Lipase catalyzed interesterification of rice bran oil with hydrogenated cottonseed oil to produce *trans* free fat

T. S. V. R. Neeharika · Ramya Rallabandi · Y. Ragini · Shiva Shanker Kaki · K. N. Prasanna Rani · R. B. N. Prasad

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Abstract Lipase catalyzed interesterification of rice bran oil (RBO) with hydrogenated cottonseed oil (HCSO) was carried out for producing a low *trans* free fat. The interesterification reaction was performed by varying parameters such as weight proportions of RBO and HCSO, reaction temperatures, time period and lipase concentration. Both non specific and specific lipases namely Novozym 435 and Lipozyme TL IM were employed for this study. Based on the data generated, the optimum reaction conditions were found to be: weight proportion of RBO and HCSO, 80:20; lipase concentration, 5 % (w/w) of substrates; reaction temperature, 60 °C; reaction time, 4 h for Lipozyme TL IM and 5 h for Novozym 435. The degree of interesterification, calculated based on the results of solid fat characteristics was used for comparing the catalytic activity of Novozym 435 and Lipozyme TL IM. It was observed that the degree of interesterification (DI) reached a near 100 % at the 4th hour for reaction employing Lipozyme TL IM with a rate constant of 0.191 h^{-1} while Novozym 435 catalyzed reaction reached a near 100 % degree of interesterification at the 5th hour with a rate constant of 0.187 h^{-1} , suggesting that Lipozyme TL IM has a faster catalytic activity.

Keywords *Trans*-free fat · Lipase catalyzed interesterification · Hydrogenated cottonseed oil · Rice bran oil · Degree of interesterification

Introduction

The innate physical and chemical properties of vegetable oils often restrict their applicability in certain food products. The need to increase their use and applicability leads to the invention of several modification strategies. Few of which include formulation, partial hydrogenation, hydrogenation, fractionation and interesterification (Rajah 2002). Each of these processes follows a different principle to achieve its goal. However, partial hydrogenation produces '*trans* fat' apart from modifying the fatty acid profile and hence is least preferred. *Trans* fat is known to have several adverse effects on the plasma lipoproteins and is also suspected to be the direct or indirect cause of several diseases ranging from cancer to cardiac disorders (Vandana et al. 2010). Interesterification is a general term for the reaction involving the redistribution of the fatty acids between and within the triacylglycerol with or without the aid of a catalyst. The modification of the physico-chemical properties of lipids by interesterification is attributed to the ester-ester exchange which results in the rearrangement of the position of fatty acids on the glycerol backbone (Rodriguez et al. 2009). The advantage of using interesterification over hydrogenation is that no *trans* fat is produced since it only involves redistribution of fatty acids (Gunstone 2002) over the glycerol backbone.

Since the early work on interesterification of edible lipids by chemical methods (Normann et al. 1920), much research has been done in this field. Lipases are preferred over chemical catalysts because they are regio- and stereo-specific and offer a better control over the final product (Marangoni and Rousseau 1995). Several positional specific and non specific lipases were exploited for the use of interesterification reaction in past 15 years. The lipases studied extensively for such type of lipid modification include commercially available lipases such as Lipozyme TL IM and *Candida antarctica* lipase or Novozym 435. Researchers have considered many fats and

T. S. V. R. Neeharika · R. Rallabandi · Y. Ragini · S. S. Kaki · K. N. P. Rani · R. B. N. Prasad (✉)
Centre for Lipid Research, CSIR-Indian Institute of Chemical Technology, Tarnaka, Hyderabad 500007, India
e-mail: rbnprasad@iict.res.in

oils as substrates for the use in lipase catalyzed interesterification. Some of these include fully hydrogenated soya bean oil, palm stearin, palm kernel oil, sesame oil, coconut oil, rice bran oil (Li et al. 2010; Adhikari et al. 2009; Shin et al. 2009; Adhikari et al. 2010; Rodriguez et al. 2009; Mayamol et al. 2009; Criado et al. 2008).

Interesterification for producing a spread like fat is normally conducted taking two triacylglycerols one of which is liquid and other one being a solid or semi solid triacylglycerol and natural oils are preferred for interesterification. Rice bran oil is well known oil which is reported to reduce harmful cholesterol [LDL-low density lipoprotein] without destroying the good cholesterol [HDL-High density lipoprotein]. Three major antioxidants occupy its composition, namely tocopherols, tocotrienols and oryzanol which not only impede the progress of melanin pigmentation but can also strengthen the immunity (Adhikari et al. 2010). The use of rice bran oil in production of value added products like margarines has been reported where interesterification of rice bran oil was carried out with different kinds oils such as palm oil, palm stearin or hydrogenated soybean oil (Adhikari et al. 2010; Mayamol et al. 2009; Malongil et al. 2009; Kaki et al. 2012). Interesterification of RBO with cotton seed oil has not been reported so far. Cottonseed oil is primarily used as salad oil, cooking oil, shortening, and margarine (Brien and Wakelyn 2005). Cottonseed oil requires less hydrogenation to achieve the same degree of hardness compared to other linoleic oils due to its fatty acid (FA) composition (Gunstone 2002). Moreover, hydrogenated cottonseed oil is popular for its longer shelf life and hence is our choice of substrate for the present study.

