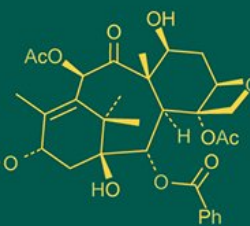


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Indirect detection of vegetable oils addition in ghee by estimating the presence of synthetic antioxidants using gas liquid chromatography approach

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Abstract

Ghee is the second largest selling dairy product after fluid milk. A protocol for the extraction of residual synthetic antioxidants from ghee samples spiked with vegetable oils was standardized. The recovery of the extraction method as calculated after gas liquid chromatography (GLC) analysis was found to be 97.73 ± 4.98 and 97.59 ± 5.38 for TBHQ and BHA, respectively. The GLC method was linear to estimate antioxidants in ghee at 0.00125% (12.5 ppm) and above. R^2 for both antioxidants was found to be above 0.99. The standardized method could detect vegetable oils in ghee at 1% and above. Storage of antioxidant-spiked ghee samples at 30°C did not show any significant degradation of the studied antioxidants. The standardized method detected the presence of antioxidants in market samples, indicating the presence of refined vegetable oils/fats in ghee.

Keywords: Ghee, vegetable oil, synthetic antioxidants, gas liquid chromatography, adulteration

Introduction

Ghee (heat-clarified anhydrous butter fat) is the second largest dairy product after fluid milk. It is reported that about 35% of the milk is converted into ghee in the Indian sub-continent [1]. In India, the ghee market has witnessed substantial growth over the past few years; the market was valued at INR 2624 billion in 2021 [2]. Ghee has already been established as a premium dairy product in the Indian sub-continent and hence sold at a higher price. The premium nature of the product attracts unscrupulous traders to adulterate it with cheaper fats other than milk fat like refined vegetable oils, body fats and vegetable fats or their concoctions [3].

The most encountered adulterant in ghee is vegetable oil because of its low cost in comparison to ghee. In order to ensure a quality product to the consumer, the Government of India has specified quality standards for ghee (FSSR, 2011) [4] and Agmark standards (1938) [5]. It is also a fact that commercially available refined edible vegetable oils have low shelf life due to unsaturation, and thus synthetic antioxidants or their mixtures are added to improve their oxidative stability [6]. As per the FSSR (2011), the maximum permissible limit of synthetic antioxidants like BHA and TBHQ in edible vegetable oils is 200 ppm. On the other hand, no synthetic antioxidant is permitted in ghee as per food standards (FSSR, 2011) [4].

In general, chromatographic and spectrophotometric methods have been recommended to estimate added synthetic antioxidants in vegetable oils [7, 8]. Several methods like High Performance Liquid Chromatography (HPLC) of sterols, Gas-Liquid Chromatography (GLC) of sterols, fatty acids and triglycerides, and Reversed-Phase Thin Layer Chromatography (RP-TLC) of sterols have been reported to detect the presence of vegetable oils in ghee. However, all these methods are time-consuming and cost-intensive. Recently, a rapid dye-based platform test was reported to check the presence of vegetable oils in milk fat, which is based on the detection of synthetic antioxidants present in refined vegetable oils, wherein addition of vegetable oil to the tune of 5% and more could be detected [9].

It has been reported that the residual antioxidant content of BHA in butter after six months of storage at -20°C was reduced to 28.4 and 62.5 ppm from its initial value of 50 and 100 ppm, respectively [10].

These observations suggest that antioxidants used to enhance the shelf life of vegetable oils can get exhausted during storage of ghee samples adulterated with such oils. This may affect the sensitivity of the rapid platform test already reported in the literature. Therefore, in the present investigation, an attempt has been made to develop a GLC-based method to check the degradation of synthetic antioxidants on account of storage and counter the malpractice of ghee adulteration.

