MATERIALS AND METHODS

Crude rice bran oil was obtained from Shri Murugharajendra Oil Industry Pvt. Limited, Chitradurga, Karnataka, India. The carbon dioxide used for extraction was of food grade from M/s Kiran Corporation, Mysore.

The following chemicals were used soy phosphatidyl choline, egg phophatidyl choline, cholesterol, 1-palmitoyl 2- linolyl phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanol amine phosphatidyl inositol were from sigma chemical company, St Louis, MO, USA. Tris (hydorxy methyl) aminomethane hydrochloride (Tris – HCL) was obtained from Merck, Chelex 100 was obtained from Bio Rad (Tokyo).

Borax and Boric acid were from Himedia laboratories, Mumbai, n-hexane, chloroform, methanol, ethanol, orthophosphoric acid and calcium chloride was from S.D. Fine chemicals, Mumbai. All the chemicals were of analytical grade.

Super critical extraction of ricebran lecithin

A high pressure extraction system of M/s Nova Swiss, Switzerland was used for the extraction of rice bran lecithin. The details of the equipment used are accordingly to Sankar, (1992). The flow diagram of the extraction system was given in fig.8 (Sankar, 1989). A 200ml sample cell fabricated in our laboratory to facilitate handling of liquid sample was used for extraction. For each extraction 100 g of crude ricebran lecithin was loaded in to the above sample cell and kept inside the above extractor vessel. Carbon dioxide was compressed by a diaphragm compressor to the desired pressure and heated to required temperature by heat exchange while the carbon dioxide entered the extractor vessel. At the extractor exit, CO_2 along with the extracted lipids was depressurized. While CO_2 was taken for recycling after

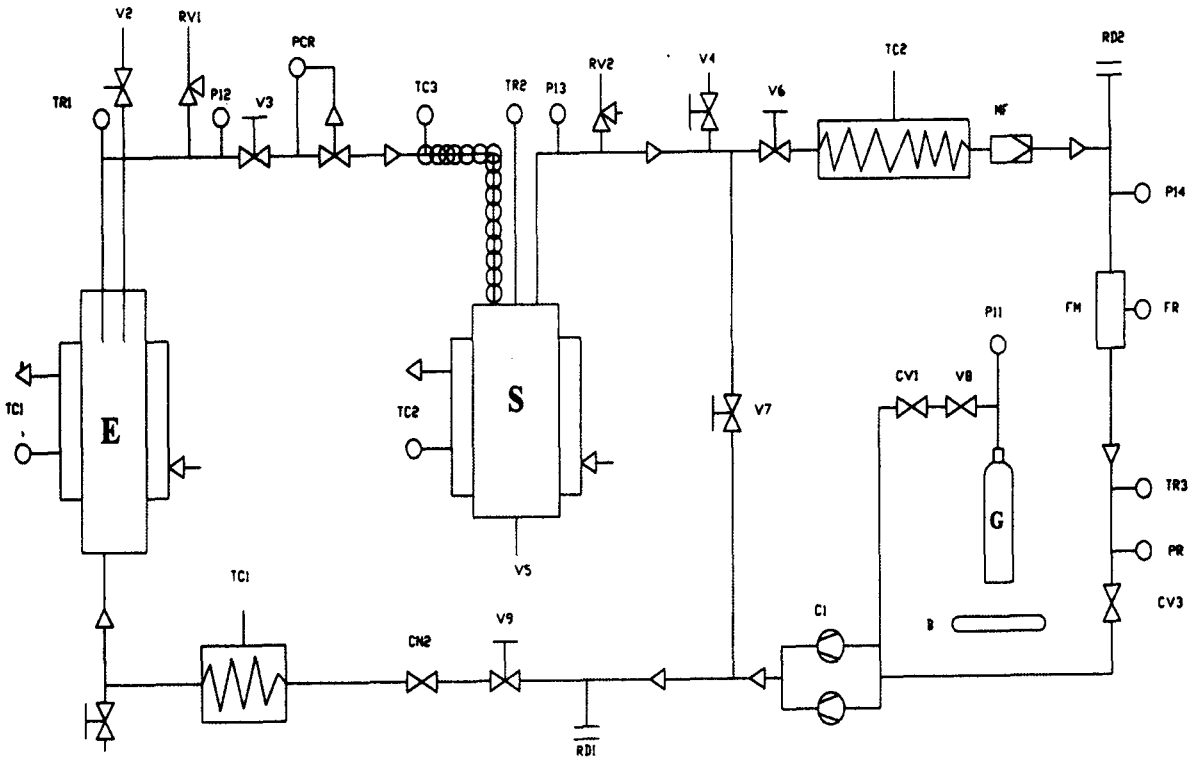


Fig 8: The flow diagram of supercritical carbon dioxide extraction apparatus.

