3. MATERIALS AND METHODS

In this chapter, the methodology used to conduct various studies has been discussed. Enzymatic degumming of rice bran oil, storage studies of physically and chemically refined rice bran oil with respect to other conventional oil and its blended oils has been evaluated. Quantification of rice bran oil was evaluated by using different methodologies. Thermal oxidation and the frying behaviour of rice bran oil was also studied.

3.1 Enzymatic degumming of crude rice bran oil

3.1.1. Materials

Crude rice bran oil was obtained from A. P. Solvex Ltd., Dhuri, Punjab. Commercially extracted crude rice bran oil was degummed under a range of experimental conditions of water concentration, temperature, time and speed of agitation. Lecitase Ultra enzyme from Novozymes south Asia Pvt. Ltd (Bangalore, Karnataka, India) was used as a source of phospholipase in the refining of oil to facilitate the removal of phospholipids. All the chemicals used in experiments were of analytical grade.

3.1.2. Methods:

3.1.2.0 Acid Degumming

Crude rice bran oil (500g) was weighed in a 1 lt beaker and heated to 60-70 °C on hot plate with a magnetic stirrer, then was treated with 2% water and food grade phosphoric acid. A critical quantity of dilute caustic soda was added to neutralize the acid and stirred at 300 rpm for 30 min using magnetic stirrer at 60-70°C to complete gum hydration. After hydration, it was kept on hold for 2 min. The hydrated gums were a complex mixture consisting mainly
of phosphatides, neutral oil and water. Gums from the oil mixture were separated by centrifugation. The supernatant layer was then decanted.

3.1.2.1. **Enzymatic Degumming**

Crude rice bran oil (500 g) was weighed in a 1 litre beaker and heated up to 30-35 °C for 15 min with continuous stirring. Then the temperature was raised up to 70 °C and citric acid monohydrate is added as 45% (w/w) solution in sample beaker. Then the solution was kept on hold for 30 min at 70 °C for gum conditioning. After 30 min, 4% NaOH solution was added and then high shear mixing was performed. Different reaction temperature was set as per the reactions requirement. High shear mixing was performed for 6 hours after adding enzyme solution. At the end of 6 hrs of retention or different reaction times, temperature was raised up to 75-80 °C and the oil was centrifuged to remove the gums. The total water content added to the process in the form of citric acid, NaOH and enzyme solution was 1.5% of weight of oil. Higher water content leads to higher losses of oil. Efficiency of degumming was evaluated based on the yield of dry gums, phospholipids and acetone insoluble gum.

3.1.3. **Analytical parameters:**

Free fatty acids (FFA) [Ca 5a-40], unsaponifiable matters (USM) [Ca 6b-53], moisture insoluble volatiles (MIV) [Ca 2f-93], phosphorous content (p-content) [Ca 19-86] and chlorophyll [Cc 13d-55(09)] were determined by using standard method (AOCS 2004). Bleachability of oil was determined by using ISO standard method (ISO/DIS 17932). Colour of crude oil and bleached oil was measured by using Lovibond tintometer (Model F, Effem Technologies Pvt. Ltd., New Delhi, India).
3.2. Storage stability of physically refined rice bran oil and its blends

The study dealing with quality characteristics and shelf life of pure rice bran oil (physically refined) and blended oils thereof while using different packaging material was undertaken.

3.2.1. Materials and Methods

Physically refined rice bran oil, sunflower oil and safflower oil were obtained from A. P. Organics Pvt. Ltd. Dhuri, Punjab. Packaging materials, used for storage of oil samples were PET, laminates consisted of typical three layer co-extruded film consisting of LD+LLD-HM HDPE- Primacor and glass bottles. The packaging materials used under study were also supplied by A. P. Organics Pvt. Ltd., Dhuri, Punjab. All the chemicals used in the study were of AR grade.

3.2.2. Preparation of samples and their storage

PRBO: Safflower, PRBO: Sunflower, oil blends were prepared in the proportions of 20:80, 60:40 respectively (Sharma et al., 1996 b and Sharma et al., 2006) and were filled in PET bottles, glass bottles and laminated pouches (typical three layer co-extruded film consists of LD+LLD-HM HDPE- Primacor). The samples were stored for a period of 11 months at a temperature of 25±5°C and relative humidity of 60-70% respectively. The samples were opened after every two months for the evaluation of various physico-chemical parameters.

