INVESTIGATION ON UNSAPONIFIABLE PHYTOCHEMICALS OF RICE BRAN OIL

Thesis submitted to
Cochin University of Science and Technology
in partial fulfilment of the requirement for the degree of

DOCTOR OF PHILOSOPHY
In
CHEMISTRY

BY
AFINISHA DEEPAM L.S.

AGROPROCESSING AND NATURAL PRODUCTS DIVISION
NATIONAL INSTITUTE FOR INTERDISCIPLINARY
SCIENCE AND TECHNOLOGY (CSIR)
THIRUVANANTHAPURAM-695019
KERALA, INDIA
MARCH-2011

DECLARATION

I here by declare that the thesis entitled **"INVESTIGATION ON UNSAPONIFIABLE PHYTOCHEMICALS OF RICE BRAN OIL"** embodies the results of investigations carried out by me at Agroprocessing and Natural Products Division of National Institute for Interdisciplinary Science and Technology (NIIST), (formerly, Regional Research Laboratory, RRL), CSIR, Thiruvananthapuram, as a full time research scholar under Dr. C. Arumughan as supervising guide and Dr. A. Sundaresan as co-guide and the same has not been submitted elsewhere for any degree.

Thiruvananthapuram
March,2011

Afinisha Deepam L.S.

CERTIFICATE

This is to certify that the work embodied in the thesis entitled **"INVESTIGATION ON UNSAPONIFIABLE PHYTOCHEMICALS OF RICE BRAN OIL"** is an authentic record of research work carried out by Mrs. Afinisha Deepam L.S. under my supervision in partial fulfilment of the requirement for the Degree of Doctor of Philosophy in Chemistry of Cochin University of Science and Technology, and further that no part of this thesis has been submitted elsewhere for any other degree.

Thiruvananthapuram
March, 2011

Dr. C. Arumughan
(Supervising Guide)
Director Grade Scientist and Head (Rtd.)
Agroprocessing and Natural Products Division
NIIST, CSIR, Trivandrum

CERTIFICATE

This is to certify that the work embodied in the thesis entitled; **"INVESTIGATION ON UNSAPONIFIABLE PHYTOCHEMICALS OF RICE BRAN OIL"** has been carried out by Mrs. Afinisha Deepam L.S. under my supervision and the same has not been submitted elsewhere for any other degree.

Thiruvananthapuram
March, 2011

Dr. A, Sundaresan
Co-guide
Scientist 'F' & Head
Agroprocessing and Natural Products Division

ACKNOWLEDGEMENTS

First and foremost, I express my deep sense of gratitude to Dr. C. Arumughan, my thesis supervisor, for giving me an opportunity to enter the fascinating world of research, for suggesting the problem, for his guidance, support and encouragement, leading to the successful completion of this work. I express my sincere thanks to Dr. A. Sundaresan, Co-guide, for his valuable suggestions and support, extended to me during the course.

I wish to thank Dr. Suresh Das, Director and former directors Prof. T.K. Chandrashekar and Dr. B. C. Pai, of the National Institute for Interdisciplinary Science and Technology [NIIST] for providing the necessary facilities for carrying out the work.

I am most grateful to Technology Mission on Oilseeds, Pulses and Maize (TMO&P), Govt. of India, that facilitated me to enter into the field of research as project Fellow with out which, this work would not have taken this shape. I am also indebted to Council of Scientific and Industrial Research (CSIR), New Delhi for granting me Senior Research Fellowship that enabled me to complete this investigation.

I would like to express my gratitude to Mr. Soban Kumar for his help and valuable suggestions.

My sincere thanks to Mr. M.M. Sreekumar, Dr. C. Balachandran, Dr. K..P. Padma Kumari Amma, Mrs. Prasanna Kumari, Dr. Beena Joy, Mrs. Annamma Mathew, Dr. P. Nisha, Mrs. M.V. Reshma, Dr. P. Jayamurthy, Mrs. Omana Kutty Amma, Mrs. Nirmala Menon, Mr. Thomas Samuel, Mr. Venu Gopal, Dr. K.G. Raghu and Mrs. Sankarikutty Amma.

My heartful thanks to Dr. Sachin, for his valuable help on the statistical analysis of the data, Dr. P.R. Jayan, Scientist, Regional Agricultural Research Station, Palakkad for his help in getting paddy varieties.

I gratefully acknowledge all the members of Knowledge Resource Centre, NIIST for their help during the course of this study and Mrs. Saumini Mathew, Mrs. Viji, Mr. Adarsh for their help in instrumental analysis.

A special word of thanks to Dr. U. Syamaprasad, Scientist, NIIST and Convenor, NIIST-CUSAT Research Committee, Mr. Harkrishna Bhatt, APC Chairman, NIIST, for their timely help.

I gratefully acknowledge the help and support from Rajam L, Dr. Renuka Devi R , Sandhya B, Neema, Simi, Sreeja, Kuttan and all the former and present members of the Agroprocessing and Natural Products Division.

I am deeply indebted to my husband Pramod. It is his unconditional love and unbounded support that helped me to complete this work successfully. I am thankful to my little loving kids Ashina and Ashima for giving me enough time to think, to write and submit thesis and also being patient in my anxious situations.

A special thanks to my Father in law who is away from this world and mother in law who supported me to complete this work. My deepest gratitude to my loving Pappa, Amma and brothers for their prayers to complete this work.

Above all I praise and worship my Almighty for raising me to this position

Afinisha Deepam L.S.

Dedicated to

My Father in Law

Who rests in peace

CONTENTS

ABBREVIATIONS

ANOVA	-	Analysis of Variance
AOAC	-	Association of Official Analytical Chemists
AOCS	-	American Oil Chemist's Society
CAC	-	Codex Alimentarius Commission
CEC	-	Capillary Electrochromatography
CRBO	-	Crude Rice Bran Oil
DAG	-	Diacylglycerols
DMRT	-	Duncan's multiple range test
DPPH	-	1,1-diphenyl-2-picryl hydrazyl
FADC	-	Food and Agriculture Division Council
FFA	-	Free Fatty Acid
FID	-	Flame Ionization Detector
FSSAI	-	Food Safety and Standard Authorities of India
GC	-	Gas Chromatography
GC-MS	-	Gas Chromatography-Mass Spectrometry
HDL	-	High Density Lipoprotein
HPLC	-	High Performance Thin Layer Chromatography
HPTLC	-	High Performance Thin Layer Chromatography
IUPAC	-	International Union of Pure and Applied Chemistry
LDL	-	Low Density Lipoprotein
LOD	-	Limit of Detection
LOQ	-	Limit of Quantification
MAG	-	Monoacylglycerols
MMT	-	Million Metric Tons
PFA	-	Prevention of Food Adulteration
PL	-	Phospholipids
RBO	-	Rice Bran Oil
RRBO	-	Refined Rice Bran Oil
RSD	-	Relative Standard Deviation
TAG	-	Triacylglycerols
TLC	-	Thin Layer Chromatography
USC	-	Unsaponifiable Constituents
USM	-	Unsaponifiable Matter

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1
INTRODUCTION

1.1 PREAMBLE

Edible oils and fats are the essential source for human health. They are the important source of various micronutrients which are beneficial to human as they can prevent various health disorders. Plants are the origin of edible oils which includes sesame, sunflower, soybean etc. As from various reports, the oil consumption is increasing per year and hence the use of non conventional oil sources is getting more importance. Among the non conventional sources, rice bran possesses an important role which is a by-product of rice milling industry. Rice bran oil is considered as healthiest edible oil due to the presence of various bioactive phytochemicals. These phytochemicals are collectively termed as unsaponifiable matter. Depending on the method of refining the amount of unsaponifiable matter varies. Crude rice bran oil is generally refined by following physical or chemical refining method. In chemical refining, deacidification is done by treatment of alkali and hence loss of major and unique unsaponifiable constituent called oryzanol was reported and hence percentage of unsaponifiable matter gets lowered. But in the case of physical refining, steam distillation is carried out for the removal of fatty acids and retains unsaponifiable constituents in oil. Also while comparing with other edible oils, the unsaponifiable matter of rice bran oil ranges from 4 to 6%. An important problem concerning with physical refined oil is the high percentage of unsaponifiable matter, often exceeding the limit which is stipulated by Food laws. Therefore it is necessary to relook the standard method of saponification for the estimation of unsaponifiable matter, since alkali treatment causes changes in constituents that may lead to distortion in weight percentage of USM. Thus the primary objective of the present work was to develop a method which is accurate and simple for the estimation of unsaponifiable constituents directly from oil without any pretreatment of the sample. Secondly comparison of constituents by newly developed method and recommended saponification method. The above aim leads to the characterization of new compounds by FTIR, ^{1}HNMR,

C^{13} NMR and Mass spectrometry. Ten paddy varieties from Kerala were extracted for oil and the profiling of unsaponifiable constituents was done by following newly developed method. Finally the effect of major unsaponifiable constituents in the stability of rice bran oil was studied.

1.2 Edible Oils

Edible oils used in cooking are the main source of fat in human nutrition. Edible oils are derived largely from oil seeds that include soybean, rape seed/mustard, cotton seed, sunflower, ground nut, coconut etc. Oil palm and olive are other large source of edible oils obtained from their fruits. The global production of oilseeds is estimated as 440 Million Metric Tons (MMT) (2010). India is one of the largest producers of oilseeds in the world and the estimated production is 34.90 MMT for the year 2010-2011 which accounts for 10-12% of the world's oilseeds production [1]. Edible oil production in India from major sources during the year 2010 was 10.12 MMT and the consumption for the same year was 16.17 MMT. The gap between domestic production and consumption has been bridged by import is about 6.05 MMT. The recent statistics indicate that demand for fats and oil in India has been increasing at rapid rate due to increase in per capita consumption and population. At this rate of increase in consumption, the demand for edible oil has been estimated as 22 MMT by 2020. This would require import of approximately about 10 MMT considering the present rate of growth in domestic production. Increasing productivity and tapping potential of non conventional sources, therefore need urgent attention to reduce the gap between demand and supply of edible oils in India.

India grows wide range of oilseed crops under different agro climatic zones which includes soybean, groundnut, mustard, sesame, safflower, linseed, nigerseed, coconut, sunflower etc. Groundnut, soybean and mustard together contribute about 85

percent of the country's oilseeds production. Coconut is an important source of edible oil in Kerala. Cotton seed and rice bran are the major non-conventional sources and primarily, the use of by-products of cotton and rice has been increasing steadily due to the increase in domestic supply of edible oils. Rice bran is being considered as the most important source for nutritionally superior edible oil and its production has been increasing at faster rate in the recent years.

1.3 Fats and oils in human health

Fat is an important part of a healthy diet. The daily intake of dietary fat must be sufficient to meet the requirements for essential fatty acids and fat soluble vitamins. There is a wide range of chain-lengths, ranging from four-carbon fatty acid in milk fat to thirty-carbon fatty acids in some fish oils. Based on fatty acids bound to acylglycerols, the biological property of fat varies. Saturated fats reported to increase blood cholesterol while mono-unsaturated and polyunsaturated fats lower the blood cholesterol, but conclusive mechanistical evidence in this regard is lacking. Long chain omega-6 and omega-3 fatty acids found in fish oil seem to have benefits for primary and secondary prevention of cardiovascular disease [2]. The saturated fatty acids - lauric, myristic and palmitic, elevate serum cholesterol and Low Density Lipoprotein (LDL) levels while stearic acid does not. Polyunsaturated linoleic acid moderately reduces serum cholesterol and LDL levels. Monounsaturated oleic acid appears to be neutral in regard to LDL, but raises High Density Lipoproteins (HDL). The type of fatty acid and the position in which it is esterified to glycerol determine the characteristics of acylglycerols. Dietary fat and oil are in the form of Triacylglycerols (TAG) which are hydrolyzed to fatty acids and Monoacylglycerols (MAG) before they are absorbed. Fatty acids are transported in the blood as lipoproteins. Lipoprotein lipase located on the interior walls of the capillary blood vessels hydrolyses the TAG, leading to the release of fatty acids. Excessive dietary fat

intake increases the risk of obesity, coronary heart disease and certain types of cancer.

The degree of risk vary according to fatty acid intakes, percentage of energy from total fat, dietary cholesterol, lipoprotein levels, intakes of antioxidants and dietary fiber.

1.4 Phytochemicals in edible oils

Dietary fats and oils are essential glycerides of fatty acids (triacylglycerols), which account for 90-98% of their mass. The remaining 2-10% consists of fat soluble phytochemicals of various classes, such as sterols, terpene alcohols, tocopherols, hydrocarbons, long chain alcohols including waxes, carotenoid pigments, sulfur and nitrogen containing flavor compounds etc. Important nonglyceridic components in oil are shown in Table 1.1. These bioactive phytochemicals provides variety of beneficial effects. Each of these classes consists of a number of compounds and their type and quantity vary from one oil to another. The major classes of phytochemicals having disease-preventing functions include dietary fiber, antioxidants, detoxifying agents, immunity-potentiating agents, neuropharmacological agents etc.

The important phytochemicals in various edible oils can be summarized as follows. Sunflower oil contains vitamin E, betaine, phenolic acid, choline, arginine, sterols, lignans etc and α-tocopherol (59 mg/100 g) is the major tocopherol [3]. Palm oil which is extracted from the pulp of the oil palm fruit contains β-carotene and tocotrienols as the major phytochemical. Sesame oil contains unusual constituents of sesamin, sesamolin, sesamol which exhibit chemical and physiological properties [4]. The sesamin content of sesame oil is reported to vary between 0.5% to 1.0%. Sesamol is the hydrolyzed product of sesamolin which is a methylene ether of oxyhydroquinone, a powerful antioxidant. Sesame oil is reported to contain 0.3% to

0.5% of sesamolin and traces of free sesamol. Virgin olive oil contains trace amounts of polyphenols and tocopherols, which have proven to exert beneficial effects on health such as prevention of coronary heart disease, cancer, immunomodulatory diseases etc [5-7]. Hydroxytyrasol is the major polyphenols in olive oil accounting more than 50% of the total polyphenols. Another health beneficial compound called Melatonin, chemically known as *N*-acetyl-5-methoxytryptamine is also found in virgin olive oil [8].

Table1.1 Nonglyceride components of fats and oils [9]

Phytochemical classes	Important constituents
Natural hydrocarbons	Squalene, short- and long chain hydrocarbons, waxes
Sterols	Phytosterols, cholesterol
Alcohols	Aliphatic and terpenoic alcohols
Tocols	Tocols
Phenolic compounds	Saponins, gossypol
Carotenoids and other pigments	β- and other carotenes
Sulfur and nitrogen compounds	Glycosylated alkaloids, isothiocyanates
Complex lipids	Phospholipids,sphingosine, diacylglyceride ethers, glycolipids

Virgin coconut oil which is obtained from fresh, mature kernel of the coconut by mechanical or natural means, with or without the use of heat [10] is rich in various phytochemicals. The major phenolic acids found in virgin coconut oil are caffeic acid, *p*-coumaric acid and ferulic acid [11]. Saturated fatty acids, lauric and myristic are higher in coconut oil and estimated 92% of the total fatty acid [12]. Mustard oil contains 42% of erucic acid which is an omega-9 mono unsaturated fatty acid, alpha-linolenic acid, an omega 3 fatty acid. Gossypol is a terpenoid phytochemical in cotton seed oil which exist in three tautomeric form gossypol- aldehyde, gossypol-lactol, gossypol -cyclic carbonyl which gives dark color to the oil [13]. In addition to this it contains tocopherols, sterols, resins, pigments etc.

1.5 Unsaponifiable Constituents (USC) in Edible Oils

Fats and oils have nonglyceride components most of which are bioactive phytochemicals. These phytochemicals are fat soluble and get extracted with the oil. The nonglyceridic constituents have distinct nutritional functions. The amount of USC varies from oil to oil and ranges from 2-8%. Even though crude oil contains high concentration of USC, the amount varies in each step of refining. Generally oils from cereals such as wheat, rice and maize have very high unsaponifiable fraction. Sterols constitute a major part of the unsaponifiable fraction in most of the oils, followed by terpene alcohols, aliphatic alcohols, squalene and hydrocarbons. The minor components are tocopherols, carotene pigments and flavor compounds.

Major sterols found in oils are campesterol, stigmasterol, β-sitosterol and brassicasterol and minor ones are 4-desmethyl, 4-monomethyl, and 4,4′-dimethyl sterols [14]. The different groups of sterols in certain oils and their relative proportions are shown in Table 1.2.

Tocopherol and tocotrienols are together called Vitamin E, present in oil and varies in a wide range. Palm oil is the major source of tocotrienol. The distribution of vitamin E in various oils are tabulated in Table 1.3 [15]. Carotenoids, which are good antioxidants are found in higher concentration in crude palm oil (700-800 ppm). Different types of carotenes have been found in palm oil which includes nonpolar compounds such as phytoene, phytofluene, ζ-carotene, neurosporene, α-zeacarotene, β-zeacarotene, lycopene, δ-carotene, α -carotene, β -carotene and γ-carotene [16-18]. α & β- carotene are the major one in palm oil and others are precursors in the biosynthesis of α & β- carotene. Other nonpolar hydrocarbons in USC include paraffins, terpenoids, polycyclic aromatic compounds, squalene etc. Olive oil is the major source of squalene.

Table 1.2. Sterols and triterpenes in different edible oils (mg/100 g) [9,14]

Oil	Campe sterol	Stigma sterol	β-sitosterol sterol	Total number of sterols [a]
Rice bran	5	271	885	6
Safflower	45	31	181	8
Corn	410	110	1180	9
Sunflower	31	31	235	8
Cotton seed	17	4	400	6
Sesame	117	62	382	6
Soybean	72	72	191	7
Groundnut	36	21	153	5
Olive	7	3	202	6
Palm	23	14	72	5
Coconut	8	21	77	4
Rapeseed	156	2	284	13

[a] Including minor sterols and cyclic terpene sterols.

Table 1.3 Tocopherol and tocotrienol fractions in vegetable oils (µg/100 g) [9]

Tocols	Palm oil	Rice bran oil	Soybean oil	Safflower oil	Corn oil	Peanut oil
Total tocopherol	642	181	958	801	782	369
α-tocopherol	256	61	101	378	112	130
β-tocopherol	-	Trace	-	-	150	-
γ-tocopherol	316	111	593	174	602	216
δ-tocopherol	70	9	244	240	18	21
Total tocotrienol	492	369	-	-	-	-
α-tocotrienol	143	49	-	-	-	-
β-tocotrienol	32	292	-	-	-	-
γ-tocotrienol	286	28	-	-	-	-
δ-tocotrienol	69	-	-	-	-	-

Terpene alcohols are usually present as free or esters in oil. Ferulic acid is the acid part in some cases forming esters. The components identified so far belong to three groups: acyclic terpenes, cyclic, di- and triterpenes. Table 1.4 shows cyclic terpenes in edible oils.

Table 1.4 Cyclic terpenes in different edible oils (mg/100 g) [9]

Oil	Cycloartanol	Cycloartenol	24-Methylene cycloartenol
Rice bran	106	482	494
Safflower	1	34	7
Corn	4	8	11
Sunflower	-	29	16
Cotton seed	-	10	17
Sesame	4	62	107
Soybean	-	168	8
Groundnut	1	11	16
Olive	1	18	31
Palm	2	60	34
Coconut	2	55	22
Rapeseed	1	54	14

1.6 Refining of fats and oils for edible use

Fats and oils are obtained by following several extraction techniques and need different treatments to make it edible. Refining is the removal of impurities and converting to edible grade. The process of refining depends on nature of impurities and purpose for which it is required. The impurities present in oil can be classified as fat insoluble substances, fat-soluble substances and colloidal suspension impurities. Fat insoluble materials can be removed by mechanical means such as settling, filtration or centrifugation. Colloidal suspensions mainly involve phosphatides,

carbohydrates, nitrogen containing compounds etc which can be removed by settling, centrifugation or filtration with adsorbents or other acids. Fatty acids, coloring substances carotenoids, chlorophylls, oxidation and decomposition products such as ketones, aldehydes etc are fat soluble impurities. Some harmful compounds such as gossypol which is present in cotton seed oil, isothiocyanic acid ester of allyl alcohol in rapeseed and mustard oil are fat soluble impurities. Edible oil should be devoid of constituents causing unpleasant taste, odour and unattractive colors. Various impurities are removed by following different steps such as degumming or desliming, deacidifying, decoloring or bleaching and deodorization.

Degumming of oil, aims at the removal of gums, resins, proteins and phosphatides from oil. Phosphoric acid has been used for degumming due to its capability of precipitating and charring the proteins, gums, coloring pigments etc. Rape seed oil is treated with 2% phosphoric acid at 100^{o}C for the removal of mineral matter, iron, lime and alumina compounds. Degumming by heating is another technique in which the precipitate formed is filtered off by heating the oil at 240-280^{o}C. However thus formed precipitate is difficult to filter and also oil undergoes changes by polymerization and thickening. Hydration techniques is also used for degumming in cases where lecithin precipitate should be isolated. For this, the oil is treated with 2-5% of warm water with vigorous stirring and the hydrated colloids are separated. Degumming with adsorbents such as kieselguhr, charcoal, fuller's earth, activated clays and with special reagents such as anhydride/water, citric and oxalic acids etc are reported [19]. In order to carry out effective deacidification process a degumming by hydration technique is more favorable.

Neutralization or deacidification step is the removal of Free Fatty Acid (FFA) by using alkali, alkali carbonate, ion exchange resins etc. Other important methods are solvent extraction, esterification and distillation. In alkali neutralization, FFA is

converted into soap along with the removal of other impurities and color compounds. But the soap thus formed causes emulsion with neutral fat leading to refining loss. Deacidification by solvent extraction process is based on the difference in solubility of fatty acids and neutral glycerides in various organic solvents [20]. Esterification is another method in which fatty acid is esterified with glycerol leading to formation of TAG at high temperature in presence of vacuum. But the process is too slow and incomplete. Deacidification by distillation is based on the principle of non volatility of neutral oil that leads to the removal of volatile fatty acid and can be distilled off in a current steam.

Distillation deacidification can only be carried out after degumming, otherwise the residual neutral oil may darken at high temperature. Deacidification by distillation is highly effective for high acidity oil.

The undesirable coloring matters are removed by the process of bleaching. Bleaching is carried out by adsorption using materials having high surface activity such as bleaching earths or activated carbons. The colored compounds can be eliminated by chemical conversion to colorless compounds or by converting through oxidation. Hydrogenation in presence of a catalyst has a strong bleaching effect.

Deodorization is the process of removing components which impart undesirable odour and taste to the oil. Short chain carbonyl compounds such as ketones and aldehydes are the cause of off flavor nature of oil which are degraded products of fatty acids. Effective deodorization can only be attained after neutralization and bleaching. The undesirable flavor compounds remain after deacidification and bleaching steps are removed by passing a current of dry steam through the neutralized oil under vacuum. Along with the off flavor compounds micronutrients such as sterols, tocopherols etc also will be distilled off during

deodorization. The fatty acid retained after neutralization is also removed in this step [20].

1.7 Specification of fats and oils for edible use

Codex Alimentarius Commission (CAC) is an international body under Food and Agricultural Organization and World Health Organization to develop food standards and guidelines. The main purpose of codex is to protect health of the consumers and ensuring fair trade practices in the food trade and also in coordination of all food standards work undertaken by international, governmental and non-governmental organizations. According to codex the definition of edible vegetable oil is "Foodstuffs, which are composed of glycerides of fatty acids". They are obtained only from vegetable sources. They may contain small amounts of other lipids such as phosphatides, USC and free fatty acids naturally present in the fat or oil. It prescribes the maximum level of antioxidants, contaminants such as heavy metal, pesticide residue etc. The methods based on International Union of Pure and Applied Chemistry (IUPAC) and Association of Official Analytical Chemists (AOAC) is cited for the physico chemical analysis of oil. The parameters relating to composition, quality, physical and chemical characteristics of the oils are also mentioned [21].

As far as India is concerned Bureau of Indian Standards (BIS) is the National standards body for the harmonious development of the activities of standardization, marking and quality certification of goods and for matters connected therewith. Indian Standard means the standard established and published by the Bureau, in relation to any article or process indicative of the quality and specification under any standard published and recognized by the bureau. Food and Agriculture Division Council (FADC) working under BIS has scope of standardization in the field of food, feed and agriculture. Bureau of Indian Standard Specification for oils and oil seeds covers the

methods of sampling and methods of test for oils and fat, specifications of oil and fats relating to the physico chemical characteristics. Prevention of Food Adulteration (PFA) deals with the quality and purity of food. PFA follows the specifications put

forward by BIS. The Food Safety and Standard Authorities of India (FSSAI) implemented an act at 2006 which deals with various acts and orders related to food in various ministries and departments. The main aim of it is to establish a single reference point for all matters relating to food safety and standards. The specifications for some of the edible oil are tabulated in Table 1.5. Table 1.6 shows the fatty acid composition [22] of edible oils.

The bioactive phytochemicals in oil which are nonglycerides get concentrated in the Unsaponifiable Matter (USM) of oil. Edible oils (refined and virgin oil) should meet all the specifications laid down by regulatory bodies following there of respective countries. USM content is one of them with permitted maximum limit. USM in Rice Bran Oil (RBO) varies widely depending on various factors. Values for USM obtained by analysis following the suggested method do not reflect the actual unsaponifiable components as far as RBO is concerned. The limit of USM in RBO is comparatively higher with that of other oils. According to food laws, RBO shall be obtained from the endosperm of rice called rice bran which is removed during the process of rice milling [23].

Table1. 5 Bureau of Indian Standard specifications of some of the refined edible vegetable oils [21]

Serial No.	Characteristics	Coconut Oil	Cotton Seed Oil	Ground nut Oil	Mustard Oil	Sunflower Oil	Palm Oil	Soybean oil	Rice Bran Oil	Sesame Oil
1	Moisture & insoluble impurities, % by mass, Max	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
2	Color on Lovibond ¼-in cell (Y+ 5 R)	2[a]	14	10[a]	15	5[a]	50 [b]	7.5	20	2
3	Refractive index at 40°C	1.448-1.449	1.463-1.466	1.462-1.464	1.464-1.466	1.464-1.480	1.449-1.455	1.465-1.470	1.460-1.470	1.464 - 1.466
4	Specific gravity at 30°/30°C	0.915-0.920	0.910- 0.920	0.909-0.913	-	-	-	-	0.910- 0.920	0.915 -0.919
5	Saponification Value	250	190-198	188-195	169-177	188-194	195-205	189-195	180-195	188-193
6	Iodine value (Wij's)	7.5-10	98-115	87-98	98-112	100-140	45-56	125-140	90-105	103-115
7	Acid value	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.75
8	Unsaponifiable Matter, % by mass, max	0.5	1.5	0.8	1.2	1.5	1.2	1.0	3.5	1.5
9	Flash Pensky-Martens(closed) , °C, Min	225	250	250	250	250	-	250	250	125

[a] color measured using 1-in cell [b] color measured using ½-in cell

Table 1.6 Fatty acid composition of edible oils (%) [22]

Oil	C6	C8	C10	C12	C14	C16	C18	C18:1	C18:2	C18:3	C20
Coconut	0-0.8	5-9	6-10	44-52	13-19	8-11	1-3	5-8	0-1	-	0-0.5
Cotton Seed	-	-	-	-	0.4	20	2	35	42	-	-
Ground nut	-	-	-	-	-	6-9	3-6	52	13-27	-	2-4
Mustard	-	-	-	-	-	1.5	0.4	22	6.8	14.2	-
Sunflower	-	-	-	-	-	3-6	1-3	14-35	44-75	-	0.6-4
Palm	-	-	-	-	0.5-2	32-45	2-7	38-52	5-11	-	-
Soybean	-	-	-	-	0.5	7-11	2-6	22-34	43-56	5-11	-
Rice Bran	-	-	-	-	0.4-1	12-18	1-3	40-50	29-42	0.5-1	-
Sesame	-	-	-	-	-	7-9	4-5	40-50	35-45	-	0.4-1

1.8 Rice Bran

Rice grain has seven distinctive parts hull, pericarp, seed coat, nucellus, aleurone layer, endosperm and embryo. Hull is the hard protective layer of rice grain, which is indigestible and comprises awn, lemma, palea, sterile lemmae and rachilla [Figure 1.1]. Hull contributes 18-20% by weight of the rice grain. Rice bran forms the immediate inner layer of the hull which is comprised of pericarp, seed coat, nucellus, aleurone layer and embryo. Hull and bran enclose starchy endosperm. Raw rice constitutes 5-8% bran. Bran is a by-product of rice milling industry. Endosperm consists of lipids which are associated with protein bodies and starch granules. The lipid involves non starch and starch lipids and majority are non starch lipids. Lipid composition of rice and its fractions are shown in Table 1.7 [24].

Bran is an important component of rice which is rich in vitamins, minerals, proteins, lipids, dietary fiber, wax, polysaccharides, trace elements etc [25-27].

Ling et al reported the feeding of rabbits with diet containing black rice and reported that the outer layer lowers the atherosclerotic plaque formation and also the malondialdehyde level of serum and aortic artery which is an important secondary product for the production of oxidized LDL [28]. Regarding the protein content of bran, albumin and globulin are the major proteins and account for 14% of bran. Rice bran proteins are reported to have hypocholesterolemic activity and also potency to fight against cancer and tumor formation [29-31]. Bran is a rich source of Vitamin E which are potent antioxidants that prevent cholesterol oxidation. There are reports showing hypocholesterolemic activity of rice bran [32]. In addition to this they have biological effects such as anti-tumor and anti-atherogenic activity.

Qureshi et al reported rice bran fiber concentrate from stabilized rice bran reduces the fasting serum glucose level in human having diabetes mellitus [33]. Phenolic acids are yet another constituent present in bran and contribute about 33-

45% of the total phenolic content in all fractions. Ferulic acid is the major phenolic acid [34]. The amount of phytonutrients in bran depends on variety, treatment of the grain before milling, type of milling system, degree of milling, fractionation during milling etc [35].

The non starch lipids in the aleurone, subaleurone and germ layers contain 86-91% neutral lipids, 2-5% glycolipids, and 7-9% phospholipids [24]. In brown rice, 14-18% of non starch lipid is in germ, 39-41% in bran, 15-21% in polish and 25-33% in milled rice [24]. The shelf life of bran is influenced by the enzymes present in the germ and outer layer of the caryopsis. Lipase, lipoxygenase and peroxidase are the potent enzymes and their activities affect quality of rice bran. Lipase is the most active enzyme in bran which converts oil in the bran into glycerol and FFA. The degree of hydrolysis depends on storage conditions. Crude Rice Bran Oil (CRBO) generally contain 5-20% FFA. The FFA content is directly responsible for refining loss. In order to reduce the formation of FFA by lipase and lipoxygenase, the bran should be stabilized by inactivating enzymes that helps to prevent hydrolytic and oxidative rancidity and there by increasing the extraction efficiency and reducing refining loss [20].

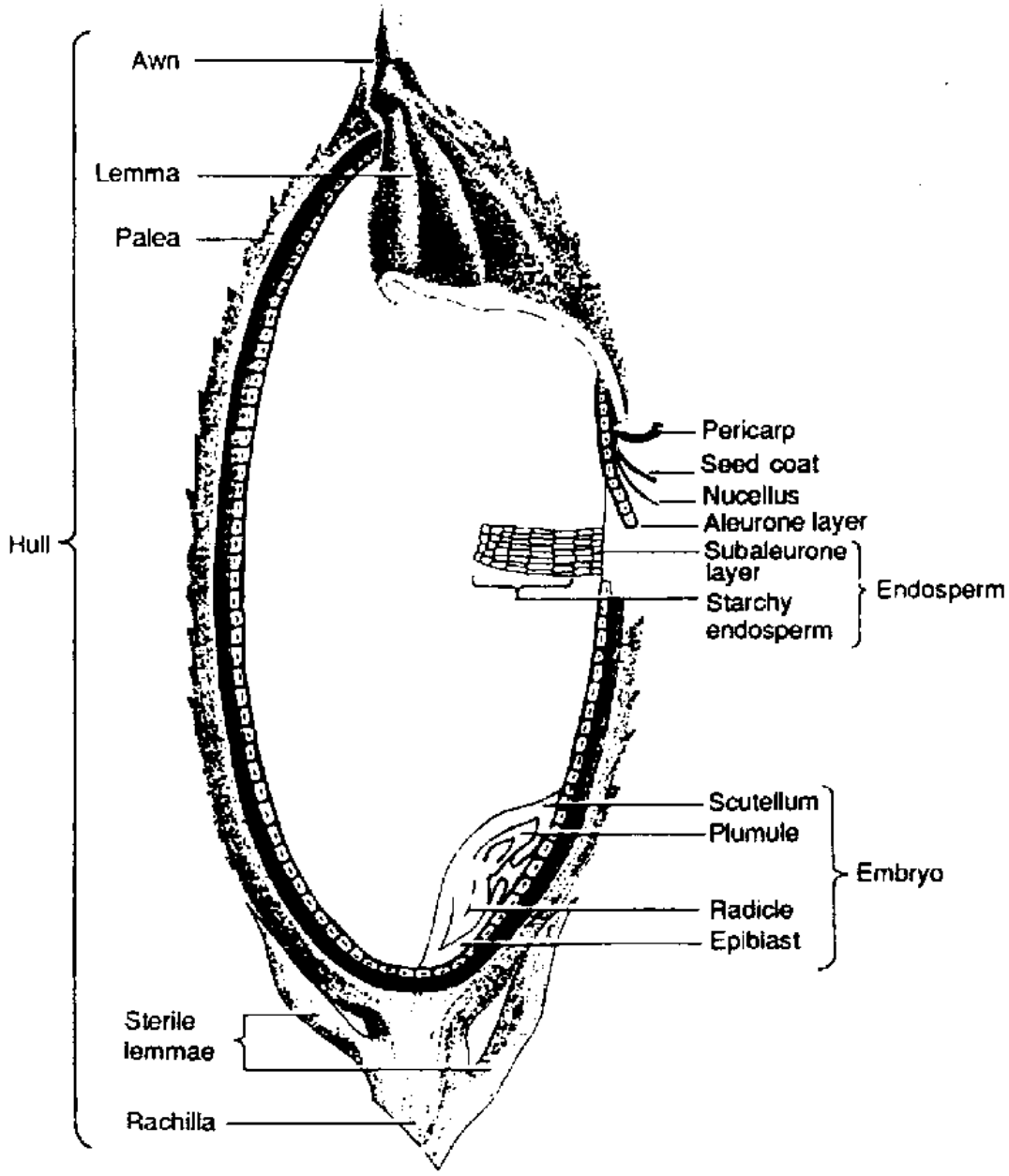


Figure 1.1 Figure showing different parts of Rice grain

Table 1.7. Lipid composition of rice and its fractions[a] [24]

Property	Non starch Lipids in Rice Fractions						Non waxy Starch Lipid in	
	Hull	Brown	Milled	Bran	Germ	Polished	Brown Rice	Milled Rice
Lipid content	0.4	2.7	0.8	18.3	30.2	10.8	0.6	.05
Saponification no.	145	181	190	184	189			
Iodine no.	69	94	100	99	101			
Unsaponifiable matter	26	6	6	6	34			
Fatty acid composition Wt% of total								
Palmitic	18	23	33	23	24	23	46	45
Oleic	42	35	21	37	36	35	12	11
Linoleic	28	38	40	36	37	38	38	40
Others	12	4	6	4	3	4	4	4
Neutral lipids, % of total lipids	64	86	82	89	91	87	28	26
Triglyceride	-	71	58	76	79	72	4	
FFAs (FFA)	2							
	- 21	7	15	4	4	5	20	
Glycolipids, % of total lipids	25	5	8	4	2	5	19	16
Phospholipids, % total lipids	11	9	10	7	7	8	53	58
Phosphatidyl choline	-	4	9	3	3	3	4	4
Phopsphatidyl ethanolamine	-	4	4	3	3	3	5	5
Lysophosphatidyl choline	-	<1	2	<1	<1	<1	21	23
Lysophosphatidyl ethanolamine	-	-	1	-	-	-	22	25

[a] Based on 6% bran-germ, 4% polish and 90% milled rice from brown rice

1.9 Rice Bran Oil

Rice bran contains 18-22 % of oil, with several bioactive phytochemicals of nutritional significance and has balanced fatty acid composition. It is generally called 'Heart Friendly Health Oil' or Heart oil. Extraction of RBO from rice bran can be achieved by methods like solvent extraction, hydraulic pressing, supercritical CO_2 extraction etc.

In hydraulic pressing oil is squeezed out of the bran by rupturing the oil cell walls when subjected to high pressure. The yield of extraction by this method is 45- 50% when compared to other methods [36] and hence commercially not viable. Super critical carbondioxide extraction is a modern extraction technique. In supercritical fluid extraction the factors, lipid solubility and diffusivity can extended up to the domain of pressure and temperature above its critical point of supercritical fluid than in solvent extraction [37]. The merits of supercritical carbondioxide extraction are ecofriendly, prevention of chemical deformation to active biomolecules, etc. RBO obtained by supercritical carbondioxide extraction has a light color, less phosphorous, wax and FFA content and high retention of essential fatty acids and micronutrients [38]. This technique is commercially not feasible at present because of high capital investment. Solvent extraction is the commercially viable and currently employed extraction technique used by industry. This includes batch, battery or continuous type of extraction. The prior step to solvent extraction is stabilization and pelletization of bran. Proctor et al [39] reported the ambient-temperature extraction of RBO with hexane and isopropanol and it was found that both the solvents are very effective. They reported oil extracted by isopropanol was significantly more stable to heat induced oxidation. However hexane is the most commonly used solvent.

The total lipid composition reported for RBO is; 81–84% Triacylglycerols (TAG), 2–3% diacylglycerols (DAG), 1–2% monoacylglycerols (MAG), 2–6% FFA, 3–4% wax, 0.8% glycolipids and 1–2% phospholipids (PL) [24]. The major saturated fatty acid in RBO is palmitic (20-22%) and unsaturated fatty acids are oleic (46-52%) and linoleic (27-31%) [40]. Among them linoleic acid is an essential n-6 fatty acid. The fatty acid composition of RBO is shown in Table 1.6. Sierra et al reported that saturated as well as unsaturated fatty acids in RBO are responsible for the immunoregulatory effects than phenolic compounds [41]. Glushenkova et al showed that 90.30% of *sn*-2 position of TAG was occupied by unsaturated fatty acids. The percentage by weight of individual type of TAG in terms of saturation and unsaturation is quantified that 39.3% and 1.2% of TAG has unsaturation and saturation in *sn*-1, 2 ,3 positions respectively. 34.7% TAG had saturation in the *sn*-1 position and unsaturation in *sn*-2 and *sn*-3 positions [42]. The amount of DAG in RBO is reported to be less compared to other vegetable oil and that the two positions of glycerol are occupied by myristic, palmitic, stearic, oleic, linoleic, linolenic and arachidic acid. In the case of phospholipids, there are hydratable and non-hydratable phospholipids. Hydratable phospholipids are phosphatidylcholine, phosphatidyl-inositol etc and non-hydratable phospholipids are calcium and magnesium salts of phosphatidic acid and phosphatidylethanolamine [43].

1.10 Unsaponifiable phytochemicals in RBO

Edible oils are source of phytochemicals of nutritional values and RBO is a rich source of these phytochemicals. Most important among them is γ-oryzanol with variety of biological properties. Chemical and biological properties of USC of RBO is briefly presented in the ensuing section.

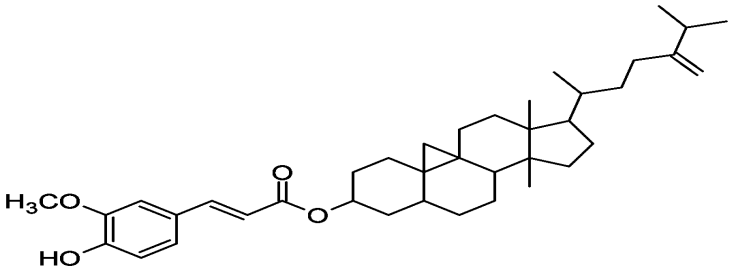
1.10.1 Chemistry behind USC

1.10.2 Oryzanol

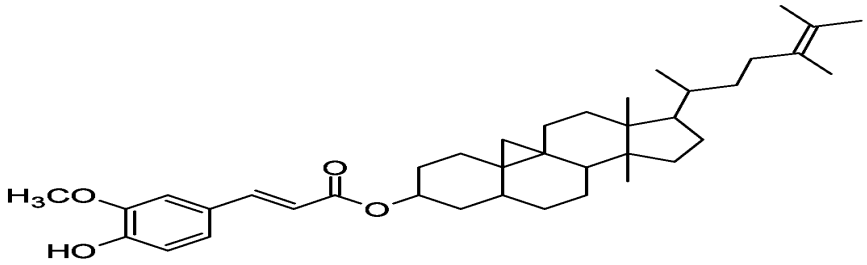
The terminology of γ-oryzanol is derived from the scientific name of rice *Oryza sativa*. The molecular structure shows oryzanol is an ester of ferulic acid with either sterols or triterpene alcohols. Sterols or triterpene alcohols forms the bulkier part of oryzanol molecule. Ferulic acid is a phenolic acid chemically called 3-hydroxy-4-methoxy cinnamic acid. It was reported first as a single compound by Kaneko et al in 1954 [44] later it was proved as mixture of compounds. Oryzanol includes cycloartenyl ferulate, stigmasteryl ferulate, campesteryl ferulate, 24-methylene cycloartanyl ferulate etc. The structures of some of the oryzanol compounds are shown in Figure 1.2.

Oryzanol has several physiological and biological effects. Previous reports revealed that they have strong antioxidant property due to the presence of ferulic acid. The antioxidant activity of oryzanol against cholesterol oxidation were analyzed by Xu et al [32] and found that all the oryzanol components had very good antioxidant activity while comparing with tocopherols and tocotrienols (tocols). Among them 24-methylene cycloartenyl ferulate having the highest activity. RBO is known to lower cholesterol, as oryzanol prevent the dietary cholesterol absorption and there by enhances fecal sterol excretion [45-46]. Oryzanol has been shown to have significant effect on the reduction of serum Thyroid Stimulating Hormone level in hypothyroid patients [47]. It increases endorphin release and aid muscle development and so used as sport supplement [48]. Yasukawa et al reported sitosteryl ferulate, cycloartenol ferulate and 24-methylene cycloartenol ferulate markedly inhibits Tetra decanoylphorbol acetate induced inflammation [49]. It has a protective role in UV-light induced lipid

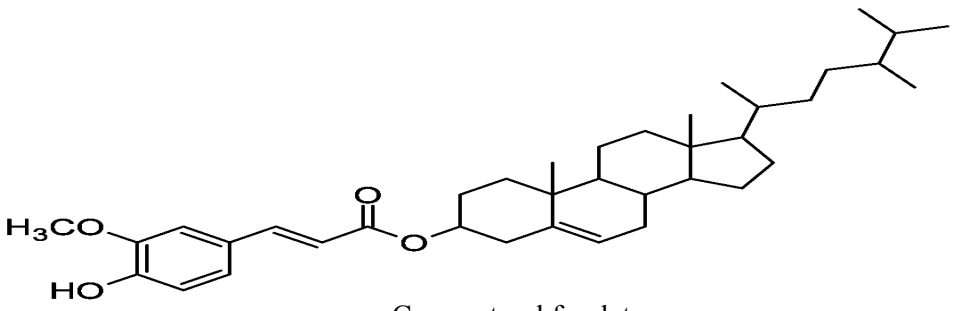
peroxidation and hence it is used as a sunscreen agent. Ferulic acid esters are reported to stimulate hair growth and prevent skin aging [50]. It is highly effective in treating autonomic imbalance and menopausal disorders [51]. Degradation of oryzanol at high temperature was reported due to oxidation in presence of other degraded oxidation products [52]. Oryzanol is highly sensitive to alkali and hence most of the oryzanol is lost through soap stock during alkali refining [53].



