Chapter -III

Materials & Methods

3.1. Materials

3.1.1. Watermelon cultivars

Fruits of ‘Namdhari-95’ and ‘Namdhari-450’ cultivars of watermelon were procured from the farm located in Kapurthala, Punjab, India and ‘Sugar Baby’ cultivar was obtained from Department of Vegetables, Punjab Agricultural University, Ludhiana, Punjab, India.

3.1.2. Chemicals

Lycopene and β-carotene standards were obtained from Sigma Chemical Co. St. Louis, Missouri, USA. The chemicals used for analysis were of analytical or HPLC grade supplied by Sisco Research Laboratories (SRL) Pvt Ltd, Mumbai, India; Thomas Baker (Chemical) Ltd, Mumbai, India; Qualigens Fine Chemicals, Mumbai, India; CDH Pvt, Ltd, New Delhi, India; Spectrochem Pvt Ltd, Mumbai, India.

3.1.3. Albino rats and diet

Healthy male albino rats were procured from local source for the in vivo studies. The standard rat diet was procured from M/S Hindustan Lever Ltd, Mumbai, India.

3.2. Methods

3.2.1 Cultivation, harvesting, storage and processing of watermelon fruit

3.2.1.1. Plant cultivation

Seeds of watermelon cultivars were sown in the first week of March. During growth atmospheric temperature, soil temperature and soil pH were 28-30°C, 25-30°C and 6.5-7.0 respectively. Plant spacing for ‘Namdhari-95’ and ‘Namdhari-450’ cultivars was 3.5 and 1.2m whereas for ‘Sugar baby’ was 2.0 and 1.0 m. Manure
around 15-22.5 ton/ha was thoroughly mixed with the soil at the time of land preparation. The chemical fertilizers were given as per directions of Punjab Agricultural University, Ludhiana, India. Watermelon crop was irrigated in initial stages up to depth of 1.5 cm and later on very less irrigation was required. The fruit yield obtained was in the range of 20-25 ton/ha.

3.2.1.2. Harvesting

Watermelon fruits were randomly harvested manually at four different development stages. Detail of development stages of watermelon fruit are as follows-

<table>
<thead>
<tr>
<th>Development</th>
<th>Fruit Size</th>
<th>Harvesting time (days after sowing)</th>
<th>Maturity Indices</th>
</tr>
</thead>
<tbody>
<tr>
<td>White (Young)</td>
<td>Small</td>
<td>55</td>
<td>White flesh and shiny waxy rind</td>
</tr>
<tr>
<td>White-pink (Premature)</td>
<td>Medium</td>
<td>62</td>
<td>White/pink flesh and clear echo</td>
</tr>
<tr>
<td>Pink (Mature)</td>
<td>Large</td>
<td>70</td>
<td>Pink flesh and green tendril</td>
</tr>
<tr>
<td>Red Ripe (Ripened)</td>
<td>Full</td>
<td>78-85</td>
<td>Red flesh, brown tendril, dull sound, yellow ground spot and sticky fruit texture</td>
</tr>
</tbody>
</table>

[Bangalore et al., 2008; Tlili et al., 2011a]

3.2.1.3. Post harvest handling and storage

Watermelon fruits were transferred from the farm to laboratory under cool conditions (4-6°C). Physical parameters like weight, diameter, length and L/D ratio of fruit at different ripening stages were determined.

Fruits were stored under 4, 14 and 24°C for 15 days and analyzed for physicochemical properties and pigment content.
3.2.1.4. Processing of watermelon fruit

Watermelon fruits (6-7) were thoroughly washed and cut longitudinally from the stem end to the blossom end through the ground spot. The images of cut watermelon at different ripening stages were taken. The rind was removed by stainless steel knife manually. Flesh was cut into small pieces and passed through the screw juice extractor (Kalsi Industries Ltd., Ludhiana, India) to obtain juice and pomace. The yield of juice, pomace and seeds was calculated. The watermelon juice and pomace were stored in the deep freezer at -20°C for further analysis. The pomace was used for drying and pigment degradation studies. The watermelon juice was further centrifuged (14000 x g/15 min) (REMI, Mumbai, India) to get pulp containing pigment and clear juice without pigment. The pulp was stored in polyethylene pouches at -20°C till used for the extraction optimization studies.

3.2.2. Physico-chemical analysis of watermelon fruit, pomace, juice and pulp

3.2.2.1. Moisture content

The moisture content was determined in a vacuum oven (Narang Scientific Pvt Ltd., New Delhi, India) at 60±2°C and 100mm Hg pressure for 24h (AOAC, 1990).

3.2.2.2. Total Soluble solids

Total soluble solids were quantified by using hand refractometer (Model A, Erma, Tokyo, Japan).