3.2.3. Analysis of the samples

Free fatty acids (FFA) [Ca 5a-40], Peroxide value (PV) [Cd 8-53], Iodine value (IV) [Cd 1-25], Saponification value (SV) [Cd 3-25], Refractive index (RI) [Cc 7-25], p-content [Ca 12-55] and p-anisidine value [Cd 18-90] and specific gravity [Cc 10a-25] were determined by using standard methods (AOCS, 2004). Color of the oil was measured by using Lovibond tintometer (Model F, Effem Technologies Pvt. Ltd., New Delhi, India). Oryzanol value
(IICT, 2008) was measured by using Spectrophotometer (UV-1700, SHIMAZDU). Fatty acids of triglycerides were analyzed by preparing methyl esters according to a conventional procedure consisting of saponification followed by acidification and finally methylation using diazomethane as per the reported method (Sharma et al., 2006). Gas chromatographic (GC) analysis of fatty acid methyl esters was carried out using a NUCON SERIES 5700 of data station 0-2.5 mV range and < 1.5s response rate. A 2m x 2 mm stainless steel 10% Silar 7C column packed with 60-120 mesh Gas Chrom Q was used. The injector and detector temperatures were maintained at 240°C. The column temperature was set at 160°C for 5 min and then ramped at a rate of 5 °C per min to a final temperature of 220°C and kept there for 20 min. The total time for analysis was 37 min. Fatty acids were tentatively identified by comparison with retention times of authentic reference samples. The data was tabulated and subjected to two ways ANOVA, test of significance, means and standard deviation using Sigma Stat 3.5 version and excel windows version.

3.3. Storage study of chemically and physically refined rice bran oil with respect to other conventional oils

3.3.1. Preparation of samples and their storage

CRBO, PRBO, Sunflower oil and safflower oil were filled in PET bottles, glass bottles and laminated pouches (typical three layer co-extruded film consists of LD+LLD-HM HDPE-Primacor). The samples were stored for a period of 11 months at a temperature of 25±5°C and relative humidity of 60-70% respectively. The samples were opened after every two months for the evaluation of various physico-chemical parameters, as per the methods given in 3.2.3
3.4. Quantification of rice bran oil in oil blends

3.4.1. Preparation of samples

Pure rice bran oil sample was blended with pure safflower and sunflower oil in different ratios i.e. 1, 3, 4, 5, 10 & 20% using high speed homogenized mixer at a temperature of 25±5°C.

3.4.2. Analysis of the samples

Iodine value (IV) [Cd 1-25], Saponification value (SV) [Cd 3-25], Refractive index (RI) [Cc 7-25] and specific gravity [Cc 10a-25] were determined by using standard methods (AOCS, 2004). Oryzanol value (IICT, 2008) was measured by using Spectrophotometer (UV-1700, SHIMAZDU). The oryzanol content of each sample was determined by measuring the optical density of the sample at 315 nm in n-Heptane in 1 cm cell using spectrophotometer.

Fatty acids of triglycerides were analyzed by preparing methyl esters according to a conventional procedure consisting of saponification followed by acidification and finally methylation using diazomethane as per the reported method (Orthoefer & Smith, 1996). Gas chromatographic (GC) analysis of fatty acid methyl esters was carried out using a NUCON SERIES 5700 of data station 0-2.5 mV range and < 1.5s response rate. A 2m x 2 mm stainless steel 10% Silar 7C column packed with 60-120 mesh Gas Chrom Q was used. The injector and detector temperatures were maintained at 240°C. The column temperature was set at 160°C for 5 min and then ramped at a rate of 5 °C per min to a final temperature of 220°C and kept there for 20 min. The total time for analysis was 37 min. Fatty acids were tentatively identified by comparison with retention times of authentic reference samples.
3.4.2.1. Measurement of ultrasonic velocity, relative association acoustic impedance, and compressibility in blended oils

