Molecular characterisation of breakfast cereals and rice to understand their digestibility

Presented by:

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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, 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.

..........................................................

Michelle Rosemarie Toutounji

September 2015
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Knowledge is power.

- Sir Francis Bacon
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<td>γ</td>
<td>Magnetogyric ratio</td>
</tr>
<tr>
<td>δ</td>
<td>Chemical shift</td>
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<tr>
<td>δ_x</td>
<td>Chemical shift of nucleus x</td>
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<tr>
<td>μ_{app}</td>
<td>Apparent electrophoretic mobility</td>
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<td>μ_{EOF}</td>
<td>Electrophoretic mobility of the electroosmotic flow</td>
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<td>a</td>
<td>Slope</td>
</tr>
<tr>
<td>b</td>
<td>Intercept</td>
</tr>
<tr>
<td>B_0</td>
<td>External static magnetic field</td>
</tr>
<tr>
<td>BC</td>
<td>Breakfast cereal</td>
</tr>
<tr>
<td>BGE</td>
<td>Background electrolyte</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>C</td>
<td>Concentration</td>
</tr>
<tr>
<td>c</td>
<td>Current</td>
</tr>
<tr>
<td>c_f</td>
<td>Final current</td>
</tr>
<tr>
<td>c_i</td>
<td>Initial current</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>CP</td>
<td>Cross-polarisation</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DAD</td>
<td>Diode array detector</td>
</tr>
<tr>
<td>DB</td>
<td>Degree of branching</td>
</tr>
<tr>
<td>DD</td>
<td>Dipolar decoupling</td>
</tr>
<tr>
<td>D_f</td>
<td>Dilution factor</td>
</tr>
<tr>
<td>DNS method</td>
<td>Dinitrosalicylic acid method</td>
</tr>
<tr>
<td>DP</td>
<td>Degree of polymerisation</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimater</td>
</tr>
<tr>
<td>E</td>
<td>Electric field</td>
</tr>
<tr>
<td>EOF</td>
<td>Electroosmotic flow</td>
</tr>
<tr>
<td>F</td>
<td>Force</td>
</tr>
<tr>
<td>FID</td>
<td>Free induction decay</td>
</tr>
<tr>
<td>FS-CE</td>
<td>Free-solution capillary electrophoresis</td>
</tr>
<tr>
<td>FSANZ</td>
<td>Food standards Australia New Zealand</td>
</tr>
<tr>
<td>FT-IR spectroscopy</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>G</td>
<td>Number of standard deviation for the Grubbs test</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GI</td>
<td>Glycaemic index</td>
</tr>
<tr>
<td>GL</td>
<td>Glycaemic load</td>
</tr>
<tr>
<td>GOD-POD method</td>
<td>Glucose oxidase/peroxidase method</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GT</td>
<td>Gelatinisation temperature</td>
</tr>
<tr>
<td>H</td>
<td>Height of peak</td>
</tr>
<tr>
<td>h</td>
<td>Half-height of peak</td>
</tr>
<tr>
<td>HILIC</td>
<td>Hydrophilic interaction liquid chromatography</td>
</tr>
<tr>
<td>HPAEC</td>
<td>High performance anion exchange chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>$I_{\alpha(1,4)}$</td>
<td>Integral of the number of branching points at $\alpha(1,4)$ signal</td>
</tr>
<tr>
<td>$I_{\alpha(1,6)}$</td>
<td>Integral of the number of branching points at $\alpha(1,6)$ signal</td>
</tr>
<tr>
<td>$I_{\text{red}}$</td>
<td>Integral of the $\alpha$ reducing end signal</td>
</tr>
<tr>
<td>$I$</td>
<td>Nuclear spin quantum number</td>
</tr>
<tr>
<td>$I_{\text{stan}}$</td>
<td>Invert sugars in the standard</td>
</tr>
<tr>
<td>$I_{\text{tot}}$</td>
<td>Total invert sugars</td>
</tr>
<tr>
<td>IUPAC</td>
<td>International union of pure and applied chemistry</td>
</tr>
<tr>
<td>L</td>
<td>Capillary length</td>
</tr>
<tr>
<td>l</td>
<td>Effective length</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantification</td>
</tr>
<tr>
<td>MAS</td>
<td>Magic angle spinning</td>
</tr>
<tr>
<td>$m_{\text{BC}}$</td>
<td>Mass of the breakfast cereal</td>
</tr>
<tr>
<td>MECK</td>
<td>Micellar electrokinetic chromatography</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>N</td>
<td>Noise</td>
</tr>
<tr>
<td>NMR spectroscopy</td>
<td>Nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>NIDDM</td>
<td>Noninsulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>NIP</td>
<td>Nutrition information panel</td>
</tr>
<tr>
<td>Non-PS</td>
<td>Nonpresweetened</td>
</tr>
<tr>
<td>NS</td>
<td>Number of scans</td>
</tr>
<tr>
<td>NSP</td>
<td>Non-starch polysaccharides</td>
</tr>
<tr>
<td>NUTTAB</td>
<td>Nutrient tables</td>
</tr>
<tr>
<td>OPM</td>
<td>Oscillations per minute</td>
</tr>
<tr>
<td>P</td>
<td>Angular momentum</td>
</tr>
<tr>
<td>PAD</td>
<td>Pulsed amperometric detection</td>
</tr>
<tr>
<td>PS</td>
<td>Presweetened</td>
</tr>
<tr>
<td>Q</td>
<td>Charge</td>
</tr>
<tr>
<td>$R^2$</td>
<td>Correlation coefficient</td>
</tr>
<tr>
<td>RDS</td>
<td>Rapidly digestible starch</td>
</tr>
<tr>
<td>$rf$</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>RH</td>
<td>Relative humidity</td>
</tr>
<tr>
<td>RI</td>
<td>Refractive-index</td>
</tr>
<tr>
<td>RID</td>
<td>Refractive-index detector</td>
</tr>
<tr>
<td>RS</td>
<td>Resistant starch</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>S</td>
<td>Signal</td>
</tr>
<tr>
<td>s</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SAXS</td>
<td>Small-angle- X-ray scattering</td>
</tr>
<tr>
<td>SDS</td>
<td>Slowly digestible starch</td>
</tr>
<tr>
<td>SNR</td>
<td>Signal-to-noise ratio</td>
</tr>
<tr>
<td>SPE</td>
<td>Single pulse excitation</td>
</tr>
<tr>
<td>$T$</td>
<td>Titre</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>$T_1$</td>
<td>Longitudinal relaxation time</td>
</tr>
<tr>
<td>$t_{EOF}$</td>
<td>Migration time of EOF</td>
</tr>
<tr>
<td>$t_m$</td>
<td>Migration time</td>
</tr>
<tr>
<td>UHT</td>
<td>Ultra-heat treatment</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>$V$</td>
<td>Voltage</td>
</tr>
<tr>
<td>$v$</td>
<td>Velocity</td>
</tr>
<tr>
<td>$x$</td>
<td>Time after which current decreases by n %</td>
</tr>
<tr>
<td>$\bar{x}$</td>
<td>Mean</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray diffraction</td>
</tr>
</tbody>
</table>
Abstract

Grains and grain products remain the main source of energy for the majority of the world’s population. These carbohydrate-rich foods also have a significant influence on digestive health which renders them ideal target foods for weight management and the prevention and management of obesity-related illnesses, especially type II diabetes and colorectal cancer. However, due to the complexity of their matrices, the mechanism behind the digestibility of grains and grain products is poorly understood. In this project, commonly consumed grain foods were analysed for sugar composition and starch structure to improve our understanding of their digestibility.

A new free-solution capillary electrophoresis (FS-CE) method with direct ultraviolet (UV) detection had been applied by our research team to plant fibre and ethanol fermentation samples. In this thesis, the FS-CE method was applied to a complex matrix food sample: breakfast cereal (BC). Sucrose was detected in all BCs, while lactose, maltose, glucose and fructose were detected in some. The quantification for 11 BCs was grouped into three categories for total sugar content: low (for example, ‘Weet-Bix’), medium (for example, ‘Corn Flakes’) and high (for example, ‘Nutri-Grain’). For total sugar content, FS-CE was significantly more repeatable and reproducible than traditional colorimetric (Fehling and 3,5-dinitrosalicylic acid) and enzymatic (glucose oxidase-peroxidase) methods, likely due to automation and lack of sample degradation before analysis. Currently, the gold standard method for individual sugar analysis in food products is high performance liquid chromatography (HPLC). However, FS-CE has a much lower running cost compared to HPLC and was shown to detect and quantify more sugars for most BCs (excluding those containing fruit). This is likely because the
The robust FS-CE method has minimal sample preparation, which only involves sample suspension in water, and therefore has a reduced risk of sample loss.

At present, glycaemic index (GI) is the only accepted measure of the digestibility of foods by the food industry (allowed on food packaging) and is the only way consumers can predict whether a product will induce a rapid or slow release of energy upon consumption. However, the measurement of GI is very costly (both money- and time-wise) and is therefore highly ineffective as a selection tool for the screening of breeding lines or during product development. Moreover, the values are not statistically significant for the population as they are based on an average blood glucose response, with a minimum requirement of only 10 people. The FS-CE method was successfully applied to digesta of three breakfast cereals. Glucose measurements by FS-CE were shown to be significantly more repeatable than enzymatic methods (glucometry and glucose oxidase-peroxidase) used to monitor in vitro digestion. Moreover, FS-CE is able to produce digestograms of sugars other than glucose, which can contribute to increased understanding of the processes involved in by enzymatic degradation of grain foods. There is great potential for future research to use the FS-CE method for online in vitro digestibility studies.

Starch digestibility of grain foods, whether measured by in vivo or in vitro methods, is related to the source and nature of starches. The structure of starch in rice flour samples (3 varieties) was analysed and associated with digestibility using an international GI database. The amylose content and gelatinisation temperature (GT) was measured by the Department of Primary Industries (Yanco, NSW, Australia). Using quantitative 1H nuclear magnetic resonance (NMR) spectroscopy the degree of branching (DB) of the same rice flour samples was determined with 2-3 % precision. Many studies have found a link between high-amylose rice starches and slower
digestibility. Whether grown at high or low temperatures, the samples of one variety had similar amylose content, DB and GT. The consistent structural properties suggest that this variety would be able to maintain a low GI value across different climates, which meets the criteria for which it was designed. For other 2 varieties (varieties B and C) grown at high temperatures, decreased DB was linked with increased amylose content and increased GT. As these varieties were also associated with a higher GI value, DB was determined to be a useful tool in the prediction of future digestibility profiles of particular rice varieties grown in a specific environment. To improve our understanding of starch digestibility the analysis of molecular and supramolecular structure (chain length distribution, single and double helices, crystalline order and lamellar spacing) should be included in future research. Useful methods for analysis of supramolecular structure include solid-state NMR spectroscopy, Fourier transform infrared (FT-IR) spectroscopy, X-ray diffraction (XRD), and small-angle-X-ray scattering (SAXS).

In conclusion, the quantification of sugars by FS-CE was successfully applied to BCs and proof-of-concept was established for monitoring in vitro digestion. The DB of 3 varieties of rice flours was measured by ¹H NMR and had associations with amylose content and GT. Thus, alongside in vitro digestion, the key to understanding digestibility of grain foods and their effects on health will require a multi-method approach to characterising starch structure.
CHAPTER 1: Grain Foods
1.1 Background

Ten thousand years of civilization began with the domestication of cereal plants to produce edible and nutritious seeds, the grains. Rice has fed more people over a longer period of time than any other grain \[1\] and currently provides 85 % energy intake to over half the world’s population (mainly in Asia).\[2\] The most common form in which people consume grain in many Western nations, after bread and pastries, is breakfast cereal (BC). In Australia, approximately 50 % of the Australian adult population that consume breakfast at least five days a week choose a breakfast cereal for this meal.\[3\]

Rice and BCs are mostly comprised of sugars and starch (the two main types of dietary carbohydrates). In recent decades, changes to the Australian diet with over-consumption of energy and decreased physical activity have fuelled the obesity epidemic. Many studies have investigated the correlation between carbohydrate-rich foods and their influence on weight gain,\[4\] insulin resistance,\[5\] diabetes\[6\]. Classification of the major dietary carbohydrates is presented in table 1. Due to their major calorific contribution to the diet and effect on digestive health, Australian rice and BCs have the potential to address this century’s key global challenge – prevention of chronic disease.

Starch structure greatly affects the quality and digestibility of food.\[7\] This study evaluated the starch structure of a number of new and existing rice varieties to speed up the screening process for rice breeding programs. For BCs, starch structure may be less influential to digestibility. While grains are still the base of BCs, sugars and other sweeteners may have a greater influence on digestibility. Thus digestibility of these grain foods depends upon molecular characterisation of both sugar and starch components.
Alternative *in vitro* methods to determine digestibility are needed given the high cost and time associated with human *in vivo* trials. This thesis is a humble contribution to understanding digestibility of rice and BCs in an era when diet-related diseases are highly prevalent.

**Table 1** The major dietary carbohydrates. Based on Food and Agriculture Organization/World Health Organisation ‘Carbohydrates in Human Nutrition’ report [8] and [9].

<table>
<thead>
<tr>
<th>Class (DP(^a))</th>
<th>Subgroup</th>
<th>Principal Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugars (1-2)</td>
<td>Monosaccharides</td>
<td>Glucose, fructose, galactose</td>
</tr>
<tr>
<td>Disaccharides</td>
<td></td>
<td>Sucrose, lactose, maltose, trehalose</td>
</tr>
<tr>
<td>Polyols (sugar alcohols)</td>
<td></td>
<td>Sorbitol, mannitol, lactitol, xylitol, erythritol, isomalt, mannitol</td>
</tr>
<tr>
<td>Oligosaccharides (3-9) (short-chain carbohydrates)</td>
<td>Malto-oligosaccharides (α-glucans)</td>
<td>Maltodextrins</td>
</tr>
<tr>
<td>Non-α-glucan oligosaccharides</td>
<td></td>
<td>Raffinose, stachyose, fructo and galacto oligosaccharides, polydextrose, insulin</td>
</tr>
<tr>
<td>Polysaccharides (≥ 10)</td>
<td>Starch (α-glucans)</td>
<td>Amylose, amylopectin, modified starches</td>
</tr>
<tr>
<td>Non-starch polysaccharides (NSPs)</td>
<td></td>
<td>Cellulose, hemicelluloses, pectin, arabinoxylans, β-glucans, glucomannans, plant gums and mucilages</td>
</tr>
</tbody>
</table>

\(^a\)Degree of polymerization or number of single sugar units.
1.2 Research in nutrition of carbohydrate foods

1.2.1 The obesity epidemic

Overweight and obesity are defined as excessive adipose tissue that may impair health.\textsuperscript{[10]} Their prevalence has been increasing at an alarming rate throughout the world, with at least 2.8 million people dying each year as a result.\textsuperscript{[11]} Australia is not only included in the obesity epidemic, but it has one of the highest incidence rates of obese people on earth,\textsuperscript{[12]} see Figure 1. The latest national survey has revealed 25 % of children and 63 % of adults are overweight or obese. For adults, 35 % are overweight and 28 % obese.\textsuperscript{[13]}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Age and gender-adjusted rates of obesity for several countries.\textsuperscript{[14]}}
\end{figure}

Obesity has many negative health consequences which include non-fatal complaints that affect the quality of life, including impaired respiratory function, infertility, skin problems and musculoskeletal disorders such as osteoarthritis. For persons with a body mass index (BMI) over 30, there is increased risk of disability and a 50 - 100 % increased risk of premature death.
by gallbladder disease, cardiovascular problems, NIDDM (Noninsulin-dependent diabetes mellitus) and some cancers.\textsuperscript{[15]}

Dietary management and preventative challenges of obesity are not currently being met. Successful weight loss, and strategies to prevent further weight gain, requires permanent changes to dietary quality, as well as changes to energy intake.\textsuperscript{[16]}

1.2.2 Relationships between energy intake, obesity and weight management

Energy balance is a state of equality between energy in versus energy out. Obesity occurs when energy intake or metabolic fuel exceeds energy expenditure and the surplus accumulates in the body as adipose tissue. The regulation of energy balance and bodyweight is complex involving numerous behavioural, hormonal and neural factors.\textsuperscript{[17]} The role of genetic influences in the development of obesity has been recognised in many studies involving identical twins and families.\textsuperscript{[18]} However, while 400 genes have been recognised to affect body weight regulation, there are a tiny proportion of people in the world with obesity caused by genetic mutations. Therefore, gene-environment interactions must be responsible for the vast majority of people who are obese.\textsuperscript{[18]} The leading environmental cause of the imbalance between energy intake and expenditure, alongside insufficient physical activity, is excess food and nutrient intake.\textsuperscript{[18,19]} Thus diet is an obvious target for body weight management in the prevention and treatment of obesity.\textsuperscript{[19]}

Diet-focused weight management places emphasis on overall calorie reduction.\textsuperscript{[20]} Although significant short-term weight loss is attainable for many people, maintenance of weight loss in the long term is a much greater challenge.\textsuperscript{[19,21]} This challenge is driven by the way diets are administered and
its potential influence on macronutrients effects on satiety and energy expenditure.\textsuperscript{19} In summary, energy intake is the combined product of the dynamics of energy density, portion size and frequency of consumption.\textsuperscript{19,22}

1.2.3 The sugar debate

1.2.3.1 Sugars as part of a healthy diet

Sugars are an ever-present component of our food supply. Human beings have evolutionary driven taste preferences for their sweetness, which is first experienced in mother’s milk, and which is the taste of the once rare energy-dense foods.\textsuperscript{23} Sugars intrinsic to a food are naturally occurring whereas sugars added to foods during processing, preparation or at the table are known as added sugars. Common definitions of sugars in the literature are listed in Table 2.

A healthy diet must at least contain naturally occurring sugars, such as glucose, fructose and lactose, because they are integral parts of whole fruit, vegetables, dairy products and various grains. Sugars have been associated with the regular consumption and enjoyment of meals and snacks.\textsuperscript{24} In this way, certain types of foods with high sugar levels have actually shown to have an overall positive impact on diets. The consumption of dairy products with added sugar was positively correlated with higher calcium intake for children and adolescents.\textsuperscript{25} Due to the nutrient fortification, consumption of presweetened BCs was associated with diets high in essential micronutrients, such as calcium, folate and iron,\textsuperscript{25} and lower in fat.\textsuperscript{26}

Sugars consumed in large amounts can displace more nourishing foods from the diet and may contribute to several diseases. Dental caries is positively correlated with sugars consumption; sucrose in particular is cariogenic.\textsuperscript{27,28} Diets high in sugars and other dietary carbohydrates have been associated with increased risk of cardiovascular disease (CVD). Several studies suggest
that relative to other dietary carbohydrates, sugar intake is correlated with increased blood pressure,\cite{29-31} increased triglyceride levels,\cite{32} formation of serum cholesterol,\cite{33} inflammation and oxidative stress.\cite{34-36} However most correlations made in these studies are inconclusive. In addition, studies have shown that a high fat and refined sugar diet is linked to impaired memory of food previously consumed, and increased incidence of Alzheimer’s disease.\cite{37,38}

Table 2 Common definitions of dietary carbohydrates adapted from Johnson, Appel, Brands, Howard, Lefevre, Lustig, Sacks, Steffen and Wylie-Rosett \cite{32}

| Simple carbohydrates (sugars) | refers to monosaccharides and disaccharides. Monosaccharides include glucose, galactose, and fructose. Dextrose is synonymous with glucose. Fructose is the most common naturally occurring monosaccharide, found in fruits and vegetables. Common disaccharides include sucrose (dimer of glucose and fructose), which is found in sugar cane, sugar beets, honey, and corn syrup; lactose (dimer of glucose and galactose), found in milk products; and maltose (dimer of glucose), found in malt. |
| Complex carbohydrates | refers to polysaccharides, such as starch. |
| Naturally occurring (intrinsic) sugars | refers to sugars that are an integral part of whole fruit, vegetable, and milk products. |
| Added (extrinsic) sugars | refers to sugars and syrups added to foods during processing or preparation and includes sugars and syrups added at the table. |
| Total sugars | are defined as all sugars (naturally occurring and added) in foods and beverages. |
| High-fructose corn syrup | is produced from corn syrup (nearly all glucose), which undergoes enzymatic processing to increase the fructose content and is then mixed with glucose. |
1.2.3.2 Sugars and obesity

There is much debate surrounding the connection between added sugar intake and obesity. Gibson [39] and Lewis, et al. [40] observed a negative correlation between sugar intake and BMI. Saris [41] reported that there was little evidence to conclude that carbohydrate content of the diet, regardless of carbohydrate type, had direct negative effects on body weight. Similarly, van Baak and Astrup [42] did not find sufficient evidence that an exchange of sugar for non-sugar carbohydrates, in an energy-restricted diet, resulted in decreased body weight. However, the assessment of added sugars is challenging because no analytical methods exist to measure sugar added to foods. In addition, it is difficult to measure added sugars in epidemiological studies due to the inconsistent use of methods.[43]

At present, epidemiological studies which measure the frequency of intake of sugar-sweetened beverages are the best type of assessment. Epidemiological studies in the US, over the past three decades, observed that energy intake in the population had risen by an average of 620 to 1300 Joules per day, and about half of this increase is attributed to liquid Joules (especially sugar-sweetened beverages).[44,45] Ludwig, et al. [46] determined a connection between sugar-sweetened beverages and childhood obesity and concluded that for every additional daily serving of sugar sweetened drink, the likelihood of obesity increased by 60 %. Flood, et al. [47] also supported the relationship between the intake of sugar-sweetened beverages and increased energy intake. Vartanian, et al. [48] concluded that, although the effect was small, there was a positive correlation between soft drink intake and energy intake. Weight gain was also observed in a study where by participants consumed self-selected diets that included 25 % of energy from beverages sweetened with glucose or fructose.[49]
For BCs, as with other solid foods, there is limited epidemiological evidence and lack of plausible mechanisms linking sugar intake and body weight\textsuperscript{[50]}. A comprehensive review of the health benefits of BCs was conducted by Williams \textsuperscript{[26]}. Analysis of data from a recent Australian national survey compared the intakes of children and adolescents consuming presweetened (PS) cereals, with ≥15 % total sugar with those consuming minimally sweetened (non-PS) cereals\textsuperscript{[51]} and found that daily energy and nutrient intakes were not found to be significantly different\textsuperscript{[26]}. Another analysis of the same survey data, specific to Australian boys, showed that BCs contributed to 34 % of total sugars of the breakfast meal but only 7 % of the total sugars for the day. \textsuperscript{[52]} In support of this finding, a recent study of 312 Australian BCs found that there was no relation between sugar content and energy density \textsuperscript{[50]}. The finding that the amount of sugar in BCs makes no difference to total daily energy is consistent with several USA studies in children\textsuperscript{[53]} although it was linked to increased daily sugar intake.\textsuperscript{[25]} Conversely, a more recent study comparing PS and non-PS BC in children (aged 4-13 y) found that daily energy and sugar intakes were higher in those that consumed PS cereals, but no correlation was found for adolescents (aged 14-18 y). Despite the higher levels of energy intake of children consuming PS cereals, BMI and waist circumference were no different to groups consuming non-PS cereals or other breakfast meals \textsuperscript{[54]}. In summary, sugars can have a positive or negative impact on diet quality and risk of obesity depending on its food source. On average, consumption of PS cereals do not increase the risk of overweight or obesity in children and adolescents (evidence of a direct link for adults has yet to be confirmed).\textsuperscript{[26]} Sugar-sweetened beverages have been shown to negatively impact the diet and increase the risk of weight gain and illnesses, such as type 2 diabetes mellitus and CVD.
1.2.4 Starch and digestive health

Starch is a homopolymer of glucose. Nutritionally, starches can be ranked into three classes according to the rate of digestion and glucose release described by the in vitro Englyst method\cite{55}: rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS).

Each of these starch fractions can impact digestive health in a number of ways. RDS is the starch digested to glucose within 20 minutes. Commonly found in highly processed foods, such as white bread, cakes and pastries, RDS causes a rapid surge in blood glucose and insulin levels. SDS is the starch fraction with a lower rate of digestion, between 20 and 120 minutes. RS is the remaining of total amount of starch and products of starch degradation after 120 minutes. RS resists digestion in the small intestine and is fermented by resident microflora in the large intestine\cite{30}.

As a highly fermentable carbohydrate, RS positively impacts digestive health in a number of ways and is associated with prevention of colon cancer\cite{56,57}. RS beneficially increases stool bulk which gives a mild laxative effect and promotes bowel regularity\cite{58}. In addition, RS releases short-chain fatty acids, especially butyrate, has shown to induce a number of positive physiological effects intestinal health\cite{59-61}. Short-chain fatty acids act as a prebiotic, a substrate for the growth of healthy bacteria in the bowel\cite{62}, which helps reduce the production of potentially harmful secondary acid biles, ammonia and phenols\cite{63}. RS also has a positive effect on the digestion system by delaying the degradation of the mucous layer within the colon. It is believed that this mucous layer protects colon cells\cite{64}.

1.2.5 Starch and glycaemic control

The digestion behaviour of starch is one of the most significant metabolic responses after meal consumption\cite{65}. Generally, SDS has been shown to elicit
a moderate postprandial (after a meal) glycaemic and insulinemic response which is considered beneficial for the dietary management of individuals with chronic and metabolic conditions, such as diabetes mellitus and hyperlipidemia.\textsuperscript{65,66}

Glycaemic response to starch digestion is, however, dependent upon both the quality (nature and food processing) and quantity and of the available carbohydrate in a food.\textsuperscript{67} First termed by McCance and Lawrence \textsuperscript{68}, ‘available carbohydrate’ is a total figure that includes starch and soluble sugars, but not the plant cell wall polysaccharides (fibre). The concept of glycaemic index (GI) was introduced by Jenkins, et al.\textsuperscript{69} as a way to classify carbohydrate-containing foods according to their postprandial response as blood glucose. GI is defined as the total glycaemic response 2 h immediately after intake of a test food (usually 50 g available carbohydrate), and is expressed relative to an equal quantity of glucose or white bread.\textsuperscript{70} GI is an average value, calculated as the area under the glucose response above the glucose baseline. As GI quantifies the rate of glucose release in the blood, it is considered a specific property of food or a measure of food quality.\textsuperscript{67}

Glycaemic load (GL) was introduced in the late 1990s in an attempt to quantify the overall glycaemic response for a typical portion of food.\textsuperscript{67} GL is calculated by multiplying the amount of available carbohydrate contained in a specified serving of food (weight in g or volume in mL) by the GI value of that food. Starch digestibility is greatly associated with both GI and GL values of a food. For example, broad beans have been shown to have a high GI, but because they contain little available carbohydrate (mainly comprised of SDS and RDS) in a typical serving, they have a low GL value.\textsuperscript{71}
1.2.6 Starch and satiety

The type and form of starch are important determinants of satiety (fully satisfied appetite) and energy intake. The concept that the postprandial glycaemic and insulinemic response from carbohydrate intake is the main regulator of satiety and energy intake is based upon the glucostatic theory by Mayer \[^{72}\]. This theory hypothesises that a high glycaemic index (GI) meal gives a high glycaemic response, followed by a hypoglycaemic period, which will trigger hunger and decrease satiety as compared to a low GI meal. A number of studies support this theory,\[^{73-75}\] giving evidence that, compared to high GI foods, low GI foods have a greater effect on satiety than energy intake or weight gain. In terms of starch components, RDS was reported to reduce sensitivity to hunger and satiety signals,\[^{37}\] with high intake of sugar from soft drinks suggested to specifically increase energy intake.\[^{76}\] In addition, SDS was shown to increase satiety by influencing blood glucose and insulin levels as well as viscosity within the gastrointestinal tract.\[^{77}\] However, some experimental studies did not support the glucostatic theory.\[^{78,79}\] Leathwood and Pollet \[^{78}\] described a delayed response in hunger after consumption of 25-40 g of SDS from bean purée compared to RDS from potato purée. Aisbitt, et al. \[^{80}\] suggested that fibre content was more responsible for satiety, rather than GI of foods, due to increasing the bulk of the diet and reducing the release time of nutrients from the digestive system.

There has been growing evidence of the role of RS in enhancing both short-term \[^{81,82}\] and long-term \[^{83,84}\] satiety. While the mechanisms responsible for these findings are not completely understood, fermentation of RS in the large intestine and short chain fatty acid production was associated with increased gene expression and plasma protein levels of satiety hormones.\[^{85}\]
In summary, sugars and dietary starch are necessary components in a healthy diet and have a specific influence on digestive health, glycaemic control, satiety and a number of chronic diseases.

1.3 Chemical structures of carbohydrates

1.3.1 Monosaccharides and disaccharides

Most ready-to-eat BCs have high levels of sugar, both naturally sourced and as an added ingredient. This has a great influence on digestibility and glycaemic response and therefore the understanding the carbohydrate composition of BCs is important. The simplest form of sugars are monosaccharides and oligosaccharides with the molecular formula \( C_n(H_2O)_n \) with \( n \) ranging from 3 to 9. Monosaccharides and short-chain oligosaccharides, which have, or are capable of forming a free hemiacetyl group (aldehyde or keto) in their structure are termed reducing sugars. Commonly occurring monosaccharides include hexoses, such as \( \alpha-d\)-glucose (\( \alpha-d\)-glucopyranose) and \( d\)-fructose (\( 1,3,4,5,6\)-pentahydroxyhex-2-one), and pentoses, such as \( d\)-arabinose (\( \alpha-d\)-arabinofuranose) and \( l\)-arabinose (\( \alpha-l\)-arabinofuranose), refer to Table 3.

Mono- and disaccharides are sugars comprised of carbon, oxygen and hydrogen atoms and have the lowest molecular weight of all carbohydrates. These sugars are biologically produced by plants and, following extraction, are commonly stored in stable crystal form. Functional properties of mono- and disaccharides in food products include their ability to increase sweetness, give colour, flavour and to improve storage stability.

