Cognitive Performance following different Postprandial Glycaemic Responses

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Abstract

Background: Optimal cognitive performance is important for learning. The brain uses glucose for fuel, so one proposed factor influencing cognition is circulating blood glucose concentrations. However, the existing body of literature on this topic is equivocal; possibly because foods and beverages used to generate differences in glycaemia, also differ in other attributes, such as fibre or macronutrient content. Some of these uncontrolled factors are found to influence glycaemia and cognition; therefore, they do not allow for any effects to be attributed solely to differences in glycaemia. More work is needed to control for confounding to clarify the relationship between cognitive performance and circulating blood glucose concentrations.

Objective: To investigate healthy adults' cognitive performance with different glycaemic responses induced by sucrose and isomaltulose: two disaccharides with identical monosaccharides, but different glycosidic bonds, resulting in differences in the rates of digestion and absorption between sugars.

Design: Randomised, double blinded, crossover, controlled trial.

Methods: To examine the cognitive effects of different glycaemic responses, a sucrose beverage was compared to an isomaltulose beverage. To match sweetness of the test beverages 0.035g of sucralose was added to the isomaltulose beverages. Healthy human nutrition students (n=70; mean age 21.9 years; mean body mass index 23.3 kg/m^2) received the lower glycaemic index (GI) isomaltulose + sucralose test beverage (ISO) and higher GI sucrose test beverage (SUC) on separate occasions. Following beverage consumption cognitive performance was examined at 30, 80, 130, and 140 minutes. Participants viewed a film in 30-minute time slots, then answered 10

questions about the film, and underwent a 25-word recall test. At 140 minutes participants completed the Reitan's Trail Making Part B test. The tests were designed to measure declarative memory (film recall); immediate recall (word recall) and executive function (Reitan's Trail Making Part B). To determine the glycaemic response of the test beverages, a subsample (n=12) from the cognitive testing population (n=70) underwent glycaemic response testing on separate days to the cognitive test days. The glycaemic response test protocol used the same beverages and timing protocol as the cognitive testing days.

Results: Between test beverages a significantly lower (mean difference (95% confidence interval) (CI) blood glucose iAUC concentration was found -44 (-70, -18) mmol/L·min (p=0.003 for ISO compared to SUC). There was no significant difference between test beverages in the sum of correct answers to the film recall (declarative memory) at 30 min 0.1 (-0.2, 0.5), 80 min -0.3 (-0.8, 0.2), and 130 min 0.0 (-0.5, 0.5). Nor were any significant differences found in the amount of words recalled in the word recall tests (immediate recall) at 30 min -0.5 (-1.4, 0.3), 80 min 0.4 (-0.4, 1.3), and 130 min -0.4(-1.1, 0.4). At 140 minutes the times taken to complete the Reitan's Trail Making Part B (executive function) was also not found to be significantly different -0.3(-6.9, 6.3).

Conclusion: Declarative memory, immediate free recall, and executive functions were not significantly different between different postprandial glycaemic responses. Our findings do not support the theory that cognitive test outcomes examined in this study will vary with different glycaemic responses.

Keywords: Glyc(a)emic response, Cognitive function, Isomaltulose (Palatinose), Sucrose, Executive functions, Memory, Glycaemic Index.

Preface

This study was conducted by Celeste Keesing (MDiet candidate) under the supervision of Dr Bernard Venn, Dr Charlene Rapsey and Dr Tracy Perry, as part of the Masters of Dietetics degree.

Dr Bernard Venn was responsible for the study design, topic, applying for ethical approval, recruitment, supervising the test days and thesis write-up. Dr Charlene Raspey was responsible for guiding the choice of cognitive tests. Dr Tracy Perry was responsible for overseeing study and thesis write up.

This study was part of a larger trial to also examine satiety with different glycaemic response test beverages. Brianna Mills (MDiet candidate) conducted the satiety part of this trial. Together we conducted preparatory screening, developing the test beverages, taste testing, making the test beverages for all test days, creating demographic, anthropometric, intake, and exercise questionnaires.

Celeste Keesing was responsible for:

- Creating a literature review out of academic literature on glycaemic response and cognitive function
- Input into the study design and preparatory procedures; timing, test day lunches, cognitive selection and testing
- Choosing and designing cognitive tests; created the word recall tests and film questions, ensured cognition tests were equal in cognitive measures between test days, chose time slots for cognitive testing to be conducted
- Created the test beverages; sought out the ingredients, equipment sourcing, ingredient and nutrient calculations, weighing ingredients
- Recruited participants for taste testing

- Anthropometric measurements of participants
- Setting up and conducting all test days
- Taking blood samples from participants on both glycaemic response test days
- Conducting the cognitive tests
- Communicating with test day facilitators over IT, blood collection and analysis, equipment and cognitive testing
- Data collection, scoring and entry
- Input into biostatistician's statistical analysis
- Interpreting trial results
- Writing this thesis

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List of Abbreviations

AUC Area under the curve

BMI Body mass index

CI Confidence interval

GI Glycaemic index

GL Glycaemic load

g grams

iAUC Incremental area under the curve

ISO Isomaltulose + sucralose test beverage

IU International units

Min Minutes

ml millilitres

mmol/L millimoles/Litre

NZ New Zealand

RCT Randomised control trial

SUC Sucrose test beverage

1. Introduction

Cognitive performance is a key factor to consider in learning (1). Adequate sleep, exercise and a healthy diet are all beneficial for cognitive function (2, 3). A significant aspect of this is examining whether cognitive function is responsive to glycaemic changes; and whether certain cognitive domains are more sensitive than others to different glycaemias. The theory that different glycaemic responses may vary in their effect on cognitive function in healthy individuals has been investigated. However, it remains unresolved, potentially due to heterogeneity and lack of consistent control among study designs, which may have led to the inconsistencies in findings.

The theory that different glycaemic responses vary in effect on cognitive function, arises from the knowledge that the brain uses glucose as its preferred and primary source of fuel in a non-prolonged fasted state (4-6). Furthermore, cognitive function decreases when healthy individuals are put in a hypoglycaemic state (7-10). Although hypoglycaemia is not common in healthy individuals, when individuals have glucose concentrations normal for an overnight fast, glucose consumption has been found to improve cognition compared to a placebo (10-13).

Carbohydrates that are rapidly absorbed, such as glucose, initially induce a rapid rise in blood glucose concentration followed by an undershoot at 150 minutes; in which blood glucose concentrations can fall below the baseline value (**Figure 2.1**.)(10, 14-16). It has been suggested that an undershoot may lead the brain's glucose supply to drop below optimal functioning concentrations (10, 17). Alternatively, lower glycaemic response carbohydrates produce a more gradual increase in blood glucose, peaking at a lower concentration compared to higher glycaemic response, and do not undershoot

below baseline after a prolonged period (**Figure 2.1.**) (18). It has been hypothesized, therefore that cognitive function would be improved with a higher glycaemic response carbohydrate in the first 30 minutes; whereas a lower glycaemic response carbohydrate would improve cognitive function at 150 minutes.

This theory is supported in some studies that have found differences in cognition with different glycaemic index (GI), glycaemic load (GL) or glycaemic response inducing foods or beverages (10, 19-28). However, in many of these studies, confounding factors such as macro and micro nutrient content (29), energy (24) , or palatability (25) was not controlled, and lack of blinding (22) may have introduced bias; additionally, the glycaemic response of the test beverage or food was not tested in some studies (20). These confounding factors and biases have been found to affect glycaemic response and/or cognitive function (30-33). Thus, until new studies control for confounding variables, and glycaemic response is tested, a limited number of studies can convincingly attribute different glycaemic responses to variations in cognitive function; leaving the relationship undetermined (20, 34).

The aim of this thesis is to determine whether the effect of different postprandial glycaemic responses (low versus medium GI beverages) alter cognitive function in healthy adults; with a study design that tests glycaemic response at times of cognitive testing, and minimises for bias and nutrition variables.

2. Literature Review

2.1 Objectives and methods

This literature review was conducted to gather evidence on the effect of postprandial glycaemic response on cognitive function.

The aims of this literature review:

1. Overview glycaemic response, measurements of glycaemic response, isomaltulose and sucrose composition, carbohydrate digestion and cellular uptake.
2. Discuss literature on the effect of postprandial glycaemia and carbohydrates on the brain and cognition.
3. Identify areas where further research is needed on the effect of postprandial glycaemia on cognitive function.

The literature examined was gathered from the data bases; Medline via Ovid, Scopus and Pubmed. Key words searched included; Glyc(a)emic response, Cognitive function, Isomaltulose (Palatinose), Sucrose, Executive functions, Memory, Glycaemic Index. Only studies written in English were included in this review.

2.2 Glycaemic response

Glycaemic response is defined as the change in plasma glucose concentration over time in response to carbohydrate consumption (35-38). Different carbohydrates induce different glycaemic responses. Research has revealed glycaemic response has associated effects both beneficial and detrimental, on cognitive function and disease risks (8, 9, 39-45). Attempts have been made to observe cognitive performance in

healthy individuals when controlling glycaemic response through diet. The literature investigating this topic has been undertaken in a variety of ways highlighted below.

2.3 Measurement of glycaemic response

Following carbohydrate consumption in healthy individuals, absorption of digested carbohydrates leads to plasma glucose level rising within 10 minutes, peaking at 30 minutes and returning to baseline by 120 minutes (10, 38, 46). Glycaemic response is measured through capillary or venous blood samples, or subcutaneously via continuous glucose monitoring systems (36, 47). Blood glucose samples are collected at specific intervals that are plotted over time, creating an area under the curve (AUC) (10, 48). Postprandial glycaemic response is often characterized by assessing the increments in glucose concentration above the baseline value, referred to as the incremental AUC (iAUC). The area can be calculated by various techniques; however, the trapezoid method is commonly used (36, 49, 50).

Different carbohydrates induce different glycaemic responses due to rate of absorption and insulinogenic effect (38, 51). Jenkins and colleagues developed a categorical ranking system to identify carbohydrates based on the glycaemic response they induced, called glycaemic index (GI) (52). Low (<55) and medium (55–69) GI foods create a respective low to medium glycaemic response and high GI foods (>70) generate a higher glycaemic response than lower GI foods (37). This GI classification method was created by 10 individual's average plasma glucose response to 50 g of a carbohydrate sample compared to 50 g of a reference carbohydrate (37, 48).

However, the amount of carbohydrate consumed influences the elevation in blood glucose, therefore applying GI to predict glycaemic response may be insufficient (37, 48, 53, 54). For this reason, glycaemic load (GL) is used to account for the amount

of carbohydrate. GL is the mathematical product of the GI of the meal or beverage and the available amount of carbohydrate (GL=GI x available carbohydrate) (37).

Measuring an individual's GL or GI to predict any glycaemic response outcome in the real-world setting is questionable. The GL and GI of carbohydrates are confirmed by measuring the carbohydrate concentration in isolation, despite real-world meals and beverages containing other nutrients. Certain fibres present in food can slow down digestion and absorption (32, 55), co-ingested fat slows gastric emptying (56), heating and cooling of certain carbohydrates slows down absorption (57), caffeine acutely decreases glucose sensitivity (58, 59) and protein can exaggerate the insulinogenic response, thereby increasing enhanced glucose removal from circulation by cellular glucose uptake (35, 60, 61).

There are numerous inter and intra-individual variations that can impact postprandial glycaemic response (61-65). The extent of mastication (resulting in different particle sizes of carbohydrate) (61, 64, 66), salivary amylase activity (61, 67), ethnicity (61, 62), age (68), time of day (61, 69), amount of sleep (61, 70, 71) and glucose tolerance (e.g normal, pre-diabetic, diabetic, or stress response state) will affect glycaemic response (57, 61, 72). It is important to consider these confounding factors especially in relation to the reliability of findings associated with cognitive function with different GL or GI (73-76).

2.4 Digestion and cellular uptake of glucose

Two major determinants of glycaemic response are, the carbohydrate molecule size, and bonds that connect the monosaccharides together (37, 61, 66, 77). Starch molecules consist of many monosaccharides, sugar molecules consist of one or two monosaccharides. Carbohydrates consisting of more than one sugar molecule are joined

together by glycosidic bonds (78). These bonds are broken down via hydrolysis into monosaccharides by specific enzymes (78). The shape of the molecule, the type of bonds, and the type and availability of hydrolysis enzymes will impact the rate of digestion; therefore, influencing glycaemic response (77-80).

Low glycaemic response carbohydrates cause less extreme changes in blood glucose than a high glycaemic response carbohydrate (**Figure 2.1**)(18, 61). Rapidly absorbed carbohydrates (high GI) can induce higher blood glucose and insulin blood concentrations (51). Carbohydrates can stimulate insulin release before or quickly once absorption has started. This higher concentration of insulin leads to rapid cellular glucose uptake (51). However, once blood glucose has been taken up by cells and fallen back to baseline, insulin levels slightly delay in returning to homeostatic concentrations for the current blood glucose concentrations. High insulin concentrations inhibit glucagons' action in glycogenolysis and gluconeogenesis, leading to blood glucose falling below baseline blood glucose concentrations, this is called an undershoot, which causes a potential for hypoglycaemia (<2.8 mmol/L) (**Figure 2.1**)(14-16, 18, 61).

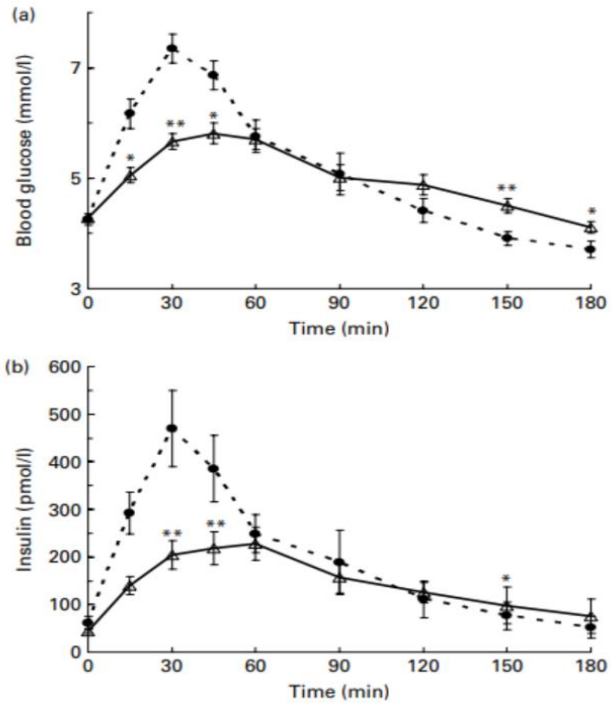

Figure 2.1 Blood glucose and insulin response to 50g of isomaltulose and sucrose in healthy volunteers. (a) Blood glucose response to 50 grams of isomaltulose (Δ) and sucrose (●) over 3 hours. (b) Insulin profiles of 50g of isomaltulose and sucrose over 3h. Mean values were significantly different: *P<0.05, **P<0.01 by Wilcoxon test for paired data (18).

2.5 Carbohydrates and the brain

Glucose is the main and preferred energy source of the brain (81, 82). The brain has multiple mechanisms to keep available energy sources within homeostatic ranges (6, 61, 83-85). Along with glucose, insulin, cortisol, lactate and glycogen have been found to influence energy availability in the brain; their effect may affect cognitive function (61, 83, 84, 86, 87).

For glucose to reach the brain it must pass through the blood brain barrier via facilitated diffusion. A glucose concentration gradient of 20% difference between brain tissue and arterial plasma is created by tight junctions, glucose transporter proteins (GLUT)1,3,4,5 and 8 and sodium glucose transporters (SGLT)1 and 2 in the blood brain

barrier, to facilitate blood glucose diffusion (82, 88). The main transporters are GLUT's and to a lesser extent SGLT's (82, 88).

Under normal circumstances this concentration gradient of 20% drives glucose across the blood brain barrier via mainly the GLUT1 route. This facilitative transport protein is at a half- saturation constant at 8mmol/L of glucose (4, 82, 89, 90). In a non-prolonged fasted state peripheral blood glucose sits between 3.9-5.5 mmol/L, and at 120 minutes post fed state at around 7.8 mmol/L (91-93). Thus, the blood brain barrier GLUT1 is barely reaching the half – saturation constant of 8 mmol/L even in the fed state. Therefore, this half saturation point of 8mmol/L indicates that even when peripheral blood glucose concentrations are high, due to being in a fed state, the brain is still not reaching its maximal glucose uptake from the periphery. This raises the question as to whether the maximum saturation point of GLUT1 is an indicator that the brain could use higher glucose concentrations for energy or glucose storage (86)?

Once glucose has entered the brain, the energy is extracted through glycolysis and oxidation with generation of adenosine triphosphate (ATP) (6, 61). Local brain tissue glucose utilization rates are defined by spikes in energy use upon engagement in demanding cognitive tasks (4, 61). Brain cells rapidly take up glucose via various GLUT and SGLT's (82, 94).

When brain glucose availability is lower, the brain has multiple homeostatic regulators for energy availability in extreme glycaemic states (6, 61, 83, 84, 86). However, it is unknown whether this can be applicable to healthy individuals in acute states where blood glucose has overshot below baseline. Some evidence suggests that a human in a hypoglycaemic state has neuronal demand for ATP buffered by the metabolic products lactate and glycogen stores in the brain (61, 83, 84, 86, 95, 96). However, lactate has a lower yield of ATP compared to glucose, and the glycogen stores

in the human brain are metabolized at a very slow rate (61, 83); thus the brain's acute energy availability may still vary.

It takes 20-30 minutes for brain glucose concentrations to decrease from normo-glycaemia to a stable hypoglycaemic state, following initiation of peripheral hypoglycaemia (<2.8 mmol/L) (61, 84). This indicates that the brain has a delayed response of 20-30 minutes through buffering, to varied peripheral blood glucose levels. In theory if given a high glycaemic response carbohydrate, peripheral blood glucose would peak at 30 minutes, fall back to baseline at 120 minutes, and undershoot at 150 minutes (**Figure 2.1**). Brain blood glucose would follow this with a 20-minute delay; thus, it would return to baseline at around 140 minutes. If blood glucose overshot below baseline after 120-minutes, which can occur with a high glycaemic carbohydrate, brain glucose concentrations may fall below baseline at 170 minutes. The brain may become depleted in energy at this point, this may be detrimental to cognitive function.

Insulin, a vital part of peripheral glucose metabolism is questioned as to whether it has the same role in the brain. Insulin concentrations measured in human brains indicate that this hormone can come from the periphery (61, 97, 98). Insulin receptors and transporters found in rat blood brain barriers are partially saturated with insulin at 10ng/ml which is within insulin concentrations found in human euglycaemic conditions (61, 99, 100). This is theorized to prevent hypoglycaemia in the mammal brain, as insulin cannot reach concentrations in the brain that could reduce blood glucose to hypoglycaemic concentrations (61, 100).

