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Research Article

Enhanced Solubility of Phytosterols (PS) in DAG-Rich Sunflower Oil and Analysis of PS Composition by Method Development and Validation through GCMS

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Abstract

Scope: In this study, PS solubility was determined in different DAG-rich sunflower oil (181.4 to 598.0 g kg^{-1}) at 10-60°C and PS composition was determined by unsaponifiables of the respective oils through GCMS.

Methods and Results: Standard of β-sitosterol, γ-sitosterol, campesterol, stigmasterol, Δ-^{5} avenasterol, Δ-^{7} stigmasterol each of with six different concentrations (2 ppm, 5 ppm, 10 ppm, 20 ppm, 50 ppm and 100 ppm) were used, prepared from 1 g kg^{-1} stock solution. Calibration were prepared and method was validated.

Experimental data revealed that Sun flower oil (SFO), phytosterol enriched sunflower oil (PS-SFO), diacylglycerol rich sunflower oil (SFDAG) and phytosterol enriched diacylglycerol richsun flower oil (PS-SFDAG) contained 7.05 g kg^{-1}, 19.31 g kg^{-1}, 5.6 g kg^{-1}, 59.4 g kg^{-1} of PS in unsaponifiable respectively. GCMS data interprets that PS-SFDAG contains 59.4 g kg^{-1} sterol that consists of ergosterol-0.34 g kg^{-1}, ethylcholesterol-0.24 g kg^{-1}, campesterol-12.4 g kg^{-1}, stigmasterol- 7.69 g kg^{-1}, β-Sitosterol- 18.79 g kg^{-1}, γ-Sitosterol-13.12 g kg^{-1}, Δ^{5}- avenasterol- 0.26 g kg^{-1} and Δ^{7} stigmasterol- 0.48 g kg^{-1}.

Conclusion: SFO and SFDAG contained 13.1 g kg^{-1} and 10.2 g kg unsaponifiables while that enriched with phytosterols showed 25.0 g kg^{-1} and 71.0 g kg^{-1} for PS-SFO and PS-SFDAG, respectively. GCMS data showed γ-sitosterol is the major sterol found in SFO and SFDAG.

Keywords: Diacylglycerol (DAG); Phytosterol (PS); Sunflower Oil (SFO); Gas Chromatography Mass Spectrometry (GCMS); β-Sitosterol; Γ-Sitosterol

Abbreviations

PS: Phytosterol; SFO: Sunflower Oil; PS-SFDAG: Phytosterol Enriched DAG-Rich Sunflower Oil; GC-MS: Gas Chromatography Mass Spectrometry; MAG: Monacylglycerol; DAG: Diacylglycerol; TAG: Triacylglycerol; FFA: Free Fatty Acids

Introduction

Phytosterols (PS) and phytostanols are tetracyclic lipid components found exclusively in plants. They are gaining importance in the food industries as nutraceuticals because of their antioxidative and anticholesterolemic effect. Dietary intake of phytosterols ranges from 0.16 gm to 0.40 gm/day for different races of humans [1]. PS have become dietary ingredients of increasing importance since Peterson first reported in 1951 [2]. PS are structurally similar with cholesterol contain an extra methyl group, ethyl group or double bond. The most common plant sterols in the human diet are the 4 desmethylsterols comprising of β-sitosterol [3], campesterol, stigmasterol [4] etc. Stanols are saturated sterols,

produced by chemical hydrogenation of sterols. PS differ from cholesterol in that they are not synthesized in humans, are poorly absorbed and are excreted faster from the liver [5].

Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and *tert*-butylhydroquinone (TBHQ) are generally added to oils to retard oxidative degradation during storage and cooking. Phytosterols are naturally occurring antioxidants hardly soluble in oil or water [6]. On the other hand, DAG-rich oil has the property to solubilise phytosterol in greater quantity due to presence of one free hydroxyl group in its structure without any esterification. Now-a-days DAG-rich oils are also popular in market due to their antiobesity effect [7-9]. The dissolved PS in different DAG concentration is difficult to measure by HPLC and UV due to lack of chromophore. Thus, they require derivatization. Recently chromatographic fingerprint technique has been accepted by World Health Organization (WHO) as an approach for the evaluation of nutraceuticals. Several analytical methods have been evolved for the quantification of naturally occurring PS in foods. They are HPLC (coupled to UV and/MS detector) [10] and or GC (coupled to an FID and/or MS detector) [11,12]. Rocco and Fanali [13] tried to determine phytosterols by nanoliquid chromatography-MS. AOAC published an official method 994.10 as "analysis of cholesterol in foods" by GC after saponification and derivatisation with trimethylchlorosilane [14].

According to [15] pharmacological activity in different drug is different due to combined effect of a group of substances. Xiong., *et al*. (2002) [16] has proposed a quantitation method for sterol from matrices of animal and plant origin. Estimation of PS from biological matrices typically requires careful selection of internal standards, extraction, and derivatization. In our previous study, DAG was prepared under previously optimised condition by enzyme catalysed glycerolysis [17]. In this study, PS solubility was determined in different DAG concentration (181.4 to 598.0 g kg^{-1}) in the oil at 10-60°C from the unsaponifiables of the respective oils. Again, individual sterol composition and its content was calculated based upon the standard calibration method, developed and validated through GCMS.

Methods

Samples (oils), reagents and enzymes

Sunflower oil was purchased from Meghdoot Oils Mill Pvt Ltd., Belapur, Navi Mumbai, India. Standard β-sitosterol, γ-sitosterol, campesterol, stigmasterol, Δ-^{5} avenasterol, Δ-^{7} stigmasterol and internal standard (5α cholestane) were all procured from Fluka (Sigma-Aldrich). Mixed phytosterol(PS) for solubilisation study was procured from Fluka (Sigma-Aldrich). Enzyme TLIM (*Thermomyces lanuginose* immobilized lipase) was supplied by Zytex India Ltd., Mumbai, India. Glycerol and all other reagents used were of analytical grade and procured from Merck India Ltd., Mumbai, India. All solvents like n-hexane, n-heptane, pet-ether, methanol, diethyl ether was of HPLC grade and obtained from Merck India Ltd., Mumbai, India. All standards were freshly prepared at six different concentrations (2 ppm, 5 ppm, 10 ppm, 20 ppm, 50 ppm, 100 ppm).

Preparation of DAG-rich sunflower oil (SFDAG)

DAG-rich sunflower oils with five different concentrations (181.4 g kg^{-1}, 275.3 g kg^{-1}, 378.6 g kg^{-1}, 511.4 g kg^{-1} and 598.0 g kg^{-1} DAG) were obtained from earlier experiment according to [17] and were used for further PS solubilisation study.

Preparation of PS enriched DAG (PS-SFDAG) and PS enriched sunflower oil (PS-SFO)

To prepare PS enriched DAG (PS-SFDAG) and PS enriched sunflower oil PS-SFO, SFDAG and SFO, each of 100g was taken in identical 250 ml conical flask placed on digital magnetic stirrer (MS-H280- pro) with 500 r.p.m at 10°C, 20°C, 30°C, 40°C, 50°C, 60°C for about 5 hr. Phytosterols (0.5 gm) was added to each of the flask at intervals of 15 min for 2 h until the undissolved particulate (PS) was obtained as precipitate. Thereafter, stirring was continued for 3 hr without any addition of exogenous PS. The contents in the conical flasks were allowed to cool, centrifuged and undissolved particulate matter was removed by membrane filtration.

Separation of unsaponifiable matter from respective oils

Unsaponifiable matter from SFO, SFDAG, PS-SFO and PS-SFDAG performed for each temperature like 10°C, 20°C 30°C, 40°C, 50°C, 60°C were separated after saponification according to the standard method [18,19]. Before saponification 0.2 ml of 100 ppm internal standard was added to each of the sample.

