Indian J Dairy Sci 77(5): 416-421
https://doi.org/10.33785/IJDS.2024.v77i05.003

RESEARCH ARTICLE

Estimation of β-sitosterol as a tool to detect ghee adulteration with palm oil

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Received: 13 January 2023 / Accepted: 08 December 2023 / Published online: 23 October 2024

Abstract: Ghee is a popular dairy product in India and most expensive fat in India so it is prone to adulteration with highly manipulated inexpensive oils/fats, especially during the lean season. Dry fractionation is the most cost-effective method of modifying the physical properties of milk fat. β-sitosterol is a principal sterol in edible oil (palm oil). The use of chromatography to separate sterols from ghee fractions could be a novel way to distinguish between β-sitosterol and cholesterol. In the current study, ghee was added with palm oil at levels of 0, 5, 10 and 20%. β-sitosterol was estimated qualitatively using the reverse phase-thin layer chromatography method (RP-TLC) and found better spot in liquid fraction than control ghee even at a 5 % level. This result was quantified using RP-HPLC and shows L_{20} with palm oil (5%) 0.1755 ppm concentration, S_{20} with palm oil (5%) 0.0755 ppm concentration.

Keywords: Reversed-phase thin layer chromatography, Reversed-phase high performance liquid chromatography, Ghee, Palm oil, β-sitosterol.

Introduction

Humans require edible oils and fats as part of their daily diet (Pitts et al. 2007). Adulteration is defined as the process of lowering the quality or nature of a given substance by adding a foreign or inferior substance and removing vital elements (Gonzálvez et al. 2010). The adulteration is a very serious problem. However, because of their higher demand in the domestic and international markets, adulteration of expensive oil with inexpensive oil is a major issue (Yadav, 2018). Adulteration is not visible because of its small scale and low impact, but it has existed in society for a long time. The main cause of adulteration is deception, which increases their income by increasing the volume of suspected products. Adulteration is also practised by some greedy businesses in order to increase their profit margins (Ayza and Yilma, 2014).

As the world's population continues to grow at an alarming rate, food is frequently tainted in order to meet the needs of this expanding population and feed the large-scale population. Most developed countries have a higher rate of food adulteration. Among them, it has become a very serious problem over the last two decades, posing serious health risks to almost all populations (Majed et al. 2016). β-sitosterol (Beta-sitosterol) is a phytosterol (plant sterol), a white waxy powder with a distinct odour that is one of the food additive components. FSSR, (2021) also recommended β-sitosterol estimation in ghee to check the purity is mandatory and it should be absent. Adulteration of fats and oils is currently a major problem all over the world, and rapid detection methods must be developed (Kou et al. 2018). It is critical to characterize milk fat for purity in order to maintain a consistent, well-defined quality. Because of the varied composition of the triglycerides contained in milk fat, detecting adulterants has always been difficult. The measurement of physico-chemical characteristics, elements of unsaponifiable matter, and evaluation of water-soluble and/or insoluble volatile fatty acids have all been used to detect foreign fats in milk fat. To detect adulteration in milk and milk products with foreign fats, TLC of unsaponifiable matter of milk fat, gas chromatography (GC) analysis of triacylglycerol (TAG) or fatty acid profile, and HPLC analysis of TAG and marker sterols of milk fat in combination with multivariate statistical data processing have been used. The majority of the above-mentioned characteristics, on the other hand, are only effective when large amounts of adulterants are employed, and they are not capable of detecting the type and level of added adulterants (Sharma et al. 2020). The resolution of cholesterol and ß-sitosterol, an index sterol in plant oils/fats, is limited in normal phase thin layer chromatography (Arun et al. 2005; Patel et al. 2011). RP-TLC methods based on silica gel-G plates impregnated with undecane (IDF 1966; Mathew and

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Kamath 1978). Thin layers of $CaCO_3$ and soluble starch (10 g + 4 g) impregnated with liquid paraffin (Ramamurthy et al. 1967), on the other hand, are time consuming and have reproducibility and sensitivity issues. β-sitosterol among the phytosterols is predominant in vegetable oil (palm oil), therefore the presence of β-sitosterol in ghee formed the basis for checking the adulteration of ghee with palm oil. Sofia (2005) detects ghee adulterated with palm oil only at a 20% level using various physicochemical characterization, especially Reichert-Meissl value. Upadhyay (2014) also detected palm oil in ghee at a 5% level using RP-TLC detects, but the spots were not clear. Therefore, in the present investigation the ghee has been fractionated in to liquid and solid fractions based on melting point. Further, the fractionated ghee used for the detection of β-sitosterol using RP-TLC at 5% level of adulteration with palm oil.