In order to obtain or tailor the desired product, it is essential to monitor the degree of interesterification. The degree of interesterification can be monitored by recording the changes in the chemical (TAG-Triacylglycerol composition) and physical (SFC-Solid fat content, DSC-Differential scanning calorimetry melting profiles) properties. It was reported that the degree of interesterification calculated based on SFC profiles was more accurate than degree of interesterification calculated on the basis of TAG composition (Rodriguez et al. 2009; Clercq et al. 2010).

Therefore, the present study aims to produce a *trans* free fat by enzymatic interesterification of rice bran oil and hydrogenated cottonseed oil with desirable physico-chemical properties employing two commercial lipases namely Novozym 435 and Lipozyme TL IM along with the determination of degree of interesterification for the two lipases during interesterification.

Materials and methods

Materials

One kilogram of fresh refined rice bran oil was procured from M/s Ramcharan Industries, Hyderabad, Telangana, India in

January 2013. Refined rice bran oil used in the study was purchased from the local market. Hydrogenated cottonseed oil with an iodine value of 5.6 was prepared in the laboratory employing traditional hydrogenation method (Simakova et al. 2008) using 10 % Pd/C as catalyst. Immobilized lipases Novozym 435 and Lipozyme TL IM (*Thermomyces lanuginosus*) were obtained from Novozymes A/S (Bagsvaerd, Denmark) and *Porcine pancreatic lipase* was purchased from Sigma Aldrich (St. Louis, USA). All other reagents were purchased from SDFCL, Mumbai, India. The solvents used for analysis (hexane, acetone, methanol, chloroform, ethyl acetate, diethyl ether) were of highest grade of purity and were obtained from Merck (Darmstadt, Germany).

Methods

Interesterification

Interesterification was carried out using rice bran oil and hydrogenated cottonseed oil in the presence of lipase. Fifty grams of blend consisting of rice bran oil and hydrogenated cottonseed oil was taken in the proportion of 80:20 (w/w) in a round bottomed flask. To this 5 % (with respect to substrate concentration) Novozym 435 was added. The reaction was carried out at a temperature of 60 °C for a reaction time of 6 h. The samples were drawn at regular intervals and for monitoring the progress of the reaction by determining the slip melting point, solid fat content and triacylglycerol species employing High Performance Liquid Chromatography (HPLC). Data was generated by carrying out interesterification reaction in a similar way by varying substrate weight proportions, lipase concentration and temperature.

Slip melting point

Slip melting point was determined in accordance with AOCS official method Cc 3–25 (AOCS 2004a). A part of sample withdrawn every hour during reaction was taken up to 1 cm height into a two side open capillary tube. Capillary tubes consisting of samples were stored in a refrigerator for 16 h before the measurements.

Gas chromatography

The fatty acid composition of rice bran oil and hydrogenated cottonseed oil and reaction products was determined by converting them to fatty acid methyl esters followed by GC. The analysis of methyl esters was carried out using Gas Chromatograph Agilent 6890 series equipped with flame ionization detector. The capillary column, DB-225 MS (i.d. 0.25 mm, length 30 m, 0.5 µm). The oven temperature was programmed as follows: 160 °C for 2 min and rose to 230 °C at 5 °C per minute and held at this temperature for 20 min. The

carrier gas used was nitrogen with a flow rate of 1 mL min⁻¹. The injector and detector temperatures were maintained at 230 and 270 °C respectively. The area percentage was recorded using HP Chem Station Data System. The fatty acids were identified by comparing the retention times with authentic standards (Kaki et al. 2012).

Regiospecific analysis of TAG by hydrolysis

Regiospecific analysis of TAG of the samples was done by subjecting the samples to hydrolysis using porcine pancreatic lipase. Briefly tris buffer (1 mol L⁻¹, pH 8.0; 4 mL) and 2.2 % calcium chloride (1 mL) and 0.05 % bile salts (1 mL) were added to the sample (20 mg). This mixture was equilibrated for 1 min at 40 °C. Porcine pancreatic lipase (10 mg) was added to this mixture and the mixture was stirred for 5 min at 40 °C. After 5 min, the reaction mixture was extracted with diethyl ether and washed with water and the diethyl ether layer was dried over anhydrous sodium sulfate. Hydrolyzed components were separated by preparative TLC method using hexane: ethyl acetate: acetic acid (70:30:2, vol/vol/vol) as developing solvent. The bands corresponding to 2-monoacylglycerol (MAG) were scrapped out separately and extracted with chloroform. The MAG was transmethyalted to fatty acid methyl esters (FAME) using 2 % sodium methoxide in methanol (2 g of sodium methoxide in 100 ml methanol) and injected in GC to determine the distribution of fatty acids at *sn*-2 position. The distribution of free fatty acids and TAG was calculated as described by Christie (1982). The mean composition of each fatty acid in positions 1 and 3 is calculated from the intact triacylglycerol and in position 2 by following the Eq. 1

$$\text{Positions 1 and 3} = \frac{3 * [\text{TAG}] - [\text{sn-2}]}{2} \quad (1)$$

High performance liquid chromatography

Reversed phase high performance liquid chromatography (RP-HPLC) was used to identify the triacylglycerol (TAG) species of samples following a reported method (Kaki et al. 2012). The analysis was performed on Agilent 1100 series high performance liquid chromatograph equipped with evaporative light scattering detector (ELSD). ELSD operating conditions were as follows: drift tube temperature, 50 °C, pressure of 1.5 bar and nitrogen flow of 1.5 L min⁻¹. About 20 µL of sample (1 mg mL⁻¹) was injected into a reversed phase column (Lichrocart C-18, 250–4.6 mm, 5 µm) column from Merck. The mobile phase used was acetone (100 %)

at a flow rate of 1 mL min⁻¹. The TAG composition was calculated based on effective carbon number (ECN) which is given by

$$\text{ECN} = \text{Carbon number} - 2(\text{number of double bonds}) \quad (2)$$

Determination of oryzanol content

Oryzanol content in sample (dissolved in hexane) was determined using Perkin Elmer lambda 35 UV–VIS spectrophotometer by recording UV absorption at 315 nm in 1 cm cell followed by calculation using specific extinction coefficient value 358.9 (Codex 1999).