Qualitative detection of phytosterols by thin layer chromatography (TLC) [20]

For qualitative analysis TLC was done to know the composition of PS in oils (4 samples, triplicate of each). Each sample was

spotted on 20×20 cm silica gel G TLC plates spread with a 0.2 mm layer of plate in equal concentration by placing uniformly along a line 1.5 cm from one edge of the plate and developed using a solvent system comprising hexane-diethyl ether (6:4 v/v) with a continuous flow on the preparative TLC. After complete run, the solvent was evaporated by drier. For visualisation the plate was placed in iodine chamber.

Determination of Sterol composition in SFO, SFDAG, PS-SFO and PS-SFDAG by GC-MS [21]

Preparation of 1 g kg^{-1} mixed stock solution

Accurately weighed 0.5 gm of each standard (β-sitosterol, γ-sitosterol, campesterol, stigmasterol, Δ-^{5} avenasterol, Δ-^{7} stigmasterol) was taken in a 100 gm volumetric flask. HPLC grade hexane was added to make the total weight 50 gm.

Preparation of calibration standard from stock solution

Six calibration standards (2 ppm, 5 ppm, 10 ppm, 20 ppm, 50 ppm and 100 ppm) were prepared from 1 g kg^{-1} stock solution. 10 ml 6 vials were taken separately to each 0.002gm, 0.005gm, 0.01gm, 0.02gm, 0.05gm and 0.1 gm stock solution was taken. Then to each vial 1 gm of internal standard was added and the weight was made upto10 gm with addition of hexane to prepare 2 ppm, 5 ppm, 10 ppm, 20 ppm, 50 ppm and 100 ppm respective calibration standard.

Build-up calibration for PS

Calibration was built up based upon the exact standard concentration. For quantitation in SIM mode, one target ion and two qualifiers ions for each sterol was selected and entered during method development very carefully to build up calibration. (r^2 for campesterol, stigmasterol, β-sitosterol, γ-sitosterol, Δ-^{5} avenasterol, Δ-^{7} stigmasterol - 0.999, 0.998, 0.999, 0.999, 0.995 and 0.996).

Analysis of PS in SFO, SFDAG, PS-SFO and PS-SFDAG

It was observed that solubility of PS in DAG-rich sunflower oil(SFDAG) was higher compared to other oils confirmed by obtained unsaponifiables from the respective oils. Again, highest solubility of PS in highest concentration was observed at 50^{o}C compared to other temperatures. Therefore, GCMS analysis was done only for those samples conducted at 50^{o}C. Quantitative detection of PS and phytostanols as their trimethylsilyl (TMS) derivatives in the presence of an internal standard was carried out by electron impact mass spectrometry using a Shimadzu GC-MS QP2010 Ultra instrument (Shimadzu, Kyota, Japan). The chromatographic column for the analysis was a 95% dimethyl polysiloxane and 5% diphenyl capillary column. (Rtx-5MS, 30m, 0.25 mm ID, 0.25µm df). The SFO, SFDAG, PS-SFO and PS-SFDAG all oils were saponified in the presence of an internal standard with potassium hydroxide in ethanol to break the ester bonds. The unsaponifiable matter was extracted with HPLC grade pet ether and evaporated to dryness under a stream of nitrogen gas. The unsaponifiable matter was plotted as band TLC on silica gel TLC plate and the all fractions were collected separately, dissolved in HPLC grade hexane/pet-ether, filtered and evaporated to collect the content in air tight vial. To get the sterol fraction corresponding sterol standard was applied to the plate and only that part was collected and repeat the steps referred early. In the mass-spectrometer the condition was as follows: ion source temperature, 200°C; interface temperature, 220°C; ionization energy, 70 ev. The MS scan range was 50-650 atomic mass unit (AMU). The carrier gas used was helium (99.999%) at a flow rate of 1.0 mL/min. Samples were analysed with the column held initially at 70ºC for 1 min and then increased to 200ºC with 10 ºC/min heating rate and held that temperature for 10 min. Finally, temperature was increased to 280ºC with increased at the rate of 10ºC/min and held for 22.13 min. Total run time was 52 min. The injection was performed in split ratio (1:20) at 250ºC. The identification of individual components was done by Wiley and NIST mass spectral library on the basis of the mass fragments and m/z values of each component as well as from the retention time(RT) of the standard.