G-CO2 cylinder; B-Balance; RD1&2-Rupture disc assemblies; MF-Micro Filter; PCR-Micro Metering Valve; TC1-3 Heat Exchangers; FM-Flow Meter; CV1-3-Check Valve Assemblies; V1-9-Valves; P1-4-Pressure indicators; FR-Flow Recorder; TR1-3-Temperature Recorder; PR-Pressure Recorder; RV1&2-Relief Valve assemblies.

recompression, the lipids were drawn out from the separator vessel at various time intervals. The CO_2 flow rate was monitored by a turbine flow meter and it varied between 0.4 to 5 Kg/hr, depending on the suction pressure and temperature of carbon dioxide. The above experiment was conducted at pressure of 160, 220 and 280 bar at temperature of 40^0, 50^0 and 60^0C. Solubility of the oil obtained from the initial slope of the curves of the total lipids collected and CO_2 consumed according to Liang and Yeh, (1991). The extractor containing the neutral lipids was called lipid fraction, while the residue, which was rich in phospholipid, was termed as lecithin fraction.

Separation of lipids from lecithin

Column chromatographic separations of the major lipids like neutral, glycolipid and phospholipid fractions were carried out by silicic acid chromatography (50 cm x 1.26 cm). Neutral lipids were eluted successively with chloroform, glycolipids with acetone and phospholipids with methanol. The percentage of each of these lipids class was determined by gravimetric analysis, direct weighing of aliquots from each column fractions after complete evaporation of solvent (Weber, 1981).

Design of experiment

The experimental design chosen for the study was that of Box – Behnken (Box and Behnken, 1960) which helped in investigating linear and quadratic effects of two factors varied at two three levels. Nine experiments were conducted at every combination of three levels of pressure and temperature. The factors and the levels at which the experiments were carried out are given in the Table 9. The actual design of experiments is presented in Table 10.

Table 9: Levels of variables chosen for the solubility study

Variables	Levels		
	+	0	-
P: Pressure, bar	280	220	160
T: Temperature, °C	60	50	40

Table 10: Box – Behnken design of experiments with three independent variables for solubility study.

Exp. No.	P bar	T°C
1	-	-
2	-	0
3	-	+
4	0	-
5	0	0
6	0	+
7	+	-
8	+	0
9	+	+

The response function used was of second order with interaction terms:

Y sol = A0 +A1 * P + A2*T + A3*P + A4* T*T + AS * P * T --------(1)

Where Y sol + Total lipids solubility, g/Kg CO_2

P = Extraction pressure, bar

T = Extraction temperature, ^{0}C

A0 = Constant

A1, A2 = Linear coefficient

A3, A4 = Quadratic coefficient

A5 = Cross - Product coefficient

The parameters of the response equation and their statistical significance were evaluated using Microsoft Excel software (Version – 5.0)

Average absolute deviation (AAD) was defined as:

AAD, % +Σ (/ (Y exp. – Y Pred)/*100 / Y exp.) /n) where Y exp. was the experimental total lipids solubility; Y pred was the predicted total lipids solubility; n was the number of experiments.

Deoiling of ricebran lecithin

The crude ricebran lecithin was de-oiled by extracting the lecithin at 220 bar and 50^{0}C using supercritical carbon dioxide as explained earlier in detail. Experiments were conducted in duplicated at pressure 160, 220, 280 bar at a temperature of 40°C.

Lecithin fractionation

Carbon dioxide extracted lecithin (CDEL) (20 g) was mixed with 125 ml of alcohol (95% ethanol, v/v) at 55°C for 30 min. The resulting soluble portions were filtered to obtain alcohol – soluble lecithin fraction (ASL). It was then concentrated in a rotary

evaporator and the weight percentage was determined. The residue remaining after filtration was alcohol insoluble lecithin (AIL).

Iodine number and acid value were determined as per standard AOCS methods (1989). The phosphorous content was determined by Bartlett method (1959). Acetone insolubles of the sample were determined according to the method of Cocks and VanRede (1966).

