MATERIALS AND METHODS
3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Oils:
Crude and refined ricebran oil samples were procured from M/s Dinesh Oils Limited, Panki, Kanpur. Refined, bleached and deodorised safflower oil (Saffola - brand) manufactured by M/s Marico Industries Ltd, Mumbai, groundnut oil (Vital - brand) manufactured by M/s Brooke Bond India Ltd., Calcutta, soyabean oil (Vegepro - brand) manufactured by Vegetable Foods and Feeds Limited, Orai and sunflower oil (Cooklite - brand) manufactured by M/s Godrej Foods Ltd., Mumbai were procured from the market.

3.1.2 Chemicals for extraction and analytical purposes:
Chemicals used for extraction and analysis were of AR grade of Qualigens Fine Chemicals make, a division of Glaxo India Ltd., Mumbai. HPLC grade iso-octane, acetonitrile, methanol, methylene chloride, water, hexane and isopropanol used were also of Qualigens Fine Chemicals make.

3.1.3 Chemicals for Refining of ricebran oil:
LR grade chemicals of Qualigens Fine Chemicals were used for refining of ricebran oil. Activated earth of Premier brand was obtained from M/s Mahajeet Clayton Industries Ltd., Mumbai for bleaching of the oil.

3.1.4 Clinical Trials:
3.1.4.1 Rats: Male albino rats for clinical trials were obtained from a local scientific store.
3.1.4.2 Cages: Tarsons make cages fitted with water bottles were used for rat colonies.

3.1.4.3 Diet for rats: The diet for rats was obtained from CDH Laboratory Chemicals, New Delhi.

3.1.4.4 Cholesterol testing kit: Total cholesterol, HDL cholesterol and triglycerides were analysed enzymatically with enzokit supplied by Ranbaxy Laboratories Limited, Nehru Place, New Delhi.

3.1.5 Reference oryzanol:
Standard oryzanol was procured from Lipid Technology Division, CFTRI, Mysore. The purity of reference oryzanol was 98%.

3.1.6 Reference tocopherol:
Reference tocopherols were purchased from M/s E. Merck, Germany. The purity of reference tocopherols was 98% - 99%.

3.1.7 Potatoes:
The potatoes used during the frying experiments were purchased from the local market. The variety of potatoes used for the entire study was G - 4 type. The average moisture content of the potatoes was 75% - 80%.

3.2 METHODS AND METHODOLOGY
3.2.1 Physico-chemical characteristics:
Physico-chemical characteristics of experimental samples of oils were determined using the following procedures:
3.2.1.1 Colour: The colour of the samples was determined with computerised lovi bond colour scan, manufactured by The Tintometer Ltd., Salisbury, U.K., by using Lovibond Tintometer scale.

3.2.1.2 Specific gravity: Specific gravity of the samples was determined against water at 30°C using a specific gravity bottle of 10 ml capacity following BIS method (261).

3.2.1.3 Refractive index: The refractive index of oil samples was determined by Abbe's refractometer at 40°C following BIS method (261).

3.2.1.4 Acid value: The acid value (AV) of the oil samples was determined by BIS method (261). Accurately weighed sample of oil was mixed with neutral ethyl alcohol, warmed and titrated against standardised sodium hydroxide solution using phenolphthalein as indicator. The AV was calculated using following relationship

\[ AV = \frac{56.1 \times N \times V}{W} \]

Where 
- \( N \) = normality of aqueous NaOH solution
- \( V \) = volume in ml of NaOH solution consumed and
- \( W \) = weight of the sample in grams

3.2.1.5 Iodine value: The IV of the oil samples was determined by BIS method (261). The oil sample was weighed accurately into erlenmeyer flask and 25 ml of carbon tetra chloride was added to
dissolve the contents. Then 25 ml of Wijs solution was added and glass stopper was replaced after wetting it with potassium iodide solution. The flask was swirled for intimate mixing and was allowed to stand in dark for 30 min. Thereafter, the flasks were taken out and 15 ml of 10% potassium iodide solution was added followed by addition of 10 ml of distilled water rinsing the stopper also. The liberated free iodine was titrated against standard sodium thiosulphate solution until the colour of the solution was straw yellow. At this stage 1 ml starch solution (1%) was added to the flask and titration was continued until the blue colour formed on addition of starch disappeared after thorough shaking with the stopper on. A blank determination was also made under similar conditions and IV of the sample was calculated using the following relationship

\[ IV = \frac{12.69 \times (B-S) \times N}{W} \]

