Western Sydney University Human Research Ethics Committee. The Approval number is *[enter approval number once the project has been approved]*.

Mail: Locked Bag 1797,
Penrith NSW 2751
Phone: 02 4736 0493,
Email: humanethics@westernsydney.edu.au

Consent Form

Benefits of low viscosity soluble fibre in gastroparesis patients

I hereby consent to participate in the above named research project.

I acknowledge that

- ☐ I have read the participant information sheet (or where appropriate, have had it read to me) and have been given the opportunity to discuss the information and my involvement in the project with the researcher/s
- ☐ The procedures required for the project and the time involved have been explained to me, and any questions I have about the project have been answered to my satisfaction.

I consent to:

- ☐ Participate in this research project as described and understand that I can withdraw at any time during the study without affecting my future health care.
- ☐ I consent for my data and information provided to be used in this project and other related projects for an extended period of time.
- ☐ I understand that my involvement is confidential and that the information gained during the study may be published and stored for other research use but no information about me will be used in any way that reveals my identity.
- ☐ I understand that I can withdraw from the study at any time without affecting my relationship with the researcher/s, and any organisations involved, now or in the future.

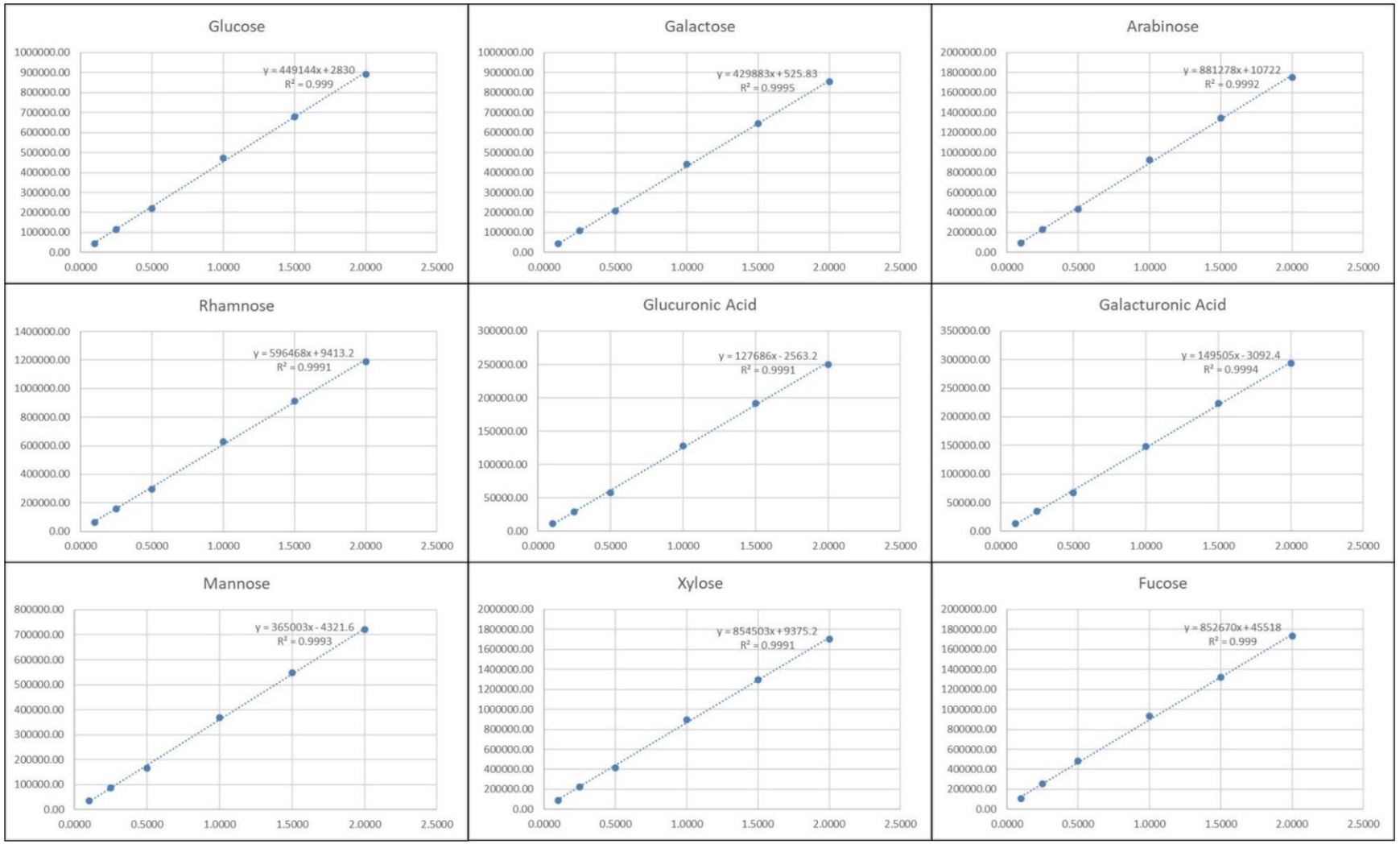
Signed: _____

Name: _____

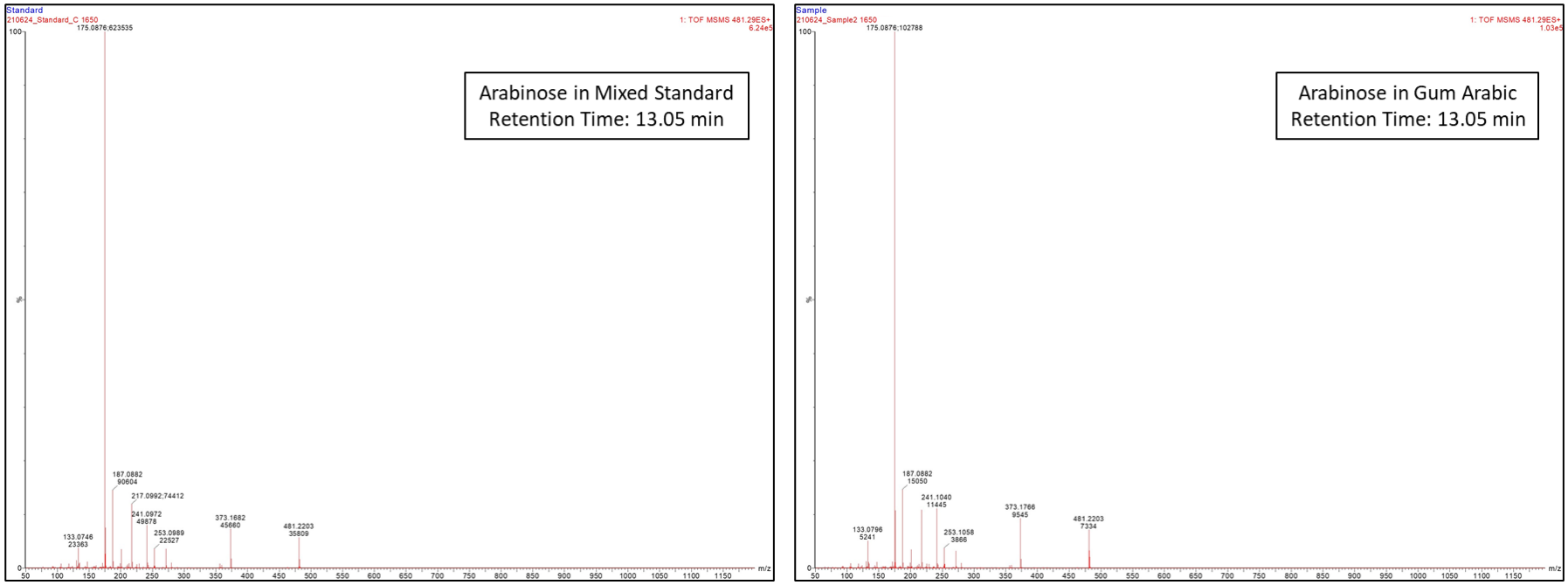
Date: _____

This study has been approved by the Human Research Ethics Committee at Western Sydney University. The ethics reference number is: H12254. If you have any complaints or reservations about the ethical conduct of this research, you may contact the Ethics Committee through Research Engagement, Development and Innovation (REDI) on Tel +61 2 4736 0229 or email humanethics@westernsydney.edu.au. Any issues you raise will be treated in confidence and investigated fully, and you will be informed of the outcome.