Disaccharides have the molecular formula \( C_n(H_2O)_{n-1} \). They are made up of two condensed monosaccharide units with the loss of one molecule of water. Three commonly found disaccharides in food are sucrose, simply
referred to as ‘sugar’ in colloquial terms, lactose and maltose (Table 3). Maltose is homogenous, having two identical monosaccharide units (4-O-α-d-glucopyranosyl-d-glucopyranose), whereas lactose (4-O-β-d-galactopyranosyl-d-glucopyranose) and sucrose (α-d-glucopyranosyl-β-d-fructofuranoside) are heterogeneous.\[88\]

Table 3 Structural formulas and ionisation constants of common mono- and disaccharides

<table>
<thead>
<tr>
<th>Monosaccharides</th>
<th>Disaccharides</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Glucose" /></td>
<td><img src="image" alt="Sucrose" /></td>
</tr>
<tr>
<td>pKa at 25°C = 12.35 [90]</td>
<td>pKa at 25°C = 12.51 [90]</td>
</tr>
<tr>
<td><img src="image" alt="Fructose" /></td>
<td><img src="image" alt="Maltose" /></td>
</tr>
<tr>
<td>pKa at 25°C = 12.03 [90]</td>
<td>pKa at 25°C = 12.39 [91]</td>
</tr>
<tr>
<td><img src="image" alt="Xylose" /></td>
<td><img src="image" alt="Lactose" /></td>
</tr>
<tr>
<td>pKa at 25°C = 12.29 [90]</td>
<td>pKa at 25°C = 11.98 [90]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Trisaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Maltotriose" /></td>
</tr>
<tr>
<td>pKa at 25°C = 12.39 [91]</td>
</tr>
</tbody>
</table>
1.3.2 Starch

Starch is a key plant metabolite and the main form in which carbohydrates are consumed, representing 80-90% of all polysaccharides in the human diet.\textsuperscript{[92]} It exists as granules in plant tissues, including seeds, pulses and tubers, and granular diameter ranges from 1 to 100 µm depending on the plant species and cultivar within the species \textsuperscript{[57]}. Chemically, starch is a glucose homopolymer linked by $\alpha$(1-4) linkages, which form the backbone, and branching points at $\alpha$(1-6) glycosidic linkages\textsuperscript{[93]} (Figure 2).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{starch_structure.png}
\caption{Chemical structure of starch}
\end{figure}

Starch is one of the most complex materials found in nature. The starch within a grain endosperm has six structural levels at both short and long range order (Figure 3). Furthermore the complexity of starch structure is caused by the starch granule heterogeneity that exists within the range of distance scales as well as natural variation between granular species.
1.3.2.1 Molecular structure

Starch is mainly composed of α-glucan in the form of two types of macromolecules: amylose and amylopectin \[^{93}\] (Figure 3, levels 1-2 in green). Amylose is a primarily linear glucan, comprised mostly (>99 %) of α(1-4) bonds with a small number of branches connected by α(1-6) bonds.\[^{95}\]

Amylopectin is a highly branched glucan that is characteristically larger than amylose. It is mainly made up of many short α(1-4) chains, which are interlinked by 4-6 % of α(1-6) bonds at the branch points.\[^{93,96}\] (Figure 2). A variety of branching types exits for amylopectin, described as A-chain (outer), B-chain (inner chains bearing A-chains) and C-chain (single chain per molecule that carries other chains as branches), see Figure 4.\[^{97}\]
1.3.2.2 Supramolecular structure

At the higher levels of structure, the starch granule was shown to be semicrystalline (Figure 3, levels 3-6 in purple). Supramolecular starch structure is largely influenced by the fundamental architecture of amylopectin, described in the ‘cluster model’ \[^{98,99}\]. According to this model, amylopectin forms crystalline regions predominantly through the pairing of adjacent linear chains which form tightly packed parallel arrays of double helices (Figure 3, level 3). Amylose tends to form either a single helical inclusion complex or a double helical structure and makes up most of the amorphous regions which are linked to one another by branch linkages of amylopectin clusters (Figure 3, level 4). This results in the formation of interblock segments that build up the alternating crystalline and amorphous lamellae, whereby each repeating cluster unit is 9 nm in length.\[^{100}\] At the larger scale, this reveals ring-like structures in the starch granule (Figure 3, level 5).

Amylopectin double helices are more predominant than amylose double helices in native starches, even though the latter forms more readily in solution.\[^{101}\] Thus, amylose trapped within granules as separate, individual molecules are only found in nature. In general, the tight manner in which the
Amylose is packed within the granule has shown to contribute to stronger crystallites and reduced digestibility compared with the large, open highly branched conformation of amylopectin.[102,103]

Native starch granules have been further classified into one of three X-ray diffraction patterns: type A, B and C. [104] While there are exceptions, the A-type polymorph is commonly found in cereals (normal maize, rice wheat and oats) and B-type is typically found in tuber starch (such as in raw potato and green banana starch). C-type starch seems to be a mixture of A-type and B-type and is contained in legume starches including smooth pea and various beans.

The outer branch (chain) length of amylopectin has found to be a basic factor for determining the type of crystalline polymorph.[101] For example, the analysis of average chain length from a wide variety botanical sources showed that A-type had relatively short branches (<19.7), B-type had longer branches (>21.6) and values between 20.3 and 21.3 lead to A, B or C-type polymorphs.[105]

A and B-type polymorphs both have double helical structures but differ in the packing arrangement and the quantity of water stabilising their helical structures, which affects the rate of digestion.[106] For example, rice or wheat starches (A-type) were hydrolysed by α-amylase over 6 times faster than banana starch (B-type) and over 20 times faster than potato starch (B-type).[107] For hydrolysis of modified potato starch, A-type polymorphs were found to be more susceptible to enzymatic action compared to B-type.[108] Greater susceptibility to enzyme hydrolysis for C-type starch was associated with a higher ratio of A-type starch. Increased digestion was attributed to A-type starches having an uneven surface, yielding a greater accessible surface area compared to B-type starches. [108]
1.4 Australian rice

1.4.1 Rice grain structure

The rice grain (rough rice or paddy) is comprised of edible fruit enclosed in a hard, protective covering called the hull or husk (see Figure 5). Underneath the hull is a thin layer of bran (pericarp, seed-coat and nucellus) as well as the embryo (germ). In brown rice and other coloured rice, pigments are contained in the pericarp and seed coat (testa).\[109,110\] The aleurone layer, mostly made of protein and lipid bodies, completely surrounds the starchy endosperm\[110\]. Starchy endosperm cells that make up the inside of the rice grain are packed with amyloplasts containing starch granules. The starchy endosperm has two distinct cell regions; the subaleurone layer and the inner endosperm. Cells in the subaleurone layer are relatively small, with more protein bodies than starch bodies. Moving towards the inside of the grain, cells become larger with more starch than protein. Inner endosperm cells are primarily large, highly compact, polygonal (hexagonal) compound starch granules (3 -9 µm in diameter).

During the milling process, rough rice is subjected to stages of dehulling and whitening, whereby polishing removes the hull and pericarp layers to reveal the starchy endosperm. Unlike other cereals which require further processing, rice is primarily consumed as milled or polished grain.

Milled rice is chemically simple, composed of ~93 % starch, ~6 % proteins and ~1 % lipids. The starch component of rice plays an important role in the functional properties of the food. One functional property of great interest is digestibility. Starch granule size, level of crystallinity,\[111\] amylose-to-amylopectin ratio and existence of other material (e.g. lipids or proteins) \[108\] are all contributing factors to the rate of digestion. Smaller proportions of protein found within the starch granules can also affect digestibility. These
proteins are mainly biosynthetic enzymes that remain in the grain after starch synthesis is complete.[112]

Figure 5 Longitudinal section of rice grain [113]

1.4.2 Australian rice varieties

Rice in Australia is primarily grown in the southern irrigation areas and districts of NSW, with a smaller proportion grown in northern Victoria[114] (see Figure 6). These regions are appropriate for rice farming due to irrigation infrastructure, large areas of flat land, appropriate clay-based soils and a temperate climate. Almost all the rice grown in Australia is classified as japonica, a colder climate sub-species that is perfectly suited to the non-tropical climate of the rice growing regions. Australian rice japonica varieties have been the product of rice-breeding programs. The purpose of these breeding programs is to ensure the stable production of rice varieties to meet consumer preferences in domestic and international markets. Many Australian varieties are old varieties, cultivated from rice varieties grown in
similar climates around the world such as California and the Mediterranean region. Australian rice breeders have also been successful in the production of new varieties. For example, “Australia has developed the Opus variety for the Japanese style of cuisine and Reiziq which is fast becoming a premium product in the Middle East”.[115] The most common rice varieties grown in Australia are listed in table 4.

**Table 4 Most common Australian rice varieties**[115]

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amaroo</td>
<td>Semi-dwarf medium grain, high yielding</td>
</tr>
<tr>
<td>Reiziq</td>
<td>Semi-dwarf medium grain, but a longer grain than Amaroo and more appealing to Middle East consumers</td>
</tr>
<tr>
<td>Quest</td>
<td>New semi-dwarf medium grain, short season</td>
</tr>
<tr>
<td>Jarrah</td>
<td>Semi-dwarf medium grain, short season</td>
</tr>
<tr>
<td>Koshihikari</td>
<td>Tall-strawed ‘Japanese quality’ short grain, low yielding</td>
</tr>
<tr>
<td>Opus</td>
<td>Semi-dwarf ‘Japanese quality’ short grain, higher yielding than Koshihikari</td>
</tr>
<tr>
<td>Illabong</td>
<td>Semi-dwarf ‘arborio’ medium grain</td>
</tr>
<tr>
<td>Langi</td>
<td>Semi-dwarf long grain, soft cooking</td>
</tr>
<tr>
<td>Doongara</td>
<td>Semi-dwarf long grain, hard cooking for premium markets</td>
</tr>
<tr>
<td>Kyeema</td>
<td>Tall strawed ‘fragrant’ long grain, low yielding</td>
</tr>
</tbody>
</table>

The Australian rice industry has the capacity to produce more than 1 million tonnes of rice per year (in a non-drought year). Climate plays a major role in the formation of rice starch structure. While rice production is small on a global scale, Australia is the world leader in water use efficiency. Rice varieties that are adapted to different growing conditions need to be generated side by side with efforts to improve their digestibility properties through better breeding management.
1.4.3 Glycaemic properties of milled rice

The quantity and rate at which rice starch is digested and absorbed into the body has significant nutritional implications. Maximum digestibility of rice is necessary for survival in many developing countries where rice constitutes the main source of energy in the diet. In developed nations, reduced starch digestibility can be considered a form of dietary fibre that has a positive influence on colonic health. The digestion\textsuperscript{[117]} and intestinal absorption\textsuperscript{[118]} of cooked rice starch has been found to be complete or near complete in humans. However the rate of starch digestion in rice can vary.

Worldwide studies on the GI of rice have given a wide range of results. Early studies on white rice produced GI values as low as 54 to as high as 121, relative to white bread (GI =100) as the reference food\textsuperscript{[119-121]}. Brand-Miller, et al.\textsuperscript{[122]} found that among Australian white rice, a Doongara variety of high-amylose content (28 %) had a low GI (64) and two low-amylose rice varieties, Calrose and Pedle, had high GI values (83 and 93 respectively). However, even among rice varieties of the same amylose content, GI has been found to vary. Cooking time and method of preparation are important determinants. Three high-amylose (26.7-27.0 %) rice varieties had GI values of 61, 72, and 91 when prepared under the same conditions.\textsuperscript{[123]} However, when cooking
time was reduced to the minimum for each variety of rice, there was no significant difference in GI (75, 78, and 81). The effects of cooking procedure on GI was also observed by Gatti, et al. [124] who observed a significant reduction in plasma glucose response of baked rice compared to boiled rice.

The variability of starch is dependent upon on genetic makeup and environmental influences.[125] As a raw agricultural product, rice grain quality fluctuates between geographical locations and from one growing season to the next. It is therefore imperative for individual countries to carry out their own digestibility measurements.[122]

### 1.5 Australian breakfast cereals

#### 1.5.1 Processing

Breakfast cereal is a processed grain food often eaten at the first meal of the day. There are two basic types of breakfast cereals: hot, which requires cooking, and ready-to-eat, which are usually consumed with cold milk.

The manufacturing process for rolled oats (the most popular hot BC in Australia) simply involves steaming hulled oat grains prior to flattening. According to Fast [126] there are twelve categories for ready-to-eat BCs by manufacturing processes: 1) flaked cereals (such as corn flakes), including extruded flakes, 2) gun-puffed whole grains, 3) extruded gun-puffed cereals, 4) shredded whole grains, 5) extruded and other shredded cereals, 6) oven-puffed cereals, 7) granola cereals, 8) extruded expanded cereals, 9) baked cereals, 10) compressed flake biscuits, 11) muesli-type products, and 12) filled bite-size shredded wheat.

The endosperm of the grain used in BCs contains unsaturated fats which have a tendency to go rancid. Many manufacturers separate the various
components of the grain, removing some or all the fat to prevent rancidity.\cite{23}

In addition, refined grains are less difficult to cook and for the consumer, they are easier to chew and are more attractively light in colour.\cite{23} However, processing conditions of ready-to-eat BCs at manufacture removes the nutritional benefits of whole-grain cereals. To compensate for this, Australian Food Standards Code, Food Standards Australia New Zealand (FSANZ) code 1.3.2\cite{127}, has allowed BCs to be fortified with a variety of vitamins and minerals. Mandatory fortification for cereals and cereal product (standard 2.1.1)\cite{128} requires the addition of thiamin and folic acid. Permitted standards for the food manufacturer allow for voluntary fortification of BC with vitamin A, thiamin, riboflavin, niacin, vitamin B6, vitamin C, vitamin E, folate, calcium, iron – except ferric sodium edetate, magnesium, and zinc. In this way, BCs can be more nutrient dense than other processed foods.

There are numerous BCs on the market and each product line has a unique combination of carbohydrates (including sugars and starch), lipids, proteins and minerals. The heterogeneity of this complex matrix can make carbohydrate analysis challenging.\cite{129}

1.5.2 Effect of processing on the digestibility of starch

Starch is the most important carbohydrate for glycaemic response of a food.\cite{130} During the manufacture of processed grain foods different treatments can cause changes to starch structure and the accessibility of amylolytic enzymes. Thus food processing has a major influence on the rate and extent to which starchy foods are hydrolysed.\cite{131,132}

Breakfast cereals are processed in a variety of ways to meet cultural and consumer preferences for taste and texture. Two popular processing techniques used by the food industry for the manufacture of breakfast
cereals are pressure cooking and extrusion. These processing techniques have been found to have specific effects on starch hydrolysis in other food products. Beans that had been pressure cooked were found to have significantly increased RDS, SDS and total starch and decreased RS. This is due to the fact that increased heat and moisture cause disruption to native starch granules and cause a greater sensitivity to amylolytic enzymes. In this same way, extrusion conditions such as temperature, moisture and pressure/shear, have shown to influence the physicochemical and nutritional properties of starch-based foods. In a study by Vasanthan, et al. an increase in the content of insoluble dietary fibre in Phoenix barley flour, at all extrusion temperatures, was reported. The increased measure of insoluble fibre could be the result of resistant starch (RS3, retrograded amylose) being formed during the process of extrusion cooking, followed by cooling.

1.5.3 Conclusion

Our understanding of digestibility of grain foods is limited. The GI of a food is an established and accepted way to measure digestibility, but in vivo methods for glucose release rate into the body are costly, time-consuming and not suitable for screening new breeding or product development lines. Chapter 2 is focussed on a new, robust method that can quantify sugars from a complex matrix such as breakfast cereals. Digestibility of grain foods is also related to the molecular structure of starch, but methods to measure this structure have not been applied a great variety of rice samples. Chapter 3 aims to use principles developed within polymer chemistry to measure the degree of branching in starch by NMR spectroscopy. Chapter 4 is a general discussion on digestibility of grain foods and ties the starch structure results of chapter 3 with a proof of concept for the new free-solution capillary electrophoresis (FS-CE) method to be used for in vitro digestibility.
CHAPTER 2: Quantification of sugars in breakfast cereals

This material is an edited version of material presented in
Quantification of sugars in breakfast cereals using capillary electrophoresis
MR Toutounji, MP Van Leeuwen, JD Oliver, AK Shrestha, P Castignolles, M Gaborieau
Carbohydrate Research 2015, 408, 134-41.

The publication is shown in Appendix A
2.1 Introduction

For sugars, total content is all that is required for the nutrition information panel (NIP), a mandatory labelling requirement of all processed foods in Australia\(^{[136]}\). As sugar is often listed as the highest ingredient contained in BC products, its accurate and precise quantification, not only during, but also prior to digestion is important. BCs, like most food products, contain various types of carbohydrates as well as lipids, proteins and minerals and the heterogeneity of this complex matrix can make total sugar analysis challenging\(^{[129]}\). This chapter aims to compare the most common methods for sugar analysis in the Australian food industry with a newer and more robust method.

2.1.1 Reducing-sugar methods

For most of the twentieth century, reducing properties were considered among the most important chemical properties of sugar analysis. Monosaccharides and short-chain oligosaccharides, which have, or are capable of forming a free hemiacetyl group (aldehyde or ketone) in their structure are termed reducing sugars \(^{[89]}\). In alkaline solutions, reducing sugars tautomerise to an enediol salt which destroys the asymmetry at carbon 2, see Figure 7.

![Figure 7 Enediol formation of d-Glucose \(^{[137]}\)](image-url)
At elevated temperatures the enediol forms of sugars are degraded into a number of reactive fragments which are readily oxidised by oxygen and oxidising agents \[^{137}\]. The Lane and Eynon \[^{138}\] method and the 3,5-dinitrosalicylic acid (DNS) method \[^{139}\] employ oxidising agents, copper salt and DNS, respectively. Determination of the oxidised compounds (by colour development) provides the basis for measuring reducing sugars. Reducing-sugar methods are often used to give estimate sucrose content in a foodstuff by determining the increase in reducing sugars after hydrolysis of the sucrose \[^{140}\].

Colour reactions based on these methods are inexpensive and technically easy to perform. This type of analyses is highly applicable to a limited range of foods where reducing-sugar content is relatively constant and total reducing-sugar values expressed as invert sugar total or glucose is adequate, e.g. for quality control\[^{141}\]. The Lane-Eynon method was one of two standard reducing sugar methods recommended by the International Commission for Unified Methods of Sugar Analysis committee \[^{142}\]. In the late 1990s, Fehling’s titration for reducing sugars was a still being employed for determining total sugars by laboratories in Australia and several southeast Asian nations\[^{143}\]. In a study of the moisture uptake of milk by a ready-to-eat breakfast cereal, Kellog’s Corn Puffs, application of the Lane-Eynon method was used as a routine analysis for total sugar determination\[^{144}\]. Similarly, the DNS method was outlined in a book on laboratory methods for the estimation of available carbohydrate content in breakfast cereals\[^{145}\].

The major disadvantage of reduction methods are that they are non-specific\[^{146}\]. For estimation of total sugar, non-reducing sugars must undergo mild acid hydrolysis prior to analysis. If adherence to experimental
conditions of reduction methods is not followed strictly, there is a high risk of lowered assay precision and accuracy [147]. In addition, for most food products, a single sugar (usually glucose) is used to produce the standard curve. For example, available carbohydrate content was determined by the DNS method using a glucose standard for breakfast cereals [145], spaghetti [148], and taro flour samples [149]. Standard curves produced from a single sugar cannot accurately be used for quantification of food products (which rarely contain a single species of sugar) and so the data should be taken as a rough estimation of reducing sugar only.[141] This is because different sugars are expected to yield different responses during the chemical reaction. Even if several sugars were used for the calibration curve, reducing sugars methods depend on a semi-quantitative knowledge of the composition of the food stuff being analysed to yield an accurate result.

2.1.2 Glucose-specific enzymatic assays

Glucose-specific enzymatic assays have most commonly been used to measure glucose concentrations in biological fluids and in various foods. In the glucose oxidase/peroxidase (GOD-POD) method, two enzymatic reactions occur simultaneously to improve the specificity of the reaction by glucose oxidase alone [150] and to allow for a more convenient analysis [137]. Glucose released from in vitro digestion of breads, biscuits, various pastries and selected Spanish breakfast cereals (Kellogg’s Ricicle and Kellogg’s Fruit’n’Fibre) was measured using the GOD-POD reagent [151]. More recently, blood-glucose monitoring meters (glucometers) have been successfully used to continuously measure glucose at set times during in vitro digestion of various starch substrates, including: raw rice starch flours, [152] sorghum and wheat starch,[153] instant rice porridges,[154] as well as unripe banana, edible canna and taro flours [155].
An obvious advantage of these methods is that they are highly specific for glucose determination in a mixture without prior separation from other simple sugars \[137\]. In addition, glucose-specific enzyme preparations are relatively inexpensive,\[140\] However analysis of samples containing a variety of sugars (such as breakfast cereals) may be subject to inaccuracies. Sucrose was found to significantly interfere with the GOD-POD reagent compared to lactose, maltose, xylose and galactose when present in large quantities \[156\]. Enzymatic methods for quantifying glucose can also be tedious and subject to error if they are not performed correctly.

### 2.1.3 Separation of sugar mixtures

More recently developed, chromatographic methods have allowed for greater sensitivity and precision of sugar analysis in foods.

#### 2.1.3.1 Gas Chromatography (GC)

Gas chromatography (GC) is a method which has found wide acceptance for the analysis for carbohydrates as a means of identification and for quantitative determination.

GC takes place in very small bore columns, packed with an inert powder support of even particle size and specific surface area, which has been previously treated with a liquid (stationary phase). The procedure is usually simple; sample components are instantly vaporised, and an inert gas is passed down the length of the column, causing the sample to be swept along. During the movement, test substances with a high affinity for the stationary phase will be dissolved into the liquid coat and emerge from the discharge end of the column later than those with less affinity for the stationary phase. Components present in the test substance move forward at differing speeds depending on their solubility, volatility and on the gas pressure. A fundamental parameter in chromatography is retention time, the
time taken from the injection of the sample to the peak maxima. For quantitative analysis, an internal standard must be used.

The major advantage of analysis of carbohydrates with GC is that it is an established method and the technique has been optimised. In addition, sample sizes required are small, ranging from 10 µg to 500 mg \[157\]. Several studies have employed GC in the separation and quantification of sugars in international BC products \[151,158,159\]. However, strong intermolecular forces (hydrogen bonding) exist between saccharides, which means they have a higher boiling point than pure water and are therefore not volatile. For this reason, carbohydrates must be derivatised (supressing hydrogen bonds), usually into aditol acetates, prior to analysis by GC. Not only is extra preparation an obvious disadvantage in terms of time, but the pre-treatment to the carbohydrates for GC is considered more tedious than sample treatment for high-performance liquid chromatography (HPLC).\[129\] Finally, if derivatisation does not proceed to completion there is a considerable risk of sample loss, and thus underestimation of sugar concentration. For these reasons, GC was not be a method used for the determination of sugars in BCs for this Masters project.

### 2.1.3.2 High-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) is at present the most popular chromatographic method for the separation and identification of carbohydrates in the food industry. Sample analysis is fast (minutes) and permits both qualitative and quantitative examinations of monosaccharides, disaccharides and oligosaccharides \[160\]. The current reference database for the nutrient content of Australian foods is the NUTrient TABble (NUTTAB 2010) released by the Food Standards Australia New Zealand (FSANZ), which lists HPLC with refractive index detection as the general method for individual sugar values in analysed foods, including BCs.\[161\]
HPLC, like GC, involves separation of a mixture of compounds on a stationary phase in specialised columns. In HPLC, both the mobile phase (eluent) containing the sample is a liquid and the stationary phase is a solid. A pump(s) is used to transport the eluent and injected sample mixture under high pressure. Unlike GC separation, the HPLC separation is based upon affinity for sample components towards the eluent and stationary phase \[129\].

**Conventional HPLC**

Amino bonded or amino modified systems are the most popular types of columns for mono-and disaccharide separation in HPLC analysis \[140\]. Anion exchange columns used for carbohydrates are coated with an anion exchange resin \[129\]. The acid is then displaced from the column by decreasing the pH or by increasing the cationic strength of the buffer by adding an eluent, typically sodium hydroxide. The increased Na\(^+\) ion concentration displaces the amino acid cation to allow for the separation of mono- and disaccharides.

The most widely used HPLC modes are ligand exchange and hydrophilic interaction liquid chromatography (HILC). While carbohydrates are suited to HPLC due to their low volatility, they do not absorb UV radiation and so extensive clean-up procedures for sample preparation as well as high quality eluents are required if UV detection is to be employed \[140\]. However, direct detection is possible with refractive-index (RI) detector, evaporative light scattering detection (ELSD)\[162\], fluorescence following derivatisation and mass spectrometry (MS).

Refractive index (RI) detectors are universal to all compounds, and are widely used in carbohydrate determination for applications in plant fibers \[166,163\] and various foods.\[164\] The inclusion of an internal standard improved repeatability of the peak area. \[137,163\] Of particular interest to this Masters project, RI detection was used to measure free sugars released from non-
starch polysaccharides (NSP) of BCs \[159\]. However, the response for RI detection was reported to lack uniformity for all sugars so that response factors for sugars in the mixture must be determined.\[140\] Issues of degradation of some disaccharides, such as sucrose in the acidic mobile phase, and poor temperature control of RI detectors was also reported.\[165\]

The experimental conditions for a number of conventional HPLC studies used for carbohydrate analysis are listed in Table 5.

**High performance anion exchange chromatography (HPAEC)**

In recent years, high performance anion exchange chromatography (HPAEC) was shown to be an invaluable type of HPLC in the analysis of carbohydrate materials.\[129\] Ion exchange columns used in HPAEC are able to contain a strongly alkaline mobile phase, which allows carbohydrates to become ionised and separated by ion exchange chromatography. The basic principle behind this mechanism involves the interaction between solute ions and ions bound to the stationary phase. Solute ions of opposite charge attracted to ions on the stationary phase are retained on the column, while solute ions of the same charge as stationary phase ions are released. Ion exchange chromatography works by altering experimental conditions, such as pH and salt concentration of the eluent and temperature of the column, so that ions retained in the column become displaced by other ions and are eluted [136]. As carbohydrates are uncharged molecules, ion exchange chromatography cannot occur without pre-treatment to the samples (so they become ionized).

In terms of detection, the pulsed amperometric detectors (PAD) is most often used for HPAEC as it has a better sensitivity than RI detectors.\[147\] PAD measures the change in current resulting from carbohydrate oxidation at a gold or platinum electrode, rather than oxidation or reduction of a compound at an electrode \[129\]. As PAD is suitable for gradient elution with
the advantage of low detection limits, it seems to provide the best quantification for carbohydrates\textsuperscript{[140,147]}.

**HPLC-Mass Spectrometry**

HPLC-MS is a technique that couples the separation and quantitative capabilities of HPLC with the mass analysis and identification potential of MS. A major downside to this method is that very little fragmentation occurs. Quantitative measurements using standards in HPLC is preferable to HPLC-MS as the likely sugars contained in BCs have already been identified by previous literature.\textsuperscript{[158,161,166]}
Table 5 Experimental parameters and applications of HPLC in carbohydrate analysis.

<table>
<thead>
<tr>
<th>Column name</th>
<th>Resin form</th>
<th>Mobile phase (eluent)</th>
<th>Flow rate (mL∙min⁻¹)</th>
<th>Temperature (column/detector)</th>
<th>Detector</th>
<th>HPLC mode</th>
<th>Applications</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partisil 5</td>
<td>Amino</td>
<td>4:1 Acetonitrile (ACN):water</td>
<td>3.0</td>
<td>RT*</td>
<td>RID**</td>
<td>HILIC^</td>
<td>Saccharides in foods</td>
<td>[164]</td>
</tr>
<tr>
<td>µBondapack NH₂</td>
<td>Amino</td>
<td>4:1 ACN:water</td>
<td>0.15</td>
<td>RT*</td>
<td>RID**</td>
<td>HILIC^</td>
<td>Reducing sugars in French fries</td>
<td>[167]</td>
</tr>
<tr>
<td>Aminex HPX-87P</td>
<td>Lead</td>
<td>Water</td>
<td>0.6</td>
<td>80 °C / N/A</td>
<td>RID**</td>
<td>Ligand exchange</td>
<td>Sugars and pre-treatment by-products of willow branches</td>
<td>[160]</td>
</tr>
<tr>
<td>Aminex HPX-87P</td>
<td>Lead</td>
<td>Water</td>
<td>0.6</td>
<td>80 °C / 60 °C</td>
<td>RID**</td>
<td>Ligand exchange</td>
<td>Monosaccharides in plant fibers</td>
<td>[163]</td>
</tr>
<tr>
<td>Aminex HPX-87C</td>
<td>Calcium</td>
<td>Water</td>
<td>0.6</td>
<td>80 °C / 60 °C</td>
<td>RID**</td>
<td>Ligand exchange</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aminex HPX-87H</td>
<td>Hydrogen</td>
<td>0.005 M H₂SO₄</td>
<td>0.6</td>
<td>60 °C / 60 °C</td>
<td>RID**</td>
<td>Non determined</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supelcosil LC-NH₂</td>
<td>Amino</td>
<td>4:1 ACN:water</td>
<td>1.0</td>
<td>25 °C / 40 °C</td>
<td>RID**</td>
<td>HILIC^</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dionex CarboPac PA1</td>
<td>PSDVB^</td>
<td>30-100 mM NaOH</td>
<td>1.0</td>
<td>N/A</td>
<td>PAD‡</td>
<td>Anion-exchange</td>
<td>Mono- and disaccharides, sugar alcohols, and ethanol in fermentation broths</td>
<td>[168]</td>
</tr>
<tr>
<td>Aminex HPX-87H</td>
<td>Hydrogen</td>
<td>0.005 M H₂SO₄</td>
<td>0.6</td>
<td>60 °C / 60 °C</td>
<td>RID**</td>
<td>Non determined</td>
<td>Common carbohydrates and alcohols in fermentation broths</td>
<td>[169]</td>
</tr>
<tr>
<td>Dionex CarboPac PA1</td>
<td>PSDVB^</td>
<td>30-100 mM NaOH</td>
<td>1.0</td>
<td>N/A</td>
<td>PAD‡</td>
<td>Anion-exchange</td>
<td>Released sugars from non-starch polysaccharides in a wide range of raw and processed food (including BCs)</td>
<td>[159]</td>
</tr>
<tr>
<td>Dionex CarboPac PA1</td>
<td>PSDVB^</td>
<td>1:1 NaOH:water</td>
<td>1.5</td>
<td>RT*</td>
<td>PAD‡</td>
<td>Anion-exchange</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Assumed to be room temperature.
** refractive index detector
† pulsed amperometric detection
^ hydrophilic interaction liquid chromatography
§ 10-µm-diameter substrate (polystyrene 2% cross-linked with divinylbenzene) agglomerated with 500-nm MicroBead quaternary ammonium functionalized latex (5% cross-linked).
2.1.3.3 Free-solution capillary electrophoresis (FS-CE)

Fundamental concepts of CE

Free-solution capillary electrophoresis (FS-CE) is a high resolution separation technique which takes place in thin, liquid-filled capillaries devoid of supporting solid or gel media \[^{[170]}\]. There are two simultaneous processes involved in FS-CE: electrophoresis and electroosmosis.

Electrophoresis describes the independent migration and separation of ions through an ionic solution towards an electrode of opposite charge (Figure 8). The force (F) involved in the one directional movement of ions in capillary electrophoresis (CE) is due to the electric field (E) acting on the charge (Q) of the molecule, see Equation 1. Species with a greater charge move faster in solution. The speed at which ions travel, or migration velocity (v), can also be influenced by molecular size.

\[
F = EQ
\]  

(1)

![Figure 8 The migration of ions in free-solution capillary electrophoresis.](image)

The electrophoretic mobility (μ\text{ep}) is the proportionality constant between v and E (equation 1), a key parameter of FS-CE. This relationship is known as the charge-to-friction ratio\[^{[170],[171]}\], see Equation 2. In the migration order of v for ionic species to the cathode, cationic species are fastest, followed by neutral species and anionic species respectively.
Electroosmosis is a “pumping” mechanism which contributes to the flow of the bulk fluid (usually buffer) across a capillary tube. This flow is generated by the electric field and a charged capillary surface. The surface charge or zeta potential of the capillary causes the formation of a double-layered, ionic distribution (Figure 9), which is important because it enables the mobility of the ions in the buffer. The most frequently used capillaries, fused-silica capillaries, have negatively charged silanol groups present on the surface of the wall which form the rigid layer, also known as the Gouy-Chapman layer. Cations from the buffer are tightly bound to the negative surface and anions are repelled \[172\]. Further from the capillary surface is a compact, mobile region of ions called the diffuse layer, or Stern layer, which is mainly comprised of cations,\[171\] Once voltage is applied, the excess of cations migrate to the cathode (negative electrode), dragging the buffer fluid along to produce an electroosmotic flow (EOF) \[172\]. The EOF is measured by the flow of neutral compounds, for example dimethyl sulfoxide (DMSO), since they have no attraction to the cathode or anode.

\[
v = \mu_e E
\]
An important factor influencing the EOF is pH. A high pH buffer promotes dissociation of silanol groups, increasing the zeta potential and density of the electrical double layer [172]. For this reason, the EOF velocity or the electroosmotic mobility increases with pH above 2 [170], see Figure 10. There are gaps in the literature about the EOF at very high pH levels, for example pH 13 where carbohydrates become ionised.