3.2.2.3. Titrable Acidity

Titrable acidity (% citric acid) was determined using a pH meter (LI120, Elico, Hyderabad, India). The titration was performed using standardized 0.1N NaOH and 2-3 drops of phenolphthalein indicator.
3.2.2.4. Total sugars, reducing sugars and non-reducing sugars

Reducing and total sugars were determined according to Lane and Eynon method (Ranganna, 1986). Non-reducing sugar was calculated from difference of total sugars and reducing sugars. Invert sugar reduces the copper in Fehling’s solution to red, insoluble cuprous oxide. The sugar content in a food sample is estimated by determining the volume of the unknown sugar solution required to completely reduce a measured volume of standardized Fehling’s solution.

3.2.2.5. Ascorbic acid (Ranganna, 1986)

Dye factor (0.5/titre value) was calculated by titrating standard L-ascorbic acid (100 mg ascorbic acid in 3% HPO₃) with standardized dye solution (50 mg of sodium salt of 2,6-dichlorophenol-indophenol+150 ml of hot distilled water + 42 mg sodium bicarbonate and diluted unto 200ml). Sample was extracted with metaphosphoric acid (30 g/L in water), filtrated and titrated against standardized dye solution up to a pink colour end point, which persisted for 15s.

Ascorbic acid (mg/100g) = \( \frac{\text{Titre (ml)} \times \text{Dye factor (mg/ml)} \times \text{Vol.made up (ml)}}{\text{Extract taken for estimation (ml)} \times \text{wrt of sample (g)}} \times 100 \) \hspace{1cm} (1)

3.2.2.6. Proximate analysis of watermelon pomace

Crude protein (Micro-Kjeldahl method), crude fat (Soxhlet method) content, crude fiber (Acid-Alkali Digestion) and total ash (Oxidation of Organic Matter at 550±5°C) of pomace were determined (AOAC, 1990). Carbohydrates were determined by difference.

3.2.2.7. Lycopene and β-carotene

The sample was extracted with acetone in a pestle and mortar till residues became colourless. Pigment was transferred into petroleum ether phase in a separating funnel, passed through sodium sulphate and evaporated under vacuum. Lycopene content was determined by HPLC (Waters, Milford, MA, USA) fitted with a photodiode array detector using YMC Carotenoids (5µm) column, methanol: ethanol: tetrahydrofuran
(15:4:1) mobile phase, 1 ml/min flow rate and 470-503 nm wavelength (Perkins-Veazie and Collins, 2006). Lycopene (Sigma Chemical Co, St. Louis, Missouri, U.S.A.) was used as standard. The extinction coefficient (1.72 x 10⁴ mol cm⁻¹) was verified with standard lycopene solution (Sigma Chemical Co, St. Louis, Missouri, U.S.A.) and was used to calculate the lycopene in sample.

For β-carotene estimation, the filtered pigment extract in petroleum ether was loaded onto a 10 cm long column of magnesium oxide overlaid by 1 cm length of anhydrous sodium sulphate. The column was washed using eluent (3 ml acetone: 97 ml petroleum ether) until β-carotene moved off the column and filtrate became colourless. Contents were diluted to 100 ml with eluent. The intensity of colour was measured at 450 nm using HPLC (Waters, Milford, MA, USA) (Ranganna, 1986) fitted with a photodiode array detector using YMC Carotenoids (5µm) column, methanol: ethanol: tetrahydrofuran (15:4:1) mobile phase, 1 ml/min flow rate and 452 nm wavelength.

3.2.2.8. Total carotenoids (Ranganna, 1986)

The total carotenoids were determined by measuring the absorbance of the extracted sample at 452 nm on UV visible spectrophotometer (2450 Shimadzu Co., Ltd., Tokyo, Japan) using β-carotene (Sigma Chemical Co, St. Louis, Missouri, USA) as standard.

3.2.2.9. Colour Analysis

Colour was measured using a Hunter Colour Lab (Ultra Scan-VIS Hunter Associates Laboratory, Reston, U.S.A.) in terms of L (lightness), a (redness (+) and greenness (-)) and b (yellowness (+) and blueness (-)). The instrument was calibrated with a standard black tile and then standard white tile (L= 90.55, a= -0.71, b= 0.39). A sample handling dish was charged with samples, placed on the analyzing port and noted the L, a, b values. The hue angle (h° = tan⁻¹b/a) represents the maximum degree of redness at 0°, yellowness at 90°, greenness at 180° and blueness at 270° (Patras et al., 2011). The chroma (C=√a²+b²) indicates the colour intensity or saturation of sample. ΔE= [(L-L*)²+(a-a*)²+(b-b*)²]¹/² indicates the magnitude of colour difference between watermelon samples with ‘L*, ‘a*’, ‘b*’ values are for ideal sample.
3.3. Concentration of watermelon juice

The soluble solids of watermelon juice were increased from initial value to 20, 40, 60 and 70 g/100g by means of a rotary vacuum evaporator (Buchi Labortechnik AG, Flawil, Switzerland) using water bath temperature of 45±2°C, vacuum of 25±2 mbar and speed of 20 revolutions per minute.