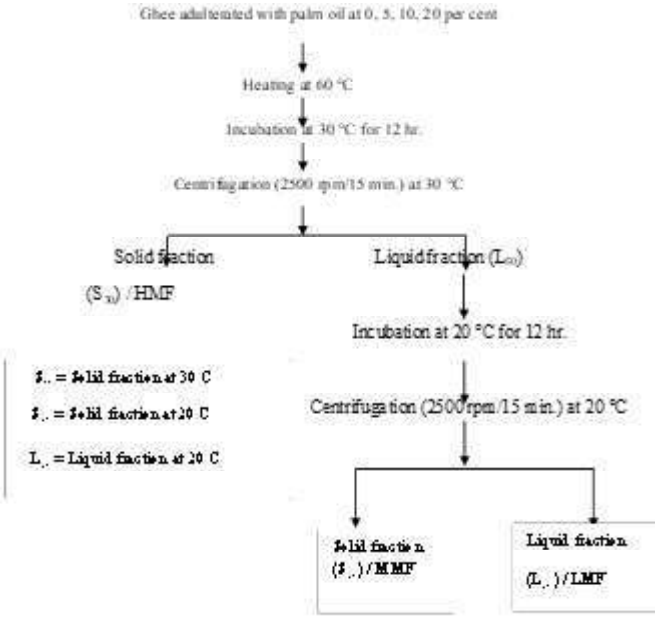
Materials and Methods

Butter of National brand was purchased from retail outlet near Hebbal, Bengaluru. Palm oil were purchased from the local Bengaluru market and used for the blending with ghee to their impact on physico-chemical characterization of ghee. β-sitosterol (Sigma Aldrich, USA), Cholesterol (Sigma Aldrich, USA) RP-TLC (reversed-phase thin layer chromatography) plates TLC silica gel 60 RP-18 F 254S (Merck Specialities Private Ltd., Mumbai, India).

Preparation of samples

Butter was then heated on direct flame in a stainless steel vessel and clarified into ghee with continous strring at a temperature of 115-117°C. Ghee was then filtered through muslin cloths, cooled, filled in airtight glass bottles for futher analysis.

Dry fractionation technique



The method of Kankare (1974) was followed fractionate ghee. The crystal memory was removed by heating ghee to 60°C. It was then progressively cooled to 30°C in an incubator for 12 hr. to crystallize. After centrifugation at 2500 rpm for 15 min. in a temperature-controlled centrifuge kept at 30°C, the liquid was separated from the crystals by decantation. At 30°C, solid fraction obtained (S_{30}) was considered a high melting fraction. The liquid fraction collected at 30°C was then incubated for a further 12 hr. at 20°C. After centrifugation at 2500 rpm for 15 min. in a temperature-controlled centrifuge kept at 20°C. The produced crystals were separated. The solid portion obtained at 20°C (S_{20}) was considered a medium melting fraction, whereas the amount that remained liquid at 20°C was referred to as the low melting fraction (L_{20}).

Extraction of Unsaponifiable Matter (USM) from the Ghee for RP-TLC

Unsaponifiable matter from fat samples was isolated essentially as per the method standardized by Sharma et al. (2009). To extract 0.2 g molten fat sample was taken in a 15 ml capacity screw capped tube followed by the addition of 5 ml of 5 % methanolic KOH. The tube was incubated in a water bath maintained at 90 °C with intermittent shaking after every 5 min., for about 20 min. After 20 min. of incubation, the tube was cooled to room temperature under tap water. One ml water and 5 ml hexane were added in the tube and tube was vortexes for 1–2 min. followed by centrifugation at 2,000 rpm for about 2 min. The upper hexane layer was pipetted out and in a small beaker of about 10 ml capacity and hexane was evaporated to get dried unsaponifiable matter. The dried unsaponifiable matter was redissolved in chloroform and volume was made to 500 µl in an eppendorf tube.