Where

- **B** = Volume in ml of standard thiosulphate solution used in blank determination
- **S** = Volume in ml of standard sodium thiosulphate solution used for sample
- **N** = Normality of sodium thiosulphate solution
- **W** = Weight of sample in grams

### 3.2.1.6 Peroxide value

The peroxide value (PV) of the sample was determined by using BIS method (261). Accurately weighed sample of the oil (approx. 5g) was taken in a 250 ml glass stoppered conical flask and dissolved in 30 ml of acetic acid - chloroform mixture and
allowed to stand for 1 min with occasional shaking. After adding 30 ml of distilled water, the mixture was titrated against 0.1N sodium thiosulphate solution with constant and vigorous shaking. The titration was continued until the yellow colour of iodine almost disappeared. Thereafter, 0.5 ml of starch solution (1%) was added and the titration was continued until the blue colour just disappeared. A blank determination of the reagents was conducted in the same way and the PV of oil samples was calculated using the following relationship

\[
\text{PV as milli equivalent per 1000 g} = \frac{(S-B) \times N \times 1000}{W}
\]

Where 
- \(S\) = Volume in ml of sodium thiosulphate solution used in the sample
- \(B\) = Volume in ml of sodium thiosulphate solution used in the blank determination
- \(N\) = Normality of sodium thiosulphate solution
- \(W\) = Weight of sample in grams

3.2.1.7 Unsaponifiable matter: The unsaponifiable matter was determined by using BIS method (261). Accurately weighed sample of oil (5.0 g) was taken into a 250 ml of conical flask and 50 ml of 1.5 N alcoholic potassium hydroxide solution was added. The contents were refluxed for 1 h to completely saponify the oil sample. After washing the condenser with 10 ml ethyl alcohol and cooling, the contents were transferred into a separating funnel. The saponified material was extracted with 50 ml petroleum ether (60° - 80° C). The two layers were separated and the soap solution was further extracted at least six times
with petroleum ether. The combined ether extract was first washed with aqueous alcohol (10% v/v) and then with distilled water until the wash water no longer turned pink on addition of a few drops of phenolphthalein indicator. The washed ether extract was transferred into a flask. The ether was evaporated and the last traces of moisture were moved by evaporating the contents with addition of 5 ml of acetone and the residue was weighed. Thereafter, 50 ml of neutral alcohol was added to the residue and warmed and then titrated against 0.02 N standard sodium hydroxide solution using phenolphthalein as indicator. The unsaponifiable matter of the oil was calculated using the following relationship

\[
\text{unsaponifiable matter (\% w/w)} = \frac{100 \times (A - B)}{W}
\]

Where \(A\) = weight in gram of the residue
\(B\) = weight in gram of the fatty acids in the extraction which equals to 0.282 \times \text{normality of standard NaOH used}
\(W\) = weight of the sample in grams

3.2.1.8 Phosphorus and phosphatide content: Appropriating the AOCS procedure (262), method no. Ca 12-55, the phosphorus content of the oils was determined by ashing the oils in presence of zinc oxide followed by its colorimetric measurement as molybdenum blue. The equivalent phosphatide contents of the oils were calculated from their phosphorus content.
The experimental oils were accurately weighed in 3.0 - 3.2 g quantities in silica crucibles and added with 0.5 g zinc oxide. The contents of the crucible were slowly heated on a hot plate until completely charred. After incinerating them at 550° - 600° C for 2 h in a muffle furnace and cooling to room temperature in a dessicator, the resultant ash of each sample was boiled with 5 ml hydrochloric acid plus 5ml distilled water for 5 min. The resultant solutions were filtered using whatman No 42 filter paper into 100 ml volumetric flasks. The sides of the crucibles and the filter papers were washed with 30 ml aliquots of distilled water and the washings were collected in the same volumetric flasks. The filtrates were cooled to room temperature and then neutralised to a faint turbidity by dropwise addition of 50% sodium hydroxide solution (v/v). Thereafter, the precipitates of zinc oxide were dissolved by dropwise addition of conc. hydrochloric acid. After adding two more drops of acid, each filtrate was made to volume with distilled water and mixed thoroughly. The filtrates in 10 ml aliquots were pipetted into clean dry 50 ml volumetric flasks and added with hydrazinium sulphate solution (0.015%, w/v) and sodium molybdate solution 2.5% (w/v) in 51.5% sulphuric acid (w/v). The contents of the flasks were mixed with well and heated for 10 min on a boiling water bath for development of colour. After cooling to room temperature, the contents of the flasks were made to volume with distilled water and mixed thoroughly. Thereafter, their absorbances were measured at 650 nm with spectronic 20 spectrophotometer (Milton Roy Company, Analytical products Division, New York, U.S.A.) adjusted to read zero absorbance for distilled water.
The absorbances of the reagent blanks, prepared without using the oils by the aforesaid procedure, were also read in a similar manner. The phosphorus contents of the aliquots of the samples of the oils and reagent blanks were read from the standard curve prepared as follows:

A standard stock solution of phosphate containing one mg phosphorus per ml was prepared by dissolving 1.0967g dry potassium dihydrogen phosphate in 250 ml distilled water. A standard working phosphate solution containing 0.01 mg P/ml was prepared by diluting the stock solution by 100 folds. Its 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 ml aliquots were taken in a 50 ml volumetric flasks. Requisite volumes of distilled water were added to the flasks to make their volumes to 10 ml. In a separate flask only 10 ml distilled water was taken for the preparation of blank. The contents of each flasks were added with hydrazinium sulphate and sodium molybdate reagents and treated as before for the development and measurement of colour at 650 nm. Using the absorbances and the contents of phosphorus in the aliquots of standard working stock solution analysed, a standard curve of phosphorus was plotted.

The phosphorus contents of the oils were determined using the following relationship:

\[
\text{Phosphorus (\%) = } \frac{10 \times (A - B)}{W \times V}
\]

Where \( A = \) Phosphorus content of the sample aliquot in mg,
B = phosphorus content of the reagent blank aliquot in mg,
W = weight of the oil sample analysed in gram and
V = volume of aliquot in ml taken for developing colour.

Equivalent phosphatides contents in oil (%) = Phosphorus content of the oil (%) x 30

3.2.1.9 Fatty acid composition: The fatty acid composition of oil samples was determined by preparing methyl esters of oil samples and then analysing them by GLC as explained in the following procedure:

(i) Preparation of methyl esters: For determination of fatty acid composition of the oils, the methyl esters of fatty acids were prepared by saponification and esterification process (263). A mixture of 10 g of oil and 50 ml of 7.8% potassium hydroxide in 95% ethanol was heated under reflux for 3h. The resulting solution was poured into an equal volume of cold water and the unsaponifiable was removed by extraction with petroleum ether (60° - 80°C). The aqueous layer containing the potassium salt of fatty acids was acidified with 2N sulphuric acid solution and the free fatty acids were extracted with 3 x 10 ml portions of diethyl ether. The combined ether extracts were dried for 30 min. over anhydrous sodium sulphate and after filtration the ether was removed by distillation.

The fatty acids thus obtained were esterified in a 100 ml round bottom flask by heating under reflux for 4h with five volumes of methanol containing 1% concentrated sulphuric acid (v/v). After esterification,
the contents of the flask were poured into an equal volume of water and the methyl esters were extracted with 3 x 10 ml portions of diethyl ether. The combined ether extract was washed with an aqueous sodium bicarbonate solution and finally with distilled water. The ether extract was dried for 30 min over anhydrous sodium sulphate and after filtration the bulk of ether was removed by distillation. The methyl esters were finally dried under vacuum. The methyl esters thus prepared were subjected to gas liquid chromatographic analysis for the determination of fatty acid composition.