3.3.1. Physico-chemical properties of watermelon concentrates

The moisture content, soluble solids, titrable acidity, reducing, total sugars and non-reducing sugar of watermelon concentrates were estimated as given in section 3.2.2.1. to 3.2.2.4.

3.3.2. Lycopene and total carotenoids of watermelon concentrates

The lycopene and total carotenoids of watermelon concentrates were quantified as given in section 3.2.2.7. to 3.2.2.8.

3.3.3. Colour Analysis of watermelon concentrates

The colour of watermelon concentrates were quantified as given in section 3.2.2.9.

3.3.4. Rheology of watermelon concentrates

A programmable Rheolab QC system (Model No.: CC27-SN 16634, Needle No.: DG26.7-SN17111, Antor Paar, Graz, Austria, Europe) was used for the rheological study of watermelon concentrates. Data on shear stress and apparent viscosity were recorded for watermelon juice concentrate up to shear rate of 1000s⁻¹. Various empirical models employed to study the flow behavior (Steffe, 1996; Kyereme et al., 1999; Chuah et al., 2008) were:

- Power Law Model: \( \tau = k_p (\gamma)^n \) (2)
- Herschel-Bulkley Model: \( \tau = \tau_0 + k_{HB} (\gamma)^n \) (3)
- Casson Model: \( \tau^{0.5} = \tau_0^{0.5} + k_c (\gamma)^{0.5} \) (4)
where $\tau$ is shear stress (Pa), $\gamma$ is shear rate (s$^{-1}$), $k$ is consistency index for the respective models (Pa s$^n$), $n$ is flow behavior index (dimensionless).

### 3.4. Preparation of watermelon juice powder

The watermelon juice was passed through 40 microns sieve and then through 15 microns sieve to remove the fibrous matter. Maltodextrin (3, 5, 7 and 10%) was added in the juice and spray dried (ADL 31, Yamato Scientific Co. Ltd., Tokyo, Japan). Atomization of the juice was done using two fluid nozzle in which one part contained juice and other one contained compressed hot air. The spray dryer was operated at 125$^\circ$C inlet temperature, 70$^\circ$C outlet temperature, 0.25 kg/cm$^2$ air pressure, 6.5 m$^3$/min aspiration rate, 4 ml/min pump speed and 3 g/min feed rate. The powder was packed in air tight Low Density polyethylene pouches and stored at 4$^\circ$C in dark.

In second drying method, the watermelon juice was passed through the 40 microns sieve, mixed with maltodextrin (3, 5, 7 and 10%), frozen in a chiller (Heto, Vedback, Denmark), dried in a freeze drier (Heto, Vedback, Denmark), packed in air tight polyethylene pouches and kept at -20$^\circ$C.

### 3.4.1. Physico-chemical analysis of watermelon juice powder

The moisture content, soluble solids, titrable acidity, reducing, total sugars and non-reducing sugar of watermelon juice powder were estimated as given in section 3.2.2.1. to 3.2.2.4. The water activity was determined using water activity analyzer (Aqua Lab 4TE, Decagon Devices, USA). For the dissolution test, 50mg of powder sample was placed in a test tube. 1ml of distilled water at room temperature was added and mixing was performed using vortex. The time (s) required to fully reconstitute the powders was recorded (Quek et al., 2007).

### 3.4.2. Lycopene and total carotenoids of watermelon juice powder

The lycopene and total carotenoids of watermelon juice powder were quantified as given in section 3.2.2.7. to 3.2.2.8.
3.4.3. Visual Colour Analysis of watermelon juice powder

The visual colour of watermelon juice powder were quantified as given in section 3.2.2.9.

3.5. Dehydration characteristics, thermal treatment and sorption isotherms of watermelon pomace

3.5.1. Dehydration of watermelon pomace

Watermelon pomace was dehydrated in fluidized bed dryer (FBD 2000, Endecotts Ltd., London, U.K.) and cabinet dryer (ESS 36 T, La Parmigiana, Fidenza, Italy) at different temperatures 50, 60 and 70°C with feed rates of 2, 4 and 6 kg/m². The initial weight as per tray load was considered at the mass at zero time. The change in weight was recorded after every 10 min and 1 h for fluidized bed dryer and cabinet dryer respectively. The drying was carried out till three concordant constant readings were obtained. The initial moisture content of watermelon pomace was determined by keeping in a vacuum oven (Narang Scientific Pvt Ltd., New Delhi, India) at 60±2°C and 100mm Hg pressure for 24h.