Materials and Methods

Chemicals and Reagents

Methanol (HPLC grade) was purchased from Thermo Fisher Scientific India Pvt. Ltd., Mumbai; n-Hexane from Honeywell International India Pvt. Ltd., New Delhi; Chloroform (AR grade), Ethanol absolute and Acetonitrile (HPLC grade) from S.D. Fine-Chem. Ltd., Mumbai, India; Petroleum Ether (AR grade) from Glaxo Laboratories India Ltd., Mumbai; Ethyl Acetate and Diethyl Ether from Merck Specialities Pvt. Ltd., Mumbai, India. Synthetic antioxidants, viz., Butylated Hydroxyanisole (BHA), Butylhydroquinone (TBHQ) and Ascorbic acid were purchased from Sigma Aldrich, St. Louis, USA; Butylated Hydroxytoluene (BHT) from Thermo Fisher Scientific India Pvt. Ltd.; Gallic Acid from Central Drug House (P) Ltd., New Delhi; and 6-O-Palmitoyl-L-ascorbic Acid from Tokyo Industry Co., Ltd., Tokyo, Japan.

Preparation of Stock and Working Solutions

Stock solution (0.02%) of BHA, BHT and TBHQ was prepared by dissolving 20 mg of each synthetic antioxidant separately in acetonitrile and making the volume to 100 ml in a volumetric flask. Working solutions of lower concentrations (0.01, 0.005 and 0.0025%) were prepared from the above stock solutions in acetonitrile.

Procurement of Pure Cow Ghee and Adulterant Vegetable Oils

Pure cow ghee was collected from the Experimental Dairy (ICAR-NDRI, Karnal, Haryana, India). Refined sunflower oil (SFO) used as adulterant was purchased from the local market at Karnal, Haryana, India.

Preparation and Storage of Adulterated Ghee Samples

Cow ghee was heated at 45 °C and mixed well to ensure homogeneity. Stock solution of cow ghee containing individual antioxidant (BHA and TBHQ) at 5% was prepared. Ghee samples with effective concentrations of antioxidants at 0.0025, 0.005, 0.01 and 0.02% were prepared by diluting the stock ghee solution with pure ghee devoid of any added antioxidant. In another set, adulterant vegetable oil (SFO) was added individually to pure cow ghee at 1, 2.5, 5, 7.5 and 10% to obtain ghee samples spiked with different adulterant vegetable oils. After addition, samples were

thoroughly mixed, filled in air-tight HDPE narrow-mouth bottles and stored at room temperature (30 °C) until further analysis.

Procurement of Market Ghee Samples

Branded (coded A, B, C, D, E and F) and unbranded (coded 1, 2, 3, 4, 5 and 6) ghee samples were purchased from the local market of Karnal, Haryana.

Extraction of Antioxidants from Ghee Samples

To ensure complete extraction of synthetic antioxidants from ghee, 1.0 ml of antioxidant-spiked ghee sample was mixed with 1.0 ml of hexane and then 1.0 ml of acetonitrile, followed by vortexing for 30 seconds and centrifugation at 2000 rpm for 5 min. The upper layer was collected and extraction was repeated with an additional 1.0 ml of acetonitrile. The upper layers from both extractions were combined and used for GLC analysis.

Gas Liquid Chromatography (GLC) of Synthetic Antioxidants

Synthetic antioxidants in ghee were measured essentially based on the method of Yang *et al.* (2002)¹¹ described for the estimation of synthetic antioxidants in vegetable oils. A GC-2010 Plus (Shimadzu, Japan) with Flame Ionization Detector (FID) and SPB-5 (30.0 m × 0.53 mm × 1.5 µm) column was used. Carrier gas: Nitrogen at a column flow of 4.00 mL/min. FID temperature: 290 °C; Injector Split: 250 °C; Column oven temperature: initial 150 °C for 1 min, then increased to 200 °C at 7 °C/min, then to 300 °C at 40 °C/min, held for 5.0 min. Injection volume: 0.2 µL.

Statistical Analysis

The data obtained were subjected to one-way and two-way Analysis of Variance (ANOVA) to check for significant differences in means via Duncan's Multiple Comparison test at a 95% confidence interval using IBM SPSS Statistics (Version 20). Results were expressed as mean ± standard deviation (SD).