Quantitation of PS in oil samples

To obtain the accurate quantitation result, each sample had been injected in triplicate. GCMS is very useful and reliable method for identification of sterols. In our study, six different concentration (2 ppm, 5 ppm, 10 ppm, 20 ppm, 50 ppm and 100 ppm) for each sterol were used to built-up calibration. All the calibrations for all sterols were built-up accurately and then samples (PS-SFDAG) were compared with this existing calibration. The results were expressed as mean ± SD of three determinants.

Limit of quantitation (LoQ)

LoQ was obtained by spiking the lowest concentration level i.e,2 ppm of calibration standard in a blank sample which gives an identifiable, discrete peak with a precision of 20% and accuracy

is 80-100%. Precision of the method was determined by taking samples (stigmasterol, campesterol and β -sitosterol, γ -sitosterol 100mg/L) prepared in three times and each of them was injected in duplicate. The precision was determined from % RSD. To establish the inter-assay and intra assay precision of the method six different concentration of standard were assayed in one day, on three different days.

Results and Discussion

Method performance and validation

The GCMS method was developed to analyse the sterol composition of experimental oils. This method is applicable for analysis of sterols in all vegetable oils. Method validation was done according to [1]. Linearity was performed by freshly prepared standard solution using six-point calibration with concentration of 2, 5, 10, 20, 50 and 100 ppm (Ergosterol, cholesterol, campesterol, stigmasterol, β-sitosterol, γ-sitosterol, Δ^{-5} avenasterol, Δ^{-7} stigmasterol). Initially run time for each individual sterol was determined by injecting 20 ppm of standard. All correlations for all standards were found to be linear, with correlation coefficients between 0.998-0.999. This indicates that 99% of the experimental variability could be explained by linear models confirming the satisfactory relationship between analytes concentration. Amount of each sterol was calculated by comparing the test sample with the existing calibration.

Table 1 showed the amount of TAG, DAG, MAG, FFA and unsaponifiable matter and sterol content in four variants of sunflower oil. Experimental data revealed that PS-SFDAG contains 71 g kg^{-1} unsaponifiable, in which 59.4 g kg^{-1} is sterol. Whereas, SFO shows only 13.1 g kg^{-1} unsaponifiable that comprises 7.05 g kg^{-1} sterol in unsaponifiable. Therefore, DAG has higher solubilising capability for PS than SFO.

Oils	TAG	DAG	MAG	FFA	Unsap	Sterol content
SFO	951.6 ± 6.1	23.4 ± 0.50	11.6 ± 0.3	0.5 ± 0.0	13.1 ± 0.2	7.05 ± 0.2
SFDAG	285.2 ± 6.5	598.5 ± 4.6	101.6 ± 1.5	5.5 ± 0.1	10.2 ± 0.1	5.6 ± 0.2
PS-SFO	945.8 ± 5.4	11.2 ± 0.6	10.5 ± 0.6	7.5 ± 0.5	25.0 ± 0.3	19.31 ± 0.4
PS-SFDAG	279.8 ± 6.2	599.0 ± 8.2	42.0 ± 0.8	7.2 ± 0.1	71.0 ± 7.2	59.4 ± 2.1

Table 1: Amount of TAG, DAG, MAG, FFA, unsaponifiable matter and sterol content (g kg^{-1}) in SFO, SFDAG, PS-SFO and PS-SFDAG[a]

[a]Values are mean ± SD of three determinations.

TAG-Triacylglycerol, DAG-Diacylglycerol, MAG-Monoacylglycerol, FFA-Free fatty acid, Unsap-Unsaponifiable.

