# CHAPTER 3

# EXPERIMENTAL

## 3.1 EXPERIMENTAL DESIGN

The effects of malting temperature (steeping and germination) and time and moisture (germination) conditions were investigated using two pearl millet varieties. Various quality parameters were measured, including: those for opaque and conventional beer brewing, and physico-chemical, nutritional and functional parameters. The assays were replicated three times.

The flow chart of the experimental design is shown in Figure 5.

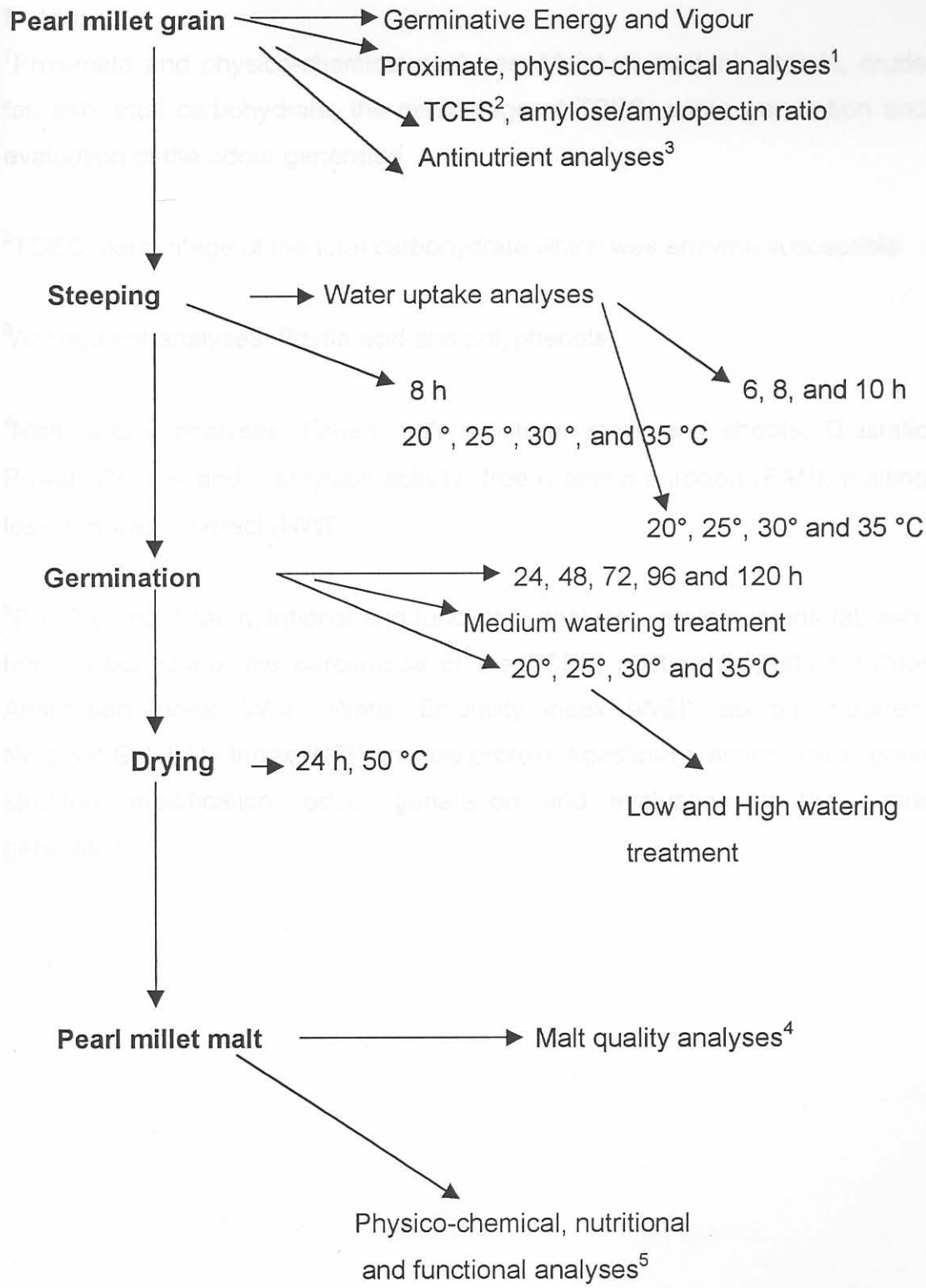


**Figure 5. - Flow chart of the experimental design of pearl millet malting**

**Notes:**

[1]Proximate and physico-chemical analyses: Moisture content, protein, crude fat, ash, total carbohydrate, the percentage of TCES, odour generation and evaluation of the odour generated.

