CHAPTER 4

EXPERIMENTAL

4.1 Materials

4.1.1 Maize

Two varieties of maize; PAN 6335 grown in 1997 in Mpumalanga, South Africa and PAN 6043 grown in the same year in Free State also in South Africa were used.

4.1.2 Sorghum

Two varieties of sorghum; white sorghum KAT 369 (grown in 1997 in Cheplambus, Baringo, Kenya) and NK 283 red hybrid (grown in 1997 in Nola, Randfontein, in South Africa) were used.

4.1.3 Pearl millet

The two varieties of pearl millet used were: SDMV 89004 and SDMV 91018 grown in 1999. These two varieties were supplied by the SADC/ICRISAT research station for sorghum and millet at Bulawayo, Zimbabwe.

4.1.4 White wheat bread

The bread used in this study was white wheat pan bread, it was bought in a fresh state and from one batch a few days before starting of the experiment on August 2000 at Pick 'n Pay Supermarket at Hatfield, Pretoria, South Africa. It was cut into slices of about 15 mm thick, each slice was vacuum sealed in a plastic bag and stored at – 20°C before defrosted for use. Only the bread crumb was used.

All the grain and grain flours were store under dry conditions at 10°C.

The proximate composition of maize, sorghum and pearl millet flours is shown in Table 5, and that of white wheat bread in Table 6.

Table 5. Proximate composition of unrefined and refined flours of maize, sorghum and pearl millet

Sample	Moisture (%)	Protein[1] (% dwb)[4]	Fat (% dwb)	Ash (% dwb)	Starch (% dwb)
Maize PAN 6043 (unrefined)	11.31^{c2} (0.36)[3]	10.76^{ef} (0.05)	4.26^{d} (0.02)	1.31^{c} (0.03)	69.45^{de} (1.59)
Maize PAN 6043 (refined)	10.06^{def} (0.34)	9.69^{g} (0.02)	0.66^{h} (0.01)	0.36^{f} (0.01)	83.74^{a} (1.00)
Maize PAN 6335 (unrefined)	11.70^{bc} (0.03)	9.69^{g} (0.12)	4.37^{d} (0.0.4)	1.15^{de} (0.02)	75.62^{bc} (2.62)
Maize PAN 6335 (refined)	10.01^{def} (0.15)	8.58^{h} (0.02)	0.70^{h} (0.01)	0.27^{f} (0.01)	87.19^{a} (3.13)
Sorghum KAT 369 (unrefined)	11.70^{bc} (0.07)	10.49^{f} (0.05)	3.35^{e} (0.04)	1.81^{a} (0.03)	68.76^{de} (1.58)
Sorghum KAT 369 (refined)	12.37^{a} (0.07)	9.47^{g} (0.08)	1.66^{g} (0.07)	1.04^{e} (0.14)	78.85^{b} (2.70)
Sorghum NK 283 (unrefined)	12.19^{ab} (0.22)	11.76^{d} (0.14)	3.54^{e} (0.12)	1.53^{b} (0.06)	72.63^{cd} (1.79
Sorghum NK 283 (refined)	12.21^{ab} (0.22)	10.92^{e} (0.31)	2.30^{f} (0.03)	1.20^{cd} (0.04)	79.26^{b} (2.52)
Pearl millet SDMV 89004 (unrefined)	10.57^{bc} (0.27)	12.64^{c} (0.11)	6.60^{a} (0.03)	1.74^{a} (0.04)	68.23e (3.67)
Pearl millet SDMV 89004 (refined)	10.27^{de} (0.02)	10.67^{ef} (0.04)	4.88^{c} (0.08)	1.24^{cd} (0.03)	77.15^{b} (0.58)
Pearl millet SDMV 91018 (unrefined)	9.89^{ef} (0.09)	15.32^{a} (0.08)	6.33^{b} (0.02)	1.70^{a} (0.03)	66.95^{e} (2.02)
Pearl millet SDMV 91018 (refined)	9.51^{f} (0.04)	13.22^{b} (0.06)	3.54^{e} (0.22)	1.03^{e} (0.03)	78.83^{b} (0.96)

