CHAPTER 3

MATERIAL AND METHODS

3.1 Apparatus

Aqua-TR II, Chopin Technologies, France

Cooling Centrifuge (Model C-25), Remi Instruments Ltd., Mumbai, India

Deep Frier, Friendz Forever, FZ-591

Deep-frying Oil Tester, Testo 270

Differential Scanning Calorimetry, Q10, Universal V4.1D TA Instruments

Electrophoresis Apparatus (Model AE-6220 and power supply model AE-8150), Atto Corporation, Tokyo, Japan

Falling Number, Model 1100, Perten Instruments, Australia

Gel Documentation System, Bio Rad, USA

Glutomatic System, Perten Instruments, Sweden

Laboratory Mill, Model CD1, Villeneuve la Garenne, France

Mixolab, Chopin Technologies, France

Pasta Machine, Atlas Electric, Marcato, Italy

PCR, Genepro, MJ Research, Inc., USA

Rapid Visco Analyser, RVA TecMaster, Perten Instruments, Australia

Rice Cooker, UL-255, Ultimate

Scanning Electron Microscopy, SEMTRAC Mini, Nikkiso, Germany

SD matic, Chopin Technologies, France

Single Kernel Characterization System, Model 4100, Perten Instruments, Australia

Solvent Extractor, SER148, Velp Scientifica, Usbate, Italy

Texture Analyzer, Model TA-XT 2i, Stable Micro Systems, U.K
3.2 Materials

3.2.1 Wheat grains

The pure seeds of fifteen Indian wheat (*Triticum aestivum*) varieties namely DBW 16, C 306, HS 490, HW 2004, PBW 343, PBW 443, PBW 550, WH 147, PBW 373, WH 283, WH 542, WH 711, WH 1021, WH 1025 and HI 977 were obtained from agricultural universities; IARI regional centres; DWR, Karnal and Central State Farm, Hisar emphasizing on their diversity for preparation of instant noodles. The wheat grains were cleaned and stored in a deep freezer (-18°C).

3.2.2 Chemicals

All chemicals used were of analytical reagent grade or molecular biology grade (for electrophoretic studies) purchased from Genei, Bangalore; Hi Media Laboratories Pvt Ltd; Sigma Aldrich; Merck Pvt Ltd; Loba Chemie Pvt Ltd and Operon Biotech. The various chemicals used during research were 1, 4 Dithiothreitol, Cetyl trimethyl ammonium bromide Acetic acid glacial, DNA ladder, DNTPs, RNAase A, Taq DNA polymerase, Protein molecular weight marker, Acetone, Agarose, Ammonium acetate, Ammonium per sulphate, Ammonium sulphate, Amylopectin, Beta-mercaptoethanol, Bisacrylamide, Boric acid, Chloroform, EDTA (disodium), Glycerol, Glycine, Isoamyl alcohol, Isopropyl alcohol, Magnesium chloride, N, N, N’, N’-tetramethylenediamine (TEMED), PCR water (nucleus free water), Sodium chloride, Sodium dodecyl sulphate, Tris buffer, Tris HCl, Xylene cyanol, Amylose standard, Bromophenol blue, Acrylamide, Coomassie brilliant blue (CBB) G-250, Ethidium bromide, Iodine (resublimed), Cupric sulphate, Ethanol, Hydrochloric acid, Lactic acid, Liquid nitrogen, Methanol, Petroleum ether, Phenol, Phenolphthalein, Potassium carbonate, Potassium chloride, Potassium iodide, Sodium acetate, Sodium carbonate, Sodium chloride, Sodium hydroxide, Sodium phosphate, Sodium sulphate, Sodium thiosulphate and Primers Set (RAPD).

3.3 Methods

3.3.1 Physical characteristics of wheat grains

Physical characteristics of grains like thousand kernel weight, test weight, kernel length and width of all the varieties were determined according to standard AACC (2000) procedures.
Kernel weight expressed as weight in grams per 1000 kernels is a function of kernel size or kernel density. After proper sampling, hundred kernels were counted, weighed and multiplied by 10 to determine the thousand kernel weight. Test weight or hectolitre weight determines the plumpness of the grain. It is a rough measure of the density of grain in terms of weight per unit volume. AQUA TR (Fig. 3.1) was used to determine the moisture content and hectolitre weight of the grains. The wheat grains were taken in beaker (1000 ml) supplied with the instrument, poured into the hopper of the instrument and tested using the calibrated protocol GJU Wheat. The kernel length was determined using a Digital Vernier Calliper. Physical parameters including kernel width, kernel hardness, kernel weight and moisture content were determined using SKCS (Single Kernel Characterization System) (Fig. 3.1). All the above physical parameters were determined in five replications and the mean of the readings has been reported.

Fig. 3.1 Instrument used for physical analysis of grains: Aqua-TR for determination of moisture content and hectoliter weight (left); Single Kernel Characterization System for analyzing hardness, kernel width, moisture content and kernel weight (right)
3.3.2 Milling of wheat grains

The grains of different varieties were tempered to 14% for soft wheat and 15% for hard wheat after determining their moisture content and milled in Laboratory mill to obtain the wheat flour/ maida. All flour samples obtained were stored at -18°C in deep freezer. The flour samples were thawed at room temperature prior to further analysis. Milling yield was recorded for different varieties. Commercial flour was purchased in a single lot from local market, Hisar for optimization of formula and process ingredients for instant noodles.