Results and Discussion

Resolution of Standard Antioxidants Using GLC

Conditions: As evident from the chromatogram (Fig. 1), upon injecting 0.2 µL of 0.02% solution of BHA, BHT and TBHQ individually and as a mixture in acetonitrile, all three antioxidants eluted at distinct retention times: 7.45, 8.0 and 8.35 min, respectively. In the mixture, all three were well-resolved at their respective retention times. This confirmed that the GLC conditions (oven temperature program, carrier gas flow rate, injector temperature, injection volume) were optimum for resolving the three antioxidants as distinct peaks and the method can be used to check the degradation of BHA, BHT or TBHQ in ghee samples spiked with vegetable oils.

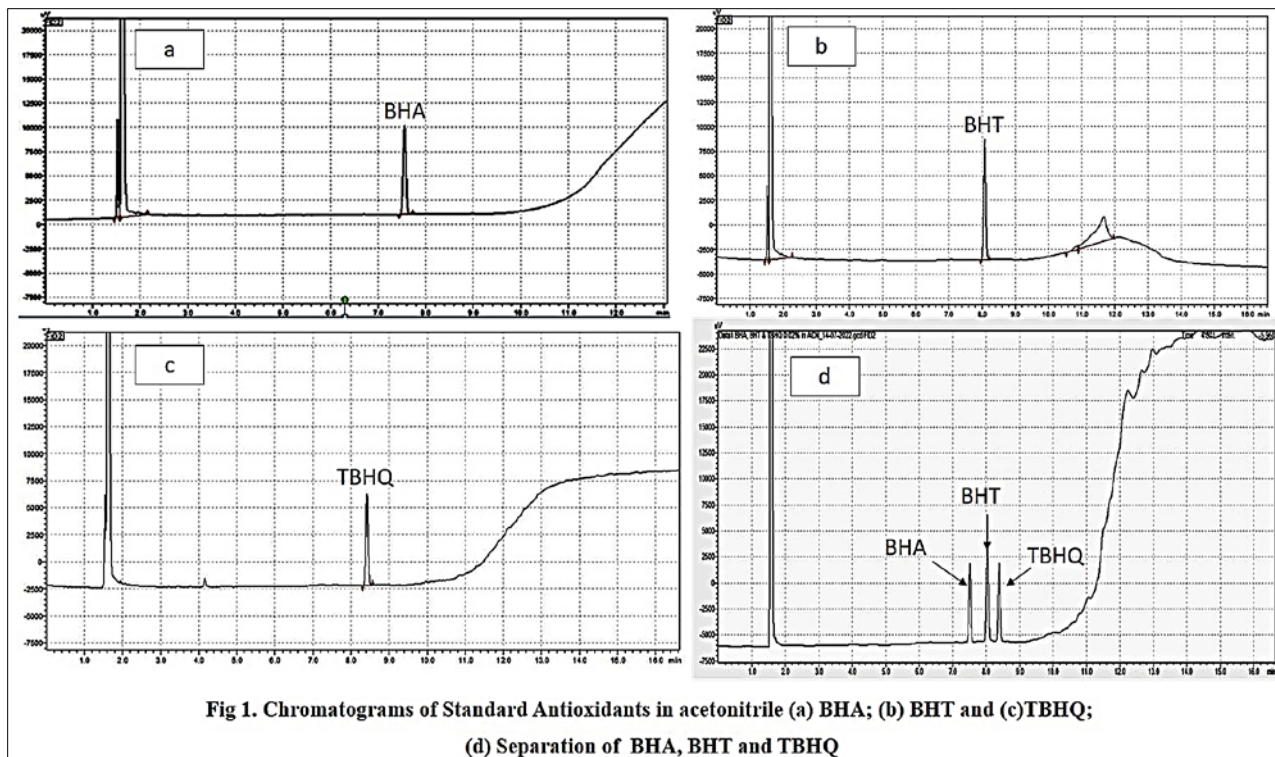


Fig 1. Chromatograms of Standard Antioxidants in acetonitrile: (a) BHA; (b) BHT and (c)TBHQ; (d) Separation of BHA, BHT and TBHQ

Fig 1: Chromatograms of standard antioxidants in acetonitrile: (a) BHA; (b) BHT; (c) TBHQ; (d) separation of BHA, BHT and TBHQ

Linearity of the Direct Method of Antioxidant Estimation in Ghee

The scatter graph of area versus concentration of added antioxidants in ghee (Fig. 2a) showed that the decrease in area with decreasing concentration of individual antioxidants (BHA and TBHQ) was not linear below 0.02% (200 ppm), and the line was haphazard. This indicated that direct injection as suggested for vegetable oils may not work effectively in samples where vegetable oil is added as an adulterant at lower concentrations.