[2]TCES: percentage of the total carbohydrate which was enzyme susceptible.

[3]Antinutrient analyses: Phytic acid and polyphenols.

[4]Malt quality analyses: Green malt moisture, roots and shoots, Diastatic Power (DP), α- and β-amylase activity, free α-amino nitrogen (FAN), malting loss, hot water extract (HWE).

[5]Physico-chemical, nutritional and functional analyses: protein, crude fat, ash, total carbohydrate, the percentage of the TCES, pasting properties, Water Absorption Index (WAI), Water Solubility Index (WSI), soluble nitrogen, Nitrogen Solubility Index (NSI), *in vitro* protein digestibility, amino acids, grain structure modification, odour generation and evaluation of the odour generated.

## 3.2 MATERIALS

The raw materials used were:

(1) Pearl millet grain, variety SDMV 910018, planted at Estação Agrária de Chockwé in Gaza Province, Mozambique, in November 1996 and harvested in April 1997.

(2) Pearl millet grain, variety SDMV 89004, (released in Zimbabwe as PMV-2), kindly donated by SADC/ICRISAT, Matopos Research Station, Bulawayo, Zimbabwe, planted at Matopos Research Station, in November 1997 and harvested in April 1998.

(3) Sorghum malt, NK 283, a condensed tannin-free hybrid from South Africa, previously malted in laboratory conditions, was used as a standard.

(4) Barley malt, variety Arapiles from Australia, kindly donated by S.A. Malsters (Pty) Ltd. (Alrode, South Africa) was used as a standard.

The raw materials were kept under cold storage (≤ 10 °C) conditions until used.

Pearl millet grains of variety SDMV 89004 are shown in Figure 6.

**Figure 6.- Pearl millet grains of variety SDMV 89004 (average mass of each kernel: 8.9 mg)**

## 3.3 PEARL MILLET MALTING PROCESS

The malting of the two varieties of pearl millet was done at CSIR – Bio/Chemtek (Division of Food, Biological and Chemical Technologies), Pretoria, South Africa. The malting process comprised three main stages: Steeping, Germination and Drying.

### 3.3.1 Steeping

Samples of pearl millet grain (5 kg) were washed 4 to 5 times, in running tap water (22-24 °C) to remove foreign material. The grain was then put in 250 x 300 mm (2.5 kg) nylon bags and closed with rubber bands and spin-dried (30 s at 300 x g) to remove excess surface-held water. After the spin-drying process, exactly 500 g of grain per sample was placed in 150 x 200 mm nylon bags and closed with rubber bands. The bag held grain was re-weighed then steeped in static water at 20°, 25°, 30° or 35 °C, with a cycle of 2h wet, 2 h dry air rest for 8 h. During the dry stands the grain was held in still air at 20–22 °C. After the steeping period, the grain in nylon bags was spin-dried (30 s at 300 x g) and weighed.

### 3.3.2 Germination

The steeped grain was then germinated in the nylon bags for 1 to 5 d at one of four different temperatures: 20°, 25°, 30° and 35 °C. Germination was carried out in a water-jacketed incubator (Forma Scientific, Marrietta, Ohio, USA) in an atmosphere of near water-saturation with a continuous flow of moist air. The nylon malting bags were covered with wet cloths to maintain the water saturation. Twice daily, the bag held grain samples were removed from the incubator, weighed and steeped for 10 min in tap water (22–24 °C), then spin-dried (30 s at 300 x g), weighed and returned to the germination cabinet (Medium watering treatment).