3.3.3 Analysis of wheat flour

3.3.3.1 Moisture content

Moisture was determined by standard AACC method (2000). Flour sample (5 g) was weighed in a preweighed moisture dish and dried in an oven at 130°C for 1 hr or till a constant weight was obtained. The sample was cooled in a dessicator and reweighed. The total loss in weight due to evaporation of moisture was calculated as moisture content and expressed in percentage.

3.3.3.2 Ash content

Ash content represents the inorganic residue remaining after ignition or complete oxidation of organic matter in a sample. Ash content of the flour samples was estimated by standard AACC method (2000). Flour sample (5 g) was taken in the silica crucible and charred on a hot plate till the smoke disappeared. The crucible was then placed in muffle furnace (550°C) for 5-6 hrs to facilitate incineration and obtain whitish grey ash. The crucible was cooled in a dessicator and weighed. Ash content was reported in percentage.

3.3.3.3 Protein content

Protein content was determined by standard AACC methods (2000). About 1g of the sample, 8 g Na₂SO₄, 500 mg CuSO₄ and glass beads (10-12) were transferred to the Kjeldahl flask, taking care that the particles do not stick on to the neck of the flask. 25 ml conc. H₂SO₄ was added and the mixture was digested till it became clear and pale green or colourless. Contents were then cooled and 200 ml distilled water was added along with a few drops of phenolphthalein indicator. The flask was then attached to Kjeldahl assembly and the dip tube was placed below the surface of the 0.1 N HCl added with 3-4 drops of methyl red indicator in a conical flask. Then NaOH (50%) was added slowly to neutralize H₂SO₄ and make the
solution alkaline. Distillation was carried out until all the ammonia passed over into the standard HCl. The distillate was titrated with 0.1 N NaOH. Blank was also titrated. Crude protein (%) was calculated using factor N × 5.7.

3.3.3.4 Falling number or α- amylase activity

The enzyme activity (α- amylase) of the wheat flour was determined using Falling Number Apparatus (Fig. 3.2) according to AACC method (2000). Flour sample (7 g) and distilled water (25 ml) were taken in a falling number tube and shaken vigorously to obtain a homogenous suspension. The tube with stirrer was kept in the previously maintained boiling water bath. After few seconds, the contents were automatically stirred for 60 s. The time taken (in seconds) by the plunger to stir the suspension and to move down through the gelatinized suspension was displayed and reported as the falling number.

![Fig. 3.2 Falling number apparatus](image)

3.3.4 Dough rheology and thermomechanical behaviour

Mixolab (Fig. 3.3) is capable of determining physical dough properties like mixing behaviour, dough strength and stability along with the pasting properties. The device senses in real time the torque (in Nm) produced by the dough mixed by the two blades. It measures the behaviour of dough as a function of time, mixing development and temperature. The tests were based on preparing a constant hydrated dough mass in order to obtain a target consistency of 1.1 Nm (±0.05 Nm). Tests were carried out using the inbuilt standard
protocols in Mixolab software i.e. Chopin S (equivalent to Farinograph) and Chopin+ protocol. Values were entered for moisture content, water absorption capacity, moisture basis etc. and the automatically calculated amount of flour was taken and tests were carried out.

3.3.4.1 Chopin S protocol

It involved mixing of flour-water dough for 30 min at 80 rpm and constant temperature i.e. 30°C. It provided the information regarding physical dough properties like water absorption capacity (%), dough development time (min), stability (min) and softening (FU) which were equivalent to Farinograph.

3.3.4.2 Chopin + protocol

It measures torque (Nm) produced by mixing dough between two kneading arms and provides comprehensive information about the protein and starch quality as well as enzymatic activity in a single test (45 min) wherein actual dough is subjected to a dual mixing and temperature constraints. A constant mixing speed of 80 rpm was used to assess the dough thermomechanical properties. It allows (i) mixing of dough at constant temperature (30°C) for 8 min providing information about the mixing behavior of dough (ii) gradual increase in dough temperature from 30°C to 90°C (8-23 min) which results in thermal weakening of dough due to continued mixing under increasing temperature initially and an increase in dough viscosity later on due to gelatinization of starch (iii) a constant temperature of 90°C for 7 min which allows for the enzymatic action demonstrating reduction in dough viscosity in presence of enzymes (iv) decrease in dough temperature from 90°C to 50°C over 10 min and then holding at 50°C for 5 min which raises dough torque owing to recrystallization of starch molecules i.e. retrogradation. During the above mentioned phases, five different torques i.e. C1, C2, C3, C4 and C5 (Fig. 3.4) were obtained which were used to measure water absorption, protein weakening, starch gelatinization, stability of hot formed gel and starch retrogradation, respectively. The various parameters obtained using Mixolab were compared for different flour samples and interpreted as according to Table 3.1.
Material and methods

Fig. 3.3 Mixolab

Fig. 3.4 Graph obtained using Mixolab
### Table 3.1 Parameters obtained using a Mixolab and their significance