![Figure 10](image)

**Figure 10** Variation with pH of the electroosmotic mobility in a fused silica capillary at constant ionic strength. The solid line represents a naked silica surface and the dashed line represents a gelling silica surface [174].

The migration and separation of ions in a FS-CE experiment is measured as a function of time. However, the actual mobility ($\mu_{ep}$) of a solute is a more precise “migration parameter” and thus a better way to present data compared to using migration time. This is because $\mu_{ep}$ requires input from the observed or apparent electrophoretic mobility ($\mu_{app}$) and the impact of EOF to be factored out of the calculation by mobility of the electroosmotic flow ($\mu_{EOF}$) [172], see equation 3.
\[
\mu_{ep} = \mu_{app} \pm \mu_{EOF}
\]

Actual mobility can also be directly calculated from migration time: see equation 4.

\[
\mu_{ep} = \frac{Ll}{V} \left( \frac{1}{t_m} - \frac{1}{t_{EOF}} \right)
\]

where \(l\) is the capillary length to the detection window (effective length), \(L\) is the total capillary length, \(V\) is the voltage, \(t_m\) is the migration time, \(t_{EOF}\) is the migration time of the electro-osmotic flow (EOF) marker.

**Application of CE for food**

Two major difficulties exist for modern analytical separation techniques of complex carbohydrates due to their inherent properties. First, since the majority of carbohydrates, including those found in BCs, lack readily ionisable charged functions they cannot undergo direct differential migration for electrophoretic separation.\(^{171,175}\) Second, very few carbohydrates have the ability to absorb UV or fluoresce, a property which excludes sensitive detection analysis.\(^{175}\) Various approaches to electrolyte systems and detection systems have been developed in order to overcome the structural limitations of carbohydrates for routine CE analysis.

The most common detection strategy for neutral sugars is based on permanent modification by derivatisation with a suitable absorbing agent (chromophore) or fluorescing agent (fluorophore).\(^{176}\) However, determination of food carbohydrates better takes place without derivatisation because the process may cause side-reactions with proteins and lipids.\(^{163}\) Derivatisation is also a time-consuming process. In addition, derivatisation with a chromophore or fluorophore is not possible with
compounds that lack a free carbonyl group, including sucrose, a common ingredient of BCs.

Complexation is a dynamic approach whereby neutral carbohydrates are converted into charged species via complex formation with ions [171,177]. The most widespread method involves association with borate ions, which allows sugars to be suitable for CE without derivatisation [177]. The stability of anionic borate complexes is influenced by pH, the configuration of the hydroxyl groups involved, and the number of hydroxyl groups. In general, pH conditions are optimised between 7 and 10 and an increased number of hydroxyl groups leads to a greater the stability of the complex. [177] As a consequence of the varying degrees of carbohydrate-borate complex formation for various sugars, borate-based buffers produce differences in electrophoretic mobilities of the complexed solutes and thus improved selectivity of separation. [171] The complexation of sugars with borate shows a 2- to 20-fold increased UV response compared to underivatised sugars at 195 nm [178], even though sensitivity is poor. [176]

In carbohydrate-metal complexes, coordinate bonds are thought to be formed between the metal cations and the hydroxyl groups of the sugars [171]. Detection of neutral sugars with CE was developed following chelation with copper (II) under alkaline conditions. [176] In this way, hydroxyl groups of the sugars displace water or ammonia ligands from the copper (II) sulphate electrolyte (increased to pH 11.6 with ammonia). It was found that sugar mobility increased as the copper (II) concentration in the electrolyte increased. Copper (II) chelation by sugars produces direct UV absorbance at 240 nm, with detection limits of 50-100 µM [176].

Another systematic approach to charging neutral carbohydrates involves alkali-metal hydroxide solutions, such as lithium, potassium or sodium
hydroxide, to produce highly alkaline pH conditions \[^{[171]}\]. Highly alkaline buffers (above pH 12) allow ionisation of the hydroxyl groups of sugars (11.9 < pKa < 12.8) to yield negatively charged species known as alcoholates which can migrate in the capillary during analysis \[^{[90]}\]. The pKa values of some typical sugars are listed in Table 3. Resolution among various saccharides was shown to increase with increasing buffer pH from 12.3 to 13.0 \[^{[179]}\]. An optimised method using alkaline phosphate solutions, showed improved resolution and baseline stability for the analysis of glucose, fructose and sucrose in soft drinks, isotonic beverages, fruit juice and sugarcane spirits \[^{[180]}\]. A highly alkaline pH electrolyte solution was also prepared using volatile organic bases, including diethylamine (DEA), for the separation and quantification of arabinose, inositol, galactose, glucose, fructose and mannitol in wine samples \[^{[181]}\]. FS-CE analysis using extremely high pH buffers should only be carried out with naked fused-silica capillaries because, under such alkali conditions, most coated capillaries will degrade \[^{[171]}\].

Micellar electrokinetic chromatography (MEKC) is another mode of CE used in which a surfactant (micelle) is added to the buffer. In this case the cationic surfactant, cetyltrimethylammonium bromide (CTAB), was employed \[^{[166]}\]. Cationic surfactants are attracted and absorbed onto the negatively charged capillary wall surface, creating a positively charged dynamic coating \[^{[182]}\]. Under the influence of an electric field, the bulk reverses flow toward the positive electrode, reversing the direction of the EOF \[^{[182]}\]. The addition of CTAB to the electrolyte was for the purpose of increased separation efficiency and reduced analysis times \[^{[166]}\]. However, the recommended CTAB concentration (0.2 mM) for the best resolution and integration for sugars, also produced flow instability of the EOF as a longer time was required for absorption equilibrium \[^{[166,183]}\].
Methods for analysis of sugars in food must reduce pre-treatment steps to compete with other high-performance analytical methodologies \[184\] such as ion-exchange liquid chromatography with pulsed amperometric detection (HPAEC-PAD), as used to detect underivatised sugars in orange juice \[185\]. The typical detection system used for underivatised sugars in highly alkaline buffer solutions is indirect UV or indirect fluorescence, whereby charged molecules in the sample displace chromophores or fluorophores in the background electrolyte (BGE), producing negative peaks \[177\]. At present, only one study was published for FS-CE analysis of carbohydrates for the application of breakfast cereals with indirect UV detection.\[166\] Details of the MEKC method and buffer composition were mentioned in the previous paragraph. The BGE composition was similar to the BGE used for CE analysis of sugar in honey samples.\[186\]

Until recently, indirect UV detection of underivatised carbohydrates was considered superior to direct UV detection, the latter being associated with low wavelengths that generated poor sensitivity. However, a direct UV detection at a wavelength of 270 nm under highly alkaline conditions (with a buffer containing sodium hydroxide and sodium phosphate) was applied to the separation of 12 different monosaccharides in beverage samples.\[187\] The method is simple, requiring only a highly alkaline pH BGE, and fast; no sample preparation is needed except for dilution. An adapted method has been applied to complex plant samples with a maximum concentration of 400 mg L\(^{-1}\) for single sugars \[163\]. The unexpected direct UV detection of sugars at 270 nm, without any complexation or derivitisation, was first observed by Rovio, Yli-Kauhaluoma and Sirén \[187\] under high-pH conditions. This phenomenon has been attributed to a fast base-catalysed photo-oxidation process that occurs in the detection window of the capillary which leads to the formation of UV absorbing substances\[188\], mainly
malonenolate \cite{189-191}. This mode of detection is very useful for sugars in food samples because it is highly specific to sugars. More importantly, it eliminates most of the physically destructive sample preparation steps, allowing for a method that is robust, that is, applicable to a wide variety of matrices.

In this thesis chapter, focus has been on critical comparison of the optimised FS-CE method optimised for complex plant samples\cite{163} with other analytical methods appropriate to sugar quantification in the application of Australian BCs. Results on the FS-CE work was obtained in this Masters project and published\cite{192} alongside GOD-POD and Fehling (Lane-Eynon) which were obtained in the Masters Candidate’s previous research project.\cite{193}

2.2 Materials and Methods

2.2.1 Materials

Milli Q quality (Millipore, Bedford, MA, USA) water was used throughout the analysis. Sodium hydroxide pellets (NaOH), disodium monohydrogen phosphate powder (Na$_2$HPO$_4$ stored in a desiccator) and lactose were obtained from Univar (Ingleburn, NSW, Australia). d-fructose was supplied by BDH AnalR, Merck Pty Limited. Sucrose, glucose ≥99.5 % and dimethyl sulfoxide (DMSO) ≥99.5% were supplied by Sigma-Aldrich (Castle Hill, NSW, Australia). Xylose ≥99 % was from Alfa Aesar (Ward Hill, MA, USA). Fused-silica capillaries (50 µm internal diameter, 360 µm outside diameter) were obtained from Polymicro (Phoenix, AZ, USA).

2.2.2 Breakfast cereal samples

Thirteen breakfast cereal (BC) products were purchased from a local Woolworths supermarket (Marayong, NSW, Australia). Approximately 80-
150 g of each BC was milled in a K-mart, m-mini glass jug blender for 20 s, speed level 1. The ground cereal was passed through a laboratory sieve with pore size 1000 µm and retained in a 500 µm sized sieve, producing BC samples with particle size between 500 to 1000 µm, see Appendix B. Samples were stored at 4 °C. The sugar content of the 13 BC samples according to their packaging label is shown in Table 6.

Table 6 Breakfast cereal samples according to total sugar content as listed on Nutrition Information Panel (NIP, or packaging label information). Sugar quantity is listed in g per 100 g of BC

<table>
<thead>
<tr>
<th>Sample Name (Brand Name)</th>
<th>Sugar (g / 100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Bran Fibre Toppers™ (Kellogg’s®)</td>
<td>19.6</td>
</tr>
<tr>
<td>Coco Pops® (Kellogg’s®)</td>
<td>36.5</td>
</tr>
<tr>
<td>Corn Flakes® (Kellogg’s®)</td>
<td>7.9</td>
</tr>
<tr>
<td>Froot Loops® (Kellogg’s®)</td>
<td>38.0</td>
</tr>
<tr>
<td>Nutri-Grain® (Kellogg’s®)</td>
<td>32.0</td>
</tr>
<tr>
<td>Rice Bubbles® (Kellogg’s®)</td>
<td>9.0</td>
</tr>
<tr>
<td>Sultana Bran (Kellogg’s®)</td>
<td>22.7</td>
</tr>
<tr>
<td>Sustain® (Kellogg’s®)</td>
<td>20.4</td>
</tr>
<tr>
<td>Weet-Bix™ (Sanitarium™)</td>
<td>3.3</td>
</tr>
<tr>
<td>Weet-Bix™ Multi-grain (Sanitarium™)</td>
<td>9.9</td>
</tr>
<tr>
<td>Oats Traditional (Uncle Tobys®)</td>
<td>1.0</td>
</tr>
<tr>
<td>Oats apple &amp; blueberry bake (Uncle Tobys®)</td>
<td>25</td>
</tr>
<tr>
<td>Oats banana bake (Uncle Tobys®)</td>
<td>22.7</td>
</tr>
</tbody>
</table>

2.2.3 Buffer and sample preparation

For FS-CE separations, disodium hydrogen phosphate (NP200 - 130 mM NaOH and 36 mM Na₂HPO₄) buffer was prepared according to [187]. This
buffer was prepared on the day of use, sonicated for 5 min and filtered with a Millipore membrane (polyethersulfone) syringe filter (0.2 µm). A stock solution of sugars (standard) was prepared in water containing 1.5 g·L⁻¹ sucrose and 0.5 g·L⁻¹ of each maltose, glucose and fructose. Standard curves were obtained using an undiluted standard and standards diluted by factors of 2, 4, 8, 16, and 32. Sample solutions at 10.0 g·L⁻¹ were prepared by adding 15.0 mg of ground sample (500-1000 µm particle size) to 1.500 mL of water, see Appendix C. To each of the standards and samples, 1.50 g·L⁻¹ xylose was added as an internal standard as well as DMSO (5 µL per 500 µL) to mark the electro-osmotic flow. Samples solutions were prepared 1 to 2 days prior to FS-CE analysis.

2.2.4 Parameters for the FS-CE system
Separations were performed on a Beckman P/ACE MDQ capillary electrophoresis system (Sciex Separations, Mount Waverley, Australia) monitoring at 191 nm, 266 nm and 270 nm with a 10 nm bandwidth. A capillary with a total length of 61.8 cm (51.8 cm effective length) was used. The capillary was preconditioned before use by flushing with 1 M NaOH, 0.1 M NaOH, water and NP200 buffer for 20 min each. The cassette temperature was set to 15 °C. Samples were injected by applying 34 mbar for 4 s followed by injection of NP200 buffer in the same manner. A voltage of 16 kV was ramped up over 2 min. Between consecutive separations, the capillary was flushed with NP200 buffer for 5 min. After the final injection, the capillary was flushed with 1 M NaOH for 1 min, followed by water and then air (10 min each). Carbohydrates were monitored at 266 nm and the EOF was monitored at 191 nm by 32 Karat software. The data was processed first using either Origin 8.5.1 (‘manual’ treatment) or 32 Karat (‘automated’ treatment). Both data treatment yielding the same results (data not shown).
the latter treatment was used for all the results presented in this work. Concentration of identifiable sugars in BCs was determined from corrected peak area (relative to the internal standard) and the standard curve. Outliers were removed, where relevant, after applying a Grubbs test (see Appendix E).

2.2.5 Verification of the FS-CE system

The FS-CE hardware and capillary were validated by comparing the current and electroosmotic mobility values after sample injections to an injection with NP200 buffer (plus 1 µL per 500 µL of DMSO). Limit of detection (LOD) corresponded to a signal-to-noise ratio (SNR) greater than 3. The SNR was calculated using the expression:

\[
SNR = \frac{2H}{h}
\]

where, S is the signal (meaningful information), N is the background noise (unwanted signal), H is the height of the peak of concern, measured from the maximum of the peak to the signal’s baseline observed at a distance approximately equal to twenty times the width at half-height, h is the range of the background noise, if possible, located near the peak concerned \(^{[194]}\). Electroosmotic mobility was measured by visual observation of the middle point between the start and end of a peak (average electroosmotic mobility was \(1.26 \times 10^6 \text{m}^2\text{V}^{-1}\text{s}^{-1}\)). Precision was determined through the repeatability of the values for current, EOF and peaks between sample triplicates.

2.2.6 Double correction of electrophoretic mobility

Electrophoretic mobility was corrected using a mobility marker as shown:
\[ \mu_{\text{normalized}} = \frac{\mu_{\text{ref mobility marker}} \times \mu_{\text{ep}}}{\mu_{\text{mobility marker}}} \]  

where \( \mu_{\text{ep}} \) is the actual electrophoretic mobility (calculated with Equation 3) which has already been determined according to the EOF marker, \( \mu_{\text{normalized}} \) refers to the electrophoretic mobility normalised by a reference mobility marker, \( \mu_{\text{mobility marker}} \) is the electrophoretic mobility of the mobility marker, \( \mu_{\text{ref mobility marker}} \) refers to the electrophoretic mobility of the reference mobility marker (taken as an average for all mobility markers for a set of injections).

The double correction of the electrophoretic mobility is an important stage of data treatment for the identification of sugars in samples. While preliminary data of breakfast cereals (October 2012) were repeatable, see Figure 11, the double correction of electrophoretic mobility is required for more precise peak assignment (sugar identification) assignment. This is especially important for experiments run on different days, by different operators and on different equipment. Identification of sugars for all other FS-CE experiments in this thesis were calculated by double correction of electrophoretic mobility.
Figure 11 Mobility electropherogram of 'Weet-Bix Multigrain'. Two repeats (black and red) were run on day 1 and the other two repeats (blue and green) were run on day 3 of the sequence run.

2.3 Results and Discussion

2.3.1 Buffer decomposition – change in current over time

The plateau value of the current, \( c \), for a given FS-CE experiment, was measured and plotted against time that had lapsed since the preparation of the buffer (see Figure 12). The initial current is noted as \( c_i \). The evolution of the current \( (c) \) with time \( (t) \) was fitted with linear regression and the slope, \( a \), and intercept, \( c_i \), were determined (see table 7). The following applies:

\[
\begin{align*}
\frac{dc}{dt} &= a \Rightarrow c &= c_i - at \\
\end{align*}
\]

(7)

This allows calculating the time after which the current decrease by 10 % as:

\[
\begin{align*}
\chi &= \frac{0.1 \times b}{a} \\
\end{align*}
\]

(8)

and the time taken to lose 15% current from initial current measurement as:

\[
\begin{align*}
\chi &= \frac{0.15 \times b}{a} \\
\end{align*}
\]

(9)
The high pH of the sodium phosphate buffer made it prone to carbonation\cite{195} and it is thus recommended to use buffer within 13 h (or 19 h) of its preparation to be within 10 % (or 15 %) of initial current measurement, see table 7 and Figure 12.

Table 7 Results of buffer decomposition

<table>
<thead>
<tr>
<th>Experiment series</th>
<th>a</th>
<th>(c_i)</th>
<th>Time x for 10 % current loss</th>
<th>Time x for 15 % current loss</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(min)</td>
<td>(h)</td>
<td>(min)</td>
<td>(h)</td>
</tr>
<tr>
<td>1</td>
<td>-0.0146</td>
<td>115.78</td>
<td>793</td>
<td>13.2</td>
</tr>
<tr>
<td>2</td>
<td>-0.0163</td>
<td>118.06</td>
<td>724</td>
<td>12.1</td>
</tr>
<tr>
<td>3</td>
<td>-0.0155</td>
<td>116.74</td>
<td>753</td>
<td>12.6</td>
</tr>
<tr>
<td>4</td>
<td>-0.014</td>
<td>115.86</td>
<td>828</td>
<td>13.8</td>
</tr>
<tr>
<td>5</td>
<td>-0.021</td>
<td>109.88</td>
<td>523</td>
<td>8.7</td>
</tr>
<tr>
<td>6</td>
<td>-0.0115</td>
<td>117.23</td>
<td>1019</td>
<td>17.0</td>
</tr>
<tr>
<td>AVERAGE</td>
<td></td>
<td></td>
<td>773</td>
<td>12.9</td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td></td>
<td>161</td>
<td>2.7</td>
</tr>
</tbody>
</table>

2.3.2 Identification of sugars by apparent mobility

To identify the carbohydrates, electrophoretic mobility is used, and not migration time (see Figure 13), since the former has a higher repeatability than the latter. Preliminary sugar identification (December 2012) was confirmed by spiking BC samples, see Figure 14 and 15. Subsequent identification of the sugars in breakfast cereals was performed by comparison of their electrophoretic mobility with that of a standard sugar solution and previous literature\cite{187} (see Table 8 and 9).
Change in current over time

Figure 12 Current against time since the buffer was prepared
Figure 13 Migration time electropherogram (A) and mobility electropherogram (B) of ‘Weet-Bix Multigrain’. From the left to the right, peaks correspond to sucrose, (unknown), maltose, glucose and fructose.
Figure 14 FS-CE with direct UV detection of 'Cornflakes', unspiked sample as well as sample spiked with lactose, galactose or mannose.

Figure 15 FS-CE with direct UV detection of 'Froot Loops', unspiked sample as well as sample spiked with maltose or fructose.
2.3.2.1 Sugar composition of breakfast cereals
Sucrose was detected in all BCs, while lactose, maltose, glucose and fructose were detected in some. Although the sample matrix is complex, other components (such as proteins and lipids) are not detected because the direct UV detection has been shown to be specific to carbohydrates, with a photo-oxidation reaction taking place at the detection window.\textsuperscript{[189,190]}

2.3.2.2 Repeatability and reproducibility of the electrophoretic mobility of standards and samples
Repeatability within the standards was sufficient with relative standard deviation (\(RSD\)) values of no more than 1.3 \%, providing a reliable set of values on which to base sugar identification. In the analysis of BC samples, higher \(RSD\) values were observed in MRT1 ‘Nutri-Grain’ and MVL ‘Weet-Bix’, yielding measurement errors of 5.6 \% and 2.4 \% respectively (see table 9). Apart from these isolated cases, the repeatability of BC sample analysis was good with \(RSD \leq 1.5\) \%.

Reproducibility of the electrophoretic mobility values has been investigated by comparing the results obtained by two different operators, MVL and MRT, or by the same operator with different sample preparation (differing only in dilution), MRT1 and MRT2, or in the literature. Comparison of analysed samples shows a reasonable level of reproducibility between values reported in reference \textsuperscript{[187]}, and those obtained by operator MVL and MRT1, showing a maximum variance between mobilities of approx. 5\% (sucrose), with MRT reporting consistently higher mobilities. Identical operator with diluted sample (MRT2) showed reduced repeatability, yielding mobility value variance of 5-10 \% compared to MRT1, while comparison with MVL showed variance of 1-5 \%, with two values yielding a 10 \% variance (glucose and fructose in ‘Weet-Bix’). Results for diluted ‘Coco-Pops’ lacked the identification of both glucose and fructose, present in previous experiments,
indicating dilution carried out to reduce impacts of overloading is negatively affecting the sensitivity for low concentration sugar identification. Results reported in reference [163] present significantly higher electrophoretic mobilities for both glucose and xylose. The mobility value of a given sugar can differ according to operator, type of equipment and from one session to the next. This illustrates the importance of using a standard sugar solution to establish the mobility values of sugars in each session to account for this inherent variation.

The results obtained in this work by both operators MVL and MRT showed a sufficient level of reproducibility between operators to yield identical sugar identifications in all cases. Same operator, with dilution showed a reduced reproducibility, yielding slightly higher variance in mobility, though still indicates a sufficient level for the identification of sugars. CE is thus a viable method for the identification of sugars in breakfast cereals.

Table 8 Reproducibility of the separation of sugars in a standard sugar solution with FS-CE. Operator MVL (n=2) and Operator MRT (n=3).

<table>
<thead>
<tr>
<th>Standard sugar solution</th>
<th>Operator or publication</th>
<th>Average $\mu_{ep}$ ($10^{-8}$ m$^2$ V$^{-1}$ s$^{-1}$) (RSD in %)</th>
<th>Sucrose</th>
<th>Lactose</th>
<th>Maltose</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Xylose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ref. [187] $^\dagger$</td>
<td>-0.772 ($u^*$)</td>
<td>-</td>
<td>-</td>
<td>-1.176 ($u^*$)</td>
<td>-</td>
<td>-1.365 ($u^*$)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ref. [163] $^\ddagger$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-1.518 (0.45)</td>
<td>-</td>
<td>-1.754 (0.40)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MVL$^*$</td>
<td>-0.720 (1.08)</td>
<td>-1.116 (0.49)</td>
<td>-1.181 (0.30)</td>
<td>-1.265 (0.30)</td>
<td>-1.395 (1.07)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRT$^+$</td>
<td>-0.801 (1.29)</td>
<td>-1.003 (0.77)</td>
<td>-1.172 (0.40)</td>
<td>-1.224 (0.74)</td>
<td>-1.285 (0.40)</td>
<td>-1.419 ($u^*$)</td>
<td></td>
</tr>
</tbody>
</table>

* $u$ stands for unavailable
$^\dagger$ mobility correction using methanol as an EOF marker
$^\ddagger$ mobility double correction using DMSO as an EOF marker and lactose as an electrophoretic mobility marker
$^*$ mobility double correction using DMSO as an EOF marker and sucrose as an electrophoretic mobility marker
$^+$ mobility double correction using DMSO as an EOF marker and xylose as an electrophoretic mobility marker
Table 9 Reproducibility of the separation of sugars in five breakfast cereal samples with FS-CE. Operator MVL (n=2) and Operator MRT (n=3).

<table>
<thead>
<tr>
<th>Breakfast Cereal</th>
<th>Operator or publication</th>
<th>Average $\mu_{eq}$ ($10^{-8}$ m² V⁻¹ s⁻¹) (RSD in %)</th>
<th>Sucrose</th>
<th>Lactose</th>
<th>Maltose</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Xylose</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Coco Pops’</td>
<td>MVL^</td>
<td>-0.720 (u*)</td>
<td>-</td>
<td>-</td>
<td>-1.152 (0.77)</td>
<td>1.237 (0.83)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRT1⁺</td>
<td>-0.810 (0.109)</td>
<td>-</td>
<td>-</td>
<td>-1.223 (0.006)</td>
<td>-1.289 (0.029)</td>
<td>-1.419 (u*)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRT2⁺</td>
<td>-0.733 (0.470)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-1.329 (u*)</td>
<td></td>
</tr>
<tr>
<td>‘Nutri-Grain’</td>
<td>MVL^</td>
<td>-0.720 (u*)</td>
<td>-</td>
<td>-</td>
<td>-1.188 (0.77)</td>
<td>-1.283 (0.79)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRT1⁺</td>
<td>-0.788 (5.59)</td>
<td>-</td>
<td>-</td>
<td>-1.212 (1.18)</td>
<td>-1.276 (0.792)</td>
<td>-1.419 (u*)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRT2⁺</td>
<td>-0.730 (0.49)</td>
<td>-</td>
<td>-</td>
<td>-1.118 (0.93)</td>
<td>-1.201 (0.19)</td>
<td>-1.333 (u*)</td>
<td></td>
</tr>
<tr>
<td>‘Sustain’</td>
<td>MVL^</td>
<td>-0.720 (u*)</td>
<td>-</td>
<td>-1.124 (1.06)</td>
<td>-1.195 (1.15)</td>
<td>-1.288 (1.46)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRT⁺</td>
<td>-0.805 (0.070)</td>
<td>-</td>
<td>-1.172 (0.051)</td>
<td>-1.224 (0.038)</td>
<td>-1.285 (0.047)</td>
<td>-1.419 (u*)</td>
<td></td>
</tr>
<tr>
<td>‘Weet-Bix’</td>
<td>MVL^</td>
<td>-0.720 (u*)</td>
<td>-</td>
<td>-</td>
<td>-1.240 (2.20)</td>
<td>1.348 (2.39)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRT1⁺</td>
<td>-0.798 (0.119)</td>
<td>-</td>
<td>-</td>
<td>-1.221 (0.062)</td>
<td>-1.285 (0.026)</td>
<td>-1.419 (u*)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRT2⁺</td>
<td>-6.834 (1.48)</td>
<td>-</td>
<td>-</td>
<td>-1.090 (0.21)</td>
<td>-1.161 (0.11)</td>
<td>1.272 (u*)</td>
<td></td>
</tr>
<tr>
<td>‘Oats: Apple and Blueberry Bake’</td>
<td>MRT⁺</td>
<td>-0.808 (1.061)</td>
<td>-1.025 (1.230)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-1.419 (u*)</td>
<td></td>
</tr>
</tbody>
</table>

* mobility double correction using DMSO as an EOF marker and sucrose as an electrophoretic mobility marker
⁺ mobility double correction using DMSO as an EOF marker and xylose as an electrophoretic mobility marker
2.3.3 Quantification of individual sugars by FS-CE

The peak area values of the standard solutions were used to produce a calibration curve for each sugar (sucrose, maltose, glucose and fructose). Concentration of identifiable sugars in BCs was calculated using the standard curve.

2.3.3.1 Linearity of the calibration curves

The calibration curve for each sugar was prepared with the sequential analyses of six sugar mixtures injected in triplicate. The linearity and repeatability were determined for 5 sugars, with xylose (0.5 g·L⁻¹) used as the internal standard (see Appendix F). Sufficient linearity was achieved for all tested sugars with correlation coefficient (R²) greater than 0.99 (Table 10), as achieved in the literature applying this FS-CE method to different matrices.¹⁶³,¹⁹⁶ The calibration for disaccharides, maltose, lactose and sucrose, had slightly better linearity than that for monosaccharides glucose and fructose.

Table 10 Calibration of response at 266 nm (y) as a function of sugar concentration (x) with its correlation coefficient (R²), for the sugars in standard (capillary of 61.8 cm total length). Xylose (0.5 g·L⁻¹) was used as the internal standard.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Linear Equation</th>
<th>R²</th>
<th>Concentration range (mg·L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>y = 0.4517 x - 0.0395</td>
<td>0.999</td>
<td>50 to 1500</td>
</tr>
<tr>
<td>Lactose</td>
<td>y = 0.3292 x + 0.0208</td>
<td>0.998</td>
<td>20 to 500</td>
</tr>
<tr>
<td>Maltose</td>
<td>y = 0.4104 x + 0.0259</td>
<td>0.995</td>
<td>20 to 500</td>
</tr>
<tr>
<td>Glucose</td>
<td>y = 0.2327 x + 0.0338</td>
<td>0.992</td>
<td>20 to 500</td>
</tr>
<tr>
<td>Fructose</td>
<td>y = 0.4263 x + 0.0324</td>
<td>0.992</td>
<td>20 to 500</td>
</tr>
</tbody>
</table>

2.3.3.2 Precision of sugar concentrations in samples

Sugar concentrations in the breakfast cereal samples show good repeatability when measured by the normalised peak area. Repeatability of sugar concentrations in measured samples is consistent with the literature (see Table 11).¹⁶³,¹⁸⁷ The use of an electro-osmotic flow marker and the addition of
an internal standard are recommended for optimal repeatability of the peak area.

Problems were reported with the sugar quantification in GC of North American BCs with sampling and/or measuring aliquots of individual cereals.\textsuperscript{[158]} The correlation of variation of most samples, however, was not greater than the standards. They state that RSD was not greater than 2 % for glucose and sucrose and not greater than 5 % for lactose and maltose. The average RSD from FS-CE reported in this work is approximately 7 % for lactose, glucose and fructose and about 13 % for sucrose and maltose concentrations. The average overall error of FS-CE at ± 9 % is comparable to that of the reported GC method\textsuperscript{[158]} at ± 7 %.

### Table 11 Precision of the quantification of sugars (concentration, C) by this FS-CE method on both breakfast cereals (this work) and in the literature

<table>
<thead>
<tr>
<th>Data from \textsuperscript{[197]}</th>
<th>This work</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Sugar</td>
</tr>
<tr>
<td>Media</td>
<td>Glucose</td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
</tr>
<tr>
<td>0 h</td>
<td>Glucose</td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
</tr>
<tr>
<td>2 h</td>
<td>Glucose</td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
</tr>
<tr>
<td>4 h</td>
<td>Glucose</td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
</tr>
<tr>
<td>6 h</td>
<td>Glucose</td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
</tr>
<tr>
<td>8 h</td>
<td>Glucose</td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
</tr>
</tbody>
</table>

*below detection limit
2.3.3.3 Sensitivity of the detection

The relative sensitivity of the detection of different sugars in FS-CE was measured through their limit of detection (LOD) and limit of quantification (LOQ). LOD and LOQ were obtained by six sigma signal-to-noise ratio (SNR) calculated automatically by 32 Karat software. The sensitivity of the direct detection in this study, with LOD values between 2.4 and 30 mg·L⁻¹, was comparable to studies that had used the same FS-CE method on different types of analytes,[158,160,188] (see Table 12). It is important to note that the sensitivity can be increased using a photo-initiator.[189]

Table 12 LOD and LOQ of the FS-CE with direct UV detection used in this work and comparison with the literature

<table>
<thead>
<tr>
<th>Sugar</th>
<th>FS-CE (this work)</th>
<th>FS-CE[188]</th>
<th>FS-CE[160]</th>
<th>GC[158]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LOD (mg·L⁻¹)</td>
<td>LOQ (mg·L⁻¹)</td>
<td>LOD (mg·L⁻¹)</td>
<td>LOQ (mg·L⁻¹)</td>
</tr>
<tr>
<td>Sucrose</td>
<td>5.88</td>
<td>21.6</td>
<td>9.93</td>
<td>0.1</td>
</tr>
<tr>
<td>Lactose</td>
<td>2.38</td>
<td>19.8</td>
<td>3.6</td>
<td>12.0</td>
</tr>
<tr>
<td>Maltose</td>
<td>20.7</td>
<td>41.7</td>
<td>5.95</td>
<td>0.01</td>
</tr>
<tr>
<td>Glucose</td>
<td>30.0</td>
<td>42.6</td>
<td>5.95</td>
<td>0.01</td>
</tr>
<tr>
<td>Fructose</td>
<td>15.9</td>
<td>44.1</td>
<td>8.47</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* the complete unit is not given in the reference.