Reversed-phase thin layer chromatography (RP-TLC) of unsaponifiable matter (USM)

Method of sterols separation on C18 stationary phase as described by Jarusiewicz et al. (2005) was adopted in the study. Developing solvent consisting of Petroleum ether: Acetonitrile: Methanol (20:40:40 v/v) was added to a TLC glass chamber lined with filter paper on the three sides. Chamber was saturated for about 15 min. 6 µl of the unsaponifiable matter solution (500 µl solution in chloroform) was spotted on TLC silica gel 60 RP-18 F 254S plate at a distance of about 1 cm from the bottom along with solutions of standard cholesterol, β-sitosterol and mixture (β-sitosterol and cholesterol) as different spots and allowed to air dry. TLC plate was then developed in the developing chamber saturated with developing solvent till the solvent front had travelled about three-quarters of the length of the plate. The plate was then removed, dried, and sprayed with phosphomolybdic acid solution (20 % solution in ethanol) and kept at 90-95°C/3 min and spot of distinct blueish bands was compared with reference standard. The Rf value was calculated

by taking the ratio of distance moved by the solute (in cm) to the distance moved by the solvent (in cm).

Extraction of Unsaponifiable Matter (USM) from the Ghee for RP-HPLC

One gram of the fat sample was weighed for the extraction of USM in a screw-capped test tube and 25 mL of 5% methanolic KOH was added to it. The tube was kept in a water bath maintained at 90°C for about 50 min with vigorous shaking at regular intervals. 5 mL of water and 15 mL hexane were then added and the contents were vortexes for 1 min followed by centrifugation at 3000 rpm for about 5 min. The upper hexane layer was pipetted out and dried to obtain USM. The dried USM obtained was then dissolved in 300 µL of chloroform and the volume was made up to 500 µL with methanol. This sample was then filtered through 0.22 µm Millipore filter paper and subjected to RP-HPLC analysis. The reference standards of β-sitosterol of 1 mg/mL concentration were also run-on RP-HPLC and peak detection was made at 205 nm.

Analytical Conditions for HPLC

RP-HPLC was used to profile the samples of sterols. HPLC conditions as described by Oh et al. (2011) were adopted for the profiling and separation of sterols from the mixture of standard sterols vegetable oils, specific adulterant oil, and adulterated ghee samples. 20 µL of sample was injected into the HPLC column (Reversed phase C-18, 4.6 × 250 mm ID, 5µ 120 Å particle size, Dionex) held at 30°C (in a temperature-controlled column oven) for separation of sterols. Chromatography was initiated at a linear solvent (Acetronitrile:Isopropanol; 9:1, v/v) flow rate of 1.5 mL per min over a period of 30 min with a UV detector probe fixed at 205 nm for the detection of sterols.

Peaks Identification and their Confirmation:

USM of pure ghee and adulterated ghee samples (20 µL) were injected to examine for the presence of cholesterol and phytosterols. The identification of the peaks in the samples was done by comparing the retention time with that of reference standards. The appearance of the peak for β-sitosterol (phytosterol) in the adulterated samples was used as an indicator to confirm the presence of vegetable oils in adulterated ghee sample**s.**

Results and Discussion

Reversed-phase thin-layer chromatography

Reversed-phase thin-layer chromatography (RP-TLC) to check the purity of milk fat at low levels of adulteration with vegetable oil (palm oil). RP-TLC protocol to resolve the β-sitosterol and cholesterol (Jarusiewics et al. 2005) has been developed, where in new generation readymade RP-18 Silica gel-G F254S TLC were used. These plates are easy to use and have good reproducibility.

Separation of standard sterols and their mixture

It is evident from chromatogram (Fig. 1) that standard cholesterol and β-sitosterol showed difference in their mobility on RP-18 TLC silica gel G F254 S plate and even the mixture of cholesterol and ß-sitosterol was also resolved into two different bands corresponding to cholesterol and ß-sitosterol. The Rf value of cholesterol standard was calculated as 0.21, whereas that of β-sitosterol as 0.17. This indicated that the standardised conditions had the potential to be used for resolving sterols, especially cholesterol and ß-sitosterol in ghee samples adulterated with vegetable oils (palm oil).