3.5.1.1. Mathematical Drying models

The moisture ratio was calculated using the following equation:

\[ M.R. = \frac{(m_t - m_e)}{(m_o - m_e)} \]  

where M.R.- moisture ratio; \(m_t\) –moisture content dry basis (% d.b.) at any given instant time \(t\); \(m_e\) - equilibrium moisture content (% d.b.); \(m_o\) - initial moisture content (% d.b.); \(t\) - time (h). The following mathematical models were applied to the drying data of watermelon pomace:

- **Lewis Model**
  \[ M.R. = \exp (-k t) \]  
- **Henderson and Pabis Model**
  \[ M.R. = a \cdot \exp (-k t) \]  
- **Logarithmic Model**
  \[ M.R. = a \cdot \exp (-k t^n) + c \]  
- **Page Model**
  \[ M.R. = \exp (-k t^n) \]  
- **Wang and Singh Model**
  \[ M.R. = 1+at+bt^2 \]
where \( a, b, c, n \), are constants in models (dimensionless number) and \( k \) is drying rate constant (1/h). The best suited drying model was used to calculate the drying time needed to reduce the initial moisture content to 8.5% d.b.

### 3.5.1.2. Effective Moisture Diffusivity

Fick’s second law of diffusion assuming uni-dimensional moisture movement without volume change, constant diffusivity, uniform initial moisture distribution and negligible resistance was used to compute moisture diffusivity from experimental drying data (Crank, 1975; Sinija and Mishra, 2009). The second law of diffusion was transformed in the form of following equation (Doymaz et al., 2004; Akpinar and Toraman, 2013, Koua et al., 2013).

\[
MR = \frac{8}{\pi^2} \times \exp \left( \frac{-\pi^2 D \cdot t}{4l^2} \right) \tag{11}
\]

where \( D \): effective moisture diffusivity (m\(^2\)/sec); \( l \): average sample thickness (2kg/m\(^2\) is 0.007m, 4kg/m\(^2\) is 0.009m, 6kg/m\(^2\) is 0.011m).

The correlation between drying rate constant and effective moisture diffusivity was also found using linear, exponential and power models. The adequacy of the model was evaluated on the basis of coefficient of determination (R\(^2\)).

### 3.5.1.3. Effect of Temperature on Drying Rate constant and Effective Moisture Diffusivity

The Arrhenius law was used to relate ‘\( k \)’ and ‘\( D \)’ with drying air temperature and to calculate the activation energy:

\[
k = k_o \exp \left( -\frac{E_a}{RT} \right) \tag{12}
\]
\[
D = D_o \exp \left( -\frac{E_a}{RT} \right) \tag{13}
\]

where \( k_o \): frequency factor (l/h); \( D_o \) – Reference diffusion coefficient at infinitely high temperature; \( E_a \): activation energy (kJ/mol); \( R \): Universal gas constant (8.314 kJ/mol.K); \( T \): Absolute temperature (K).
3.5.2. Thermal degradation of pigment in watermelon pomace

The watermelon pomace was sealed in glass cultured tubes (19 mm internal diameter × 930 mm length) and was immersed in a water bath (TC-2000 Brookfield Laboratories, Middleboro, USA) for preset times (0, 1, 2, 3, 4, 5, 6 and 7 h) at 50, 60, 70, 80, 90°C. The desired temperature was considered to have achieved when the temperature of water-bath reached the set value.

The kinetics of degradation of pigments has been reported to follow first order reaction adequately (Weemaes et al., 1999; Ahmed et al., 2000). The first order kinetic model for lycopene degradation of watermelon pomace is:

\[ \ln \left( \frac{L}{L_0} \right) = -k_D t \]  
(14)

where, \( L \) is the concentration of lycopene at time ‘t’ (mg/100g), \( L_0 \) is the initial concentration of lycopene (mg/100g), \( k_D \) is the degradation rate constant (1/h), and \( t \) is heating time (h).

3.5.3. Sorption Isotherm of dehydrated watermelon pomace waste

The static method was used to determine equilibrium moisture content (EMC) of the dehydrated watermelon pomace (Brooker et al., 1974; Ranganna, 1986) at 20-50°C. Samples were placed into the desiccators containing saturated salt solutions (LiCl, KC2H3O3, MgCl2, NaCr2O7, NaBr, Na2C2H2O2, (NH4)2 SO4, KNO3) to maintain RH in the range of 11-90%. The desiccators containing salt solution were kept in incubator at selected temperature (20-50°C). Samples were allowed to equilibrate for 21 days and changes in weight were used to determine EMC. At higher water activities (\( a_w >0.70 \)) crystalline thymol was placed in the desiccators to prevent the microbial spoilage of the dehydrated watermelon pomace. The EMC at given temperature were plotted against the corresponding RH values to achieve sorption isotherm.

3.5.3.1. Sorption Models

Sorption models employed to describe behavior of food product can be classified into two groups. Type I (Eq. 15-24) models based on dependence between the equilibrium moisture content (EMC) and the relative humidity (RH) of the air while Type –II (Eq. 25-28) models used to determine the relationship among RH, EMC and
temperature (Rahman, 1995) (Table 1). The criteria used to select the most appropriate models were based on coefficient of determination ($R^2$), standard error (SE) and pattern of residual plot.