At the germination temperature of 25 °C, beside this Medium watering regime, two additional regimes were used:

1) Low- a very small amount of water was applied to maintain the green malt at constant fresh weight.

2) High- as for Medium watering regime, but the spin-drying process was omitted; so as to keep the malt wet.

Figure 7 shows the green malt moisture content of the two pearl millet varieties.

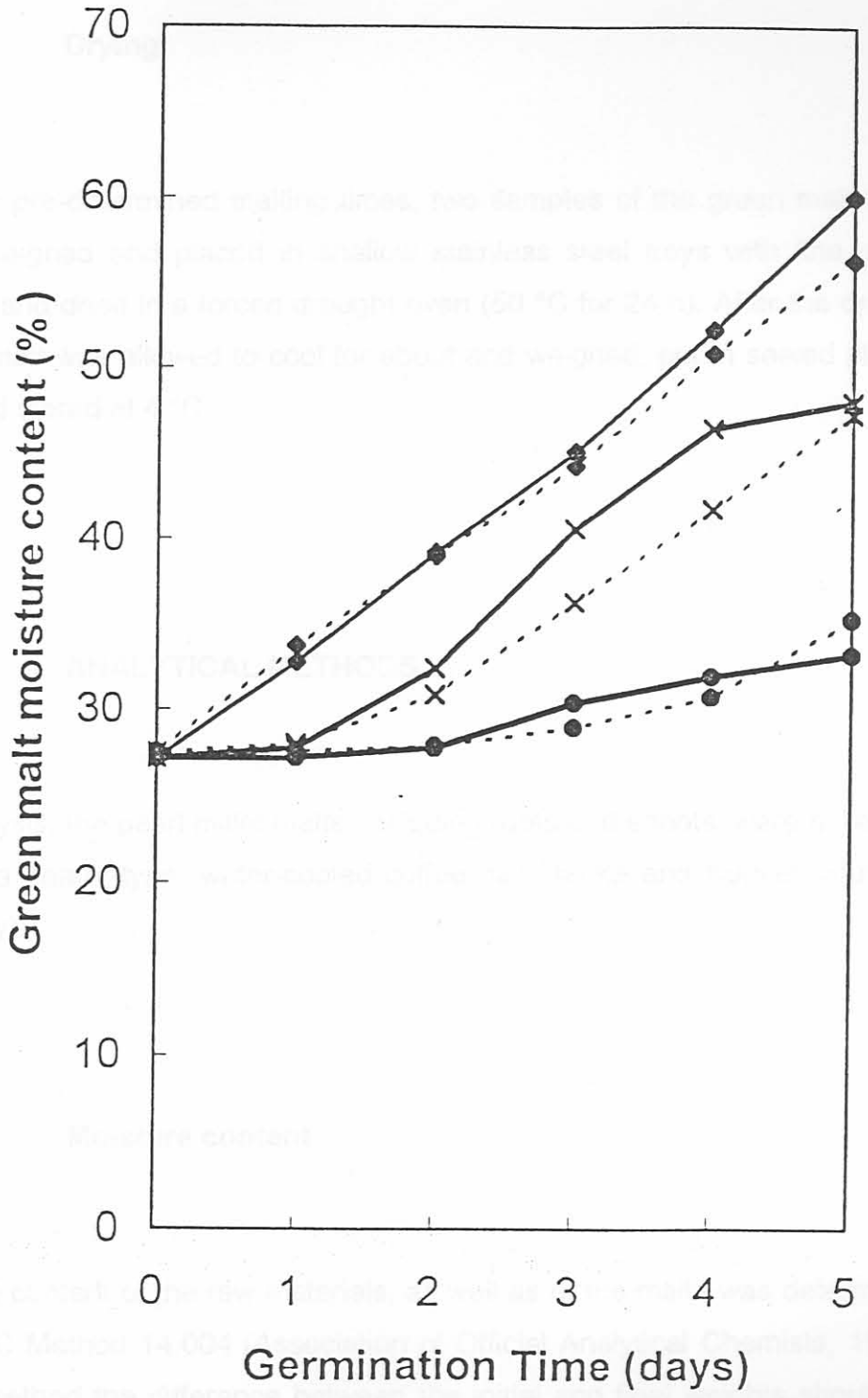


**Figure 7.- Green malt moisture content at low, medium and high watering treatment of pearl millet at 25 °C (variety SDMV 89004(– ) and variety SDMV 91018 (--) at various watering treatments (•- Low watering; ×- Medium watering; ♦- High watering)**