2.3.3.4 Quantification of sugars in breakfast cereals

Figure 16 presents the sugar concentration results for eleven BCs determined by FS-CE. Each BC contained sucrose at a higher concentration than any other sugar detected (lactose, maltose, glucose and fructose). This is likely due to the amount of sugar added during manufacture of the product. BCs with high sucrose concentrations (measured above 15 g / 100 g), including
‘Oats Apple & Blueberry Bake’, Nutrigrain’, ‘Coco Pops’, listed sugar as the second highest ingredient after the cereal component on their packaging.

Nutritionally insignificant concentrations of maltose, glucose and fructose were detected in 9 of the 11 BCs analysed by FS-CE. Barley malt extract is listed as an ingredient on BCs ‘Sustain’, ‘Corn Flakes’ and ‘Sultana Bran’ thus the low concentration values (1.8, 0.9 and 0.6 g / 100 g respectively) for maltose could be expected. Similarly, trace levels of lactose detected in ‘Oats Apple & Blueberry Bake’ (0.5 g / 100 g) and ‘Oats Banana Bake’ (0.3 g / 100 g) are in agreement with the addition of the milk powder ingredient in these BCs.

Figure 16 Individual sugar quantification of 11 BCs by FS-CE (n=3 or 5 for all BCs).
2.3.4 Comparison of FS-CE with literature for total sugar quantification

Total sugar content is a legal measurement requirement for food labelling in Australia and many other countries. Total sugar content of 11 BCs was determined by the traditional Fehling (Lane-Eynon) method and the high performance FS-CE separation method, and compared with the NIP on the BC packaging label as well as with available HPLC data \cite{161} (see Figure 17). Comparison of methods is important to determine the accuracy of sugar content in BCs, both to ensure label information is correct and to highlight differences between method cost and efficiency. However it must be noted that the following discussion on the comparison of the methods has practical limitations simply due to differences in sample preparation. The FS-CE and Fehling methods did not actually measure “total sugar” for some of the BCs as the fruit pieces were removed prior to analysis. Thus for the cereals containing fruit pieces, any comparison of methods undertaken in this project with the nutritional label of the cereal is not accurate. In the same way, strong conclusions cannot be drawn between experimental data and HPLC data taken from the literature as the sample preparation for the latter is unknown.
The 11 BCs presented in Figure 17 can be grouped into three categories for total sugar content: high (>12.5 g / 100 g), medium (5 – 12.5 g / 100 g) and low (<5 g / 100 g) sugar content. The high sugar content cereals as measured by FS-CE, in increasing order, were ‘Sultana Bran’, ‘Sustain’, ‘Nutri-Grain’, ‘Coco Pops’ and ‘Oats Apple and Blueberry Bake’. ‘Sultana Bran’ and ‘Sustain’ had lower levels of total sugar measured by FS-CE and Fehling method in this work compared to the packaging and available HPLC data \cite{161} likely due to removal of fruit during sample preparation (as mentioned in section, 2.3). ‘Nutrigrain’ was consistently ranked amongst the top three BCs for high sugar content among all methods compared. The total sugar content for ‘Coco Pops’ measured by FS-CE had a relatively high RSD of 20 % and
was therefore not significantly different from that measured with other methods in the comparison. ‘Oats Apple and Blueberry Bake’ was determined to have the highest total sugar content of all BCs by FS-CE, 15% more than labelled on the NIP.

The medium sugar content BCs as determined by FS-CE, in increasing order, were ‘Rice Bubbles’, ‘Corn Flakes’, ‘Oats Banana Bake’ and ‘All Bran Fibre Toppers’. All methods, except Fehling which gave a large degree of variability between replicates, determined ‘Rice Bubbles’ to have between 6 and 9 g/100 g total sugar. The FS-CE results for ‘Corn Flakes’ were in agreement with the NIP which was more than double the amount reported by HPLC; no conclusions could be drawn from Fehling data of this BC due to the large degree of error. Sugar content of ‘Oats Banana Bake’ and ‘All Bran Fibre Toppers’ measured by FS-CE was approximately half of that reported on label or measured by the Fehling method.

The low sugar content BCs determined by FS-CE were ‘Oats Traditional’ and ‘Weet-Bix’. ‘Oats Traditonal’ had the lowest sugar content measured by FS-CE at 0.2 g/100 g. Interestingly, the NIP labelled this BC to have 1 g/100 g total sugar, however HPLC data\(^{[161]}\) reported no sugar at all. Also, sugar contained in ‘Oats Traditional’ was below the limit of detection for the Fehling method. The total sugar content of ‘Weet-Bix’ was consistent between the NIP, the HPLC data and the FS-CE method in this study. At this low level of sugar, the Fehling method had very poor repeatability ($RSD = 89\%$) as one of the repeats was below LOD.$^{[161]}$

The overall error for the quantification of sugars in BCs by the FS-CE method was much lower than that of the Fehling method, especially for the BCs with low sugar content. The error for the Fehling method is mainly caused by human technique (such as over-titration), whereas FS-CE is automated and is
more affected by operation error. In the case of the low sugar content BCs, the Fehling method was not sensitive enough to produce precise and accurate results. The most criticised aspect of FS-CE in terms of operation error is related to volume variation between injections. Injections in FS-CE are achieved by inserting a capillary into a sample solution vial and using pressure to draw sample solution into the capillary (hydrodynamic injection). Pressure variations lead to differences in injection volume and thus to relatively poor peak area precision. As previously mentioned, an internal standard was used in this work (to correct the peak area) and this eliminated this type of error and greatly improved precision as it had been observed with the FS-CE method applied to plan fibers\textsuperscript{[163]} or fermentation monitoring\textsuperscript{[168]}. There was no available data on the precision of the NIP and HPLC measurements. However, FS-CE was more comparable to HPLC than NIP or the Fehling method.
2.3.5 Comparison of FS-CE and HPLC for determination of individual sugars

Available data of individual sugars in Australian BCs using the HPLC method was obtained on the Food Standards Australia New Zealand (FSANZ) NUTTAB 2010 database. This data was compared with FS-CE results from this work (see Figure 18).

2.3.5.1 Sucrose concentration
Samples with added fruit, ‘Sultana Bran’ and ‘Sustain’, were omitted from the comparison study due to the large possible variation in sample matrices (sample preparation for the HPLC method was not provided). Among the six BCs included in this comparison, both HPLC and FS-CE determined ‘Oats Traditional’, ‘Weet-Bix’ and ‘Corn Flakes’ to have the lowest sucrose.
concentrations. The HPLC method did not detect any sugar in Oats Traditional whereas FS-CE measured 0.2 % sucrose. FS-CE also detected approximately 36 % more sucrose in ‘Weet-Bix’ and 48 % more in ‘Corn Flakes’ compared to HPLC (see Figure 18A). Sucrose content of ‘Rice Bubbles’ as measured by FS-CE was not significantly different from that from HPLC data. For the two BCs with the highest sucrose concentration (‘Nutri-Grain’ and ‘Coco Pops’), less sucrose was measured by FS-CE compared to HPLC. Some of these variations may be due to changes in the recipes or even batch to batch variations. The HPLC data from individual quantification of sugars in BCs are available online to the public. However, the exact methodology is unpublished and unreported. It is very likely that some filtration and / or centrifugation is required to prepare samples for carbohydrate analysis by HPLC. Thus sample loss could have occurred during sample preparation and may have caused an underestimation of sugar content for ‘Weet-Bix’ and ‘Corn Flakes’. FS-CE analysis also yielded a significantly higher sucrose concentration of 12.5 g / 100 g for ‘Sustain’ compared to HPLC data at 0.2 g / 100 g (see Figure 18A).

2.3.5.2 Glucose and fructose concentration
FS-CE data for glucose and fructose concentrations in ‘Sustain’ were significantly lower (0.9 and 1.1 g / 100 g) compared to HPLC data (7.4 and 8.7 g / 100 g), see Figure 18B. This may be due to differing sample preparation, in a sample which is even more heterogeneous than the other BCs due to the presence of pieces of fruit. During initial sample preparation of BCs for all experimental methods in this study, the fruit pieces and some other large particulate ingredients were resisting grinding and did not pass through laboratory sieves (see Appendix B). Thus BCs with added pieces of fruit measured in this study, such as ‘Sustain’ and ‘Sultana Bran’, are acknowledged as not being representative of the whole sample.
The free form of glucose and fructose is found naturally in plants, including many fruits and vegetables. Typical sugar composition of Australian sultanas, for example, is 38 % fructose and 35 % glucose by dry weight. The majority of sultanas and other pieces of fruit were selectively removed from the BC samples in this study. This likely caused an underestimation of glucose and fructose concentration, as seen in ‘Sustain’ and ‘Sultana Bran’ (Figure 18B).

More extensive sample preparation of BCs that contained fruit led however to up to 55 % error on the measured sugars in the case of GC. The extraction procedures of the BCs including fruits for GC requires aqueous methanol. However, this still led to larger error, especially for maltose quantification. As the total sugar measured by FS-CE and HPLC for ‘Sustain’ is similar, 16.3 and 18.2 g / 100 g respectively (see Figure 17), the significantly lower sucrose levels by HPLC (see Figure 18A) may be attributed again to sample loss during preparation for HPLC or to a change in the BC composition due to different time of purchase.

Lower level of individual sugars quantified by HPLC compared to FS-CE with direct UV detection has been observed for other samples with complex matrices, namely plant fiber and ethanol fermentation.

2.4 Conclusions

Free solution capillary electrophoresis (FS-CE) with direct UV detection was shown to be advantageous for measuring sugar content in breakfast cereals compared to traditional reducing sugar and glucose-specific methods for several reasons: (1) BCs can contain as many as 5 sugars; FS-CE is able to separate and quantify all sugars in a sample compared to traditional methods which give an estimate of total sugar after inversion of sucrose (a
great disadvantage when sample composition is unknown), (2) BCs with the lowest measured sugar levels, ‘Oats Traditional’ and ‘Weet-Bix Original’, had sugar contents below the LOD of the Fehling method, (3) the precision of data collected by FS-CE is greater than that from traditional methods likely due to automation of the FS-CE. While GC has been used to measure individual sugars in BCs, multi-step sample preparation involving derivatisation\textsuperscript{158} or enzymatic removal of starch and acid hydrolysis of the non-starch polysaccharides\textsuperscript{151,159} is time consuming and presents a high risk of sample loss.

At present, high performance liquid chromatography (HPLC) is the gold standard for measuring individual sugars in food. This study showed that, for most BCs (excluding those containing fruit), FS-CE was able to detect more sugars compared to data measured with HPLC\textsuperscript{161}. Moreover, FS-CE was observed to produce a higher total sugar content compared to HPLC, for most BCs. The discrepancy between values of obtained by FS-CE and HPLC are not only attributed to differences in the analytical method, but also differences in sample preparation (HPLC sample preparation was not given). Nevertheless, FS-CE is a more flexible method, has a much lower running cost and requires much less sample preparation than HPLC. High performance anion exchange chromatography (HPAEC) has some of the inherent disadvantages associated with normal phase HPLC, but has a greater sensitivity range than FS-CE.

In summary, free solution FS-CE is, with HPAEC, the method to recommend for the analysis of carbohydrates in breakfast cereals. FS-CE would be a simple and robust method to monitor the enzymatic hydrolysis in foodstuffs as previously done by NMR spectroscopy \textsuperscript{199}. Chapter 4 determines the
applicability of FS-CE to measure the sugars in digested breakfast cereal samples and the possibility of its use in online *in vitro* digestibility studies.
CHAPTER 3: Characterisation of starch structure by NMR spectroscopy
3.1 Introduction

3.1.1 General concepts of NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is one of the most versatile and informative spectroscopic techniques available for elucidation of molecular and supramolecular structure. NMR spectroscopy has the unique ability to (1) identify chemical bonds within a molecule to determine molecular structure and (2) detect spatial proximities between nuclei within a molecule (or between molecules) and thus configuration and conformation,\textsuperscript{[200]} and (3) investigate molecular dynamics.\textsuperscript{[201]}

The basic principles of NMR spectroscopy are grounded in two important physical properties of the atomic nucleus: magnetism and spin. Nuclear magnetism describes the interaction of nuclei with magnetic fields. Such magnetic fields may originate from surrounding electrons, from other nuclear spins in the same molecule or from an external source. The nuclear spin phenomenon is a highly abstract concept that, in very simple terms, describes the spinning behaviour of the atomic nucleus. While nuclear magnetism and nuclear spin have no effect on the physical and chemical behaviour of substances, their study (which is non-invasive) provides valuable information on the microscopic and internal structure of objects.\textsuperscript{[202]}

The nuclei of all atoms possess a nuclear spin quantum number, \( I \), which may have values greater than or equal to zero and are multiples of \( \frac{1}{2} \).\textsuperscript{[200]} Nuclei with spin 0 are not NMR active. Native starch contains a high natural abundance of hydrogen-1 (proton) and carbon-12 isotopes. While the proton nuclide has nuclear spin that can produce NMR signal, carbon-12 has no nuclear spin \( (I = 0) \) due to having an even number of protons and an even number of neutrons\textsuperscript{[200]} (that is, an even atomic weight and an even atomic
number). Thus carbon-12 is termed ‘NMR silent’. Fortunately, there are a large number of NMR active isotopes. Carbon-13 has a nuclear spin-1/2 and is thus NMR active. However in natural abundance, only 1% of carbon atoms are carbon-13, which results in poor sensitivity.\cite{203} Starch also contains oxygen atoms, however the most abundant, oxygen-16, is also NMR silent. Only oxygen-17 is NMR active but it has a low natural abundance of 0.03 %, giving very poor sensitivity, and is therefore rarely used for NMR spectroscopy experiments.

Nuclear spin and nuclear magnetism are closely related. All nuclear spins possess an angular momentum, $P$, which is not produced by a rotation of the particles but is an inherent property of the particles themselves.\cite{204} In addition, all substances are magnetic, enabling them to interact with magnetic fields. This interaction, otherwise known as the magnetic moment of nuclear spin, $\mu_{mag}$, reads as:

$$\mu_{mag} = \gamma P$$  \hspace{1cm} (10)

where the magnetogyric ratio, $\gamma$, is the proportionality constant for a given nuclide and can be used to describe how ‘magnetic’ a particular nuclide is.\cite{200} When the nuclear spin is placed in an external static magnetic field, $B_0$, the microscopic magnetic moments align to the field, revolving around the field in a circular motion. The rate of this motion or precession is known as the Larmor frequency ($\nu$), and is specific to each nucleus ($x$). The relationship can be described by:

$$\nu_x = -\gamma B_0$$  \hspace{1cm} (11)

NMR occurs when the nucleus changes its spin state, driven by absorption (resonance) of radiation at the radio frequency, $rf$, region of the electromagnetic spectrum. The frequency of electromagnetic radiation must
match the Larmor frequency to enable the process of resonance with the energy involved. As shown in equation 11, the Larmor frequency, \( \nu_x \), is directly proportional to the magnetic field \( B_0 \) and the magnetogyric ratio, \( \gamma \).\(^{[200]}\) Thus a specific irradiation frequency is required depending on the magnetic field of the instrument and nuclide of interest (\(^1\)H or \(^1\)C).

When rf pulses are applied at the specific Larmor frequency, a net amount of nuclei move to a higher energy spin state (consuming energy).\(^{[204]}\) After excitation, the magnetisation vector oscillates at the Larmor frequency. This produces a weak oscillating voltage in the detection coil surrounding the sample.\(^{[200]}\) The detected NMR signal is known as a free induction decay (FID) as the oscillating signal decays with the time constant, \( T_2 \) (transverse relaxation). When the ‘relaxation’ process returns the system to thermal equilibrium, this is referred to as the time constant, \( T_1 \) (longitudinal relaxation).\(^{[200,204]}\) This time domain data is made more useful by converting to frequency domain NMR spectra through a Fourier transform.

Each nucleus has a slightly different Larmor frequency due to differences in their environment. This is due to neighbouring atoms in the molecule or surrounding solvent molecules affecting the value of the magnetic field \( B_0 \) experienced by each nucleus. In an NMR spectrum, signal frequencies (acquired in Hz) are recorded as chemical shifts (\( \delta \))\(^{[205]}\) which represent the Larmor frequency of a specific nucleus relative to a standard (equation 12). The chemical shift is universally used to record the x-axis of an NMR spectrum, rather than Larmor frequency, because on this scale spectra can be compared irrespective of the magnetic field strength of the instrument.\(^{[204,205]}\)

The relationship between chemical shift and Larmor frequency is shown:

\[
\delta_{x, \text{sample}} = \frac{\nu_{x, \text{sample}} - \nu_{x, \text{reference}}}{\nu_{x, \text{reference}}} \quad (12)
\]
where $\delta_x$ sample is the chemical shift of nucleus x in the sample in ppm, $\nu_x$ sample is the Larmor frequency of nucleus x in the sample in Hz and $\nu_x$ reference is the Larmor frequency of a reference in Hz. Proton chemical shifts are commonly referenced with respect to tetramethylsilane (TMS) at 0 ppm. TMS in dilute solution, is recommended by the International Union of Pure and Applied Chemistry (IUPAC).[206]

3.1.2 Degree of branching in starch by solution-state 1H NMR spectroscopy

In the first chapter of this thesis, the structure of starch was described (section 1.3.2). It important to re-emphasise that starch macromolecules consist of glucose monomer units connected by $\alpha$(1,4) glycosidic bonds with branch points involving $\alpha$(1,6) linkages. Branched chains are regularly arranged into tightly packed clusters that create crystalline regions in the supramolecular structure [207], see Figure 3. The open nature of amylopectin clusters is more susceptible to enzymatic attack compared to amylose.[96,208] Thus the degree of branching ($DB$) is an important molecular feature of starch as it is expected to play a role in granular crystallinity[209] and thus may be a useful predictor of digestibility.

$DB$ in starch can be quantified as a relative number (fraction) of branching points with respect to the number of glucose units.[210] In $^1$H NMR spectra, the branching signal is the signal of the anomeric proton (protons attached to carbon number) which is involved in the $\alpha$(1,6) linkage at the branching point, see Figure 19. $^1$H NMR spectroscopy can differentiate between anomeric protons involved in $\alpha$(1,4) and $\alpha$(1,6) linkages, see Figure 19, and has been used to estimate the $DB$ of d-glucans glycogens[211], oligosaccharides[207,212] (Figure 20 and 21), and starches[209].
Figure 19 Chemical structure of α-d-glucose

Figure 20 Partial $^1$H NMR spectrum of SBE-catalyzed branched α-glucan obtained by reaction of synthetic linear α-glucan with a starch branching enzyme. Solvent mixture was 80:20 DMSO-$d_6$:D2O v/v. The spectrum was recorded at 300 MHz and 60 °C with 5362 scans were recorded. $\alpha$(1,4) and $\alpha$(1,6) signals are observed at 5.15 and 4.8 ppm, respectively. [207]
Figure 21 Partial $^1$H NMR spectra in D$_2$O at 90 °C of (a) original Amylofax (cationically-modified starch), (b) Amylofax after debranching by isoamylase, and (c) Amylofax after random cleavage by $\alpha$-amylase. $\alpha$(1,4), $\alpha$(1,6) and $\alpha$ reducing signals are observed at 5.34, 4.94 and 5.19 ppm, respectively \[212\]

The DB of starches is calculated as a ratio of the number of $\alpha$(1,6) branching points to the total number of glucose monomer units and is expressed as a percentage, as shown:

$$DB \,(\%) = \frac{I_{\alpha(1,6)} \cdot 100}{I_{\alpha(1,4)} + I_{\alpha(1,6)} + I_{\alpha\text{red}}}$$  \hspace{1cm} (13)
Where $I_y$ is integral (peak area) of the $^1$H NMR signal of the anomeric proton of type $y$. For example, the $y$ signals observed for a cationically modified starch were $\alpha(1,4)$ at 5.34 ppm, $\alpha(1,6)$ at 4.94 ppm and $\alpha$ reducing signal at 5.19 ppm\textsuperscript{212} (see Figure 21). In the case of native starch, the alpha reducing end signal is too weak to be detected and is therefore not taken into account. The following equation was used in this project to calculate a $DB$ from signal integrals of the standard starches and rice samples:

$$DB \, (\%) = \frac{I_{\alpha(1,6)}}{I_{\alpha(1,4)} + I_{\alpha(1,6)}} \cdot 100$$ \hspace{1cm} (14)

Assuming the peaks of interest are completely resolved (for example in Figure 20), the relative peak area can be measured for that sample. To ensure a quantitative spectrum, there must be sufficient repetition time between rf pulses to allow the nuclei to return to their equilibrium state.\textsuperscript{200}

The $SNR$ is measured to estimate the precision of the values of $DB$, which is expressed in terms of relative standard deviation ($RSD$). The $RSD$ is inversely related to the $SNR$ of the proton signal. Repeated acquisition and summation of a FID causes an overall increase in $SNR$ as an average of the NMR signal builds over the total number of scans ($NS$). The general rule is that the $SNR$ increases with the square root of the $NS$.\textsuperscript{200} For example, four times the $NS$ is required to quadruple the intensity of the signal. However, the intensity of the background noise increases with the square root of the $NS$ (that is, more slowly than the signal). In this way, four times the $NS$ doubles the $SNR$, that is, the $SNR$ only increases by a factor of 2. The precision of the $DB$ can be estimated using the $SNR$ of the branching point signal. Theoretically the $RSD$ is inversely proportional to the $SNR$, however an exponent of -1.28 was empirically derived from work on polyethylene in melt-state $^{13}$C NMR spectroscopy (equation 16).\textsuperscript{213} In the case of polyacrylate
samples, this equation was found to yield accurate RSD values for melt-state $^{13}$C NMR spectroscopy and good RSD estimates for solution-state $^{13}$C NMR spectroscopy.$^{[214]}$

$$RSD = \frac{238}{SNR^{1.28}}$$  (15)

### 3.1.3 Basic principles of solid-state NMR spectroscopy

Solid-state NMR spectroscopy is preferred for the investigation of samples in their solid state, especially for the crystalline structure (as solution state destroys the molecular packing). It is also useful for samples with limited or no solubility.$^{[214]}$ Similar to solution-state, solid-state NMR spectroscopy is able to give information about the sample, including composition, molecular structure and dynamics, and in addition it characterises the molecular packing.

#### 3.1.3.1 Line broadening

Liquids experience significant motions and by the fast molecular tumbling, the NMR interactions such as quadrupole interaction, dipole-dipole interaction and chemical shift anisotropy are averaged, often resulting in narrow spectral lines (signals). Due to motion restriction, most NMR experiments in the solid state have the inherent issue of strong anisotropic NMR interactions. This causes resonance line broadening which makes high-resolution analysis impossible in solid-state NMR spectroscopy without optimisation. Line broadening describes the undesirable spread of sample signal.$^{[215]}$ There are three line-broadening types caused by different coherent and incoherent processes (see Figure 22).$^{[215]}$ Inhomogeneously broadened line shapes are often observed with chemical shift anisotropy, which consist of a continuum of independent lines that are caused by differences in chemical shift for molecules in different parts of the sample. This is often
observed in a powder sample where the tiny crystallites have different orientations (e.g., powder average). Homogenenously broadened lines have the same broadening and are indivisible, i.e. have no difference in the chemical shifts of the individual components. Heterogeneous line shape broadening involves the coupling of lines with shifted frequency positions. In order to obtain high resolution in solid-state NMR spectra, special techniques must be used, including Magic Angle Spinning (MAS) and multiple / shape pulse decoupling.

Figure 22 Visualisation of inhomogeneous, heterogeneous, and homogeneous line broadening. For homogeneous line broadening, coupled lines that behave as one entity are indicated by hatching. [215]

3.1.3.2 Magic-angle spinning
As mentioned in the previous section, line broadening can be reduced by macroscopic sample rotation and thus one can reveal the structural information contained in the otherwise “hidden” isotropic chemical shifts. Magic-angle spinning (MAS) is a well-known technique used to reveal the isotropic chemical shift and obtain high resolution spectra. [203,216,217] MAS involves uniform rotation of a solid sample at high speed at a particular angle to the external static magnetic field ($B_0$). According to theory, the anisotropic NMR interactions such as chemical shift anisotropy, dipole-dipole coupling and the first order quadruple interaction produce spectral
broadening with an angular dependence of $3 \cos^2 \theta - 1$, where $\theta$ is the angle between $B_0$ and the rotation axis.\cite{203} Therefore in principle, spectral broadening should be reduced to zero as long as the rotor containing the sample is held at the magic angle of 54°44’ (for which $3 \cos^2 \theta - 1 = 0$), see Figure 23, and is spinning at a higher frequency than the largest anisotropic interaction from which the spectral broadenings originate.\cite{216} A comparison of $^1$H solid-state NMR spectra of a polyacrylate shows significant loss in signal detection under static conditions (top) and under MAS (bottom), see Figure 24.

![Figure 23 Magic angle spinning (MAS) of a solid-state NMR spectroscopy sample. Adapted from Blümich\cite{203}](image)
Figure 24 Comparison of $^1$H solid-state NMR spectra of a polyacrylate recorded under static conditions (top) and under MAS (bottom) $^{[218]}$

3.1.3.3 Recording 13C solid-state NMR spectra

The two most often used methods to obtain $^{13}$C spectra are single pulse excitation (SPE) and cross-polarisation (CP), Figure 25 and 26 respectively.

Figure 25 Pulse scheme of $^{13}$C single pulse excitation (SPE). Adapted from Berger and Braun $^{[217]}$
SPE is the simplest pulse sequence used in NMR spectroscopy experiments. First, the $^{13}$C spins are irradiated by a 90° pulse which flips their magnetisation by an angle of 90°, from the axis of the main magnetic field to its perpendicular plane. Driving sample magnetisation in a direction that is perpendicular to the direction of the main magnetic field, rather than parallel to it, allows for its measurement without interference from the (much larger) main magnetic field. Immediately following the 90° pulse, the $^{13}$C signal is recorded. During the data acquisition, $^{13}$C nuclei are decoupled from the surrounding $^1$H nuclei to eliminate line broadening caused by the strong dipolar coupling between $^1$H and $^{13}$C.

CP techniques were developed to improve signal sensitivity in rare nuclei. Although the $^{13}$C isotope is magnetically active, only 1% exists in natural abundance, and so the signal in $^{13}$C spectra is very weak compared to NMR spectra from a system of spins with high natural abundance, such as $^1$H.
Unlike SPE which obtains $^{13}$C magnetisation directly, CP is an indirect method which transfers the magnetisation from $^1$H nuclei to $^{13}$C nuclei. CP provides higher polarisation and is therefore able to produce more signal.$^{[217]}$ Also, the time needed for full relaxation between scans is shorter for hydrogen nuclei compared to carbon nuclei. $^{13}$C SPE require full relaxation of $^{13}$C nuclei, while $^{13}$C CP spectra require full relaxation of $^1$H nuclei. Therefore a higher number of scans can be recorded for CP than for SPE in the same measuring time. Thus a similar SNR found in a SPE spectrum can be obtained by CP in a much shorter amount of time.

Experimentally, the CP pulse program involves three stages$^{[203]}$ (see Figure 26). Initially, a 90° pulse in the hydrogen channel flips the $^1$H magnetisation by an angle of 90°, from the axis of the main magnetic field to its perpendicular plane (a). This is followed by continual irradiation of both $^1$H and $^{13}$C nuclei in a process called cross-polarisation (b). During cross-polarisation, the $^1$H and $^{13}$C spins are spin-locking at the same frequency (i.e., Hartman-Hahn match) and a significant amount of polarisation is exchanged between $^1$H and $^{13}$C spins. Finally, after a defined contact time, during dipolar decoupling on the $^1$H channel, the $^{13}$C signal is recorded (c).

### 3.1.4 Molecular organisation of starch by solid-state 13C NMR spectroscopy

Starch granules are semi-crystalline, made up of amorphous and crystalline (ordered) domains, and may have some transitional regions. The molecular order of starch has been studied by a variety of techniques including microscopy, X-ray diffraction, neutron scattering and solid-state NMR spectroscopy.$^{[219]}$ The long-range molecular order of starches, otherwise known as crystallinity, was originally determined by X-ray diffraction. It is important to remember the three types of crystalline phases of starch
(previously mentioned in section 1.3.2.2 of this thesis), which differ according to their packing and association with water. In native starch, type A is mostly densely packed whereas type B is loosely packed and significantly more hydrated. Type C is believed to be a mixture of type A and B in various proportions and is attributed to lipid-amylose complexes in some starches.[220]

X-ray diffraction has the ability to observe crystallinity through regular packing of double helices formed from branching side chains (not involving branching points).[221] The use of solid-state $^{13}$C NMR spectroscopy allows for a non-invasive probe of starch granule organisation at shorter range compared to X-ray diffraction because it is more sensitive at the molecular level. The amorphous and the different crystalline phases exhibit different $^{13}$C NMR spectra (see Figure 27). Separation of the spectra of into amorphous and ordered phases has been demonstrated in polyethylene by subtraction of amorphous signals from the original sample spectrum [222] (see Figure 28). The same technique has been applied to native starch samples [223-225] $^{13}$C NMR spectroscopy is able to provide relative proportions of double helices (at both crystalline and non-crystalline regions), single helices and amorphous regions within solid starch samples (see Figure 29).
Figure 27 Subtraction of the amorphous signature (extruded starch) from native A-type starch $^{13}$C CP-MAS NMR spectrum and resulting spectrum of partially ordered and crystalline components of starch.\[223\]
Figure 28 $^{13}$C CP-MAS NMR spectra of a polyethylene sample at $T = 67 ^\circ$C.\cite{222}

Figure 29 The separation of the $^{13}$C CP-MAS NMR spectra of waxy maize starch (black) into contributions from the amorphous (red) and ordered phases (green) by subtraction of the subspectrum for the amorphous phase.\cite{224}
3.2 Materials and Methods

3.2.1 Materials
Deuterium oxide ≥99 % (100 g bottles), deuterium oxide ≥99.96 % (1 g glass ampules) and dimethyl sulfoxide-d$_6$ ≥99 % were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Lithium bromide ≥99 % was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

3.2.2 Standards and rice samples
Three types of commercial maize starches with different amylose contents, Waxy (3.4 % amylose), Regular (24 % amylose) and Gelose 80 (83 % amylose), were obtained from Penford Australia Limited (Lane Cove, NSW 2066, Australia). The amylose content of these starches was determined by the iodine binding method. \[^{[224]}\]

Three varieties of rice flours, A, B and C, were sourced from the Department of Primary Industries at Yanco, NSW, Australia. Varieties A, B and C are examples of rice with different end-quality attributes, with behaviour linked to the genetic classification by Ward \[^{[226]}\] Jane, et al. \[^{[227]}\] and Chen, et al. \[^{[228]}\]. Information on the culture, harvesting and processing conditions of the rice varieties were provided:

- Five days after flowering, each variety was transferred to one of two temperature-controlled headhouses. The first headhouse had a day/night temperature of 26/17 °C (low temperature) and the second headhouse was at 36/27 °C (high temperature).