Determination of solid fat content by pulsed-NMR

The solid fat content was determined by following AOCS official method Cd 16b-93 (AOCS 2004b). An mq20 NMR analyzer minispec supplied by Bruker Company was used to determine the SFC profiles of the samples. The samples were analyzed by NMR at a temperature range of 0 to 50 °C with regular intervals of 5 °C.

Differential scanning calorimetry

A Perkin Elmer differential scanning calorimetry was used to determine thermal properties of samples in accordance with AOCS official method Cj 1–94 (AOCS 2004c). The samples were heated to 80 °C and maintained at this temperature for 10 min. Thereafter, the temperature was cooled to 60 °C at a flow rate of 10 °C min⁻¹ where they were held for 30 min. This was followed by heating to 80 °C at 5 °C min⁻¹.

Statistical analysis

The data, presented as mean±SD, was analyzed by a paired Student's *t*-test to evaluate the level of statistical significance. A *p*-value of less than 0.05 was considered significant.

Results and discussion

In the present study, preparation of a trans free fat employing rice bran oil and hydrogenated cotton seed oil was carried out employing lipases as catalyst. The reaction system was employed served as a model to determine the interesterification activity of lipases based on degree of interesterification which was calculated by reported methods (Nathalie et al. 2012).

Different set of interesterification experiments were carried out for the generation of data under different reaction

conditions to arrive at optimum process conditions. Firstly, the weight proportion of RBO:HCSO were varied in the order 60:40, 70:30, 80:20, 90:10 (RBO:HCSO) by keeping lipase concentration constant at 5 % and temperature at 60 °C. Based on the optimum substrate proportions, the lipase concentration was varied in order of 2.5 %, 5 %, 7.5 %, and 10 % at a temperature a 60 °C. Finally, the experiments were carried out by varying temperature as 50 °C, 55 °C, and 60 °C by optimizing lipase concentration as 5 % and weight proportion as 80:20. The same sets of reactions were repeated with Lipozyme TLIM. The analysis was carried out by duplicating each sample.

Fatty acid composition

Table 1 shows fatty acid composition of rice bran oil and hydrogenated cottonseed oil (HCSO) selected as substrates. Rice bran oil is rich in palmitic, oleic and linoleic acids. These three fatty acids represent more than 90 % of the oil. Compared to rice bran oil, hydrogenated cottonseed oil is high in stearic acid which amounted to about 69 % of the total quantity followed by palmitic acid. The fatty acid composition of HCSO shows the presence of oleic acid in minor quantities (6.6 %) along with traces of lauric and behenic acids. The nutraceutical and antioxidant components present in RBO was the main reason to select it as one of the substrates anticipating that the product derived from it can have better shelf life.

The fatty acid compositions of all the fat blends were different from one another. However, all fat blends were composed mainly of palmitic, stearic, oleic and linoleic acids. The fatty acid composition changed depending upon the proportion of RBO and HCSO used in the mixture. As the quantity of HCSO was increased the amount of saturated fatty acids namely, palmitic and stearic acids were also found to be increased.

Figure 1 shows the chromatograms of TAG molecular species profiles analyzed by reverse-phase HPLC. The analysis was done by considering the tentative equivalent carbon number. It is evident from chromatogram **a** that the hydrogenated cottonseed oil consists of TAG species of four ECN composed of – C48, C50, C52 and C54 while TAG species having ECN of C42, C44, C46 and C48 are prominently seen in chromatogram **b** representing rice bran oil. The

chromatogram **c** represents the TAG species in the physical blend of RBO and HCSO (80:20; wt/wt) which show two distinct sets of molecular species corresponding to RBO and HCSO. The chromatograms **d** and **e** represent TAG species of interesterified products using Novozym 435 and Lipozyme TL IM respectively.

From chromatograms **d** and **e**, it can be observed that there is a decrease in C52 and C54 molecular species present in HCSO in both the products of reactions catalyzed by Lipozyme TL IM and Novozym 435 compared with the initial physical blend. The newer molecular species observed in the interesterified products indicate the exchange of fatty acids between HCSO and RBO compared to the initial blend.

Slip melting point

The effect of various reaction parameters (weight proportions of the blends, lipase concentration, and reaction temperature) on the slip melting point (SMP) was studied and the reaction conditions were optimized when the desired melting range was achieved. The lipase concentration of both Novozym 435 and Lipozyme TL IM was studied in the range of 2.5 to 10 % and in both cases and lipase concentration of 5 % was found to be optimum. Similarly four physical blends of RBO and HCSO were prepared ranging from 60:40 to 90:10 and the effect of the weight proportion on interesterification showed that a weight proportion of 80:20 was found to be optimum. Further advances in optimizing the reaction was done by studying the effect of reaction temperature in a range of 50 to 60 °C and it was observed that a reaction temperature of 60 °C was optimum for interesterification.