### 3.3.3 Drying

After the pre-determined malting times, two samples of the green malt were taken, weighed and placed in shallow stainless steel trays with fine mesh bottoms and dried in a forced draught oven (50 °C for 24 h). After the drying, the dry malt was allowed to cool for about and weighed, put in sealed plastic bags and stored at 4 °C.

## 3.4 ANALYTICAL METHODS

For analysis, the pearl millet malts, including roots and shoots, were milled for 45 s in a beater-type, water-cooled coffee mill (Janke and Kunkel, Staufen, Germany).

### 3.4.1 Moisture content

Moisture content of the raw materials, as well as of the malts was determined by AOAC Method 14.004 (Association of Official Analytical Chemists, 1980). In this method the difference between the initial and final weights should be equal to the weight of free moisture in the sample.

### 3.4.2 Protein

Protein content (N x 6.25) was determined by the Kjeldahl method, as modified in AOAC Method 2.057 (Association of Official Analytical Chemists, 1980). Samples are digested with concentrated sulphuric acid in the presence of Kjeldahl tablet (potassium sulphate, selenium and copper) catalyst for the conversion of nitrogen to ammonium hydrogen sulphate. Then the digested samples are neutralised with concentrated NaOH and volatile ammonium was distilled off into a solution of boric acid. An amount of borate anions equivalent to the ammonium is formed which is then titrated against standard hydrochloric acid. The distillation and titration steps were performed using an automated Büchi 322 Distillation Unit (Büchi, Flawil, Switzerland).

### 3.4.3 Fat

Fat content was determined by AOAC Method 14.018, procedure 7.056 (Association of Official Analytical Chemists, 1980). This method is based on the principle of gravimetric extraction of fat from a sample by petroleum solvent, followed by recovery of the fat by evaporation of the solvent.

### 3.4.4 Ash

Ash was determined by AOAC Method 14.006 (Association of Official Analytical Chemists, 1980) by differential weighing after incinerating at 550 °C until a light grey ash was obtained to constant weight.

### 3.4.5 Total carbohydrate and the total carbohydrate which was enzyme-susceptible

To determine total carbohydrate and the percentage of the TCES an enzymic method using α-amylase and amyloglucosidase was used (Taylor, 1992). This method measures total starch by gelatinization of the starch by pressure-cooking followed by hydrolysis to glucose by α-amylase and amyloglucosidase and colorimetric determination of glucose. The percentage of the total carbohydrate which was enzyme susceptible was measured in the same way except that the gelatinization step was omitted.

### 3.4.6 Fibre

Fibre was calculated by difference:

***100 - (% moisture + % carbohydrate + % fat + % ash + % protein)***

### 3.4.7 Phytic acid

The determination of phytic acid was done by a modification of the method of Garcia-Villanova, Garcia-Villanova & Ruiz de Lope (1982). The method involves reaction of phytic acid with iron (III) solution. Excess iron (III) is complexed with sulphosalicylic acid and determined by complexometric back

tritation with standard disodium ethylenedinitrilotetraacetate (EDTA) solution. The time allowed to stand after adding HCl and $Na_2SO_4$ was 2 h instead of 90 min.

### 3.4.8 Total polyphenols

Before the determination of the total polyphenol content, the pearl millet grains were submitted to the Chlorox bleach test. In this test, the grain is immersed in a sodium hypochlorite solution (bleach) containing alkali. The solution at 70 °C dissolves away the out pericarp layer of grain, revealing the presence of a black pigmented testa layer in the case of tannin grains, or its absence in the case of non-tannin grains (Waniska, Hugo & Rooney, 1992).

