Enzymatic Preparation and Characterization of *Trans*-free Fat from Palm Stearin and Natural Vegetable Oils

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ABSTRACT

Trans-free fat was prepared by enzymatic interesterification of palm stearin (PS) and vegetable oils exemplified by rice bran oil (RBO), sesame oil (SMO) and groundnut oil (GNO). Reactions were optimized with respect to molar proportions of palm stearin and vegetable oils to achieve a desired slip melting point of less than 40^{0}C in 8 hr. Lipozyme TL IM was used as a biocatalyst and the prepared fat was analyzed for slip melting point and triacylglycerols molecular species. Most of the interesterification reaction was observed during first four hours as suggested by the changes in the slip melting point and the molecular species composition. The optimum molar proportions required for obtaining a fat with desired slip melting points were 0.8:1 for the blends of PS:RBO and PS:SMO, whereas for PS:GO the molar ratio needed was 0.6:1. There was gradual decrease in the molecular species of tripalmitin during interesterification with increase in molecular species containing unsaturated fatty acids. The products contain sufficient amounts of palmitic and oleic acids which impart plastic consistency needed for spreadable fat or vanaspati and have the added advantage of presence of the micronutrients and antioxidants from the vegetable oils used in the study.

KEYWORDS*: Trans-free fat, interesterification, lipase, slip melting point, HPLC, molecular species.*

INTRODUCTION

Vanaspati, an alternative plastic fat is an vegetable oil based product originally developed in the 1930s in India as an alternative to ghee which is made from cow milk fat. Vanaspati is widely used in the Indo-Pakistan subcontinent, Middle Eastern countries and South East Asia for cooking and frying[1]. There is a growing interest over the production of vanaspati with minimum or zero content of *trans* fatty acids. The *trans* fatty acids have a negative impact on plasma lipoprotein profile by lowering high-density lipoprotein (HDL) cholesterol and raising the low-density lipoprotein (LDL) cholesterol[1,2]. Most of the natural vegetable oils and fats which are reported to posses nutritional benefits have limited application in fat based products due to their specific chemical natures like fatty acid composition and triacylglycerol distribution. Food industries are looking forward to prepare fat products employing the vegetable oils which have proven nutritional and health benefits. Addressing this issue there are few reports on the preparation of *trans* free fat by modifying the native oils either physically, chemically or enzymatically[3]. Interesterification is the main method currently being employed for the production of fats containing no *trans* fatty acids. There are reports on the preparation of *trans* free fat using palm oil fraction and rice bran oil where the prepared fat exhibited characteristics similar to commercial vanaspati[4]. It would be interesting to prepare and characterize *trans* free vanaspati employing palm stearin (PS) as solid fat and nutritionally important oils such as rice bran oil (RBO), sesame oil (SMO) and ground nut oils (GNO) as palm stearin has been included in the list of edible fats for use in vanaspati production in the recent draft notification of Food Safety and Standards Authority of India (FSSAI)[5]. Rice bran oil is produced from the bran of the rice grain and it contains unique nutraceutical components, such as oryzanol, tocotrienols, and phytosterols which have been reported to have beneficial effects on human health. It was shown that rice bran oil reduces the harmful cholesterol (LDL) without reducing good cholesterol (HDL)[6]. Similarly sesame oil (also called as gingelly oil or til oil) contains antioxidants like sesamin, sesamolin and sesamol which can prevent oxidation of oils on storage. Sesame oil contains 43% polyunsaturated fatty acids and 40 mg of Vitamin E per 100 grams of oil[7]. The higher oxidative stability exhibited by the sesame oil is not alone due to tocopherols, but is mainly due to lignan compounds.

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Two types of lignan compounds are reported to be present in sesame seeds namely, oil soluble lignans and water soluble lignan glycosides. The unique nutritional properties have been attributed to oil soluble lignans such as sesamin and sesamolin and oil insoluble lignans are present as lignan glucosides. It was indicated that sesame oil helps to reduce blood pressure in hypertensive patients taking diuretics or beta-blockers, and that sesame oil helped people with diabetes regardless of high blood pressure[7, 8]. It has been recently reported that the blends of sesame oil with palm or soy bean oils enhanced the oxidative stability[9]. Groundnut oil or peanut oil is very popular in cooking medium in India and has a mild flavour. It is reported that diets high in groundnuts and its oil are as effective in protecting against heart disease as diets high in olive oil or even low-fat diets. It is attributed to phytosterols and phenolics present in groundnut oil that absorb dietary cholesterol in the blood and thus protects against cardio vascular diseases and cancer[10].