The densities of all oil samples were measured using a single capillary pycnometer. The measured densities were reproducible within ± 0.2 kg m\(^{-3}\). The ultrasonic velocities were measured at room temperature (26±1\(^\circ\)C) using a single crystal variable-path interferometer at 1 and 2 MHz with an accuracy of ±0.05%. The criteria of purities of chemicals and accuracies were checked by comparing the experimental values with distilled water sample. The isotropic compressibility, relative association, acoustic impedance and adiabatic compressibility were calculated by using equation 1, 2, 3\& 4 respectively (Mehra & Israni, 1999).

\[
K_s = u^2 \rho^{-1} \quad ........... (1)
\]

\[
RA = \left(\frac{\rho}{\rho^0}\right) \left(\frac{u^0}{u}\right)^{1/3} \quad ........... (2)
\]

\[
Z = u \rho \quad ........... (3)
\]

\[
\beta = 1/ (u^0)^2 \rho^0 \quad ........ (4)
\]

Ks isotropic compressibility: \(\rho^0\) densities of control sample: \(U^0\) ultrasonic velocity of control sample: RA relative association: Z acoustic impedance: \(\beta\) adiabatic compressibility: \(\rho\) density of sample: \(u\) ultrasonic velocity of sample.

3.4.2.2. Oryzanol estimation by HPLC

The oryzanol content (CD13CS-11) was estimated as per the standard method (AOCS, 2004). The estimation was carried out using HPLC system of Agilent technologies, connected with a injector having a 20 \(\mu\)l sample loop. The sample was weighed to 5gm and transferred to a 50ml volumetric flask and the volume was made up with n-hexane. Then the solution was filtered through 0.45\(\mu\)m filter. The filtered sample was used for HPLC analysis.
The sample, 20μl was injected into HPLC column for quantification of oryzanol. The column used was of Zorbax eclipse plus C\textsubscript{18} (4.6 X 250mm, 5μm). The mobile phase was ACN: MeOH: IPA in ratio 55:35:10 respectively, which had a run time of 25min with flow rate, 1.2ml/min. The oryzanol content was detected at a wave length of 325nm by using Diode array (DAD) detector by Agilent technologies and it was quantified as per the retention time and peak area/height of standard and sample.

3.4.2.3. Oryzanol estimation by spectrophotometer

Oryzanol value (IICT, 2008) was measured by using Spectrophotometer (UV-1700, SHIMAZDU). The oryzanol content of each sample was determined by measuring the optical density of the sample at 315 nm in n-Heptane in 1 cm cell using spectrophotometer. An accurately weighed amount of RBO (about 0.03 – 0.05 g) was dissolved in n-heptane and the volume was made up to 25 ml. After thorough mixing, the O.D. was recorded at 315 nm by spectrophotometer (UV 1700, Shimadzu Scientific Instruments, Columbia, North America). The oryzanol content was calculated as:

$$\text{Oryzanol (ppm)} = \frac{\text{OD} \times 25}{359 \times \text{sample wt.}}$$

3.4.2.4. Statistical analysis

Each value is the mean of three repetitions. Standard deviation was applied by using Microsoft excel software. Sigma stat 3.5 versions were used for analysing all the experimental data and for finding regression equation and correlation coefficient.
3.5. Thermal oxidation of rice bran oil during oven test and microwave heating

3.5.1. Materials and methods

Physically refined rice bran oil, without addition of any antioxidant was obtained directly from A. P. Solvex Ltd., Dhuri, Punjab. TBHQ, BHA, BHT and CA all antioxidants used in the study were of Milestone Preservatives Pvt. Ltd., Vadodara (India). Oryzanol was procured from Sigma Aldrich, Saint Louis, US. All the solvents used in the study were of HPLC grade and the reagents were of analytical grade.

3.5.2. Sample preparation

The antioxidant tertiary-butyl hydro quinone (TBHQ), butyl hydroxy-anisole /butyl hydroxy toluene (BHA/BHT), citric acid (CA) and BHA/BHT + CA were prepared as: BHA/BHT- 200mg/kg, BHA/BHT (200mg/kg) + CA(100mg/kg), CA (100mg/kg), TBHQ (100mg/kg and 200 mg/kg) and pure rice bran oil for control sample. Requisite amount of antioxidants were added in a small amount of the oil sample at elevated temperature and then the sample was added directly to the oil which was homogenized mechanically at ambient temperature for 10 min.

