Journal of Scientific & Industrial Research
Vol. 81, May 2022, pp. 549-560

Development of Phytosterol Enriched Functional Edible oils: Study of Physical, Chemical, Thermal and Structural Properties

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Received 23 January 2022; revised 06 April 2022; accepted 08 April 2022

Phytosterol plays a major role in reducing the bad cholesterol in blood resulting in decreasing the chance of cardiovascular and other chronic diseases in human. The objective of this work was to develop a functional edible oil by incorporating phytosterol into different edible oils, such as, sunflower, soybean, rice bran, canola, and coconut oil to enhance their nutritional and functional value. In this work, thermal and structural properties of the functional oils were evaluated using Differential Scanning Calorimeter (DSC), X-Ray Diffraction (XRD) and Confocal Laser Scanning Microscopy (CLSM), respectively. The physicochemical properties like specific gravity, color, refractive index, peroxide value, acid value and antioxidant activity of the functional oils were studied. Specific gravity, acid value and peroxide value of the functional oils increased slightly as compared to the pure oils which are within acceptable range. The antioxidant capacity of the functional oil increased from 28.85 to 98.25% due to the addition of phytosterol. Onset melting, peak melting and offset melting temperature along with the melting enthalpy were evaluated during thermal transition by using DSC. The melting temperature of the functional oils varied according to the presence of saturated and unsaturated fatty acid content. For example, a lower melting point was obtained for oils containing lower saturated fatty acids and vice versa. XRD results showed significant variations in the crystallinity values in functional oils. From the above study taking all the properties into account, functional soybean oil can be considered as the best oil for various food application due to its higher oxidative stability.

Keywords: Beta-sitosterol, Cholesterol, Functional properties, Oil stability, Soybean oil

Introduction

Phytosterols are naturally derived plant-based sterol alcohols, which have gained considerable attention in various fields due to their different bioactive properties namely, lowering cholesterol absorption rate and contribution to prevent various types of cancer, cardiovascular and metabolic diseases. However, phytosterol effectiveness mainly depends on physical state, compositional variation, and dosage level.[1,2]

Phytosterols are structurally analogous to cholesterol, but the only difference is the presence or lack of a double bond at 22nd carbon on side chain and an additional methyl or ethyl group at 24th carbon.[3] So far, 250 categories of phytosterols have been identified and mentioned in various studies; however, the most abundantly available sterols are campesterol, sitosterol and stigmasterol.[4] They are widely distributed in various plant-based foods especially vegetable oils followed by nuts, cereals, stems, roots, grains, vegetables, and fruits.[5,6] However, average daily consumption can only provide 167 to 473 mg of phytosterol per day to humans. Moreover, European Food Safety Authority (EFSA) and United States Food and Drug Administration (US FDA) recommend consuming around 1–3 g/day of phytosterols to reduce cholesterol concentration in blood serum.[7] Therefore, phytosterols must reach the humans in the form of functional foods or medication for full health benefits.

In this regard, phytosterols have become an essential component in food and pharmaceutical industries. In 1995, scientists identified that phytosterols could reduce low-density lipoprotein (LDL) levels in humans. Later, several studies have been conducted using both free and esterified sterols to reduce cholesterol levels in human blood.[8] In most of the cases, esterified sterols are commonly used to develop various functional foods (like spreads, dairy beverages, juices, and chocolates) due to their lower melting point and high solubility in fat-based medium.[7] However, the main drawback is that an additional 20% of oil is needed to be consumed which isn't desired as part of the health regime. Furthermore, the esterification process can cause an un-imaginary absorption rate leading to an uneven cholesterol-

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lowering profile.[9] Moreover, United States Department of Agriculture (USDA) 2010 recommended to reduce consumption of saturated fatty acids and trans-fat in the diet. Thus, there is need to replace the semi-solid fats since it is linked to various diseases like cardiovascular diseases and various types of cancer.[10] In this regard, free phytosterols are being evaluated as a viable option for development of various phytosterol-enriched foods and to lower fatty acid intake percentage.[11] However, only few studies have been conducted on free sterols based functional foods.[7] For example, Hayes *et al.* indicated that free sterols recrystallize easily when cooled and become bioavailable, which can aid to lower cholesterol absorption rate. In another study, Gomes Silva *et al.* found that higher free phytosterol concentration in supersaturated liquids such as sunflower oil was critical in the reduction of huge agglomerates of phytosterol crystals. Solubility, crystallization kinetics and phase transition behavior of phytosterols and phytostanols in corn oil was investigated by Vaikousi *et al.* and found that phytosterol was more soluble and form stable blend in corn oil compared to the phytostanol-based blend. Co-crystallization of phytosterols with fully hydrogenated soybean oil was accepted as an alternative to form trans-free fat for various food applications. From all these studies, it was concluded that inclusion of free-sterols in fats and oils would be cost-effective and convenient process because of their higher bioavailability and solubility in fat-based food. Incorporation of phytosterols in various edible oils will be helpful for development of various phytosterol enriched formulations.

Investigation of physical and chemical properties of various phytosterol enriched functional oils will provide knowledge about its quality to both researchers and the food industry at large. Consequently, to enhance the phytosterol application as an oil structuring agent, it is essential to understand the phytosterols' melting and crystallization behavior in various oil blends using techniques such as X-ray Diffraction (XRD) and Differential Scanning Calorimeter (DSC), respectively. This work aims to study the effect of adding phytosterol on the physical and chemical properties of functional oils (pure oils blended with phytosterols). Furthermore, the investigation of melting and crystalline behavior of functional oils was performed which can be used as a functional component in using oil for the development of different food products.

Materials and Methods

Raw Materials

Five different refined edible oils (fortified with antioxidants), namely, sunflower, soybean, rice bran, canola and coconut oils were purchased from the local market in Rourkela, Odisha, India. Beta-sitosterol (typically containing 10% campesterol, 75% beta-sitosterol) was purchased from Thermo Fisher Scientific Pvt. Ltd., Mumbai, India.

Dispersion of Phytosterol in Edible Oils

Initially, the oils were heated in a water bath at 90°C for approximately 5 min. Then, based on preliminary experiments, a 10% (w/v) concentration of phytosterol was added to the pure oils and the temperature was maintained at 60°C under continuous stirring in a magnetic stirrer (Remi elektrotechnik, Pvt. Ltd., Vasai, India) for approximately 15 min at 400 rpm until all the phytosterol crystals got dissolved. The solution was then cooled at ambient temperature (~25°C) and stored at refrigerated conditions (<4°C) until further analysis. Before analysis, functional oils were brought back to room temperature (~25°C). The blends were visually stable and formed homogeneous mixture with no visible liquid-liquid phase separation.