- Each variety was grown in 2 replicates for each of the high and low temperature headhouses.
Grain was harvested at maturity, dehulled (THU35A 250V 50Hz Test Husker, Satake, 23-25 Marigold Street, Revesby, PO Box 4065, Milperra, NSW, 1891, Australia), milled (No.2Mill, McGill, Seedburo Equipment Company, 1022 W Jackson Blvd, Chicago, IL 60607, USA) and then ground (Cyclotec 1093 Sample Mill, Tecator AB, Box 70 S-263 01, Hoeganaes, Sweden) to pass through a 50 µm sieve.

3.2.3 Solution state 1H NMR spectroscopy

3.2.3.1 Sample preparation
Dry starch samples and rice flours (10 mg) were suspended in 2.24 g D2O (5 mg·mL⁻¹) in round bottom flasks (50-250 mL), rinsed with anhydrous D2O prior to use. The flasks were sealed with Parafilm M® and placed at room temperature with continuous stirring (200 rpm) for 8 to 17 h, followed by freeze-drying. The stirring and freeze-drying process was repeated once. This was to ensure complete exchange of hydroxylic protons in order to reduce resonance interference from residual solvent. The dried, deuterated sample (4.5 mg) was dissolved in 0.45 mL of a 5 wt% LiBr DMSO-d₆ solution and heated in glass vials at 80 °C overnight (approximately 15 h). Then 0.15 mL of D2O was added using a precision pipette to give a final sample concentration of 10 mg·mL⁻¹ in DMSO-d₆/ D2O (75/25). The NMR spectroscopy samples were always prepared immediately before measurements and never cooled below 70 °C in order to prevent retrogradation prior to analysis.

3.2.3.2 Methodology
The solution state ¹H NMR measurements were performed with a Bruker DRX300 spectrometer (Bruker Biospin Ltd, Sydney) equipped with a 5 mm dual ¹H/¹³C probe, at Larmor frequency of 300.13 MHz. ¹H NMR spectra were recorded using a 10 000 Hz spectral width, 30° flip angle, and acquired
at either 70 or 90 °C. The probe was tuned and the spectrometer was shimmed for each sample to ensure optimal SNR and resolution, respectively. Spectra were recorded and treated using Topspin software. Longitudinal relaxation \((T_1)\) was estimated using the one-dimensional inversion recovery pulse sequence, which involved a 180° pulse followed by a variable delay and then a 90° pulse. The chemical shift scales were calibrated with respect to the signal of DMSO-\(d_6\) at 2.541 ppm at 70 °C, and at 2.526 ppm at 90 °C. These values were calculated using the 2\(^{nd}\) order polynomial equation reported for the DMSO-\(d_5\) peak in the pure DMSO-\(d_6\) solvent.\(^{229}\)

### 3.2.4 Solid state 13C NMR spectroscopy

#### 3.2.4.1 Materials

3 singly isotopically labelled samples, \(l\)-alanine (1-13C, 99%), \(l\)-alanine (2-13C, 99%), \(l\)-alanine (3-13C, 99%), were obtained from Cambridge Isotope Laboratories, Inc. (Andowver, MA, USA). Adamantane was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

#### 3.2.4.2 Sample preparation

Gelose 80 powder was placed in a sealed glass container for one week. The enclosed vesicle contained a saturated solution of \(K_2\)\(CO_3\) at 20 °C, providing an environment with a relative humidity (RH) of 44%. The sample (~100 mg) was packed in a 4-mm diameter, cylindrical, zirconium oxide rotor with a KelF end cap. Sample packing was performed with a packing tool, to ensure maximum compaction and uniformity (2 mm space was left at the top of the rotor for the cap). Prior to analysis, the outside of the rotor was cleaned with an ethanol-rinsed tissue, and then half of the bottom edge of the rotor was daubed with a black permanent marker for spinning speed detection.
3.2.4.3 Methodology
The solid-state $^{13}$C CP-MAS NMR spectroscopy experiments were performed at a $^{13}$C Larmor frequency of 50.33 MHz on a Bruker DPX200 spectrometer. The rotor was spun at 10 kHz MAS (54.7°). 90° pulses of 4 µs for $^1$H and $^{13}$C were optimised with adamantane. Cross-polarisation conditions were optimised with a mixture of 3 singly $^{13}$C labelled alanines. The contact time was optimized for Gelose 80 (see section 3.3.2). Spectra were recorded and treated using Topspin software. The $^1$H and $^{13}$C chemical shift scales were externally referenced to tetramethylsilane (TMS) at 0.0 ppm using adamantane by setting the CH resonance to 1.64 and 38.5 ppm, for $^1$H and $^{13}$C, respectively. [230]

3.3 Results and Discussion

3.3.1 Degree of branching by solution-state $^1$H NMR spectroscopy

3.3.1.1 Test for sufficient purity of D2O used in sample preparation
$^1$H NMR spectroscopy experiments of starches were obtained using DMSO-$d_6$/D$_2$O 75/25 as solvent. Prior to analysis, samples were exchanged with D$_2$O over two days to reduce interference from the non-deuterated proton content of the solvent. This solvent suppression method is ideal for quantitative analysis, as other techniques (e.g. selective irradiation to suppress the solvent signal) may lead to distortions in the branching signals,[221]

High grade D$_2$O ($\geq$99.96 %) in sealed 0.5 mL ampoules are considered superior for use in sample preparation. This is because D$_2$O ($\geq$99 %) from large bottles (multiple time use) have the risk of becoming wet and contributing a large residual HDO signal.
Figure 30 $^1$H NMR spectra of Waxy Maize-d prepared with a sealed ampule of D$_2$O (solid line) and a non-sealed bottle of D$_2$O (dashed line). Samples were measured at 70 °C (a) and 90 °C (b).
Waxy Maize-d prepared with a sealed ampoule of D$_2$O was compared with a sample prepared with a non-sealed bottle of D$_2$O (see Figure 30). The water signal for all samples was observed between 3.7 and 3.4 ppm. In samples prepared with sealed D$_2$O it was observed at 0.1 ppm higher than samples prepared with non-sealed D$_2$O (for both measurement temperatures of 70°C and 90°C). To quantify the DB in starch, the ppm scale for both signals, $\alpha$(1,4) and $\alpha$(1,6), ranged between 5.3 and 4.7 ppm. Thus, the use of non-sealed D$_2$O did not adversely affect the branching signal on the NMR spectrum. In fact, the measurement temperature (70 or 90 °C) was observed to have a greater effect on the chemical shift of the water signal than the purity of D$_2$O used in sample preparation.

In conclusion, non-sealed D$_2$O is a sufficient solvent to use during sample preparation of $^1$H NMR experiments for DB measurement in starch. It has the added advantage of being lower in cost than sealed D$_2$O – an important factor for routine analysis.

### 3.3.1.2 Sample degradation test

The error in branching measurements is generally reduced with longer acquisition times. However starch degradation may be occurring with measurement temperatures of 70 °C or 90 °C. As the standard starch sample Gelose 80 has the lowest amylopectin content (83 % amylose) compared to Regular (24 % amylose) and Waxy Maize (3 % amylose) starches, it requires longer acquisition time to obtain a satisfactory SNR. A single sample of Gelose 80 was measured sequentially for the duration of: 2 h and 10 min (light blue), then 7 h and 12 min (dark blue) and finally 2 h and 10 min (black), see Figure 31. No degradation was observed between the first and last experiments. Thus it can be concluded $^1$H NMR spectroscopy experiments conducted at 70 °C for less than 11 h and 30 min do not appear
to degrade the starch and adversely affect the starch branching. In addition, the second experiment of Gelose 80-d (7 h and 12 m) had a SNR of 46 (1-2 % instrument error), giving sufficient sensitivity, and thus acquisition time for future quantification experiments can be reduced, saving time.

![Partial 1H NMR spectra](image)

**Figure 31** Partial 1H NMR spectra of a Gelose 80 sample measured sequentially (1) to (3) for a total of 11 h and 32 min at 70° C. The duration time of the experiments was: 2 h 10 min (1, light blue), 7 h 12 min (2, dark blue) and then 2 h 10 min (3, black).

### 3.3.1.3 Test for optimal LiBr concentration used in sample preparation

Complete dissolution is essential for the characterisation of whole starch molecules. This is because solution-state NMR spectroscopy is only able to measure dissolved starch, whereas solids are not detected and gels give broad signals. The assumption that dissolution is complete upon visual
inspection of a clear dispersion of starch has been disproved as it does not account for the presence of small microgels or aggregates.\textsuperscript{[231]}

Polar organic solvents have been shown to be good for starch dissolution, with less degradation and retrogradation issues. For high amylose starches, which are known to be more difficult to dissolve than regular starches, pure DMSO-$d_6$ in mild conditions was shown to be more effective than water.\textsuperscript{[231,232]} Lithium salts have been used to dissolve polysaccharides in organic solvents by disrupting hydrogen bond interactions.\textsuperscript{[233]}

Starch samples dissolved in 0.05 wt\% LiBr were compared with dissolution in 5 wt\% LiBr (see Figures 32 and 33). A slightly higher intensity of the spectrum was observed in all standard starch samples with 0.05 \% LiBr compared to 5 \% LiBr (see Figure 32). The concentration of LiBr (whether 0.05 \% or 5 \%) did not have a significant effect on the $DB$ in the low amylose starch standard, Waxy Maize. However less branching was observed in the Regular Maize starch and Gelose80 when dissolved in 5 \% LiBr compared to 0.05 \% LiBr. It is likely that the increased amount of LiBr added to Regular Maize starch and Gelose80 lead to improved dissolution, releasing amylose from within the starch structure. This increase in amylose would increase the peak area of the $\alpha(1,4)$ (normalised by number of scans), which is consistent with the observed decreased $DB$ values. This is in agreement with a previous study on the extent of starch dissolution reported 85\%±2 \% of starch in a Gelose80 dissolved with 0.5 \% LiBr solvent and complete dissolution was achieved with 5 \% LiBr.\textsuperscript{[231]}
Figure 32 Partial $^1$H NMR spectra of starch samples Gelose 80 (a), Regular Maize (b) and Waxy Maize (c) prepared using different concentrations of LiBr, measured at 70 °C. Sample in DMSO-$d_6$ solvent was dissolved using either 0.05 % LiBr (blue) or to 5 % LiBr (navy blue).
3.3.1.4 Testing optimal measurement temperature

The temperature of samples affects the measurement of $DB$ by $^1$H NMR spectroscopy. In this project, dissolution was conducted at 80 °C the night prior to analysis. Thus the measurement temperature must be kept at a high temperature to ensure the starch does not form aggregates. It is important to highlight the other main advantage of operating at high temperatures: increased mobility. Peaks are sharper, resulting in better resolution and there is a shift in the residual solvent (HOD) signal to a lower chemical shift, which reduces interference with the signals of interest. [209]

To optimize experimental conditions for the degree of branching by $^1$H NMR spectroscopy, Regular and Waxy Maize starches were measured at different temperatures. Regular Maize was measured at 50, 70 and 90 °C and Waxy...
Maize was measured at 20, 70 and 90 °C, see Figures 34 and 35 respectively. The HOD signal for experiments that carried out at higher temperatures were furthest from the signals of interest, but temperature did not affect the chemical shift of signals of interest, α(1,4) and α(1,6) at operational temperatures of 70 and 90 °C (Figure 30).

**Qualitative observations**

Measurements at 20 °C yielded a very broad α(1,4) signal (most broad among all tested measurement temperatures) and the α(1,6) was not detected (Figure 34). At 50 °C, the α(1,4) and α(1,6) signal was slightly broader than the measurements performed at 70 and 90 °C, with the α(1,6) signal being split in two (Figure 35).

Sharper, better resolved peaks were shown in the samples measured at 70 and 90 °C compared to those measured below 50 °C. Most comparative spectra of standard starches (Figure 34, 35 and 41) and rice flours (Figure 43) showed slightly sharper peaks at 90 °C compared to 70 °C. Consistent with literature,[209] the observed narrowing of the signals with increasing measurement temperature is likely due to increased mobility of all the molecules in solution (decreased viscosity), which improves the detection of branching by 1H NMR.

**DB measurements**

The highest DB was observed through 1H NMR experiments measured at 90 °C (4.56 ± 0.2 %) for Waxy Maize and the difference between the DB values at 50 (3.19 ± 0.3 %) and 70 °C (3.51±0.2 %) was not significant, see Figure 36. For Regular Maize, no branching was detected at 20 °C, compared to DB at higher measurement temperatures, 70 °C (2.68 ± 0.06 %) and 90 °C (3.09 ± 0.06 %), see Figure 36. From Figure 34, it is easy to mistakenly conclude that for the Regular Maize measured at 20 °C (blue), a lower α(1,4) signal was
observed than for measurements at 70 °C (orange) and 90 °C (red) due to reduced solubility (also responsible for the undetected α(1,6) signal). However the peak area of the α(1,4) signal was similar all measurement temperatures: 0.24 AU at 20 °C, 0.26 AU at 70 °C and 0.23 AU at 90 °C. Thus reduced mobility, rather than incomplete dissolution, of the starch molecules is likely responsible for the broadening of the α(1,4) and α(1,6) signals in Regular Maize measured at 20 °C. A similar, but less pronounced effect was observed for the measurement of Waxy Maize at 50 °C. Therefore, the DB measurements at 20 and 50 °C were not trusted.

At higher operating temperatures, a significantly higher degree of branching was observed at 90 °C compared to experiments measured at 70 °C for Regular (0.4 %) and Waxy Maize (1.1 %), see Figure 36. However, on another day of experimentation, the measurement temperature did not seem to have a significant effect on DB for Waxy Maize and Gelose 80, see Figure 42. This inconsistency could be due to slightly improved mobility of the starch molecules 90 °C, leading to decreased viscosity and an improvement to the data treatment (especially baseline correction and shimming). Moreover reproducibility studies should be conducted to determine the extent that error from overlapping signals has on the DB in starches with varying amylose content.
Figure 34 Partial $^1$H NMR spectra of Regular Maize-d measured at 20, 70 and 90 °C in 3:1 DMSO-$d_6$ (5 % wt% LiBr)/D$_2$O with 580 scans.

Figure 35 Partial $^1$H NMR spectra of Waxy Maize-d measured at 50, 70 and 90 °C in 3:1 DMSO-$d_6$ (0.05 wt% LiBr)/D$_2$O with 48 scans.
Figure 36 The degree of branching was measured for 2 starch standards at different temperatures. Waxy Maize-d (red) was measured at 50, 70 and 90 °C in 3:1 DMSO-d$_6$ (0.05 wt% LiBr)/D$_2$O with 48 scans. Regular Maize-d (black) was measured at 20, 70 and 90 °C in 3:1 DMSO-d$_6$ (5 wt% LiBr)/D$_2$O with 580 scans.

3.3.1.5 Sample temperature equilibration test
Poor temperature control can lead to less signal being produced and an underestimation of the branching measurement, see previous section. A sample temperature equilibration test series was performed to determine how long it would take to properly heat up Waxy Maize starch to 70 °C from the time of insertion into the NMR spectrometer. Prior to the day of NMR spectroscopy analysis, the Waxy Maize starch solution was heated overnight at 80 °C. On the day of measurement, the NMR spectrometer was set to 70 °C and ramped at 5 °C per min. After 6 min the sample was inserted (starting point of experiment timing). Each experiment ran for 2 min 6 s, with a total experimental time of 67 min. Sequential spectra of the α(1,4) and the α(1,6) branching signal in Waxy Maize-d (see Figure 37) reveal that the peak shape and signal intensity after 8.2 min (light blue) is consistent with all other
experiments from 10.3 to 67 min. Thus the maximum time for sample temperature to reach equilibrium, from insertion into the NMR spectrometer (already at 70 °C) was 8.2 min.

![Chemical Shift (ppm) vs. minutes](image)

Figure 37 Sequential, partial $^1$H NMR spectra of $\alpha(1,6)$ branching signal in Waxy Maize-d measured at 70 °C. Each experiment ran for 2 min 6 s. Total experimental time was 67 min, calculated from insertion of the sample tube into the spectrometer until final experiment measurement.

### 3.3.1.6 Comparison of high and low number of scans

Good sensitivity and precision of the degree of branching in starch requires the summation of a sufficient NS. In general, instrumental error of < 5 % is acceptable and < 3 % is ideal for quantification.

A comparison was made between experiments with high and low NS to observe the differences in error (calculated from SNR) to determine an adequate NS required for samples with differing levels of amylose. Samples used in this test were: 2 standard starch samples, Waxy Maize and Gelose 80,
and 2 rice flours, variety C (grown at high temperature, replicate 2) and variety A (grown at low temperature).

In this test, quantification of branching in low amylose starches required less NS than high amylose starches: Waxy Maize, with 2-3 % instrumental error, required acquisition time of around 12 min (NS = 304) and Gelose 80, with 1-2 % error, required an acquisition time of 4-5 h (NS = 6664). Starch branching measurements in the two rice varieties tested, with 1-3 % error, had an acquisition time of 1-2 h, see table 13. These results agree with the theory that signal averaging improves the SNR by the square root of the number of scans \[^{[200]}\]. Figure 38 shows a positive correlation \((r^2 = 0.95)\) between SNR (normalised by DB) and NS. Contributing factors to a non-perfect correlation \((r^2 = 1)\) was due to differences in data treatment (such as shimming, phasing and baseline corrections), in addition the Gelose 80 used in this test was measured at 70 °C compared to other samples which were measured at 90 °C.

For the branching signal, a minimum SNR of 30, giving 3 % error, is an ideal number to aim for when calculating the adequate NS for an experiment, see equation 14. Thus Figure 38 could also be used as a tool to predict NS required for precise and accurate measurement of the (1,4) and α(1,6) signals in starch-based samples, when a rough DB is known. It is important to note that when instrumental error is relatively low, other factors such as broad signals, poor resolution, poor phasing and poor baseline correction can contribute significantly to the overall error.

In all measurements that had a low NS (9 – 42 % instrument error), there was less branching observed and a higher degree of error compared to measurements that had a high NS (1 – 5 % instrument error), see table 13 and Figure 38.
Table 13 Degree of branching, \( DB \) (% of glucose units), of 2 starch standards and 2 rice varieties. A comparison of error between measurements taken with a high and low number of scans (\( NS \)).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Low ( NS )</th>
<th>High ( NS )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( NS ) DB SNR RSD</td>
<td>( NS ) DB SNR RSD</td>
</tr>
<tr>
<td>Waxy Maize-d</td>
<td>48 3.47 13.7 8.35</td>
<td>304 3.96 32.3 2.79</td>
</tr>
<tr>
<td>Gelose 80-d</td>
<td>64 1.32 3.89 41.8</td>
<td>6664 1.21 22.9 4.33</td>
</tr>
<tr>
<td>Rice variety C-d (grown at high temperature, replicate 2)</td>
<td>32 2.63 8.73 14.9</td>
<td>1624 3.29 35.5 2.47</td>
</tr>
<tr>
<td>Rice variety A-d (grown at low temperature)</td>
<td>88 2.12 12.7 9.17</td>
<td>2352 2.91 60.2 1.26</td>
</tr>
</tbody>
</table>

Figure 38 The linear relationship between of \( SNR \) (normalised by \( DB \)) and measurements taken with a high (red) and low (black) number of scans (\( NS \)) for 2 starch standards and 2 rice varieties.
3.3.1.7 T1 Test

The intrinsic longitudinal relaxation time, $T_1$, is a value for a particular NMR signal for specific solvent, temperature and magnetic field. For the $\alpha(1,4)$ signal and $\alpha(1,6)$ branching signal in Waxy Maize, measured at both 70 and 90 °C, $T_1$ was tested for 0.5 s. Figure 40 shows that signals of interest were positive (on the y scale), thus giving an overestimated value for $T_1$. In order to quantify the integral of this signal, the repetition delay (between scans), $AQ + d_1$, should be at least five times longer than the $T_1$ value. Thus a repetition delay of 2.5 s was used for branching in all quantification experiments.

Figure 39 The degree of starch branching between experiments measured with a low (black) versus a high (red) number of scans for Waxy Maize, Gelose 80, rice variety C (grown at high temperature, replicate 2) and rice variety A (grown at low temperature).
Figure 40 $^1$H NMR spectra of a $T_1$ test for Waxy Maize starch measured at 70 °C and 90 °C, $AQ +d_1 = 2.5$ s; $T_1$ tested for = 0.5 s

3.3.1.8 Quantification of the degree of branching in standard starch samples

While both $^1$H NMR and $^{13}$C NMR spectroscopy can be used to give the same information, the former method is preferred in starch due to improved sensitivity and a significantly reduced acquisition time.$^{[209]}$ $^1$H NMR spectroscopy has been used to obtain the degree of branching in standard starch samples and rice varieties grown in different conditions.

As expected, the standard starches were observed to have increasing levels of branching with decreasing levels of amylose, see Figures 41 and 42. When measured at 90 °C in 3:1 DMSO-$d_6$ (5 wt% LiBr)/D$_2$O, Waxy Maize (low amylose content) had the highest $DB$ 3.96 ± 0.1 %, followed by Regular Maize at 3.09 ± 0.1 % and Gelose 80 (high amylose starch) at 1.31 ± 0.01 %.
Figure 41 Partial $^1$H NMR spectra of standard starch samples measured at 70 °C (dotted line) and 90 °C (solid line).

Figure 42 Degree of branching in standard starch samples, measured at 70 °C (square) and 90 °C (triangle) in 3:1 DMSO-$d_6$ D$_2$O with 5 wt% LiBr (black) or 5 wt% LiBr (red).
Figure 43 Partial spectra of variety A (a), variety B (b) and variety C (c) rice samples grown at low (purple) and high temperatures (orange and red). $^1$H NMR spectroscopy measurement temperatures were 70 °C (dashed line) and 90 °C (solid line).
The degree of branching in Waxy Maize (3.96 ± 0.1 %) is lower compared to measurements by Gidley [209] (4.76 %). While both standard samples are commercial Waxy Maize samples, this project was carried out 30 years after the Gidley [209] study and therefore the differences in branching may be due to differences in the samples and solvent, whereby Gidley [209] used pure D2O compared to this project which used 3:1 DMSO-d6 (5 % wt% LiBr)/D2O.

3.3.1.9 Quantification of the degree of branching in rice samples
The degree of branching in starch, alongside more popular properties such as amylose content and gelatinisation temperature, can be used as a tool to define the quality characteristics of the end product. A goal of rice breeding programs is to obtain varieties that perform consistently across different environments and over the years so the consumers’ expectations are met. The DB of three rice varieties (A, B and C) grown at low or high temperatures was measured by 1H NMR spectroscopy, see Figure 43.

For most rice varieties, a higher DB was observed for samples grown at high temperatures (3.13 ± 0.2 %) compared to low temperatures (2.73 ± 0.2 %), see Figure 44. An exception to this was the samples of variety A which showed no significant difference in DB when grown at two different temperature conditions. At low temperature, the DB for rice variety A was 2.9 % and at high temperature it was 2.9 to 3.0 %. Thus compared to other rice varieties, variety A had with the most consistent branching across environments and thus fits the criteria of being a consistent product over time.

In addition, a general trend was observed for DB among rice varieties grown at high temperatures, with reasonable precision between replicates (Figure 44). Variety B had the most DB (3.2-3.4 %), followed by variety C (3.0-3.3 %) and variety A (2.9-3.0 %). There is no prior expectation about branching
frequency in these varieties, it is postulated that the DB may be linked to amylose structure (this will be further discussed in chapter 4).

Figure 44 Degree of branching in rice samples grown at different low or high temperatures (rep = replicate), $^1$H NMR spectroscopy measurement temperatures were 70 °C (triangle) and 90 °C (circle).

3.3.1.10 Comparison of the degree of branching with amylose content in rice samples

Amylose content is considered the most important biochemical property of milled rice as a determinant of rice end-use quality.[234,235] Variation in grain amylose content is known to be linked to genetic mutations within the \textit{waxy} gene.[226,228,236,237] Amylose content is also largely determined by environmental factors, especially temperature, during seed development.[226,235]
The amylose content of three rice varieties (A, B and C), grown at low or high temperatures, was measured by the Department of Primary Industries (Yanco, NSW, Australia) using colourmetric determination (AACC International Approved Method 61-03.01) based on Juliano[234]. As a relatively new approach, the degree of starch branching in rice measured by $^1$H NMR spectroscopy was used to understand digestibility by comparing to the amylose content data, see Figure 45.

![Figure 45](image)

**Figure 45** Comparison between degree of branching and amylose content in different rice varieties grown at low and high temperatures (rep = replicate).

A general trend was observed where 2 out of 3 rice varieties grown at high temperature showed a reduction in amylose content and an increase in DB when compared to samples grown at low temperature. The decreased amylose content of variety B and C samples grown in a warmer climate is consistent with literature[226,228,236] and the associated increase in DB is a new
observation that confirms inferences made by Ward \[226\]. If the increased $DB$ at lower amylose is consistent with greater digestibility then breeders will have another tool to predict the digestibility profile of a particular rice variety grown in a specific environment. As this observation is based on a molecular fingerprint, there is potential to screen at early generations of the breeding program.

Whether grown at high or low temperatures, samples of variety A had a similar amylose content and $DB$ (no general trend). Variety A samples had a $DB$ around 3% and small amylose content range from 26 to 28, giving a clustering effect, see blue points on Figure 45. Another goal for rice breeders is to produce a sound variety of rice which can maintain quality across different climates. Thus the ability of variety A to maintain particular biochemical properties (amylose content and $DB$) across different climates meets the criteria for which it was designed.

### 3.3.1.11 Comparison of the degree of branching with gelatinisation temperature in rice samples

Gelatinisation is an irreversible process in which starch granules, heated in the presence of water, swell and form gel particles. First water enters the amorphous regions, degrading the amylose chains that hold the (often) many layers of amylopectin. The absorption of water in amylopectin (through hydrogen bonding) causes swelling of the granule. Eventually the starch supramolecular structure disintegrates: double helices of amylopectin split into single helices, disassociate from other neighbouring chains (in other helices) and unwind.\[238\] Amylose molecules of then diffuse out of the swollen granules into the surrounding water to contribute to the continuous gel phase.\[239\] The gelatinisation temperature ($GT$) is the temperature at which starch crystals melt and lose their supramolecular structural order. Normally in rice varieties this value ranges from 55 to 79 °C.\[240\]
The GT of three rice varieties (A, B and C), grown at low or high temperatures, was measured by the Department of Primary Industries (Yanco, NSW, Australia) using a Differential Scanning Calorimeter (DSC, Mettler Toledo DSC822e), see methodology in Appendix G. GT was determined by integrating the area of endotherm peak, which marks the maximum point at which the endothermic reaction occurred. Provided GT data was compared with DB data of the same samples as measured by ¹H NMR spectroscopy in this project (see Figure 45).

![Figure 46 Comparison between degree of branching and gelatinisation temperature in different rice varieties grown at low and high temperatures (rep = replicate).](image)

GT occurs with the melting of the B-chains in amylopectin.[241] A general trend between GT and DB was observed for 2 out of 3 rice varieties. For varieties B and C, GT was 6-7 °C higher in samples grown at high temperature compared to those grown at low temperatures. The increased GT for samples grown in warmer climates was associated with an increase in DB. The same varieties, when grown at high temperatures, were also
associated with a decrease in amylose content (discussed in the previous section). Thus it can be suggested that the increased DB in rice varieties B and C (grown in a warmer climate) is likely due to changes to the structure of amylose rather than amylopectin or a change to the amylose-to-amylopectin ratio.

These results are in agreement with previous published studies which have reported that non-waxy (higher amylose) rice starch and starches with longer branch chain length, restrict the hydration of amorphous regions and therefore require more energy to gelatinize (higher GT) compared to waxy (higher amylopectin) rice starch.\[^{242,243}\]

As mentioned in the previous section, the quality of variety A was designed to be constant over different climates. Consistent with the other biochemical properties (amylose content and DB) variety A has the least change in GT across different growing temperatures (72-79 °C) compared to variety B (74-81 °C) and variety C (67-75 °C), giving a clustering effect (Figure 46). Therefore GT is another tool of analysis which was able to successfully determine the behavioural changes (or lack thereof) of this particular rice variety across different climates.

### 3.3.2 Starch crystallinity by solid-state 13C NMR spectroscopy: optimisation of contact time

\(^{13}\)C NMR spectroscopy was planned to be used to determine relative proportions of amorphous, single and double-helical components in standard starch samples and native starch samples (rice flours). In this Masters project, the only completed work was some method optimisation in a standard starch sample due to time constraints.
The high amylose starch Gelose 80 was measured by solid-state NMR spectroscopy at six different contact times: 0.2, 0.5, 1, 2, 5 and 8 ms (Figure 47). Gelose 80 spectra revealed 9 peaks which was consistent with Gelose 80 spectra by Tan, Flanagan, Halley, Whittaker and Gidley [224]. Contact time of 1 ms was optimal for this starch, giving the highest intensity compared to other contact times, especially for C6 (peak 2) at 60 ppm (Figures 47 and 48). In future work, this contact time will be used in the measurement of crystallinity, starting with at least 2 standard samples. Comparison with literature[224] will confirm the accuracy of relative proportions of amorphous, single and double-helical components of starch. This method will then be applied to all rice flour samples.

Figure 47 $^{13}$C CP-MAS NMR spectra of Gelose80 acquired using different contact times (Tcp)
Figure 48 Evolution of contact time with intensity for 9 signals of Gelose 80 by solid-state $^{13}$C NMR spectroscopy, normalised by number of scans.

### 3.4 Conclusions

Three rice flour varieties (A, B and C), grown at low or high temperatures, were obtained from the Department of Primary Industries (Yanco, NSW, Australia). The DB of these rice flours was measured by solution-state $^1$H NMR spectroscopy to better understand the amylose structural changes across different growing climates. In a relatively new approach, the DB by $^1$H NMR was compared with provided information on amylose content and gelatinisation temperature of the same samples. Determination of starch crystallinity of the rice flours by solid-state $^{13}$C NMR was also intended; however, due to time constraints of the project only some optimisation of the method was performed.
Initial \(^1\)H NMR spectroscopy experiments for the measurement of DB in rice flours involved optimisation of the method using standard starch samples (Gelose 80, Regular and Waxy Maize). During sample preparation, the use of non-sealed bottled D\(_2\)O (rather than high-grade, sealed D\(_2\)O) gave satisfactory low solvent signal. The minimum time for sample temperature to reach temperature equilibrium, from insertion into NMR spectrometer was 8.2 min.

Improved resolution was observed with increasing measurement temperatures due to increased mobility of starch molecules in solution (decreased viscosity). The use of lower temperatures (<50 °C) during \(^1\)H NMR experiments was concluded to yield inaccurate results. The measurement temperature for DB by \(^1\)H NMR at 70 °C was acceptable but 90 °C was optimal. In a sample degradation test, experiments conducted at 70 °C for more than 11 h did not seem to adversely affect the starch branching measurements. In addition, slightly improved resolution (leading to more repeatable data treatment) is suggested to be responsible for the observed increase in DB for some samples measured at 90 °C compared to 70 °C. Starch branching measurements for rice flours, with 1-3 % error, required an acquisition time of approximately 1-2 h (repetition delay of 2.5 s).

Quantification of the DB in standard starches gave expected results with an observed decrease in DB in samples with increasing amylose content. Waxy Maize (3.4 % amylose) had the highest DB at 3.96 ± 0.1 %, followed by Regular Maize (24 % amylose) at 3.09 % ± 0.1 and Gelose 80 (83 % amylose) at 1.31 % ±0.01.