### 3.5.3.2. Isosteric Heat of Sorption

The net isosteric heat of sorption or enthalpy of sorption ($q_{st}$) is defined as the difference between the total heat of sorption and the heat of vaporization of water (Gabas et al., 2007). The net isosteric heat of sorption was determined from $a_w$ at different temperatures using Eq. (1), which is derived from Clausius-Clapeyron equation (Rizvi, 1995).

$$a_w = a_o \exp \left( \frac{-q_{st}}{RT} \right)$$

(29)

where $a_w$ is the water activity (dimensionless); $q_{st}$ is the net isosteric heat of sorption (kJ/mol); $a_o$ is a dimensionless constant; $R$ is Universal gas constant (8.314 J/mol.K) and $T$ is the absolute temperature (K).
Table 1: Models employed to describe the sorption behavior for dehydrated watermelon pomace at selected temperatures (20-60°C) and water activity (0.11 to 0.92).

<table>
<thead>
<tr>
<th>Sorption model</th>
<th>Equations</th>
<th>Eq No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freundlich (1926)</td>
<td>( m = A (a_w)^{1/B} )</td>
<td>15</td>
</tr>
<tr>
<td>B.E.T. (1938)</td>
<td>( a_w / [(1-a_w)m] = A + B(a_w) )</td>
<td>16</td>
</tr>
<tr>
<td>Harkins &amp; Jura (1944)</td>
<td>( \ln(a_w) = A + B(1/m^2) )</td>
<td>17</td>
</tr>
<tr>
<td>Oswin (1946)</td>
<td>( m = A [a_w/(1-a_w)]^B )</td>
<td>18</td>
</tr>
<tr>
<td>Smith (1947)</td>
<td>( m = A + B \ln(1-a_w) )</td>
<td>19</td>
</tr>
<tr>
<td>Hasley (1948)</td>
<td>( a_w = \exp[A(1/m)^B] )</td>
<td>20</td>
</tr>
<tr>
<td>Henderson (1952)</td>
<td>((1-a_w) = \exp[-Axm^B])</td>
<td>21</td>
</tr>
<tr>
<td>Chung &amp; Pfost (1967)</td>
<td>( \ln(a_w) = -A \exp(-Bxm) )</td>
<td>22</td>
</tr>
<tr>
<td>Khun (1967)</td>
<td>( m = A / \ln(a_w) + B )</td>
<td>23</td>
</tr>
<tr>
<td>Iglesias and Chirife (1981)</td>
<td>( m = A + B [a_w/(1-a_w)] )</td>
<td>24</td>
</tr>
<tr>
<td><strong>Type II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Modified Hasley</td>
<td>( a_w = \exp[-\exp(A+Bet)/m^C] )</td>
<td>25</td>
</tr>
<tr>
<td>Modified Chung-Pfost</td>
<td>( a_w = \exp[(-A/(t+B))\exp(-Cm)] )</td>
<td>26</td>
</tr>
<tr>
<td>Modified Oswin</td>
<td>( m = (A+B)(a_w/(1-a_w))^C )</td>
<td>27</td>
</tr>
<tr>
<td>Modified Henderson</td>
<td>( 1-a_w = \exp[-A(t+B)m^C] )</td>
<td>28</td>
</tr>
</tbody>
</table>

where \( m \) – equilibrium moisture content (% dry basis); \( a_w \) – water activity; \( A, B \) and \( C \) – coefficients; 
\( t \) – temperature [°C]; B.E.T- Brunauer-Emmett- Tetter
3.6. Extraction optimization of lycopene from watermelon pulp

The effect of four independent variables $X_1$ (solvent/meal ratio), $X_2$ (number of extractions), $X_3$ (temperature) and $X_4$ (extraction time) were investigated on extracted lycopene yield (dependent variable) using central composite design (Table 2). Thirty two combinations of the independent variables were selected as per experiment design for four independent parameters as shown in Table 3 (Gomez & Gomez, 1984). Experimental data were used to get a multiple regression equation which was used to calculate the predicted value of dependent variable.

$$Y = b_0 + \sum_{n=1}^{4} b_n X_n + \sum_{n=1}^{4} b_{mn} X_n^2 + \sum_{n<m}^{4} b_{nm} X_n X_m$$  \hspace{2cm} (30)$$

where $Y$: Dependent variable i.e. lycopene content (mg/100g); $X$: Independent variables such as solvent/meal ratio, number of extractions, temperature and extraction time; $b_0$: constant value at central point; $b_n$, $b_m$ and $b_{mn}$ : linear, quadratic and cross product coefficients.

Experimental and predicted data were analyzed to judge the accuracy of the model based on significance of different coefficients of Analysis of Variance (ANOVA) technique. The predicted values were obtained from the regression equation and analyzed for coefficient of determination ($R^2$), standard error (SE), root mean square error and residual plot.