(ii) **Gas liquid Chromatography**: The gas chromatograph used for the analysis was Treemetrics - 540 (U.S.A.) having flame ionisation detector. The column used for analysis was 3 mm x 2 m stainless steel column packed with 20 per cent D.E.G.S. on Chromosorb W (80 - 100 mesh). The temperature of the injection port was maintained at 230°C and that of column oven at 190°C. Chromatographic grade purified nitrogen was used as a carrier gas. Hydrogen gas of extra high purity was used for the flame ionisation detector. The flow rates of hydrogen and nitrogen were kept at 30 ml/min. The methyl esters of fatty acids were dissolved in diethyl ether to a specific concentration (10%) and 0.4 µl of this solution was injected to the gas chromatograph by the help of high pressure hypodermic syringe. The fatty esters were identified by their retention times and then clear peaks were obtained in the chromatograms of the samples. The fatty acid composition was directly calculated from the area per cent obtained as calculation from the printer.
3.2.1.10 Conjugated fatty acids: The content of conjugated fatty acids in ricebran oil samples were determined by AOCS spectrophotometric method (262) no. Cd 7 - 58. An accurately weighed sample of the oil (20 - 30 mg) was dissolved in 100 ml of purified iso-octane and further diluted, if necessary, with iso-octane to get it's absorbance between 0.2 to 0.8 at 233 nm in a 1cm quartz cell on UV spectrophotometer (Chemito 2500 model). The extent of conjugation in the oil sample, as per cent conjugated acids, was calculated using the following relationship:

Conjugated diene (%) = 0.91 x a

Where a = absorptivity at 233 nm corrected for absorption by ester group which is equal to 0.07.

\[
A = \frac{A}{b \times c}
\]

A = observed absorbance at 233 nm
b = path length in cm, and
c = concentration of sample used for the absorbance measurement (g/l).

3.2.1.11 Dielectric constant: Dielectric constant of various oil samples was determined by measuring the capacitance of a liquid cell with and without oil samples to be studied (264). GR 1620 AP (USA) capacitance measuring assembly was used to measure the capacitance. Three thermal measurements were made to avoid stray capacitances. The leads were of co-axial wires for this purpose. Lead
capacitance (1 pf) was subtracted from the measured capacitance before calculating the dielectric constant.

3.2.2 **Analysis of oryzanol by UV spectrophotometer**

The identification and quantitation of total oryzanol content was done by UV spectrophotometer (Chemito UV-2500 model). An accurately weighed quantity of the sample was dissolved in petroleum ether (60°-80°C) and its absorbance was measured at 315 nm. Quantitation was done by taking specific extinction coefficient $E_{1cm}^{1\%}$ as 358.9 (20).

3.2.3 **Identification and quantitation of oryzanol by HPLC**

(i) **Apparatus**: The HPLC system consisted of a Kontron Pump 322, a rheodyne injector with a 20 µl sample loop, a 332 UV detector and a PC integrator connected to a printer. The column used was Lichrosorb 10 RP 18 (25 cm x 4.6 mm) of Kontron Analytical model.

(ii) **Preparation of standard and sample**: A solution of accurately known concentration of about 0.5 mg/ml of reference γ-oryzanol was prepared in the mobile phase. Samples of rice bran oil were prepared by dissolving a known amount of oil in the mobile phase. The samples were shaken vigorously and centrifuged for 10 min. The supernatent was taken for HPLC analysis.

(iii) **Eluents**: The following mobile phase was used:

(a) acetonitrile : methanol : methylene chloride (45:45:5 by volume)
(b) water (5% by volume)
(iv) Solvent Gradient: A gradient system was used which was as follows:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
<th>Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>95</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>95</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>95</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

The flow rate was kept at 1 ml/min and the wavelength was 315 nm. The temperature of the column oven was 40°C.

(iv) Quantitation of γ-oryzanol by HPLC: For quantitation reference sample of γ-oryzanol was run on HPLC as per the above standardised analytical technique. Oil samples were run on HPLC under identical conditions and quantitation was done by electronic integration of chromatographic peaks and their comparison with the peak area of the reference sample.

3.2.4 Identification and quantitation of tocopherols by HPLC:
This was done by the earlier reported technique (73).
(i) **Apparatus**: The HPLC system consisted of a Kontron Pump 322, a rheodyne injector with a 20 µl sample loop, a 332 UV detector and a PC integrator connected to a printer. The chromatographic separation was performed on a 4.6 x 25 cm Spherisorb column (Kontron Analytical model).

(ii) **Eluents**: The mobile phase used was 1.5% iso-propyl alcohol (IPA) in hexane.