Insulin receptors and glucose transporters have been found in areas of human brain, including insulin sensitive GLUT4 (61, 98, 101-103). Some small human studies that induced states of hypoglycaemia to euglycaemia have found insulin to be associated with the increased brain cell glucose uptake in the prefrontal cortex and

ventral stratum (61, 104-106). These brain areas have a significant role in cognitive function (1). Whether the increase in glucose metabolism is because insulin stimulates glucose uptake directly, or indirectly via stimulation of neurotransmitter release, is not yet clarified.

Insulin increases norepinephrine concentrations (neurotransmitter involved in cognitive processes) in the brain influencing cognitive function, independent of glucose concentration (85, 107-110). Insulin improves attention and memory in humans (85, 110). The consumption of a food or beverage that raises peripheral blood glucose, and thus insulin levels from a hypoglycaemic state to a fed state, and vice versa, may also lead to varied insulin concentrations and glucose metabolism in the human brain (85). This raises the question, does the varied insulin concentrations from different glycaemic foods or beverages independently impact cognitive function?

Acutely higher cortisol concentrations reduce hippocampal (area involved in memory) glucose metabolism, impair memory retrieval, but improve emotional memory and attention (110-114). A randomised crossover study, found higher circulating cortisol concentrations in n=74 children who consumed a high GI food compared to a lower GI food (26). However, although high GI raised cortisol, it cannot be attributed to the glycaemic response, as there was no significant difference in glycaemic response between the high and low GI test meals (26). These results highlight a lack of knowledge around the physiological effects of different GI foods.

Another factor found to influence cognition is the sweet taste; for example sucrose and sucralose (115, 116). However, different sweeteners vary in the brain areas that they activate. Sucrose compared to low-calorie sweeteners, has a greater effect on reward centres in the brain (117-119). Activation of this mid brain dopaminergic centre is associated with enhanced memory formation (118). High glycaemic foods and

beverages are often sweeter than low glycaemic foods or beverages, due to sucrose being a common ingredient. This indicates that masking a low glycaemic carbohydrate, such as isomaltulose's less sweet taste with artificial sweetener to match the sweetness of sucrose, may produce a different brain response. However, due to limited research, it is unknown whether different sweeteners with identical sweetness taste, do have an effect on cognition.

Carbohydrate's effect on cognition is poorly understood; mainly due to the brain's inaccessibility, requiring indirect methods to understand its processes. However, cognitive function is found to be affected by fluctuating glucose concentrations, taste, and glycaemic related hormones. Thus, cognitive performance being influence by different postprandial glycaemic responses, appears possible; therefore, this topic is worth investigating further.

2.6 Carbohydrates and cognition

An individual's cognitive performance should be theoretically poorer if the areas of the brain required to perform a cognitive task have insufficient energy provision (9). This raises the question as to how higher glycaemic response carbohydrate's acute cognitive effects compare to the cognitive effects of lower glycaemic response carbohydrates?

Although healthy individuals do not usually reach a hypoglycaemic state; theoretically, in a fasted state cognitive performance would acutely improve with a high glycaemic response carbohydrate compared to a low-calorie sweetener (13). Ginieis et al. examined n=49 participants' cognitive performance between glucose, sucrose, fructose and non-calorie sweetener following an overnight fast; conflicting results were reported (120). Although the beverages varied in energy content, cognitive performance

at 20 minutes was poorer for those who fasted and consumed sucrose or glucose compared to low glycaemic response fructose, or a low-calorie sweetener (p=<0.001) (120). This indicates that a rapidly available energy source for the brain is not always a predictor of improved cognitive performance. It also identifies the need for improved understanding of not just glycaemic response, but also the other effects carbohydrates have on cognitive performance.

2.6.1 The effect of GI and GL on cognitive performance in children and adolescents

In studies examining healthy children and adolescents' cognitive performance with different glycaemic foods or beverages, findings vary (10, 22, 25, 27, 28, 121) (**Table 2.1**). Low compared with a high GI or GL test beverage or food improved executive functions (working memory and attention) at 120-minutes (22, 25); secondary memory at 0 and 120-minutes (22); declarative memory at 100-minutes (26), 120 and 180-minutes (25); reaction times at 120-minutes (28) and immediate recall at 120 and 180-minutes (23).

In contrary, other studies found a high GI compared to a low GI meal was found to improve executive functions (26) and delayed recall at 100-minutes (27) in children and adolescents. Additionally, some studies found executive functions and immediate recall was not affected by different GI or GL foods and beverages (22, 26-28).

In summary, research of low versus high predicted glycaemic response food and beverages on cognitive performance have produced inconsistent results. Declarative memory (episodic and semantic) was consistently improved in children and adolescents (25, 26). However, declarative memory has only been examined by two studies, whereas executive functions have been examined by seven studies. More studies need

to examine the specific cognitive domains; declarative memory, immediate recall and executive function before conclusions can be made.

2.6.2 The effect of GI and GL on cognitive performance in adults

There are less studies examining healthy adults and findings are inconsistent (**Table 2.2**). Low compared to high GI or GL test foods or beverages; improved declarative memory at 30, 105, 195-minutes (20), executive functions at 90-minutes (21), immediate recall at 120 and 150-minutes (24, 29) and delayed recall at 150 and 210 minutes (24). In contrary, further studies evaluating the effect of low glycaemic foods or beverages on these cognitive performance domains failed to demonstrate statistically significant results (14, 20, 29). This leaves effect of GI and GL on cognitive performance in adults inconclusive.

2.6.3 The effect of glycaemic response on cognitive performance in adults, adolescents and children

Test foods or beverages that are classified by their GL or GI, can deviate from their predicted glycaemic response value (27, 28). This may be due to non-carbohydrate nutrients in the test food or beverage, not accounted for in the GI or GL classification. Glycaemic response is influenced by non-carbohydrate nutrients; for example, fat and fibre can slow down absorption of carbohydrates (56). Certain studies attributing GI or GL to cognitive differences did not test glycaemic response, or glycaemic response was not significantly different at cognitive test times between groups (20, 21, 23). By attributing cognitive performance outcomes to test foods and beverages conveyed in their classified GI categories, it suggests that their findings are due to what the classification recognises: glycaemic response (20, 24, 26-29). It is insufficient to attribute glycaemic response to cognitive performance outcomes in those studies

findings that did not test or find significant differences in glycaemic response at cognitive test times.

Of the nine studies that tested glycaemic response (10, 14, 21, 24, 26-29, 122); seven found significant differences in glycaemic response between test foods or beverages (10, 14, 21, 24, 26, 29, 122). Of this group only three found significant differences in cognitive function when glycaemic response was significantly different (14, 21, 122). A lower glycaemic response meal improved some executive functions at 120 minutes in adolescents ($p=0.013$) (122), and 170 minutes in adults ($p=<0.017$) (21). Yet a study conducted on adults found a high glycaemic response beverage improved executive functions at 35-minutes when baseline performance was lower ($p=0.0034$) (14). Despite these findings, some executive functions are found to not be significantly different with different postprandial glycaemic responses (10, 21, 29). In conclusion, a limited number of studies can attribute significant differences in postprandial glycaemic response to significant differences in cognitive performance. In most circumstances studies have contradicted these results which leaves this topic inconclusive.

2.6.4 Possible confounders influencing results

The differences in cognitive findings between and within studies, may be due to differences in test foods' or beverages' energy, macronutrients and micronutrients (22, 24, 25, 27). The nutrients and energy that varies between tests foods may affect cognitive performance (30, 31, 123). Higher protein is found to improve short-term memory (123), and greater accuracy is associated with higher dietary fibre (30). Certain studies have compared foods with varied nutrient composition; Coco Pops have been compared with All-Bran (22), low-fat yoghurt and walnuts have been compared with white bread and jam (29). Thus, the test foods used in some studies do not define

whether the benefits found with lower glycaemic foods and beverages are a result of glycaemic response or/and other nutritional components (63, 76, 124).

Only two studies found significant differences in cognitive function with test beverages that were controlled for nutrient variation, and created significant differences in glycaemic response at cognitive testing times. A small (n=24) cross-over designed study by Dye et al. on adult males, found executive functions better at 35-minutes with a higher glycaemic response milk beverage, when baseline cognitive performance was lower (p=0.0034) (14). Whereas, the larger (n=40), cross-over study by Nilsson et al. on healthy adults, found some executive functions at 170-minutes significantly better (p=0.017) with a lower glycaemic response beverage (21). However, some executive functions (21) and immediate recall (14) were found to not be significantly different with different glycaemic responses.

Cognitive findings may also have been subject to the effect of between group baseline cognitive function. Several studies compared cognitive function between individuals by using a parallel design (20, 24, 27). These studies did not have a large sample size, such as in Smith et al. (n=38) (27). Therefore, confounding due to differences in participant's baseline cognition between groups may have influenced results. A crossover design compares cognitive function within individuals to minimize this confounding.

Blinding is another factor that may impact cognitive results. This is a key factor to quality research, as cognitive tests are subjective and may be influenced by investigators' and/or participants' opinions about one test food or beverage (21). The study mentioned previously by Nilsson et al., that found a lower post prandial glycaemic response improved cognitive function in adults; imitated a lower glycaemic response beverage by participants sipping glucose dissolved in water at 30-minute

intervals, while the higher glycaemic response group consumed the beverage as a bolus (21). The author recognised participants and investigators were not blinded; thus, investigators' and participant knowledge of consuming carbohydrates at different rates, could have impacted the application and attempt of cognition testing; additionally, tasting a sweet beverage is shown to affect cognitive behaviour (115, 116). Many un-blinded studies have found differences in cognitive function with specific glycaemic foods or beverages (21, 22, 24-29, 122). Therefore, their cognitive findings need to be viewed with some caution.

Additionally, as mentioned previously, insulin and cortisol are found to differ within different glycaemic foods and beverages, and affect cognitive function (26, 53, 85, 111, 112). Therefore, due to the lack of blinding and controlling for confounding variables, cognitive performance is found to not be strongly related with differences in glycaemic response (22, 26-29, 122). Larger blinded studies that control for confounding and test glycaemic response; and studies that consider additional biochemical pathways in the brain associated with glycaemic response, need to be researched. Then mechanisms can be clarified around the cognitive performance effects of different glycaemic responses.

2.7 Isomaltulose effect on humans

Isomaltulose is a sugar that naturally occurs in honey and sugar cane juices (125, 126). Japan has used isomaltulose since 1980 as an alternative sweetener to sucrose, due to it not being cariogenic (127-129). Isomaltulose and sucrose are composed of the same two sugar monosaccharides; fructose and glucose (18, 55). These monosaccharides are completely absorbed; thus, isomaltulose provides the same amount of energy as sucrose (18, 80, 130, 131). This sugar is distinct as it produces a

lower glycaemic and insulin response when compared to sucrose (18, 122, 130). Isomaltulose's novel glycaemic response compared to common table sugar and the energy it provides, raises interest for industries and individuals focused on prevention and interventions for health, performance and disease (18, 130-132).

Compared to sucrose's GI of 65, isomaltulose has a GI of 32, and is about half the sweetness of sucrose (18). This is due to differences in glycosidic bonds connecting 6-*O*-α-D-glucopyranosyl-D-fructofuranose together (**Figure 2.2**)(18, 126). Unlike sucrose which binds its monosaccharides via an α-1, 2 glycoidic bond, isomaltulose glycosidic bond is α-1, 6 (**Figure 2.2**) (78, 126). Although isomaltulose is completely absorbed in the small intestine, the brush border enzyme isomaltase that hydrolyses isomaltulose does it at V-max 26-45% the rate of sucrose's enzyme invertase (18, 78). This leads to isomaltulose's monosaccharide constituents entering the blood at a slower rate than sucrose (18, 78, 80, 126, 133). These varied rates of hydrolysis result in isomaltulose taking longer to break down into absorptive monosaccharides, and therefore induces a lower glycaemic response (18, 133).

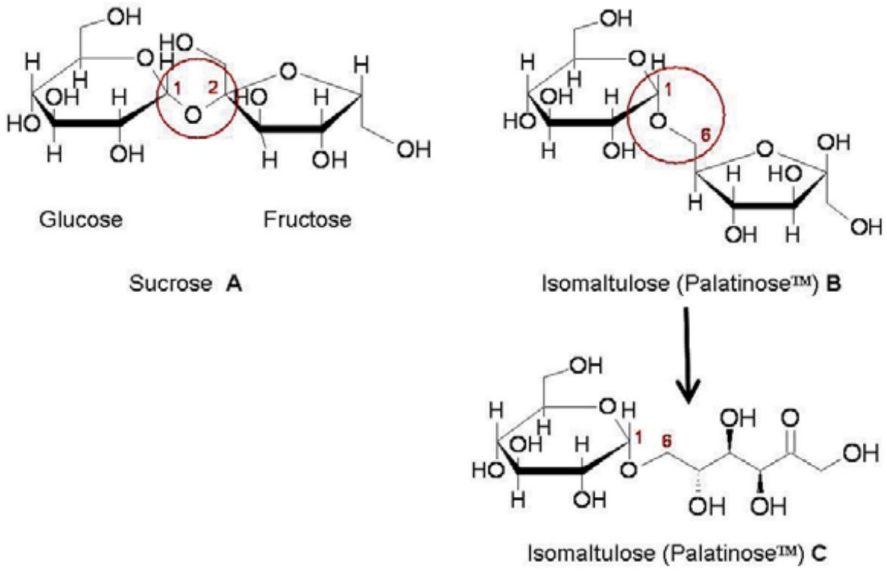

Figure 2.2 Structure of sucrose and isomaltulose (Palatinose TM) (122, 134)

Some evidence suggests isomaltulose can reduce metabolic disease risk factors. Isomaltulose is found to significantly lower triglycerides (125, 135), increase fat oxidation during exercise compared to sucrose (130, 136, 137), and reduce diabetes risk factors (fasting blood glucose, fasting blood insulin, and a marker of insulin resistance (HOMA-IR) (138)). The isolated effects of low glycaemic response carbohydrates, specifically isomaltulose, on metabolic disease risk factors in healthy people requires further research to make conclusive opinions.

2.6.1 Isomaltulose and cognition

Isomaltulose is a sugar that can be used in research to reduce confounding and confer blinding when compared to similar carbohydrate alternatives. It induces a lower glycaemic response than sucrose, yet contains the same monosaccharides as sucrose. Because it contains no other nutrients, test foods or beverages can be designed to not be detectably different in nutrients, appearance and taste. This allows for double blinding and control for confounding.

Some studies have found foods and beverages predicted to produce a lower glycaemic response, to improve cognitive performance compared to higher glycaemic foods and beverages (**Figure 2.1**) (20-24). However, when examining the cognitive effects that the lower glycaemic response carbohydrate isomaltulose can produce, there are a sparse number of studies and they vary in design (14, 20, 23).

Test foods and beverages containing isomaltulose have been compared to similar nutrient profile test foods and beverages with higher GI's. In the study by Young et al. conducted on (n=155) adults, declarative memory (episodic memory) was improved at 30, 105 and 195 minutes ($p=<0.01$) with isomaltulose compared to sucrose and glucose (20). Yet, the Dye et al. study found significantly improved executive functions (at 35 minutes ($p=0.0034$)) with sucrose milk beverage compared to

isomaltulose milk beverage; in those with poorer baseline executive functions scores (14). In the study on n=75 children by Young et al., immediate recall at 180 minutes ($p=<0.0001$) was significantly improved with isomaltulose (23, 139). Dye et al's. findings that incorporate baseline cognitive scores, may indicate that those with poorer cognitive function at baseline may be more sensitive to glycaemic changes, and suggests that baseline cognitive performance should be tested. From the small amount of studies available lower glycaemic isomaltulose may improve memory. However, this is a weak conclusion and should be taken lightly until further research is conducted.

2.8 Conclusions and rationale for research

In the studies that found cognitive function to be significantly different with different glycaemic foods or beverages; cognitive function was better with a lower glycaemic food or beverage. However, when considering all studies, the findings are inconsistent; the inconsistencies could be due to lack of control for confounding, not blinding and minimal consideration for the molecules associated with glucose availability. A standardised protocol that minimizes confounding and bias, would reduce the uncertainty around this topic. Isomaltulose compared to sucrose would assist in reducing confounding and allows for double blinding.

Table 2.1 Effect of different glycaemic test foods or beverages on cognitive domains frequently measured in children and adolescents

	Test foods or beverages		**Glycaemic category**	**Declarative memory**		**Immediate recall**	**Executive functions**	
Reference	Glycaemic category			Episodic memory	Semantic memory		Attention	Working memory
(Ingwersen et al., 2007)	High GI[1]	Coco Pops	Low GI				✓[3]	×[4]
	Low GI	All Bran						
(Young et al., 2015)	High GI	Cornflakes, semi-skim milk, glucose sweetened fruit, glucose orange drink.	Low GL			✓	×	
	Low GI	Cornflakes, semi-skim milk, Palatinose, Palatinose sweetened fruit, Palatinose orange drink.						
(Benton et al., 2007)	High GL[2]	Cornflakes, semi-skim milk, sugar, waffle, maple syrup.	Low GL		✓		✓	
	Medium GL							
	Low GL	Scrambled egg, bread, jam, low fat spread, low-cal yoghurt.						
		Ham slice, cheese slice, Burgen bread, low fat spread.						
(Micha et al., 2011)	High GI	Cornflakes, semi-skim milk, apple juice.	Low GI	✓	✓	×		
	Low GI	Muesli, semi-skim milk, apple juice.						
	High GL	Cornflakes, semi-skim milk, sugar.	High GI				✓	✓
	Low GL	Muesli, semi-skim milk, sugar.						
(Taib et al., 2012)	High GI	Standard growing up milk.	Low GI					✓
	Low GI	Reformulated growing up milk.						
	Low GI	Isomaltulose enriched growing up milk.						
(Cooper et al., 2011)	High GI	Cornflakes, white bread, margarine, 1% fat milk.	Low glycaemic response				✓	✓
	Low GI	1% fat milk, muesli, apple.						

(Continued)

	Test foods or beverages		Glycaemic category	Declarative memory		Immediate recall	Executive functions	
Reference	Glycaemic category			Episodic memory	Semantic memory		Attention	Working memory
(Cooper et al., 2015)	High GI Low GI	Cornflakes, white bread, margarine, 1% fat milk. 1% fat milk, muesli, apple.	Low GI				×	×
(Smith et al., 2008)	Low GI High GI	All Bran 1.8% fat milk. Cornflakes 1.8% fat milk.	High GI			×		

[1]Glycaemic index (GI)
[2]Glycaemic load (GL)
[3]Ticks indicate improvements in cognitive function
[4]Crosses indicate no differences in cognitive function

Table 2.2 Effect of different glycaemic test foods or beverages on cognitive domains frequently measured in adults

	Test foods or beverages		**Glycaemic category**	**Declarative memory**		**Immediate recall**	**Executive functions**	
Reference	Glycaemic category			Episodic memory	Semantic memory		Attention	Working memory
(Sanchez-Aguadero et al., 2018)	High GI[1]	White bread, strawberry jam, grape juice.	Low GI			✓[3]	×[4]	×
	Low GI	Low fat natural yoghurt, apple, walnuts, dark chocolate.	Low glycaemic response			×	×	×
(Young et al., 2014)	High GL[2]	Wholemeal bread, low carbohydrate jam, low-cal yoghurt, glucose, glucose drink.	Low GL	✓	×		×	
	Medium GL	Wholemeal bread, low carbohydrate jam, low-cal yoghurt, sucrose, sucrose drink.						
	Low GL	Wholemeal bread, low carbohydrate jam, low-cal yoghurt, isomaltulose, isomaltulose drink.						
(Benton et al., 2003)	Low GI	Diet 1 Biscuit.	Low GI			✓		
	Medium GI	Diet 2 Cereal bar.						
	Low GL	Diet 3 Biscuit.						
	Medium GI	Diet 4 Breakfast cereal.						