1 N x 6.25

2 Values with different letters in the superscript are statistically significantly different ($p < 0.05$)

3 Values in brackets are the standard deviations

4 Percentage dry weight basis

Table 6. General proximate composition of bread

Sample	Moisture (%)	Protein[1] (% dwb)[5]	Fat (% dwb)	Ash (% dwb)	Starch (% dwb)
White wheat bread	45.3[6] (0.2)[2]	13.1[3]	2.77[3]	2.94[4]	69.7[6] (1.2)

1 N x 5.70 for wheat

2 Values in brackets are the standard deviations

3 According to South African food composition tables (Langenhoven, Kruger, Gouws and Faber, 1991)

4 According to Van Heerden, Anderson, Van Niekerk and Wight (1990)

5 Percentage dry weight basis

6 According to the analyses carried out in this study.

4.2 Methods

Unrefined and refined flours of two varieties of each cereal grain (maize, sorghum and pearl millet) were prepared. The steps involved in the preparation are discussed below.

4.2.1 Removal of the bran and germ

4.2.1.1 Degerming maize

Degerming was done to separate the germ and the bran from the maize kernel so that the resulting endosperm could produce highly starch-concentrated refined flour after grinding. Before degerming, the moisture content of the maize kernels was increased to toughen the bran, which makes it easier to remove together with the germ from the endosperm (Uhlig & Bhat, 1979). The conditioning was done in two steps: the maize grains were conditioned to 16% moisture for overnight, and then 30 min before milling the grains were conditioned to a moisture content of 18%.

Degerming was done with a small-scale maize degermer designed by the Council for Scientific and Industrial Research (CSIR) in collaboration with the South African Maize Board. This small-scale maize degermer, works in the same principles as the industrial-scale Robinson or Beall degermer.

4.2.1.2 Decortication of sorghum and pearl millet

Sorghum and pearl millet were decorticated at CSIR using a rice polisher machine, unlike maize there was no conditioning of the grains. According to Rooney and Miller (1982) and Serna-Saldivar and Rooney (1995), the germ in sorghum and pearl millet is deeply interred in the grain and therefore making it difficult to remove through decortication/dehulling. High milling losses, up to 40%, may be incurred if the fat content of the meal is to be reduced to about 1%, a fat content that would ensure reasonable shelf stability of the meal. To make sure that no excessive endosperm was removed, an attempt was made to limit the amount of bran and germ removed to about 20% of the grain weight, which is figure recommended by the FAO according Kent and Evers (1994). Due

to the lack of suitable decorticating machine to remove exactly the outer surface material equivalent to 20% of the grain weight, it was found eventually that, the amounts of the bran and germ together removed from sorghum and pearl millet grains ranged from 13.96% to 29.94%. Sorghum and pearl millet remained with a high proportion of the germ after decorticating as per the above explanation.

4.2.2 Milling maize, sorghum and pearl millet to flour

- Unrefined flour was prepared by grinding the whole grain of the two varieties of each cereal.
- The refined flour was prepared by grinding degermed and decorticated maize grain and decorticated sorghum and pearl millet grains from the two varieties from each cereal.
- Grinding was done by a laboratory hammer mill using 800 μm sieve.

4.2.3 Maize, sorghum and pearl millet stiff porridge cooking procedure

The recipe used in this experiment is the one which is commonly used in most parts of East and Southern Africa. Since the quantities of water and flour had to be reduced, the suitable method was that of adding the whole amount of flour into the boiling water at once. Unlike the alternative method in which a thin porridge is made first before adding the rest of the remaining flour, this method had the advantage of reducing excessive evaporation and hence minimizing the amount of variation. Every day two samples of stiff porridge were prepared by picking a pair of flour samples in a complete randomized table. Every sample was cooked in triplicate. The steps involved were as follows:

Flour (60 g) for each sample was weighed into a 250 ml plastic beaker.