Physical Properties of Pure and Functional Oils

Specific Gravity and Density

The specific gravity of pure and functional oils was measured by first taking the weight of the clean and dry specific gravity bottle which was marked as W_0 and the weight of the standard flask with oil sample (10 g) which was noted as W_1. Then, the oil was replaced with water and the weight of the standard flask with water was labelled as W_2. The density of different pure oils was calculated as the mass to volume ratio of oil. The specific gravity as well as density were measured using the Eqs (1) and (2), respectively.

$$\text{Specific Gravity} = \frac{(W_1 - W_0)}{(W_2 - W_0)} \quad \ldots (1)$$

$$\text{Density } (g/cm^3) = \frac{m}{V} \quad \ldots (2)$$

where, m, quantity of oil (g); V, volume of oil (cm^3)

Color

The color values were determined according to[14,15], a Hunter lab colorimeter (Color flex EZ, Reston, Virginia, USA) was used to measure the color values of pure and functional oils. After calibration of the instrument using white and black plates at room

temperature (~25°C) the L^* (lightness), a^* (redness to greenness) and b^* (yellowness to blueness) values were measured at constant light conditions. The total color value was calculated to quantify the overall color change in functional oils compared to pure oils. It was calculated using Eq. (3):

$$\Delta E = \sqrt{(L_0 - L_1)^2 + (a_0 - a_1)^2 + (b_0 - b_1)^2} \quad \ldots (3)$$

where, L_0, a_0, and b_0 represented the color values of pure oils (control); L_1, a_1, b_1 were the color values of functional oils.

Refractive Index

A bench-top digital refractometer (Model RFM 700, Bellingham and Stanley, UK) was used to calculate the refractive index of pure and functional oils. In this process, water was taken as standard and small amount of oil sample was placed on the prism and refractive index values were measured in triplicates.

Chemical Properties of Pure and Functional Oils

Peroxide Value

The peroxide value (PV) is a standard chemical method used to calculate the milli equivalent of peroxide oxygen per 1kg of oil. About 1g of potassium iodide and 30 mL of solvent (acetic acid and chloroform, 2:1) were added in 10g of oil sample and mixed vigorously for 1 min. Later, the mixture was diluted with 30 ml of pure water and titrated with 0.01 N $Na_2S_2O_3$ until the desired pale-yellow color was obtained. Subsequently, 0.5 ml starch mixture was added and titrated until the color faded. Finally, the peroxide value was determined using Eq. (4):

$$\mathrm{PV(meq/Kg)} = \frac{V*N*1000}{W_S} \quad \ldots (4)$$

where V_1, initial burette reading (ml);V_2, final burette reading (ml); N, normality of $Na_2S_2O_3$ (0.01N), and W_s, weight of the oil (g);V, change in volume (initial to final).

Acid Value

About, 2 g of oil samples were mixed with 50 ml of methanol followed by the addition of 2–3 drops of phenolphthalein indicator. The solution was then titrated against 0.1 N of KOH until pale pink color emerges. Finally, the acid value was estimated using Eq. (5) was followed by American Oil Chemists' Society.

$$\mathrm{AV(mg\ KOH/g)} = \frac{\mathrm{MW\ of\ KOH}*N*V}{W_S} \quad \ldots (5)$$

where MW, molecular weight of KOH; V, change in volume of final and initial reading; N, normality of KOH; W_s, weight of oil (g).

Antioxidant Activity

The antioxidant activity is considered as the ability of compounds to delay and reduce the oxidation process, which is mainly caused by the reactive oxygen species. The antioxidant activity assay was measured according to Singha & Muthukumarappan, with slight modification. About 0.2ml of oil sample, 2 ml of DPPH solution, and 2 ml of methanol (99%) were added and incubated for 30 minutes in the dark. Separately, 2 ml each of both DPPH and methanol was prepared and used as blank. A UV-vis spectrophotometer was used to measure the absorbance of oil and a blank at 517 nm. The percentage inhibition was calculated using Eq. (6):

$$\text{Antioxidant assay (\%)} = \frac{(A_{blank} - A_{oil})}{(A_{blank})} \times 100 \quad \ldots (6)$$

where, A_{blank}, absorbance of blank solution (methanol, 100%); A_{oil}, absorbance of sample (oil).

Melting Characteristics by Differential Scanning Calorimeter (DSC)

Melting characteristics of the beta-sitosterol, pure and functional oils were performed by using DSC (DSC-200 F3 MAIA, Netsch, Germany). An empty aluminum pan was considered as a reference and nitrogen was used as purge gas at a flow rate of 4 ml/min. About 10–30 mg of oil samples were placed into hermetically sealable aluminum pans. After attaining equilibrium state, samples were analyzed from −30 to 250°C temperature at a heating rate of 5°C/min. The offset, mid, onset melting temperatures and enthalpies were calculated.

Structural Properties of Pure and Functional Oils

X-Ray Diffraction (XRD) Analysis

The crystallinity (%) and crystalline size of pure and functional oils were analyzed using X-ray diffractometer (XRD, D8 advance A25, Brucker, USA). The scattering angle was adjusted from 5–45° in 1°/min increment. The Origin Lab (Version, 2020B) software was used to analyze the obtained data and all analyses were performed at ambient temperature (25°C). The relative crystallinity was calculated using Eq. (7):

$$\text{Relative crystallinity (\%)} = \frac{Ac}{Ac+Aa} \times 100 \quad \ldots (7)$$

where, A_c, oils relative crystallinity; A_c and A_a represent the area under crystallinity and amorphous region, respectively in the XRD graphs.

The crystalline size of both pure and functional oils was calculated using the Scherrer Eq. (8):

$$D = \frac{K \times \lambda}{\beta \times \theta} \quad \ldots (8)$$

where, *D,* diameter; *K*, Scherrer constant (0.9 as the shape of crystalline is unknown); λ, Copper K alpha wavelength of 1.5418; β, full width at half maximum of the diffractive peaks in radians; θ, Braggs diffractive angle.