(Continued)

	Test foods or beverages		Glycaemic category	Declarative memory		Immediate recall	Executive functions	
Reference	Glycaemic category			Episodic memory	Semantic memory		Attention	Working memory
(Nilsson et al., 2007)	High GI Low GI	Glucose in water. Glucose in water.	Low GI					✓
			Low glycaemic response				✓	×
(Dye et al., 2010)	High GR Low GR	Isomaltulose and milk Sucrose and milk	High glycaemic response			×		✓

[1]Glycaemic index (GI)
[2]Glycaemic load (GL)
[3]Ticks indicate improvements in cognitive function
[4]Crosses indicate no differences in cognitive function

3. Objective Statement

The aim of this study is to examine the effects on cognitive function of different postprandial glycaemic responses.

This study's objectives:

- Develop two beverages that vary in glycaemic response by containing two different glycaemic index sugars, with minimal additional nutrients and are indistinguishable in taste, appearance and energy.
- Test that the beverages are not notably different in taste.
- Test the two beverages' glycaemic response.
- Compare the effects of the two beverages on participants' declarative memory, immediate free recall and executive functions.

4. Methods

This study was conducted over four weeks during March 2018 in the Department of Human Nutrition, University of Otago, Dunedin, New Zealand.

4.1 Ethical approval

The University of Otago Human Ethics Committee (health) granted ethical approval for this study in October 2017 (Ethics committee reference number 17/011) (**Appendix A**)**.** The trial was registered at Australian and New Zealand Clinical Trials (ACTRN12618000901202)

4.2 Study design

This randomised double-blinded controlled crossover trial, examined the cognitive effects of two beverages differing in glycaemic response (Isomaltulose + sucralose beverage (ISO) low glycaemic index (GI); sucrose (SUC) medium GI)), on healthy adults over three hours.

Prior to the cognitive testing randomised control trial (RCT) the test beverages were tested for differences in taste with a randomised triangle sensory test (**Appendix C**). Once the two beverages were confirmed to be indistinguishable in taste, this allowed the beverage recipe to be finalised.

Once the test beverage recipe was finalised the glycaemic response of test beverages' was tested, over the same time of day as the cognitive testing. A random subset of participants from the cognitive testing participants volunteered to take part in the glycaemic response testing. Participants were blinded to their blood glucose test results. Glycaemic response test days were conducted each week prior to the cognitive test days (**Figure 4.1**).

The cognitive testing consisted of a cross-over design in which participants were randomised to the order that they received the two test beverages. During each test day a film was shown in 30-minute time slots. The cognitive tests, comprising of recall associated with the film and immediate word free recall, were conducted between the film time slots over the three-hour test period (**Appendix F, Appendix G**). Reitan's Trail Making Part B test was conducted in the last testing time slot on each test day (**Figure 4.3**) (**Appendix H**).

Along with cognitive testing, a fellow Master of Dietetics candidate (MDiet) collected satiety data. The satiety results are not included in this thesis.

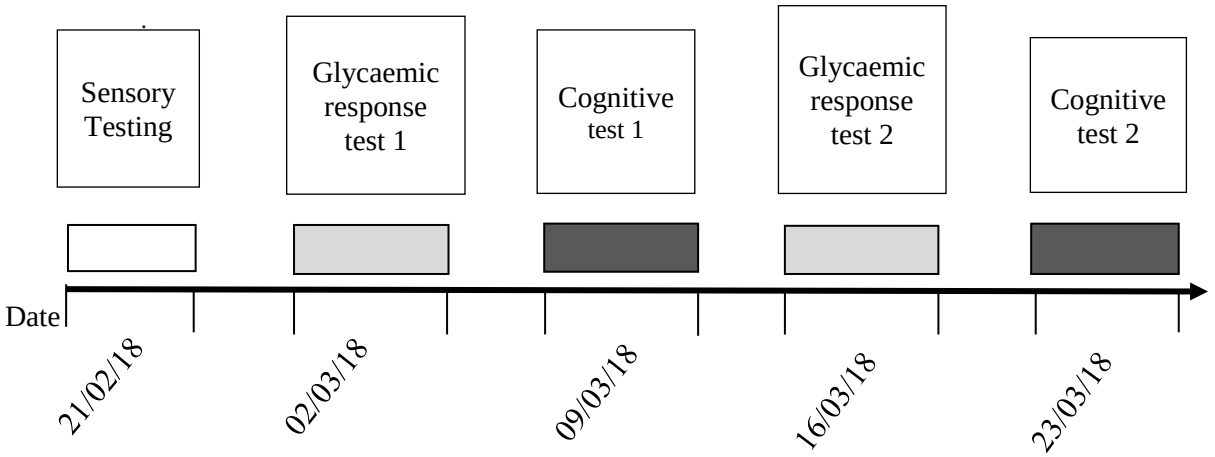

Figure 4.1 Timeline of trial

4.3 Development of sweetened beverages

The following section describes the formulation and sensory testing that occurred prior to the RCT.

4.3.1 Composition of test beverage

The two beverages were aimed to be isocaloric and be indistinguishable in taste. The beverages contained the same sugar concentration (10%) to frequently consumed beverages such as sports beverages and sweetened carbonated beverages (140). To

create a medium glycaemic response beverage sucrose (Glycaemic index (GI) 65) was used, and to create a low glycaemic response beverage isomaltulose (GI 32) was used (141). Sucrose and isomaltulose were used as they both contained the same two sugar monosaccharides fructose and glucose, and they contribute the same number of calories per gram (18, 142). To match the sweetness of sucrose, the isomaltulose beverage also contained the non-caloric sweetener sucralose (126). According to the sucralose patent, 0.88g of sucralose is equivalent to 1000g of sucrose (141, 143).This amount is shown to be safe for consumption (144). A taste testing session including the investigators involved in this study (n=4), determined that 0.035g of sucralose was to be use in the isomaltulose beverage. To make the beverages more palatable, carbonated water and an artificial lemon flavour were used (**Table 4.1.**).

Table 4.1 Composition of test beverages

Beverage type	**Lemon flavour (ml/500ml)**	**Sucrose (g/500ml)**	**Isomaltulose (g/500ml)**	**Sucralose (g/500ml)**
Isomaltulose	0.05		50	0.035
Sucrose	0.05	50		

4.3.2 Beverage formulation

The sucrose (SUC) and isomaltulose + sucralose (ISO) beverages were made up in the University of Otago Human Nutrition Mellor laboratory kitchen.

The day before test day: 50.00 grams of sucrose (Caster Sugar 172323, Smart Choice; New Zealand) and 50.00 grams of isomaltulose (Unflavoured Palatinose®, Myprotein; United Kingdom) were weighed using calibrated electronic scales (Sartorius 0.01/0.1mg). The pre-measured sugars were placed in sealed plastic containers.

The morning of test day: To make the test beverages equal volume (500 ml) the following procedures occurred. A summary of the beverage making process is present in **Figure 4.2** The test beverages were made in 500ml bottles containing 500ml of carbonated water (Pure NZ sparkling water, NZ drinks Ltd; New Zealand). To accommodate the sugars to be added, a volume of carbonated water was removed from the bottles. This amount removed was measured to the meniscus mark of a volumetric flask to maintain volume consistency between test beverages.

The 500ml bottles to become SUC had 80 mls of carbonated water removed, and the bottles to become ISO had 85mls removed. The extra five millilitres of carbonated water was removed from the ISO bottles as isomaltulose expanded in the carbonated water solution, leading to five millilitres more volume.

Before the premeasured sugars were added to the carbonated water bottles, the sugars were dissolved in boiled water. The premeasured sugars were added to the isomaltulose or sucrose volumetric flask, and filtered boiling water was added up to the 80ml meniscus mark. This mixture was stirred to solution and added to the carbonated water bottle.

To ensure the beverages did not differ in taste, lemon flavour (50uL) (Lemon 59223, lot:1002802470, Invita NZ Ltd; New Zealand) was pipetted (P100, 20-100uL, Gilson; France) into every beverage. Additionally, 1ml of sucralose + water solution containing 0.035g of sucralose (98% sucralose powder, J66736, lot:T21D050 Alfa Aesar; China) was pipetted (P1000, 200-1000uL Gilson; France) into the ISO beverages. The cap was sealed tightly for the beverage to be inverted four times and placed on the designated tray labelled SUC or ISO, to be refrigerated three hours prior to the tests commencing. This was to ensure that they were chilled upon consumption.

Food safety measures comprising hand washing, hair tied back, and clean benches were adhered to throughout beverage making (145).

Day of trial

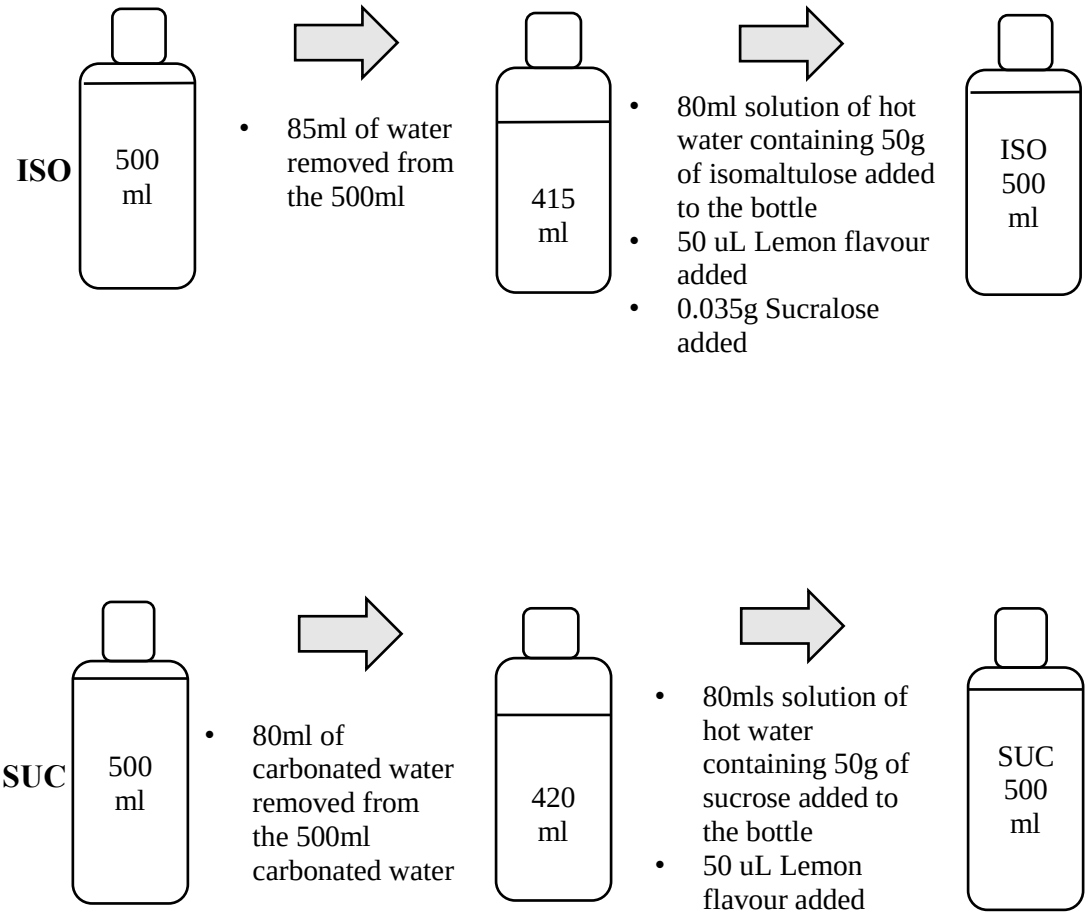

Figure 4.2 Beverage making process

4.3.3 Sensory testing of sweetened beverages

Following the formulation of the beverages, we undertook a sensory taste testing panel (**Appendix C**). This preceded the glycaemic response testing (n=12) and cognitive testing (n=70) RCT. This taste test was conducted in the University of Otago Sensory Science Centre. The test design was a blinded randomised controlled standard Triangle Sensory Test (146). Participants (n=6) aged between 21 and 30, were not participating

in the cognitive or glycaemic response test days of the main study, and were required to be without a sensitivity to artificial sweeteners.

Sensory testing consisted of four computer-generated, random coded taste tests per participant (**Appendix C**). Each taste test involved participants ingesting four lots of three 10ml samples. Of these samples, two of the samples contained the same test beverage (ISO or SUC) and the third contained the alternative test beverage (ISO or SUC). Participants answered questions related to notable differences in the beverages. (**Appendix C**). Based on the scoring results of the taste test questions, the beverages were found to be indistinguishable in taste**.**

4.4 Recruitment for cognitive testing and glycaemic response testing

Otago University students (n=77) from a 300-level human nutrition paper (HUNT) were recruited for the cognitive RCT in February 2018. Potential participants were invited verbally during a lecture by Dr. Bernard Venn. Verbal invitation included a brief presentation outlining the testing and participation requirements. From the n=77 HUNT students who volunteered to participate, a sub-sample group (n=12) further randomly volunteered for the glycaemic response testing aspect of this trial. Written consent for all participants was gained prior to the trial commencing (**Appendix B**).

4.4.1 Exclusion criteria

Participants were excluded if they were outside the ages 18 to 60 years, had sensitivities to artificial sweeteners, or were unable to eat sushi. The recruitment information sheet that asked about these dietary conditions was sent out prior to the study (**Appendix B**).

4.5 Double blinding

Sensory testing concluded that the beverages were indistinguishable in taste. The transparent beverages were served in their original identical unmarked bottles to ensure they were indistinguishable in appearance. For the glycaemic response and cognitive test days, a laboratory technician allocated the unmarked beverages to the participants. This resulted in the participants and testing facilitators being blinded to the treatment in both the glycaemic response and cognitive test days.

4.6 Test day procedures

The glycaemic response testing (n=12) and cognitive (n=77) RCT occurred over two test days. Each glycaemic response test day occurred a week before each cognitive test day (**Figure 4.1.).** For every test day, participants consumed sushi at 12:00. On the first cognitive test day at 13:50 the anthropometric and demographic questionnaire was completed (**Appendix E**). At 14:00 participants consumed the test beverage and blood testing, or cognitive testing commenced. At 17:00 participants filled in the intake and exercise questionnaire (**Appendix D**). Please note that test day preparatory beverage procedures, lunch, timing of beverage consumption and testing duration were the same for all test days.

4.6.1 Test day lunch

On glycaemic response and cognitive performance test days: To control for confounding factors of diet on glycaemic response, participants were given eight pieces of Wasabi's maki sushi (all sushi was the same size and made by Wasabi employees) and water (ad libitum) at 12:00 on the days of the glycaemic response and cognitive testing. Prior to the first test day participants chose their eight pieces of sushi, that would be provided for both test days. The sushi arrived at the laboratory pre-refrigerated in packaged plastic lidded trays. Once participants had eaten their sushi in the Otago

University Human Nutrition Mellor laboratory, they could freely leave, to be back at the laboratory by 13:45.

4.6.2 Demographics and anthropometrics

All participants completed a demographic and anthropometric questionnaire before the consumption of sushi and beverages (**Appendix E).** This included date of birth, sex, the ethnicity to which participants were affiliated, weight and height measurements. Weight and height were taken by research assistants following standardised procedures. Participants removed shoes, jackets, heavy items in pockets, and took down hair styles inhibiting accurate height measurements. Height was taken from a freestanding calibrated stadiometer (Holtan Limited, Britain). Weight was measured with calibrated electronic scales (Seca Alpha, model 770, Germany). Weight and height measurements of participants were used to calculate body mass index (BMI) using the equation: weight in kilograms/height in meter squared (kg/m^2).

4.6.3 Compliance diet, fluid and exercise questionnaire

Participants were instructed via email prior to the test days, to fast from 10:00 to 12:00. Once the glycaemic response (n=12) and cognitive testing (n=77) participants had consumed the sushi and water, they were instructed to fast again and to not partake in any vigorous physical activity until testing commenced at 14:00.

To assess compliance to instructions, participants were required to fill in a questionnaire at 17:00 on each test day, around exercise and diet, fluid and alcohol intake (**Appendix D**). On the cognitive testing days, this questionnaire also included a question as to whether they had ever viewed the film another time outside of the study period. This was asked because if participants were familiar with the film, seeing it a second time may mean their recall would be better compared to those who had only seen it on the test day.

4.6.4 Glycaemic response testing

The purpose of this test was to compare the glycaemic and insulinaemic responses of isomaltulose + sucralose test beverage (ISO) with the sucrose test beverage (SUC). This test was conducted in the University of Otago Human Nutrition Mellor Laboratory on a small subsample of n=12 participants from the main cognitive testing study. Glycaemic response of the test beverages was tested to be able to attribute any cognitive test differences to glycaemic response differences. It was not conducted on the same day as the cognitive testing, because the participant's response (fear) to finger pricking, may alter cognitive performance. This study incorporated the same protocol as the cognitive test days, apart from not including the cognitive testing and film.

4.6.4.1 Blood analysing

Baseline capillary blood sampling occurred between 13:50 and 14:00 prior to consumption of the sweetened beverage. Following participants' consumption of their allocated beverage within 10 minutes, blood samples were collected at 30, 60, 90, 120, 150 and 180 minutes.

Finger pricking glucose and insulin measurement procedures were standardised (**Appendix I**): To measure blood insulin and glucose response, seven 500uL capillary blood samples were collected from participants by trained dietetics candidates and laboratory technicians. Participants' fingers were pricked with a contact-activated 1.5 mm x 2.0 mm disposable lancet (BD Microtainer®, United States of America). Blood was collected in a microtainer containing anti-coagulant (BD Microtainer® Tube with BD Microgard™ Closure. K2EDTA anticoagulant Additive, 250-500 uL fill volume, code number 365975; United States of America).

Each blood sample was centrifuged at 2500 x G for 10 minutes at room temperature. Plasma was transferred to a Hitachi cup and stored at -20 ◦C until analysed.