SMP of the interesterified fats and physical blends are presented in Table 2. The physical blends showed higher SMP than interesterified product in all cases. This decrease is attributed to the extensive rearrangement of FAs among triacylglycerols and it was reported that enzymatic interesterification of high melting point triacylglycerols with liquid oils results in a product with lower melting point (Ghosh and Bhattacharyya 1997). In the present study, it was observed that decrease in SMP was more pronounced in the reaction catalyzed by Lipozyme TL IM suggesting that the reaction rate was higher in Lipozyme TL IM catalyzed interesterification over Novozym 435 catalyzed reaction. This is also

Table 1 Fatty acid composition of rice bran oil (RBO) and hydrogenated cottonseed oil (HCSO) and physical blend (RBO: HCSO in 80:20; wt/wt)

Sample	Fatty acid composition (wt %)												
	12:0	14:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3	20:0	21:0	22:0	24:0
RBO	–	0.3	18.3	0.4	–	2.0	43.4	33.7	1.0	0.1	0.8	0.3	0.3
HCSO	0.8	–	23.7	–	–	69.1	6.6	–	–	–	–	0.2	–
Blend		0.4	20.3	–	–	15.4	35.9	25.6	0.8	0.8	0.3		0.5

Fig. 1 Chromatogram **a**- hydrogenated cottonseed oil **b**-rice bran oil **c**-physical blend, interesterified product using **d**- Novozym 435 and **e**-Lipozyme TL IM. Note : C₄₂-LLL; acid; O-oleic C₄₄-OLL, PLL; C₄₆-

SLL, OOL, OLP, PLP; C₄₈-SOL, OOO, LPS, POO, POP, PPP; C₅₀-SLS, SOO, PSO, PSP; C₅₂-SOS, PSS; C₅₄-SSS (L-linoleic acid; P-palmitic acid; S-stearic acid)

supported by the HPLC analysis where it can be observed that the saturated molecular species (C₅₄) was found in higher amounts in the product catalyzed by Novozym 435 compared to Lipozyme TL IM. Therefore, the differences observed in SMP could be due to the remaining saturated species in the interesterified products. Figure 2 portrays the differences in the SMP profiles of physical blends and interesterified product under optimized conditions (weight proportion of 80:20, reaction temperature 60 °C and lipase concentration of 5 %) using both Novozym 435 and Lipozyme TL IM.

The experiments were run in duplicates for experimental error estimation and the mean values are represented in Table 2. A paired Student's *t*-test statistical approach has been used to analyse the S.M.P data, and a *p*-value of 0.00026 was obtained which was considered significant.

SFC profile

The effect of various reaction parameters on the SFC profile was studied by varying weight proportions (RBO: HCSO - 60:40, 70:30, 80:20, 90:10), lipase concentrations (2.5 to 10 %) and reaction temperatures (50 to 60 °C), and are depicted in Table 2. An increase in the amount of HCSO in the initial blend is responsible for an increase in the solid fat content in the interesterified product. Hence, the solid fat was observed least in the interesterified product of 90:10 (RBO: HCSO) while the interesterified product of 60:40 (RBO: HCSO) was found to contain highest SFC. Table 2 shows that the SFC profile of interesterified product of the optimized condition decreased from 29.60 % at 0 °C to 0.9 % at 45 °C. It can be noticed that an increasing in lipase concentration

