CHAPTER 3

MATERIALS AND METHODS

3.1 INTRODUCTION

The present investigation entitled “Efficacy of Natural Antioxidants of plant origin on shelf life of edible oils” was carried out in the laboratories of Department of Food Technology, Guru Jambheshwar University of Science and Technology, Hisar and Department of Food Science and Technology, Chaudhary Devi Lal University, Sirsa. This chapter enlists relevant information relating to the research design and methodological steps followed in the present investigation.

3.2 MATERIALS

3.2.1 Raw material

Fruits (Pomegranate, Papaya, Mango, Sweet Orange,) were purchased from local market of Sirsa (Haryana). The fruit peels were washed from tap water initially and then with distilled water to remove any debris or dirt particles and allowed to dry in an oven at around 55°C for 48 hours (Xu et al. 2008). The dried peels were grinded to powder and stored in airtight packs until used for further studies. The oil samples (mustard, sunflower and soybean) were collected from a local oil extractor mill from Bathinda and the olive oil was purchased from local market of Sirsa. The oils were kept stored in amber colored glass bottles until used for further studies.

3.2.2 Chemicals and reagents

The organic solvents (acetone, chloroform, ethanol, methanol, ethyl acetate) were of analytical grade and obtained from Qualigens (Fischer). Folin ciocalteu reagent was also obtained from Qualigens (Fischer). Other chemicals viz. DPPH, BHA, BHT, Trichloroacetic acid, NaOH, HPO3, Potassium phosphate buffer, 2,6 dichlorophenol in...
iodate, isopropanol and all other reagents were obtained from Sigma-Aldrich Chemicals Pvt. Ltd.

3.2.3 Glassware

All glasswares used in the experimental work were of Borosilicate. Before using, glasswares were washed with labolene detergent and rinsed with distilled water and dried in oven at 180°C for three hours.

3.2.4 Apparatus

During the course of research work the following instruments and apparatus were used:

1. Vaccum oven
2. UV chamber (Camag)
3. Refrigerater (LG)
4. Ph meter
5. Uv-Vis Spectrophotometer
6. Electronic balance
7. Magnetic stirrer
8. Weighing balance (Sartorius)

3.3 PREPARATION OF EXTRACTS

Hot water extract of dried fruit peels were prepared by the procedure given by Xu et al. (2008). The sample peel powder (5g) were added to 100 ml of boiling distilled water and infused for 30 minutes. Then the extract was cooled to ambient temperature and centrifuged at 5000 rpm for 20 minutes. The supernatant was collected and made a final volume of 100 ml by addition of distilled water and the liquor of each sample were analyzed for total phenolic content, radical scavenging activity (RSA%), total carotene content and β-carotene by spectrophotometric methods.
3.3.1 Total carotene content

Total carotene content of samples were determined using method described by Arnon, (1949). The samples (100-500 mg) were homogenized with 10-15 ml of 80% acetone in pestle—mortar until tissue become colorless. A pinch of calcium carbonate was also added to avoid the destruction of chlorophyll and other pigments. The liquid was centrifused at 2000 rpm for 10-15 minutes and filtered it. The volume was made to 10 ml with acetone and the absorbance was recorded at 480 and 510 nm using spectrophotometer.

Calculations

\[
\text{Total carotenoids} = \frac{7.6 \times A_{480} - 14.9 \times A_{510} \times V}{a \times 1000 \times w}
\]

where,

\(A\) = Absorbance (nm)

\(a\) = Light path (cm)

\(V\) = Volume (ml)

\(W\) = Weight of sample (mg)

3.3.2 \(\beta\)-Carotene content (BCC)

The \(\beta\)-carotene content of fruit peel was evaluated using the method described by Srivastava et al. (2001). The sample (5g) was crushed in 10-15 ml of acetone and followed by addition of anhydrous sodium sulphate, with the help of pestal and mortar. Decanted the supernatant in a beaker. The process was repeated twice and the combined supernatant was transferred to a separatory funnel, 10-15 petroleum ether added and thoroughly mixed. Two layers will separated out on standing. The lower layer was decanted off and upper layer was collected in a 100 ml volumetric flask then the volume was made to 100 ml with petroleum ether and optical density was recorded at 452 nm using petroleum ether as blank in spectrophotometer.
Calculations

\[ \text{B-carotene (µg/100g)} = \frac{\text{O.D.} \times 13.9 \times 10^{-4} \times 100}{\text{Wt. of sample} \times 560 \times 1000} \]