24-methylene cycloartanyl ferulate



Cycloartenyl ferulate



Campesteryl ferulate

Figure1. 2 Structure of oryzanol

1.10.3 Tocopherol/Tocotrienols

Vitamin E is the generic term used for tocols. It was discovered in 1922 in green leafy vegetables by Herbert Evans and Katherine Bishop [54]. In 1924 Sure named it as Vitamin E, related to fertility. The name tocopherol was derived from Greek word *tokos* means childbirth, *phero* means to bring forth. Tocopherols consist of a chromanol ring and a 15 carbon tail derived from homogentisate and phytyl diphosphate. Tocopherols and tocotrienols having same structure except tocotrienols have three trans double bonds in the isoprenoid units. The substituents at 5, 6, 7 and 8 positions are different forming α,β,γ and δ-derivatives. The free radical reacts with hydrogen of hydroxyl group to form a stable form of vitamin E. The mechanism is illustrated in Figure 1.3.

The important sources of vitamin E are wheat germ oil, almond, sunflower oil, peanut, corn oil, soybean oil, canola oil etc. It is a fat soluble vitamin and very good radical scavenger. It react with reactive oxygen species. It prevents or delay coronary heart diseases by preventing the blood clots in heart that could lead to heart attack [55].

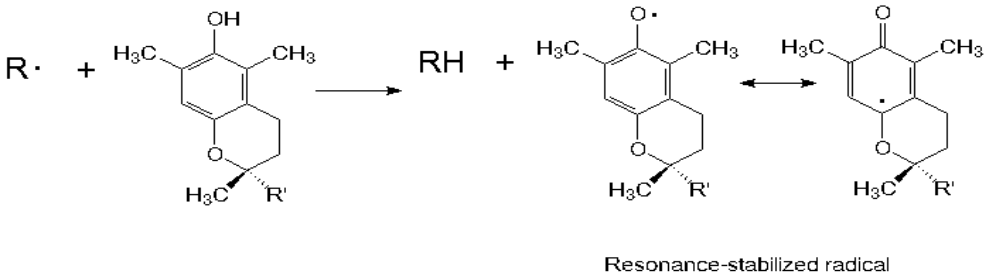


Figure 1.3 Mechanism of formation of stable free radical of vitamin E

They can interrupt free radical in reactions by capturing it and hence antioxidant. α and γ tocopherols have shown numerous biophysical and biochemical mechanisms against reactive oxygen species and radicals. The hydroxyl group of

the ring donates hydrogen to peroxyl radical (LOO·) of an unsaturated lipid to form hydroperoxide and a tocopheroxyl radical (TO·). Thus formed tocopheroxyl radical does not propagate the reaction while comparing with other radicals.

$$LOO\cdot + TOH \rightarrow LOOH + TO\cdot$$

Tocopheroxyl radical combine with other radical in the medium to form stable products [56].

$$TOH + O_2 \rightarrow \text{unknown products}$$

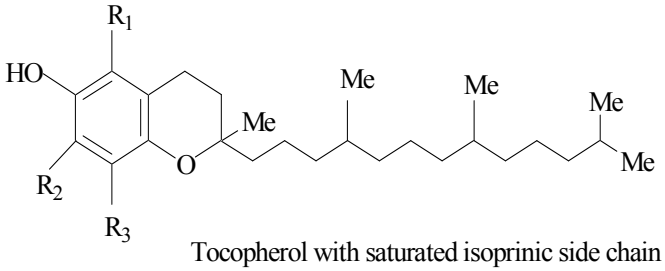
$$TO\cdot + LH \rightarrow TOH + L\cdot$$

$$TO\cdot + LOOH \rightarrow TOH + LOO\cdot$$

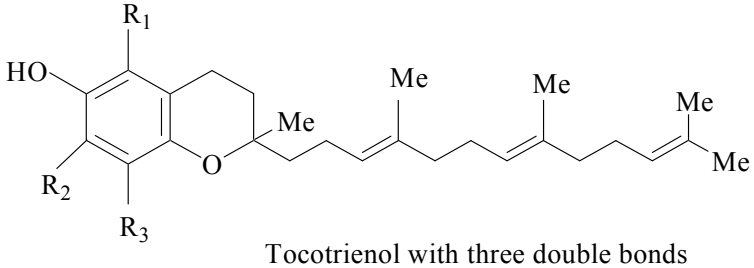
Burton et al reported α-tocopherol has more capacity to donate hydrogen than γ- tocopherols in various lipid systems [57].There are supporting and opposing results regarding the antioxidant effect of α-tocopherol. Some of the reports showed the prooxidant effect of α-tocopherol at varying concentrations and solvent conditions [58]. The studies on prooxidant nature of α-tocopherols explains that they are not acting as prooxidants but its efficacy get lost in presence of other prooxidants such as transition metal ions, oxidizing agents and by participating in other side reactions [59]. Vitamin E is known to block the formation of nitrosamines from nitrites of food in stomach and thereby modulating predisposal to cancer. Antioxidant effect of tocopherols at microwave heating conditions were analyzed in edible vegetable oils and its effect in the decreasing order is found to be $\alpha>\beta=\gamma>\delta$ [60].

Tocotrienols are formed in the endosperm of seed monocots of cereal grains such as wheat, rice and barley. Since tocotrienols have double bonds in the isoprenoid units they are reported to have more biological activity than tocopherols. It has various functions such as anti inflammatory, inhibition of platelet aggregation, increased immune function etc. It possesses greater neuroprotective, anticancer and cholesterol lowering properties compared to

tocopherols. Delta tocotrienol is the most potent cholesterol oxidation inhibitor among tocotrienols followed by γ-tocotrienol and α-tocotrienol. Temperature has greater influence on the activity of tocols and reports suggest α-tocopherol reduces rate of autoxidation of lard TAG when temperature increases [61]. The antioxidant activity of vitamin E keeps the oils stable by preventing autoxidation by undergoing oxidation itself to quinones and dimers [62]. Rossi et al reported the stability of tocols depends on polyunsaturated fatty acid composition of oil and the type of tocols in it [63]. The chemical structures of tocols are shown in Figure 1.4.



Tocopherol with saturated isoprinic side chain



Tocotrienol with three double bonds

Tocopherol/ Tocotrienol	R_1	R_2	R_3
α	CH_3	CH_3	CH_3
β	CH_3	H	CH_3
γ	H	CH_3	CH_3
δ	H	H	CH_3

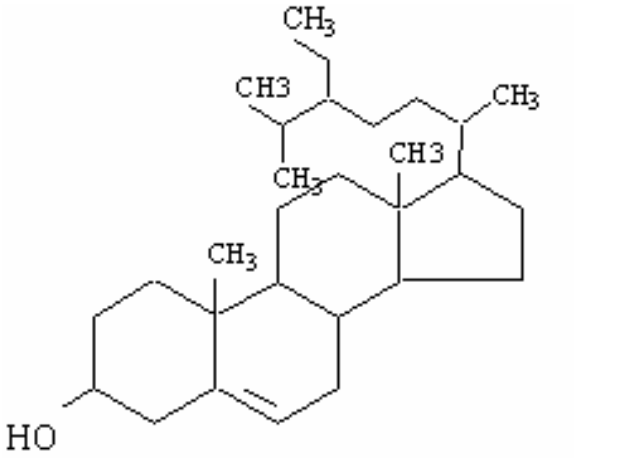
Figure 1.4 Structures of tocopherols and tocotrienols

1.10.4 Sterols

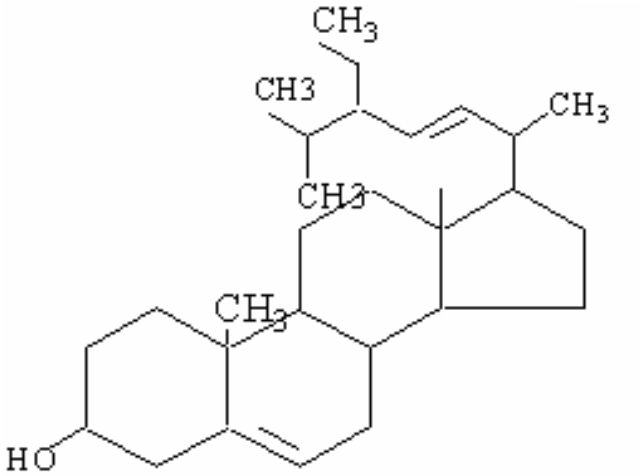
Sterols are a group of organic molecules that occur in plants, animals and fungi. More than 250 sterols and related compounds are reported [64]. Sterols in

animals are called zoo sterols and that in plants are called phytosterols. The most familiar animal sterol is cholesterol which has vital role in cellular function and acts as precursor of fat soluble vitamins and steroid hormones. A major sterol which is present in the cell membrane of fungus is ergosterol. Apart from cell functions they are also precursors of plant growth factors. Sterol molecules consist of three fused ring with a cyclopentane ring and the first ring having hydroxyl group at third position and a side chain at C-17 carbon. Sterols are mostly nonpolar and soluble in most of the non polar organic solvents. Sterols are derived from squalene. The common nucleus present in sterol is hydrogenated 1,2-cyclo pentanophenanthrene system. The chemical structures of few important sterols are shown in Figure 1.5.

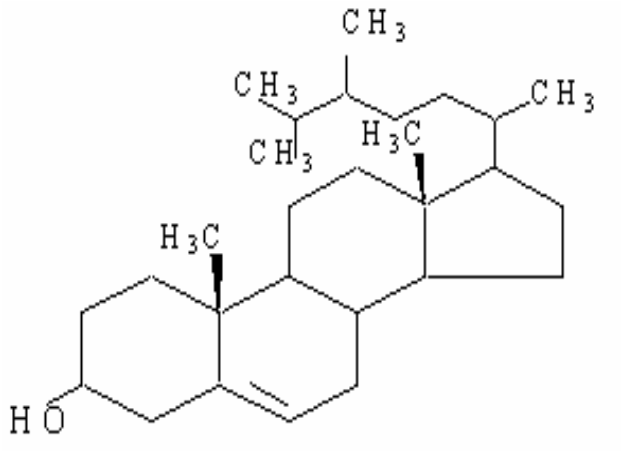
Edible oils such as soybean oil, corn oil, sunflower oil etc contain sterols in significant amount. In vegetable oil it mainly occurs in free form, esters or steryl glycosides. A 5α-saturated derivative of sitosterol called sitostanol is reported to reduce intestinal absorption of cholesterol and serum cholesterol more effectively than sitosterol [65]. Structurally sterols can be classified into 4-desmethyl sterols, 4-α methyl sterols and 4,4- dimethyl sterols. The occurrence of 4-α methyl sterols and 4, 4- dimethyl sterols are at lower level in plants than 4-desmethyl sterols [66]. The 4-desmethyl sterols categorized into Δ^5-sterols, Δ^7-sterols and $\Delta^{5,7}$- sterols according to the position and number of double bonds in the second ring. Sterols are reported to have cholesterol lowering activity and hence functional foods with greater sterol content is more nutritive. Sterols are the major USC in RBO present as free as well as esters. Sterols have biological effects such as decrease in absorption of cholesterol by lowering total plasma cholesterol and LDL acts as anti-inflammatory, anti-oxidative, anticarcinogenic, antibacterial, antifungal etc [67].



β-sitosterol



Stigmasterol



Campesterol

Figure 1.5 Structure of sterols

1.10.5 Steryl esters

Steryl esters are complex groups of phytochemicals in which sterols esterified to fatty acid at different combinations. Vegetable oils are the major source of steryl esters and other sources are cereals and cereal by-products. Soybean oil deodorizer distillate is an important source for the production of steryl esters [68]. The fatty acid steryl esters isolated from soybean oil deodorizer distillate show the following fatty acids 31.74% palmitic acid (C16:0), 9.99% stearic acid (C18:0), 0.46% elaidic acid (C18:1 *trans*-9), 27.51% oleic acid (C18:1 *cis*-9), 0.19% linoleaidic acid (C18:2 *cis*-9, *trans*-12), and 29.19% linoleic acid (C18:2 *cis*-9, *cis*-12) [69]. In the case of steryl esters in oils, the unsaturated fatty acid esterified with sterol is higher when compared with fatty acid composition of TAG of a particular oil [70]. Steryl esters have wide applications in cosmetic, pharmaceuticals and neutraceuticals. It has water holding properties and used as ingredients of cosmetics and bath additives. It lowers the plasma cholesterol concentration by inhibiting the absorption of cholesterol from small intestine [71]. Steryl esters gained much attention recently as neutraceuticals because of its effective role in lowering of blood cholesterol level, as steryl esters are oil soluble [69]. Structure of palmitate sitosteryl ester and stearate sitosteryl ester is shown in Figure 1.6.

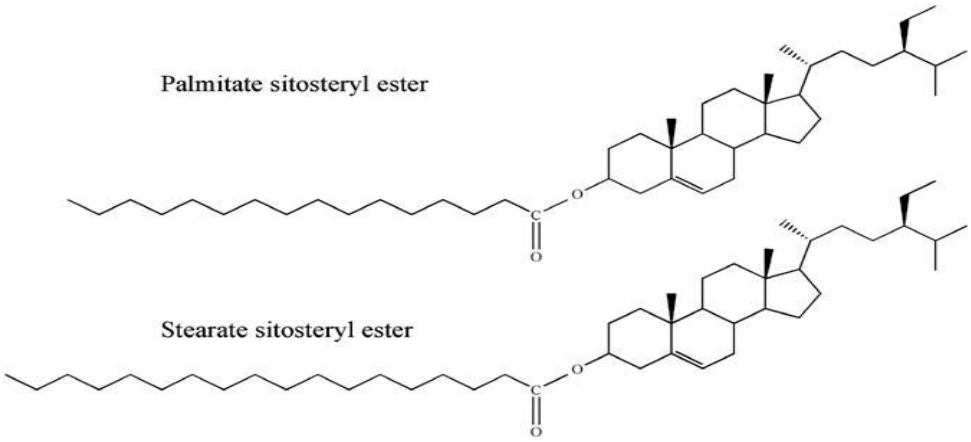


Figure 1.6 Structure of steryl esters

1.10.6 Squalene

Major hydrocarbon present in RBO is squalene. It is a triterpenic hydrocarbon with six non-conjugated double bonds belonging to terpenoid family. Squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexane) is the biochemical precursor of all steroids. Squalene is converted to 2, 3-squalene oxide by the oxidation of the terminal double bond and further undergoes cyclization in presence of enzyme to form other products. It is an intermediate compound in the biosynthesis of sterols in plants and animals. It is usually found in cartilaginous fish such as shark, whale etc and in amaranthus grain, olive oil, wheat germ oil etc. Olive oil is a rich source of squalene ranging from 200 to 7000 mg/Kg [72]. It contains two methylene interrupted double bonds which forms hydroperoxides and further decomposition with carbon-carbon scission in oxidation reactions [73]. It is a major dietary chemo preventive source against some type of cancer cells and also acts as hypocholesterolemic agent [74-76]. Squalene has protective effects on human mammary epithelial cells in lowering the intracellular reactive oxygen species level, prevention of H_2O_2 induced oxidative injury and also protection against oxidative DNA damage [77]. It is an important ingredient in

skin cosmetics as it reduces free radical oxidative damage occurring in skin due to its photo protective role. It is used as a lubricant in computer disk due to its thermo stability [78-79]. Structure of squalene is shown in Figure 1.7.

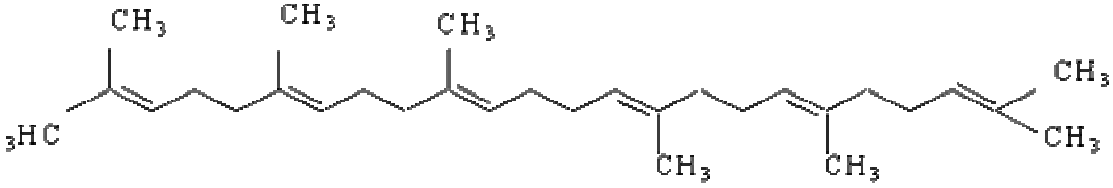


Figure 1.7 Structure of squalene

1.10.7 Wax

Wax is a group of compounds formed by the esterification of long chain fatty alcohols with long chain fatty acids resulting in a carbon chain of C_{34} to C_{46}. The fatty acids of rice bran wax contain carbon chain ranging from C_{16} to C_{32} and the fatty alcohols ranges from C_{24} to C_{38}. The hydrocarbon chains of fatty acids and fatty alcohols provide strong hydrophobic character and the ester functionality in the middle provides hydrophilic character. On cooling, wax crystallizes due to its low solubility in oil causing turbidity at low temperatures and often they are removed during dewaxing and winterization step. The clarity and the quality of the refined oil depend on the amount of wax in it. Rice bran wax is a rich source of high molecular weight aliphatic alcohols known as policosanol. The composition of soft and hard waxes of RBO obtained by the tank settling of CRBO show melting point of 79.5 ^{o}C and 74^{o}C for hard and soft waxes respectively. The important fatty alcohols and fatty acids of hard waxes are C_{24},

C_{26} and C_{30} ; C_{22}, C_{24} and C_{26} respectively [80]. Kodali suggested the oxidative stability of polyunsaturated ω-3 oils enhanced by the treatment of rice bran wax. When polyunsaturated oil is mixed with wax esters at low concentrations it results in encapsulated solids with large volumes of liquid oil called organogels. The results showed menhaden oil added with 1.5% wax has oxidative stability similar to 500 ppm of tocopherols and 200 ppm of tert-butylhydroquinone [81]. Rice bran wax has potential applications in cosmetics such as cold cream, hair-conditioners and in pharmaceutical, food, polymer, and leather industries [82]. Structure of lignoceric acid (24:0) esterified to triacontanol (30:0) is shown in Figure 1.8.

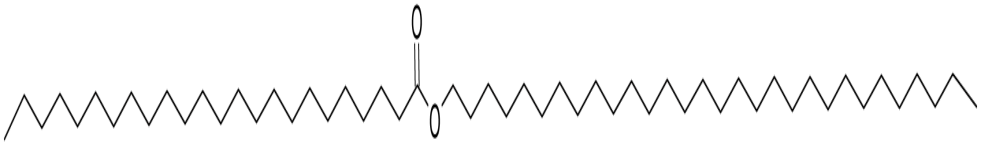


Figure 1.8 Structure of wax ester of lignoceric acid and triacontanol

Turbidity in RBO is due to presence of wax. According to BIS, solvent-extracted RBO should be refined for edible purpose. The refined oil should be clear and free from rancidity, adulterants, sediment, suspended and other foreign matter, added colorings and flavoring substances. The clarity of the sample is judged, after heating the sample to 50^{o}C, filtered and maintaining the filtrate at 35 ^{o}C for 24 hrs which should be free of turbidity [23].

1.11 Refining of RBO

Refining is the process for removal of undesirable impurities from crude thereby meeting with specifications for edible Refined Rice Bran Oil (RRBO).

CRBO contains high level of wax, gum, phosphatides, FFA, coloring materials etc that give haziness in appearance and poor color. RBO extracted by hexane usually contains 2–3% wax, 1–2% phosphatides, 5–25% FFA and coloring pigments [83-84].The important objective of refining is to remove such impurities without causing changes in the composition of TAG and micronutrients. Refining of CRBO is tedious process as compared to other edible oil due to high FFA, wax, gums and coloring pigments. Moreover, in the refining process the refining loss is very high for RBO particularly when chemical refining is employed. The important prerequisites of refining are it should be cost-effective by controlling loss and improving quality through maximum retention of micronutrients. Generally refining of RBO can be carried out by two methods: a) chemical refining and b) physical refining. Main difference between physical and chemical refining is in the deacidification step. In chemical refining alkali is used and in physical refining steam distillation is carried out to remove the FFA.

1.11.1 Chemical Refining/Alkali refining

In this process FFA are deacidified by alkali treatment and thus the process of refining is called chemical refining or alkali refining. Deacidification is the

neutralization step carried out by the reaction of NaOH with fatty acid which forms soap. Excess alkali is used after calculating the amount of FFA in crude. In alkali refining, the total refining loss is very high to the extent of three times of initial FFA value [24, 85-87]. Ichimatsu and Hidaka et al carried out refining with an indicator to monitor P^H during neutralization process to reduce the refining loss [86-87]. Moreover alkali refining causes loss of micronutrients especially

oryzanol. Hoed et al reported the changes occurring on major and minor components of RBO and observed the significant loss of oryzanol. Changes in phytosterol composition, isomerization of 24-methylene cycloartenol, stripping of phytosterols and tocotrienols are also reported to be occurring during deodorization [88]. Krishna et al reported oryzanol get reduced mainly during neutralization step [53]. Formation of steradienes is a consequent effect by this method which was reported by Ferrari et al [89].

Miscella refining is modified process of alkali refining. Bhattacharya et al reported miscella refining involving hexane and alkali which is very efficient [90]. The method is advantageous due to the production of lighter colored oil without bleaching and less refining loss [91]. Another benefit of this method is without desolventization, oil can be degummed, dewaxed and get refined [92]. But cost of equipment is higher than that for normal refining process and the process control was also difficult and hence commercially not practiced.

Bhattacharya et al reported mixed solvent refining for high fatty acid and dark colored oil by making use of hexane as the main solvent and ethanol or isopropanol as the second solvent [93].

Thus alkali refining has following drawbacks: high refining loss, elimination of antioxidant phytochemicals, produces environmentally polluting acid water during splitting of soap etc. In order to overcome the drawbacks a new effective refining method that retains micronutrient has been developed namely physical refining that avoid treatment with alkali.

1.11.2 Physical refining/steam refining

Steam distilling of fatty acid is the principal of physical refining [94]. Important advantage of this method is retention of oryzanol, the most important bioactive and antioxidant compound in RBO. The reports on physical refining indicates, retention to an extend of 1-1.7% of oryzanol as against 0.1-0.2% by chemical refining [53]. As compared with chemical refining, the refining loss is reported to be 1.1 to 1.2% of FFA in crude as against three times in the case of chemical refining. It does not produce soap stock and thereby effluent treatment problem could also be avoided.

However for successful physical refining pretreatment of CRBO to bring down phosphorous content to less than 5 ppm and wax content to very low level are the challenges. Various steps of physical refining of CRBO are presented below.

1.11.2.1 Degumming

This process step is performed to remove phospholipids that imparts final color to the oil. Over the years, work has been carried out for the effective elimination of phosphorous compounds through various degumming process. Water degumming is the simplest method but it removes only hydratable phospholipids leaving behind non hydratables (80-200 ppm) [95-96]. Studies have been established in relation to variations in water concentration, temperature, rate of agitation and time of extraction in degumming. Indira et al reported water degumming through a response surface approach [97]. The report explains that increase in temperature has a negative effect on the yield of gums because high temperature reduces the yield of gums due to the presence of wax which will be

retained in oil at high temperatures. Water concentration levels leads to the increase in the yield of gums up to about 4% and agitation with speed of 215 rpm is found to be more effective [97]. Additives such as citric, phosphoric, acetic, oxalic, nitric, boric and tannic acids have also been studied and found to facilitate degumming.

Bhattacharya and Bhattacharya conducted studies using various degumming agents such as organic acid, inorganic acid, and inorganic salt to degum CRBO, and found that degumming agents reduced the phosphorus content of CRBO to 52–72 ppm. The degumming and dewaxing carried out with an inorganic acid required further neutralization and water washing which resulted in a loss of neutral oil and the formation of soap. The degumming agent $CaCl_2$ reduced the phosphorus content of CRBO to 56 ppm after degumming [98].

Enzymatic degumming is another degumming process by which both hydratable and non hydratable phospholipids are converted into water-soluble lysophospholipids. Roy et al reported reduction of phosphorous content to 15-18 ppm by enzymatic degumming using phospholipase A_1 [95]. Membrane degumming is reported to remove phospholipids through the formation of inverse micelle which has large dimensions formed by hydrophobic group turned outside and the hydrophilic group turned inside. Since these micelles have large dimensions their osmotic pressure is rather low and can easily be removed by membrane filtration at moderate pressure through suitable semi-permeable membranes [99]. Degumming, dewaxing and decolorizing steps were done simultaneously as reported by Manjula et al using non porous membranes and the results showed 99% removal of phosphatides [100]. Simultaneous degumming and dewaxing using water and aqueous $CaCl_2$ were reported by Rajam et al and explained the importance of divalent calcium ion in thermotropic phase transfer of lecithin forming liposome with hydratable phospholipids [84].

1.11.2.2 Dewaxing

Waxes are high melting esters of fatty alcohols and fatty acids with low solubility in oil. Vegetable oils including sunflower, corn and linseed oils contain higher amount of wax which makes the oils cloudy at low temperatures. To get oil with sufficient cold stability, the wax content has to be reduced to a level of about 10 ppm. Dewaxing is based on the principle of crystallization by slow cooling. Removal

of wax is tedious by cooling below $10^{o}C$ due to the presence of high melting saturated fatty acids. Usually in dewaxing step oil is heated to around $90^{o}C$ and cooled slowly to $20^{o}C$ to separate wax. Rajam et al reported simultaneous degumming and dewaxing by heating oil at $75^{o}C$ after adding 1% $CaCl_2$ and slow cooling to $20^{o}C$ at a rate of 0.4°C/min to remove gum and wax by centrifuging [84]. Degummed oil treated with 5% water containing small amount of sodium lauryl sulphate was shown to have better effect in dewaxing [101]. Similarly De et al reported combined degumming and dewaxing at $10^{0}C$ in presence of 5% water with yield of better quality oil [102].

1.11.2.3 Bleaching

Coloring compounds in oil such as chlorophyll, oxidized products, metallic salts of fatty acids are removed from oil by adsorbing in activated carbon or clay. Under high vacuum in presence of bleaching earth and activated charcoal, coloring compounds and oxidation products are adsorbed. Bleaching earth acts as an ion exchange for the removal of metal and the activated carbon there by reducing the red pigment in CRBO. Generally bleaching earth is of high cost and a study on economic analysis of membrane bleaching suggests that it is more economical [103]. The color of CRBO can be lowered by silica gel treatment either by shaking the miscella with the gel or by column percolation. The results

showed column percolation leads to 30-72% of color reduction [104] but these methods not employed for economic reasons.

1.11.2.4 Deacidification/ Deodorization

Deacidification is the process of removal of FFA from oil. Chen et al suggested supercritical carbondioxide deacidification process in which about 97.80% of removal of fatty acids was attained [105]. These methods are not commercially viable and hence not employed by industry. De et al reported reesterification of FFA with monoacylglycerols leading to the reduction of FFA [106]. Kale et al reported deacidification by methanol extraction using the principle of difference in solubility of FFA and neutral lipid in appropriate solvents followed by a nano filtration using commercial membrane. This technique reduced 16.5% FFA to 0.33% by two steps of

extraction [107]. Based on the molecular weight difference, FFA can be separated by using of porous or nonporous membranes. Under vacuum and at high temperature deacidification/ deodorization carried out by steam distillation in which FFA along with volatile components were removed and this method is widely practiced in industry.

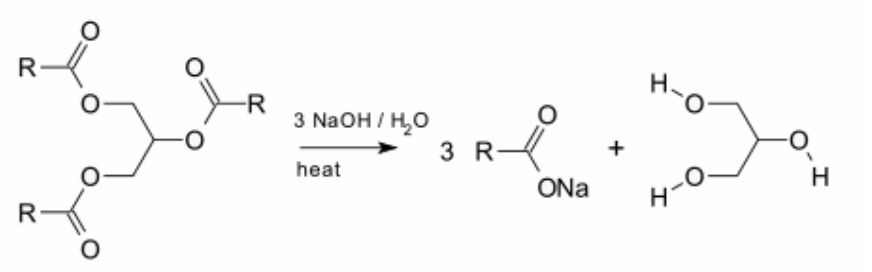
The specifications for RBO are shown in Table 1.8. Physically refined RBO agrees all specifications except for Unsaponifiable Matter (USM). The phytochemical profile of RBO shows very high content of USC which get concentrated in USM and hence responsible for high USM in RBO.

Table 1.8 Specifications for RBO

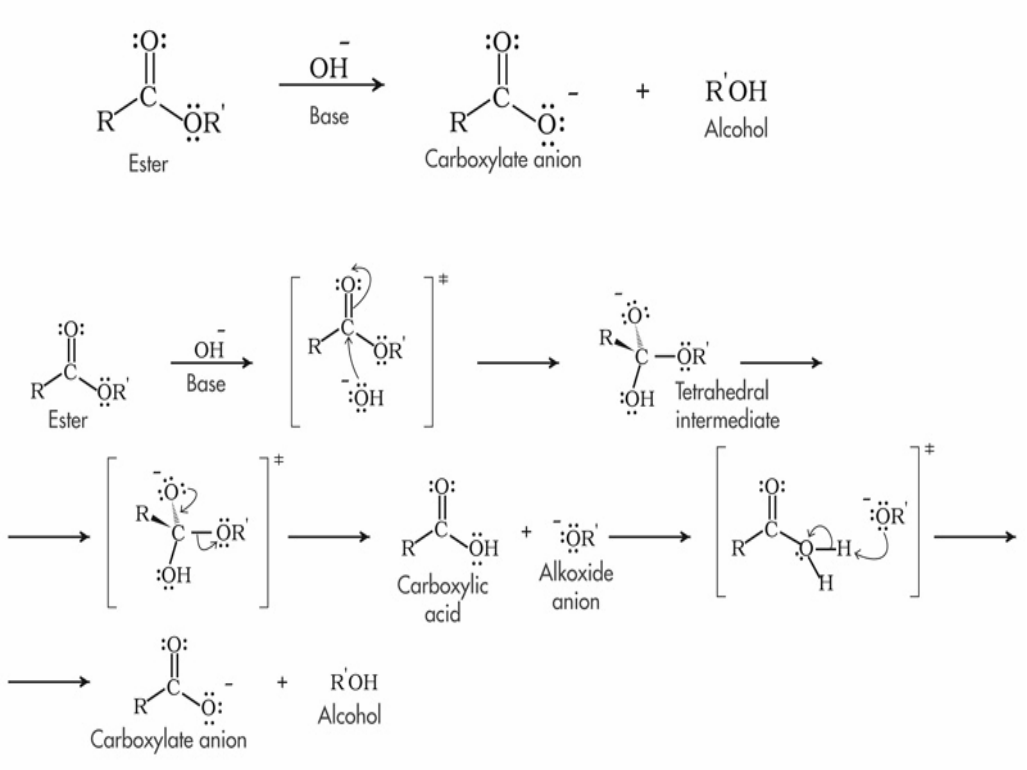
Serial No.	Characteristics	Requirement for		
		Refined grade	Raw grade 1	Raw grade 2
1	Moisture and insoluble impurities, percent by mass, Max	0.1	0.5	1
2	Color in a l-in Cell On the Lovibond scale expressed as R+ 5 R, not deeper than	20	-	-
3	Refractive index at 40^{o}C	1.460-1.470	1.460-1.470	1.460 - 1.470
4	Specific gravity at 30^{o}/30^{o}C	0.910- 0.920	0.910- 0.920	0.910 -0.920
5	Saponification Value	180-195	175-195	175-195
6	Iodine value (Wij's)	90-105	85-105	85-105
7	Acid value	0.5, max	20, max	Above 20
8	Unsaponifiable Matter, % by mass, max	3.5	6	6
9	Flash Pensky-Martens(closed) , ^{o}C,Min	250	100	90

1.12 Unsaponifiable Matter (USM)

USM of oil or fat are those components which are extracted together after saponification using solvents such as petroleum ether, diethyl ether etc. USC here refers to individual constituents in oil. Oil contains glycerides and during saponification acyl bonds are cleaved to form glycerol and alkaline salt of fatty acids (soap). The reaction can be expressed as follows.



Saponification reaction usually takes place in aqueous solutions with miscible organic solvents, such as methanol, ethanol, tetrahydrofuran, dioxane, dimethoxyethane etc to dissolve both, esters and hydroxides (KOH, NaOH, LiOH). The reaction temperature varies from room temperature to the boiling point of the mixture and the duration of the reaction varies from 30 min to 24 h, or more. Volume of alkali used is generally in excess in the range of 0.1 - 2 N [108]. The mechanism of saponification of an ester is shown below.



The attacking species in the base catalyzed ester hydrolysis is powerful nucleophile OH^- First step of neucleophilic acyl substitution involves the attack of hydroxyl anion on the carbonyl group of the ester giving an ortho ester. The orthoester leads to the expulsion of alkoxide generating carboxylic acid. The alkoxide ion abstract proton which is more basic than carboxylic acid leads to the formation of alcohol and carboxylate anion.

Generally, USM of edible oil comprised of sterols and their esters, tocopherols, hydrocarbons (wax, squalene etc) triterpene alcohols, lignin's, polyphenols etc. Most of the vegetable oil contains 0.5–2.5% USM but RBO is exceptional having 5–6% of USM [109].

1.12.1 USM in RBO

RBO differ from other oils in terms of its high USM content which has beneficial effects in human health as they are composed of biologically active phytochemicals. The nonsaponifiable fraction of RBO contains components which includes tocols (0.08%), γ-oryzanol (1.6%), sterols etc [110-111]. RBO is the unique source of oryzanol among the edible oil. It also provides other biologically active compounds sterols, hydrocarbons, triterpene alcohols etc. There are various types of sterols and among them campesterol, β-sitosterol and stigmasterol are the major ones. Sterol content reported in edible oils are; rice bran (1055 mg/100g), corn (952mg/100g), wheat germ (553mg/100g), flax seed (338mg/100g), cottonseed (327mg/100g), soybean (221mg/100g), peanut (206mg/100g) and olive (176mg/100g) [112]. Other USC in RBO are squalene **(**120 mg/100 g), tocopherols (40 mg/100 g), tocotrienols (70 mg/100 g) and triterpene alcohols (1.2 g/l00 g) [113]. Depending on the genetic and environmental factors the tocols and oryzanol concentrations in RBO varies and it ranges from 0.10–0.14% and 0.9–2.9% respectively [114-116].

1.13 Effect of refining on the USM content of RBO

USM content mainly depends on method of refining, estimation etc. Hoed et al reported major and minor constituents accounted 5.4% USM before chemical refining and reduced to 2.7% in the final refined oil [88].They also reported chemical refining caused loss of oryzanol and also change in individual phytosterols. They reported the formation of isomers of 24-methylene cycloartenol after bleaching. Because of the high volatility of phytosterols and tocotrienols, they get striped off from the oil during deodorization and get concentrated in the deodorizer distillate [88].Tocopherols, tocotrienols and squalene were affected by alkali neutralization and deodorization steps. Deacidification, bleaching, deodorization causes loss of γ-oryzanol and sterols. About 93.0-94.6% loss of oryzanol was reported by Krishna et al by chemical refining method [53]. In general USC in RBO are affected by chemical refining process. Reports reveal that physical refining limits the loss of USC. Usually oil undergoes various chemical and physical steps during refining and this leads to change in profile of USC in refined oil. In a chemical refining process thermal degradation of tocols occur and causes reduction of 50% and 70% of tocopherols present in crude and refined oil respectively [117]. Mezouari et al reported chemical refining caused changes in sterol and sterol esters. They reported decrease in sterols (65% and 72%), sterol esters (14% and 46%) of CRBO and RRBO, respectively, after eight hours of heating [117]. Pestana et al investigated the quality and changes in bioactive phytochemicals at different steps of refining [118].

Deacidification by steam refining process showed retention of natural antioxidants and low neutral oil loss [119]. Krishna et al reported degumming and dewaxing steps removed only 1.1 and 5.9% oryzanol respectively, while in alkali

treatment 93.0 to 94.6% of oryzanol is lost [53]. Thus majority of the nutritional components present in rice bran oil are destroyed or removed during traditional alkali refining. Refining lose can be considerably reduced by following physical refining. The Central Committee of Food Standards, Government of India through PFA restricts, the USM content of physically refined RBO should be with in the limit of 3.5% and this legal limit has come in the way of wide spread adaptation of physical refining of RBO till recently [120-121]. Presently the limit of USM is enhanced to 4.5% by the corresponding organization without any scientific scrutinizes.

1.14 Legal dimensions of USM in RRBO

The limits of USM for different vegetable oils were given in Table 1.9 [122]. RBO differs from other edible oils in terms of richness in its phytochemical profile, which is responsible for the higher percentage of USM.

Under PFA Act of 1954 the maximum limit for USM is 3.5% [120] which is recently changed to 4.5% by sixth amendment of PFA rules 2008. Selling of RRBO having USM exceeding this limit is punishable. Apart from refining methods, the procedure of estimation of USM is a significant step which should be clarified with scientific proofs.

Table 1.9. USM limits of vegetable oils [122]

Oil	Codex USM limit	USM limit (%) by Bureau of Indian standards	
		Raw	Refined
Palm	< 1.2	1.2	1.2
Peanut	<1	-	-
Rapeseed	<1.2	2	1.2-1.5
Mustard	<1.5	-	-
Rice bran	-	6	3.5
Safflower	<1.5	-	1.5
Sesame	<2	2.5	1.5
Soybean	<1.5	1.5-2	1.1-1.2
Sunflower	<1.5	1.5	-
Cotton seed	<1.5	-	-
Corn	<2.8	2.5	1.5
Olive	<1.5	-	-

1.15 Method of Estimation of USM

Gravimetric estimation of USM is usually done by following American Oil Chemist's Society (AOCS), AOAC and IUPAC methods. The above cited methods are used for quantitation of all USC present in oil. By these methods individual constituents cannot quantified and quantification attained by use of various analytical instruments.

The method of estimation of USM by AOCS method is as follows: 95% alcohol and 50% aqueous KOH is mixed with 5g sample and boiled gently with a reflux condenser for one hour. The refluxed sample is extracted after neutralization using petroleum ether containing 10% ethyl alcohol. All fractions of petroleum ether mixed and washed with distilled water, till the wash water is neutral to phenolphthalein. Petroleum ether evaporated to dryness and finally dried in vacuum. A blank is also conducted and the residue from sample and blank are weighed. The sample is added with 50 mL of warm 95% alcohol, phenolphthalein indicator and titrate against 0.02 N NaOH to faint pink color [123] to estimate FFA.

AOAC method of estimation of USM is as follows; 2-2.5 g sample is added with 25 ml alcohol, 1.5 ml KOH and boiled with occasional swirling in a steam bath for 30 min under reflux air condenser. Extraction of USM by ether and combined ether extracts are washed with water, followed by washing with 0.5 M aqueous KOH and water. Finally water washed till it become free of alkali, evaporated and dried under vacuum. The contents are dissolved in 10ml neutralized alcohol and titrated with 0.1 M alcoholic NaOH to determine the FFA [124].

IUPAC method describes, 5g sample is treated with 50 ml of 1 mol/L ethanolic KOH fitted with reflux condenser and kept in boiling water bath for 1 h. The contents extracted with diethylether along with water. After the complete diethylether extraction it is washed successively with 0.5 mol/L KOH solution and water until free of hydroxide finally evaporated. The residue dissolved in acetone and dried constantly at 100^{o}C. In order to estimate FFA residue dissolved in ethanol and titrated with 0.1mol/lit ethanolic KOH solution [125].

One of the consequences of these methods is the use of high concentration of alkali which results in loss of oryzanol. The other draw back as evidenced by the literature is the correction of FFA to obtain the actual USM percentage. A study conducted related to the acid value of refined oil showed oryzanol influenced acid value [126]. Takeshita et al reported soybean oil with added oryzanol that increased the acid value and hence presence of oryzanol has been shown to increase acid value of samples [126]. The currently practiced method of estimating USM based on alkali saponification (AOCS) when applied to RBO may distort the results, as oryzanol, tocopherols etc are destroyed which are sensitive to alkali and temperature. A reliable and accurate method to quantify USC directly from RBO without saponification is important from regulatory and nutritional points of view.

1.16 Relevance and objectives of the present investigation

RBO is attaining importance because of the presence of various phytochemicals. Present investigation focuses on the areas to analyze the increase in percentage of USM of physical refined oil.

It is necessary to relook the standard methods for the estimation of USM in rice bran oil by saponification. Saponification particularly when applied to RBO destroys, the alkali and heat sensitive constituents, leading to large variation in USM content. Thus the primary objective of the present work was to develop a method which is accurate and simple for the estimation of USC to obtain their actual values directly from oil without alkali treatment.

It was followed by quantitative and qualitative comparison of constituents obtained by newly developed method and saponification method. The changes occurring during saponification were verified and the gap between the quantitative values was also accounted after characterizing new compounds by FTIR, ^{1}HNMR, C^{13} NMR and Mass spectrometry analysis.

Varietal studies were conducted for ten paddy varieties collected from Regional Agricultural Research Station, Kerala. The variations in USC of different varieties not reported so far. The bran separated and oil was extracted for the physico chemical characteristics, and analysis of variations in USC of the oil.

Stability of rice bran oil was monitored by adding known concentration of tocols, sterols and oryzanol, in striped oil (without micronutrients) by individual mixings and two combinations. Usually the stability of RBO in blends was analyzed to point out the stability effect of RBO. The current study refers to the stability effect of major and minor antioxidants on RBO itself.

1.17 Structure of the present Investigation

Chapter 1: Introduction-objectives and literature review

Chapter 2: Materials and Methods

Chapter 3: Results and Discussions (divided into five chapters)

3.1. Development of a Method for Simultaneous Estimation of Unsaponifiable Constituents of Rice Bran Oil using HPTLC

3.2. Effect of Saponification on Composition of Unsaponifiable Matter in Rice Bran Oil

3.3. Detection, Isolation and Characterization of *sn*-2 alk-1'-enyl ethers of Glycerol from Rice Bran Oil

3.4. Quality of oils and unsaponifiables from paddy varieties

3.5. Contributions of oryzanol, tocols and sterols to the stability of RBO

Chapter 4: Summary and Conclusion

References

CHAPTER-2
MATERIALS AND METHODS

2.1 Materials

Crude Rice Bran Oil (CRBO) is obtained from Chakkiyathumoodu Solvent Extractors, Angamali, Kerala, India.