Linearity of the Indirect Method of Antioxidant Estimation in Ghee: Since the study focused on detecting residual antioxidants in ghee adulterated with vegetable oils

(wherein the permissible limit of BHA and TBHQ does not exceed 0.02% in edible oils except ghee and butter), the method needed to be workable at approximately 0.002 mg/ml (2 ppm) in ghee spiked with at least 1% vegetable oil. Upon drawing the scatter plot of area versus concentration after extracting antioxidants from spiked ghee samples (Fig. 2b), the line was found to be linear up to 0.00125% (12.5 ppm). Standard curves of BHA and TBHQ (Fig. 3) drawn using the extraction approach yielded R^2 values above 0.99 for both antioxidants. These findings confirmed that the standardized method can detect the presence of vegetable oils in ghee at a minimum spiking level of 1% and above.

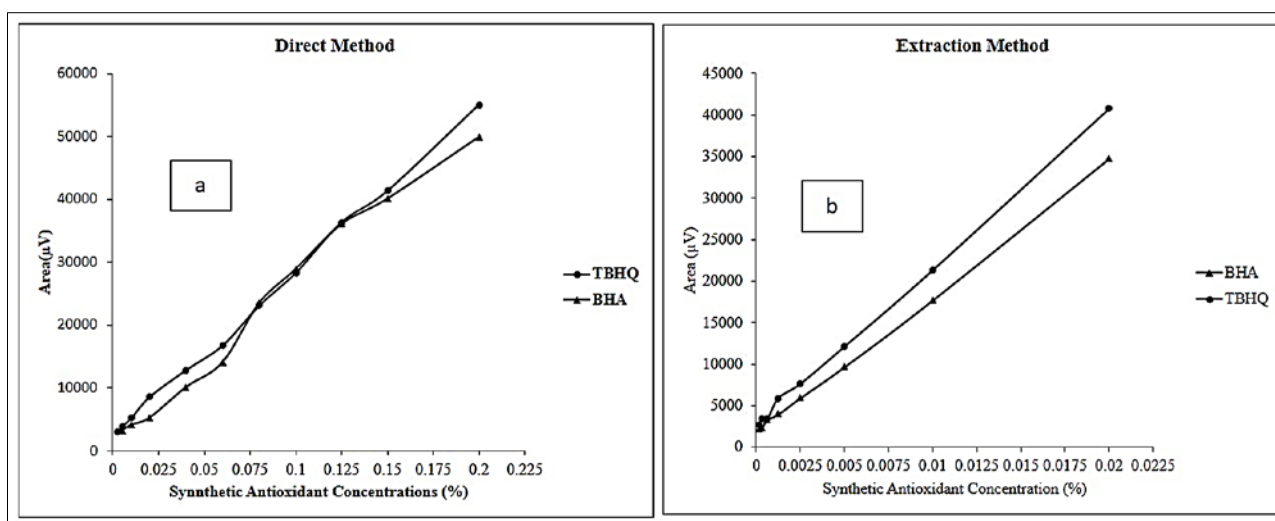


Fig 2: Scatter plots of BHA and TBHQ: (a) Direct GLC method; (b) GLC of extracted antioxidants