RP-TLC Profile of Sterols in USM of Ghee Samples

It can be seen from the chromatogram that in case of control ghee samples (cow and buffalo) the position of prominent band was corresponding to the Rf value of cholesterol and there was no band corresponding to the Rf value of β-sitosterol (Fig. 1). However, in case of ghee with palm oil (5%), L_{20} with palm oil (5%) and S_{20} with Palm Oil (5%) a prominent band corresponding to the Rf value of β -sitosterol appeared as evident in Fig. 1. These observations clearly indicated that β-sitosterol was the prominent sterol in palm oils selected for the study, thereby the appearance of any β-sitosterol band in ghee sample could be considered as an indicator of adulteration of ghee with vegetable oils (palm oil).

Validity and specificity of RP-TLC method

The unsaponifiable matter from these samples was subjected to RP-TLC analysis to obtain the profile of sterols. It is evident from the RP-TLC chromatogram (Fig. 1) that control ghee samples not showed any band corresponding to the band of β-sitosterol. This clearly indicated that standardized protocol was very specific in detecting the added vegetable oils (palm oil) in ghee and method could be used to detect the adulteration of ghee with vegetable oils without showing any false positive results.

Detection of adulteration of ghee with palm oil

The unsaponifiable matter of the ghee samples adulterated with palm oils at different levels was subjected to the above standardized RP-TLC method. RP-TLC chromatograms (Fig. 1), clearly showed that the adulteration of ghee with palm oil could easily be detected even at the level of ghee with palm oil (5%), L_{20} with palm oil (5%) and S_{20} with Palm oil (5%) due to the presence of spot corresponding to the β-sitosterol which were not there in case of the control of ghee. Finding from the present work clearly demonstrated that the concentration of palm oil in the samples of milk fat increased the intensity of spots (both cholesterol and β-sitosterol) were prominent in fractionated ghee (especially liquid