To determine the total polyphenol content of the samples, the modified method of the International Standardisation Organisation (ISO), (1988) was used. In this method the Jerumanis ferric ammonium citrate reacts with the phenolic compounds under alkaline conditions and the absorbance of the products is linearly related to the concentration of the phenolic acid compound in the samples.

### 3.4.9 Amylose/amylopectin ratio

The amylose/amylopectin ratio in the native starches was measured colorimetrically by a method of Knutson (1986) as modified by Faulks & Bailey (1990). This method is based on the principle of the reaction of iodine when it is dissolved in mixtures of water and dimethyl sulphoxide where triicodide ion

is formed. This reaction is utilised to form the blue amylose-iodine complex. Amylose is then determined by measurement of the absorbance of the complex at 620 nm.

### 3.4.10 Germinative Energy (GE) and Germinative Vigour (GV)

Germinative Energy and Germinative Vigour were measured as described by Dewar, Joustra & Taylor (1993). In order to produce good quality malt, a prerequesite is that a high (>90%) proportion of the grain is germinable. Germinative Energy (GE) is a measure of the percentage of grains, which can be expected to germinate if the grain is malted normally at the time of the test. Germinative Vigour (GV) is a measure of how energetically the grain germinates.

### 3.4.11 Malting loss

The total malting loss was calculated according to the method described by Gomez, Obilana, Martin, Madzvamuse & Monyo (1997):

***Malting loss (%)=[(Initial dry grain weight- Dry malt weight)/(Initial dry grain weight)] x 100***

### 3.4.12 Roots and shoots

Where stated, these were separated from the pearl millet malt kernels by rubbing the grain in a nylon bag of coarse mesh size, which allowed the roots and shoots to escape while retaining the kernels, as described by Morrall, Boyd, Taylor & Van der Walt (1986). The weight of roots and shoots was expressed as a percentage of the total malt weight.

### 3.4.13 Green malt moisture

The green malt moisture content was calculated as follows, Gomez, Obilana, Martin, Madzvamuse & Monyo (1997):

***Green malt moisture (%) = [(Green malt weight – Dry malt weight)/Green malt weight] x 100***

### 3.4.14 Water uptake

Water uptake of the pearl millet grains was calculated as a percentage of the weight gained by the pre-washed, non-steeped grain at the end of the 8 h of steeping.

### 3.4.15 Grain hardness

Pearl millet grain hardness was estimated according to Rooney & Miller (1982). Pearl millet grains were cut into halves longitudinally. One half was viewed with the naked eye and the proportion of horny endosperm was determined by reference to a standard. On the basis of the proportion of horny endosperm, grains were classified into hard, intermediate and soft. The hardness was rated in a scale of 1 to 5, with a 1 rating meaning that the kernel contains very little floury endosperm (almost completely horny) and a 5 rating meaning essentially all floury.

### 3.4.16 Diastatic Power (DP)

Diastatic Power is a measure of the joint α- and β-amylase activity. The standard method for sorghum malt Diastatic Power (South African Bureau of Standards (SABS), 1970) was used. In this Diastatic Power determination, an aqueous extract of milled malt is prepared which contains amylase enzymes. The enzyme extract is reacted with an excess of starch under specific conditions of time, temperature and pH. The amount of sugar product produced by hydrolysis of starch is determined by titration in terms of its reducing power.

The following amendments to the SABS method were included:

The peptone extraction was replaced by distilled water extraction since the pearl millet malt used was prepared from tannin-free grains.

Two g whole malt flour was used instead of 25 g and the extraction volume

was 40 ml instead of 500 ml.

The extraction was carried out in centrifuge tubes and the extraction time was reduced from 2.5 to 2 h.

The results were expressed as Pearl Millet Diastatic Units (PMDU/g dry wt malt), where PMDU is equivalent to Kaffircorn Diastatic Units (KDU).

### 3.4.17 Alpha-amylase activity

Determined according to the method of Novellie (1962a) by inactivating the β-amylase activity at 70 °C in the presence of $Ca^{2+}$ ions. The malt extracts were prepared as for DP. Calcium acetate (0.02 g) was added to the 10.0 ml of the malt extract. The extracts were incubated at 70 °C for 15 min. After incubation, extracts were cooled in ice for 10–15 min at 30 °C. Enzyme activity was then determined by the DP procedure and expressed as PMDU.

