III METHODOLOGY

The methodology of the study entitled, “Determination of Glycemic Index of Selected Foods and Formulation of Low Glycemic Index Food Products” was carried out in five phases:

PHASE 3A: Glycemic index determination of commonly consumed carbohydrate containing foods and composite meals

PHASE 3B: Determination of Amylose and Amylopectin of Selected Rice Varieties

PHASE 3C: Profiling of glycemic index of rice samples using High Resolution Melting (HRM) technology

PHASE 3D: Formulation of Low Glycemic Index Food Products

PHASE 3E: Assessment of Knowledge and Awareness of Glycemic Concept and Development of Educational Tools on GI
PHASE 3A: Glycemic index determination of commonly consumed carbohydrate containing foods and composite meals

The flow chart of the methodology (Phase A) is given in the flow chart below (Figure1).

3A.1. Reference, Test Foods and Reagents

Thirty different varieties of test foods were tested for GI. A 50-g dextrose anhydrous load was used as the reference. All the test food samples were prepared as per instructions on the packaging or as they are usually prepared for consumption. The portion size of each test food contained 50g available carbohydrate (ACHO) defined as total carbohydrate minus dietary fibre.

3A.2. Methods - In-vivo Glycemic Index Determination

Glycemic Index of the foods and composite meals were tested in-vivo using accredited GI test method.

3A.2.1. Eligibility for Participation in the Study

Sixty healthy volunteers aged between 21 and 60 inclusive with no known medical condition and otherwise who fulfilled the inclusion and exclusion criteria were recruited for the study.

Inclusion criteria

- Subjects must be healthy males or non-pregnant females at least 6 weeks postpartum, non-lactating and aged 21-60yrs old in both groups.
- They should not suffer from any chronic diseases.
- No known food allergy or intolerance.
- No medications known to affect glucose tolerance (excluding oral contraceptives).
- Note: stable doses of oral contraceptives, acetylsalicyclic acid, thyroxin, vitamins and mineral supplements or drugs to treat hypertension or osteoporosis are acceptable.

Exclusion criteria

- Age less than 21 years old and greater than 60 years old.
• Known history of diabetes mellitus or the use of antihyperglycemic drugs or insulin to treat diabetes and related conditions.
• Known history of AIDS, hepatitis, renal or heart disease or any other serious complications that may interfere with glucose metabolism.
• Subjects using any medication (eg steroids, protease inhibitors or antipsychotics, etc.) that would interfere with the digestion and nutrient absorption,
• Subjects having gastrointestinal diseases that may interfere with nutrient absorption, distribution, metabolism, excretion and have no known food allergies.
• A major medical or surgical event requiring hospitalisation within the preceding 3 months.

3A.2.2. Study Protocol

Healthy volunteers with normal glucose tolerance took part in the study. Each of the test food samples were tested in minimum 10-12 subjects. The inclusion of at least 10 subjects will provide a reasonable degree of power and precision for measuring GI (Brouns et al., 2007). Their ages ranged from 21 to 44 y and their body mass index (BMI; in kg/m2) from 18.5 to 25. The subjects took 50 g available carbohydrate portions of each of the test food sample in random order on separate mornings after an overnight fast. Foods were consumed over 12 -15 minutes. Finger-prick capillary blood samples were taken at -5, 0 (fasting), 15, 30, 45, 60, 90, and 120 minutes after the test food/meal was consumed. Safe-T-Pro lancets were used to puncture the finger sites and capillary blood was drawn and transferred into minicollect tubes which were coated with an anticoagulant.

The reference food was tested three times and the test food was tested only once. The Incremental area under the curve for test and reference foods were analysed and the GI values were calculated by expressing each subject’s AUC of the test food as a percentage of the same subject’s mean reference AUC. The mean of the resulting values was the GI of the test food. If any individual subject’s GI value for a food sample fell outside the group mean (average) GI value plus or minus two standard deviations the value was classified as an outlier (unusual observation) and removed from the dataset. The group mean was expressed as mean GI. The final GI of the test food was expressed as GI ± S.E.M; where GI is
the mean GI value of all subjects, excluding outliers and S.E.M is the standard error of the mean. Ethics approval was and all the subjects signed an informed consent form.

3A.2.4 Calculation of GI and GL

Glycemic Index of the foods tested was calculated as follows:

\[
\text{Glycemic Index} = \frac{\text{Incremental Area Under Curve (IAUC) of Test Food}}{\text{Incremental Area Under Curve (IAUC) of Reference Food}} \times 100
\]

There are a number of different methods that have been used to calculate the area under the curve. For most glycemic data, the area under the curve has been calculated as the incremental area under the blood glucose response curve (IAUC), ignoring the area beneath the fasting concentration. This can be calculated geometrically by applying the trapezoid rule. When a blood glucose value falls below the baseline, only the area above the fasting is included. It is recommended to calculate GI as the mean of the individual ratios (Brouns et al., 2007).