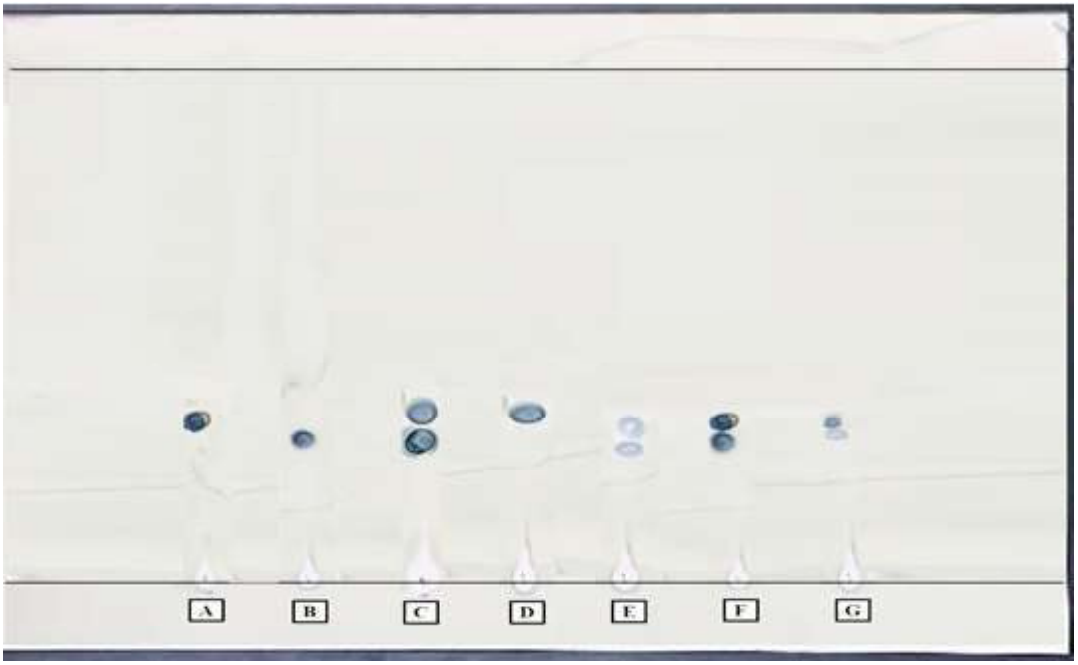


Fig. 1 Reverse phase thin layer chromatography (A) cholesterol (B) β-sitosterol (C) Mixture (β-sitosterol and cholesterol) (D) Control ghee (E) Ghee with palm oil (5%) (F) L_{20} with palm oil (5%) (G) S_{20} with palm oil (5%)

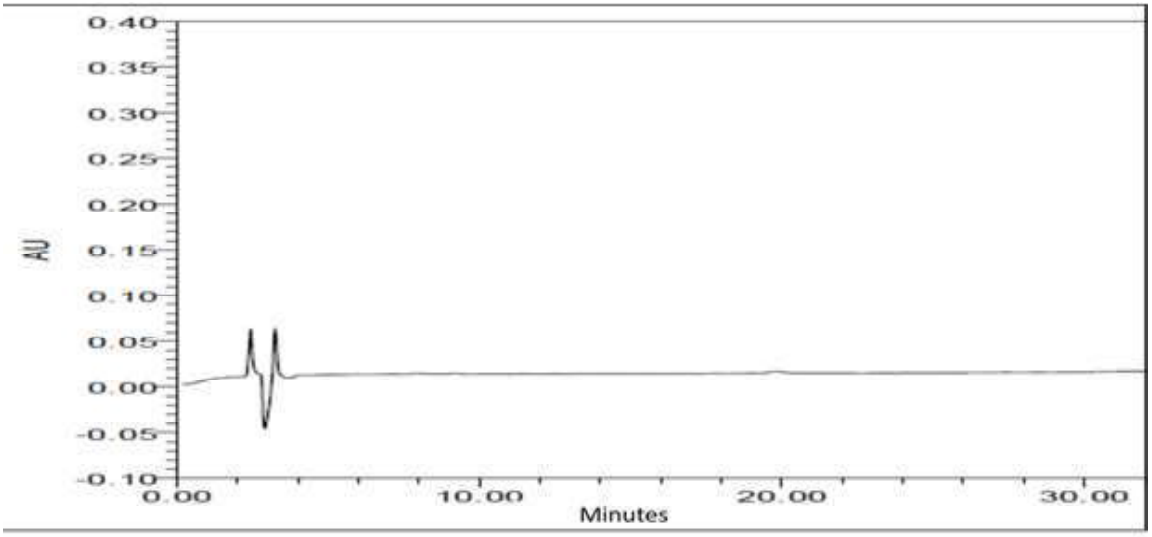


Fig. 2 Chromatography graph for control ghee

fraction) compared with control ghee. The result obtained are comparable with those of Rani et al. (2015) reported that RP-TLC chromatograms clearly showed that coconut oil added to ghee at 5 % level a very faint band were visible in the chromatogram, but at 7.5 % level, the visibility of band corresponding to β-sitosterol band were more. Similar results were also found by Upadhyay, (2014) reported that the concentration of vegetable oil in the samples of milk fat increased (from 5 to 15 % groundnut oil) the intensity of spot increased indicating the increase in the amount of β-sitosterol in the samples and thus enabling the detection of milk fat adulteration. Physicochemical characterization (Reichert-Meissl value) detects palm oil in ghee only at 20%. (Sofia, 2005) In the current investigation, the spots clear by using dry fractionation to detect the ghee adulterated with palm oil at a 5% level in less than 2 hours.

Reverse phase high performance liquid chromatography (RP-HPLC)

An efficient, fast and reliable reversed phase high-performance liquid chromatography-based method was developed to detect specific adulterant in ghee (clarified butterfat) samples. The method is based on the detection of cholesterol and β-sitosterol as markers in the unsaponifiable matter of pure ghee and adulterated ghee samples, respectively. Validation of the method revealed that it was fast, economical, highly reliable, and comparable with the reversed-phase thin layer chromatography method with no false negatives.