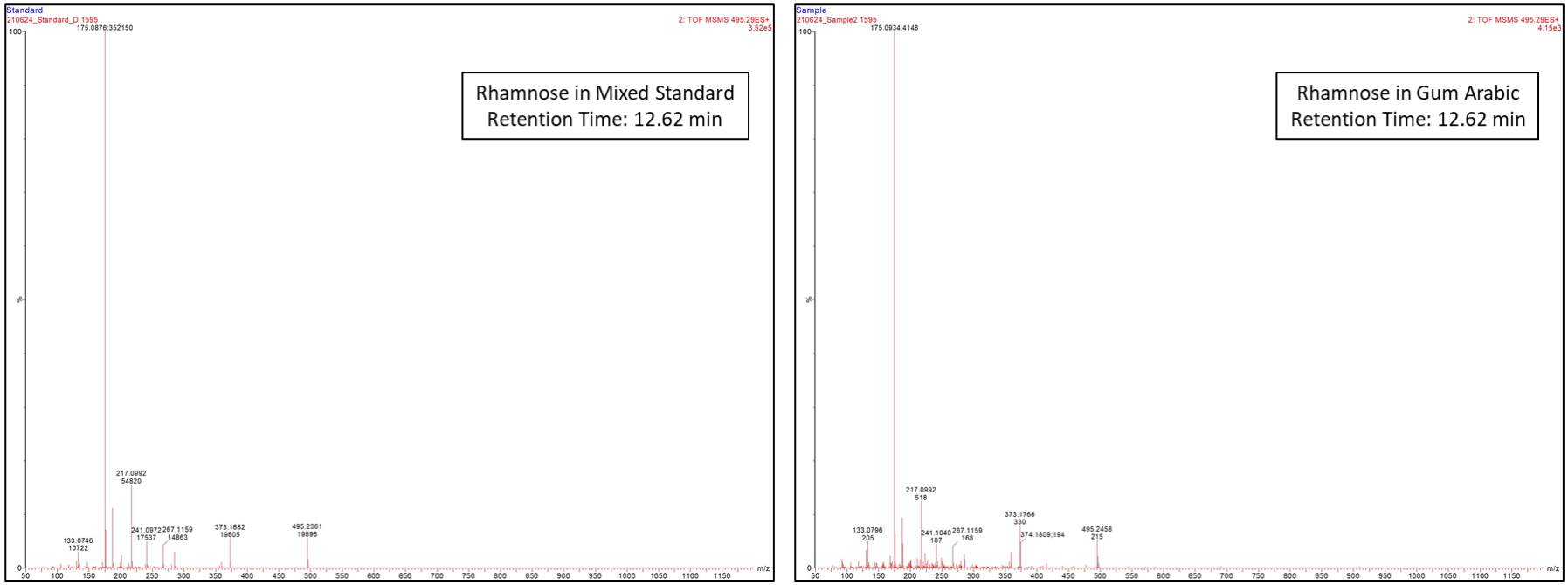
Appendix B2. Consent form used in clinical study.



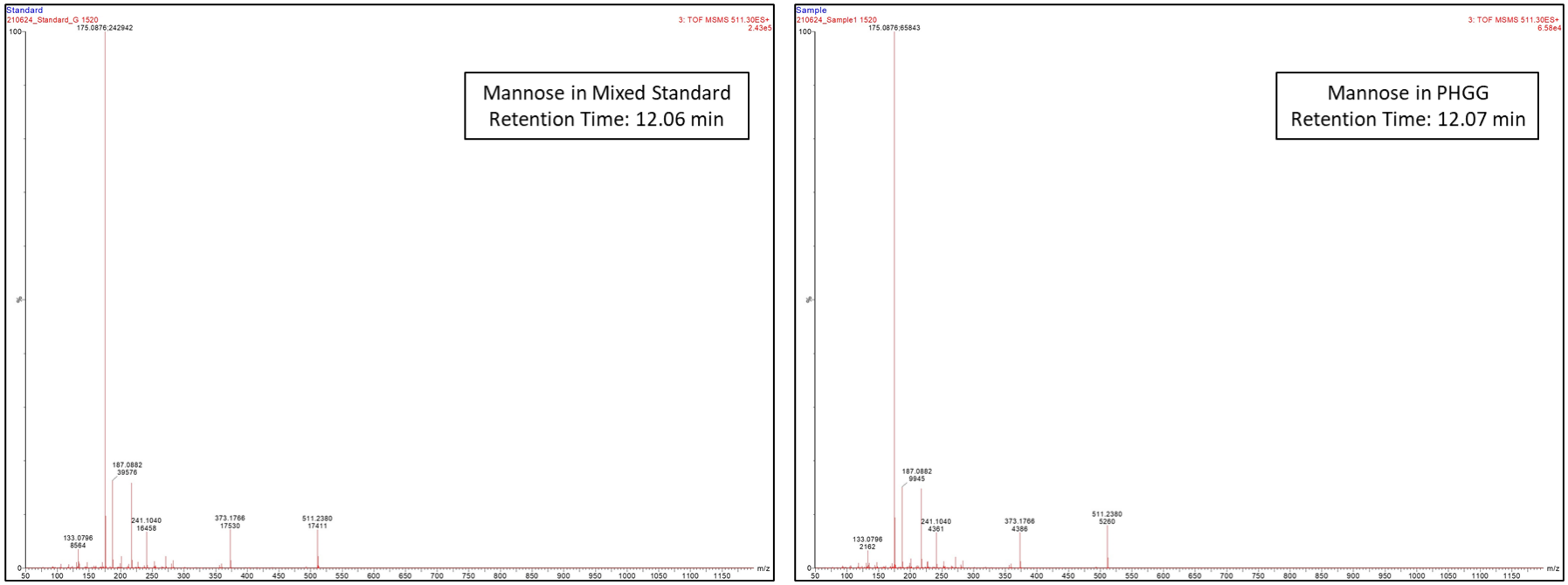
Appendix C1. Standard curves for glucose, galactose, arabinose, rhamnose, glucuronic acid, galacturonic acid, mannose, xylose and fucose in mixed standard (calibrated stock solution). The x-axis units for concentration are (µg/mL), and the y-axis units for absorbance are (counts).