Most of the reported literature was focussed on the preparation and application of the prepared fat products in bakery and other food products with minimum emphasis on the distribution of molecular species and on the slip melting point behaviour[11]. It is reported that melting point of natural oils do not show a clearly defined sharp melting point like pure substances. They consist of several molecular species of triacylglycerols that undergo gradual melting according to the individual melting points until they become completely liquid and thus possess melting ranges[12]. The rearrangement of fatty acyl residues on the triacylglycerol molecules during interesterification generally modifies the melting points of fats and this depends on the initial raw materials taken for interesterification. Reports on interesterification of RBO and PS for production of *trans*-free fat are available[13] whereas SMO and GNO were not employed for such modification previously using PS. These oils have either balanced fatty acid profile or have natural micronutrients and therefore the products prepared from these oils would be potentially more beneficial than the most of the available *trans*-free fats in terms of essential fatty acids, micronutrients and stability of the product.

The objective of the present study is to prepare *trans*-free vanaspati having a slip melting point of below 40^{0}C. PS was blended with vegetable oils, namely rice bran, sesame and groundnut oils. This was followed by enzymatic interesterification using suitable enzymes. Hydrogenated or natural fats with high melting points, when interesterified with liquid oils generally show a decrease in the melting point in relation to the original material due to a decrease in the proportion of trisaturated triacylglycerols. This idea was employed for the preparation of *trans*-free fat with melting points of below 40^{0}C and the prepared fats will be mainly analyzed for the slip melting point profile and also for the change in the molecular species as the changes in the molecular species effect the slip melting point of the fat involved in the enzymatic modification.

MATERIALS AND METHODS

Materials

Rice bran oil and palm stearin (slip melting point, 55-58°C) were procured from M/s Ramcharan Oil Industry, Hyderabad. All other oils were purchased from local market. Reference triacylglycerols (Cat. No. 17811), fatty acid methyl ester mixtures (Cat. No. 18919) were purchased from Sigma Chemical Co., USA. Commercial immobilized 1,3-specific lipase, *Lipozyme* TL IM [granulated silica preparation of a microbial lipase from *Thermomyces lanuginosa* with an activity of 75 IUN/g (IUN is Interesterification Unit Novo/g)] was a gift sample from M/s Novozymes South Asia Pvt. Ltd., Bangalore, India. Acetone of HPLC grade was purchased from Merck, Germany. Open tube capillaries and all other solvents were purchased from SD fine chemicals, Mumbai and were of analytical grade. The reactions were performed on Heidolph (GmbH, Germany) magnetic stirrers equipped with a temperature controlled oil bath system.

Methods

Enzymatic interesterification: Blends of PS with vegetable oils at different molar proportions (molar equivalents of PS and vegetable oils were taken according to the mean molecular weight) were stirred for 30 min at 70°C under reduced pressure (10 mm Hg) for the preparation of physical blend. *Lipozyme* TL-IM (4% wt/wt of total substrate) was added into the reaction mixture and stirred for 8 hr at 65-70°C keeping the reaction mixture under reduced pressure for an additional 0.5 hr. Aliquots were collected at zero, 1, 2, 4, 6 and 8 hr for HPLC and GC analysis as well as for slip melting point determination. Before sampling, stirring was stopped and the enzyme was allowed to fully settle to the bottom and then sample was withdrawn from the top for further analysis.

Preparation of fatty acid methyl esters: Fatty acid composition of the substrate oils and the blends were determined after methylation with methanol-sulphuric acid reagent as described by Christie[14].

Gas chromatography (GC): GC was performed on Agilent 6890 series Gas Chromatograph equipped with flame ionization detector (FID) and the capillary column DB-225 MS (30 m length, 0.25 mm i.d, 0.5 μm film thickness.). The oven temperature was programmed for 2 min at 160°C, raised at 6°C/min to 180°C, held for 2 min at 180°C, raised again at 4°C/ min to 230°C and finally held at 10 min at 230°C. The carrier gas N_2 flow was adjusted at 1.5 ml/ min and split ratio was 50:1. The injector and detector temperature were maintained at 250°C and 230°C respectively.