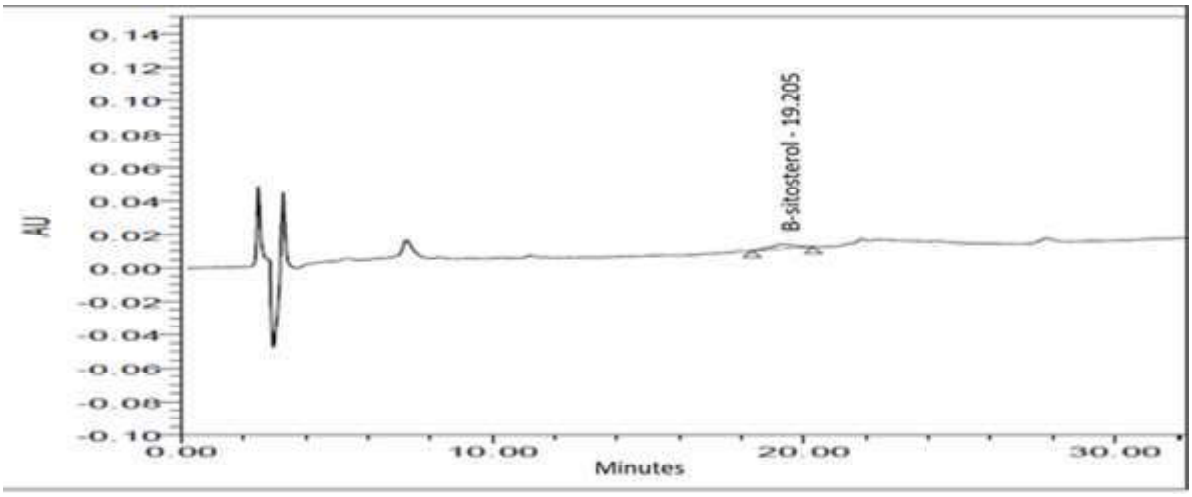


Fig.3 Chromatography graph for S_{20} with palm oil (5%)

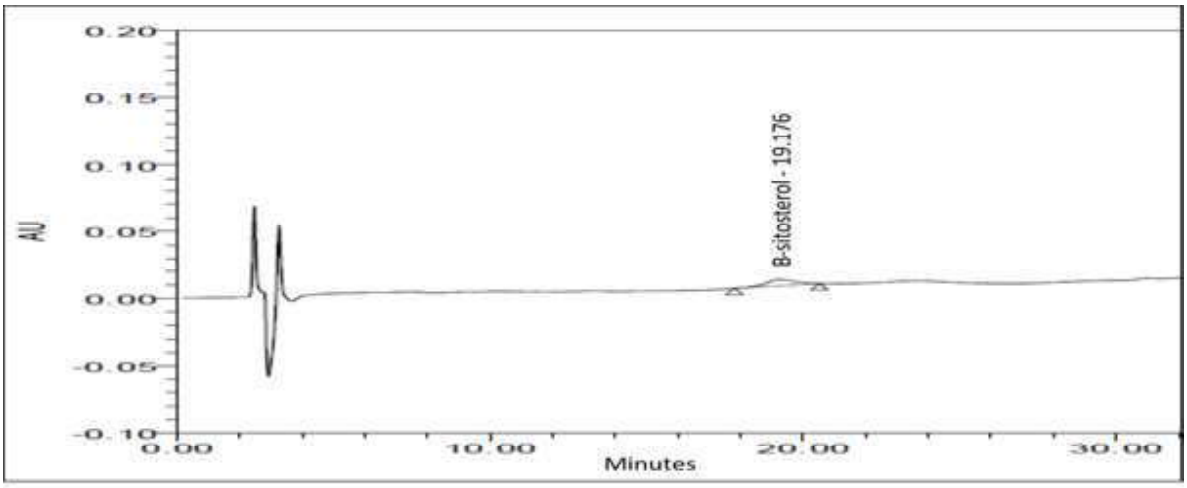


Fig.4 Chromatography graph for L_{20} with palm oil (5%)