The Glycemic Load (GL) of the foods tested was calculated as follows:

\[
GL = \frac{\text{GI} \times \text{ACHO present in one serving of the food item}}{100}
\]

Classification of GI and GL

<table>
<thead>
<tr>
<th>Glycemic Index*</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤55.0</td>
<td>56-69</td>
<td>≥70.0</td>
<td></td>
</tr>
<tr>
<td>Glycemic Load**</td>
<td>≤10.0</td>
<td>11-19</td>
<td>≥20.0</td>
</tr>
</tbody>
</table>

(* - Brand-Miller, 2003; ** - Salmeron et al., 1997)

The GI of the composite meals was calculated using the formula proposed by (Wolever, 2006). The GI of individual foods was weighted according to the amount of carbohydrate each food contributed to the composite meal, to estimate the GI of composite meal. The method of calculating meal GI (GI_m) is shown in Table 1 as given below.
Table 1
Method of Calculating Meal GI (GI_M)

<table>
<thead>
<tr>
<th>Food</th>
<th>Food GI</th>
<th>ACHO</th>
<th>Proportion of carbohydrate</th>
<th>Meal GI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>GI_a</td>
<td>g_a</td>
<td>P_a g_a /G</td>
<td>M_a P_a x GI_a</td>
</tr>
<tr>
<td>B</td>
<td>GI_b</td>
<td>g_b</td>
<td>P_b g_b /G</td>
<td>M_b P_b x GI_b</td>
</tr>
<tr>
<td>C</td>
<td>GI_c</td>
<td>g_c</td>
<td>P_c g_c /G</td>
<td>M_c P_c x GI_c</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>G</td>
<td>1.0</td>
<td>GI_M</td>
</tr>
</tbody>
</table>

Ref = (Wolever, 2006)

PHASE 3B: Determination of Amylose and Amylopectin of Selected Rice Varieties

3 B.1. Reference, Test Foods and Reagents

Fourteen different varieties of rice (test foods) were tested. A 50-g dextrose anhydrous load was used as the reference. All the rice samples were prepared as per instructions in a rice cooker. Amylose and amylopectin was analysed using the K-AMYL 07/11 enzyme assay kit (Megazyme, 2011a).

3B.2. Methods

3B2.1. Glycemic Index Determination of rice samples

The GI of the rice samples were determined in Phase A. Please refer to Phase A for the details and protocol.

3B2.2. Determination of amylose and amylopectin

The flow chart of the methodology is presented in Figure 2. A modification of Concavalin A (Con A) method was used (Yun and Matheson, 1990). The rice samples were completely dispersed by heating in dimethyl sulphoxide (DMSO). Lipids were removed by precipitating the starch in ethanol and recovering the precipitated starch. After dissolution of the precipitated sample in an acetate/salt solution, amylopectin was specifically precipitated by the addition of ConA and removed by centrifugation. Amylose in the supernatant was enzymatically hydrolysed to D-glucose, which is analysed using glucose oxidase/peroxidase reagent. The total starch in a separate aliquot is similarly hydrolysed to D-glucose and measured colorimetrically by glucose oxidase/peroxidase. The concentration of amylose in the starch sample is estimated as the ratio of GOPOD absorbance.
at 510nm of the supernatant of the Con A precipitated sample, to that of the total starch sample.

Calculation of amylose content (%)

\[
\text{Amylose, } \% (\text{w/w}) = \frac{\text{Absorbance (Con A supernatant)}}{\text{Absorbance (Total Starch Aliquot)}} \times 66.8
\]

**PHASE 3C: Profiling of glycemic index of rice samples using High Resolution Melting (HRM) technology**

HRM is a new, post-PCR method that can be used to identify variations in nucleic acid sequences. It can be used for genotyping, mutation scanning and sequence matching (Reed, Kent, and Wittwer, 2007). Recently, it is introduced as a closed tube method for mutation scanning and genotyping (Liew et al., 2004). HRM is also known to be sensitive and specific for detecting methylation. It characterizes DNA samples according to their dissociation behavior as they transition from double-stranded DNA (dsDNA) to single-stranded DNA (ssDNA) with increasing temperature (Montgomery, Wittwer, Palais, and Zhou, 2007). The region of interest is amplified using standard PCR techniques. A specialized dsDNA binding dye is used and it is highly fluorescent when bound to dsDNA and fluorescents poorly in the unbound state.