(iii) **Conditions**: The flow rate was 1.8 ml/min., temperature 35°C and wavelength was 295 nm.

(iv) **Standards and samples**: An accurately weighed sample of reference tocopherols were prepared in the mobile phase and were run on HPLC. 5 g of oil samples were weighed in 100 ml volumetric flasks. The samples were brought to volume with the mobile phase and run on HPLC under same conditions. Quantitation was done by comparison of peak areas of oil samples with reference tocopherols.

3.2.5 **Isolation of oryzanol from crude ricebran oil**

Isolation of oryzanol from crude oil was done in four steps viz. chromatography, rechromatography, purification and crystallisation. Chromatography was done on a silica gel (60-120 mesh) column. 60 g silica gel was used during chromatography and 30 g during rechromatography. Different fractions and volumes of hexane and ether were used as eluents. All the fractions obtained by chromatography and rechromatography were distilled, vacuum dried, weighed and analysed for their oryzanol content. The oryzanol rich
fraction obtained by rechromatography after purification gave pure oryzanol.

3.2.6 **Blending and deep fat frying:**

3.2.6.1 **Blending**: Refined ricebran oil was blended with safflower oil and groundnut oil in the ratios of 20:80, 40:60, 60:40 and 80:20 and these blends were taken for the studies on deep fat frying experiments.

3.2.6.2 **Deep fat frying:**

(i) Procurement of potato chips: G-4 variety of potatoes were peeled and sliced manually into elliptical chips (4.0 - 6.0 cm, thickness 1.0 mm) and kept under cold water until used for frying.

(ii) Moisture content of potato chips: The moisture content of the potato chips was determined from the weight loss from 10 g of chips after drying them at 105°C for two hours.

(iii) Frying apparatus: The assembly used during the experiment is given in Figure 3.1.

(iv) Procedure followed: About 1 kg oil was taken in a stainless steel frying pan and heated to 180°C with a thermostatic control device. A 25 g batch of potato chips (dried on a filter paper) was then fried and four batches at 15 minutes interval were fried each hour for 6h/day.
Fig. 3.1 - Assembly used in deep-fat frying experiments
and the process was continued for 6 days. At the end of each day i.e. after every 6h frying about 50 ml of the oil sample was taken out, vacuum filtered and kept in cool and dark place in the refrigerator until analysed. The frying medium was filtered, cooled and kept in refrigerator for next day’s frying operations.

3.2.7 **Clinical trials**:  
Clinical trials were done to study the hypolipidemic effect of ricebran oil and its blends in comparison with other edible oils. For this three different sets of experiments were carried out in the following order:

(i) In the first set of experiments forty male albino rats weighing between 90 -110 g were taken and divided into five groups of eight animals each. They were fed cholesterol free control diet (219) as given in Table 3.1. The control diet included 10% of individual edible oils tried in the study namely ricebran oil, groundnut oil, sunflower oil, safflower oil and soyabean oil. Their dietary regimen was maintained for eight weeks. At the end of the experiments, animals were killed after overnight fast and 3 - 4 ml of blood was withdrawn after the cardiac puncture. The blood samples were centrifuged to obtain serum for determination of lipid profiles.

(ii) During the second set of experiments rats were fed cholesterol rich diet as depicted in Table -3.2 (219) using the same oils as mentioned above. The rest of the experiments were done in the similar manner given in (i) for lipid profile determination.
(iii) In the third set of experiments blends of ricebran and safflower oil (as given in Table - 3.3) were used in cholesterol rich diet instead of pure oils. The experiments were done in the similar manner as mentioned above.

**TABLE - 3.1**

**COMPOSITION OF CHOLESTEROL FREE DIET (%w/w)**

<table>
<thead>
<tr>
<th>Dietary components</th>
<th>Control Group</th>
<th>Experimental Group</th>
<th>Experimental Group</th>
<th>Experimental Group</th>
<th>Experimental Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
</tr>
<tr>
<td>Casein</td>
<td>15.0</td>
<td>15.0</td>
<td>15.0</td>
<td>15.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Starch</td>
<td>67.8</td>
<td>67.8</td>
<td>67.8</td>
<td>67.8</td>
<td>67.8</td>
</tr>
<tr>
<td>Groundnut oil</td>
<td>10.0</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Soyabean oil</td>
<td>-</td>
<td>10.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>-</td>
<td>-</td>
<td>10.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10.0</td>
<td>-</td>
</tr>
<tr>
<td>Ricebran oil</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Salt mixture</td>
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<td>4.0</td>
<td>4.0</td>
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<td>4.0</td>
</tr>
<tr>
<td>Vitamin mixture</td>
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<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Cellulose</td>
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<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Choline chloride</td>
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<td>0.2</td>
<td>0.2</td>
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</tr>
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</table>