Total unsap composition was investigated (Table 2) which revealed that unsaponifiables of all four variants of SFO (SFO, SFDAG, PS-SFO and PS-SFDAG) contained four different fractions *viz*. fraction I- sterols, fraction II- triterpene alcohols, fraction III- fatty alcohols and fraction IV- hydrocarbons. The variations in composition in constituents of unsaponifiables of different oils may be due to different method of preparation and processing of oils. SFO was naturally obtained. SFDAG was prepared from SFO by enzymatic glycerolysis with lipase. PS-SFO and PS-SFDAG were prepared by addition of exogenous PS. All the oils undergo saponification followed by washing with water to remove soap content. Washing with water may lead to loss of some of the water-soluble components that may affect in case of all samples. Triterpene alcohols like α-amyrin and β-amyrin are also tetracyclic compounds similar to sterols. Fatty alcohols are higher molecular weight alcohols like oleyl alcohols etc. They are not tetracyclic compounds. Among the various fractions of unsaponifiable only the sterol fraction was collected and was further proceeded for GCMS analysis.

Figure 1 shows the TLC profile of PS (1), SFO (2), PS-SFO (3) and PS-SFDAG (4). SFO showed the presence of triacylglycerols (TAG) as well as traces of free fatty acids (FFA). No other significant spots corresponding to PS standard and DAG was seen on TLC plate. PS-SFO indicated the presence of TAG. In this case too, no significant spot was seen corresponding to PS standard which

Oils	Fraction unsaponifiables (g kg^{-1})			
	Methyl sterol and sterol	Triterpene alcohol	Fatty alcohol	Hydrocarbon
SFO	538.7 ± 1.4	283.3 ± 3.0	103.2 ± 1.0	76.6 ± 2.3
SFDAG	549.2 ± 0.2	231.3 ± 1.8	140.1 ± 4.1	80.6 ± 3.2
PS-SFO	772.4 ± 0.4	156.2 ± 1.6	58.1 ± 3.1	12.3 ± 4.3
PS-SFDAG	836.6 ± 2.1	147.5 ± 5.1	14.0 ± 7.6	1.8 ± 3.0

Table 2: Composition of total unsaponifiable matter in different oils used under study.

[a]Values are mean ± SD of three determinations.

implied that SFO had poor solubility for phytosterols. However, PS-SFDAG showed the presence of DAG as well as a dark prominent spot corresponding to PS standard which clearly implied a better solubility of PS in SFDAG. Table 3 clearly indicates the composition of sterols in sterol fraction of four oils. Data of Table 3 was derived from Figure 2 that represents the GCMS chromatogram of unsaponifiables of four variants of sunflower oil. *viz*.(a) SFO, (b) SFDAG, (c) PSSFO, and (d) PS-SFDAG. PS-SFDAG contains 59.4 g kg^{-1} sterol that consists of ergosterol-0.34 g kg^{-1}, ethylcholesterol-0.24 g kg^{-1}, campesterol-12.4 g kg^{-1}, stigmasterol- 7.69 g kg^{-1}, β-Sitosterol-18.79 g kg^{-1}, γ-Sitosterol- 13.12 g kg^{-1}, Δ^5- avenasterol- 0.26 g kg^{-1} and Δ^7 stigmasterol- 0.48 g kg^{-1}. Whereas, PS-SFO showed 19.31 g kg^{-1} sterols in oil that consists of ergosterol- 0.11 g kg^{-1}, ethylcholesterol-0.10 g kg^{-1}, campesterol- 4.85 g kg^{-1}, stigmasterol-4.47 g kg^{-1}, β-Sitosterol- 5.40 g kg^{-1}, γ-Sitosterol-3.08 g kg^{-1}, Δ^5-avenasterol- 0.15 g kg^{-1}, Δ^7 stigmasterol- 0.42 g kg^{-1}. This analysis is essential for detailed characteristics of oil. It also still remains unknown why do plant require a mixture of phytosterols instead of only one kind as cholesterol generally present in animals. GCMS analysis of sterols demonstrates the uniqueness of the composition of the specific oils. Analysis of mixed phytosterol (exogenous PS) in GCMS revealed presence of ergosterol-36.3 g kg^{-1}, methylcholesterol-49.1 g kg^{-1}, campesterol-274.3 g kg^{-1}, stigmasterol-219 g kg$^{-1,}$ β-sitosterol-420.75 g kg^{-1} of PS. It did not contain any γ-sitosterol. Analysis of PS-SFO and PS-SFDAG showed the presence of both β-sitosterol as well as γ-sitosterol as because β-sitosterol was present in original SFO whereas γ-sitosterol was obtained from mixed exogenous PS.