Composition of fatty acid by gas chromatography

The methyl esters of the fatty acids were prepared by treating the lipids with boron trifluoride methanol according to Morrison and Smith (1964). Gas chromatographic analysis of the fatty acid esters was carried out on a Shimadzu Gas chromatography – 150 fitted with an FID detector, and data 25 processor CR 4A. The column (3 m x 4mm ID stainless steel) was packed with 15% diethyl glycol succinate (DEGS) on solid support of chromosorb W (mesh size 60/80, acid washed). The operating conditions of GC were as follows. Temperature at the injection port and at the detector port was maintained at 240°C, while the column temperature was 175°C. The flow rates of hydrogen, nitrogen and air were 40, 40 and 300 ml/min, respectively. Peak identification was performed by comparing the relative retention time of each compound with the of standard fatty acid methyl esters.

Composition of phospholipids by HPLC

The composition of phospholipids was determined by HPLC (Shimadzu). The HPLC system consisted of an CR-4A chromatopack data integrator, a rheodyne injector, a SCL-6A system controller, LC 6A pump and a SPD 6AV UV visible

spectrophotometric detector set at a wavelength of 206nm. The HPLC column was a 4.6mm x 15cm microporasil. The HPLC mobile phase was acetonitrile : methanol : 85% 0-phosphoric acid (780 : 10 : 0.9) and the flow rate 0.5 ml/min (Hurst and Martin, 1984).

Lecithin enrichment

De-oiled lecithin (1g) was mixed with a known quantity of ethanol in a test tube for a known period. The temperature of extraction was maintained by placing the tube in thermostat – controlled hot water bath. The mixture was then filtered and the ethanol extract was evaporated and dried under vacuum (30^0C) to a constant weight. The PC content of the dried extract was determined by HPLC. The above process was carried out for all experiments described under design of experiments.

Experimental design

The experimental design chosen for this study was that of Box and Behnken (1960) a fractional factorial design for three independent variables at three levels, as described elsewhere (Sattur and karanth, 1989). This design was preferred because relatively few experimental combinations of the variables were adequate to estimate complex response functions. The various process parameters involved in the purification of ricebran lecithin are : ethanol concentration, extraction time (X1), temperature of extraction (X2) and ethanol to lecithin ratio (X3). Ethanol of 100% concentration was used for all the experiments. Three levels (low, medium and high denoted as -, 0 and + respectively) of variables chosen for the experiments are given in the Table 11. The actual design of the experiments is presented in Table 12. For each of the experiment done, PC enrichment (PCE) was determined:

PCE% = (PC% in extract * amount of extract) * 100/ (PC % in crude lecithin * amount of crude lecithin)

The response function used was a quadratic polynomial equation as given below

PCE : A0 + A1 *X1 + A2*X 2 + A3* X 3 + A4 * X 1 * X2 + A5 * X 2 * X 3 + A6 * X 3 * X 1 + A7 * X 1 *X1+ A8 * X2 * X2 + A9* X 3* X 3

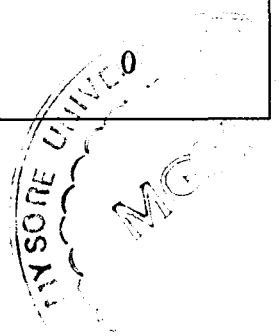
Where, PCE = PC enrichment (%), A0 = constant, A1 A2 and A3 = linear coefficients, A4, A5 and A6= cross – product coefficients, A7, A8 and A9 = quadratic coefficients. A multiple regression analysis was performed to obtain the coefficients and the equation was used to predict responses. The maximum value of the response function was searched by the 'successive variation parameter method' (Jenson and Jeffreys, 1977).

Table 11: Levels of variables chosen for the enrichment study

Variables	Levels		
	+	0	-
X_1 : Time (min)	9	6	3
X_2 : Temperature (^oC)	60	45	30
X_3 : Ethanol to lecithin ratio (l /Kg)	50	30	10

Table 12: Box – Behnken design of experiments with three independent variables for enrichment study

Exp. No.	X_1	X_2	X_3
1	+	+	0
2	+	-	0
3	-	+	0
4	-	-	0
5	+	0	+
6	+	0	-
7	-	0	+
8	-	0	-
9	0	+	+
10	0	+	-
11	0	-	+
12	0	-	-
13	0	0	0