For rice varieties B and C a higher DB was observed for samples grown at high temperatures (3.13 ± 0.2 %) compared to those grown at low temperatures (2.73 ± 0.2 %). The same varieties grown at higher temperature
were also associated with an increase in \( GT \) and a decrease in amylose content. From these observations, changes to amylose structure rather than amylopectin or a change to the amylose-to-amylopectin of these samples is likely responsible for the observed increase in \( DB \). Moreover, if the increased \( DB \) is consistent with greater digestibility then breeders will have another tool to predict the digestibility profile of a particular rice variety grown in a specific environment. Moreover, as this observation is based on a molecular fingerprint, there is potential to screen at early generations of the breeding program.

Rice variety A, whether grown at higher or lower temperatures, had the most consistent \( DB \), amylose content and \( GT \) compared to all other varieties. The \( DB \) was shown to be another possible tool of quality control as it successfully confirmed that variety A fulfilled the criteria from which it was designed: to maintain quality across different climates.
CHAPTER 4: General discussion on digestibility of grain foods
FS-CE was successfully shown to be a robust and cost-effective separation method for quantification of individual sugars in Australians BCs. In this chapter, FS-CE has been assessed as a batch technique to measure 3 digested samples of Australian BCs characterised in chapter 2. The first aim of this chapter was to give a proof-of-concept for the application of FS-CE for offline \textit{in vitro} digestibility of BCs. GOD-POD and glucometry analysis of the same digested BCs samples were included for comparison of FS-CE with conventional methods. The second aim of this chapter was to address the challenging problem of understanding the link between the structure of starch and its digestibility in grain foods. The degree of branching in starch in rice samples discussed in chapter 3 (as measured by NMR spectroscopy) was compared with \textit{in vivo} GI data from literature.

\section*{4.1 Introduction: Improving \textit{in vitro} digestion methods}

\subsection*{4.1.1 Why measure \textit{in vitro}?}

Digestibility of grain foods (being mainly starch-based) depends on factors that control various degrees of resistance against acids and enzymatic action during hydrolysis of starch into soluble products. The health benefits of incorporating RS (section 1.2.4) and low GI carbohydrates in the diet have increased interest in starch digestibility research. Many studies on \textit{in vivo} and \textit{in vitro} starch digestion have focussed on the correlation between the two to predict physiological responses.\cite{55,244-247} The measurement of GI is a physiological test of particular importance to the food industry as health concerns for carbohydrate foods are an increasing consumer trend.
In vitro testing is preferred due to time, cost and ethical advantages, particularly for screening food products for their predicted GI during product development. However, due to the complexity of the human digestive system, no in vitro method has been able to replace in vivo testing, even though in vitro digestibility studies on high starch foods have been shown to estimate the GI of high starch foods.

4.1.2 In vitro digestibility methods

At present, an international standard method for in vitro starch digestibility does not exist. A review of the literature reveals differences in initial mechanical breakdown procedure, enzyme-to-substrate ratio, enzyme concentration, type and pH of buffer, degree of agitation, as well as incubation temperature and duration. The common types of enzymes used include α-amylase, amyloglucosidase and pancreatin. Another key difference in in vitro digestion methods is whether a test tube or dialysis system is used. Both systems have shown good correlations between in vitro starch digestibility and in vivo GI for grain foods. The various procedures for in vitro starch digestibility can be performed as a few single-points or as a detailed time-course measurement. For digestion profiles (digestograms) by a few single-point measurements, analysis of data involves selected specific time points of amount of glucose released. While digestion studies that use single-point measurement data can be used to roughly compare different sample sets, there is not enough information to bring real understanding about the kinetics of digestion. Apparent first-order rate equations have shown to describe digestograms and were used as a tool to estimate GI. In comparison, the Lineweaver-Burk plot has been shown to be a more accurate fitting procedure for analysing rate of starch digestion.
4.1.3 Monitoring glucose

For the present, glucose is the only product monitored during in vivo digestion methods. Therefore, many in vitro digestion methods also focus on the release of glucose as an estimate of digested starch. Common glucose determination methods are based on techniques that use chemical analysis, spectrophotometry and chromatography with limited studies on NMR spectroscopy. Spectrophotometry is the most widely used technique for measuring products of in vitro starch digestion. Assays involving glucose oxidase are popular because they are cheap and highly glucose specific, often involving chemical reactions with the use of enzyme glucose oxidase.

Glucose oxidase-peroxidase (GOD-POD) reactions have been used for in vitro starch digestibility of BC and rice samples. The use of spectrophotometry relies upon analytes being taken from the digesta at several time points during the digestion and batch-measurement of glucose afterwards. However, each analyte removed from the digesta contains a proportion of enzyme (concentrating the substrate in the digesta), with inherent error caused by a fluctuating enzyme-to-substrate ratio.

Glucometers, which traditionally measure blood glucose levels in diabetic patients, have been adapted for use during in vitro digestibility of starchy foods. Glucometers are relatively cheap, have the advantage of giving quick results (available in seconds) and analyte volumes required are less than 10 µL so enzyme-substrate ratios in digesta are not substantially altered. However, glucose is not the only product of starch digestion and so using glucose-specific enzymatic assays (e.g. GOD-POD and glucometry) for digestibility ignores the measurement of other products of digestion which could allow for a better understanding of the processes involved.
4.1.4 Monitoring all sugars using FS-CE

FS-CE has been discussed in chapter 2 as a robust method for the separation and quantification of sugars in BCs. As a relatively new technique, FS-CE has great potential to be used for *in vitro* starch digestibility studies of grain foods. Besides rate of glucose release (as a predictor for *in vitro* GI), FS-CE can be used to quantify the release of other sugars in a sample in order to better understand *in vivo* characteristics of digestion. Understanding the digestibility of a foodstuff is paramount to ensuring the accuracy of its given GI value and could help to explain other physiological responses important to maintaining good health.

4.2 Materials and Methods

4.2.1 Sample preparation and storage

4.2.1.1 Fresh BC samples

Three breakfast cereals, ‘All Bran Fibre Toppers’, ‘Coco Pops’ and ‘Weet-Bix Original’, were ground into a particle size range of 500–1000 µm, as described in section 2.2.2.

4.2.1.2 Digested BC samples

In a previous project[193] fresh BCs were digested *in vitro* using a method adapted from published work[225,237,258] (see Appendix H).

**Online (real-time) method:** 1-2 µL aliquots of digesta were removed from each bottle at different incubation times (0, 15, 30, 60, 90, 120, 150 min), added to water and measured by glucometry in real time (see Appendix I). Dilution factor for sample digesta to water was either 1:1 or 1:2 to ensure the maximum limit of detection for glucose (33.3 mmol·L⁻¹) was not exceeded. Samples were measured in duplicates.
Offline methods: 3 mL digesta were removed from each Schott bottle at a
different incubation times (0, 15, 30, 60, 90, 120, 180 min), placed into glass
vials and pure ethanol was immediately added to stop the digestion. The
dilution factor for sample digesta to pure ethanol was 1:3 for 0, 15, 30 min,
1:5 for 90 and 120 min and 1:6 for 180 min. Digesta were placed in a
refrigerator at 1-2 °C.

After 6 days, 50 µL aliquots of refrigerated digesta at each incubation time
point were collected and analysed using the GOD-POD method (see
Appendix J). Digesta remained in refrigeration for approximately 9 months
before being placed in a freezer at -18 °C. The frozen digesta were later
thawed and analysed by FS-CE (see section 4.2.2.3).

4.2.2 remained there Monitoring in vitro digestion

4.2.2.1 Glucometry
Glucometry data from a previous project[193] (see Appendix I for materials
and methods) for the BC, ‘Coco Pops’, was re-analysed in this thesis.

4.2.2.2 GOD-POD
GOD-POD data from a previous project[193] (see Appendix J for materials and
methods) for the BCs, ‘Coco Pops’, ‘All Bran Bran Fibre Toppers’ and ‘Weet-
Bix Original’ was re-analysed in this thesis.

4.2.2.3 FS-CE
In this Masters work, the frozen digesta were thawed and analysed by FS-
CE. Separations were performed on an Agilent 7100 capillary electrophoresis
system (Agilent, Waldbronn, Germany) with a Diode Array Detector (DAD)
monitoring at 191 nm and 270 nm with a 10 nm bandwidth. A capillary with
a total length of 60.0 cm (51.5 cm effective length) was used (materials and
methods as otherwise described in section 2.2). In addition, a sugar standard
was added to the standard solution: maltotriose ≥98 % was obtained from Alfa Aesar (Ward Hill, MA, USA).

The FS-CE method was also applied to long-life milk samples: ultra-heat treatment (UHT) full cream and skim milk (Devondale brand) purchased from a local supermarket. The conditions and FS-CE instrument used for the analysis of milk was exactly the same as in section 2.2.

4.2.3 Solution state 1H NMR spectroscopy

The DB was measured by 1H NMR for three varieties of rice flours, A, B and C, see chapter 3 (section 3.2) for materials and methods.

4.3 Results and Discussion: Monitoring in vitro digestion: results and discussion

The FS-CE method (discussed in chapter 2) was applied to the monitoring of digested breakfast cereal samples. The glucose digestograms produced by FS-CE data was compared with old (re-treated) digestograms from glucometry and GOD-POD data.

4.3.1 Glucometry

In food research, glucometers have been used to measure glucose at set times during in vitro digestion of various starch substrates, including: sorghum and wheat starch [153], instant rice porridges [154], unripe banana, edible canna and taro flours [155], and instant rice noodles [256]. However the application of glucometers for monitoring glucose release during in vitro digestion of breakfast cereals has not been found in the literature.
Figure 49 shows a digestogram of Coco Pops monitored by glucometry (single-point measurements at 0, 15, 30, 60, 90, 120, 150 min) as well as a blank sample. The blank sample corresponds to an undigested sample of Coco Pops measured by FS-CE after 24-48 h of extraction in water (see chapter 2). The fastest increase in glucose release occurred within the first 15 minutes of digestion (8 g/100g at 0 min to 46 g/100g at 15 min). In this in vitro digestion method, time 0 marks the beginning of simulated digestion of the small intestine. Thus the rapid increase in glucose release from Coco Pops within the first 15 minutes of digestion is likely due to increased dissolution of the glucose (from the sample) during the simulated mouth and stomach phase prior to time-point measurement at 0 min. This is consistent with the blank (undigested) sample having a higher level of glucose than the 0 min time-point measurement. Another likely contributing factor to rapid digestion (within the first 15 min) is that during the 30 min acidified “stomach” incubation period, sucrose was likely hydrolysed into glucose and fructose, thus causing a spike in glucose levels (from 0 to 15 min). This is consistent with the blank (undigested) sample having a lower level of glucose than the 15 min time-point measurement.
Figure 49 Digestogram of Coco Pops monitored by glucometry (measurements at 0, 15, 30, 60, 90, 120, 150, 210 min). The blank measurement is an undigested sample measured by FS-CE (see chapter 2).

After 30 min, no increase in glucose concentration, or any variation in general was observed (within experimental error). This is because the glucose concentration measured by glucometry had poor precision, as shown by the large error bars, (Figure 49). As the samples were measured in duplicates, the error bars were determined by the difference of the two measurements.

In a previous study,\cite{153} good repeatability was shown for in vitro digestion of barley, sorghum and starch-whey mixtures (see Appendix K) measured in duplicate. This may indicate that high precision results by glucometry may only be obtainable on less complex starch-based samples. For starch foods in general, little confidence can be drawn from the repeatability or reproducibility of the glucometry method for in vitro digestibility as most studies do not include error bars on their digestograms at all.\cite{154,155,256}
study which measured a mixture of sugars by glucometry, a synergistic effect was observed whereby displayed concentrations were consistently higher than expected on the basis of the individual calibration curves. The quantification of glucose by glucometry was recommended as a useful (quick and convenient) alternative to conventional methods in non-clinical samples where a high degree of accuracy was not essential. Thus the sample matrix of breakfast cereal, as a processed grain foods which contains sugars other than glucose (mostly sucrose), may be too complex for this type of assay.

In addition, the use of a glucometer on this high-sugar sample was not as quick compared to methodology described in the literature. Digesta taken at each time-point had to be diluted to fall within the detection range of the glucometer (0.6 to 33.3 mmol·L⁻¹). This added dilution step in the method was easily estimated for Coco Pops because an average sugar and carbohydrate content is printed on the packaging; however trial-and-error optimisation for glucometry assays would be required for lesser known samples.

### 4.3.2 GOD-POD

In the glucose oxidase/ peroxidase (GOD-POD) method, specificity for glucose is due to the action of glucose oxidase which only reacts with the β-anomer of d-glucose. Glucose released from *in vitro* digestion of breads, biscuits, various pastries and selected Spanish breakfast cereals (Kellogg’s Ricicle and Kellogg’s Fruit’n’Fibre) was measured using the GOD-POD reagent. Compared to the BCs analysed in this thesis, it is obvious that BCs from the literature differed by country of origin, brand and product line. More significantly, the digesta from the samples in the literature also had three additional treatment steps (gelatinisation, treatment with strong
alkali and hydrolysis with amyloglucosidase) to degrade and hydrolyse the starch prior to glucose quantification. Thus this particular study\textsuperscript{[151]} cannot be fairly compared with the data in this thesis as the samples have been significantly altered before measurement of glucose by GOD-POD.

Figure 50 shows the digestograms of All Bran Fibre Toppers, Weet Bix and Coco Pops monitored by GOD POD assay. For the first measurement of glucose (time 0 minutes), Coco Pops had the highest amount, followed by Weet Bix and All Bran Fibre Toppers (42, 20 and 8 g/100 g breakfast cereal, respectively). This trend is consistent with increasing levels of sugar indicated on packaging as well as blank measurements on undigested samples (see chapter 2).

Figure 50 Digestogram of All Bran Fibre Toppers (black), Weet Bix (red) and Coco Pops (blue) monitored by glucose oxidase peroxidase (GOD POD) assay. The blank measurement is an undigested sample measured by FS-CE (see chapter 2).
For the GOD-POD measurements, Coco Pops and All Bran Fibre Toppers were measured in triplicate and Weet-Bix Original had 4 repeats. A typical monophasic digestogram was observed for All Bran Fibre Toppers, with a seemingly slow release of glucose.

Coco Pops and Weet-Bix Original suffered from very poor precision (error bars produced from SD), see Figure 50. For Coco Pops, precision was worst at the time-point measurement at 0 min. It has been shown that, when present in large quantities (~1 g.L\(^{-1}\)), sucrose can interfere significantly with the glucose oxidase-peroxidase reagent.\(^{[156]}\) An explanation for this phenomenon is that β-fructosidase activity (which causes inversion of sucrose) can be detected in the oxidase, leading to an overestimation of d-glucose concentration in the presence of sucrose.\(^{[140]}\) Thus the high levels of sucrose in Coco Pops (blank = 28.8 g/ 100 g BC) may have interfered with the GOD-POD reagent and significantly reduced the accuracy and precision of the measurements. Considering the large error bars on the digestograms, there was no significant difference between time-point measurements, and thus it is impossible to comment on the rate of digestion for Coco Pops and Weet-Bix Original.

4.3.3 Free-solution capillary electrophoresis

FS-CE has been shown to be a robust way to separate and quantify sugars in breakfast cereals, see chapter 2.\(^{[192]}\) Digesta of breakfast cereals All Bran Fibre Toppers, Weet Bix and Coco Pops from a previous study (2012) were injected in triplicate using the FS-CE method \(^{[192]}\), see chapter 2. Peak areas of glucose (Figure 51) and sucrose (Figure 52) were plotted as a function of digestion time.
Figure 51 Peak area of glucose as a function of time during *in vitro* digestibility assay of All Bran Fibre Toppers (black), Weet-Bix (red) and Coco Pops (blue) monitored by free solution capillary electrophoresis.

Figure 52 Peak area of sucrose as a function of time during *in vitro* digestibility assay of All Bran Fibre Toppers (black), Weet-Bix (red) and Coco Pops (blue) monitored by free solution capillary electrophoresis.
4.3.3.1 General trend exhibited by digestograms

Starch digestibility is often determined by glucose released over time (by enzymatic hydrolysis). An expected monophasic trend for glucose release was for observed for all BCs measured by FS-CE, with an initial rapid increase in glucose peak areas for all BCs (within 15 min for ‘Weet-Bix Original’ and 30 min for ‘All Bran Fibre Toppers’ and ‘Coco Pops’). The spike in glucose peak area within the first 15 to 30 min of digestion is likely due to the fact that at this period of digestion, the substrate (starch) to enzyme ratio is at its highest.

Sucrose also exhibited a monophasic digestogram profile for all BCs. This trend is surprising as it is chemically impossible for sucrose (composed of a glucose and fructose molecule) to be released from starch (a glucose homopolymer). Similar to the trend for glucose release, an initial rapid increase was observed for peak areas of sucrose for all BCs. For ‘Weet-Bix Original’, a slight decrease in sucrose peak area was observed between 15 and 30 min, followed by a gradual increase. Following this period, a more gradual increase in glucose and sucrose was observed until 180 min (for Weet-Bix and All Bran Fibre Toppers). The sucrose peak area from 30 to 60 min was constant for Coco Pops, however a general trend for rate of sucrose release cannot be confirmed after 60 min due to incomplete data. This unexpected general trend of increasing sucrose concentration over time is likely to be due to physical reactions occurring within the food matrix. It is important to note that Weet-Bix Original had a slight decrease in sucrose peak area, between 15 and 30 min, which may be due to delayed release of sucrose within the matrix after 30 min.

This particular data set represents the first trial of these digested breakfast cereal monitored by FS-CE for proof-of-concept, with peak area (and not
individual sugar concentration) determined so far. Thus the accuracy of the method cannot be compared with GOD-POD or glucometry, however the precision is significantly improved (smaller error bars, calculated by $SD$ of triplicates) for every sample. Research on this proof-of-concept work has been continued by another member of the research team[259].

4.3.3.2 Milk and cereal
All breakfast cereals characterised in this thesis, with the exception of the oats, are ready-to-eat products that are usually consumed with milk. Ideally, an in vitro digestion method for breakfast cereals would be measured as suspension in milk. The feasibility of measuring sugars contained in milk by FS-CE was assessed. Devondale brand UHT, full cream and skim milk were injected as pure samples and analysed using FS-CE at 195 nm and 266 nm, see Figure 53. As mentioned in chapter 2, the unusual and unexpected detection of sugars occurs at the 266 nm wavelength, whereas and the low wavelength (191 nm) is non-specific.

Compared to the standard sugar solution measured at 266 nm, the pure milk (full cream) sample had an overloaded peak that appeared between 24 and 28 min as well as decreased amounts of unidentified sugars that were observed at slightly longer migration times. The overloaded peak is likely lactose, the main sugar naturally occurring in milk. It does not appear to be an artefact or contamination because it was only observed at 266 nm (in the same UV conditions required to detect the degradation products of sugars) and it does not appear at 191 nm. In addition, the same overloaded peak shape was observed in skim milk (0 % fat), confirming it not a lipid.
Figure 53 Electropherogram of sugars in a standard solution (red), full cream milk (black) and skim milk (green) at 266 nm as well as full cream milk (blue) at 191 nm as measured by free-solution capillary electrophoresis.

The method used to measure of sugars with the FS-CE method\textsuperscript{192} was applied to pure milk samples, with adequate repeatability. Skim milk and full cream milk had 0.51 and 3.58 % relative difference respectively (n = 2). Unfortunately, the capillary was so overloaded that peak area could not be accurately quantified. In addition, if a sample of milk was mixed with BC, it is likely that maltose and glucose (but not sucrose and fructose) would co-migrate with the milk’s peak. Nevertheless, adjustment of the method, perhaps by measuring milk and subtracting it as a background, would be necessary in the application of \textit{in vitro} digestibility of breakfast cereal meals.

4.4 Discussion: Relation between starch structure and digestibility
4.4.1 Starch structure and their influence on digestibility

Starch digestibility of grain foods, whether measured by *in vivo* or *in vitro* methods, is related to the source and nature of starches.\[260\] Several studies have determined that the increased digestibility of rice starch is associated with decreased amylose content.\[261-265\] This is because amylose inherently has a more linear and flexible structure than amylopectin and can form double helices after cooking (retrogradation), which have a higher resistance towards amylase hydrolysis than does amorphous starch.\[102\]

Many factors other than amylose content also have a significant impact on starch digestibility. In an *in vitro* digestibility study,\[103\] high-amylose rice starches were associated with increased total dietary fibre and RS, but were also associated with decreased SDS and had almost no correlation to RDS. It was postulated that amylose molecules in the amorphous regions may initially be hydrolysed by amylases but hydrolysed molecules may associate and become resistant to enzyme digestion. The exception to this was low-amylose rice, which, compared with waxy rice had lower RS content as well as lower GT and lower crystallinity and therefore (even with increased amylose content) was more susceptible to α-amylase digestion. Similarly, another study\[102\] demonstrated the effect of amylose in slowing the digestion rate of starch but also emphasised the significance of the fine structural features of both amylose and amylopectin in the process. In particular, cooked rice grains that contained starches with shorter amylose branches and long chains in amylopectin were shown to have a decreased *in vitro* digestion rate due to their ability to form more stable double helices and stronger crystallites.\[102\] The same features were observed in a high-amylose rice starch that exhibited the type B crystalline pattern and semi-compound starch granules.\[103\]
4.4.2 Producing a consistent, low GI rice product

One important agenda for rice breeders is to produce a low GI (below 55) variety that performs consistently from one season to the next. In the process of rice breeding programs, the selection of a trait of interest (low GI) can involve hundreds or even thousands of breeding lines. Thus it is highly impractical and costly to employ 10 human subjects (minimum) in order to obtain a GI measurement for every breeding line. The measurement of biochemical properties of rice breeding lines is an essential tool used in the screening process, even though the link to digestibility is poorly understood.

4.4.3 Comparing measured structural properties of rice with GI values from the literature

In this project, flours of three rice varieties (A, B and C), grown at low or high temperatures, were obtained. In chapter 3 of this thesis, associations between the growing environment (low or high temperature) and structural properties (DB, amylose content and GT) of these varieties was investigated. This chapter section is intended as general discussion about the relationship between measured structural properties of starch in rice varieties A, B and C and their in vivo digestibility.

The GI values of 38 rice varieties of rice were obtained from the international GI database [266] and were classified according to their food name as either having a low or high amylose content. On average, rices with a low amylose content (<24 %) had a higher GI value (72 ± 12) compared to those with high amylose (>25 %) which had a lower GI value (51 ± 5). Variety A had the highest amylose content (27 %) compared to variety B (24 %) and C (20 %), see Figure 45. In a weak association between these values and published GI values of rices of differing amylose content[266], variety A could have a low
Gl, variety C could have a high Gl and variety B could have a high Gl (but on the borderline, very close to being low Gl).

Measurements of DB (alongside amylose content and GT), were shown to be a useful tool for end-use quality analysis, particularly digestibility. Among rice varieties B and C, a general trend was observed increased DB was associated with a reduction in amylose content and a reduction in GT. As these properties were also associated with a higher Gl value (increased digestibility), breeders should be able to use structural features to predict future digestibility profiles of a particular rice variety grown in a specific environment. As a high amylose grain with a higher DB than other varieties, variety A could have a low Gl and thus meet the goal of rice breeders’ to produce a low Gl variety that has the potential to be digested slowly in the human body, contributing to positive health. Variety A also had the most consistent amylose content, DB and GT compared to the other varieties when grown at different temperatures. Thus, rice variety A is likely an adaptable crop variety that is able to maintain Gl across environments and years so that consumer expectations are met.

4.5 Conclusions

The health benefits of incorporating RS (section 1.2.4) and low Gl carbohydrates in the diet influenced the recent consumer trend for low Gl food products and have increased interest in starch digestibility research. In vitro digestibility testing is preferred over in vivo digestibility testing due to time, cost and ethical advantages, particularly for screening food products for their predicted Gl during product development.
A new, robust, free-solution capillary electrophoresis method\textsuperscript{[192]} was successfully applied to digested samples, with higher precision than traditional glucose monitoring methods (glucometry and GOD-POD). Using FS-CE to monitor sugars after or during digestion has been proposed as a way to better understand the kinetics of digestion by measuring the rate of release of all carbohydrates products,\textsuperscript{[192]} not only glucose. This method has been exhibited as proof-of-concept (successfully applied to three breakfast cereals) and with adjustments could be applied to breakfast cereal suspended in milk.

The starch digestibility of grain foods is influenced by the morphology, molecular and crystalline structure of starch.\textsuperscript{[103,261,265,267]} Besides increased amylose content, compound granules and long chains in amylopectin were attributed to increased resistance to enzyme digestion.\textsuperscript{[102,103]} Increased amylose content, decreased $DB$ and higher $GT$ were found for 2 out of 3 observed rice varieties. According to amylose content alone, in association with published $GI$ values of rices \textsuperscript{[266]}, variety A could have a low $GI$, variety B and C could have a high $GI$ (with variety B being very close to having low $GI$).
CHAPTER 5: Conclusions and Future Research
5.1 Predicting digestibility of rice through its link with starch molecular structure

5.1.1 Conclusions

A current priority of rice breeding programs is to produce low GI (<55) varieties that perform consistently over the years so consumers’ expectations are met. Rice flour samples (varieties A, B and C) were obtained from the Department of Primary Industries (Yanco, NSW, Australia), for which information on amylose content and GT was provided. According to the international GI database\[266\], rices with a low amylose content (<24 %) were, on average, classified as high GI (72 ± 12) and rices with a high amylose content (>25 %) were considered low GI (51 ± 5). Variety A had the highest amylose content compared to variety B and C. In a weak association between amylose and published GI values\[266\], variety A would be classed as low GI and variety B and C would be classed as high GI (with variety B being close to having low GI).

\(^1\)H NMR spectroscopy can differentiate between anomeric protons involved in \(\alpha(1,4)\) and \(\alpha(1,6)\) linkages, see Figure 19, and has been used to measure the DB in rice flour samples (same as above), with 2-3 % precision. Whether grown at high or low temperatures, samples of variety A had similar amylose content, DB and GT. The consistent structural properties suggest that variety A would be able to maintain a low GI value across different climates, which meets the criteria for which it was designed. For the other 2 varieties an increase in temperature resulted in an increase in DB, which was linked with increased amylose content and increased GT. The amylose content for these varieties was also weakly associated with having a higher GI value. As this observation was linked to DB, DB was determined to be a
useful tool in the prediction of future digestibility profiles of particular rice varieties grown in a specific environment.

5.1.2 Future Research

Determination of the degree of branching by $^1$H NMR spectroscopy enabled associations between starch molecular structure and GI in three varieties of rice flours. After optimisation of the starch dissolution for BC samples, the same $^1$H NMR method could be used to determine $DB$ in BCs.

Provided data of the amylose content was shown to have an important influence on digestibility of the rice flours, consistent with literature. However, these measurements were based on iodine binding using colorimetric techniques, which can suffer from an overestimation of amylose content due to overlapping absorption bands. More specifically, in the analysis of a mixture of amylose and amyllopectin, the bands from amylose and amyllopectin complexes with iodine are overlapping. Future work on this project will involve the complete separation and quantification of amylose and amyllopectin components of starch by an iodine affinity capillary electrophoresis method in order to determine the amylose-to-amylopectin ratio.

To improve the understanding of starch digestibility, associations must be made to the supramolecular structure, that is, the arrangement of starch molecules: single and double helices, and crystalline order and lamellar spacing. Many factors other than amylose content have been correlated to starch digestibility, notably: amyllopectin chain length and Type B crystalline pattern. A $^{13}$C NMR spectroscopy method for determining relative proportions of amorphous, single and double-helical components was optimised on a UWS spectrometer for a high amylose
starch sample (Gelose 80). In the future, this method as well as quantitative $^1\text{H}-^{13}\text{C}$ CP/MAS experiments recently developed by Johnson and Schmidt-Rohr [271] will be applied to native starch samples (rice flours) and possibly BCs. In addition, other methods, including Fourier transform infrared (FT-IR) spectroscopy, [272] X-ray diffraction (XRD), and small-angle-X-ray scattering (SAXS) may contribute to the understanding of starch supramolecular structure and its influence on starch digestibility. In addition, as samples were rice flours rather than starches, investigation into the influence of lipids and proteins should also be considered.

5.2 Predicting digestibility of breakfast cereals using an improved in vitro digestion method

5.2.1 Conclusions

A FS-CE method with direct UV detection was established for the quantification of several sugars in breakfast cereals. [192] The separation and characterisation of individual sugars is especially important when sample composition is unknown. As a separation method, FS-CE produced more reliable data than data from traditional colorimetric (Fehling and 3,5-dinitrosalicylic acid) and enzymatic (glucose oxidase-peroxidase). Moreover the total sugar content determined by the FS-CE method (as a summation of individual sugars) had significantly better precision as the method was automated and had no risk of sample loss (sample was suspended in water and prepared in the CE vial itself). In comparison to HPLC [161], FS-CE was shown to detect more sugars and give a higher total sugar content for most BCs (excluding those containing fruit). Nevertheless, FS-CE is a more flexible method, has a much lower running cost and requires much less sample
preparation than HPLC. The robustness of FS-CE analysis, without the need for extensive sample pre-treatment (for example, derivatisation, hydrolysis, filtration, centrifugation) has been successfully demonstrated for the direct injection from the plant tissues of zucchini, mushroom and apple samples.[273] Thus FS-CE is an ideal method to monitor the enzymatic hydrolysis in foodstuffs (a more complex matrix) as previously done by NMR spectroscopy [199].

The gold standard for measuring in vivo digestibility of foods is GI. In vitro testing is preferred due to time, cost and ethical advantages,[153,248] particularly for screening food products for their predicted digestibility during product development.[249] Frozen digesta of breakfast cereals were obtained from an in vitro method adapted from published work.[225,257,258] For the present, glucose is the only product measured during digestion.

As a proof-of-concept, both sucrose and glucose in the digested samples were analysed by the FS-CE method.[192] An expected monophasic trend for glucose release was observed for all BCs measured by FS-CE, with an initial rapid increase in glucose peak areas for all BCs. The spike in glucose peak area within the first 15 to 30 min of digestion is likely due to the fact that at this period of digestion, the substrate (starch) to enzyme ratio is at its highest. However, for all BCs, sucrose also exhibited a monophasic digestogram profile which was unexpected as sucrose (composed of a glucose and fructose molecule) cannot be released from starch (a glucose homopolymer). Similar to the trend for glucose release, an initial rapid increase was observed for peak areas of sucrose for all BCs. This unexpected general trend of increasing sucrose concentration over time is likely to be due to physical reactions occurring within the food matrix. ‘Weet-Bix Original’ had a slight decrease in sucrose peak area, between 15 and 30 min,
which may be due to delayed release of sucrose within the matrix after 30 min.

This particular data set represents the first trial of these digested breakfast cereal monitored by FS-CE for proof-of-concept, with peak area (and not individual sugar concentration) determined so far. Thus the accuracy of the method cannot be compared with GOD-POD or glucometry, however the precision is significantly improved (smaller error bars, calculated by SD of triplicates) for every sample.

Consistent with other digestograms in the literature, the trend for glucose release was monophasic for all breakfast cereals measured by FS-CE. Unexpectedly, the trend for release of sucrose was also shown to be monophasic. An initial rapid increase was observed for glucose and sucrose peak areas for all BCs likely due to the fact that at this period of digestion (simulating the beginning of small intestine), the substrate to enzyme ratio is at its highest. As the substrate to enzyme ratio slowly decreased, less enzymatic action would have occurred, which was consistent with the observed slower increase in glucose and sucrose until 180 min. An outlying time point-measurement was exhibited in the digestogram for Weet-Bix Original which had a slight decrease in sucrose peak area, between 15 and 30 min. This may be caused by delayed dissolution of sucrose molecules found within the matrix of the food due to delayed release of sucrose within the matrix.

5.2.2 Future Research

At present, an international standard method for in vitro starch digestibility does not exist. Breakfast cereals, with the exception of BC containing oats,
remain an ideal food product for optimisation of the \textit{in vitro} digestibility method because cooking (a highly influential factor of digestibility) is not required prior to consumption.