3.3.3 Radical scavenging activity (RSA %)

RSA was evaluated by method given by Brand-Williams et al. (1995), based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH. The methanolic solution (3.9 ml) of 0.063 mM DPPH was added to the test tube and 0.1 ml of each extract was added and shaken vigorously then the test tube was allowed to stand at 27ºC for 20 minutes. A control reaction was prepared as above without any extract and methanol was used for baseline correction and the absorbance was measured at 515 nm in spectrophotometer.

Conclusions

Radical scavenging activity (RSA %) was expressed as the inhibition % and was calculated by using the following equation:

\[ \% \text{RSA} = \left( \frac{\text{control Abs} - \text{sample Abs}}{\text{control Abs}} \right) \times 100 \]

3.3.4 Ascorbic acid content

Reagents

1. Metaphosphoric acid (3%).
   
   It was prepared by dissolving the pellets of HP\(\text{O}_3\) in distilled water

2. Ascorbic acid standard
   
   L-Ascorbic acid (100 mg) was dissolved in 3% HP\(\text{O}_3\) and made the solution to 100 ml with 3% HP\(\text{O}_3\).

3. Dye solution (2,6-dichlorophenol –indophenol)
   
   2,6-dichlorophenol –indophenols (50 mg) dissolved in 150 ml of distilled water containing 42 mg of sodium bicarbonate, after that the solution was
cooled and made the solution to 20ml with distilled water and stored in refrigerator.

**Procedure:**

**Standardization of Dye:**

Standard Ascorbic acid (5ml) was added to 5ml of HPO₃ and the burette was filled with dye. Then the Standard Ascorbic acid solution was titrated with dye solution to pink color, which had persisted for 15 seconds.

Dye factor = 0.5/titre

**Preparation of sample:**

Sample (10mg) was mixed with 3% HPO₃ and made the volume to 100ml with HPO₃ and after, it was centrifuged at 2000 rpm for 20 minutes.

**Assay of Extract:**

The Aliquot (4ml) of HPO₃ extract was taken and titrated with the standard dye to pink end point which had persisted for 15 minutes.

**Conclusions:**

\[
\text{Ascorbic acid (mg/100g)} = \frac{\text{Titre} \times \text{Dye factor} \times \text{volume made up}}{\text{Aliquot of extract for estimation} \times \text{wt. of sample for estimation}}
\]

3.4 DETERMINATION OF PEROXIDE VALUE (PV)

Determination of PV of olive oil samples were made according to the analytical method described in European Official Method of Analysis (Commission Regulation EEC N·2568/91 - Determination of peroxide value). The PV was expressed in milli equivalents of oxygen per kg of oil (equiv. of O₂/kg).

For standardization of 0.01M sodium thiosulphate; 2 g of potassium iodate (KIO₃) was dried in an incubator at 90-100 °C for 1-2 hours. After 1-2 hours, 0.001 mol/L KIO₃ solutions (0.1070 gm KIO₃/500 ml dH₂O) was prepared with potassium iodate taken from the incubator. Exact weight of KIO₃ was recorded. In order to prepare 0.5 M H₂SO₄ solutions, 2.8 mL of H₂SO₄ (96% purity) was diluted to 100 mL with deionized water. For preparation of starch solution; 1 g of starch was
weighed and dissolved in 10 mL of deionized water. 90 mL of boiling deionized water was added to starch boiling continued for 2-3 minutes.

Before titration, 0.2 g of potassium iodine (KI) was weighed and 1 mL of 0.5 M H₂SO₄, 50 mL of 0.001 M potassium iodate (KIO₃) solution was added. Reddish brown solution was titrated with sodium thiosulphate (0.01 mol/L) until the solution has turned to its initial reddish brown color and has become pale yellow. 2 mL of starch indicator was added into pale yellow solution and titration was completed when the sodium thiosulphate M mol solution becomes colourless and sodium thiosulphate spent during titration was recorded.