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Method of calculation</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water Absorption (%)</td>
<td>Quantity of water required to obtain C1 = 1.1 Nm +/-0.07</td>
<td>Quantity of water that the flour can absorb to obtain a given consistency during the constant temperature phase.</td>
</tr>
<tr>
<td>Time for C1 (min)</td>
<td>Time required to obtain C1</td>
<td>Dough formation time- the stronger the flour, the longer it lasts.</td>
</tr>
<tr>
<td>Stability (min)</td>
<td>Time during which torque is &gt; to C1 – 11% (constant T° phase)</td>
<td>Dough resistance to kneading- the longer it lasts, the &quot;stronger&quot; the dough.</td>
</tr>
<tr>
<td>Amplitude (Nm)</td>
<td>Curve width at C1</td>
<td>Dough elasticity- the higher the value, the greater the flour elasticity.</td>
</tr>
<tr>
<td>Slope α</td>
<td>Slope of the curve between the end of the 30°C period and C2</td>
<td>Speed of the weakening of the protein network due to the effects of heat.</td>
</tr>
<tr>
<td>Slope β</td>
<td>Slope of the curve between C2 and C3</td>
<td>Starching speed.</td>
</tr>
<tr>
<td>Slope γ</td>
<td>Slope of the curve between C3 and C4</td>
<td>Enzymatic degradation speed.</td>
</tr>
<tr>
<td>C1</td>
<td>Dough temperature and the time taken for different torques to appear at their respective peaks</td>
<td>Used to calculate water absorption</td>
</tr>
<tr>
<td>C2</td>
<td>Measures protein weakening as a function of mechanical work and temperature</td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>Measures starch gelatinization</td>
<td></td>
</tr>
<tr>
<td>C4</td>
<td>Measures the stability of the hot-formed gel</td>
<td></td>
</tr>
<tr>
<td>C5</td>
<td>Measures starch retrogradation during the cooling period</td>
<td></td>
</tr>
</tbody>
</table>

#### 3.3.5 Isolation and characterization of wheat gluten

##### 3.3.5.1 Gluten isolation, gluten content and gluten index

Gluten was isolated from the wheat flour as per standard ICC method using Glutomatic (Fig. 3.5). Wheat flour sample (10 g) and 4.5 ml of 2% NaCl solution were mixed automatically in the mixing chamber of the instrument for 10 seconds, followed by washing with 2% NaCl solution. Wet gluten obtained was weighed and further subjected to centrifugation to determine the gluten index. The dry gluten was obtained by drying the wet gluten for 4 min in gluten dryer.

Wet gluten (%) = Weight of wet gluten/ Weight of sample taken × 100

Gluten Index (%) = Weight of gluten retained on sieve/ Weight of wet gluten × 100

Dry gluten (%) = Weight of dry gluten/ Weight of sample taken × 100
3.3.5.2 Gluten extensibility

Gluten extensibility of isolated gluten was analysed by Kieffer dough/gluten extensibility rig (A/KIE) using Texture Analyzer. Wet gluten was placed into the grooved base and upper block was tightened in the mould until the two blocks came together. The excess gluten from sides was removed by spatula and the gluten was rested for 40 min. After completion of resting time, the strips of gluten were analyzed by setting Pre-Test Speed 2.0 mm/s, Test Speed 3.3 mm/s, Post-Test Speed 10.0 mm/s and data acquisition rate 200 pps. The test mode of the instrument used was force in tension. Resistance to extension, g (R) and Extensibility, mm (E) were the two main parameters measured by the gluten extensibility test.

3.3.5.3 SDS sedimentation volume

Sodium dodecyl sulphate (SDS) sedimentation volume of flour samples was estimated by the method of Axford et al. (1979). SDS-sedimentation volume test gives an indirect measure of quantity and quality of gluten proteins. The SDS-lactic acid reagent was prepared by dissolving 20 g of SDS in 1 l of distilled water and adding to it 20 ml of stock diluted lactic acid solution (one part lactic acid and eight parts distilled water by volume). Flour sample (5 g) was taken in a 100 ml stoppered measuring cylinder and 50 ml distilled water to it. The contents were shaken vigorously for 15 s to achieve complete dispersion of flour in water. The contents were re-shaken for 15 s at 2 min and 4 min, respectively. After the last shake, 50 ml of SDS- lactic acid reagent was added, and the contents were mixed by inverting the cylinder four times. Inversion (four times) was repeated at 2, 4 and 6 min. The contents of the cylinder were allowed to settle for 40 min and sedimentation volume (ml) was recorded.
3.3.5.4 Glutenin/gliadin ratio

Modified Osborne method (1907) was used to fractionate the gluten proteins into glutenins and gliadins fractions. The wheat flours were defatted using chloroform as described by MacRitchie (1984). The extraction was repeated thrice and the contents were filtered. The defatted flour was dried at room temperature. Gluten was isolated from defatted flour dough by washing it manually using distilled water (15°C). The gluten obtained was freeze-dried and then ground to powder using a pestle and mortar. Powdered gluten (10 g) was suspended in 200 ml of 70% (v/v) ethanol and stirred on magnetic stirrer for 3 h at room temperature (~22°C) followed by centrifugation at 1000 x g for 30 min in a cooling centrifuge at 4°C. The extraction was repeated thrice. The precipitant was collected as glutenins and the supernatant was subjected to rotary evaporator at 30°C to remove ethanol to obtain the gliadins. Gliadin content (%), glutenin content (%) and gliadins/glutenin ratio were determined.