Confocal Laser Scanning Microscopy (CLSM)

The micro-structural images of the functional oils were measured using Leica TCS-SP8 Confocal Laser Scanning Microscopy (Leica Microsystems, Heidelberg, Germany) attached to a Leica DMi8 inverted epifluorescence microscopy. The functional oils were pipetted into a concave slip (diameter 20 mm, depth 2.8 mm) and sealed with a cover slip. The images were captured using the 514 nm laser. For each sample, five different images were taken to avoid the non-homogenization distribution of the phytosterols in oils.

Statistical Analysis

All the analyses were conducted in triplicates. The data was illustrated as mean ± standard deviation and the various parameters were analyzed using statistical software (SPSS, version 24, IBM). Duncan test was performed to measure the variation among the mean values of samples. The statistical significance was taken at $P < 0.05$.

Results and Discussion

Specific Gravity and Density

Specific gravity is an essential physical property which affects the purity of oil. The specific gravity of oils varies due to their various structural composition. The specific gravity values of pure oils ranged from 0.9145 ± 0.0012 to 0.9335 ± 0.0007 (Table 1). The highest specific gravity was observed for rice bran oil as 0.9335 ± 0.0007, whereas the lowest value was found for sunflower oil as 0.9145 ± 0.0012. These specific gravity values were in line with other studies but did not show significant difference ($P > 0.05$). The difference in specific gravity in the pure oils could be due to difference in fatty acid composition, degree of unsaturation and total solid content.[18]

In the case of functional oils, the specific gravity values ranged from 0.9721 ± 0.0096 to 1.4242 ± 0.0007. The highest value was observed in rice bran oil (1.4242 ± 0.0007) and the lowest specific gravity value was found in canola oil (0.9721 ± 0.0096). Thus, it was observed that functional oils exhibited higher specific gravity values compared to pure oils and this could be due to their higher concentration of phytosterols in it.

The density of edible oils is proportional to the degree of unsaturation and inversely proportional to the chain length of the fatty acids present in their composition.[19] In our investigation, density of pure oils ranged from 0.91084 ± 0.001 to 0.9307 ± 0.0007 g/cm^3. Rice bran oil had highest density, which could be attributed to its higher unsaturated fatty acid content.

The density of functional oils ranged from 0.96918 ± 1.0177 to 1.41984 ± 1.2565 g/cm^3. The highest value was found in functional rice bran oil 1.4933 ± 1.2567g/cm^3, while the lowest values were observed for functional coconut (0.9691 ± 1.0177g/cm^3) and canola (0.9692 ± 1.002 g/cm^3) oils. There was a significant difference ($P < 0.05$) between pure and functional oils, which could be related to the inclusion of phytosterols in the edible oils. Similar to specific gravity, the difference in density values among the pure and functional oils can be due to an increase in solute concentration and variation in the unsaturated fatty acid content of edible oils.

Color

Color is one of the most critical parameters that determine the visual acceptance and adulteration of the oils. The color values of pure oils significantly differed from the functional oils ($P < 0.05$). The lightness value was highest for pure soybean oil (9.35 ± 0.17), followed by rice bran oil (6.25 ± 0.01), coconut oil (6.12 ± 0.03), canola oil (5.91 ± 0.03) and sunflower oil (5.37 ± 0.05).

The functional oils showed lighter color compared to pure oils. According to Katkade *et al.*, functional oils exhibiting lighter color are considered as purer and shows better quality. Among all the functional oils, sunflower oil (29.37 ± 0.99) was the lightest among others, whereas coconut oil (10.05 ± 0.462) exhibited a darker color due to the smoggy texture caused by the addition of phytosterols. Except coconut oil, other oils exhibited '*L**' values in the range of pure oils. The above results are in agreement with Waghmare *et al.*, which can be due to a lower carotenoid pigment content compared to other oils.

The '*a**' value indicates the (−) greenness to (+) redness color of a particular oil. The redness values of oils showed less impact; however, after transport, storage and reuse, edible oils become darker resulting in development of a deep yellow and light red color.

A significant ($P < 0.05$) decrease in redness values for functional sunflower, soybean and rice bran oils was observed. However, functional canola and coconut oils exhibited higher redness values compared to pure oils (Table 1). On the other hand, functional rice bran oil showed the lowest redness values due to its lower carotenoid content compared to other oils making it appear greener.

The '*b**' value indicates blueness (−) to yellowness (+) of the oil samples. Like *a** value, the yellowness values of oils increased with the addition of phytosterol except for coconut oil which can be due to the formation of a smoggy mixture. Rice bran oil was the most yellowish in color. It has been reported that compared to other oils even after refining, rice bran oil remains yellow in color, which is a challenging issue faced by edible oil industries.[22]

Refractive Index

The refractive index plays critical role in determination of the rancidity and aging of edible oils. However, in this study, comparative estimation of the refractive index of pure and functional oils indicated the amount of polymerized and un-oxidized triacylglycerol present in both the oils. Furthermore, the pure and functional oils exhibited almost similar refractive index values ranging from 1.4372 ± 0.006 to 1.476 ± 0.0045 (Table 1). The refractive index values of pure oils were in line with other qualitative assessment studies of edible oils.[20] The addition of phytosterol had no significant impact on the refractive index of the edible oils because there is no change in unsaturated fatty acid content after adding phytosterols to edible oils.

Peroxide Value

Peroxide value is mostly used to assess the degree of oxidation that occurs during the heating and storage of oils. In general, fresh oils have a peroxide value of less than 10 mEq of O_2/kg of oil but oils with peroxide value greater than 20 mEq of O_2/kg are considered highly rancid.[23] Therefore, oils with higher peroxide values are not suitable for consumption since they accelerate various diseases like cardiovascular and inflammatory diseases due to the development of secondary oxidative products and rise in reactive oxygen species (ROS).[24] The peroxide values of pure oils ranged from 2.21 ± 0.14 to 8.25 ± 0.19 mEq of O_2/ kg (Table 2), which fall in the good quality range. Among all, rice bran oil had the highest peroxide