### 3.4.18 Beta-amylase activity (by inactivation of α-amylase)

The contribution of β-amylase to the diastatic activity was estimated by inactivating α-amylase activity with 0.2 M ammonium oxalate which binds with calcium ions in the α-amylase molecule (Taylor & Von Benecke, 1990). Pearl millet extracts were prepared with 1.00 g malt and 40.0 ml 0.2 M ammonium oxalate. Enzyme activity was then determined by the DP procedure and expressed as PMDU.

### 3.4.19 Beta-amylase activity (Betamyl method)

The Betamyl Method (beta-amylase assay kit, from Megazyme, Ireland) was used. This method measures soluble and total β-amylase activity using a mixture of p-nitrophenyl maltopentaoside and p-nitro maltohexaoside as substrate (McCleary & Codd, 1989). Cysteine is used to extract the "insoluble" β-amylase. This method is based on hydrolysis of p-nitrophenyl maltopentaoside to maltose and p-nitrophenyl maltotrioside by β-amylase, the nitrophenyl trioside is immediately cleaved to glucose and free p-nitropehenyl by the α-glucosidase present in the substrate mixture. Thus, the rate of the release of p-nitrophenol relates directly to the rate of release of maltose by β-amylase (Mathewson & Seabourn, 1983). Beta-amylase activity was expressed as Betamyl Units.

### 3.4.20 Free α-amino nitrogen (FAN)

Free amino nitrogen consists of free amino acids and small peptides produced by proteinase and peptidase activity in the malt. The FAN content was determined according to the ninhydrin method described by Morrall, Boyd, Taylor & Van der Walt (1986), except that 1 g of the sample was used instead of 5 g. This method measures the pre-formed FAN malt. An aqueous extract of milled malt is heated with ninhydrin and the colour produced measured colorimetrically. Ninhydrin is an oxidising agent and causes oxidative decarboxylation of α-amino acids, producing $CO_2$, $NH_3$ and an aldehyde with one less carbon atom than the parent amino acid. The reduced ninhydrin then reacts with the unreduced ninhydrin and the liberated ammonia, forming a purple complex.

The intensity of purple colour is determined colorimetrically and the amount of FAN calculated by the use of a standardised solution of an amino acid. The results were expressed as mg/ 100 g dry weight malt.

### 3.4.21 Hot water extract (HWE)

Hot water extract is a measure of the soluble solids in solution and gives an estimate of how much of the malt will solubilise during the brewing process (Briggs, Hough, Stevens & Young, 1981). Hot water extract gives an indication of the modification of the malt during the malting process. Hot water extract was measured as described by Morrall, Boyd, Taylor & Van der Walt (1986). In this method the amount of malt solubilised is measured by determining the specific gravity of clear wort obtained by centrifugation under standard conditions.

### 3.4.22 Water Absorption Index (WAI)

Water Absorption Index was determined according to the method of Anderson, Conway, Pfeifer & Griffin (1969). This method is based on the determination of the amount of pearl millet starch dispersed in an excess of water (supernatant) after centrifugation.

### 3.4.23 Water Solubility Index (WSI)

Water Solubility Index was measured according to the method of Anderson, Conway, Pfeifer & Griffin (1969). In this method the amount of milled pearl millet solubilised in water is measured by determining the amount of dry matter recovered after the supernatant is evaporated from Water Absorption determination (3.4.22).

### 3.4.24 Percentage of soluble nitrogen and Nitrogen Solubility Index (NSI)

Nitrogen Solubility Index is a measure of the amount of water soluble nitrogen in a sample. The amount of the total nitrogen and water soluble nitrogen in non- and germinated pearl millet was determined according to AACC Method 46-23 (American Association of Cereal Chemists, 1983). This method is based on the determination of the amount of nitrogen in water extract from pearl millet samples after centrifugation.