High performance liquid chromatography (HPLC): The triacylglycerols molecular species were analyzed by reversed phase HPLC. The reverse phase (RP) HPLC was performed on a HP-1050 series HPLC equipped with an ELSD (Alltech ELSD-2000). About 25μl of the reaction mixture (0.5-1 mg/ml) was injected in the Merck RP column [RP-18 (5μm) 250-4]. The molecular species were eluted within 15 min. using a mobile phase of 100% acetone at a flow rate of 1 ml/min. The operating conditions for ELSD: drift tube temperature, 40°C, flow of N_2 1.5 l/min, with impactor on mode.

Slip melting point (SMP): SMP was determined according to open capillary slip melting point method (AOCS method Cc 3-25)[15]. Capillary tubes were filled with the fat upto 1 cm and the tubes were stored at 4^0C for about 16 hr. Then the tubes were placed in a beaker containing cold water and the temperature of the water was slowly raised while the water was stirred during which the temperature was recorded when the fat in the tube was melted and raised slowly.

RESULTS AND DISCUSSION

In the present study, three types of *trans*-free fats were prepared by enzymatic interesterification of palm stearin as hard fat and three vegetable oils e.g.,rice bran oil, sesame oil and groundnut oil. These oils are well known for the presence of different types of antioxidants. Groundnut oil is well known for its flavour and balanced fatty acid profile. The fatty acid compositions of the substrate oils are give in Table 1. The oils used in the study were not having any *trans* fatty acids.

The lipase used was a 1,3-specific lipase *Lipozyme* TL IM which acts on the *sn*-1 and *sn*-3 positions of triacylglycerols and the fatty acids in *sn*-2 position are undisturbed. This is a well established methodology to prepare structured lipids containing health beneficial fatty acids in the *sn*-2 position of triacylglycerols[16]. Enzymatic routes are desirable to prepare food based products as they have advantages of easy product separation, milder reaction conditions and especially attractive when the product is intended for edible use.

TABLE 1
Fatty Acid Composition (wt %) of the Substrate Oils

Fatty acid	Palm stearin	Rice bran oil	Sesame oil	Groundnut oil
14:0	1.2	0.3	–	–
16:0	60.7	21.0	9.3	13.8
18:0	4.6	2.0	6.3	4.0
18:1	25.9	43.7	40.9	39.9
18:2	7.2	31.5	42.0	35.8
18:3	–	1.0	–	–
20:0	0.4	0.5	0.7	1.5
20:1	–	–	0.2	0.9
22.0	–	–	0.2	3.1
24:0	–	–	–	1.0

Interesterification of palm stearin (PS) and vegetable oils: PS and RBO were taken in 1:1 molar ratio and interesterification was carried over a period of 8 hr. The change in slip melting point was monitored during the process of interesterification. The slip melting point at the end of 8 hr was found to be 44^0C. In order to get the desired slip melting point of $<40^0$C, another blend of PS and RBO with a molar ratio of 0.8:1 was prepared and interesterification was carried out and the change in slip melting point was monitored. The molar proportions of PS and RBO in 0.8:1 was found to be the optimum ratio to obtain the fat with slip melting point of below 40^0C. The same behavior was also observed during the interesterification of PS and SMO as the desired slip melting point was achieved with molar ratio of 0.8:1. In the case of groundnut oils, three molar ratios were examined as both blends of PS and GNO in 1:1 and 0.8:1 were not successful to produce an interesterified fat having slip melting point of 40^0C. It was found that reaction with PS and GNO in molar ratio of 0.6:1 could produce the product with desired slip melting point. The change in slip melting points with different blends during interesterification is given in Table 2.