Table 1 —The physical properties of pure and functional oils

Oils	Specific gravity		Density		Color						Refractive Index	
	Pure	Functional	Pure	Functional	L*		a*		b*		Pure	Functional
					Pure	Functional	Pure	Functional	Pure	Functional		
Sunflower	0.915 ± 0.0012^{a}	1.225 ± 0.0120^{g}	0.911 ± 0.0013^{a}	1.222 ± 0.0125^{g}	5.370 ± 0.0473^{a}	29.370 ± 0.9906^{e}	−0.636 ± 0.0513^{e}	−0.883 ± 0.0115^{d}	0.440 ± 0.0600^{c}	1.173 ± 0.1750^{e}	1.473 ± 0.0013^{b}	1.475 ± 0.0024^{b}
Soybean	0.916 ± 0.0015^{b}	1.247 ± 0.0126^{h}	0.915 ± 0.0014^{b}	1.243 ± 0.0123^{h}	5.370 ± 0.0473^{b}	19.576 ± 1.4420^{c}	−0.900 ± 0.0346^{d}	−1.540 ± 0.0346^{c}	1.467 ± 0.0404^{e}	2.633 ± 0.4252^{f}	1.437 ± 0.0060^{b}	1.476 ± 0.0045^{b}
Rice bran	0.934 ± 0.0007^{e}	1.424 ± 0.0129^{i}	0.931 ± 0.0006^{e}	1.420 ± 0.0134^{i}	5.370 ± 0.0473^{a}	21.683 ± 0.6170^{b}	−1.817 ± 0.0351^{b}	−2.016 ± 0.0306^{a}	2.863 ± 0.0808^{f}	5.647 ± 0.2303^{g}	1.472 ± 0.0062^{b}	1.474 ± 0.0593^{b}
Canola	0.918 ± 0.0003^{c}	0.972 ± 0.0201^{f}	0.916 ± 0.0002^{c}	0.969 ± 0.0231^{f}	5.370 ± 0.0473^{a}	20.246 ± 0.7427^{cd}	−0.917 ± 0.0593^{d}	−0.853 ± 0.0321^{d}	−0.240 ± 0.0200^{b}	0.7667 ± 0.0115^{d}	1.472 ± 0.0052^{b}	1.475 ± 0.0054^{b}
Coconut	0.919 ± 0.0097^{d}	0.972 ± 0.120^{f}	0.917 ± 0.0093^{d}	0.969 ± 0.0120^{f}	5.370 ± 0.0473^{a}	10.050 ± 0.4620^{b}	−0.273 ± 0.0404^{f}	−0.007 ± 0.1250^{g}	−0.363 ± 0.0208^{b}	−0.863 ± 0.1850^{a}	1.474 ± 0.0046^{a}	1.467 ± 0.0813^{a}

†Note: L* = Lightness, a* = yellowness to redness, b* = blueness to greenness; Different letters (a–i) represent a significant difference ($P<0.05$) among different oils with and without phytosterols; Values are mean ± standard deviation

Table 2 — The chemical properties of pure and functional oils

Parameters	Oil type	Sunflower oil	Soybean oil	Rice bran oil	Canola oil	Coconut oil
Peroxide value (mEq of O_2/kg)	Pure	4.490±0.0755^{c}	2.210±0.1442^{a}	8.260±0.1914^{g}	5.400±0.0115^{e}	5.000±0.0503^{d}
	Functional	6.250±0.3905^{f}	4.330±0.3004^{b}	10.760±0.3188^{h}	7.100±0.1825^{g}	11.100±0.1400^{i}
Acid value (mg KOH/g)	Pure	0.259±0.0053^{a}	0.253±0.0338^{a}	0.663±0.0401^{b}	0.738±0.0106^{c}	1.895±0.0252^{f}
	Functional	0.985±0.0176^{d}	1.393±0.006^{e}	2.035±0.0014^{g}	2.103±0.1198^{h}	4.154±0.0140^{i}
Antioxidant (%)	Pure	3.475±0.0396^{a}	12.949±0.0686^{c}	16.712±0.0792^{e}	16.049±0.0686^{d}	3.681±0.0396^{b}
	Functional	28.829±0.2772^{f}	59.001±0.5536^{h}	95.267±0.3160^{j}	69.616±0.3900^{i}	51.578±0.1851^{g}

†Note: Different letters (a–i) represents a significant difference ($P<0.05$) among different oils with and without phytosterols; Values are mean ± standard deviation

value of 8.260 ± 0.1914 mEq of O_2/ kg, while soybean oil had the lowest value of 2.210 ± 0.1442 mEq of O_2/ kg. This suggests that rice bran oil is less stable than other pure oils.

The addition of phytosterols in oils also increased the peroxide value and it ranged from 4.33 ± 0.30 to 11.1 ± 0.14 mEq of O_2/kg. The coconut oil had highest peroxide value of 11.1 ± 0.14 mEq of O_2/kg, while soybean oil had lowest values of 4.33 ± 0.30 mEq of O_2/kg. The oils should have a peroxide concentration of less than 10 mEq of O_2/kg to avoid rancidity and off-flavor development. However, coconut oil was slightly rancid as indicated by peroxide value more than 10 mEq of O_2/kg. Alternately, olive oils cannot be considered as rancid until it reaches 20 mEq of O_2/kg, while fish oil becomes rancid within <1 mEq of O_2/kg.[25] Moreover, the functional oils exhibiting higher peroxide value can be due to slight heating during blending process.[24] Hence, even though peroxide values increased upon addition of phytosterol in pure edible oils, the values were in acceptable range.

Acid Value

The acid value measures free fatty acid content present in oils. Also, it is considered as one of the essential characteristics to determine the degree of refining and quality of edible oils. The acid values of pure oils ranged from 0.253 ± 0.033 to 1.895 ± 0.025 mg KOH/g (Table 2). Coconut oil had a higher acid value of 1.895 ± 0.025 mg KOH/g, whereas soybean oil had a lower acid value of 0.253 ± 0.033 mg KOH/g. The values were in comparison to those reported elsewhere.[26] The difference in acid values of pure oils can be attributed to natural free fatty acids present in fresh oil. However, higher the acid value higher will be the oil deterioration, which affects the functional characteristics and quality of the final product.