Tap water (150 ml) for each sample was measured by using a measuring cylinder and poured into a saucepan. The saucepan was covered and put on a hotplate (double solid hot plate with settings 0, 1, 2, 3, 4, 5, and 6) and temperature was set at 4 mark. The stop watch set at 20 min was started counting down when the power was switched on from the socket and after approximately 6 min and 10 s the water was brought to boiling point.

The flour was poured in and the stirring was done gently for 30 s and then covered for 40 s before performing another stirring which unlike the first one, this was vigorous for 30 s and then covered for 40 s. While the first saucepan was still covered the second saucepan which was started 1 min and 40 s later was coming to the boiling point and the same procedure was followed. After 5 min and 10 s, for each saucepan the temperature settings was turned down to 2 while stirring and covering continued for another 4 min and 5 s when the temperature settings was turned to 0. Quickly the saucepan was removed from the hot plate and the porridge was pulled together and covered to avoid drying on the surface while cooling at room temperature. The same was done to the porridge on the second saucepan.

4.3 Analyses

4.3.1 Hardness of the grains

Before milling, the hardness of the grains was visually characterized. This method involved cutting the whole grain into longitudinal section and making visual comparison of the ratios of the vitreous to floury areas with standard sections developed from sorghum grains (Rooney and Miller, 1982). The standard sections are grouped into three categories; namely floury, intermediate and corneous and use a scale from 5 to 1. The category of floury uses a scale of 5, category of intermediate to corneous uses a scale from 4 to 2 while the category of corneous uses a scale from 1 to 2.

4.3.2 Weight of the grains (1000 kernel weight)

The grains were counted by seed counter (Numigral seed counter, Tripette & Renaud). Only the whole grains and decorticated grains of sorghum and pearl millet varieties were counted. It was impossible to count decorticated and degermed maize grains because it was converted to maize samp which consisted mainly of broken maize kernels.

4.3.3 Proximate analysis

The proximate composition of the samples was expressed on dry weight basis. Quantities calculated from the experiments were converted to dry weight basis by the equation below:-

$$\text{Quantity (\% dry weight basis)} = \frac{\text{Quantity (\% as is)} \times 100}{(100 - \text{moisture content (\%)})}$$

4.3.3.1 Moisture

AACC Method 44 – 15A (American Association of Cereal Chemists, 1983b) was used. For samples that with moisture less than 13% (maize, sorghum and pearl millet flours), the one-stage air oven method was used. Approximately 2 –3 grams of well-mixed sample was placed into a moisture dish that had previously been dried, cooled in a dessicator and weighed. The dish was covered with its lid and weighed. The sample was put in an air oven at 103 ± 1°C while the lid was put under the dish. The sample was heated for 60 min counting from when the oven regained the temperature of 103 ± 1°C after introducing the samples. The dish was covered with its lid and the sample was placed in a dessicator to cool to room temperature. The moisture content was determined as a loss in moisture using the following equation:

$$\% \text{ Moisture} = \frac{A \times 100}{B}$$

In which A = moisture loss in grams, B = original weight of sample

In the case of the bread and the maize, sorghum, and pearl millet stiff porridges, the two-stage air oven procedure of the same method was followed. The pre-weighed moisture dish was filled nearly full with a representative portion of the sample. The sample weight was recorded. The sample was placed in a ventilated air oven at 30°C over night to reduce the moisture content to about 10%. The sample was taken out of the oven and left outside for 2 hours to equilibrate to atmospheric moisture content. The air-dried sample was

weighed and the percentage loss due to air-drying recorded. The particle size of the air-dried sample was then reduced using a clean, dry food liquidiser. The one-stage procedure described above was then followed. The total moisture content was calculated with the following equation:

$$\% \text{ Total moisture} = X + \frac{(100 - X)\,Y}{100}$$

Where X = percent moisture loss on air drying, Y = percent moisture loss as determined by oven drying.