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TABLE - 3.2
COMPOSITION OF CHOLESTEROL CONTAINING DIET (% w/w)

<table>
<thead>
<tr>
<th>Dietary components</th>
<th>Control Group</th>
<th>Experimental Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Casein</td>
<td>15.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Starch</td>
<td>66.3</td>
<td>66.3</td>
</tr>
<tr>
<td>Groundnut oil</td>
<td>10.0</td>
<td>-</td>
</tr>
<tr>
<td>Soyabean oil</td>
<td>-</td>
<td>10.0</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Safflower oil</td>
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<td>-</td>
</tr>
<tr>
<td>Ricebran oil</td>
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<td>Salt mixture</td>
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<tr>
<td>Vitamin mixture</td>
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<tr>
<td>Cellulose</td>
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<td>2.0</td>
</tr>
<tr>
<td>Choline chloride</td>
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<td>0.2</td>
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<tr>
<td>Cholesterol</td>
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</tr>
<tr>
<td>Cholic acid</td>
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TABLE - 3.3
BLEND OF RBO AND SAF

<table>
<thead>
<tr>
<th>S.NO.</th>
<th>GROUP</th>
<th>BLEND RATIO (RBO:SAF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>30:70</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>50:50</td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td>70:30</td>
</tr>
<tr>
<td>4</td>
<td>D</td>
<td>90:10</td>
</tr>
</tbody>
</table>

3.2.8 Estimation of lipid profiles

3.2.8.1 Estimation of total cholesterol: Total cholesterol was enzymatically estimated according to the method of Allain et. al. (265).

Contents:
Reagent 1
- Buffer
- Active ingredient - Phenol

Reagent 2
- Enzyme
- Active ingredients
- Cholesterol esterase
- Cholesterol oxidase
- Peroxidase
- 4 - Amino antipyrene
- Sodium Cholate
Reagent 3 : Standard
(Cholesterol 200 mg/dl)

Preparation of working solution: Contents of one bottle of reagent 2 was dissolved with contents of one bottle of reagent 1, mixed well and stored at 2° - 8° C. This was the chromogen reagent.

Procedure

<table>
<thead>
<tr>
<th>Blank</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipette into test tubes chromogen reagents</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Sample</td>
<td></td>
</tr>
</tbody>
</table>

They were mixed well and incubated at 37° C for 10 min., thereafter 2.0 ml of distilled water was added and reading was taken at 500 nm in spectrophotometer.

For the preparation of standard curve 0.01, 0.02, 0.03, 0.04 and 0.05 ml of the standard cholesterol solution was taken in individual test tube as triplicate set. Then 1 ml of chromogen reagent was added and mixed well and incubated at 37° C for 10 min. Then 2.0 ml of distilled water was added to each test tube so that the total volume of the solution was made upto 3.02 ml in each case. The absorbance of the standards were taken against reagent blank at 500 nm wavelength.

3.2.8.2 HDL - Cholesterol Separation :

Reagents: 1. Chromogen reagent: as described under the reagents of total cholesterol.

Procedure: 1.0 ml of chromogen reagent was pipetted into a test tube and 0.20 ml of clear supernatent solution (obtained from HDL -
cholesterol separation) was added. It was mixed and incubated at 37°C for 10 min., thereafter 2.0 ml of distilled water was added. Absorbance of the test sample was measured against a reagent blank at 500 nm wavelength in spectrophotometer (266). A standard curve was prepared with the supplied standard (200 mg/dl).