With the addition of phytosterols the acid values of oils increased, and it was highest for functional coconut oil (4.154 ± 0.013 mg KOH/g) and lowest for functional sunflower oil (0.985 ± 0.017 mg KOH/g). The values for the functional oils were slightly higher than those of pure oil due to heating which can be attributed to the separation of fatty acids from triglycerides by breaking the bond. Also, there is chance of activation of lipolytic enzymes that may be responsible for the fatty acid separation.[27] Moreover, higher acid values of functional oils are attributed to the low acidity of three C-linked hydroxyl groups on the stigmasterol ring.[28] Most of the commercial phytosterols are free or esterified with fatty acids extracted from natural oils.[29] This may account to higher amounts of free fatty acid estimated in the blends eventually increasing the acid value of the blends. Even though the acid value almost fits within the recommended range, a higher acid value can cause faster quality deterioration.[30]

Antioxidant Activity

The presence of antioxidants is essential in providing stability of edible oils. Stability also depends on the processing methods and type of thermal treatment. The antioxidant content of the pure oils ranged from 3.082 to 16.826% (Table 2). The highest antioxidant value was found in rice bran oil, whereas the lowest value was found in sunflower oil. Many research studies have declared that highest antioxidant activity is found in rice bran oil compared to other oils.[31,32]

Like other natural antioxidants such as the tocopherol, phytosterols protect the unsaturated fatty acids from peroxidation in vegetable oils.[28] Phytosterols' antioxidant effect are expressed through the free radical scavengers. It is hypothesized that the donation of hydrogen atoms from the allyl methyl group in the sterol side chains followed by isomerization results in a relatively stable tertiary allylic free radical responsible for phytosterol scavenging activity.[33] Functional oils showed higher antioxidant values compared to pure oil and it ranged from 28.85 to 98.25%. The functional oils followed a similar trend of antioxidant activity as that of the pure oils, wherein the lowest antioxidant value was observed in coconut oil and the highest antioxidant activity was observed in rice bran oil.

Differential Scanning Calorimeter

In this study, various melting characteristics of phytosterols, pure oils and functional oils were measured. These parameters includes i) onset temperature of melting that is considered as the starting phase of thermal transition; ii) peak melting temperature represents the temperature at which the thermal effect is maximum; iii) offset temperature of melting represents the end of thermal phase transition; and iv) enthalpy of melting is estimated by integrating the melting curve.[34]

In case of pure phytosterol (powder form), single melting peak was observed with a peak melting temperature of 137.60 ± 0.09℃. Similar results of melting peak for pure phytosterols were observed by

Gomes Silva *et al.* According to Vaikousi *et al.*, a single melting peak indicates presence of similar crystalline form and melting points for all the components in a phytosterol blend. Likewise, the pure oils also demonstrated a single melting peak with values ranging from −16.83 ± 0.01 to 30.75 ± 0.012°C as shown in Fig. 1. The single and multiple peaks mainly depend on saturated and unsaturated fatty acids present in oils and type of triglycerides presented in the sample. A study by Ong *et al.* reported that canola oil which contains higher unsaturated fatty acid content displayed a single peak melting curve and triglycerides melt in a narrow temperature range. Whereas palm oil which contains more saturated fatty acids showed multiple peaks over a wider temperature range. Additionally, saturated fats have a higher melting point than those with higher unsaturated fatty acid content. In this study, pure coconut oil had the highest melting point attributed to its higher saturated fat content, whereas pure soybean and rice bran oils had the lowest which indicates a higher level of unsaturated fatty acids.

In functional oils, two distinct peaks were found

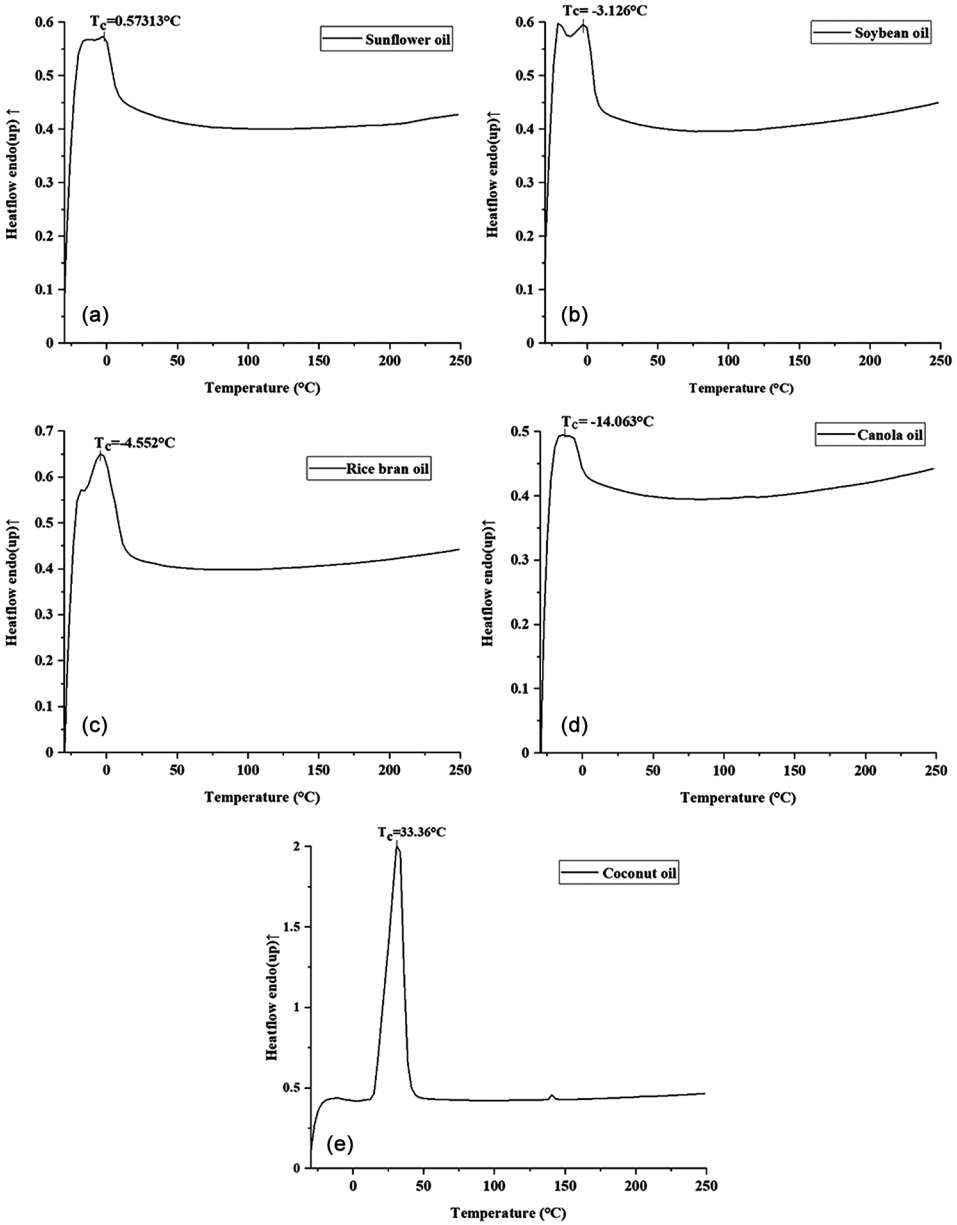

Fig. 1 —DSC melting graphs of pure: a) Sunflower oil, b) Soybean oil, c) Rice bran oil, d) Canola oil and e) Coconut oil; T_c = Crystallisation temperature

in which one peak was related to oils, and another peak was related to the presence of added phytosterols (Fig. 2). Similar trends of double peaks for phytosterol blends were observed by Gomes Silva *et al.* The different endothermic peaks in functional oils were mainly due to the higher and lower melting points of edible oils, phytosterols and due to polymorphic transition.