3.5.3. Oven test

Samples (50 ml) of each oil treatment were placed in separate 100 ml open beakers, without stirring and held in an oven at 63°C temperature during 1, 2, 3, 4, 5 and 6 days. Immediately after each storage period, oil samples were analysed.
3.5.4. Microwave heating

Samples (50 ml) of each treatment were placed in 100 ml beakers and covered with PVC film. Samples were heated in Onida power-grill 25, microwave oven (900W effective power) for 0, 2, 4, 6, 8, 10, 12, 16, 20, 24, 28 and 32 min. After each heating period, the oil temperature was determined with a thermocouple, and all the samples were withdrawn and analysed.

3.5.5. Analytical procedures

Free fatty acids, Iodine value, Peroxide value (PV) and p-anisidine value were determined as per the standard analytical methods, Ca5a-40 Cd1-25 Cd8-53 and Cd18-90 (AOCS, 2004). Colour of the oil samples was determined by Tintometer (Model F, Effem Technologies Pvt. Ltd, New Delhi, India). Oryzanol content (IICT, 2008) was determined by spectrophotometric (UV- SHIMAZDU) method. Absorptivity at the wavelength of 232 and 270 nm were determined by using spectrophotometric method, II.D.23 (IUPAC, 1979).

3.5.6. Statistical analysis

Each value is the mean of three repetitions. The ANOVA and Tukey’s test were applied. The Microsoft office excel software was used for analysis.
3.6. Effect of frying conditions on the physico-chemical properties of rice bran oil and its blended oil

3.6.1. Materials and methods

Physically refined rice bran oil and sunflower oil samples were procured from the leading oil industry A. P. Organics Ltd located in Dhuri, Punjab, India. Rice bran oil and sunflower oil blend was prepared in the ratio of 60% rice bran oil (RBO) and 40% SnFO (sunflower oil) as the ratio had shown the better suitability during repeated deep fat frying than the remaining blended oils (Sharma et al., 2006). The oils in the specific proportions were blended using high shear mixer. The blending was carried out at 150 rpm and 60°C temperature for 30 minutes.

3.6.2. Preparation of Potato Chips:

The potato (variety- Kufri Jyoti) chips were prepared from the conventional method consisting of scrubbing, washing and then slicing using the slicer. The slices were immersed in water and washed repeatedly till the removal of starch. The washed slices were then dipped in the boiled water, consisting of salt, 1% for nearly 20 minutes. The slices obtained were wrapped in ordinary filter paper to remove the surface water. These potato chips, having the moisture of 64.77% were used for frying under the designed conditions.

To observe the frying behaviour of the moistened chips and the dried chips, the drying of the potato chips was carried out under the sun (till the moisture of 1.09%) followed by drying in an oven at a temperature of 80°C till the constant weight. The finally dried chips obtained had the moisture of 0.5% (wb). Thickness of dried potato chips and moistened chips was 1.28mm and 2.29mm respectively.
3.6.3. Deep Fat Frying of Potato Chips:

The deep fat frying process was carried out in the similar way as reported by Sharma et al., (2006). The frying of a known weight (50 g) of potato chips was carried out by drawing 500 ml of oil sample from control as well as blended oils separately in a frying domestic fryer (diameter 28 cm, depth 6 cm) at a deep fat frying temperature of 210°C for 8 sec. Deep fat frying time and temperatures were decided based on preliminary experiments carried out in the laboratory. Another frying operation was carried out in 500 ml of oil under the same frying conditions. After frying, the oil samples from control as well as blended oils were cooled to room temperature and stored separately in PET bottles for three days for further frying. After 3 days, 80 ml of oil sample was taken from control and blended oils for analysing physico-chemical parameters and lipid profile. After every frying cycle, the volume of all oil samples was again made up to 500 ml by adding oil from another frying operation carried out under similar conditions. The same frying processes were repeated six times with each and every oil sample after successive storage of oils for three days and withdrawing 80 ml oil sample. The total time for an experiment was 18 days.