Glucose was analysed on the Roche/Hitachi Cobas c311 (Roche, Indianapolis, IN, USA) using an enzymatic colorimetric method. Insulin was analysed using an electrochemiluminescence immunoassay (ECLIA) on a Roche/Hitachi Cobas e411.

The blood insulin and glucose tests were performed with quality control measures following Westguard and manufacturers' rules **(Appendix J)**. Pooled Roche controlled serum samples of insulin and glucose were used during testing to determine repeatability and accuracy of tests and intra and inter-assays variation. Insulin coefficients of variation for Precinorm U control one and two were 5.17% and 1.98% respectively, and for glucose PeriControl ClinChem Multi two were 0.98% and 2.58%.

4.6.5 Cognitive testing

The acute cognitive effects of low glycaemic response ISO compared to medium glycaemic response SUC beverage were examined over three hours. Participants ingested the sweetened beverages within 10 minutes, then watched a film displayed on two projector screens in 30-minute segments. After each 30-minute film segment participants completed cognitive tests, on assessment papers given following each film segment (**Appendix F, Appendix G, Appendix H**). The tests were collected immediately after completion. A summary of cognitive tests is presented in **Table 4.2.**

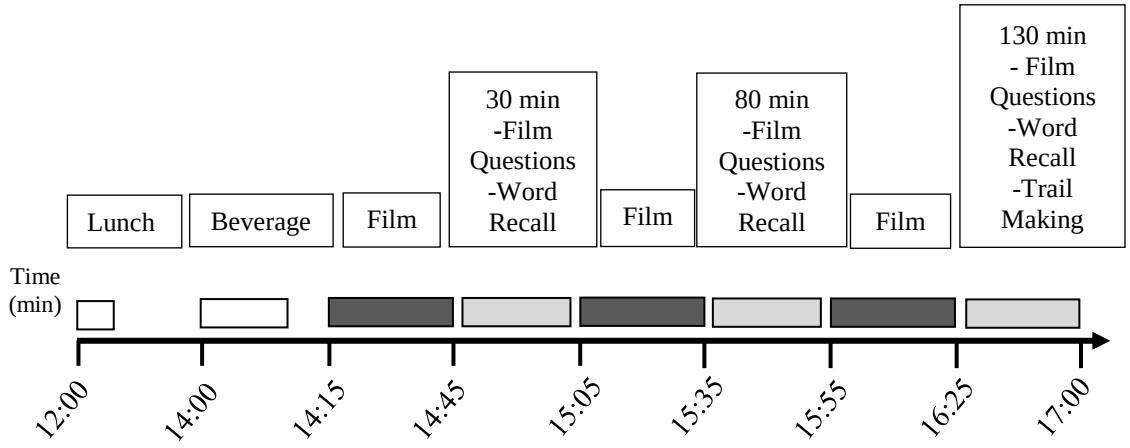

Figure 4.3 Timeline of cognitive testing day

Cognitive testing occurred at 30-minutes post-test beverage consumption then at 50-minute intervals up until three hours (**Figure 4.3).** At 30 minutes film questions one and word recall one were performed (**Appendix F, Appendix G**). At 80 minutes film questions two, and word recall two were performed. At 130 minutes, film questions three, word recall three were performed and at 140 minutes Reitan's Trail Making Part B was performed (**Appendix H**).

Table 4.2 Summary of cognitive tests

Test	Cognitive measure	Outcome	Max. score
Film questionnaire	Declarative memory	Number of questions answered correctly	10
Word recall	Immediate free recall	Number of words recalled	25
Reitan's Trail Making Part B	Executive functions	Time to complete	N/A

4.6.5.1 Film recall questions

During the cognitive testing days, watching a film and recalling details was used to assess declarative memory (**Appendix F**) (147, 148). Declarative memory includes semantic and episodic memory (149). Studies have used this method to assess declarative memory previously (150, 151). The films Sugar Versus Fat and Fat Versus Carbs for test day one and That Sugar Film for test day two were shown in three 30-minute segments. Participants were told prior to the film commencing that after each film segment they were required to fill in a questionnaire regarding the 30-minute segment most recently watched. The three questionnaires included in each cognition test day comprised 10 questions, consisting of three multi-choice questions; four auditory written answer questions; and three visual written answer questions.

4.6.5.2 Immediate word recall

Word recall has previously been used to assess immediate recall in glycaemic associated studies (8, 121, 152, 153). The six wordlists for the two cognitive testing days (three wordlists for each test day) (**Appendix G**) were based on the standardised and validated word categories and methods from Hopkins Verbal Learning Test (154, 155).

The Hopkins Verbal Learning Test uses 12-word wordlists; however, each wordlist in this study contained 25 words to increase the difficulty of the task and avoid ceiling effects. Ceiling effects reduce the ability to detect differences in performance between test days. Each wordlist consisted of equal amounts of words from 13 different Category Norms from Battig and Montague (1969), that were each randomised in a unique way to reduce category sequence learning. A wordlist was read out at the 30, 80, and 130-minute time slots post film question completion. The words were read out one word per two seconds.

4.6.5.3 Trail making

Reitan's Trail Making Part B has been used to assess executive functions (**Appendix H**) (156, 157). Executive functions include working memory and attention (158, 159). This test requires participants to alternate joining letters in alphabetical order with numbers counting up, e.g 1 to A to 2 to B so on to letter L, without lifting the pen off the paper. Participants were individually timed from start to finish by test facilitators. The outcome measure being time taken to complete. This test was conducted at 140 minutes following the third film questionnaire and word recall. For the second cognitive testing day the mirror image of Reitan's Trail Making Part B was used, to reduce learning effects.

On the first cognitive test day n=27 participants were instructed incorrectly to only join the letter with the number, not number to letter, e.g 1 to A, 2 to B; thus, the line was not continuous. This method still requires the same cognitive abilities (executive functions) to switch between number to letter in order; therefore, this method measured the same cognitive domains (156, 157). The 27 participants completed this task the same way for the second cognitive testing day, to control for any time differences between the two different methods to complete the test. Additionally, this would allow detection of inter-individual changes in cognition between test days due to the cross-over study design.

4.7 Statistical analysis

Participants' inter-individual differences in cognitive test scores at 30, 80 and 130 minutes with ISO compared to SUC, were analysed using mixed-regression. Participants with incomplete data and those who did not consume the beverage at a test day, were not included in the analysis (**Figure 5.1.**). Data was adjusted for dietary intake of macronutrients in the form of kcal between 12:00 and 14:00 (not including sushi lunch).

4.7.1 Randomisation

The n=77 participants in the cognitive testing were computer randomised using Microsoft Excel, to be either in the group to receive ISO beverage (n=39) or SUC beverage (n=38) for the first test day, and have the alternate beverage in the second test day. The n=12 participants in the glycaemic response testing were also randomised in the same way.

4.7.2 Sample size calculation

In-house data indicate that the glycaemic response testing sample size of n=12, was sufficient to detect a 33% change in glycaemic iAUC using the 5% level of significance with 80% power.

Previous published data specify that the cognitive trial sample size of n=60 is adequate to detect a difference of 0.5SD for all standardised outcomes, with the significance level of $p<0.01$ providing 90% power.

5. Results

The cognitive test data of 70 participants (81% female), and the blood glucose data of 12 participants (83% female) were analysed. Participant randomisation, allocation and exclusion information is present in **Figure 5.1**. Those who did not complete both test days (n=7) were not included in the analysis due to the cross-over design.

The cognitive tested participants' (n=70) demographic data are presented in **Table 5.1** The participants' average body mass index (BMI) was within the healthy range (18.5-24.9 kg/m²) and 62% were New Zealand (NZ) European.

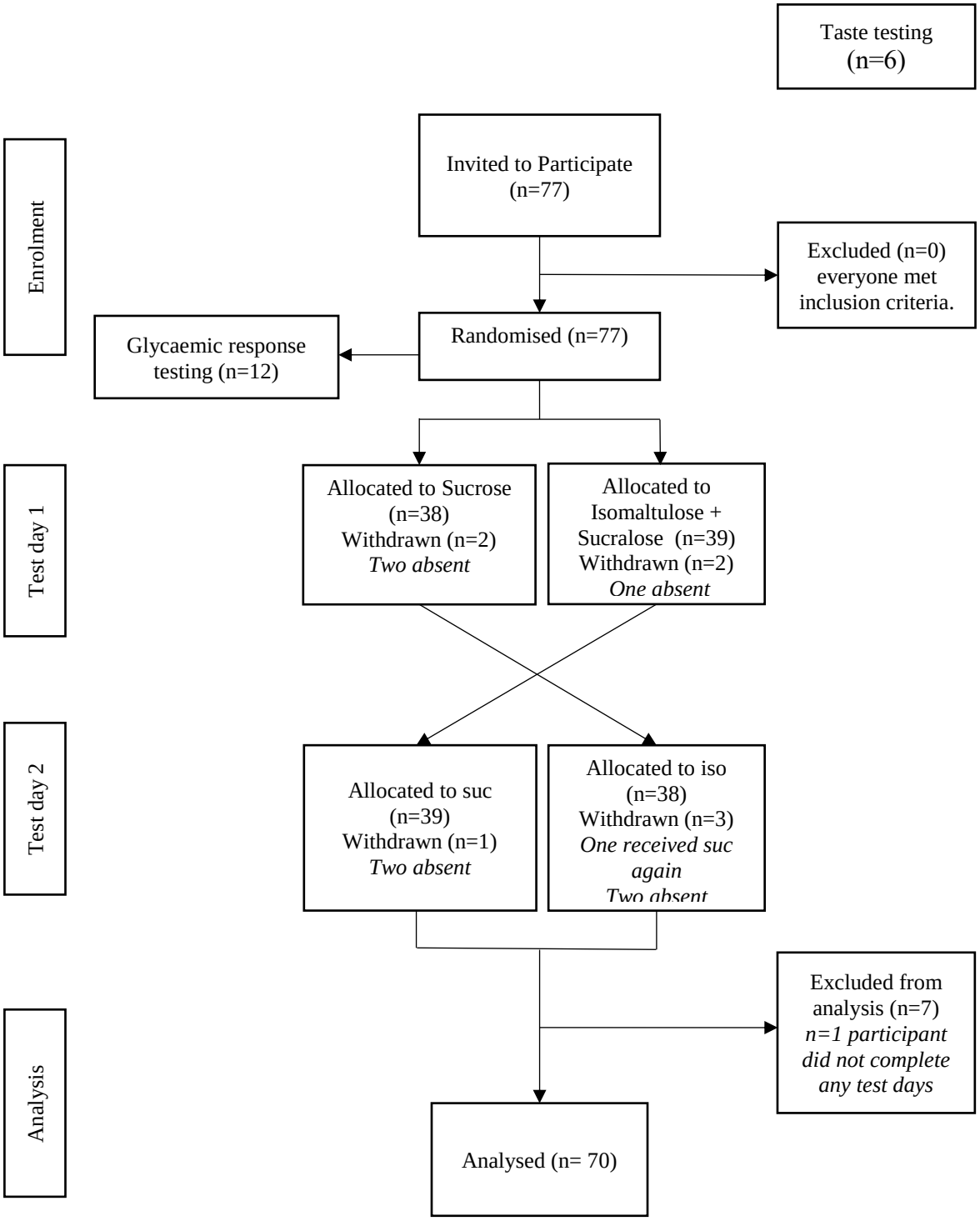

Figure 5.1 Study design and participant flow

Table 5.1 Cognitive tested participant characteristics

Characteristics		**Participants (n=70)**
Sex, n (%)	Female	57 (81)
	Male	13 (19)
Age, years (SD)[1]		21.9 (0.64)
Ethnicity, n (%)	NZ European	44 (62)
	Māori	4 (6)
	Chinese	12 (17)
	Other	11 (15)
BMI kg/m^2 (SD)[1]		23.3 (2.7)

[1]Results presented as Mean (SD).

Glycaemic response participants' (n=12) demographic data is available in **Table 5.2**. The participants' average BMI was within the healthy range (18.5-24.9 kg/m^2) and the majority were NZ European.

Table 5.2 Glycaemic response tested participant characteristics

Characteristics		**Participants (n=12)**
Sex, n (%)	Female	10 (83)
	Male	2 (17)
Age, years (SD)[1]		21.2 (1.4)
Ethnicity, n (%)	NZ European	7 (58)
	Chinese	5 (42)
BMI kg/m^2 (SD)[1]		21.9 (3.7)

[1]Results presented as Mean (SD).

The mean differences in insulin and blood glucose concentrations with isomaltulose + sucralose (ISO) compared to the sucrose test beverage (SUC) are presented in **Table 5.3**. ISO induced a significantly lower blood glucose iAUC

(p=0.003) compared with SUC (**Figure 5.2**). The main time points of difference were at 30 minutes when SUC induced a mean (95% CI) 2.0 mmol/L (-1.4, 2.6) higher glucose concentration than ISO, and at 150 minutes when ISO induced an average 1.1mmol/L (-0.49,1.7) higher glucose concentration than SUC (**Figure 5.2**).

Table 5.3 Mean difference in glycaemic and insulin response of isomaltulose + sucralose compared to sucrose (n=12)

Response	**SUC[2,3]**	**ISO[1,3]**	**Mean difference (95 % CI)**
Glucose (mmol/L·min)	61 (52)	17 (52)	-44 (-70, -18)
Insulin (µIU/L·min)	2492 (1540)	609 (1115)	-1883 (-2845, -921)

[1]SUC is sucralose beverage
[2] ISO is isomaltulose + sucralose beverage
[3] Mean (SD)

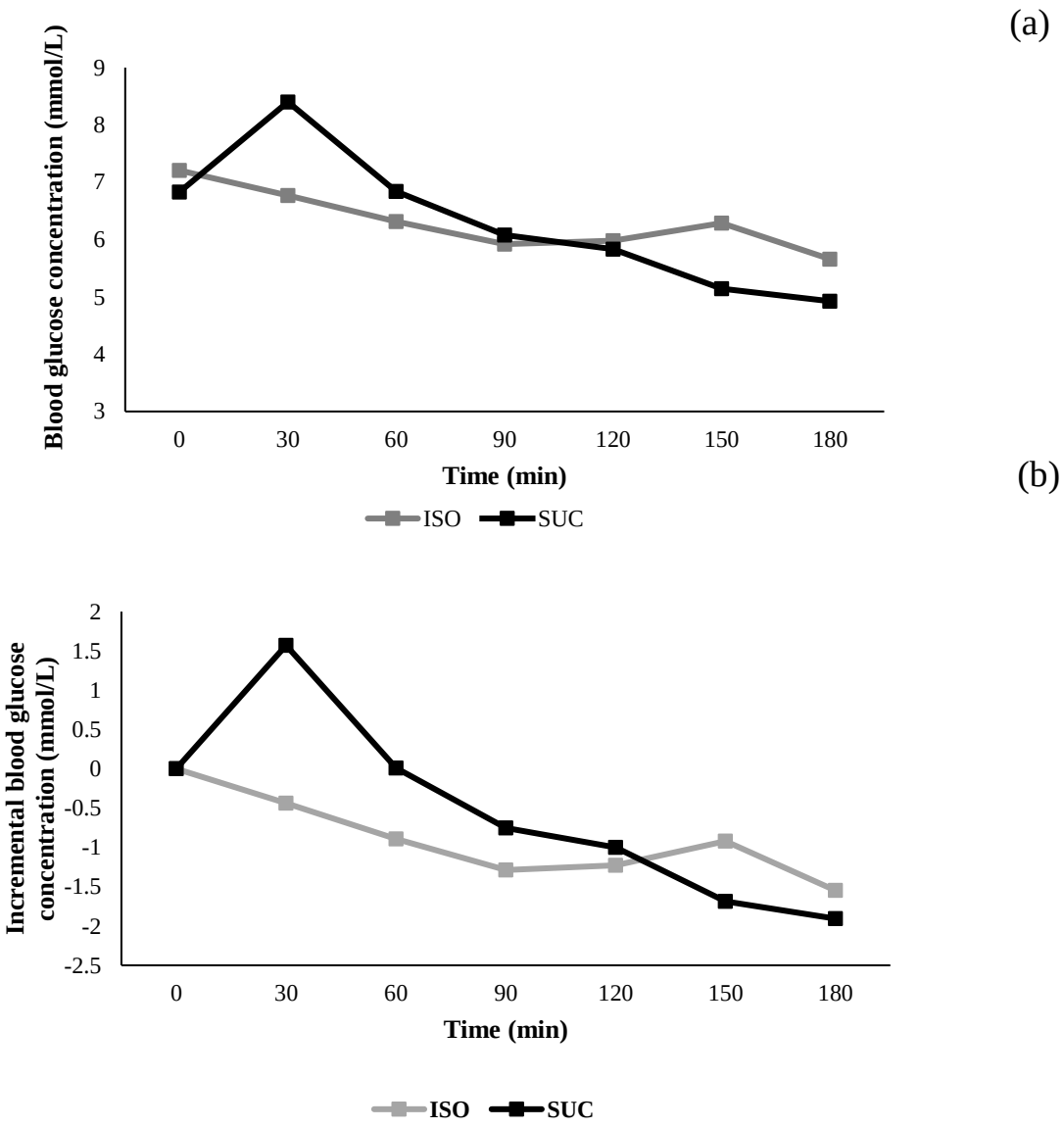

Figure 5.2 Isomaltulose + sucralose (ISO) and sucrose (SUC) beverages blood glucose (a)AUC (b)iAUC

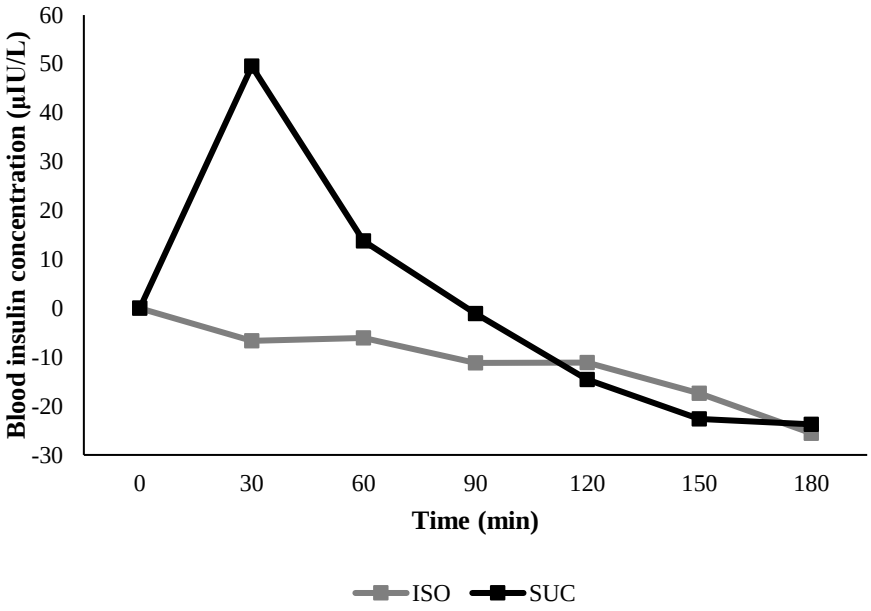

Figure 5.3 Isomaltulose + sucralose (ISO) and sucrose (SUC) beverages blood insulin AUC

Results from the dietary and fluid intake and exercise questionnaire, indicated compliance with instruction to not eat between 12:00 and 14:00 was 79% and 82% respectively for the two cognitive test days. Of this sample five of the participants broke their fast on both cognitive test days.