For functional oils, melting temperature ranged from −16.37 ± 0.05°C to 29.67 ± 0.11°C which was almost similar to the pure oils. The second peak temperature ranged from 55.84 ± 0.09°C to 75.53 ± 0.01°C. The highest peak temperature was found in functional canola oil, whereas the lowest melting temperature was observed in functional coconut oil. The presence of saturated and unsaturated fatty acid content in the oils might cause a difference in melting points of functional oils. The higher melting temperature of functional canola oil could be due to its higher unsaturation, while, functional coconut oil had a sharp melting point because of its higher saturated fatty acid content. When considering the geometry of saturated fats, they have linear rather than zigzag tetrahedral carbon atom bond angles. This intermolecular interaction allows them to "stack" together ultimately increasing the melting point. On the other hand, the unsaturated fatty acids when introduced with double bonds in the unsaturated fatty acid hydrocarbon chain result in "bends" in the molecules. The double bond geometry always remains a cis-configuration, which blocks stack formation ultimately reducing the melting point due to weak intermolecular interaction.

The melting enthalpy of the edible oils ranged from 2.2 ± 0.019 J/g to 16.48 ± 0.12 J/g. On the other hand, the enthalpy of phytosterols were 25.75 ± 0.12 J/g and enthalpy values of functional oils ranged from 14.95 to 27.49 J/g, which indicated that the functional oils released more energy during thermal phase transition in melting compared to pure oils.

X-Ray Diffraction (XRD)

The X-ray diffraction patterns of pure and functional oils were analyzed using XRD as shown in Figs 3 and 4. Various XRD characteristics such as crystallinity (%), amorphous (%), full width at half maximum (FWHM), crystalline size (nm) are presented in Table 3. The graph shows a relative difference in crystallinity behavior of pure and functional oils. For all oils, background intensity was in the regular range of 12–25° (2θ).[10] The variation in peak intensity affects the crystalline morphology of oils. Spacing between peaks also influence the

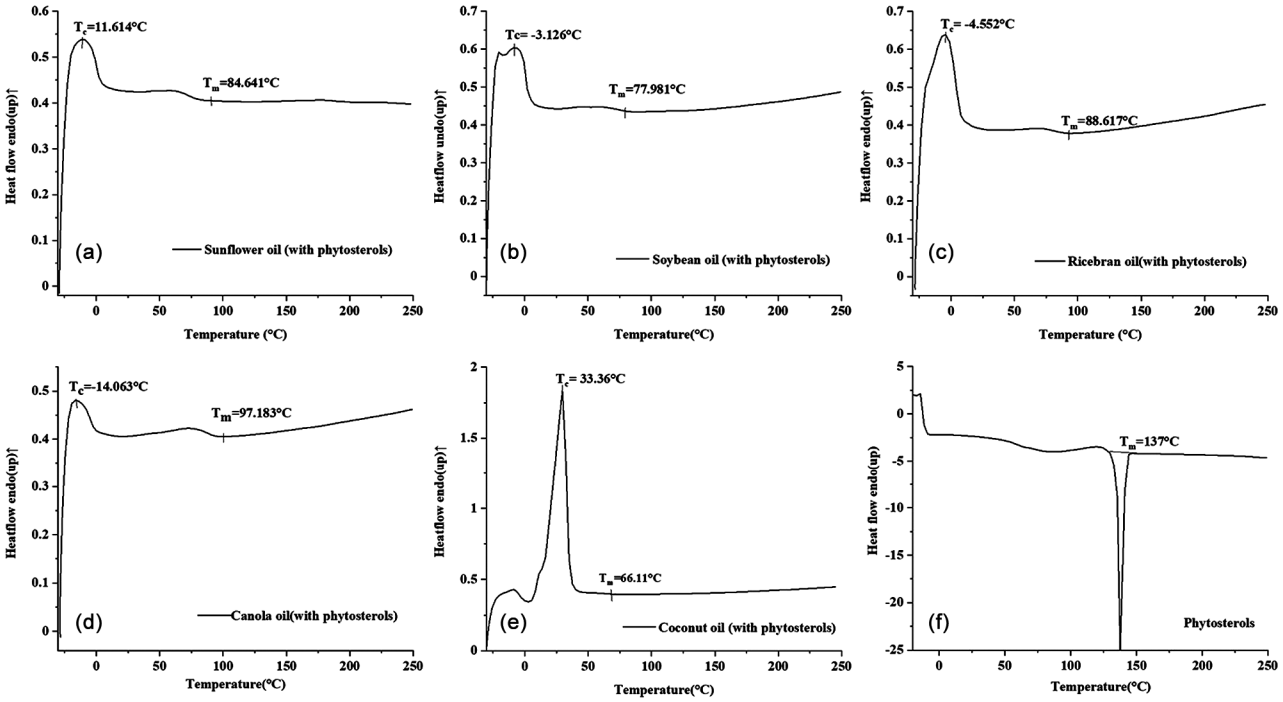

Fig. 2 — DSC melting graphs of: a) Sunflower oil (with phytosterols), b) Soybean oil (with phytosterols), c) Rice bran oil (with phytosterols), d) Canola oil (with phytosterols), e) Coconut oil (with phytosterols), f) Phytosterols; Note: "with phytosterols" means functional oils; T_c = Crystallisation temperature, T_m =Melting temperature