Optimum values of independent variables were determined by varying two independent variables while keeping remaining two at zero level. Each pair of independent variables was plotted as surface graph to get optimum value. The experiment was run again at optimum level of independent variable for the confirmation of the results.
Table 2: Independent variables and their levels used for central composite rotatable design for extraction optimization of lycopene from watermelon pulp.

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>Symbol</th>
<th>Coded variable levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-2</td>
</tr>
<tr>
<td>Solvent/meal ratio, v/w</td>
<td>(X_1)</td>
<td>4</td>
</tr>
<tr>
<td>Number of extractions</td>
<td>(X_2)</td>
<td>1</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>(X_3)</td>
<td>20</td>
</tr>
<tr>
<td>Extraction time, min</td>
<td>(X_4)</td>
<td>4</td>
</tr>
</tbody>
</table>
Table 3: Central composite arrangement for coded and uncoded independent variables $X_1$ (Solvent/meal ratio, v/w), $X_2$ (Number of extractions), $X_3$ (Temperature, °C) and $X_4$ (Extraction time, min).

<table>
<thead>
<tr>
<th>Run</th>
<th>$X_1$ (Solvent/meal ratio, v/w)</th>
<th>$X_2$ (Number of extractions)</th>
<th>$X_3$ (Temperature, °C)</th>
<th>$X_4$ (Extraction Time, min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-1 (6)</td>
<td>-1 (2)</td>
<td>-1 (30)</td>
<td>-1 (8)</td>
</tr>
<tr>
<td>2</td>
<td>1 (10)</td>
<td>-1 (2)</td>
<td>-1 (30)</td>
<td>-1 (8)</td>
</tr>
<tr>
<td>3</td>
<td>-1 (6)</td>
<td>1 (4)</td>
<td>-1 (30)</td>
<td>-1 (8)</td>
</tr>
<tr>
<td>4</td>
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<td>5</td>
<td>-1 (6)</td>
<td>-1 (2)</td>
<td>1 (50)</td>
<td>-1 (8)</td>
</tr>
<tr>
<td>6</td>
<td>1 (10)</td>
<td>-1 (2)</td>
<td>1 (50)</td>
<td>-1 (8)</td>
</tr>
<tr>
<td>7</td>
<td>-1 (6)</td>
<td>1 (4)</td>
<td>1 (50)</td>
<td>-1 (8)</td>
</tr>
<tr>
<td>8</td>
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<td>1 (50)</td>
<td>-1 (8)</td>
</tr>
<tr>
<td>9</td>
<td>-1 (6)</td>
<td>-1 (2)</td>
<td>-1 (30)</td>
<td>1 (16)</td>
</tr>
<tr>
<td>10</td>
<td>1 (10)</td>
<td>-1 (2)</td>
<td>-1 (30)</td>
<td>1 (16)</td>
</tr>
<tr>
<td>11</td>
<td>-1 (6)</td>
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3.7. Preparation, stability and in vivo studies of watermelon colouring

3.7.1. Preparation of watermelon colouring

Pulp was used for extraction with ethyl acetate 30:1 solvent/meal ratio (v/w) using shaker incubator (REMI, Mumbai) at 50°C for 20 min. The extraction was repeated four times, all extracts were combined and it was evaporated by using rotary vacuum evaporator (Buchi Labortechnik AG, Flawil, Switzerland) using water bath temperature of 45±2°C, vacuum of 25±2 mbar and speed of 20 revolutions per minute. The extract was evaporated to semi solid mixture called watermelon colouring.

3.7.2. Stability of colouring under different storage conditions

Watermelon colouring (5 ml) was placed in culture tubes (25 × 57 mm), sealed and kept at different temperatures such as 20, 40, 60 and 80°C, with or without agitation (27°C and 90 rpm) and under different light effects such as dark, light day (60 lux) and 50,000 lux. Samples were analysed for 9 days for total carotenoids, lycopene and antioxidant activity present in the colouring. All the procedures were performed in reduced light to minimize isomerization and photodegradation and in triplicates.

3.7.2.1. Antioxidant activity of watermelon colouring

3.7.2.1.1. DPPH free radical scavenging assay

The DPPH (2, 2-diphenyl-1-picryl hydrazyl) radicals scavenging activity of the watermelon colouring was determined spectrophotometrically using the DPPH method (Leong & Shui, 2001, Szabo et al., 2007). 10µL of sample was dissolved in 1ml acetone. Four ml of DPPH (0.1mM) were added and samples incubated for half an hour in dark. After half an hour, the absorbance values were measured at 517nm using UV visible spectrophotometer (2450 Shimadzu Co., Ltd., Tokyo, Japan). DPPH scavenging activity of sample was measured as a decreased in the absorbance. The decrease in absorbance was measured at different time intervals until the absorbance remains constant.
Antioxidant activity (%) = $\frac{A_0 - A_1}{A_0} \times 100$ \hspace{1cm} (31)

where $A_0$ is the absorbance of the control reaction and $A_1$ is the absorbance in the presence of extract measured at different time intervals.