It is observed from Table 2, that the interesterified fat obtained from 0.8:1 molar ratio of PS and RBO had resulted in the product of desired melting characteristics. It has been earlier reported that enzymatic interesterification of high-melting point palm stearin with liquid oils resulted in a decrease of the melting point of non-interesterified blends[17]. A similar and detailed study performed earlier on palm stearin

TABLE 2

Changes in the Slip Melting Point (^{0}C) during Interesterification of Different Blends

Time (hr)	PS:RBO (SMP, ^{0}C)		PS:SMO (SMP, ^{0}C)		PS:GNO (SMP, ^{0}C)		
	1:1	0.8:1	1:1	0.8:1	1:1	0.8:1	0.6:1
0	58	53	54	52	55	54	50
1	52	43	49	49	52	50	47
2	49	42	48	47	49	48	41
4	47	41	44	43	47	45	38
6	45	40	43	41	45	44	37
8	44	39	42	39	43	43	35

PS: Palm stearin; RBO: Rice bran oil; SMO: Sesame oil

and RBO reported that the interesterified fat prepared from a blend containing PS and RBO in equal ratios exhibited characteristics similar to that of commercially available vanaspati[13]. The decrease in SMP could be due to the increase of unsaturated fatty acids and the decrease in saturated fatty acids (SFA) in the blends. The lower amounts of palm stearin that could have randomized during interesterification resulted in lower SMP. The amount of SFA in the 1:1 and 0.8:1 blends of PS and RBO are found to be around 51 and 45.8% respectively whereas in the blends comprising PS and SMO, the amount of SFA in the 1:1 and 0.8:1 blends were about 44.4 and 41.4% respectively. The amount of SFA in the blends of PS and GNO also decreased with increasing proportion of GNO.

Reaction conditions were optimized with respect to molar ratios of palm stearin and oil, time of interesterification to obtain a fat with desired slip melting point of <40^{0}C. Table 3 shows the fatty acid composition of the physical blends studied and optimum molar proportions of PS and the oil required for obtaining the desired slip melting point after interesterification.

RBO and SMO contain 76.3 and 82.9% of unsaturated fatty acids respectively whereas the GNO contains about 65.7% of unsaturated fatty acids.

The change in triacylglycerol (TAG) molecular species during interesterification was monitored by RP-HPLC analysis and the results are compared with physical blends as well as with individual molecular species of PS and the three oils in the study. The products which resulted from the blend giving the desired SMP were taken up for RP-HPLC analysis and the molecular species composition is compared for the three products. The molecular species composition of the substrate oils, the respective

TABLE 3

Fatty Acid Composition (wt %) of the Blends of Palm Stearin (PS) and Vegetable Oils

Time (hr)	PS:RBO		PS:SMO		PS:GNO		
	1:1	0.8:1	1:1	0.8:1	1:1	0.8:1	0.6:1
14:0	0.7	0.7	0.5	0.6	0.6	0.5	0.5
16:0	45.9	41.2	37.8	34.8	39.0	36.8	33.1
18:0	3.8	3.3	5.6	5.5	4.4	4.5	4.3
18:1	32.5	34.4	31.2	32.3	31.3	31.7	33.3
18:2	16.5	19.8	24.4	26.3	20.6	22.1	24.1
18:3	0.4	0.4	-	-	-	-	-
20:0	0.2	0.1	0.5	0.5	1.0	1.1	1.1
20:1	-	-	-	-	0.5	0.5	0.5
22.0	-	-	-	-	1.9	2.1	2.3
24:0	-	-	-	-	0.7	0.7	0.8

RBO: Rice bran oil; SMO: Sesame oil; GNO: Groundnut oil

physical blend and the interesterified products of all the reactions are shown in Tables 4, 5 and 6. During interesterification, significant changes were observed from the TAG molecular species of the physical blends. Interesterification resulted in randomization and formation of new molecular species was observed from the blend. This was also evident from the decrease of slip melting point of the interesterified product. The new molecular species may have been distributed evenly in the range of C46 and C48 as it was difficult to say which molecular species is newly formed because most of the TAGs in the initial blend were in C46 and C48. All of the interesterified products had melting points of <40^{0}C when compared with their respective blends which had a slip melting point in the range of 50 to 60^{0}C (Table 2). Similar observations were also made previously where the authors suggested that the decrease in SMP was associated with randomization of saturated TAGs with liquid oils[18].