Noncompliance did not substantially influence effect sizes or the significance of results. The adjusted effects of ISO compared to SUC on cognitive tests are presented in **Table 5.4.**

Declarative memory (film questions), immediate free recall (word recall), and executive functions (Reitan's Trail Making Part B) performance were not significantly different between ISO and SUC. In the word recall tests, no participants were able to recall all 25 words. Additionally, neither methods (incorrect or correct) of completing Reitan's Trail Making Part B were significantly different between treatments (p=0.823 for incorrect method, p=0.627 for correct method).

Table 5.4 Adjusted mean difference of cognitive test results with isomaltulose + sucralose compared to sucrose over three hours[1]

Cognitive test		Time minutes	SUC[2] (n=70)	ISO[2] (n=70)	Mean difference (95 % CI)	P-value
Film Question (n)	**1**	30	5.6 (1.5)	5.6 (1.4)	0.1 (-0.2, 0.5)	0.463
	2	80	5.5 (1.8)	5.2 (1.8)	-0.3 (-0.8, 0.2)	0.252
	3	130	5.0 (1.5)	5.0 (1.7)	0.0 (-0.5, 0.5)	0.927
Word Recall (n)	**1**	30	11.4 (3.4)	10.7 (3.0)	-0.5 (-1.4, 0.3)	0.198
	2	80	10.7 (3.2)	11.1 (3.5)	0.4 (-0.4, 1.3)	0.301
	3	130	11.0 (3.5)	10.5 (3.3)	-0.4 (-1.1, 0.4)	0.357
Trail Making Part B (sec)		140	52.3 (26.1)	53.0 (23.9)	-0.3 (-6.9, 6.3)	0.928

[1] Isomaltulose + sucralose (ISO) and sucrose (SUC), results adjusted for dietary intake between 12:00 and 14:00 hours of the test days.
[2] Mean (SD)

6. Discussion

This study was designed to compare cognitive function under two different glycaemic conditions, using low glycaemic index (32) (GI) isomaltulose + sucralose (ISO) and moderate GI (65) sucrose (SUC)(18). Although differences in postprandial glycaemia were achieved, tests of cognitive performance were not significantly different.

6.1 Comparison of findings

Our study's findings are in agreement with some previous research. That is, foods confirmed to induce a significant difference in glycaemic response, did not create significant differences in executive functions (10, 21, 29), declarative memory (semantic) (20) and immediate recall (14, 29, 122). A study conducted by Dye et al. on n=24 blinded adult males used the same cross-over design and glycaemic sugars; isomaltulose compared to sucrose as in our study. This study found immediate recall was not significantly different between beverages (14). In another cross-over study of n=39 children by Brindal et al., executive functions were also found to not be significantly different with meals varying in protein, fat and carbohydrates (10). Executive functions (21, 23, 29), immediate recall(14, 26, 27), and declarative memory (semantic memory) were also not significantly different with different GI or glycaemic load (GL) foods (20) in studies did not test, or find significant differences in glycaemic response.

Possibly due to heterogeneity in design, others have reported significant differences in the same cognitive domains with different glycaemic foods or beverages. Low compared to high GI and GL test foods or beverages improved executive functions (14, 139), immediate recall (23, 24, 29) and declarative memory (20, 25). On the other

hand, executive functions have also been shown to be better with a high GI meal (26). Executive functions are also found to be improved with lower glycaemic response food or beverage in two studies (p=0.033) (21, 122) and high glycaemic response in one study (p=0.023) (14).

The null and significant findings reported in the literature maybe be an outcome of considerable inter-study variation in designs. Some of these studies did not examine the same cognitive domains, had unblinded participants' and investigators (10, 22, 26-28), compared test foods of varying macro and micro-nutrients or/ and did not measure glycaemic response. This could have introduced bias or confounding, particularly if the nutrient composition of the test foods differed; for example, white bread and jam compared to low fat yoghurt and walnuts (10, 22, 26, 28, 29, 139). Thus, there has been little standardization in the literature to enable direct comparisons among studies.

6.2 Nutrients' influence on cognitive function and glycaemic response

Cognitive performance in some studies may have been directly influenced by different nutrients between test foods (30, 31, 33, 55-60, 123, 160). Fibre and protein are associated with variations in cognitive performance (30, 123).

Interestingly, studies have compared foods containing different amounts of protein and fibre. Despite not finding differences in glycaemic response at cognitive test times, significant differences in cognitive performance were attributed to glycaemic response (22, 24). Controlling nutrient variation is vital to attribute glycaemic response to cognitive performance; as fat and fibre can influence the glycaemic response (32, 56). Some studies acknowledge their findings cannot be distinguished from potential effects of differences in macronutrient and energy content between test foods. However, specific explanations of mechanism behind the test foods or beverages macronutrient

or energy effect are limited (24, 26). Future research needs to control for nutrient variation to minimise confounding.

In the body of literature that did test glycaemic response (nine studies)(10, 14, 21, 24, 26-29, 139), only three reported altered cognitive performance depending on glycaemic response (14, 21, 122). Of this group only two studies controlled for nutrient variation; the study by Dye et al. found executive functions better at mainly 35-minutes with a higher glycaemic response beverage ($p=0.0034$) (14). Whereas, a study conducted on n=40 older healthy adults (aged 49-70 years) some executive functions at 170-minutes were significantly better ($p=0.017$) with a lower glycaemic response beverage (21). However, some executive functions (21) and immediate recall (14) were found to not be significantly different.

In the current study, test beverages only varied in sucralose, and glycaemic response was different at times when cognitive tests were conducted. Thus, the inconclusive findings from the body of literature are possibly due to uncontrolled nutrients in test foods or beverages, affecting glycaemic response and cognitive function (22, 24-29).

6.3 Blinding

Despite considering the significance of nutrient variation, cognitive findings may still not be attributed to glycaemic response, due to potential bias from not blinding. Cognitive testing is subjective; thus, it is important to minimize inter-individual subjective variation in cognitive tests. Some studies are subject to bias due to variation between the tests food or beverage consumption methods (21), visual appearance or taste (10, 22, 24, 27). Our study was able to double blind due to the test beverages being consistent in these areas. Un-blinded trial investigators or participants may hold views towards one test food or beverage improving or worsening cognition

(161, 162), possibly leading to investigators unintentionally providing one test group with more attention during cognitive tests (163). Cognition is sensitive to perceived ability; thus, participant's bias may lead to altered perception of ability to perform cognitively demanding tasks (164). Blinding of participants' and/or trial investigators reduces the likelihood of bias affecting cognitive outcomes. Unblinded trials associating cognitive improvements solely to postprandial glycaemic response is questionable. Our study was able to minimise participant and investigator bias through double blinding the glycaemic response testing and cognitive testing. This allowed bias to be considered to be an unlikely rationale for our findings.

6.4 Strengths and limitations

6.4.1 Sucralose effect on cognitive function

Although our study controlled for nutrient variation, the ingredient that did vary between test beverages, sucralose, is found to affect different areas of the brain compared to sucrose. Unlike the taste of sucralose, the taste of sucrose is found to have an effect on areas of the brain associated with memory formation (117, 118). However, a study by Frank et al used significantly more sucralose (0.042g/100g) and sucrose (32g/100ml) than what was compared in our study (0.007g/100ml sucralose and10g/100ml sucrose) (117). Thus, this sucrose amount used in our study is not considered likely to have had a major effect on cognitive function.

6.4.2 Glycaemic response testing of beverages

Glycaemic response testing was conducted on a small subset (n=12) of cognitive testing participants. This small sample size raises the risk of inter-individual variation influencing results and limits the power of the study, reducing the likelihood of true effect. Future studies should test glycaemic responses of all participants included in the cognitive testing to improve the quality of results.

Glycaemic response testing of the beverages was carried out on different days to cognitive testing due to the likelihood of finger pricking effecting cognitive performance. For some individuals receiving finger pricks is an unpleasant stressful experience. Participants may be distracted during cognitive testing due to anticipation of the next finger prick. During times of stress cortisol (a stress hormone) can be released. Cortisol is found to have an impact on cognition (111-114). Thus, to minimise confounding, glycaemic response testing was not tested on the same day as cognitive testing. However, this decision increases the potential for intraindividual variation between test days to cause different glycaemic responses for the same test beverage; for example; varied duration of sleep between test days (71). Thus, the glycaemic response findings cannot directly apply to cognitive performance test days.

In our study, measurable cognitive differences may have been produced if a greater difference in glycaemic response was created. The sushi meal consumed by participants two hours prior to testing, had a *glycaemic* effect at baseline; and the test sugars being low and medium GI, predicted a smaller glycaemic response difference compared to a high and low GI. Food and beverage studies have found cognitive improvements with high and low GI (26, 27). However, because this current study's investigators valued controlling for nutrient variation; low GI isomaltulose and medium GI sucrose were used in the test beverages, allowing both test beverages to contain the same monosaccharides fructose and glucose.

Despite the smaller difference between the sugar GI's; our study created a greater difference between test beverages in blood glucose, of 2 mmol/L (-2.6,1.4) (mean (95% CI)) at 30 minutes compared to some studies. A study that had a mean difference in blood glucose of 1.2mmol/L at 30 minutes found significant differences in cognitive performance with isomaltulose compared to sucrose at 30-40 minutes (14).

Studies that compared high with low GI test foods created a 0.5 mmol/L and 0.4mmol/L mean difference at 30 minutes (28, 122). The difference in GI in our study was predicted to be smaller compared to some studies. However, our study created a greater difference in glycaemic response compared to others who found significant differences in cognition while examining the same sugars and in low compared to high GI foods and beverages.

The sushi meal at 12:00 affected baseline blood glucose at 14:00; this may have reduced possible differences in cognitive function. Mean baseline(CI) blood glucose levels (7.2 mmol/L(-6.4,7.9) were above healthy fasting blood glucose concentrations (3.9-5.5 mmol/L) (91). Although the effect of the previous meal on cognitive function is minimally understood, this may have meant the brain already had an adequate supply of energy; therefore, subsequent rises in blood glucose above this range may have had no effect (165). Testing cognitive performance following an overnight fast would be ideal in future research. However, previous research has found improved cognition in individuals who had their usual breakfast prior to consuming a glucose beverage, compared to placebo (12). This indicates that cognitive function may still benefit from additional glucose following a meal.

The timing of our study provides a real-world examination of glycaemic response on intellectually demanding tasks, when cognition may be fatigued in the afternoon. In contrast, in most studies cognitive performance was examined with different glycaemic foods and beverages after an overnight fast (10, 14, 20-28). Due to the timing of our study, making direct comparisons with others is difficult; as a 10-12 hour fast may prime cognition to be more sensitive to glycaemic response changes (63, 161). Despite this theory, cognitive performance has been found to be improved with a

higher glycaemic beverage following an overnight fast, and in the afternoon, following a meal two hours prior (166).

Hence, the prediction that the timing of our study, the smaller difference in test beverages GI, and the baseline glycaemic effect of the meal two hours prior; would lead to the glycaemic response difference having less of an effect on cognitive performance, is unlikely.

6.4.3 Cognitive domains measured

Another aspect regarding the likelihood of finding cognitive differences, is testing cognitive domains that are already found to differ with different glycaemias. Executive functions are found to be sensitive to lower glycaemic foods and beverages at 120 (122) to 180 (139) minutes, and higher glycaemic foods at 100 minutes (26). Reitan's Trail Making Part B tests executive functions and is found to detect differences in cognition with different glycaemic foods (7, 29, 156). Our study used Reitan's Trail making Part B only at 140 minutes. Testing executive functions after 100 minutes allowed our investigators to examine whether cognitive performance will be better over a longer period with a lower compared to a higher glycaemic response beverage (26). However, due to only testing at this time, we cannot conclude that executive functions are not affected by different glycaemic responses prior to 140 minutes.

Measuring declarative memory with film was used in this study to create a real-world learning setting, like being in a lecture. Like lectures, film involves visual and auditory information processing (167, 168). Although watching a film and recalling details has been previously used as a tool to measure declarative memory; this method has not been used in glycaemic studies (150, 151, 169).

To keep the film questions consistent in what they measured, the question types were the same for both test days; however, each test day used a different film. The

difference in film content may have caused intra-individual variation in memory; individuals may have remembered more from one film due to personality rather than the effect of SUC or ISO (170). In future research a standardised cognitive test that is found to distinguish differences in cognition with different glycaemia's (Stroop test) in replace of the film questionnaire, may detect differences in cognitive function (14, 26, 28, 121).

If cognitive function is related to postprandial glycaemic response, it was likely to have been found in our study; as two of the cognitive tests used are found to be sensitive to different glycaemias; glycaemic response was different at cognitive testing times; test beverages contained minimal additional nutrients; and the large sample size, double blind randomised cross-over design controlled for confounding (20, 24, 27, 171).

7. Conclusion

From this current study we conclude that is no evidence that declarative memory, executive functions, and immediate free word recall are affected by differences in glycaemic responses; induced by different GI beverages in the afternoon.

Nevertheless, a standardised study design protocol that controls for confounding, is conducted in the morning following an overnight fast, uses the same cognitive tests, and is double blinded, needs to be conducted to confirm the null findings.

8. Application of Research to Dietetic Practice

This study can contribute to the nutrition evidence pool used in dietetic practice. Analysing the effect that diet can have on cognitive function, is relevant for dietitians working with healthy and diseased individuals and in the food industry. Patients coming to see a dietitian may hold opinions about improving cognitive function with specific foods from popular unsubstantiated media messages (172). Their opinions may lead to their diet being nutritionally inadequate. Dietitians can use evidence-based nutrition to provide the patient with the correct knowledge; this study adds to that pool of evidence on cognitive function and diet.

The literature that has examined the relationship between different glycaemic foods and beverages and cognitive function in healthy individuals is conflicting. This study found that declarative; immediate free recall; and executive functions did not vary with different glycaemic responses. These findings are reliable in that our study size and design controlled for confounding variables. However, due to our study's novel design, these findings need to be repeated.

Most of the studies that found a cognitive performance effect with low GI, low glycaemic load or low glycaemic response foods or beverages, did not control for nutrients; therefore, the additional nutrients that those test foods or beverages contained may be what is beneficial for cognitive function. Additionally, low GI and low glycaemic load diet is not a reflection of a good diet. Dietitians should suggest to those looking to improve cognitive performance through diet, to meet nutritional requirements by adhering to the Ministry of Health dietary guidelines (3, 31, 173). This would ensure micronutrients and macronutrients vital for normal brain functioning are met (31). From this study and the body of evidence regarding this topic, dietitians

should hold the position that the evidence is limited and until these findings are repeated no conclusions should be made.

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10. Appendices

10.1 Appendix A: Ethics approval letter

H17/011

Academic Services
Manager, Academic Committees, Mr Gary Witte

16 October 2017

Dr B Venn
Department of Human Nutrition
Division of Sciences

Dear Dr Venn,

I am again writing to you concerning your proposal entitled "**HUNT311 clinical nutritional laboratory; a repeated teaching activity**", Ethics Committee reference number **H17/011**.

Thank you for your request for the amendment to give students a sweet beverage containing 50mg of sucralose instead of a "trifle". Thank you for keeping the Committee informed.

Your proposal continues to be fully approved by the Human Ethics Committee. If the nature, consent, location, procedures or personnel of your approved application change, please advise me in writing. I hope all goes well for you with your upcoming research.

Yours sincerely,

Mr Gary Witte
Manager, Academic Committees
Tel: 479 8256
Email: gary.witte@otago.ac.nz

c.c. Professor S Samman Department of Human Nutrition

10.2 Appendix B: Consent form for participants

University of Otago Human Ethics Committee (Health)

HUNT311 clinical nutritional laboratory; a repeated teaching activity

Principal Investigator: Dr Bernard Venn (bernard.venn@otago.ac.nz tel 034795068)

CONSENT FORM FOR PARTICIPANTS

Following signature and return to the research team this form will be stored in a secure place for ten years.

Name of participant:……..

1. I have read the Information Sheet concerning this study and understand the aims of this research project.
2. I have had sufficient time to talk with other people of my choice about participating in the study.
3. I confirm that I meet the criteria for participation which are explained in the Information Sheet.
4. All my questions about the project have been answered to my satisfaction, and I understand that I am free to request further information at any stage.
5. I know that my participation in the project is entirely voluntary, and that I am free to withdraw from the project at any time without disadvantage.
6. I know that as a participant I will be asked to provide demographic data and have my height and weight measured. I will provide blood samples via fingerprick and participate in tests of cognition.
7. I know that the laboratory will explore the effect of consuming beverages sweetened with sucrose or isomaltulose+sucralose on blood glucose, satiety and cognition. If I feel hesitant or uncomfortable I may decline to answer any particular question(s) , and /or may withdraw from the project without disadvantage of any kind.
8. I understand the nature and size of the risks of discomfort or harm which are explained in the Information Sheet.
9. I know that when the project is completed all personal identifying information will be removed from the paper records and electronic files which represent the data from the project, and that these will be placed in secure storage and kept for at least ten years.
10. I understand that the results of the project may be published and be available in the University of Otago Library, but that either (i) I agree that any personal identifying information will remain confidential between myself and the researchers during the study, and will not appear in any spoken or written report of the study ☐
11. I know that no commercial use will be made of the data.

10.3 Appendix C: Triangle Sensory Test

5.2.2.1 Triangle test

Objective: To determine if a difference exists between two samples.

Procedure: Assessors are presented with three samples and told that two samples are the same and one is different. They are asked to assess the samples in the order provided and determine which sample is 'the odd one out'. They may also be asked to describe the difference. Appropriate palate cleansers should be used between each sample. Samples are labelled with three-digit codes (blind coded).

Experimental design: There are six possible orders of sample presentation.

They are

AAB BBA

ABA BAB

BAA ABB

In some instances, only one half of the design is used, for example if the quantity of one of the samples is limited, or if one of the samples is the standard/reference and, therefore, presented as the duplicate sample. It is good practice to use each possible presentation order an equal number of times with 24–30 assessors, although the absolute number chosen depends on the overall aim and the significance level selected. Larger panels are more discriminating and are commonly used when the differences are very small or when the aim of the test is to determine similarity (see Section 5.2.4).

Questionnaire: See Figure 5.1.

Data analysis: The total number of responses correctly identifying the 'odd' sample is counted. There are two ways of analysing the data. If analysing the data by hand, the number of correct responses is compared to statistical tables (see Appendix 3). The table states the minimum number of correct identifications required (at different levels of significance) before a significant difference can be concluded from the test. The total number of correct responses must exceed the critical minimum value from the table.