The change in fluorescence can be used to monitor the DNA amplification during PCR. In order to obtain a distinct melting profile, amplified target will be gradually denatured by small increments of the temperature after PCR. As the amplified target denatures, the dye is released and thus resulting in a drop in fluorescence. HRM is a sensitive method which is capable of detecting a single base change between identical nucleotide sequences.

Genome sequences of rice were sourced from gene bank, conserved sequences were located and specific primers were designed to distinguish low, medium and high GI of rice samples.
PHASE 3D: Formulation of Low Glycemic Index Food Products

3D.1. Product concept

The product concept was based on the following criteria:

i. Products those are suitable for all age groups.

ii. Products those are healthier than their market counterparts

iii. Developed products should be tested in an accredited GI testing laboratory.

iv. Products that are most suited for commercialization (this also includes cost effectiveness)

3D.2. Methods

The low GI products that were developed included: Multigrain cookie, Muffins, Fried rice and a Beverage.

A thorough search on the low GI ingredients was carried out using the Sydney University’s GI database (The University of Sydney, 2011). In addition to it information on GI of ingredients were sourced from ingredient suppliers/product specifications. The ingredients were purchased from reliable suppliers, with certificate of analysis (COA).

The glycemic index value of the product formulations were calculated using the method proposed by (Wolever, 2006). Nutrient analysis of the trial formulations were computed using nutrition information on the packaging and Food works nutrient database. Product development trials were conducted if the calculated GI values were below 55 and the computed nutritional information confirmed the formulations to be healthier than their market counterparts. Preliminary sensory analysis and invivo-Glycemic Index testing (only in 5 human subjects) was conducted to ascertain that the developed products are lower in GI and are also palatable. Many product trials were conducted until the product formulations were finalized. Before conducting in-vivo glycemic index testing the available carbohydrate content (digestible or glycemic carbohydrate) of the developed products were analysed. Final sensory evaluation was conducted in atleast 30 subjects using the 7-point hedonic scale
All the rest of the products were tested for their GI only among healthy subjects.

3D.3. In-vivo Glycemic Index Determination of the Developed Food Products

The GI for all the developed products was tested among healthy subjects. Refer to the detailed protocol for GI testing. The GI testing was also conducted among diabetics as there was a keen interest to include the muffins as a part of the hospital menus for diabetics. However, it should be noted that subject characteristics that have been examined specifically and were found to have no significant effect on mean GI include: normal v. diabetic subjects (Jenkins et al., 1983). Subject characteristics do not appear to have a significant effect on mean GI values but the variation of the values may differ in various groups, being highest in individuals suffering from type 1 diabetes. Therefore, for routine testing, healthy human volunteers are recommended (Brouns et al., 2007).

PHASE 3E: Assessment of Knowledge and Awareness of Glycemic Concept and Development of Educational Tools on GI

3E.1. Materials and Methods

3E.1.1. Construction and administration of survey questionnaires

Three different types of questionnaires namely: one for the general public, one for health professionals and another one for food industry representatives were constructed and administered during 2009/10 and 2012/13. All the questionnaires were pre-tested to check for glitches in wording of questions, clarity of instructions and the order of presentation of questions before administration. Pre-testing was conducted systematically with potential respondents using the same method of administration, before finalising the questionnaires. These questionnaires were administered to gather information on the knowledge and awareness of the GI concept. The questions relating of knowledge and awareness about the GI concept, preferred low GI information sources were some of the questions that were kept the same for all the three questionnaires administered. In addition to the common questions, the questionnaire constructed for the health professionals included specific questions about the use of GI concept while counselling, menu planning with regards to disease condition, ranking the importance of the use of GI concept in their daily practice. The questionnaire
administered to food industry representatives included additional questions that targeted on whether they were interested to manufacture new low GI foods.

The survey questionnaires to the public were administered face-to-face and through online survey mode. The online survey was shared in various social media platforms such as Facebook and Twitter. The survey questionnaires were sent by snail mail to the health professionals and food industry representatives.

Survey conducted among the general public included 2139 participants who were randomly selected to complete the survey. The first phase of the survey was conducted among 1000 respondents during 2009/2010 and the second phase of the survey was conducted between September 2012 and February 2013. Convenience and stratified sampling were used to collect the data. Stratified sampling was one of the sampling methods that were used to divide the entire target population into different subgroups. During the second phase 130 surveys that were conducted via the online mode were excluded from the data analysis as they were incomplete. In addition to the survey conducted among the general public, survey questionnaires were also administered to 62 health professionals and 50 food industry representatives.

3F. Statistical Analyses

All statistical analyses were carried out by using the SPSS statistical package (version 21.0), SPSS Inc. and the tests used a significance level of 0.05.