3.2.8.3 Estimation of triglyceride: Estimation of triglyceride was carried out by the method of Bucolo and David (267).

Contents

<table>
<thead>
<tr>
<th>Reagent 1</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Active reagents</td>
</tr>
<tr>
<td></td>
<td>Pipes buffer</td>
</tr>
<tr>
<td></td>
<td>ESPAS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reagent 2</th>
<th>Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Active ingredients</td>
</tr>
<tr>
<td></td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td></td>
<td>Glycerokinase</td>
</tr>
<tr>
<td></td>
<td>Glycerol - 3 - phosphate oxidase</td>
</tr>
<tr>
<td></td>
<td>Peroxidase</td>
</tr>
<tr>
<td></td>
<td>4 - Amino antipyrene</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Reagent 3</th>
<th>ATP</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Standard (triglyceride 200 mg/dl)</td>
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</table>

Preparation of working solution: Contents of one bottle of reagent 2 was dissolved with contents of one bottle of reagent 1. This chromogen reagent was mixed well and stored at 2°C - 8°C.
Procedure: A standard was prepared using aliquots of supplied stock solution (200 mg/dl) containing 10 µg, 20 µg, 30 µg, 40 µg and 50 µg. The rest of the process was same as described above. A standard curve was drawn plotting absorbance against concentration of triglycerides standard. Triglyceride content of the unknown was estimated from the standard curve.

1 ml of the chromogen reagent was taken in each test tube, 0.02 ml of sample was added, mixed well and incubated at 37°C for 10 min. Distilled water was added so that the total volume of the solutions became 3.02 ml. The absorbance was read in a spectrophotometer at 546 nm wavelength against a reagent blank.

3.2.9 Refining of ricebran oil:
Refining of ricebran oil was done as follows (268):

3.2.9.1 Degumming: Weighed quantity of experimental ricebran oil (100g) was taken in a beaker and heated under stirring to about 45°C. Thereafter, a 10% solution of phosphoric acid was added to the sample. Further heating and stirring was continued to attain a temperature of 70°C of the mixture. At this stage heating was discontinued. However, the mixture was kept under stirring for next 45 min. Thereafter, the stirring was also discontinued and the mixture was washed with hot water (15% weight of the oil). After the settling time of 10 min. the gums settled at the bottom, along with wash water, were siphoned off and the clear degummed oil was recovered.
3.2.9.2 Dewaxing: Degummed oil samples in 500 g batch was kept in a temperature controlled chamber and cooled to 10°C for 24 h for crystallisation of waxy material in the oil. Thereafter, the samples at their temperatures were centrifuged at 4000 rpm for 30 min to effect settling of waxes at the bottom of the centrifuge tubes. The supernatent layer of the oil was decanted off and the dewaxed oil was thus obtained.

3.2.9.3 Bleaching: The oil sample in 1 kg quantities were taken in a three necked flask equipped with arrangements for heating and stirring under vacuum. Initially, the oil samples were heated, while stirring under vacuum to 90°C to make them free from moisture. Thereafter, these were supplemented with a slurry of adsorbent mixture consisting of clayton bleaching earth and activated carbon in 4:1 ratio at 2.5% level of the weight of the oil. The slurry was made by mixing the adsorbent mixture with dried ricebran oil taken out from the flask. The slurry was sucked into the flask under vacuum. The temperature was raised to 115 ± 2°C and the stirring was continued under vacuum for another 30 min. Thereafter, the oil was filtered to obtain clear samples of bleached ricebran oil.

3.2.9.4 Alkali refining: The ricebran oil samples in 1 kg. quantities were taken in a beaker and heated to 70 ± 1°C under stirring. Thereafter, caustic lye of 22° Be' in 10% excess was added to the oil samples under stirring. A little quantity of common salt was added for better graining of soap formed during neutralisation. The stirring was put off and a silent hot water wash, using 15% water on weight of the oil was given and the contents were allowed to settle for three hours.
After settling the soap solution was siphoned off and the oil was again given hot water wash to remove the entangled soap from the neutral oil. The hot water washing, settling and siphoning off soap water were repeated until the oil became completely free from soap and neutralised oil was obtained.

3.2.9.5 **Physical deacidification of ricebran oil**: The experimental samples of ricebran oil were degummed by bleaching with 3% bleaching earth at 105°C. The oil thus obtained was subjected to steam distillation at 3 mm Hg pressure and 260°C temperature. The refined, bleached and deodorised oil was thus obtained.