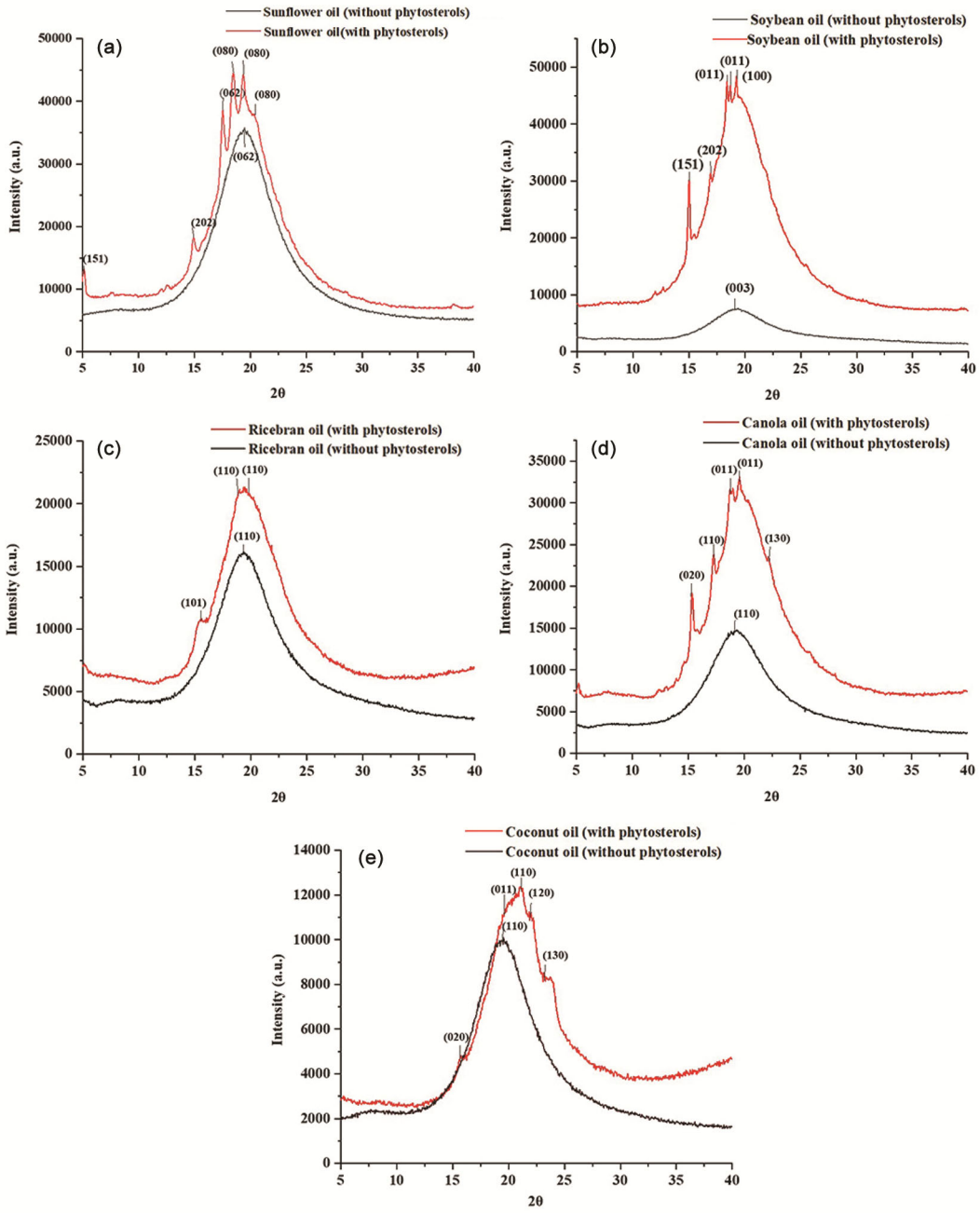

Fig. 3 — X-Ray Diffraction (XRD) patterns of: a) Sunflower oil, b) Soybean oil, c) Rice bran oil, d) Canola oil, e) Coconut oils; Note: "with phytosterols" means functional oils and "without phytosterols" means pure oils

morphology. The graph with short spacing peaks shows needle like morphology compared to the large spacing peaks. From Figs 3 and 4, functional sunflower oil had less peak spacing resulting in needle like morphology compared to other functional oils. However, functional coconut oil had low-intensity peaks and exhibited spherulitic morphology.

The average crystalline size (D) was evaluated using the Scherrer equation to better understand the distribution of phytosterols in different food oils (Eq. 8). The crystalline size of edible oils remained nearly constant ranging from 11.437 to 11.932 nm because pure oils were liquid at ambient temperature resulting in same crystalline sizes in all oils. The addition of phytosterols to different edible oils resulted in diverse crystalline sizes showing that the addition of phytosterols induces a change in the physical state of the sample making it more solid.

Additionally, solubility of phytosterols in various oils mainly depends on oil type and purity of phytosterols. Again, temperature plays an important role on solubilization of phytosterols. The crystallinity and crystal size are inversely proportional to the solubility. Functional sunflower oil showed the highest crystalline size of 19.72 nm, while functional rice bran oil showed the lowest crystalline size of 12.06 nm. The variation in crystalline sizes implies that the gaps between crystal grains have altered resulting in greater spacing between grains. This behavior can be attributed to the presence of phytosterols as well as differences in the dispersion effect of the various edible oils.

Confocal Laser Scanning Microscopy

The microstructures of five functional oils were analyzed using confocal laser scanning microscopy (Fig. 5). The pure oils are optically isotropic and seemed to be darker, while the phytosterol portion appeared as brighter area. In general, microstructure of pure phytosterols, especially β-sitosterol and stigmasterol are spherulites, which are formed through the aggregation of the needle and plate-like structure grown from the nucleation center and shows various order of branching resulting in larger crystal structure. Similar results were observed by Acevedo & Franchetti. From CLSM images, it was observed that addition of free phytosterols into various edible oils reduced larger agglomerations and disintegration of the crystalline aggregates. This may be due to the difference in oil type (degree of saturation and unsaturation), fatty acid composition and thermal behavior (melting and crystalline behavior), respectively.