2.2 Standards, chemicals and solvents

Authentic **s**tandards of squalene, phytosterols (stigmasterols, betasitosterols, campesterols) lupeol, TAG (tripalmitin, triolein and tristearin), DAG (*sn*-1, 2 -dioleoyl-rac-glycerol, *sn*-1,2-linoleoyl-rac-glycerol), MAG (1-monopalmitoleoyl glycerol), fatty acids (Stearic acid, oleic acid) phospholipids (L-α-phosphatidyl ethanolamine-β-Linoleoyl-γ-palmitoyl, L-α- Lysophosphatidyl choline oleoyl, L- α-phosphatidyl choline) and heptadecanoic acid methyl ester from Sigma-Aldrich (Steinheim, Germany); α, β, γ and δ-tocopherols and tocotrienols from Calbiochem (Merck, Darmstadt, Germany). Oryzanol (cycloartenyl ferulate and 24-methylene cycloartanyl ferulate) are received as gift from M/s. Tsuno Rice Fine Chemicals, Wakayama, Japan. Spectroscopic solvents $CDCl_3$, deutrated methanol are from Sigma Chemical Company. All chemicals and analytical grade solvents are from Merck, chemicals, India. HPLC solvents used are from JT Baker (USA).

2.3 Refining of CRBO

Physical refining of CRBO has been done following the method reported by Rajam et al [84] at laboratory scale as briefly described below.

2.3.1 Simultaneous degumming and dewaxing

CRBO (500 g) is heated to 75^{o}C in a 1000 mL beaker on a heating mantle with a magnetic stirrer. It is filtered through ordinary filter paper using buchner funnel and continued stirring for 1h at 75 ^{o}C after adding 2% water for first 30 min and 1% (vol/wt) $CaCl_2$ (6% wt/vol) for another 30 min. Thus obtained oil is cooled for crystallization from 75°C to 20°C at a rate of 0.4°C/min using chilled

water. The crystallized gum and wax in oil mixture is separated by centrifuging in a cooling centrifuge at 7,500 × *g* for 20 min and the supernatant layer is decanted.

2.3.2 Bleaching

Degummed and dewaxed oil is heated to 95°C under 3 mmHg pressure after adding 0.25% (vol/wt) of aqueous citric acid solution (25%wt/vol) in a 1000 mL RB flask. 4% (w/w) fuller's earth and 0.4% (w/w) of activated charcoal are added to the preheated oil and heated to 105°C under 3 mmHg pressure for 20 min with stirring and finally filtered using buchner funnel.

2.3.3 Winterization

The bleached oil is cooled to 20°C in a time-temperature programmable crystallizing unit and maintained at this temperature for 24 h to separate residual gum and wax. The crystallized wax and gum are removed by vacuum filtration.

2.3.4 Deodorization

For deodorization the oil is taken in a three necked 1000 mL round bottomed flask in which a thermometer placed in one neck, a bent adapter in another neck which is connected to a water condenser/distillate receiver (500 mL RB flask fitted with a bent vacuum adaptor). The steam passed through the other neck by heating water in a RB flask (1000 mL) and the oil is heated on a sand bath to 180-200^{o}C for 30 min under 3mm Hg pressure for deodorization and the temperature is raised from 200 to 250^{o}C for 60 min under 3 mm Hg pressure for deacidification.

2.4 Chemical characteristics of RBO

FFA, iodine value, saponification value is determined according to the procedure of IUPAC [127].

2.5 HPLC analysis

HPLC analysis is performed in a Shimadzu binary system (LC-10A) (Kyoto, Japan) with a LC-10AD model pump, a 7125 model Rheodyne injector (Cotati, California, USA) fitted with a 20 µl sample loop. A SPD-10A UV-Visible detector with a C-R7 Ae plus integrator is used for data acquisition and display.

2.5.1 Tocols composition

Samples are prepared in hexane (1mg/ml) and the quantitation has been done in a phenomenex NH_2 column (5µm, 4.6 X 250 mm; Luna) with a mobile phase of *n*-hexane:isopropnol (96:4 v/v) at a flow rate of 1 mL/min. The UV detector is set at 297 nm. The separated compounds are analyzed using the authentic standards and quantitated based on the peak areas relative to standard calibration plots [128].

2.5.2 Sterols composition

To determine the sterols composition, USM of RBO was analyzed. The sample is prepared in methanol:chloroform (7:3) and separated using a reverse phase C-18 Zorbax column (4.6 X 250 mm) (Rockland Technologies, Newport, USA). The mobile phase used for the separation is methanol:water (96.5:3.5 v/v) at the flow rate of 1.5 mL/min. UV detector set at wavelength 206 nm. Quantitation of samples is carried out by calculating the peak area of sample with that of standard calibration plots [129].

2.5.3 Oryzanol composition

Oryzanol in RBO is analyzed using Waters µ-bondapak ^{TM}C18 column (4.6 mm i.d. X 25 cm) using a binary solvent system of acetonitrile:dichloromethane:acetic acid (88:6:6 v/v/v); methanol:n-butyl alcohol: water (90:2:8 v/v/v) in the ratio of 75:25 (v/v) at a flow rate of 1 mL/min. The detector is set at 325 nm. Using the authentic standards R_t of the compounds are identified and from the calibration plots the quantity was estimated [130].

2.6 Isolation and purification of steryl esters and wax from RBO

CRBO (500mg) dissolved in chloroform is adsorbed in silica gel and transferred to a silica gel column (75 g silica gel of mesh 60-120) of 45 cm length and 2.5 cm i.d. The column is eluted with hexane (150ml) as first fraction. Subsequently 200 ml of 2% diethyl ether in hexane was collected as second fraction, which contained wax and steryl esters as a mixture. The second fraction is concentrated and separated on 20 x 20 silica coated (0.2mm) glass Thin Layer Chromatographic (TLC) plates on preparative scale (20 mg per plate) and developed in petroleum ether : diethylether in the ratio 25:0.5. Bands corresponding to steryl esters and wax are collected and eluted from silica using chloroform for steryl esters and hexane for wax and further confirmed by spectroscopic analysis. Thus purified compounds were used as standards.

2.7 Optimization of chromatographic conditions for the separation of USC

2.7.1 Preparation of standard solutions

A stock solution containing 1mg/ml of sterols, oryzanol, tocols, squalene, steryl esters and wax, are prepared in chloroform. Mixture of phytosterols contains stigmasterols, betasitosterols, campesterols in the ratio 2:1.8:1, oryzanol mixture cycloartenylferulate and 24-methylene cycloartanylferulate in the ratio 1: 0.5 and equal contribution of α, β, γ, δ-tocopherols and tocotrienols. Stock solutions of the standards are diluted in such a way to obtain concentrations of sterols 0.25 mg/ml, oryzanol 0.025 mg/ml, tocols 0.05 mg/ml and squalene, steryl esters, wax 0.01 mg/ml in chloroform and used as working solutions.

2.7.2 HPTLC; Instrumentation and Conditions [131]

Analytical Instrument: CAMAG HPTLC system, Switzerland

Spotter: Linomat 5 Automatic Sample Spotter

Scanner: CAMAG TLC Scanner3

Software:	CAMAG "win CATS" 1.3.0 planar chromatography manager
Plate:	HPTLC aluminium sheets coated with silica gel $60F_{254}$ (E.Merck) (0.2mm thickness)
Chamber:	CAMAG glass twin trough chamber (10 x10 cm)

2.7.3 TLC-1 sterols, oryzanol and tocols

For calibration the standard solutions were spotted on precoated HPTLC plates (10 ×10 cm) using semi-automatic band wise spray using CAMAG Linomat 5 system in 6 mm bands with 12 mm spacing between two tracks. Chromatographic conditions for sterols, oryzanol, and tocols are optimized to obtain maximum resolution of the compounds. Solvent system; benzene: chloroform in the ratio 24:2 is found to be optimum. The amount of samples applied are: sterols, 0.50-3.00 μg (mixture of stigmasterols, betasitosterols and campesterols in the ratio 2:1.8:1); oryzanol 0.15-0.50 μg (cycloartenyl ferulate and 24-methylene cycloartanyl ferulate in the ratio 1:0.5); tocols, 0.40-1.20 μg (equal contribution of mixture of α, β, γ and δ-tocopherols and tocotrienols). The plates are developed in a CAMAG glass twin trough chamber. After development, the spots have been visualized under UV and scanned at its λ_{max} sterols at 206 nm, oryzanol at 325 nm and tocols at 297 nm using the slit dimension 5 x 0.45 mm.

2.7.4 TLC-2 steryl esters, wax and squalene

For calibration of TLC-2 components chromatographic separation of steryl esters, wax and squalene are optimized using solvent system petroleum ether: diethyl ether and the ratio 25:0.5 were arrived at for maximum resolution. The sample spotted on precoated HPTLC plates (10 ×10 cm) using CAMAG Linomat 5 system in 6 mm bands with 12 mm spacing between two tracks and the sample applied were; squalene, 0.4-1.2 μg ; steryl esters, 0.4-1.0 μg (mixture of betasitosteryl ester, stigmasteryl ester and campesteryl ester); wax, 0.75-1.2 μg. The plates have been developed in a CAMAG glass twin trough chamber and

scanned for squalene at its λ_{max} 214 nm. Since steryl esters and wax were not UV sensitive they are quantified using charring method. The same plate is charred after dipping in 5% methanolic sulphuric acid for 3 s and heated in an oven at temperature 110^{o}C for an hour to develop the spot. The spots have been scanned at 439 nm (λ_{max}) to quantify steryl esters and wax.

2.7.5 Validation

Using the above TLC conditions the parameters validated are linearity, detection limit, quantification limit, precision and accuracy. Validation is performed in following the international standards [132-134] using adequate statistics estimated [CV%, standard error].

2.7.6 Quantification of USC in CRBO and RRBO by direct application of oil

For the quantitative analysis of USC 0.5, 2 and 5% solutions of CRBO and RRBO have been prepared in chloroform. For the quantification of USC in CRBO and RRBO, sterols, 6 µl (0.5%); oryzanol, 2 µl (0.5%) and tocopherol, 12 µl (2%); in chloroform have been applied using semi-automatic band wise spray using CAMAG Linomat 5 system in 6 mm bands with 12 mm spacing between two tracks and developed using the solvent system in TLC-1 and it is scanned at respective wavelengths as described in TLC-1.

For the quantification of steryl esters 12 µL (0.5%) and squalene 10 µL (5%) of CRBO and RRBO in chloroform have been applied. In order to quantify wax 18 µl (0.5%) CRBO and 10 µl (5%) RRBO oil samples are applied and developed using the solvent system in TLC- 2 and first it is scanned for squalene and then charred to quantify steryl esters and wax, as described in TLC-2.

2.8 Isolation of USM by saponification

USM is prepared from CRBO & RRBO following the AOCS [123] official method as described in methods of estimation of USM in section 1.14. Method in brief as follows: 5 g sample has been mixed with 30 mL of 95% alcohol and 5 mL of 50% aqueous KOH. The mixture was boiled gently with a reflux condenser for

one hour. The refluxed sample is transferred to the extraction cylinder using alcohol and extracted with petroleum ether (50 ML). Extraction continued for six times and the petroleum ether fraction has been washed with 25 ML portion of 10% ethyl alcohol in distilled water. Washing continued till it became failed to give pink color in phenolphthalein. Petroleum ether evaporated to dryness and finally dried in vacuum for constant weight. A blank also conducted. The sample is added with 50 mL of warm (50^{o}C) 95% alcohol containing phenolphthalein indicator and titrate against 0.02 N NaOH to faint, pink color [123] to estimate FFA. 1mL of 0.02 N NaoH is equivalent to 0.0056 g of oleic acid.

$$\text{Unsaponifiable matter, \%} = \frac{A-(B+C)}{\text{Mass of sample, g}} \times 100$$

.

A = Mass of residue, g

B = Mass of fatty acids, g

C = Mass of blank, g

2.9 Standardization of triterpene alcohol

A stock solution containing 1mg/ml of lupeol is prepared and diluted to obtain concentrations of 0.25 mg/ml. The amount of samples applied is 0.5-4µg.

2.10 Standardization of policosanol

A stock solution containing 5mg/ml of lupeol is prepared and diluted to obtain concentrations of 1 mg/ml. The amount of samples applied is 11-21µg.

2.11 Sample preparation of USM and HPTLC quantification

USM samples in chloroform have been prepared at concentrations 3, 12 and 30 mg/mL for CRBO and 2, 8 and 20 mg/mL for RRBO.

2.11.1 TLC-1

Sterols, oryzanol, triterpene alcohols and tocols have been separated and estimated in TLC-1 of CRBO and RRBO USM. The samples are applied on precoated HPTLC plates (10 ×10 cm) using CAMAG Linomat 5 system in 6 mm

bands with 12 mm spacing between two tracks and the plates have been developed in a CAMAG twin trough chamber using benzene : chloroform in the ratio 24:2 (v/v). Sterols, oryzanol, triterpene alcohols and tocols have been separated at R_f values of 0.09, 0.15, 0.2 and 0.25, respectively and identified using reference standards. The bands are scanned at 206 nm for sterols and triterpene alcohols, at 325 nm for oryzanol and at 297 nm for tocols.

2.11.2 TLC-2

For the separation of steryl esters, wax and squalene; petroleum ether and diethyl ether in the ratio 25:0.5 (v/v) is used and the sample application is same as described for TLC-1. The R_f of steryl esters, wax and squalene are 0.36, 0.46 and 0.74, respectively as confirmed by their respective reference standards. In order to quantify squalene, the spots have been scanned at its λ_{max} 214 nm. After squalene quantification the plate was dipped in 5% methanolic sulfuric acid for 3 s to quantify steryl esters and wax which are not UV active, followed by 1 h heating in an oven at 110^{o}C, finally scanning at λ_{max} 439 nm.

2.12 Identification and estimation of potassium salt of oryzanol from USM

Potassium salt of oryzanol has been separated from USM by preparative TLC (silica coated 0.2 mm, 20x20 glass TLC plates) after developing in methanol. The band retained in origin was collected. For confirmation the sample has been hydrolyzed using 20% ethanolic hydrochloric acid by refluxing (10 h). The hydrolyzed sample is analyzed by spectroscopic techniques such as ^{1}HNMR, ^{13}CNMR and FT-IR. In order to confirm the formation of potassium salt of oryzanol a model experiment has been conducted following the same procedure for saponification using standard oryzanol. Estimation of potassium has been done according to the AOAC method by flame photometry [135].

2.13 Characterization and identification of unknown compounds from USM

2.13.1 Isolation by TLC from USM

For the characterization, identification and quantification of unknown bands in USM, the sample is applied on 20x20 silica coated (0.2 mm thickness) glass plates and developed using the solvent system hexane : diethyl ether : acetic acid (8:2:0.1v/v/v). A band at an R_f 0.56 was found to be unknown and therefore collected for further separation by preparative TLC using the solvent system toluene: chloroform (7:3 v/v).The bands with R_f of 0.52 (Compound 1) and 0.68 (Compound 2) separately collected and eluted from silica gel and subjected to ^{1}HNMR, ^{13}CNMR, FT-IR and mass spectrometric studies [136-137] for confirmation and used as reference standards.

2.14 Fatty aldehyde (Compound 1) quantitation by Gas Chromatography (GC)

The aldehyde esters has been separated in gas chromatography (GC-2010) Shimadzu, Japan using a DB-23 capillary column [30 m length, 0.32 mm id (wide bore) with 0.25 μm film thickness] (Agilent Technologies, USA). The initial temperature is fixed as 180 ^{o}C for 2 mins and then increased to 200 ^{o}C at the rate of 5 ^{o}C /min and held at 200 ^{o}C for 5 mins. The carrier gas flow is 1.2 mL/min and the FID detector used is set at 300 ^{o}C. The fatty aldehyde obtained by saponification of TAG has been converted into dimethyl acetal esters by refluxing it with 2% anhydrous methanolic sulfuric acid for 3 h. Heptadecanoic acid methyl ester (1mL) was added as internal standard (1mg/mL) for quantification. The reaction mixture is cooled, extracted with 25 mL hexane (3 times) and washed with 3% sodium bicarbonate solution (3 x 10 mL) followed by distilled water (3x10mL) and evaporated to obtain dimethyl acetals of fatty aldehyde.

2.15 Gas Chromatography-Mass Spectrometric analysis of compound 2

GC-MS of compound 2 has been performed to analyze whether the fraction is a mixture as it was evidenced as fatty alcohol by spectroscopic data. A Shimadzu GC-17A Gas Chromatograph equipped with QP-5050 (Quadruple) (Japan) mass spectrometer fitted with a fused silica BP21 polar column (30 m x 0.25 mm i.d., Japan) was used. The detector temperature set at 270^{o}C and the oven temperature is programmed to start and hold at 180^{o}C for 3 min and then increased to 220^{o}C by raising 5^{o}C/min.

2.15.1 Quantification of policosanol (compound 2) by HPTLC (TLC-3)

For the estimation of policosanol, USM sample is methylated by refluxing with 2% methanolic sulphuric acid for 3 h, extracted with hexane and washed with 2% $NaHCO_3$ and distilled water. Evaporated sample has been applied and developed in the solvent system hexane : diethyl ether : acetic acid in the ratio 8:2:0.1(v/v/v). The band is identified using the reference compound (R_f 0.52) and estimated at its λ_{max} 206 nm.

2.16 Isolation of ether lipid from RBO

2.16.1 Isolation by column chromatography

RRBO (10 g) has been adsorbed in silica gel and packed in a glass column (60 cmx3.5 cm) having 60-120 mesh (350 g) silica gel. The column is eluted starting with hexane (300 mL), followed by hexane/diethyl ether in the ratio 95:5 (300 mL) and finally with 80:20 ratio. The third fraction is evaporated and re-column in different ratios of hexane and diethyl ether. Six fractions have been collected viz: 100:0, 94:6, 92:8, 90:10, 88:12 and only chloroform. All fractions are evaporated, dissolved in $CDCl_3$ and subjected to ^{1}H NMR, ^{13} CNMR, FT-IR and Mass spectrometry analysis.

2.16.2 Spectral Studies

Perkin Elmer Spectrum 100 FT-IR instrument (UK), ^{1}H and $^{13}CNMR$ ($CDCl_3$) on Bruker AVANCE DPX-300 spectrometer & 500 MHz (Germany) at room temperature. Mass analyzed by GC-17A Gas Chromatograph equipped with QP-5050 (Quadruple) (Japan) mass spectrometer by Direct Inlet Probe (DIP) method.

2.16.3 *sn*-2 Positional Analysis of ether linked TAG in RBO by Pancreatic Lipase-Catalyzed hydrolysis and TLC fractionation

Sn-2 positional analysis has been done by pancreatic lipase-catalyzed hydrolysis [138] and the method in brief is as follows. 1M tris buffer (2 mL), 2.2% $CaCl_2$ (2 mL), 0.05% bile salt (0.5 mL) have been added to 100 mg TAG and equilibrated at 40^{o}C in a water bath for one min before adding standardized amount of 5 mg pancreatic lipase. The amount of pancreatic lipase is optimized so as to occur 35% hydrolysis of the TAG (partial hydrolysis). The mixture is shaken vigorously at 40^{o}C using mechanical shaker for 2-4 min. The reaction has been stopped by the addition of 1M ethanol followed by the addition of 6M HCl (mL). The whole mixture is extracted with 10 mL diethyl ether for 3 times and pooled. The solvent layer is washed with distilled water and dried over anhydrous sodium sulphate. The solvent is then evaporated. The sample thus obtained has been separated on preparative TLC using solvent system hexane / diethyl ether/acetic acid in the ratio, 8:2:0.1 using silica gel coated TLC glass plates (0.2mm). The bands corresponding to TAG, FFA, DAG and MAG have been collected after identification of band using reference standards. Schematic presentation for the identification procedure of ether linked TAG is shown in Figure 2.1.

2.16.4 FT-IR analysis of *sn*-2-MAG obtained by pancreatic lipase hydrolysis

2-MAG obtained has been analyzed by FT-IR for the confirmation of characteristic peaks and also after hydrolysis in alkaline media. The aldehyde obtained by hydrolysis is converted to dimethyl acetals as explained in the section

2.14 and analyzed in GC to compare the alkyl groups in 2-MAG (lipase hydrolyzed) and that obtained from USM [138].

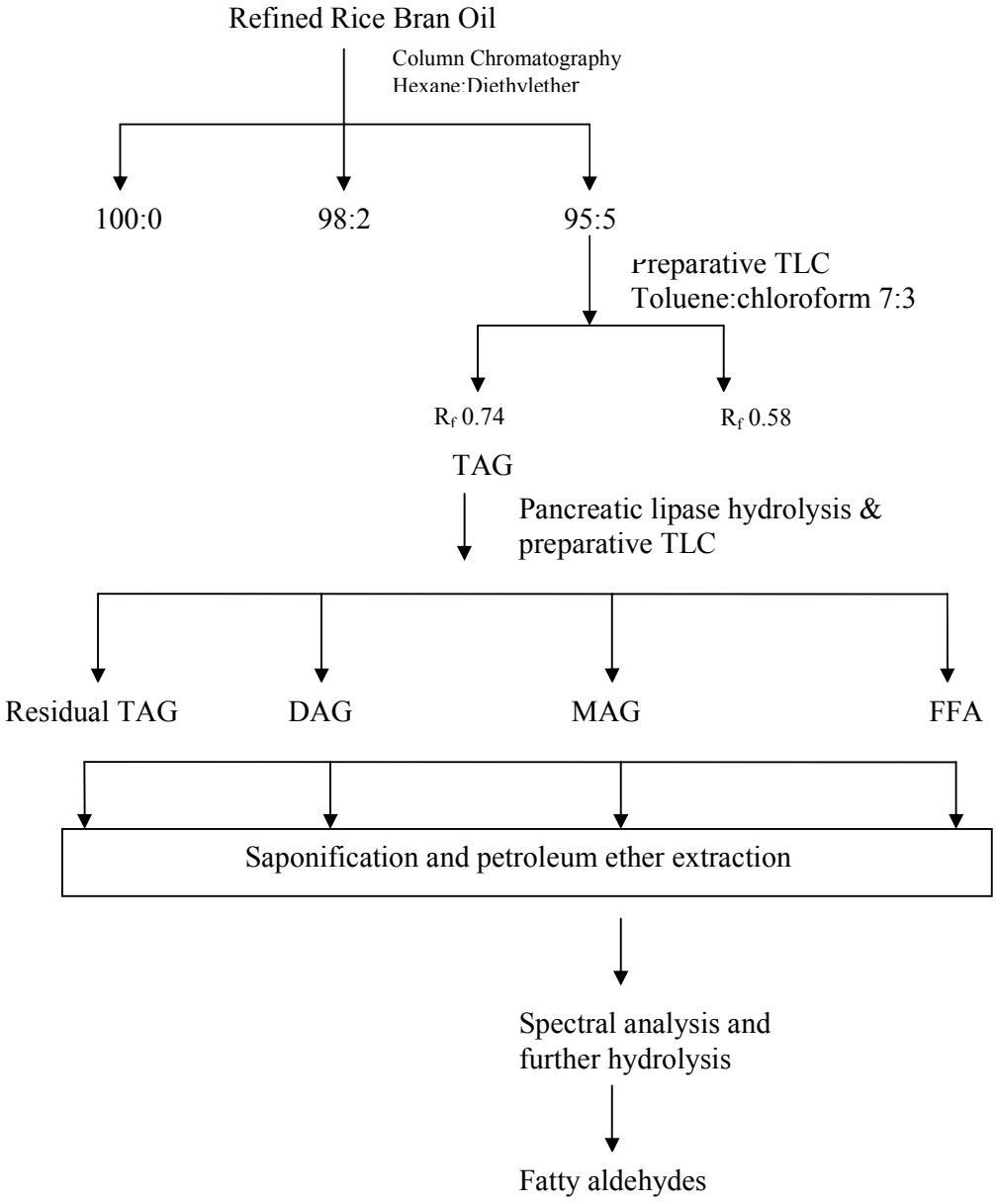


Figure 2.1 Schematic presentation of identification of ether linked TAG

2.17 Variety studies

2.17.1 Sampling methods of rice bran

Rice varieties; Annapoorna, Aiswarya, Geerakasala, Gandhakasala, Jyothi, Uma, RM-1, Makaram, Karuna and Varsha have been boiled in a steam kettle and dried in a hot air oven. The samples are de husked and milled in an ordinary dehuller and the bran is separated by sieving through a 20-mesh size sieve so as to separate the broken pieces of rice and husk. It has been stored in moisture proof containers to extract oil. Oil was extracted by soxhlet using hexane as the solvent, evaporated and dried under vacuum.

2.17.2 Color value, Carotene and Chlorophyll estimation in CRBO of various varieties

Color value, carotene and chlorophyll have been measured using Lovibond PFX 995 Tintometre (Amesburg, UK) using 10 mm cuvettee following AOCS procedure [139]

2.17.3 Fatty acid composition of variety sample

Fatty acid composition of samples have been done by using GC-2010, Shimadzu, Japan, using DB-23 capillary column (30 m length, 0.32 mm id (wide bore) with 0.25 μm film thickness) (Agilent Technologies, USA). The column temperature is programmed from 100 to 180^{o}C at the rate of 5^{o}C/min and 2 min hold time at 150^{0}C. The injection temperature was 250^{o}C and the detector temperature was 300^{o}C. The flow rate of carrier gas is 20 mL/min. Fatty acid methyl esters have been identified by comparing with authentic standards.

2.18 Stability of RBO in terms of tocols, sterols and oryzanol [140]

2.18.1 Striping of RBO: Removal of oryzanol, tocols and sterols

A glass column (110 cm length and 4cm wide) has been packed with silica gel of 60-120 mesh (750 g). RRBO (20 g) is adsorbed in silica gel and packed in

the column. For the separation of oryzanol, tocols and sterols, solvents used were hexane and diethyl ether in the ratios 100:0, 95:5, 80:20, 70:30, 65:35 and finally washed with methanol and acetone. All the fractions are evaporated and checked in TLC (silica gel 60 coated glass plates 0.2mm thickness) for neutral and polar lipids after developing in the solvent system hexane:diethylether:acetic acid (80:20:1) and for oryzanol, tocols and sterols developed in the solvent system benzene:chloroform (24:2) and compared with reference standards.

The concentrated fractions of tocols, oryzanol and sterols has been 80:20, 70:30 and 65:35 respectively along with minor amount of acyl glycerols. Therefore it is again purified by recolumn chromatography. A column of length 90 cm and 2 cm width has been chosen and packed with silica of 60-120 mesh. Recolumn of 80:20 fraction was done using the solvent hexane and diethyl ether and the fractions separated are viz: 92:8, 86:14, 84:16, 82:18 and 80:20. Fraction 70:30 and 65:35 are recolumn using 95:5, 90:10, 85:15, 80:20, 75:25 and 65:35 ratios of hexane and diethyl ether. All fractions has been evaporated and analyzed by TLC using the solvent system hexane:diethyl ether: acetic acid (80:20:1) and benzene :chloroform (24:2). The individual components sterols, oryzanol and tocols are quantified by HPTLC [131]. Table 2.1. and 2.2. shows the column separation of bioactive phytochemicals.

Table 2.1. Isolation of oryzanol, tocols and sterols from RBO by column

Eluent Hexane:diethylether	Volume (mL)	Weight of fraction (g)	% of micronutrients		
			Oryzanol	Tocols	Sterols
100:0	1000	15.20	-	-	-
95:5	900	2.69	2	-	-
80:20	1000	0.12	18	10	-
70:30	1000	0.86	29	0.46	14
65:35	800	0.61	6	-	24

2.18.2 Addition of bioactive phytochemicals in oil and heating of the samples

Sterols, oryzanol and tocols have been added in striped oil (column separated oil) at two different concentrations as individual and also as mixtures of two combinations as shown in Table 2.3. Micronutrients added in each 30 g of striped oil weighed in glass bottles (50 ml) and the samples are sonicated for 30 min at 35^{o}C.The samples added were the calculated amount of 100% and 50% of the total micronutrient originally present in the oil taken for the analysis. In order to study the stability parameters, Schall oven test is conducted for which the samples were heated in an oven at 60^{o}c for five days and analyzed every day for peroxide value, [141], p-anisidine value [142] and diene value [143]. Samples with (control oil) and with out micronutrients (control sample) are also kept at same conditions for the analysis.

Table 2.2. Recolumn chromatography of oryzanol, tocols and sterols rich fractions and % by HPTLC

Eluent Hexane:diethylether		Volume (mL)	Weight of fraction (mg)	% of Oryzanol	% of tocols	% of sterols
80:20	92:08	500	60.00	-	-	-
	86:14	400	20.00	-	-	-
	84:16	400	5.00	-	-	-
	82:18	400	12.20	-	98.30	-
	80:20	400	22.00	98.18	-	-
70:30	95:05	500	218.00	-	-	-
	90:10	400	136.00	-	-	-
	85:15	400	144.00	-	-	-
	80:20	400	5.00	-	80.00	-
	75:25	400	253.00	97.98	-	-
	65:35	400	97.00	-	-	97.52
65:35	95:05	500	215	-	-	-
	90:10	400	114	-	-	-
	85:15	400	78	-	-	-
	80:20	400	2	-	-	-
	75:25	400	45	81.33	-	-
	65:35	400	151	-	-	96.02

Table2.3. Percentage of micronutrients added as individual and combinations on striped RBO

Micronutrient	Individual mixing		Micronutrient	Combinations	
	% of Mixing	Sample code		% of Mixing	Sample code
Oryzanol (O)	1.60	O_1	Oryzanol+Tocols	1.60 + 0.04	OT_1
	0.80	O_2	O + T	0.80 + 0.08	OT_2
			Oryzanol+Sterols	1.60 + 0.60	OS_1
			O + S	0.80 + 1.20	OS_2
Tocols (T)	0.08	T_1	Tocols + Sterols	0.08 + 0.60	TS_1
	0.04	T_2	T + S	0.04 + 1.2	TS_2
Sterols (S)	1.20	S_1			
	0.60	S_2			

2.18.3 DPPH radical scavenging activity

The antioxidant activity of the samples (first and fifth day) have been analyzed by DPPH assay which is widely using to analyze the activity of various samples. The method [144] followed was briefly explained as follows. The oil samples ranging from 0-40 mg (before heating) and 0-60 mg (after heating) weighed and dissolved in ethyl acetate and 1ml of 0.01mM DPPH (in ethyl acetate) is added. The decrease in absorbance is determined at 517 nm after 10 min. DPPH solution was taken as control sample without oil and as the blank. The % DPPH RSA has been calculated as follows:

% DPPH RSA= [(control absorbance- oil absorbance)/ (control absorbance)] x 100The % DPPH RSA has been plotted against concentration of the oil sample (mg) and IC_{50} value was calculated.

2.19 Statistical analysis

All measurements have been duplicated on duplicate samples (2 x 2). The results are statistically analyzed by Analysis of Variance (ANOVA) and Duncan's multiple range test (DMRT). Statistical significance is accepted at a level of $P < 0.05$ [145].The statistical program Origin Pro 8 was used for data analysis. One way ANOVA with Tukey's multiple range test has been applied to determine the statistical significance.

CHAPTER-3.0
RESULTS AND DISCUSSIION

3.1. Development of a Method for Simultaneous Estimation of Unsaponifiable Constituents (USC) of Rice Bran Oil using HPTLC

The major constituents in edible oil are acylglyerols of saturated and unsaturated fatty acids which form soap when treated with alkali. USC are bioactive phytochemicals in oil which do not form soap on alkali treatment. RBO contains a variety of USC which include sterols, oryzanol, tocopherols, tocotrienols, squalene etc. These phytochemicals belong to various classes of chemical compounds and hence differ in their physical as well as chemical properties. USC are usually quantified by following, various analytical techniques such as GC, High Performance Liquid Chromatography (HPLC), GC-MS etc. Quantification of majority of USC was done after the pretreatment of the sample, which bring about drastic changes in the original composition of USC. In order to eliminate the changes occurring in USC due to pretreatment, development of a new method is conceived for the direct estimation of USC in oil in their native form.

HPTLC has been employed here to develop a method which is highly accurate in terms of resolution, quantification, reproducibility, fastness and its capability to analyze number of samples simultaneously etc.

3.1.1 High Performance Thin Layer Chromatography [131]

HPTLC is a modern chromatographic technique which has greater separation efficiency. Principle of HPTLC is similar to conventional TLC in which sample is migrated on a stationary phase. One of the important differences of HPTLC and normal TLC is in the particle size of stationary phase, which is 5µm in HPTLC while that of conventional TLC, it is 12-20 µm. It is widely applied in natural products and pharmaceuticals for finger printing marker compounds due to its ability to separate complex samples to individual

components with minimal time, low mobile phase consumption, detection and quantification of non UV active compounds by post-chromatographic derivatization, use of selective reagents for detection and confirmation without interference of the mobile phase, repeated detection and quantification at any time by using previously analyzed sample. The important steps in HPTLC are application of sample in TLC layer, development, scanning and documentation of chromatoplate. Various stationary phases such as cellulose TLC, silica gel 60 F 254 TLC, LiChrospher® Si 60 F 254s TLC, dipotassium hydrogen phosphate impregnated silica gel TLC, amino bonded TLC etc are available which has to be chosen depending on the nature of compound to get separated. Sample application is a critical step in which, maximum resolution of TLC bands should be obtained for detection and quantification and is done by automatic sample applicator. The second step is the development of the applied bands in a suitable mobile phase by using a developing chamber and finally detection and documentation using scanner and visualizer respectively. HPTLC 0analysis of components enables separation, detection and quantitation of complex mixture of samples which could not be easily separated by using any other single analytical technique.

According to convention, a method need to be validated before it is accepted for routine analysis. Method validation is the process used to confirm an analytical procedure for its capability to yield consistent results. The usual parameters for validation include linearity, precision, accuracy, recovery, robustness, limit of detection (LOD) and limit of quantitation (LOQ). The linearity of an analytical method implies the test results are directly proportional to the concentration of analyte in samples within a given range obeying Beer Lambert's law. Precision is the measure of how the data values are close to each other for a number of measurements under same analytical conditions. Repeatability, intermediate precision and reproducibility are the three components

which are measured under precision. Repeatability is evaluation of reliability of the method under varying environment other than that used during development of the method. Depending on time and resources, the method can be validated for intra and inter day precision. Reproducibility expresses the precision between laboratories in collaborative studies. Sensitivity is the ability to detect small changes in the concentration of the analyte in samples. Intermediate precision is expressed as Relative Standard Deviation (RSD) of which multiple measurements of a sample by the same analyst under the same analytical conditions. Accuracy is the measure of how close the experimental value with the true value. This is checked by adding known amount of standards by weight or volume to the sample. Recovery is expressed as the amount/weight of the compound of interest analyzed as a percentage to the theoretical amount present in the medium. Robustness acts as a measure of the method's capability to remain unaffected by small, but deliberate variations of analytical parameters.

3.1.2 Creation of calibration data using standard compounds

Calibration is a general technique which is used in analytical methods to determine reliability of analytical instrument/ method, using known standard. It gives the analytical signal of the instrumental response with the change in concentration of the analyte. A plot of instrument response verses analyte concentration will show a linear relationship by using linear regression analysis according to Beer Lambert law. According to Beer Lambert law there is a logarithmic dependence between the transmission of light through a substance and the product of absorption coefficient of the substance and the distance of light travel through the substance.

$$A = -\log_{10} (I/I_0)$$

A-absorbance

I_0-Intensity of incident radiation

I-Intensity of emitted radiation

Standard compounds of the respective USC are spotted in different concentrations and the calibration curve has been plotted. Calibration graph is found to be linear and the linearity is checked for minimum four concentrations of each standard. Peak area and concentration have been subjected to linear regression analysis to calculate the calibration equation and correlation coefficients. The linearity of calibration graphs is validated by high value of correlation coefficient. Sterols which is a major USC showed correlation coefficient of 0.99924 with a linear regression equation y = 564.91 x -15.93. Similarly all others showed a very good correlation coefficient.

The R_f with standard deviation, linearity, Relative Standard Deviation (RSD), equations of the regression lines are shown in Table 3.1. HPTLC chromatograms of standard compounds of TLC-1 sterols, oryzanol and tocols which are medium polar compounds, showed R_f ranges from 0.09-0.42 in Figure 3.1. TLC-2 components, squalene, wax and steryl esters are shown in Figure 3.2. Even though sterols, oryzanol and tocols are mixtures, they were separated as single bands with R_f of 0.12 for sterols, 0.21 for oryzanol and 0.39 for tocols. Non-polar compounds in TLC-2 (steryl esters, wax and squalene) have been separated well with R_f of 0.36 for steryl esters, 0.46 for wax and 0.74 for squalene.

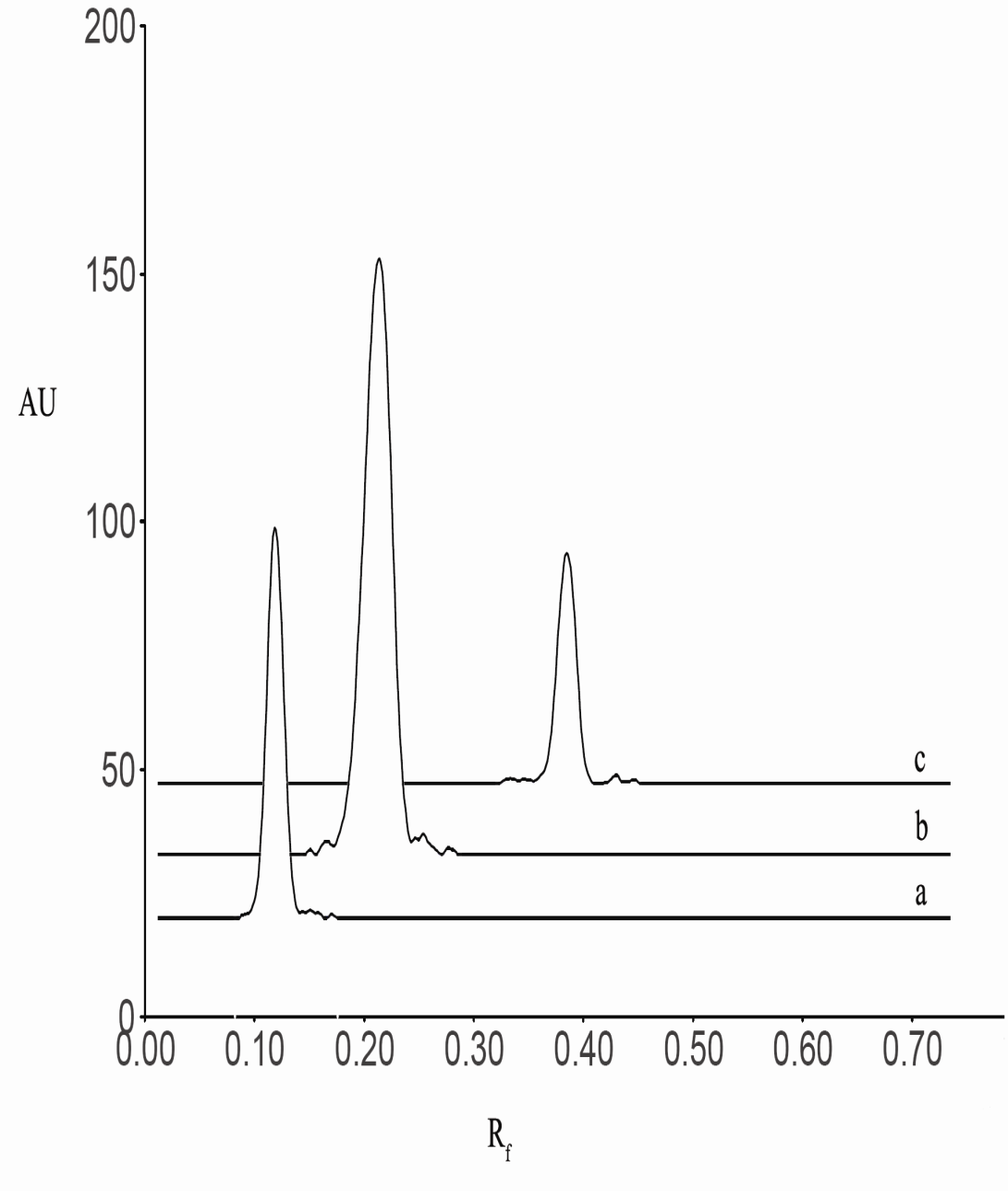


Figure 3.1. HPTLC densitograms of standards a. sterols b. oryzanol c. tocols with mobile phase benzene: chloroform (24:2 v/v), detection of sterol λ=206 nm, oryzanol λ=325 nm and tocols λ=297 nm.

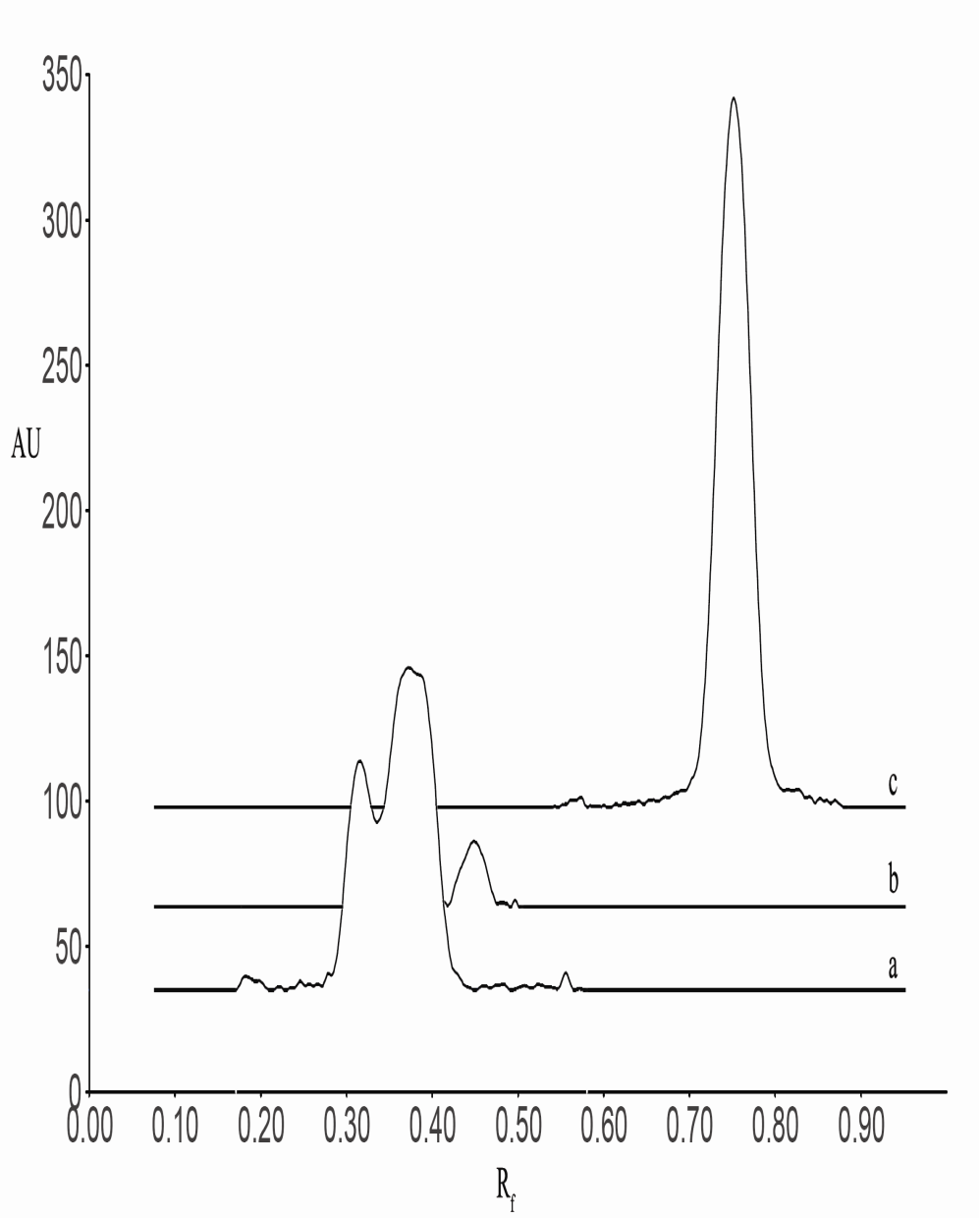


Figure 3.2. HPTLC densitograms of standards a. steryl esters b. wax c. squalene with mobile phase petroleum ether : diethyl ether (25:0.5v/v), detection before charring at λ=214nm after charring steryl esters, wax at λ = 439nm.