3.3.5.5 Extraction of glutenins using acetone precipitation for electrophoretic study

Total glutenins were extracted according to the procedure described by Khatkar (2006). Flour sample (1g) was taken and 0.5 M NaCl solution was added to it in order to remove albumins and globulins. The pellet was dispersed in distilled water thrice to remove the residual salt. The pellet was further suspended in 70% ethanol, stirred on a magnetic stirrer and centrifuged at 15,000 x g for 30 min to remove gliadins. The procedure was repeated twice. The residue obtained was suspended in 5 ml of 50% (v/v) propan-2-ol, 0.08 M Tris HCl (pH 8.0), 1% (w/v) dithiothreitol (DTT) for extraction of glutenins. The samples were kept in water bath at 60°C for 90 min with intermittent shaking and vortexing every 15 min. The contents were centrifuged at 15,000 x g for 30 min at 20°C. To the supernatant obtained, 20 ml pure acetone was added to achieve a final concentration of 80% (v/v) to precipitate total glutenins. The contents were centrifuged (15,000 x g for 30 min, 20°C). The total glutenin residue was dried in an oven at 60°C for 5 min. Total glutenins (4 mg) were suspended in 1 ml SDS sample buffer containing 62.5 mM Tris/ HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue and 5% (v/v) 2 mercaptoethanol. The protein buffer mixtures were vortexed for 2 min and kept at room temperature for 3 h. The samples were heated directly in a boiling water bath for 3 min. The clear supernatant so obtained was cooled to room temperature and used for electrophoresis.
3.3.5.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using slab gel electrophoresis apparatus (M/S ATTO, Japan). Glass plates were cleaned with ethanol, dried and assembled in gel casting assembly. A 12% polyacrylamide separating gel containing 1.35% bisacrylamide crosslinker was used according to the procedure of Laemelli (1970). After cooling to room temperature, 15 µl of total glutenin samples extracted using acetone precipitation method (as stated above) were loaded into the wells. Gels were run at constant current of 40 mA for 4 h or till the sample dye reached the end of the gel. The gels were stained overnight in the staining solution {80 ml of 0.1% (w/v) Coomassie Brilliant Blue G-250 in 2% (w/v) phosphoric acid, 10% (w/v) ammonium sulphate and 20 ml methanol adjusted to a final volume 100 ml}. The gels were then briefly washed with the destaining solution {25% (v/v) methanol in distilled water}. Gels were stored in 20 % (w/v) ammonium sulphate solution.

3.3.6 Isolation and characterization of wheat starch

3.3.6.1 Isolation of wheat starch

Pure starch was isolated from wheat flour samples of different wheat varieties according to the method described by Singh et al. (2010). Stiff dough ball was prepared by mixing wheat flour with water and was kept covered with moist cheese cloth for 1h. Starch was then washed manually using distilled water. The starch slurry collected was sieved, settled and centrifuged. Tailing starch was removed using spatula and pure starch fraction was purified by repeatedly suspending the starch in distilled water and centrifugation. The pure starch obtained was dried at 40°C in a hot air oven and was uniformly ground using pestle and mortar (Fig. 3.6).

3.3.6.2 Swelling power and solubility

Swelling power (g/g) and solubility (%) were determined using method of Leach et al. (1959). Aqueous starch suspension (2% w/w) was heated at 90°C for 30 minutes with constant stirring and cooled to room temperature. Samples were transferred to pre-weighed centrifuge tubes and centrifuged at 3000 rpm for 15 minutes. Supernatant and sediments were separated. Sediment obtained was weighed and supernatant was poured into pre-weighed moisture dishes, which were then dried at 110°C for 24 hours. All measurements were taken in triplicates. Solubility was expressed as the percentage of dried supernatant weight based on
weight of starch sample taken. Swelling power was calculated as the ratio of weight of residue to weight of starch sample.

Swelling power (g/g) = weight of sediments / weight of sample

Solubility % = Weight of the dried supernatant / Initial weight of the dry starch × 100

Fig. 3.6 Flow chart for the isolation of wheat starch

3.3.6.3 Light transmittance

Light transmittance (%) was measured as described by Craig et al. (1989). An aqueous suspension (1%) of starch was heated in a water bath at 90°C for 1 h with constant stirring. The suspension was cooled and held for 1 h at 30°C. The sample was then stored for 7 days
at 4°C, during which the light transmittance was determined every 24 h by measuring the absorbance at 640 nm against water blank with a spectrophotometer.