Incorporation of the free phytosterols into the sunflower, canola and rice bran oil showed elongated crystals with lower density aggregates contributing to formation of larger number of sites for binding among

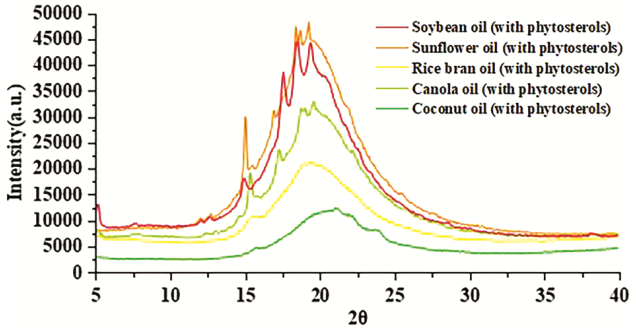

Fig. 4 — Comparative X-Ray Diffraction (XRD) patterns of the different functional oils

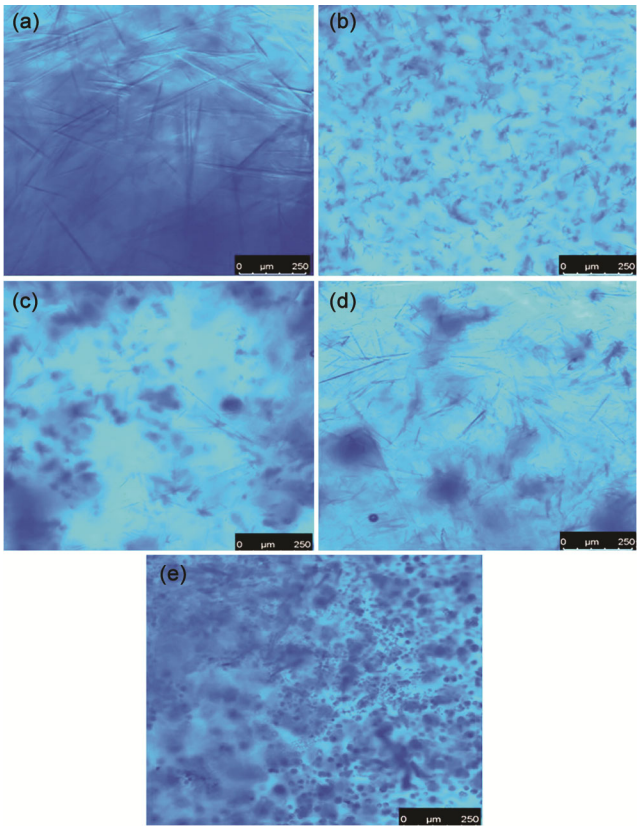

Fig. 5 — Confocal laser scanning micrographs of functional oils: a) Sunflower oil, b) Soybean oil, c) Rice bran oil, d) Canola oil, e) Coconut oil

Table 3 — XRD characteristics of pure and functional oils

Parameters	Oil type	Sunflower oil	Soybean oil	Rice bran oil	Canola oil	Coconut oil
Crystallinity(%)	Pure	8.00±0.27^{a}	17.20±0.30^{b}	24.08±0.31^{c}	30.26±0.21^{e}	32.67±0.17^{f}
	Functional	51.78±0.30^{h}	79.26±0.36^{i}	26.53±0.21^{d}	35.43±0.28^{g}	83.04±0.24^{j}
Amorphous(%)	Pure	92.00±0.25^{j}	82.80±0.23^{i}	75.92±0.37^{h}	69.74±0.28^{f}	67.33±0.25^{e}
	Functional	48.22±0.17^{c}	20.74±0.14^{b}	73.47±0.23^{g}	64.57±0.11^{d}	16.96±0.18^{a}
FWHM	Pure	6.28±0.25^{e}	6.33±0.29^{f}	6.47±0.33^{j}	6.56±0.32^{l}	6.41±0.19^{i}
	Functional	5.96±0.14^{b}	6.16±0.11^{c}	6.20±0.21^{d}	6.52±0.27^{k}	5.91±0.24^{a}
Crystalline size(nm)	Pure	11.93±0.30^{c}	11.83±0.36^{b}	11.44±0.39^{a}	11.43±0.32^{a}	11.44±0.33^{a}
	Functional	12.56±0.17^{e}	15.26±0.21^{g}	12.07±0.29^{d}	14.69±0.25^{f}	17.73±0.32^{h}

†FWHM: Full width at half maximum; Different letters (a–i) represent a significant difference ($P<0.05$) among different oils with and without phytosterols; Values are mean ± standard deviation.

the crystals. This could be due to the low percentage of saturated fatty acid content. The similar results were observed by Gomes Silva *et al.* According to their results incorporation of the free phytosterols in high oleic sunflower oil showed smaller aggregates with less denser network. However, dispersion of free phytosterols in soybean oil showed shiny flower like crystals, which was different from the original pure phytosterol structure. Similar results were observed by Acevedo & Franchetti. Furthermore, coconut based functional oils showed that large aggregates resulted in denser crystalline network, which could be attributed to a change in the physical condition caused by the presence of a significant amount of saturated fatty acids. Whereas morphology of crystals in functional canola oil demonstrated a different crystal network, which may be due to a small tightly packed crystal trapped within a large fibrillar crystal, a typical morphology of non-esterified phytosterol.

Conclusions

The physical, chemical, thermal, and structural characteristics of pure and oils containing free phytosterols were analyzed using various techniques. The degree of unsaturation greatly influences the ultimate quality of pure and functional oils. Except refractive index, all other characteristics differed significantly for pure and functional oils. According to chemical characteristics data, soybean oil had a lower peroxide value and acid value indicating improved oil quality and suitability for phytosterol inclusion. The melting peaks of functional oils mainly depend on saturated or unsaturated fatty acids present in the blend. According to XRD analysis, the crystallinity and crystal sizes of oils increased with the addition of phytosterols, with rice bran oil having the lowest crystallinity, which was further consistent with CLSM images. Based on all the results, soybean oil can be used for dispersion of phytosterol and food fortification owingto its better stability compared to other oils.

Acknowledgments

The authors are thankful to Science and Engineering Research Board (SERB), New Delhi, India, for financial support to conduct the research (Grant no. SRG/2019/000998). The authors are grateful to PhD research scholars, (Ms Niveditha Asaithambi and Mr Dibya Ranjan Dash) for their assistance in preparing the manuscript. The authors are also thankful to the Centralised Instrumentation Facility (CIF) of the National Institute of Technology Rourkela, India, to provide the facility to use analytical instruments like CLSM, X-ray diffraction system, and DSC.

Declaration of Competing Interest

The authors of the present work declare no conflict of interests.

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Graphical Abstract