It can be observed that PS has higher amount of palmitic acid rich species of which tripalmitin is about 55% whereas the three oils showed higher content of unsaturated TAG molecular species. The molecular species which showed a change during interesterification are considered responsible for the change in the slip melting points and the same will be dealt here. In the present study, PPP is one such molecular species which can be compared for the difference in composition during interesterification.

From the composition in Table 4, it can be observed that there was a decrease in the concentration of LLL, LLO and LOO with an increase

TABLE 4

RP-HPLC Analysis of TAG Molecular Species of Palm Stearin (PS), Rice Bran Oil (RBO), Physical Blend (PB) and Interesterified Fat

ECN	EMS	PS	RBO	Interesterified Fat (wt %)				
				PB	2 hr	4 hr	6 hr	8 hr
C42	LLL	-	3.3	1.3	0.4	0.3	0.2	0.3
C44	LLO	-	9.7	3.5	1.4	1.1	1.3	1.1
C44	PLL	0.3	7.3	3.0	3.9	4.0	3.9	4.0
C46	LOO	0.3	16.2	6.7	4.2	4.2	4.2	3.8
C46	POL/PPL	2.1	23.4	12.1	19.3	21.1	21.2	21.0
C48	OOO/SOL	4.3	20.7	12.0	13.8	14.3	14.4	13.8
C48	POO	9.7	16.7	15.2	18.9	19.0	19.0	19.1
C48	POP	23.2	-	14.9	25.6	26.2	26.7	27.0
C48	PPP	55.1	-	29.0	10.7	8.1	7.6	7.9
C50	SOO	-	2.7	-	0.5	0.7	0.7	0.8
C50	POS	4.9	-	2.3	1.3	1.0	0.9	1.2

ECN: effective carbon number; EMS: expected molecular species; PS: palm stearin; RBO: rice bran oil; SMO: sesame oil; GNO: groundnut oil; L: linoleic; O: oleic; P: palmitic; S: stearic

TABLE 5

RP-HPLC Analysis of TAG Molecular Species of Palm Stearin (PS), Sesame Oil (SMO), Physical Blend (PB) and Interesterified Fat

ECN	EMS	PS	SMO	Interesterified Fat (wt %)				
				PB	2 hr	4 hr	6 hr	8 hr
C42	LLL	-	18.1	8.0	3.3	2.0	1.5	1.4
C44	LLO	-	31.5	14.8	7.4	5.8	4.7	4.3
C44	PLL	0.3	1.2	0.8	5.7	7.0	7.3	7.4
C46	LOO	0.3	25.8	11.8	7.7	7.2	7.1	7.2
C46	POL	-	5.5	4.9	22.0	26.3	29.1	28.9
C46	POL/PPL	2.1	-	-	-	-	-	-
C46	PLP		12.2	6.2	9.8	11.7	12.0	12.2
C48	OOO/SOL	4.3	2.0	-	-	-	-	-
C48	OOO/POO	-	-	7.8	13.4	13.9	13.9	14.1
C48	POO	9.7	-	-	-	-	-	-
C48	POP	23.2	3.6	11.3	15.8	16.1	15.6	15.8
C48	PPP	55.1	-	32.6	16.8	11.8	5.5	5.3
C50	SOO	-	-	-	1.4	1.9	2.1	2.1
C50	POS	4.9	-	1.8	1.7	1.3	1.2	1.3

TABLE 6

RP-HPLC Analysis of TAG Molecular Species of Palm Stearin (PS), Groundnut Oil (GO), Physical Blend (PB) and Interesterified Fat

ECN	EMS	PS	GNO	Interesterified Fat (wt %)				
				PB	2 hr	4 hr	6 hr	8 hr
C42	LLL	-	5.8	2.8	1.4	1.2	1.1	1.1
C44	LLO	-	23.3	11.9	6.5	4.2	3.4	3.7
C44	PLL	0.3	2.9	1.3	5.0	5.8	6.2	6.3
C46	LOO	-	-	-	7.7	7.6	7.3	7.4
C46	POL	0.3	23.4	12.2	25.1	28.1	29.9	28.5
C46	PPL	-	16.1	10.7	10.9	12.1	12.6	12.8
C48	OOO	-	-	-	15.2	15.8	15.5	15.5
C48	SOL	4.3	6.2	8.4	18.5	17.7	17.6	17.1
C48	POO	9.7	4.2	9.7	-	-	-	-
C48	POP	23.2	0.6	14.3	9.0	5.6	4.5	4.3
C48	PPP	55.1	2.8	26.1	1.7	1.9	1.9	1.9
C50	SOO	-	0.7	-	-	-	-	-
C50	POS	4.9	0.8	2.6	-	-	-	-