3.3.6.4 Amylose content

Amylose content of the isolated starches was determined by iodine binding method as described by Williams et al. (1970). The starch sample (20 mg, on dry basis) was dispersed in 10 ml KOH (0.5 N), vortexed for 5 min, and the volume was made to 100 ml using distilled water. 10 ml of the aliquot was taken and added 5 ml of HCl (0.1 N) and 0.5 ml of iodine reagent (20 g KI + 2 g resublimed iodine were dissolved in 100 ml distilled water, further 10 ml of this solution diluted to 100 ml was used as iodine reagent) were added and made up to 50 mL. Absorbance was measured at 625 nm. The quantity of amylose was determined from a standard curve developed using amylose and amylopectin blends. The absorbance was read on three replications per sample and averaged. Amylose content (%) was determined from a standard curve developed using amylose and amylopectin blends.

3.3.6.5 Pasting properties of wheat starch

The pasting properties of starch samples were determined using a Rapid Visco Analyser. Starch sample (3.0g, 14%mb) was weighed in the canister and distilled water was added to obtain a sample weight of 28.0g. The temperature-time conditions included a heating step from 50 to 95°C at 6°C/min (after an equilibration time of 1 min at 50°C), a holding phase at 95°C for 1.5 min, a cooling step from 95 to 50°C at 6°C/min, and a holding phase at 50°C for 2 min. Pasting temperature, peak viscosity, trough viscosity, breakdown, final viscosity, and setback were recorded.

3.3.6.6 Starch gel characteristics

The gelatinized mixture in the canister, after the RVA measurement, was sealed with paraffin film to prevent moisture loss and kept overnight at room temperature to allow gelation. The gel was then subjected to texture profile analysis following the method of Wu et al. (2006) and the textural parameters hardness (the maximum force on first cycle, g), adhesiveness (the total negative area between the first and the second peak, g/s), springiness (the ratio of height at second compression to the height at first compression), cohesiveness (the ratio of the positive force area between the second and first compression), gumminess (hardness × cohesiveness), chewiness (gumminess × springiness) were computed from the graph obtained.
3.3.6.7 Thermal properties of wheat starch

Thermal properties of isolated starches were analyzed using Differential Scanning Calorimetry. Starch samples (3.5 mg, db) were weighed in an aluminium pan and distilled water was added with the help of a Hamilton microsyringe to obtain a starch-water suspension containing 70% water (w/w). The pan was hermetically sealed and allowed to equilibrate for 1 h before analysis. The instrument was calibrated using indium and an empty aluminum pan was used as a reference. The sample pans were heated from 40 to 110°C at the rate of 10°C/min. The onset of gelatinization (T_o), the temperature at peak (T_p), the temperature at the end of gelatinization (T_e) and enthalpy of gelatinization (Δ H_{gel}, J/g) were determined.

3.3.6.8 Scanning electron microscopy

Scanning electron micrographs of starch granules were taken using Scanning Electron Microscope (Fig. 3.7). Starch samples were suspended in ethanol (1%), mounted on the aluminium stubs using double-sided sticky tape, and coated with gold. An accelerating potential of 5 kV was used during microscopy.

Fig. 3.7 Scanning electron microscope
3.3.6.9 Damaged starch

Damaged starch content of wheat flour was estimated using SDmatic. The reaction mixture (120 ml distilled water, 3 g boric acid, 3 g of potassium iodide and 1 drop of 0.1 mol/l sodium thiosulphate) was prepared, mixed well and poured into the reaction bowl. Flour sample (1 g) was weighed in the sample pan and inserted into the sample pan holder. The moisture, protein content and weight of sample taken were entered and test was started. The test takes around 10 min for its completion. On completion of the test, the results of damaged starch were displayed and noted in AACC units.

3.3.7 Instant noodle preparation

3.3.7.1 Optimization of formula ingredients

Preparation of noodles with varying formula ingredients: The instant noodles were prepared using the process described by Wu et al. (2006) with some modifications. The formula ingredients including salt (1-2%), alkaline salt (potassium carbonate and sodium carbonate, 1:1) (0.1-0.3%), guar gum (0.2-0.6%) and water (30-35%) were varied at levels selected on the basis of previous studies (Park and Baik 2004b; Wu et al. 2006; Yu and Ngadi 2004). The amount of water to be incorporated was also judged on the basis of minimum and maximum water for acceptable noodle sheet formation. Wheat flour 100 g (on 14% moisture basis) and water containing dissolved salts and guar gum were mixed thoroughly using mixer for 5 minutes. The crumbly dough was then formed into sheet using noodle machine by passing it through roll no. 1 four times and folding in half each time. The dough sheet formed was divided into two halves and rested for 10 minutes in ziplock pouches. The dough sheet was then passed five times through roller unit attachment with the regulating knob set at position no. 2, 3 and 4 respectively. After passing through the final roll the dough sheet with final thickness of 1.5 mm was again rested for 30 min in polybag to avoid the moisture loss. The dough sheet was then cut through the cutter attachment. The resulting noodle strands were placed uniformly on a sieve and put into a preheated (100°C) steamer, and steamed for 5 min. The steamed noodle strips were placed in a wire basket and immersed in oil (refined soyabean oil) at a temperature of 145°C for 90 s in a deep fat fryer. The fried noodle strips were allowed to cool for 15 min and excess oil was drained from the surface. The cooled samples were stored in ziplock pouches for further analysis.
Experimental design and statistical analysis: The study was based on the hypothesis that oil uptake, cooked weight, cooking loss, texture and overall acceptability of instant noodles are functionally related to the level of incorporation of ingredients used for preparation of instant noodles, and attempts to fit a multiple regression equation describing these attributes were made. The levels of variation were chosen within the reasonable range based on some preliminary studies and other studies conducted by other previous researchers.