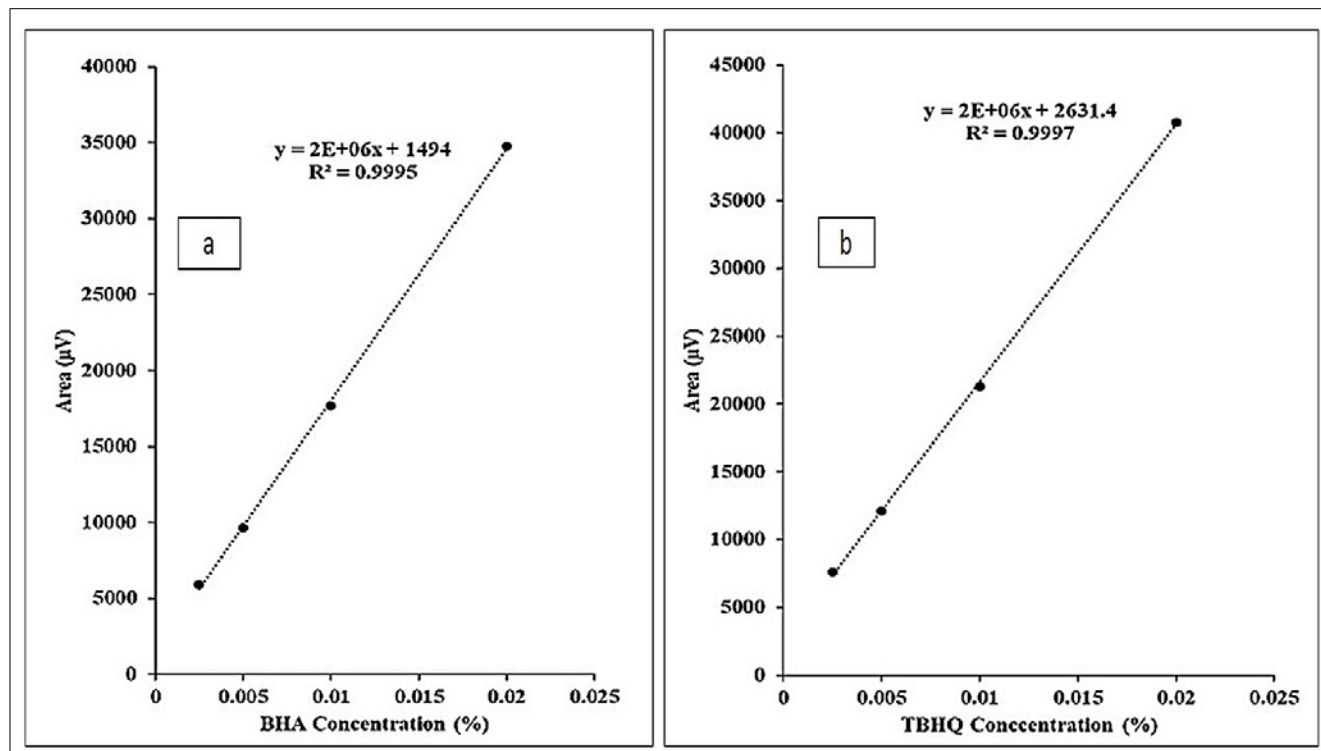


Fig 3: Standard curves of (a) BHA and (b) TBHQ in ghee using the extraction method

Table 2: Recovery (%) of extracted antioxidants from ghee samples

Antioxidants	Added concentration (%) in ghee	Concentration (%) calculated in spiked ghee samples from standard curve	Recovery (%)	Avg Recovery (%)±SD
TBHQ	0.02	0.0180	90.60	97.73±4.98
	0.01	0.0098	98.02	
	0.005	0.0050	100.00	
	0.0025	0.0026	104.00	
BHA	0.02	0.0185	92.63	97.59±5.38
	0.01	0.0093	93.25	
	0.005	0.0051	102.25	
	0.0025	0.0027	106.88	

Concentrations are expressed as percentage (%) by weight of ghee. Recovery (%) = (Calculated concentration/Added concentration) × 100. TBHQ: tert-Butylhydroquinone; BHA: Butylated hydroxyanisole; SD: Standard deviation.

Effect of Storage on the Status of Antioxidants in Antioxidant-Spiked Ghee Samples

The concentration of TBHQ at 0 days of storage at ambient temperature was 0.0212, 0.0112, 0.0053 and 0.0026% in ghee samples spiked at 0.02, 0.01, 0.005 and 0.0025%, respectively. After 270 days of storage, the measured concentration in the same samples was 0.0207, 0.0093, 0.0047 and 0.0023%, respectively. Similarly, for BHA-

spiked ghee samples, the concentration at 0 days was 0.019, 0.0097, 0.0048 and 0.0024% for the same spiking levels. After 270 days of storage, the concentrations were 0.019, 0.0097, 0.0048 and 0.0024%, respectively, indicating negligible degradation. The chromatograms (Fig. 4) clearly indicated a marginal but gradual reduction in antioxidant concentration during storage at 30 °C.