3.7.2.2. Total carotenoids and lycopene of colouring

The total carotenoids and lycopene of colouring were estimated as given in section 3.2.2.7. to 3.2.2.8.

3.7.2.3. Kinetics of antioxidant activity, total carotenoid and lycopene degradation

The kinetics of degradation of both pigment and colour has been reported to follow first order reaction (Steet & Tong, 1996; Weemaes et al., 1999; Ahmed et al., 2000; Gunawan & Barringer, 2000). The first order kinetic model based on different components was followed as given in eq 14 for lycopene.

3.7.2.4. Effect of temperature on lycopene and colour degradation.

The Arrhenius model was applied to describe the temperature dependence of lycopene/colour reaction rate constant as given in eq 12.

3.7.3. In vivo bioavailability of lycopene

3.7.3.1. Feeding Trial

Male albino rats with average body weight and age 21–28 days were selected and housed in individual cages. The animals were fed with standard rat diet (M/S Hindustan Lever Ltd, Mumbai, India) and conditioned to the laboratory environment for 7 days. The animals were segregated into five groups, allocating three rats each, with similar mean body weights to each group. The four groups were fed with lycopene enriched diet prepared by mixing the watermelon colouring dispersed in carboxymethyl cellulose (CMC) with rat diet whereas the fifth group was fed with control diet without lycopene.
The different concentrations of lycopene in the diet fed to four groups of rats were 5, 20, 35 and 50 mg/100g for the next seven days. After this treatment, the rats were fed with the control diet for seven days. Observations on daily feed intake, body weight changes, weight of faecal matter, lycopene intake and lycopene absorbed were made during the feeding trial.

3.7.3.2. Proximate Composition of animal feed

The moisture content, crude protein, crude fat, crude fiber, total ash, and carbohydrates were determined as per section 3.2.2.6.

3.7.3.3. Lycopene Content of feed and faecal matter

The lycopene content of faecal matter was quantified as given in section 3.2.2.7.

3.8. Utilization of lycopene in food products

3.8.1. Preparation of lycopene crystals

The watermelon colouring was saponified using alkaline propylene glycol solution (colouring: propylene glycol: alkali: water: 5:3:1:1) at 65°C for 2 h. Finally the water was added to disperse the impurities and filtered (Whatman No. 4). The lycopene crystals were dried in freeze drier (Heto, Ved.b.ack, Denmark) and kept at -20°C (Ausich & Sanders, 1999; Hartal et al., 1999).

3.8.2. Microscopy of lycopene crystals

Lycopene crystals were mounted on a slide in immersion oil, covered with cover slip and observed under the light microscope (Olympus, Tokyo, Japan). The images generated in the light microscope were captured by digital camera (C-5060, Olympus, Tokyo, Japan).
3.8.3. Utilization of lycopene crystals in food products

Lycopene crystals were incorporated into different food products such as mayonnaise, ice-cream and butter. These products contain high fat content and lycopene is fat soluble pigment. Mayonnaise, ice-cream and butter were prepared in laboratory and lycopene crystals extracted from watermelon colouring were incorporated which act as biocolour and antioxidant. The lycopene crystals were added at 2.5, 5, 7.5 mg lycopene/100 g finished product formed. The samples prepared were compared with commercial market samples.

3.8.3.1. Mayonnaise

Various ingredients used for the mayonnaise preparation are egg yolk (15%), vinegar (6%), mustard flour (0.5%), refined oil (74%), white pepper (0.2%), potassium sorbate (0.1%), sugar (2%), salt (1.2%) and lycopene crystals at different levels. All the ingredients were mixed together with help of blender (4.5cm and 6 blades) at high speed for 2 min. Yolk was beaten initially followed by addition of dry ingredients and blending for 3-4 min. Vinegar (one third part) was added initially for the stable emulsion and then added oil containing dissolved lycopene crystals. The oil was added with continuous shaking and remaining vinegar was incorporated. The rest of the ingredients were added and mixed to form emulsion. The product was packed in plastic jars and stored at 4-6ºC. The market sample – Cremica (Mrs. Bector’s Cremica Enterprises Limited, Phillaur, Punjab, India) containing tomato paste was used for comparison with test sample.

3.8.3.2. Ice cream

Ingredients used for formulation of ice cream were milk fat (10%), milk solid not fat (12%), sugar (15%), stabilizers (0.4%), emulsifier (0.4%) and lycopene crystals at different levels. Mix was initially preheated to a temperature of 54.4ºC, homogenized, pasteurized (68.3ºC/30 min) and aging (-4ºC) for 24 h. Whipping of ice cream was performed in order to achieve 75% overrun followed by packaging and frozen storage (-20ºC). Commercial sample of Amul (Gujarat Cooperative Milk Marketing Federation Ltd, Anand, Gujarat) was used for comparison.
3.8.3.3. Butter

Cream was pasteurized at 68.5°C/30 min, cooled to 10°C in ice bath and churned using electric churn in order to form butter kernels. The butter was separated from butter milk and washed with cold water. Different levels of lycopene crystals were incorporated into butter along with 2% salt and mixed for 10-12 min for uniform distribution. The butter was packed in plastic jars and stored at 4-6°C. The product prepared was compared with market sample of Amul (Gujarat Cooperative Milk marketing Federation Ltd, Anand, Gujarat) containing annatto as colouring agent.