The design and levels of factors are shown in Table 3.2. The main advantage of using experimental design approach was the ability to reduce the number of experimental runs required to provide sufficient information for statistically acceptable results. Response surface methodology was chosen to analyze the effect of variables including water absorption (A), alkaline salt (B), guar gum (C) and salt (D) on quality properties (Oil uptake, cooking loss, cooked weight, texture and overall acceptability) of instant noodles. The experiments were designed and processed using Design Expert 8.0.5 software (Stat Ease Inc.). Experiments were conducted for four variables using box benkehn design involving 29 combinations with five centre points (see Table 4.2). The runs were randomized in order to minimise the effects of unexplained variability in the observed responses due to extraneous factors.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Experimental Factor</th>
<th>Coded Value</th>
<th>Actual Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Water absorption (%)</td>
<td>-1 0 +1</td>
<td>30.0 32.5 35.0</td>
</tr>
<tr>
<td>B</td>
<td>Alkaline salt (%)</td>
<td>-1 0 +1</td>
<td>0.1 0.2 0.3</td>
</tr>
<tr>
<td>C</td>
<td>Guar gum (%)</td>
<td>-1 0 +1</td>
<td>0.2 0.4 0.6</td>
</tr>
<tr>
<td>D</td>
<td>Salt (%)</td>
<td>-1 0 +1</td>
<td>1.0 1.5 2.0</td>
</tr>
</tbody>
</table>

After the selection of model suggested, analysis of variance was done. To evaluate the fitness of model, F-values were determined. Further details have been mentioned in Chapter 4.

3.3.7.2 Optimization of noodle making process

Preparation of noodles using varying processing conditions: The same commercial flour which was used for ingredient optimization was utilized to study the effect of processing variables. The instant noodles were prepared using the standardized ingredient formulation i.e. water absorption, alkaline salt, guar gum and salt 30.97, 0.23, 0.28 and 1.54%, respectively as optimized in Chapter 4. The processing variables were varied and levels were selected on the basis of previous studies (Park and Baik 2004b; Wu et al. 2006; Yu and Ngadi 2004). The wheat flour 100 g (on 14% moisture basis) and water containing dissolved salts
and guar gum were mixed thoroughly using KitchenAid mixer for 4, 8 and 12 min at medium speed. The crumbly dough obtained was then formed into sheet using noodle machine by passing it through roll no. 1 (3.2 mm) four times and folding in half each time. The dough sheet formed was divided into two halves and rested for 10 min in ziplock pouches. The dough sheet was then passed five times through roller unit attachment with the regulating knob set at position no. 2 (2.5 mm), 3 (2.0 mm), 4 (1.5 mm), 5 (1.2 mm), and 6 (1 mm), respectively in order to attain desired dough sheet thickness as per experiment design. After achieving desired final thickness the dough sheet was again rested for 30 min in polybag to avoid any moisture loss. The dough sheet was then cut through the cutter attachment. The resulting noodle strands were steamed into a preheated (100°C) steamer, and cooked for 3, 5 or 7 min. The steamed noodle strips were placed in a wire basket and immersed in oil (Refined soyabean oil, Nutrella Soyumm) at temperature and time combination obtained using RSM in a deep fat fryer. The fried noodle strips were cooled for 15 min and excess oil was drained from the surface. The samples were then stored in plastic bags for further analysis.

**Experimental design and statistical analysis for process optimization:** The design and levels of factors studied are shown below in Tables 3.3. Response surface methodology was chosen to build some mathematical models using box benkehn design, making it possible to quantitatively interpret and describe the relationship between process variables and quality properties viz. oil uptake, cooking time, cooked weight, cooking loss, hardness and overall acceptability of instant noodles. The experiments were designed and processed using Design Expert 8 software (Stat Ease Inc). Experiments were conducted with five independent variables (mixing time, dough sheet thickness, steaming time, frying temperature and frying time) resulting in 46 random combinations (including five centre points) according to box benkehn design (see Table 5.1).

**Table 3.3 Levels of examined processing variables according to box-benkehn RSM design**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Experimental Factor</th>
<th>Coded Value</th>
<th>Actual Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Mixing time (min)</td>
<td>-1</td>
<td>4.0</td>
</tr>
<tr>
<td>B</td>
<td>Dough sheet thickness (mm)</td>
<td>-1</td>
<td>1.0</td>
</tr>
<tr>
<td>C</td>
<td>Steaming time (min)</td>
<td>-1</td>
<td>3.0</td>
</tr>
<tr>
<td>D</td>
<td>Frying temperature (°C)</td>
<td>-1</td>
<td>130.0</td>
</tr>
<tr>
<td>E</td>
<td>Frying time (min)</td>
<td>-1</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Responses were analysed using ANOVA and F-values were determined. Lack of fit for the response models in each case was not significant indicating the validity of fitness of models. Predicted equations for the responses were derived using backward regression analysis in order to eliminate the insignificant (p>0.1) model terms (Chapter 5, Table 5.3).