Alternatively, software packages calculate the probability of making a type I error (α risk) should it be concluded that a significant difference exists between the samples. In this instance, a probability of less than 0.05 (equivalent to 5% level of significance) is used as a 'cut-off ' although common sense should be used when interpreting this data, e.g. would it be sensible to ignore a result of p 0.056 and conclude that no significant difference exists just because this value is not less than 0.05?

Conclusion: From a triangle test, the conclusion is that a significant difference does OR does not exist between the two samples. In either case, the significance level of the test, e.g. p 0.05, must also be stated. In addition a comment may be made about the nature of the difference.

Questionnaire: See Figure 5.1.

Triangle test

Assessor: Date:

You are provided with three samples, each labelled with a three-digit code. Two samples are the same and one is different. Assess each sample in the order provided, from left to right, and select the 'odd' sample. Record your result below.

Cleanse your palate with cracker and water after each sample. You are not permitted to retaste the samples. Please comment on how the odd sample is different.

Sample	Different sample (please tick)
219	
470	
593	

Comments:

Figure 5.1 Example of a questionnaire for the triangle test.

5.2.3.2 3-Alternative forced choice

Objective: To determine if a difference exists between two samples with regard to a specified attribute, e.g. sweetness, hardness and intensity of fragrance.

Procedure: Assessors are presented with three blind coded samples. Two samples are the same and one is different, although the assessor is not made aware of this fact. They are asked to assess the samples in the order provided and determine which sample has the highest intensity of a specified 'attribute'. Assessors may be pretrained on the attribute, depending on the test objectives. Appropriate palate cleansers should be used after each sample. As with the 2-alternative forced choice (2-AFC) test, samples should vary only in intensity of the attribute in question, although practically this is very hard to achieve. If there are too many differences between samples, overall discrimination tests should be used, e.g. triangle test. This method is commonly used to determine threshold values, i.e. the lowest concentration of a compound that can be detected, whereby the 'same' samples are the diluent or carrier (water, air) and the 'different' sample contains the stimulus in the diluent or carrier (see ISO 13301:2002).

Experimental design: There are only three possible orders of sample presentation. They are

AAB

ABA

BAA

It is good practice to use each possible presentation order an equal number of times with a minimum of 24 assessors, although the absolute number chosen depends on the overall aim and the significance level selected. Typically, the sample assumed to be the most intense is presented as the 'odd' sample; however, when the most intense sample cannot be predicted, the test may need to be performed twice

with each sample being presented as the 'odd' sample.

Questionnaire: See Figure 5.7.

Data analysis: Determine the total number of times the 'odd' sample is selected. There are two ways of analysing the data. When calculating by hand, the number of 'correct' responses is compared to statistical tables (see Appendix 3). The table states the *mini-mum* number of 'correct' responses before a significant difference can be co ncluded from the test. The significance level of the test must also be specified (typically 5%). Alternatively, software packages calculate the probability of making a type I error should it be concluded that a significant difference exists between the samples.

Conclusion: From a 3-alternative forced choice (3-AFC) test, the conclusion is either that one sample was significantly more intense than the other with regard to the specified attribute or that there was no significant difference between them with regard to the specified attribute. The significance level of the test, e.g. p _ 0.05, must also be stated.

Sample test: lavender aroma

Assessor: Date:

You are provided with three shower gel samples, each labelled with a three-digit code. Assess each sample in the order provided, from left to right, and determine which sample has the most intense lavender aroma. Record your result below.

Do not sniff the samples too vigorously and leave 10 seconds between samples to give your nose a chance to recover. You may resniff the samples.

DO NOT CONSUME THE SAMPLES

Sample	Most intense aroma (please tick)
219	
470	
593	

Comments:

Figure 5.7 Example of a questionnaire for the 3-AFC test. (*Note*: The ballot does not use the name of the test to avoid providing too much information and biasing the assessors.)

10.4 Appendix D: Intake and exercise questionnaire

1. **Did you consume any alcohol last night?**

   Please circle YES / NO

   If yes how many standard drinks?

2. **Did you consume any beverages apart from water or food between 10am and 12pm today?**

   Please circle YES / NO

   If yes what was it and how much? Please provide in serving sizes eg medium apple, 1 cup.

3. **Did you consume any beverages apart from water or food between 12 and 2pm?**

   Please circle YES / NO

   If yes what was it and how much? Please provide in serving sizes eg medium apple, 1 cup.

4. **Did you do any exercise apart from walking between 12 and 2pm?**

   Please circle YES / NO

   If yes please describe the activity and how long you were exercising for.

10.5 Appendix E: Demographic and anthropometric questionnaire

Demographics questionnaire

What is your date of birth? (DD/MM/YYYY)

Are you female or male? (please circle)

What ethnicity do you affiliate with?

Weight: _____ kg

Height: _____ cm

Thanks for completing the questionnaire!

10.6 Appendix F: Film questions and answers

Fat vs Carbs with Jamie Owen

Film questions 1

1. 0.01.20 What types of diet/ diets has the presenter already tried?

A Lower calorie exercising more*

B Reduced carbohydrate

C Low fat

D A and B

E All of the above

2. 0.02.36 How many g of carbohydrates does Zoe Harkham want Jamie to eat per day?

A 25 g or 5% of daily intake

3. 0.03.25 During Dr Zoe Harken showing Jamie what the low carbohydrate diet consisted of, what did she pull out of the oven?

A Pork and crackling.

4. 0.07.49 What did Jamie's first breakfast on the diet consist of?

A Bacon and eggs, no bread.

5. 0.08.45 What does Dr Nadim Haboubi claim is the most critical component of a diet?

A Sustaining it in the long term.

6. 0.10.19 How much weight had Paul Henwood lost?

A 20kg

B 17kg (230 to 213)*

C 15kg

D 23 kg

7. 0.17.50 Where were the spokesperson for the British clinical dietetics association and Jamie sitting while speaking to each other?

A In a Garden/ outside on a lawn

8. 0.24.40 Who wouldn't talk to Jaime about dietary guidelines?

A Chief medical officer and health minister.

9. Did Jaime's cholesterol change following completing the diet?

A It increased

B It decreased*

C It stayed the same

D It increased then went back to the range before the diet.

10. 0.26.42 What colour is the GP office?

A Red

Sugar versus Fat

Film questions 1

1. 0.00.47 What was the name of Xand's cat?

A Tiger

2. 0.02.09 What does Chris specialize in?

A Infectious diseases

3. 0.05.08 What are two things that Xand mentioned he is concerned about going on the high fat diet?

A constipated (won't poop for a month), low fibre, bad breath, craving fresh greens

4. 0.08.52 What colour swim cap was the first twin to go into the Bodpod wearing?

A Red

B Blue

C Orange

D Yellow

5. 0.10.37 Xand was eating turkey rolled up in what for lunch?

A Cheese

6. 0.15.20 Why is a high fat, low carbohydrate diet suggested to compromise cognitive function?

A Have limited glucose available for energy use.

7. 0.18.39 Where does Xand meet Dr Robert Lustig?

A Carnival pier 39

8. How much energy did Xand consume during the all you can eat trial?

A 825kcal

9. 0.24.50 How much energy did Chris consume during the all you can eat trial?

A 950kcal

B 1500kcal

C 1250kcal*

D 820kcal

10. 0.26.00 In the film it is stated that protein supresses ------ for longer than sugar?

A Ghrelin*

B Leptin

C Glucagon

D Neuropeptide Y

Sugar versus Fat

Film questions 2

1. 0.30.12 Where was the last blood glucose test taken during the final blood test during the cycling exercise challenge?

A Under trees next to the telly board.

B On the side of the road at the top of the hill next to the telly board.

C In the car park next to the telly board.

D At the top of the hill in an open field on grass next to the telly board.

2. 0.33.21 What was Chris's and Xand's blood glucose in the final blood test?

A 3.4 and 2.3 consecutively

B 2.2 and 3.1 consecutively

C 5.1 and 7.0 consecutively

D 7.1 and 5.1 consecutively *

3. 0.37.00 How much weight did Xand loose?

A 3.5kg

4. 0.38.45 Which biochemical tests were checked first following weighing?

A Cholesterol then blood glucose

5. 0.46.52 What was one of the twins holding a tray of while standing in the streets of New York?

A Doughnuts

6. 0.48.39 In what scene did Prof Paul Kenny first get introduced in the film?

A On the roof top holding rats

7. 0.49.54 Did the rats on the high fat diets consume different energy compared to their usual diet?

A No similar energy intake

8. 0.54.00 What did Prof Paul Kenny find to be the most powerful macronutrient ratio to impact caloric intake and weight in rats?

A fat to sugar ratio of 50:50

9. 0.56.30 What suggestions did Prof Susan Jebb provide to prevent weight gain and reduce health risks?

A Modest changes to diet

B Remove treats that have become part of our daily diet

C Overall balanced diet

D All of the above*

10. 1.00.00 Who produced and directed the documentary?

A David Stewart

That sugar film

Film Questions 1

1. 0.00.18 What is the opening scene?

A Crop field

2. 00.01.42 What was Damon living off before he went healthy?

A Cigarettes, homemade pizza, sugar*

B Added sugar, takeaways, homemade lasagne

C Cigarettes, bakery lunches, added sugar

D Takeaways, sugar, homemade pizza

3. 00.02.33 How much sugar does the average Australian family of four eat per week?

A 6kg

4. 0.03.12 When the film title was introduced, what was the film title made out of?

A Cereal boxes

5. 0.07.07 How much sugar does the average Australian eat per day?

A 30 tsp

B 40 tsp*

C 20 tsp

D 50 tsp

6. 0.10.07 What colour were Damon's knickers when he was getting his biochemical test?

A yellow/ mustard

7. 0.15.30 For Damon's breakfast how many teaspoons of sugar was he having (including the juice)?

A 17 tsp

B 25 tsp

C 20 tsp*

D 15 tsp

8. 0.19.00 How much weight did Damon gain in the first 12 days of his high sugar diet?

A 3.2 kg

9. 0. 27.22 What was the first symptom that Damon noticed while on the sugar diet?

A Mood swings, feel good for 45min then he felt like he was having a sugar crash.

10. 00.29.09 What was the name of the psychologist?

A Dr Nick Allen

That sugar film

Film Questions 2

1. 0.31.29 Where was Coca Cola's highest selling region per capita in the world in 2008?

A Australia's northern territory.

2. 0.34.30 What was the name of the programme that was introduced to ensure Aborigines were introduced to fresh produce?

A MAI WIRU

3. 0.35.01 When Damon tested whether Power aid would make him 'crazy', what animal was watching him?

A Camels

4. 0.36.00 Why did the Aboriginal program end?

A The community fought back for the sugary foods

B Sugar consumption didn't decline

C Government cut funding*

D All of the above

5. What colour was the colour of John's car that Damon was being driven around in while in the Aboriginal community?

A White

6. 0.40.24 When talking to the nutritionist Sharon Johnson, Damon says he is?

A Eating less than normal

B Eating the same amount as usual

C Drinking more often than usual

D Eating a lot more than usual

7. 0.41.18 What did the t shirt that Damon's partner gave him, have written on the front?

A Sugar daddy

8. 0.47.08 The dentist Larry who drives around fixing teeth has a specific name for the brown decay around children's front teeth?

A Mountain Dew mouth

9. 0.49.45 How many teeth is Larry going to have extracted?

A 26*

B 14

C 25

D 18

10. 0.55.00 What does the food industry call the optimum amount of added sugar in a product?

A Bliss point

That sugar film

Film Questions 3

1. 1.01.06 What neurotransmitter is released when a sugary cue is presented?

A Dopamine

2. 1.03.00 How does the food industry think people should control their weight?

A Take personal responsibility

3. 1.06.58 How much sugar is global trade worth?

A 50 Billion dollars

4. 1.08.57 What brand of cigarettes did Barney offer Fred?

A Lucky strike

B Malboro

C Benson and Hedges

D Winston*

5. 1.13.30 On the sugar diet Damon says his fuse is?

A longer

B Thinner

C Lower

D Shorter *

6. 1.17.09 What was the name of the delicatessen on the advertisement in Times Square?

A Roxy

7. 1.20.00 Did Damon’s triglycerides change following the diet, if so what way?

A They doubled *

B They halved

C They stayed the same

D They tripled

8. 1.21.50 What does the film state that pimples are commonly related to?

A Liver function

9. 1.25.20 How long does it take for the desire of sugar to disappear?

A 2-4 weeks

10. 1.33.55 What was the closing scene?

A Him in a mall on an elevator singing and dancing

10.7 Appendix G: Word recall lists

Word list 1 Trial 1

	Test 1	**Test 2**	**Test 3**
1	Diamond	Pistol	Pan
2	Rifle	Sugar	Diesel
3	Vanilla	Shoes	Robin
4	Socks	Potato	Golf
5	Spinach	House	Cow
6	Hut	Pot	Rum
7	Knife	Kerosene	Harmonica
8	Oil	Eagle	Saw
9	Canary	Soccer	Engineer
10	Tennis	Lion	Pencil
11	Horse	Beer	Foot
12	Wine	Violin	Father
13	Trumpet	Wrench	Acorn
14	Saw	Dentist	Tablet
15	Engineer	Pearl	Ruby
16	Tractor	Bomb	Whiskey
17	Ocean	Garlic	Drum
18	Cola	Pants	Screwdriver
19	Rice	Lettuce	Teacher
20	Sapphire	Apartment	Opal
21	Gun	Spoon	Sword

22	Cinnamon	Gasoline	Garlic
23	Skirt	Sparrow	Pants
24	Carrot	Baseball	Lettuce
25	Cave	Tiger	Hotel

Trial 2

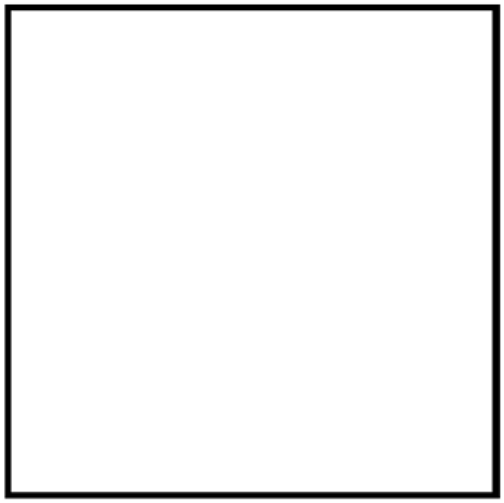
	Test 1	**Test 2**	**Test 3**
1	Hammer	flute	bourbon
2	Lawyer	Chisel	Clarinet
3	Football	Crow	Coal
4	Tent	Corn	Shirt
5	Arrow	Emerald	Wolf
6	Bluebird	Electricity	Spatula
7	Cucumber	Celery	Scarf
8	Hat	Cape	Arrow
9	Butter	Cheese	Lawyer
10	Jade	Amber	Iron
11	Table	Chair	Torch
12	Bowl	Stove	Jug
13	Tui	Duck	Falcon
14	Rugby	Skate	Bike
15	Spider	Shark	Sheep
16	Coffee	Tea	Juice
17	Guitar	Piano	Recorder
18	Trowel	Shovel	Camera
19	Accountant	Judge	Police
20	Pasta	toe	peanut
21	pill	fern	fence
22	butterfly	carpet	flute

23	Rose	file	Corn
24	Grass	Laptop	Stove
25	Ice	Umbrella	Window

10.8 Appendix H: Reitan's Trail Making Part B

Trail Making

The following pages contain the forms used in the trail making test. If your pages are thick and you can't see through to images on the reverse side, you can print pages 3-6 back to back so that slides 3 and 4 are on one sheet and slides 5 and 6 are on a second sheet. Otherwise, print single side. Your printer may complain about the margins. Go ahead and print. So long as the image does not clip, you are OK. Scaling the printout is problematic as the Trail Making test is normed for set dimensions between the numbers.



To check that your copy machine scales the Trails printout correctly, the box above should measure 3x3 inches when printed.

Trail Making Instructions

Follow these instructions exactly as the time includes the time for the instructor to correct errors made by the subject.

Equipment: Trail Making forms, pen or pencil, stopwatch

1. Using the Trail Making Part A SAMPLE, demonstrate the test to the subject. "On this page are numbers. Begin at number 1 and draw a line to 2, then to 3, then to 4 and so on until you reach End. without lifting your pencil from the paper. You should draw the lines as fast as you can. Like this." (demonstrate on the Sample)

2. Give subject pen or pencil and Trail Making Part A. "Now it is your turn. Do you have any questions? Ready. Begin."

3. Time the subject. Stop the subject if an error is made and return subject to last correct circle. The clock keeps running during corrections, but the subject should not be penalized if the examiner takes too long to explain the error. If the subject misses a circle, remind subject to touch all circles, but do not stop the subject. Stop the clock when End is reached.

4. Write time in seconds on the form and. Write subject number and date on the form

5. Using the Trail Making Part B SAMPLE, demonstrate the test to the subject. "This time the page has both letters and numbers. Begin at number 1 and draw a line to the letter A, then to the number 2, then to the letter B and so on until you reach End. without lifting your pencil from the paper. You should draw the lines as fast as you can. Like this." (demonstrate on the Sample)

6. Give subject pen or pencil and Trail Making Part B. "Now it is your turn. Do you have any questions? Ready. Begin."

7. Time the subject, correcting errors along the way. Stop the clock when End is reached. Write time in seconds on the form. Write subject number and date on the form.