Table 3.1. Calibration data [correlation coefficient (r), RSD% and regression equation] and R_f of standard USC

USC	R_f [a]	r	RSD [%]	Linear range[µg]	Regression equation
TLC 1 components					
Sterol	0.12 ± 0.01	0.99924	7.62	0.50-0.30	[b] y = 564.91 [c] x -15.93
Oryzanol	0.21 ± 0.02	0.99768	0.37	0.15-0.50	y = 5398.53 x-632.01
Tocols	0.39 ± 0.03	0.99912	3.08	0.40-1.20	y =1536.00 x-192.20
TLC 2 components					
Sterylester	0.36 ± 0.01	0.99385	3.62	0.40-1.00	y = 2548.15 x+527.62
Wax	0.46 ± 0.01	0.99899	1.05	0.75-1.20	y = 882.73 x+221.03
Squalene	0.74 ± 0.01	0.99665	0.96	0.40-1.20	y = 4610.88 x-520.23

[a] Standard deviation (n=3) [b] y is the peak area of calibration plot of standard USC [c] x is the concentration of calibration plot of standard USC.

3.1.3 Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD is calculated based on the standard deviation of the response (SD) and the slope of the calibration curve. The values of SD and slope can be obtained from the linear function when creating calibration curve. LOD is the lowest concentration of an analyte in a sample that can be detected but cannot be used for the quantification, as it falls below the linear range. LOD for the standard compounds was calculated using the equation,

$$LOD = 3 \times N/B$$

Where N is the standard deviation of the 'y' intercept of peak area (n=3) of calibration plot, B is the slope of the calibration plot.

LOQ is the lowest concentration of the analyte in a sample that can be quantified with acceptable precision and accuracy under the operating condition. LOQ based on the calibration graph for the standard compounds was calculated using the equation,

$$LOQ = 10 \times N/B.$$

Among the USC, oryzanol has the lowest LOD and LOQ with 1 ng and 4 ng respectively due to the presence of a chromophore, ferulic acid, its absorbance λ_{max} is 325 nm could be responsible for the high sensitivity for oryzanol. Squalene, a long chain hydrocarbon with six double bonds, showed LOD and LOQ, 3 ng and 10 ng respectively. In the case of sterols the LOD and LOQ values are 6 ng and 20 ng respectively.

In the case of steryl esters and wax, ester is the functional group that has no characteristic UV absorbance and hence charring method was employed. The compounds quantified by charring method have been found to have LOD and LOQ greater than those for components quantified by direct scanning at their absorption maxima (λ_{max}).

The charred spots have been scanned at its λ_{max} of 439 nm in the visible region and thus showed the lowest sensitivity among the USC. LOD for steryl esters and wax are 22 ng and 19 ng and LOQ, 73 ng and 65 ng respectively. LOD and LOQ values of USC are shown in Table 3.2.

A few authors reported LOD and LOQ for some unsaponifiable constituents in edible oils. Reported LOD and LOQ values for sterol are 50 and 70ng by HPLC [146]. In the present method it is 6 and 20ng respectively. The LOD values by HPLC for α,γ and δ tocopherols have been 0.56 ng, 0.16 ng and 0.16 ng respectively. The LOQ values for these compounds are 1.68 ng, 0.48 ng and 0.48 ng respectively by HPLC [147]. The present method showed LOD of 11ng and LOQ of 38 ng for tocols. Regarding the accuracy of HPLC and present method (HPTLC) the recovery reported are identical or comparable for sterols and oryzanol [146,148].

Table 3.2 LOD and LOQ of USC in RBO

USC	LOD (ng)	LOQ (ng)
Sterol	6	20
Oryzanol	1	4
Tocols	11	38
Steryl esters	22	73
Wax	19	65
Squalene	3	10

3.1.4 Accuracy

To verify the accuracy of the method, recovery studies have been performed, by the method of addition of known amount of standards to the samples. Single concentration of each standard compound is added to the previously analyzed oil sample and the recovery is calculated and repeated three times. The accuracy has been then calculated from the test results as the percentage of recovery and the data is shown in Table 3.3.

Recovery results ranged from 93.45% to 101.97%, which showed the reproducibility of the method and 100% recovery has been shown by squalene. Steryl esters and wax, which were quantified using charring method, showed recovery values of 97.23% and 93.45% respectively. Recovery values obtained here are found to be within the limits recommended for chemical analysis, which indicated the reliability and reproducibility of the method.

Table 3.3 Recovery studies of USC in RBO.

USC	Concentration of oil taken (μg)	Amount present in oil (μg)	Amount added (μg)	Amount found (μg)	Recovery (%)[a]
Sterol	100	1.08	1.00	1.99	95.88± 2.72
Oryzanol	12.5	0.17	0.20	0.37	98.87± 3.09
Tocols	400	0.56	0.40	0.9288	96.75 ± 1.60
Steryl esters	80	0.56	0.30	0.8361	97.23 ± 8.40
Wax	60	0.79	0.15	0.8802	93.45 ± 3.36
Squalene	1000	0.50	0.50	1.0197	101.97±3.27

[a] Mean ± SE (n =3)

3.1.5 Precision

Repeatability is determined by the measurement of instrumental, inter and intra assay precision. Instrumental precision has been measured by scanning the same spot of a single concentration for seven times. The concentration of standards used for instrumental precision are sterols, 0.5 μg; oryzanol, 0.16 μg; tocols 0.4 μg; steryl esters and wax, 1μg; squalene, 0.42 μg. The repeatability or intra assay precision has been studied by analyzing repeatedly, in the same laboratory and on the same day, on three concentrations. For intra assay precision the concentrations used for each standard are: sterols, 0.50, 2.00, 4.00 μg; oryzanol, 0.16, 0.32, 0.48 μg; tocols, 0.40, 0.80, 1.20 μg; steryl esters, 1.00, 1.40, 1.90 μg; wax, 1.00, 1.57, 2.14 μg; squalene, 0.42, 0.80, 1.60μg. Inter assay precision on the same three concentrations have been analyzed on different days as did for intra assay. Instrumental, intra assay and inter

assay precision are shown in Table 3.4. as expressed in CV%. Precision criterion for instrumental analysis is ≤1 CV% which is considered to be very good. Instrumental precision studies conducted here showed values between 0.30 -1.18 CV% and for oryzanol it exceeded the limit, 1.18 CV%. Studies of intra assay precision showed values ranging from 0.52-1.94 CV% and all the values are within the limit prescribed for intra assay, which could be ≤2 CV% [133]. Inter assay precision ranged from 0.87 to 2.27 CV% in the present study.

Table 3.4 Instrumental, inter and intra assay precision of USC.

USC	Instrumental Precision [a] CV(%)	Concentration (μg)	Intraday precision [b] CV (%)	Interday precision [c] CV (%)
Sterol	0.30	0.50	1.31	1.56
		2.00	1.62	1.81
		4.00	1.87	2.27
Oryzanol	1.18	0.16	1.48	1.55
		0.32	1.58	1.77
		0.48	1.94	1.92
Tocols	0.77	0.40	0.90	1.27
		0.80	1.12	1.54
		1.20	1.29	1.35
Steryl esters	0.77	1.00	1.16	1.28
		1.40	1.63	1.89
		1.90	1.78	1.98
Wax	0.56	1.00	0.97	1.18
		1.57	1.76	2.00
		2.14	1.79	2.17
Squalene	0.49	0.42	0.52	0.87
		0.80	0.83	1.32
		1.60	1.23	1.40

a) CV (n= 7); analyzed on the same day for single concentration.

b) CV (n=3); analyzed on the same day (for each concentration)

c) CV(n=3); analyzed on three different days (for each concentration)

3.1.6 Chemical Characteristics of CRBO and RRBO

Table 3.5 shows the chemical characteristics of the CRBO and RRBO taken for the study

Table 3.5 Chemical characteristics of RBO

Chemical Characteristics	CRBO	RRBO
Saponification Value	176±2.00	182± 1.00
Iodine Value	102±1.00	105±2.00
FFA (%)	4.20±0.50	0.4±0.10
USM (%)	5.95±0.21	4.03±0.15
Oryzanol (%)	1.91±0.11	1.51±0.21
Tocols (ppm)	1800 ± 6.0	1200±3.0
Sterol (%)	1.10±0.24	0.98±0.14

3.1.7 Application of the method: Separation of USC of RBO samples by HPTLC

Development of this method is aimed at quantitative determination of USC directly from oil without the pretreatment of the sample. Saponification is the usual method followed for the gravimetric estimation of USC, together as USM which causes drastic changes in USC. The newly developed HPTLC method is thus applied to RBO samples so as to validate its suitability to estimate unsaponifiables without saponification.

Due to the complexity of USC, they are quantified by using two mobile phases in two separate TLC (TLC-1 and TLC-2). For the separation of sterols, oryzanol and tocols (TLC-1), various ratios of benzene:chloroform is checked and finally fixed 24:2 as optimum ratio. Since squalene, steryl esters, and wax are nonpolar components, mobile phase, petroleum ether:diethylether have been optimized and the ratio 25:0.5 (v/v) showed maximum resolution of components (TLC-2). In TLC-2 after developing, the plate was firstly scanned for the estimation of squalene and secondly the plate is charred after dipping in methanolic sulphuric acid followed by heating for 1h at 110^0C in a hot air oven for the quantification of steryl esters and wax

since both of them were UV inactive. From the experiments it is noticed that resolution of all constituents is very good with distinct R_f values. Quantification of individual components was done by scanning the spots at its λ_{max} viz: 206 nm for sterols; 297 nm for tocols; 325 for oryzanol; 214 for squalene and 439 nm for steryl esters and wax after charring. The identity of individual USC in RBO is confirmed using reference standards and by overlaying their UV absorption spectra with those of the standards.

Using the HPTLC standardized method direct estimation of USC in CRBO and RRBO are carried out. The RRBO sample used here is produced in the laboratory following the physical refining method recommended by Rajam et al to retain maximum amount of USC [84]. RBO is reported to contain TAG (68-71%), DAG (2-3%), MAG (5-6%), FFA (2-3%), waxes (2-3%), glycolipids (5-7%), phospholipids (3-4%) and unsaponifiables 3-4% [149]. These acyl glycerols or the saponifiables did not interfere with the USC primarily due to the selection of solvent system and the optimization of the conditions. The mobile phase used in TLC-1, separated saponifiables of the oil samples (polar lipids, DAG, MAG and FFA) from unsaponifiables and got retained at the origin. The major saponifiable TAG moved with an R_f of 0.48 and the USC sterols, oryzanol and tocols got resolved with distinguishable R_f as shown in Figure 3.3. In TLC-2 polar lipids, DAG, MAG, FFA, phytosterols, oryzanol, tocols were retained in origin, TAG moved just above the origin and steryl esters, wax, squalene have been well separated as shown in Figure 3.4. with out interfering with other constituents.

The amount of oil sample taken for HPTLC separation is adjusted so as to fall within the sensitivity range of the USC. Since sterol and oryzanol contents are high in the oil samples quantity of the sample is optimized to fall within the linear range, (30 μg and 10 μg oil respectively). However concentrations of tocols and squalene are low as compared to sterols and oryzanol and hence higher amount of oil sample (240 μg and 500 μg oil respectively) was used. Results on the USC estimation using HPTLC method developed here are presented in Table 3.6.

Oryzanol, one of the unique compound in RBO is a major USC obtained by HPTLC analysis and its content varies in refined oil as a result of refining methods and conditions employed. Depending on the method of refining, the amount of oryzanol ranges from 0.2-2.72%; phytosterols and tocols ranges from 1.0 -2.8 % and 0.1-0.2%, respectively [150,53]. The previous reports on quantitative determination of oryzanol are mainly by absorptiometry, normal- phase and reverse- phase HPLC methods. The most basic method of determination of oryzanol is by absorptiometry reported by Tsuchiya et al [151] in which oil sample was UV scanned at λ_{max} 315 nm for quantification. Tanabe et al [152] reported normal-phase HPLC by using cholesterol ferulate as the external standard using Zipax-BOP column with hexane:diethylether (85/15 v/v) as mobile phase. But it is observed that there is wide difference in the estimated value while comparing with absorptiometric measurements reported by Yoshie et al [153]. A reverse phase HPLC method reported by Britz et al showed highly precise recovery of oryzanol using YMC C-30 reverse-phase carotenoid column [154]. Reverse–phase HPLC using ODS column was reported by many researchers [155-157]. From the review of various methods it is recommended that before analysis the sample should be purified by liquid–liquid fractionation, low-pressure column chromatography or any other purification methods [158]. The high temperature gas chromatographic analysis requires pre-purification by liquid–liquid fractionation followed by preparative TLC, and silylation. Even though such complex steps are followed, only poor resolution of trimethylsilyl ether derivatives of 24-methylenecycloartanyl ferulate and cycloartenyl ferulate was achieved in GC [159]. On reviewing the methods of oryzanol reported so far, merits of HPTLC method presented here are low consumption of mobile phase, high resolution, no need of derivatization of sample, many etc. The present HPTLC method showed 18.80 mg/g of oryzanol in CRBO and 13.80 mg/g of oryzanol in RRBO as shown in Table 3.6. The reported amount of oryzanol in CRBO and RRBO by HPLC analysis is in the range 16 mg/g to 27.20 mg/g [53, 160].

Verleyen et al reported the analysis of free and esterified sterols of vegetable oils after purification of sample by silica gel column chromatography, eluting with *n*-

hexane/ethyl acetate (90:10 vol/vol) followed by *n*-hexane/diethyl ether/ethanol (25:25:50 v/v/v) and thus obtained fractions are saponified separately and further analyzed in GC [161]. The majority of sterol determinations have been carried out with non-polar stationary phases such as 100% polysiloxane capillary columns using Flame Ionization Detectors (FID). Even though method for direct estimation of sterols is available by GC, the resolution of peaks is not good as compared to that obtained for trimethylsilyl derivatives or acetates. Therefore prior to GC analysis sterols are conventionally transformed into their derivatives to get peaks with improved, resolution, sensitivity and stability. Capillary GC quantification of trimethylsilylether derivatives of sterols from edible oils and fats is reported by Toivo et al [162]. Capillary electrochromatography (CEC) of sterols and related steryl esters derived from vegetable oils has been analyzed by Abidi using octadecyl silica columns and triacontyl silica columns after saponification of the oil sample [163]. CEC with either phase facilitates not only separations of epimers but also the separation of campesterol–stigmasterol pair which is very difficult to resolve by HPLC. The major draw back of GC analysis of sterols is it needs thermally stable columns and chemical derivatization prior to analysis. Normal-phase and reverse- phase HPLC techniques also used for the quantitation of sterols using UV detectors, photodiode array detector, refractive index detector, evaporative light scattering detector etc. Ultra performance liquid chromatography (UPLC) with atmospheric pressure chemical ionization mass spectrometry equipped with Acquity UPLC BEH C18 column using acetonitrile/water (0.01% acetic acid) for the estimation of saponified samples of sterol by Lerma - Garcia et al [164]. HPLC-MS analysis of saponified samples have been done using 5 mm dC18 column with a gradient of acetonitrile/water (0.01% acetic acid) by Carretero et al [165]. HPTLC analysis as demonstrated here is capable of using oil directly. Sterol contributed 11.20 mg/g in CRBO and 10.80 mg/g in RRBO by the present HPTLC method which agreed closely with the reported values of 14.80 mg/g in CRBO and 14.00 mg/g to 28.0 mg/g in RRBO by HPLC method [97, 150].

Alkali treatment affect tocols and therefore their estimation directly from oil is recommended [128, 166]. Stogg et al reported the separation and detection of

tocopherols, carotenoids, and γ-oryzanol in single run using RP-LC. The detector used is photodiode array and the separation has been achieved by using C_{18} and C_{30} silica columns. The results showed C_{30} column is more efficient as it separated β- and γ –tocopherols which is not obtained by C_{18} column [157]. HPTLC analysis of tocols showed CRBO and RRBO had 2.00 and 1.40 mg/g respectively. According to the previous reports RBO contained tocols ranges from 0.5-0.8 mg/g [150,167].

In the case of steryl esters the amount present in CRBO and RRBO are found to be almost same, 7.2 mg/g in CRBO and 7 mg/g in RRBO. Usual procedure for the estimation of steryl esters requires various steps for the quantitative determination. But the present method avoids the pretreatment steps thereby enabling quantification directly from oil. Cunha et al reported quantification of esterified sterols based on solid-phase extraction (SPE), trans esterification and GC–MS [168]. Capillary electrochromatography of sterols and steryl esters was also reported by Abidi [163].

Regarding wax, CRBO has high wax content (1.0 -3.0%) and almost all analytical methods reported for the estimation of wax needed various pretreatment of the sample. Nots et al reported the separation of wax in silica gel column by using carbon tetrachloride solvent and followed by GC analysis using TX-65 TG capillary column [169 Nota]. Kanya et al reported the separation of fatty esters by TLC and further identification by GC using a Dexil-300 column [170]. Quantification of wax in CRBO and RRBO using HPTLC reported here showed 13.20 mg/g in CRBO and not in detectable amount in RRBO as the refined oil taken for the analysis was physically refined [84].

The hydrocarbon squalene obtained here is 0.40 mg/g in CRBO and 0.48 mg/g in RRBO by HPTLC. Lau et al reported the simultaneous estimation of sterols, squalene, and acylglycerols after silylating the sample by GC analysis using BPX5 fused silica capillary column at higher temperature conditions [171]. Choo et al reported supercritical fluid chromatograph technique for the analysis of carotene, squalene, vitamin E in which isolation of the minor components of palm oil done by

less than 20 min, when compared to HPLC and GC [172]. Grigoriadou et al reported solid phase extraction of squalene and tocopherols by HPLC [173]. A simple and reliable procedure is presented for the quantification of alkanols, squalene, α-tocopherol and sterols in olive oils by GC analysis of the unsaponifiable fraction after silylation [174].

Polarity of USC in RBO is different and therefore quantification of all constituents cannot be done by using single equipment. Present method favors the quantification of constituents without any chemical change, low consumption of solvent, time saving etc with reproducible results. Even though the method allows quantification of constituents of different classes, the separation of individual component with in a class cannot be attained. For example, sterol as a single class can be quantified but not individual as stigmasterol, campesterol, betasitosterol etc. Similarly, in the case of tocols, oryzanol, steryl esters etc. However, the method is very reliable, accurate and reproducible which is proved by the validation parameters.

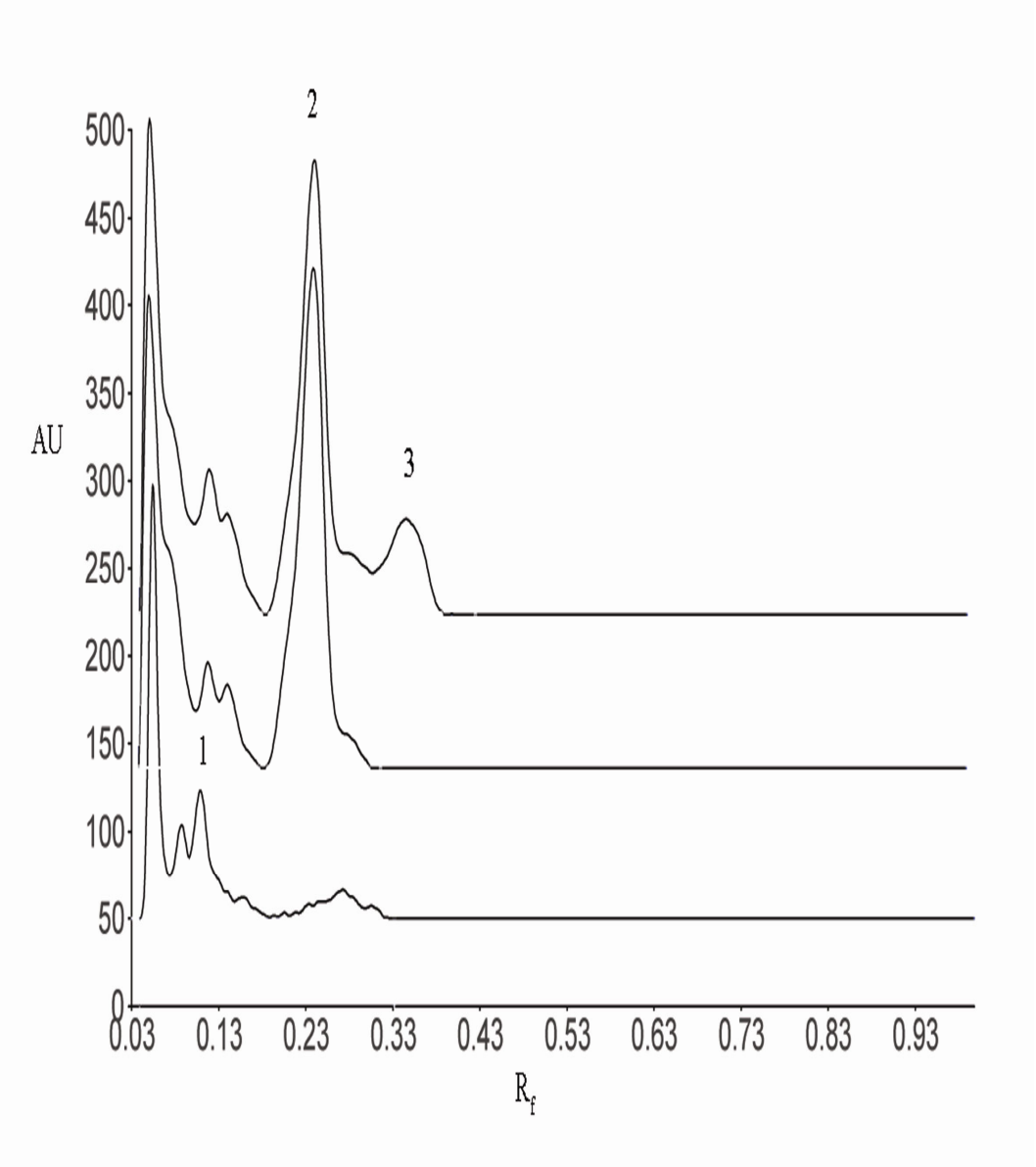


Figure 3.3. HPTLC densitograms of USC in RBO sample (TLC-1) 1. sterols 2.oryzanol 3. tocols, detection of sterol λ=206 nm, oryzanol λ=325 nm and tocols λ=297 nm, mobile phase benzene : chloroform (24:2 v/v).

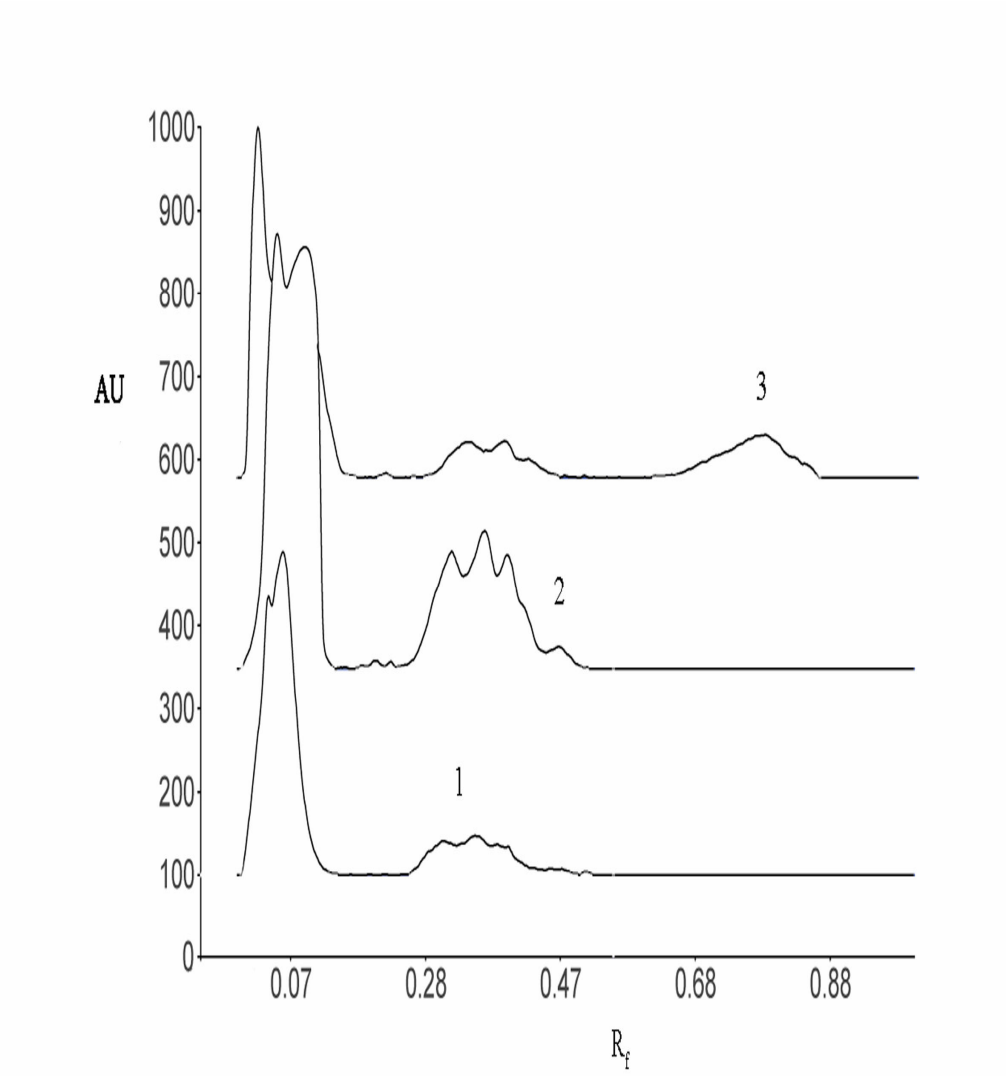


Figure 3.4. HPTLC densitograms of USC in RBO sample (TLC-2) 1.sterylester 2.wax 3. squalene, detection before charring at λ=214nm after charring steryl esters, wax at λ = 439nm, mobile phase petroleum ether : diethyl ether (25:0.5v/v).

Table 3.6 USC in CRBO and RRBO.

USC	CRBO [mg/g][a)]	RRBO [mg/g] [a)]
Sterol	11.20 ± 0.19	10.80 ± 0.37
Oryzanol	18.80 ± 0.21	13.80 ± 0.12
Tocols	2.00 ± 0.07	1.40 ± 0.03
Squalene	0.40 ± 0.01	0.48 ± 0.02
Steryl esters	7.20 ± 0.22	7.00 ± 0.30
Wax	13.20 ± 0.24	ND [b)]

a) Mean ± SE, n=3

b) ND = Not Detected

3.2 Effect of Saponification on Composition of Unsaponifiable Matter in Rice Bran Oil

USM is estimated by following recommended methods such as Official methods and recommended practices of the AOCS, Official Methods of AOAC and IUPAC. All the recommended methods for the estimation of USM refers the use of high concentration of alkali (saponification) under high temperature. As evidenced from many reports high alkali concentration and high temperature causes degradation and lose of compounds. Oryzanol which is a unique USC in RBO has been greatly affected by high concentration of alkali as evidenced from earlier reports. Because of the affinity of oryzanol towards alkali, during chemical refining almost oryzanol lost through soap stock. Saponification at high temperature causes degradation of tocols yielding lower amount in USM. Based on these facts, changes occurring in USC were investigated. The term USC referred here is those constituents quantified directly from oil by HPTLC method. USM is defined as those constituents obtained gravimetrically after saponification.

Food laws prescribe limit to certain parameters such as color value, wax content USM etc. Refined oil which would not meet the quality parameters prescribed by food laws could not be sold as edible oil. Though RBO has been recognized as healthy dietary oil there are some limitations to physically refined oil, in terms of USM content. Physical refined oil is reported to have high concentration of USC than chemical refined oil and the USM content exceed the limit prescribed by food laws. Because of this reason the industrial physical refining process is limited and there by lessen use of physical refined oil. Food laws such as PFA, Codex Alimentarius Commission prescribes 3.5% as the maximum limit and now it is revised by PFA as 4.5% without any scientific support. At this context, the method of estimation of USM should be validated in terms of its reaction conditions and at various dimensions. The changes occurring

in USC should be analyzed quantitatively. A comparison of constituents which are directly quantified from RBO by HPTLC method and that in USM obtained after saponification (USM) is needed. The following section analyzes the above objectives.

3.2.1. Quantification of constituents in USM of CRBO and RRBO by HPTLC

USM sample is prepared according to AOCS procedure and made up in chloroform for analysis. Direct oil estimation of USC have been performed by using two solvent systems: sterols, oryzanols and tocols separated in benzene:chloroform (24:2v/v) (TLC-1) : and squalene, steryl esters and wax in petroleum ether: diethyl ether (25:0.5 v/v) (TLC-2) as described in the previous section (3.1). In order to compare the results same method is followed for the estimation of USM components.

In TLC-1 it is found that, along with sterols, oryzanols and tocols an unknown peak detected at an R_f of 0.2. Using standards, it was identified as triterpene alcohol and from calibration graph, triterpene alcohol has been quantified. Table 3.7 shows the standardization parameters of lupeol which is used as standard for triterpene alcohol. The solvent system petroleum ether: diethyl ether is used for the separation and estimations of squalene, steryl esters and wax in TLC-2 of USM. HPTLC densitogram of TLC-1 is shown in Figure 3.5 and TLC- 2 is same as that done in direct oil estimation (Figure 3.4).

Table 3.7 Parameters evaluated by HPTLC for the standardization of lupeol

Parameters	Lupeol
R_f[a]	0.2±0.01
Linear regression, r	0.99836
RSD [%]	3.72
Linear range[μg]	0.5-4 μg
Regression equation	Y= 601.07x+37.64
LOD	18 ng
LOQ	61 ng
Recovery %	98.85±1.2

The method applied for the estimation of USM constituents is same as that done for direct estimation of constituents in oil. But one difference noticed that even after the estimation of those constituents achieved by direct estimation of oil could not account the total weight percentage of USM. Therefore the study designed to isolate, characterize and quantify rest of the constituents in USM. Thus the composition of USM appeared to be more complex than that of USC. It could be seen that there are tremendous variations between USC and USM in terms of their quality and quantity.

3.2.2 Characterization of unknown compounds in USM by spectroscopic techniques

Isolation of unknown compounds from USM has been done by preparative TLC using the solvent system hexane: diethyl ether: acetic acid in the ratio 8:2:0.1

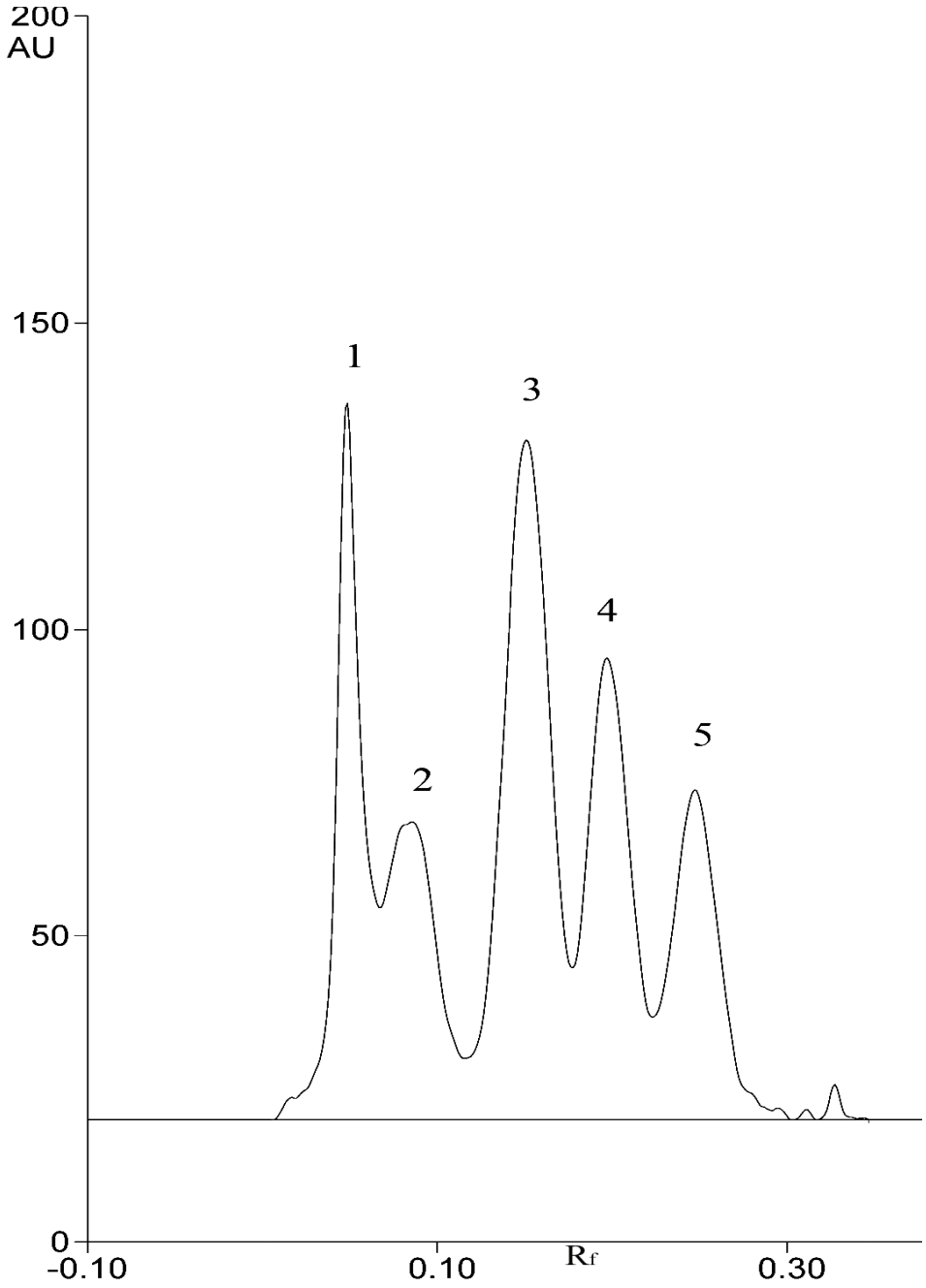


Figure 3.5. HPTLC densitogram of USM:TLC-1. Peak:1, base spot; peak 2, sterol; peak 3, oryzanol; peak 4, triterpene alcohols; peak 5, tocols. Mobile phase: benzene: chloroform (24:2, v/v)

(v/v/v) by comparing with standards. The band corresponding to the R_f 0.56 is separated preparatively, eluted from silica gel using chloroform, evaporated and checked in TLC for its purity. The extracted sample found separated in toluene:chloroform in the ratio 7:3 (v/v) at an R_f of 0.52 (Compound 1) and 0.68 (Compound 2).

Compound 1 is characterized by FTIR, ^{1}HNMR, ^{13}CNMR and mass spectrometric methods. The ^{1}HNMR showed a characteristic singlet signal at 9.92δ corresponding to aldehydic proton, olefinic and methylene protons showed delta values at 5.36 and 2.77 δ (br t, *J*=10Hz) respectively. The methylene protons adjacent to carbonyl carbon showed a signal at 2.4 δ (t, 2H) and the spectrum showed a strong aliphatic region. ^{13}C NMR showed values δ: 180 (C=O), 129(-CH=CH-), 34 (-CH_2), 29 (-CH_3). FTIR spectra showed the characteristic peak of aldehydic –CO stretching at 1717 cm^{-1} corresponding to C=O vibration, two moderately intense bands at 2851 cm^{-1} and 2929 cm^{-1} which corresponds to aldehyde C-H stretching and at 1465 cm^{-1}C-H bending. GC-MS analysis showed $[M]^+$ peaks at m/z, 212, 240, 268, 266 and 264 and peaks corresponding to $[M-18]^+$, $[M-44]^+$ confirming the presence of long chain aldehydes C_{14}, C_{16}, C_{18}, $C_{18:1}$, $C_{18:2}$ respectively. Thus the spectroscopic data confirmed the presence of fatty aldehydes.

3.2.3. Quantification of fatty aldehydes by GC

Characterization of aldehydes in USM leads to its quantification and by using HPTLC it cannot be quantified and hence GC method was optimized. Aldehyde in USM separated from other components as described in 3.2.2. and the sample has been methylated using 2% methanolic sulphuric acid. During methylation heptadecanoic acid methyl ester is added as internal standard to quantify the dimethyl acetals of aldehydes. The dimethyl acetal composition of fatty aldehydes in CRBO and RRBO USM is shown

in Table 3.8 and its chromatogram in Figure 3.6.The values obtained in GC analysis showed relative % of $C_{18:1}$ is more in CRBO USM. USM of RRBO showed higher percentage of C_{16}. Contribution of $C_{18:1}$ gets decreased in refined may be due to its volatile nature during refining process. Moreover C_{16} get increased from 30.66% (CRBO) to 61.73% (RRBO).

Vali et al reported a resinous matter separated from rice bran wax which is proved as aldehydes in saponified samples [175]. Usually refined oil is devoid of aldehydes since it undergoes drastic refining conditions that lead to the removal of aldehydes.

Table 3.8 Relative percentage by GC analysis of fatty aldehydes obtained from USM of CRBO and RRBO

Aldehyde	R_t	CRBO	RRBO
C_{14}	1.8	1.21 ± 0.01	3.36 ± 0.40
C_{16}	2.8	30.66 ± 0.53	61.73 ± 0.81
C_{18}	4.2	3.71 ± 0.18	6.67 ± 0.06
$C_{18:1}$	4.5	47.42 ± 0.93	25.79 ± 0.86
$C_{18:2}$	4.9	17.00 ± 0.36	2.44 ± 0.28

[a] Mean ± SE (n =3)

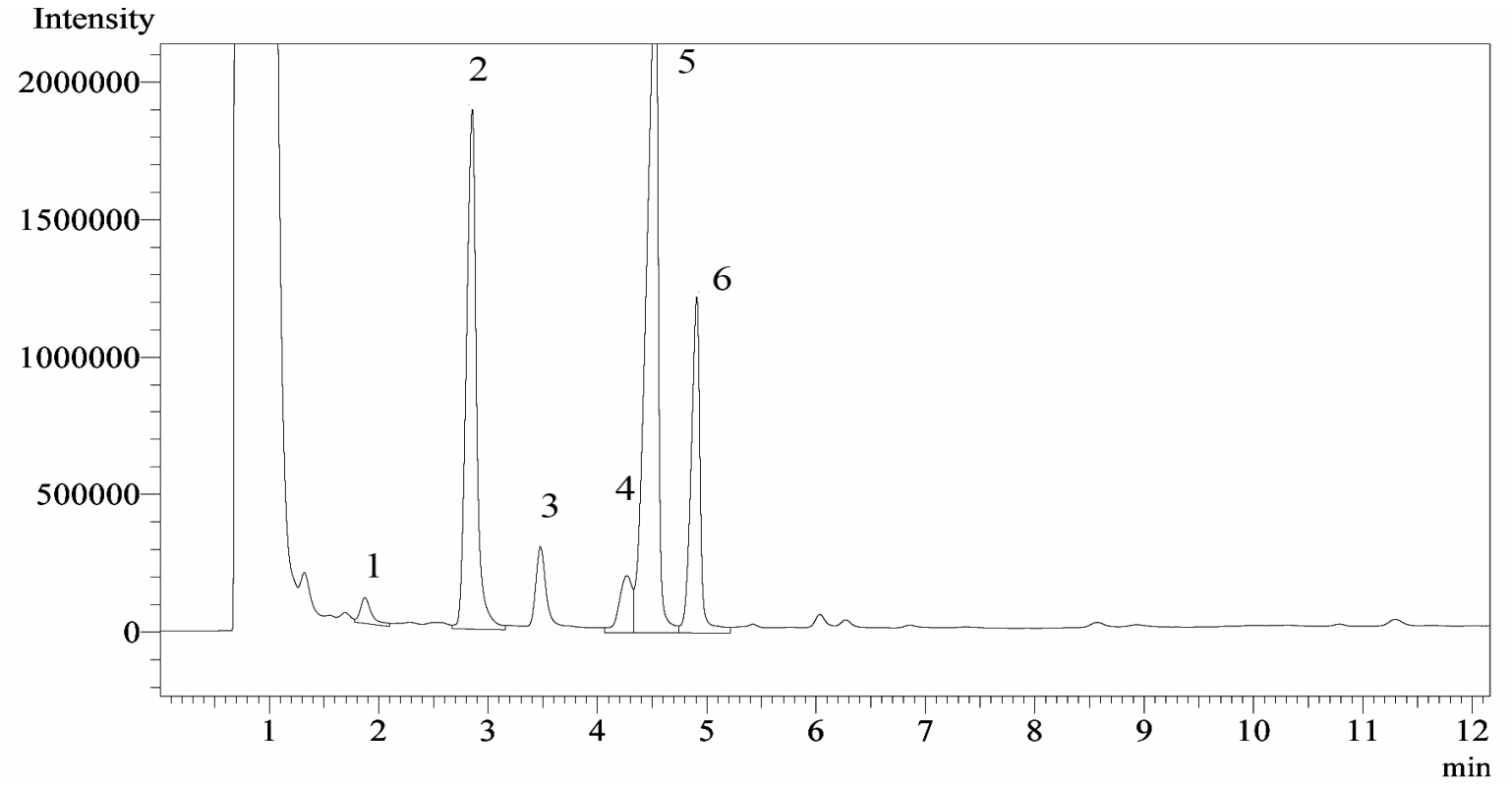


Figure 3.6. Gas chromatogram of dimethyl acetals of fatty aldehydes 1. C_{14} 2. C_{16} 3. C_{17} (internal standard) 4. C_{18} 5. $C_{18:1}$ 6. $C_{18:2}$ obtained from USM of RBO.