3.3.7.3 Preparation of noodles of different wheat varieties

Instant noodles were prepared using the standardized formulation and processing conditions (Chapter 4 and 5). The formula ingredients including water (30.97%), alkaline salt (potassium carbonate and sodium carbonate, 1:1) (0.23%), guar gum (0.28%) and salt (1.54%) were used for preparation of noodles. Wheat flour 100 g (on 14% moisture basis) and water (based on calculated water absorption as per optimal water absorption obtained from Mixolab and multiplying it with the factor derived from the standardized water absorption for the commercial flour) containing dissolved salts and guar gum were mixed thoroughly for 4 min. After mixing, the crumbly dough was formed into sheet using noodle machine by passing it through roll no. 1 four times and folding in half each time. The dough sheet formed (3.2 mm) was divided into two halves and rested for 10 min (25°C) in ziplock pouches. The dough sheet was then passed five times through roller unit attachment with the regulating knob set at position no. 2 (2.5 mm), 3 (2.0 mm), 4 (1.5 mm) and 5 (1.2 mm), respectively. After passing through the final roll the dough sheet with final thickness of 1.2 mm was again rested for 30 min (25°C) in polybag to prevent moisture loss. After the final dough resting, dough sheet was cut through the cutter attachment. The resulting rectangular noodle strands (2.0 mm × 1.2 mm) were placed uniformly on a sieve and put into a preheated (100°C) steamer, and steamed for 6.4 min. The steamed noodle strips were placed in a wire basket and immersed in oil (refined soyabean oil) at a temperature of 142°C for 2 min in a deep fat fryer. The fried noodle strips were allowed to cool for 15 min and excess oil was drained from the surface. The cooled samples were stored in ziplock pouches for further analysis.

During frying of noodles quality of fried oil was monitored and judged by using Deep frying oil tester (Testo 270) (Fig. 3.8) which measured the total polar matter (TPM) in the oil developed as a result of repeated frying.
3.3.8 Assessment of instant noodle quality

Quality factors important for instant noodles are colour, texture, cooking quality, rehydration rates during final preparation and the presence or absence of rancid taste after extended storage.

3.3.8.1 Oil uptake

Oil uptake was estimated according to approved AACC (2000) method using solvent extractor. The fried noodles were uniformly ground and oil extraction was performed with petroleum ether (60-80°C) using a solvent extractor (SER148, Velp Scientifica, Usmate, Italy). Three replicates were made for each measurement and the mean has been reported. Oil uptake was expressed in terms of percentage on dry basis.

![Monitoring the quality of frying oil using Testo 270 (deep-frying oil tester)](image)

3.3.8.2 Cooking time, cooking loss and cooked weight

Fried instant noodles (10 g) were added to 400 ml of boiling water in a 500 ml beaker and cooked to the optimum cooking time according to the method of Oh et al. (1983) by crushing the noodle strands between glass petriplates. The cooked noodles were cooled in running tap water for 1 min. The noodles were drained and wiped to remove excess water from their surface, reweighed, and then stored in a covered petriplate at room temperature for texture
analysis. The water left after cooking along with rinsings was collected and an aliquot of 50 ml was evaporated in oven at 100°C for 4 h to determine cooking loss. Results were reported as per cent weight loss during cooking. The cooked weight was recorded as per cent increase in weight of noodles after cooking as demonstrated by Wang et al. (2011).

**3.3.8.3 Texture analysis of cooked noodles**

Texture measurements were carried out using Texture Analyser (Stable Micro Systems TA-XT 2i, U.K.). The instrument was calibrated using 5 kg load cell and probe distance was 15 mm. The settings were: mode- texture profile analysis (TPA); pre-test speed, test speed and post-test speed- 2.0, 3.0 and 3.0 mm/sec respectively; distance- 1 mm; trigger type- auto 5 g; and the probe compression plate of 45 mm × 30 mm was used. Five noodle strands were arranged completely flat close to each other. Three replicates were taken for each sample completed within 15 min after cooking and the results have been presented as noodle hardness in Newton (N) obtained from the peak of the graph. The textural parameters i.e. springiness, adhesiveness, cohesiveness and chewiness were calculated.

**Table 3.4 Reference score sheet used by sensory panel to evaluate instant noodle quality**

<table>
<thead>
<tr>
<th>Quality parameter</th>
<th>Product Characteristic</th>
<th>Score Point</th>
<th>Product Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour (15)</td>
<td>Yellow</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amber</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Milky</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Smooth surface</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Shape and Appearance (15)</td>
<td>Coarse surface</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Very coarse surface</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Very good</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Bite Characteristics (15)</td>
<td>Good</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Excellent</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Chewiness (15)</td>
<td>Good</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Non-sticky</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Stickiness (15)</td>
<td>Slightly sticky</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Very sticky</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Excellent</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Taste (15)</td>
<td>Good</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bland</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Very easy to rehydrate</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Rehydration (10)</td>
<td>Easy to rehydrate</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hard to rehydrate</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>
3.3.8.4 Overall acceptability

Sensory analysis was done to determine the overall acceptability of instant noodles using five panel members previously trained about noodle characteristics using commercial noodle samples in order to differentiate effectively between prepared samples. Sensory analysis was done using Composite scoring method (detailed in Table 3.4) in which scores were given to various quality characteristics of the product i.e. Colour (15), Shape and appearance (15), Bite characteristics (15), Chewiness (15), Stickiness (15), Taste (15) and Cooked Weight (10). The mean total score assigned out of 100 by panelists was used to assess the overall acceptability of the product.