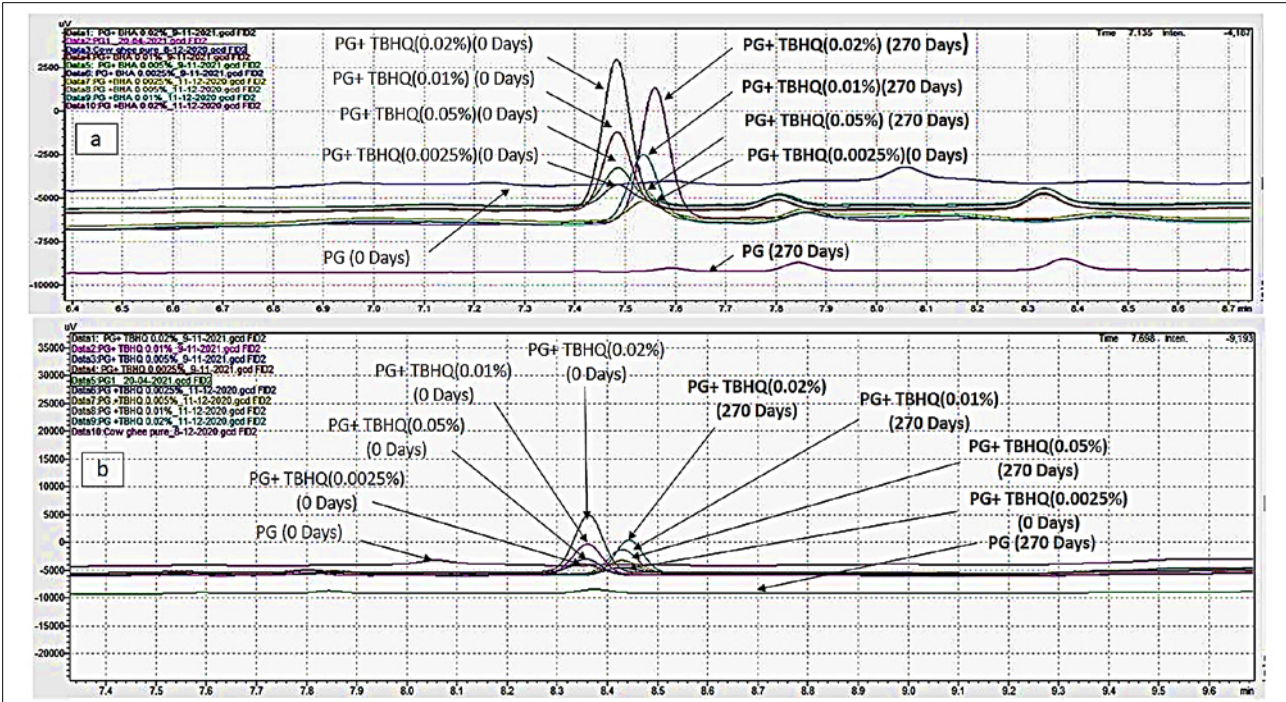


Fig 4: Reduction of antioxidants in stored (a) BHA and (b) TBHQ spiked cow ghee samples from 0 to 270 days at ambient temperature (30 °C)

These findings were consistent with earlier reports.¹⁰ Ozturk and Cakmakci (2006) ^[10] reported that BHA and BHT added at 50 and 100 ppm in butter degraded upon increasing temperature and storage period. Similarly, Martínez *et al.* (2013) ^[7] observed greater degradation of TBHQ in walnut oil stored under light compared to dark conditions. Ribeiro and Jorge (2017) ^[8] reported degradation of BHA and TBHQ at 200 ppm in soybean oil stored at 60 °C. In lard containing BHA, only a 10% change in residual antioxidant level was observed on the 42nd day of storage at 50 °C ^[12].