in concentrations of PLL, POL, PPL, OOO, SOL and POO. The major increase in concentration was observed in the case of POP from an initial content of 14.9 to 23.6% within 1 hr. After 4 hr interesterification no significant change in the molecular species was observed. The TAG species PPP was found to be decreased from an initial content of 29% in the blend to 7.9% in the interesterified product. A similar pattern of molecular species was observed when PS, RBO and coconut oils were used for interesterification to produce *trans* free margarine stock using lipase[19].

The results of RP-HPLC analysis showed a decrease in concentration of LLL, LLO and LOO molecular species and an increase in POL, POP and POO molecular species for interesterification reaction of PS and SMO and the results are shown in Table 5. The TAG species of PPP decreased from an initial content of 32.6 to 5.3% in the interesterified product.

As the molar ratio of 0.6:1 of PS:GO was giving the desired SMP, the RP-HPLC analysis for the same was carried out. The changes observed in the distribution of molecular species are tabulated in Table 6.

It was observed that there was an increase in the concentration of LOO, OOO and SOL molecular species with decrease in the concentration of LLL, LLO and PPP. Some new molecular species were observed in the range of C44 to C46 which suggests that the saturated TAG species could have been randomized with unsaturated TAG species producing mixed TAG species containing both unsaturated and saturated fatty acids.

In all the three blends, there was a decrease in the molecular species of PPP with concomitant

increase in the newer molecular species containing unsaturated fatty acids. This could probably be the main reason for the observed decrease in the slip melting point when compared with the starting blend. From Figure 1, it can be seen that there is a large decrease in PPP in the case of blend of GNO compared with the other two oils. This can be attributed to the difference in the palmitic acid content in the blends of PS:GNO compared with the other two blends. Though the difference in composition of PPP for starting blends of PS:RBO and PS:GNO is not significant, the composition of PPP in product with GNO is only 1.9% compared with PS:RBO product which had 7.9% PPP. It is also observed that there was an overall decrease in the content of the molecular species with ECN C42, C44 and C48 with concomitant increase in the molecular species of C46 due to interesterification.

Blends and Products of Palm Stearin (PS) with Different Oils

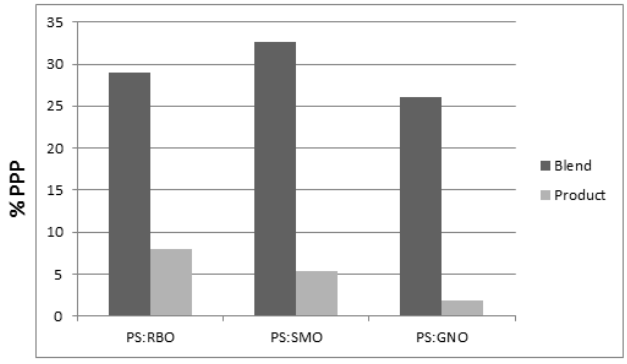

Fig. 1: Difference in Tripalmitin Molecular Species between Starting Blends and Interesterified Fat

The interesterified fat produced from RBO and SMO showed a similar profile in the changes of some selected molecular species that exhibited significant difference in composition. Figure 2 shows the change in composition of some molecular species which had significant changes during interesterification. The selected molecular species are PPP, POP, POO and POL and the changes during interesterification are shown in Figure 2. Plots **A** and **B** are from the products obtained from RBO and SMO respectively and **C** is from the product obtained from GNO. The TAG molecular species of POP, POO and POL showed an increase in composition in both A and B, where as POP showed a decrease in composition in **C**. This difference in pattern of molecular species in **C** may be due to the difference in composition of groundnut oil blend which has complex TAG molecular species as it shows a balanced fatty acid profile. It can be observed that the changes were very minor after 4 hr of interesterification in all the reaction products of the present study.