Offline monitoring of digested breakfast cereals was demonstrated as proof-of-concept method using FS-CE\textsuperscript{[192]}, with the ability to analyse different sugars (not only glucose). Future research in this area should focus on online monitoring of food samples as this prevents the issue of possible sample alteration between digestion of the sample and analysis of the digesta aliquots. Optimisation of the \textit{in vitro} digestion method presented in this thesis could be developed using tests that aim to improve the understanding of the enzymatic action (by monitoring the digestibility of one sugar at a time or only injecting one enzyme per sample) and the complexity of the sample matrix (by monitoring the kinetics of online dissolution).

As breakfast cereals are normally consumed with milk, incorporating samples suspended in milk should be considered in future developments of an \textit{in vitro} digestion method. As a proof-of-concept, pure milk was injected into the FS-CE instrument (no sample preparation) and gave repeatable separation of sugars. However, a large peak, presumed to be lactose, was observed in the electropherogram, likely caused by overloading in the capillary and thus adjustment to the method is required in future work. One possible solution would be to inject pure milk samples and subtract their electropherogram from the electropherograms of the BC and milk mixtures.

Beyond BC samples, monitoring \textit{in vitro} digestion by FS-CE could be applied to a number of other starch-based foods. In future work, the rice varieties that were characterised through the \textit{DB} in this thesis would be the first priority samples. The rice grains of these varieties could be cooked, ground and then digested the same conditions as the breakfast cereals: within bottles
submerged in a shaking water bath with the sequential addition of enzymes (simulating in vivo conditions of the human digestive system). A CE instrument could be modified to connect the sample bottles to the injection vials so that monitoring of the sugars could occur online during the digestion, similar to bioprocess monitoring in the literature [274].

The kinetics of in vitro digestion of starch-based foods could also be monitored online by time-resolved 1H NMR spectroscopy [199]. While glucose is likely the only measurable product of digestion, a measurement could theoretically be taken every two minutes, yielding a statistically significant digestogram.

5.3 The larger context

In this project, commonly consumed grain foods were evaluated to improve our understanding of their digestibility. Rather than measuring the in vivo response after food consumption, digestibility work was focussed on measuring the food itself and finding associations with published in vivo data. While much more work is required in the future (increased number of samples and method validation with other techniques), this molecular characterisation approach to understanding digestibility of foods is promising. In particular, the work on FS-CE presented in this thesis opens the gateway to solutions that could be used to address issues (such as mimicking the process of gastric emptying) faced by in vitro digestion studies.

An improved understanding of the rate of enzymatic hydrolysis depends on having a much higher number of points on the digestogram. At present, blood glucose levels are only measured 4-8 times within the first 2 hours of
digestion for the measurement of GI. Thus, *in vitro* digestion by FS-CE could be measured at many more time-points to give a more precise GI value. In addition, improved data treatment is possible for *in vitro* digestibility experiments whereby the slope as well as the area under the digestibility curve could be measured for glucose and the other sugars contained in food.

Moreover, there is great potential for FS-CE techniques to be miniaturised and then applied *in vivo*. For example, lab on a chip technology could be implanted into diabetic patients (perhaps at several stages of the digestive system) to measure digestibility of particular foods after ingestion. This type of technology would be especially relevant for Type 1 diabetic patients who could use sugar measurements (especially maltotriose) taken at initial stages of digestion to predict blood glucose levels in the later stages of digestion. FS-CE lab on a chip technology could be useful for telemedicine applications and would be an improvement from GI measurements which can only estimate a fast or slow glycaemic response.

In conclusion, a better understanding of the digestibility of foods would improve prediction rates of digestion made by consumers, health advisors and the food industry, and ultimately have implications for the improvement of public health and prevention and management of disease (especially obesity, diabetes and colorectal cancer).


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Appendices
Quantification of sugars in breakfast cereals using capillary electrophoresis

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3,5-Dinitrosalicylic acid (DNS) assay

Abstract

About 80% of the Australian population consumes breakfast cereal (BC) at least five days a week. With high prevalence rates of obesity and other diet-related diseases, improved methods for monitoring sugar levels in breakfast cereals would be useful in nutrition research. The heterogeneity of the complex matrix of BCs can make carbohydrate analysis challenging or necessitate tedious sample preparation leading to potential sugar loss or starch degradation into sugars. A recently established, simple and robust free solution capillary electrophoresis (CE) method was used in a new application to 13 BCs (in Australia) and compared with several established methods for quantification of carbohydrates. Carbohydrates identified in BCs by CE included sucrose, maltose, glucose and fructose. The CE method is simple requiring no sample preparation or derivatization and carbohydrates are detected by direct UV detection. CE was shown to be a more robust and accurate method for measuring carbohydrates than Fehling method, DNS (3,5-dinitrosalicylic acid) assay and HPLC (high performance liquid chromatography).

1. Introduction

About 80% of the Australian adult population consume breakfast cereal, either cooked or ready-to-eat, at least five days a week. Breakfast cereals, like most food products, contain a variety of carbohydrates as well as lipids, proteins and minerals. The heterogeneity of this complex matrix can make sugar analysis in breakfast cereals challenging.

For sugars, total content is all that is required for the nutrition information panel (NIP), a mandatory labeling requirement of all processed foods in Australia. Methods for the measurement of sugar in foodstuffs were often developed before carbohydrate chemistry was established. Earlier quantitative chemical analytical assays often relied upon the reducing properties of aldehyde or keto group found in monosaccharides and short-chain oligosaccharides. In alkaline solutions, at elevated temperatures, these reducing sugars tautomerase to enediol forms, which are then readily oxidized by oxygen and oxidizing agents (such as metallic salts). An estimate of glucose content was based on the colorimetric measurement of the oxide or the free metal formed. The empirical nature of this reaction allowed analysts to develop methods such as the Fehling method and the 3,5-dinitrosalicylic (DNS) assay, which can produce reproducible and accurate results for samples with simple matrices. Such methods are also inexpensive, technically easy to perform and highly applicable to routine quantification. However, a strict control of experimental conditions (rate of heating, alkalinity and strength of the reagent) in a non-automated setting is necessary to obtain repeatable and reproducible results.

Due to their specificity and ease of operation, enzymatic assays are the preferred reducing sugar method over Fehling method and DNS assay. Glucose and sucrose content has been determined in 79 dry, North American BCs using the glucose-oxidase peroxidase (GOD-POD) method. The sugar content of these samples was previously assayed by the colorimetric condensation reaction with anthrone, which gave unsatisfactory reproducibility.
Most foods, including BCs, contain a mixture of sugars rather than a single type of sugar. Therefore, the methods previously discussed are innately flawed by being either glucose-specific (e.g., GOD-POD) or unable to distinguish between different reducing sugars as is the case for the Fehling method and the DNS assay. In reducing-sugar assays the quantity of product formed and measured is not exactly equivalent to sugar content, and different sugars yield different color intensities; this shows that the chemistry involved in the assay is considerably more complicated than it appears. For certain foods in which the composition of the sugars is known and the requirement of the analysis is routine, e.g. quality control, an estimate of total sugar values expressed as invert sugar or glucose may be sufficient. However, most BCs have sucrose added during manufacture and thus total sugar determination requires a preliminary hydrolysis of non-starch polysaccharides (by acid or enzyme), which may cause sample loss or overestimation of reducing sugars. In addition, mineral ions have been reported to interfere with some reducing-sugar assays, a problem for most Australian BCs, which have been fortified.

In the area of nutrition research, the intrinsic accuracy of the quantities of the different carbohydrates present in a diet is often required for correlation with their metabolic behavior. Separation is used for this purpose. Separation methods have allowed for greater accuracy of sugar analysis in foods. Individual sugars measurements can be summed to calculate the ‘total sugar content’ for the NIP. Gas chromatography (GC) is a popular method for carbohydrate analysis and is very sensitive. It is the only chromatography method published in the peer-reviewed literature so far for sugar quantification of BCs. The sample preparation requires multiple steps: grinding to pass through a 30-mesh (0.59 mm) screen, drying under vacuum, defatting with n-hexane, extraction with water for some of the sample and with aqueous methanol for the rest, centrifugation. In order to make the carbohydrate volatile a multistep derivatization was then needed: concentration under nitrogen flow and then drying under vacuum, reaction with pyridine, hexamethyldisilazane in presence of trifluoroacetic acid, followed by another centrifugation. The sample preparation for GC is thus time consuming, laborious and has a significant probability of sample loss. High performance liquid chromatography (HPLC) is the other established analytical method for measuring individual sugars in many foods. A number of columns have been tested for normal phase HPLC of carbohydrates, for example ion-exchange columns for BCs, but they all have their own disadvantages including co-elution, tedious sample preparation and intolerance to salt or acid leading to short column life.

For starchy-food sample matrices, such interfering substances not only disrupt the analysis, they can damage the column leading to a non-reproducible result. One way and then drying under vacuum, due to a photo-oxidation reaction taking place at the detection window. To identify the carbohydrates, electrophoretic mobility is used, and not migration time, since the former has a higher reproducibility than the latter (Table 1). Preliminary sugar identification was confirmed by spiking BC samples (see Figs. S-2 and S-3). The samples, ‘Corn Flakes’, ‘Froot Loops’ and ‘Weet-Bix Multigrain’ were selected as they contained the greatest number of peaks among the seven BCs used in the first set of experiments. Repeatability within the standards was sufficient with relative standard deviation (RSD) values of no more than 1.3%, providing a reliable set of values on which to base sugar identification. In the analysis of BC samples, higher RSD values were observed in MRT (series1) ‘Nutri-Grain’ and MVL ‘Weet-Bix’, yielding measurement errors of 5.6% and 2.4%, respectively (see also Fig. S-1). Apart from these isolated cases, the repeatability of BC sample analysis was good with RSD ≤1.5%

Reproducibility of the electrophoretic mobility values has been investigated by comparing the results obtained by two different methods: FSC and DNS assay. Available data from the Food Standards Australia New Zealand (FSANZ) NUTTAB database of individual sugar quantities for relevant Australian breakfast cereals was also included for a comparison to our findings.

2. Results and discussion

2.1. Detection of individual sugars in breakfast cereals using capillary electrophoresis (CE)

The ground breakfast cereals (BC) were simply suspended in water and injected in CE. The sugars in breakfast cereals were separated (Fig. 1) by CE. Identification of sugars was validated by comparison of the electrophoretic mobility of observed peaks with that of a standard sugar solution and previous literature (Table 1). A double correction was used to precisely determine the electrophoretic mobility of each sugar peak. The first correction was using a neutral species (DMSO) as an EOF marker (see Equation S-2). The second correction involved a monochromat transformation with an electrophoretic mobility marker (Equation S-3). Table 1 demonstrates how the mobility value of a sugar, much like elution time in HPLC, is useful in peak identification. Sucrose was detected in all BCs, while lactose, maltose, glucose and fructose were detected in some. Other components are not detected even though the matrix is complex: proteins, lipids are also present but the direct UV detection has been shown to be specific to carbohydrates since it is due to a photo-oxidation reaction taking place at the detection window. To identify the carbohydrates, electrophoretic mobility is used, and not migration time, since the former has a higher repeatability than the latter (Table 1). Preliminary sugar identification was confirmed by spiking BC samples (see Figs. S-2 and S-3). The samples, ‘Corn Flakes’, ‘Froot Loops’ and ‘Weet-Bix Multigrain’ were selected as they contained the greatest number of peaks among the seven BCs used in the first set of experiments. Repeatability within the standards was sufficient with relative standard deviation (RSD) values of no more than 1.3%, providing a reliable set of values on which to base sugar identification. In the analysis of BC samples, higher RSD values were observed in MRT (series1) ‘Nutri-Grain’ and MVL ‘Weet-Bix’, yielding measurement errors of 5.6% and 2.4%, respectively (see also Fig. S-1). Apart from these isolated cases, the repeatability of BC sample analysis was good with RSD ≤1.5%

Reproducibility of the electrophoretic mobility values has been investigated by comparing the results obtained by two different
The results obtained in this work by both operators MVL and MRT showed a sufficient level of reproducibility between operators to yield identical sugar identifications in all cases. Same operator, with dilution showed a reduced reproducibility, yielding slightly higher variance in mobility, though still indicates a sufficient level for the identification of sugars. CE is thus a viable method for the identification of sugars in breakfast cereals.

2.2. Quantification of individual sugars in breakfast cereals using capillary electrophoresis (CE)

The calibration curve for each sugar was prepared with the sequential analyses of six sugar mixtures injected in triplicate. The linearity and repeatability were determined for 5 sugars, with xylose (0.5 g L\(^{-1}\)) used as the internal standard. Sufficient linearity was achieved for all tested sugars with correlation coefficient \(R^2\) greater than 0.99 (Table 2) and reasonable standard error on the \(y\) estimates (see Figs. S-5 to S-6 in Supplementary data), as achieved in the literature applying this CE method to different matrices.\(^{18,19}\) The calibration for disaccharides, maltose, lactose and sucrose, had slightly better linearity than that for monosaccharides glucose and fructose. The sugar concentrations determined by CE also show good repeatability (see error bars on Fig. 2) of the peak area (normalized with respect to the peak area of the internal standard, each peak area being also divided with the corresponding migration time) consistent with the literature (see Table S-3).\(^{18,30}\) The use of an electro-osmotic flow marker and the addition of an internal standard are recommended for optimal repeatability of the peak area. The high pH of the NP200 buffer made it prone to carbonation\(^{40}\) and it is thus recommended to use buffer within 13 h (or 19 h) of its preparation to be within 10% (or 15%) of initial current measurement (Equation S-5 to S-8, Table S-4 and Fig. S-7 in Supplementary data). Table 2 lists the relative sensitivity of the detection of different sugars along through the limit of detection (LOD) and limit of quantification (LOQ). LOD and LOQ were calculated from the signal-to-noise ratio (SNR) obtained with 32 Karat software. The sensitivity of the direct detection in this study, with LOD values between 2.4 and 30 mg L\(^{-1}\), was comparable to studies that had used the same CE method on different types of analytes.\(^{31,41}\) (see Table S-1).

The method was applied to the quantification of sugars in several commercial breakfast cereals. Since fewer than six sugars have been identified in BCs previously, a short capillary, total length 61.8 cm, was employed for all experiments to decrease analysis time to 40 min (including xylose internal standard). Fig. 2 presents the sugar concentration results for ten BCs determined by CE. Each BC contained sucrose at a higher concentration than any other sugar detected (lactose, maltose, glucose and fructose). This is likely due to the amount of sugar added during manufacture of the product. BCs with high sucrose concentrations (measured above 15 g/100 g), including ‘Oats Apple & Blueberry Bake’, ‘Nutrigrain’, ‘Coco Pops’, listed sugar as the second highest ingredient after the cereal component on their packaging.

Nutritionally insignificant concentrations of maltose, glucose and fructose were detected in 9 of the 11 BCs analyzed by CE. Barley malt extract is listed as an ingredient on BCs ‘Sustain’, ‘Corn Flakes’ and ‘Sultana Bran’ thus the low concentration values (1.8, 0.9 and 0.6 g/100 g, respectively) for maltose could be expected. Similarly, trace levels of lactose detected in ‘Oats Apple & Blueberry Bake’ (0.5 g/100 g) and ‘Oats Banana Bake’ (0.3 g/100 g) are in agreement with the addition of the milk powder ingredient in these BCs.

Problems were reported with the sugar quantification in GC of North American BCs with sampling and/or measuring aliquots of individual cereals.\(^{12}\) The variation of most samples, however, was not greater than the standards. They state that RSD was not greater than 2% for glucose and sucrose and not greater than 5% for lactose and maltose. The average RSD from CE reported in this work is approximately 7% for lactose, glucose and fructose and about 13%
Table 1
Reproducibility of the separation of sugars in five breakfast cereal samples with CE. Operator MVL (n=2) and Operator MRT (n=3)

<table>
<thead>
<tr>
<th>Breakfast cereal</th>
<th>Operator or publication</th>
<th>Average ( \mu_m ) (10^{-9} \text{ m}^2 \text{ V} \text{ s} \text{ cm}^{-2} ) (RSD in %)</th>
<th>Sugar LOD (mg L^{-1})</th>
<th>Sugar LOQ (mg L^{-1})</th>
<th>Linear equation</th>
<th>Sugar LOD (mg L^{-1})</th>
<th>Sugar LOQ (mg L^{-1})</th>
<th>Linear equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard sugar solution</td>
<td>Ref. 30 (^b)</td>
<td>-0.772 (u')</td>
<td>-0.810 (0.109)</td>
<td>-1.172 (1.16)</td>
<td>y=0.4517x-0.0395</td>
<td>0.999</td>
<td>50-1500</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ref. 18 (^c)</td>
<td>-0.720 (1.08)</td>
<td>-1.116 (0.49)</td>
<td>-1.724 (0.74)</td>
<td>y=0.3295x-0.0208</td>
<td>0.998</td>
<td>20-500</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MVL(^d)</td>
<td>-0.801 (1.29)</td>
<td>-1.181 (0.30)</td>
<td>-1.224 (0.74)</td>
<td>y=0.4104x+0.0259</td>
<td>0.995</td>
<td>20-500</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRT(^e)</td>
<td>-0.720 (u')</td>
<td>-1.116 (0.49)</td>
<td>-1.224 (0.74)</td>
<td>y=0.4263x-0.0324</td>
<td>0.992</td>
<td>20-500</td>
<td></td>
</tr>
<tr>
<td>‘Coco Pops’</td>
<td>MVL</td>
<td>-0.772 (u')</td>
<td>-1.124 (1.06)</td>
<td>-1.195 (1.15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRT (series 1)</td>
<td>-0.788 (5.59)</td>
<td>-1.172 (0.051)</td>
<td>-1.224 (0.038)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRT (series 2)</td>
<td>-0.733 (0.47)</td>
<td>-1.188 (0.77)</td>
<td>-1.283 (0.79)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Nutri-Grain’</td>
<td>MVL</td>
<td>-0.720 (u')</td>
<td>-1.188 (0.77)</td>
<td>-1.283 (0.79)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRT (series 1)</td>
<td>-0.810 (0.109)</td>
<td>-1.223 (0.006)</td>
<td>-1.289 (0.029)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRT (series 2)</td>
<td>-0.733 (0.47)</td>
<td>-1.212 (1.18)</td>
<td>-1.276 (0.792)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Sustain’</td>
<td>MVL</td>
<td>-0.720 (u')</td>
<td>-1.212 (1.18)</td>
<td>-1.276 (0.792)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRT (series 1)</td>
<td>-0.805 (0.070)</td>
<td>-1.118 (0.93)</td>
<td>-1.201 (0.19)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRT (series 2)</td>
<td>-0.733 (0.47)</td>
<td>-1.172 (1.16)</td>
<td>-1.288 (1.46)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Weet-Bix’</td>
<td>MVL</td>
<td>-0.720 (u')</td>
<td>-1.172 (1.16)</td>
<td>-1.288 (1.46)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRT (series 1)</td>
<td>-0.798 (0.119)</td>
<td>-1.189 (0.93)</td>
<td>-1.201 (0.19)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRT (series 2)</td>
<td>-0.805 (0.070)</td>
<td>-1.221 (0.006)</td>
<td>-1.285 (0.026)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Oats: Apple and Blueberry Bake’</td>
<td>MVL</td>
<td>-0.720 (u')</td>
<td>-1.201 (0.19)</td>
<td>-1.285 (0.026)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRT (series 1)</td>
<td>-0.808 (1.001)</td>
<td>-1.276 (0.792)</td>
<td>-1.333 (u')</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) u stands for unavailable.
\(^b\) Mobility correction using methanol as an EOF marker.
\(^c\) Mobility double correction using DMSO as an EOF marker and lactose as an electrophoretic mobility marker.
\(^d\) Mobility double correction using DMSO as an EOF marker and sucrose as an electrophoretic mobility marker.
\(^e\) Mobility double correction using DMSO as an EOF marker and xylose as an electrophoretic mobility marker.

Table 2
Calibration of response at 266 nm (y) as a function of sugar concentration (x) with its correlation coefficient (\( R^2 \)), for the sugars in standard (capillary of 61.8 cm total length). Xylose (0.5 g L^{-1}) was used as the internal standard.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>LOD (mg L^{-1})</th>
<th>LOQ (mg L^{-1})</th>
<th>Linear equation</th>
<th>( R^2 )</th>
<th>Concentration range (mg L^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>5.88</td>
<td>21.6</td>
<td>y=0.4517x-0.0395</td>
<td>0.999</td>
<td>50–1500</td>
</tr>
<tr>
<td>Lactose</td>
<td>2.38</td>
<td>19.8</td>
<td>y=0.3295x-0.0208</td>
<td>0.998</td>
<td>20–500</td>
</tr>
<tr>
<td>Maltose</td>
<td>20.7</td>
<td>41.7</td>
<td>y=0.4104x+0.0259</td>
<td>0.995</td>
<td>20–500</td>
</tr>
<tr>
<td>Glucose</td>
<td>30.0</td>
<td>42.6</td>
<td>y=0.2327x+0.0338</td>
<td>0.992</td>
<td>20–500</td>
</tr>
<tr>
<td>Fructose</td>
<td>15.9</td>
<td>44.1</td>
<td>y=0.4263x-0.0324</td>
<td>0.992</td>
<td>20–500</td>
</tr>
</tbody>
</table>

\(^a\) u stands for unavailable.
\(^b\) Mobility correction using methanol as an EOF marker.
\(^c\) Mobility double correction using DMSO as an EOF marker and lactose as an electrophoretic mobility marker.
\(^d\) Mobility double correction using DMSO as an EOF marker and sucrose as an electrophoretic mobility marker.
\(^e\) Mobility double correction using DMSO as an EOF marker and xylose as an electrophoretic mobility marker.

Fig. 2. Individual sugar quantification of 11 BCs by CE (n=3 or 5 for all BCs).
for sucrose and maltose concentrations. The average overall error of CE at ±9% is comparable to that of the reported GC method at ±7%.

2.3. Comparison of CE and HPLC for determination of individual sugars

The quantity of individual sugars in Australian BCs as measured by HPLC is available on the Food Standards Australia New Zealand (FSANZ) NUTTAB 2010 database. This data was compared with CE results from this work (see Figs. 3–5). Among the eight BCs included in this comparison, both methods determined ‘Oats Traditional’, ‘Weet-Bix’ and ‘Corn Flakes’ to have the lowest sucrose concentrations. For these cereals, the HPLC method did not detect any sugar in ‘Oats Traditional’ whereas CE measured 0.2% sucrose. CE also detected approximately 36% more sucrose in ‘Weet-Bix’ and 48% more in ‘Corn Flakes’ compared to HPLC (see Fig. 3A). The sucrose content of ‘Rice Bubbles’ as measured by CE was not significantly different from that from HPLC data. For the two BCs with the highest sucrose concentration (‘Nutri-Grain’ and ‘Coco Pops’), less sucrose was measured by CE compared to HPLC. Some of these variations may be due to changes in the recipes or even batch to batch variations. The HPLC data from individual quantification of sugars in BCs are available online to the public. However, the exact methodology is unpublished and unreported. It is very likely that some filtration and/or centrifugation is required to prepare samples for carbohydrate analysis by HPLC. Thus sample loss could have occurred during sample preparation and may have caused an underestimation of sugar content for ‘Weet-Bix’ and ‘Corn Flakes’. CE analysis also yielded a significantly higher sucrose concentration of 12.5 g/100 g for ‘Sustain’ compared to HPLC data at 0.2 g/100 g (see Fig. 3A). In addition, CE data for glucose and fructose concentrations in ‘Sustain’ were significantly lower (0.9 and 1.1 g/100 g) compared to HPLC data (7.4 and 8.7 g/100 g), see Fig. 3B. This may be due to differing sample preparation, in a sample, which is even more heterogeneous than the other BCs due to the presence of pieces of fruit. During initial sample preparation of BCs for all experimental methods in this study, the fruit pieces and some other large particulate ingredients were resisting grinding and did not pass through laboratory sieves. Thus BCs with added pieces of fruit measured in this study, such as ‘Sustain’ and ‘Sultana Bran’, are acknowledged as not being representative of the whole sample. The
free form of glucose and fructose is found naturally in plants, including many fruits and vegetables. Typical sugar composition of Australian sultanas, for example, is 38% fructose and 35% glucose by dry weight. The majority of sultanas and other pieces of fruit were selectively removed from the BC samples in this study. This likely caused an underestimation of glucose and fructose concentration, as seen in ‘Sustain’ and ‘Sultana Bran’ (Fig. 3B). More extensive sample preparation of BCs that contained fruit led however to up to 55% error on the measured sugars in the case of GC. The extraction procedures of the BCs including fruits for GC requires aqueous methanol. However, this still led to larger error, especially for maltose quantification. As the total sugar measured by CE and HPLC for ‘Sustain’ is similar, 16.3 and 18.2 g/100 g, respectively (see Fig. 5), the significantly lower sucrose levels by HPLC (see Fig. 3A) may be attributed again to sample loss during preparation for HPLC or to a change in the BC composition due to different time of purchase. Lower level of individual sugars quantified by HPLC compared to CE with direct UV detection has been observed for other samples with complex matrices, namely plant fiber and ethanol fermentation.

2.4. Estimation of total sugar by CE and Fehling (Lane–Eynon) method

Total sugar content is a legal measurement requirement for food labeling in Australia and many other countries. Total sugar content of 11 BCs was determined by the traditional Fehling (Lane–Eynon) method and the high performance CE separation method, and compared with the NIP on the BC packaging label as well as with available HPLC data. Comparison of methods is important to determine the accuracy of sugar content in BCs, both to ensure label information is correct and to highlight differences between methods in cost and efficiency.

The 11 BCs presented in Fig. 4 can be grouped into three categories for total sugar content: (high (>12.5 g/100 g), medium (5–12.5 g/100 g) and low (<5 g/100 g) sugar content). The high sugar content cereals as measured by CE, in increasing order, were ‘Sultana Bran’, ‘Sustain’, ‘Nutri-Grain’, ‘Coco Pops’ and ‘Oats Apple and Blueberry Bake’. ‘Sultana Bran’ and ‘Sustain’ had lower levels of total sugar measured by CE and Fehling method in this work compared to the packaging and available HPLC data likely due to removal of fruit during sample preparation (as mentioned in Section 2.3). ‘Nutritrail’ was consistently ranked amongst the top three BCs for high sugar content among all methods compared. The total sugar content for ‘Coco Pops’ measured by CE had a relatively high RSD of 20% and was therefore not significantly different from that measured with other methods in the comparison. ‘Oats Apple and Blueberry Bake’ was determined to have the highest total sugar content of all BCs by CE, 15% more than labeled on the NIP.

The medium sugar content BCs as determined by CE, in increasing order, were ‘Rice Bubbles’, ‘Corn Flakes’, ‘Oats Banana Bake’ and ‘All Bran Fibre Toppers’. All methods, except Fehling, which gave a large degree of variability between replicates, determined ‘Rice Bubbles’ to have between 6 and 9 g/100 g total sugar. The CE results for ‘Corn Flakes’ were in agreement with the NIP, which was more than double the amount reported by HPLC; no conclusions could be drawn from Fehling data of this BC due to the large degree of error. Sugar content of ‘Oats Banana Bake’ and ‘All Bran Fibre Toppers’ measured by CE was approximately half of that reported on label or measured by Fehling.

The low sugar content BCs determined by CE were ‘Oats Traditional’ and ‘Weet-Bix’. ‘Oats Traditional’ had the lowest sugar content measured by CE at 0.2 g/100 g. Interestingly, the NIP labeled this BC to have 1 g/100 g total sugar, however HPLC data reported no sugar at all. Also, sugar contained in ‘Oats Traditional’ was below the limit of detection for the Fehling method. The total sugar content of ‘Weet-Bix’ was consistent between the NIP, the HPLC data and the CE method in this study. At this low level of sugar, the Fehling method had very poor repeatability (RSD—85%) as one of the repeats was below LOD.

The overall error for the quantification of sugars in BCs by the CE method was much lower than that of the Fehling method, especially for the BCs with low sugar content. The error for the Fehling method is mainly caused by human technique (such as over-titration), whereas CE is automated and is more affected by operating conditions. In the case of the low sugar content BCs, the Fehling method was not sensitive enough to produce precise and accurate results. The most criticized aspect of CE in terms of operation error is related to volume variation between injections. Injections in CE are achieved by inserting a capillary into a sample solution vial and using pressure to draw sample solution into the capillary (hydrodynamic injection). Pressure variations lead to differences in injection volume and thus to relatively poor peak area precision. As previously mentioned, an internal standard was used in this work (to correct the peak area) and this eliminated this type of error and greatly improved precision as it had been observed with the CE method applied to plant fibers or fermentation monitoring. There was no available data on the precision of the NIP and HPLC measurements. However, CE was more comparable to HPLC than NIP or the Fehling method.

2.5. Estimation of reducing sugar by CE and DNS assay compared with HPLC

A reducing sugar is classified as any sugar that contains or is capable of forming an aldehyde functional group that can be oxidized to a carboxylic acid functional group. Though the largest proportion of sugar contained in BCs is sucrose, rather than reducing sugars (lactose, maltose, glucose and fructose), the quantification of these sugars is important—especially glucose (key product of digestion). The same ground samples (with fruit removed) were used to measure reducing sugars with both CE and DNS methods (Fig. 3), which may explain the significantly lower sugar content in reducing sugars in ‘Sultana Bran’ and ‘Sustain’ measured by CE and DNS compared to HPLC data. As previously discussed, individual CE results confirmed the reducing sugars quantified by HPLC were fructose and glucose (see Fig. 3B). The use of the DNS assay for the estimation of reducing sugars is a widely practiced assay and also recommended by the International Union of Pure and Applied Chemistry (IUPAC). There was no significant difference between the sugar concentrations from CE and DNS assay. The sugar concentrations were found to agree with that from CE and HPLC in some cases, but not for the most complex matrices (plant fiber). Overall, the repeatability of the CE results was better than that of the DNS data in this work. During the DNS assay, relatively harsh reaction conditions (pH 13.0, 100 °C, 5–10 min) is likely to cause starch degradation. In addition, other side reactions (especially involving minerals) may compete for the availability of the DNS reagent. The simple suspension in water used for sample preparation in CE ensure no or much more limited degradation for the use of CE on starchy food.

3. Conclusion

FSCE with direct UV detection was shown to be advantageous for measuring sugar content in breakfast cereals compared to traditional reducing sugar and glucose-specific methods for several reasons: (1) BCs can contain as many as 5 sugars; CE is able to separate and quantify all sugars in a sample compared to
4. Materials and methods

4.1. Materials

Milli Q quality (Millipore, Bedford, MA, USA) water was used throughout the analysis. Sodium hydroxide pellets (NaOH), disodium monohydrogen phosphate powder (Na₂HPO₄ stored in a desiccator), 100% pure glacial acetic acid hydrochloric acid and methylene blue (C.I. 52015) were sourced from Ajax Chemicals (Auburn, NSW, Australia). Copper (II) sulfate was purchased from Fisons (Homebush, NSW, Australia). Citric acid (anhydrous) was obtained from Chem-Supply Pty Ltd (Gillman, SA, Australia). Zinc acetate ≥99% was supplied by BDH AnalaR, Merck Pty Limited (Poole, Dorset, England). Sodium potassium tartrate 99%, sodium bisulfite, phenol, 3,5-dinitrosalicylic acid, Glucose ≥99.5% and dimethyl sulfoxide (DMSO) ≥99.5% were supplied by Sigma–Aldrich (Castle Hill, NSW, Australia). Xylose ≥99% was from Alfa Aesar (Ward Hill, MA, USA). Fused-silica capillaries (50 μm internal diameter, 360 μm outside diameter) were obtained from Polymicro (Phoenix, AZ, USA). Infinity™ glucose oxidase liquid stable reagent, pH 7.5±0.1 at 20 °C, was obtained from Thermo Scientific (TR-15221, Worthing, West Sussex, UK).