Effect of Storage on Antioxidants in Vegetable Oil-

Spiked Ghee Samples: The antioxidant concentration in SFO as determined by GLC was 0.013% (130 ppm) on day zero of storage, marginally reducing to 0.010% (100 ppm) after 210 days of storage at ambient temperature. In ghee samples spiked with SFO at 1, 2.5, 5, 7.5 and 10%, the antioxidant concentrations corresponded to the level of spiking and showed almost no significant change during storage at 30 °C, indicating that BHA and TBHQ are quite stable under the tested storage conditions ^[12].

Analysis of Market Ghee Samples for Synthetic Antioxidant Presence

Table 1: Reduction of antioxidants in stored pure ghee, sunflower oil and sunflower oil-spiked pure ghee samples at ambient temperature (30 °C)

Storage Days	PG	SFO	Wa	Wb	Wc	Wd	We
0	0	0.013 ^A	0.0016 ^c	0.0012 ^B	0.0004 ^A	0.0006 ^c	0.0002 ^A
15	0	0.013 ^A	0.0016 ^c	0.0012 ^B	0.0004 ^A	0.0006 ^c	0.0002 ^A
30	0	0.013 ^A	0.0009 ^A	0.0010 ^A	0.0010 ^B	0.0002 ^A	0.0002 ^A
45	0	0.013 ^B	0.0017 ^c	0.0016 ^D	0.0016 ^A	0.0002 ^A	0.0002 ^A
60	0	0.012 ^B	0.0013 ^B	0.0016 ^D	0.0010 ^B	0.0004 ^B	0.0002 ^A
75	0	0.012 ^B	0.0016 ^c	0.0016 ^D	0.0016 ^L	0.0010 ^L	0.0006 ^B
90	0	0.012 ^B	0.0016 ^c	0.0016 ^D	0.0012 ^c	0.0004 ^B	0.0002 ^A
105	0	0.012 ^B	0.0016 ^c	0.0016 ^D	0.0016 ^L	0.0008 ^D	0.0006 ^B
120	0	0.012 ^B	0.0019 ^L	0.0018 ^L	0.0016 ^L	0.0016 ^O	0.0010 ^D
135	0	0.012 ^B	0.0019 ^L	0.0012 ^B	0.0012 ^c	0.0010 ^L	0.0008 ^c
150	0	0.012 ^B	0.0018 ^D	0.0014 ^c	0.0012 ^c	0.0008 ^D	0.0008 ^c
165	0	0.012 ^B	0.0018 ^D	0.0016 ^D	0.0012 ^c	0.0014 ^P	0.0008 ^c
180	0	0.012 ^B	0.0020 ^L	0.0016 ^D	0.0012 ^c	0.0012 ^O	0.0012 ^L
195	0	0.011 ^c	0.0023 ^O	0.0020 ^O	0.0014 ^D	0.0016 ^O	0.0012 ^L
210	0	0.010 ^c	0.0019 ^D	0.0016 ^D	0.0012 ^c	0.0008 ^D	0.0010 ^D

Values are represented as Mean ± SD, n = 3; values in the same column with different superscript letters (A, B, C...) differ significantly (p<0.05). PG: Pure Ghee; SFO: Sunflower Oil; Wa: PG spiked with SFO @ 10%; Wb: PG spiked with SFO @ 7.5%; Wc: PG spiked with SFO @ 5%; Wd: PG spiked with SFO @ 2.5%; We: PG spiked with SFO @ 1%.

Profiling of branded and unbranded ghee samples for synthetic antioxidants using the standardized GLC method is depicted in Fig. 5. A peak corresponding to the retention time of TBHQ (8.35 min) was observed in branded ghee samples coded C, D, E and F, and in unbranded samples coded 1, 2, 3 and 5. Since no antioxidant is permitted in ghee as per FSSR (2011) ^[4], the appearance of a TBHQ

peak in these samples raises suspicion of milk fat adulteration with vegetable oils. While the possibility of leaching from packaging materials cannot be entirely ruled out for branded samples, the fact that branded samples A

and B did not show a similar peak supports the conclusion that the primary cause is adulteration with refined vegetable oils.