3.8.4. Storage Studies of food products

The prepared samples and commercially market sample of mayonnaise and butter were stored at refrigeration conditions (4-6°C) and ice cream samples were stored at -20°C in a deep freezer. The test and control samples were analyzed for peroxide value, free fatty acids, hunter colour values and sensory characteristics for three months.

3.8.5. Preliminary preparation of sample for estimation and analysis

Mayonnaise (15 g) was added in an Erlenmeyr flask and 50 ml of petroleum ether was added. The flask was covered and mixture was stirred for 15-20 min. The sample was filtered out using anhydrous sulphate to remove water from the mixture. The ether present in the sample was evaporated using rotary vacuum evaporator (Buchi Labortechnik AG, Flawil, Switzerland).

Ice cream sample (15-20g) was taken in a separating funnel. After addition of 100 ml of hot water, flask was shaken for 5 min to break the emulsion. 10 ml of ammonium hydroxide was added and sample was heated (60°C/15-20 min) in a water bath (TC-2000 Brookfield Laboratories, Middleboro, USA). 25 ml of neutralized ethanol was added and sample was continuously shaken for 10 min. 40 ml of diethyl ether and 60 ml of petroleum ether was added to the sample. Solvent layer was transferred to already tared flask/dish. The aqueous layer was added back to separating funnel and extracted with diethyl ether (40 ml) and petroleum ether (60 ml). This extraction was repeated thrice. The solvent containing fat was evaporated followed by heating at 100°C for 1h (AOAC, 1990).
The butter sample was heated at 60ºC for 2-3 h so as to separate out the water and curd completely to get supernatant fat.

3.8.5.1. Peroxide value

Dissolved 5.0 g of sample in 30ml of glacial acetic acid-chloroform (3:2, v/v). Added 0.5ml of saturated potassium iodide, kept for one min in dark and to this solution added 30 ml of water. Titrated with standardized sodium thiosulphate (0.1N) using starch indicator till deep blue colour disappeared. The peroxide value was expressed as milli equivalent O₂/kg sample (AOAC, 1990).

3.8.5.2. Free fatty acids (AOAC, 1990)

Dissolve 5 g of fat in 50 ml of neutralized ethanol and titrating with 0.1 N NaOH to obtain a light pink colour. Results were expressed as percent FFAs as % oleic acid (AOAC, 1990).

\[
\text{FFA (\% oleic acid)} = \frac{\text{ml of alkali} \times \text{Normality of alkali} \times 56.1}{\text{weight of sample (g)}}
\]  

(34)

3.8.5.3. Colour

Visual colour was measured as given in section 3.2.2.9.

3.8.5.4. Sensory Evaluation (Ranganna, 1986)

Organoleptic evaluation of samples was performed using Hedonic scale test values ranging from 1 to 9 where 1 refers to extremely dislike and 9 to extremely like by 15 semi trained panelists. The sample was judged on the basis of colour, flavour, texture, aroma, appearance and overall acceptability.

3.9. Statistical Analysis

Two-way analysis of variance was applied on the data of physico-chemical values, pigment content, Hunter colour parameters of watermelon flesh, concentrates, juice
powder, pomace samples, bioavailability and storage studies. The difference between
the means was compared by Honestly Significant Difference (HSD) values using
Tukey’s Test (Daniel, 1991) analyzed at p≤0.05. Least significant difference values
were also calculated to find significant difference. The goodness of linear fit was
evaluated by coefficient of determination (R²), adjusted R², p-values and root mean
square error computed using Minitab-15 (Mini Tab Inc., Coventry, U.K.) software and
Microsoft Excel (Microsoft Inc., Redmond, USA).

Linear, exponential and power regressions were applied to determine the
relationship between the lycopene and ripening parameters, soluble solids with
consistency index and lycopene content of watermelon samples. Respective empirical
models were applied on rheological, drying, sorption (Type I and II models), effective
moisture diffusivity and thermal degradation experimental data by nonlinear regression
and coefficient of determination (R²), standard error and residual plots were used to
judge the adequacy of the models using Microsoft Excel (Microsoft Inc., Redmond,
USA) and Statistica-5 software (Statsoft Inc., Tulsa, USA) at level of p≤ 0.05.

The CCRD was used to make coordinates of variables for central composite
rotatable design (Cochran & Cox, 1957). The coefficients and analysis of variance were
computed using Minitab-15 (Mini Tab Inc., Coventry, U.K.) software. Three
dimensional surface graphs were plotted for the predicted value of lycopene obtained
from the models.