Table 2 SFC and SMP profiles of physical blends and interesterified product

Sample	SFC%										SMP (°C)			
	0 °C	5 °C	10 °C	15 °C	20 °C	25 °C	30 °C	35 °C	40 °C	45 °C	50 °C			
90/10 BLEND	12.7±0.6	10.4±0.3	9.7±0.7	8.7±0.4	7.9±0.6	6.8±0.6	4.7±0.4	2.9±0.7	1.9±0.5	0.9±0.3	0.9±0.4	40.6±0.4		
90/10 5 % 60 °C TL IM	15.6±0.4	13.6±0.4	12.3±0.1	9.5±0.6	6.9±0.6	4.8±0.1	3±0.3	1.8±0.6	1±0.3	0.8±0.4	0±0	25.4±0.3		
90/10 5 % 60 °C N-435	14.8±0.3	12.2±0.6	11.1±0.6	9.2±0.9	7.2±1.1	4.9±0.7	3.2±0.6	2.8±0.6	1.4±0.4	0.9±0.2	0±0	29.8±0.2		
80/20 BLEND	15.9±0.9	15.2±0.5	14.4±0.4	13.9±1.1	13.7±0.3	12.9±0.6	10.7±0.7	8.6±0.5	5.9±0.9	3.2±0.3	2.3±0.3	50.2±0.3		
80/20 5 % 60 °C TL IM	29.6±0.4	26.8±0.9	19.4±0.6	14.2±0.3	10.4±0.6	6.9±0.5	3±0.2	2±0.4	1.3±0.4	0.9±0.4	0±0	32.1±0.1		
80/20 5 % 60 °C N-435	26.8±0.4	24.8±0.9	19.2±0.9	15.5±0.5	10.9±1.1	6.6±0.9	3.8±0.9	2.3±0.5	1.3±0.3	0.9±0.6	0±0	35.1±0.1		
70/30 BLEND	29.8±1.1	26.8±0.5	25.3±0.4	19.5±0.6	12.8±0.4	12.7±0.9	10.9±0.8	7.4±0.8	5.4±0.6	4.9±0.5	2.9±0.9	52.2±0.5		
70/30 5 % 60 °C TL IM	34.5±0.5	32.4±0.4	27.2±0.5	20.3±0.2	14.2±0.4	10.5±0.6	7±0.1	4.8±0.3	2.3±0.4	1.1±0.3	0.1±0.1	37.8±0.2		
70/30 5 % 60 °C N-435	32.4±0.7	30.1±0.8	27.4±0.6	22.6±1.2	15.2±1.0	12.1±1.0	9.8±1.0	6.4±0.6	4.4±0.6	2.7±0.2	1±0.3	40.2±0.4		
60/40 BLEND	36.8±0.4	32.3±0.7	26.9±0.5	22.7±1.0	21.5±0.7	17.4±0.9	13.3±0.4	10±0.4	6.5±0.7	4.6±0.4	2.2±0.3	53.9±0.6		
60/40 5 % 60 °C TL IM	39.7±0.4	35.7±0.4	29.1±0.4	20.6±0.6	15.9±0.5	12.9±0.2	8.6±0.3	6.5±0.4	3.1±0.6	1.9±0.5	1±0.4	39.7±0.7		
60/40 5 % 60 °C N-435	38.9±0.6	33.6±0.5	27.4±0.8	23.9±0.9	19.6±0.9	16.9±0.7	12.6±0.9	9.5±0.9	5.9±0.5	3.6±0.4	2±0.7	43.8±1.0		
80/20 10 % 60 °C TL IM	23.5±0.2	21.7±0.1	16.2±0.3	10.8±0.4	8.1±0.6	5.8±0.4	2.9±0.5	1.9±0.5	1±0.3	0.9±0.6	0±0	30.2±0.1		
80/20 10 % 60 °C N-435	21.3±0.4	19.8±0.6	14.8±0.9	11.8±0.9	7.1±0.9	4.3±0.6	2.9±0.9	1.1±0.3	1±0.6	0.9±0.4	0±0	31.6±0.9		
80/20 7.5 % 60 °C TL IM	28.5±0.3	26.3±0.6	18.8±0.6	13.3±0.4	8.6±0.5	6.3±0.4	3.3±0.4	2±0.4	1.5±0.6	0.9±0.1	0±0	30.8±0.1		
80/20 7.5 % 60 °C N-435	28.6±0.9	27.1±1.3	20.6±0.9	12.5±0.6	8.6±0.3	6.1±0.9	4.6±0.7	2.9±0.6	1.4±0.3	0.9±0.3	0±0	33.4±0.3		
80/20 2.5 % 60 °C TL IM	31±0.4	27.1±0.4	19.6±0.5	14.4±0.4	10.3±0.4	7.2±0.9	4.9±0.3	3.5±0.4	1.7±0.3	1±0.4	0.9±0.3	33.3±0.1		
80/20 2.5 % 60 °C N-435	30.3±0.7	29.9±0.9	24.5±0.7	15.5±0.7	10.7±0.8	8.4±0.6	6±1.0	4.7±0.7	3±0.4	1.2±0.1	0.9±0.1	39.3±0.6		
80/20 5 % 55 °C TL IM	29.4±0.1	27±0.7	21.1±0.4	14.3±0.6	10.6±0.1	7.3±0.3	4.9±0.6	3.6±0.3	1.9±0.5	1.2±0.5	0.8±0.5	38.8±0.1		
80/20 5 % 55 °C N-435	26.5±0.6	25.8±0.8	20.2±0.3	15.5±1.0	11.1±0.9	7.6±0.9	5.6±0.9	3.9±0.6	2±0.6	1±0.6	0.9±0.1	38.8±0.4		
80/20 5 % 50 °C TL IM	32.5±0.4	30±0.4	22.5±0.6	14.5±0.9	10.6±0.4	7.9±0.4	5±0.4	3.8±0.2	2±0.3	1.8±0.6	0.9±0.4	42.8±0.6		
80/20 5 % 50 °C N-435	28.4±0.6	25.5±0.6	20.7±1.1	15.5±1.8	11.2±1.0	8±0.7	5±0.7	3.8±0.6	1.9±0.6	1.2±0.3	1±0.5	42.8±0.1		

Values are expressed as mean±SD

Fig. 2 SMP profiles of interesterified product under optimized conditions. S.M.P of physical blend- 50.2

resulted in higher decline of the solid fat in the interesterified products. Therein, the interesterification reaction catalyzed by 10 % lipase showed a lesser solid fat content while that of 2.5 % lipase catalyzing the reaction showed a greater solid fat content. An increase in reaction temperature from 50 to 60 °C decreased the solid fat content in the interesterified product considerably. Hence, the product of interesterification reaction carried out at 60 °C had the least solid fat content while the reaction product of 50 °C had the highest solid fat content. It has been reported that the decrease in SFC is attributed to the decrease in the TAG rich in saturated fatty acids (Mayamol et al. 2009). Figure 1 shows that the initial blend of 80:20 (RBO: HCSO) is composed on C52 and C54 TAGs rich in saturated fatty acids were decreased in the interesterified samples confirming the exchange of saturated fatty acids with unsaturated ones.