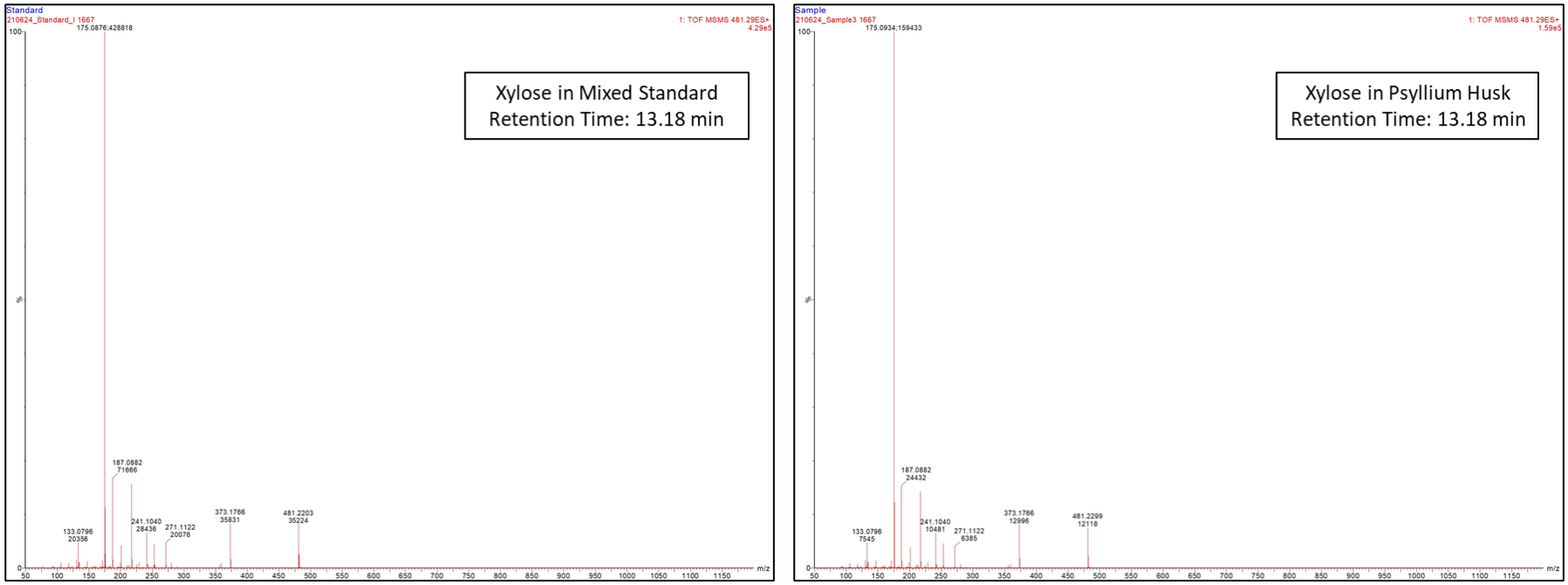
Appendix C2. Comparison of MS/MS spectra of the arabinose peak in the mixed standard (calibrated stock solution) and the arabinose peak in the gum Arabic supplement.



Appendix C3. Comparison of MS/MS spectra of the rhamnose peak in the mixed standard (calibrated stock solution) and the rhamnose peak in the gum Arabic supplement.



Appendix C4. Comparison of MS/MS spectra of the mannose peak in the mixed standard (calibrated stock solution) and the mannose peak in the PHGG supplement.



Appendix C5. Comparison of MS/MS spectra of the xylose peak in the mixed standard (calibrated stock solution) and the xylose peak in the psyllium husk supplement.

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