3.2.4 Characterization of Compound 2 by spectroscopic techniques

Compound 2 isolated from USM has been subjected to spectral analysis FT-IR, ^{1}HNMR, ^{13}CNMR and Mass spectrometry.

^{1}H-NMR (300MHz $CDCl_3$): δ 3.64 (2H, t, J = 6.7 Hz), 1.59 (2H, m, CH2–2), 1.25 [(CH2)n], 0.88 (3H, t, J =6.9 Hz, CH3) ppm. The compound showed a multiplet at δ1.59 corresponds to $–CH_2$ protons, a triplet at δ3.64 showing the presence of hydroxyl group, singlet at δ1.25-1.37.

^{13}C NMR in CDCl3 produced 15 carbon signals at % 63.08, 32.77, 31.91, 30.33, 29.87, 29.68, 29.59, 29.68, 29.42, 29.34, 26.44, 25.99, 25.71, 25.41,14.10 and 13.98 for methylene attached to hydroxyl group, all methylenes, and one methyl carbon, respectively.

FTIR spectrum displayed intense absorption bands at 3338, 2916, 2848, 1465, 1257 cm^{1}, of which 3338 cm^{-1} indicating the presence of hydroxyl group, 2916cm^{-1} and 2848cm^{1} C-H stretching frequencies.

The spectral values are similar to that reported earlier which shows single fatty alcohol or a mixture of alcohol [176-177]. GC and GC-MS showed the compound is a mixture of fatty alcohols ranges from C_{24}-C_{34} and the individual alcohols identified were C_{24}, C_{26}, C_{28}, C_{30}, C_{32} and C_{34}. The mass fragmentation pattern of aliphatic alcohols evidenced that it is difficult to get molecular ion peak of the high molecular weight aliphatic primary alcohols as it is too active to give [M+] ion. GC-MS of the sample showed characteristic peak corresponds to $[M-18]^{+}$ by loss of a water molecule and $[M-46]^{+}$ by loss of an ethene and a water molecule by rearrangement. Its relative area percentage by GC analysis and GC-MS is shown in Table 3.9 and the gas chromatogram of alcohols is shown in the Figure 3.7. From the spectral and GC analysis policosanol USM is similar to that reported in rice bran wax [178].

Table 3.9 GC and GC-MS data of policosanol from USM of RRBO

Peak	compound	R_t	m/z	molecular weight	Relative area %[a] by GC
1	tetracosanol, C_{24}	5.0	336, 308	354	12.83 ± 0.08
2	hexacosanol,C_{26}	8.7	364, 336	382	13.33 ± 0.65
3	octacosanol,C_{28}	11.0	392, 364	410	18.07 ± 0.55
4	triacontanol,C_{30}	13.8	420, 392	438	26.66 ± 0.65
5	duotriacontanol,C_{32}	18.4	448, 420	466	23.70 ± 0.43
6	tetratriacontanol,C_{34}	22.4	476, 448	494	5.88 ± 0.25

[a] Mean ± SE (n =3)

Policosanol in USM is quantified by HPTLC and the standardization parameters were shown in Table 3.10. Methylated USM sample was separated using the solvent system hexane: diethylether: acetic acid (8:2:0.1 v/v/v) and scanned at wavelength 206 nm for the quantification of policosanol. The densitogram is shown in Figure 3.8.

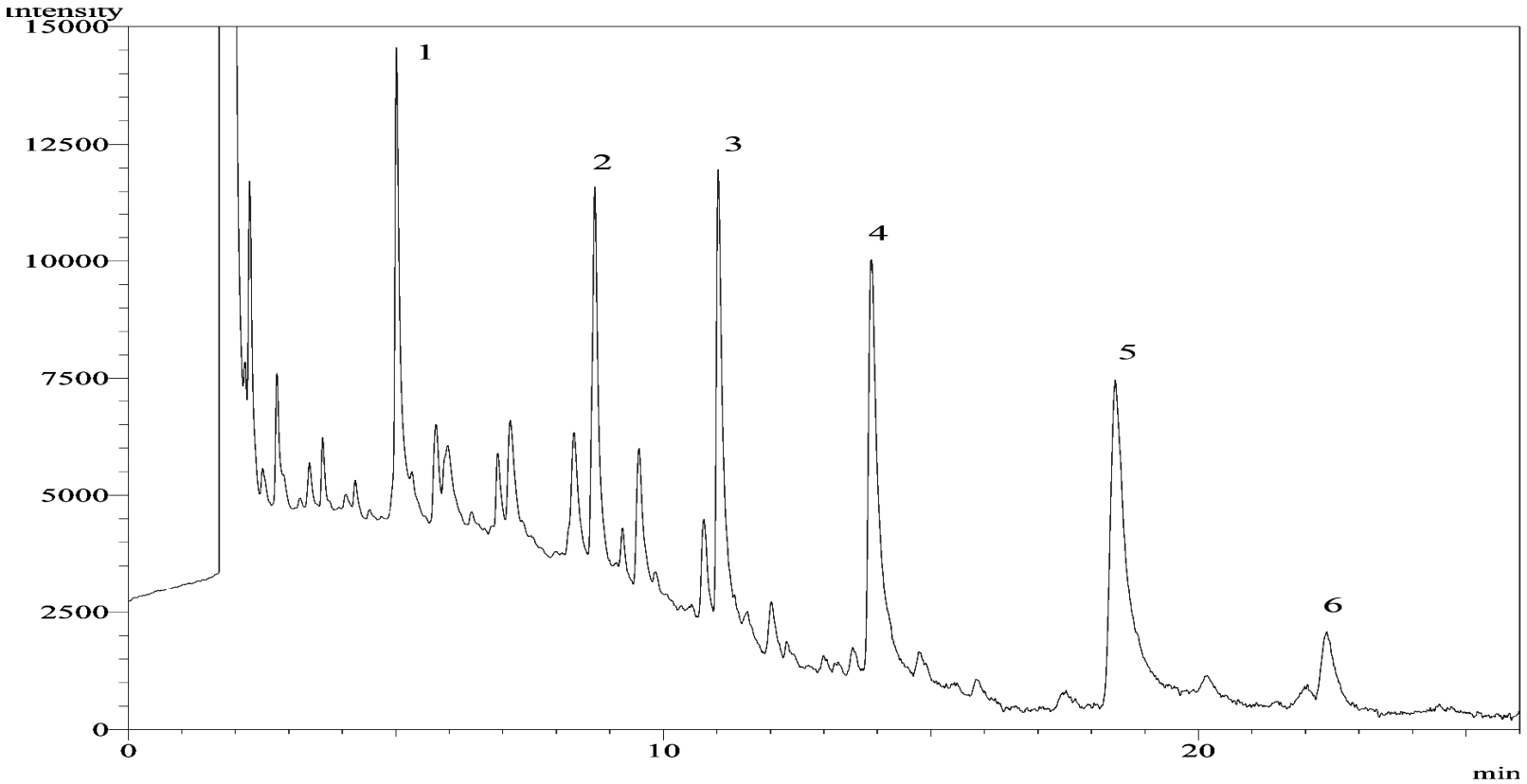


Figure 3.7 Gas chromatogram of fatty alcohol mixture 1. tetracosanol (C_{24}) 2. hexacosanol (C_{26}) 3. octacosanol (C_{28}) 4. triacontanol (C_{30}) 5. duotriacontanol (C_{32}) 6. tetratriacontanol (C_{34}) obtained from USM of RBO.

Table 3.10 Standardization parameters of policosanol

Standardization parameters	Policosanol
R_f	0.5±0.02
Linear regression, r	0.94252
RSD [%]	4.77
Linear range[µg]	11-21 µg
Regression equation	Y= 16.26x+179.21
LOD	86 ng
LOQ	289 ng
Recovery %	97.45±1.9

[a] Mean ± SE (n =3)

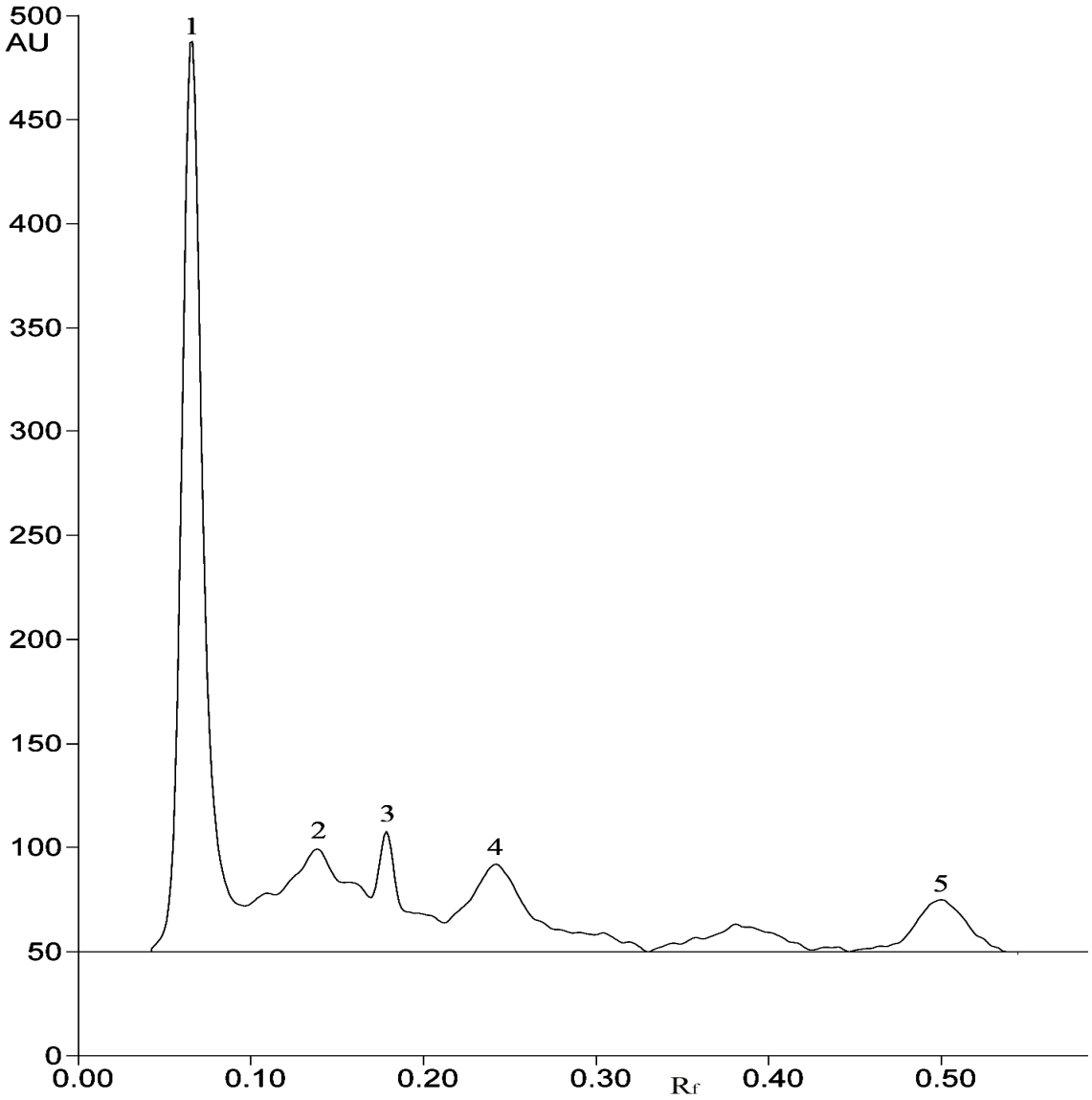


Figure 3.8 HPTLC densitogram of TLC-3. Peak:1, base spot peak 2, sterol; 3, oryzanol; 4, triterpene alcohols; 5, policosanol. Mobile phase hexane: diethyl ether: acetic acid 8:2:0.1(v/v/v).

3.2.5 Contribution of Policosanol and fatty aldehydes as major constituents in USM of RBO

Detailed investigation on USM of RBO revealed policosanol and fatty aldehydes are the major constituents contributing newly to the weight percentage of USM. The previous reports showed policosanol as an unsaponifiable component in certain oils. A recent report on unsaponifiable fraction of pomegranate seed oil showed higher amount of policosanol [179]. Similarly Sakouhi et al reported unsaponifiable fraction of olive oil contains policosanol as the major constituent [180]. The other sources of policosanol are wheat germ oil, grain sorghum kernels, dried distillers grain, sugar cane wax etc [181-182]. An important observation arouse from the major reports about policosanol is that wax is the main source of policosanol, which are esters of higher fatty alcohols and higher fatty acids. The fatty alcohols in wax get cleaved during hydrolysis there by forming a group of fatty alcohols called policosanol. Ester bond is more prone to hydrolysis under high temperature and alkaline conditions. Therefore, occurrence of policosanol in USM is due to the hydrolysis of wax during saponification. CRBO had high amount of wax which would give high percentage of policosanol in USM. HPTLC analysis showed 43.39% of policosanol in USM of CRBO on USM weight basis. While considering policosanol content in RRBO a problem arises whether policosanol is present in oil in free form rather than part of wax. Because, RRBO taken for the present study does not contain wax but it accounted 28.46% of policosanol showing oil contains free policosanol. It is by this reason CRBO contain higher amount of policosanol (both from wax and free alcohol) than RRBO.

Table 3.11 and 3.12 shows the analytical value of policosanol in USM of both crude and refined oil. Some of the reports explains the presence of free alcohol which are part of policosanol such as octacosanol (C28), triacontanol (C30) etc [182-183]. The presence of free policosanol in RRBO therefore could be explained as unutilized

ones for biosynthesis of wax [178]. In the present study, free policosanol in CRBO and RRBO could not be quantified directly by HPTLC because of the complexity of neutral lipids as policosanol move together with major saponifiable TAG. The reported method for the estimation of policosanol is capillary GC method by Wang et al from saponified sample of rice bran wax, using a Varian CP-sil 8 CB column [178]. In short policosanol as a major constituent in USM of RBO and as free form in oil has not been reported before and its presence in oil increased the nutritional quality of oil, as they possess various biological activities which add a new dimension to the health benefits of RBO [184].

Aldehydes are the other major unsaponifiable constituent contributed to high percentage of USM in RBO. The results showed that USM from CRBO and RRBO contained 13.23% and 8.10% fatty aldehydes respectively. Long chain aldehyde has not been reported until now in USM from RRBO. Fatty aldehydes in USM are presumed to be derived either from ether linked triacylglycerols or in free form [185-186] and its detailed investigation is explained in section 3.3.

Table 3.11 Comparison of compositions of the Unsaponifiable Constituents in crude rice bran oil and of unsaponifiable matter obtained by saponification

Constituents	#USC from CRBO		##USM from CRBO	
	Fractional wt of constituents (mg/g)	Relative % of USC	Fractional wt of constituents (mg/g)	Relative % of constituents
Sterols	12.30	22.64±1.20 [a]	14.10	23.77 ± 0.64 [b]
Oryzanols	18.20	33.64±1.78 [a]	0.15	0.25 ± 0.01 [b]
Tocols	2.12	3.91±0.09 [a]	0.39	0.66 ± 0.06 [b]
Squalene	0.36	0.66±0.07 [a]	0.15	0.26 ± 0.02 [b]
Wax	13.80	25.40±0.57 [a]	0.02	0.03 ± 0.01 [b]
Steryl esters	7.40	13.59±0.45 [a]	0.42	0.71 ± 0.06 [b]
Triterpene Alcohols	-	-	4.60	7.65 ± 0.25
Policosanol	-	-	25.80	43.39 ± 1.71
Fatty aldehydes	-	-	7.90	13.23 ± 0.18
Potassium salt of oryzanols	-	-	4.00	6.80 ± 0.17
Total USM % by HPTLC method	54.18	100±4.17	57.53	96.75 ± 3.11
Total USM wt% (AOCS) method	-	-	59.50	100

#USC-Unsaponifiable Constituents estimated directly in crude rice bran oil (CRBO).
##USM-Unsaponifiable matter obtained by saponification (AOCS) and further analyzed for its individual constituents.
Values within the same row with different superscripts indicate that mean values are significantly different (Tukeys Multiple-range test, $P<0.05$).

Table 3.12 Comparison of compositions of Unsaponifiable Constituents in refined rice bran oil and of unsaponifiable matter obtained by saponification.

Constituents	# USC from RRBO		##USM from RRBO	
	Fractional wt of constituents (mg/g)	Relative % of constituents	Fractional wt constituents (mg/g)	Relative% of constituents
Sterols	11.60	33.42±2.15[a]	14.80	36.79 ± 1.39[b]
Oryzanols	14.40	41.48±2.08[a]	0.02	0.05 ± 0.07[b]
Tocols	1.40	3.98±0.22[a]	0.10	0.22 ± 0.06[b]
Squalene	0.50	1.44±0.16[a]	0.10	0.25 ± 0.06[b]
Wax	ND	ND	ND	ND
Steryl esters	6.90	19.73±1.16[a]	0.50	1.10 ± 0.14[b]
Triterpene Alcohols	-	-	3.80	9.50 ± 0.13
Policosanol	-	-	11.50	28.46 ± 0.25
Fatty aldehydes	-	-	3.30	8.10 ± 0.12
Potassium salt of oryzanols	-	-	3.00	7.53 ± 0.08
Total USM % by HPTLC method	34.80	100±4.77	37.12	92.00 ± 2.31
Total USM wt% (AOCS method)	-	-	40.30	100

#USC-Unsaponifiable Constituent estimated directly in refined rice bran oil (RRBO).
##USM-Unsaponifiable matter obtained by saponification (AOCS) and further analyzed for its individual constituents. Values within the same row with different superscripts indicate that mean values are significantly different (Tukeys Multiple-range test, $P<0.05$)

3.2.6 Variation in the composition of USC in oil by direct estimation and of USM obtained by saponification method

The results presented in Table 3.11 and 3.12 afford comparison of composition of USC in CRBO and RRBO with that of the corresponding USM obtained by saponification method. RBO is exceptionally rich in unsaponifiables that may account for 4-6% by weight of the oil [149] as compared to the normal range of 0.5-2% in other edible oils. It is evident from the results that most of the constituents that are considered as unsaponifiables has undergone drastic changes in their quantity and quality during saponification. New compounds that are not detected in oil are also appeared in USM. The total USC in CRBO was 5.42% by weight and USM by saponification from the same oil was 5.95% showing a substantial increase by weight.

Among the unsaponifiables, oryzanol underwent the most drastic changes in USM. Oryzanols contributed 33.64% (1.82% in oil) of the USC in CRBO and their content came down to negligible amount of 0.25% (0.015% in oil) in USM, indicating that more than 99% of oryzanol is lost during alkali saponification for the preparation of USM. The relative percentage of oryzanol in RRBO showed 41.48% (1.44% in oil) and 0.05% (0.002% in oil) in USM. Krishna et al reported, alkali treatment removed 93.0 to 94.60% of oryzanol from crude oil [53]. The alkali treatment causes partial lose of oryzanols through soap stock partially during chemical refining under drastic conditions [187]. A similar mechanism may be operating during saponification where the alkali concentration recommended is far higher than that used for chemical refining which explains the almost complete removal of oryzanols. A report on USM of RBO explained oryzanol and wax upon saponification yield greater amount of unsaponifiable fractions accounting to high percentage of USM [160]. But the present investigation thoroughly established oryzanol is not contributing to the high percentage of USM as evidenced from Tables 3.11 and 3.12. Sayer and Saunders reported 28% by weight of USM is contributed by oryzanol following the usual

recommended procedure for the estimation of USM [113 Sayer]. Krishna et al reported, physical refined oil having 1.06% of oryzanol yielded 4.5% of USM and suggested increased content of oryzanol is the cause of higher value of USM [160]. The same authors reported another study having oryzanol 1.1057-1.3902% yielded 2.7-3.2% USM [188] that is lower USM and reporting oryzanol is the cause of increase of USM. From this it is evident that even though same method was followed for the estimation having same content of oryzanol the value of USM changes without any reason.

Triterpene alcohols estimated in USM by the present HPTLC method is 7.65% of USM (0.46% in oil) of CRBO, which is formed by the hydrolysis of oryzanols since oryzanol is esters of ferulic acid and sterols or triterpene alcohols. RRBO yielded 9.5% (0.38% in oil) of triterpene alcohols in USM. Triterpene alcohol forms one of the important USC in oils such as sheanut oil, olive oil, tea seed oil etc. Krishna et al reported 0.86% of triterpene alcohol in physically RRBO [160]. Rukmini and Sugano et al reported cycloartenol and 24-methylene cycloartenol in unsaponifiable fraction of RBO [189-190].

Potassium salt of oryzanols contributed about 6.80% of USM from CRBO. Occurrence of potassium salt of oryzanols in USM may be due to the reaction of potassium with oryzanols during saponification, which got extracted in petroleum ether during the preparation of USM. A model experiment conducted using standard oryzanols supports this conclusion.

Wax and steryl esters together accounted 1.38% and 0.74% in CRBO is reduced to 0.002 and 0.042% respectively in USM of CRBO. In the case of RRBO, wax is not in a detectable amount while steryl esters of RRBO is 0.69% and 0.05% in USM. Gunawan et al reported fatty acid steryl esters and wax esters together accounted 4% in CRBO, among them 2.8–3.2% steryl esters and 1.2–1.4% wax by GC analysis [191]. Ito et al showed that steryl esters separated from neutral lipid fraction of RBO had 6.9% fatty acid

steryl esters, which are 4-desmethylsteryl esters of fatty acid [192]. From the values by direct analysis and saponified samples there are variations in the quantitative values of steryl esters, after saponification, both in CRBO and in RRBO.

Tocols are group of compounds which are major antioxidants in RBO. Comparing with sterols and oryzanol tocols are minor components in USM. Rajam et al reported amount of tocols in RBO ranges up to 0.2% and percentage of tocotrienols is higher [84]. Various reports regarding the effect of temperature on tocols shows that there are tremendous changes occurring in it chemical structure. Bruscatto and his co-authors reported, α-tocopherol which is higher in RBO got reduced to almost one third of its content after heating at 100^{o}C for 432 h. They also reported heating at 180^{o}C for 240 h lead to complete loss [193]. Vaidya et al reported heating causes degradation as well as decrease in content of tocols [194]. Prolonged heating at high temperature causes degradation of tocopherols which yields products such as α-tocopherolquinone, 4a,5-epoxy- α – tocopherolquinone and 7,8-epoxy- α tocopherolquinone [161]. Similarly concentration of alkali also has severe effects on tocols. A report on alkaline saponification by Czauderna et al showed the influence of alkaline saponification on αγδ-tocopherols and α-tocopherol acetates. They reported KOH treatment causes removal of tocopherols as evidenced from the recovery studies, by adding known amount of tocopherol in cow milk and bovine blood plasma. The results obtained showed marked decrease in the concentration of added tocopherols [195]. Ryynanen and his co-authors optimized three solvent mixtures for extracting tocols after optimized saponification reaction, as concentration of KOH has greater effect [196]. From the review of above reports the decrease in concentration of tocols in USM (after saponification) is due to high temperature and alkaline conditions. HPTLC analysis showed 0.212% of tocols in CRBO which is reduced to 0.039% in USM, similarly 0.14% in RRBO decreased to 0.01% in USM of RRBO.

Sterol accounted for 1.23% in CRBO and increased to 1.41% in CRBO USM. Similarly 1.16% of sterol in RRBO increased to 1.48% in RRBO USM. Sterol is the only constituent whose percentage increased after saponification. At this context increase in sterol concentration can be explained by correlating decrease in concentration of steryl esters. Steryl esters in presence of alkali undergo hydrolysis yielding sterol and salt of fatty acid and this lead to the increase in percentage of sterol in USM. Gunawan et al studied about the trans-cis fatty acid in fatty acid steryl esters from soybean oil deodorizer distillate by saponifying the sample to yield sterol and fatty acid for GC analysis [191]. This report supports that saponification of steryl esters causes hydrolysis at ester bond to yield sterol and fatty acid. There are reports on the quantitation of sterol and steryl esters in fortified food and beverages by GC after converting it into free sterol by saponification [197-198]. Therefore during the estimation of USM, steryl esters hydrolyzed to sterols and fatty acid, resulting in the increase of percentage of sterols.

Quantitation of squalene also showed changes in percentage after saponification both in CRBO and RRBO. In CRBO squalene accounted for 0.036% which got reduced to 0.015% in USM. Similarly in RRBO, 0.05% reduced to 0.01% in RRBO USM. Lau et al reported squalene which is a highly unsaturated hydrocarbon got partially destroyed during saponification and reduced in the isolated fraction [171].

3.2.7 Consequences of the estimation of USM in RBO by saponification method

The chemical reaction involved in USM estimation is saponification in which tri, di and mono acylglycerol's have been converted to potassium salt and retains other chemical compounds which could not form soap. The concentration of alkali and the temperature of the reaction mentioned by the recommended methods are very high that would cause changes in chemical moiety of bioactive phytochemicals. For example the major USC in RBO is oryzanol which has very

affinity to alkali and also tendency to lose through potassium salt of fatty acids that leads to alteration in quantitative results. Heat sensitive easily degradable compounds such as tocopherols and tocotrienols are also affected by the conditions of estimation.

Depending on the refining method, the percentage of USC varies and also changes in USM content. According to Indian food laws, such as PFA the maximum limit prescribed for the USM of chemical refined oil is, not more than 2.5% and that for physical refined RBO is not more than 3.5% which is recently revised as not more than 4.5% [120-121]. By practical evidences, it was observed that USM of RBO obtained by the recommended method is high and exceeds the limit of 3.5% but with in 4.5%. The changes that occurring to USC during saponification and their actual contribution in USM are not studied in qualitative as well as quantitative terms before. There are many reports dealing with the quantification of individual USC in CRBO and RRBO such as sterols, oryzanols and tocols by using HPLC, GC, GC-MS etc [199-200]. But characterization of constituents in USM (by saponification method), including identification and quantification as together has not been attempted before. One of the previous report showed that wax and oryzanol are the two constituents responsible for the high percentage of USM in RBO [160] due to its high content.

However overall review of the reports and the analytical data obtained by the present analysis confirms that estimation of USM by the currently practiced method [123] based on alkali saponification, when applied to RBO distort the results in various dimensions. Saponification causes qualitative as well as quantitative changes in individual components of USM which is evident from the comparison of direct oil estimation and USM obtained by saponification. An important inference is constituents which are considered as unsaponifiable is not contributing significant amount in the percentage of USM after saponification. Instead new compounds are contributing to the weight percentage of USM. From

the results it is evident that profound variations are occurring and there by the values of USM got distorted.

In conclusion RBO contains USC which are very sensitive to alkali which causes deviation in results and do not reflect the true quantitative values of unsaponifiables by saponification method. HPTLC quantification as reported in the previous chapter (3.1) is a new analytical approach to estimate the total USC in RBO in its natural form.

3.3 Detection, Isolation and Characterization of *sn*-2 alk-1'-enyl ethers of Glycerol from Rice Bran Oil

Occurrence of fatty aldehydes in edible oils including RBO is reported before [201-202]. However in the present study, as part of the detailed characterization of USC/USM, fatty aldehydes have been identified and quantified from USM of RBO as discussed in the previous section (3.2). The results thus obtained prompted us to investigate the source of fatty aldehydes in RBO. The fact that aldehydes are detected in USM and their absence in USC presumed to be forming during saponification of acylglycerols. The investigation therefore is designed to examine first, the TAG fraction of RBO which is a neutral lipid of high percentage. Therefore the important objective of this chapter is isolation, identification and characterization of TAG yielding aldehyde by chromatographic and spectral techniques such as ^{1}HNMR, ^{13}CNMR, FT-IR, and Mass spectrometry.

3.3.1 Isolation of ether lipid from RBO

Isolation and characterization of ether lipid have been achieved by repeated column chromatographic purification of RRBO by using different ratios of hexane and diethyl ether. The fraction corresponding to 80:20 is rechromatographed by using various fractions of hexane and diethyl ether and finally column was eluted with chloroform. Each fraction is subjected to spectral analysis and the chloroform fraction showed values peculiar spectroscopic values while comparing with other fractions. The following are the spectral data by ^{1}HNMR, FT-IR, ^{13}CNMR and Mass spectrometric analysis.

^{1}HNMR: ^{1}HNMR (500 MHz, $CDCl_3$) showed following signals (s, d, t and m indicate singlet, doublet, triplet and multiplet):δ/ppm: 5.83(1H, d, J=15Hz, H-1^{1}), 5.26 (1H, d, J= Hz, H-2^{1}), 5.09 (t, J=10Hz, CH=CH), 4.23 (1H, q, J=6Hz, J=6Hz,

H-2[1]), 4.11 (1H,m, H-2), 3.91(2H,dd, J=3.5Hz, J=5Hz, H-3a,3b), 3.71 (2H, d, J=5.5Hz, H-1a,1b), 2.06 (m,CH_2CH=CH-CH_2), 1.62 (m, CH_2),0.97 (2H, q, J=6.5Hz, J=7Hz).

^{13}C NMR δ: 173(C-1), 129 (-CH=CH-), 77 (C-O-C), 33 (-CH_2), 29 (-CH_3).

FT-IR: v_{max} = 2854 and 2923 (CH stretching), 1743 (C=O), 1459(-CH_2), 1377(-CH_3), 1161(OC-O).

Mass Spectra: Mass spectra of ether lipid by Direct Inlet Probe method showed m/z of fragment ions at 601, 575, 337, 339, 313 and 285 which showed the presence of myristic ($C_{14:0}$), palmitic($C_{16:0}$), palmitoleic ($C_{16:1}$), stearic($C_{18:0}$), oleic ($C_{18:1}$) and linolenic ($C_{18:2}$) as the bound fatty acid in the *sn*-1,3 position of TAG with ether bond at the *sn*-2 position. The data obtained here is agreement with a previous report, regarding HPLC-Atmospheric Pressure Chemical Ionization mass spectrometric analysis of oil samples [136].

3.3.2 Isolation of fatty aldehydes from TAG fraction of RBO

The ether lipid fraction isolated as explained above was saponified under the same conditions under which USM was prepared. After saponification the sample was extracted with petroleum ether, water washed, evaporated and the fraction was subjected to spectral studies. The spectral values obtained are as follows:

^{1}HNMR: ^{1}HNMR showed a singlet signal at 9.93 δ characteristic of aldehydic proton, olefinic protons at δ 5.38, methylene protons at 2.76 (br t, *J*=10Hz), methylene protons adjacent to carbonyl carbon 2.4 (t, 2H) and with a strong aliphatic region.

^{13}C NMR δ : 180 (C=O), 129(-CH=CH-), 34 (-CH_2), 29 (-CH_3).

FT-IR: FT-IR spectra showed the characteristic peak of aldehyde at 1717 cm^{-1} corresponding to C=O vibration, two moderately intense bands at 2851 cm^{-1} and 2929 cm^{-1} corresponds to aldehyde C-H stretching and at C-H bending 1465 cm^{-1}.

GC-MS: GC-MS of fatty aldehydes showed $[M]^+$ peaks at m/z, 212, 240, 268, 266 and 264 corresponding to $[M-18]^+$, $[M-44]^+$ suggesting the presence of aldehyde of C_{14}, C_{16}, C_{18}, $C_{18:1}$, $C_{18:2}$ respectively. The above spectral values are similar to those obtained from USM fraction.

3.3.3 Spectral evidence of ether linked TAG in RBO

Glycerol esterified to alkyl moiety to form an ether bond instead of an acyl bond are called ether lipid. This group of lipid generally termed as plasmologens having a vinyl ether bond at *sn*-1 position and a phospholipid at one of the carbon atom of glycerol [203]. Other major group are alkyl and alk-1-enyl glyceryl ethers having saturation and unsaturation on the alpha-beta carbon of *O*-ether linkage with glycerol [204]. Acyl glycerol with ether bonds are not common in edible oils.

^{1}HNMR showed characteristic peaks corresponding to three distinct regions of olefinic protons, aliphatic protons and protons born by carbon bound to oxygen. A doublet at 5.83δ with one hydrogen having the coupling constant of 15Hz corresponded to $H^{1'}$ and 5.26 δ corresponded to $H^{2'}$ of Figure 3.9 Fauconnot et al studied the chemical synthesis and characterization of structured polyunsaturated TAG using NMR and they reported delta values at 5.10-5.28 δ corresponding to olefinic (-CH=CH-) proton of 1,3-dipalmitoyl-2-arachidonoyl-glycerol [205]. In the present study the spectroscopic values at δ 5.83 and 5.26 showed the downfield shift showing attachment to an electronegative atom adjacent to olefinic proton which in turn to an oxygen atom. A pentet at 5.09 δ suggested olefinic proton on fatty chain attached to it in the upfield region compared to other values. A multiplet at 4.11δ (H-2), doublet at 3.91 δ and 3.71 δ

corresponded to glyceryl protons [206-207]. ^{1}HNMR is shown in Figure 3.10. ^{13}C NMR (Figure 3.11) showed the carbonyl stretching at 172.6-173.6 ppm indicating the presence of palmitic or oleic acid in *sn*-1,2,3 positions of glycerol. Simova et al studied ^{13}C NMR spectra in detail to quantify acyl positional distribution of oleoyl, palmitoyl combinations in *sn*-1,2,3 positions of TAG by analyzing values between 172-173 ppm [208]. Signal at 127.7-130.0 ppm corresponded to –CH=CH- which is in agreement with that of a previous report of Fauconnot et al [205] in which they reported olefinic signal at 127.4-132.5 ppm. The glyceryl group –CH2-CH-CH2 showed signal between 61.1-68.7 ppm and C-O-C at 77 ppm. The values at 33.8-34.11ppm corresponded to $–CH_2COO$ and the aliphatic carbons of $-CH_2CH_2CH_3$ at 31.37-31.77ppm. The signals at 28.9-29.6 ppm is indicative of $–(CH2)_n$, 27.04 ppm of carbon on –CH2 CH=CH, 24.7- 25.4 ppm of $–CH_2CH_2COO$, 22.4-22.5 ppm of CH_2CH_3 carbons and 13.9 ppm of CH_3 carbon.

The FT-IR spectral data (Figure 3.12) supported the presence of carbonyl functional groups with a sharp symmetric stretching at 1743 cm^{-1}, acyl CH_2 scissoring at 1459 cm^{-1}, terminal CH_3 bending mode at 1377 cm^{-1} and C-O-C stretching at 1161 cm^{-1}. A previous report on the characterization of TAG and DAG by atmospheric pressure chemical ionization mass spectrometry by Holcapek et al reported masses of characteristic fragment ions of TAG and also structure and notation of the fragment ions of TAG and DAG formed during mass analysis [136]. By comparing values of this report with results of the present fragmentation ions, it could be stated that fragmentation occurring in such a way that a positive ion was forming at the second position of glycerol carbon by cleaving the ether bond giving rise to ions at m/z 601, 575, 339, 313 and 285 corresponding to myristic ($C_{14:0}$), palmitic ($C_{16:0}$), stearic ($C_{18:0}$), oleic ($C_{18:1}$) and linolenic ($C_{18:2}$) at the *sn*-1 and *sn*-3 positions of glycerol [136]. This is in agreement with the values reported by Gorgas et al [209]. The present investigation thus strongly suggests the presence of ether lipids, as alk-1'-enyl ethers of glycerol.

After the spectroscopic characterization, the fraction is hydrolyzed to yield aldehydes to confirm the spectroscopic results. Zoellar et al demonstrated release of aldehydes on hydrolysis of ether lipids using strong alkali [4]. In order to confirm the formation of aldehydes [203] in USM, the ether lipid fraction is alkaline hydrolyzed [210]. Mild alkaline hydrolysis did not cleave ether bonds but the previous reports suggested that alkali metals highly favored the heterolytic cleavage of carbon-oxygen bond by chemical mode. In addition to this, presence of -CH=CH- adjacent to the ether bond is reported to stabilize the ion formed by electron transfer thereby forming aldehyde [211-212].Release of aldehydes by alkali hydrolysis of ether lipids further supported the spectral data.

The formation of aldehydes has been substantiated by spectroscopic data. ^{1}HNMR, ^{13}C NMR, FT-IR showed well correlation with that of aldehyde obtained from USM as explained in section 3.2.2. The spectral values confirms the presence of alk-1'-enyl glycerol ethers in RBO. However position of ether linkage in TAG could not be deduced from the results as discussed above and to substantiate the position of ether linkage it was hydrolyzed by controlled pancreatic lipase hydrolysis.