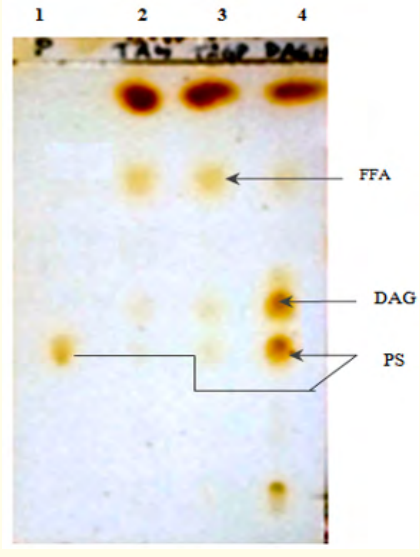
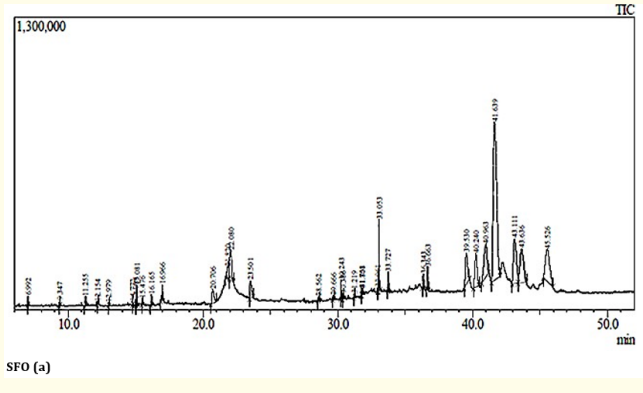


Figure 1: TLC chromatogram of (1) PS(phytosterol), (2) SFO (3) PS-SFO and (4) PS-SFDAG.