8. Enter Trail Making times on the Clinical Evaluation Data Collection Form

Scoring

	Average	Deficient	Rule of Thumb
Trail A	29 seconds	> 78 seconds	Most in 90 seconds
Trail B	75 seconds	> 273 seconds	Most in 180 seconds

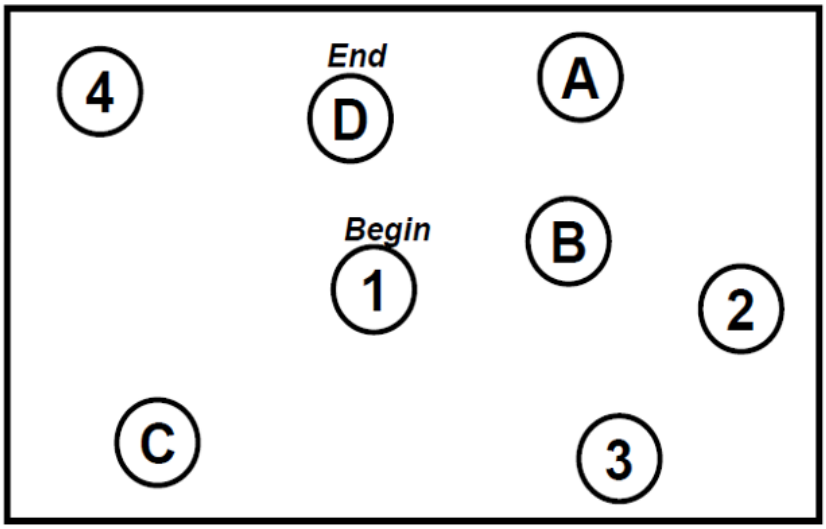
Citations

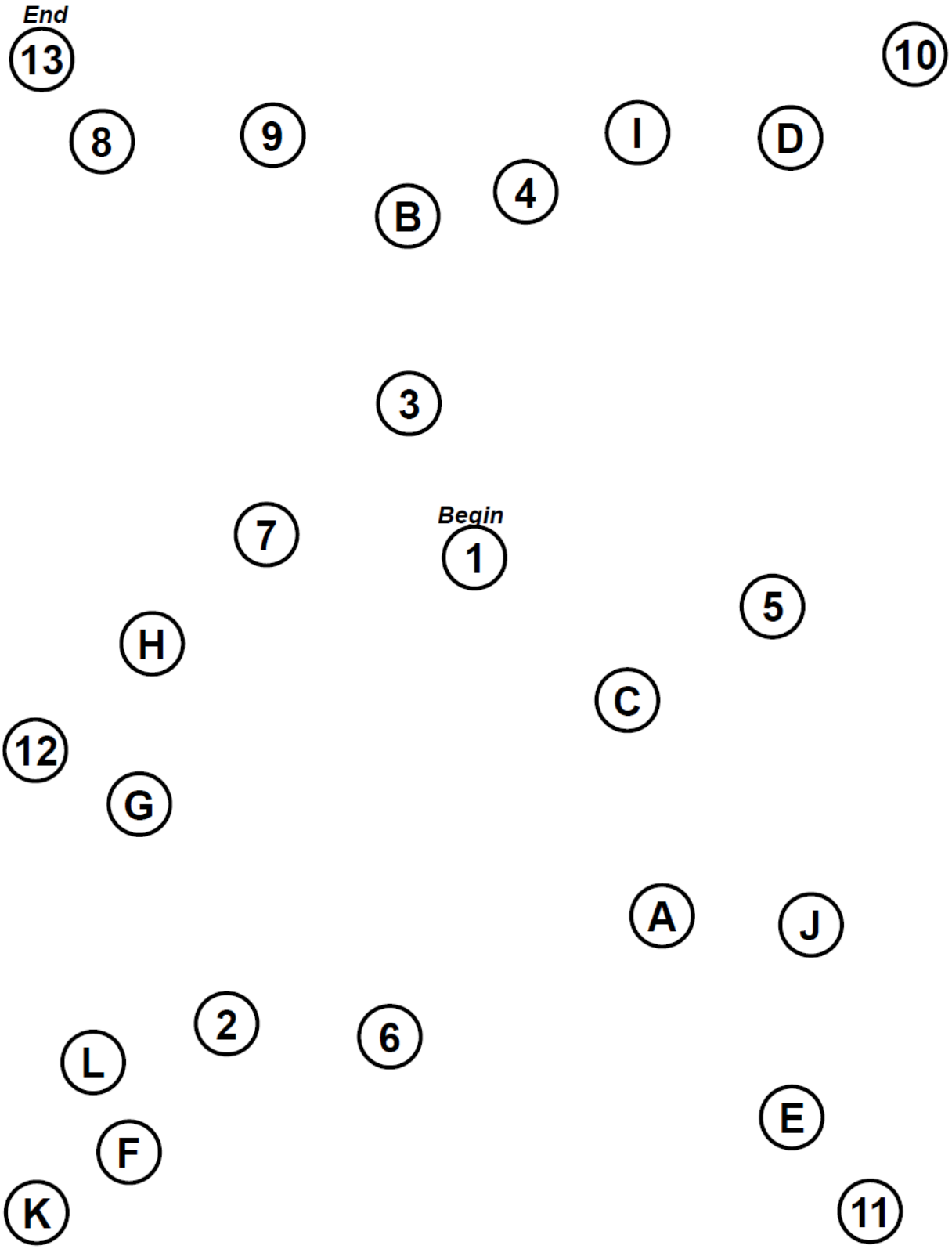
1. Reitan RM. Validity of the Trail Making test as an indicator of organic brain damage. Percept Motor Skills 1958; 8: 271-276.
2. Lezak MD (1995) Neuropsychological assessment, 3rd edn. New York: Oxford University Press.
3. Corrigan JD, Hinkeldey MS. Relationships between Parts A and B of the Trail Making Test. J Clin Psychol 1987;43:402–9.

TRAIL MAKING

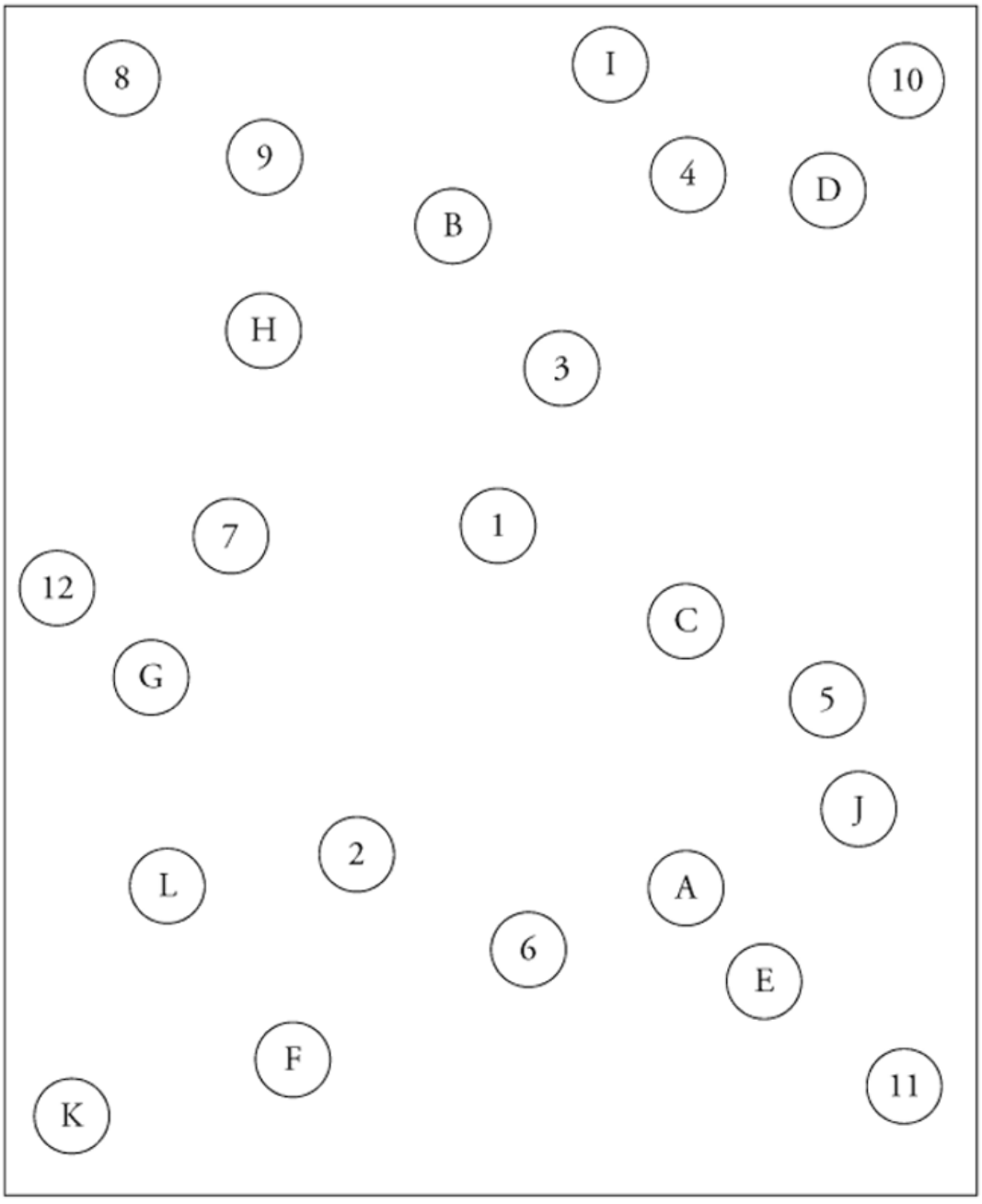
Part B

SAMPLE



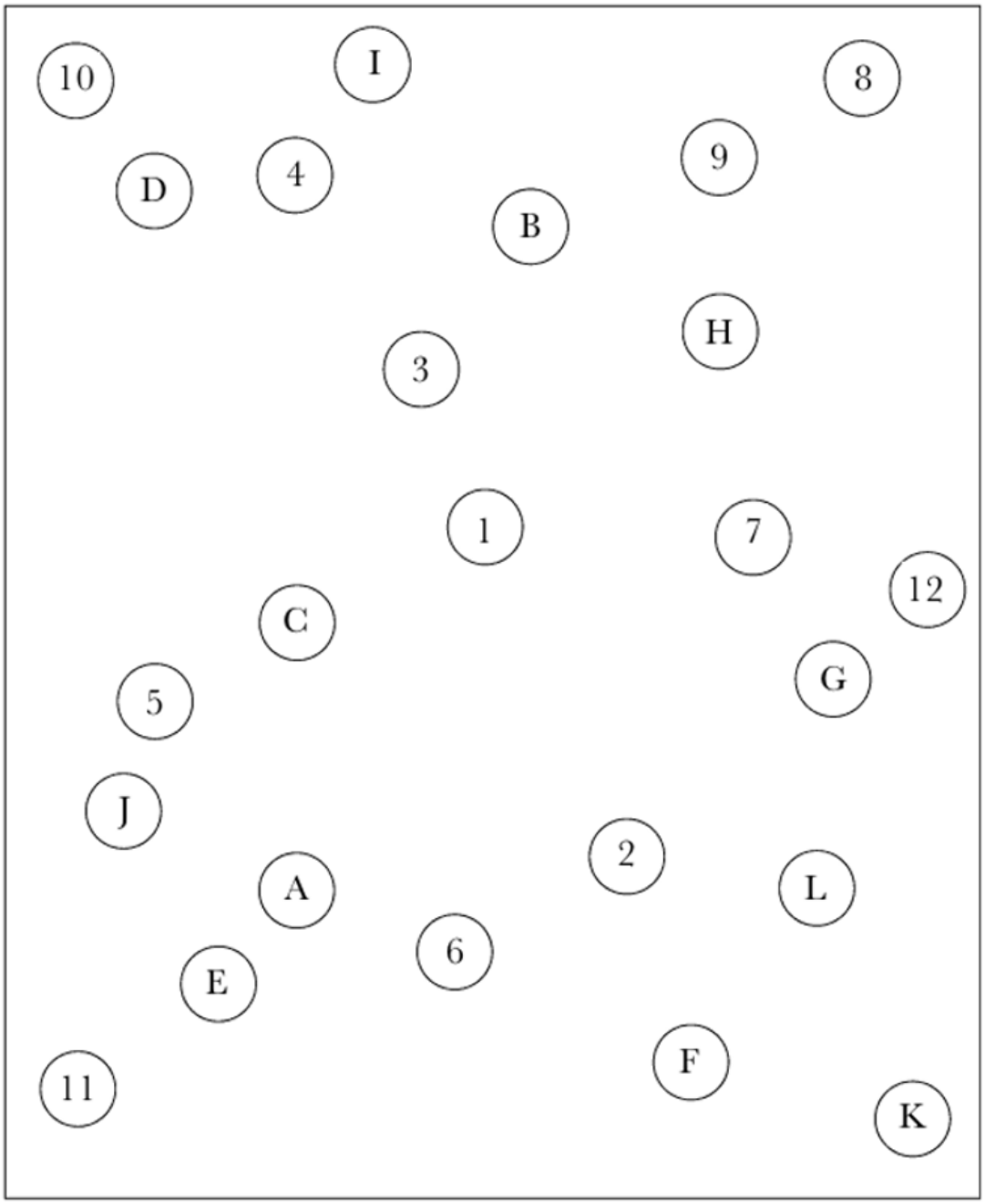

End
13
10
8
9
I
D
4
B
3
7
Begin
1
5
H
C
12
G
A
J
2
6
L
E
F
K
11

Trail Making Test **60 minutes only**



(marker only) Score _____

Trail Making Test **60 minutes only**



(marker only) Score _____

10.9 Appendix I: Blood glucose and insulin control

GLUC3

cobas®

HK
≥ 300 µkat/L; G-6-PDH (E. coli): ≥ 300 µkat/L; preservative

R1 is in position B and R2 is in position C.

Precautions and warnings
For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Safety data sheet available for professional user on request.
Disposal of all waste material should be in accordance with local guidelines.

Reagent handling
Ready for use.

Storage and stability

GLUC3

Shelf life at 2-8 °C:	See expiration date on cobas c pack label.
On-board in use and refrigerated on the analyzer: *Diluent NaCl 9 %*	8 weeks
Shelf life at 2-8 °C:	See expiration date on cobas c pack label.
On-board in use and refrigerated on the analyzer:	12 weeks

Specimen collection and preparation
For specimen collection and preparation, only use suitable tubes or collection containers.
Only the specimens listed below were tested and found acceptable.

Serum.
Plasma: Li-heparin, K_2-EDTA, NaF/Na_2EDTA, KF/Na_2EDTA, NaF/K-Oxalate. Collect blood by venipuncture from fasting individuals using an evacuated tube system. The stability of glucose in specimens is affected by storage temperature, bacterial contamination, and glycolysis. Plasma or serum samples without preservative (NaF) should be separated from the cells or clot within half an hour of being drawn. When blood is drawn and permitted to clot and to stand uncentrifuged at room temperature, the average decrease in serum glucose is ~ 7 % in 1 hour (0.28 to 0.56 mmol/L or 5 to 10 mg/dL). This decrease is the result of glycolysis. Glycolysis can be inhibited by collecting the specimen in fluoride tubes.[1]

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect Glucose the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Stability (no hemolysis):[5] 8 hours at 15-25 °C
72 hours at 2-8 °C

Stability in fluoride plasma:[6] 3 days at 15-25 °C

Urine
Collect urine in a dark bottle. For 24-hour urine collections, glucose may be preserved by adding 5 mL of glacial acetic acid to the container before collection. Unpreserved urine samples may lose up to 40 % of their glucose after 24-hour storage at room temperature.[3] Therefore, keep samples on ice during collection.[5]

CSF
Cerebrospinal fluid may be contaminated with bacteria and often contains other cellular constituents. CSF samples should therefore be analyzed for glucose immediately or stored at 4 °C or -20 °C.[3,5]

Centrifuge samples containing precipitates before performing the assay.

Materials provided
See "Reagents - working solutions" section for reagents.

Materials required (but not provided) See "Order information" section. General laboratory equipment

Assay
For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions. The performance of applications not validated by Roche is not warranted and must be defined by the user.

Application for serum, plasma, urine and CSF
cobas c 311 test definition

Assay type	2 Point End		
Reaction time / Assay points	10 / 6-32 (STAT 7 / 6-32)		
Wavelength (sub/main)	700/340 nm		
Reaction direction	Increase		
Units	mmol/L (mg/dL, g/L)		
Reagent pipetting		Diluent (H_2O)	
R1	28 µL	141 µL	
R2	10 µL	20 µL	
Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	2 µL	–	–

HK

Decreased	10 µL	15 µL	135 µL
Increased cobas c 501/502 test definition	4 µL	–	–
Assay type	2 Point End		
Reaction time / Assay points	10 / 10-47 (STAT 7 / 10-47)		
Wavelength (sub/main)	700/340 nm		
Reaction direction	Increase		
Units	mmol/L (mg/dL, g/L)		
Reagent pipetting		Diluent (H_2O)	
R1	28 µL	141 µL	
R2	10 µL	20 µL	
Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	2 µL	–	–
Decreased	10 µL	15 µL	135 µL
Increased	4 µL	–	–

Calibration	
Calibrators	S1: H_2O S2: C.f.a.s.
Calibration mode	Linear
Calibration frequency	2-point calibration

- after reagent lot change
- as required following quality control procedures

Traceability: This method has been standardized against ID/MS.

Quality control

For quality control, use control materials as listed in the "Order information" section.

In addition, other suitable control material can be used. The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

Follow the applicable government regulations and local guidelines for quality control.

Calculation

Roche/Hitachi cobas c systems automatically calculate the analyte concentration of each sample.

Conversion factors: mmol/L x 18.02 = mg/dL mmol/L x 0.1802 = g/L mg/dL x 0.0555 = mmol/L

Limitations - interference

Criterion: Recovery within ± 10 % of initial value at a glucose concentration of 3.9 mmol/L (70.3 mg/dL).

Serum/plasma

Icterus:[7] No significant interference up to an I index of 60 (approximate conjugated and unconjugated bilirubin concentration: 60 mg/dL or 1026 µmol/L). Hemolysis:[7] No significant interference up to an H index of 1000 (approximate hemoglobin concentration: 1000 mg/dL or 621 µmol/L).

Lipemia (Intralipid):[7] No significant interference up to an L index of 1000. There is poor correlation between the L index (corresponds to turbidity) and triglycerides concentration.

Drugs: No interference was found at therapeutic concentrations using common drug panels.[8,9]

In very rare cases, gammopathy, in particular type IgM (Waldenström's macroglobulinemia), may cause unreliable results.

Urine

Drugs: No interference was found at therapeutic concentrations using common drug panels.[9]

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

NOTE: Glucose values achieved on some proficiency testing materials, when evaluated against a glucose oxidase-oxygen electrode comparison method, demonstrate an approximate 3 % positive bias on average.

ACTION REQUIRED

Special Wash Programming: The use of special wash steps is mandatory when certain test combinations are run together on Roche/Hitachi cobas c systems. The latest version of the carry-over evasion list can be found with the NaOHD/SMS/Multiclean/SCCS or the NaOHD/SMS/SmpCln1 + 2/SCCS Method Sheets. For further instructions refer to the operator's manual. cobas c 502 analyzer: All special wash programming necessary for avoiding carry-over is available via the cobas link, manual input is not required. Where required, special wash/carry-over evasion programming must be implemented prior to reporting results with this test.

Limits and ranges

Measuring range Serum, plasma, urine and CSF

0.11-41.6 mmol/L (2-750 mg/dL)

Determine samples having higher concentrations via the rerun function. Dilution of samples via the rerun function is a 1:2 dilution. Results from samples diluted by the rerun function are automatically multiplied by a factor of 2.

Glucose

Lower limits of measurement

Lower detection limit of the test

0.11 mmol/L (2 mg/dL)

The lower detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying three standard deviations above that of the lowest standard (standard 1 + 3 SD, repeatability, n = 21).