4.2. Initial sample preparation

Thirteen breakfast cereals (BC) products were purchased from a local Woolworths supermarket (Marayong, NSW, Australia). Approximately 80–150 g of each BC was milled in a K-mart, m-mini glass jug blender for 20 s, speed level 1. The ground cereal was passed through a laboratory sieve with pore size 1000 μm and retained in a 500 μm sized sieve, producing BC samples with particle size between 500 and 1000 μm. Samples were stored in a cold room at 4 °C. Basic information of the 13 BC samples, including sugar content, is shown in Table 3.

4.3. Capillary electrophoresis

For CE separations, disodium hydrogen phosphate (NP200—130 mM NaOH and 36 mM Na₂HPO₄) buffer was prepared according to Ref. 30. This buffer was prepared on the day of use, sonicated for 5 min and filtered with a Millipore membrane syringe filter (0.2 μm). A stock solution of sugars (standard) was prepared containing 1.5 g L⁻¹ sucrose and 0.5 g L⁻¹ of each maltose, glucose and fructose in water. Standard curves were obtained using an undiluted standard and standards diluted by factors of 2, 4, 8, 16, and 32. Sample solutions of 10.0 g L⁻¹ were prepared by adding 15.0 mg of ground sample (500–1000 μm particle size) to 1.500 mL of water. To each of the standards and samples, 0.5 g L⁻¹ xylose was added as an internal standard as well as DMSO (5 μL per 500 μL) to mark the electro-osmotic flow.

Separations were performed on a Beckman P/ACE MDQ capillary electrophoresis system (AB Sciex Separations, Mount Waverley, Australia) monitoring at 191 nm, 266 nm and 270 nm with a 10 nm bandwidth. A capillary with a total length of 61.8 cm (51.8 cm effective length) was used. The capillary was preconditioned before use by flushing with 1 M NaOH, 0.1 M NaOH, water and NP200 buffer for 20 min each. The cassette temperature was set to 15 °C. Samples were injected by applying 34 mbar for 4 s followed by injection of NP200 buffer in the same manner. A voltage of 16 kV was ramped up over 2 min. Between consecutive separations, the capillary was flushed with NP200 buffer for 5 min. After the final injection, the capillary was flushed with 1 M NaOH for 1 min, followed by water and then air (10 min each). Carbohydrates were monitored at 266 and 270 nm and the EOF was monitored at 191 nm by 32 Karat software. The data was processed first using either Origin 8.5.1 (‘manual’ treatment) or 32 Karat (‘automated’ treatment). Quantification of the carbohydrates was not done at 270 nm as in earlier literature but at 266 nm since it gave the optimal signal-to-noise ratio as also observed in the most recent literature. Both data treatment yielding the same results (data not shown) the latter treatment was used for all the results presented in this work. Concentration of identifiable sugars in BCs was determined from normalized peak area (relative to the internal standard) and the standard curve. Outliers were removed, where relevant, after applying a Grubb test (see Equation S-4).

4.4. DNS assay in micro plate format

Reducing sugars in BCs were quantified by the DNS assay in microplate format. The DNS reagent was prepared exactly according to Ref. 9. Sample solutions of 10.0 g L⁻¹ were prepared by adding 15 mg of ground sample (500–1000 μm particle size) to 1.500 mL of water. Triplicates of 100 μL were made up as both undiluted samples and diluted samples (with dilution factors of 1, 2, 4, 8, 16, 32). Glucose standard solutions of 0.2, 0.4, 0.6, 0.8, 1.0 g L⁻¹ were prepared in triplicates for the purpose of procuring a

<table>
<thead>
<tr>
<th>Sample name (Brand name)</th>
<th>Sugar (g/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Bran Fibre Toppers™ (Kellogg’s®)</td>
<td>19.6</td>
</tr>
<tr>
<td>Coco Pops® (Kellogg’s®)</td>
<td>36.5</td>
</tr>
<tr>
<td>Corn Flakes® (Kellogg’s®)</td>
<td>7.9</td>
</tr>
<tr>
<td>Frost Loops® (Kellogg’s®)</td>
<td>38.0</td>
</tr>
<tr>
<td>Nutri-Grain® (Kellogg’s®)</td>
<td>32.0</td>
</tr>
<tr>
<td>Rice Bubbles® (Kellogg’s®)</td>
<td>9.0</td>
</tr>
<tr>
<td>Sunflana Bran (Kellogg’s®)</td>
<td>22.7</td>
</tr>
<tr>
<td>Sustain® (Kellogg’s®)</td>
<td>20.4</td>
</tr>
<tr>
<td>Weet-Bix® (Sanitarium™)</td>
<td>3.3</td>
</tr>
<tr>
<td>Weet-Bix® Multi-grain (Sanitarium™)</td>
<td>9.9</td>
</tr>
<tr>
<td>Oats Traditional (Uncle Tobys®)</td>
<td>1.0</td>
</tr>
<tr>
<td>Oats apple &amp; blueberry bake (Uncle Tobys®)</td>
<td>25.0</td>
</tr>
<tr>
<td>Oats banana bake (Uncle Tobys®)</td>
<td>22.7</td>
</tr>
</tbody>
</table>

Table 3

Breakfast cereal samples according to total sugar content as listed on Nutrition Information Panel (NIP, or packaging label information). Sugar quantity is listed in g per 100 g of BC.
standard curve. A blank and a set of standards were included with the samples tested on each microtitre plate. The contents of the plates were then mixed on a plate mixer for 5 s, sealed and incubated at 100 °C in a water bath for 10 min. The plates were then cooled to room temperature to stop the reaction by being placed on ice. Absorbance values were measured at 640 nm on a microplate reader with the standard containing no glucose as the blank.

4.5. Fehling (Lane–Eyon) method

Estimation of total sugar content in BCs was carried out using Fehling’s solution reagents as described in Ref. 45. Fehling solution A contained 69.3 g of copper sulfate in 1 L of water. Fehling solution B contained 346 g of Rochelle salt (potassium sodium tartrate) in 1 L of water. Carrez solution 1 contained 21.9 g of zinc acetate and 3 mL of glacial acetic acid in 100 mL of water. Carrez solution 2 contained 10.8 g of potassium ferrocyanide in 100 mL of water. Each ground BC sample (5 g) was added to 100 mL water, mixed with 5 mL each of Carrez solutions 1 and 2 and made up to a total volume of 250 mL with water. The mixture was then decanted and filtered through Whatman® 540 filter paper. The clarified solution (25 mL of it) was transferred to a conical flask, mixed with 2.5 g citric acid and gently boiled on a hot plate for 5 min to ensure the inversion of any sucrose present. The sample solution was then neutralized to pH 6.5–7.5 with 1 M NaOH and made up to a total volume of 250 mL with water. This gave an approximate sample concentration in the standard glucose solution until only a faint blue color remained.

Acknowledgments

The authors acknowledge a UWS School of Science and Health Equipment Grant for the CE instrument.

Supplementary data

The supplementary data includes the details of the calculation of total sugars in Fehling method, CE electropherograms with spiked BC samples, repeatability of CE electropherograms and tables with the RSDs of the electrophoretic mobility, the comparison of LODs and LOQs with published values and the statistics applied to detect outliers. Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.carres.2015.03.008.

References and notes

7. Shannon IL. Brand name guide to sugar: sucrose content of over 1,000 common foods and beverages. Chicago, USA: Nelson-Hall; 1977.
34. Thevarajah JJ, Gaborieau M, Castignolles P. 2014;1185.
SUPPLEMENTARY DATA

Quantification of sugars in breakfast cereals using capillary electrophoresis

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Supplementary information relating to the Fehling method and then Capillary Electrophoresis is given below.

1. Calculation of total sugars in Fehling method.

The total sugar content was determined as total invert sugars \( (IS_{\text{tot}}, \text{ in g per 100 g of BC}) \) as a function of the mass of invert sugars in the standard \( (IS_{\text{stan}}, \text{ in g}) \), the dilution factor \( D_f \), the measured titre \( T \) (in mL), and the mass of the BC sample \( (m_{BC}, \text{ in g}) \) according to Equation S-1. The initial volume of the sugar solution is 100 mL.

\[
IS_{\text{tot}} = \frac{IS_{\text{stan}} \times D_f \times 100}{T \times m_{BC}} \quad \text{(Equation S-1)}
\]
2. Capillary electrophoresis.

2.1 Sample preparation and ageing

Suspensions of breakfast cereals in water were injected as 1 to 2 days old samples throughout this work. It was observed that samples aged for several months contain additional peaks (data not shown) which are assumed to be degradation products.

2.2 Electrophoretic mobility

Electrophoretic mobility ($\mu$) is calculated relative to the migration of a neutral molecule representing the electro-osmotic flow, calculated by equation S-2.

\[ \mu = \frac{l_d l_t}{V} \left( \frac{1}{t_m} - \frac{1}{t_{eof}} \right) \]  

(Equation S-2)

where $l_d$ is the capillary length to the detection window (effective length), $l_t$ is the total capillary length, $V$ is voltage, $t_m$ is migration time, $t_{eof}$ is migration time of the electro-osmotic flow (EOF) marker.

Double correction of electrophoretic mobility:

Electrophoretic mobility is then normalized using a mobility marker as shown in equation S-3.

\[ \mu_{\text{normalized}} = \frac{\mu_{\text{ref mobility marker}} \times \mu_{\text{mobility marker}}}{\mu_{\text{mobility marker}}} \]  

(Equation S-3)

where $\mu$ is the electrophoretic mobility (calculated with Equation S-2) which has already been corrected according to the EOF marker, $\mu_{\text{normalized}}$ refers to the electrophoretic mobility normalised by a reference mobility marker, $\mu_{\text{mobility marker}}$ is the electrophoretic mobility of the mobility marker, $\mu_{\text{ref mobility marker}}$ refers to the electrophoretic mobility of the reference mobility marker (taken as an average of all mobility markers for a set of injections).
Figure S-1. Mobility electropherogram of 'Weet-Bix Multigrain' (4 repeats)

2.3 Spiking

Figure S-2. CE with direct UV detection of 'Cornflakes', unspiked (blank) as well as spiked with lactose, galactose and mannose.
Figure S-3. CE with direct UV detection of 'Froot Loops', unspiked (blank) as well as spiked with maltose and fructose.

2.4 Sensitivity

Table S-1. Limit of detection (LOD) and limit of quantification (LOQ) of the CE with direct UV detection used in this work and in comparison with the literature (see page before last for reference list). LOD is defined as the concentration for which the signal-to-noise ratio (SNR) is equal to 3, LOQ as the concentration for which SNR is equal to 10.1

<table>
<thead>
<tr>
<th>Sugar</th>
<th>CE (this work)</th>
<th>CE (this work)</th>
<th>CE3</th>
<th>CE4</th>
<th>CE5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LOD (mg L⁻¹)</td>
<td>LOQ (mg L⁻¹)</td>
<td>LOD (mg L⁻¹)</td>
<td>LOQ (mg L⁻¹)</td>
<td>LOD (mg L⁻¹)</td>
</tr>
<tr>
<td>Sucrose</td>
<td>4.50</td>
<td>19.0</td>
<td>5.88</td>
<td>21.6</td>
<td>-</td>
</tr>
<tr>
<td>Lactose</td>
<td>8.21</td>
<td>20.9</td>
<td>2.38</td>
<td>19.8</td>
<td>3.6</td>
</tr>
<tr>
<td>Maltose</td>
<td>13.3</td>
<td>30.4</td>
<td>20.7</td>
<td>41.7</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>23.5</td>
<td>33.8</td>
<td>30.0</td>
<td>42.6</td>
<td>3.6</td>
</tr>
<tr>
<td>Fructose</td>
<td>14.4</td>
<td>35.9</td>
<td>15.9</td>
<td>44.1</td>
<td>-</td>
</tr>
</tbody>
</table>

* Noise level in 130 mM NaOH = 0.01 mAU.

¶ Calculated manually by calculating SNR from raw data (peak height and average baseline noise height visually read from computer screen).

† Calculated manually by automatic SNR produced by 32 Karat software.
2.5 Linearity of the calibration curves

Figure S-4. Calibration curve for sucrose with the error bars from the standard deviation (n=3)
Figure S-5. Calibration curves for lactose and fructose with the error bars from the standard deviation (n=3)

Figure S-6. Calibration curves for maltose and glucose with the error bars from the standard deviation (n=3)
2.6 Determination of outlier – Grubbs Test.

This test was used to determine outliers for CE data. The mean, $\bar{x}$, and standard deviation, $s$, were calculated of the sample with the point included. If more than one point was a possible outlier, then the furthest from the mean was taken in priority.

Hypothesis – statement of truth “selected data point comes from the same sample as all data points in set” i.e. the point, in question, is not an outlier.

Calculation of $G$, the statistic value which represents the “number of standard deviation” the point in question is from the mean, see Equation S-2. 

$$G = \left| \frac{value\ of\ outlier - \bar{x}}{s} \right|$$ (Equation S-2)

If $G >$ critical value, the hypothesis is false and the suspect point is an outlier and is omitted.

Table S-2. Critical values of $G$

<table>
<thead>
<tr>
<th>Sample size</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Critical value</td>
<td>1.15</td>
<td>1.481</td>
<td>1.715</td>
<td>1.887</td>
<td>2.02</td>
<td>2.126</td>
<td>2.215</td>
<td>2.298</td>
</tr>
</tbody>
</table>

Sample: Oats Traditional, 0.349188679, 0.195632075, 0.191679245, 0.214537736, 0.1625

$Mean = 0.222707547 \quad s = 0.073118069 \quad Result = 0.349188679$

Determine if point 0.349188679 is an outlier. $N = 5$; critical value $=1.715$

$$G = \left| \frac{0.349188679 - 0.222707547}{0.073118069} \right| = 1.730 > 1.715 \quad (critical\ value)$$

$=>$ hypothesis is false $=>$ point is NOT from the sample population $=>$ point is an outlier

New sample population (outlier discarded) 0.195632075, 0.191679245, 0.214537736, 0.1625

$Mean = 0.19109 \quad s = 0.02151 \quad Result = 0.19563$

Determine if point 0.19563 is an outlier. $N = 4$; critical value $=1.481$
\[ G = \frac{0.196 - 0.19109}{0.02151} = 1.730 > 1.715 \text{ (critical value)} \]

=> hypothesis is true => point is from the sample population => point is NOT an outlier

### 2.4 Precision

**Table S-3.** Precision of sugar concentration values obtained by CE for breakfast cereals (this work) compared to complex fermentation samples sourced from literature

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Sugar</th>
<th>( C ) (g L(^{-1} ))</th>
<th>RSD (%)</th>
<th>Sample</th>
<th>Sugar</th>
<th>( C ) (g L(^{-1} ))</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>10.9</td>
<td>14.8</td>
<td>All Bran Fibre Toppers</td>
<td>Sucrose</td>
<td>11.0</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
<td>9.24</td>
<td>3.14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fermentation sample 1</td>
<td>Glucose</td>
<td>9.56</td>
<td>11.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
<td>10</td>
<td>3.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fermentation sample 3</td>
<td>Glucose</td>
<td>7.36</td>
<td>4.77</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
<td>7.86</td>
<td>2.01</td>
<td>Sultana Bran</td>
<td>Glucose</td>
<td>6.98</td>
<td>3.9</td>
</tr>
<tr>
<td>Fermentation sample 4</td>
<td>Glucose</td>
<td>0.93</td>
<td>2.75</td>
<td>Nutri-Grain</td>
<td>Glucose</td>
<td>0.937</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
<td>7.02</td>
<td>2.38</td>
<td>All Bran Fibre Toppers</td>
<td>Glucose</td>
<td>2.81</td>
<td>2.9</td>
</tr>
<tr>
<td>Fermentation sample 5</td>
<td>Glucose</td>
<td>0.57</td>
<td>2.01</td>
<td></td>
<td>Fructose</td>
<td>3.07</td>
<td>3.0</td>
</tr>
</tbody>
</table>
Fructose | 2.13 | 8.13 | Sultana Bran | Maltose | 0.616 | 8.2
---|---|---|---|---|---|---
Glucose | BDL | BDL | Coco Pops | Glucose | 0.682 | 1.4
Fructose | 0.54 | 0.56 | 3

*Fermentation media was incubated for different lengths of time with a microbe to change the carbohydrate composition.*

^ BDL stands for “below detection limit”.

### 2.5 NP200 Buffer Decomposition – change in current over time

The plateau value of the current, $c$, for a given CE experiment, was measured and plotted against time that had lapsed since the preparation of the buffer (see Figure S-3). The initial current is noted as $c_i$ while the final current is noted as $c_f$. The evolution of the current with time was fitted with linear regression and the slope, $a$, and intercept, $b$ were determined. The following applies:

$$
\begin{align*}
c_i &= a \times 0 + b = b \quad \text{Equation S-5} \\
c_f &= 0.9 \times c_i \quad \text{Equation S-6}
\end{align*}
$$

This allows calculating the time after which the current decrease by 10 % as:

$$
x = -\frac{0.1 \times b}{a} \quad \text{Equation S-7}
$$

and the time taken to lose 15% current from initial current measurement as:

$$
x = -\frac{0.15 \times b}{a} \quad \text{Equation S-8}
$$

**Table S-4.** Results of Buffer Decomposition

<table>
<thead>
<tr>
<th>Experiment series</th>
<th>a</th>
<th>b</th>
<th>Time $x$ for 10 % current loss</th>
<th>Time $x$ for 15 % current loss</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td></td>
<td></td>
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</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>1</td>
<td>-0.0146</td>
<td>115.78</td>
<td>793</td>
<td>13.2</td>
</tr>
<tr>
<td>2</td>
<td>-0.0163</td>
<td>118.06</td>
<td>724</td>
<td>12.1</td>
</tr>
<tr>
<td>3</td>
<td>-0.0155</td>
<td>116.74</td>
<td>753</td>
<td>12.6</td>
</tr>
<tr>
<td>4</td>
<td>-0.0140</td>
<td>115.86</td>
<td>828</td>
<td>13.8</td>
</tr>
<tr>
<td>5</td>
<td>-0.0210</td>
<td>109.88</td>
<td>523</td>
<td>8.7</td>
</tr>
<tr>
<td>6</td>
<td>-0.0115</td>
<td>117.23</td>
<td>1019</td>
<td>17.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVERAGE</td>
<td></td>
<td></td>
<td>773</td>
<td>12.9</td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td></td>
<td>161</td>
<td>2.7</td>
</tr>
</tbody>
</table>

References

Figure S-7: Current against time since the buffer was prepared.
Appendix B: Grinding and sieving breakfast cereal samples

Figure 54 Grinding (A), sieving (B) of ‘Rice Bubbles’ breakfast cereal to achieve 500-1000 µm particle size (C).

Figure 55 Sieving of ‘Sultana Bran’ breakfast cereal. Note: most of the fruit pieces are not included in the collected sample in the bottom laboratory sieve (500-1000 µm particle size).
Appendix C: Dissolution of sugars with breakfast cereals

Figure 56 Suspension of ground breakfast cereal samples (500-1000 µm particle size) in MQ water.
Appendix D: Calculation of total sugars with Fehling method

The total sugar content was determined as total invert sugars ($IS_{tot}$, in g per 100 g of BC) as a function of the mass of invert sugars in the standard ($IS_{stan}$, in g), the dilution factor $D_f$, the measured titre $T$ (in mL), and the mass of the BC sample ($m_{BC}$, in g) according to Equation 17. The initial volume of the sugar solution is 100 mL.

\[
IS_{tot} = \frac{IS_{stan} \times D_f \times 100}{T \times m_{BC}}
\]  

(16)
Appendix E: Determination of outlier – Grubbs Test.

This test was used to determine outliers for CE data. The mean, $\bar{x}$, and standard deviation, $s$, were calculated of the sample with the point included. If more than one point was a possible outlier, then the furthest from the mean was taken in priority.

Hypothesis – statement of truth “selected data point comes from the same sample as all data points in set” i.e. the point, in question, is not an outlier.

Calculation of $G$, the statistic value which represents the “number of standard deviation” the point in question is from the mean:

$$G = \left| \frac{\text{value of outlier} - \bar{x}}{s} \right|$$  \hspace{1cm} (17)

If $G > \text{critical value}$, the hypothesis is false and the suspect point is an outlier and is omitted.

Table 14 Critical values of $G$

<table>
<thead>
<tr>
<th>Sample size</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Critical value</td>
<td>1.15</td>
<td>1.481</td>
<td>1.715</td>
<td>1.887</td>
<td>2.02</td>
<td>2.126</td>
<td>2.215</td>
<td>2.298</td>
</tr>
</tbody>
</table>

**Sample: Oats Traditional, 0.349188679, 0.195632075, 0.191679245, 0.214537736, 0.1625**

$Mean = 0.222707547 \quad s = 0.073118069 \quad Result = 0.349188679$

Determine if point 0.349188679 is an outlier. $N = 5$; critical value =1.715

$$G = \left| \frac{0.349188679 - 0.222707547}{0.073118069} \right| = \frac{1.361}{0.073118069} = 1.730 > 1.715 \quad \text{(critical value)}$$  \hspace{1cm} (18)

=> hypothesis is false => point is NOT from the sample population => point is an outlier

New sample population (outlier discarded) 0.195632075, 0.191679245, 0.214537736, 0.1625

$Mean = 0.19109 \quad s = 0.02151 \quad Result = 0.19563$

Determine if point 0.19563 is an outlier. $N = 4$; critical value =1.481

$$G = \left| \frac{0.19563 - 0.19109}{0.02151} \right| = \frac{0.454}{0.02151} = 1.730 > 1.715 \quad \text{(critical value)}$$  \hspace{1cm} (19)

=> hypothesis is true => point is from the sample population => point is NOT an outlier
Appendix F: Linearity of the calibration curves

Figure 57 Calibration curve for sucrose with the error bars from the standard deviation (n=3)

Figure 58 Calibration curves for lactose and fructose with the error bars from the standard deviation (n=3)
Figure 59 Calibration curves for maltose and glucose with the error bars from the standard deviation (n=3)
Appendix G: DPI (Yanco) method for measuring \textit{GT} of rice

- Differential Scanning Calorimeter – Mettler Toledo model 822e
- STARRe Software 12.00
- Weigh and record 5-8 mg (aim for 6 mg) of rice flour into the pan. \textit{To see more obvious curve, simply weigh bigger amount of rice flour (6-10 mg)}
- Add 10 \( \mu \)L of R.O. water and seal crucible with lid convex side up using the supplied crimper, twice.
## Appendix H: Rapid *in vitro* digestibility assay

### Materials
Water was of MilliQ quality (Millipore, Bedford, MA, USA). Sodium hydroxide pellets (NaOH), glacial acetic acid, calcium chloride dehydrate ≥ 98 %, magnesium chloride ≥ 99 %, sodium acetate ≥ 99 %, potassium chloride ≥ 99 % and sodium hydrogen carbonate were obtained from Univar (Auburn; Ingleburn, NSW, AUS). Hydrochloric acid was sourced from Ajax Chemicals (Auburn, NSW, AUS). D+glucose ≥ 99.5 % was supplied by Sigma-Aldrich (Castle Hill, NSW, AUS). Xylose ≥ 99 % was from Alfa Asear (Ward Hill, MA, USA). A summary of enzymes used for in vitro digestibility is listed in table A-1.

### Table 15 Summary of enzymes used for in vitro digestibility assay by declared activity, optimum conditions and source

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Commercial brand name/ number</th>
<th>Declared Activity</th>
<th>Conditions for optimum activity</th>
<th>Company source</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Amylase (from porcine pancreas)</td>
<td>A-3176</td>
<td>13.4 units amylose/mg solid at pH 6.9</td>
<td>20 °C at pH 6.9</td>
<td>Sigma Chemical Company (St. Louis, MO, USA)</td>
</tr>
<tr>
<td>Pepsin (from gastric porcine mucosa)</td>
<td>P-7000</td>
<td>871 units of activity per mg protein</td>
<td>37 °C at pH 1.5-2.5</td>
<td>Sigma-Aldrich (Castle Hill, NSW, AUS)</td>
</tr>
<tr>
<td>Pancreatin (from porcine pancreas)</td>
<td>P-1625</td>
<td>3 x USP</td>
<td>40 °C at pH 7.5</td>
<td>Sigma Chemical Company (St. Louis, MO, USA)</td>
</tr>
<tr>
<td>Amyloglucosidase (from Aspergillus niger)</td>
<td>AMG® 300 L Brew Q</td>
<td>300 AGU/mL</td>
<td>60 °C at pH 4.5</td>
<td>Novozymes (Bagsvaerd, Denmark)</td>
</tr>
</tbody>
</table>

1Information obtained from company application notes (Novozymes)
Table 16 Preparation of reagent solutions used in for rapid in vitro digestibility assay

<table>
<thead>
<tr>
<th>Reagent solution*</th>
<th>Concentration</th>
<th>Composition for a batch of 10 bottles (according to activity listed on label)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artificial saliva</td>
<td>250 units of α-amylase (porcine pancreas) per mL carbonate buffer (1)</td>
<td>300 mg α-amylase, 20 mL carbonate buffer*</td>
</tr>
<tr>
<td>Acidified Pepsin</td>
<td>1 mg pepsin (from gastric porcine mucosa) per mL of 0.02 M HCl, pH 2.0</td>
<td>55 mg pepsin, 55 mg 0.02 M HCl</td>
</tr>
<tr>
<td>Pancreatin/amyloglucosidase (AMG) enzyme mixture</td>
<td>2 mg of pancreatin (from porcine pancreas) and 28 units of AMG per mL of sodium acetate buffer (2)</td>
<td>110 mg pancreatin, 5.1 AMG, 55 mL sodium carbonate buffer*</td>
</tr>
</tbody>
</table>

*As specified in the CSIRO standard operation procedure 195-5[257]

Buffer preparation

Carbonate buffer: A 100 mL carbonate buffer (pH 7.0) was prepared from 14.4 mM sodium hydrogen carbonate, 21.1 mM potassium chloride and 0.2 mM magnesium chloride dissolved in MilliQ water. This buffer was stored at 4 °C and kept for up to five days.

Sodium acetate buffer: Sodium acetate buffer at 0.2 M (pH 6.0) was made by dissolving 11.8 mL of 100 % pure glacial acetic acid in 900 mL MilliQ water. The pH of the solution was adjusted to 6.0 with 2 M NaOH, followed by 4 mL of 1 M calcium chloride and 100 µL of 4.9 M magnesium chloride. This buffer was made up to 1 L with water and stored at 4 °C.

Methodology

This assay was conducted as a three-stage enzymatic hydrolysis under physiological conditions of digestion in the mouth, stomach and small intestine. In the first stage, 0.5 g of ground BC samples (500 - 1000 µm particle size) was mixed with an equal amount artificial saliva solution (see Table 16 for preparation of artificial saliva) in a 250 mL Schott Duran® bottle. After 15 - 20 s, to mimic human mastication before swallowing, stage 2 of the
assay proceeded as 5 mL of acidified pepsin (see Table 16 for preparation of acidified pepsin) was added. The bottles were then incubated in a Julabo SW22 shaking water bath (Julabo GmbH, Seelbach, Germany) at 37 °C and 85 oscillations per min (OPM) for 30 min. At the third stage of digestion, following incubation, 5 mL of 0.02 M NaOH was added to neutralise the samples. 25 mL of 0.2 M sodium acetate buffer, pH 6, and 5 mL of pancreatin/ Amyloglucosidase (AMG) enzyme mixture (see Table 16 for preparation of pancreatin/ AMG enzyme mixture) was added. Starch digestion and α-amylase activity is highest at the third stage (simulation of small intestine). For this reason, the glucose reading taken at 0 min was immediately after the enzyme mixture was added. Each bottle was then loosely capped and incubated at 37°C in a shaking water bath set at 85 OPM.
Appendix I: Glucometry

Materials
D+glucose ≥ 99.5 % was supplied by Sigma-Aldrich (Castle Hill, NSW, AUS).

Methodology
The glucose concentration of all BC samples was measured before and in real-time (at specific periods) during the in vitro digestibility assay with a glucometer. The Accu-Chek® Active glucometer (Roche Diagnostics Australia Pty. Ltd., Castle Hill, NSW) uses a photometric test strip whereby the measurement method relies upon conversion of reflected light to a current. The test strip contains the enzyme glucose dehydrogenase (GHD) which oxidises glucose to gluconolactone. Glucose is oxidised by the mediator system quinoneimine / phenylenediamine, which then reduces an indicator (phosphomolybdic acid) for colour development. The coenzyme involved in this reaction was pyrrolo quinolone quinone (PQQ). Storage temperature recommendation for the strips is 2-30 °C. The strips are heat and light sensitive and during use temperature should be kept at 10-40 °C. Sample amount required for the meter was 1-2 μL and glucose concentration results (mg·dL⁻¹) were produced in 5 s. The measuring range of the Accu-Chek® Active is 10-600 mg·dL⁻¹ (0.6 - 33.3 mmol·L⁻¹).
Appendix J: GOD-POD method (Trinder)

Materials

d-glucose ≥ 99.5 % was supplied by Sigma-Aldrich (Castle Hill, NSW, AUS). Infinity™ glucose oxidase liquid stable reagent, pH 7.5 ± 0.10 at 20 °C, was obtained from Thermo Scientific (TR-15221, Worthing, West Sussex, UK).

Methodology

The quantitative determination of glucose by the GOD-POD method was performed using Infinity™ glucose oxidase liquid stable reagent (see Table 17 for glucose oxidase reagent composition). A 1.0 g·L⁻¹ stock glucose solution, made with 100 mg of dry glucose and 100 mL water, was diluted to prepare the working glucose standards (see Table 18). Sample solutions were obtained from the in vitro digestibility assay and clarified by centrifugation. From standard glucose solutions and digested samples, a 50 µL of aliquot was transferred into test tubes followed by 1 mL of glucose oxidase reagent. All tubes were kept at 25 °C, out of direct sunlight, for 20-30 min and measured at 505 nm in UV-visible spectrophotometer (Shimadzu, UV-1601PC).

Table 17 Composition of Infinity™ glucose oxidase liquid stable reagent*

<table>
<thead>
<tr>
<th>Active Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose oxidase</td>
<td>&gt; 15 000 U / L</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>&gt; 100 U / L</td>
</tr>
<tr>
<td>4-aminoantipyrine</td>
<td>0.5 mmol / L</td>
</tr>
<tr>
<td>4-hydroxybenzoic acid</td>
<td>10 mmol / L</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>119 mmol / L</td>
</tr>
</tbody>
</table>

*Also contains non-reactive fillers and stabilizers.
Table 18 Glucose working standards for GOD-POD assay

<table>
<thead>
<tr>
<th>Standard #</th>
<th>Stock solution (µL)</th>
<th>Water (µL)</th>
<th>Working standard concentration (µL)</th>
<th>Volume taken (µL)</th>
<th>Assay amount (µg glucose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>25</td>
<td>75</td>
<td>0.25</td>
<td>50</td>
<td>12.5</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>25</td>
<td>0.5</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>75</td>
<td>25</td>
<td>0.75</td>
<td>50</td>
<td>37.5</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>0</td>
<td>1</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>
Appendix K: Digestograms of starch measured by glucometry

Figure 60 Digestograms of starch-whey mixtures\textsuperscript{153}
Figure 61 Digestograms of barley and sorghum showing differences in grain genotype.\textsuperscript{[153]}