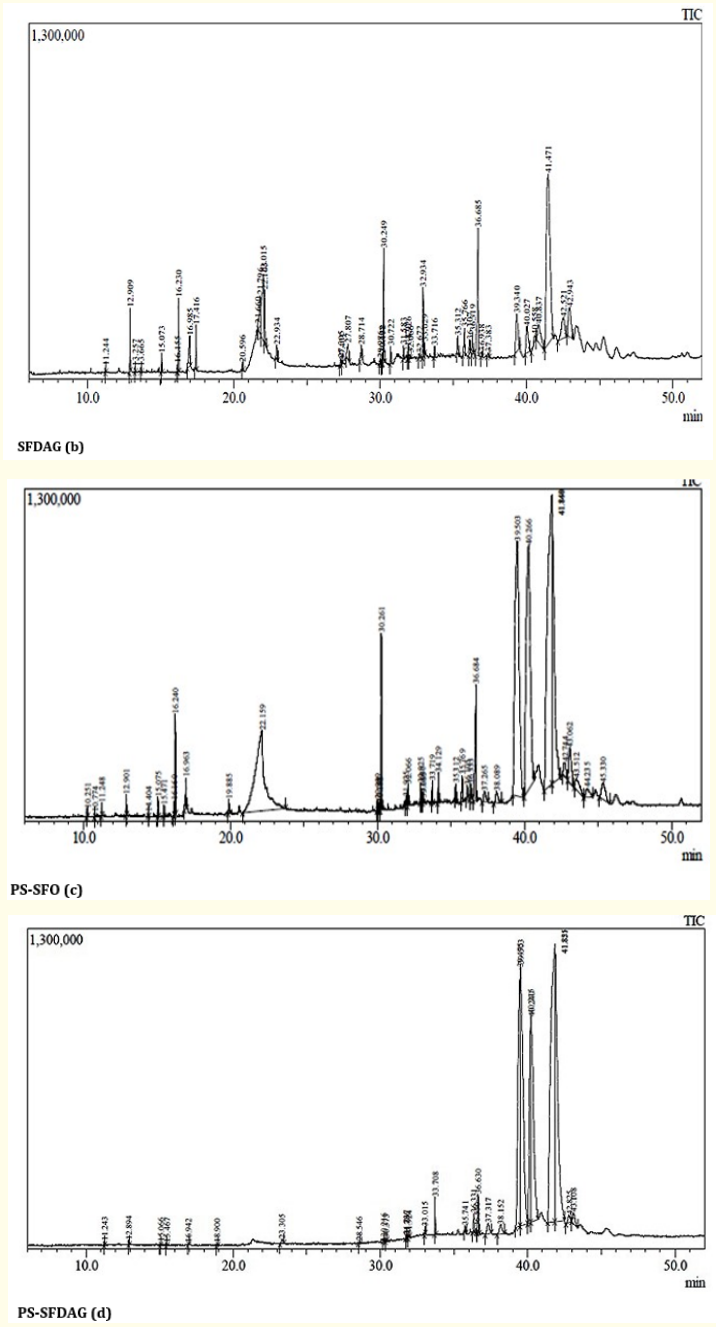


Figure 2: Total ion count (TIC) profile of the unsaponifiable part of (a) SFO, (b) SFDAG, (c) PSSFO, and (d) PS-SFDAG in the full-mode scan at 70 ev.

Oils	Methyl cholesterol	Ergosterol	Campes-terol	Stigmas-terol	β-Sitosterol	γ-Sitosterol	Δ^5-Aven-asterol	Δ^7-Stig-masterol	Others
SFO	-	90.9 ± 2.5	-	68.3 ± 0.06	-	483.7 ± 5.5	-	120.7 ± 1.3	236.4 ± 7.4
SFDAG	8.3 ± 3.5	14.5(γ) ± 0.2	-	57.9 ± 2.3	-	630.1 ± 3.8	-	102.3 ± 2.2	186.9 ± 5.3
PS-SFO	5.5 ± 0.2	5.9 ± 0.05	251.6 ± 1.5	232.1 ± 2.8	279.4 ± 8.5	160.1 ± 1.5	7.89 ± 0.05	21.8 ± 1.2	35.71 ± 1.5
PS-SFDAG	4.2 ± 0.05	5.8 ± 1.02	209.6 ± 5.5	129.6 ± 1.2	316.4 ± 1.4	221.1 ± 3.5	4.4 ± 0.03	8.2 ± 1.5	100.7 ± 2.6

Table 3: Composition of (g kg^{-1}) sterols in sterol fractions of four oils under study[a].

[a]Values are mean ± SD of three determinations.

Table 4 represents the m/z along with the mass fragments of different sterols. The retention time and the characteristic fragments of the electron ionisation mode mass spectra were determined by multiple reactions monitoring mode. For quantification, the most abundant product ions were selected, and the second was selected for confirmation. In this method one parent ion(target) and two daughter ions(qualifiers) were selected during method development. The unique MS fragmentation of each sterol was operated in the electron ionisation mode at 70 ev (SCAN mode). Identity of sterols was confirmed by comparison with pure standards and by MS-library. The mass spectrum of the β-sitosterol indicated the molecular ion at m/z 414, along with the mass fragments corresponding to the ions at 213,232,255,273,303,329,396 and 414 respectively. Whereas, campesterol showed molecular ion at m/z 400 and the mass fragments corresponding to the ions at 213, 231,273,289,315,382,400. Stigmasterol showed molecular ion at m/z 412. Δ^5-avenasterol, Δ^7-stigmaterol and Δ^7-avenasterol all showed their molecular ion at 412 but their mass fragments were different. Selection of proper target and qualifiers (ions) for each sterol will help to identify the exact compound though the co-elusion may exist.

Sterols	MW	Molecular Foumula	Mass fragments
Campesterol	400	$C_{28}H_{48}O$	107,133,145,213,231,273,289,315,382,400
Stigmasterol	412	$C_{29}H_{48}O$	107,123,145,159,213,255,271,351,369,394,412
Fucosterol	412	$C_{29}H_{48}O$	107,159,229,299,314,412
β-Sitosterol	414	$C_{29}H_{50}O$	107,231,255,273,303,329,381,396,414
γ-Sitosterol	414	$C_{29}H_{50}O$	29,43,81,133,173,213,231,329,369,414
Δ^5-Avenasterol	412	$C_{29}H_{48}O$	107,145,159,229,271,299,314,394,412
Δ^7-Stigmasterol	412	$C_{29}H_{48}O$	107, 119, 161, 173, 213,255,273,399,412
Δ^7-Avenasterol	412	$C_{29}H_{48}O$	107,145,159,229,271,299,314,394,412

Table 4: GC-MS analysis of sterols from the oils analyzed under study.