3.6.4. Analytical Procedures:

After each frying cycle, the oil samples were analysed for Free fatty acids (FFA) by titrating the free fatty acid with alkali in presence of ethyl alcohol as solvent [Ca 5a-40]. Peroxide value (PV) was estimated by using sodium thiosulfate solution as titrating agent against the evolved iodine in the sample, after reacting the peroxides present in the sample with salt of iodine (KI) [Cd 8-53]. Iodine value (IV) was determined by treating the sample with an excess of solutions of iodine monochloride (ICl) in glacial acetic acid. Unreacted iodine monochloride reacted with potassium iodide, converting it to iodine, whose concentration was determined by titration with sodium thiosulphate [Cd 1-25]. Saponification value (SV)
was determined by treating the sample with alkali and the unreacted parafins were then titrated against 0.5N hydrochloric acid [Cd 3-25] and Refractive index (RI) was determined by using refractometer with temperature adjusted to 37°C [Cc 7-25]. The phosphorus content was determined spectrophotometrically. The procedure was based on formation of ionic—associate of molybdophosphate with zinc oxide in an acidic medium and compared with the standard vanadomolybdate and molybdenum blue methods [Ca 12-55]. The p-anisidine value of the sample was estimated by using iso-octane as the solvent under 350nm of wavelength [Cd 18-90] and specific gravity [Cc 10a-25] was determined by using standard methods (AOCS 2004). Color of the oil was measured by using Lovibond tintometer (Model F, Effem Technologies Pvt. Ltd., New Delhi, India). Oryzanol value of the sample was measured by using Spectrophotometer (UV-1700, Shimadzu Scientific Instruments, Columbia, North America) at 350nm in presence of n-heptane solvent (Vijayalakshmi, 2008). The presence of starch was indicated by the appearance of blue color from the addition of a drop of one percent iodine solution.

3.6.4.0. Determination of trans fats and fatty acid composition:

Fatty acids of triglycerides were analyzed by preparing methyl esters according to a conventional procedure consisting of saponification followed by acidification and finally methylation using diazomethane as per the reported method (Sharma et al., 2006). The contents of trans-fatty acids were calculated by composition analysis using an integrator. Peaks of trans fats were identified by comparing their retention time with those of predetermined standards. Trans fatty acids mainly consisted of C18:1t, C18:2t, C18:3t and C20:1t and were expressed as percentages rounded to the second decimal place.
3.6.4.1. Esterification and quantification:

Methyl Esterification of samples used in the analyses was performed by BF3-MeOH method after alkaline hydrolysis. Mixture of 0.3 gm of sample oils and 10 ml of NaOH-methanol solution was heated at 80-90°C for 20 min. After cooling, 5 ml of BF3-MeOH reagent was added then the vessel was sealed and heated at 80-90°C for 5 minute. After cooling, 10 ml each of petroleum and saturated NaCl solution were added, followed by a thorough shaking. The resulting petroleum ether layer was used as a sample solution for GC.

The analysis of fatty acid methyl esters was carried out using Gas chromatography (GC) (Agilent Technologies J&W, 7820A) with full electronic pneumatics control (EPC) for inlets and detectors, ensuring excellent reproducibility, as well as reliable accuracy and precision. A DB-23 capillary column of high polarity phase (Agilent Technologies J&W) with 60m length, 0.250µm internal diameter, and 0.25 µm film thicknesses was used. This column is specially designed for trans fat estimation and was made from fused silica (stationary phase) and were coated with a thin uniform liquid phase (50% Cyanopropyl-methylpolysiloxane). The injector and detector temperatures were maintained at 250°C. The column temperature was set at 140°C for 5 min and then ramped at a rate of 6°C/min to 180°C and then kept for 7 min, then again ramped at a rate of 2°C/min to 230°C and kept for 17 min. The total time for analysis was 60 min. Fatty acids were tentatively identified by comparison with retention times of authentic reference samples.

3.6.4.2. Estimation of Oil Uptake by the Fried Food Products:

The extraction of fat in presence of hexane 100ml/5gm sample, (solvent) was carried out by rapid Soxtec extractor (Socs Plus, Pelican Equipment, Model No. SCS-6, Chennai) and the percent oil uptake was determined on the basis of the sample taken and residue left out after extraction.
3.6.4.3. Statistical Analysis

The ANOVA and Tukey’s test was applied to the experimental data. The Microsoft office excel software was used for analysis. Three replicates were conduct for each analysis.