Expected values

Plasma[10]

Fasting	4.11-6.05 mmol/L	(74-109 mg/dL)
Urine[11]		
1st morning urine	0.3-1.1 mmol/L	(6-20 mg/dL)
24-hour urine	0.3-0.96 mmol/L (average of 1350 mL urine/24 h)	(6-17 mg/dL)
acc. to Tietz:[5]		
Serum, plasma		
Adults	4.11-5.89 mmol/L	(74-106 mg/dL)
60-90 years	4.56-6.38 mmol/L	(82-115 mg/dL)
> 90 years	4.16-6.72 mmol/L	(75-121 mg/dL)

Children	3.33-5.55 mmol/L	(60-100 mg/dL)
Neonates (1 day)	2.22-3.33 mmol/L	(40-60 mg/dL)
Neonates (> 1 day)	2.78-4.44 mmol/L	(50-80 mg/dL)
Urine		
24-hour urine	< 2.78 mmol/24 h	(< 0.5 g/24 h)
Random urine	0.06-0.83 mmol/L	(1-15 mg/dL)
CSF		
Children	3.33-4.44 mmol/L	(60-80 mg/dL)
Adults	2.22-3.89 mmol/L	(40-70 mg/dL)

CSF glucose values should be approximately 60 % of the plasma values and must always be compared with concurrently measured plasma values for adequate clinical interpretation.

Roche has not evaluated reference ranges in a pediatric population. Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Specific performance data

Representative performance data on the analyzers are given below. Results obtained in individual laboratories may differ.

Precision

Precision was determined using human samples and controls in an internal protocol. *Serum/plasma:* Repeatability* (n = 21), intermediate precision** (3 aliquots per run, 1 run per day, 21 days); *urine/CSF:* Repeatability* (n = 21), intermediate precision** (3 aliquots per run, 1 run per day, 10 days). The following results were obtained: *Serum/plasma*

*Repeatability**	*Mean*	*SD*	*CV*
	mmol/L (mg/dL)	*mmol/L (mg/dL)*	*%*
Precinorm U	5.49 (98.9)	0.05 (0.9)	1.0
Precipath U	13.6 (245)	0.1 (2)	0.9
Human serum 1	7.74 (139)	0.05 (1)	0.7
Human serum 2	5.41 (97.5)	0.04 (0.7)	0.7
Intermediate	*Mean*	*SD*	*CV*
*precision***	*mmol/L (mg/dL)*	*mmol/L (mg/dL)*	*%*
Precinorm U	5.38 (96.9)	0.07 (1.3)	1.3
Precipath U	13.4 (241)	0.2 (2)	1.1
Human serum 3	7.61 (137)	0.09 (2)	1.2
Human serum 4	5.28 (95.1)	0.06 (1.1)	1.1
Urine			
*Repeatability**	*Mean*	*SD*	*CV*
	mmol/L (mg/dL)	*mmol/L (mg/dL)*	*%*
Control level 1	1.54 (27.8)	0.02 (0.4)	1.1
Control level 2	15.7 (283)	0.1 (2)	0.9
Human urine 1	5.00 (90.1)	0.05 (0.9)	1.0
Human urine 2	10.5 (189)	0.1 (2)	1.1
Intermediate	*Mean*	*SD*	*CV*
*precision***	*mmol/L (mg/dL)*	*mmol/L (mg/dL)*	*%*
Control level 1	1.51 (27.2)	0.01 (0.2)	1.0
Control level 2	15.4 (278)	0.1 (2)	0.8
Human urine 3	4.86 (87.6)	0.05 (0.9)	1.0
Human urine 4	10.3 (186)	0.1 (2)	0.8
CSF			
*Repeatability **	*Mean*	*SD*	*CV*
	mmol/L (mg/dL)	*mmol/L (mg/dL)*	*%*
Precinorm U	5.43 (97.8)	0.04 (0.7)	0.8
Precipath U	13.6 (245)	0.1 (2)	0.8
Human CSF 1	3.04 (54.8)	0.03 (0.5)	0.9
Human CSF 2	8.43 (152)	0.08 (1)	1.0
Intermediate	*Mean*	*SD*	*CV*
*precision***	*mmol/L (mg/dL)*	*mmol/L (mg/dL)*	*%*
Precinorm U	5.37 (96.8)	0.07 (1.3)	1.3
Precipath U	13.4 (241)	0.2 (4)	1.1
Human CSF 3	3.00 (54.1)	0.04 (0.7)	1.5
Human CSF 4	8.30 (150)	0.10 (2)	1.2

* repeatability = within-run precision

** intermediate precision = total precision / between run precision / between day precision

Method comparison

Glucose values for human serum, plasma, urine and CSF samples obtained on a Roche/Hitachi cobas c 501 analyzer (y) were compared with those determined using the same reagent on a Roche/Hitachi MODULAR P analyzer (x).

Serum/plasma

Sample size (n) = 75

Passing/Bablok[12] Linear regression y = 1.000x + 0.118 mmol/L
y = 0.996x + 0.179 mmol/L
τ = 0.983 r = 1.000

The sample concentrations were between 1.64 and 34.1 mmol/L (28.8 and 614 mg/dL).

Urine

Sample size (n) = 75

Passing/Bablok[12] Linear regression y = 1.000x + 0.060 mmol/L
y = 1.001x + 0.045 mmol/L
τ = 0.972 r = 1.000

The sample concentrations were between 0.16 and 39.5 mmol/L (2.88 and 712 mg/dL).

CSF

Sample size (n) = 75

Passing/Bablok[12] Linear regression y = 1.000x - 0.020 mmol/L y = 1.001x - 0.038 mmol/L

τ = 0.980 r = 1.000

The sample concentrations were between 0.92 and 38.0 mmol/L (16.6 and 685 mg/dL).

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Passing/Bablok[10] Linear regression y = 0.89x - 0.62 y = 0.93x - 1.02

τ = 0.935 r = 0.981

The sample concentrations were between approx. 1 and 118 µU/mL (approx. 7 and 820 pmol/L).

Analytical specificity

For the monoclonal antibodies used, the following cross-reactivities were found:

f) n.d. = not detectable

Results for cross-reactivity with recombinant insulin analogs in a number of insulin methods have been published for example by two groups in France and the USA.[9,11,12] The following results were published by Owen et al.[11] for the Elecsys Insulin assay:

Insulin lispro, insulin aspart, and insulin glargine were each tested in concentrations of 30, 100, 300, and 1000 mIU/L in the absence of insulin. The results obtained were below the detection limit of the Elecsys Insulin assay (< 0.2 µU/mL or < 1.39 pmol/L) at all the concentrations tested. Moreover, these results also correlate with those published earlier by Sapin et al. for insulin lispro.[9] References

1. Lang DA, Matthews DR, Peto J, et al. Cyclic oscillations of basal plasma glucose and insulin concentrations in human beings. N Engl J Med 1979;301:1023-1027.
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5. Clark PM. Assays for insulin, proinsulin(s) and C-peptide. Ann Clin Biochem 1999;36(5):541-564.

	Substance tested	% cross reactivity
Bovine insulin	17360 pmol/L	25.0
Porcine insulin	8334 pmol/L	19.2
Human proinsulin	1000 ng/mL	0.05
C-peptide	100 ng/mL	n.d.[f]
Glucagon	1000 pg/mL	n.d.
Somatostatin	100 pg/mL	n.d.
Insulin-like growth factor I	6579 pmol/L	0.04

6. Cheyenne D, Letailleur A, Trivin F, et al. Effect of Hemolysis on the Concentration of Insulin in Serum Determined by RIA and IRMA. Clin Chem 1998;44(2):354-356.
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For further information, please refer to the appropriate operator's manual for the analyzer concerned, the respective application sheets, the product information, and Method Sheets of all necessary components.

Elecsys and cobas e analyzers

Insulin

cobas®

Store the Elecsys Insulin reagent kit upright in order to ensure complete availability of the microparticles during automatic mixing prior to use.

Stability:

unopened at 2-8 °C	up to the stated expiration date
after opening at 2-8 °C	12 weeks
on the analyzers	4 weeks

Specimen collection and preparation

Only the specimens listed below were tested and found acceptable. Serum collected using standard sampling tubes or tubes containing separating gel.

Li-heparin, K_3-EDTA, and sodium citrate plasma. Hemolysis interferes, as insulin-degrading peptidases are released from erythrocytes.[6]

Criterion: Recovery within 90-110 % of serum value or slope 0.9-1.1 + intercept within < ± 2 x analytical sensitivity (LDL) + coefficient of correlation > 0.95. Stable for 24 hours at 2-8 °C, 6 months at -20 °C. Freeze only once.[7] The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer. Centrifuge samples containing precipitates before performing the assay. Do not use heat-inactivated samples. Do not use samples and controls stabilized with azide.

Insulin

Ensure the samples, calibrators, and controls are at ambient temperature (20-25 °C) before measurement.

Due to possible evaporation effects, samples, calibrators, and controls on the analyzers should be analyzed/measured within 2 hours.

Materials provided

See "Reagents - working solutions" section for reagents.

Materials required (but not provided)

- REF 12017504122, Insulin CalSet, for 4 x 1 mL
- REF 05341787190, PreciControl Multimarker, for 3 x 2 mL each of PreciControl Multimarker 1 and 2 or
  REF 11731416190, PreciControl Universal, for 2 x 3 mL each of PreciControl Universal 1 and 2
- REF 11731416160, PreciControl Universal, for 2 x 3 mL each of PreciControl Universal 1 and 2 (for USA) or
  REF 05341787160, PreciControl Multimarker, for 3 x 2 mL each of PreciControl Multimarker 1 and 2 (for USA)

General laboratory equipment

- Elecsys 2010, MODULAR ANALYTICS E170 or cobas e analyzer

Accessories for Elecsys 2010 and cobas e 411 analyzers:

- REF 11732988122, ProCell, 6 x 380 mL system buffer
- REF 11732970122, CleanCell, 6 x 380 mL measuring cell cleaning solution
- REF 11732346122, Elecsys SysWash, 1 x 500 mL washwater additive
- REF 11731159001, Adapter for SysClean
- REF 11706802001, Elecsys 2010 AssayCup, 60 x 60 reaction vessels
- REF 11706799001, Elecsys 2010 AssayTip, 30 x 120 pipette tips

Accessories for MODULAR ANALYTICS E170, cobas e 601 and cobas e 602 analyzers:

- REF 04880340190, ProCell M, 2 x 2 L system buffer
- REF 04880293190, CleanCell M, 2 x 2 L measuring cell cleaning solution
- REF 03023141001, PC/CC-Cups, 12 cups to prewarm ProCell M and CleanCell M before use
- REF 03005712190, ProbeWash M, 12 x 70 mL cleaning solution for run finalization and rinsing during reagent change
- REF 12102137001, AssayTip/AssayCup Combimagazine M, 48 magazines x 84 reaction vessels or pipette tips, waste bags
- REF 03023150001, WasteLiner, waste bags
- REF 03027651001, SysClean Adapter M

Accessories for all analyzers:

- REF 11298500316, Elecsys SysClean, 5 x 100 mL system cleaning solution
- REF 11298500160, Elecsys SysClean, 5 x 100 mL system cleaning solution (for USA)

Assay

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions. Resuspension of the microparticles takes place automatically prior to use. Read in the test-specific parameters via the reagent barcode. If in exceptional cases the barcode cannot be read, enter the 15-digit sequence of numbers.

Bring the cooled reagents to approx. 20 °C and place on the reagent disk (20 °C) of the analyzer. Avoid foam formation. The system automatically regulates the temperature of the reagents and the opening/closing of the bottles.

Calibration

Traceability: This method has been standardized using the 1st IRP WHO Reference Standard 66/304 (NIBSC).

Every Elecsys Insulin reagent set has a barcoded label containing the specific information for calibration of the particular reagent lot. The predefined master curve is adapted to the analyzer by using the Insulin CalSet.

Calibration frequency: Calibration must be performed once per reagent lot using fresh reagent (i.e. not more than 24 hours since the reagent kit was registered on the analyzer). Renewed calibration is recommended as follows:

- after 1 month (28 days) when using the same reagent lot
- after 7 days (when using the same reagent kit on the analyzer)
- as required: e.g. quality control findings outside the defined limits

Quality control

For quality control, use PreciControl Multimarker or PreciControl Universal.

Other suitable control material can be used in addition.

Controls for the various concentration ranges should be run individually at least once every 24 hours when the test is in use, once per reagent kit, and following each calibration. The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits.

Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

Follow the applicable government regulations and local guidelines for quality control.

Please note: Commercial controls may contain insulin of animal origin. When assessing results, the corresponding cross-reactivity of this test must be taken into account; see under "Analytical specificity".

Calculation

The analyzer automatically calculates the analyte concentration of each sample (either in µU/mL or pmol/L).

Conversion factors: µU/mL x 6.945 = pmol/L
pmol/L x 0.144 = µU/mL

Limitations - interference

REF

REF

Insulin

cobas®

The assay is unaffected by icterus (bilirubin < 1539 µmol/L or < 90 mg/dL), lipemia (Intralipid < 1800 mg/dL) and biotin (< 246 nmol/L or < 60 ng/mL).

Criterion: Recovery within ± 10 % of initial value. Hemolysis interferes. Samples should not be taken from patients receiving therapy with high biotin doses (i.e. > 5 mg/day) until at least 8 hours following the last biotin administration.

No interference was observed from rheumatoid factors up to a concentration of 18900 IU/mL.

There is no high-dose hook effect at insulin concentrations up to 20000 µU/mL or 138900 pmol/L.

In vitro tests were performed on 20 commonly used pharmaceuticals. No interference with the assay was found.

Samples from patients treated with bovine, porcine or human insulin sometimes contain anti-insulin antibodies which can affect the test results.[2,8,9] In rare cases, interference due to extremely high titers of antibodies to analyte-specific antibodies, streptavidin or ruthenium can occur. These effects are minimized by suitable test design.

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

Limits and ranges

Measuring range

0.2-1000 µU/mL or 1.39-6945 pmol/L (defined by the lower detection limit and the maximum of the master curve). Values below the lower detection limit are reported as < 0.2 µU/mL (< 1.39 pmol/L). Values above the measuring range are reported as > 1000 µU/mL (> 6945 pmol/L).

Lower limits of measurement

Lower detection limit of the test

Lower detection limit: 0.2 µU/mL (1.39 pmol/L)

The lower detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying two standard deviations above that of the lowest standard (master calibrator, standard 1 + 2 SD, repeatability study, n = 21).

Dilution

Not necessary due to the broad measuring range.

Expected values

Studies with the Elecsys Insulin assay conducted in a clinical center in Germany with samples from 57 healthy, fasting individuals gave the following results (5th-95th percentile range): 2.6-24.9 µU/mL (17.8-173 pmol/L)

Status: Elecsys Insulin MCE, study No.: B99P027 of 29 March 2001.

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

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Insulin

Specific performance data

Representative performance data on the analyzers are given below. Results obtained in individual laboratories may differ.

Precision

Precision was determined using Elecsys reagents and pooled human sera in a modified protocol (EP5-A) of the CLSI (Clinical and Laboratory Standards Institute): 6 times daily for 10 days (n = 60); repeatability on MODULAR ANALYTICS E170 analyzer, n = 21. The following results were obtained: Elecsys 2010 and cobas e 411 analyzers

			Repeatability[b]			Intermediate precision		
Sample	Mean		SD		CV	SD		CV
	µU/mL	pmol/L	µU/mL	pmol/L	%	µU/mL	pmol/L	%
HS[c] 1	6.36	44.2	0.122	0.847	1.9	0.163	1.11	2.6
HS 2	20.9	145	0.391	2.71	1.9	0.593	4.10	2.8
HS 3	747	5188	15.1	105	2.0	18.6	129	2.5

b) Repeatability = within-run precision

c) HS = human serum

MODULAR ANALYTICS E170, cobas e 601 and cobas e 602 analyzers

	Repeatability					Intermediate precision				
Sample	Mean		SD		CV	Mean		SD		CV
	µU/mL	pmol/L	µU/mL	pmol/L	%	µU/mL	pmol/L	µU/mL	pmol/L	%
HS 1	5.93	41.2	0.09	0.62	1.5	6.85	47.6	0.336	2.33	4.9
HS 2	14.5	101	0.13	0.92	0.9	16.7	116	0.616	4.28	3.7
HS 3	49.9	346	0.58	4.05	1.2	55.1	383	1.86	12.9	3.4
HS 4	399	2768	3.32	23.1	0.8	425	2949	10.0	69.6	2.4

Precision was determined using Elecsys reagents and controls in a protocol (EP5-A2) of the CLSI (Clinical and Laboratory Standards Institute): 2 runs per day in duplication each for 21 days (n = 84). The following results were obtained: Elecsys 2010 and cobas e 411 analyzers

			Repeatability[d]			Intermediate precision		
Sample	Mean		SD		CV	SD		CV
	µU/mL	pmol/L	µU/mL	pmol/L	%	µU/mL	pmol/L	%
PC MM[e]1	23.7	165	0.270	1.88	1.1	0.834	5.79	3.5
PC MM2	81.7	567	1.14	7.92	1.4	3.04	21.1	3.7

d) Repeatability = within-run precision

e) PC MM = PreciControl Multimarker

MODULAR ANALYTICS E170, cobas e 601 and cobas e 602 analyzers

			Repeatability			Intermediate precision		
Sample	Mean		SD		CV	SD		CV
	µU/mL	pmol/L	µU/mL	pmol/L	%	µU/mL	pmol/L	%
PC MM1	21.9	152	0.712	4.94	3.2	0.926	6.43	4.2
PC MM2	74.3	516	2.72	18.9	3.7	3.42	23.8	4.6

Method comparison

Elecsys and cobas e analyzers 2012-04, V 13 English

a) A comparison of the Elecsys Insulin assay (y) with the Enzymun-Test Insulinmethod (x) using clinical samples gave the following correlations (µU/mL): Number of samples measured: 99

Passing/Bablok[10] Linear regression y = 1.00x - 1.16 y = 0.92x + 0.59

τ = 0.844 r = 0.958

The sample concentrations were between approx. 3.9 and 80 µU/mL (approx. 27 and 550 pmol/L).

b) A comparison of the Elecsys Insulin assay (y) with a commercially available Insulin test (x) using clinical samples gave the following correlations (µU/mL):

Number of samples measured: 99

Passing/Bablok[10] Linear regression y = 0.89x - 0.62 y = 0.93x - 1.02

τ = 0.935 r = 0.981

The sample concentrations were between approx. 1 and 118 µU/mL (approx. 7 and 820 pmol/L).

Analytical specificity

For the monoclonal antibodies used, the following cross-reactivities were found:

f) n.d. = not detectable

Results for cross-reactivity with recombinant insulin analogs in a number of insulin methods have been published for example by two groups in France and the USA.[9,11,12] The following results were published by Owen et al.[11] for the Elecsys Insulin assay:

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	Substance tested	% cross reactivity
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Porcine insulin	8334 pmol/L	19.2
Human proinsulin	1000 ng/mL	0.05
C-peptide	100 ng/mL	n.d.[f]
Glucagon	1000 pg/mL	n.d.
Somatostatin	100 pg/mL	n.d.
Insulin-like growth factor I	6579 pmol/L	0.04

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Elecsys and cobas e analyzers

Insulin

Insulin

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