Molarity of standardize sodium thiosulphate was calculated by means of the following equations.

\[ M_{KIO_3} = \frac{m_{KIO_3} \cdot MW_{KIO_3} \cdot \text{g/mol}}{V_{KIO_3} \cdot \text{ml/solution}} \]  

\[ M_{\text{sodium thiosulphate}} = \frac{\delta \cdot M_{KIO_3} \cdot (\text{mol/L}) \cdot V_{KIO_3} \cdot (\text{ml})}{V_{\text{sodium thiosulphate}} \cdot (\text{ml})} \]  

\[ M_{KIO_3} = \text{weight of KIO}_3 \ (0.1070 \ \text{g}) \]

\[ MW_{KIO_3} = \text{molecular weight of KIO}_3 \ (214 \ \text{g/mol}) \]

\[ V_{KIO_3} \ \text{solution} = \text{total volume of KIO}_3 \ \text{solution} \ (500 \ \text{mL}) \]

\[ V_{KIO_3} = \text{volume of KIO}_3 \ \text{solution} \ (50\text{mL}) \]

\[ V_{\text{sodium sulphate}} = \text{amount of sodium thiosulphate used in titration} \ (\text{mL}) \]

After standardization part of the experiment; 10 mL of chloroform, 15 mL of acetic acid and 1 mL of potassium iodide solution were added into 3 g of an oil sample and mixed for 1 minute. Then, the sample was kept in dark and at the room temperature for 5 minutes. Lastly, 75 mL of deionized water and 0.5 mL of starch solution were added to oil sample. Titration of free iodine was carried out with 0.002 M sodium thiosulphate solution until the dark blue color of solution turns to

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colorless and the amount of total sodium thiosulphate solution spent during the titration was recorded.

The calculation of peroxide values in terms of meq O₂ / kg oil;

\[ P V = \frac{V(mL) \times M(mol/L)}{m} \times 1000 \]

V: volume of sodium thiosulphate solution spent during titration (mL)
M: molarity of sodium thiosulphate solution
m: the weight of the sample (g)

3.5 DETERMINATION OF FREE FATTY ACID VALUE

European Official Methods of Analysis (EEC 1991) was used for the determination of free fatty acid value in terms of % oleic acid. Approximately 1 g of potassium hydrogen phthalate (KHC₈H₄O₄) was weighed and dried in an oven at 110°C for 2 hours. Accurately 0.4 g of potassium hydrogen phthalate was weighed into an erlenmayer flask. 75 mL of deionized water and 3 drops of phenolphthalein indicator (0.5 g phenolphthalein in 50 mL 95% ethanol (v/v)) were added into the flask before titration with potassium hydroxide (KOH). 1 mol/L potassium hydroxide (KOH) was prepared with deionized water and it was standardized with potassium hydrogen phthalate.

50 mL of 95% ethanol-water solution (95:5 v/v) and 50 mL of diethyl ether mixture (1:1 v/v) were prepared. 3 drops of phenolphthalein indicator was added into the mixture. The ether-ethanol mixture was titrated with KOH solution until a sudden color change has occurred. 20 g of olive oil sample was weighed. The titrated etherethanol mixture was added to the 20 g of sample and 3 drops of phenolphthalein indicator was added into the mixture before titration. Then, the mixture was titrated with 0.1 mol/L solution of KOH and the volume of solution spent was recorded.

Acidity was expressed as percentage of oleic acid with the equation given
\[ V \times c \times \frac{M}{1000} \times \frac{100}{M} = \frac{V \times c \times M}{10 \times M} \]

V : the volume of titrated KOH (mL)

c: exact concentration of the titrated solution of KOH (mol/L)

M: the molar weight of the oleic acid (282g/mole)

m: weight of the sample (g)

3.6 STATISTICAL ANALYSIS

The data obtained from the laboratory experiments was subjected to Analysis of Variance (ANOVA) using OP stats. A significance level of 0.5 was selected. The variables selected for the extraction of different antioxidants were the solvents systems. Whereas for the studies of shelf life of edible oils, factors namely concentration of extracts (800, 1600, 2400 ppm) and temperature (room temp. and 45°C) were selected as factorial arrangements in completely randomized block design. Each mean value presented in tables is the average of three replicates.