Purification of phosphatiyl choline

The column chromatographic fractionation of the alcohol – enriched lecithin extract was done on a gradifrac system containing a column with a water jacket, which measured 2cm i.d. by 30 cm in length. The jacket was connected with a thermostat. Experiments were carried out at 40°C – 45°C. Silica gel was slurried with 95% ethanol, 35 g in 100 ml, heated to boiling under reflux condenser for 15 min and

then the slurry was added to the column as one portion. Solvent flow was adjusted to 2-3 ml/min and an additional 300 ml (95% ethanol) was added to wash the silica gel. Alcohol enriched lecithin (5g) with an adequate temperature was added to the column. The extract was washed onto the bed and then eluted with 1000ml of 95% ethanol. A total of 25 fractions was colleted. Out of 25 fractions, first five fractions of 30 ml each, followed by fourteen fractions of 100 ml each and the last six fractions of 250 ml each were collected. Each fraction was evaporated to dryness with a rotary vacuum evaporator. Each fraction was qualitatively analysed by thin layer chromatography and phosphatidyl choline content was analyzed by HPLC.

Purification of soybean lipoxygenase

Soybean LOX was isolated according to the method of Axelrod et al., (1981) with some modifications as described by Sudharshan and Appu Rao, (1997).

Substrate preparation

Tween – 20 solubilised substrate

Linoleic acid or phosphatidyl choline with equal amount of Tween – 20 (w/w) were weighed and dissolved in 4.0 ml of oxygen – free water. The resulting suspension was clarified by adding the 0.2 M NaOH. Final volume was made upto 25 ml with 0.2 M borate buffer. This was stored in 1.5 ml capacity eppendorf tubes under nitrogen at 4°C until used (Axelord et al., 1981).

Assay of lipoxygenase

It was assayed by following the appearance of conjugated diene hydroperoxide absorbing at 234 nm using Shimadzu UV 160 A° spectrophotometer and a molar

extinction coefficient of 25,000 M^{-1} cm^{-1}. One unit of enzyme was defined as the formation of 1 μmole of product per min at 25°C under assay conditions. The protein concentration was determined using the value $E^{1\%}_{280} = 14.0$ (Axelrod et al., 1981).

Preparation of lipid hydroperoxides and alcohols

PLPC hydroperoxide (PLPC – 00H) was prepared by the aerobic oxidation of PLPC with lipoxygenase as described by Nagata and Yamamoto (1996) 6 μmol of PLPC was dissolved in 20 ml of 0.1 mg sodium borate buffer of p^H 9 containing 3 mM sodium deoxycholate and mixed with 2 mg of soybean lipoxygenase. The solution was stirred for 45 mins at room temperature and purified by HPLC (Superiorex ods column 20 x 250 mm Shimadzu, Japan) using 0.02 % triethylamine in methanol as mobile phase with a flow rate of 8 ml / min. The corresponding alcohol (PLPC – OH) was purified by HPLC separation as described above after the reduction with sodium borohydride.

Preparation of unilamellar liposomes

Small unilamellar liposomal suspension was prepared by drying under reduced pressure from a methanolic solution of phosphatidyl choline and butylated hydroxy toluene (BHT) followed by the addition of 50 mM Tris – HCl (pH 7.4) pretreated with chelex 100 shaking vigorously and sonication (sonicator IKA, model) on an ice bath for 3 min. Final concentration of phosphatidyl choline and butylated hydroxy toluene were 1 mM and 100 μM, respectively. When required 5 mM calcium chloride ($CaCl_2$) was also added.

Thermal studies on distribution of cholesterol in phosphatidyl choline bilayers

Calorimetric experiments were performed on a Rheometric DSC – 3.0 apparatus operating at a heating rate of 5°C/ min at range 0.5 as described by De Kruyff et. al., 1973. 0.3 ml of methanolic solution containing 1 mM phosphatidyl choline with or without cholesterol was evaporated in a 1ml test tube to dryness. Residual solvent was removed by storing the tube overnight under high vacuum followed by addition of 25 mM Tris HCl – glycol (1:1 v/v) shaking vigorously and sonicated on an icebath for 3 min. About 15 µl of liposomal dispersion with or without cholesterol was sealed in an aluminum sample pan. After the calorimetric scans the amount of phosphatidyl choline present in the pan was determined by phosphorus determination (Fiske and Subba Row , 1925). Each sample was scanned at least 4 times to show the complete reversibility of the transitions. Storing the sample at room temperature up to 24 hrs did not significantly influence the temperature and energy contents of the transitions. The variation in the transition temperature between various scans was 1°C or less. The maximal variation in the determination of the energy content of the phase transition was 10%. Only heating curves are presented in this study. The pre-transitional endotherms were not found. This might be caused by the presence of glycol in the lipid dispersion. The temperature and energy contents of the main transition were not affected by the presence of glycol.