### 3.4.25 *In vitro* protein digestibility

The *in vitro* protein digestibility method of Mertz, Hassen, Cairns-Whittern, Kirlies, Tu & Axtell (1984) as modified by Hamaker, Kirleis, Butler, Axtell & Mertz (1987) was used. The principle of this method is that pepsin is used to hydrolyse the insoluble protein into soluble amino acids and peptides. The

percentage protein hydrolysis is related to the protein digestibility. Protein was determined by the Kjeldahl method (3.4.2).

The percentage of protein digestibility was calculated as:

***% Protein Digestibility = (Total protein – Residual protein)/Total protein x 100***

### 3.4.26 Amino acid analysis

The amino acid content of non- and malted pearl millet was analysed using the Pico.Tag Method (Bidlingmeyer, Cohen & Tarvin, 1984) in a Pico.Tag Column for hydrolysate amino acid analysis Part no. 88131 (3.9 mm x 15 cm). This method is based upon formation of a phenylthiocarbamyl derivative of the amino acids for analysis of free amino acids from acid-hydrolysed proteins. The amino acids are separated based on their hydrophobicity. Hydrochloric acid is used to hydrolyse proteins into amino acids. This derivatization method is very sensitive and specific for primary and secondary amino acids in protein hydrolyzates. The reversed-phase liquid column chromatographic system allows for the rapid, bonded-phase separation with ultraviolet detection of the common amino acids with 12-min analysis time and a 1-pmol sensitivity (Bidlingmeyer, Cohen & Tarvin, 1984).

### 3.4.27 Scanning electron microscopy (SEM)

Samples for SEM were germinated for 1 to 7 days as described in Germinative Energy and Germinative Vigour methods (3.4.10). Grains of non- and germinated pearl millet were fixed in 2.5% gluteraldehyde in 0.075 M phosphate buffer; pH 7.4-7.6 for 1 h. Samples were rinsed 3 times, 15 min each in 0.075 M phosphate buffer. After this, samples were dehydrated in 50%, 70% and 90% ethanol for 15 min each concentration, then dehydrated 3 times with 100% ethanol for 15 min each, respectively. After fixing the samples were fractured by cutting longitudinally using a sharp blade.

The samples were subjected to critical point drying in liquid $CO_2$. The critical-point-dried samples were mounted on stubs and sputtered coated with gold. The samples were examined in a JEOL JSM-U3 microscope (JEOL, Tokyo, Japan).

### 3.4.28 Odour generation and evaluation of the odour generated

Non- and germinated milled pearl millet were mixed with water 30% (w/v) and placed in Petri dishes. The samples were then air-dried in a fume cupboard at ambient temperature overnight. About 3 g samples were sealed in 27 ml glass bottles "polytops" for at least 1 h before being evaluated for odour generation. Twelve trained panellists carried out evaluations of treated pearl millet samples. These panellists were trained using fresh samples (no odour) and treated, i.e. wetted and dried grits (odour present). The trained panellists were asked to sniff the head space over treated samples and compare it with a control samples. Panellists were asked to rate each coded sample on numeric

scale of 1 (least intense, i.e. no odour) to 9 (most intense, i.e. strong odour present). Appendix A shows the score sheet used to evaluate the odour generated by pearl millet.

### 3.4.29 Pasting properties

The pasting behaviour of the grain and germinated pearl millet was determined using a Rapid Visco-Analyser model 3D (RVA) (Newport Scientific, Warriewood, Australia). Four g whole flour of grain and germinated of pearl millet (14% moisture basis) were separately mixed with 25 ml of distilled water. A programmed heating and cooling cycle was used. The suspension was held at 50 °C for 1 min, heated to 90 °C for 7.5 min at the rate of 6 °C/min, held at 90 °C for 5 min before cooling to 50 °C in 7.5 min and holding at 50 °C for 1 min. Peak viscosity (PV), hot paste viscosity (shear thinning) and setback were recorded.

### 3.4.30 Statistical analysis

Analysis of variance (ANOVA) with the least significant difference test (LSD-Test) was applied. The level of 95% was considered as significantly different.