Figure 3 shows the relationship between the physical blends and the interesterified products of both Novozym 435 and Lipozyme TL IM. The difference in SFC profiles of the physical blends and their interesterified product is evident from the graph. The solid fat content of the interesterified product was greater than the physical blend but only at temperatures below 15 °C, while the SFC profile of the

Fig. 3 SFC profiles of physical blends and interesterified product of optimized conditions

interesterified product above 15 °C was observed to be lower than that of the physical blends. The SFC profile of Lipozyme TL IM catalyzed product was higher than that of Novozym 435 catalyzed product but only below 15 °C. At higher temperatures, the product of interesterification reaction involving Lipozyme TL IM was softer. However, in the first hour the solid fat content profile decreased first at low temperatures (below 5 °C) and gradually exceeded the solid fat content of the initial blend.

Table 3 represents the monitoring of interesterification reaction based on the changes in the solid fat content profile. From Table 3, we observe that the highest SFC profile was recorded as ~29 % in case of Lipozyme TL IM catalyzed reaction product and ~26 % in the product of interesterification reaction catalyzed by Novozym 435. The SFC% of interesterified sample however decreased to a 0 % gradually after 15 °C.

On the basis of the results obtained from the above observations, the DI was calculated (Xu et al. 2009) for the temperature range of 0 °C to 50 °C, using the solid fat content profile of randomly interesterified product as a reference (equilibrium). Table 3 shows the changes in the solid fat content profiles at each hour based on which the DI was calculated as follows

$$X(\%) = \frac{SFC_0 - SFC}{SFC_0 - SFC_\infty} \times 100 = (1 - e^{-kt}) \times 100 \quad (3)$$

Where $X(\%)$ or $DI(\%)$ is the conversion degree, SFC_0 is the SFC at time 0 (in the blend), SFC is the SFC at time 't' of the reaction and SFC_∞ is the SFC at the equilibrium stage. The degree of interesterification calculated was used to compare the catalytic activity of Novozym 435 and Lipozyme TL IM. It is inferred from Figs. 4 and 5 that degree of interesterification of near 100 % was attained by 4th hour in case of Lipozyme TL IM catalyzed interesterification while in case of Novozym 435 catalyzed interesterification reaction the degree of interesterification of near 100 % was attained by the 5th hour. Further, the DI% at 0 °C was found to be around 60 % for Lipozyme TL IM while for Novozym 435, the DI% reached a mere 40 % suggesting that Lipozyme TL IM was faster than Novozym in its catalytic activity. For both the reactions i.e. using Lipozyme TL IM and Novozym 435, the degree of interesterification based on SFC profile was observed to be least at 0 °C while a maximum conversion was found to be at 50 °C.

Based on the degree of interesterification, the reaction rate constant k was derived from the SFC results by fitting the following equation:

$$DI\% = (1 - e^{-kt}) \times 100 \quad (4)$$

Table 3 SFC and SMP profile of hourly samples of interesterification reaction

Temp (°C)	Blend	NOVOZYM 435						k (h ⁻¹)	LIPOZYME TL IM						k (h ⁻¹)
		1 h	2 h	3 h	4 h	5 h	6 h		1 h	2 h	3 h	4 h	5 h	6 h	
0	15.9±0.5	15.4±0.6	19.3±0.3	21.3±0.2	25.3±0.4	26.1±0.1	26.8±0.4	0.088	15.5±0.4	20.2±0.3	24.6±0.4	27.8±0.4	29.1±0.1	29.6±0.6	0.115
5	14.9±0.4	14.5±0.2	15.7±0.1	18.9±0.3	22.9±0.1	23.9±0.5	24.8±0.5	0.095	12.7±0.3	18.9±0.6	21.9±0.1	23.5±0.4	25.8±0.6	26.8±0.1	0.118
10	13.8±0.4	13.6±0.7	14.5±0.4	15.9±0.6	17.4±0.3	18.8±0.3	19.2±0.3	0.116	13.4±0.1	15.7±0.3	17.1±0.1	17.9±0.4	18.8±0.3	19.4±0.3	0.129
15	13.9±0.3	12.8±0.4	13.1±0.1	13.4±0.6	13.4±0.6	13.0±0.4	15.5±0.7	–	11.3±0.4	13.8±0.8	14.1±0.5	14.0±0.6	13.9±0.8	14.2±0.3	–
20	13.7±1.0	11.6±0.3	11.2±0.2	10.4±0.8	9.7±0.2	6.8±0.3	10.9±0.9	–	11.8±0.4	10.4±0.2	9.9±0.3	9.3±0.1	9.3±0.4	10.4±0.5	–
25	10.9±0.6	10.0±0.1	9.3±0.4	8.9±0.1	7.9±0.3	6.8±0.6	6.6±0.3	0.16	10.2±0.3	9.4±0.5	8.3±0.2	7.3±0.6	7.0±0.3	6.9±0.6	0.169
30	10.7±0.4	8.5±0.4	6.6±0.4	5.8±0.9	4.8±0.7	3.3±0.4	3.8±0.1	0.17	9.2±0.6	7.3±0.3	5.6±0.6	4.2±0.6	4.1±0.1	3.0±0.3	0.172
35	5.5±0.7	5.0±0.9	4.2±0.3	3.0±0.4	2.8±0.4	2.3±0.1	2.3±0.1	0.187	5.3±0.1	4.0±0.3	3.1±0.6	2.4±0.3	2.2±0.3	2.0±0.7	0.191
40	4.9±0.8	3.9±0.6	3.7±0.6	2.9±0.2	1.8±0.3	1.3±0.3	1.3±0.2	0.182	4.7±0.5	4.0±0.6	2.1±0.3	2.0±0.7	1.3±0.6	1.3±0.4	0.184
45	3.2±0.3	2.2±0.3	2±0.1	1.9±0.6	1.6±0.1	0.9±0.4	0.9±0.4	0.18	3.0±0.3	2.3±0.1	2.0±0.1	1.2±0.3	0.9±0.6	0.9±0.5	0.185
50	1.3±0.1	1.3±0.4	1.2±0.3	1.1±0.1	0.7±0.3	0±0	0±0	0.152	1.3±0.1	1.1±0.1	0.6±0.3	0±0	0±0	0±0	0.187
SSMP (°C)	50.2±0.3	47.0±0.4	42.3±0.3	39.9±0.4	37.5±0.4	35.3±0.3	35.1±0.1		40.1±0.1	38.8±0.3	37.5±0.4	36.2±0.2	34.3±0.3	32.1±0.4