4.3.3.2 Ash

AACC Method 08-01 (American Association of Cereal Chemists, 1983a) was used to determine the ash content of the maize, sorghum and pearl millet flours. Approximately 4 grams of sample was weighed accurately into a silica ashing crucible which had previously been ignited, cooled in a dessicator and weighed. The samples were incinerated in a muffle furnace until a light grey ash was obtained, cooled in a dessicator and weighed. Ash content was calculated as follows:

$$\% \text{ Ash} = \frac{(\text{weight of crucible} + \text{ash}) - \text{weight of an empty crucible} \times 100}{\text{weight of sample}}$$

4.3.3.3 Protein content

Samples were analysed for crude protein using a Kjeldahl method (modified AACC Method 46 – 12, American Association of Cereal Chemists, 1983c). Approximately 0.5 g sample was weighed accurately into a digestion tube. One Kjeltab (Thompson & Capper, Cheshire, UK), a 5 g tablet consisting of 100 parts K_2SO_4, 6 parts $CuSO_4.5H_2O$ and parts selenium was added. To that, 20 ml of concentrated H_2SO_4 was added. Samples were digested for approximately 1.5 h using a Buchi 430 Digestor (Buchi, Flavil, Switzerland). Distillation of ammonia, reaction with boric acid and titration with standard HCl (0.1 M)

were done with a Buchi 322 Distillation unit (Buchi, Flavil, Switzerland). The crude protein content was calculated using the following equation:

$$\% \text{ Protein} = \frac{(\text{ml std NaOH} \times \text{M of NaOH}) \times 1.4007 \times \text{factor}}{\text{sample weight (g)}}$$

The same factor of 6.25, was used for maize, sorghum and pearl millet.

4.3.3.4 Crude fat

The crude fat contents of the flours of maize, sorghum and pearl millet were determined by the modification of AACC Method 30 – 25 (American Association of Cereal Chemists, 1983d). Approximately 20g of well-mixed sample was weighed accurately onto a thimble. A piece of fat-free absorbent cotton wool was placed on top to prevent escape of the flour from the thimble and distribute solvent as it dropped on the sample. The sample was extracted with petroleum ether which was put into a flask previously dried in an oven, cooled in dessicator and weighed. The extraction was done for 5 h in a Soxhlet extractor and maintained at condensation rate of 5 – 6 drops per second. The solvent was evaporated on a water bath in a fume cupboard. The flask with fat was then dried completely in an oven at 103°C for approximately 1h. The flask was then cooled in a dessicator and weighed. The crude fat content was calculated as follows:

$$\% \text{ Crude fat} = \frac{((\text{weight of flask} + \text{fat}) - (\text{weight of flask})) \times 100}{\text{weight of sample}}$$

4.3.3.5 Total starch

Total starch assay kit, α-amylase/amyloglucosidase method (AA/AMG 9/97, Megazyme International Ireland Limited, Wicklow, Ireland, www. Megazyme.com). This method has been adopted first action by the AACC (Method 76.13).

The analysis included solubilisation of the starch with 80% ethanol and then dimethyl sulphoxide, digestion with thermostable α-amylase and digestion with amyloglucosidase. The formed glucose was then determined with a glucose oxidase/peroxidase reagent

(GOPOD) and the absorbance read at 510 nm. The total starch was calculated by the following equation:

$$\% \text{ Starch} = \frac{\Delta E \times F \times 90}{W}$$

Where ΔE is the absorbance read against the reagent blank, F is the conversion from absorbance to μg glucose, 90 is the adjustment from free glucose to anhydrous glucose (as occurs in the starch) and W is the weight of the sample.

4.3.4 Amylose content

A modification of the method of Knutson (1986) was used. Flour sample (50 mg) was dissolved in 10 ml 90% dimethyl sulphoxide containing 6 x 10^{-2} M iodine and heated at 50°C overnight in a shaking water-bath. The aliquots of this solution was diluted x 200 with distilled water and the absorbance was measured at 620 nm. The amylose content was determined by comparison with a standard curve. Mixtures of pure amylose and amylopectin from Sigma in different ratios between 0 and 100% was used to plot the standard curve. The purity of the amylose and amylopectin used for calibration was not checked. Thus, the amylose/amylopectin ratios can be considered as precise rather than accurate. The values of the amylose content obtained were percentages of dry weight flour. These values were converted to percentage weight of total starch by multiplying them with the following factor: $\frac{100}{TS_f}$

Where 100 is a constant and TS_f is the amount of the total starch contained in the dry flour sample.