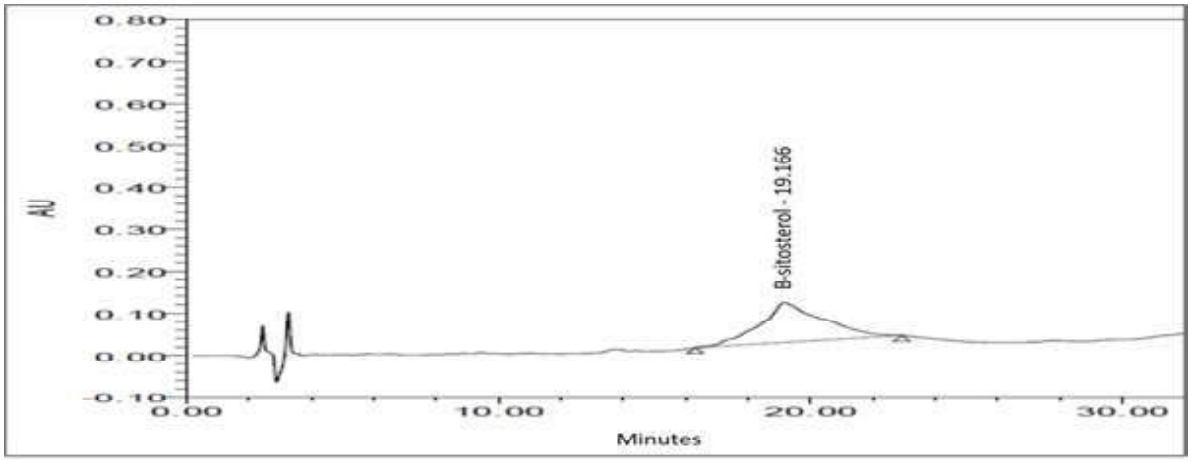


Fig.5 Chromatography graph for standard

Reversed-Phase High Performance Liquid Chromatographic (RP-HPLC) Conditions for the Profiling of USM

RP-HPLC conditions for the separation of standard sterols as described by (De, 2011) were adopted and standardized for the profiling of sterols in USM of ghee, adulterated samples. The retention time of individual standard sterols (β-sitosterol), was 19.166 min. (Figure 5). The results indicated that the β-sitosterol resolved properly as evident from their Rt values. Since β-sitosterol resolved properly using the standardized HPLC method and β-sitosterol served as principle markers sterol for vegetable

oils. Therefore, this standardized method was selected to compare the results with that of the RP-TLC method.

Validation of Standardized RP-HPLC Method

The USM from these samples were subjected to RP-HPLC analysis to obtain the profile of sterols which is depicted in Figure 5. A small variation in the retention time of β-sitosterol was observed in the samples used for the validation and was found to be 19.166 min. control ghee sample no peak corresponding to the Rt of β-sitosterol It is evident from the RP-HPLC chromatograms that L_{20} with palm oil (5%) and S_{20} with palm oil (5%) samples showed peak corresponding to the peak of β-sitosterol (Figure 3, 4). It was amply clear that in L_{20} with palm oil (5%) and S_{20} with palm oil (5%) samples peak corresponding to the Rt of β-sitosterol was 19.205, 19.176 min. observed, hence the standardized method was found to be very specific in detecting the adulterant oil in ghee, wherein major sterol, i.e., β-sitosterol, was the selected tracer component.

Detection of Adulterant Oils in Ghee

RP-HPLC chromatograms that L_{20} with palm oil (5%) and S_{20} with palm oil (5%) samples. Quantified using RP-HPLC shows L_{20} with palm oil (5%) 0.1755 ppm concentration, S_{20} with palm oil (5%) 0.0755 ppm concentration.

Conclusions

Reversed-phase thin layer chromatographic protocol has been standardized in the present study. The adulteration of ghee with adulterant oils such as palm oil could be detected up to 5 % level. Physicochemical characterization (Reichert-Meissl value) detects palm oil only at 20% for ghee adulterated with palm oil. Without fractionation, the intensity of the band on RP-TLC is not clear. As a result, fractionation using RP-TLC the intensity of band is clear in L_{20} with palm oil (20%) and RP-HPLC could be suggested in the current study even at a 5% level.

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