3.3.8.5 Noodle microstructure

Microstructure of the good and poor instant noodles were analysed using Scanning Electron Microscopy (SEMTRAC Mini, Nikkiso, Germany). The fried instant noodles strands were fractured to expose their inner structure and placed on to the stubs using double-sided sticky tape (Dexter et al. 1979). The exposed surface was coated with gold and then the inner surface was scanned at 5kV potential and 100 X magnification.

3.3.9 Molecular studies using RAPD primers

3.3.9.1 DNA extraction

Genomic DNA was isolated from young leaves of 15 days old plants (raised in a green house using pure wheat grains) using CTAB method (Doyle and Doyle 1987). Five grams of macerated leaf tissue was dispersed in 10 ml of pre-warmed CTAB extraction buffer (65°C) by gentle and thorough inversion of the tubes. The samples were incubated in a water bath at 65°C for 90 minutes to assist cell lysis with an intermittent mixing of tube contents at an interval of 15 minutes. The samples were then cooled to room temperature and 10 ml of phenol: chloroform: isoamyl alcohol (25:24:1) was added to denature the proteins and facilitate phase separation. The contents were centrifuged at 10,000 rpm for 10 min at 15 °C. The supernatant was collected and mixed with two volumes of chilled iso-propanol to precipitate the DNA. DNA pellet was obtained by centrifugation at 8000 rpm for 10 min. The pellet was washed with 70% ethanol, air dried and resuspended in TE buffer (Tris pH 8.0 10 mM, EDTA pH 8.0, 0.5 mM). RNA was removed by treating with enzyme RNAase A (10 µg/ml) for 60 min at 37 °C, while the protein was digested using Proteinase-K for 60 min at
55 ºC. Gel electrophoresis of the purified DNA samples was done using 0.8% agarose gel and TBE buffer to assess the quality of DNA.

### 3.3.9.2 PCR conditions and gel analysis of PCR products

PCR amplification parameters including primer, MgCl₂ concentration, annealing temperature and no. of cycles were varied to determine the optimal conditions for the reaction. PCR was carried out in 25 µl reaction volumes using a Thermocycler. A total of 15 RAPD primers (Operon Technologies, Alameda, USA) were used for PCR amplification. The PCR reaction mixture (25 µl) contained 1x PCR buffer, 1.5 µl MgCl₂, 2 µl dNTPs, 1.0 µl of primer, 0.5 unit of Taq DNA polymerase and 1 µl (50 ng) template DNA. The PCR regime consisted of an initial denaturation (94ºC for 10 min), 40 cycles each consisting of a denaturation step (94ºC for 1 min), annealing at 40ºC (1 min), and an elongation step at 72ºC (1 min). At the end of the run, a final extension period was included (72ºC for 7 min). PCR products were stored at 4ºC until they were analysed. For gel electrophoresis, a 2 µl aliquot of PCR product was combined with 2 µl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water) and electrophoresed on 1.2% agarose gel in Tris Borate EDTA buffer at 80 V for 2.5h. A 1 kb and 100bp DNA ladder was used as a molecular weight standard on each gel. The DNA bands were visualized and photographed under UV light using the gel documentation system.

### 3.3.10 Statistical analysis

All determinations were made in triplicate. Data was analyzed using SPSS software version 16.0 (SPSS Inc.). Correlation among various parameters were derived using Pearson’s test (p<0.05). The mean comparison was carried out using one way ANOVA with Duncan’s multiple range test. The statistical significance was observed at p < 0.05. Multiple regression equations for noodle characteristics were derived using SPSS employing linear stepwise regression to retain the most significant variables influencing the quality parameter.

Multivariate hierarchical cluster analysis (HCA) was done using SPSS software version 16.0 (SPSS Inc.) to show the average linkage between the genotypes through a dendrogram. Four dendograms were obtained on the basis of physical (length, breadth, thousand kernel weight, hectoliter weight and grain hardness), protein (protein content, SDS sedimentation volume, gluten content, R/E ratio of gluten and gluten index), starch (amylose content, damage starch, solubility, swelling power and starch paste peak viscosity) and
noodle quality parameters (oil uptake, cooking time, cooking loss, cooked noodle hardness and overall acceptability score). The frequency of RAPD polymorphism between the wheat cultivars was assessed based on the presence (1) and absence (0) of band. Data was analysed using NTSYS-pc (numerical taxonomy and multivariate analysis) software. The 0/1 matrix was used to calculate the similarity matrices using ‘Simqual’ subprogram of software NTSYS-pc. Dendogram representing the genetic variation among the wheat cultivars was built based on the unweighted pair group method.