In our study, all the calibrations for all sterols (Ergosterol, cholesterol, campesterol, stigmasterol, β-sitosterol, γ-sitosterol, Δ-^{5} avenasterol, Δ-^{7} stigmasterol) had been built accurately prior to the analysis of experimental oils and then DAG samples were compared with those existing calibration. It clearly indicates that PS-DFDAG has higher solubility for PS than SFO. Using GCMS one can easily identify the mixtures of isomers as because isomeric mass fragmentations are different in mass spectra.

Figure 3 depicts the solubility of PS in different temperature at different DAG concentration. In our previous study, we optimized the method parameters for the production of DAG-rich sunflower oil by enzyme catalysed glycerolysis. In present study, we have used all the five different concentrations of DAG viz. 181.4 g kg^{-1}, 275.3 g kg^{-1}, 378.6 g kg^{-1}, 511.4 g kg^{-1} and 598.0 g kg^{-1} that we obtained from our earlier experiment [17]. It is evident from Figure 2 that PS solubility was increased with increase in DAG concentration. It is an established fact that polar solutes easily dissolved in polar solvents. Presence of free hydroxyl group makes DAG oil more polar than TAG rich oil [22]. So, DAG-rich oil being a more polar solvent solubilise higher amount of PS(solutes) quantitatively compared to TAG. Therefore, according to Figure 2, '59.4' is indicated as the highest solubility for PS with DAG concentration 598.0 g kg^{-1}.

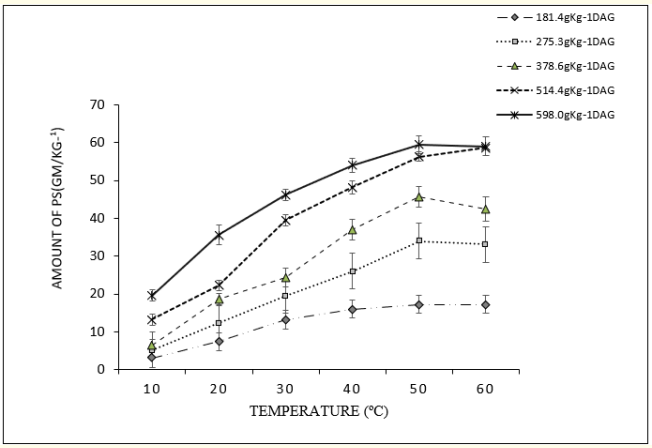


Figure 3: Effect of temperature on solubility of phytosterols at different DAG concentration.

Reaction temperature always influences the rate of reaction. Higher temperature increases the solubility of PS in DAG. Initially, when temperature is increased PS solubility is also increased. After 4-5 hrs, PS solubility did not increase significantly as the reaction reached the equilibrium. It is evident from the Figure 2 that PS solubility is increased with increase in temperature up to 50 ºC. After 60ºC no change was observed in solubility. This also proved that SFDAG had better solubility for PS than SFO.

In summary, SFO and SFDAG contained 13.1 g kg^{-1} and 10.2 g kg^{-} unsaponifiables while that enriched with PS showed 25.0 g kg^{-1} and 71.0 g kg^{-1} for PS-SFO and PS-SFDAG, respectively. This was possible due to higher solubility of PS in DAG, as confirmed by TLC as well as in GC-MS. Experimental data showed γ-sitosterol is the major sterol found in SFO and SFDAG. The higher solubility of PS in SFDAG as compared to SFO enables PS-SFDAG a double active nutraceutical which can control obesity as well as hypercholesterolemia. Ongoing research in this field will reveal new information and will enrich our knowledge in near future.

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