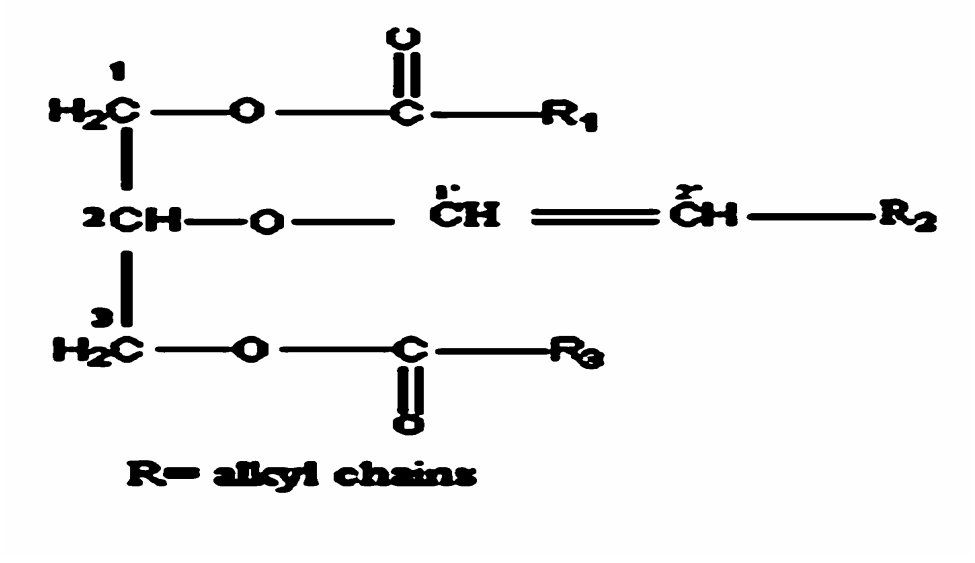


Figure 3.9 Proposed Structure of ether linked lipid isolated from RBO

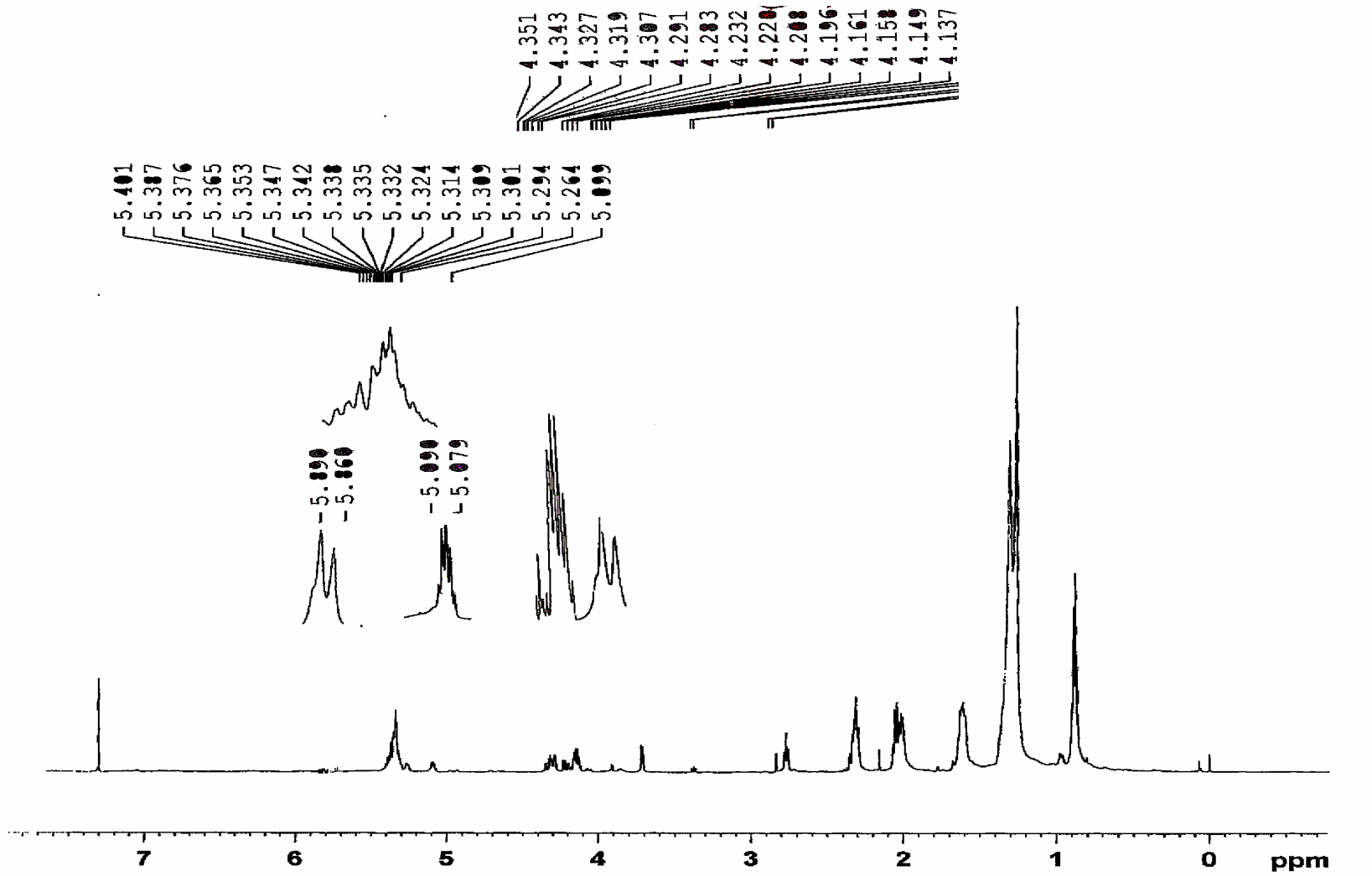


Figure 3.10 [1]HNMR spectra of ether lipid from RBO

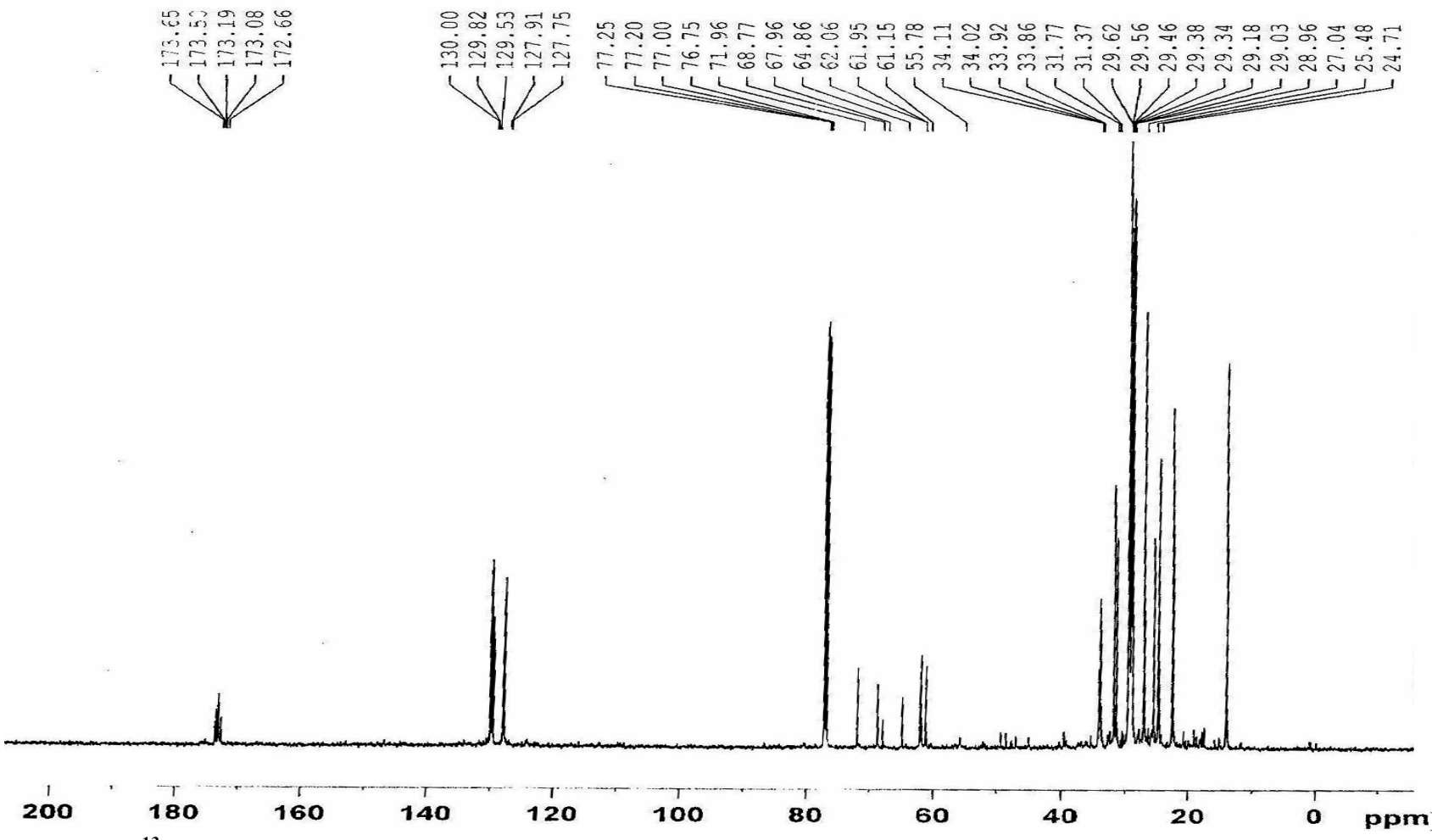


Figure 3.11 ^{13}CNMR of ether lipid from RBO

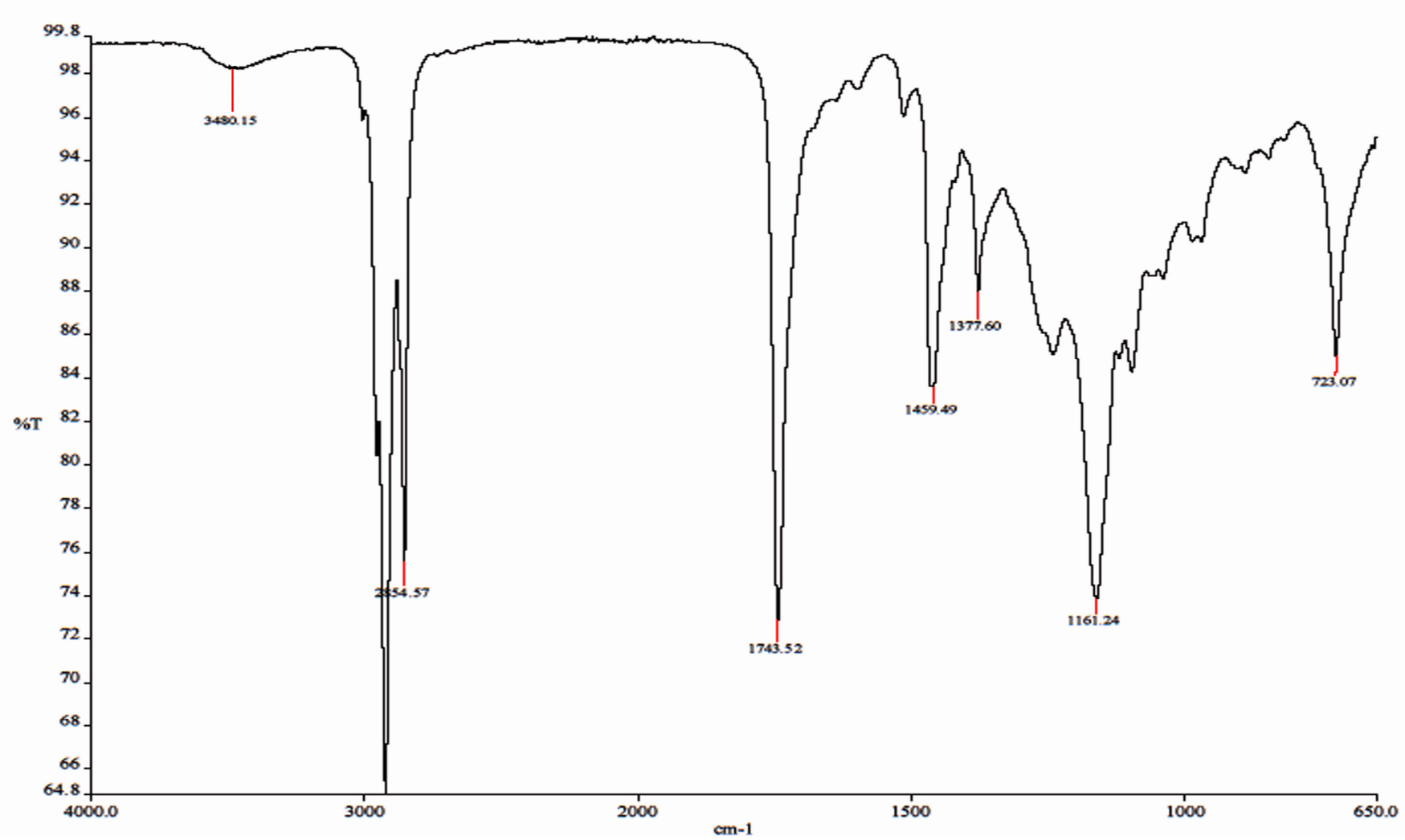


Figure 3.12 FT-IR of ether lipid from RBO

3.3.4 Pancreatic lipase hydrolysis

Having established the presence of ether linked TAG, this experiment is conducted to elucidate the position of ether bond in TAG using pancreatic lipase. Pancreatic lipase cleaves primary ester bonds (*sn*-1 and *sn*-3) preferentially when subjected to partial hydrolysis of TAG [213]. The reaction conditions have been optimized so as to occur 35% hydrolysis of TAG which is isolated from RBO by column chromatography. The hydrolyzed products in the reaction mixture are extracted with diethyl ether to obtain a mixture of TAG, DAG (*sn*-1, 2 and *sn*-1,3), MAG (*sn*-2). Further separation of individual constituents has been achieved by preparative TLC (silica coated glass plates) using hexane:diethylether:acetic acid (8:2:0.1 v/v/v). The bands are identified using standards and eluted with chloroform. The *sn*-2 MAG is methylated using 2% methanolic sulphuric acid, extracted with hexane and washed to analyze the alkyl chain composition by GC. It is observed that the fraction corresponding to *sn*-2 MAG is similar to aldehydes obtained from USM by GC analysis in its composition. Also each fraction was analyzed by FT-IR to observe the changes in characteristic peak of functional group. Among the fractions, *sn*-2-MAG showed an intense peak at 1215 cm^{-1} which corresponded to asymmetrical C-O-C stretching of vinyl ether [214] that confirmed the position of ether bond at *sn*-2 of glycerol back bone. The enzymatic hydrolysis of TAG and products are shown in Figure 3.13

For the subsequent confirmation of ether linked TAG the same fraction is hydrolyzed in presence of alkali at the same conditions from which USM has been prepared to confirm the release of fatty aldehydes. After hydrolysis the product extracted showed an intense peak at 1709 cm^{-1} in FT-IR, which is a characteristic peak of aldehyde =C=O group.

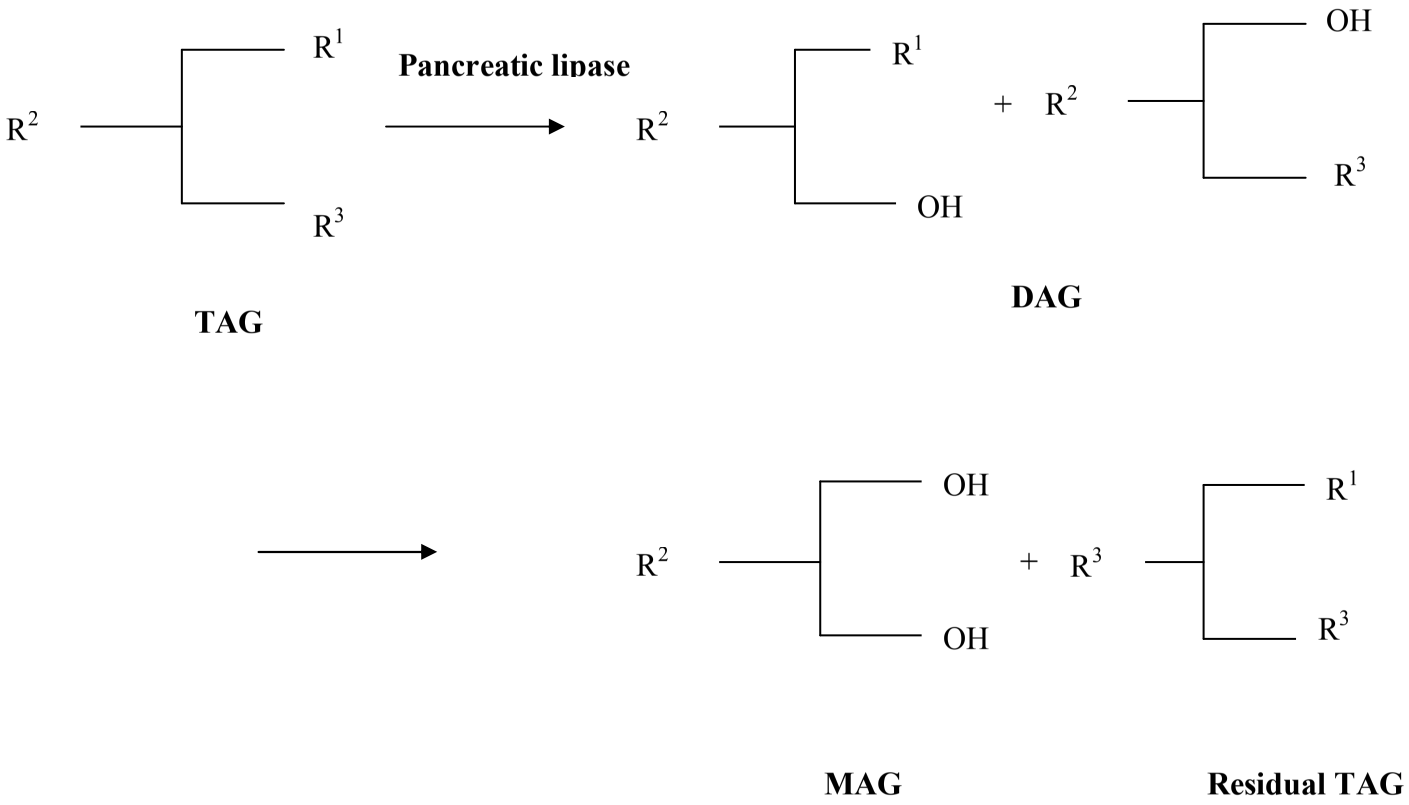


Figure 3.13 Pancreatic lipase hydrolysis of TAG and hydrolytic products

This substantiated the release of aldehyde on hydrolysis of ether lipids. The hydrolysis occurred here is heterolytic cleavage of bond between carbon and oxygen, in presence of alkali, which favors reductive cleavage of ethers. Ether bonds are very reactive to alkali [210] and the method followed for the USM estimation recommends high concentration of alkali (50% ethanolic KOH) which lead to the fast cleavage of ether bonds. It is reported that lipid having an ether bond with glycerol back bone and having unsaturation adjacent to ether bond lead to the formation of alkenal as the primary product which is unstable and rearranged to form aldehydes [203]. Figure3.14. explains the formation of aldehyde from enzyme hydrolyzed MAG. Spectra b of Figure 3.14 shows, standard MAG fatty acyl bond and 3.14 (c) corresponds to enzyme hydrolyzed MAG. While comparing both the spectra, it is noticed that an additional sharp peak at 1215 cm^{-1} for enzyme hydrolyzed MAG (spectra c). Figure 3.14. (a) shows the saponified sample of enzyme hydrolyzed TAG. By comparing (a) and (c), after saponification the sharp peak at 1215cm^{-1} disappeared, instead a peak at 1709 cm^{-1} has been observed which is characteristic stretching frequency of aldehyde that formed after hydrolysis of ether bond.

Earlier reports indicated presence of free aldehydes in CRBO [185] but its presence in refined oil is not reported. Doleschall et al reported that aldehydes are removed during deodorization step [215] of refining process. Refining at high temperature and vacuum removes aldehydes in refined oil. But the present study showed USM of RBO had aldehydes, which are formed from ether lipids during saponification. The amount of fatty aldehydes in CRBO is significantly greater than that in RRBO that supports the occurrence of free aldehydes before refining as reported earlier. Volatile nature of aldehydes is responsible for their reduction during deacidification and deodorization steps of refining. The structure of ether linked TAG based on the data obtained from the present investigation is proposed as shown in Figure 3.12.

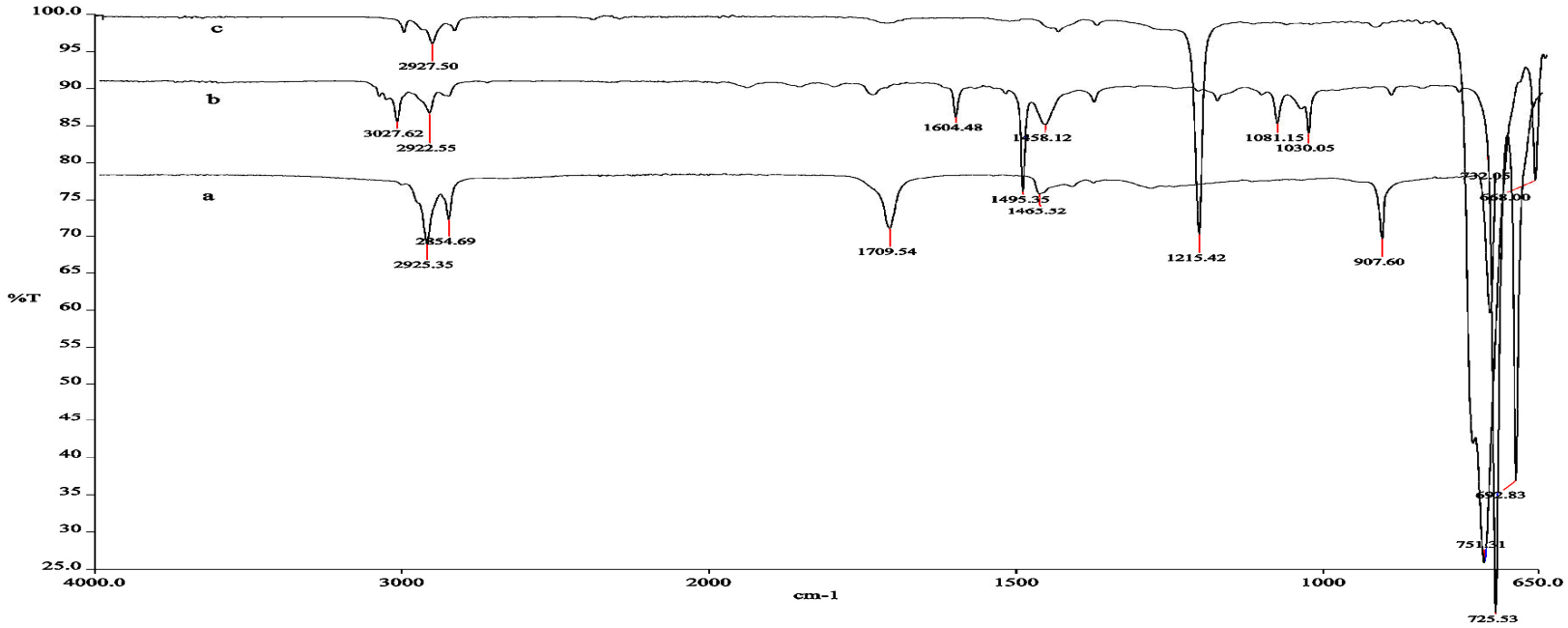


Figure 3.14. FTIR spectra of MG a) Fatty aldehyde obtained after saponification of enzyme hydrolyzed MG showing characteristic peak of aldehyde b) standard MG c) MG obtained after enzyme hydrolysis showing peak of ether linkage.

3.3.5 Biological significance of ether lipids

Ether lipids can act against singlet oxygen, peroxyl radical because of the presence of vinyl ether bond, a target of oxidative attack by radicals and hence protecting the other part of lipid against oxidative damage [216]. Ether lipids are reported to have biological functions such as cholesterol transport, reduction of radiation damage, suppression of tumor growth, increased haemopoesis, acceleration of wound healing, prevention of cataract etc [217]. It has been reported that dietary intake of ether glycolipids helps in the synthesize of membrane plasmalogen in most tissues [218]. Their deficiency is reported to be associated with diseases such as Zellweger syndrome, Alzheimer's, Down syndrome etc [219-221].

3.4 Quality of oils and unsaponifiables from paddy varieties

Indica and Japonica are the two rice subspecies differ in morphological traits having numerous varieties cultivated all over the world. Depending on the variety, the yield of rice grain varies. Rice grain constitutes 5-6% of rice bran, and the nutrients of rice are concentrated mostly in bran. The composition of rice bran depends on the method and degree of milling, variety, geo-climatic conditions etc. The oil content of different varieties of bran may also vary and therefore variations in the amount and composition of USC. Investigation on different varieties of bran with regard to its physical as well as chemical properties is limited. This section deals with the study of RBO extracted from bran of ten varieties of paddy collected from Regional Agricultural Research Station, Pattambi, Palakkad under Kerala Agricultural University. Paddy varieties used for the study are Annapoorna, Aiswarya, Geerakasala, Gandhakasala, Jyothi, Uma, RM-1, Makaram, Karuna and Varsha. The physical characteristics such as color value, cloud point; chemical analysis such as carotene, chlorophyll, saponification value, iodine value and FFA are measured. The USC in oil viz: oryzanol, tocols, sterol, squalene, steryl esters and wax have been quantified by newly developed HPTLC method.

3.4.1 Physico chemical characteristics of RBO from bran of different paddy varieties

3.4.1.1 Physical characteristics

The color of crude extracted oil varied and therefore its color value, carotene and chlorophyll have been measured by using Lovibond Tintometre. The results showed that varieties Annapoorna, Aiswarya and Makaram have higher red value; Jyothi, RM-1 and Varsha have higher yellow value. Table 3.13 and 3.14 presents the color value and cloud point. Figure 3.15 presents chlorophyll and carotene content of oils. The carotene content is higher for Jyothi, (85.49 ppm)

followed by Varsha (82.44 ppm) and Annapoorna (74.60 ppm). Chlorophyll content is greater for Jyothi variety. Cloud point of Gandhakasala is found to be higher, however others have comparatively similar values within the range of 44-49^{o} C. Malekisn et al reported the physical and chemical characteristics of rice bran varies with milling techniques and source of rice bran [222].

Table 3.13 Color value of oil from ten paddy varieties

Variety	Color value
Annnapoorna	7.3R + 7.9Y
Aiswarya	7.2R+48.2Y
Gandhakasala	6.0R+57.0Y
Geerakasala	5.3R+51.0Y
Jyothi	5.5R+70.0Y
Karuna	5.8R+29.9Y
Makaram	8.7R+42.2Y
RM-1	5.1R+70.0Y
Uma	6.4R+57.0Y
Varsha	7.0R+69.0Y

Table 3.14 Cloud point of oil from ten paddy varieties

Variety	Cloud point (^{0}C)
Annnapoorna	49±1.0
Aiswarya	45±1.0
Gandhakasala	51±1.0
Geerakasala	45±0.5
Jyothi	46±0.5
Karuna	44±0.5
Makaram	45±0.5
RM-1	46±1.0
Uma	49±0.5
Varsha	44±0.5

3.4.1.2 Chemical characteristics of oil from paddy varieties

Chemical characteristics analyzed were iodine value, saponification value and FFA. Figure 3.16 shows iodine value and saponification value, Geerakasala has iodine value higher than other varieties (100.31) suggesting higher percentage of unsaturated fatty acids Annapoorna and Jyothi variety appeared to have low unsaturation with proportionally low iodine value. Saponification value is an index for average chain length of fatty acids and hence molecular weight. Aiswarya and Uma have high percentage of short chain fatty acids, 205.63 and 205.38 respectively. Regarding the content of FFA, the values ranged from 1.71 to 4.20% indicating the degree of lipase hydrolysis. It could be seen (Figure 3.17) that Gandhakasala and Makaram have high FFA value.

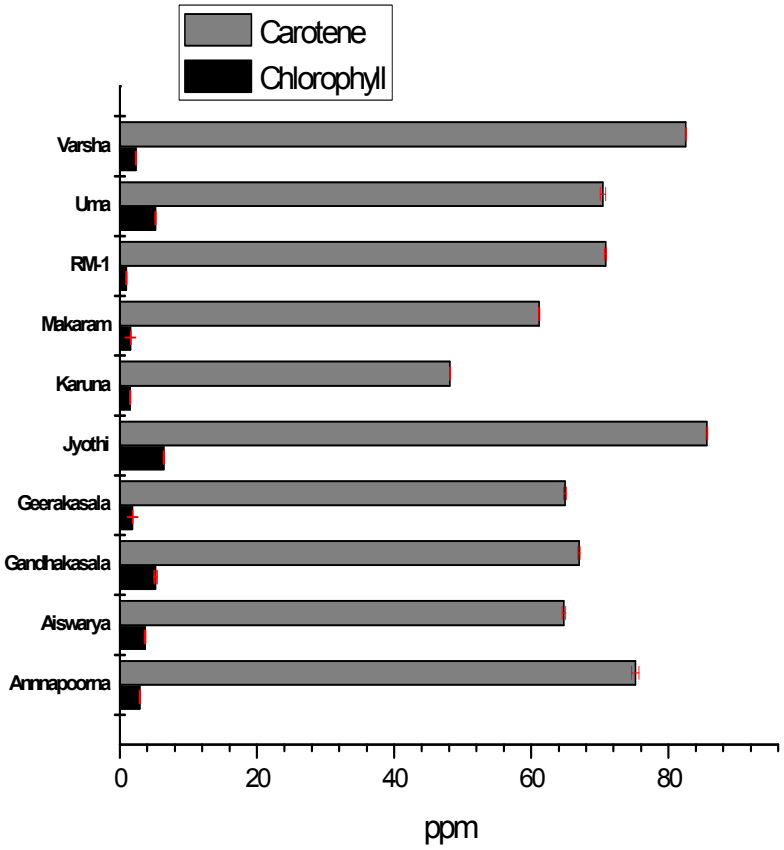


Figure 3.15 Chlorophyll and carotene content of oil from ten paddy varieties

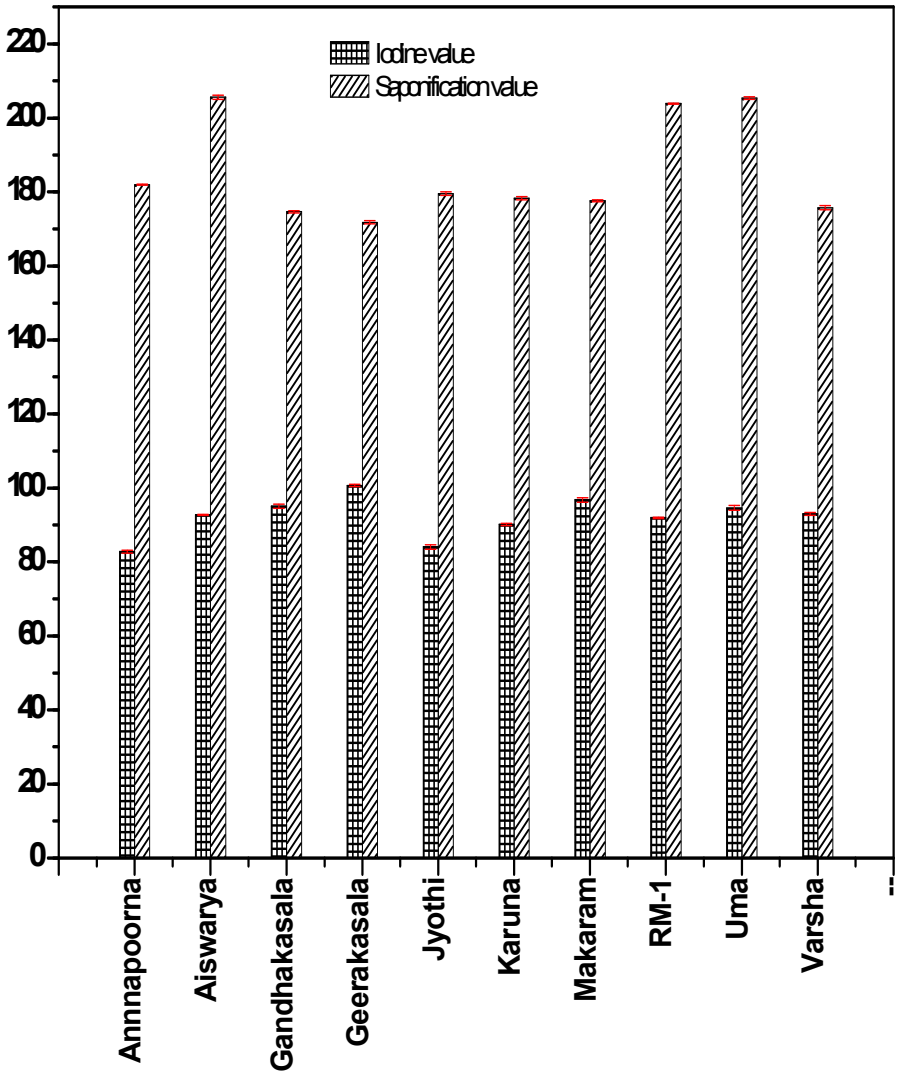


Figure 3.16 Iodine value and Saponification value of oil obtained from paddy varieties

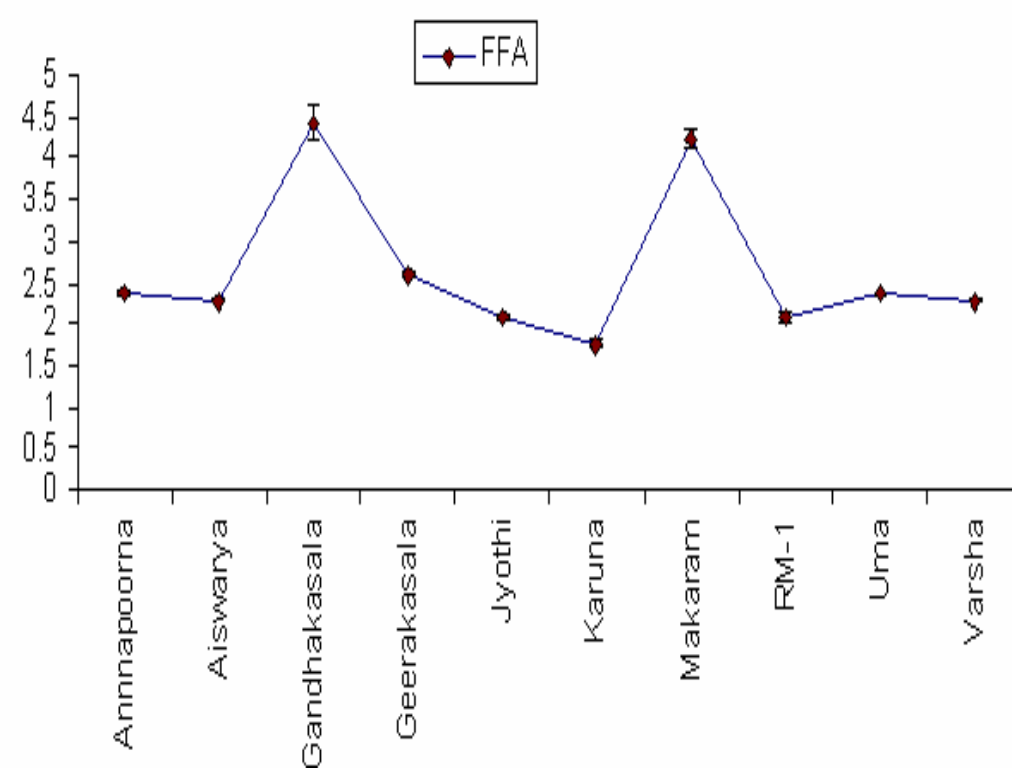


Figure 3.17 Free Fatty acid of oil from ten paddy varieties

3.4.2 Fatty acid composition of RBO from different paddy varieties

RBO has been subjected to methylation by using methanolic sulphuric acid and fatty acid methyl esters are analyzed by GC. The fatty acid composition is presented in Table 3.15 and chromatogram is shown in Figure 3.18 RBO is known for its balanced fatty acid profile of saturated, unsaturated and polyunsaturated fatty acids. It is rich in linoleic acid an essential fatty acid required for human.

Major fatty acids in RBO are 16:0, 18:1, and 18:2 [223] and minor amounts of 12:0, 14:0, 16:1, 18:3 and 20:0 were present. The fatty acid profile in the present study showed: palmitic (17-23%), oleic (32-44%) and linoleic (30-36%) in high percentage. It is reported that there are variations with in the variety as reported for RBO by Gaydou et al [224]. Gandhakasala and Geerakasala have lower palmitic acid (C16:0) compared to that of other varieties. Karuna, Makaram, RM-1, Uma and Varsha contain C16:1 in minor amount. C18:0 fatty acid was minor saturated acid in all the varieties reported here. C18:1 and C18:2 are the major unsaturated fatty acids in RBO and the present study shows high content of these unsaturated fatty acids in all varieties. RM-1 has high content of C18:1(43.74%) with closer value for Gandhakasala, Geerakasala and Varsha; 42.38, 42.52 and 42.39% respectively. Geerakasala contain 36.44% of linoleic acid (C18:2) and all other varieties have 32-35% of this acid. Arachidic acid (C20:0) is found in Aiswarya, Gandhakasala, Makaram, RM-1, Uma and Varsha, among them Aiswarya and Gandhakasala have less amount. From the fatty acid compositions Gandhakasala, Geerakasala and Varsha have higher content of unsaturated fatty acids compared to those of other variety. The total content of saturated and unsaturated fatty acid is shown in Figure 3. 19

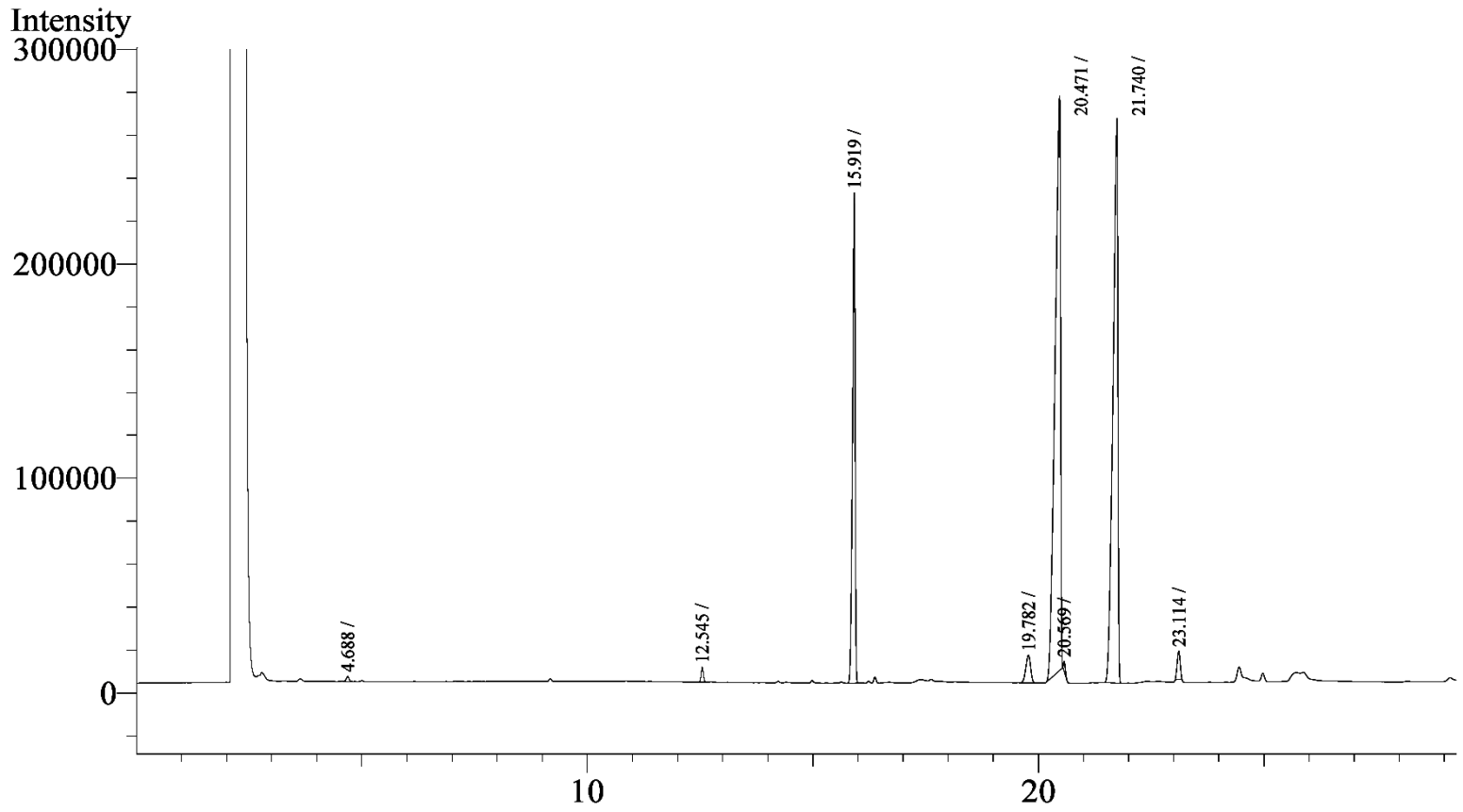


Figure 3.18 Fatty acid profile of RBO by GC

Table 3.15 Fatty acid composition by GC (area%) of ten varieties of RBO

Fatty acid	$C_{12:0}$	C_{14}	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	C_{20}
R_t	10.9	12.5	16.0	16.4	20.0	20.7	21.6	23.1	25.9
Variety									
Annnapoorna	-	0.76 (± 0.02)[a0]	22.58 (±0.07)	-	2.37 (±0.05)	32.59 (±0.47)	35.72 (±0.28)	1.66 (±0.08)	3.3 (±0.08)
Aiswarya	-	0.79 (± 0.02)	23.21 (±0.05)	-	2.08 (±0.05)	37.52 (±0.37)	33.74 (±0.17)	1.19 (±0.04)	1.17 (±0.01)
Gandhakasala	0.21 (±0.02)	0.56 (± 0.02)	18.73 (±0.09)	-	2.36 (±0.04)	42.38 (±0.34)	33.52 (±0.09)	1.18 (±0.04)	1.40 (±0.02)
Geerakasala	0.39 (±0.02)	0.49 (±0.03)	17.26 (±0.02)	-	1.81 (±0.05)	42.52 (±0.12)	36.44 (±0.08)	1.35 (±0.02)	-
Jyothi	-	0.48 (±0.01)	21.75 (±0.06)	-	2.15 (±0.03)	38.64 (±0.36)	34.90 (±0.45)	1.6 (±0.03)	-
Karuna	-	0.48 (±0.02)	21.32 (±0.09)	0.23 (±0.02)	1.79 (±0.02)	41.65 (±0.52)	32.48 (±0.62)	1.33 (±0.3)	-
Makaram	0.05 (±0.01)	0.29 (±0.03)	22.83 (±0.07)	0.20 (±0.01)	1.8 (±0.02)	40.63 (±0.10)	32.44 (±0.27)	1.04 (±0.06)	0.65 (±0.02)
RM-1	-	0.24 (±0.05)	20.18 (±0.02)	0.17 (±0.01)	2.44 (±0.06)	43.74 (±0.08)	30.42 (±0.10)	1.2 (±0.03)	0.86 (±0.01)
Uma	-	0.35 (±0.02)	22.02 (±0.01)	0.19 (±0.01)	2.09 (±0.07)	40.4 (±0.19)	32.91 (±0.26)	1.03 (±0.06)	0.75 (±0.01)
Varsha	-	0.35 (±0.02)	20.83 (±0.08)	0.17 (±0.07)	1.26 (±0.02)	42.69 (±0.51)	33.33 (±0.16)	1.18 (±0.01)	1.00 (±0.08)

a) Mean ± SE, n=3

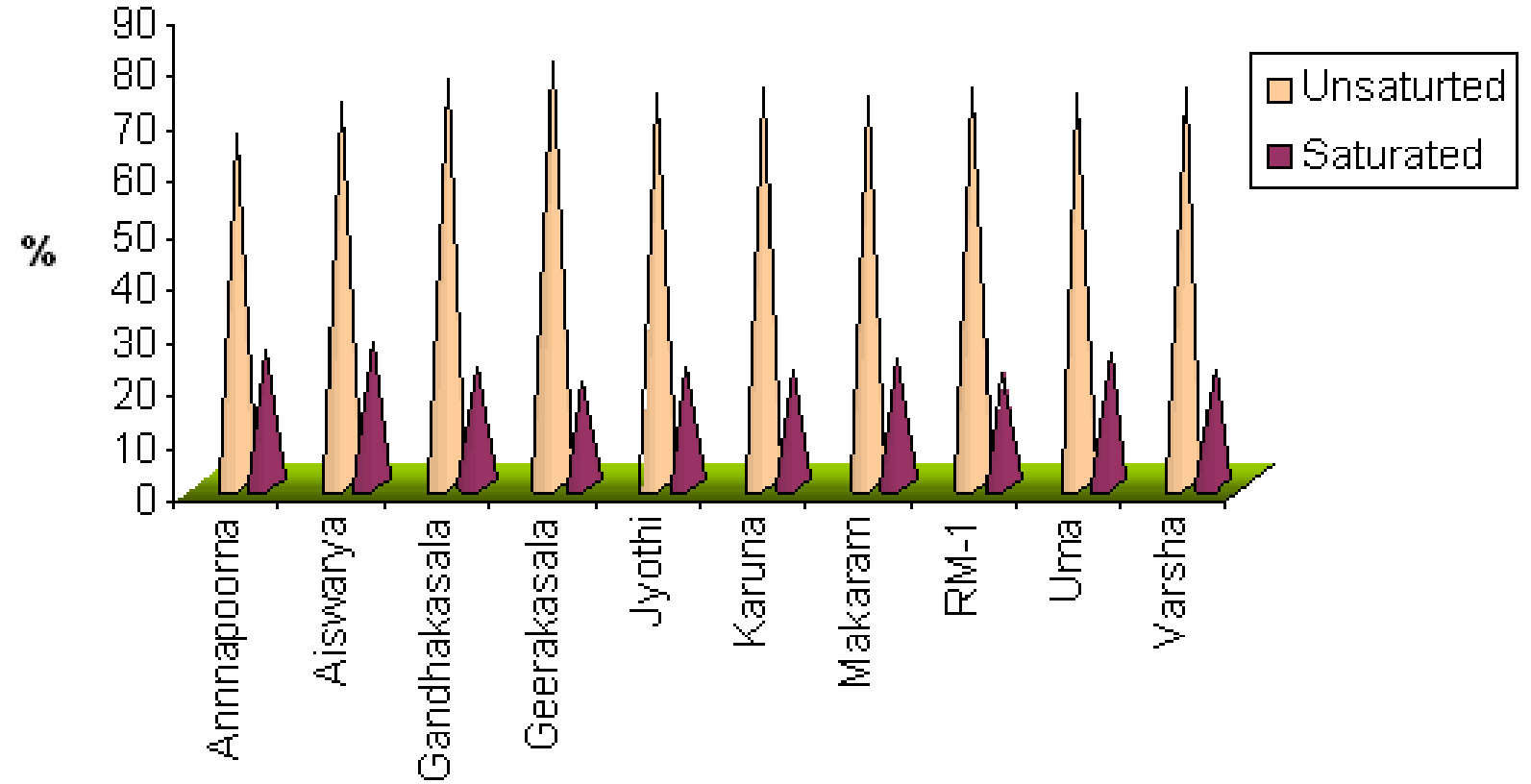


Figure 3.19 Total unsaturated and saturated profiles of oil from ten varieties of RBO

3.4.3 Unsaponifiable Constituents in RBO from paddy varieties

The nutritional composition of bran varies with nature of the soil, environmental and agronomic practices [225]. Studies on micronutrients in RBO of different paddy varieties are limited. In the present study RBO obtained from ten paddy varieties were analyzed by HPTLC for the quantification of USC [131]. Among the varieties Aiswarya has the highest amount of USC (6.09%) and Jyothi variety showing the lowest amount of USC (3.86%). USC of Aiswarya has higher amount of sterols (1.33%) and oryzanols (2.61%). The percentage of sterol varied from 0.92 to 1.35% and most of the varieties have more than 1.0% of sterols showing variation within narrow range among the variety. Gaydou et al reported the composition of sterols in Malagasy oil and the study showed variety did not have significant effect on sterol composition [224].

Oryzanol is a unique and major constituent in RBO with nutritional antioxidant properties. Varietal difference shows variations in oryzanol quantity in ten varieties. The varieties analyzed in the present study exhibited higher amount of oryzanol ranging from 1.09-2.79%, Uma having the highest (2.79%) and Jyothi had lowest content (1.09%) of oryzanol. Gandhakasala and Geerakasala with 2.14 and 1.81 % of oryzanol are aromatic varieties. Lowest amount of oryzanol in Jyothi could be the reason for its reduced USC (3.86%) when compared to other varieties Uma, Aiswarya, Varsha and Karuna which are hybrid varieties.