Fig. 4 Evolution of DI-Lipozyme TL IM

Table 3, shows the derived rate constants for interesterification reaction using Lipozyme TL IM and Novozym 435. The reaction rate calculated based on the SFC% at different temperatures confirmed the fact that the effect of acyl migration was sensitive at low temperature SFC where the reaction rate was lower. The variations in the reaction rate during the interesterification reaction using Lipozyme TL IM and Novozym 435 is studied. It is observed that the reaction rate increased gradually from 0.115 to 0.187 h⁻¹ for Lipozyme TL IM and from 0.088 to 0.152 h⁻¹ for Novozym 435. The reaction rate for interesterification reaction using Lipozyme TL IM was greater than Novozym 435 suggesting that Lipozyme TL IM was a better catalyst than Novozym 435. The highest reaction rate was recorded as 0.191 h⁻¹ for Lipozyme TL IM and 0.187 h⁻¹ for Novozym 435 at 35 °C.

DSC melting profile

The DSC melting profiles of blend of 80:20 weight proportions of RBO and HCSO and their interesterified products (optimized conditions) were studied. A set of three peaks called the LMP (low melting peak), MMP (medium melting peak and HMP (high melting peaks) were observed (Fig. 6). The LMP arises between –30 and 13 °C, the MMP between

Fig. 5 Evolution of DI- Novozym 435

14 and 25 °C and the HMP is observed between 30 and 50 °C. It can be assumed that the LMP corresponds to the melting of SUU and UUU components, the MMP to the melting of SUS and SUU components and HMP to the melting of SSS and SUS.

From Fig. 6 it can be observed that the HMP and MMP have transited to lower temperatures for interesterified products compared to the initial blend. The reason is attributed to a decrease in saturated fatty acid due to fatty acid exchange during interesterification. This change is more pronounced while comparing the 80:20 physical blend and interesterified product of Lipozyme TL IM than that of Novozym 435. Similar kind of observation was earlier reported where rice bran oil was interesterified with palm stearin (Adhikari et al. 2010). The results obtained from SMP, SFC, DSC and HPLC indicate that the changes in the melting behavior were due to the extensive rearrangement of fatty acids over the glycerol backbone.

Gas Chromatography was employed to determine both the overall distribution of fatty acid residue and the manner in which they are distributed among various positions on the glycerol backbone. In case of interesterification reaction carried out using Novozym 435, the percentage of saturated residue (14:0, 16:0, 18:0 and 20:0) increased at the *sn* 2

position as the reaction time decreased where as little change was observed in the percentage of saturated fatty acid in the *sn* 2 position of the initial mixture and the interesterified product as the reaction time increased for the reaction using Lipozyme TL IM. The stearic acid in case of Novozym 435 interesterification decreased from an initial percentage of 25 to 10 % by the end of 6th hour of interesterification. The amount of stearic acid in case of interesterified product using Lipozyme TL IM was restricted to 25 %, indicating the regiospecificity of Lipozyme TL IM. A decrease in saturated fatty acid at the *sn* 2 position was accompanied by an increase in percentage of unsaturation (18:1 and 18:2). The initial percentage of oleic acid at the *sn*2 position increased from 28 to 44 % while the linoleic acid increased from 18 to 33 % in the interesterified product using Novozym 435 while oleic acid in case of interesterified products using Lipozyme TL IM, showed no change and were stagnant at a range of 28–30 %.

Rice bran oil is rich in oryzanol, a potent antioxidant and nutraceutical that inhibits the formation of peroxides during processing and storage of fat and hence its levels were closely monitored during interesterification. The study lead to a conclusion that enzymatic interesterification in general does not effect the oryzanol content of the oil (the oryzanol content of the oil was 3,000 ppm and for interesterified product it was

Fig. 6 DSC melting profile of physical blend (80:20) and its interesterified product (Novozym 435 and Lipozyme TLIM)

found to be 2,898.8 ppm) and hence can still have an antioxidant quality to it.

Conclusion

Interesterification of RBO and HSCO was optimized in terms of weight proportion of substrate mixture, lipase concentration, reaction temperature and time. The study revealed that a weight proportion of 80:20 (RBO: HSCO), lipase concentration of 5 % and reaction temperature of 60 °C yield the desired product for both the lipases Novozym 435 and Lipozyme TL IM. Based on the degree of interesterification, our results indicate that that Lipozyme TL IM showed a faster degree of conversion with a reaction rate of 0.191 h^{-1} at the 4th hour. Novozym 435 on the other hand showed a reaction rate of 0.187 h^{-1} at the 5th hour. Hence, it is concluded that TL IM produces the structured fat with desired SMP at a faster rate and has a higher catalytic activity.