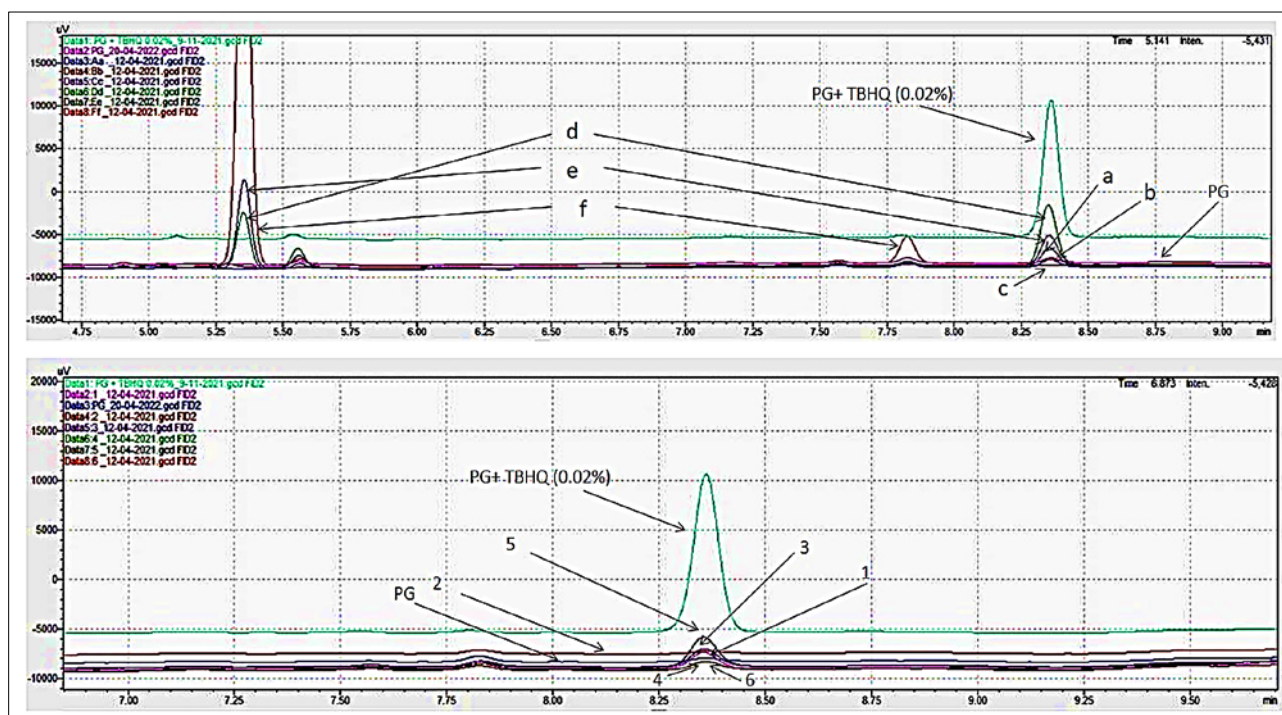


Fig 5: Antioxidant profile of (a) branded ghee samples; (b) unbranded ghee samples

Conclusion

The SPB-5 (30 m × 0.53 mm × 1.5 μm) column successfully resolved synthetic antioxidants with retention times of 7.45, 8.0 and 8.35 min for BHA, BHT and TBHQ, respectively. The double extraction approach was effective in detecting vegetable oils added to ghee at 1% or above. The standardized GLC method was nonlinear below 0.00125% antioxidant concentration. The method was stable over prolonged storage with negligible antioxidant degradation at 30 Cover 270 days. The standardized method successfully detected the presence of synthetic antioxidants in both branded and unbranded market ghee samples, confirming its utility in detecting vegetable oil adulteration in ghee.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Author Contributions

Yugal Kishor Naik: Experimental work; investigation; methodology; statistical analysis. Vivek Sharma: Conceptualization; data curation; investigation; methodology; supervision; writing-original draft. Sumit Arora: Conceptualization; methodology; resources; writing-review and editing. Raman Seth: Resources; writing-review and editing.

Data Availability Statement

Research data supporting this publication are not publicly available.

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