Iqbal et al studied commercially available varieties of rice bran in Pakistan and they quantified oryzanol by HPLC. The results showed 511-802 ppm of oryzanol in Rice bran-Super kernel (RB-kr), Rice bran-Super 2000 (RB-s2) etc [226]. Antioxidant properties of solvent extracts from Japonica rice bran was studied by Li and his co-authors and found out 1.31% of oryzanol in TK 9 rice bran hexane extract [227]. Khatoon et al quantified 1.07-1.43% of oryzanol from

brown rice [228]. Krishna et al reported oryzanol content in eighteen different paddy cultivars and showed 1.63-2.72% [53]. By a partial extraction technique, total lipid and oryzanol content of nine varieties of rice bran were analyzed by Lilitchan et al and the values obtained were in the range of 1.95-3.07% [229].

Tocols ranged from 0.01-0.20% and its highest content recorded in Varsha (0.21%) and lowest in Geerakasala (0.01%).The total USC is highest for Aiswarya but its tocols content is lower (0.09%). The previous reports shows hexane extract of TK9 rice bran has 0.021% of tocols in Japonica variety [227]. The total tocopherol content of Basmati brown rice is 31.19 ppm and Jaya brown rice is 26.23 ppm, parboiled brown rice has 2.18 ppm of tocols as reported by Khatoon et al [228]. Krishna et al [230] reported a total tocopherol content of 31.30–48.70 ppm for four varieties of brown rice, Madhu (improved), Jaya, Pushpa, and IR-20. A large variation of tocols contents is observed among 109 rice bran samples analyzed by Sookwong et al [231].

Squalene content ranged from 0.16- 0.23% which is a major hydrocarbon in RBO. Geerakasala has the highest content of squalene (0.23%) among the ten varieties studied here. Moreover most of them have values in between 0.16-0.19%. Regarding the steryl esters, Karuna and Varsha have the highest content of 1.48 and 1.49% respectively. The percentage of wax is greater in Gandhakasala (1.17%), all others have values ranging from 0.55-0.94%.Both Karuna and Varsha have almost identical composition of USC in terms of oryzanol, squalene, steryl esters and wax. Table 3.16 shows the USC in ten varieties of oil analyzed by HPTLC.

Table 3.16 USC of RBO from ten varieties

Variety	Sterol (%)	Oryzanol (%)	Tocols (%)	Squalene (%)	Steryl esters (%)	Wax (%)	Total USC (%)
Annnapoorna	0.95±0.01[a)]	1.84±0.01	0.08±0.01	0.16±0.01	1.42±0.02	0.72±0.01	5.17±0.01
Aiswarya	1.33±0.01	2.61±0.02	0.09±0.01	0.16±0.02	1.34±0.02	0.56±0.02	6.09±0.01
Gandhakasala	1.28±0.02	2.14±0.02	0.05±0.02	0.18±0.01	0.69±0.02	1.17±0.01	5.52±0.01
Geerakasala	1.13±0.01	1.81±0.02	0.01±0.01	0.23±0.03	1.25±0.02	0.94±0.03	5.37±0.01
Jyothi	0.93±0.01	1.09±0.02	0.07±0.01	0.21±0.02	0.79±0.02	0.77±0.01	3.86±0.02
Karuna	1.35±0.04	2.36±0.02	0.13±0.01	0.18±0.01	1.48±0.01	0.55±0.02	6.05±0.01
Makaram	1.11±0.02	1.86±0.01	0.11±0.03	0.19±0.03	1.05±0.02	0.54±0.01	4.86±0.01
RM-1	1.18±0.06	1.62±0.02	0.07±0.01	0.17±0.01	1.33±0.01	0.77±0.01	5.15±0.02
Uma	1.09±0.02	2.79±0.02	0.08±0.01	0.18±0.02	0.97±0.01	0.63±0.01	5.74±0.02
Varsha	0.99±0.01	2.55±0.01	0.20±0.01	0.19±0.03	1.49±0.01	0.55±0.02	5.97±0.01

a) Mean±SE (n=3)

3.5 Contributions of Oryzanol, Tocols and Sterols to the Stability of Rice Bran Oil

Oxidative stability of edible oils have nutritional and health implications. Auto oxidation of oils and fats, caused by extrinsic and intrinsic factors lead to off flavor due to oxidized constituents such as ketones, aldehydes, alcohols etc. Fats and oils differ widely in their oxidative stability and it depends on chemical composition, degree of unsaturation, amount of antioxidants and oxidants, processing conditions employed etc. RBO in this context is comparatively stable due to the presence of high levels of antioxidants which are unsaponifiables viz: tocols, sterols, oryzanol etc. However, the content of these nutritionally significant compounds varies widely in CRBO and RRBO as influenced by varying factors. CRBO extracted under good conditions may contain 2-3% of oryzanols, 1-2% of sterols and 800-1500 ppm of tocols. However their potential, either individually or in combinations to influence oxidative stability of RBO has not been the subject of investigation before. The present study is designed, to separate oryzanol, tocols and sterols from RBO and the stripped oil is reconstituted with these constituents individually and combinations at known concentrations. The samples thus prepared have been subjected to accelerated storage study with periodical monitoring by analyzing various parameters to study the state of oxidation.

3.5.1 Antioxidant activity of the bioactive compounds in RBO

The constituents separated from RBO are added to 30 g of the stripped oil in glass bottles (50 ml) and the samples are sonicated for 30 min at 35^{o}C. The concentrations of the constituents added have been selected to match with those present naturally in RBO taken for the study. The concentration of samples mixed is presented in Table 2.3 in the materials and methods section. Two individual concentrations of oryzanol O_1, O_2: two combinations of oryzanol and tocols, OT_1, OT_2: two combinations of oryzanol and sterol OS_1, OS_2 have been selected.

Similarly two individual concentration of tocols and sterols, T_1, T_2 and S_1, S_2 and two combinations of tocols and sterols TS_1 and TS_2 are used. In order to study the stability parameters, Schaal oven test method was employed. The samples are kept in an oven at 60^{o}C and have been analyzed every day for peroxide value [141], *p*-anisidine value [142] and diene value [143] for 5 days. Samples with (control oil) and with out micronutrients (control sample) are also kept at same conditions for the analysis.

3.5.2 Effect of oryzanol on the stability of RBO

The five day storage studies of RBO with added oryzanol, shows variations in its oxidative stability. Figure. 3.20 shows peroxide values of individual oryzanol, tocols and sterols with that of control oil and control sample. Figures 3.21 and 3.22 shows oryzanol in combinations with tocols and sterols respectively. The control oil (with micronutrients) shows peroxide values ranging from 7.7 to 5.5 mequiv/kg from initial to final day and that of control sample (without micronutrient) 8.1 to 52.2 mequiv/kg, respectively. Samples with oryzanols (O_1 and O_2) did not show any concentration dependent oxidation. However O_2 samples have lower peroxide values, from the initial to fourth day (Figure 3.20). Combinations of oryzanol and tocols at the 0.04% tocols (OT_1) level has lower peroxide values from initial to fourth day compared to OT_2 0.08% (Figure 3.21). The lower peroxide value for oryzanol and tocols combinations compared to those of individuals O_1 and O_2 may be due to the balanced action of tocols and oryzanol against oxidation of lipids. Juliano et al [232] studied the antioxidant activity of oryzanol and confirmed that activity of oryzanol is enhanced in presence of other natural antioxidants. Comparing samples O_1 and OT_1 (1.6% oryzanol), peroxide value of OT_1 is lower than that of O_1. A similar trend is also observed for O_2 and OT_2. This indicated an additive effect for oryzanol and tocols. In the case of combinations of sterol and oryzanol, OS_1 and OS_2 have high peroxide formation when compared with O_1 and O_2. Sterol appeared to promote radical formation, which in turn would favor the radical

chain reactions [233]. The peroxide formation is lower in OS_2 (17.3 mequiv/kg) than that in OS_1 (41.8 mequiv/kg) as shown in Fig 3.22. It is inferred that addition of tocols to oryzanol, decreases the formation of peroxides when compared to the value of control sample, O_1 and O_2. Compared to tocols, oryzanol has less effect on radical suppression [232]. But there are additive effects between oryzanol and tocols, which is substantiated by analyzing the values of control sample, O_1 and O_2.

Diene value is a measure of conjugated double bonds formed prior to peroxide formation. The hydrogen abstraction from allylic position of lipid causes the formation of stable allylic radical. Figures. 3.24, 3.25 and 3.26 show the diene values related to samples with individual and combinations of USC. Comparing diene value of O_1 and O_2 with control oil, O_2 has lower value than O_1. Diene formation in OT_1 and OT_2 showed decreasing trend during the first three days but on fifth day OT_1 had lower diene value than OT_2 suggesting lower peroxide formation in OT_1 than in OT_2 (Figure 3.25). The combinations of oryzanol and tocols in general thus has lower conjugated diene formation. Though, O_2 showed lower diene value but at higher concentration with tocols appeared to be less beneficial. A previous report about oryzanol shows that it was more effective than tocopherols (alpha) in inhibiting the formation of conjugated dienes [234]. OS_1 and OS_2 have higher conjugated diene values than O_1 and O_2 on final day but they had almost comparable values till third day indicating that sterols neither improve the activity of oryzanol nor it has antioxidant activity. However sterol and oryzanol combinations have lower diene value than control sample (Figure 3.26).

The secondary oxidation products have been monitored by *P*-anisidine value. The control oil had *p*-anisidine value of 24.4 while that of control sample (devoid of unsaponifiables) reached 162.9. Oryzanol with tocols (OT_1) has lower *p*-anisidine value through out the study period. Oryzanol alone had (O_1 and O_2) *p*-anisidine value greater than that of OT_1 and OT_2, indicating that oryzanol and tocols combinations is more effective against oxidation. Among the oryzanol-

tocols combinations, OT_1 has lower *p*-anisidine value and therefore better stability compared to the control oil. Among oryzanol-sterol combinations, OS_2 has lower *p*-anisidine value than OS_1. However, oryzanol combinations OT_1 (53.8) has lower *p*-anisidine value than that with oryzanol (O_1: 62.2, O_2: 56.8) with stripped RBO. The results suggest that values obtained for oxidative stability of RBO has good correlation with the USC under the experimental conditions of the present study. The maximum stability is for the samples with oryzanol and tocols (OT_1 > OT_2). Figure 3.28 shows the *p*-anisidine value of individual mixing of sterol, oryzanol and tocols, 3.29, 3.30 and 3.31 shows its combinations.

3.5.3 Effect of tocols on the stability of oil

Figures 3.20, 3.21, 3.23 show the peroxide values of samples with tocols alone and tocols in combination with oryzanols and sterols. Tocols content ranges from 0.04 to 0.08% in refined RBO which is known to be a major antioxidant responsible for the stability of RBO. Two concentrations of tocols, 0.04% (T_1) and 0.08% (T_2) added as individual concentrations. It has been observed that T_1 has greater effect in lowering the peroxide formation than that by T_2 as evidenced by the first day peroxide values, 6.1 and 8.7 mequiv/kg respectively (Figure 3.20). The current study supports the previous report that tocols are more effective at lower concentrations as antioxidants [235]. From Figure 3.20 it is evident that peroxide value of T_2 (final day) (9.69 mequiv/kg) is almost double while comparing with T_1 (5.7 mequiv/kg), which clearly indicated, tocols at higher concentration could less effective. Even though OT_1 (0.04% tocols) has same tocols concentration as in T_1, peroxide value of OT_1 doubled (10.6 Mequiv/kg). Similarly the effect is observed between T_2 and OT_2 with closer values (T_2:9.7 Mequiv/kg, OT_2:10.42 Mequiv/kg). As compared to control sample and combinations (Figure 3.21), tocols- oryzanol combinations have significant antioxidant effect. However, tocols alone shows more effect than in combination with oryzanol. As in the case of tocols with sterol combinations, sterols did not

exert any beneficial effect to enhance the activity of tocols as [236] evident from the values of T_1 with TS_2 (Figure 3.20 and 3.23). At higher concentration of sterols with tocols, increase in peroxide is observed as evident from the values of T_1, T_2 with TS_1, TS_2. Previous report suggests that sterols having ethylidene group in the side chain has antioxidant property rather than stigmasterol, β-sitosterol and campesterol, which are higher amount in RBO. The mechanistic aspects of this could be, free hydrogen atom in the allylic carbon of the ethylidene group is more prone to radical formation and this radical isomerizes to tertiary radical to become more stable. But in the case of stigmasterol, β-sitosterol and campesterol even though a tertiary radical is formed it does not exhibit antioxidant activity due to steric effects [237]. The overall effect of individual and combinations of tocols on the stability of RBO is in the order $T_1>T_2>OT_1>OT_2>TS_1>TS_2$ based on the final day values.

Tocols mentioned in the present study includes both tocopherols and tocotrienols. Various previous reports mentioned the antioxidant activity of tocols in RBO and in blends of RBO [63, 238]. A blend of sunflower oil and RBO of equal volumes is found to have protective effect due to the presence of various antioxidants in RBO and a blend of coconut oil and RBO reported to retard formation of peroxides [239-240]. It has also been reported that γ-oryzanols components have greater antioxidant activity than α and *γ* tocopherols and tocotrienols [32]. Inhibition of lipid peroxidation by RBO tocols was also reported earlier [241]. However Contribution of tocols and oryzanol to the stability of oil is analyzed in quantitative terms and their potential on oil stability in the present study. It could be seen (Figure 3.24) that diene formation in both T_1 and T_2 are less than control oil. This shows the greater antioxidant activity of tocols in the absence of other micronutrients naturally present in RBO. Samples having same concentration of tocols when in combinations with other unsaponifiables registered higher diene values viz: T_2 (4.03) and OT_2 (7.1), T_1 (4.27) and OT_1 (5.86) (Figure 3.24 and 3.25). The results indicated that tocols and oryzanol

combinations show lower peroxide value than sterol combinations. This could be because though initially diene formation is greater by abstracting hydrogen from lipid, further propagation of radicals is arrested due to the strong antioxidant activity of oryzanol and tocols.

Secondary oxidation products have been measured by *p*-anisidine value and shown in Figures 3.28, 3.29, 3.31. of tocols samples. The control sample shows very high *p*-anisidine value compared to other samples indicating rapid formation of secondary oxidation products in the absence of antioxidant compounds. *p*-anisidine value of T_1 and T_2 were 48.6 and 41.7 respectively (final day) which is very lower than control sample (162.9) but higher than control oil (24.4). Tocols as individual and combinations, T_1, OT_1 and OT_2 have significant effect on the stability of oil, with T_1 having values similar to control oil (Peroxide value: T_1; 5.7 Mequiv/kg, control oil; 5.5 Mequiv/kg: Diene value: T_1; 4.3, Control oil; 6.1: *p*-anisidine value: T_1 ; 48.6; control oil; 24.4).

3.5.4 Effect of sterol on the stability of oil

Stigmasterol, campesterol and betasitosterol are the major sterols in RBO which have diverse effect on the stability of oil. Previous reports shows stigmasterol is not acting as antioxidant but Δ^5 avenosterols are effective antioxidants [237]. Present investigation focused on the stability effects of sterol in striped RBO by adding two concentrations viz: 0.6 and 1.2% and as combinations with tocols and oryzanol. From the sample S_1 (46.1 mequiv/kg) and S_2 (47.0 mequiv/kg) show values nearer to control sample (52.3 mequiv/kg) on the final day indicating, sterols have no effect on suppression of peroxide formation (Figure 3.20) but in combinations with tocols (Figures 3.22 and 3.23), showed lower peroxide values than that of S_1 and S_2. Among sterol combinations, TS_2 (25.3 Mequiv/kg) has greater peroxide value than TS_1 (10.1 mequiv/kg). The order of activity against peroxide is $TS_1 > OS_2 > TS_2 > OS_1$. In conclusion TS_1 has lower peroxide value among the sterol combinations.

In the case of diene values a similar trend is observed. Diene value of S_2 (14.4) is highest among sterol samples. Comparing the sterol samples S_1, S_2, OS_1, OS_2, TS_1 and TS_2, the order of diene value is as follows TS_1(5.5) < OS_2 (6.3) TS_2 (6.6) < S_1 (7.2) < OS_1 (9.6) < S_2(14.4), which indicates combination with oryzanol and tocols, sterol samples show lesser diene value. Diene value is shown in Figures 3.24, 3.26 and 3.27.

Sterol alone did not have any significant effect on lowering oxidation as evidenced by diene value, peroxide value and from the evaluation of *p*-anisidine value. Final day analysis shows S_2 had *p*-anisidine value very close to control sample indicating no effect of sterol against lipid oxidation and its value reaches maximum (159.5). Comparing S_2 (159.5), with TS_2 (78) and OS_2 (47.7): S_1 (61.4) with TS_1 (49.0) (same sterol concentration), the lower *p*-anisidine values shows the effect of tocols and oryzanol in combinations (Figures 3.28, 3.30 and 3.31).

3.5.5 Total Radical Scavenging assay (DPPH)

The antioxidant activity of oil samples in presence of oryzanol, tocols and sterols as individual and in their combinations have been evaluated for the radical scavenging property of DPPH assay. The IC_{50} values of samples at the time of mixing and after conducting Schall oven test are analyzed and results are shown in Figure 3.32. The control oil shows IC_{50} value of 5.53 mg on initial day. Samples with individual constituent are oryzanol (O_1 and O_2), tocols (T_1 and T_2) and sterols (S_1 and S_2) among them O_2 (0.8%) and T_2 (0.08%) show almost same antioxidant activity on first day of storage period. The result indicate that oryzanol has radical scavenging activity comparable with that of tocopherols at identical concentrations [232]. The order of IC_{50} values on first day of analysis is T_2> O_2>T_1>O_1. Sterol S_2 shows the lowest activity with IC_{50} value 21.17 mg. Among combinations of T_2, OT_2: T_1, OT_1: O_1, OT_2: O_2, OT_1 having and oryzanol at same concentrations at each sample, the IC_{50} values are close to all samples on first day.

The maximum activity has been observed for OT_2 with IC_{50} value of 10.13 mg which is significantly lower compared with other combinations on the first day. Result of OT_2 having highest radical scavenging activity indicated the synergistic effect of oryzanol and tocols. Among tocols and sterol combinations, TS_2 shows low IC_{50} value (9.93 mg). Same is observed for oryzanol and sterol combinations with lower IC_{50} value than sterol individual mixing [237].

The result on DPPH radical scavenging activity of samples at the end of study period shows the following trend. In individual mixing, T_1 shows IC_{50} value of 14.24 mg and T_2, 21.43 mg. This supported the result of oxidative parameters that tocols have higher activity against radical at lower concentrations. An important observation is that T_1 has same IC_{50} value of control oil on the final day which is lower than other samples. Next to T_1, OT_1 show lower IC_{50} value than OT_2 suggesting even in presence of oryzanol, tocols are dominant.

RBO, which is a very stable and nutritive oil having tocols, oryzanol and sterol in abundance owes its stability primarily due to tocols and oryzanols synergistic effect. Sterols do not contribute substantially to the stability of RBO.

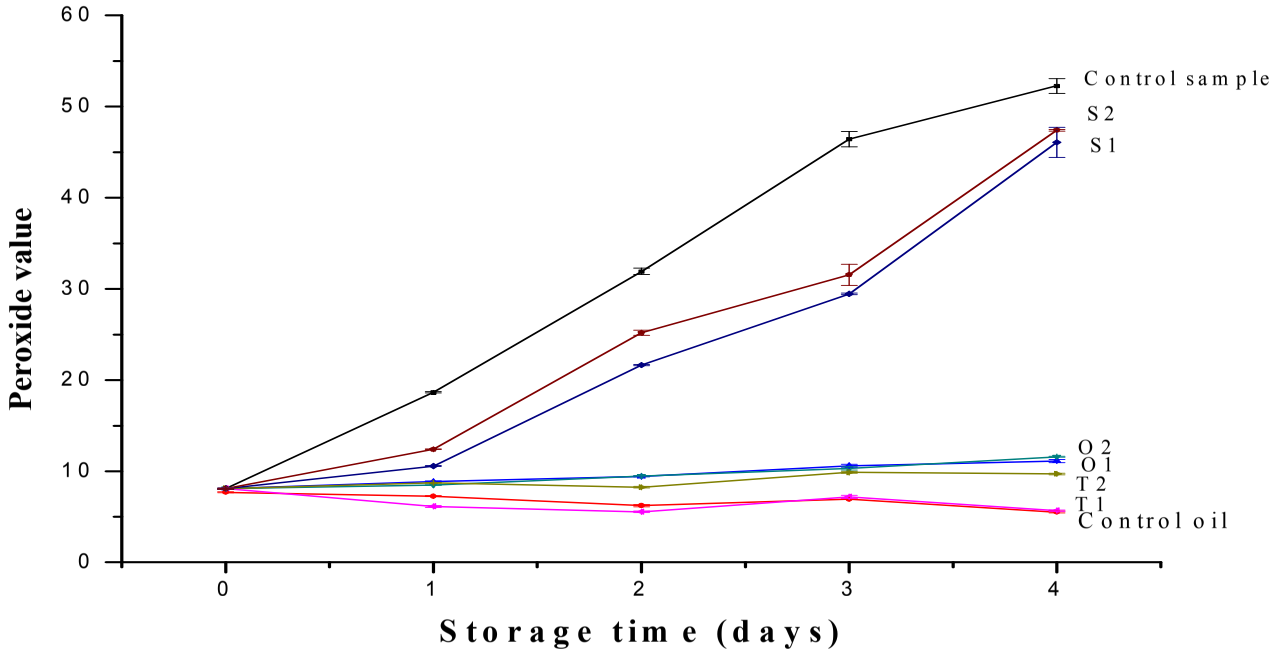


Figure 3.20 Effect of oryzanol, tocols and sterols at various concentrations on the peroxide value of stripped RBO by Schaal oven method at 60^0C

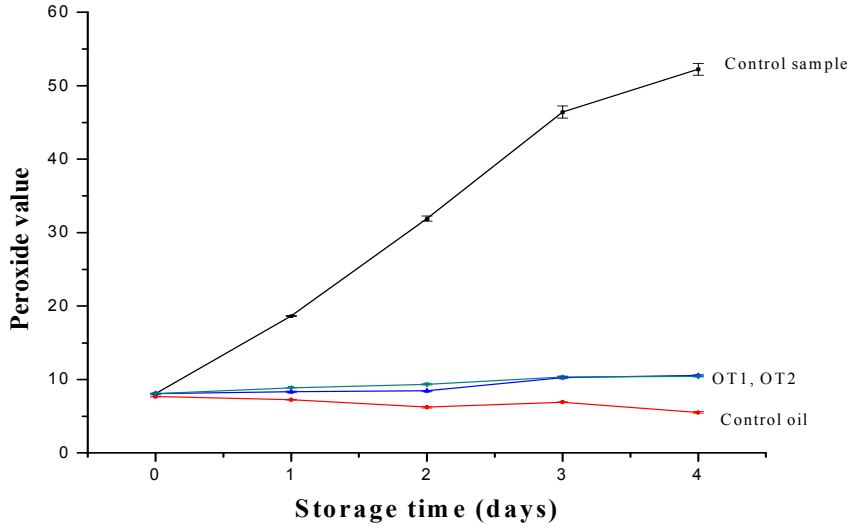


Figure 3.21 Effect of oryzanol and tocols in striped RBO at two combinations on the peroxide value by Schaal oven method (60^0C)

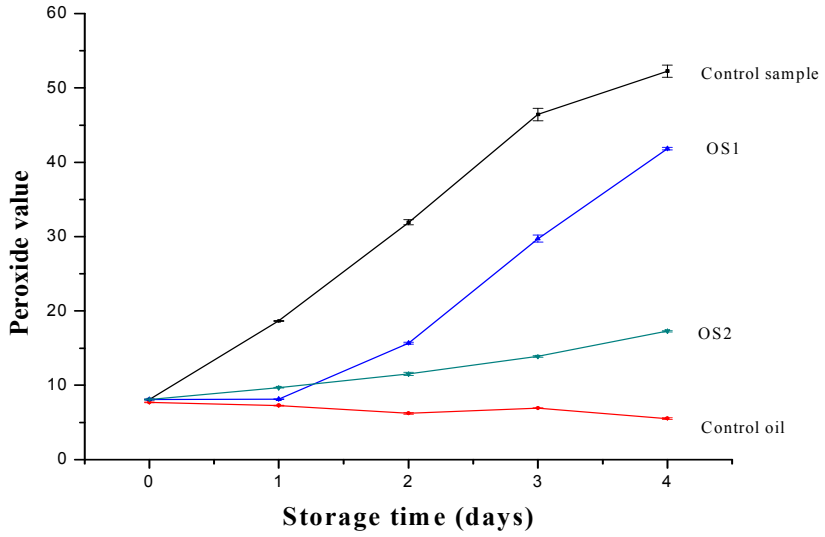


Figure 3.22 Effect of oryzanol and sterols in striped RBO at two combinations on the peroxide value by Schaal oven method (60^0C)

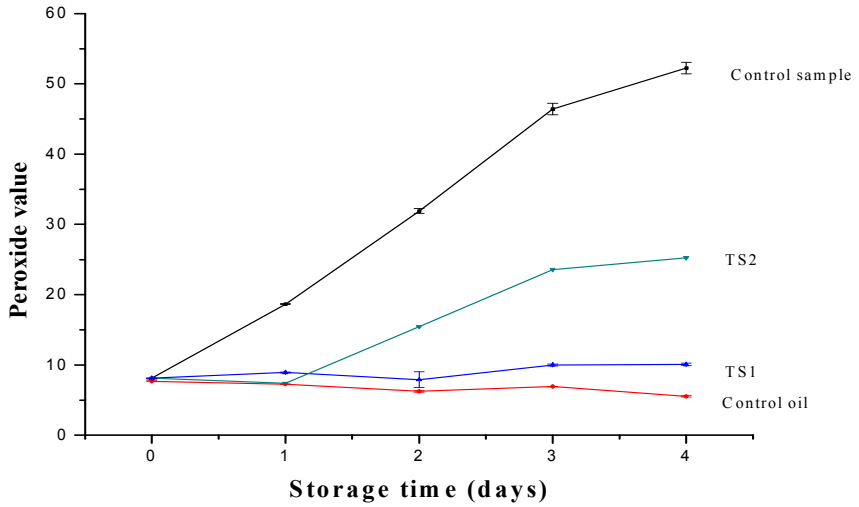


Figure 3.23Effect of tocols and sterols in striped RBO at two combinations on the peroxide value by Schaal oven method (60^0C)

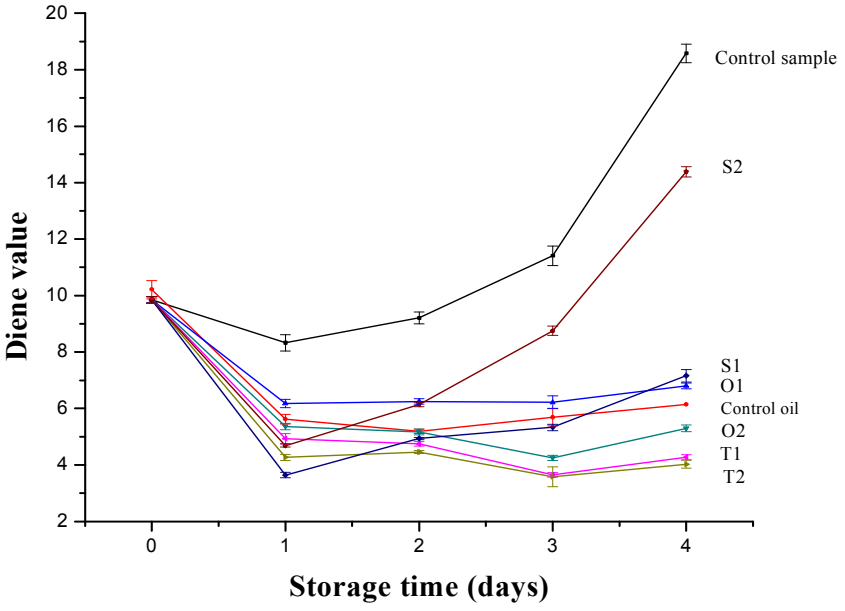


Figure 3.24 Effect of oryzanol, tocols and sterols at various concentrations on the diene value of striped RBO by Schaal oven method at 60^0C.

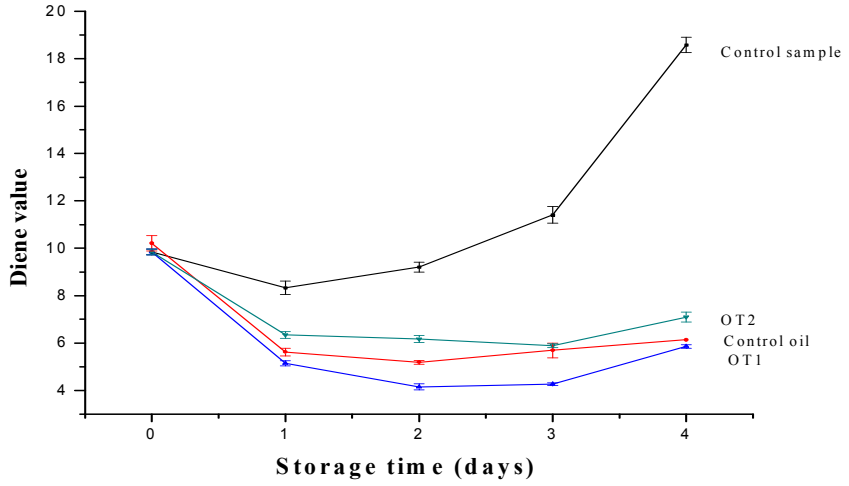


Figure 3.25 Effect of oryzanol and tocols in striped RBO at two combinations on the diene value by Schaal oven method (60^0C)

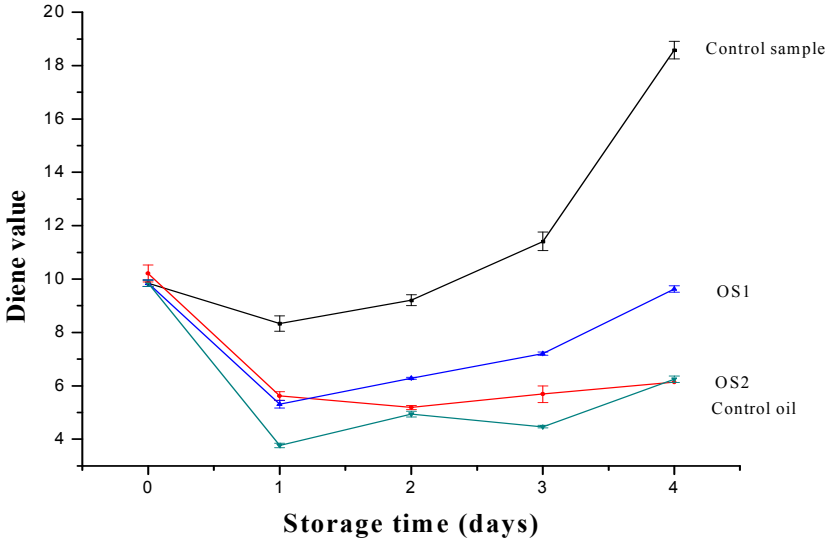


Figure 3.26 Effect of oryzanol and sterols in striped RBO at two combinations on the diene value by Schaal oven method (60^0C)

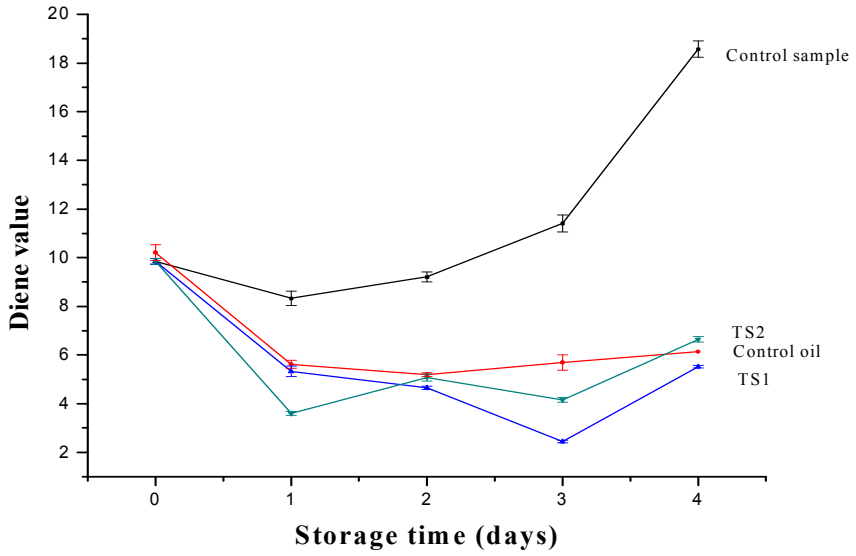


Figure 3.27 Effect of tocols and sterols in striped RBO at two combinations on the diene value by Schaal oven method (60^0C

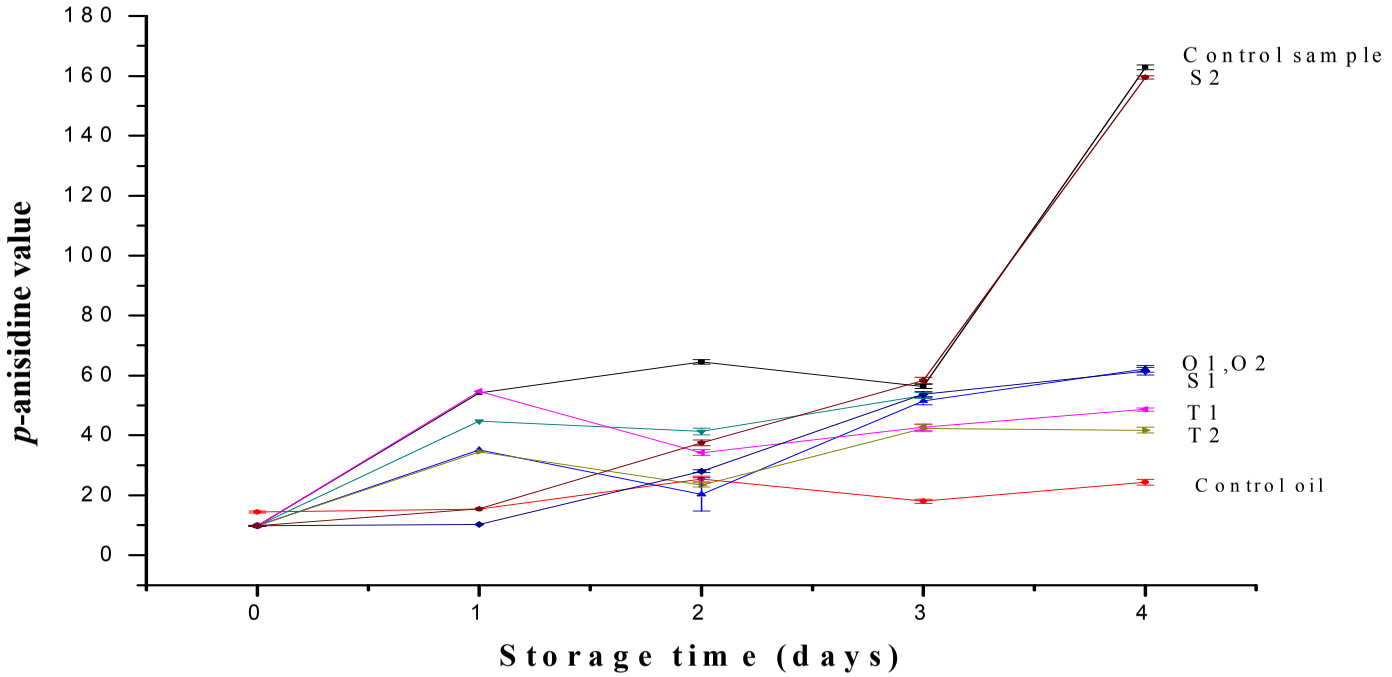


Figure 3.28 Effect of oryzanol, tocols and sterols at various concentrations on the *p*-anisidine value of striped RBO by Schaal oven method at 60^0C.

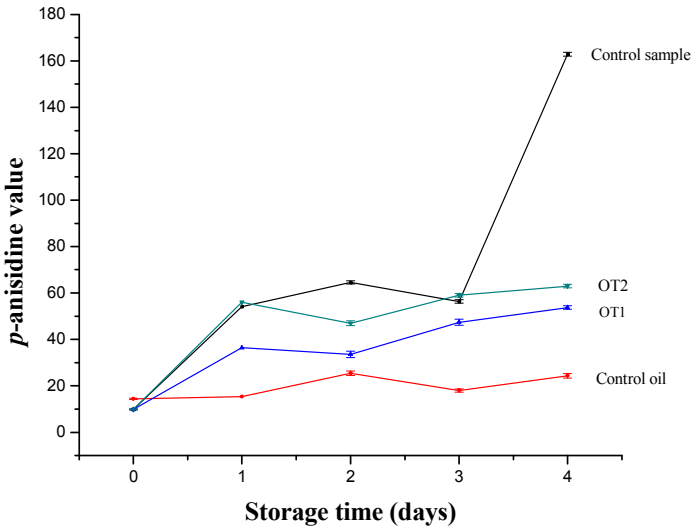


Figure 3.29 Effect of oryzanol and tocols in striped RBO at two combinations on the *p*-anisidine value by Schaal oven method (60^0C)

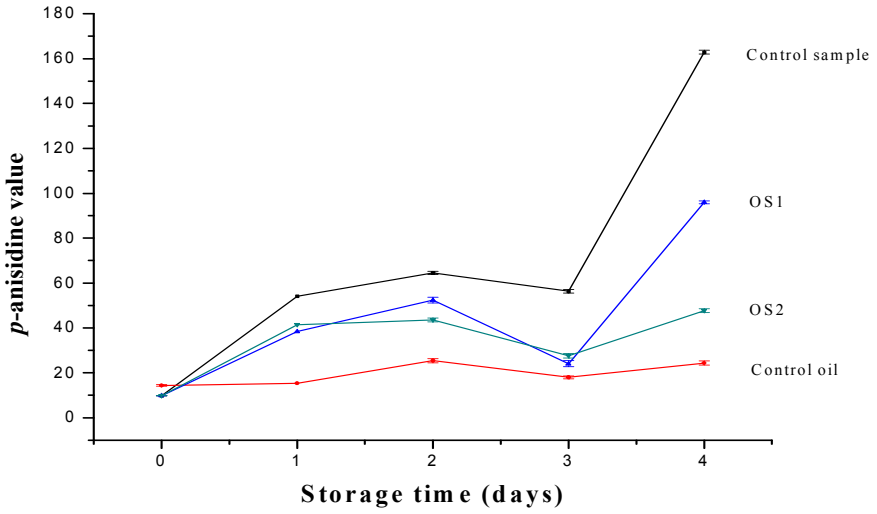


Figure 3.30 Effect of oryzanols and sterols in striped RBO at two combinations on the *p*-anisidine value by Schaal oven method (60^0C)

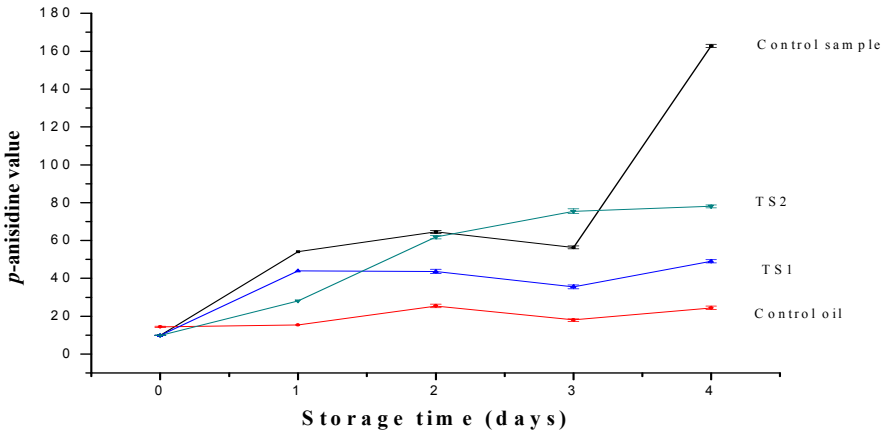


Figure 3.31 Effect of tocols and sterols in striped RBO at two combinations on the *p*-anisidine value by Schaal oven method (60^0C)

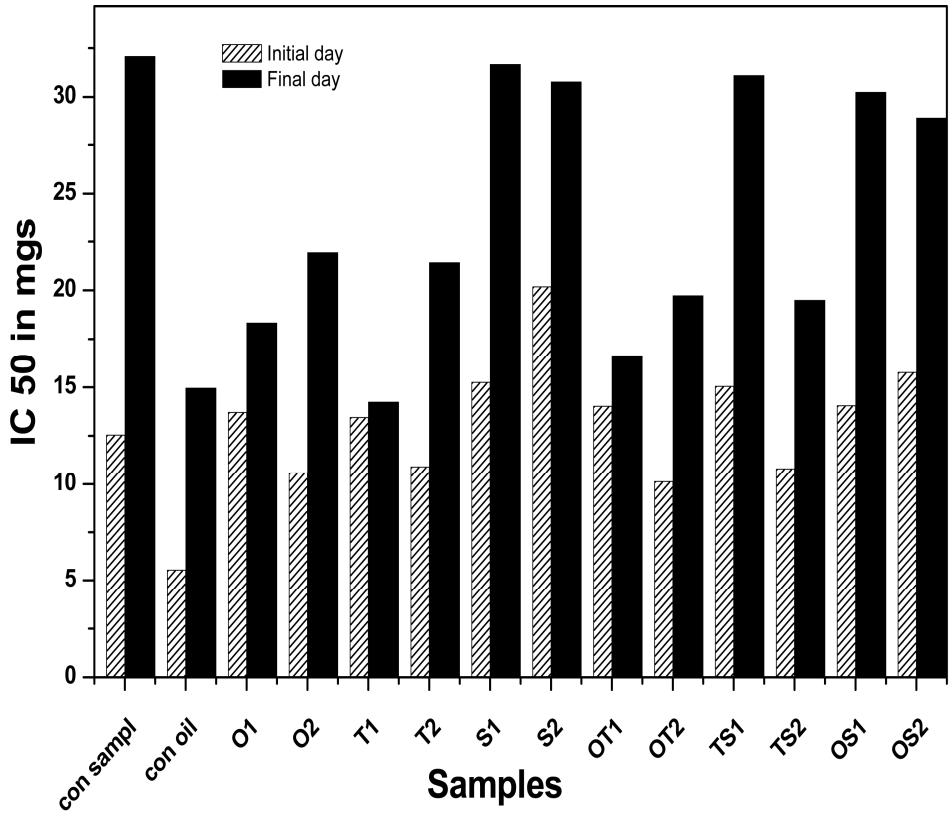


Figure 3.32 DPPH radical scavenging effects of stripped RBO added with oryzanol, tocols and sterols at various concentrations and combinations on initial and final day of mixing

CHAPTER 4
SUMMARY AND CONCLUSION

4.0. Unsaponifiable constituents in edible oils are group of bioactive phytochemicals that has various nutritional importance. Important phytochemicals in edible oil include sterols, tocopherols, tocotrienols, etc. These constituents are not saponified by the treatment of alkali and together contribute weight percentage of Unsaponifiable Matter (USM). The percentage of USM is an important criterion for crude oil which is undergoing refining process. Physical refining and chemical refining are the two important refining methods generally followed now. Food laws limits the percentage of USM in edible oils. In general USM of edible oil varies within the range of 0.5 -2.5% and among them RBO has high percentage of USM ranging from 4-6%. The percentage of USM depends on the source material, treatments, process of refining etc .It is noticed that physically refined oil has high percentage of USM that exceeds the limit prescribed by the food laws. One of the objectives of the present study therefore was, development of an analytical protocol for the complete estimation of Unsaponifiable Constituents (USC) directly from oil with out any pretreatment. Knowledge on changes occurring during saponification on USC (direct oil) and composition of the resulting USM (saponification method) were another objective of this study. Isolaton, identification and characterization of compounds hitherto not reported in USM, profiling of USC in oil of different paddy varieties and effect of USC on the stability of RBO were the other objectives of this investigation. The results obtained were discussed and summary of the findings and conclusions drawn there from are presented below.