4.3.5 Total polyphenols

Total polyphenols were determined by a modification of the method of Daiber (1975). 250 mg of the flour sample was mixed with 5.0 ml of the dimethyl formamide extractant and stirred vigorously in a vortex mixer after every 10 min for 1 h at room temperature. The polymeric phenols extracted were reacted with ferric ions (green ammonium ferric citrate) in an alkaline solution and the green/yellow dye produced was measured in a spectrophotometer at 525 nm.

4.3.6 Texture

Porridge was analysed by using TA – XT2 Texture analyser (Stable Micro Systems, Godalming: England) which consists of a computer with a texture expert programme and up and down movable vertical device mounted with a compressing probe. All the samples were subjected to the same conditions. Each porridge sample was prepared from the same amount of flour (60 g) and water (150 ml) and packed in strong and rigid small cylindrical plastic containers of 32 mm internal diameter and height of 40 mm. Thin steel wire for cutting cheese was used to cut the porridge bulging at the top of the porridge cylinders to make a smooth flat surface and then cooled at room temperature for 30 min. The texture of the porridges were measured by penetrating a 20 mm cylindrical probe with a flat end, 4 mm deep in the porridge at a speed of 0.2mm/s. The units of the porridge's texture was expressed as compression force in Newtons exerted during penetration.

4.3.7 In vitro starch digestibility

The Granfeldt *et al.* (1992) method involving pre-chewing of the food was used. The flow diagram in Figure 7 summarises the procedure. The whole *in vitro* starch digestibility experiment consisted of 12 treatments or samples of stiff porridges which were done in triplicate, and each replicate was divided into three portions. All the stiff porridges were cooked on a hot plate under the same conditions.