Conflict of interest The authors declare no conflict of interest.

References

- Adhikari P, Shin JA, Lee JH, Hu JN, Hwang KT, Lee KT (2009) Enzymatic production of trans-free hard fat stock from fractionated rice bran oil, fully hydrogenated soybean oil, and conjugated linoleic acid. *J Food Sci* 74:E87–E96
- Adhikari P, Shin JA, Lee JH, Hu JN, Zhu XM, Akoh CC, Lee KT (2010) Production of *trans*-free margarine stock by enzymatic interesterification of rice bran oil, palm stearin and coconut oil. *J Sci Food Agric* 90:703–711
- AOCS (2004a) Slip melting point. Cc 3–25. In: Firestone D (ed) Official methods and recommended practices of the American Oil Chemists' society, 4th edn. American Oil Chemists' Society Press, Champaign
- AOCS (2004b) Solid Fat content. Cd 16b-93. In: Firestone D (ed) Official methods and recommended practices of the American Oil Chemists' society, 4th edn. American Oil Chemists' Society Press, Champaign
- AOCS (2004c) Differential scanning calorimetry. Cj 1–94. In: Firestone D (ed) Official methods and recommended practices of the American Oil Chemists' society, 4th edn. American Oil Chemists' Society Press, Champaign
- Brien RD, Wakelyn PJ (2005) Cottonseed oil: an oil for trans free options. *Inform* 16:677–679
- Christie WW (1982) Lipid analysis. Pergamon Press, New York
- Clercq ND, Danthine S, Nguyen MT, Gibon V, Dewettinck K (2010) Enzymatic interesterification of palm oil and fractions: monitoring the degree of interesterification using different methods. *J Am Oil Chem Soc* 89:219–229
- Codex Standard for Named Vegetable Oils, CODEX STANDARD 210–1999, 5.20, Determination of gamma oryzanol content
- Criado M, Martin EH, Hernandez AL, Otero C (2008) Enzymatic interesterification of olive oil with fully hydrogenated palm oil: characterization of fats. *Eur J Lipid Sci Technol* 110:714–724
- Ghosh S, Bhattacharyya DK (1997) Utilization of high-melting palm stearin in lipase-catalyzed interesterification with liquid oils. *J Am Oil Chem Soc* 74:589–592
- Gunstone FD (2002) Cottonseed oil. *Vegetable Oils in Food Technology, Composition, Properties, Uses*. Blackwell, Oxford 203–230
- Kaki SS, Reddy KK, Kanjilal S, Prasad RBN (2012) Enzymatic preparation and characterization of trans-free Fat from palm stearin and natural vegetable oils. *J Lipid Sci Technol* 44:79–85
- Li D, Adhikari P, Shin JA, Lee JH, Kim YJ, Akoh CC, Lee KT (2010) Lipase-catalyzed interesterification of high oleic sunflower oil and fully hydrogenated soybean oil comparison of batch and continuous reactor for production of zero *trans* shortening fats. *LWT – Food. Sci Technol* 43:458–464
- Malongil BR, Reddy SRY, Belur RL (2009) Changes in triacylglycerol molecular species and thermal properties of blended and interesterified mixtures of coconut oil or palm oil with rice bran oil or sesame oil. *Eur J Lipid Sci Technol* 111:346–357
- Marangoni AG, Rousseau D (1995) Engineering triacylglycerols: the role of interesterification. *Trends Food Sci Technol* 6:329–335
- Mayamol PN, Balachandran C, Samuel T, Sundaresan A, Arumughan C (2009) Zero *trans* shortening using rice bran oil, palm oil and palm stearin through interesterification at pilot scale. *Int J Food Sci Technol* 44:18–28
- Nathalie DC, Sabine D, Mai TN, Veronique G, Koen D (2012) Enzymatic interesterification of palm Oil and fractions: monitoring the degree of interesterification using different methods. *J Am Oil Chem Soc* 89:219–229
- Normann W, Germania GmbH FO, German Patent. (1920) 417, 215; verfahren zur Umesterung von fettsaureestern 1920
- Rajah KK (2002) Fats in food technology. Blackwell, London
- Rodriguez RC, Gibon V, Verhe R, Greyt WD (2009) Chemical and Enzymatic interesterification of a blend of palm stearin: soybean oil for low trans-margarine formulation. *J Am Oil Chem Soc* 86: 681–697
- Shin JA, Akoh CC, Lee KT (2009) Production and physicochemical properties of functional-butterfat through enzymatic interesterification in a continuous reactor. *J Agric Food Chem* 57:888–900
- Simakova IL, Simakova OA, Romanenko AV, Murzin DY (2008) Hydrogenation of vegetable oils over Pd on nanocomposite carbon catalysts. *Ind Eng Chem Res* 47:7219–7225
- Vandana D, Gulia N, Kulveer SA, Bhupender SK (2010) Trans fats - sources, health risks and alternative approach—a review. *J Food Sci Technol* 48:534–541
- Xu X, Balchen S, Hoy CE, Nissen AJ (2009) Production of specific-structured lipids by enzymatic interesterification in a pilot continuous Lipase bed reactor. *J Am Oil Chem Soc* 75:1573–1579