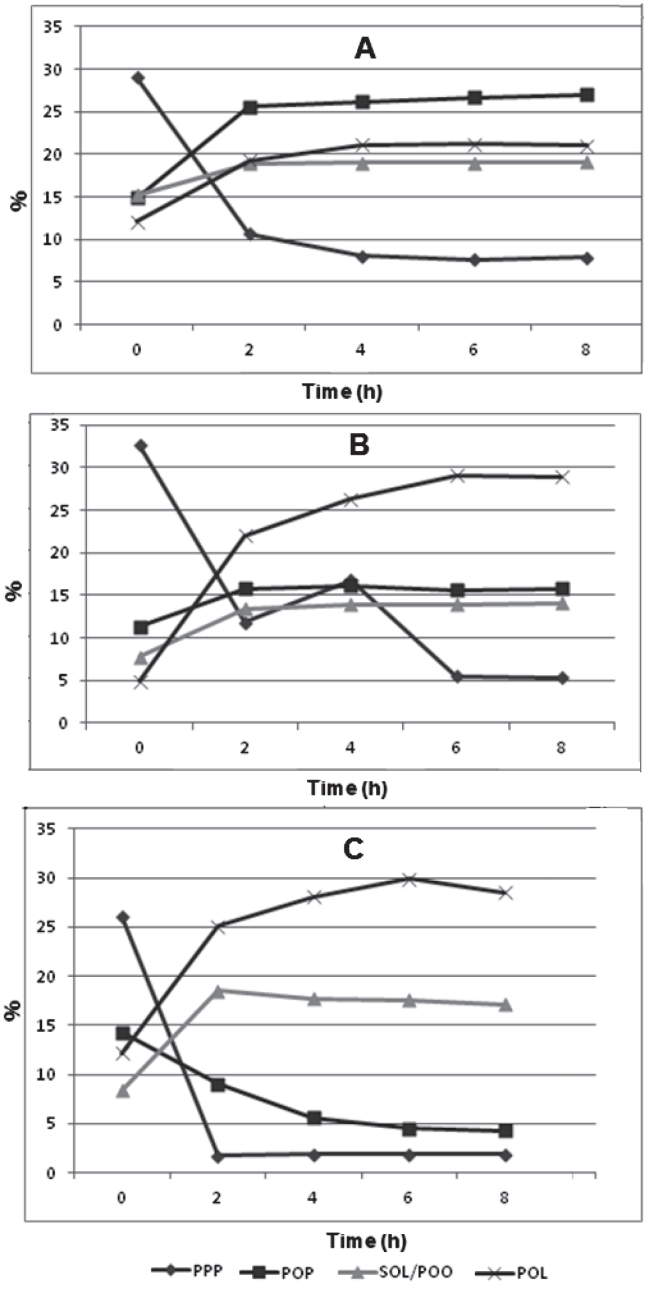

Fig. 2: Change in Composition of Molecular Species during Interesterification

These products have sufficient quantities of palmitic and oleic acids to impart smooth consistency and they are devoid of *trans*-fatty acids unlike commercially available hydrogenated fats. They also contain essential fatty acids and micronutrients which

act as antioxidants and nutraceuticals. The products prepared can have potential combined benefits of the oils and palm stearin used in the present study.

CONCLUSION

The present study showed that *trans*-free fats having slip melting point less that 40°C can be prepared with palm stearin and vegetable oils by enzymatic interesterification. The results reveal that the blends of PS with RBO (0.8:1), SMO (0.8:1) and with GO (0.6:1) after interesterification produced fats with desired slip melting points. Thus *trans*-free fats suitable for products like margarines, confectionaries and bakery products can be prepared from the above oils and palm stearin. The *trans*-free fats produced contain considerable amounts of saturated fatty acids which ensures the oxidative stability and shelf life of the products. In addition, the micronutrients and phenolic antioxidants present in the oils can impart the additional stability to the products and increase its value as nutraceutical product.

ACKNOWLEDGEMENTS

This work is part of Networking Project on "Positioning Nutraceuticals & Nutragenomics in Global Platform" executed with the financial grant of Council of Scientific and Industrial Research, New Delhi.

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