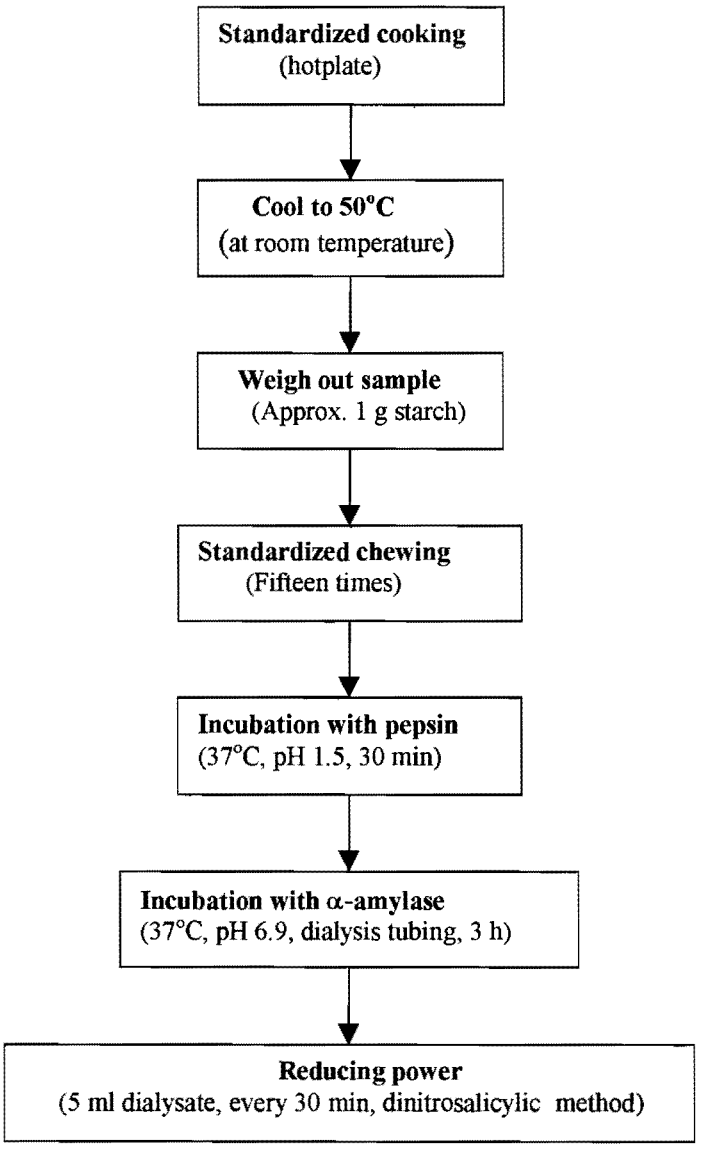

Figure 7: Flow diagram of the procedure used to determine the *in vitro* starch digestibility of stiff porridges from maize, sorghum and pearl millet.

4.3.7.1 Preparation of dialysis tubing

Dialysis tubing (Visking ex Labretoria, Pretoria) with a dry flat width of 42 mm and a molecular weight cut-off of 12 – 14 kDa was cut into 15 cm strips. The tubing was soaked in distilled water at 15°C overnight. One end of the tube was closed by tying it with a piece of string. The tubing as well as the extra pieces of string later used to close the other end of the tube were boiled in distilled water for 5 min to remove the sulphur used as preservative by the manufacturers. The tubing was then covered with fresh distilled water and used the same day. There was no tubing that was stored for more than a day, and therefore the preservative sodium benzoate acid (0.2 % m/v) that is commonly used to inhibit cellulolytic micro-organisms was not used.

4.3.7.2 Sample preparation

Two samples were cooked at a time on a saucepan, but with a difference of 2 min between the two in-terms of starting and finishing. After finishing cooking, porridge of each sample was pulled together in the form of a ball and covered the saucepan with a lid. The porridge was allowed to stand for about 18 min, the time by which it cooled to about 50°C at room temperature. Three portions from each sample containing about 1 g starch were weighed into the weighing boats and put inside a warm container with lid. The inside of the container was kept warm by a hot water sealed in a plastic bag. Weighing always started with the first sample that had been cooked.

Along with the two samples, there was also a bread which was used as a standard. Cubes of about 20 x 20 x 15 mm were cut from defrosted crumb of white wheat bread slice which had been stored in vacuum plastic bags at – 20°C. Two portions containing about 1 g starch were again cut from the cubes and weighed into the weighing boats and put together with the other portions from the two samples inside the warm container.

4.3.7.3 Chewing

Throughout the experiment no food was consumed in the 2 h before chewing took place. The mouth was rinsed with tap water before and after chewing any sample. After chewing, the sample was carefully expectorated into a 50 ml glass beaker containing 50

mg pepsin (2000 FIB-U/g, Merck, Darmstadt, Germany) and 6 ml of 0.05 M Na,K-phosphate buffer (containing 0.4 g/l NaCl) adjusted to pH 1.5 with HCl. The mouth was rinsed with 5 ml phosphate buffer (pH 6.9) for 30 s and the rinsing solution was also expectorated into the beaker. Twelve samples were handled per day.

4.3.7.4 Enzyme incubation

Before incubating with pepsin, the sample pH was adjusted to 1.5 with 2 M HCl and the beaker was covered with aluminium foil. It was incubated in a 37°C water bath for 30 min to simulate digestion in the stomach. The sample was mixed three times during incubation.

Following the incubation with pepsin, the pH was adjusted to 6.9 with 2 M NaOH. Porcine pancreatic α-amylase (A 6255 Sigma) was then added. The enzyme (35 µl) was dissolved in 9 ml of 0.05 M phosphate buffer and pH 6.9, and 1 ml of this solution was added to the sample. This represented approximately 147 Sigma units per gram of starch.

The sample was transferred to dialysis tubing. The beaker was rinsed with an amount of phosphate buffer that would bring the final volume in the dialysis tube to 30 ml (this volume varied between 7 ml to 8 ml according to sample). The dialysis tube was then tied and suspended in a 1 l beaker with 800 ml of 0.05 M phosphate buffer and pH 6.9. The beaker with the dialysis tube was then covered with aluminium foil to reduce evaporation and incubated in a water bath at 37°C for 3 h.