USC in oil is quantified by developing a new method using High Performance Thin Layer Chromatography (HPTLC). The method provides simultaneous estimation of six USC by using two TLC plates in two different mobile phases. The main advantage of the new method is by using a single analytical instrument six USC can be quantified rather than using GC, GC-MS, HPLC etc as required by previous USC analysis. It avoids the pretreatment such

as extraction, derivatization etc and therefore changes occurring in constituents can be avoided. Presently recommended method for the estimation of USM uses high concentration of alkali and further extraction with solvents. As oryzanol is the major unsaponifiable constituent in RBO, alkali treatment converts it to other compounds and also lose through soap stock. Other constituents such as tocopherols, tocotrienols, steryl esters also undergo change and thereby the USM quantitation does not reveals the actual unsaponifiables in RBO. The newly developed method rectifies these anomalies and provides quantification of USC actually present in oil.

Saponification is the usual method followed for the estimation of USM as recommended by AOCS, IUPAC etc. An important observation about physically refined RBO is the USM exceeds the legal limit. In order to study the variations during saponification, USM is quantified separately for individual constituents by following the HPTLC method developed here used for direct oil estimation. It is noticed that major and minor components in RBO were very sensitive to alkali and temperature. The actual content of USC obtained by HPTLC method and by saponification method show a wide difference. Accordingly, the amount of sterols after saponification increased and contribution of other constituents were negligible to the weight percentage of USM. The increase in percentage of sterol is due to decrease in percentage of steryl esters. Steryl esters are esters of fatty acids and sterols and by using high concentration of alkali, ester bond is hydrolyzed and forms free sterols and potassium salt of fatty acid. Sterol thus formed was adding up to the percentage of free sterols and thus total percentage of sterols increased.

A unique compound in RBO called oryzanol was completely lost during saponification. The loss of oryzanol is investigated in order to compute the total percentage in oil. It is found that oryzanol undergoing changes during saponification viz: degrading to triterpene alcohol, derivatizing to potassium salt

of oryzanol and loss through soap stock. The formation of triterpene alcohol could be substantiated as oryzanols are esters of ferulic acid with triterpene alcohol or sterols. Since the USM estimation follows high concentration of alkali, causing degradation of oryzanol to triterpene alcohol and ferulic acid. Also temperature in presence of high alkali concentration, leads to derivatization of potassium salt. The formation of potassium salt of oryzanol is confirmed by conducting saponification with standard oryzanol compounds. According to earlier reports, oryzanol has a tendency to retain in soap and lost. However oryzanol which should have contributed to the weight percentage of USM is found to be eliminated almost completely during saponification. Tocopherols and tocotrienols also reduced to negligible amount because of alkali and temperature. In the case of wax, which are esters of long chain fatty alcohols and fatty acids, not contributing to the weight percentage of USM even though it is present in high percentage in crude RBO. The total weight percentage of USM in terms of actual USC estimated directly(sterols, oryzanols, tocols, steryl esters, wax and squalene) from oil was significantly low as compared to direct estimation. New compounds contributing to the quantity of USM was then studied. By different chromatographic and spectroscopic techniques the presence of a group of fatty alcohols called policosanol in USM was confirmed. Wax was found to be the source of policosanol in USM in the case of crude RBO. But in the case of physically refined RBO the oil taken for the study was devoid of wax. Even though it did not contain wax the refined oil estimated was found to have high percentage of policosanol. From this data it was confirmed that RBO contain free fatty alcohols and also as part of wax. The present study also confirmed the presence of long chain fatty aldehydes in USM from crude and refined RBO hitherto not reported. Source of fatty aldehydes was then investigated. However the percentage of USM is not accounting to the percentage of individual USC and also profile of USM constituents also varies.

Previous reports indicated that alkenyl ether lipids yield aldehydes on hydrolysis. Following this lead Triacylglycerols (TAG) in RBO is subjected to various analysis. TAG was saponified and subjected to spectroscopic analysis for the formation of aldehydes. The aldehyde fraction obtained from USM is also analyzed in a similar way and spectroscopic data confirmed the presence of long chain aldehyde. To identify the source of aldehydes, RBO has been fractionated by column chromatography and each fraction was analyzed as such and also after saponification. The results showed the presence of ether linked TAG fraction in RBO. In order to confirm the position of ether bond this fraction is subjected to controlled pancreatic lipase hydrolysis and confirmed the presence of *sn*-2 alk-1'-enyl ethers of glycerol.

Studies were also conducted to understand variations in USC in RBO from ten paddy varieties using the newly developed HPTLC method. Annapoorna, Aiswarya, Geerakasala, Gandhakasala, Jyothi, Uma, RM-1, Makaram, Karuna and Varsha were the paddy varieties selected which included high yielding medium duration and traditional aroma varities. Characteristics such as iodine value, saponification value, free fatty acid value have been measured. Fatty acid composition obtained by GC shows unsaturated fatty acid is greater in traditional variety Geerakasala and saturated fatty acid was higher in Aiswarya. Total USC quantification shows Aiswarya has high percentage of USC and Jyothi has the lowest.

Unsaponifiable constituents as antioxidants contribute to the stability of RBO. Antioxidant capacity of major USC was investigated. The effect of each USC on the stability of RBO has not been reported so far. In order to study the effect of individual constituents sterols, oryzanols and tocols (tocopherols and tocotrienols) on the stability of RBO, the constituents were separated from RBO by column chromatography and then reconstituted using oil stripped off these

constituents with addition of known concentrations both individually and in combination. The results showed among the individual constituents, addition of tocols showed concentration dependent effect on the stability. Among the combinations oryzanol and tocols additions was more effective than any other combinations. Sterol had little effect on the stability of RBO.

In conclusion, RBO as an edible oil has unique combination of bioactive phytochemicals at levels higher than other edible oils that contribute to high USM content. From this study it may be concluded that, HPTLC method developed here could be recommended for estimation of unsaponifiable constituents in RBO using single instrument. Identification of policosanol and fatty aldehydes in USM and their contribution to USM content hitherto not reported are salient findings of this investigation. Identification and confirmation of *sn*-2 alk-1'-enyl ethers in RBO through the present study is reported for the first time.

REFERENCES

1. India Oil seeds and products Annual Report, *USDA foreign Agricultural service*, IN 1031, April 2010.
2. Adkins, Y & Kelley, DS., *J Nutr Biochem* 21: 781-792, 2010.
3. Schwartz, H., Ollilainen, V., Piironen, V., & Lampi A., *J Food Compos Anal* 21: 152-161,2008.
4. Budowski, P., *J Am Oil Chem Soc* 41:280–285, 1964.
5. Perona, J S., Cabello-Moruno, R., & Ruiz-Gutierrez, V., *J Nutr Biochem* 17: 429–445,2006.
6. Perez-Jimenez, F., *Eur J Clin Invest* 35: 421–424, 2005.
7. Bogani, P., Galli, C., Villa, M., & Visioli, F., *Atherosclerosis* 190:181-186, 2007.
8. Puerta, C., Carrascosa-Salmoral, MP., Garcı´a-Luna,PP., Lardone,PJ., Herrera, JL., Ferna´ndez-Montesinos, R., Guerrero, JM., Pozo, D., *Food Chem* 104:609-612, 2007
9. Rao, BSN., Nonglyceride components of edible oils and fats. 2. Nutritional and health significance, *Food and Nutrition Bulletin* 22: 82-84, 2001.
10. Villarino, BJ., Dy, LM., & Lizada, CC., *Food Sci Technol* 40:193-199, 2007.
11. Seneviratne, KN., & Dissanayake, MS., *Int J Food Sci Technol* 43:597-602,2008
12. Nandakumaran, M., Al-sarraf, H., Al-fadhli1,R., Al-shammari1, M., Al-harmi1, J., & Al-saleh, E., *Nutritional Therapy & Metabolism* 27:183-188, 2009.
13. Kitts, D., edited by Hui, YH., *Bailey's Industrial Oil and Fat Products*, 5th edn., John Wiley & Sons, New York, 1: 256, 1996.
14. Kochhar, SP., *Prog Lipid Res* 22:161–85, 1983.
15. Packer L., *Am J Clin Nutr* 1991; 53(suppl):1050S–1055S.
16. Tay, BYP., & Choo, Y M., *Palm Oil Developments* 33: 13–17, 2007.
17. Tan, B., Grady, CM., & Gawienowski, AM., *J Am Oil Chem Soc* 63:1175–1179, 1986.
18. Yap, SC., Choo, YM., Ooi, CK., Ong, ASH., & Goh, SH., *Elaeis* 8: 369–378,1991
19. Thomopoulos, CD., & Tzia, C., *http://grasasyaceites.revistas.csic.es* 44:259-262, 1983.
20. Andersen, AJC., *Refining of oils and fats for edible purposes*, 2nd edn., Pergamon press, London, page 38, 1962.
21. Bureau of Indiana Standards, Indian Standard Specifications of Oil, 2nd edn, IS 3448 (1984), IS 4276 (1977), IS542- IS543, IS545.IS547 (1998), IS546 (1975), IS4277 (1975), IS8323 (1977).
22. Hui, YH., *Bailey's Industrial Oil and Fat Products*, 4th edn., Wiley Inter science, New York, 2: 97-676, 2005.
23. Bureau of Indiana Standards, Indian Standard Specification of Rice Bran Oil, 2nd edn, page 71, 1998.
24. Shahidi, F., *Bailey's Industrial Oil and Fat Products*, 6th edn., 2:467, 2005.
25. Godber, J., Xu, Z,, Hegsted, M., & Walker, T., *Louisiana Agriculture* 45**:**9-10, 2002.
26. Qureshi, A., Mo. H,, Packer, L., & Peterson, D., *J Agr Food Chem* 48**:**3130-3140,2000.
27. Rohrer, C., & Siebenmorgen, T., *Bio Syst Eng* 88**:**453-460, 2004.
28. Ling, WH., Wang, LL., & Ma, J., *J. Nutr* 132:20-26, 2002.
29. Han, BS., Park,CB., Takasuka, N., Naito,A., Sekine,K., Nomura,E., Taniguchi,H., Tsuno, T.,& Tsuda, H., *Jpn. J. Cancer Res*. 92: 404-409, 2001.
30. Helm, RM., & Burks,AW., *Cereal Food World* 41: 839-843, 1996.
31. Kawamura, Y., Muramoto, M., Waldron, KW., Johson, IT., & Fenwick, LR., *Chem. Biol. Aspects.,* 54: 331-401, 1993.
32. Xu, Z., Hua, N., & Godber, JS., *J. Agric. Food Chem* 49:2077-2081, 2001.
33. Qureshi, AA., & Sami, SA., Khan, FA., *J Nutr Biochem,*13:175-187, 2002.
34. Butsat,S., & Siriamornpun, S., *Food Chem* 119:606–613,2010.
35. Bergman, C., Xu, Z., *Journal of Cereal Chemistry* 80:446-449, 2003.
36. Sival,K.,Mukherjee, RK., & Bhole, NG., *J food Eng* 20: 215-222, 1993.
37. Kuk, MS., & Dowd,MK., *J Am Oil Chem Soc* 75:623-625,1998.
38. Kim, H., Lee, S., Park, K., & Hong, I., *Sep Purif Technol* 15:1–6, 1999.
39. Proctor, A., & Bowen, DJ., *J Am Oil Chem Soc,* 73:811-813, 1996.

40. Lee,JH., Yu,F., Vu,PL., Choi,MS., Akoh,CC., & Lee, KT., *J Food Sci* 72:C163-C167, 2007.
41. Sierra,S., Lara-Villoslada, F., Olivares, M., Jiménez, J., Boza,J., & Xau, J., *Eur J Nutr* 44: 509–516,2005.
42. Glushenkova, AI., Ul'chenko, NT.,Talipova, M., Mukhamedova, KS., Bekker, NP.,& Tolibaev, I., *Chemistry of Natural Compounds* 34: 275-277, 1998.
43. Hvolby, A., *J Am Oil Chem Soc*, 48:503–509, 1971.
44. Kaneko, R., & Tsuchiya, T., *Journal of Industrial Chemistry* 57: 526, 1954.
45. Cicero, AFG., & Gaddi, A., *Phytother Res*, 15:277-289, 2001.
46. Rong, N., Ausman, LM., & Nicolosi, R., *FASEB J.* 8:A162, 1994.
47. Kobayashi, SYI., Maruto, S., Ohshima, K., Mori, M., Kamio, N., & Fukuda, H., *Endocrinol Jap* 12: 83-86, 1980.
48. Bucci, LR., Black man, G., & Defoyd, W., *J Applied Sport Sci Research* 4: 110,1990.
49. Yasukawa, K., Akihisa, T., Kimura, Y., Tamura, T., & Takido, M., *Biol Pharma Bull* 21: 1072-1076, 1998.
50. http://onlinelibrary.wiley.com/doi/10.1002/(SICI)1097-0010(19990301)79:3%3C476::AID-JSFA270%3E3.0.CO;2-L/abstract -fn1Saija, A., Tomaino,A., Cascio, RL., Trombetta, D., Proteggente, A., Pasquale, AD., Uccella, N., & Bonina, F., *J Sci Food Agric* Special Issue: Proceedings from Ferulate '98, 79:476–480, 1999.
51. Lau, CB., Hom TC., Chan, TW., & Kim, SC. *Menopause*12:734–40, 2005.
52. Khuwijitjaru, P., Yuenyong, T., Pongsawatmanit, R., & Adachi, S. *Journal of Oleo Science* 58: 491-497,2009.
53. Krishna,AGG., Khatoon,S., Shiela, PM., Sarmandal, CV., Indira, TN., & Mishra, A., *J Am Oil Chem Soc* 78: 127-131, 2001.
54. Sen, CK., Khanna, s., & Roy, s., *Life Sci.* 78: 2088–2098, 2006.
55. Glynn, RJ., Ridker, PM., Goldhaber, SZ., Zee, RY., Buring, JE., *Circulation* 116:1497-1503, 2007.
56. Lampi, A., Kataja, L., Kamal-Eldin, A., & Vieno, P., *J Am Oil Chem Soc* 76: 749–755, 1999.
57. Burton, G.W., & Ingold, KUI., *J. Am.Chem. Soc.* 103:6472–6477 (1981).
58. Cillard, J., Cillard, P., & Cormier, M., *J. Am. Oil Chem. Soc.* 57:255–261 (1980).
59. Fuster, MD., Lampi, A., Hopia, A., & Kamal-Eldin, A., *Lipids*, 33:715-722, 1998.
60. Yoshida, H., Kajimoto, G., & Emura, S., *J Am Oil Chem Soc* 70: 989-995, 1993.
61. Marinova, M., &Yanishlieva, NV., *J. Sci Food Agric* 60: 313–318, 1992.
62. Hoffmann, G., *The chemistry of edible fats* Academic Press, London, page 1–28, 1989 .
63. Rossi, M., Alamprese, C., & Ratti, S., *Food Chem* 102: 812–817, 2007.
64. Akihisa, T., Kokke, W., & Tamura, T., *Naturally occurring sterols and related compounds from plants*, American Oil Chemists' Society, Champaign, IL, page 172-228,1991.
65. Heinemann, T., Kullak-Ublick, G., Pietruck, B., & Bergmann, VK., *Eur J Clin Pharmacol* 40: S59-S63, 1991.
66. Hartmann, M., & Benveniste, P., *Method Enzymol* 148:632-650, 1987.
67. de Jong, N., Plat, J., & Mensink, RP., *J Nutr Biochem* 4:362–369, 2003.
68. Hirota, Y., Nagao, T., Watanabe, Y., Suenaga, M., Nakai, S., Kitano, M., Sugihara, A., & Shimada, Y., *J. AmOil Chem Soc* 80: 341-346, 2003.
69. Gunawan, S., Melwita, E., & Ju, Y., *Food Chem* 121: 752–757, 2010.
70. Ferrari, RA., Esteves, W., Mukherjee, KD., & Schulte, E., *J Agric Food Chem* 45:172-228 1997.
71. Sierksma, A., Weststrate, JA., & Meijer, GW., *Br J Nutr* 82:273–282, 1999.
72. Perrin, JL., *Rev Fr Corps Gras* 39: 25-32, 1992.
73. Scott, G., *Atmospheric Oxidation and Antioxidants*, Elsevier Publishing Co, Amsterdam, 2: 16, 1965.

74. Rao, CV., & Newmark, HL., *Carcinogenesis* 19: 287-290, 1998.
75. Khor, HT., & Chieng, DT., *Asia Pac J Clin Nutr* 6: 36-40, 1997.
76. Miettinen, TA., & Vanhanen, H., *Am J Clin Nutr* 59:356-363, 1994.
77. Warleta, F., Campos,M., Allouche, Y., Sánchez-Quesada,C., Ruiz-Mora,J., G., & Gaforio, JJ., *Food Chemical Toxicol* 48:1092-1100, 2010.
78. Budin, JT., Breene, WM., & Putnam, DH., *J Am Oil Chem Soc* 73: 475-481, 1996.
79. Sun, H., Wiesenborn, D., Rayas-Duarte, P., Mohamed, A., & Hagen, K., *J Am Oil Chem Soc* , 72: 1551-1555, 1995.
80. Yoon, SH., & Rhee, JS., *J Am Oil Chem Soc* 59: 561-563.
81. Kodali, DR., *Lipid Technol* 21: 254-256, 2009.
82. Buffa, CW., *Cosmet Toiletries* 91:14–16, 1976.
83. Liu, SX., & Mamidipally, PK., *Cereal Chemistry* 82: 209-215, 2005.
84. Rajam, L., Kumar, DRS., Sundaresan, A., & Arumughan, C., *J Am Oil Chem Soc* 82: 213-220, 2005.
85. Ramaswamy, KG., Gopalakrishna, AG., & Sen, DP., *J Oil Tech Assoc India* 12:109–113, 1980.
86. Ichimatsu, I., & Ichimatsu, S., Refining of rice bran oil. JP7026288, 1995.
87. Hidaka, I.,& Tsuchiya, N., Preparation of rice bran oil. JP11968, 2000.
88. Hoed, VV., Depaemelaere, G., Ayala, JV., Santiwattana, P., Verhé, R., & Greyt, WD., *J Am Oil Chem Soc* 83: 315-321, 2006.
89. Ferrari, RA., Schulte, E.,Esteves, W., Brühl, L., & Mukherjee, KD., *J Am Oil Chem Soc* 73:587–592,1996.
90. Bhattacharyya, AC., Majumdar, S., & Bhattacharyya, DK., *J Am Oil Chem Soc* 63: 1189-1191, 1986.
91. Canavag, GC., *J Am Oil Chem Soc* 53:361–363,1976.
92. Nogima, S., Miscella refining of rice bran oil. JP 1819, 1950.
93. Bhattacharyya, AC., Majumdar, S., & Bhattacharyya, DK., Oleagineux 42:431–43, 1987.
94. Andersen, AJC., *Refining of fats and oils for edible purposes*, 2nd edn., Pergamon press, Oxford, page 40, 1962.
95. Jahani, M., Alizadeh, M., Pirozifard, M., & Qudsevali, A., *Food Sci Technol*, 41:1892-1898, 2008.
96. Gibon, V., & Tirtiaux, A., *inform* 11:524–535, 2000.
97. Indira, TN., Hemavathy, J., Khatoon, S., Krishna, AGG., & Bhattacharya, S., *J Food Eng* 43: 83-90, 2000.
98. Bhattacharya, AC., & Bhattacharya, DK.,. *J Oil Tech Assoc India*, 17: 27-30, 1985.
99. Bhowmick, DN., *Membranes –Proceedings of India-EC Workshop*. Oxford & IBH, New Delhi, page 157–164,1992.
100. Manjula, S., & Subramanian, R., *Sep Purif Technol* 66: 223-228, 2009.
101. Sullivan, FE., *J Am Oil Chem Soc* 57:845A, 1980.
102. De, BK., & Bhattacharyya, DK., *J Am Oil Chem Soc* 75: 1683-1686, 1998.
103. Koseoglu, SS., & Engelgau, DE., *J Am Oil Chem Soc* 67:239–249, 1990.
104. Krishna, AGG., *Am Oil Chem Soc* 69: 1257-1259, 1992.
105. Chen,C., Wang, C., Wang, L., Hong,Z., Chen,S., Ho, W., & Chang, CJ., *J Supercrit Fluid* 45: 322-331,2008.
106. De, BK., & Bhattacharyya, DK., *J Am Oil Chem Soc* 76: 1243-1246, 1999.
107. Kale, V., Katikaneni, SPR., & Cheryan, M., *J Am Oil Chem Soc* 76: 723–72, 1999.
108. Theodorou, V., Skobridis, K., Tzakos, AG., & Ragoussis, V., *Tetrahedron Lett* 48: 8230–8233,2007.
109. Elmadfa, I., *Fat Science Technology*, 97:85, 1995.
110. Saunders, RM., *Cereal Foods World* 35:632–636, 1990.
111. Kim, JS., & Godber, JS., *J Food Qual* 24:17–26, 2001
112. Kritchevsky, D. Phytosterols. In Bonfield Kristchevsky (Ed.). *Dietary fiber in health and disease*. New York: Plenum Press, 427:235–242, 1997.

113. Sayre, B., & Saunders, R., *Lipid Technol*. 2:72-76, 1990.
114. Diack, M., & Saska, M., *Journal of the American Oil Chemists' Society,* 71: 1211–1217, 1994.
115. Lloyd, BJ., Siebenmorgen, TJ., & Beers, KW., *Cereal Chemistry,* 77:551–555, 2000.
116. Patel, M., & Naik, SN., *J Sci Ind Res India,* 63:569–578, 2004.
117. Mezouari,S., Eichner, K., Kochhar, SP., Brühl,L., & Schwarza, K., *Eur J Lipid Sci Technol* 108:193–199, 2006.
118. Pestana, VR., Zambiazi, RC., Mendonc¸CRB., Bruscatto, MH., Lerma-Garcı,MJ., & Ramis-Ramos, G., *J Am Oil Chem Soc* 85:1013–1019,2008.
119. Kim, SK., Kim, CJ., Cheigh, HS., & Yoon, SH., *J Am Oil Chem Soc* 62: 1492-1495, 1985.
120. The Prevention of Food Adulteration Act, Central Law Agency, University Road, Allahabad, 1954.
121. Codex Alimentarius standard for named vegetable oils, Ref. CL2005/47FO.FAO and WHO. Rome, 2005.
122. Gunstone, FD., Harword, JL. & Padley, FB., *The Lipid Handbook.* Chapman and Hall, London, 1986.
123. Official methods and recommended practices of the AOCS, AOCS official method, 5th edn, *Unsaponifiable Material*, Ca 6a-40, Washington DC, 1997.
124. Official methods of analysis of AOAC international AOAC official method Residue (unsaponifiables) of oils and fats, 17th edn., Gaithersburg, USA, page 42, 2000.
125. International Union of Pure and Applied Chemistry (IUPAC), Standard Methods for the Analysis of Oils, Fats and Soaps, 5th edn. Blackwell Scientific, London, II.D.5.1 and II.D.5.3, 1966.
126. Takeshita, Y., & Okubo, O., Trans. Kokushikan Univ. Dep. Eng. No. 14, 1981.
127. International Union of Pure and Applied Chemistry (IUPAC), Standard Methods for the Analysis of Oils, Fats and Derivatives, 7th edn. Blackwell Scientific, London, 1987.
128. Devi, RR., Suja, KP., Jayalekshmy, A., & Arumughan C., *J. Oil. Tech. Asso. India* 32: 176-182, 2000
129. Holen, B., *J.Am.Chem. Soc* 62: 1344-1346, 1985.
130. Devi, RR., Jayalekshmy, A., & Arumughan,C., presented at the 5th Annual convention and seminar on Indian Oil seeds and Edible oil sector, Challenges ahead, IICT, Hyderabad, India, 2000.
131. Deepam, LSA., Kumar, DRS., Sundaresan, A., & Arumughan, C., *J sep sci* 30:2786-2793, 2007.
132. Ghulam, A., & Shabir., J., *Chromatogr. A* 987: 57-66, 2003.
133. FDA Reviewer, *Guidance Validation of Chromatographic Methods*, CDER, USA, 1994.
134. Ferenczi-Fodor, K., Vegh, Z., Nagy-Turak, A., Renger, B., & Zeller, M., *J. AOAC Int*. 84:1265–1276, 2001.
135. AOAC, Official Methods of Analysis of AOAC International, 17th edn. Gaithersburg, USA, 2000.
136. Holcapek, M., Jandera, P., Zderadicka, P., & Huba, L., *J chomatogr A* 1010: 195-215, 2003.
137. Hartvigsen, K., Ravandi, A., Bukhave, K., Holmer, G., & Kuksis, A., *J. Mass Spectrom.* 36: 1116-1124, 2001.
138. Deepam, LSA., & Arumughan, C., *J Oleo Sci* 59: 521-526, 2010.
139. Official methods and recommended practices of the AOCS, AOCS official method Cc 13e-92, Cc13i-96, Washington DC, 1997.
140. Deepam, LSA., Sundaresan, A., & Arumughan, C., *J Am Oil Chem Soc* DOI:10.1007/s11746-010-1744-1,2011.
141. Official methods and recommended practices of the AOCS, 5th edn. Method Cd 8-53 Champaign, IL, USA, 1997.
142. Jirusova, J., *Nahrung* 19: 319, 1975.

143. Wettasinghe, M., & Shahidi, F., *J Agric Food Chem* 47: 1801–1812, 1999.
144. Espın, JC., Soler-Rivas, C., & Wichers, HJ., *J Agric Food Chem* 48: 648-656,2000.
145. Duncan, DB., *Biometrics*, 11:1–42, 1955.
146. Nair, VDP., Kanfer, I.,& Hoogmartens, J., *J Pharm Biomed Anal* 41:731-737,2006.
147. Gliszczy´nska- ´Swigło, A., & Sikorska, E., *J Chromatogr A* 1048: 195-198, 2004.
148. Miller, A., Frenzel,T., Schmarr, H.,& Engel, K., *J. Chromatogr. A,* 985:403-410, 2003.
149. Subrahmanyam, CV., Rao, M., Balasubrahmanyam, V., & Bhowmick, DN., *Eur J Lipid Sci Technol* 108: 746-752, 2006.
150. Mezouari, S., & Eichner, K., *Eur J Lipid Sci Technol* 109:198–205, 2007.
151. Tsuchiya, T., & Kaneko, R., & Ohkubo, O., Komenukayuchuuno oryzanol ganryou nituite Tokyokougyou sikensho Houkoku 52: 1-3, 1957.
152. Tanabe, K., Tanaka, A., & Kato, A., *J Jpn oil Chem Soc* 30:512-514, 1982.
153. Yoshie, A., Kanda, A., Nakamura, T., Igusa, H., & Hara, S., *J oleo sci* 58: 511-518, 2009.
154. Britz, SJ., Prasad, PVV., Moreau, RA., Allen, LH., Kermer, DF., & Boote, KJ., J Agric Food Chem 55:7559-7565, 2007
155. Rogers, EJ., Rice, SM., Nicolosi, RJ., Carpenter, DR., Clelland, MCA., & Romanczyk, LJ., *J Am Oil Chem Soc* 70: 301-307, 1993.
156. Terashima, K., Kishimoto, K., Kamata, K., Nishijima, M., & Ohnishi, K., *Ann. Rep.* Tokyo Metr. Res. Lab P.H. 48: 81-85, 1997.
157. Stoggl, W., Huck, C., Wongyai, S., Scherz, H., & Bonn, G., *J. Sep. Sci.* 28: 1712–1718, 2005.
158. Norton, RA., *Lipids* 30:269, 1995.
159. Evershed, RP., Spooner, N., Prescott, MC., & Goad, LJ., *J. Chromatogr* 440:23, 1988.
160. Krishna, AGG., Prashanth, PA., Pragasam, A., Raghavendra, KV., & Khatoon, S., *J.Food Lipids,* 10: 329-340, 2003.
161. Verleyen, T., Forcades, M., Verhe, R., Dewettinck, K., Huyghebaert, A., & Greyt, WD., *J Am Oil Chem Soc* 79:117-122, 2002.
162. Toivo,J., Piironen, V., Kalo, P., & Varo, P., *Chromatographia* 48;745-750 1998.
163. Abidi, SL., *J. Chromatogr. A* 1059: 199–208, 2004.
164. Lerma-Garci, M.J., Simo-Alfonso, EF., Me ndez, A., Lliberia, JL., & Herrero-Martinez, JM., *J. Agric. Food Chem.* 58: 2771–2776, 2010.
165. Carretero1,AS., Carrasco-Pancorbo1,A., Cortacero1,S., Gori,A., Cerretani,L., & Alberto Fernández-Gutiérrez1., *Eur. J. Lipid Sci. Technol* 110: 1142–1149, 2008.
166. Sookwong, P., Nakagawa, K., Murata., K., Kojima, Y., & Miyazawa, T., *J. Agric. Food Chem.* 55: 461-466, 2007.
167. Asaf, A., Qureshi., Mo, H., Packer, David, L., & Peterson, M., *J Agric Food Chem* 48: 3130-3140, 2000.
168. Cunha, SS., Fernandes, JO., & Oliveira, BPP., *J Chromatogr A* 1128: 220-227, 2006.
169. Nota, G., Naviglio, D., Romano, R., Sabia, V., Musso, S.S., & Improta, C., *J Agric Food Chem* 47: 202-205, 1999.
170. Kanya, TCS., Rao, LJ., & Sastry, MCS., *Food Chem* 101:1552–1557,2007.
171. Lau, HLN., Puah CW., Choo YM., Ma AN., & Chuah CH., *Lipids* 40: 523-528, 2005.
172. Choo, YM., Ng, MH., Ma, AN., Chuah, CH., & Hashim, MA., *Lipids* 40: 429-432, 2005.
173. Grigoriadou, D., Androulaki, A., Psomiadou, E., & Tsimidou, MZ., *Food Chem* 105:675–680, 2007.
174. Giacometti, J., *Analyst*, 126: 472–475, 2001.
175. Vali, SR., Ju, Y., Kaimal, TNB., & Chern, Y., *J Am Oil Chem Soc* 82: 57-64, 2005.
176. Chung, I., Hahn, S., & Ahmed, A., *J Chem Ecol* 31:1339-1352, 2005.
177. Hamill, FA., Apio, S., Mubiru, NK., Mosango, M., Bukenya-Ziraba, R., Maganyi, OW., & Soejarto, DD., *J Ethno Pharmacol* 87:15–19, 2003.
178. Wang, M., Lian, H., Mao, L., Zhou, J., Gong, H., Qian, B., & Fang, Li, J., *J Agric Food Chem* 55: 5552-5558, 2007.

179. Caligiani, A., Bonzanini, F., Palla, G., Cirlini, M., & Bruni, R., *Plant Foods Hum Nutr* 65:277-283, 2010.
180. Sakouhi F., Absalon, C., Flamini, G., Cioni, PL., Kallel, H., & Boukhchina, S., *Comptes Rendus-Biologies,* 333: 642-648, 2010.
181. Irmark, S., & Dunford, NT., & Milligen, J., *Food Chem* 95: 312-318, 2006.
182. Hwang, KT., Weller, CL., Cuppett, SL., & Hanna, MA., *Cereal Chem* 81: 345-349, 2004.
183. Avato, P., Bianchi, G., & Murelli, C., *Phytochemistry* 29: 1073–1078, 1990.
184. Taylor, JC., Rapport, L., & Wood, GBL., *Nutrition* 19: 192–195, 2003.
185. Bianchi,G., Lupotto,E., & Russo, S., *Experientia* 35: 1417-1540, 1979**.**
186. Sjovali, O., Kuksis, A., & Kallio, H., *Lipids*, 37:81-94, 2002.
187. Christianne, EC., Pedro, R., Filho, AP., & Meirelles, AJA., *Fluid Phase Equilib* 216:271-283, 2004.
188. Krishna, AGG., Hemakumar, KH., & Khatoon, S., *J Am Oil Chem Soc* 83: 117-120, 2006.
189. Rukmini, C., & Raghuram, TC., *J Am Coll Nutr* 10: 593-601, 1991.
190. Sugano, M., & Tsuji, E., VII th Asian Conference of Nutrition: Lipid Symposium Proceedings, Journal of nutrition, 521S-524S, 1997.
191. Gunawan, S., Vali, SR.,& Ju, Y., *J Am Oil Chem Soc,* 83:449-456, 2006.
192. Ito, S., Shzuki,t., & Fujino, Y., *Cereal Chem* 60:252–253, 1983.
193. Bruscatto, MH., Zambiazi, RC., Sganzerla, M., Pestana, VR., Otero, D., & Lima, R., *Paiva, F. J Chromatogr Sci* 47: 762-765, 2009.
194. Vaidya, B., & Choe, E., *J Am Oil Chem Soc* 88:83-90, 2010.
195. Czauderna, M., & Kowalczyk, J., *J Chromatogr B*, 858:8-12, 2007.
196. Ryynänen**,** M., Lampi**,** AM., Salo-Väänänen**,** P., Ollilainen**, V., &** Piironen**, V.,** *J Food Compos Anal* 17: 749-765, 2004.
197. Toivo, J., Phillips, K., Lampi, AM., & Piironen, V., *J Food Comp Anal* 14:631–643,2001.
198. Clement, LM., Hansen, SL., Costin, CD., & Perri, GL., *J Am Oil Chem Soc* 87:973–980, 2010.
199. Marini, F., Balestrieri, F., Bucci, R., Magr, AL., & Marini, D., *MicrocheM J* 74: 239-242, 2003.
200. Zigoneanu, IG., Williams, L., Xu, Z., & Sabliov, CM., *Bioresour Technol* 99: 4910-4918, 2008.
201. Lkeda, RM., Rolle, LA., Vannier, SH., & Stanley, WL., *JAgricFoodChem* 10:98-102, 1964.
202. Wang, C., Chen, C., Wu, J., Wang, L., Chang, CM., & Ho, WJ., *J Sep Sci* 31:1399-1407,
203. Rizzo, WB., Heinz, E., Simon, M., & Craft, DA., *Biochim. Biophys. Acta, Gen. Subj.* 1535: 1-9 2000.
204. Stoffel, W., & Lekim, D., *Hoppe-Seyler's Zeitschift fur Physiologische Chemie* 352: 501-511 1971.
205. Fauconnot, L., Robert, F., Villard, R., & Dionisi, F., *Chem Phys Lipids*, 139:125-136, 2006.
206. Smith, GM., & Djerassl, C., *Lipids* 22:236-240, 1987.
207. Dick, D., Pluskey, S., Sukumaran, DK., & Lawrance, DS., *J Lipid Res* 33: 605-609,1992.
208. Simova, S., Ivanova, G., & Spassov, SL., *Chem Phys Lipids*, 126:167-176, 2003.
209. Gorgas, K., Teigler, A., KomLjenovic, D., & Just, WW., *Biochimi. Biophysi. Acta* 1763: 1511-1526, 2006.
210. Qu, L., Zhu, S., Liu, M., & Wang, S., *J. Appl. Polym. Sci.* 97: 2279-2285, 2005.
211. Grobelny, Z., *Eur J Org Chem* 14: 2973-2982, 2004.
212. Casado, F., Pisano, L., Farriol, M., Gallardo, I., Marquet, J., & Melloni, G., *J Org Chem* 65: 322-331, 2000.

213. Hartvigsen, K., Ravandi, A., Bukhave, K., Holmer, G., & Kuksis, A., *J Mass Spectrom* 36**:** 1116-1124, 2001.
214. Silverstein,RM., Webster, FX., & Kiemle, DJ., *Spectrometric Identification of Organic Compounds,* 2nd edn. Oxford university press, page 91 2005.
215. Doleschall, F., Kemeny, Z., Recseg, K., & Kovari, KJ., *Microcolumn Separations*, 13: 215-220, 2001.
216. Sindelar, PJ., Guan, Z., Dallner, G., & Ernster, L., *Free Radical Biol Med* 26:318-324, 1999.
217. Pugliese, PT., Jordan, K., Cederberg, H., & Brohult, JJ., *Altern Complem Med* 4: 87-99, 1998.
218. Hoffman- Kuczynski, B., & Reo, NV., *Neurochem Res* 29: 843-855, 2004.
219. Schakamp, G., Schutgens, RB., Wanders, RJ., Heymans, HS., Tager, JM., & Bosch, HV., *Biochim. Biophys. Acta* 833**:**170-174,1985.
220. Ginsberg, L., Rafique, S., Xuereb, H., Rapoport, SI., & Gershfeld, NL., *Brain Res* 698: 223-226 1995.
221. Murphy, EJ., Schapiro, MB., Rapoport, SI., & Shetty, HU., *Brain Res* 867:9-18, 2000.
222. Malekian, F., Rao, RM., Prinyawiwatkul, W., Marshall, W. E., Windhauser, M., & Ahmedna, M., Bulletin, Agricultural Experiment Station, Louisiana, 870:1-68, 2000.
223. Denev, R., Kuzmanova, I., Panayotova, S., Momchilova, S., Kancheva, S., & Lokesh, BR., *Comptes Rendus de L'Academie Bulgare des Sciences*, 62: 709-716, 2009.
224. Gaydou, EM., Raonizafinimanana, R., & Bianchini, JP., *J Am Oil Chem Soc* 1980:141-142, 1980.
225. Juliano, BO., Albano, EL., & Cagampang, GB., *Philipp Agricultur*, 48, 234–456, 1964.
226. Iqbal, S., Bhange, MI., & Anwa, F., *Food Chem* 93: 265–272, 2005.
227. Lai, P.,Li, KY., Lu,S., & Chen, HH., *Food Chem* 117:538–544,2009.
228. Khatoon, S., & Gopalakrishna, AGG., *J Am Oil Chem Soc* 81:939-943, 2004.
229. Lilitchan, S., Tangprawat, C., Aryusuk, K., Krisnangkura, S., Chokmoh, S., & Krisnangkura, K., *Food Chem* 106:752–759, 2008.
230. Krishna, AGG., Prabhakar, JV., & Sen, DP., *J Food Sci Technol* 21:222–224, 1984.
231. Sookwong, P., Nnakagawa, K., Murata, K., Kojima, Y.,& Miyazawa, T., *J. Agric. Food Chem*., 55:461-467, 2007
232. Juliano, C., Cossu, M., Alamanni, MC., & Piu, L., *Int J Pharm* 299:146-154, 2005.
233. Soupas, L., Juntunen, L., Lampi, AM., & Piironen, V., *J Agric Food Chem* 52, 6485–6491, 2004.
234. Kim, JS., Suh, MH., Yang, CB., & Lee, HG., *J Food Sci* 68: 2423-2429, 2003.
235. Yanishlieva, NV., Kamal-Eldin, A., Marinova, EM., & Toneva, AG., *Eur J Lipid Sci and Technol* 104: 262-270, 2002.
236. Plaza, LA., Sancheez-Moreno, CN., Pascual-Teresa, SD., Ancos, BAD., & Cano, MP., *J Agric Food Chem* 57: 3204-3209, 2009.
237. Gordon, MH., & Magos, P., *Food Chem* 10: 141-147, 1983.
238. Nystrom, L., Achrenius, T., Lampi, A., Moreau, RA., & Piironen, V., *Food Chem* 101: 947-954, 2007.
239. Mezouari, S., & Eichner, K., *Eur J Lipid Sci Technol* 109: 531-535, 2007.
240. Bhatnagar, AS., Kumar, PKP., Hemavathy, J., & Krishna, AGG., *J Am Oil Chem Soc* 86: 991-999, 2009.
241. Yoshida, Y., Niki, E., & Noguchi, N., *Chem phys Lipids* 123: 63-75, 2003.

LIST OF PUBLICATIONS

1. A new method for simultaneous estimation of unsaponifiable constituents of rice bran oil using HPTLC**, *Journal of Separation Science*,** **Afinisha Deepam L.S.**, Kumar, DRS., Sundaresan, A., Arumughan, C., 30: 2786-2793, 2007.

2. Characterization of *sn*-2 alk-1-enyl ethers of glycerol from refined rice bran oil, ***Journal of Oleo Science,*** **Afinisha Deepam L.S**., C. Arumughan. 59, 521-526, 2010.

3. Stability of Rice Bran Oil in terms of oryzanol, tocopherols, tocotrienols and sterols **Afinisha Deepam L.S**., Sundaresan, A., Arumughan, C., ***Journal of American Oil Chemists Society, Accepted, article in press, DOI:****10.1007/s11746-010-1744-1***.**

SEMINARS ATTENDED / PRESENTED

1. Paper presented in 8th National Seminar on Rice Bran Oil, Unsaponifiable Matter in Rice bran Oil: Scientific and Legal Dimensions C. Arumughan, D.R. Soban Kumar, **Afinisha Deepam L.S**., 29th July 2005, New Delhi.

2. Paper presented in National Seminar on Nutritional phytochemicals. Oryzanol in rice bran a nutritional phytochemical. **Afinisha Deepam L.S**., C.Arumughan., A.Sundaresan, 25th June 2010, Trivandrum.

3. Policosanol as major unsaponifiable constituent in refined rice bran oil **Afinisha Deepam,L.S. C. Arumughan.** "97th Indian Science Congress", Agriculture and Forestry Sciences, Page No. 253. 2010, Thiruvananthapuram.

4. Antioxidant Activity of Rice Bran Oil Micronutrients at Different Combinations **Afinisha Deepam L.S**., C.Arumughan., A.Sundaresan. International Conference on Advances in Free Radical Research, Natural Products, Antioxidants and Radioprotectors in Health and Ninth Annual Meeting of the Society of Free Radical Research, page No. 133, 2010, Hyderabad.

5. 5th Mid CRSI Symposium in Chemistry, held at National Institute of Interdisciplinary Science and Technology) CSIR, Trivandrum, Kerala, India, July, 2010.

****