4.3.7.5 Measurement of products of digestion

Every 30 min a 5 ml aliquot of the dialysate was removed after thorough stirring of the contents in the beaker with a table spoon. It was analysed for reducing power by the 3,5 – dinitrosalicylic (DNS) acid method (Bernfeld, 1955). The aliquot was added to 5 ml DNS reagent (1% DNS in 0.4 M NaOH containing 30% w/w sodium potassium tartrate) in a 25 ml volumetric flask. The flask was immersed in a boiling water bath for 5 min to develop the colour, cooled and the sample was made up to volume. Absorbance was measured at 540 nm. A maltose standard curve was also constructed and used to convert

the absorbancy readings to maltose concentration (mg/ml). From maltose concentration the starch digestibility was calculated as follows:

Maltose liberated (mg) = maltose concentration (mg/ml) x 830

Where 830 ml is the total volume of the contents of the dialysis tube plus the buffer in the beaker.

Starch in porridge sample (mg) = mass of sample (g) x solids of porridge x starch content of sample x 1000

$$\text{Starch digestibility (\%)} = \frac{\text{mg maltose liberated x 100.}}{\text{mg starch in porridge sample}}$$

4.3.7.6 Blanks and reference sample

With every set of samples, a blank porridge sample was run in duplicate. The blank sample was not chewed, instead it was transferred to the beaker containing 50 mg pepsin in 6 ml of 0.05 M phosphate buffer and pH 1.5. Addition of 5 ml of 0.05 M and pH 6.9 buffer as it was done for the samples, was also done for the blank and it was broken up slightly with a glass rod. With the exception of chewing and addition of 1 ml of enzyme solution of which 1 ml of phosphate buffer (pH 6.9) was added, the blanks were treated in the same way as the samples.

White wheat bread was used as a reference, because it is often used as a reference when GI is determined (Granfeldt *et al.*, 1992; Perlstein *et al.*, 1997)

4.3.7.7 Calculation of Hydrolysis Index (HI) and predicted GI

A Hydrolysis Index (HI) was calculated according to Granfeldt *et al.* (1992):

$$\text{HI} = \frac{\text{Area under digestibility curve of sample (0 – 180 min) x 100}}{\text{Area under digestibility curve of white bread reference (0 –180 min)}}$$

Granfeldt (in her Ph.D. thesis, 1994) found a significant correlation (r = 0.862) between HI and GI which was also used by Akerberg *et al.* (1998) to predict GI by using the following equation:

$$GI = 0.862HI + 8.198$$

The result was converted to a glucose reference basis by multiplying by 0.7, as was done by Foster-Powell & Brand-Miller (1995).

4.3.8 Statistical analysis

Statistical analysis was done by using Statistica for Windows Release 5.0 (StaSoft Inc. 1984 – 1995, Tulsa, USA) and Microsoft Excel 97 (Microsoft Corporation, 1985 – 1997). Mrs. L Swart (Department of Statistics, University of Pretoria) and Prof. Van der Linde (Department of Information Technology, University of Pretoria) were consulted regarding the experimental design and statistical analysis.

Significant differences between means were obtained with both Tukey's honest significant difference and least significant difference tests. Significant differences between the regression coefficients (slopes) of straight lines after linear regression were determined by using the following equation:

$$t\ (student) = \frac{b_1 - b_2}{(S^2b_1 + S^2b_2)^{1/2}}$$

Where b_1 and b_2 were the regression coefficients and S_1 and S_2 were the standard errors of b_1 and b_2. The degrees of freedom of the t-distribution were calculated as $n_1 + n_2 - 4$, where n_1 and n_2 were the number of measurements taken in 1 and 2 respectively and 4 is for the two regression coefficients and intercepts that had to be estimated. The p-value of the test was 1 minus the p obtained from a t-table. In all the significance tests, a p-value of smaller than 0.05 was considered to be statistically significant.